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THE PATH OF CARBON IN PHOTOSYNTHESIS. XIV.

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It seems hardly necessary to repeat to an audience of this kind the importance of the process known as photosynthesis in the interaction and the interdependence of organisms and in the very existence of life as we know it. This 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.

Not very long ago I had occasion to witness the very direct relationship between the amount of photosynthesis taking place in a limited area and the amount and variety of all processes depending upon it. During the winter the surface waters of the North Atlantic are relatively poorly populated with life. With the coming of spring, bringing with it more suitable conditions for the development of photosynthetic ogranisms (warmer temperatures and the increase in the mineral nutrients required), there is a relatively great and rapid increase in the population of the photosynthetic microorganisms (algae, diatoms, etc.). Almost concomitant with this, and following it very closely, is a corresponding increase in the population of microanimals which feed upon these primary producers and, in turn, larger organisms, fish and even mammals increase as the summer proceeds. The cycle is brought to a close with the gradual diminution of the mineral supply and the sunlight, and the cooling of the waters as the winter again approaches and the supply of diatoms exhausted. A cycle very similar to this can be observed in any backyard garden in the northeastern part of our country.

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, I think it is still 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

$$CO_2 + H_2O \underset{-\ \text{Energy}}{\overset{+\ h\nu}{\rightleftharpoons}} O_2 + (CH_2O)$$

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:

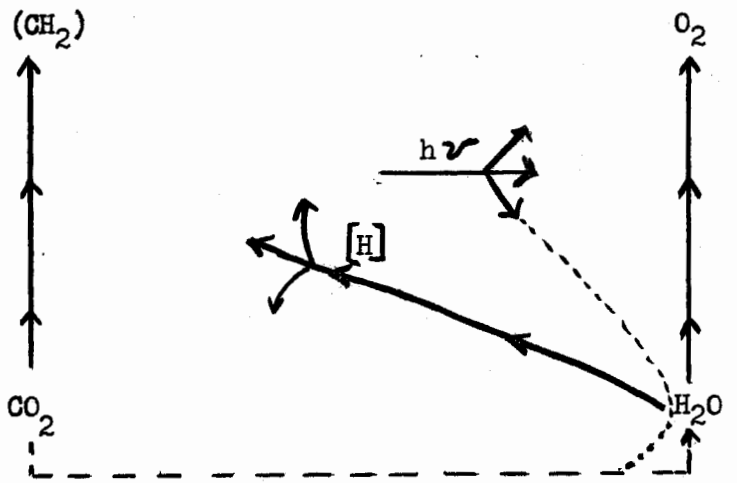


Figure 1

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.[1,2,3]

The scheme itself is an outgrowth of proposals of some fifteen years ago by Van Niel[4] 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 experiments of Hill.[5]

He was able to make preparations of chloroplasts and chloraplastic 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[6] was 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[7] showed that the molecule of oxygen evolved in photosynthesis had its proximate 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[8] 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 even 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. 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 coenzyme.

Very recently it has been reported[9,10,11] that both the higher plants and isolated chloroplasts emit a chemiluminiscence following cessation of illumination. This chemiluminiscence 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 limuniscence 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 discussion this evening 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 reduced 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 phytosynthesis. 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

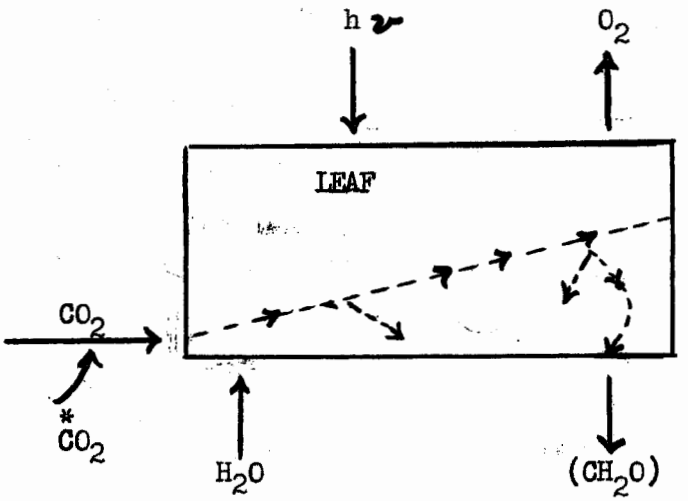


Figure 2

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 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 of a 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 compound which might act as an intermediate. Assuming that such a chain of intermediates exists, it is quite clear that by setting up some photosynthetic ogranism, 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.

Very early it became clear that we would be required to perform many experiments, and in order to have available as reproducible a biochemical material as possible it was advisable for us to grow our own plants. In order that the material be as nearly constant as possible it was necessary that the conditions of growth be easily and accurately reproducible. These requirements are most easily fulfilled by the unicellular algae such as Chlorella and Scenedesmus, the former being the

organism that had been used for many previous quantitative studies of photosynthesis. We therefore established in one corner of the laboratory what we call our algae farm, a photograph of which is shown in Figure 3. The green vessels contain the two culture of green algae mentioned, the red ones contain the purple bacterium, Rhodospirillum rubrum. These flasks are mounted on a shaker in a thermostat over light cylinders. They are, in effect, continuous cultures which may be harvested every day or every two days or over longer intervals. Such cultures have been maintained for very long periods extending beyond three or four months. The organisms so derived are quite reproducible and a good deal of the work here represented was originally done with such algae. However, as may be apparent, a variety of higher plants were also used such as barley shoots and soy bean leaves. Although there are differences from plant to plant, the general character of the result is the same for all of them. We will not at this time go into the comparative biochemistry of the different plants but rather emphasize that behavior which is common to all.

In performing an experiment a sample of algae was harvested and washed of its nutrient medium and resuspended in solutions placed in a flat vessel illuminated from both sides as shown in Figure 4. A stream of carbon dioxide containing air was put through the algae during the course of illumination for a suitable period of time until a steady state of photosynthesis was assured. To start the experiment, a suitable amount of sodium carbonate was injected into the algae suspension and the photosynthesis allowed to proceed for a predetermined number of seconds, after which the large stopcock at the bottom of the flask was opened and the algae run into boiling alcohol so as to stop all enzymatic reaction as rapidly as possible. When

leaves were used, the lollipop was replaced by a flat cell from which one of the faces might be easily removed. The reaction was stopped by removing the face and plunging the leaf into a suitable killing medium. It is now a simple enough matter to determine the total amount of carbon which has been fixed in the particular experiment. This is done by removing an aliquot of the entire extract and suspension of the material and mounting it on a plate to be counted by a Geiger counter.

The larger problem now presents itself, namely, to determine the nature of the compounds into which the carbon has been fixed. The first, and simplest, step of the fractionation which was performed was to separate the soluble from the insoluble material. Alcohol extracts (80%) followed by water extracts were made and it was very early learned that for short periods of photosynthesis all of the fixed carbon is in some relatively soluble form, as shown in Figure 5 (total and soluble product for 20° algae). It is only after more extended periods that the carbon finds its way into insolubles such as protein, cellulose and starch. Since at this stage we are primarily interested in the very early products of incorporation, we have, therefore, to be concerned only with soluble material. Unfortunately, this criterion of solubility does not limit the possibilities very greatly. The soluble constituents of plants are myriad and the ordinary methods of analysis would be extremely slow and laborious indeed.

We have, therefore, turned to the very elegant method of separation which was recently developed, particularly for amino acids, by the biochemists, Consden, Martin and Synge[12], known as paper partition chromatography. The method has since been applied to a wide variety of

substances, the only limits being that substances be non-volatile so that a piece of filter paper may be dried and treated without evaporation of the substance. The method is undoubtedly familiar to most of you and will not be described again in detail.[13,14] The only change which we have added was made possible by the radioactivity of the atoms which we seek to find. It will be remembered that we are not interested necessarily in all of the compounds of plant extract but particularly in those which carry radioactive atoms. These may be detected after they have been spread on the paper because of the fact that they carry radioactivity and that this radioactivity will affect photographic film. Thus, after compounds have been spread in two dimensions on a piece of filter paper, this paper is dried and placed in contact with a corresponding sheet of photographic film. Wherever, on the paper, there is a radioactive carbon-containing compound, the film will be bombarded by the beta particles emitted by these atoms, and after a suitable period of time the film may be developed and will show exposed areas wherever it has been in close contact with radioactivity. We are thus able to locate precisely those compounds in which we are expecially interested. The photographs of the radioactive products produced by 60 second exposure of the algae, Scenedesmus, to radioactive carbon is shown in Figure 6.*

(*) In this and all other radiograms or paper chromatograms shown in this paper the origin spot is the lower right hand corner, the horizontal direction was run from right to left in phenol-water and the vertical direction from bottom to top in butanol-propionic acid-water. The

photographic film used was Eastman "No-Screen" X-ray film. The time of exposure of the films varied and the absolute intensities on different films are not to be compared as significant, although, of course, spots on a single film are comparable.

The position occupied by the radioactive spot with respect to the origin is, in principle, the means of identifying it chemically. By comparing the position occupied by a radioactive spot with that taken by an authentic sample of the material, a proximate indication is given, at least, of the nature of the compound in the spot. Ultimate identity, however, rests upon the sum of a wide variety of observations in which the unknown spot containing the fixed carbon compound is eluted from the paper, a chemical determination performed and the resulting material chromatographed a second time to determine whether or not the supposed chemical change has occurred in the unknown compound. Finally, a mixture of the unknown radioactive material with an authentic sample of the proposed substance is chromatographed together and complete coincidence of the radioactivity with some colored or other visible product of the known compound achieved. The nature of the color spots produced on the paper is dependent, of course, on the particular types of compounds that are being investigated.

Methods of detecting very nearly all varieties of substances which are capable of being chromatographed have been devised, ranging from the simple case in which the compound itself is colored and can be excellently seen and the original reaction performed on the paper for detecting amino acids, namely, the purpose color produced when alpha-amino acids

react with ninhydrin, to a number of different reagents producing colors with aldoses and ketoses, as well as the reaction of acids and bases with ordinary pH indicators. With methods such as these a wide range of the early compounds produced in photosynthesis have been identified as shown in Figure 7. 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. This has been done with the soluble products of Figure 5 and the result shown in Figure 8. It is clear that the variety of products synthesized at room temperature by Scenedesmus (as well as by all other plants tried) is very great, even in a very short time such as a minute or somewhat less. 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 reaction and enable us to see more clearly the earliest products.

Figure 9 shows a plot of the concentration of radioactivity per unit of algae for three of the major early compounds, while Figure 10 is a plot made from the same data, but given in terms of the percentage in each compound of the total fixed radioactivity as in Figure 8. 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 negative slope; that is to say, for short enough periods of time the first isolable products formed must represent one hundred percent of the total carbon fixed. This is certainly the case for phosphoglyceric acid and possibly 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.[15]

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 derived from the hexose phosphates. This has been done for the glyceric acid, and hexose obtained from an experiment in which barley shoots had been allowed to photosynthesize in radioactive carbon dioxide for 15 seconds with the result shown in Figure 11 in which

the numbers and bar lengths represent the percentage of total radioactivity in the compound which is to be found in the indicated carbon atoms. 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 distribution in these two compounds (glyceric acid and hexose) is compared for a variety of different times, as may be seen in the data contained in Table I.[16]

Table I

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

Conditions[a]	Glyceric Acid			Glycolic Acid		Sucrose		
	-COOH	-CHOH	$-CH_2OH$	-COOH	$-CH_2OH$	C3,4	C2,5	C1,6
Barley								
Preillum: 2 min. dark	96.	2.6	1.7					
4 sec. PS (photosynthesis)	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[b]	75.	6.	9.					
40 sec. PS				47.	53.			
60 sec. PS	44.[c]	30.	25.					

Table I (Continued)

Conditions[a]	Glyceric Acid			Glycolic Acid		Sucrose		
	-COOH	-CHOH	$-CH_2OH$	-COOH	$-CH_2OH$	C3,4	C2,5	C1,6
Scenedesmus:								
5 sec. PS	95.[d]	2.5	1.2					
30 sec. PS						87.[e]	7.	6.
30 sec. PS[f]	81.	7.	10.					
30 sec. PS[f]MI[g]	73.	12.	15.					
60 sec. PS[f]	51.	24.	25.					
60 sec. PS[f]MI	48.	24.	28.					
60 sec. PS[f]								
60 sec. PS[f]MI	43.	27.	30.					

a. Experiments are steady-state photosynthesis 10,000 footcandles unless otherwise stated.

b. 1,000 footcandles.

c. Alanine obtained from this extract was 48% carboxyl-labeled.

d. Under the same conditions, Chlorella produced phosphoglycerate labeled 95%, 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.

With this clear cut 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 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. That this is indeed the case is demonstrated in Figure 12 which shows the radioautograph of a paper chromatogram of some hydrolyzed pure sucrose obtained from barley which had been photosynthesizing for 15 seconds in radioactive carbon dioxide. A direct count of the spots on this paper showed the fructose to contain roughly twice as much radioactivity as the glucose. While these data in and of themselves do not unequivocally demonstrate that the formation of sucrose in photosynthesis is precisely the reverse of the glycolytic sequence in all its details, they do indicate that the general pattern of compounds lying between glyceric acid and sucrose is much the same in the two cases.

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 appears that they acquire it at equal rates, at least within the present accuracy of the experiments.

It thus appears that at high light intensities the first reaction which the carbon dioxide can undergo corresponds to a C_2-C_1 addition leading to carboxyl-labeled glyceric acid, since the C_1 is carbon dioxide or some one-carbon isoximer of it. The problem is now one of determining the origin of the C_2 carbon dioxide acceptor. A further examination of the data in Table I shows that the α- and β-carbon atoms of the glyceric acid, which presumably originate as the C_2 acceptor, become labeled very early. Thus, we are constrained to devising a sequence of reactions by which this C_2 compound not only is continually generated but also generated in such a manner as to acquire labeled carbon dioxide almost equally in both carbon atoms at a very early stage.

In examining the radiograms of early products for compounds which might give some clue as to the character of a compound which might serve as or be related to a C_2 acceptor we have found as yet only two compounds containing two carbon atoms, namely, glycine and glycolic acid. (Phosphoglycolic acid also was identified as a spot just above phosphoglyceric acid in the radiograms.) Furthermore, the condition under which the amounts of these two compounds could be enhanced was illumination of the plant system, after exposure to radioactive carbon dioxide, under carbon dioxide limitation.

One might expect that under these conditions any C_2 acceptor produced by the photochemically generated reducing power would tend to accumulate since there would be little carbon dioxide with which it could react to produce glyceric acid. On the other hand, if after a period of illumination and following a very short period of darkness in the presence of

carbon dioxide, one would expect very little of the labeled glycolic acid and glycine; this is indeed the case.[17]

If the glycolic acid is closely related or actually a precursor to the two-carbon acceptor, one would expect that the distribution of radioactivity between the two carbon atoms would always correspond to that of the α- and β-carbon atoms of the glyceric acid in the same experiment. An examination of the glycolic acid column in Table I shows this to be the case even in the shortest experiments that have been performed, namely, the 4-second photosynthesis in barley.

In an attempt to gain a further insight into the relation of glycolic acid to the photosynthetic carbon cycle some feeding experiments were performed in which the two possible labeled glycolic acids were fed to Scenedesmus while they were photosynthesizing in the presence of unlabeled carbon dioxide. The results are shown in the chart of Figure 13. Thus, when α-labeled glycolic acid is fed, the glyceric acid derived from it is found to be equally labeled in the α- and β-carbon atoms and to contain very little radioactivity in the carboxyl group. Similarly, when carboxyl-labeled glycolic acid is fed, again the label is equally distributed between the α- and β-carbons of the glyceric acid but a very appreciable quantity appears in the carboxyl group of the glyceric acid. This might be due to a partial oxidation of the glycolic acid resulting in some labeled carbon dioxide within the cell which would then be incorporated in the usual fashion. There are, of course, other routes by which this might be achieved. With whole cells, unfortunately, feeding experiments are not very satisfactory since one must always be concerned with the

question of permeability as well as the possibility of the added metabolite entering into a wide variety of reactions other than the one being tested.

If we accept the above two observations as indicating that glycolic acid is either on a direct line to the two-carbon carbon dioxide acceptor or else very closely related to it as a side product, and some times in practical equilibrium with it, then we must presume that there exists a symmetrical two-carbon compound between glycolic acid and the two-carbon acceptor. The above observations as yet give us very little clue as to the origin of the two-carbon piece.

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 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. It will be recalled that along with glyceric acid one of the earliest labeled compounds to appear in photosynthesis experiments is the C_4 compound, malic acid. This fact, taken together with the lack of any appreciable amounts or label in the compounds of the tricarboxylic acid cycle[18], led us to the supposition that malic acid was either, again, a precursor to or very closely related to a four-carbon compound which could be split to produce the required two-carbon fragment.

An experiment designed to test whether or not malic acid lay in the direct line leading to the two-carbon fragment was performed. The formation of labeled malic acid during photosynthesis was largely inhibited by the addition of malonic acid, and the rate of appearance of radioactive carbon in the α- and β-carbon atoms of the glyceric acid was determined.[19] It was found that even under greatly inhibited production of malic acid the rate of appearance of labeling in the α- and β-carbons of glyceric acid was hardly affected; if anything, it appeared to be somewhat accelerated. This seemed to preclude the possibility that malic acid lay directly in the two-carbon regenerating cycle, and we were tentatively forced to the supposition that the sequence of reactions leading to the regeneration of the two-carbon fragment began with oxaloacetic acid which we cannot observe on our chromatograms. A number of intermediates lying between oxaloacetic acid and the compound which would ultimately be split were proposed, but not any of them have as yet been found.

In the course of this search for both the two-carbon acceptor and its immediate precursors the techniques of paper chromatography of phosphates,

in particular, were improved, and the device of enzymatically hydrolyzing the phosphates so as not to destroy or alter the carbon skeleton appreciably was introduced. These improvements in technique, and continued work with a variety of organisms, resulted in the recognition of at least two rather important compounds formed very early in photosynthesis by all the organisms which we have so far studied.

In fact, curiously enough, the first realization of the possible importance of these two unknown compounds began to grow as a result of studies of photosynthesis with Rhodospirillum. It was found, for example, that when the phosphates from relatively short photosynthetic experiments were hydrolyzed, either with a malt-phosphatase preparation or with a commercial "Polidase," and then chromatographed, a wide variety of organic substances appeared, most of which had already been identified as glucose, fructose, glyceric acid, glycolic acid and triose. There were, however, two unknown spots whose importance seemed to increase relatively as the photosynthetic period was shortened. One of these lay between glucose and fructose on the chromatogram and the other lay just beyond alanine. For the first of these we used the symbol U_s until it was identified and for the second we used the symbol U_a (Figure 14).

The story of the work leading up to the identification of these two spots is indeed an interesting one. In fact, it occupied the major portion of the laboratory's effort for well over a year and acted more or less as a brake upon any further progress. The struggle, however, did force the development of a technique of structure determination when only microgram amounts or less of the unknown material were available and those only in the form of spots on the paper, not as isolated crystalline substances. This technique depends upon the accumulation of several varieties of evidence, all involving

the radioactive property of the unknown compound.

In order to give some idea of the nature of this technique it might be worthwhile at this point to outline briefly the particular sorts of evidence leading to the identification of the spot known as U_s. The earliest work constituted simply recognizing that the spot gave no color with ninhydrin and was, therefore, either not an amino acid or perhaps might be one present in amounts so small as to fail to react with ninhydrin; secondly, recognizing from the particular position occupied by U_s that it might very well be a sugar type of molecule.

From the known behavior of a wide variety of sugars it could be expected that if U_s were a sugar it would have more than five carbon atoms. That it was not any of the common hexoses was very soon determined by cutting out the spot and rechromatographing it mixed with authentic samples of a wide variety of hexoses which could be detected by color reactions. It was found to run extremely closely with mannose and especially sorbose. However, it did not coincide exactly with any of the hexoses which we had available.

A considerable amount of the chemistry of U_s was tested by simply cutting out the spot as defined by the radiogram, performing a chemical operation upon the solution containing the tracer amount of material, and then rechromatographing the resulting product to determine the nature of the changes which might have been brought about.

I cannot at this time describe the many failures and repetitions which were performed on U_s. Rather it seems better to list those chemical properties which were definitely established just prior to the recognition of its identity.

1. U_S is quite sensitive to relatively dilute acid. Upon heating for five minutes at 100° in 1 N hydrochloric acid it is converted almost completely into a new compound which moves on the chromatogram considerably further than the original U_S in the horizontal (phenol) direction and about the same distance in the vertical (butanol-propionic acid) direction. This conversion product we called U_H, the product from U_S by acid treatment (Figure 15).

2. When U_S is heated with phenylhydrazine hydrochloride to try and form an osazone, most of the product appeared as U_H.

3. When U_S, formed in 5 minute photosynthesis by soy beans, is oxidized with periodate after adding carbon dioxide, formate and formaldehyde as carriers, 14.5% of the activity contained in the sample appeared as formaldehyde, 55% as formic acid, a negligible amount as carbon dioxide, and the remainder (about 25%) as non-volatile activity in the oxidation flash. Since 5 minute photosynthesis in soy bean is ample time to saturate the unknown compound with radioactivity, it can be presumed that all of its carbon atoms are of equal specific activity. If that be the case and the oxidation reactions complete, the presence of 14.5% of the radioactivity in a single carbon atom requires that there be at least six carbon atoms in the compound and possibly seven if we accept 14.5% as being a very accurate determination.

4. U_S if not fermented by Lactobacillus.

5. U_H is very insensitive to most reagents and relatively stable to acid and alkali and to nitrous acid and is a neutral compound.

6. U_H resists reaction with hydroxylamine and phenylhydrazine.

7. Perhaps the most interesting change that has taken place in U_H is its behavior with respect to periodate oxidation. It gives no formaldehyde,

and only 14% of its radioactivity appears in formic acid, the remainder being in the non-volatile residue in the oxidation flask.

8. U_S, and especially U_H, is resistant to bromine oxidation, although here, again, the acidity apparently converts a small amount of U_S to U_H.

9. U_S can be hydrognated, and the hydrogenation product, upon periodate oxidation, shows approximately twice as much of the activity in formaldehyde, the remainder being formic acid (~70%).

10. U_H is easily susceptible to acetylation.

An examination of these properties seems to pretty definitely require a molecule of carbohydrate character, containing at least six and possibly seven carbon atoms. The outstanding reaction in the whole list above is the ease with which U_S is converted to U_H at tracer concentrations. (One molecule of U_S is involved in the transformation). This latter seems to indicate that U_H is a cyclic anhydride of some sort and the ease of its formation is the crucial piece of information which leads to the suggestion that U_S is a heptose of the altrose series, in particular, sedoheptulose.[20] As soon as this realization was achieved, the acquisition of an authentic sample of sedoheptulose followed by co-chromatography of both the original sugar and the anhydride formed from it, as well as the hepitotol obtained upon reduction, all confirmed the identification. (Figure 15). In a similar manner, the identification of U_a was made as the ketopentose, ribulose.[21]

There seems to be very little question that the phosphates of the five- and seven-carbon carbohydrate acquire the label at least as early, and probably earlier, than the hexoses. Some indication of this may be seen in

Figures 16 and 17. These are radiograms showing enzymatic hydrolysis products of phosphates which have been cut out of the total chromatograph as indicated. Thus, two seconds of photosynthesis in barley produces practically no labeled glucose phosphates; all the monophosphates that are labeled are fructose and sedoheptulose. Similarly, there is very little labeled fructose diphosphate; most of the labeled diphosphate being that of ribulose. (The intensities of these two spots on the radiograms of Figures 16 and 17 are not to be compared since the aliquot of the material and the exposure time is much greater in the ribulose chromatogram.) In this same way it is clear that in soy bean the heptose phosphate becomes labeled at least as fast as the fructose, and in all probability, if shorter experiments were performed, it would be the only labeled phosphate present among the monophosphates.

It is perhaps worth spending a few moments at this point to discuss the significance of the rate of appearance of radioactivity in the particular compound as we can observe it by this chromatographic method. It is clear that we do not easily get, by this method alone, the specific activity of the particular compound involved. We get only the concentration of radioactivity in a particular compound; that is, the amount of radioactivity per unit of organisms which is in a particular form. Actually, this quantity as a function of time is precisely the quantity which is needed in order to determine the sequence of events. The specific activity as usually determined (counts/minute/milligram of compound) is not necessarily significant in establishing a precursor-product relationship when the compound is isolated from a complete organism, as it is in this case. Almost certainly there are a number of different sources for any particular compound and these sources may be more or less isolated and not in equilibrium with each other, so that although

the specific activity of a particular precursor in a certain physiological area of the organism might be very high, it would not appear that way when that compound is isolated from the whole organism and thus diluted by the inert reservoirs from other sources. A more precise, rigorous and general criterion would be to repeat the type of plot made in Figure 10; that is, percentage in a given compound of a group of compounds, but instead of using carbon dioxide as the starting point, as we did in Figure 10, to use phosphoglyceric acid as the starting point and determine which compounds appear percentage-wise with negative slopes from this. It is clear that this procedure will give us the next step, or steps, in the transformation of the phosphoglyceric acid. If phosphoglyceric acid is transformed entirely into one other compound there will be only one compound appearing following it with a negative slope on such a plot and representing one hundred percent of the first product of transformation of phosphoglyceric acid. If, however, there are two or more independent paths for the transformation of phosphoglyceric acid there will be two corresponding negative sloped lines for them. Another direct kinetic way which we have at the moment of determining the order of entry is the rate at which a particular reservoir becomes saturated with radioactivity in terms of its specific concentration, as mentioned earlier.

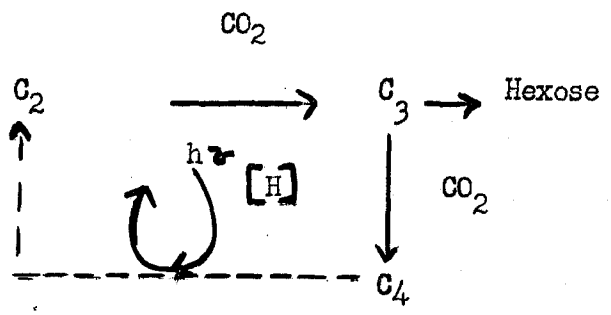
Although a kinetic experiment of sufficient accuracy to determine unequivocally the position occupied by the heptose and pentose in the sequence of events has not yet been performed, it is very likely on the basis of the data of Figures 16 and 17 that they precede any of the hexoses (with the possibility that heptose and fructose come in simultaneously), especially in view of the easily established fact that the

stationary state concentration of hexose derivatives is certainly much larger than that of the heptose in most of the plants we have examined.

The question as to the relative order of the heptose and pentose is, however, not so easily answered. In Figure 18 is shown a complete chromatogram from the soluble material from a 15 second photosynthetic barley experiment. Here, there has been considerable phosphate hydrolysis in the extract itself (presumably by the resistant phosphatases present), and if we make the not unreasonable assumption that the relative amount of free sugars we see in this chromatogram reflects the relative amount of sugar phosphates that were originally present it would appear that the heptose is coming in prior to the pentose. A comparison of the rate of approach to saturation of the pentose and hexose in the rather rough kinetic data shown in Figure 19 also leads to a similar implication.

As yet, the only degradation data which we have available for short-term heptose and pentose indicate that the label appears in these two compounds somewhere in the center of the chain first, later coming into the terminal carbon atoms. All that can be said, then, is that the pentose and heptose are not likely to have been formed via a terminal carboxylation of a tetrose or hexose, respectively.

It is perhaps now worthwhile to reconsider what modifications, if any, may be made in the originally proposed photosynthetic cycle of some years ago, which consisted, in essence, of the following sequence of steps:*



* It should be re-emphasized[22] at this point that practically all the compounds discussed in connection with any proposed photosynthetic cycle are phosphorylated compounds, many of them of the anhydride or enol ester type (high energy phosphates). Since the only mechanism as yet known for the production of such high energy phosphate involves an oxidation reaction (not necessarily directly connected with molecular oxygen) it is clear that at least the reduction of carbon dioxide involves the cooperation of energy derived from oxidation reactions. What is oxidized seems to be some of the compounds which constitute the primary or secondary stored "reducing power" mentioned above. Since there is an appreciable reservoir of this which may be stored for some seconds to minutes, it is clear that for very short periods of time the apparent quantum requirement, at least for carbon dioxide reduction, need have no direct relationship to the real efficiency of the photochemical energy transformation. That such is indeed the case has been recently demonstrated by Burk and Warburg.[23] In fact, it would appear from this work that the actual evolution of molecular oxygen also requires a redistribution of the primarily photochemically produced chemical energy. Here, again, it is easily possible to imagine systems in which oxidation or oxygenation reactions taking place in the short dark intervals can contribute to the evolution of oxygen in the immediately following light intervals. Furthermore, this conclusion is not dependent upon the assumption made by Burk and Warburg that the oxygen absorption and carbon dioxide evolution observed in the dark periods continues unchanged in the light periods.

The particular nature of the C_4 compound and the route by which it might return to C_2's has been the subject of speculation, and, as mentioned earlier, as yet no compounds had been isolated which might definitively be placed along that route other than glycolic acid in close connection with the C_2 compounds.

It now appears not at all unlikely that the pentose and heptose which have just been described might actually be part of the path by which the two-carbon carbon dioxide acceptor is regenerated. It is possible to visualize the condensation of a tetrose derived from the initial four-carbon compound with a triose derived from the initial three-carbon compound to form the heptose. This, in turn, would then lose a two-carbon fragment, possibly twice in succession, producing two two-carbon compounds and regenerating the triose molecule. Since both heptose and pentose are 2-ketoses the split presumably would take place between the number two and number three carbon atoms in each case, by a reverse acyloin type of reaction, as has already been suggested.[14] In fact, recently some very significant evidence has been presented[24] that such a reaction can take place when arabinose-1-C^{14} is converted into acetic acid and lactic acid by Lactobacillus pentoaceticus. The acetic acid was all labeled in the methyl group.

It might be mentioned here that there is distinct evidence for the presence of both tetrose (presumably erythrose) and the corresponding aldonic acid (presumably erythronic acid) in the extracts. It is not, however, possible to be sure that they are primary products and not formed as oxidation or breakdown products of the pentose and heptose on the paper.

Assuming for the moment that these two four-carbon compounds are genuine intermediates it might be proposed that the four-carbon acid (erythronic acid) would be formed by a single reductive carboxylation of dihydroxyacetone in a manner exactly paralleling the formation of malic acid from pyruvic acid by the widely distributed malic enzyme.[6,25] This, in turn, would be reduced to erythrose by an enzyme system comparable to the one which can reduce glyceric acid to triose, thus providing the tetrose precursor to heptose.

We could thus incorporate the pentose and heptose into our basic scheme which would, in effect, be the splitting of the four-carbon fragment into two two's as before, but carrying along three more carbons to provide the mechanism for it. Quite obviously, a definitive answer to the many questions which arise must await more detailed information.

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20. Benson, A.A., Bassham, J. A. and Calvin, M. J. Am. Chem. Soc., 73, 2970 (1951).

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Fig. 3. Algae "Farm". Scenedesmus, Chlorella, Rhodospirillum Rubrum.

Fig. 4. "Lollipop" for exposing algae (leaves) to $C^{14}O_2$.

Fig. 5. Photosynthesis by Scenedesmus in $C^{14}O_2$ at 20° C.

Fig. 6. Radiogram of the soluble porducts formed in 60 sec. of photosynthesis in $C^{14}O_2$ by Scenedesmus.

Fig. 7.

Fig. 8. Percentage distribution of radioactivity as a function of time among the compounds formed by Scenedesmus.

Fig. 9. Distribution of radioactivity among some of the compounds formed by Scenedesmus during photosynthesis at 2° C.

Fig. 10. Percentage distribution of radioactivity among compounds formed in photosynthesis by Scenedesmus at 2° C.

Fig. 11.

Fig. 12. Radiogram of the total hydrolysis products from pure sucrose formed by barley photosynthesizing for 12 sec. in $C^{14}O_2$.

Fig. 13.

Fig. 14. Phosphatase hydrolysis, Rhodospirillum rubrum. U_S (sedoheptulose), U_a (ribulose).

Fig. 15. Left - Sprayed paper containing several micrograms each of sedoheptulose and sedoheptulosan. Right - Radiogram of the same paper showing positions of U_S and U_H. Lower - The correspondence between the pair of sugars and the radioactivity is complete in every detail.

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 19. Rate of appearance of radioactivity in a number of sugars during photosynthesis by Scenedesmus at 20° C. These sugars have been determined after liberation from their phosphates by phosphatase.

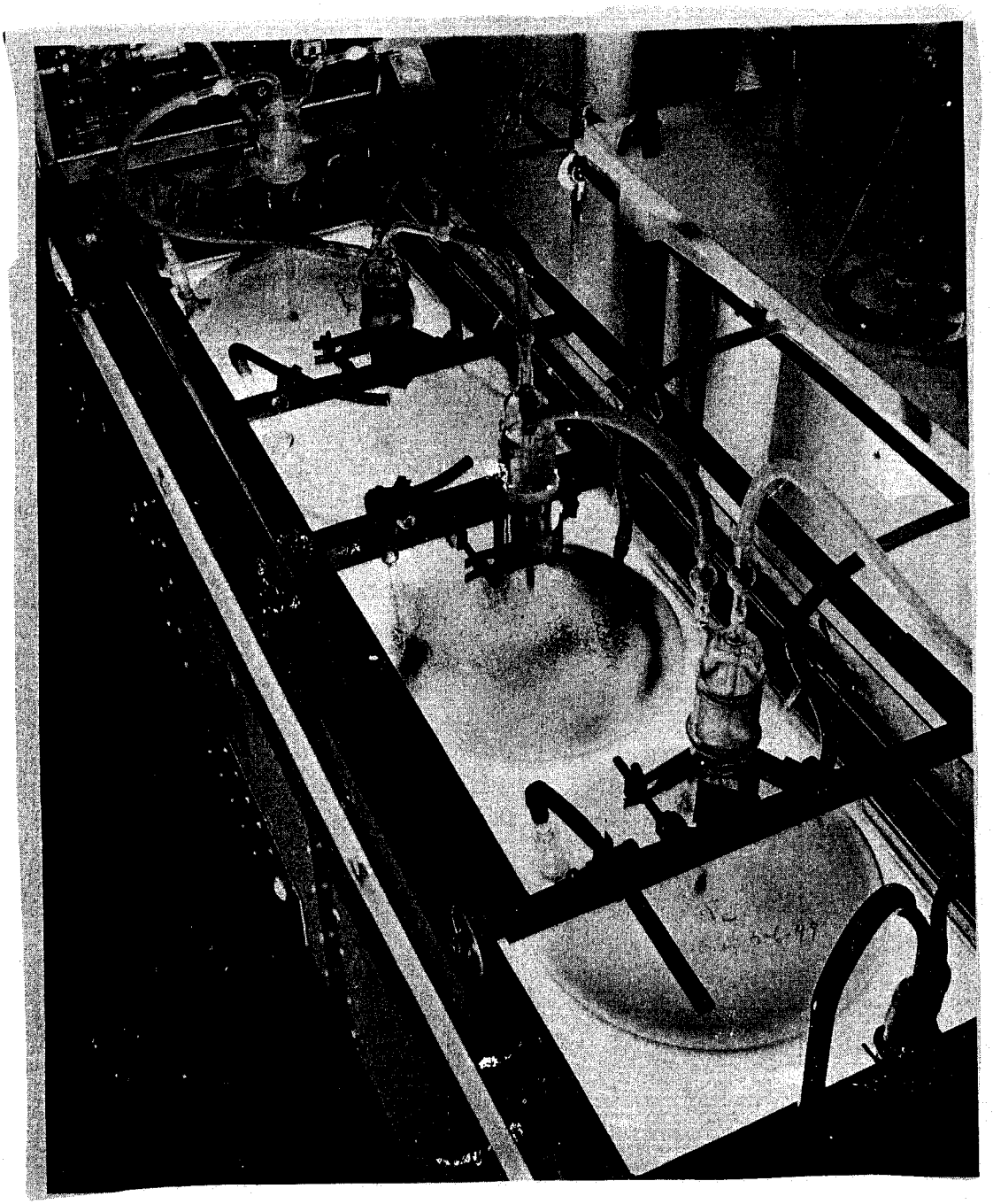


Fig. 3

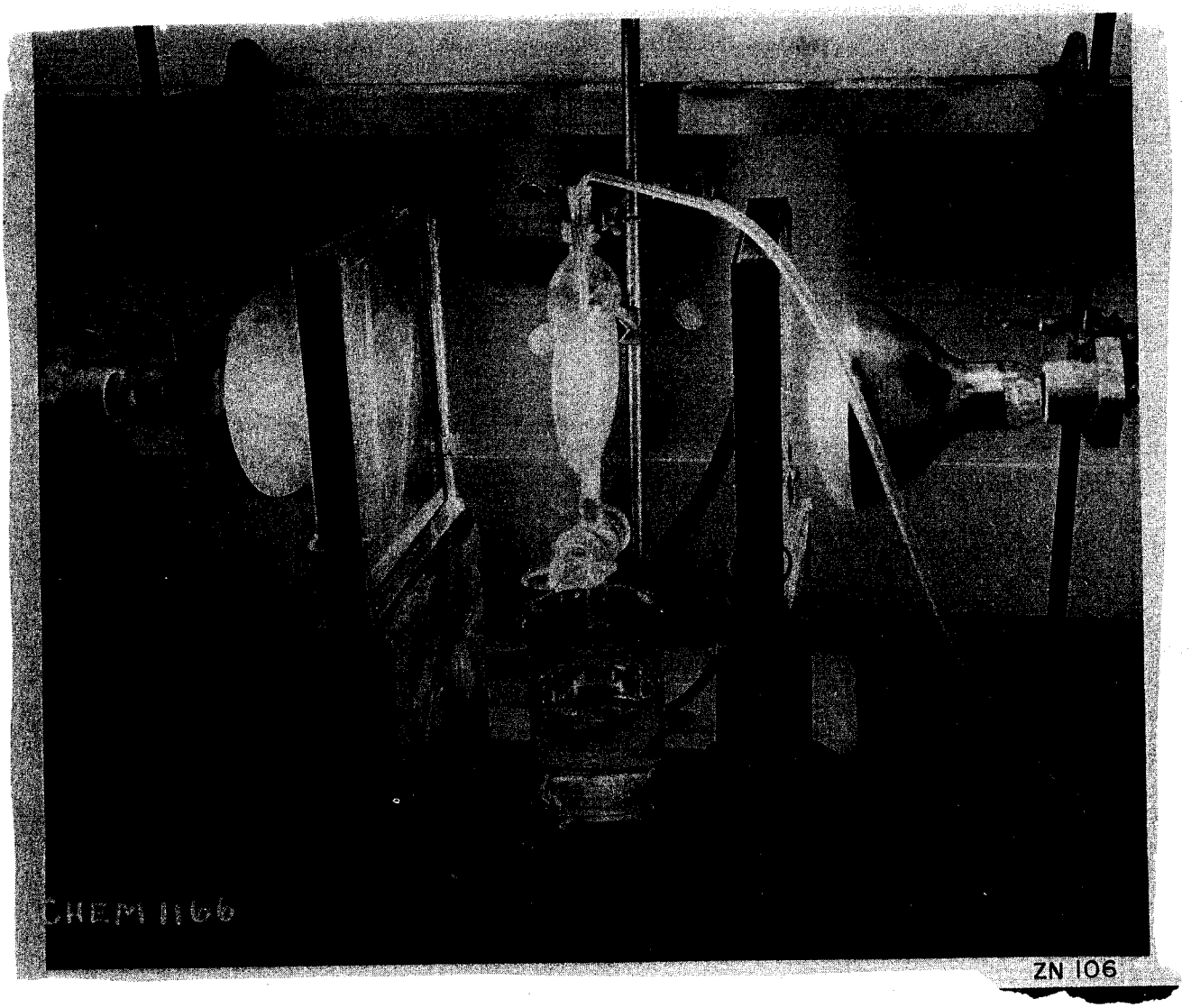


Fig. 4

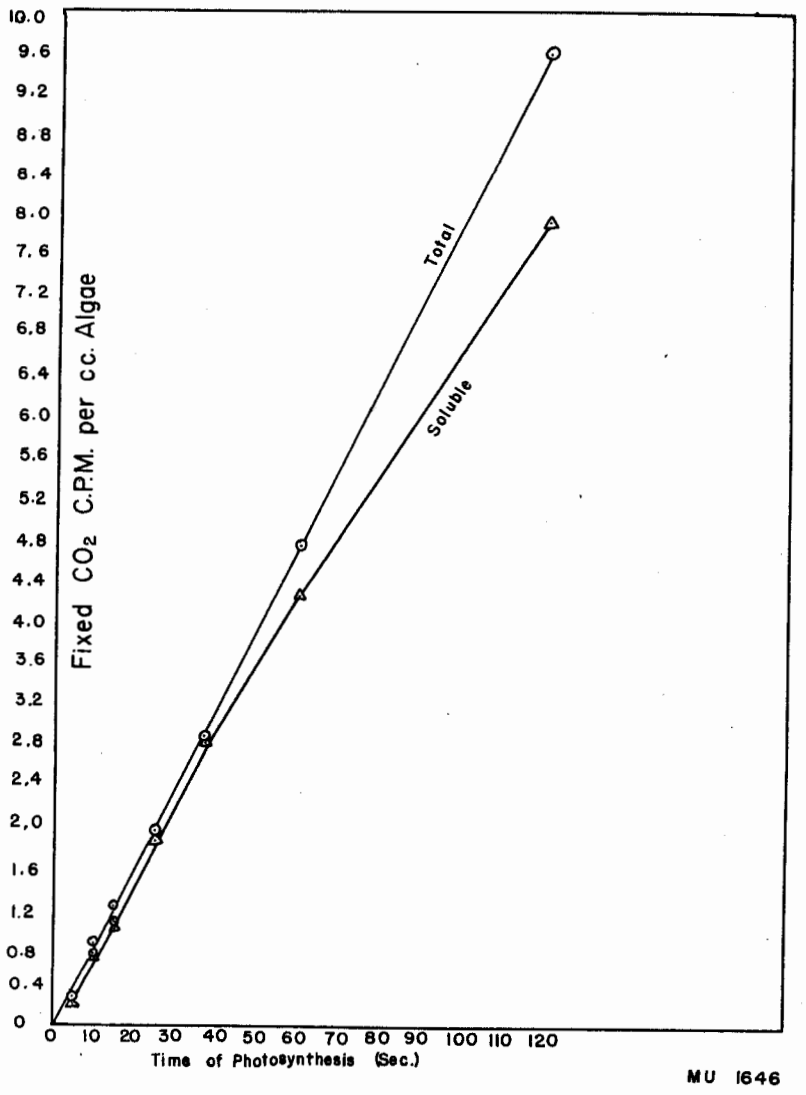

10.0
9.6
9.2
8.8
8.4
8.0
7.6
7.2
6.8
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
Fixed CO_2 C.P.M. per cc. Algae
Total
Soluble
0 10 20 30 40 50 60 70 80 90 100 110 120
Time of Photosynthesis (Sec.)
MU 1646

Fig. 5

Fig. 6

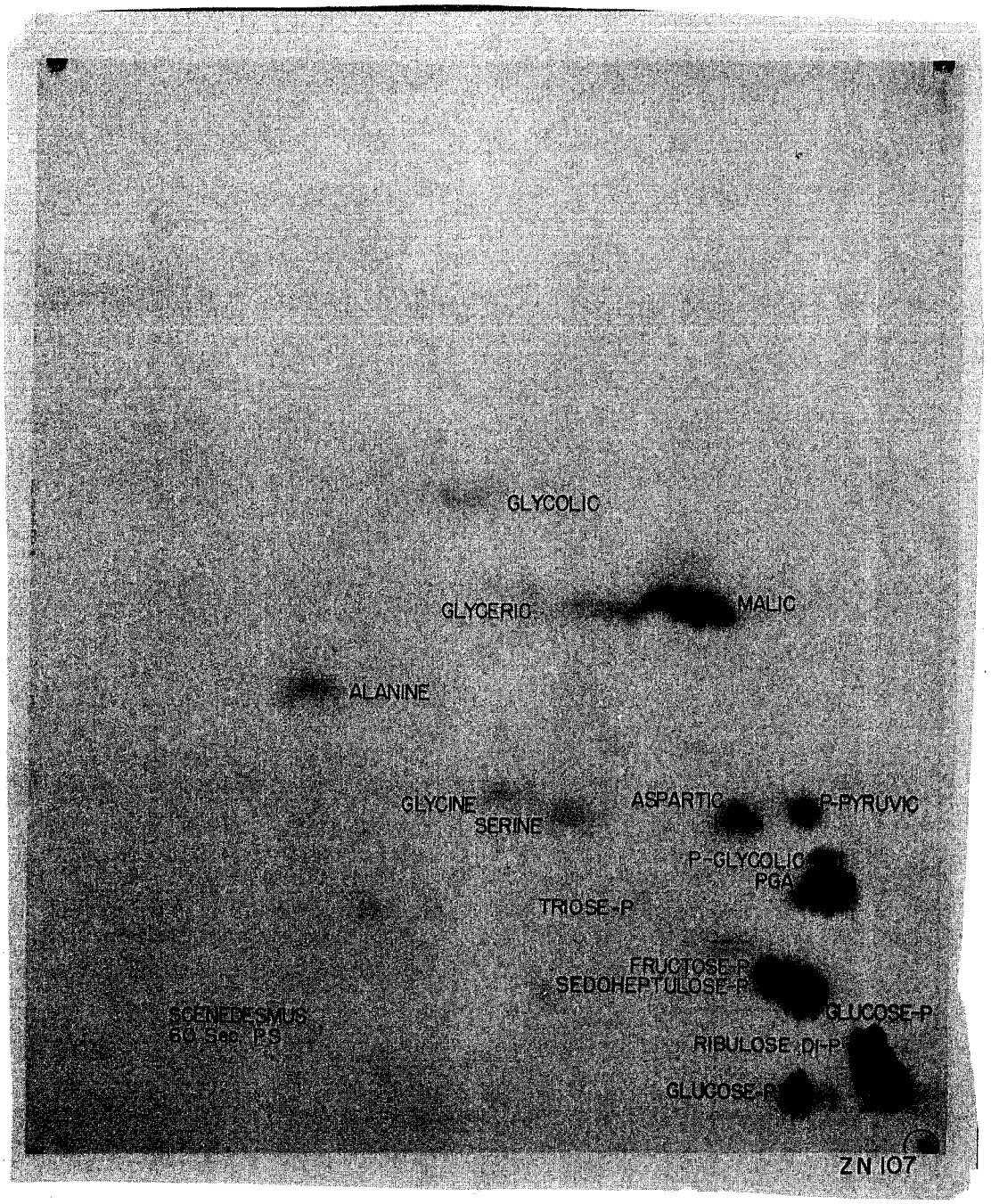

GLYCOLIC
GLYCERIC
MALIC
ALANINE
GLYCINE
SERINE
ASPARTIC
P-PYRUVIC
P-GLYCOLIC
PGA
TRIOSE-P
FRUCTOSE-P
SEDOHEPTULOSE-P
GLUCOSE-P
RIBULOSE DI-P
GLUCOSE-P
SCENEDESMUS
60 Sec. P.S.
ZN 107

Fig. 7

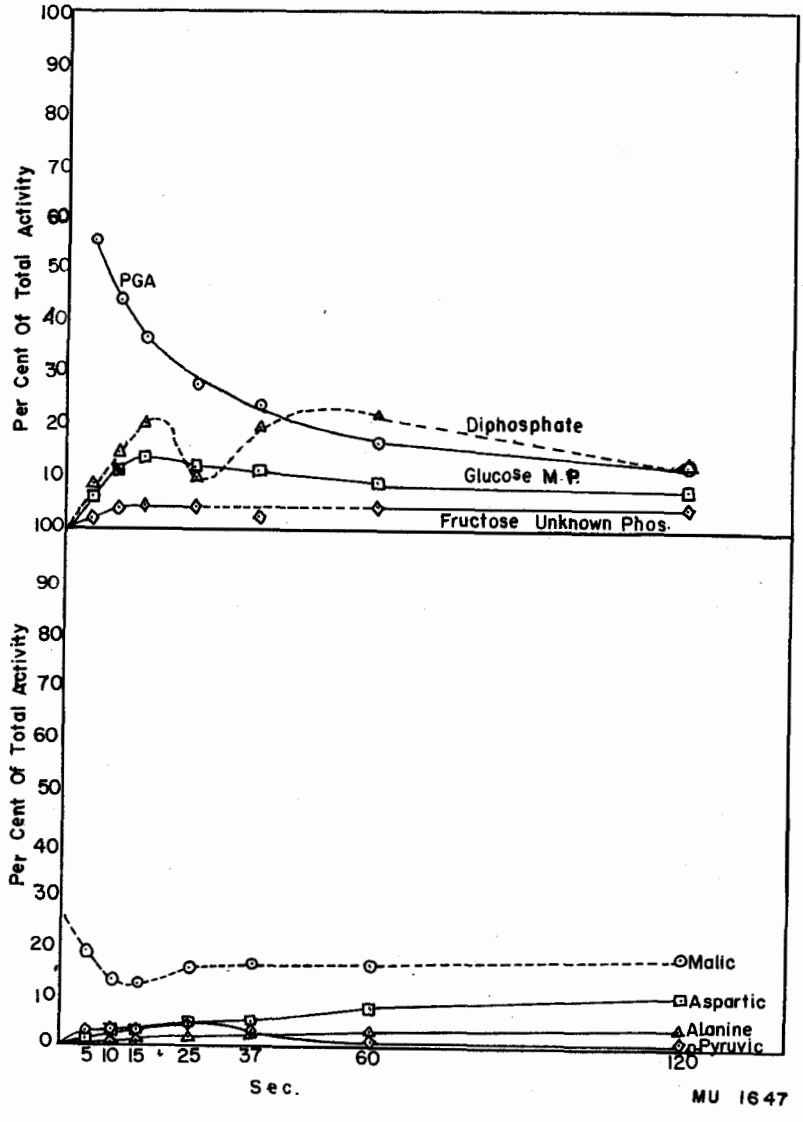

Per Cent Of Total Activity
PGA
Diphosphate
Glucose M.P.
Fructose Unknown Phos.
Per Cent Of Total Activity
Malic
Aspartic
Alanine
Pyruvic
5 10 15 25 37 60 120
Sec.
MU 1647

Fig. 8

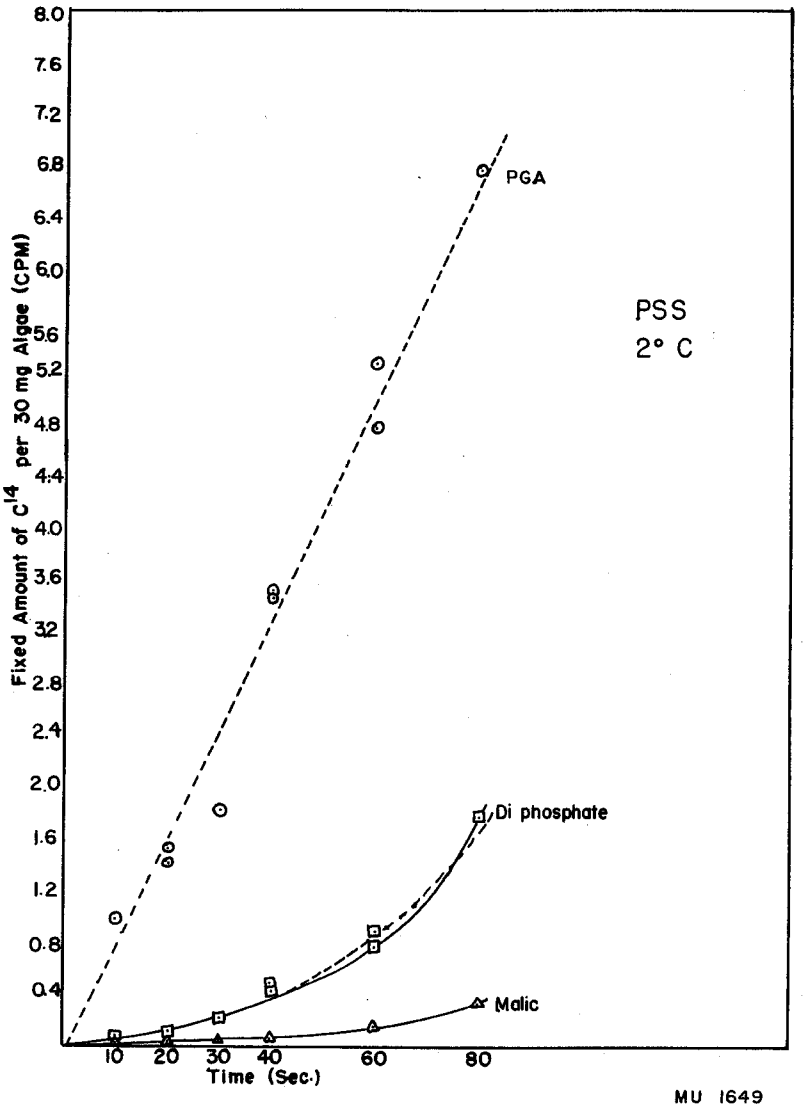

8.0
7.6
7.2
6.8
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Fixed Amount of C14 per 30 mg Algae (CPM)
PGA
PSS
2° C
Di phosphate
Malic
10
20
30
40
60
80
Time (Sec.)
MU 1649

Fig. 9

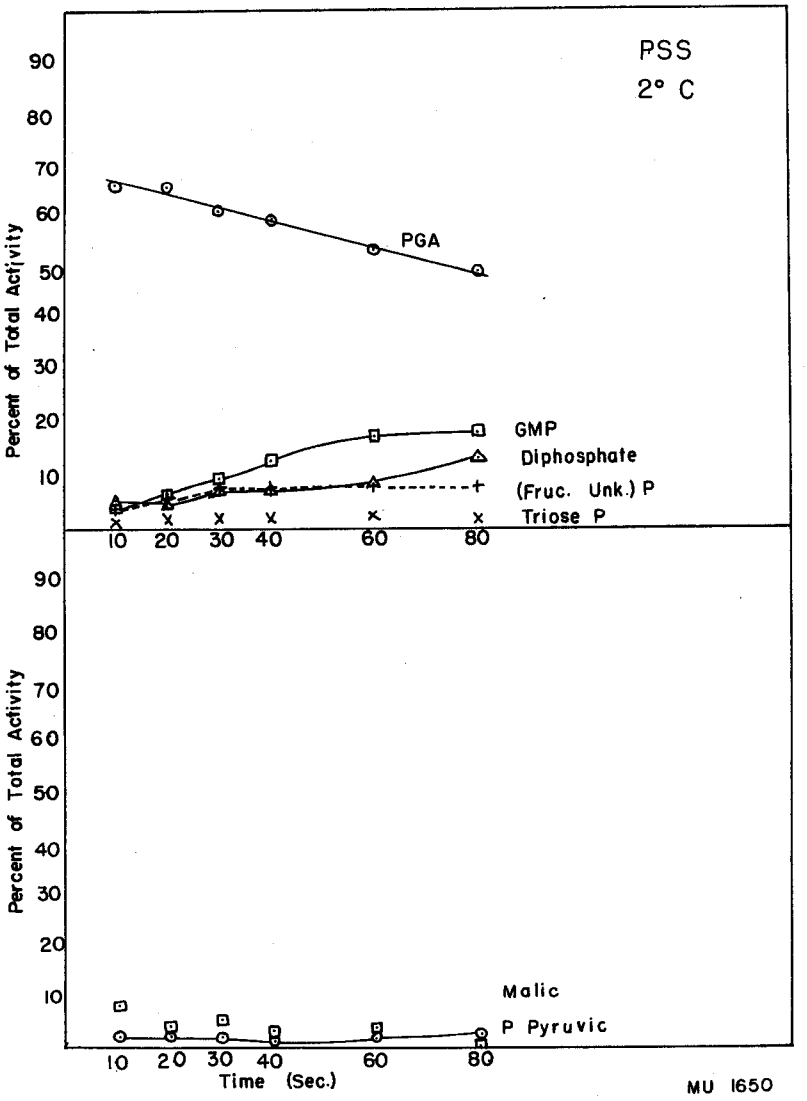
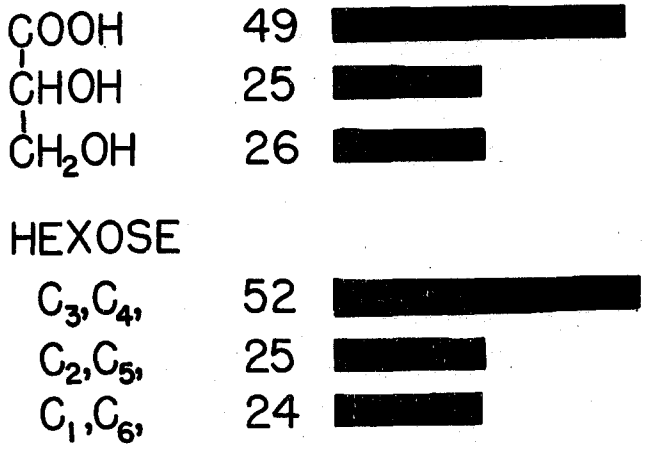

PSS
2° C
Percent of Total Activity
PGA
GMP
Diphosphate
(Fruc. Unk.) P
Triose P
Malic
P Pyruvic
Time (Sec.)
MU 1650

Fig. 10



15 SEC. P.S. BARLEY

Fig. 11

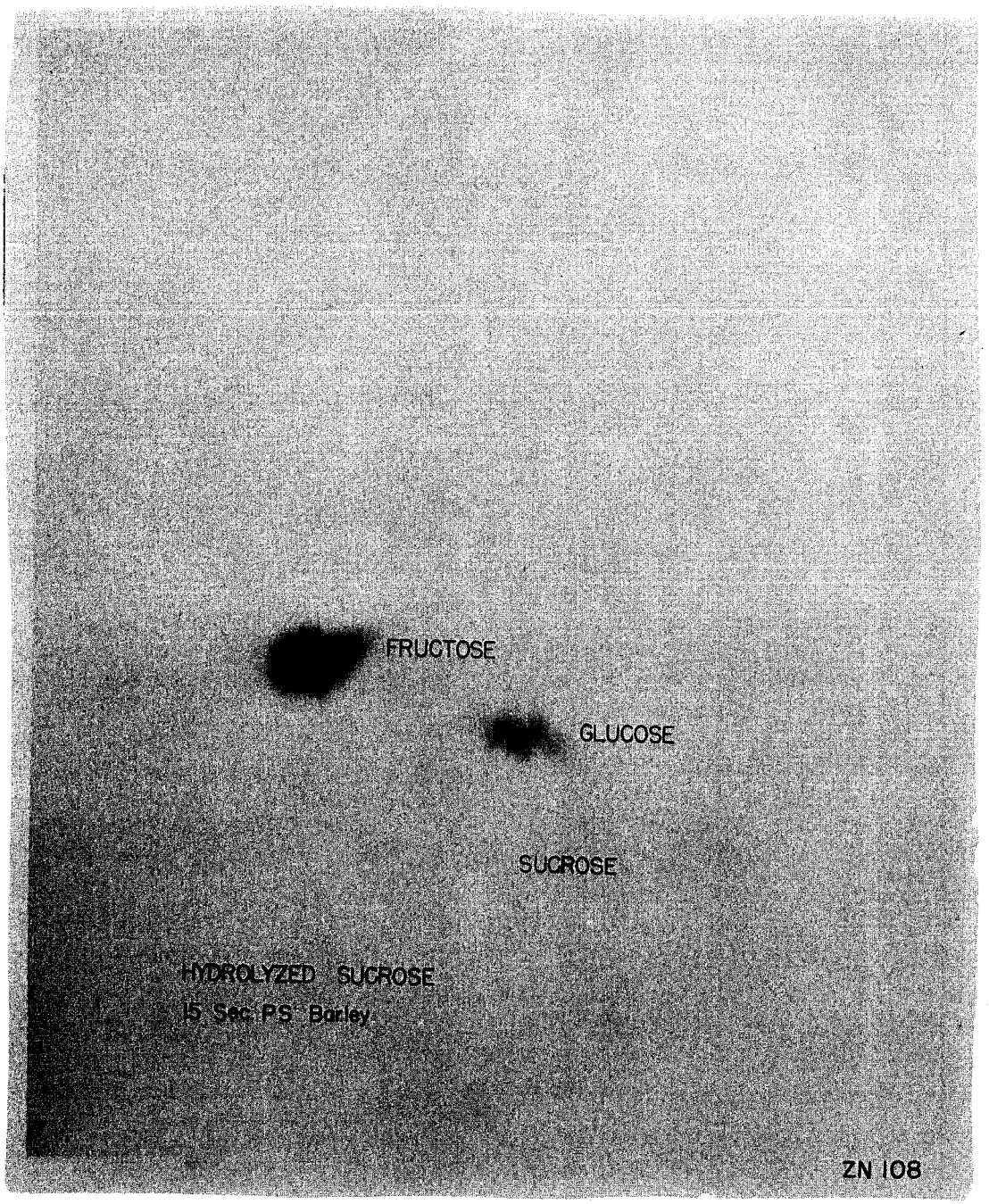

FRUCTOSE
GLUCOSE
SUCROSE
HYDROLYZED SUCROSE
15 Sec. P.S. Barley
ZN 108

Fig. 12

C^{14} DISTRIBUTION IN GLYCOLIC ACID AND GLYCERIC ACID

	4 Sec. PS BARLEY $C^{14}O_2$	10 Min. PS SCENEDESMUS, N_2 $C^{14}H_2OH-COOH$	10 Min. PS SCENEDESMUS, AIR $CH_2OH-C^{14}OOH$
GLYCOLIC			
CH_2OH	51	100	0
$COOH$	49	0	100
GLYCERIC			
CH_2OH	6.8	48	29
$CHOH$	6.5	56	24
$COOH$	87	7.2	46
Percentages Given	in Terms of Measured Starting Activity		

Fig. 13

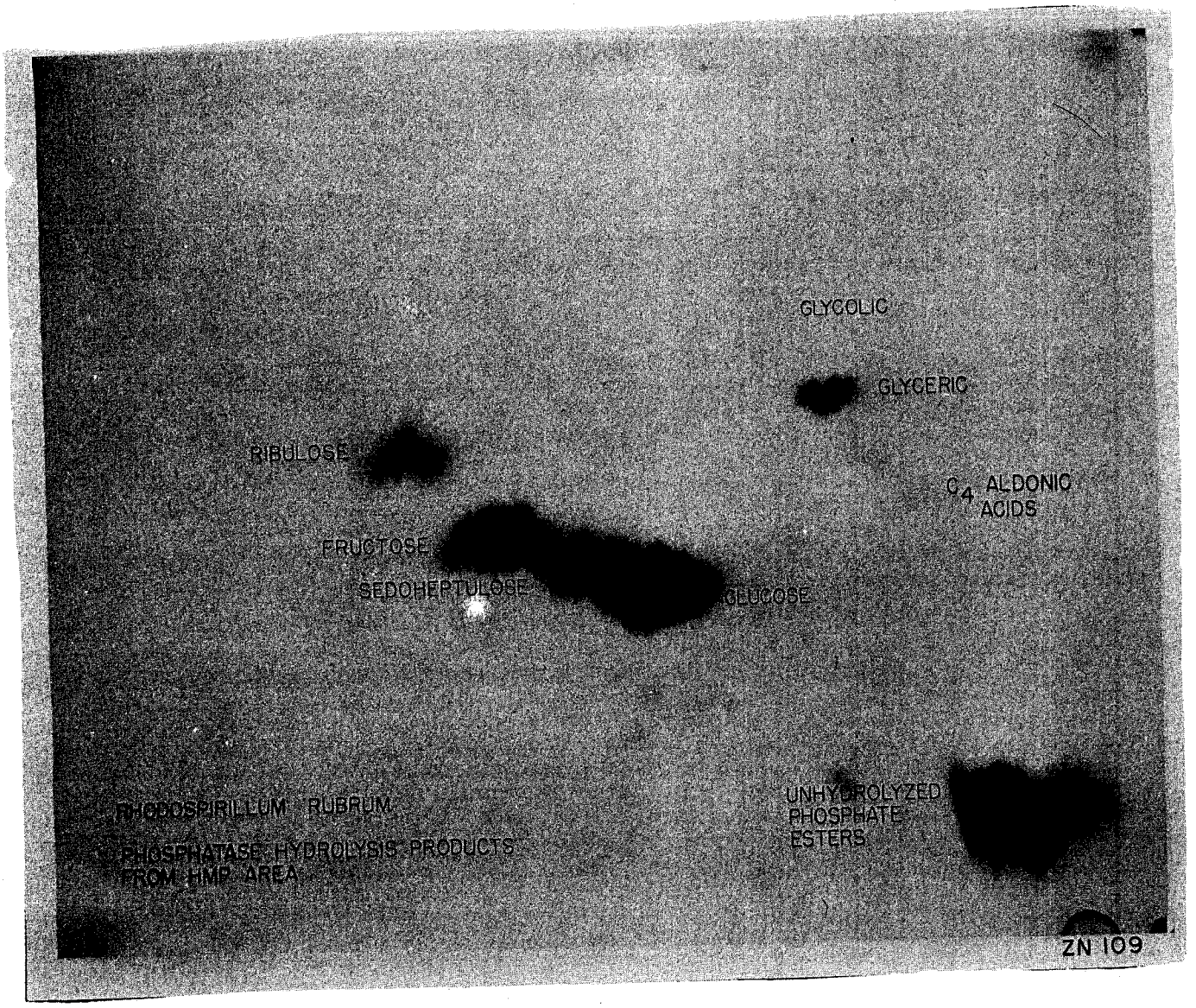

GLYCOLIC
GLYCERIC
RIBULOSE
C4 ALDONIC ACIDS
FRUCTOSE
SEDOHEPTULOSE
GLUCOSE
UNHYDROLYZED PHOSPHATE ESTERS
RHODOSPIRILLUM RUBRUM
PHOSPHATASE HYDROLYSIS PRODUCTS FROM HMP AREA
ZN 109

Fig. 14

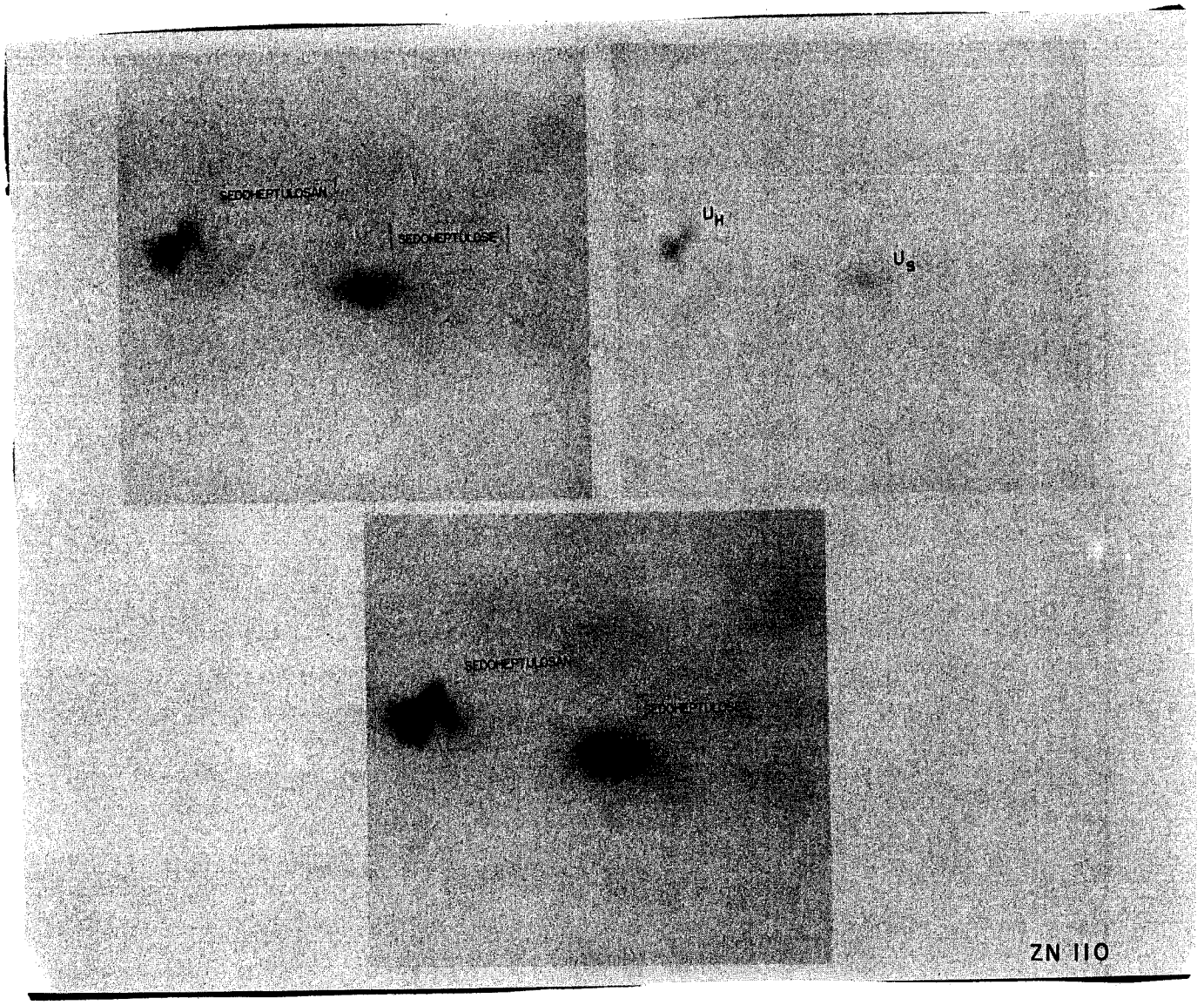

SEDOHEPTULOSAN
SEDOHEPTULOSE
U_H
U_S
SEDOHEPTULOSAN
SEDOHEPTULOSE
ZN 110

Fig. 15

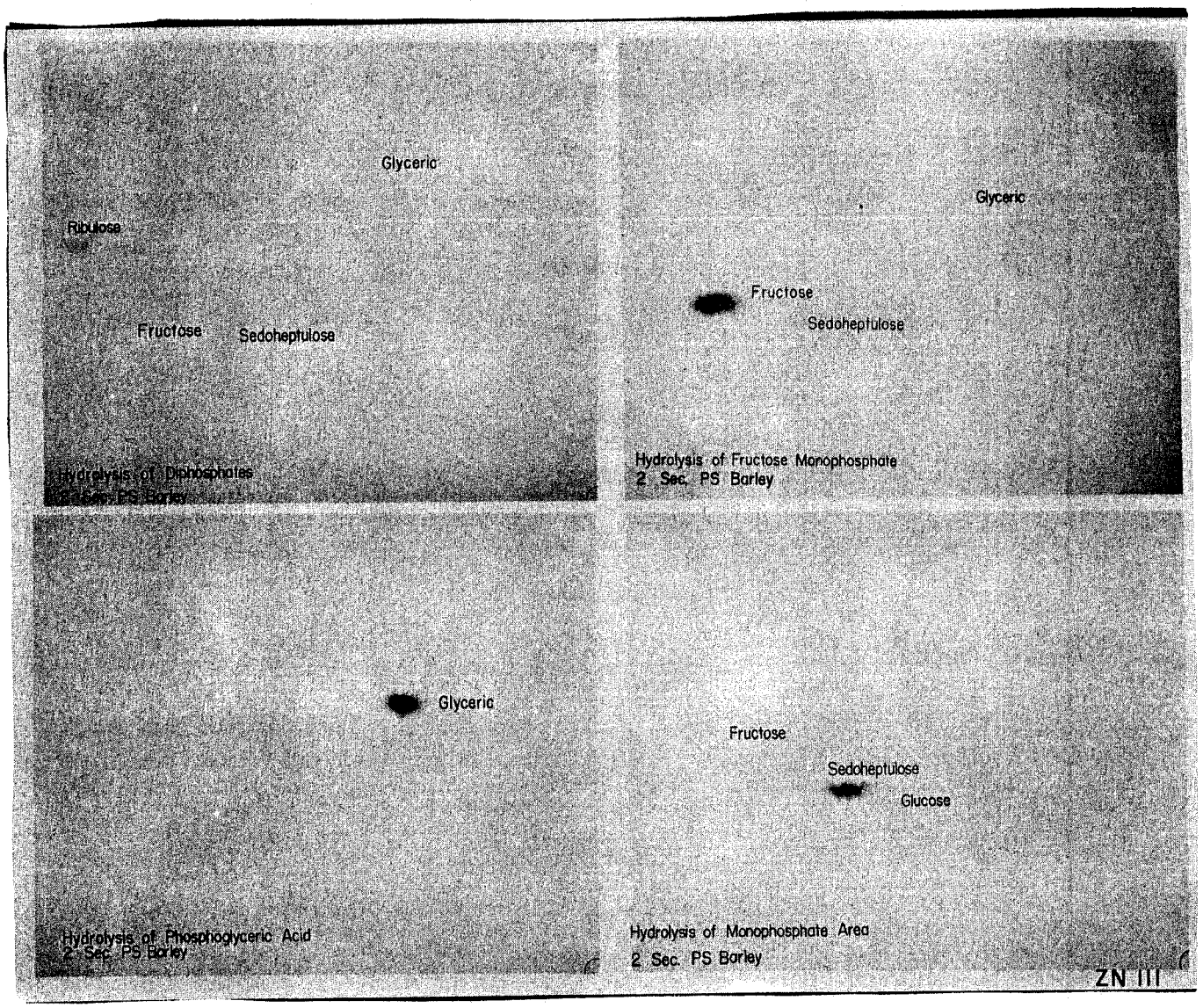

Glyceric
Ribulose
Fructose
Sedoheptulose
Hydrolysis of Diphosphates
2 Sec. PS Barley
Glyceric
Fructose
Sedoheptulose
Hydrolysis of Fructose Monophosphate
2 Sec. PS Barley
Glyceric
Hydrolysis of Phosphoglyceric Acid
2 Sec. PS Barley
Fructose
Sedoheptulose
Glucose
Hydrolysis of Monophosphate Area
2 Sec. PS Barley
ZN III

Fig. 16

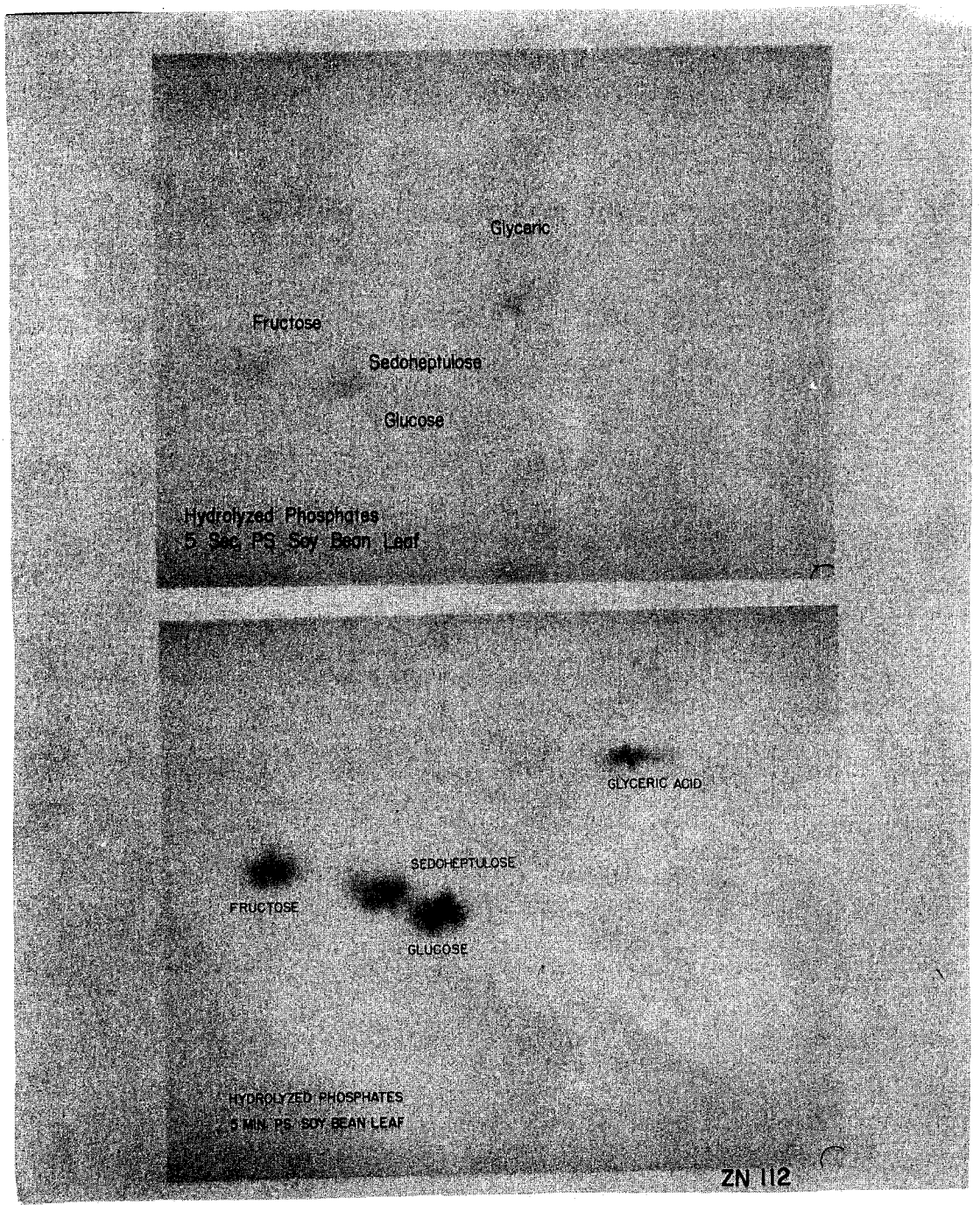

Glyceric
Fructose
Sedoheptulose
Glucose
Hydrolyzed Phosphates
5 Sec. PS Soy Bean Leaf
GLYCERIC ACID
SEDOHEPTULOSE
FRUCTOSE
GLUCOSE
HYDROLYZED PHOSPHATES
5 MIN. PS SOY BEAN LEAF
ZN 112

Fig. 17

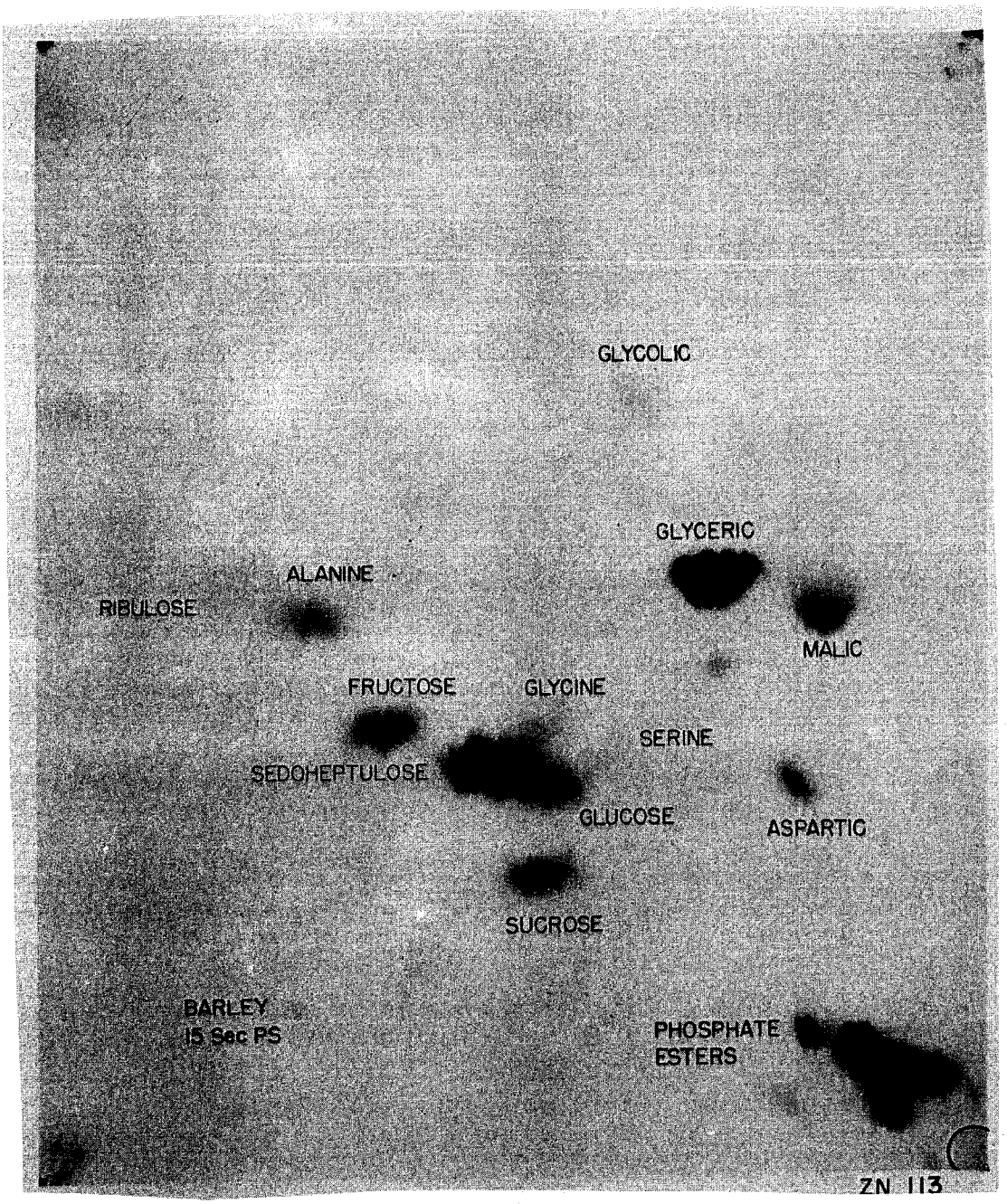

GLYCOLIC
GLYCERIC
ALANINE
RIBULOSE
MALIC
FRUCTOSE
GLYCINE
SERINE
SEDOHEPTULOSE
GLUCOSE
ASPARTIC
SUCROSE
BARLEY
15 Sec PS
PHOSPHATE
ESTERS
ZN 113

Fig. 18

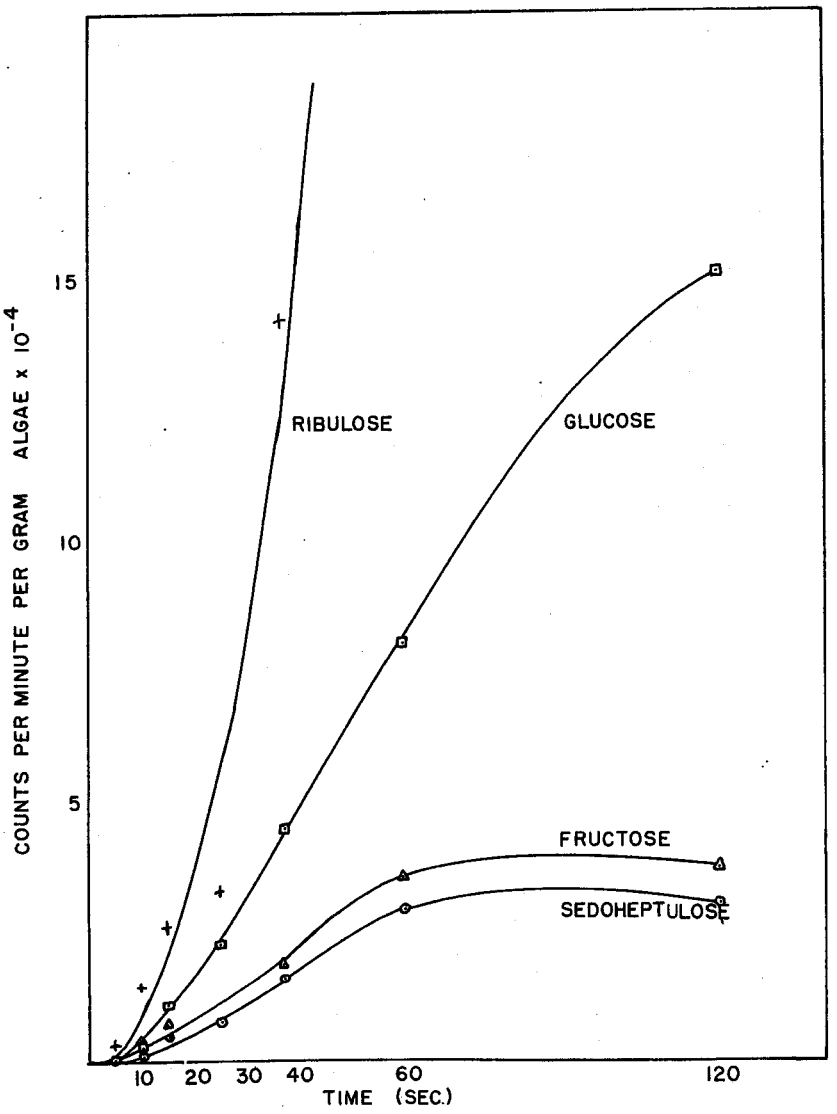

COUNTS PER MINUTE PER GRAM ALGAE x 10^{-4}
15
10
5
RIBULOSE
GLUCOSE
FRUCTOSE
SEDOHEPTULOSE
10
20
30
40
60
120
TIME (SEC.)

Fig. 19