

UCRL-1950  
Unclassified-Chemistry Distribution

UNIVERSITY OF CALIFORNIA

Radiation Laboratory

Contract No. W-7405-eng-48

THE PATH OF CARBON IN PHOTOSYNTHESIS.

XX. THE STEADY STATE

M. Calvin and Peter Massini

September, 1952

Berkeley, California

THE PATH OF CARBON IN PHOTOSYNTHESIS.

XX. THE STEADY STATE

M. Calvin and Peter Massini\*

Radiation Laboratory and Department of Chemistry,  
University of California, Berkeley\*\*

---

(\*) Fellow of the Swiss Foundation, "Stiftung für Stipendien auf dem Gebiete der Chemie," 1951-1952.

(\*\*) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

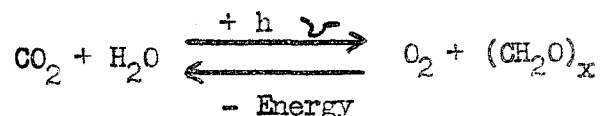
---

ABSTRACT

The separation of the phenomenon of photosynthesis in green plants into a photochemical reaction and into the light-independent reduction of carbon dioxide is discussed. The reduction of carbon dioxide and the fate of the assimilated carbon were investigated with the help of the tracer technique (exposure of the plants to the radioactive  $C^{14}O_2$ ) and of paper chromatography. A reaction cycle is proposed in which phosphoglyceric acid is the first isolable assimilations product. Analyses of the algal extracts which had assimilated radioactive carbon dioxide in a stationary condition ("steady-state" photosynthesis) for a long time provided further information concerning the proposed cycle and permitted the approximate estimation, for a number of compounds, of what fraction of each compound was taking part in the cycle. The earlier supposition that light influences the respiration cycle was confirmed. The possibility of the assistance of  $\alpha$ -lipoic acid, or of a related substance, in this influence and in the photosynthesis cycle, is discussed.

THE PATH OF CARBON IN PHOTOSYNTHESIS. XX. THE STEADY STATE

Photosynthesis, the process by which green plants are able to capture electromagnetic energy in the form of sunlight and transform it into stored chemical energy in the form of a wide variety of reduced (relative to carbon dioxide) carbon compounds provides the only major source of energy for the maintenance and propagation of all life. For this and other reasons, the study of the nature of this process has been a very attractive area for many years and a wide variety of scientific interest and backgrounds have been brought to bear upon it. These range from the purely biological to the strictly physical with the biochemical and physicochemical area lying between. Important contributions to the understanding of the phenomenon have come from all these areas, but in spite of the enormous amount of work and study that has gone into the problem, relatively little is known, or rather understood, about the fundamental character of the process even today. It is perhaps pardonable that one engaged in studies in this area tends to the conclusion that most of the knowledge has been acquired in the relatively recent past. Discounting that tendency, it still seems fair to say that we have only just begun in the last decade or so to gain some understanding of the intimate details by which the basic process represented in the overall reaction



has come to be understood. The recognition of this overall reaction as written, to represent the basic nature of the process of photosynthesis, and, further, that its reversal represents the basic reaction of respiration is, of course, an old one.

As a result of more recent study, it has been possible to separate the process of photosynthesis into two distinct and separate parts. The general features of this separation may be represented in the following chart:

(Figure 1). The essential feature of the separation is the independence of

the photochemical part of photosynthesis from the carbon dioxide reduction part. We shall not here even try to outline all of the various forms of evidence which have been adduced in support of such a scheme but only to point out additional bits which have been added in recent years and particularly those which stem from our own work.<sup>1,2,3</sup>

The scheme itself is an outgrowth of proposals of some fifteen years ago by Van Niel<sup>4</sup> resulting from his studies of the comparative biochemistry of photosynthesis. More recently, the photochemical apparatus has been shown to be separable from the rest of the plant by the experiment of Hill.<sup>5,6</sup>

He was able to make preparations of chloroplasts and chloroplastic fragments which, upon illumination in the presence of suitable oxidizing agents other than carbon dioxide, were able to evolve molecular oxygen. Still more recently, Ochoa and others<sup>7,8,9</sup> were able to demonstrate that these same preparations were capable of using coenzyme I and II (DPN and TPN) as suitable oxidizing agents leading to the evolution of oxygen. Furthermore, the experiments of Ruben<sup>10</sup> showed that the molecule of oxygen evolved in photosynthesis had its approximate origin in the oxygen of the water molecule and that the oxygen atom associated with the carbon dioxide must first pass through water before arriving at gaseous oxygen. From the chart it may be seen that the ultimate result, then, of the photochemical reaction initiated by the absorption of light by the chlorophyll molecule is the division of the water molecule into an oxidized part which ultimately leads to molecular oxygen and some reduced parts represented in the chart by  $[H]$ .

This reduced part  $[H]$  we have called "reducing power" because as yet it is not possible to state specifically what form or forms it may be in. This reducing power is capable of reducing carbon dioxide in the absence of light; that is to say, that the reduction of carbon dioxide itself is a dark reaction. This was indicated first in the earlier experiment of McAlister<sup>11</sup>

in which he was able to show that following a period of photosynthesis a number of plants continued to absorb carbon dioxide for a short period (seconds to minutes) after cessation of illumination. We were able to demonstrate this in an ever more direct and unequivocal fashion and generalize it for all plants so far tried when we were able to show that not only did all of these plants absorb quantities of carbon dioxide in the dark after illumination but that the products formed in the dark were qualitatively and under certain conditions quantitatively similar to those formed in a fairly comparable light period.<sup>12</sup> The method used for this demonstration was the same as those to be described later in the review. The lifetime in the dark of this reducing power which is generated by light is also of the order of seconds to minutes and almost certainly corresponds to a concentration of one or more definite chemical species. It is quite conceivable, as mentioned earlier, that some of it might be in the form of reduced coenzymes.

Very recently it has been reported<sup>13,14</sup> that both the higher plants and isolated chloroplasts emit a chemiluminescence following cessation of illumination. This chemiluminescence has a decay time which corresponds very closely to that which we have observed for the reducing power. In fact, it would seem almost surely to represent the reversal of the conversion of electromagnetic into chemical energy, namely, the transformation of at least some of the chemical energy stored in the reducing power into the electromagnetic energy of luminiscence. Furthermore, the luminiscence is reduced by the presence of carbon dioxide in those cases in which the carbon dioxide fixing system is still present. However, when the carbon dioxide system has been removed, as is true in the case of chloroplasts, the luminiscence becomes independent of carbon dioxide.

While it thus appears that the unique problem of photosynthesis lies in the right hand half of the chart of Figure 1, the present discussion will be

limited to the other side of the chart, that is, the path through which carbon passes on its way from carbon dioxide to all the raw materials of the plant. It is essentially a study of what we now believe to be entirely dark reactions and might best be characterized as photosynthesis. This area not only has a great interest for its own sake but would almost certainly cast some light upon the nature of the reducing agents which arrive from the photochemical part of the reaction and drive the carbon cycle toward reduction. The reason for this particular interest lies in the fact that we have, in recent years, come into possession of a tool which is especially suited for this study, namely, labeled carbon atoms in the form of a radioactive isotope of carbon,  $C^{14}$ . All of the results that will be described later were made possible through the use of this labeled carbon dioxide. With such a labeled molecule available, the design of an experiment for determining the sequence of compounds into which the carbon atoms of carbon dioxide may pass during the course of their incorporation in the plant is, in its first phase, a straightforward one.

We may visualize the problem in terms of the chart in Figure 2 in which the green leaf is represented schematically as a closed opaque container into which stream the raw materials of photosynthesis, namely, carbon dioxide, light and water containing the necessary mineral elements. From this container are evolved the products of photosynthesis - oxygen gas and the reduced carbon compounds constituting the plant and its stored reserves. Heretofore, it has been possible to study in a quantitative way the nature of the process going on inside the opaque container only by varying external conditions and noting variations in the final products. Although there has been no serious doubt that the formation of sugar did not take place by the aggregation of six molecules of carbon dioxide, six molecules of water and the requisite number of light quanta into a single unit followed by the rearrangement into hexose and

molecular oxygen, no specific information was available as to the compounds which might act as intermediates. Assuming that such a chain of intermediates exists, it is quite clear that by setting up some photosynthetic organism, leaf or other suitable material, in a steady state of photosynthesis in which the various ingredients are being absorbed and products formed in some uniform manner and injecting the labeled carbon dioxide into the entering carbon dioxide stream, we should find the label appearing successively in time in that chain of intermediates. This can be observed by stopping the entire process after a suitable lapse of time and examining the incorporated labeled carbon to determine the nature of the compounds into which it has been built. It is also clear that in addition to the identity and sequence of the compounds into which the carbon is incorporated, we may also determine the order in which the various carbon atoms within each compound acquire the label. With this type of information at hand it should be possible to reconstruct the sequence of events from the time of entry of the carbon atom into the plant as carbon dioxide until it appears in the various more or less finished products of the plant.

While photosynthetic experiments have been done with a wide variety of plant materials, the major kinetic work has been carried out with suspensions of unicellular green algae. The reason for this lies in the fact that these algae may be obtained in a reproducible biological form relatively easily and in any amount. They are grown in the laboratory in a continuous culture arrangement shown in Figure 3. The algae may be harvested from these flasks daily or every other day, depending upon the type of material desired. Such cultures have been maintained in a continuous fashion over periods extending beyond several months. Most of our experiments have been performed with the unicellular green algae Chlorella or Scenedesmus. After harvesting the algae are washed with distilled water and resuspended in the medium in which the

experiment is to be done. This suspension is placed in a flat vessel called a "lollipop", a photograph of which is shown in Figure 4. A stream of air containing carbon dioxide is passed through the algae while they are being illuminated so as to achieve a steady state of photosynthesis.

In order to begin the experiment the air stream is interrupted and the labeled bicarbonate is injected into the algal suspension. After the pre-selected period of time, the algae are killed by opening the large stopcock at the bottom of the flask, allowing the algal suspension to fall into alcohol in order to stop the reaction and extract the photosynthesized material. Although a variety of killing and extracting procedures have been tested, most of the experiments were performed by dropping the algae into alcohol so as to result in an 80% alcohol solution. The total amount of carbon fixed is then determined by taking an aliquot of this entire suspension, evaporating it to dryness on an aluminum disk and counting it on a Geiger counter.<sup>15</sup> The fraction soluble is determined by either filtering or centrifuging the suspension and then recounting the clear supernate or filtrate.

The distribution of the fixed radiocarbon among the various compounds must now be determined. Since in relatively short periods of time most of the fixed radioactivity is found in the soluble components, the problem is one of analyzing for the distribution in the soluble fraction. This has been done by application of the method of paper chromatography introduced and developed for amino acid analysis by Consden, Martin & Synge.<sup>16,17</sup> It has been applied to a wide variety of compounds and no detailed description of it will be given here. The unique extension to our work lies in the ability to locate particularly those compounds which contain the radioactive carbon atoms on the paper by means of a radioautograph of the resulting paper chromatogram obtained by allowing an X-ray film to remain in contact with the paper for a suitable period of time. These areas of the paper which are



occupied by radioactive compounds will, of course, expose the X-ray film. Such a map of the disposition of the radioactive compounds contained in an extract is shown in Figure 5. The chemical nature of the compounds defined by the exposed areas can be inferred from the position occupied by a compound with respects to the origin of the chromatogram. More precise determination of the chemical character is assisted by chemistry performed on the material eluted from the spot defined by the radiogram and rechromatography. Final identification, however, is usually dependent on the co-chromatography of the unknown, or questioned, radioactive material eluted from the paper with an authentic specimen of the suspected compound and the demonstration of the complete identity of the carrier material as determined by some visible test on the paper with the pattern of radioactivity in the co-chromatogram. The amount of radioactivity incorporated in these compounds can be determined quite accurately by using the X-ray film as a means of defining that area of the paper containing the compound, thus permitting the particular spot to be cut out from the larger piece and eluted from the paper and mounted on a plate to be counted.

A much simpler means would be to count the spot right on the paper with a Geiger counter. The fraction of the total amount of radioactivity in the spot which is thus registered by the Geiger counter is fairly constant for all compounds for any given chromatographic system. Thus, for most purposes it is sufficient simply to expose the paper to X-ray film in order to determine just where the radioactive spots are, and then having so defined them, to count them right on the paper for quantitative comparison, by the Geiger counter. It is clear from Figure 5 that the variety of products synthesized at room temperature by Scenedesmus (as well as by all other plants tried) is great, even in a very short time such as ten seconds. But even so, it is clear that the predominant compound as the time gets shorter is phosphoglyceric acid.

This is even more strongly demonstrated when the experiment is carried out at reduced temperature, for instance 2° C., so as to slow down all of the reactions and enable us to see more clearly the earliest products. Figure 6 shows a plot of the concentration of radioactivity per unit for algae for three of the major early compounds, formed at 2° C. On such a plot as this, it is clear that those substances which are formed directly from carbon dioxide with no appreciable intermediates lying between them and carbon dioxide will be the only ones that will show a finite slope; all others should start with a zero slope. A finite slope is certainly the case for phosphoglyceric acid and possible for malic acid, indicating at least two independent carbon dioxide fixing reactions, one leading to a three-carbon compound and the other producing a four-carbon compound.<sup>18,19</sup>

Since the hexose phosphates appear extremely early in all of these photosynthesis experiments and because of the known close relationship between the hexose phosphates and phosphoglyceric acids in the glycolytic sequence, it seemed most reasonable to suppose that these hexose phosphates were formed from the phosphoglyceric acid by a combination of the two three-carbon fragments derived from phosphoglyceric acid in an overall process very similar to, if not identical with, the reversal of glycolysis.

One means of testing this suggestion would be a comparison of the distribution of radioactivity in the three carbon atoms of glyceric acid with those in the hexose as shown in Table I. It thus appears that the hexose is indeed formed by the combination of two three-carbon molecules derived from the glyceric acid in such a manner that carbon atoms three and four of the hexose correspond to the carboxyl-carbon of the glyceric acid; carbon atoms two and five with the alpha-carbon; and carbon atoms one and six with the beta-carbon of the glyceric acid. This correspondence is maintained when the

TABLE I

 $C^{14}$  Distribution in Photosynthetic Products of Barley and Scenedesmus

Conditions <sup>a</sup>	Glyceric Acid			Glycolic Acid		Hexose		
	-COOH	-COH	-CH <sub>2</sub> OH	-COOH	-CH <sub>2</sub> OH	C3,4	C2,5	C1,6
<u>Barley</u>								
Preillum: 2 min. dark	96.	2.6	1.7					
4 sec. PS	87.	6.5	6.8	48.5	51.5			
15 sec. PS	56.	21.	23.	50 ± 5	50 ± 5			
15 sec. PS	49.	25.	26.			52.	25.	24.
30 sec. PS				48.	52.			
30 sec. PS <sup>b</sup>	75.	6.	9.					
40 sec. PS				47.	53.			
60 sec. PS	44. <sup>c</sup>	30.	25.					
<u>Scenedesmus</u>								
5 sec. PS	95. <sup>d</sup>	2.5	1.2					
30 sec. PS						87. <sup>e</sup>	7.	6.
30 sec. PS <sup>f</sup> MI <sup>g</sup>	73.	12.	15.					
60 sec. PS <sup>f</sup>	51.	24.	25.					
60 sec. PS <sup>f</sup> MI	48.	24.	28.					
60 sec. PS <sup>f</sup>								
60 sec. PS <sup>f</sup> MI	43.	27.	30.					

- a. Experiments are steady-state photosynthesis, 10,000 footcandles unless otherwise states.
- b. 1,000 footcandles.
- c. Alanine obtained from this extract was 40% carboxyl-labeled.
- d. Under the same conditions, Chlorella produced phosphoglycerate labeled 93%, 3% and 2%, respectively.
- e. In this extract, malic acid was labeled 6.5% and aspartic acid 4% in the non-carboxyl carbons.
- f. 3,000 footcandles.
- g. Malonate inhibited.

distribution in these two compounds (glyceric acid and hexose) is compared for a wide variety of different times.

With this clear out indication of the similarity between the path of hexose synthesis and the known path of its breakdown, another means of testing how closely this parallelism might be followed suggests itself. The hexose derivative which is last in the sequence of changes prior to the breakdown of the carbon skeleton during glycolysis is the fructose-1, 6-diphosphate. Correspondingly, then, it presumably would be the first hexose derivative to appear in the reverse direction. If this is the case and, furthermore, if the hexose derivative reservoirs involved in sucrose synthesis are more or less isolated from those involved in storage and glycolysis, the radioactivity should appear in the fructose half of the sucrose molecule prior to its appearance in the glucose half. This is indeed the case.<sup>19</sup> However, sucrose does not seem to be formed by the simple reversal of the sucrose phosphorylate system which was described for certain bacteria,<sup>20,21</sup> since for this to be the case, free fructose would have to be apparent in the photosynthesizing organism, whereas it is never so found, nor has the enzyme itself ever been isolated from any green plant.

The recent identification<sup>22,23</sup> as uridine diphosphoglucose (UDPG) of the spot which had been previously<sup>19</sup> called "the unknown glucose phosphate spot" has lead to another suggestion as to the mode of formation of sucrose. Glucose-labeled UDPG appears very early in the sequence of compounds formed. Furthermore, it has been possible to demonstrate the presence in the hexose monophosphate area of a sucrose phosphate by using a carefully selected phosphatase, containing no invertase, in the treatment of this entire phosphate area.<sup>23,24</sup> We have suggested, therefore, that UDPG may be involved in sucrose synthesis in a manner similar to that of glucose-1-phosphate in the numerous phosphorylase reactions, with the difference, however, that the

acceptor of the glucose moiety would be some phosphate of fructose, thus producing a sucrose phosphate. Recent work by Putnam & Hassid<sup>25</sup> gives further support to the idea that only phosphorylated derivatives of glucose and fructose are involved in sucrose synthesis in higher plants. They found that in sucrose synthesis, from labeled glucose in leaf punches, no free fructose was formed, although the sucrose becomes equally labeled in both the glucose and fructose portions. Conversely, when labeled fructose is used, no free labeled glucose appears, while the sucrose is uniformly labeled in both moieties.

It is possible that compounds of the UDPG type could be concerned in the transformation of sugars and the subsequent incorporation into polysaccharides. Uridine diphosphate would thus serve as a carbon carrier in the same way that pyridine nucleotides and flavonucleotides are involved in hydrogen transfer; the adenylic acid system in phosphate transfer; and coenzyme A in the transfer of acetyl groups. There is already some evidence for the existence of other members of the uridine diphosphate group from our own work, as well as that of other.<sup>26,27,28</sup>

We may now turn our attention from the fate of the glyceric acid to the problem of its origin. An examination of Table I indicates quite clearly that the first position in the glyceric acid to become labeled is the carboxyl group. As time proceeds, the other two carbon atoms in the glyceric acid acquire radioactivity and it thus appears that they acquire it at equal rates, at least within the present accuracy of the experiments.

It thus appears that the most rapid reaction which carbon dioxide can undergo at least at high light intensities, is a condensation with a C<sub>2</sub> fragment leading directly to phosphoglyceric acid. An examination of the chromatograms of a very short photosynthetic period shows glycine and glycolic acid only as the two-carbon compounds present. The distribution of

radioactivity among the carbon atoms of these two compounds is always equal and the same and corresponds very well with that in the alpha- and beta-carbon atoms of the glyceric acid, as may be seen from Table I. This suggests that either glycolic acid is in the direct line for the formation of the  $C_2$  carbon dioxide acceptor, or is very closely related thereto.

The question now arises as to the source of this  $C_2$  carbon dioxide acceptor. There are, of course, only two possibilities for its origin. Either it results from a one-plus-one combination or it must result from the splitting of a four-carbon compound or a larger one. In order for it to result from the combination of two one-carbon fragments there must exist as an intermediate some one-carbon compound more reduced than carbon dioxide which, in turn, may combine either with itself or with carbon dioxide. Furthermore, the reservoir of this one-carbon intermediate would have to be vanishingly small since all attempts to find labeled, reduced, one-carbon compounds, such as formic acid or formaldehyde, in the early stages of photosynthesis have failed and, in addition, the resulting two-carbon fragment is very nearly equally labeled in both carbon atoms.

One would also expect that these one-carbon compounds would tend to disappear under conditions of low carbon dioxide concentrations leading to the disappearance of the two-carbon condensation product resulting from them. This leads us to the supposition that the formation of glycolic acid would be expected to drop off under conditions of low carbon dioxide concentration which is the reverse of what is observed.

We are thus left with the following possibility for the origin of the  $C_2$  compound - the cleavage of some  $C_4$  or larger structure. The fact of the early appearance of label in malic acid, taken together with the lack of any appreciable amounts of label in the compounds of the tricarboxylic acid cycle, led us to the supposition that malic acid was either a precursor to, or very

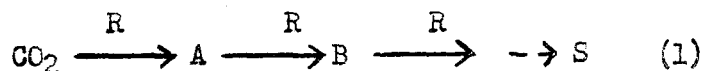
closely related to, a four-carbon compound which could be split to produce the required two-carbon fragment.

In the course of the search for the two-carbon acceptor, and its immediate precursors, two new compounds were identified as early products of carbon dioxide incorporation which seem to have little to do with the direct synthesis of hexoses and, therefore, had a very likely function in the regeneration of the two-carbon acceptor. These were the phosphates of the seven carbon sugar sedoheptulose and of the five carbon sugars ribulose, ribose and arabinose.<sup>30</sup>

The question immediately presents itself as to the relation between these two compounds along the path of carbon assimilation, not only with each other but with the precursors which are already known and the possible products that might be formed from them. The attempt to answer this question focusses our attention once again upon some of the shortcomings and limitations of the method of observation that we are using and the nature of the experiment which we are performing. Our initial hope of determining the sequence of intermediates by a simple observation of a sequence of compounds into which radioactivity has been incorporated in steady state experiments is now complicated by the uncertainty as to the amount of compound present during the steady state. It is easy to visualize a situation in which the actual amount of intermediate present during the steady state is so small as to escape observation by our methods, or perhaps even to be so unstable as to be lost by our methods of observation. This complete failure of a compound to appear on a chromatogram, although it might conceivably be an intermediate, is, of course, an extreme case. The more usual situation is one in which most of the intermediates are present but in varying concentrations in the steady state. Under such conditions a single or even several observations of the relative amount of radioactivity incorporated into a variety of compounds

would not necessarily be any real criterion of the relative order of these compounds in the sequence of events.

In order to achieve the full value of the method of observation then, it becomes necessary to perform rather extended kinetic experiments in which the appearance of radioactivity in all compounds is plotted as a function of time at sufficiently short intervals to enable a rather accurate and detailed curve to be obtained. Furthermore, the distribution of radioactivity among the atoms within each compound should also be determined as a function of time. The validity of any proposed sequence of events could then be determined by a comparison of the calculated appearance and distribution curves with those actually observed. In order to calculate such appearance curves, as well as the distribution curves amongst the atoms in each compound, one can set up a system of linear differential equations based upon the following model:



where  $CO_2$  represents the entering carbon dioxide

A, B, etc. represent intermediates involved in carbon dioxide assimilation

S represents more or less final storage product

R is a measure of the total rate of carbon dioxide assimilation in the steady state expressed in moles of carbon per minute

The rate of change of the specific activity of a single carbon atom in A, given by  $X_A$ , is then expressed by Equation (2). (The specific activity of the entering carbon dioxide is here taken as unity.  $[A]$ , the concentration of the compound A, is independent of time.)



$$\frac{dX_A}{dt} = \frac{R}{[A]} (1 - X_A) \quad (2)$$

The specific activity of the corresponding atom in compound B is given by an exactly similar Equation (3).

$$\frac{dX_B}{dt} = \frac{R}{[B]} (X_A - X_B) \quad (3)$$

Equations of identical form may be written for every atom of every compound that might be considered an intermediate. These equations may be solved explicitly by means of a differential analyzer provided two parameters are known. These are the total rate of entry of carbon into the system during the steady state, R, and the steady state concentration of each atom which might be considered as lying along the path of carbon assimilation, [A], [B], etc.

It is clear that if such compounds (whose prime function it is to serve as carbon carriers between the entering carbon dioxide and the final storage products in the plant) do indeed exist in biological systems they would very soon become saturated with radioactivity. By this is meant that the amount of radioactivity observed in that particular compound would very soon reach a maximum value and remain that way. The reason for this is that by definition the amount of these intermediate compounds is not changing, and also is small compared to the total amount of carbon the plant assimilates during the experiment. Since all of the carbon, or at least most of it, must pass through these reservoirs of intermediates they will very soon acquire the same specific activity as the entering carbon dioxide. In contrast to this, those materials which are not functioning as simple intermediates but rather are functioning as storage reservoirs, or are very distant from the immediate photosynthetic intermediates, will not acquire radioactivity as rapidly, or if they do they will not become saturated as rapidly as those which are directly involved in

the path of carbon assimilation. The amount of radioactivity found in those compounds which saturate in a relatively short time now provides a relatively easy method of determining the size of the actively functioning reservoirs of these compounds which are directly engaged in the path of carbon assimilation. A simple measurement of this amount compared to the specific activity of the entering carbon dioxide will provide a measure, in moles per unit volume of the biological material, of the compound in question. Furthermore, having once achieved a relatively uniform label in these photosynthetic intermediates, it becomes possible to follow the behavior of the reservoir size as a function of change in external variables, for example, light intensity. We have chosen to include in this review a more or less detailed description of just this determination of the effect of light intensity upon reservoir sizes as a means of describing the general experimental technique which is involved.

#### STEADY STATE & RESERVOIR SIZES - METHODS & RESULTS

The apparatus used for these experiments was constructed to permit the algal suspension to be left under controlled external conditions (illumination intensity, temperature, carbon dioxide and oxygen concentration) while photosynthesizing for at least one hour. Furthermore, it was required that the change from natural to radioactive carbon dioxide, which was to be circulated in a closed system, and the withdrawal of several samples at given time intervals be accomplished with a minimum of change in these conditions.

The apparatus consisted of:

(a) A square illumination vessel A (Figure 7) made out of Lucite (polyacrylic plastic), 49 cm. high, 11 cm. wide and 0.7 cm. thick (inside dimensions). The bottom was provided with a gas inlet tube with five small holes to allow good contact between gas and liquid and a drain tube closed

with a screw clamp. The top of the vessel was provided with a gas outlet tube. A water-alcohol mixture from a constant temperature bath was allowed to flow over the outer surfaces of the vessel in order to control the temperature of the suspension.

(b) Two illumination banks (represented by B), each with four fluorescent tubes (General Electric, quality white, 20 watts each), providing an almost uniform illumination over the whole surface of the vessel, of  $7 \times 10^4$  ergs/cm.<sup>2</sup>-sec. (roughly 700 footcandles).

(c) An ionization chamber C, connected to a recording vibrating reed electrometer, to record the activity of the gas leaving the vessel continually during the run.

(d) Three gas traps D, to permit the addition of a known amount of radioactive carbon dioxide to the system, and trap the remaining radioactivity after the run.

(e) A flask E, of 5 liter volume, containing a mixture of 1% radioactive carbon dioxide in air. The reservoir contained so much carbon dioxide that the algae assimilated no more than 20% of it during a run.

(f) A gas circulating pump F of the rubber tubing type, and a flow meter G.

(g) A system of four-way stopcocks H, which permitted the vessel to be flushed with a mixture of 1% ordinary carbon dioxide in air, from the cylinder I. The assembly is shown in Figure 8.

In a typical experiment, 2 cc. (wet packed) of one-day old Scenedesmus, washed and resuspended in 200 cc. of de-ionized water, were placed in the vessel and aerated with the ordinary gas mixture for at least one-half hour, while the mixture of radioactive carbon dioxide circulated in the gas system for thorough mixing, without passing through the vessel. The suspension was kept at 24° C. After this time, during which a steady state of photosynthesis

had been reached, the radioactive mixture was passed through the vessel in place of the ordinary gas mixture, by a manipulation of the pair of stop-cocks at H, and samples of 20 cc. of the suspension withdrawn at intervals of five or ten minutes. These samples were dumped into 80 cc. of alcohol of room temperature, to make an extraction in 80% alcohol. After thirty minutes of photosynthesis, the lights were turned off and the suspension allowed to remain in the dark for a period of five minutes, during which time again several samples were withdrawn, and treated in the same manner. In one experiment another light period followed the dark period.

The samples were shaken for one hour and centrifuged. The residue was re-extracted in 50 cc. of 20% alcohol at room temperature, centrifuged, and re-extracted again with 20cc. of water. The extracts were concentrated together to 0.5 cc.

An aliquot of the concentrate equivalent to 30  $\mu$ l. of packed cells was evaporated on a corner of a filter paper (Whatman #1), and the chromatogram run with water-saturated phenol in one direction and n-butanol-propionic acid-water in the other. The chromatograms were exposed to X-ray film for about two weeks.<sup>12</sup> The labeled compounds appeared on it as black spots. Figure 9 shows the radiogram for ten minute photosynthesis of Scenedesmus. The amount of radioactivity contained in the different compounds was determined by counting the corresponding spots on the paper directly with a large-area Geiger-Mueller tube with thin mica window. The compounds were identified by a combination of the following criteria: (a) Their position on the paper; (b) the spot was cut out, eluted from the paper with water and run again in suitable solvents, together with such an amount of the suspected compound that it could be detected by a specific spraying reagent. The black spot on the film had to coincide accurately with the color reaction; (c) the eluted spot was chemically transformed (e.g. treating the sugar phosphates with phosphatase)

and the resulting compound co-chromatographed with carrier detectable by spray.

Figure 10 shows the total and the extracted amounts of radiocarbon fixed by 1 cc. cells during thirty minutes of photosynthesis followed by five minutes of darkness. The slope in the total fixation curve in the light corresponds to an assimilation of 13 cc. CO<sub>2</sub> (NTP) per hour.

Figure 11 shows the amount of radioactivity incorporated into sucrose and three phosphorus compounds for the experiment of Figure 10.

Figure 12 gives the number of counts in sucrose, glutamic, malic and citric acid, for a different experiment of fifteen minutes photosynthesis, followed by ten minutes dark, and again five minutes of photosynthesis.

Although the variation between experiments is quite high, there are some striking features which are common to all:

(1) The curves of some of the compounds show a marked decrease in slope after five minutes of photosynthesis. This quite clearly indicates the presence of rapidly turning-over reservoirs in the photosynthesis cycle which are then thoroughly labeled and reach the specific activity of the fed carbon dioxide: Diphosphate area (mainly ribulose diphosphate); hexose-monophosphate area (50% glucose-, 26% sedoheptulose-, some fructose- and mannose-monophosphate); phosphoglyceric acid. The leveling off of these curves permits the calculation of the concentration of the reservoirs of those compounds in the photosynthesis cycle, by dividing the measured amount of radioactivity per carbon atom by the specific activity of the fed carbon dioxide.\*

---

\* The efficiency factor of the counting of spots on papers has been determined by converting three cut out spots to barium carbonate and measuring their activity in an ionization chamber as carbon dioxide. It is 19 disintegrations per count.

---

Table II gives the steady state concentrations during photosynthesis for some compounds determined by this method.

TABLE II

Steady State Concentrations of Some Compounds Involved in  
the Photosynthesis Cycle

Scenedesmus, experimental conditions as in Figure 10

Substance	$\mu\text{moles/cc. cells}^*$
Phosphoglyceric acid	1.4
Dihydroxyacetone phosphate	0.17
Fructose phosphate	0.12
Glucose phosphate	0.4
Mannose phosphate	0.05
Sedoheptulose phosphate	0.18
Ribulose diphosphate	0.5
Alanine	0.2
* Volume measured as wet packed cells.	

(2) The fact that the activity vs. time curves show a definite yet low slope for as long as thirty minutes can be taken to indicate that the breakdown of carbohydrates continues throughout the illumination, i.e., their formation from photosynthetic intermediates is reversible. Thus, there are two sources of the intermediates: (a) the carbon dioxide fed; the amount of compound formed from this source reaches the maximum specific activity in five to ten minutes; (b) the carbohydrate pool of the cells; the amount formed

from this source is labeled only slowly since the specific activity of the carbohydrate pool rises slowly due to the large size of the pool.

(3) Other compounds show almost constant rate of labeling during the whole period of photosynthesis; sucrose, malic and glutamic acid. For this and other reasons it is clear that these compounds are not in the photosynthesis cycle, but are formed during the photosynthesis at a constant rate. Their large reservoirs in the cells are labeled only slowly.

(4) When illumination is interrupted there appears a sudden great increase in the concentration of phosphoglyceric acid (followed by a slow decrease after two minutes), and an almost complete depletion of the diphosphate area. Analysis of the monophosphate area showed that the amount of sedcheptulose phosphate decreased also (cf. Table III). The concentration of malic acid decreases as well. The rate of labeling of glutamic acid is increased greatly after a short induction period; citric acid, which contains little activity during the whole light period, shows a sudden increase in the dark, followed by a slow decrease. The labeling of sucrose continues at the same rate as in light for about two minutes, after which it is stopped almost completely.

Both experiments gave the same picture for most of the compounds, with the two exceptions: In the second experiment the diphosphate area, which in the first contained almost the same number of counts as phosphoglyceric acid during the light, had only about 15% of it. This value dropped to 5% in the dark. The phosphoglyceric acid showed a hardly significant rise in the dark during the first two minutes, but again a slow decrease after five minutes. Although we do not know why in this experiment the concentration of ribulose diphosphate was so low in the light, the coincidence with the lack of increase of phosphoglyceric acid points to a connection between both effects.

TABLE III

Phosphatase Treatment of HMP Area after 30 Minutes  
 Photosynthesis and 30 Minutes Photosynthesis Followed  
 by 5 Minutes Dark

Substance	Number of counts/min. on Paper	
	30 min. PS	30 min. PS 5 min. D
Glucose	3140	4280
Fructose	910	1040
Sedoheptulose	1600	1210*
Mannose	460	

\* An appreciable fraction of this count is certainly hexose so that one may estimate a maximum value of the heptose at around 800 counts/min.

(5) In the light following the dark, the diphosphates, phosphoglyceric and malic acid increase again.

The effect of dark on the labeling of glutamic and citric acid was already reported in an earlier paper<sup>12</sup> and studied more closely in the following experiment: 0.2cc. wet packed algae (*Chlorella pyrenoidosa*) were suspended in 200 cc. distilled water, illuminated in a flat circular vessel of 1 cm. thickness by incandescent lights through an infra red filter (intensity  $1.6 \times 10^5$  ergs/cm.<sup>2</sup>-sec.) and aerated with 0.08% carbon dioxide in air. The low concentration of cells was chosen to avoid shading of cells in the suspension, so that during the light period all the cells were illuminated continually.

After one-half hour, the aeration bubbler was taken out and a suitable amount of radioactive bicarbonate (sodium) solution added. (The algae which

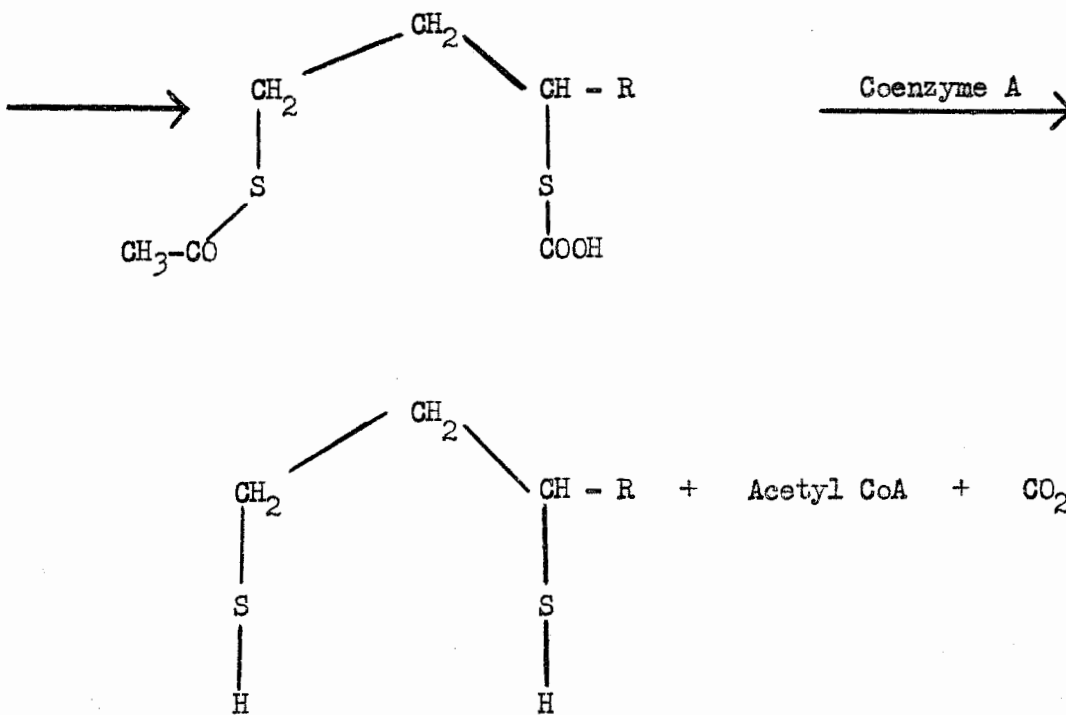
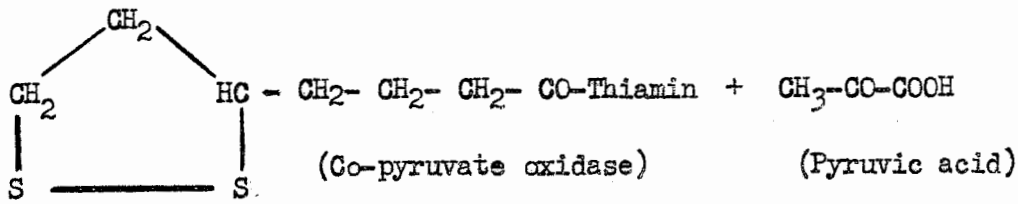


were grown in slightly acid medium, had enough buffering capacity to convert the bicarbonate to carbon dioxide.) The vessel was immediately stoppered and shaken in the light. After one minute, the suspension was drained into a darkened flask, and after another minute poured into four times its volume of boiling alcohol. Control samples were treated in the same way, but kept in the light, in contact with radioactive carbon dioxide for one and two minutes, respectively. The analysis of the fixed radioactivity was performed by paper chromatography and radioautography with the technique already described. The results are shown in Figure 13.

#### DISCUSSION

It has already been pointed out that photosynthesis is not a mere reversal of respiration; this was supported by the observation that the carbon of newly formed photosynthetic intermediates is not available for respiration while the light is on.<sup>12,31</sup> We may thus represent the relationship between photosynthesis and respiration by the following scheme: (See Figure 14) The labeling of the Krebs cycle intermediates through the storage products (carbohydrates, fats, proteins) of the cells is a slow process, due to the relatively large size of the storage pools. The fact that the photosynthesis intermediates find their way into the tricarboxylic acid cycle very rapidly after the light is switched off means that there is another connection between the two cycles which is blocked as long as the light is on but becomes accessible in the dark. This was interpreted in earlier work<sup>12</sup> in terms of the action of the light in maintaining at low concentration the intermediate required for entry into the tricarboxylic acid cycle. A closer specification of how this is accomplished is now possible since the discovery that alpha-lipoic acid is a cofactor for the oxidative decarboxylation of pyruvic acid to an active acetyl group<sup>32-36</sup> which is the one reaction known to feed the Krebs

cycle.<sup>37,38</sup> The mechanism of the reaction may be written this way:



The reduced lipoic acid complex would then be reoxidized to the disulfide form by a suitable oxidant (e.g., pyridine or flavin nucleotides). In order that the oxidation of pyruvic acid can proceed, the enzyme has to be present in its oxidized form. If it is kept in its reduced form under the influence of the light-produced reducing power, the reaction cannot proceed and the pyruvic acid formed during photosynthesis will not find its way into the respiratory cycle. The reaction is inhibited because only a small amount of the enzyme catalyzing it exists in the required form, most of it being kept in the other form under the "pressure" of the reducing power generated by the

light energy. This recalls a similar phenomenon which has been known for a long time, i.e., the suppression of the fermentation of carbohydrates in favor of their oxidation under aerobic conditions (Pasteur effect). This effect has been explained in a manner similar to the one used here to account for the inhibition of the respiration of photosynthetic intermediates.<sup>39</sup> The reduction of acetaldehyde to alcohol requires a dehydrogenase in its reduced form; under aerobic conditions the dehydrogenase exists primarily in its oxidized form, and the acetaldehyde instead of being reduced is oxidized to acetic acid.

The sudden rise in phosphoglyceric acid and the decrease in ribulose diphosphate and sedoheptulose phosphate in the dark period, together with the observation that the dark rise in phosphoglyceric acid is absent when the ribulose diphosphate concentration was low during the light, confirms the earlier suggestion that the phosphates of the C<sub>7</sub> and C<sub>5</sub> sugars are precursors of the C<sub>2</sub> carbon dioxide acceptor.<sup>30</sup> This, together with evidence gathered in previous work<sup>19,40</sup> leads to the following scheme for the photosynthetic cycle: \* (Figure 15)

---

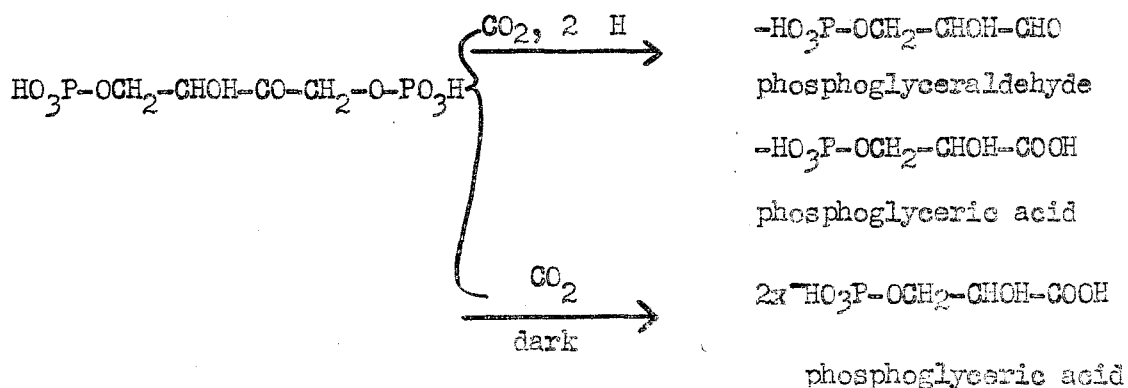
\* This scheme is intended to represent only changes in the carbon skeletons. The reducing equivalents are indicated only to show redox relationships, between the known compounds. A number of the isolated compounds are isoximers and have not been included.

---

Upon this basis an attempt might be made to relate the two effects as follows; when the light is turned off, the reduction reactions requiring light are stopped, whereas cleavage and carboxylation reactions continue until their substrates are exhausted. Presumably, this would lead to a depletion of the C<sub>5</sub> and C<sub>7</sub> sugars, the synthesis of which requires reduction steps (particularly the six-equivalents leading to the tetrose which itself is a very small reservoir), and a rise of phosphoglyceric acid, the further fate of which is

also dependent upon reduction. However, a number of arguments seem to contradict this view: (1) The observation that plants fix radiocarbon in the dark immediately following a light period at low carbon dioxide concentration to form a similar pattern of compounds as the one found in photosynthesis shows that the sequence following phosphoglyceric acid is not blocked at once upon cessation of illumination, but that the cells contain sufficient reducing power to transform some phosphoglyceric acid into carbohydrates; (2) the cleavage of the pentoses and heptose into the C<sub>2</sub> carbon dioxide acceptor and a triose and pentose respectively is dependent on a reduction step as well.

We are thus led to the suggestion that the rise in phosphoglyceric acid is not to be explained by a mere interruption of the sequence, but that the rate of production of phosphoglyceric acid at some time in the first minute of darkness is actually higher than it is in the steady state photosynthesis. This would be the case if the C<sub>3</sub>-C<sub>2</sub> cleavage of ribulose diphosphate, which in photosynthesis presumably yields a triose phosphate molecule beside the C<sub>2</sub> carbon dioxide acceptor, in the dark yields a molecule of phosphoglyceric acid instead of the triose molecule. The overall reactions may be represented as follows (not a mechanism):



This hypothesis is supported by the fact that the triose phosphate is decreased too in the dark.

The fact that the net result of the reaction sequence in the light from ribulose diphosphate to phosphoglyceric acid and triose phosphate is a reductive carboxylation and thus the reversal of the oxidative decarboxylation which, in the case of pyruvic acid, requires the presence of a cyclic disulfide compound leads to the idea that the former sequence might be catalyzed by a similar enzyme. This idea seems to be supported by the results of an experiment performed in this laboratory some time ago, which were difficult to explain.<sup>41</sup>

In order to examine the relation between photosynthesis and the glycolytic cycle, a series of experiments similar to those described previously were performed with added iodoacetamide which is known to inhibit the action of triose phosphate dehydrogenase<sup>42</sup>, presumably through a reaction with its sulfhydryl group.<sup>43</sup> A 1% suspension of Chlorella in phosphate buffer was allowed to photosynthesize in light of 2500 footcandles and an atmosphere of 1% carbon dioxide, 5% oxygen and 94% nitrogen. At various times before adding the radioactive bicarbonate solution, iodoacetamide was added to give a  $1.5 \times 10^{-4}$  M solution. One minute after adding the radiocarbon, the cells were killed and extracted.

After eight minutes contact with iodoacetamide, the cells were still able to fix 75% as much carbon dioxide as non-poisoned cells otherwise treated the same way (control). The amount of radioactivity in phosphoglyceric acid was 50% of the control, and the amount in sucrose had reached a sharp maximum of 3.5 times that in the control. There was practically no radioactivity in the ribulose diphosphate. After 90 minutes of exposure to the poison the cells had practically lost their ability of photosynthesis.

If, in the proposed photosynthetic cycle, the cleavage of the heptose and pentose phosphates is dependent on an enzyme containing sulfhydryl groups, which were more sensitive to iodoacetamide than the triose phosphate dehydrogenase, a picture similar to the one described would be expected: After short

exposure to the poison, in relatively low concentration, the lack of  $C_2$  carbon dioxide acceptor would slow down the photosynthetic cycle. The synthesis of carbohydrates, however, would proceed almost without inhibition, thus decreasing the concentrations of the intermediates in the cycle. This would allow the compounds to reach a higher specific activity during the period of exposure to radiocarbon (cf. equation(2)), change of specific activity inversely proportional to concentration). At some time after administration of the poison, the sucrose would be labeled faster than in the control due to the higher specific activity of its precursors. After a longer period, however, the rate of synthesis of sucrose would decrease because the pool of its precursors would be exhausted.

References

- (1) M. Calvin & A. A. Benson, *Science*, 107, 476 (1948).
- (2) A. A. Benson & M. Calvin, *Cold Spring Harbor Symposia on Quantitative Biology*, 13, 6 (1948).
- (3) M. Calvin & A. A. Benson, *Science*, 109, 140 (1949).
- (4) C. B. Van Niel, Chapter 22, "Photosynthesis in Plants", Iowa State College Press, Ames, Iowa (1949), pp. 437-495.
- (5) R. Hill, *Nature*, 139, 881 (1947); *Proc. Roy. Soc. (Lond.)*, 127B, 192 (1939).
- (6) R. Hill & R. Scarisbrick, *Nature*, 146, 61 (1940).
- (7) W. Vishniac & S. Ochoa, *J. Biol. Chem.*, 195, 75 (1952).
- (8) D. I. Arnon, *Nature*, 167, 1008 (1951).
- (9) L. J. Tolmach, *Arch. Biochem. Biophys.*, 33, 120 (1951).
- (10) S. Ruben, M. Randall, M. D. Kamen & J. Hyde, *J. Am. Chem. Soc.*, 63, 877 (1941).
- (11) E. D. McAlister & J. Myers, *J. Smithsonian Inst. Publ. (Misc. Coll.)*, No. 6, 99 (1940).
- (12) M. Calvin, *J. Chem. Education*, 26, 639 (1949).
- (13) B. L. Strehler & W. Arnold, *J. Gen. Physiol.*, 34, 809 (1951).
- (14) B. L. Strehler, *Arch. Biochem. Biophys.*, 34, 239 (1951).
- (15) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert & P. E. Yankwich, "Iostopic Carbon," John Wiley & Sons, Inc., New York, New York (1949).
- (16) R. Consden, A. H. Gordon & A. J. P. Martin, *Biochem. J.*, 38, 224 (1944).
- (17) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas & W. Stepka, *J. Am. Chem. Soc.*, 72, 1710 (1950).
- (18) E. J. Badin & M. Calvin, *J. Am. Chem. Soc.*, 72, 5266 (1950).
- (19) S. Kawaguchi, A. A. Benson, M. Calvin & P. M. Hayes, *J. Am. Chem. Soc.*, 74, (1952), in press.
- (20) W. Z. Hassid, M. Doudoroff & H. A. Barker, *J. Am. Chem. Soc.*, 66, 1416 (1944).
- (21) M. Doudoroff, H. A. Barker & W. Z. Hassid, *J. Biol. Chem.*, 168, 725 (1947).
- (22) J. G. Buchanan, et al., in press.

- (23) J. G. Buchanan, J. A. Bassham, A. A. Benson, D. F. Bradley, M. Calvin, L. L. Daus, M. Goodman, P. M. Hayes, V. H. Lynch, L. T. Norris and A. T. Wilson, "Phosphorus Metabolism," Volume II, Johns Hopkins Press, Baltimore, Maryland (1952), in press.
- (24) J. G. Buchanan, in press.
- (25) E. W. Putnam, Thesis, 1952, University of California, Berkeley.
- (26) R. Caputto, L. F. Leloir, C. E. Cardini & A. C. Paladini, J. Biol. Chem., 184, 333 (1950).
- (27) A. C. Paladini & L. F. Leloir, Biochem. J., 51, 426 (1951).
- (28) J. T. Park, J. Biol. Chem., 194, 885 (1952).
- (29) A. A. Benson & M. Calvin, J. Exptl. Botany, 1, 63 (1950).
- (30) A. A. Benson, J. A. Bassham, M. Calvin, A. G. Hall, H. E. Hirsch, S. Kawaguchi, V. H. Lynch & N. E. Tolbert, J. Biol. Chem., 196, 703 (1952).
- (31) J. W. Weigl, P. M. Warrington & M. Calvin, J. Am. Chem. Soc., 73, 5058 (1951).
- (32) L. J. Reed, I. C. Gunsalus, et al., J. Am. Chem. Soc., 73, 5920 (1951).
- (33) E. L. Patterson, et al., J. Am. Chem. Soc., 73, 5919 (1951).
- (34) I. C. Gunsalus, L. Struglia & D. J. O'Kane, J. Biol. Chem., 194, 859 (1952).
- (35) L. J. Reed & B. G. DeBusk, J. Am. Chem. Soc., 74, 3457 (1952).
- (36) M. W. Bullock, et al., J. Am. Chem. Soc., 74, 3455 (1952).
- (37) S. Ochoa, J. R. Stern & M. C. Schneider, J. Biol. Chem., 193, 691 (1951).
- (38) S. Korkes, A. Del Campillo, I. C. Gunsalus & S. Ochoa, J. Biol. Chem., 193, 721 (1951).
- (39) C. Meyerhof, Amer. Scientist, 40, 483 (1952).
- (40) M. Calvin, The Harvey Lectures, Vol. 45 (1951), in press.
- (41) W. Stepka, Thesis, University of California, June, 1951.
- (42) C. Meyerhof & W. Kiessling, Biochem. Z., 281, 249 (1935).
- (43) L. Rapkins, Compt. rend. soc. biol. (Paris), 112, 1294 (1933).



Captions to Figures

Figure 1 -

Figure 2 -

Figure 3 - Algae Plant

Figure 4 - "Lollipop"

Figure 5 - Radiogram of a paper chromatogram from 10 sec.  $C^{14}O_2$  fixation in the light by Scenedesmus.

Figure 6 - Behavior of radioactivity in specific compounds in extracts of Scenedesmus, exposed to radioactive carbon dioxide at 2°C.

Figure 7 - Diagram of the assembly for steady state photosynthesis.  
(For explanation of the letters, see text.)

Figure 8 - Assembly for steady state photosynthesis.  
(For explanation of letters, see text.)

Figure 9 - Radiogram of a paper chromatogram from 10 minute  $C^{14}O_2$  fixation in light by Scenedesmus. 1% suspension, 1%  $CO_2$  in air; light intensity  $7 \times 10^4$  ergs/cm.<sup>2</sup>-sec.

DHAP - dihydroxyacetone phosphate; PEB - phosphoenolpyruvic acid;

PMP - pentose monophosphates; PGoA - phosphoglycolic acid;

PGA - phosphoglyceric acid; HMP - hexose monophosphates;

DP - pentose & hexose diphosphates

Figure 10 -  $C^{14}O_2$  fixation by Scenedesmus. 1% suspension, 1%  $CO_2$  in air, light intensity  $7 \times 10^4$  ergs/cm.<sup>2</sup>-sec.

Figure 11 - Behavior of radioactivity in specific compounds in the extract from the experiment of Figure 10.

Figure 12 - Behavior of radioactivity in specific compounds in the extract from an experiment done under conditions corresponding to those of Figure 10.

Figure 13 - Effect of light and dark on the labeling of glutamic and citric acid. 0.1% suspension, light intensity  $1.6 \times 10^5$  ergs/cm.<sup>2</sup>-sec. (Numbers: counts/min.  $\times 10^{-3}$  on paper per cc. cells).

Figure 14 -

Figure 15 -

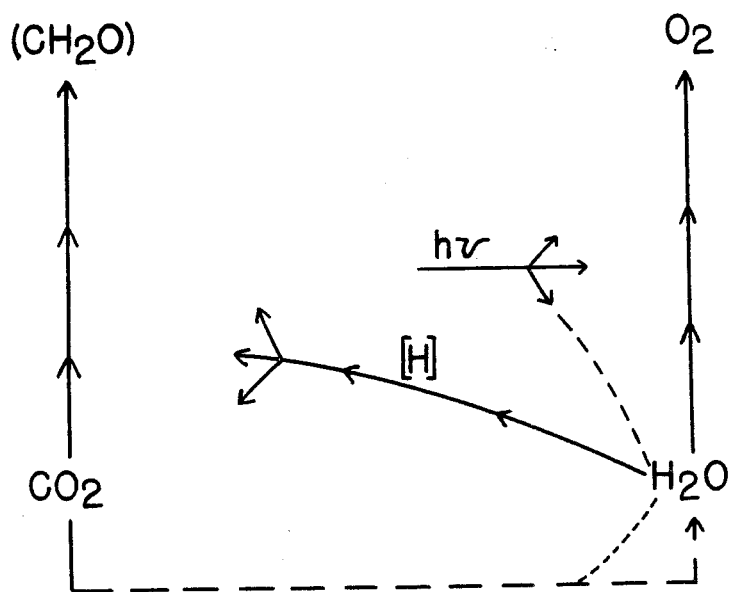


Fig. 1

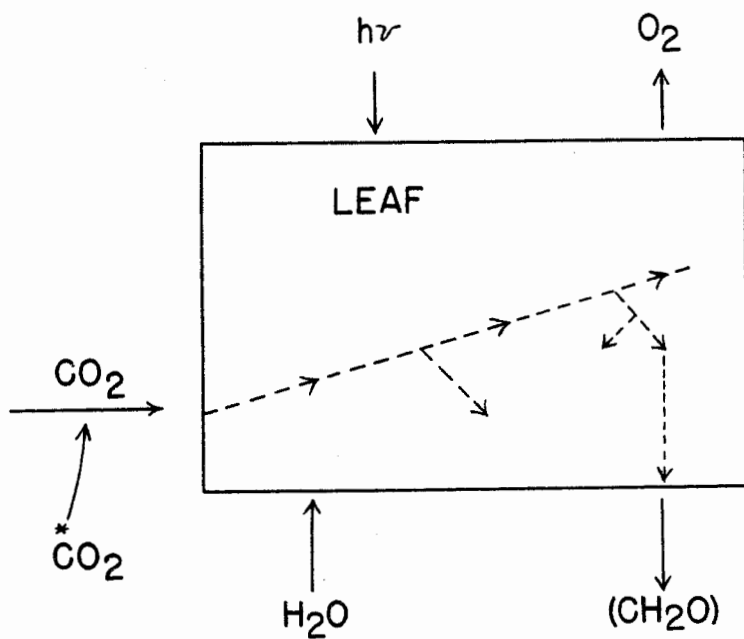


Fig. 2

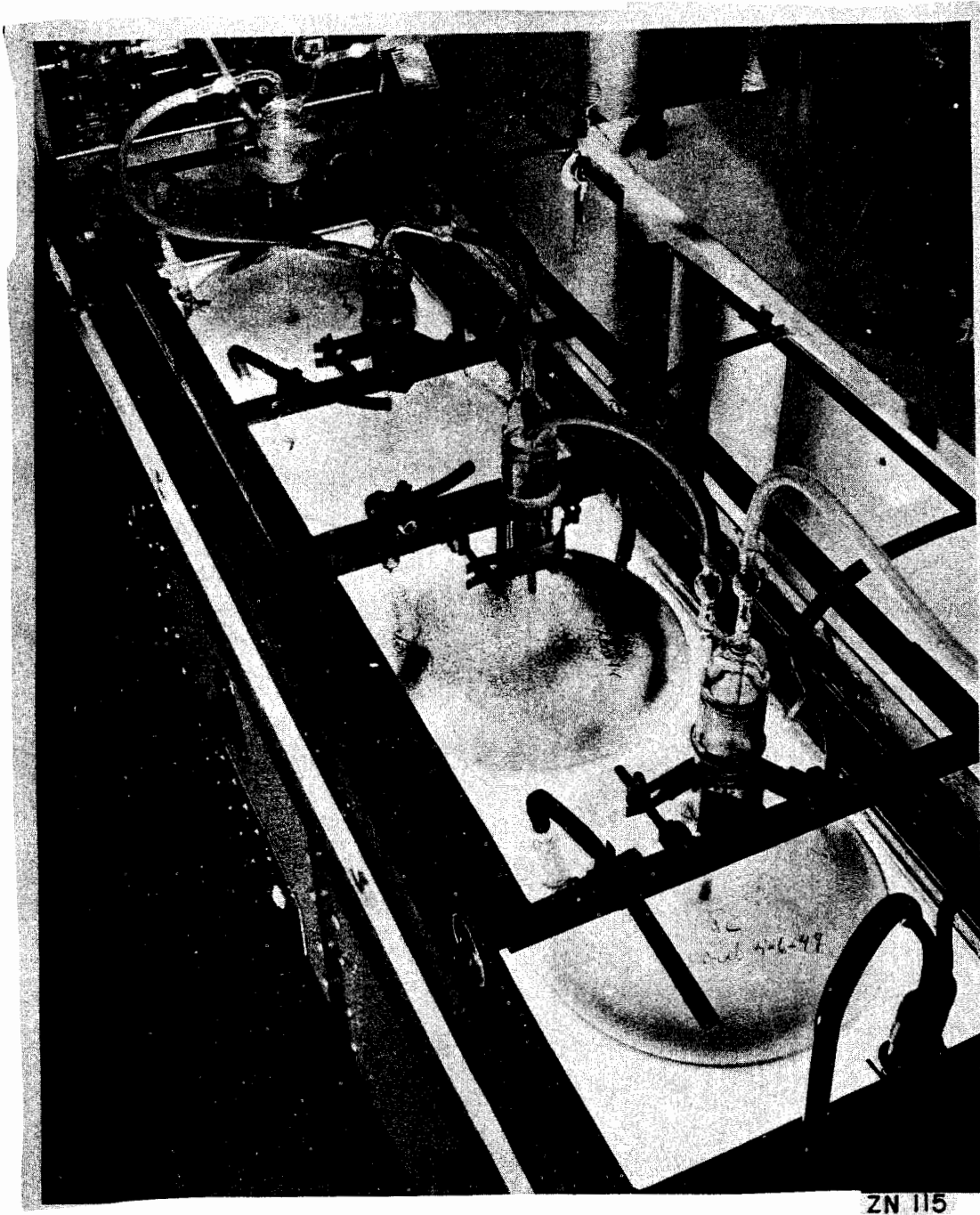
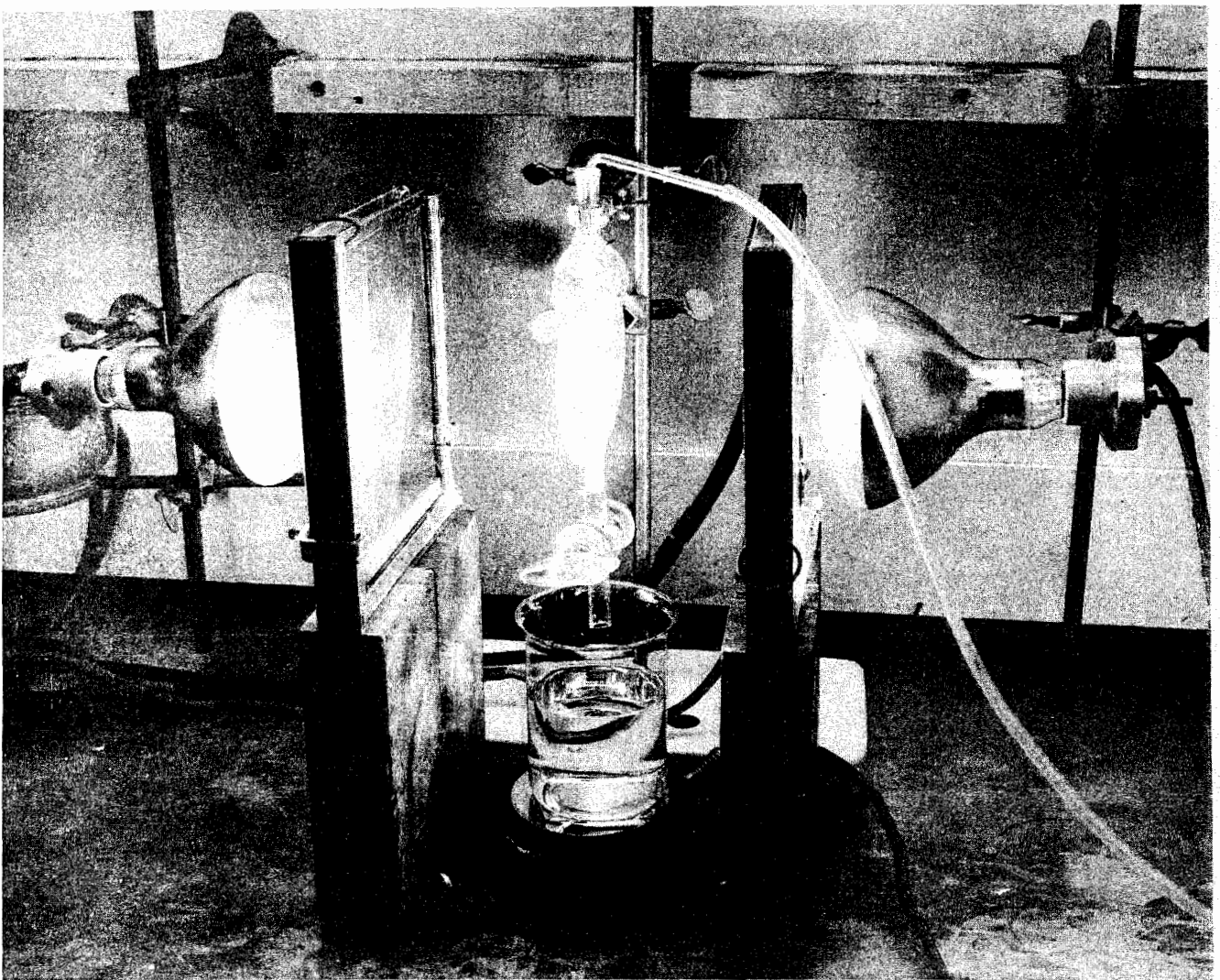


Fig. 3



ZN-481

Fig. 6

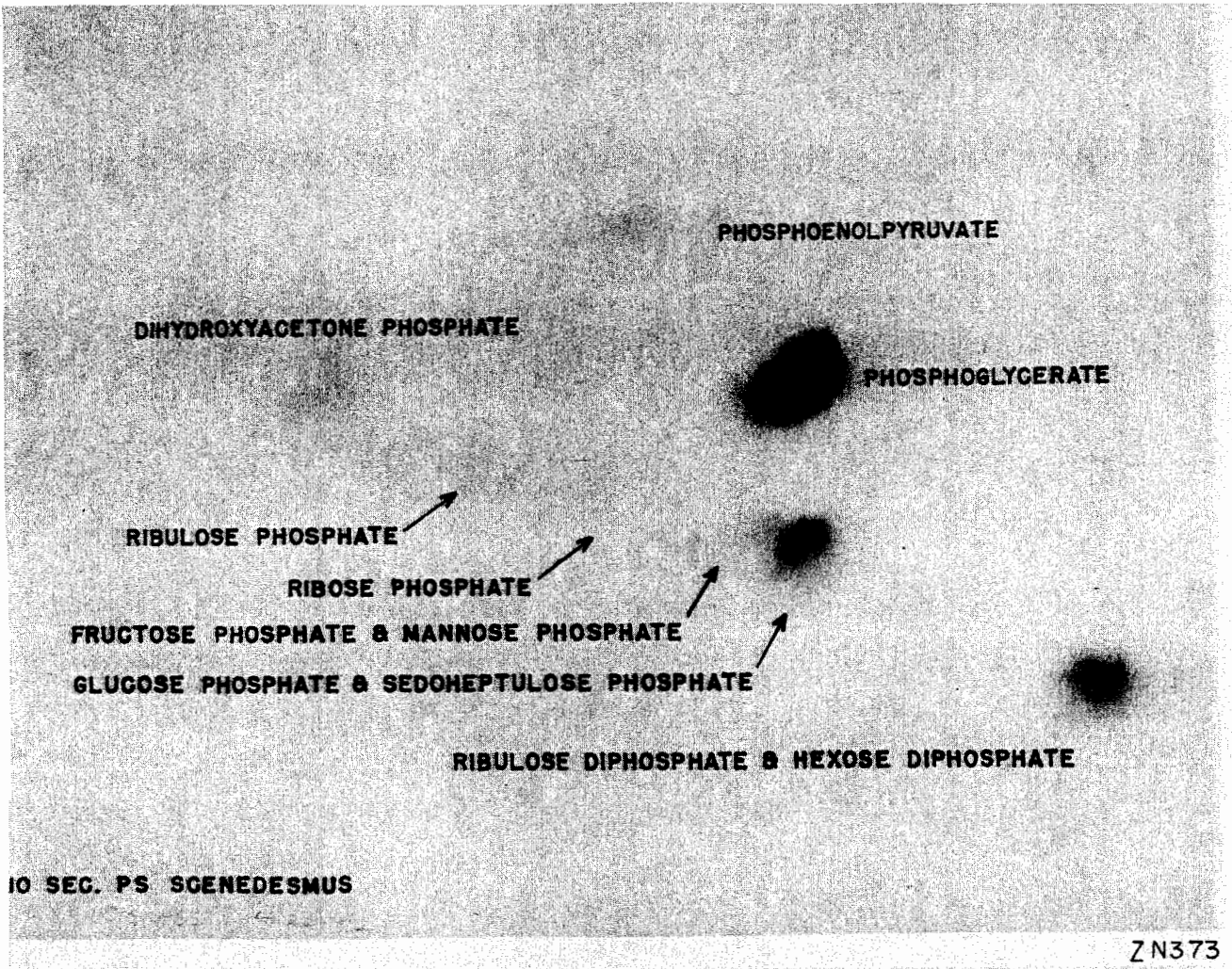


Fig. 5

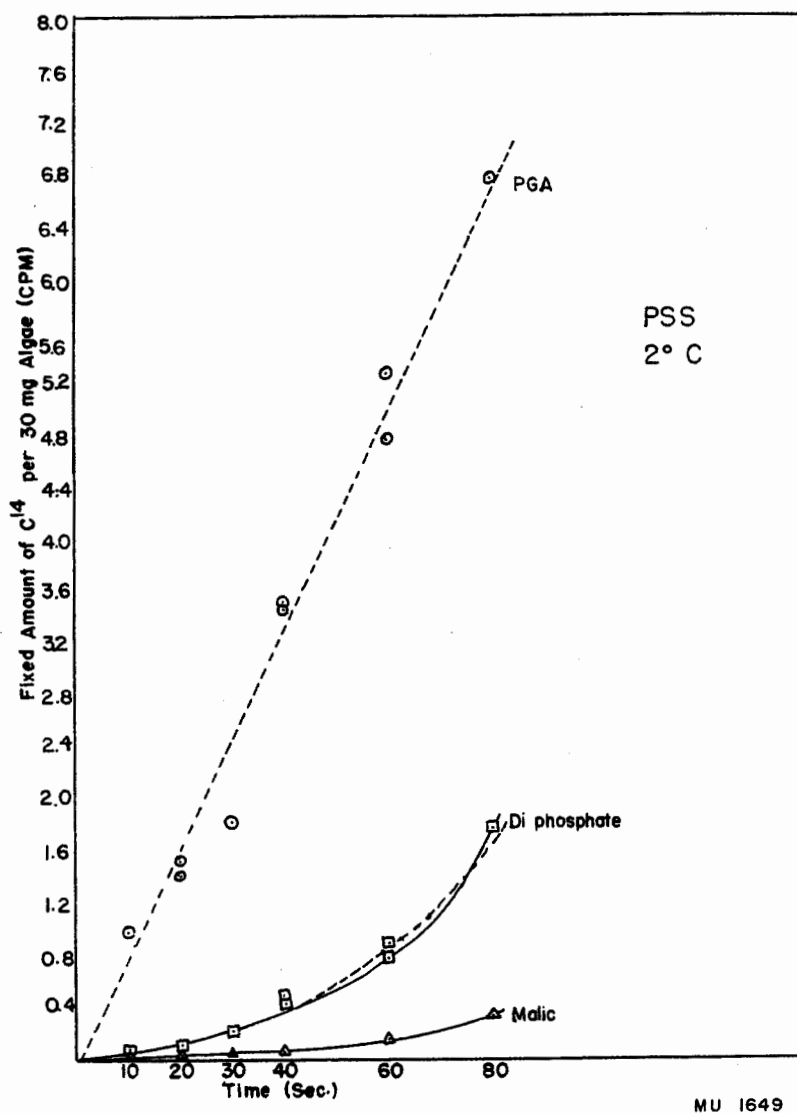


Fig. 6



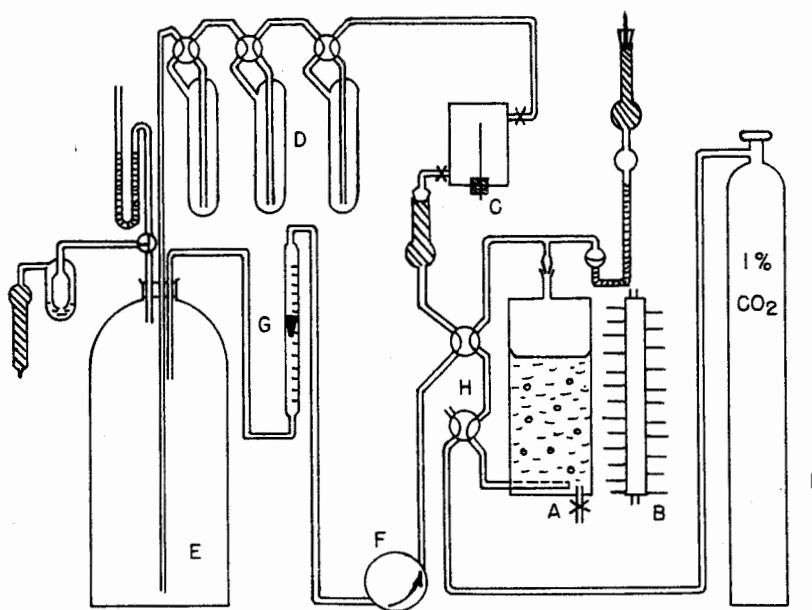
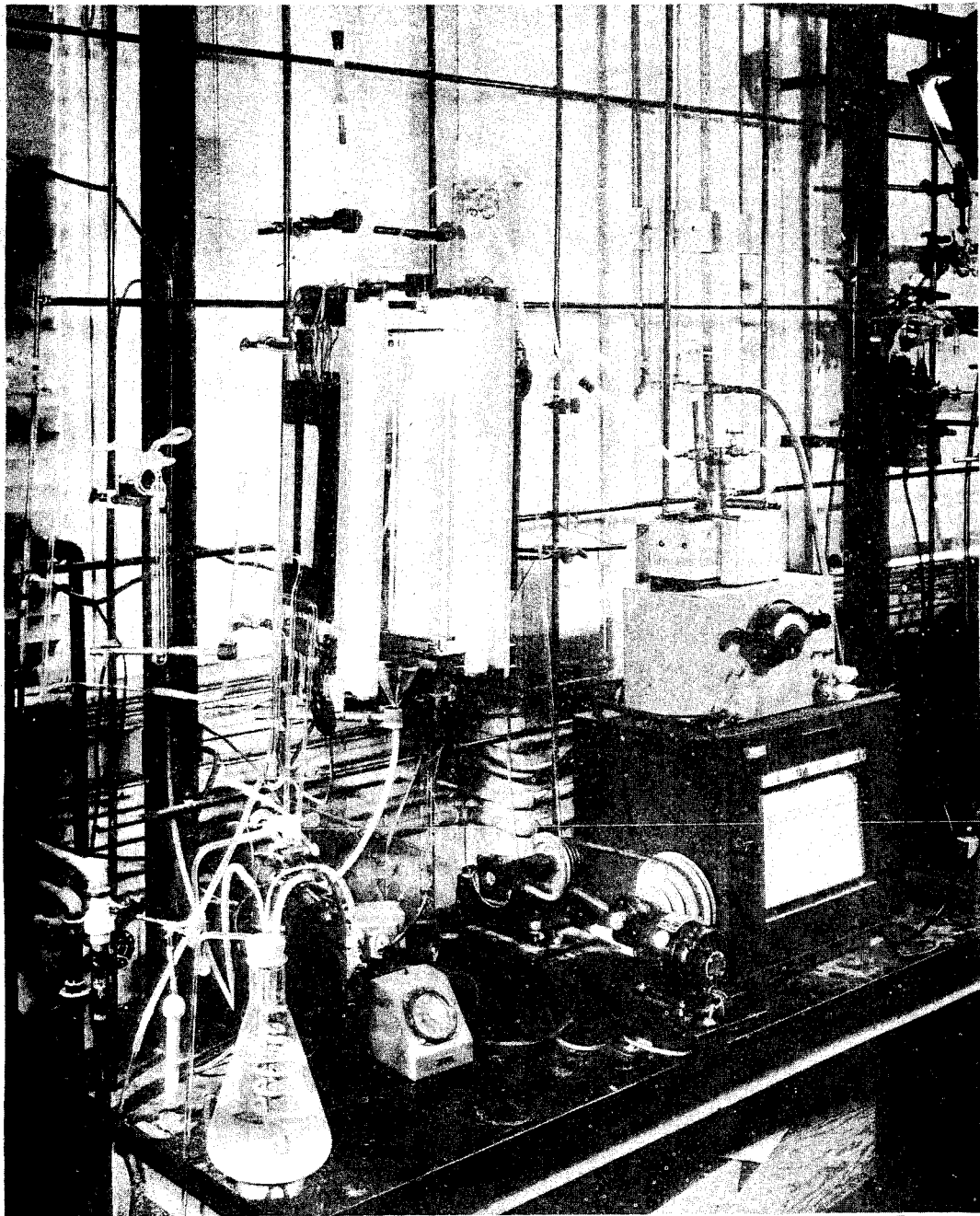


Fig. 7



ZN356

Fig. 8

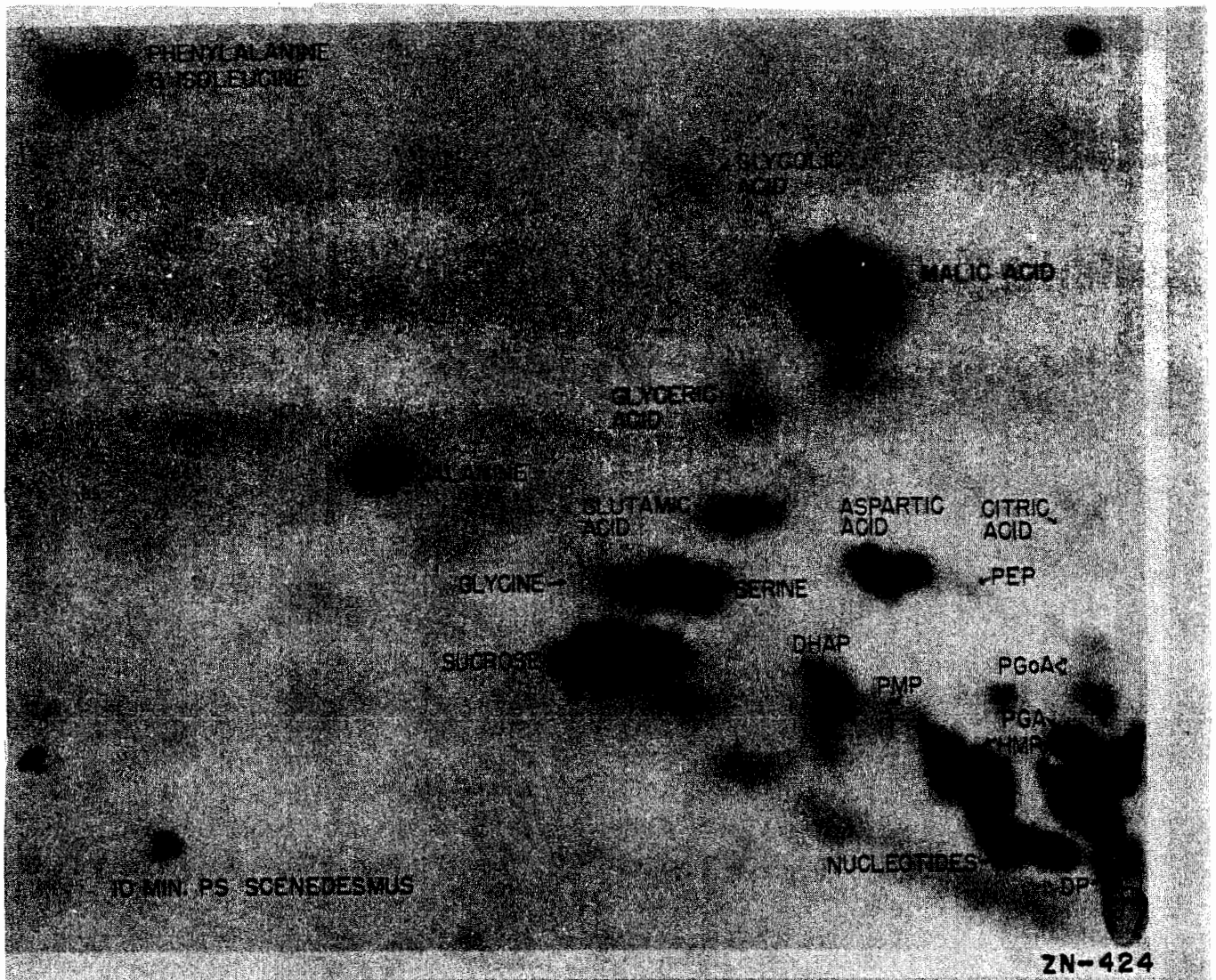


Fig. 9

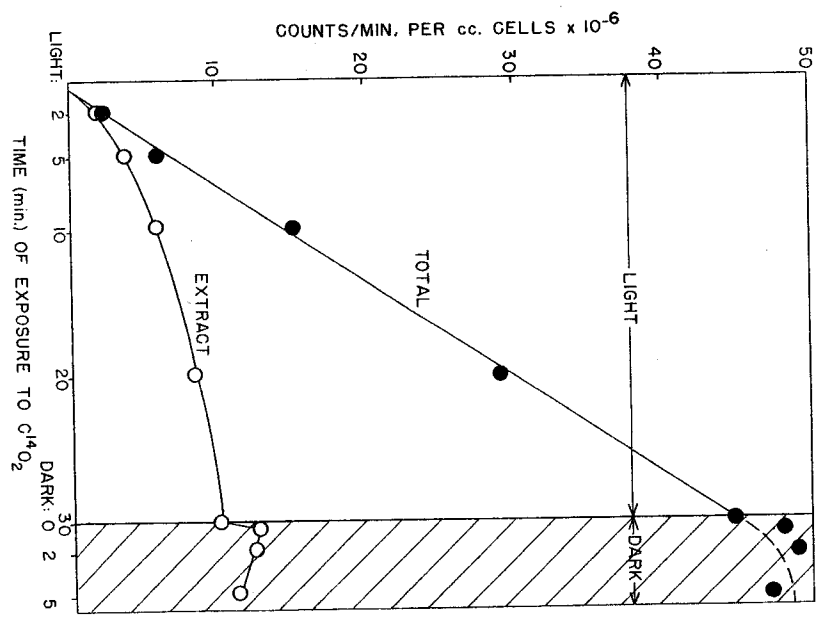


Fig. 10

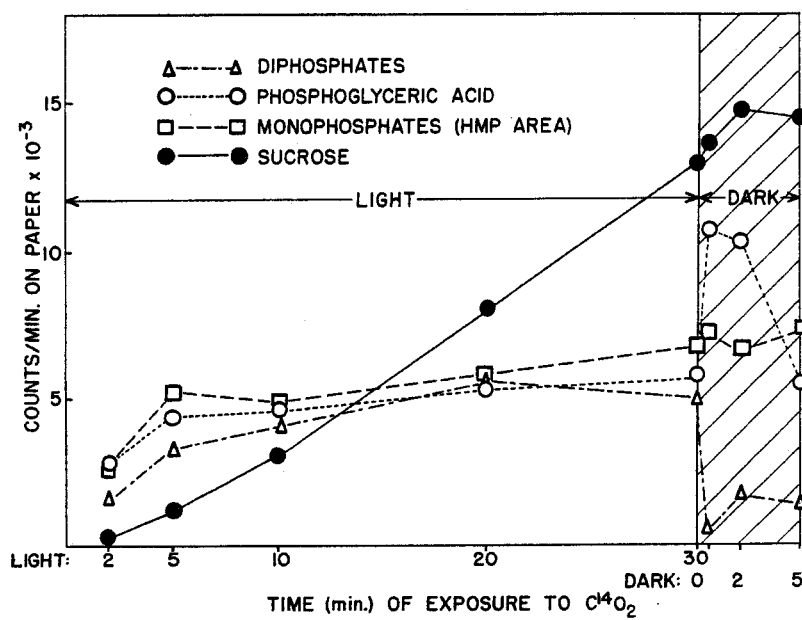


Fig. 11

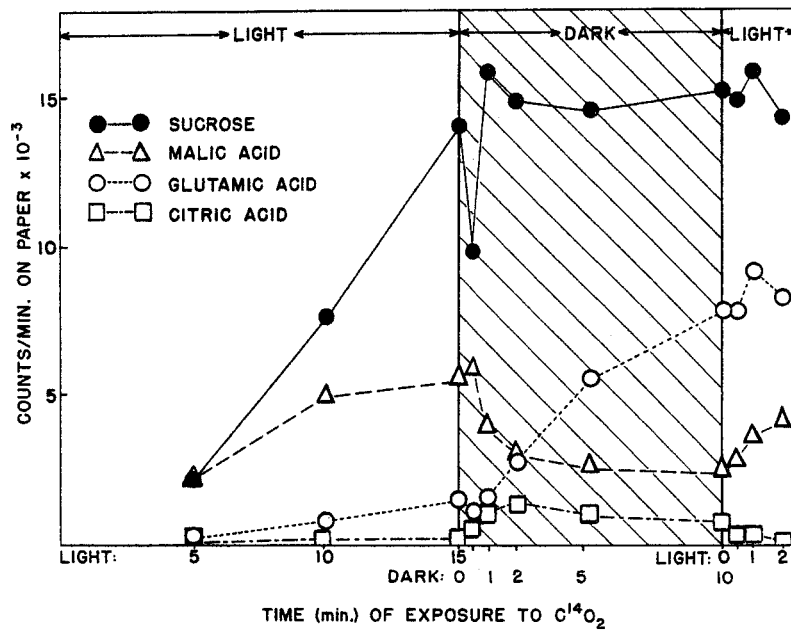


Fig. 12

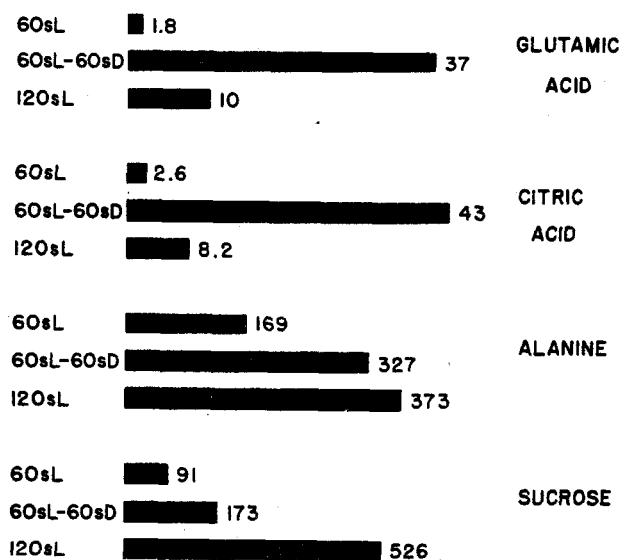


Fig. 13

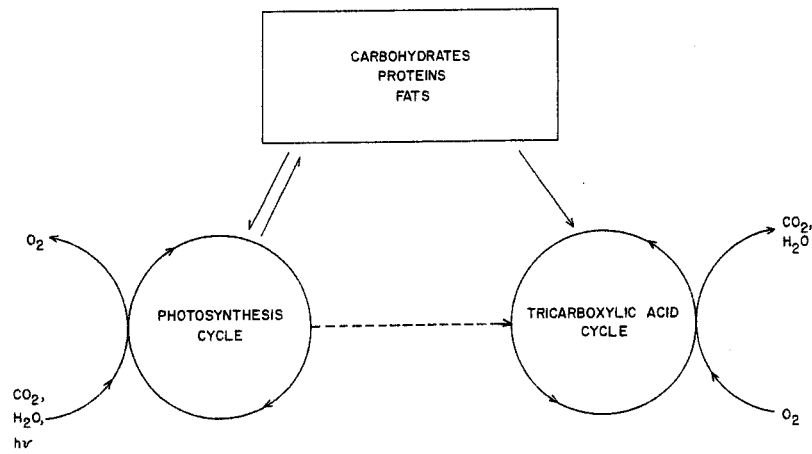


Fig. 14



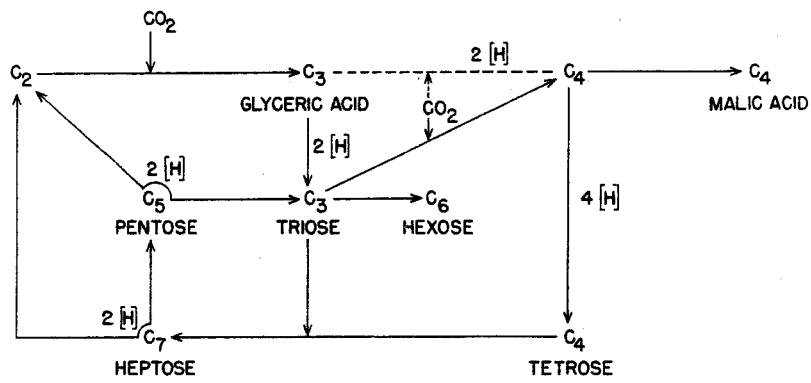


Fig. 15