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M. Calvin

June 30, 1949

Berkeley, California

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

ABSTRACT

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This paper is a compilation of the essential results of our experimental work in the determination of the path of carbon in photosynthesis. There are discussions of the dark fixation of photosynthesis and methods of separation and identification including paper chromatography and radioautography. The definition of the path of carbon in photosynthesis by the distribution of radioactivity within the compounds is described.

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* Presented as the Peter Reilly Lectures, University of Notre Dame, April, 1949.

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VI.

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INTRODUCTION

Tonight I hope to introduce to you a subject in which I have been very interested for about ten years. The experiments I am going to describe during the next few days have all been done in the last three years, since January of 1946, by a group of men working with me in the Radiation Laboratory and Department of Chemistry at the University of California in Berkeley. Foremost among these men is Dr. Andrew Benson who has been associated with me in this work right from its inception. A great deal of what I have to say is due to his very considerable experimental skill and to the collaboration of Mr. J. A. Bassham.

HISTORICAL BACKGROUND

The problem of photosynthesis is an old one. Ever since its recognition in about 1840, chemists have been concerned with an attempt to understand it. Perhaps it would be wise to give you some general idea of its fundamental character. I am sure that an audience such as this is fiarly well versed in the importance of the process. All life on earth as we know it stems from or depends upon this process which is the con-

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version of carbon dioxide and water into organic materials through the agency of light by certain specialized green organisms.

As early as 1850 it was recognized that the overall process was just this - the conversion of carbon dioxide and water into reduced carbon, that is, the substance of the living organism, and oxygen. And so we know for certain, and have known for a long time, that the basic character of the process is

$$CO_2 + H_2O \xrightarrow{\text{Light}} \text{Reduced carbon (carbohydrates, etc.)} + O_2$$

We have also known that ordinary animal respiration is very nearly the reverse of this process, supplying the energy to keep us warm, do mental and physical labor, etc. These, then, are the fundamental life processes: Photosynthesis and its reverse, Respiration.

In the mid-nineteenth century some of the well-known names of organic chemistry were associated with attempts to understand what this process was, and names like Liebig and Baeyer are quite closely connected with the early guesses as to what the nature of the process might be. Then, toward the end of the nineteenth century, the organic chemists, and physical chemists for that matter, became more concerned with synthetic processes and with simple physical processes and left the field of photosynthesis alone for quite a long time. There were, of course, the names of Willstätter and Stoll who determined what was in the plant, but not how it worked. They did make some suggestions as to how it worked, but most of their activities were concerned with the determination of the nature of the compounds that one finds in plants. Of course, the name of Warburg is very closely associated with theories of the process of photosynthesis.

All of this progress was persistently hindered by the nature of the process itself. It was as though chemists were trying to discover what was going

on in a sealed box by examining only what went in and what came out. They were trying to deduce the various things that might be happening inside this closed area which they could not see and had no way of examining, by looking only at the factors which determined the rate at which carbon dioxide would go in and oxygen come out, the effect upon it of light intensity, wave length, etc. They were examining the outside of the process and not the inside.

There was no way of looking on the inside until in 1939 Ruben and Kamen used the isotope of carbon called Carbon 11. The isotope provided a method of putting a mark on the atom. Such a mark would remain with it no matter what happened to the atom. No matter what transformations it underwent, no matter what complicated processes it might be engaged in and how it might be converted, that label remained. It was now possible to follow this carbon atom through the various and devious routes that it took in the plant on the way from carbon dioxide to sugar and other plant materials. It was as though the scientists had been given an eye which could look into the plant cells and which could see the actual processes taking place.

The first such experiments were done with the labeled carbon atom, Carbon 11, which was made in the Berkeley cyclotron by Kamen. These men worked under very great difficulties. The amount of the label that was present in the carbon was reduced by one-half every twenty minutes. This meant that the longest experiment they could do would be four or five hours.

Shortly after the discovery of Carbon 11, Ruben and Kamen discovered another isotope of carbon, Carbon 14. This isotope has a half-life of about five thousand years, so we have ample time to do any experiments. Unfortunately, however, Carbon 14 could not be made in large quantities on the cyclotron

in the pre-war days. It could only be made in very small amounts, and for this reason the early experiments of Ruben were done mostly with Carbon 11 which could be made in fairly high intensity on the cyclotrons.

After the development of the nuclear piles during the war, Carbon 14 became available in large quantities made either at Hanford or at Oak Ridge, and now at the end of the war in 1945 we undertook to resume the work of Ruben which was interrupted by the war and by Ruben's death in 1944. In January, 1946 the work was resumed using the now much more available Carbon 14 with its half-life of five thousand years. The experimental time was now no longer a factor in the experiments designed to discover the complex process by which carbon dioxide is converted in the plant from its initial state into its final state through the agency of light.

DARK FIXATION OF PHOTOSYNTHESIS

It was clear that the simplest kind of an experiment would be to feed a plant in the light some carbon dioxide with a label on it and give it only a very short time for photosynthesis. By examining the compounds present in the plant after exposures of various durations, it should be possible, by reducing the period of exposure, to eventually find only one compound formed with the labeled carbon. The beginning of the process would thus be known and by then lengthening the period of exposure, under careful control it should be possible to follow the labeled carbon atom into each succeeding compound. It would thus be possible to understand the entire process of conversion within the plant cell.

In an effort to reduce the time of exposure to a minimum in order to obtain the very first compound, we finally cut the light time down to zero,

that is, we gave the carbon dioxide not while the light was still on, but immediately after the light was turned off. This involved the following conception of the nature of the reaction: namely, that the absorption of carbon dioxide is not directly connected with the act of light absorption, that is, the two are separate acts and that light absorption can create something in the plant which, after the light is turned off, can pick up the carbon dioxide.

This was not a new idea. It was proposed by a number of investigators including Dr. C. B. Van Niel of Pacific Grove. The idea which Van Niel suggested was based not upon the study of green plants alone but on a comparison of the biochemistry of various colored organisms, bacteria, algae, etc. Through a study of the comparative biochemistry of these organisms he postulated that the process of light absorption was concerned only with the splitting of the water molecule and the removal of the hydrogen from the oxygen and that this hydrogen was in some form which could then later reduce the carbon dioxide molecule. Also, this active hydrogen could be produced in a number of ways other than through the agency of light as in the sulfur bacteria, blue-green algae, etc. The thing that was unique about green plant photosynthesis was the production of this reducing hydrogen by the absorption of light by chlorophyll and the splitting of water. This was proposed by Van Niel as early as 1936 and has been reiterated by him several times, and I will describe some of our evidence which leads us to the same opinion, albeit in more detail by bringing it to an experimental definition much closer than that of Van Niel's comparative biochemistry of algae and sulfur bacteria.

Fig. 1 represents the overall process of photosynthesis as it appears now. Carbon dioxide reacts with water through the agency of light to produce reduced carbon and oxygen. The primary energy conversion results in the photolysis of water, producing oxygen from the water, the hydrogen passing through a series of hydrogen transfer systems of some sort and then reducing the carbon dioxide to sugars, fats, carbohydrates, etc. That the oxygen comes from the water rather than carbon dioxide was shown by the experiments of Ruben with Oxygen 18. He fed the plant water containing the labeled oxygen and noted that the oxygen as it evolved had the isotopic compesition of water rather than that of the carbon dioxide. This means, of course, that the oxygen of the carbon dioxide must pass through the water stage before it is evolved as gaseous oxygen. This was one question answered by the isotope technique.

Another question is answered by the recent experiments of Hill and Scarishrick in England, in which for the first time the green cell has been broken down into a smaller fragment which can reproduce, at least in part, the photosynthetic process. By grinding up green leaves to destroy the cells one can extract small green fragments which are called chloroplasts, or chloroplast fragments which are called grana, which contain the green pigment and which will evolve oxygen when they are illuminated in the presence of a proper oxidizing agent other than carbon dioxide, such as quinone or ferricyanide. This process has exactly the same characteristics as the evolution of oxygen in photosynthesis.

It appears that some of Van Niel's suggestions are being established experimentally from several points of view - the isotopic one presented

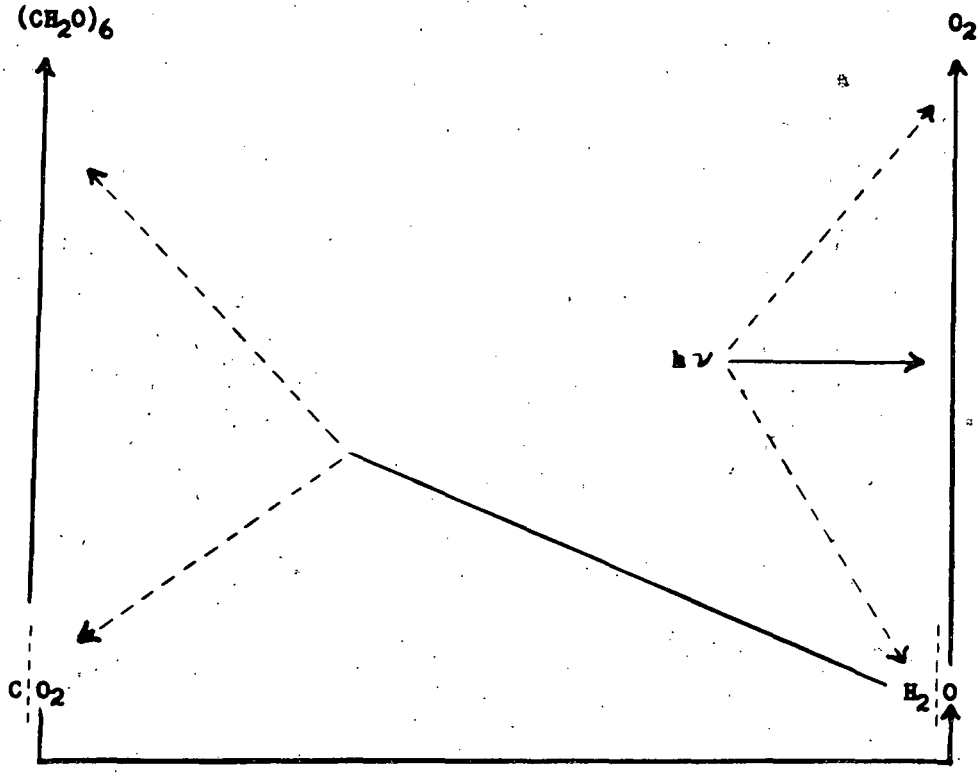


Fig. 1

The Process of Photosynthesis.

here and the fact that it is possible to isolate the oxygen-producing reaction and tie it to the primary photochemical act in some way.

Our experiments have been designed to begin the determination of the Path of Carbon in Photosynthesis and to determine the relationship between the carbon path and the completely independent act of light absorption. In order to do this one would have to demonstrate that carbon can pass up from one to the other of the compounds in the left-hand vertical line in Fig. 1 in total darkness, but that the rate at which it passes from one end to the other and the amount which passes will depend upon the immediate prehistory of the plant. If the passage from the bottom to the top depends upon a reducing agent made in the light by splitting water, it is clear that if one were to illuminate the plant for a period of time (as yet unknown) in the absence of carbon dioxide so that the reducing hydrogen which is split from the water has nowhere to go, and then immediately after turning off the lights give the plant carbon dioxide, the reducing agent which had been generated could carry the carbon dioxide at least part way along the path and perhaps the whole way.

The first experiments were done to test this idea. The plant material which we used for the early experiments were the green algae Chlorella and Scenedesmus. The reason for this is two-fold. First of all, a great deal of photosynthetic research had been done with these algae, Chlorella and Scenedesmus, their kinetics have been studied, quantum yields have been determined, etc. Secondly, being chemists, we liked to have something quantitative to work with, and one can make up a quantitative suspension of algae and treat it, within limits, like an ordinary chemical.

In order to get the algae we had to set up a little "farm" to grow them. The continuous culture apparatus is shown in Fig. 2. We can harvest the algae every day, taking out nine-tenths and leaving one-tenth behind as an inoculum in the inorganic nutrient solution. Having harvested the algae from this "farm," they are now ready for the experiment. The algae are centrifuged out of the nutrient bath, washed once and resuspended in a suitable buffer for the performance of the experiments. The flask into which the suspension is placed is shown in Fig. 3 and the complete apparatus is shown in Fig. 4 Pure helium is bubbled through the algal suspension in the upper flask to sweep out the carbon dioxide of the air. The lights are turned on to illuminate the algae for varying or selected periods of time in the absence of carbon dioxide. An aliquot part of the algae is taken in the evacuated sampling tube, and the sample then flows down into the partially evacuated blackened vessel which contains a known amount of radioactive carbon dioxide. The whole transfer operation can be done in less than a second, the turning of two stopcocks being all that is necessary (Fig. 4). The radioactive carbon dioxide is in the form of sodium bicarbonate at a small partial pressure of carbon dioxide, there being no other gas in the blackened flask. The black vessel with the radioactive carbon dioxide and the algae in it is shaken for various periods. After a chosen period of time, acid is injected to kill the algae. An aliquot of the resulting suspension is counted to see how much radiocarbon the algae have picked up.

Fig. 5 shows how much carbon dioxide is fixed as a function of the time the flask is shaken in the dark after the algae have been put into the vessel containing radioactive carbon dioxide. Curve B is that one which is obtained when the algae have been illuminated for ten minutes in the absence

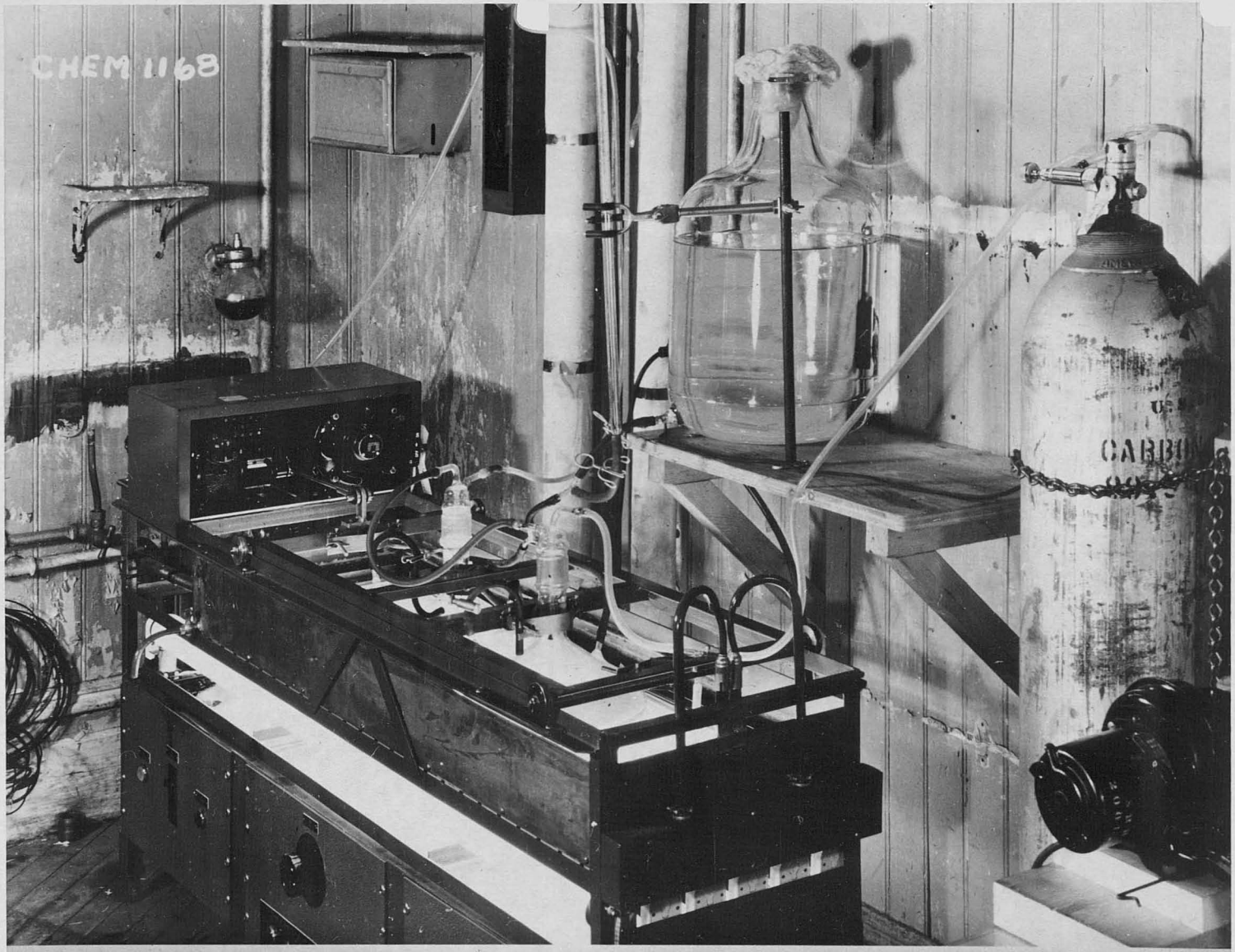


Fig. 2

"The Farm" - Apparatus for the Continuous Culture of Algae.

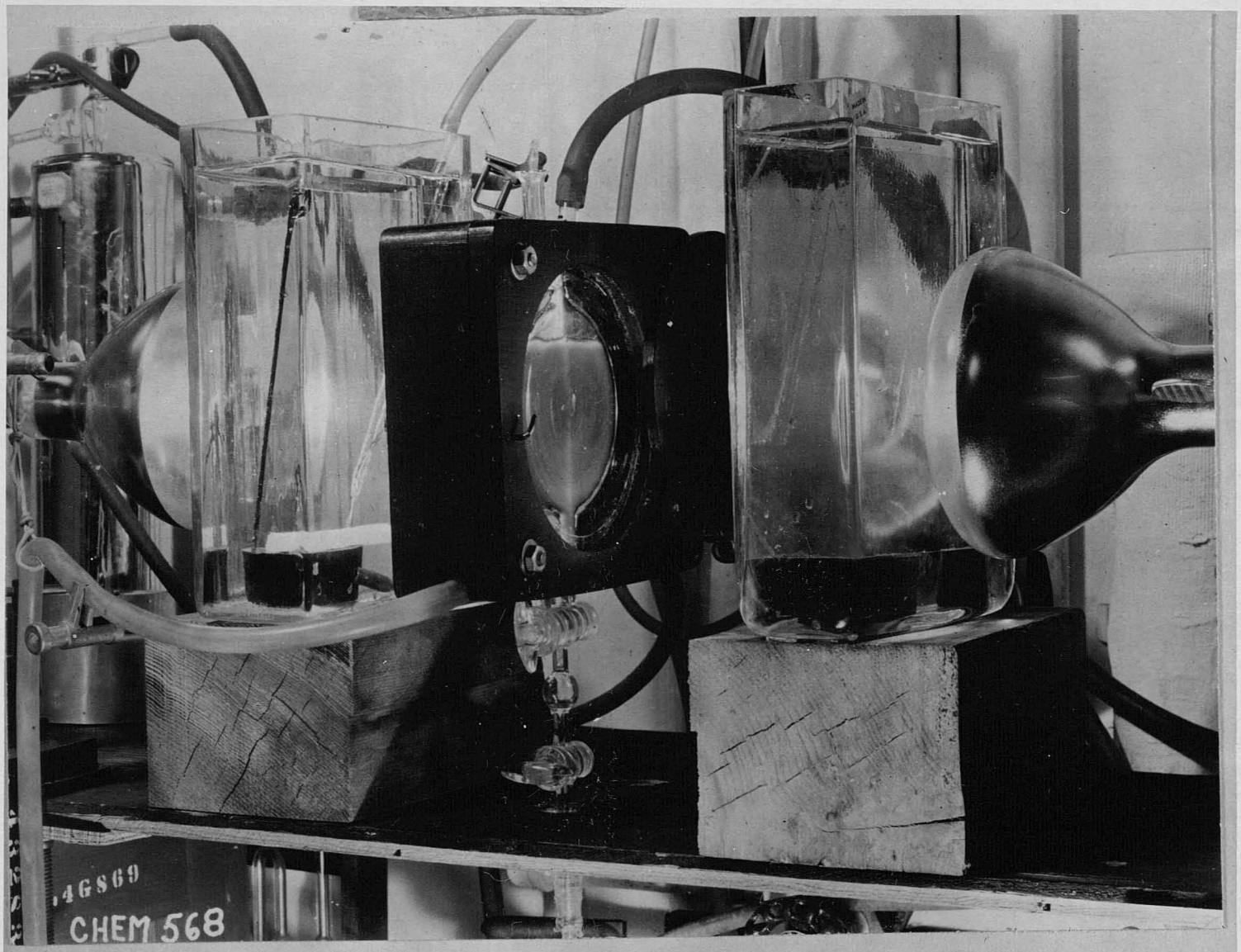


Fig. 3

Exposure Vessel and Sampling Tube for Preillumination Experiments.

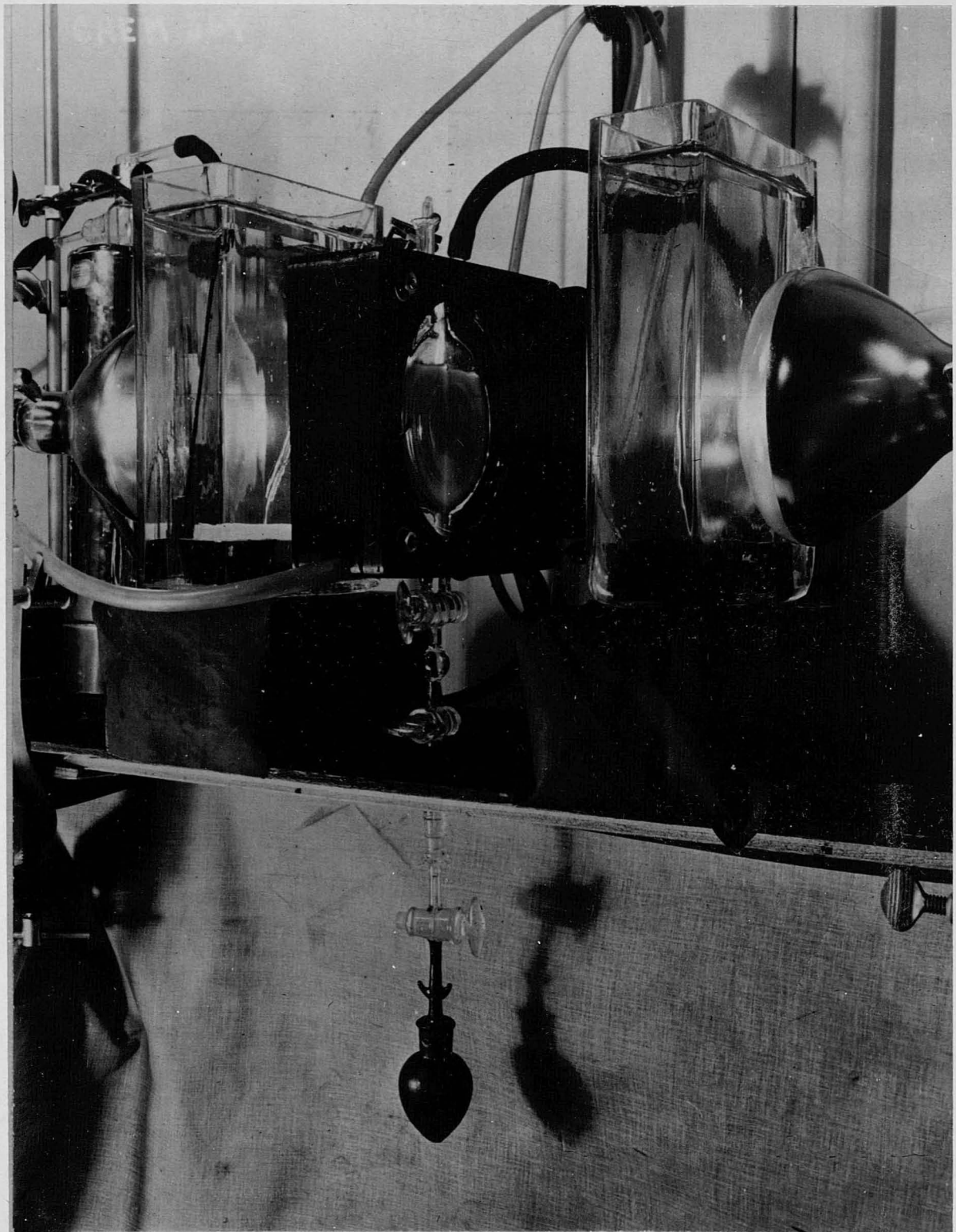


Fig. 4

Complete Preillumination Apparatus

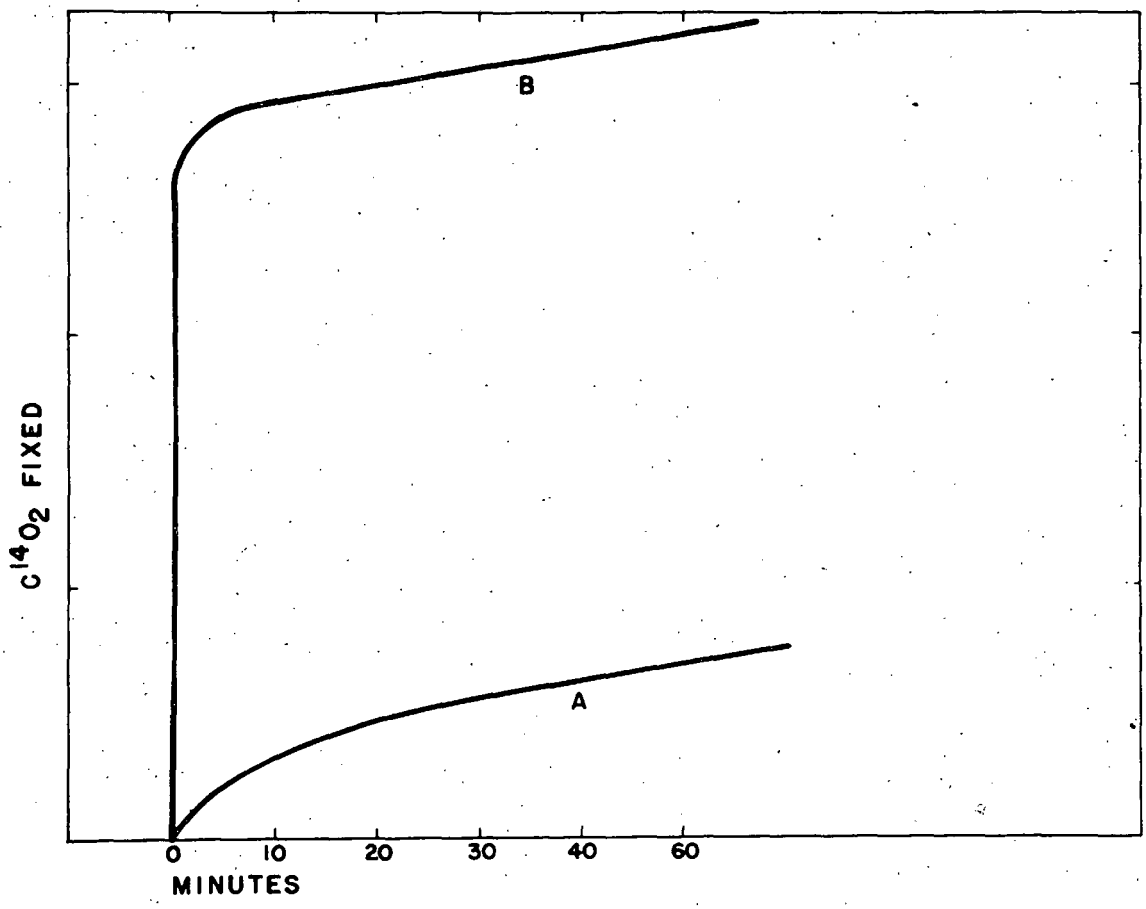


Fig. 5

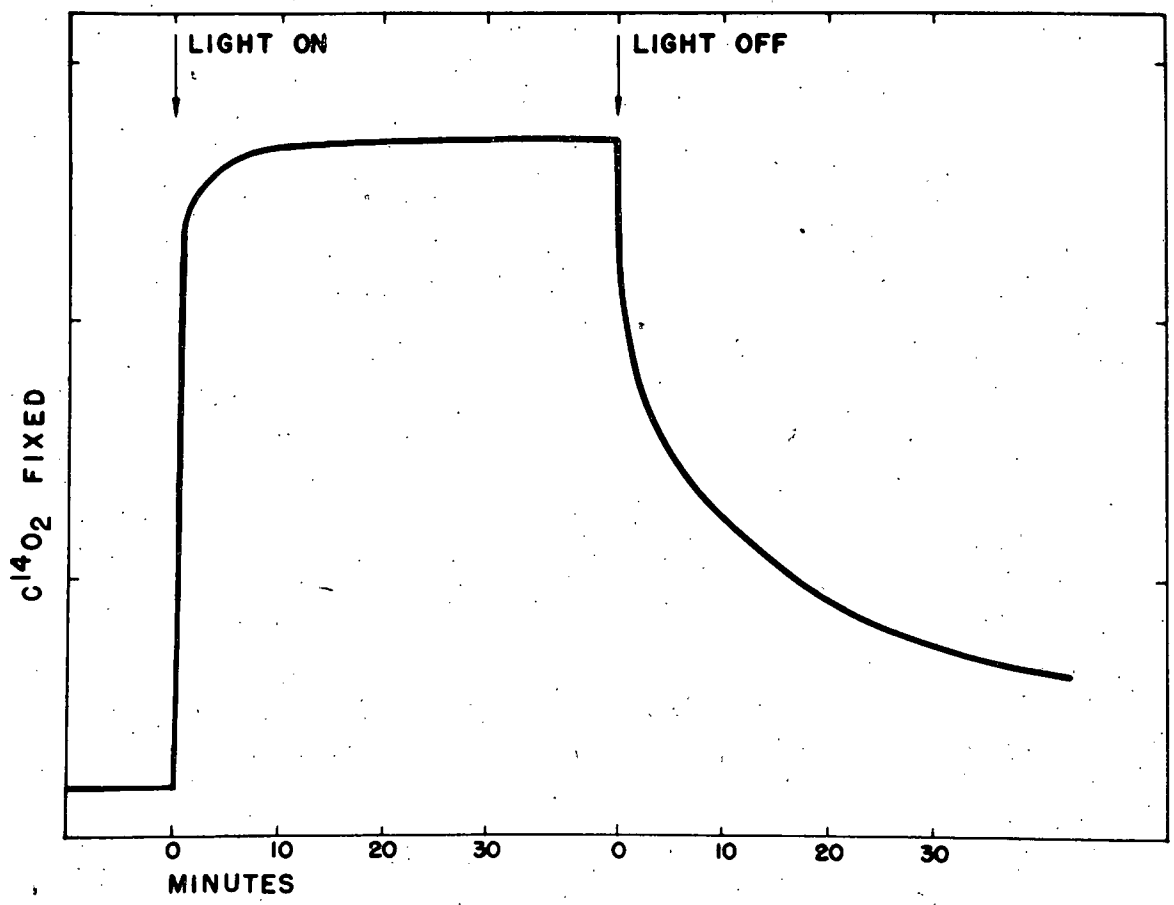
Dark Fixation of Radioactive CO_2 by Algae.

A. Dark Fixation by Non-Preilluminated Algae.
B. Dark Fixation by Algae Which Have Been Preilluminated Ten Minutes in the Absence of CO_2.

carbon dioxide prior to giving the algae the radioactive carbon dioxide. For Curve A the algae were saturated in the dark with orginary carbon dioxide just prior to giving them the radioactive carbon dioxide. The effect of preillumination is there in great measure. If the algae are illuminated in the absence of carbon dioxide and then, in the dark, given the radioactive carbon dioxide they do indeed reduce, that is, fix, a great deal more carbon dioxide than they do under the reverse conditions.

The question arises as to how long the cells must be illuminated in the absence of carbon dioxide in order to get this reducing power generated. It is obvious that there is a limited amount of reducing power generated (Fig. 6) and that it is almost completely used up by a one-minute exposure to carbon dioxide in the dark. A second experiment was done to discover how long the illumination had to be in order to generate this reducing power. It was determined by simply measuring one-minute dark fixations after different lengths of time of preillumination. The result is shown in Fig. 6 which has for abcissa the time in the light prior to dropping the cells for one minute into the darkened flask containing the radioactive carbon dioxide. It is apparent that the reducing power reaches ninety percent of saturation in about twenty seconds. The decay of the reducing power after turning the light off in the upper vessel requires about ten minutes. It may be used up by respiratory intermediates or respiratory carbon dioxide.

If this reducing power is the same as that which functions in ordinary photosynthesis, then as long as the reducing power is there in large amount the rate of absorption of carbon dioxide by the reducing power should be the same as though the light were on. Thus, the dependence of the rate of



EFFECT OF PRE-ILLUMINATION UPON 1 MINUTE DARK FIXATION (SCENEDESMUS)

Fig. 6

The Growth and Decay of Dark Fixing Power.

dark fixation by preilluminated algae on the partial pressure of carbon dioxide should reflect the dependence of carbon dioxide absorption in ordinary photosynthesis on the partial pressure of carbon dioxide.

Fig. 7 shows just this variation of dark fixation rate (one-minute) as a function of carbon dioxide pressure both for un-preilluminated cells and preilluminated cells. Curve B shows the rate of carbon dioxide fixation as a function of carbon dioxide pressure for preilluminated algae and corresponds to the dependence of ordinary photosynthesis on carbon dioxide pressure. The rate of dark fixation of carbon dioxide by the un-preilluminated cells (Curve A) is very small and shows very little dependence on carbon dioxide pressure. From the kinetic point of view, at least, we have demonstrated that the dark fixation after preillumination resembles very closely the steady-state photosynthetic picture as it is affected by carbon dioxide pressure.

You will notice that all of these measurements are measurements of the total amount of carbon dioxide fixed by the plant. No information has yet been given as to what has happened to the carbon dioxide. Fig. 8 will give an idea of what is to be found. The relative area and density of the spots is a rough measure of the relative amount of those compounds which have been formed under the specified conditions. The compounds found in fixation in the dark by preilluminated algae (Fig. 8b) were very different from those formed by fixation in the dark by algae which were not preilluminated (Fig. 8a) and, in fact, these compounds formed by the preilluminated algae were exactly the same as those formed by the algae in the light (Fig. 8c). The complete separation of carbon dioxide reduction from the primary photochemical act has thus been established both kinetically and chemically.

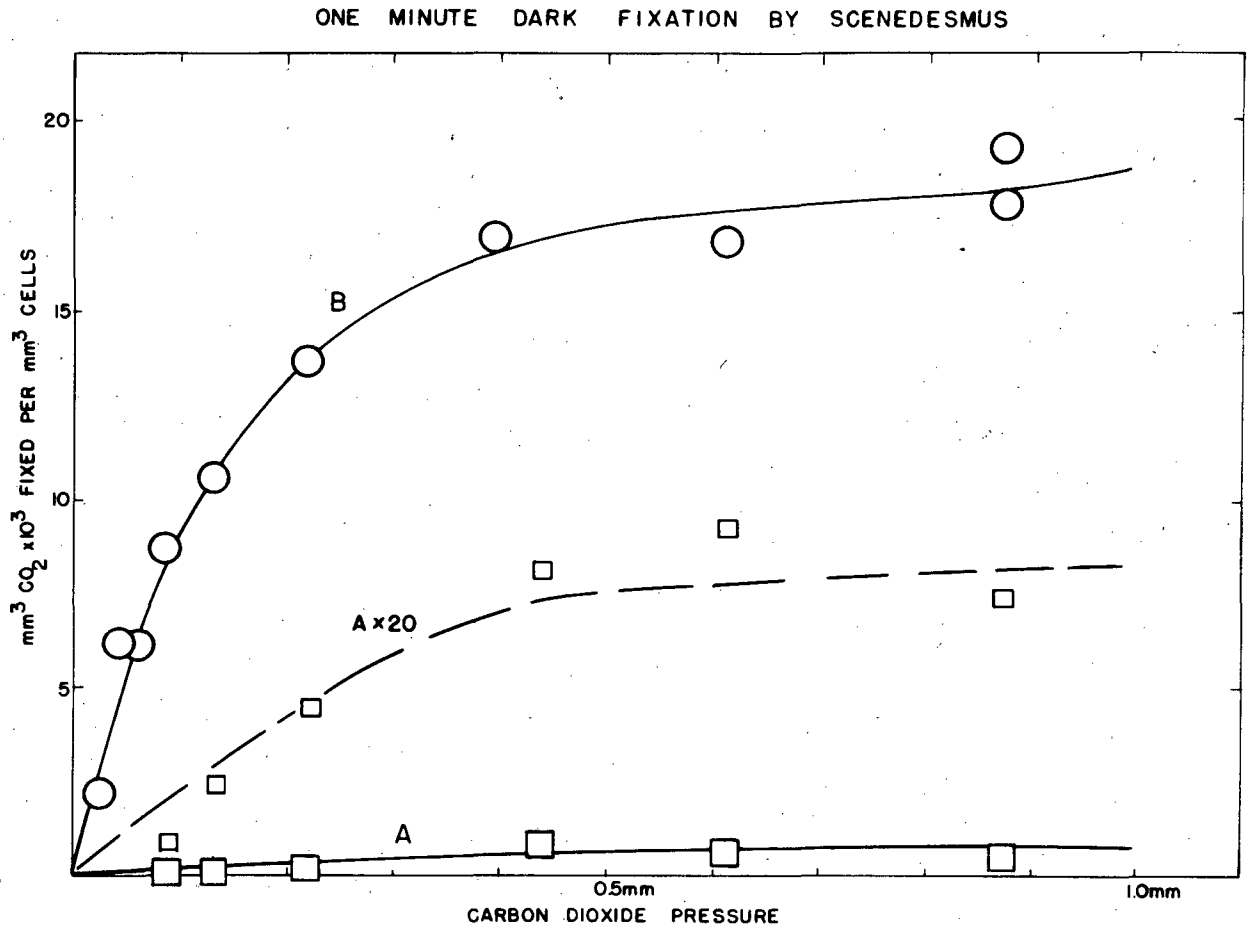
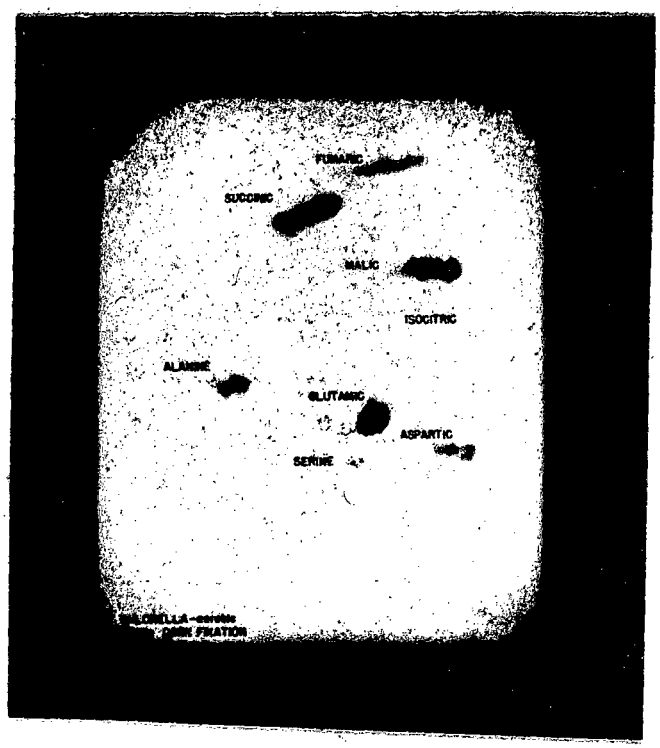


Fig. 7

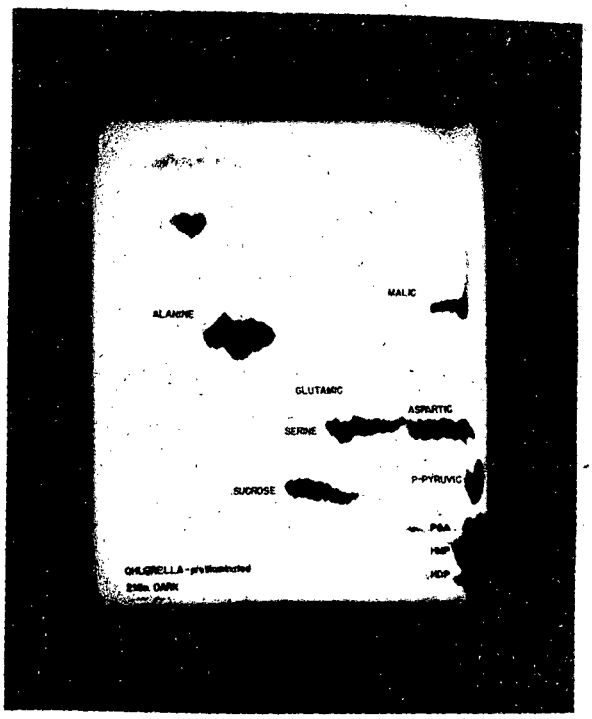
The Dependence of Dark Fixation on CO_2 Pressure
A. Non-Preilluminated Algae.
B. Preilluminated Algae.



Radiograms of Dark Fixation Products

Fig. 8a

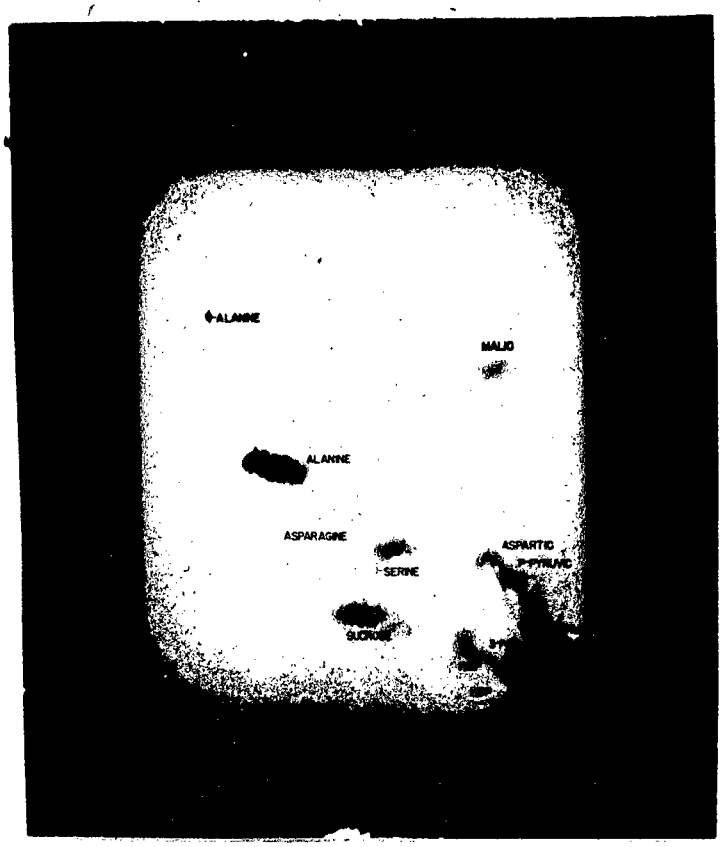
45 Minutes of Dark Fixation by CO_2-Saturated Algae



Radiograms of Dark Fixation Products

Fig. 8b

2 Minutes Dark Fixation by Algae Preilluminated 5 Minutes in the Absence of CO_2.



Radiograms of Dark Fixation Products

Fig. 8c

The Products of 30 Seconds Photosynthesis in Radioactive CO_2.

We can now return to the study of the order of appearance of the compounds in time and the method of analysis represented in Fig. 8.

In Fig. 9 is shown the apparatus in which the algae are exposed to radioactive carbon for the chemical isolation experiments. There is a "lollipop" in which the algae are suspended with light sources on either side separated from the "lollipop" by infra-red filters to absorb the heat. The algae are allowed to photosynthesize for a short period of time (one-half to one hour) in the presence of 4% ordinary carbon dioxide in air which comes into the tube at the top of the algae flask. After the algae have achieved a steady-state of photosynthesis in the normal carbon dioxide, radioactive carbon dioxide in the form of an aqueous solution of sodium bicarbonate is injected into the flask after the removal of the bubbling tube. The flask is then stoppered and shaken in the light beam for the prescribed period of time (5 seconds, 30 seconds, 90 seconds, 5 minutes, etc. as the case may be), at the end of which the large stopcock at the bottom is opened and the algae suspension is forced into hot alcohol as rapidly as possible to stop the reaction. For the higher plants (leaves) we have a similar flask in which, instead of a stopcock at the bottom, the whole front face comes off and the leaves drop out into the alcohol at the given instant.

Now, after having exposed the plant to the radiocarbon for the desired length of time and having stopped the activity by immersing it in hot 80% alcohol and cooling it as fast as is reasonable, we have a suspension and a solution; that is, there are denatured proteins and insoluble cellulose suspended in the alcohol which has extracted from the plant all the soluble materials. For very short periods of photosynthesis (less than periods of the order of 90 seconds) all of the carbon which has been fixed

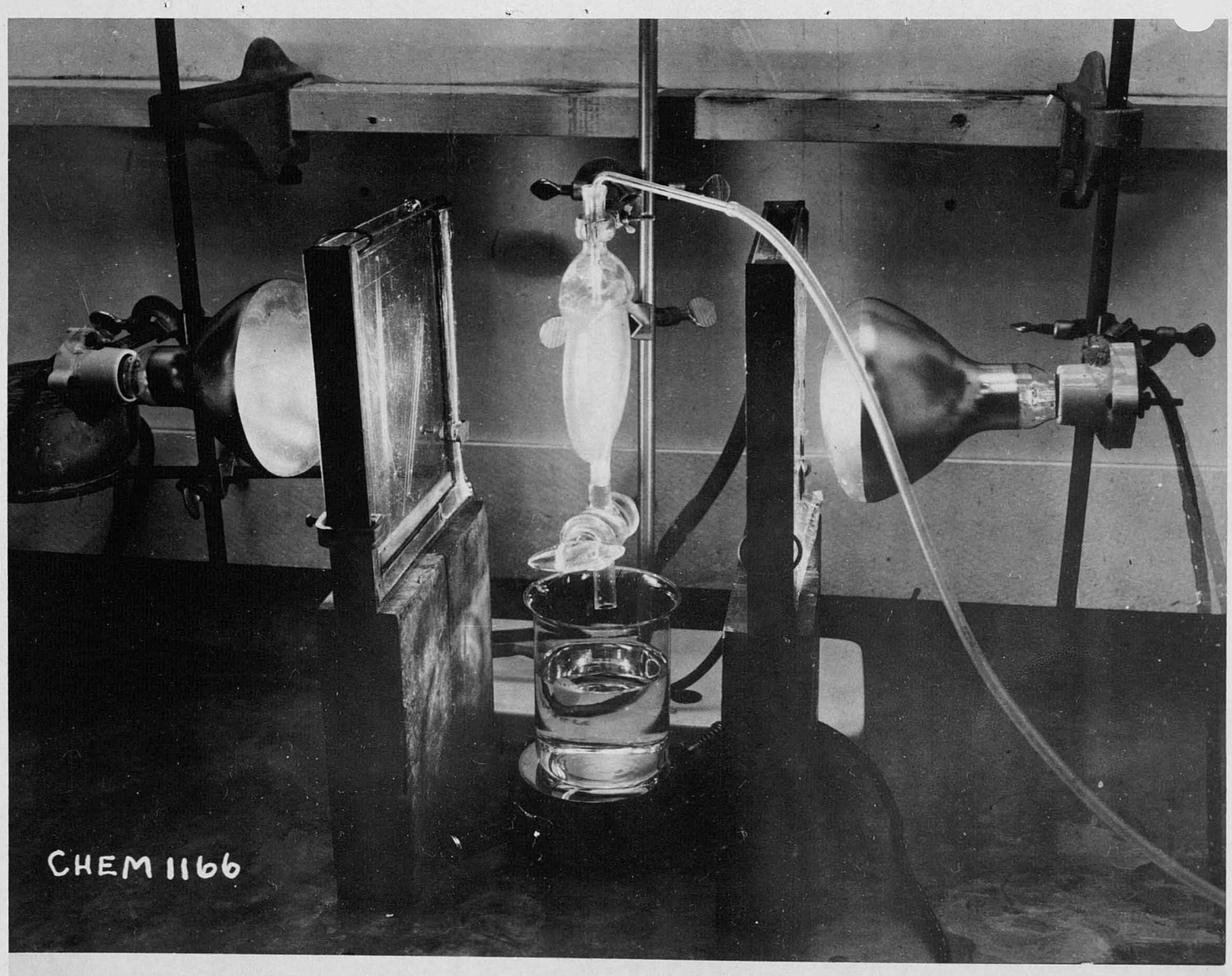


Fig. 9

"Lollipop" - Apparatus for the Exposure of Algae to Radiocarbon in the Light Followed by Rapid Killing.

in that short period of time, namely all of the radioactive carbon, is still in the soluble fraction and will be found in the clear supernatant solution or filtrate from the alcohol extract.

All subsequent operations are on this alcohol extract from which has been filtered the insoluble denatured proteins and cellulose-like materials.

METHODS OF SEPARATION AND ANALYSIS

When this work was started in the summer of 1946, we began by using classical methods of analysis, that is, to make extracts, crystallize, distill and use the usual methods of separation and identification which were common among organic chemists. We started out this way, and, as a matter of fact, worked for about a year and at the end of that year we had identified one compound. This was very slow progress. Since we knew that there were many compounds in the plant and since we had not accounted for anything like all the radioactivity, it seemed like a good idea to seek other analytical methods.

Ion Exchange: - About this time we began the work with the ion exchange resins. They have, of course, proved their usefulness in inorganic chemistry and are only beginning to show how useful they may be in organic separations. It is possible to use the ion exchange resins to separate the organic materials into cations, or potential cations, and into anions, or potential anions, and into neutral substances. Such a fractionation is, indeed, of value when dealing with a very complex mixture. It did give us a great many leads and a great deal of information.

Using ion exchange resins we were able, for example, to recognize the presence of sugars because these passed through both the cation exchanger and the anion exchanger. We were able to determine about how much was potentially cationic. There are not a great many organic substances which are potentially cationic except the amine bases. There are a few other organic cations like oxonium ion, but most of them are amine bases of one sort or another. We were able to find that there was a group of compounds which contained radioactivity from these short periods of photosynthesis which were retained on the cation resin and which, as a matter of fact, are amino acids, as was later shown. A very large fraction of the material was retained on the anion resin. This could be a wide variety of compounds including carboxylic acids and esters of inorganic acids such as sulfuric and phosphoric acids.

The use of ion exchange resins did help a great deal, and in the following year up until the Spring of 1948 we were able to identify unequivocally two or three more compounds. However, we still had not accounted for the major fraction of the fixed radioactivity in one minute of photosynthesis. It appeared that other methods still more rapid would have to be found.

Paper Chromatography and Radioautography: - It was at this time that we undertook to use the method of paper chromatography developed by a number of British biochemists. It was developed for the purpose of analyzing amino acid mixtures and protein hydrolysates. It depends upon a rather old principle which had been known for a long time. The principle is readily demonstrated by a drop of ink on a piece of filter paper; if it is a good big drop you will notice that it spreads out from where the drop hit the paper,

and if it is the right kind of ink it will eventually appear as three concentric rings around the middle spot and the three rings will be of different colors. Our particular ink gives a red ring, a green ring and a blue one, which, when mixed together, will give a black ink. The point is that the three different components of the ink move at different rates and actually achieve separation.

If this is done in a quantitative way, it is apparent how it might be used for identification purposes. The three British chemists, Consden, Martin and Synge, worked with the method for the separation of amino acids, and one of the reasons that they had such great success is that they had an almost universal method of detecting the amino acids on the filter paper.

The easiest way to show how the method can be used in the quantitative way is to describe the way it is done. To prepare the extract (from which the insoluble materials have been separated) for analysis it is concentrated to a small volume, of the order of 2-3 cc. The analysis is performed by placing a small aliquot part of this 2-3 cc. of solution, perhaps 50 or 100 μl., on the corner of a large piece of filter paper (Whatman No. 1, 17" x 24") as shown in Fig. 10. The sample is squeezed out of the pipette into the circle and there is a fan blowing on it to evaporate the solvent and leave the compounds in the circle. Next, the paper is removed from the frame and hung up in a box as shown in Fig. 11. This shows the top view of a box with four papers. There are two or three frames in each box which contain troughs. The fold in the paper is laid down in the bottom of the trough and a glass rod is placed on top of the fold to hold the paper down in the trough. The paper lies over the edge and hangs down into the bottom of the box. After the papers are placed, a solvent, chosen

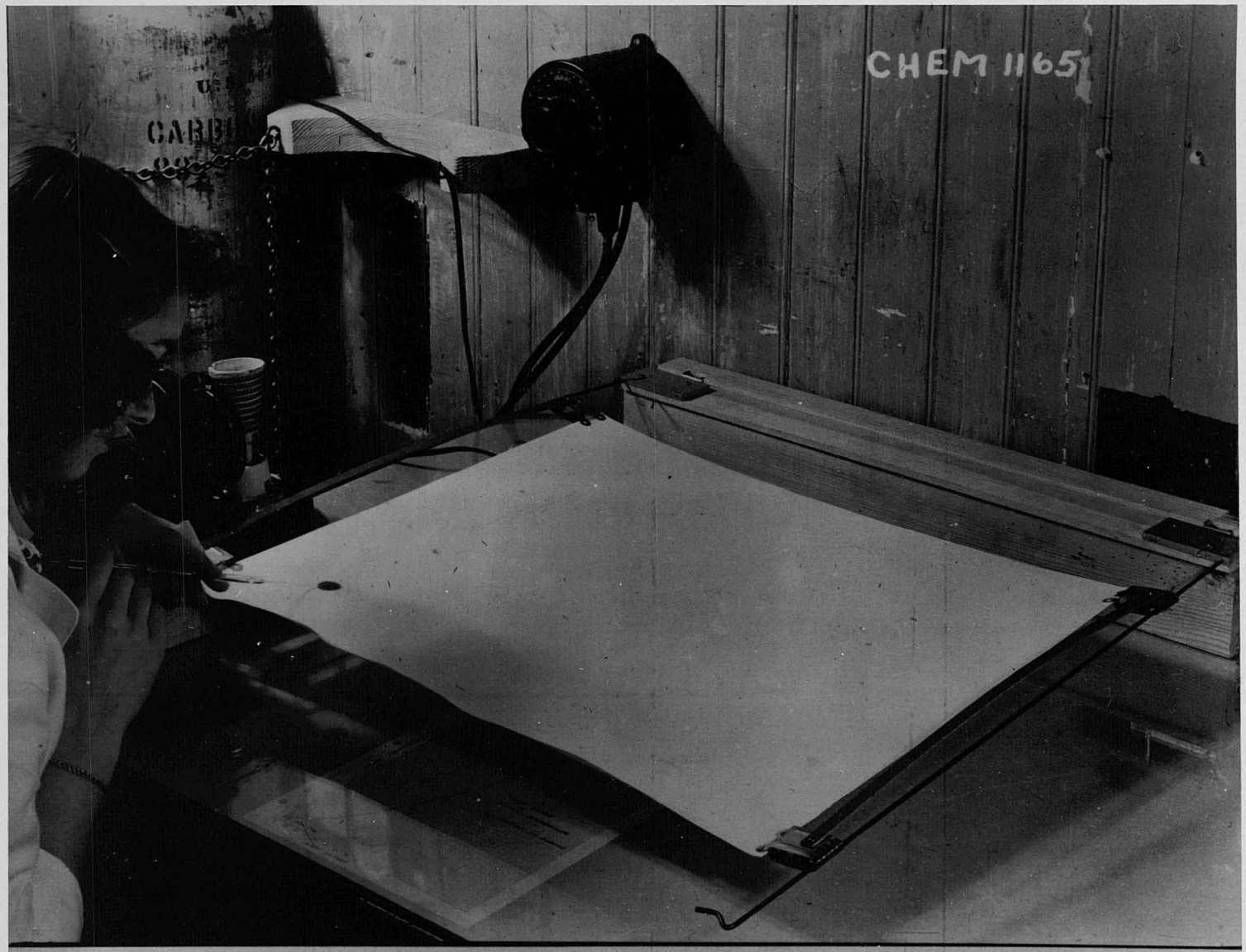


Fig. 10

Placing the Sample on the Paper for Chromatographing

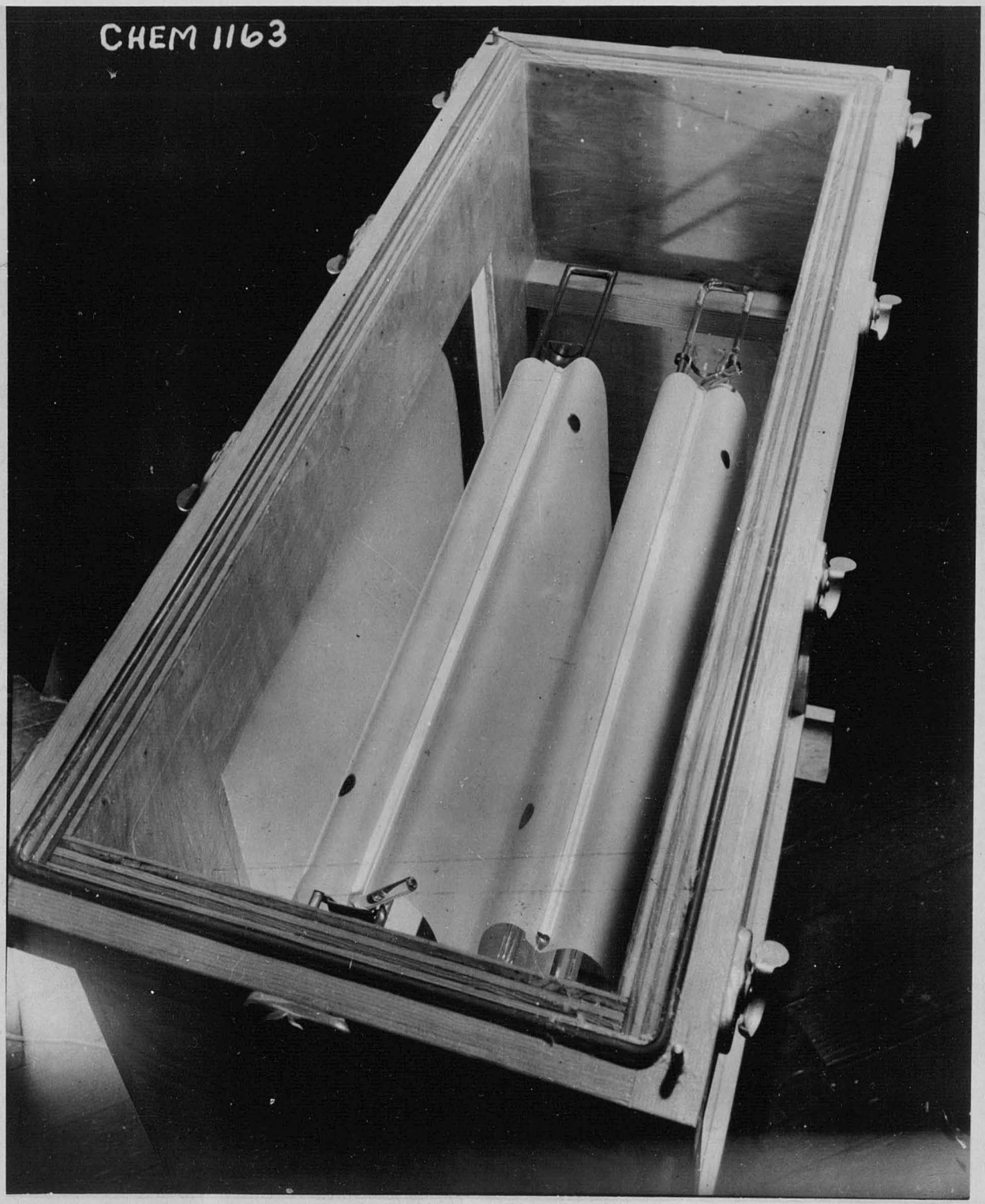


Fig. 11

Top View of Chromatographing Chamber Containing Two Trays Holding Two Papers Each.

OZ 505

for a specific purpose, is poured into the trough. It passes up through the paper by capillarity and down to the edge of the paper (Fig. 12).

As the solvent runs down toward the bottom of the paper, the compounds that were put on the spot are spread out in a line. The compounds do not all move at the same rate; they have different relative solubilities in the organic solvent and therefore different rates of progress. Thus, we shall have spread along one edge of the paper a series of spots, each one representing a different compound or group of compounds. It may be that two compounds have very nearly the same relative solubilities in a particular solvent, in which case they move together. In that case, it is possible to achieve separation of those compounds by taking the paper out of the box and drying off this particular solvent, turning the paper around so that we now have instead of a single spot a whole row of spots spread along the top of the paper and a different solvent is placed in the trough. Those compounds which failed to separate in the first solvent will have an opportunity to separate in the second solvent.

The result of this will be, then, a pattern of spots each of whose coordinates with respect to the origin will be characteristic for that particular compound in the same way that a melting point, index of refraction, specific rotation or any other property of an organic substance is characteristic of it. The extraction coefficient, upon which the chromatographic coordinates depend, is particularly useful since it can be easily determined on tracer amounts of material. In other words, from the coordinates of the compound (spot) with respect to its origin and the solvents used, one can say, in many cases unequivocally, what the compound is and in other cases specify within certain limits a group of compounds it might be.

Fig. 12

Side View of Chromatographing Chamber

In order to do this, however, it is quite obvious that one must have a method of locating the compound on the paper. This is essential; without it the method of paper chromatography is of no use. A number of methods have been used, and one of themajor reasons for the success of paper chromatography for the separation of amino acids is that there exists an almost universal reagent for amino acid identification, namely triketohydrindene hydrate (ninhydrin). When ninhydrin in alcohol solution is sprayed on the paper and the paper is placed in a warm air current, a purple color develops wherever there is an amino acid. By use of this method one can locate almost all the amino acids on the paper.

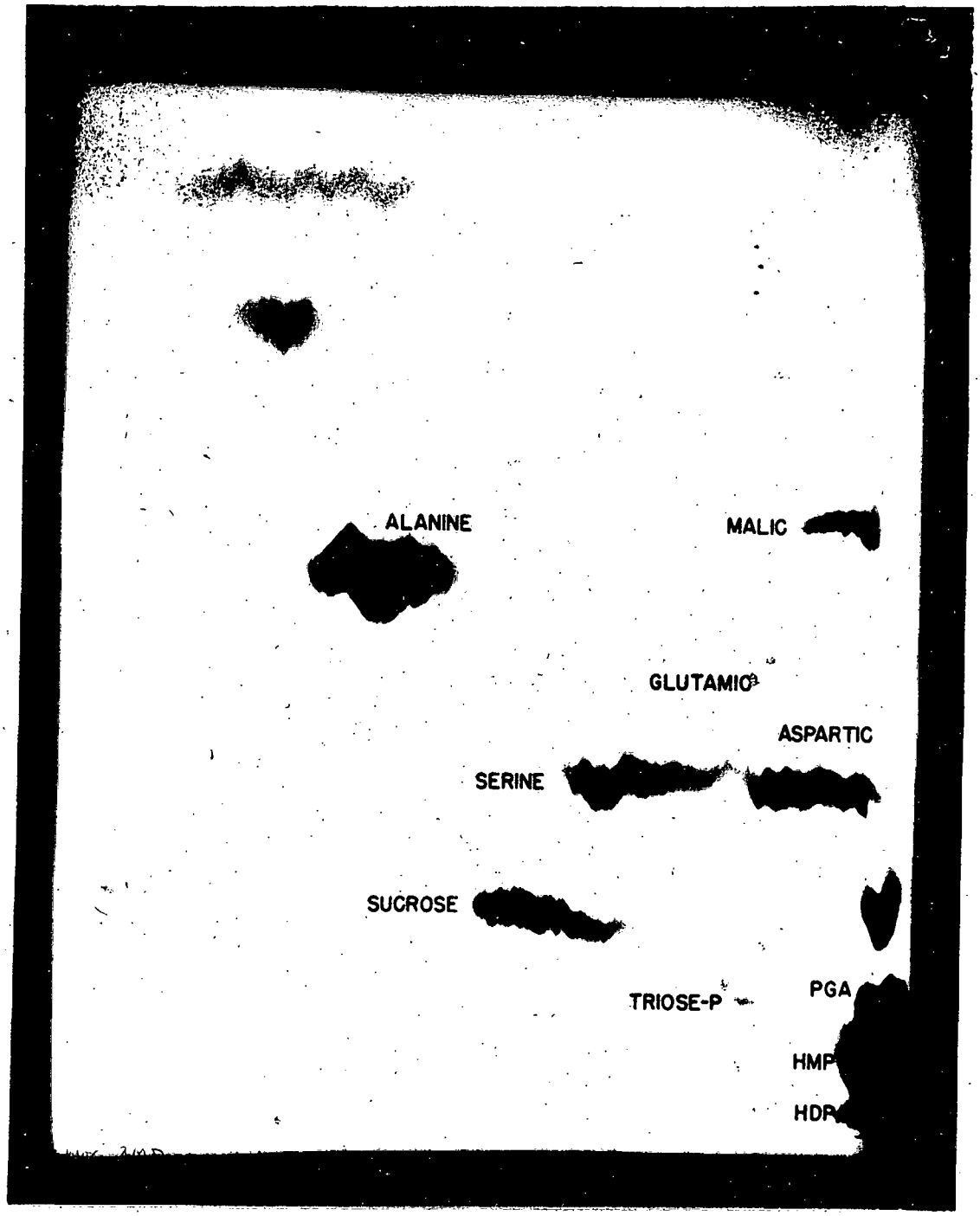
Another method that has been used, with some degree of success, is fluorescence. When the paper is thoroughly dried and it is good clean paper to start with, one can sometimes distinguish compounds by the fluorescent areas they produce on the paper by holding the paper in an ultraviolet light beam.

There have been a few other reagents that have been used with some success in locating specialized compounds. For example, a reducing sugar can be located with a couple of reagents. Naphtharesorcinol locates the keto-sugars, fructose and sucrose, very nicely since they form red spots. The molybdenum blue test can be used for certain forms of phosphate; ammonium molybdate is sprayed on the paper followed by the addition of a buffer solution which will give a blue color for inorganic phosphate. Very easily hydrolyzable organic phosphate will also show up because the molybdate is sprayed on in a nitric acid solution which hydrolyzes the phosphate to form inorganic phosphate. We have used all of these methods, but none of them is universal for all compounds.

However, we do have a method in our particular case which is universal for all compounds, particularly the ones we wanted to find. We are looking for those compounds which contain the radioactive carbon. They may be found by laying the filter paper down on a table and going over it with a Geiger counter to determine where the radioactivity is on the paper. As a matter of fact, we do this very often, but the resolving power of this procedure per unit time is very small. It would take a long time to define all of the areas which are radioactive on the paper by this procedure.

Fortunately, there is another easy way of doing this all in one operation, and that is simply to lay an X-ray film on the filter paper, press it down flat to make good contact with the chromatogram and let it expose for a given period of time, depending upon the amount of radioactivity that is on the chromatogram. Then, the X-ray film is removed and developed. Everywhere there is a radioactive compound on the chromatogram there will be a dark spot on the film. Fig. 13 shows such a result.

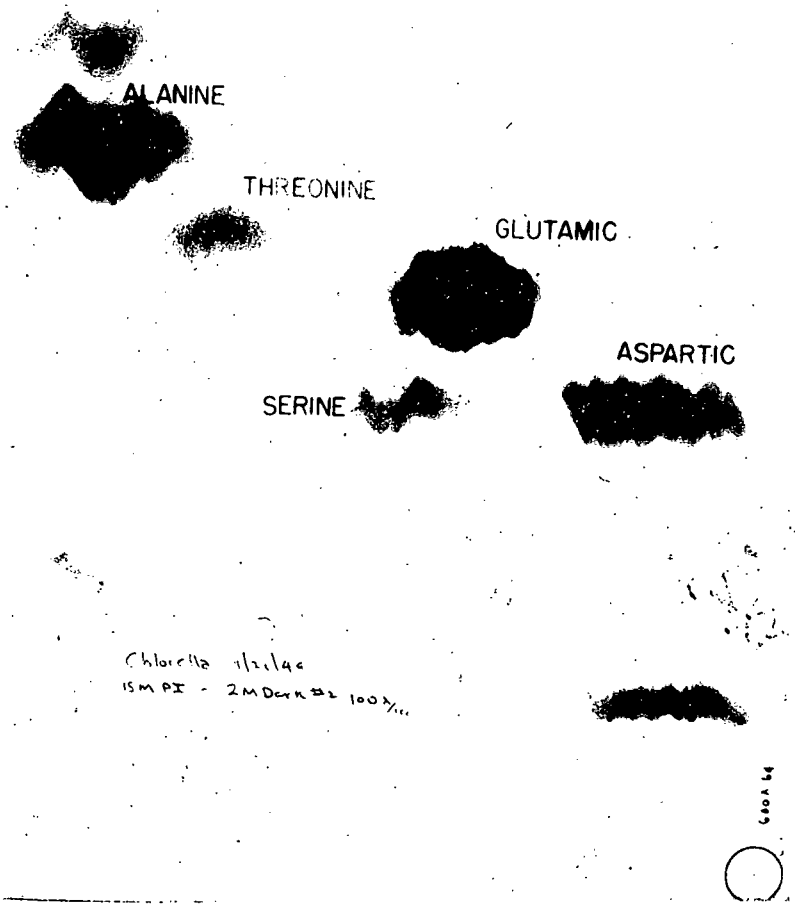
Fig 13a shows the paper chromatogram itself after it has been sprayed with ninhydrin to locate the amino acids which are present in this particular extract. Before spraying the paper, we laid on an X-ray film for a couple of days and took the X-ray film off and developed it, resulting in Fig. 13b. Here you will notice fifteen or twenty black spots corresponding to radioactive areas on the paper. After the paper is sprayed, the exposed X-ray film is laid down on the paper. The perfect correspondence between a radioactive spot on the film and the ninhydrin spot on the paper which is alanine is evident. A similar correspondence is to be noted for aspartic acid, serine and glycine. Note also the absence of any radioactivity corresponding to the rather large ninhydrin spot for glutamic acid.



Method of Identification of Radioactive Amino Acids

Fig. 13a

The paper showing ninhydrin spots corresponding to those amino acids present.



Method of Identification of Radioactive Amino Acids

Fig. 13b

Film obtained from paper of 13z showing radioactive compounds.

You will notice that there are many spots on this film in areas which do not correspond to amino acids. Our major problem is the identification of these spots, especially the group near the origin which contains such large amounts of radioactivity. As was mentioned earlier, there are spot tests for a few other compounds. Using the naphtharesorcinol test for sucrose together with separate chromatograms of genuine sucrose it was relatively easy to demonstrate that one of these unknowns corresponded to sucrose.

The spots near the origin, however, were not so easy to identify. The very fact that these spots are down near the origin tells us something very definite about the properties of these molecules. Whatever these substances are, they are extremely water soluble and very insoluble in organic solvents. If their properties were otherwise, they would have been found farther from the origin on the paper. The lipids, for example, or anything that has a very large solubility in organic solvents, will move a long way on the chromatogram.

We had indications from the ion exchange work and from the extraction properties that there were sugar phosphate and various phosphate esters present. In order to find out where on the paper one would find these phosphate esters we did a rather obvious experiment. We allowed the algae to photosynthesize in radioactive phosphorus (Phosphorus 32) for a period of time and made a radiogram of these extracts which would thus show only the phosphorus compounds. Fig. 14 is a radiogram (radioautograph of a paper chromatogram) made of an extract of Scenedesmus algae which had been photosynthesizing for one hour in radiophosphorus; the black spots were made by radiation from phosphrous and not from carbon and each of these spots corresponds to a phosphorus compound of some sort. Thus, it is evident that

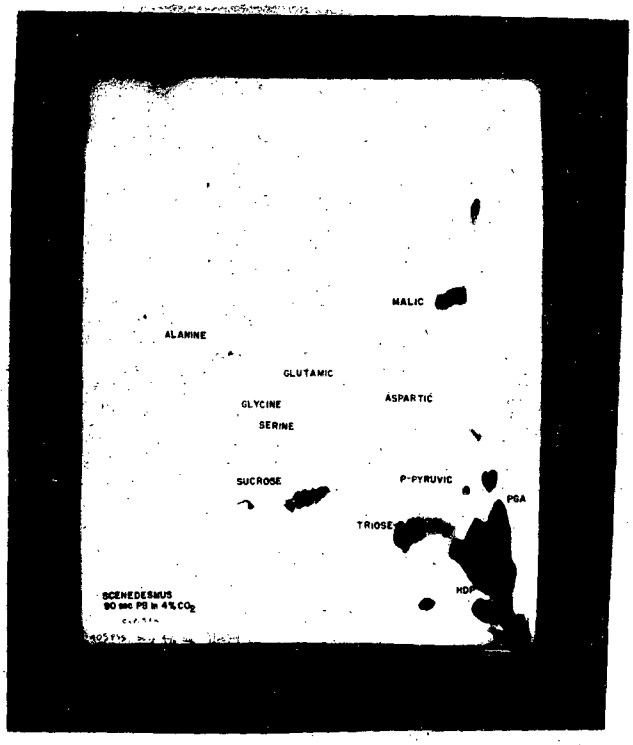
Fig. 14

Radiogram showing position taken by phosphorus-containing compounds.

most of the phosphorus compounds are where we expected them to be.

In order to be more specific a number of known phosphate esters were prepared radioactive by a yeast fermentation in radiophosphorus. We isolated the fructose-diphosphate, fructose-6-phosphate and phosphoglyceric acid and made a one-dimensional chromatogram in the butanol direction of the mixture. This radiogram is shown in Fig. 15 beside one from algae photosynthesizing in $C^{14}O_2$. This correspondence all by itself is no proof of the identity of the photosynthesized compounds.

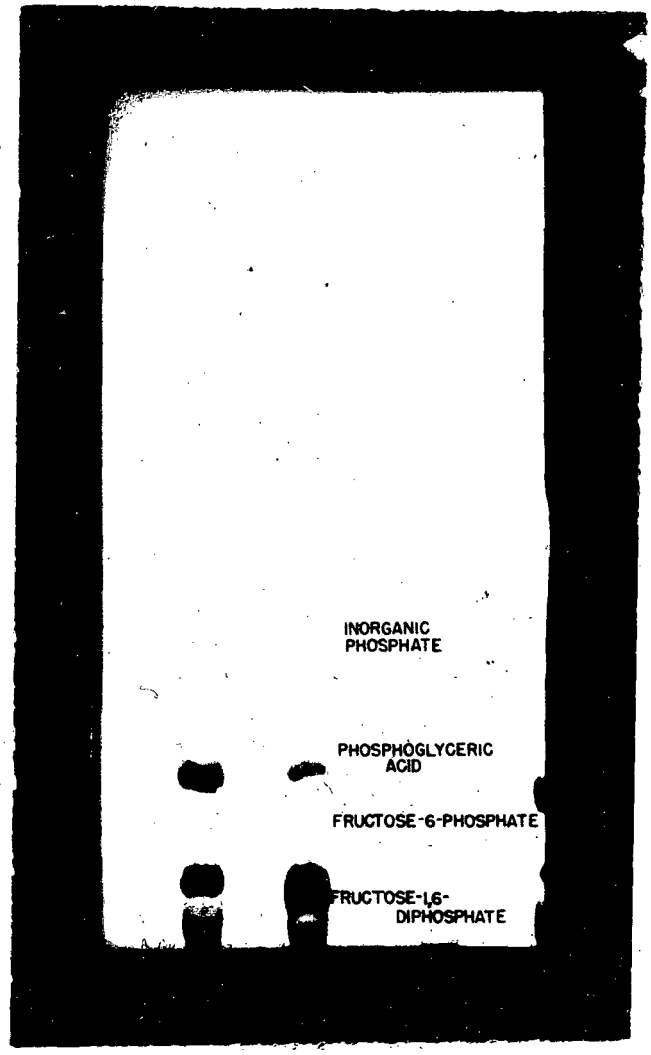
This can be confirmed in a number of ways. Perhaps the best way is to cut out a spot, elute it and mix it with a little of a known substance. Then run a chromatogram on the mixture in two directions and see if there is any separation between the carbon radioactivity on the one hand and the phosphorus radioactivity on the other. It is very easy to tell the difference between the two radioactivities. The radiation from Carbon 14 does not penetrate through the X-ray film, whereas the radiation from Phosphorus 32 is a much stronger beta-ray and goes right through the X-ray film. Since the X-ray film has emulsion on both sides, one can tell which one is carbon as it exposes only one side of the film while the phosphorus exposes both sides of the film. This sort of thing has been done to demonstrate the presence of fructose-diphosphate, glucose-1-phosphate and fructose-6-phosphate. Both glucose-1-phosphate and fructose-6-phosphate come in the area marked HMP; they do not separate very well. The presence of G-1-P (glucose-1-phosphate) in the photosynthesis experiment has been further demonstrated by the appearance of free glucose after 10 minutes hydrolysis in 0.1 N hydrochloric acid. Mixed chromatograms have also been made for phosphoglyceric acid.



Comparison of known phosphates with compounds formed in photosynthesis.

Fig. 15a

Two-dimensional radiogram showing products formed from $C^{14}O_2$.



Comparison of known phosphates with compounds formed in photosynthesis.

Fig. 15b

One-dimensional chromatogram of radioactive phosphate prepared by yeast fermentation.

We have thus identified about fifteen compounds as having been formed in 90 seconds of photosynthesis and these contain over 90% of the carbon fixed in that time. However, this information by itself allows very little to be said about their sequence of formation. There are still many compounds even in as short a period as 30 seconds (Fig. 16). Not until the time of photosynthesis is reduced to 5 seconds do we have only four or five compounds formed (Fig. 17). Most of the radioactivity fixed in 5 seconds is in the two lowest spots. It would appear, then, that these are the first compounds into which radiocarbon is incorporated by the green plant. Their position corresponds with that for which we already had evidence was the phosphoglyceric acid area, thus indicating that in 5 seconds the material constituting 65% of the total fixed radioactivity is phosphoglyceric acid.

Carrier-free Isolation of Phosphoglyceric Acid: - It seemed wise at this stage to make at least one point of contact with the classical methods of identification, that is, a direct isolation and determination of properties of the type to which most chemists are accustomed. It should be mentioned that if the spot on the right (Fig. 17) is heated in 0.1 N hydrochloric acid for 1-1-1/2 hours it becomes the spot on the left. Thus, they are related, and our suggestion was that the one on the right was 2-phosphoglyceric acid and the other was 3-phosphoglyceric acid.

Furthermore, if the temperature of the algae is lowered to about 4° when they are given radioactive carbon dioxide for about 10 seconds, one gets a single spot which is the one labeled 2-phosphoglyceric acid. We thus have, tentatively, the following scheme representing the first steps in The Path of Carbon in Photosynthesis.

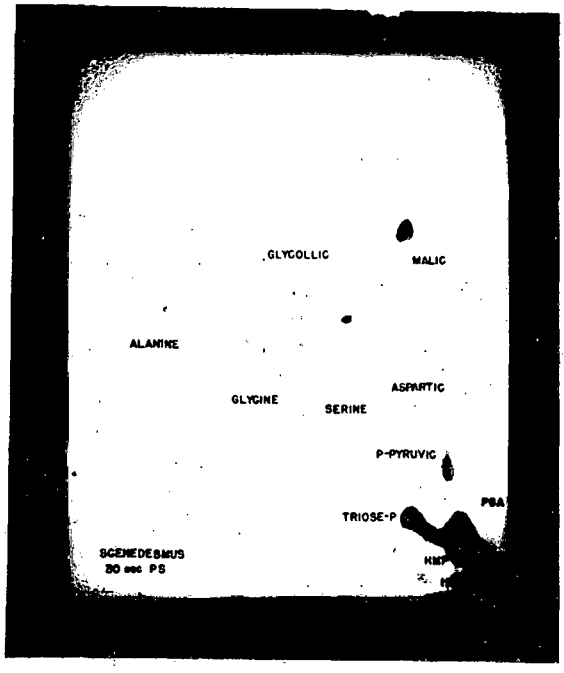


Fig. 16

Compounds formed in 30 seconds by Scenedesmus

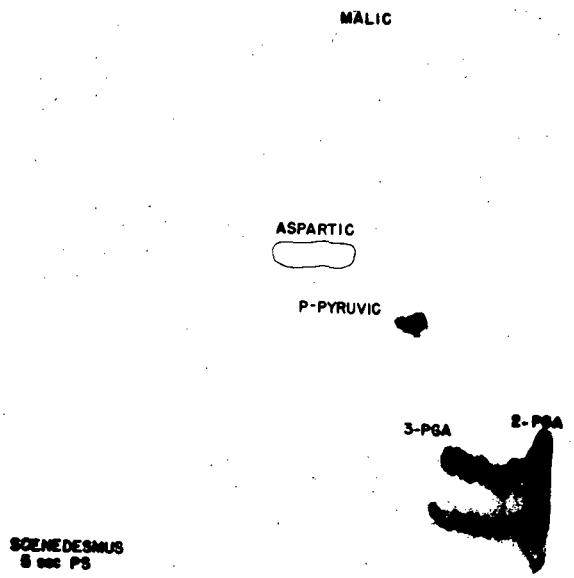
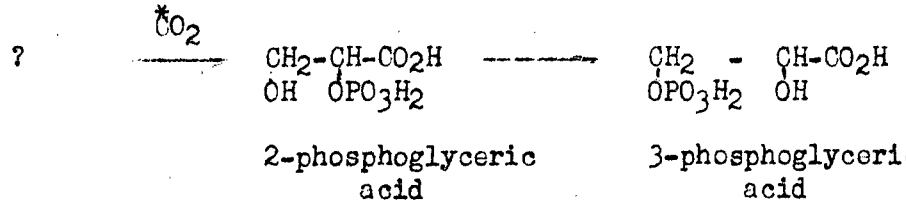


Fig. 17

Compounds formed in 5 seconds by Scenedesmus.



It was obvious that we had now established the conditions for preparing from the plant a single compound or, at most, a pair of compounds which would represent the earliest products of incorporation of carbon dioxide into the plant. Although we had this body of evidence already mentioned, that these two products were 2-phosphoglyceric acid and 3-phosphoglyceric acid, we thought it desirable to go about their identification by the more usual methods of isolation.

We prepared 25 cc. of packed algae which had been exposed to radiocarbon in the light for 5 seconds corresponding to Fig. 17. We proceeded to isolate that radioactivity on the assumption, partly at least, that it was phosphoglyceric acid. Now I will outline for you very briefly what that procedure was. An extract of the algae was made using a mixture of concentrated hydrochloric acid and glacial acetic acid. If the product were phosphoglyceric acid, the acetic acid-hydrochloric acid mixture would not hurt it in any way because the phosphoglyceric acids are very stable compounds. One of the reasons for killing the algae with hydrochloric and acetic acids was to get rid of as much of the protein and cellulose as possible in the early stage and not to include the lipid material and pigment, which occurred if we killed them in alcohol. We thus obtained a perfectly clear, colorless solution containing all the radioactivity. This extract of the 25 cc. of cells was taken almost to dryness to get rid of the hydrochloric and acetic acids, and then taken up again in 8-10 cc. of water. Although most of the acid was gone it was still an acidic solution. The pH was adjusted to 7 by adding 1 N alkali. There was a very heavy flocculant precipitate (histone-

like material) which carried with it most of the radioactivity at pH 7. If the pH is raised to 9, the radioactivity redissolved in the supernate. This was interpreted to mean that the precipitated material forms a salt with the phosphoglyceric acid which carries it out of solution at pH 7, the phosphoglycerate ions being displaced into the solution at pH 9.

This gelatinous histone-like material was centrifuged out and we had a clear supernate containing nearly all of the radioactivity. The solution is brought back to an acid condition, about pH 1, and barium chloride is added to take out some acid-insoluble barium salt. Here, again, a great deal of the radioactivity comes out with the barium salt; it was washed thoroughly with warm dilute hydrochloric acid to wash the activity back into solution out of the precipitate. The precipitate does not dissolve, but the radioactivity does come out when washed with warm dilute hydrochloric acid. The supernate, together with the washings, is then made alkaline to get a trace of an alkali-insoluble barium salt. At this point, the volume is great enough to retain the small amount of barium phosphoglycerate. The solution is now made up to 50% alcohol and the radioactivity comes out in a barium salt. The barium salt is redissolved in 0.05 N acid and made up to 50% alcohol to reprecipitate it, and this operation is repeated half a dozen times. As the operation is repeated, the precipitate begins to appear crystalline. Finally, we recovered 10 mg. of the salt which contained one-third of the initial radioactivity. The last four supernates from these crystallizations contained another 35% of the radioactivity, making a total of 65-70% of the radioactivity in this white crystalline material which had 8.6% phosphorus compared to 8.7% for barium phosphoglycerate. It had a molar rotation in molybdate solution of $-650 \pm 100^{\circ}$, the reported rotation for 3-phosphoglyceric acid being -720°. The rotation of the pure solution is

measured and then ammonium molybdate is added. The molybdate greatly enhances the rotation and this has been described by Meyerhof as almost specific for 3-phosphoglyceric acid. A sample of this isolated phosphoglyceric acid was chromatographed and it appeared in the proper position.

A final characterization of this material was made as follows. We obtained from Prof. Carl Neuberg a sample of 100 mg. of the barium salt of what he said was pure 3-phosphoglyceric acid. We absorbed it on a column of strong base anion resin in the chloride form and eluted that genuine sample of 3-phosphoglyceric acid with 0.2 N sodium chloride. The elution curve is shown in Fig. 18. It is evident that it was not quite pure 3-phosphoglyceric acid; there was a little easily eluted phosphorus, but a very small amount. Most of the phosphorus came out in a single band at an eluate volume of 6.2 cc. This constituted the calibration of this particular column. Five milligrams of our isolated material was added to an equal amount of Neuberg's phosphoglyceric acid to make a total of 10 mg. This mixture is absorbed on the identical column and the same elution with 0.2 N sodium chloride is performed with the results shown in Fig. 19. The solid points represent the total phosphorus coming out per unit volume of eluate. Note that in addition to the trace of impurity added with Neuberg's salt there is only a single peak representing the bulk of both salts. The open circles represent the radioactive carbon counts per unit volume of solution coming through, and you see that the radioactivity coincides exactly with the 3-phosphoglyceric acid curve. Thus, not only is there only a single peak for the mixture but the radioactivity coincides with it. This, taken with the paper chromatograms, the optical rotation, the extraction coefficient and derivative formation is about as complete a comparison between two compounds, one authentic and one unknown, as need be made to determine identity without doing an absolute structure determination.

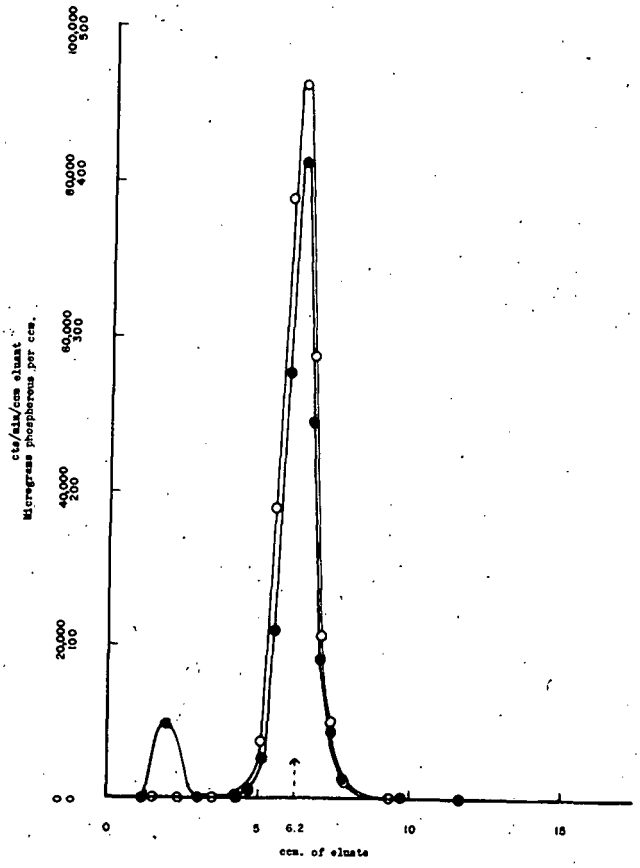


Fig. 18

Elution curve of 3-phosphoglyceric acid (Neuberg) from (6 x 80 mm.) column of Dowex A1 resin in the chloride form. Eluant is 0.2 N sodium chloride (neutral)

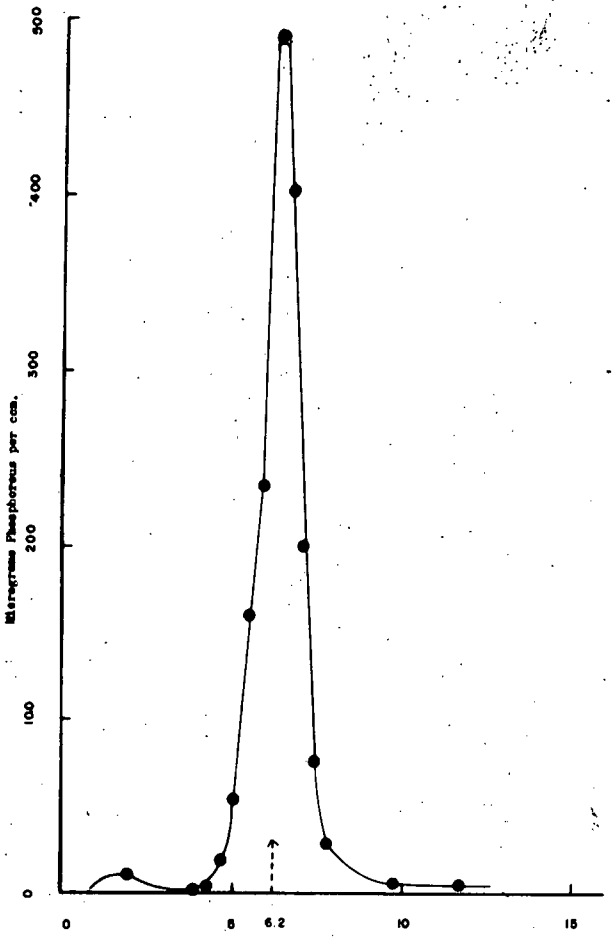


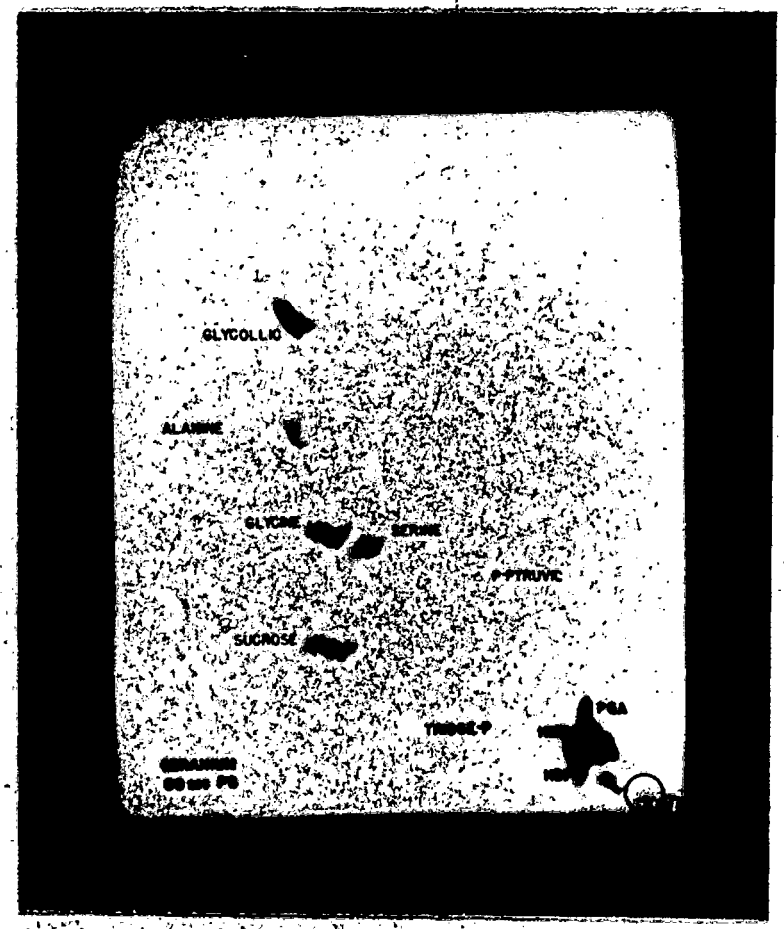
Fig. 19

Elution curve of equal mixture of unknown with authentic barium 3-phosphoglycerate. Conditions identical with those of Fig. 18. Solid points, phosphorus elution; open circles, radioactivity elution.

Thus, we have evidence that the first compound to appear in the fixation of carbon dioxide by the green plant is phosphoglyceric acid. The same kind, but not nearly as complete a determination, has been adduced for all of the other compounds. Many of them we had to synthesize directly with radioactive carbon in order to determine their position and make mixed radiograms with the suspected compounds. The next two figures (Fig. 20 and Fig. 21) will show some of the other chromatographing that has been done using barley and geranium leaves.

We were beginning to get some clues as to the order of appearance of compounds in the process of photosynthesis. Fig. 20 is one showing 60 second photosynthesis for geranium leaves. This is an interesting one as it shows the effect of solvent. The one on the left was done with the solvents used for most of the other chromatograms and the phosphate groups did not separate well. The one on the right is the same extract developed with a different solvent in a vertical direction. The solvent had a little more water in it which made the phosphate areas spread out more; the hexose diphosphate has separated from the hexose monophosphate which, in turn, has separated from the phosphoglyceric acid. The hexose monophosphate area actually consists of three spots. Obviously, one should use different solvents for different areas or different types of compounds.

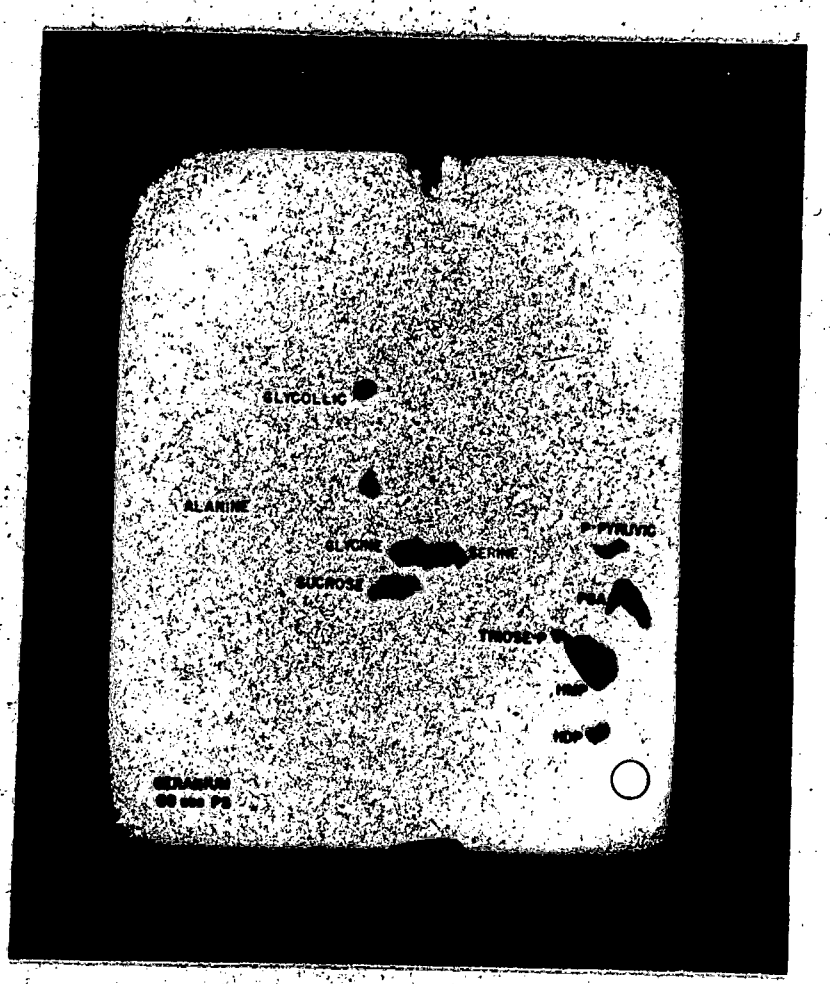
Now that we have identified some twenty compounds in the process of photosynthesis and we have shown which are the first two or three of them, we can make some guesses as to what the order of appearance was. There is another set of data which can be obtained which is even more crucial and critical in defining the Path of Carbon in Photosynthesis, and that is not only the determination of which compounds contain the radioactive carbon atoms but also which atoms in the compounds are the radioactive ones.



Compounds formed by geranium leaf in 60 seconds.

Fig. 20a

Vertical direction was run in the usual solvent (butanol-propionic acid-water)



Compounds formed by geranium leaf in 60 seconds.

Fig. 20b

Vertical direction was run in butanol-acetic-acid-water.

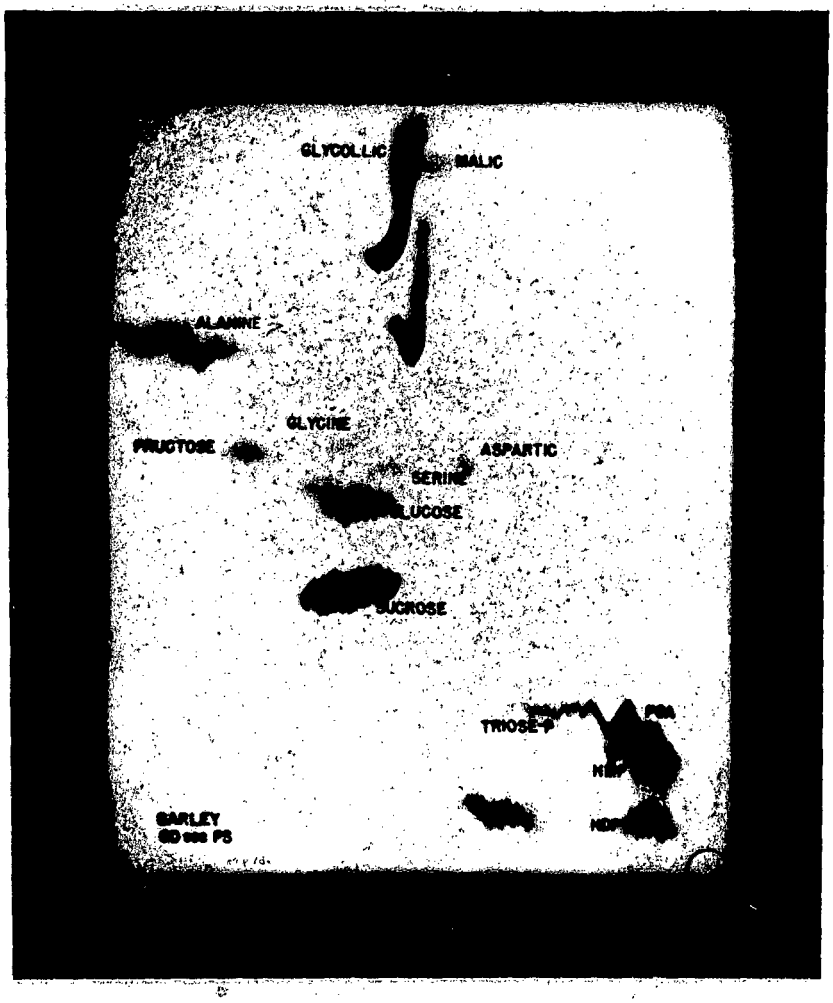


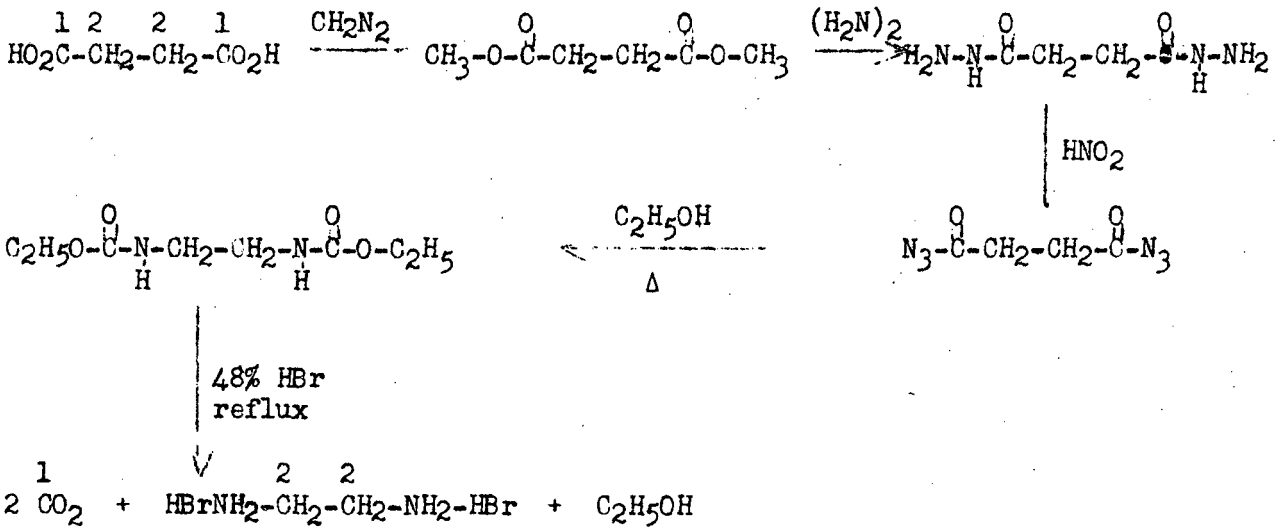
Fig. 21

Compounds formed by barley leaf in 60 seconds.

DISTRIBUTION OF RADIOACTIVITY WITHIN THE COMPOUNDS

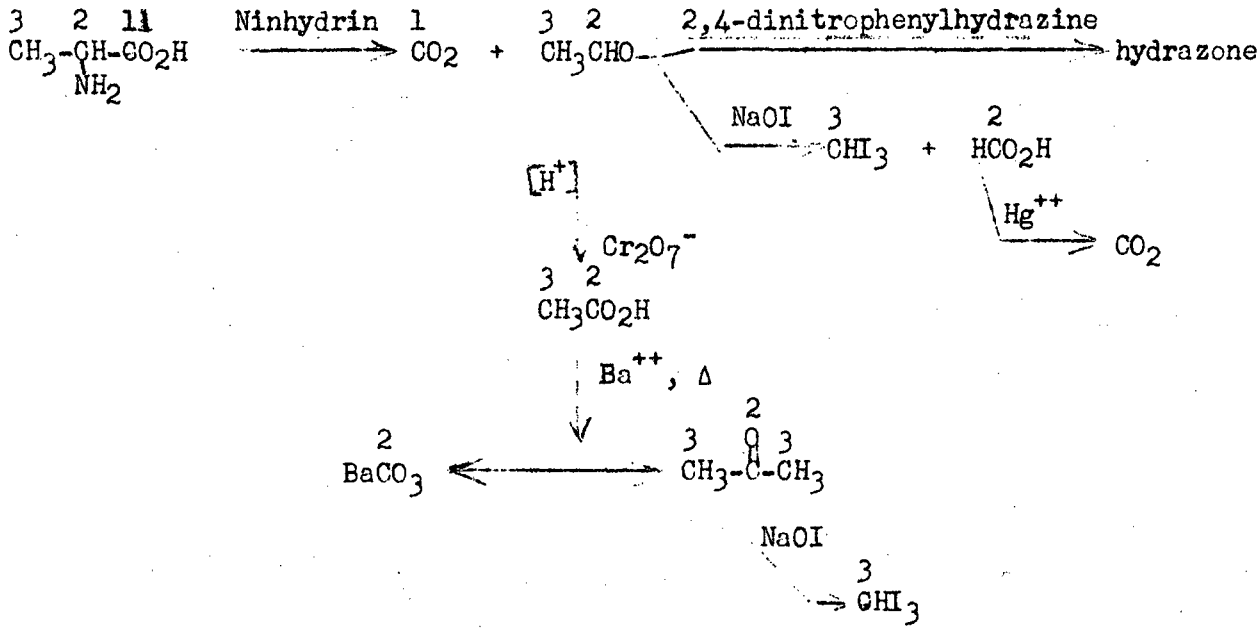
In order to do this we will have to devise degfadative methods for several of the various compounds that are shown in the radiograms which will give us one carbon atom at a time specifically.

Degradation of Succinic Acid: - The Curtius degradation of succinic acid has been modified to give good yields on small amounts. Methyl succinate, prepared using diazomethane, is converted to the diqzide through the dihydrazide. Rearrangement of the diazide in ethanol gives ethylenediurethan which was hydrolyzed to give carbon dioxide from the carboxyl groups and ethylenediamine from the methylene groups of the original succinic acid.

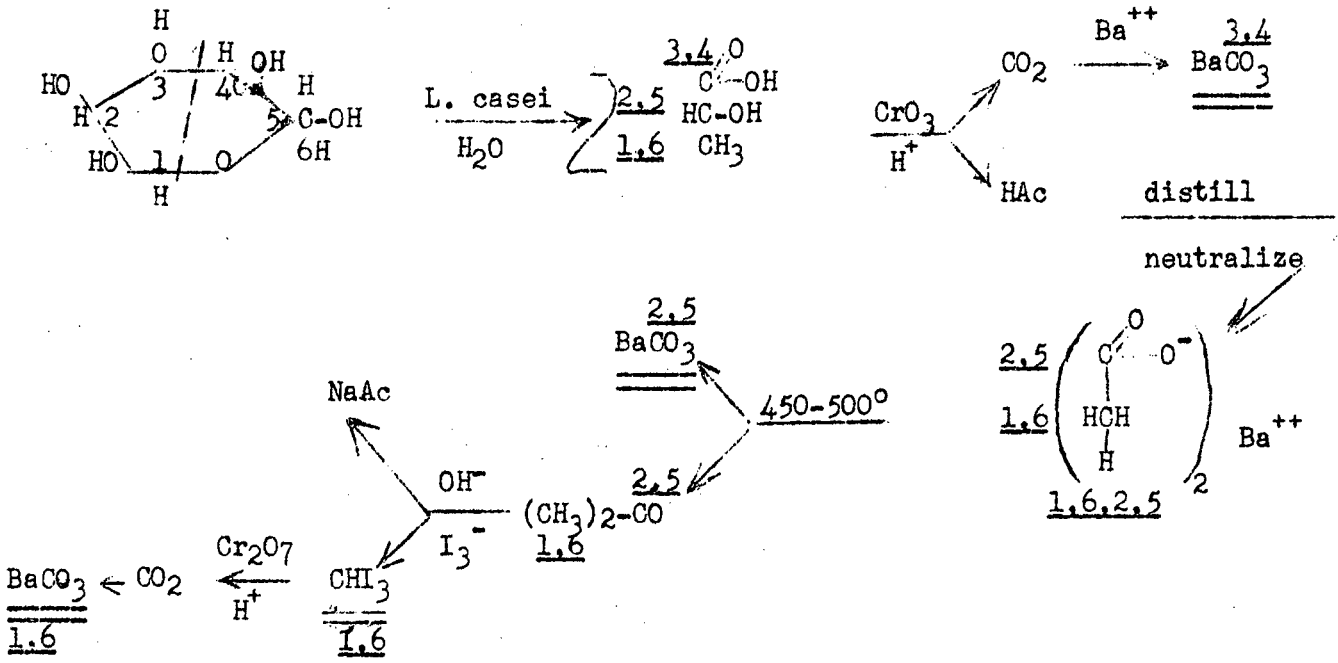


The carbon dioxide from the carboxyl groups is counted as barium carbonate and the methylene carbons are counted as ethylenediamine dihydrochloride.

Degradation of Alanine: - Alanine has been degraded by either of the following methods:



Degradation of Hexose: - There are chemical methods of degrading the hexoses that have been used, but the one used here is somewhat easier. The hexose is fermented by Lactobacillus casei and it can be shown, and has been shown by chemical degradation of the methyl glucoside, that the lactic acid which is formed from the glucose by L. casei represents the carbon atoms in the following way:



* Doubly underlined compounds are those upon which measurements were made.

Indepedent chemical degradations carried out by Wood at Western Reserve University showed that the radioactivity was the same in each of these pairs, that is, the three- and four-, two- and five- and one- and six-carbon atoms.

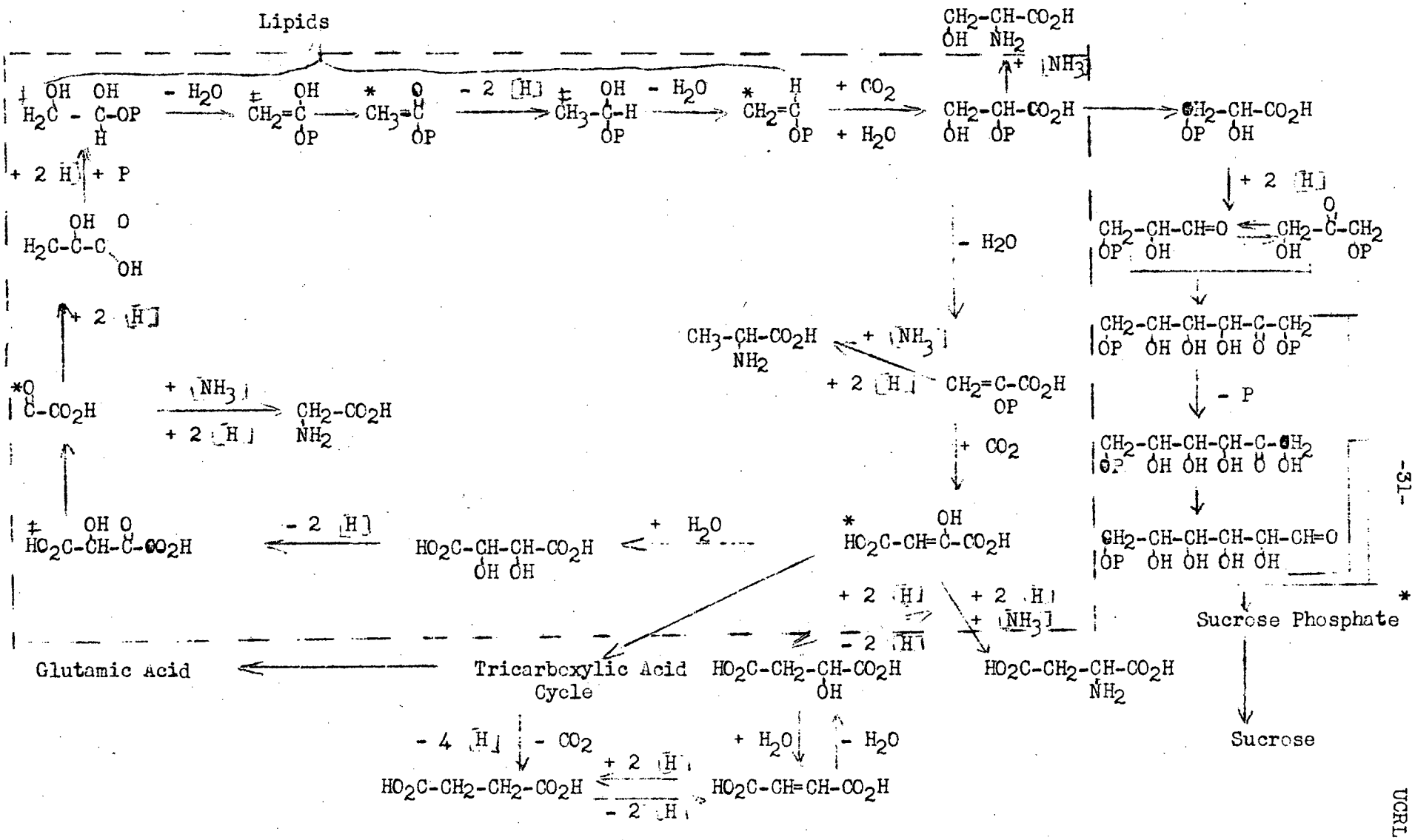
Degradation of Malic Acid: - Malic acid and aspartic acid have been oxidized with chromic acid to yield two molecules of carbon dioxide from the carboxyl groups and one molecule of acetic acid from the alpha- and beta-carbon atoms.

$$\underset{4\ 3\ \ 2\ \ 1}{HOOC\text{-}CH_2\text{-}CHOH\text{-}COOH} \xrightarrow{CrO_3} \underset{1 \text{ and } 4}{2\ CO_2} + \underset{3\ \ 2}{CH_3\text{-}COOH}$$

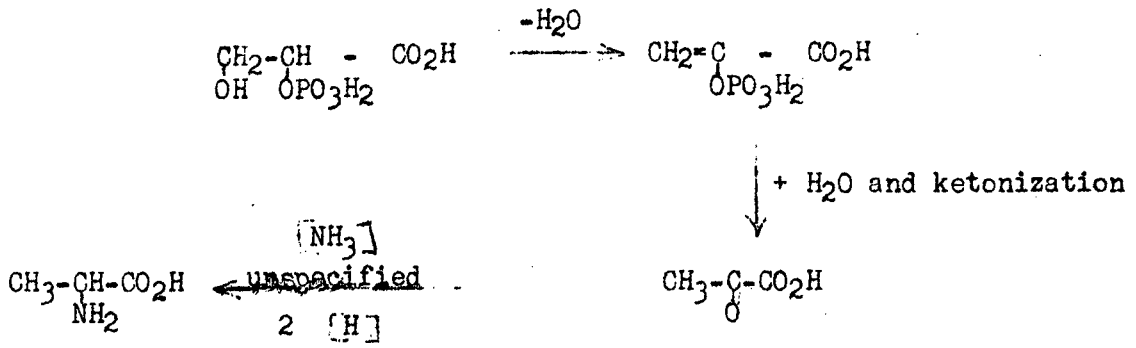
These constitute about all the degradations that we have done up to this time, but you will see how it was possible with just this much information to be able to deduce the necessary attributes of The Path of Carbon in Photosynthesis.

The Argument: - The first thing we discovered was that the six-carbon sugar achieved its radioactivity in a very definite pattern. The first carbon atoms that become radioactive, shown in a 30 second photosynthesis, are the three- and the four-carbon atoms. As the photosynthesis proceeds for a few more minutes, the two- and five-acquire radioactivity and finally after a longer period (of the order of 5 minutes) we can see radioactivity in the one- and six-positions. This is a most important sequence of events, and I might s y that this result has also been observed by Wood at Western Reserve University and Burr at the University of Hawaii. In the succinic acid, first the carboxyl groups become radioactive and then the methylene groups. The same sort of thing holds for malic acid, and presumably since the malic acid consists of four different carbon atoms we should find four different orders of radioactivity. However, we did not degrade the malic acid in such a way as to determine this; all we did was to break it down into the two carboxyl groups and the two center atoms and exactly this pattern appeared, first on the end and then in the middle. The alanine is, perhaps, one of the most interesting compounds. It shows a radioactivity pattern as follows: first the carboxyl group, then the alpha-carbon atom and finally the beta-carbon atom appear radioactive.

These, then, are the actual facts as we have them at this time. What can we deduce from them? Remember that we have found two-, three- and four-carbon fragments prior to the sugar. We do not see five- or six-carbon acids. The first compound we see is 2-phosphoglyceric acid which is very closely related to the three-carbon alanine and it will, in all probability, have very nearly the distribution that the alanine has. The link between them is certainly through the phosphopyruvic acid, which is already evident in the 5-second experiment, and a rapid reductive equilibrium.

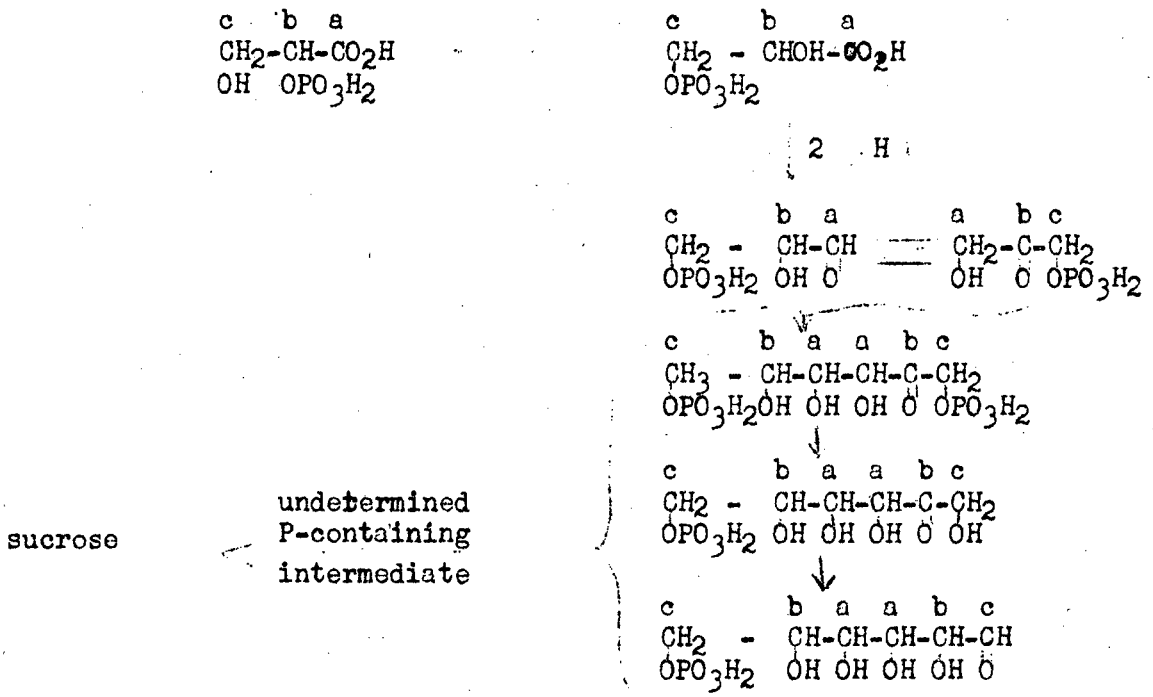


* There is indirect evidence for these compounds (marked with an asterisk), but their presence has not been unequivocally demonstrated.
These compounds (marked with a dagger) are as yet completely hypothetical and are inserted to complete the cycle.
All unmarked compounds have been unequivocally identified except FDP, G-6-P.



Furthermore, we have degraded the aspartic acid and it has almost exactly the same distribution as the malic acid.

Keeping in mind the distribution of radioactivity in the hexose and in the three-carbon compounds and noting that most of the radioactivity fixed in a short time (30 seconds) is found in the hexose monophosphates, hexose diphosphate, triose phosphate and phosphoglyceric acids, it seems fairly certain that the six-carbon skeleton is formed by a combination of two three-carbon compounds. Since the only compounds involved are those of the accepted glycolytic sequence, the obvious conclusions is that the hexose is formed by a simple reversal of this sequence.

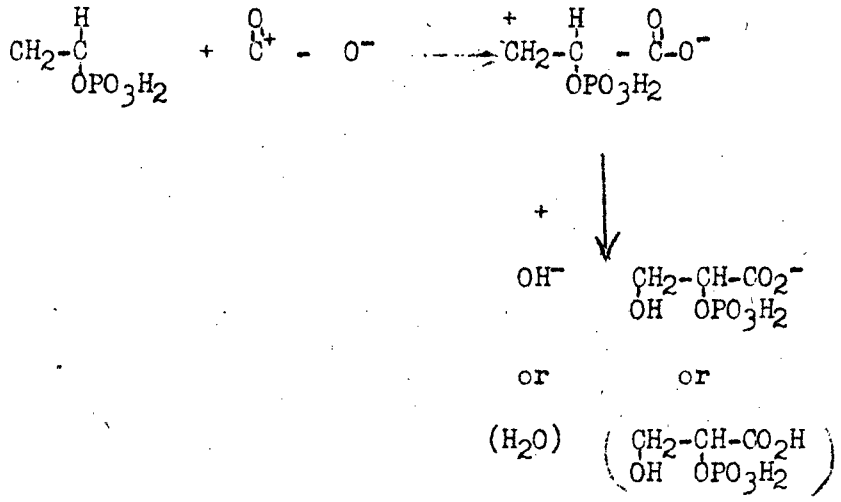


A confirmation of this suggested sequence was found in the fact that the sucrose formed in 30 seconds photosynthesis is made up of fructose having at least twice the specific activity of the glucose. After longer periods of photosynthesis (~90 seconds) the glucose and fructose moieties of sucrose have equal specific activities. Since all of the intermediates are originally present unlabeled in the plant, the fructose derivative which is labeled before the glucose part will at first find only unlabeled glucose compounds with which to combine, thus producing initially the unsymmetrical sucrose. It is of interest to note here that the sucrose appears radioactive without the appearance of free radioactive glucose or fructose, indicating that it is formed not from free hexose but from some hexose derivatives, probably phosphates. It is thus quite easy to see how the distribution of the radioactivity in the hexose is achieved by the combination of two properly labeled triose phosphates formed from the 3-phosphoglyceric acid, a reversal in sequence of the well-known glycolytic, or respiratory, reactions.

The problem now is to construct the three-carbon compounds in such a way that the carboxyl carbon will be the first one that is radioactive followed by the alpha-carbon atom and finally the beta-carbon atom. Remember that the very first thing we see is 2-phosphoglyceric acid, and the question is thus directed to this compound. If the first compound one sees is a three-carbon fragment and if one of the reactants is carbon dioxide, a one-carbon fragment, it seems only reasonable and, as a matter of fact hardly possible otherwise, that the other reactant must be a two-carbon fragment, either free or very loosely attached to something larger. Now, what is this two-carbon fragment and how is it formed? Furthermore, not only must it be formed but it must get there continuously, i.e., it must be continuously

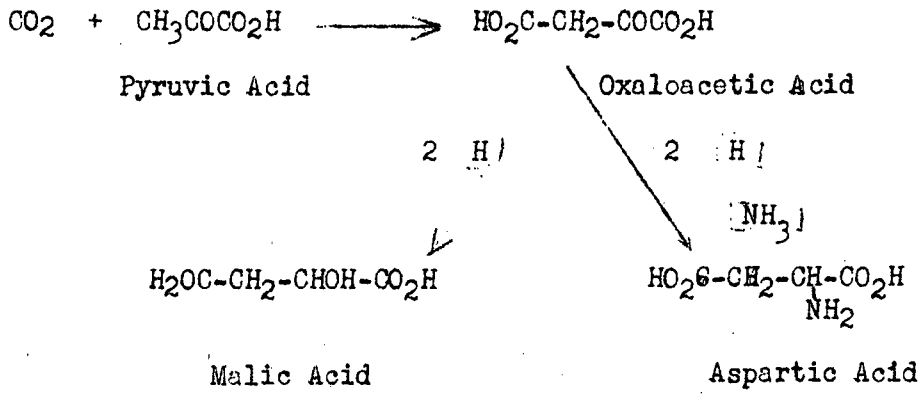
regenerated from other early intermediates. In addition, the two-carbon atoms must not be equivalent. The one to which the carbon dioxide is to be attached must acquire radioactivity before the other one does.

Although we have not as yet unequivocally established the precise structure of this two-carbon carbon dioxide acceptor, there are two arguments which are very suggestive that it might well be vinyl phosphate. The first of these is the structure of the first observable carbon dioxide fixation product itself, 2-phosphoglyceric acid, viewed in the light of the generally simple nature of the individual reaction steps of any biological synthesis. Its synthesis from vinyl phosphate and carbon dioxide can be formulated simply as follows:



The second is an experimental observation that extracts of organisms which have been photosynthesizing for one minute or more contain an originally non-volatile compound, which after 10 minutes hydrolysis in 1 N hydrochloric acid at 80° C, liberates radioactive acetaldehyde. It is difficult to visualize many things which would behave in this manner beside a non-volatile vinyl ester, presumably of phosphoric acid. Since this accords so well with the other compounds so far identified in this scheme, we are tentatively calling it vinyl phosphate until further experiment confirms or denies it.

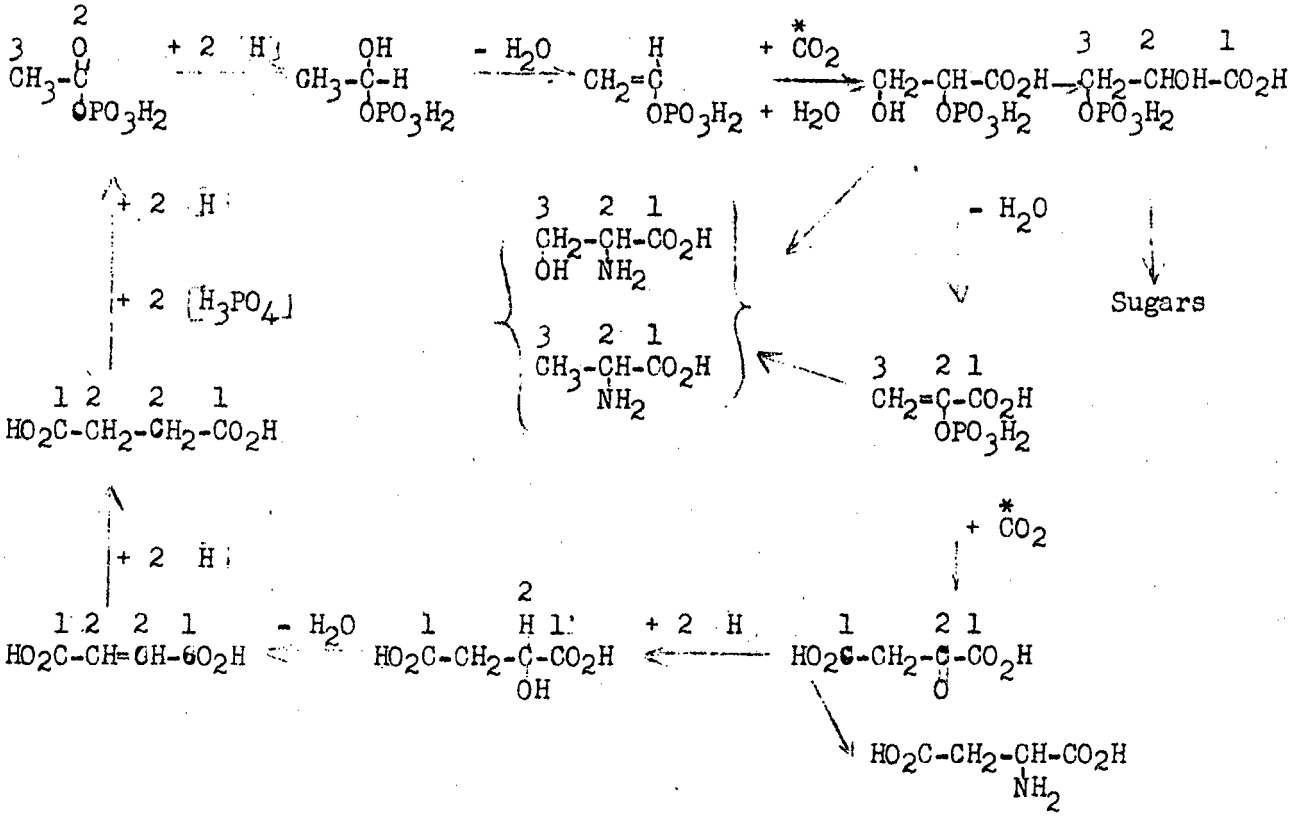
Recall that the only compounds that we have seen other than the sugars are the two-, three- and four-carbon fragments, and that the very first product is a three-carbon fragment which is formed by the addition of carbon dioxide to a two-carbon fragment. The next things we see are two four-carbon compounds, malic and aspartic acids, which are very closely related to oxaloacetic acid. The latter compound, too unstable to appear in the paper chromatograms, is readily formed through carbon dioxide fixation by pyruvic acid (or simple derivative), the well-known Wood-Werkman reaction.



Since no larger carbon skeleton is found, it seems altogether reasonable and, in fact, impossible otherwise that a four-carbon compound must be split to a two-carbon fragment ultimately to regenerate the two-carbon carbon dioxide acceptor.

Since oxaloacetic acid would be the first four-carbon compound to be formed, it now remains for us to discover by what route this is converted to vinyl phosphate. In our early work with Chlorella, we had seen, in addition to malic and aspartic acids, small amounts of radioactive fumaric and succinic acids, but had not identified any radioactive two-carbon compounds on the chromatograms. This led to the supposition that the two-carbon compounds were volatile or easily decomposed to give volatile substances such as

acetate and acetyl phosphate as well as acetaldehyde and vinyl phosphate. Since a direct relationship between acetate and succinate has been suggested in the literature many times, especially in connection with yeast fermentation, we proposed to use it as the splitting reaction to regenerate the two-carbon compounds, thus completing the cycle.



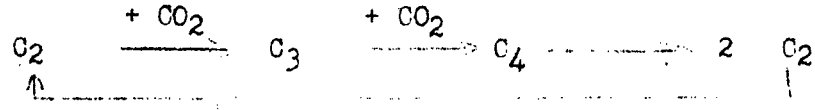
The Path of Carbon in Photosynthesis

An examination of this cycle shows how the distribution of radioactivity among the carbon atoms comes about in accordance with the experimental facts. These compounds are all present non-radioactive and the cycle is operating at the instant of the injection of radioactive carbon dioxide. The first reaction in which it takes part is the carboxylation of vinyl phosphate to form 2-PGA (2-phosphoglyceric acid), thus leading to the group of three-carbon compounds labeled in the carboxyl group. The carboxyl-labeled 3-PGA formed from it goes on through the glycolytic sequence to produce 3,4-labeled

hexose. Simultaneously with this carboxylation, another one takes place in a different part of the cycle, possibly at a different rate, to form oxaloacetic acid from pyruvic acid (Wood-Werkman reaction). The oxaloacetic acid thus formed is labeled in the γ-carbon group. This, then, passes on through malic acid to fumaric acid, which being symmetrical is labeled in the carboxyl groups, as is the succinic acid formed from it. By a reductive splitting of the succinic acid between the methylene groups, two molecules of acetate would be formed labeled in the carboxyl group. This, in the form of acetyl phosphate, is then reduced and dehydrated to form vinyl phosphate labeled in the number one carbon atom and the cycle is ready to start over again. Carboxylation of this vinyl phosphate produces 2-PGA labeled in both the numbers one (1) and two (2) carbon atoms. But it is important to note that the specific activities of these two-carbon atoms are as yet very unequal. The radioactivity in the number two (2) carbon atoms has been very much diluted by passing through the reservoir of non-radioactive intermediates which were present to start with, while the newly formed carboxyl group approaches in specific activity that of the carbon dioxide from which it has just been formed. Some of this phosphoglyceric acid, with a high specific activity in the carboxyl group and a lower specific activity in the alpha-carbon atom, can then go on to produce a hexose carbon skeleton having a high specific activity in the numbers two- and five-carbon atoms. Some of it will go to form the other three-carbon compounds, serine, alanine and phosphopyruvic acid, having high specific activity in the carboxyl group and lower specific activity in the alpha-carbon atom. The pyruvic acid so formed is again carboxylated and passes on to form succinic acid labeled in the methylene groups as well as in the carboxyl groups, but, of course, with the lower specific

activity in the methylene groups. It is thus apparent that as the cycle runs, the three-carbon compounds will become labeled in the order (1), (2), (3) with the two-, four- and six-carbon skeletons acquiring radioactivity in a corresponding fashion. This cycle was first proposed about two years ago and can still account for all the presently existing degradation data.

However, as the range of organisms was extended we found less and less of succinic and fumaric acids until in some cases we found alanine labeled in the alpha- and beta-positions and no radioactive succinic and fumaric acids at all. This raised some doubts as to whether this was the only cycle, if it were the cycle at all, by which the plant was operating. Furthermore, especially in the case of barley leaves, very little radioactive malic acid was found but radioactive glycolic acid and glycine appeared. While these facts in no way relieved the requirement for the fundamental sequence



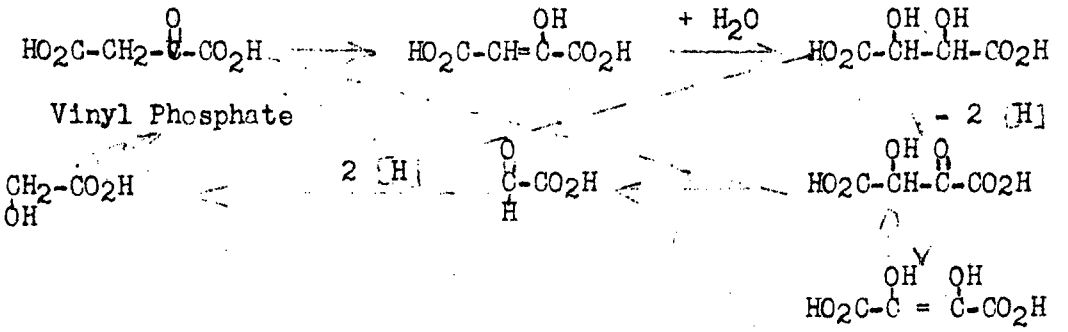
it did suggest that our selection of succinic acid as the four-carbon compound to be split might not be the most important path, if indeed it took place at all.

The appearance of two-carbon fragments which are in a higher oxidation level than acetic acid, namely glycolic acid and glycine which are at the same oxidation level, and glycine presumably representing the presence of glyoxylic acid, suggested that the splitting took place on a four-carbon compound more oxidized than succinic acid. This is not an unwelcome change, since the direct reductive splitting of succinic acid to acetic acid does not all fit into the presently known pattern of chemical reactions.

There are a number of ways in which the split might occur without going through malic, succinic and fumaric acids. For example, oxaloacetic acid may be hydrolyzed directly to produce one molecule of glycolic acid and one molecule of glyoxylic acid. This would account for the presence of glycolic acid and glycine. Although we yet have not identified a glyoxylic acid, the presence of glycine seems very good evidence for glyoxylic acid. The glycolic and glyoxylic acids could go through the reduction cycle in the two-carbon state instead of in the four-carbon state, finally reaching vinyl phosphate.

The advantage of this scheme is that it does not require the presence of any new four-carbon intermediates which we have not yet found. The disadvantage is twofold, namely that the type of hydrolysis suggested is the inverse of that shown in alkali splitting of beta-keto acids which for oxaloacetic acid would give oxalic acid and acetic acid; and the undesirability of forming two different compounds in the split which must ultimately feed back again to the same one, the two-carbon carbon dioxide acceptor.

There is another somewhat more plausible scheme involving the preliminary oxidation of oxaloacetic acid to dioxymaleic acid which then splits to two molecules of glycoxylic acid by a reversal of a benzoin condensation. It is indicated by the following scheme in which no attempt has been made to specify the actual reacting species such as phosphate esters, etc. The dotted arrows indicate possible alternate paths.



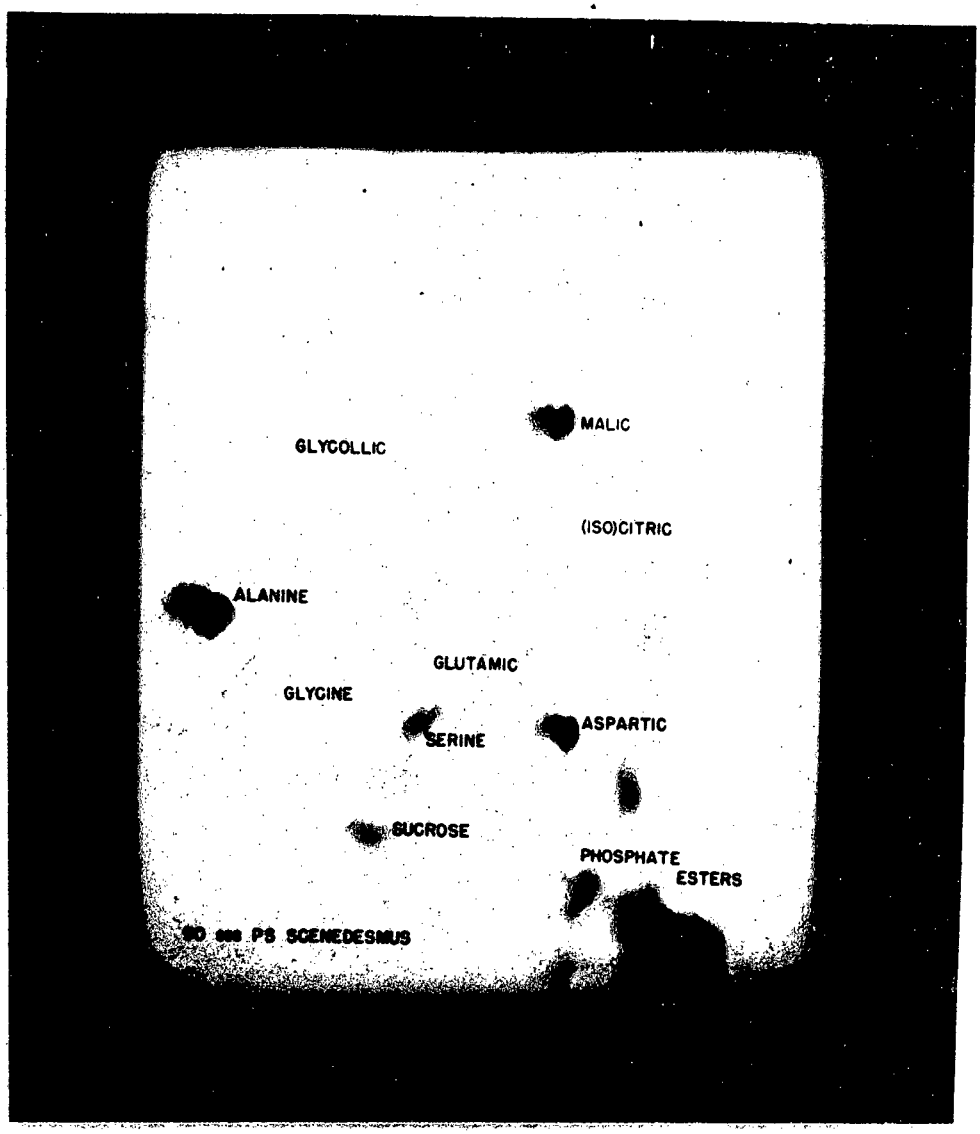
A path such as this is preferred for the following reasons. First of all, the reactions are all simple hydrations or hydrogenations in accord with the presently known habit of biosynthesis reactions, and the formation of carbon-carbon bonds by the benzoin type reaction is known in biochemistry. There are enzymes in various organisms and bacteria which, for example, can form acetoin from acetaldehyde which is the reverse of the one here suggested.

The second piece of evidence is in terms of dioxymaleic and glyoxylic acids. There is a very labile enzyme system in fresh juices of green plants which will act on dioxymaleic and at least one of the products is glyoxylic acid. A more distantly related fact is the demonstrated conversion, in both animal and bacterial studies, of the methylene carbon atom of glycine into both carbon atoms of acetic acid. This could be achieved through the oxidative deamination of glycine to glyoxylic acid followed by the reversal of the above sequence to produce oxaloacetic acid in which the two central carbon atoms have originated from the methylene carbon atoms of the glycine. The oxaloacetic acid is then decarboxylated to pyruvic which, in turn, is oxidatively decarboxylated to acetic acid.

This scheme suggests the presence of two more four-carbon compounds which we have not yet identified, tartaric acid and dioxymaleic acid. However, they may both be present as phosphates of some sort, or the dioxymaleic acid, if free, might be too unstable to be found on the paper. In either case, there are still a sufficient number of unidentified minor spots on the radiograms to allow for their presence.

Malonate Inhibition: - There is a relatively simple way of determining whether or not the malic, fumaric, succinic acid sequence is part of the photosynthesis cycle. It is well known that malonate will inhibit the fumaric-succinic conversion, and we now know that it will block the oxaloacetic-malic conversion as well. If it could be determined whether or not the photosynthetic cycle were operating under conditions of malonate poisoning known to exist within the cells, an answer to the question would be forthcoming. The experiment was performed as follows: A sample of algae (Scenedesmus) was split into two parts. One (the normal control) was suspended in the usual buffer (fumarate or phosphate), the other was suspended in 0.05 M malonate buffer at the same pH. Both were allowed to photosynthesize for one hour in 4% carbon dioxide-in air and then given the same amount of radioactive carbon dioxide for 90 seconds in the usual way. The total amount of carbon fixed was the same in both cases, indicating very little if any effect of the malonate on overall carbon dioxide assimilation. The chromatographic results are shown in Fig. 22. On the left is shown the radiogram of the control and on the right is the one for the malonate-inhibited algae.

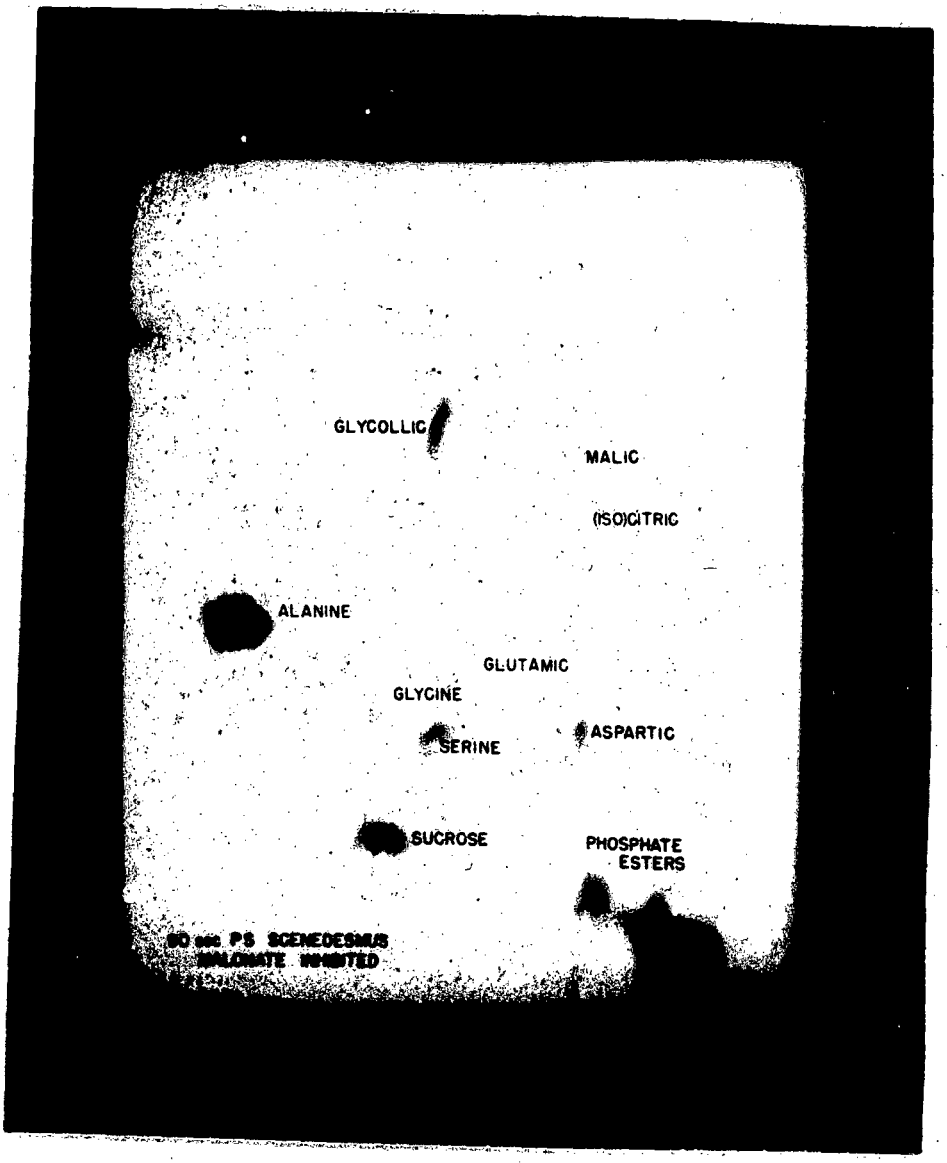
The most pronounced effect is the almost complete absence of radioactive malic acid from the malonate-inhibited algae. The disappearance of malic acid can be taken as proof that the malonate had actually penetrated the cell walls and was providing an effective inhibiting environment within the cells. The other compounds, including sucrose, are all present as in the control. However, this fact by itself would not be proof that the complete cycle was still operative in the presence of malonate. It would be possible to get radioactivity into all but the glycine and glycolic acid without the operation of the $C_4 \longrightarrow C_2$ split. In that case, the radioactivity would be



Effect of malonate on products formed.

Fig. 22a

Control



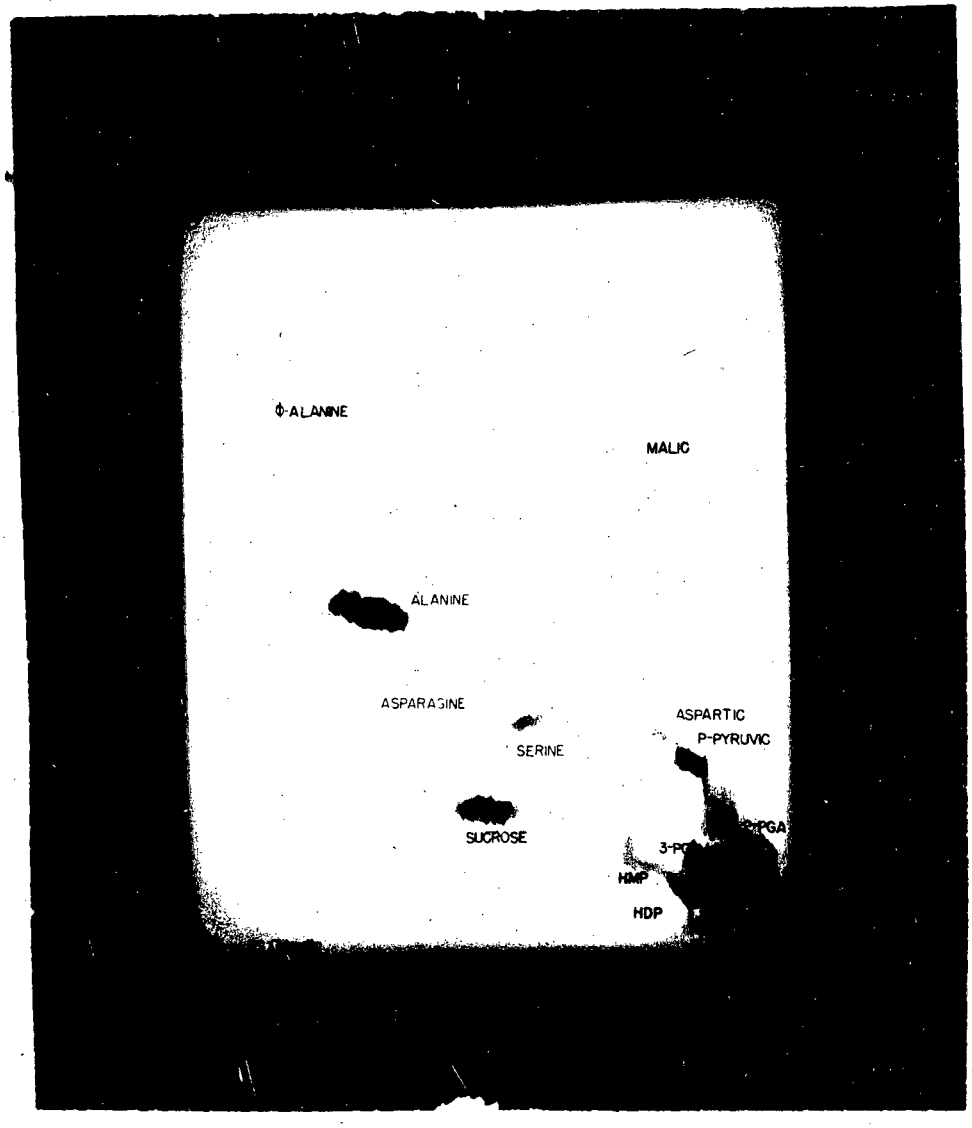
Effect of malonate on products formed

Fig. 22b

Malonate inhibited

present only in carboxyl groups of the three- and four-carbon compounds and the numbers three- and four-carbon atoms of the hexose. The mere presence of radioactive glycine and glycolic acid is indicative of the $C_4 \rightarrow C_2$ split unless they are formed by some entirely independent route. The continued operation of the cycle was finally demonstrated by degrading the alanine found in each of the two experiments. In both cases, about 35-40% of the radioactivity in the alanine was in the alpha- and beta-carbon atoms, the remaining 60-65% being in the carboxyl group. We, therefore, know that the $C_4 \rightarrow C_2$ split can and does take place from a four-carbon compound formed prior to malic acid.

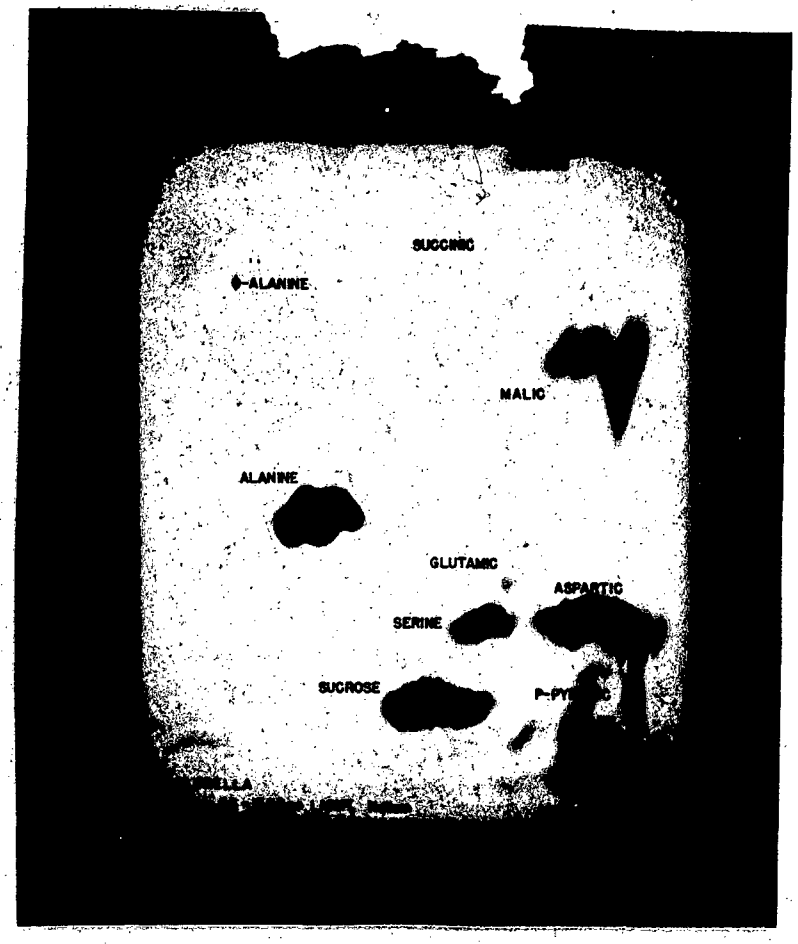
Effect of Light on Respiration: - There is perhaps another observation which can be interpreted. It consists of the fact that the five-carbon and six-carbon acids are not formed in the light but are formed immediately after the light is turned off. This is shown in Fig. 23. The upper left is an ordinary 30 second photosynthesis. The lower left is 30 seconds of photosynthesis in radiocarbon dioxide followed by 150 seconds in the light with helium flowing through the suspension; that is, we have allowed the plant to photosynthesize for 30 seconds and then, instead of turning off the light and killing them as we did for the radiogram above, we switched to a tank of helium to let it sweep out the radiocarbon so that we had only 30 seconds of photosynthesis, but we kept the light on to see what the effect was of having the light there without photosynthesis. In the lower right is shown the result of an experiment consisting of 30-second photosynthesis after which we switched to helium but, at the same time, we turned the light off. The most oustanding difference between the two is that while the light is on



Effect of Light on Respiration

Fig. 23a

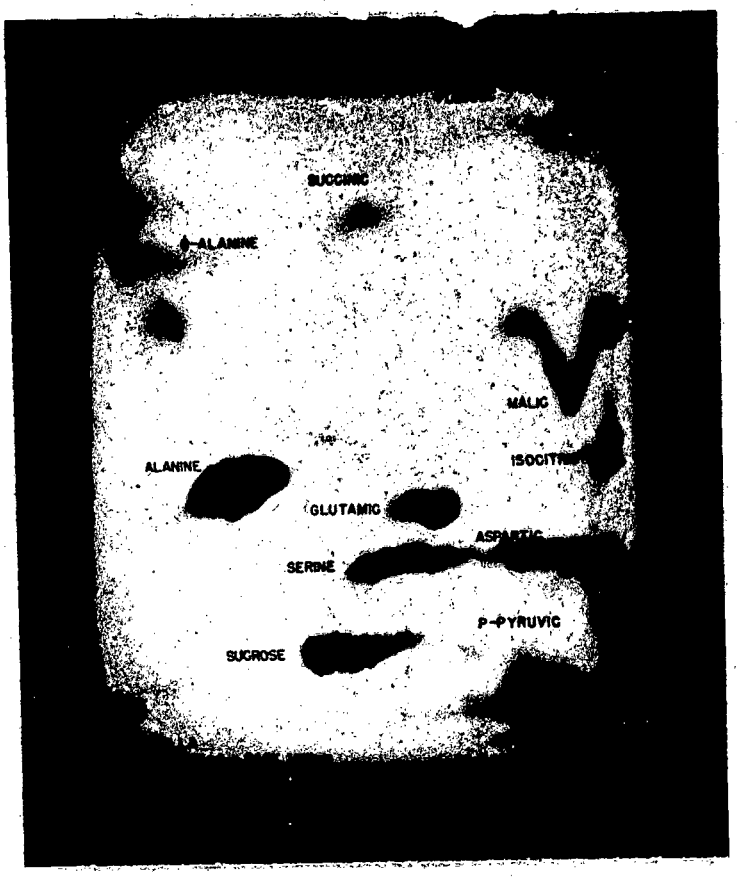
Control



Effect of light on respiration

Fig. 23b

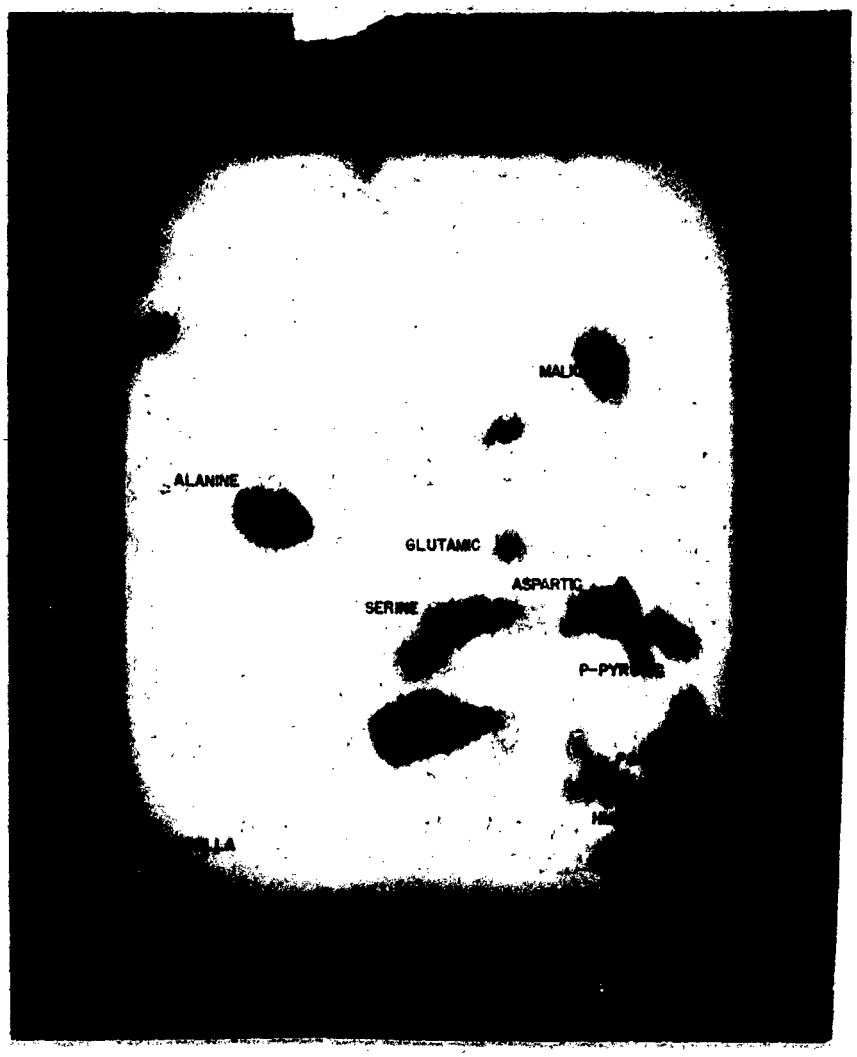
30 seconds of photosynthesis followed by 150 seconds in the light with helium sweep (absence of CO_2)



Effect of light on respiration

Fig. 23c

30 seconds of photosynthesis followed by 150 seconds in the dark with helium sweep (absence of CO_2)



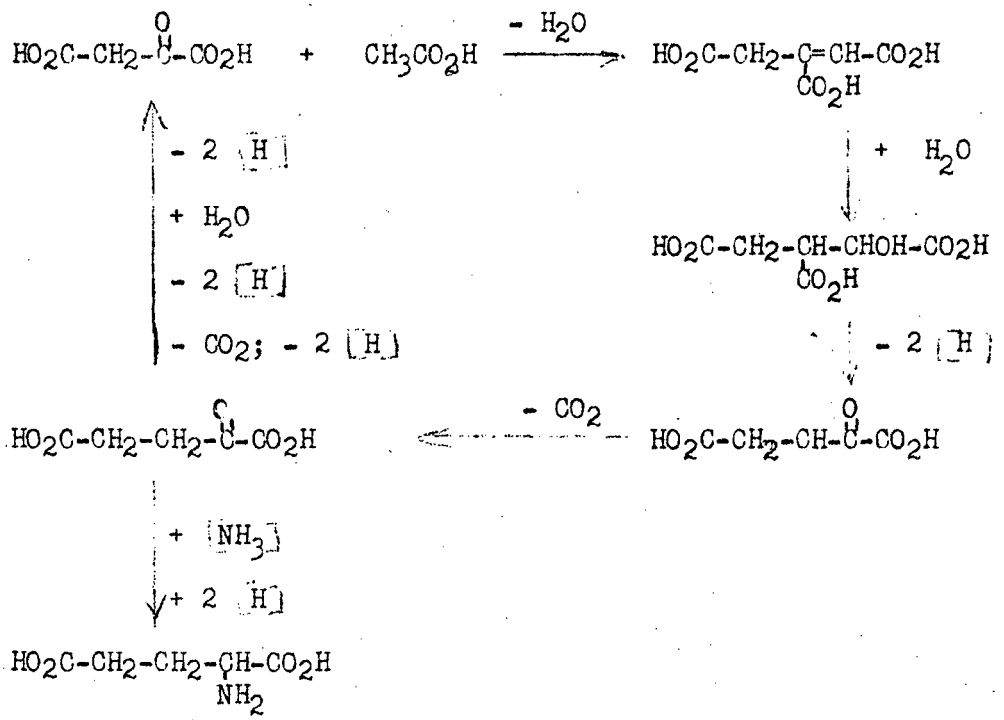
Effect of light on respiration.

Fig. 23d

5 minutes of photosynthesis.

on there is practically no glutamic, citric and/or isocitric acids formed. Even in 5 minutes (upper right) of photosynthesis there is very little glutamic or isocitric acid formed. In 150 seconds in the dark, those products which had just been made by photosynthesis and were radioactive obviously immediately got into the Krebs cycle and were converted to glutamic, isocitric and, presumably, ketoglutaric, aconitic and other acids of the Krebs cycle.

I would like to suggest the following interpretation. In order for the Krebs cycle to operate it is presumed that some form of acetate is required to condense with oxaloacetate. This forms aconitic acid and the aconitic acid is hydrated to isocitric which is oxidized to oxalsuccinic and so down to ketoglutaric acid, etc.



Now if there is no radioactive acetate or its equivalent present, the compounds of this sequence cannot acquire radioactivity. I believe that the reason, then, that the light prevents this from occurring is that the presence of a high intensity of light keeps the acetate concentration at a

minimum by keeping it all in the form of vinyl phosphate which presumably cannot enter the Krebs cycle. Quite apart from its interpretation, the phenomenon itself is most interesting because it indicates that not only does the light initiate a reduction process but it also inhibits certain oxidation processes or at least prevents the freshly formed photosynthate which is made, in this case in 30 seconds, from getting into the Krebs cycle and forming glutamic acid and isocitric acid. It is not unreasonable to suppose that this direct effect of light on the course of plant metabolic reactions in addition to overall carbon dioxide assimilation may be directly connected with the phenomenon of photoperiodism. A more immediate effect would be an inhibition of respiration by light. This has indeed been observed in quite independent kinetic experiments and will require the revision of certain concepts which have depended upon allowing for respiration in the light by assuming that it is the same as it is in the dark.

It now seems worthwhile to gather together in a single chart what we presently believe about the early fate of carbon dioxide in phytosynthesis and examine it in general terms. (See chart on the following page.)

This chart makes no attempt to define the actual reacting species involved. All it purports to show is the path by which the carbon skeletons are constructed, i.e., what is happening, not how it happens. It remains for future work to isolate the enzyme systems and determine the details of the atomic migrations. That part enclosed in the squqre is the new proposal for the four-carbon cycle. This cycle provides a path, (as did the earlier one shown) for the conversion of two molecules of carbon dioxide into one molecule at the oxidation level of acetaldehyde using ten equivalents of reducing power. Some or all of this reducing power has its ultimate origin

in the primary photochemical act and the splitting of water. We cannot as yet say which ones do, but there is a predisposition to suppose that those reducing equivalents involved in the direct reduction of the carboxyl groups, for example, in the conversion of acetate to vinyl phosphate, are the most likely ones.

The four-carbon cycle thus provides the structural raw materials for the synthesis of the three major constituents of plants - fats, carbohydrates and proteins. It is interesting to note that radioactivity appears in all three of these groups in as short a time as 120 seconds of photosynthesis.

Ultimately, as we lengthen the time of exposure we should be able to determine how the proteins and fats are built up and, incidentally, how the rarer compounds found in plants are constructed.