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PHOSPHORYLATED SUGARS

A. A. Benson

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A. A. Benson

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

INTRODUCTION

Phosphorylated sugars and hydroxyacids are the intermediates in the primary functions of the plant: sucrose and polysaccharide synthesis. Almost all the intermediates and products of phytosynthesis are phosphorylated during their inverconversions in the highly organized complex of chemical systems maintained in the living cell. Their formation, mediated by enzymes (kinases, phosphorylases and phosphatases), serves as a step in the conservation of energy and in the synthesis of the cell itself. The number of known compounds in this group increases with our understanding of biosynthesis and it is likely that the list will eventually include many compounds now known only in the hydrolyzed form.

Sucrose synthesis is almost universal in photosynthetic organisms. It proceeds via the intermediates of glycolysis well known in animal and microbial metabolism. Sucrose, as well as fructose, sedoheptulose and glucose, are freely transported through the plant to the roots or rapidly metabolizing regions. The oxidation of these sugars through a series of phosphorylated intermediates provides the free energy necessary for absorption of nutrients and synthesis of new

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tissue. The sugars are always phosphorylated, however, when acting as enzyme substrates during synthesis and degradation.

The sugar phosphates are not commonly encountered in plant analysis for two reasons. Their concentrations are usually ten or a hundred times lower than those of sucrose or the free hexoses. The very active plant phosphatases rapidly hydrolyze these phosphate esters unless they can be very quickly denatured during isolations.

The metabolic state of the plant determines the concentrations of these intermediates. Since their concentrations are low and the amount of material passing through these metabolic pools in a short time is great, there may be very rapid and profound variations resulting from external changes prior to the analysis. The influence of external conditions upon these interrelationships is not yet thoroughly understood. The value of the results for compounds of such transient nature can only progress with our understanding of their metabolism.

Analytical methods for sugar phosphates in animal and isolated enzyme systems are well known. Leloir (1951) has written an excellent review on the isolation, structure, properties and analyses of sugar phosphates. Previous reviews by Courtois (1941) and Robison and Macfarlane (1941) are also available. A recent summary of transformations of sugars in plants by Hassid and Putman (1950) deals particularly with polysaccharide synthesis. Occurrence and metabolism of phosphorylated compounds in plants are reviewed by Albaum (1952). The identifications of photosynthetic intermediates of carbon dioxide reduction are summarized by Buchanan, et al. (1952) and their transformations are discussed by Bassham, et al. (1954). As yet there is no comparative biochemical study of the distribution of phosphate esters in plants. A preliminary survey by Norris and Calvin (1954) covers the plant kingdom quite well but much investigation of the identity and concentrations of phosphates remains to be done.

This section is particularly dedicated to the development of an understanding of the transient status of sugar phosphates in plant material.

THE SUGAR PHOSPHATES IN PHYTOSYNTHESIS

Phytosynthesis is a complex network of energy and material transfer. Each reaction is dependent upon all the others and the status quo is a function of the external conditions. Synthesis and degradation, therefore, are interdependent and it is not surprising that the intermediates of the two processes are often identical.

The first product of CO_2 assimilation by the plant is phosphoglycerate. It is reduced to triose phosphate and thence the newly incorporated carbon passes through the members of the glycolytic sequence to the condensation resulting in sucrose phosphate. Plants appear to have a very active sucrose phosphatase system which produces free sucrose as the major free sugar in most plants and a very low concentration of sucrose phosphate. The major products of phytosynthesis, starch and cellulose, result from condensation of glucose-1-phosphate which itself exists in very low concentration during the synthesis. Similarly the polypentoses are probably formed from xylose- and arabinose-1-phosphates. Aromatic nuclei appear to be formed from heptose phosphates by way of cyclized carbohydrate intermediates, possibly shikimic acid and its phosphates. The known number of such phytosyntheses which use sugar phosphates as substrates grows with our understanding of the identity and quantitative relationships of compounds involved in this very active system.

Some of the known relationships of sugar phosphates in plants are described in Figure 1. That these include only a small fraction of the phosphorylated compounds is borne out by a very simple experiment. If the combined phosphorylated compounds in a leaf which has photosynthesized from $C^{14}O_2$ are hydrolyzed

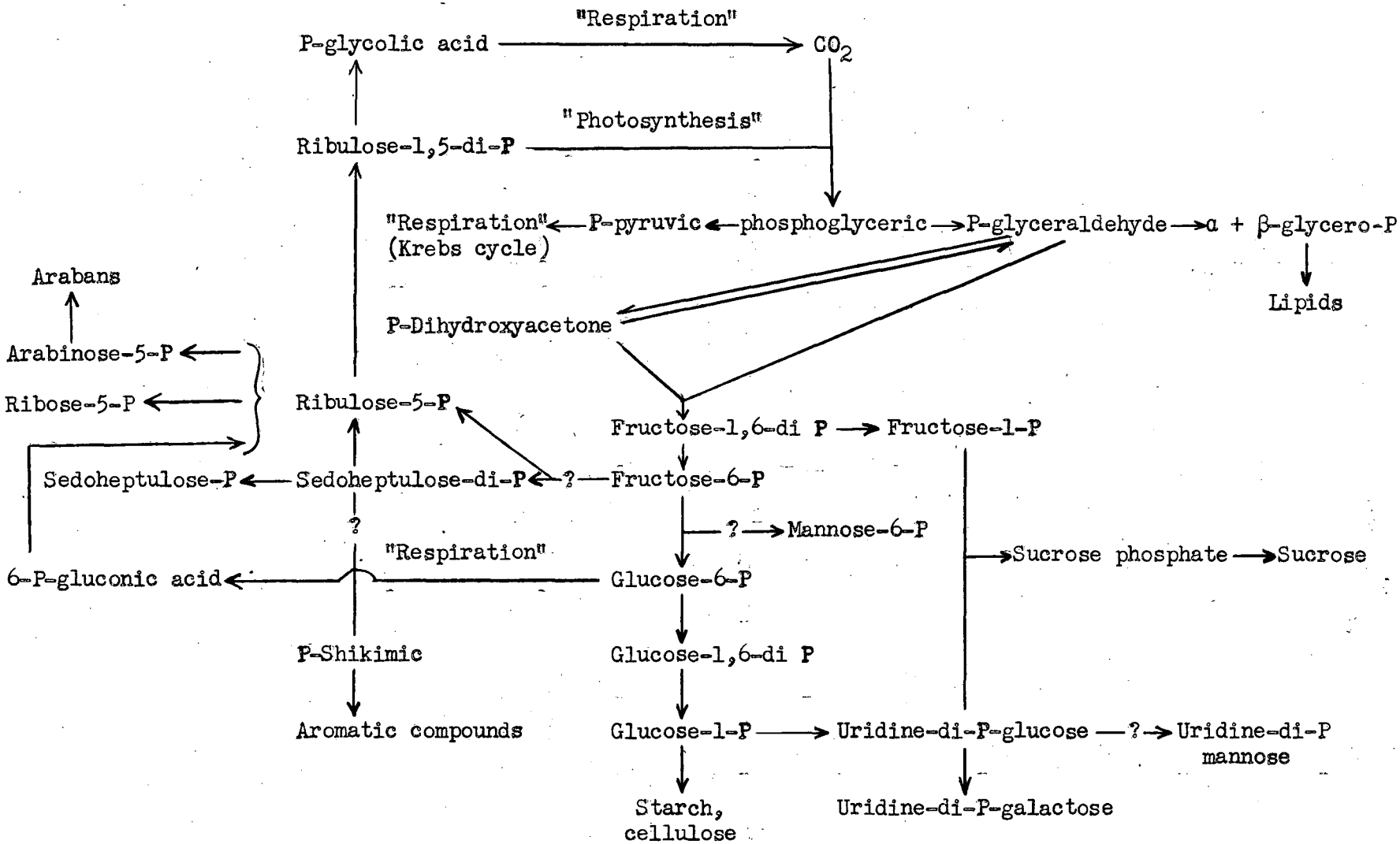

Fig. 1 - Transformations of sugar phosphates in plants. This tentative scheme represents only a small fraction of plant metabolism involving sugar phosphates.

with phosphatase, the resultant sugars, polysaccharides and hydroxy acids can be separated by two-dimensional paper chromatography. The radioautograph of such a hydrolysate shows at least sixty discernable products besides the major ones, glucose, fructose and glyceric acid. This is a challenge to those studying the interconversions of phosphorylated compounds. The end is not yet in sight.

Oxidative Pathways in Hexose Metabolism. - There appear to be two pathways for glucose oxidation in plant systems as well as in animal tissues. The Embden-Meyerhof glycolytic sequence has been observed in a variety of experiments which (Stumpf, 1951; Holzer and Holzer, 1952) demonstrated the presence of the necessary enzyme systems. The same systems were suggested by Hartt's work (1944) on sucrose synthesis in sugar cane. She found evidence for the glycolytic mechanisms by studying the effects of specific inhibitors upon sucrose synthesis.

Evidence for glycolytic function is reviewed by Stumpf (1952, 1952). The identification of all of the intermediates involved constitutes a second line of evidence for the function of the Embden-Meyerhof system (Albaum, 1952).

As in animal systems (Horecker, 1951; Dickens and Glock, 1951), it seems likely that a major fraction of glucose oxidation proceeds via the "pentose shunt" from 6-P-gluconate to ribulose-6-P (Axelrod, Bandurski, Greiner and Jang, 1953; Conn and Vennesland, 1951). The high activity of this enzyme in plant systems suggests the major role of this mechanism (Figure 2). Ribulose and sedoheptulose may split to give P-glycolic acid, which can be readily oxidized to carbon dioxide by the ubiquitous glycolic oxidase (Clagett, Tolbert and Burris, 1949; Zelitch and Ochoa, 1952), and triose phosphate which may be oxidized through pyruvate and the tricarboxylic acid cycle. The exact mechanism of ribulose oxidation is yet undetermined but it is undoubtedly responsible for

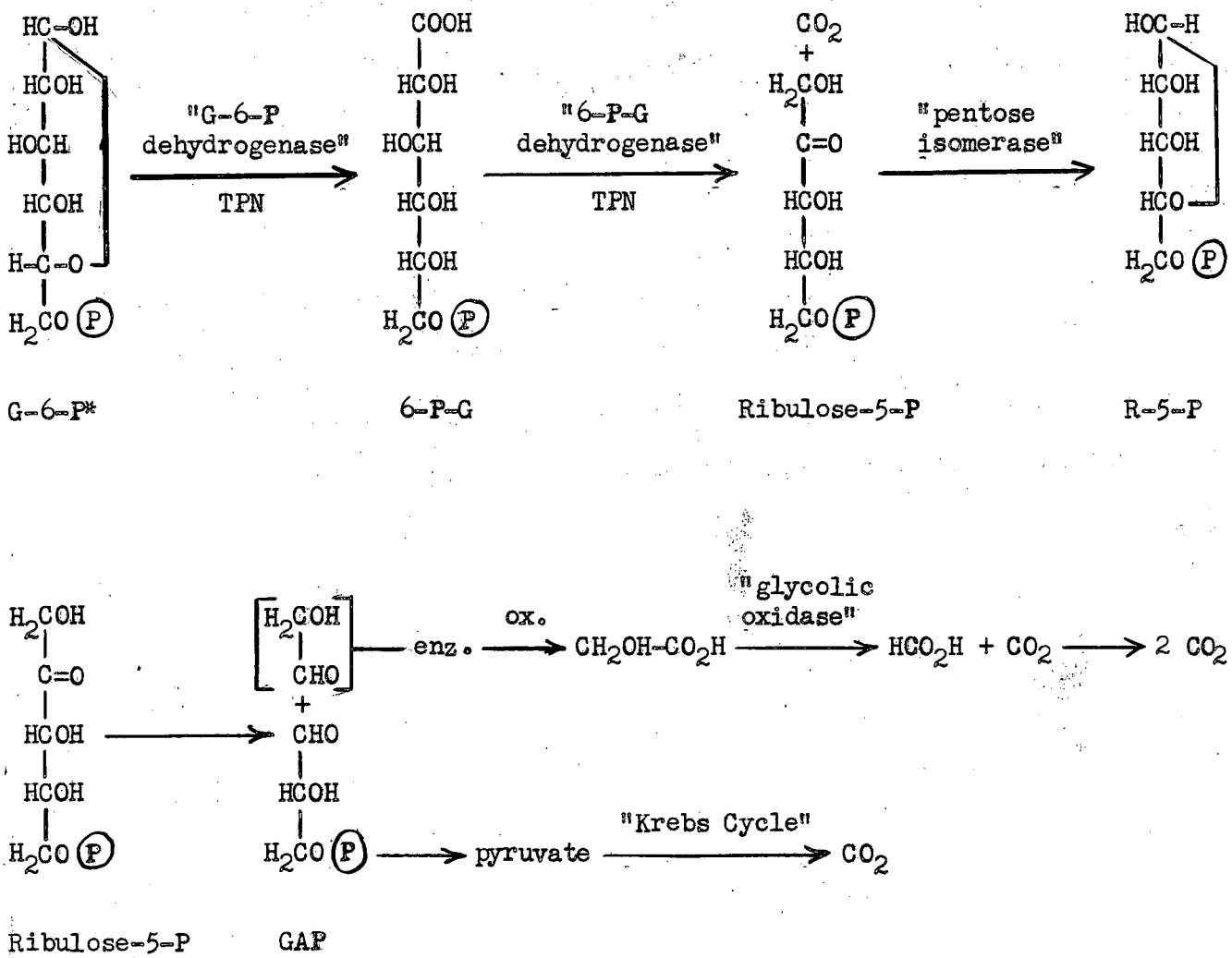

Fig. 2 - Alternate Path of Hexose Oxidation in Leaves

(*) The following currently adopted abbreviations for the common phosphate esters will be used. TPN, triphosphopyridine nucleotide; TCA, trichloroacetic acid; PGA, 3-phosphoglyceric acid; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; R-1-P, ribose-1-phosphate; R-5-P, ribose-5-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconic acid; F-6-P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; ATP, adenosine triphosphate.

the observed copious formation of glycolic acid when leaves or algae accumulate ribulose. This occurs especially when plants are exposed aerobically to light in the absence of carbon dioxide, but also can occur during normal photosynthesis when the light intensity is high and the normal CO_2 pressure in the air is rate limiting.

APPLICATIONS OF THE TRACER METHOD IN DEVELOPMENT OF NEW ANALYTICAL METHODS

The great sensitivity of the tracer method and its capacity of allowing one to discriminate between like substances from different sources have stimulated the development of all analytical fields. Techniques of separation of sugar phosphates, particularly, have profited from tracer application. At the same time tracer applications demanded improved analytical methods.

With the simultaneous advent of the "chromatographic" and "tracer" techniques analysis became keyed to the requirements of both, and the synergistic effect of the two disciplines has been phenomenal. The ability of the tracer technique to detect and measure impurities in the classically prepared sugar phosphates is matched by the elegance of paper chromatographic separation of these components. The completeness of extraction methods, stability of the compounds and validity of observed physical constants now can be much better known. The techniques required for tracer work are simple and readily adapted to the well-known methods of fractionation and analysis of sugar phosphates. The work of Axelrod and Bandurski serves as an excellent example of an application of the tracer method for control and extension of the classical separations of metabolic intermediates. By simply labeling their phosphorylated compounds by exchange with radiophosphate in the metabolizing enzyme system they achieved a great increase in sensitivity and reliability of subsequent separation and assay methods.

Radiophosphate as an analytical tool. - When all the phosphorus in a tissue is equally labeled it is only necessary to separate the phosphorus-containing compounds, since the others do not interfere, and measure the radioactivity in each. The specific radioactivity (counts per minute per μg. P) is

obtained by ordinary phosphorus analysis and radioactivity measurement on an identical sample. With this figure, the amounts of sugar phosphates corresponding to their observed radioactivities can be calculated. The advantage of this method is primarily its sensitivity. The micrograms of compounds in a few milligrams of plant material can be accurately and reliably determined.

The major problem is that of achieving a uniformly phosphorus-labeled plant sample. This problem can be solved with microorganisms or small plants where the phosphorus turnover is rapid. Our knowledge of phosphorus turnover rates in tissue of higher plants is not yet established well enough to render this a universally applicable method.

Phosphorus activation analysis. - The problem with which the reader is left in the preceding section is not without solution. Again we must resort to a tool of the atomic era. The high and uniform neutron flux available in the nuclear reactor can synthesize P^{32} from the P^{31} of the phosphate esters in a plant extract.

$$n + P^{31} \xrightarrow{\text{synthesis}} P^{32} + \gamma \xrightarrow{\text{decay}} \beta^- + S^{32}$$

To apply this method one need only irradiate a paper chromatogram of separated phosphates or merely a sample containing a trace amount of phosphorus compound in the nuclear reactor. Such services are already available. The resulting P^{32} radioactivity depends upon the time and flux of the irradiation and the initial amount of P^{31}. It can be readily counted with a Geiger counter or the paper chromatogram may be exposed to a film for a permanent record of the radioactivity and its position. A series of phosphate samples or spots on the paper could be used to standardize the radioassay.

The major difficulty in this approach is that of radioactive byproducts. The presence of elements with large neutron-capture cross sections even in

trace quantities results in a variety of radioactive species. Many and possibly all of these may be separated by paper chromatography. The isotopic purity of the P^{32} can be estimated by observing its decay rate. The amount of radioactive impurity can be minimized by careful control of adsorbent purity and partial purification of the sample. The literature on this type of phosphorus analysis is not yet in existance but it will certainly accumulate rapidly as the obstacles are overcome.

TRANSIENTS IN SUGAR PHOSPHATE CONCENTRATIONS

The reliability of a determination of sugar phosphates is determined by the control we may have over the constancy and reproducibility of their concentrations while the sample is being prepared. Such control requires an understanding of the many factors affecting their concentration. We can draw some conclusions from the available information and develop a respect for the possible errors and difficulties arising from such transient concentrations.

The concentrations of some of the intermediates of photosynthesis and respiration were measured by Benson (1952) and Calvin and Massini (1952) using P^{32} and C^{14}. The former grew green algae (Scenedesmus) in P^{32}-labeled nutrient and measured the radioactivity in the paper-chromatographically separated sugar phosphates. The specific radioactivity (counts per minute per gram atom P) was derived from a phosphorus analysis and radioactivity measurements on a sample of the uniformly P^{32}-labeled plant material. From the specific radioactivity, the known number of phosphorus atoms in the compounds and their radioactivity the results of Table I were calculated. A similar measurement was obtained by saturating the sugar phosphates with C^{14} as the algae photosynthesized in $C^{14}O_2$ of known specific radioactivity. When C^{14} concentration in the compounds became constant after ten minutes the radioactivity observed on the chromatogram was a measure of the concentration of these esters.

The gross discrepancies between the analyses for the compounds in Table I can be attributed only partly to errors in the method. The concentrations of these phosphates are known to depend on pH (Ouellet and Benson, 1952), light intensity (Calvin and Massini, 1952) and carbon dioxide pressure (Wilson, 1954). Age of the organisms, temperature and the cultural conditions have similar pronounced effects.

Table I

CONCENTRATIONS OF PHOSPHORYLATED COMPOUNDS

IN PHOTOSYNTHESIZING SCENEDESMUS

Compound	P^{32} Analysis 4% CO_2, 20°	C^{14} Analysis 1% CO_2, 20°	C^{14} Analysis 1% CO_2, 6°	C^{14} Analysis 0.003% CO_2, 6°
Phosphoglycerate	5.7×10^{-3} M	1.4×10^{-3} M	1.0×10^{-3} M	2.9×10^{-4} M
Glucose monophosphate	1.1×10^{-3} M	4.0×10^{-4} M	8.8×10^{-3} M	8.8×10^{-4} M
Fructose monophosphate	2.7×10^{-4} M	1.2×10^{-4} M	3.3×10^{-4} M	1.4×10^{-4} M
Sedoheptulose monophosphate	1.0×10^{-4} M	1.8×10^{-4} M	1.8×10^{-4} M	..
Ribulose diphosphate	1.0×10^{-3} M	5.0×10^{-4} M	5.0×10^{-4} M	7.5×10^{-4} M

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Figure 3 describes the changes occurring in C^{14}-saturated phosphate reservoirs, i.e. the concentrations of the sugar phosphates, when the light is turned off. Phosphoglycerate doubles in concentration while ribulose diphosphate, labeled "diphosphates," decreases in an equimolar amount within a few seconds. The result is interpreted as being caused by the dark carboxylation of ribulose diphosphate to form two moles of phosphoglycerate. Figure 4 shows the reverse shift in concentrations occurring when the photosynthesis substrate is suddenly changed from 1% $C^{14}O_2$ to 0.003% $C^{14}O_2$. The accumulation of ribulose diphosphate again suggests that it is the carboxylation substrate accumulating as the CO_2 pressure is reduced. These sudden changes certainly should tend to make one cautious in interpreting the meaning of a single determination of a phosphate in a rapidly metabolizing system.

The concentrations of sugar phosphates in plants, then, are highly dependent upon the metabolic state and can change profoundly within a few seconds. Their analyses offer valuable information on the function of the plant. Since this is the case, analyses for phosphates should be accompanied by a complete description of the condition of the plant material and preparation of the sample.

EXTRACTION OF PHOSPHATE ESTERS FROM PLANT MATERIAL

One of the current problems in phytochemistry is the effect of treatment of fresh plant material upon its sugar phosphates. One may assume that most currently used preservation methods result in rapid autohydrolysis of the phosphorylated sugars by the very active widely distributed plant phosphatases. The free sugar analysis, then, is in fact a measure of free and phosphorylated sugar. The relationship between these is a subject not yet reliably determined, or soundly understood. Since the concentrations of the sugar phosphates are usually very small compared to those of free sugars in plant tissue the errors in free sugar determinations are negligible.

The vigorous activity of plant phosphatases may be described with the results of a $C^{14}O_2$ photosynthetic experiment on barley seedling leaves. After one minute photosynthesis in $C^{14}O_2$, the leaves were plunged into boiling ethanol; only phosphorylated sugars were observed on the radiogram (Figure 5a). On the other hand, when killed by extraction with cold (-50°) ethanol or by grinding in liquid nitrogen and then killed in hot ethanol (Figure 5b) a major fraction of the sugar phosphates were hydrolyzed and observed as free sugars. Neither the extent nor the precise conditions for obtaining these results are yet well understood. The results show quite clearly, however, that the plant phosphatases are very active over a great temperature range, even in non-aqueous medium. Results from this laboratory have demonstrated that the most stable phosphatase activity remains attached to the plant tissue.

Extraction of phosphate esters, then, requires that the phosphatases be quickly and permanently destroyed and that cellular material be separated from the extracts as soon as possible. This is commonly done by extraction with dilute trichloroacetic acid (TCA) in the manner used for extraction of animal

tissue. Albaum (1952) has reviewed the differences in extractability of phosphates from animal and plant tissues. Rapid denaturation in boiling 80% ethanol or methanol insures that the phosphates obtained are in the concentration existing at the instant of killing.

TCA Extraction Method (Umbreit, Burris and Stauffer, 1945). - Extraction by successive portions of cold (0°) 5% TCA is continued until the extract contains no phosphorus. It is advisable to use an equal volume 10% TCA for the initial extraction of wet plant tissue. The rate of the extraction depends upon temperature and degree of subdivision of the material. With bacterial cells, for example, it was necessary to treat with acetone to break down the cells before adequate extraction was obtained. Several long extractions were necessary to remove all extractable phosphorus. In general it is not yet possible to avoid checking completeness of extraction of organic phosphorus from unfamiliar sources.

The rate of enzyme denaturation of green plants by TCA extraction is not known. At the low temperatures employed it can be expected to be rapid compared to the possible changes in concentrations of intermediates during the extraction. Direct paper chromatography of TCA extracts has not given very good results without intermediate precipitation of barium salts.

Hot Ethanol Extraction. - Boiling one or two minutes in ten to fifty volumes of 80% ethanol is sufficient to denature plant phosphatases. Extraction of free sugars, amino acids, and plant acids is essentially complete when the chlorophyll appears to be extracted. A major fraction of the sugar monophosphates are extracted under these conditions. The tissue still contains a large fraction of the PGA and almost all of the ribulose diphosphate and hexose diphosphates.

These are extracted by boiling in 20% ethanol. In some cases ribulose diphosphate is more firmly adsorbed and requires a hot water extraction. The separation in these two extractions suffices to make it a preparative method for obtaining ribulose diphosphate in a partially purified state. Plant tissues apparently contain varying amounts of more firmly bound phosphates which are liberated by subsequent TCA extractions (L. P. Zill and N. E. Tolbert, Personal communication). The identity of the acid-extractable phosphorus and its relation to that extracted by alcohol-water mixtures is not yet clear.

The presence of high concentration of certain polysaccharides may interfere with extraction of phosphate esters. The galactan content of Iridophycus (Bean, 1953) selectively adsorbs the phosphate esters. The usual extraction methods failed. As an example, it was necessary to use phosphatase in the liberation of dihydroxyacetone for identification of triose phosphate.

Effect of Killing Methods on Soy Bean Leaves (R. Bean, Private communication. - The newly formed sugar phosphates of soy bean leaves were stable when killed in hot alcohol even after grinding in liquid nitrogen. When extracted at -20° C. in 80% alcohol followed by centrifugation, a major fraction of the sugar phosphates were hydrolyzed. Similar effects have been observed with Scenedesmus. Prompt separation of the extracted cells presented undue phosphatase action.

Effect of Dark Periods Before Killing Soy Bean Leaves (J. A. Bassham, Private communication). - When the dark period is increased from zero to 60 sec. after a brief photosynthesis in $C^{14}O_2$ the fraction of free glyceric acid/phosphoglyceric acid increased from 5% to 75%. Similar effects have been observed in barley seedling leaves (Benson and Calvin, 1950), suggesting an ATP-light relationship.

SEPARATION OF SUGAR PHOSPHATES

PRECIPITATION METHODS FOR FRACTIONATION OF SUGAR PHOSPHATES. - LePage and Umbreit (1943) developed previously applied methods for separation and identification of specific phosphate esters in extracts of Thiobacillus thiooxidans. The procedure consisted of a deproteinization with TCA and treating the acid-soluble fraction with barium ion at pH 7 or 8.2. The precipitate contained barium phosphoglycerate, fructose diphosphate, ATP and ADP. Under these conditions barium glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate, adenylate and DPN were soluble. Phosphoglycerate was determined by the method of Rapoport (1937). Hexose diphosphate was measured by fructose assay (Roe, 1934). In the acid-soluble fraction, fructose was estimated again to give a value for fructose-6-phosphate. Glucose-1-phosphate was hydrolyzed by 7 minutes boiling in N HCl and the new orthophosphate and freed reducing sugar were determined. Glucose-6-phosphate was calculated from the phosphorus remaining after the subtraction of the DPN value and from its measured reducing value. This general method was found well adapted to analysis of animal tissues. With Thiobacillus over 90% of the soluble phosphorus was accounted for in the above compounds. Albaum (1952) points out from considerable experience, that such procedures preclude a knowledge of the constituents of the mixture before the results can be valid. It cannot yet be assumed that the major fraction of water-soluble phosphorus compounds in plants are known.

Albaum and Umbreit (1947) naturally expected that this procedure should be applicable for the fractionation of extracts from oat seedlings. It was immediately clear that the procedure was not to be directly successful. The large amounts of co-precipitated starch gave a charred dark color in the PGA estimation instead of the characteristic blue color. When all the possible

corrections were made there still remained a large amount of unaccounted-for phosphorus. Part of this turned out to be phytic acid. The compounds identified with certainty were orthophosphate, fructose-6-phosphate, fructose diphosphate and phytic acid. A survey of the possibly labile phosphorus compounds in Euglena revealed the source of complication, the presence of the highly acid-labile inorganic pyrophosphate ($H_2P_2O_7^{=}$) (Albaum, Schatz, Hutner and Hirschfeld, 1950). Inorganic metaphosphate (PO_3^{-}) was found in a polysaccharide fraction and identified by its purple toluidine-blue reaction in acid solution. The presence of the inorganic ortho-meta- and pyrophosphates, glucose-1-phosphate, fructose-6-phosphate, hexose diphosphate, PGA were determined in the presence of ATP, ADP, OPN and riboflavin phosphate.

Large amounts of material are necessary for separations and identifications by precipitation methods (200 l. Euglena culture, 5-10 lb. mung beans). While the scale can be reduced somewhat the specificity must be improved over that attainable in these early experiments. This is largely due to the greater number of known compounds which are present. The many complementary methods now available have not yet superseded the rather simple separation of phosphates by virtue of the solubilities of their barium salts. However, the rapid development of purer primary standards and more quantitative separation methods has placed certain reservations upon our interpretation of results obtained by precipitation methods alone.

CHROMATOGRAPHY OF SUGAR PHOSPHATES. - Our knowledge of the identity and interrelationships between the phosphate esters of plants has been expanded tremendously by the application of paper and column chromatography. The multitude of phosphorylated compounds in plants and the very low concentration of some of these require a method of great sensitivity and versatility. Paper chromatography meets this exacting requirement and when augmented by ion

exchange resin column chromatography the sensitivity and scale of operation are almost limitless.

Paper chromatography of the phosphates requires special conditions for increasing the R_f values of these hydrophilic and readily adsorbed compounds. Early chromatograms of plant extracts (Calvin and Benson, 1949) showed almost no separation of the phosphates under conditions where the sugars, hydroxy acids and amino acids were well separated. As the importance of these compounds in plant metabolism became more obvious their chromatographic separation was slowly improved.

The major difficulty lay in the adsorption by impurities in the paper such as calcium, magnesium or iron. This was most pronounced when attempts were made to chromatograph minute amounts of oxalic acid-C^{14}. The compound was progressively adsorbed until no more remained to move at its characteristic R_f. When the paper was first washed with oxalic acid to saturate or elute immobile cations the result was striking. Labeled oxalic acid and the sugar phosphates were not adsorbed and chromatographed satisfactorily in commonly used solvents like phenol-water and butanol-acetic acid-water. Besides reducing adsorption the treatment resulted in an acidic paper (even after thorough water rinsing) which gave higher R_f values for the acidic compounds (Figure 6). Hanes and Isherwood (1949) solved the problem by pre-washing with hydrochloric acid, 8-hydroxy quinoline and by saturation with hydrogen sulfide. Mortimer (1952) replaced the 8-hydroxy quinoline by 0.02% aqueous versene (Bersworth Chemical Co., Framingham, Mass.) with improved results.

R_f Values of Sugar Phosphates. - The measurement and comparison of absolute R_f values is hardly practical for identification of compounds in systems of many components. Variations in paper hydration, temperature and solvent saturation of the atmosphere all affect the absolute R_f values but

only rarely can they affect relative R_f values of similar compounds. For this reason Mortimer (1952) has used the concept of position constant where the movement of a compound is compared to that of orthophosphate.

A number of reported position constants for sugar phosphates are given in Table II. It must be borne in mind that the relative values of the R_f values and position constants are useful in identifying an unknown but not necessarily unequivocal. Successful cochromatography, then, does not preclude identity of the two substances. Chemical evidence is always invaluable in identifying an unknown. One or more observations of chemical transformation of a compound is more likely to provide conclusive identification than chromatography in many solvent systems. Two-dimensional chromatography of a product of a chemical reaction of the unknown often provides a simple and unequivocal identification, particularly when the properties of the suspected product are known.

Paper Purification. - Single sheets can be washed chromatographically while suspended in the trough. A saturating rinse with 1% oxalic acid or other reagent followed by a complete water washing can be done in a few hours. Larger quantities (100-200 sheets) are washed in a rectangular filter with a perforated bottom for applying suction (Hanes and Isherwood, 1949). Whatman No. 4 gives much faster development of sugar phosphates relative to the amino acids than No. 1 (see Figure 7). Schleicher and Schuell No. 589 paper was used by Bandurski and Axelrod (1951) without prior washing and was found to give good results.

Solvents for Paper Chromatography. - Hanes and Isherwood examined the properties of a variety of solvents. The following are typical:

Table II-a

R_f VALUES AND POSITION CONSTANTS FOR
PHOSPHATE ESTERS IN CHROMATOGRAPHIC SOLVENTS

Solvent	Ethyl acetate 3, Acetic acid 3, Water 1		Methyl cellosolve 7, Methylethyl ketone 2, 3 N NH_4OH 3		Ethylacetate 1, Formamide 2, Pyridine 1	
Temperature	4°		26°		26°	
Paper	Whatman No. 1		Whatman No. 1		Whatman No. 1	
Reference	Mortimer 1952		Mortimer 1952		Mortimer 1952	
	a	b	a	b	a	b
Orthophosphate	33	100	21	100	50	100
Phosphoglycoladehyde	••	••	••	••	••	••
Phosphoglycolic acid	••	••	••	••	••	••
Glycerol-1-phosphate	26	79	39	192	54	114
Glyceraldehyde-3-phosphate	7	22	19	90	••	••
Dihydroxyacetone phosphate	••	••	••	••	••	••
2-Phosphoglyceric acid	27	81	41	200	23	47
3-Phosphoglyceric acid	23	71	22	116	28	57
2,3-Diphosphoglyceric acid	11	35	7	36	15	30
Phosphopyruvic acid	••	••	••	••	••	••
Phosphoerythronic acid	••	••	••	••	••	••
Ribose-1-phosphate	15	45	40	197	50	110
Ribose-5-phosphate	••	••	••	••	••	••
Ribulose-5-phosphate	••	••	••	••	••	••
Ribulose-1,5-diphosphate	••	••	••	••	••	••
Glucose-1-phosphate	14	37	36	170	44	89
Glucose-6-phosphate	12	29	29	140	50	100
Uridine diphosphate glucose	••	••	••	••	••	••
Glucose-1,2-cyclic phosphate	••	••	••	••	••	••
Glucose-1,6-diphosphate	••	••	••	••	••	••
Fructose-1-phosphate	••	••	••	••	••	••
Fructose-6-phosphate	17	48	36	171	54	108
Fructose-1,6-diphosphate	8	25	8	37	13	26
Galactose-1,2-cyclic phosphate	••	••	••	••	••	••
Mannose-6-phosphate	••	••	••	••	••	••
Mannoheptulose phosphate	••	••	••	••	••	••
Sedoheptulose-7-phosphate	••	••	••	••	••	••
Sedoheptulose diphosphate	••	••	••	••	••	••
Sucrose phosphate	••	••	••	••	••	••

(a) Numbers give R_f values in % of solvent travelled
(b) Numbers are P-constants relative to orthophosphate

Table II-b

Solvent	t-Butanol 80 Picric acid 2 g. Water 20	i-propylether 90, 90% formic acid	Phenol 72 g. Water 28	
Temperature	22°	20°	22°	
Paper	Whatman No. 1	Whatman No. 1	Whatman No. 4	
Reference	Wilson 1954.	Hanes and Isherwood 1949		
	b	b	a	b
Orthophosphate	100	100	22	100
Phosphoglycolaldehyde	..	..	..	-170
Phosphoglycolic acid	106	..	23	102
Glycerol-1-phosphate	..	..	..	..
Glyceraldehyde-3-phosphate	..	..	..	..
Dihydroxyacetone phosphate	..	..	..	170
2-Phosphoglyceric acid	..	..	..	..
3-Phosphoglyceric acid	85	59	22	100
2,3-Diphosphoglyceric acid	..	..	..	..
Phosphopyruvic acid	120	..	26	110
Phosphoerythronic acid	..	..	..	74
Ribose-1-phosphate	..	..	..	..
Ribose-5-phosphate	..	..	..	139
Ribulose-5-phosphate	..	..	..	147
Ribulose-1,5-diphosphate	58	..	8	26
Glucose-1-phosphate	..	18	..	..
Glucose-6-phosphate	40	18	..	113
Uridine diphosphate glucose	dec.	..	26	111
Glucose-1,2-cyclic phosphate	54	..	..	170
Glucose-1,6-diphosphate	..	..	..	26
Fructose-1-phosphate	..	..	..	135
Fructose-6-phosphate	61	28	29	125
Fructose-1,6-diphosphate	..	15	..	26
Galactose-1,2-cyclic phosphate	45	..	..	170
Mannose-6-phosphate	52	..	29	125
Mannoheptulose phosphate	..	..	..	113
Sedoheptulose-7-phosphate	52	..	27	113
Sedoheptulose diphosphate	..	..	..	26
Sucrose phosphate	32	..	..	113

Table II-c

Solvent	Butanol 100, Propionic acid 50 Water	Methanol 80 88% Formic acid 15 Water 5		Methanol 60 28% NH_4OH 10 H_2O 30	
Temperature	22°	2°		2°	
Paper	Whatman No. 1	S and S No. 589		S and S No. 589	
Reference		Bandurski, Axelrod 1951		Bandurski, Axelrod 1951	
Orthophosphate	100	63	100	28	100
Phosphoglycolaldehyde	-73	••	••	••	••
Phosphoglycolic acid	75	••	••	••	••
Glycerol-1-phosphate	••	••	••	••	••
Glyceraldehyde-3-phosphate	••	••	••	••	••
Dihydroxyacetone phosphate	59	••	••	••	••
2-Phosphoglyceric acid	••	46	13	18	64
3-Phosphoglyceric acid	65	50	79	35	125
2,3-Diphosphoglyceric acid	••	••	••	••	••
Phosphopyruvic acid	92	52	82	46	165
Phosphoerythronic acid	51	••	••	••	••
Ribose-1-phosphate	••	••	••	••	••
Ribose-5-phosphate	49	••	••	••	••
Ribulose-5-phosphate	53	••	••	••	••
Ribulose-1,5-diphosphate	22	••	••	••	••
Glucose-1-phosphate	••	27	43	60	215
Glucose-6-phosphate	40	38	60	48	170
Uridine diphosphate glucose	15	••	••	••	••
Glucose-1,2-cyclic phosphate	49	••	••	••	••
Glucose-1,6-diphosphate	22	••	••	••	••
Fructose-1-phosphate	46	••	••	••	••
Fructose-6-phosphate	46	34	54	44	156
Fructose-1,6-diphosphate	22	40	63	24	86
Galactose-1,2-cyclic phosphate	49	••	••	••	••
Mannose-6-phosphate	46	••	••	••	••
Mannoheptulose phosphate	40	••	••	••	••
Sedoheptulose-7-phosphate	40	••	••	••	••
Sedoheptulose diphosphate	22	••	••	••	••
Sucrose phosphate	40	••	••	••	••

Acid, water-immiscible solvents - 90 ml. t-amyl alc./90 ml. H_2O/30 ml. 90% formic acid; 60 ml. t-amyl alc./30 ml. H_2O/2 g. p-toluene sulfonic acid

Acid, water-miscible solvent - 80 ml. t-butanol/20 ml. H_2O/4 g. picric acid

Basic, water-miscible solvent - 100 ml. ethyl acetate/40 ml. pyridine/100 ml. H_2O

A radical development in solvents was introduced by Mortimer (1952) when he replaced water with formamide. Ethylacetate-formamide-pyridine development resulted in a different distribution pattern for which R_f values are given in Table II. It must be borne in mind that separation methods developed for equimolar mixtures of pure phosphates may not always be applicable for naturally occurring mixtures where concentrations of the phosphates vary by a factor of a hundred and inorganic and polyphosphorylated impurities are encountered.

While reagent-grade phenol is satisfactory for sugar and amino acid separations, further purification is often necessary for the phosphates and hydroxy acids. Freshly distilled phenol is mixed with the calculated amounts (28% w/w) of deionized (Dowex 50) distilled water and stored in the cold until used to prevent accumulation of oxidized impurities. Benson, et al. (1950) used phenol-water and butanol-propionic acid-water (freshly prepared 1:1 mixture of n-BuOH 1246 ml. + H_2O 84 ml. and propionic acid 620 ml. + H_2O 790 ml.) for two-dimensional separations of plant extracts. The initial plant extracts contain phosphate esters at ca. pH 6 and hence in the ionized form. With oxalic-washed Whatman No. 4 paper these solvents separate a wide variety of amino acids, sugars, fats, polysaccharides and carboxylic acids in addition to the sugar phosphates (Figure 7).

An excellent two-dimensional method was developed by Bandurski and Axelrod (1951) for separation of phosphate esters in plant extracts. It has been found superior to the above methods by a number of workers. The mixture of free phosphoric acids on S and S No. 589 paper is developed in an acid solvent (80 ml. methanol and 15 ml. 88% formic acid) and a basic solvent (60 ml. methanol, 10 ml. ammonium hydroxide (sp. gr. 0.9015) and 30 ml. H_2O) at 2° C. with pre-equilibration of the paper in solvent vapor. The separations obtained are illustrated in Figure 8.

Paper Electrophoretic Separation of Phosphate Esters. - The separation of phosphate esters by virtue of their differences in transport rates by paper electrophoresis has been reported by Schild and Buttenbruch (1953). Direct electrophoretic separation of plant phosphates by this method does not yet offer practical possibilities. Similar successful separation of borate complexes of the free sugars (Consden and Stanier, 1952) could well be applied to separation of the sugar phosphates.

DETECTION OF SUGAR PHOSPHATES ON PAPER. - Acidity, phosphorus content and sugar content may be used to detect these compounds on paper.

Acid Spray. - Bromcresol green (0.05% in 90% ethanol) adjusted to a blue tint (ca. pH 5.5) gives yellow spots for PGA and other acids after development in an acid solvent. It may be necessary to autoclave the paper a few moments in order to remove acidic solvents effectively.

Phosphorus Spray. - Hanes and Isherwood (1949) reagent, 5 ml. of 60% w/w perchloric acid, 25 ml. of 4% w/v ammonium molybdate, 10 ml. N HCl and 60 ml. H_2O is sprayed on the dry chromatogram. Inorganic orthophosphate appears as a yellow spot immediately. The paper is dried one minute at 85° whereupon glucose-1-phosphate appears as a yellow to blue spot. Irradiation with a germicidal u.v. lamp for 10 minutes gives blue spots for all organic

phosphate compounds while inorganic phosphate becomes yellow green (Bandurski and Axelrod, 1951). Alternatively the paper may be heated 5 minutes at 85° and autoclaved two minutes at 110°. Treatment with ammonia vapor removes the blue background leaving phosphomolybdate blue spots. Exposure to H_2S intensifies the blue spots and reduces the background.

Sugar Reagents. - Aniline-TCA-acetic acid, resorcinol-HCl-ethanol, orcinol-TCA-acetic acid or Tollen's reagent may be used to detect the sugar moiety in the usual manner. Since the amounts of phosphate esters which paper chromatography will separate well are usually small (unless a phosphate fraction is chromatographed) such reagents have limited use.

The characteristic colors exhibited by these reagents with the free sugars are generally formed with the phosphate esters as well. The sugar phosphates are hydrophilic; spray reagents should be dissolved in alcohol, acetic acid or other solvent which will not distort the shape of the original spot. Glucose-6-phosphate and fructose diphosphate gave brown spots when sprayed with aniline phthalate in butanol and heated (Mortimer, 1952).

ION EXCHANGE SEPARATION OF SUGAR PHOSPHATES. - A sharp digression from the classical methods of separating sugar phosphates was made possible with the development of strong base-anion exchange resins. The limitations of partition chromatography, low capacity and appreciable extent of irreversible adsorption, are overcome in anion exchange resin chromatography. The capacity of the resin determines the quantities of material which can be separated but this is very high indeed compared to that of cellulose adsorbents. The adsorption of phosphates on paper which becomes critical with tracer quantities of diphosphates appears to be non-existant in the resins. One may compare the exceedingly minute amounts of cations which have been chromatographed quantitatively in the separation of uranium fission products by cation exchange resins.

Procurement of authentic compounds for characterization of unknowns has been a major difficulty for all those studying their metabolism and chemical properties. Exchange resin chromatography shows great promise even though it has been used in but a few laboratories in experiments with a limited number of compounds. It will undoubtedly make it possible to obtain large quantities of analytically pure phosphate esters from plant material with a fraction of the effort required by the method of relative solubilities. Even though the process is still in the developmental stage, the method demands a detailed discussion. With suitable adaptations one may expect any mixture of phosphates to be separable.

Ion exchange separation of phosphorylated compounds was developed by Cohn and Carter (1950) (nucleotides), Benson, et al. (1950) (PGA and HMP), Goodman (1952) (PGA, HDP and HMP) and by Khym and Cohn (1953) (borate complexing). The methods used by Goodman and by Khym and Cohn utilize the wider range of resin affinities exhibited by the borate complexes. The structural similarity and nearly identical dissociation constants of the biologically important sugar monophosphates appear to be obstacles in their direct ion exchange separation. The application of boric acid in paper chromatography solvents by Cohen and Scott (1950) was successful in separating configurationally isomeric pentose phosphates and suggested the application in ion exchange.

Principles of the Method. - A strong base anion resin in the chloride form acts as a stationary cation surrounded by a field of mobile chloride ions. The mobility of the chloride ions differs from that of other anions, such as glucose-6-phosphate ion and an equilibrium between the anions is established:

$$G\text{-}OPO_3H^- + Resin^+ \_\_ Cl^- \rightleftharpoons Resin^+ \_\_ {}^-HO_3PO\text{-}G + Cl^-$$

Under batchwise equilibrium conditions, then,

$$\frac{\text{Fraction of solute on resin}}{\text{Fraction of solution in solution}} = \text{Constant}$$

and

$$K_{\text{distribution}} = \frac{\text{Fraction of solute on resin}}{\text{Fraction of solute in solution}} \times \frac{\text{Solution volume}}{\text{Resin mass}}$$

is a characteristic and measurable quantity for each phosphate under a given set of resin and pH conditions.

The separation of polyacidic compounds, PGA and HDP, from the HMP's and orthophosphate was found relatively simple by virtue of great differences in their adsorption on the resin. The two acidic groups of PGA and the diphosphates greatly decrease the probability of the compound being released from the resin. At low pH the carboxyl dissociation of PGA is repressed and the elution properties resemble that of a monophosphate.

The formation of borate complexes in dilute borate solutions was applied by Khym and Cohn (1953) for separation of the monophosphate esters. While the borate form of an anion resin can be successfully used (Goodman, 1952) for separation of hexose monophosphates the interference of the excess borate in subsequent operations presents an unnecessary difficulty.

Separation Method of Khym and Cohn (1953). - The sample of phosphate esters in the free acid form was prepared using Dowex-50 cation exchange resin. The amount corresponding to 5 to 10 mg. of free sugar in 25 ml. dilute ammoniacal solution at pH 8.5 was adsorbed on a 12 cm. x 0.86 sq. cm. column of 200-400 mesh strong base anion exchanger (Dowex 1, trimethylammonium polystyrene) in the chloride form. Free sugars were washed out with 100 ml. of 0.001 M ammonium hydroxide. A succession of different eluting agents in the order described by

Figure 9 and Table III was passed through the column at 3.5 ml. per minute to desorb selectively the components of the adsorbed mixture.

Very small amounts of borate ion radically change the affinities of the sugar phosphates in ammonium hydroxide-ammonium chloride buffers. Ribose-5-phosphate is affected markedly by 10^{-5} M borate followed by fructose-6-phosphate at 10^{-4} M and glucose-6-phosphate at 10^{-2} M. An elution sequence of borate solutions in that order will separate these monophosphates. A simple pH and ionic strength adjustment suffices to accomplish the separation of several polyacidic phosphates from the monophosphates.

Ion Exchange Separation of Polyacidic Phosphates. - Goodman (1952) separated radioactive phosphoglycerate, phosphoglycolate and ribulose diphosphate on a tracer scale. No difficulties were encountered even though carrier phosphates were not used. The mixture of pure C^{14}-labeled phosphates eluted from a paper chromatogram was placed on a 28 x 0.6 cm. Dowex-2 (dimethylethanol-ammonium polystyrene) chloride column and eluted with 0.15 N NaCl + 0.05 N HCl at a rate of 0.11 ml./min. The radioactivity observed in the effluent is described by Figure 10. When the carrier phosphates, PGA and FDP, were added to the radioactive extract the result was not seriously distorted by the presence of the added materials, Figure 11.

Identification of Chromatographed Sugar Phosphates. - In general the acid hydrolysis used to determine stability of the phosphate linkage does not release the organic moiety in a form suitable for identification. Levulinic acid obtained from the hexoses may be identified as a preliminary identification step. Phosphatase hydrolysis (Benson, et al., 1951) gives the free sugars which can be identified paper chromatographically. Unless the phosphatase is quite free of sugars precautions must be taken in interpreting the result.

Table III

ANALYTICAL DATA FOR SEPARATION DEMONSTRATED IN FIG. 9

Compound	Assay method	Wave length used, mμ	Approx. amount added,[a] -mg.	Recovered, %
Glucose	Anthrone	620	5	101
Glucose-1-PO_4	Anthrone	620	10	99
Glucose-6-PO_4	Anthrone	620	10	93
Fructose-6-PO_4	Anthrone	620	5	92
Fructose-1,6-DiPO_4	Anthrone	620	10	95
Inorg. PO_4(K_2HPO_4)	Phosphate	660	2	105
2-PHA	Phosphate	660	4	95
Ribose-5-PO_4	Orcinol	660	5	90
AMP	U.V. absorption	260	8	95
ADP	U.V. absorption	260	5	102
ATP	U.V. absorption	260	6	100

(a) The mg. quantities given for the sugar phosphate represent the free sugar content of these substances. The quantities given for inorganic phosphate and 2-PGA are calculated as total phosphorus present. The amount of each adenosine derivative was calculated from extinction coefficients.

Identification of C^{14} or H^3 labeled sugars is not affected by sugar impurities in the phosphatase preparation. The free glucose and amino acids in Polidase S (Schwarz Laboratories, Inc., 202 East 44th Street, New York, N.Y.) for example act as excellent identifying aids for comparing positions of the unknown spots.

Phosphatase Hydrolysis. - The unknown sugar phosphate on a wedge-shaped cutout is eluted with 200-300 μl. water by attaching it to a wet paper wick hanging from a vessel of water. This is two or three times that required for eluting a sugar or amino acid. The sample (pH 5) collected in a small centrifuge tube is concentrated, if necessary, with a nitrogen stream and 100-200 μg. Polidase-S added in 10 μl. After 1-3 days at 35° under toluene the hydrolysate is chromatographed and the position of the sugars compared with those of the enzyme preparation alone. Larger samples of exchange resin eluates are hydrolyzed in the same way except that desalting of the sugar before paper chromatography may be necessary. Such desalting is best effected by treatment with a cation resin (Dowex-50) and a weak base anion resin. Horecker, Smyrniotis and Seegmiller (1951) used phosphatase in the identification of ribulose-5-phosphate. Horecker and Smyrniotis (1953) used a potato phosphatase preparation to hydrolyze tetrose phosphate.

METHODS OF ESTIMATION OF SUGAR PHOSPHATES

The available methods for estimations of sugar phosphates require a knowledge of the components of the mixture and their possible reactions during the analysis. The problems of estimating a certain ester in a plant extract, then, are quite different than those encountered in determining the purity of a preparation or the components of an enzymatic reaction in which one may predict what the products are. This situation places more stringent requirements upon the separation method used. Chromatographic separations give the selectivity required to yield products pure enough for analysis by moderately specific methods.

Analysis of phosphate esters has applied a wide variety of their physical and chemical properties. Salt solubilities, partition coefficients, optical activity, hydrolysis rates, acid strengths, and infrared spectra are physical properties which are exploited in analytical methods. Infrared spectra have not been seriously investigated but will be increasingly useful. The chemical properties of sugar phosphates are used in their color reactions, reducing values, borate complexing characteristics and enzymatic reactivity.

Leloir (1951) has reviewed the physical and many of the chemical properties of the natural and synthetic sugar phosphates. Analytical methods for compounds involved in glycolysis are given by Umbreit, Burris and Stauffer (1945). The compounds of known and suspected importance in plant metabolism, and currently applied analytical methods for their determination, are given in the following sections.

Anthrone Method for Analysis of Sugar Phosphate. - The Anthrone reagent of Dreywood (1946) has considerable value as a reliable non-specific method for determination of hexose phosphates. Morris' (1948) quantitative method

was applied by Khym and Cohn (1953) for analysis of effluant samples in ion exchange separation of the sugar phosphates. In this case, a specific method was not necessary and the non-specificity of the method was a decided advantage.

Four to 5 ml. of solution to be analyzed is mixed rapidly and thoroughly with 8-10 ml. of anthrone reagent (2 g. anthrone in 1 l. 95% H_2SO_4) in a 19-25 mm. diameter test tube. After standing 10 minutes the color is measured either photometrically at 620 mμ. against a water blank or by comparison with glucose standards. The range over which the color developed obeys Beer's law is 8-200 μg. glucose for the 620 mμ. filter.

The nature of the reaction is dependent upon the temperature rise upon mixing the reagent and sample and upon the temperature fall before measurement. Hence the dimensions of the test tube are critical. With adequate temperature control the test could readily be adapted to smaller samples and colorimeter tubes. There is some variation in sensitivity for sugars, galactose having a low (54%) glucose value.

Colorimetric Phosphorus Analysis (Allen, 1940). - Aliquot portions containing five to forty micrograms phosphorus can be readily and accurately determined. The sample and 0.4 ml. of 72% $HClO_2$ and a drop of 30% H_2O_2 were heated in a micro Kjeldahl flask until a yellow color appeared and then disappeared and heavy fumes of perchloric acid rose in the tube. The orthophosphate, so obtained, was determined by adding 0.4 ml. of a reducing solution [10 g. $NaHSO_3$ (meta), 0.5 g. diaminophenol hydrochloride (acrol) in 50 ml. distilled water] followed by 0.2 ml. of 8.3% ammonium molybdate. After 5 minutes development, the blue solution was diluted to 5 ml. in a colorimeter tube and the density at 660 mμ. determined (Klett-Sommersen colorimeter). The amount of P is read from the linear-calibration curve.

Phosphoglycolic acid. - A low concentration of phosphoglycolic acid, about 5% of that of PGA, is often isolated by paper chromatography from C^{14} or P^{32} labeled Scenedesmus. Part of this arises from oxidative degradation of ribulose diphosphate (Benson, 1951) and part appears to fluctuate with the concentration of C_2 carbon dioxide acceptor in photosynthesis.

Phosphoglycolic acid has been separated from PGA on a tracer scale by elution chromatography on Dowex 2 anion exchange resin by Goodman (1952). Figure 11 shows separation of phosphoglycolic acid and PGA from ribulose diphosphate in the presence of fructose diphosphate. The radioactive components alone (without carrier PGA and FDP) were separated even more cleanly.

3-Phosphoglyceric Acid. - The extreme stability toward acid hydrolysis and the susceptibility to phosphatase hydrolysis are characteristic properties of PGA. It is only 2% hydrolyzed after 3 hours at 100° in N HCl while it is 50% hydrolyzed after 1.5 hours in 1 N HCl at 125° (Kiessling, 1935). By comparison, ribose phosphates from ATP in a barium-insoluble fraction are hydrolyzed to the extent of 59% in 3 hours at 100°. Free glyceric acid is observed in widely varying amounts in extracts of most leaves and usually occurs in appreciable concentrations. The ratio of free glyceric acid to PGA is often as high as 10, being dependent upon the treatment of the tissue before extraction and upon the manner of its extraction. The highest fraction of phosphorylated glyceric acid is obtained when leaves are rapidly killed at high CO_2 pressure, low light intensity, and the extract separated from the cell walls as soon as possible. Free glyceric acid should be included in most values for PGA since it could well have been derived from PGA during the sample preparation. The barium-insoluble fraction, then, may not always be a true indicator of the amount of PGA contained in the plant tissue.

PGA is strongly adsorbed upon organic plant material as well as upon anion exchange resins by virtue of its divalent structure. Extraction of PGA from plant material is not complete with 80% alcohol and requires 20% alcohol or even stronger extraction solvents. In a TCA extraction, the repressed dissociation of the carboxyl group certainly enhances its extraction rate. The presence of pectic substances in Scenedesmus made it difficult to apply the normal extraction methods for PGA during its isolation and identification as primary carboxylation product of photosynthesis (Benson, et al., 1950).

Isolation of 3-PGA from Scenedesmus. - The acidic extract of 24 grams of packed cells was neutralized with sodium hydroxide. A small amount of PGA was adsorbed by the precipitate at pH 7 but was readily eluted when the solution was made pH 10. The extract, after several washes at pH 10 (where other adsorbed anions apparently displaced the PGA), was acidified and barium chloride added. The acid precipitate contained PGA which was removed by careful washing with 1 N HCl. The extract was adjusted to pH 10 without further precipitation due to the large volume. The solution was made 60% alcohol and the precipitate separated. It was extracted with a small volume of 0.05 N HCl which dissolves the PGA readily. It is reprecipitated by adding an equal volume of ethanol. The barium salt was purified by repetition of the precipitation. Fructose diphosphate and ATP which would be obtained in these precipitates were hydrolyzed by the repeated warming with 0.05 N HCl.

3-Phosphoglycerate. - The barium salt (1 gram) of PGA isolated from the barium insoluble fraction can be purified by dissolving it in 0.05 N HCl. The solution is warmed and filtered. An equal volume of warm alcohol is added. Crystals of barium phosphoglycerate separate upon standing in the cold room.

Meyerhof and Schulz (1938) characterized PGA by its phosphorus content (theoretical, 8.7% P), its molar optical rotation in N HCl $[\alpha]_D^{18} = -13.27^\circ$ (strongly dependent on acidity) and its very high molybdate ion-enhanced rotation $[\alpha]_D = -745^\circ$ (4% w/v ammonium molybdate). The latter is the most specific for PGA and only a few α-hydroxy acids interfere. It can be used in extracts without extensive purification.

The colorimetric method of Rapoport (1937) is specific for PGA in the presence of a wide variety of compounds. Its great sensitivity (10 μg.) is apparently identical for glyceric acid, 3-PGA, 2-PGA and 2,3-diPGA.

The sample of free phosphoglyceric acid (purified through the lead salt and H_2S) containing 50-400 μg. was pipetted into a short wide test tube and dried on the water bath with two drops of HCl (to remove HNO_3). The residue was evaporated again with two drops HCl and kept on the water bath one half hour to remove free HCl. Two ml. of a freshly prepared 0.1% naphthoresorcinol solution in dry H_2SO_4 was added and the vessel kept one hour on the water bath. The blue solution was transferred to a 25 ml. volumetric flask using fresh portions of conc. H_2SO_4. The color was determined in a colorimeter and compared to a standard. Calculated concentrations varied from 96 to 106% of theoretical over the sample range of 150 to 900 μg. PGA. According to Meyerhof and Schulz (1938) and Neuberg and Lustig (1943) the method requires considerable preliminary purification of PGA. Carbonization results from polysaccharide impurities which frequently coprecipitate with PGA obtained from plant extracts. The purity of the H_2SO_4 is critical for obtaining a blue solution free of brown color (attributed to nitrate impurities). Emerson, Stauffer and Umbreit (1944) concluded that the reliability of this colorimetric method must be checked for each plant tissue. Apparently there may be other compounds in

plant extracts which give the characteristic blue color, whether or not free glyceric acid is included in the sample.

2-Phosphoglyceric Acid. - The acid hydrolysis rates of 2- and 3-PGA are identical. Acid catalyzed migration of the 2-phosphate group is apparently responsible for this identity and for many of the difficulties involved in obtaining pure 2-PGA. Neuberg (1943) found $[\alpha]_D^{22} = +23.2^\circ$ for the barium salt. Meyerhof and Schulz found the molybdate-enhanced rotation $[\alpha]_D^{20} = -68^\circ$. The great difference between -68° and -745° allows one to readily calculate the ratio of the two isomers in a mixture.

Bandurski and Axelrod (1951) separated 2- and 3-PGA paper chromatographically. R. W. Cowgill (personal communication) improved the separation by using molybdate-saturated paper. With an isopropanol-ammonia-water solvent 2-PGA moved 20% faster than 3-PGA. Ion exchange separations have not been reported.

Phosphoenolpyruvic acid. - Phosphopyruvic acid is hydrolyzed rapidly in neutral or acid solution at room temperature in the presence of $HgCl_2$. In N HCl at 100° it is half hydrolyzed after 9 minutes and 93% hydrolyzed in 30 minutes.

After acid hydrolysis, pyruvic acid may be determined by colorimetry of the 2,4-dinitrophenylhydrazone in alkaline solution by the method of Lu (1939). The hydrazone is extracted by ethylacetate. The ethylacetate solution is then extracted by 10% sodium carbonate solution and N NaOH is added to give a stable red color which is measured with a Wrattan No. 62 filter after ten minutes. The method estimates 2 μg. of pyruvate in 10 ml. with an error of $\pm 1.5\%$.

Phosphopyruvic acid reacts with alkaline hypoidite using six equivalents of iodine forming iodoform and orthophosphate. This is the basis for the

method of Lohmann and Meyerhof (1934). They obtained barium phosphopyruvate in the barium-soluble alcohol-insoluble fraction and purified it by recrystallization as the silver-barium salt. A neutralized 3 ml. sample of it or of the initial TCA extract was treated with 1.0 ml. of N/10 iodine and 1.5 ml. of N/10 NaOH for 10-20 minutes at room temperature. After acidifying with 1.0 ml. of N HCl the excess iodine was titrated with N/100 or N/200 thiosulfate solution. One ml. of N/100 thiosulfate is equivalent to 0.146 mg. pyruvic acid.

Radioactive phosphopyruvate is readily identified by the rate of appearance of labeled C^{14}-pyruvate or labeled P^{32}-orthophosphate. Since pyruvic acid in small quantities is very volatile it is only necessary to determine the non-volatile C^{14} radioactivity in the solution as a function of time.

Triose Phosphates. - The concentrations of triose phosphates are generally quite low in plant tissues. By far the major ester of the two is dihydroxyacetone phosphate. It is the source of free dihydroxyacetone often observed in sugar concentrates. Utter and Werkman (1941) were able to distinguish between dihydroxyacetone and glyceraldehyde phosphates. However the equilibrium ratio of the two gives only 4% for P-glyceraldehyde which is corroborated by the observed preponderance of DHAP. It does not yet seem expedient to differentiate between the two except in studies of triose phosphate isomerase activity. Analyses for triose phosphate have been reported by Arreguin-Lozano and Bonner (1949), Allen (1940) and James, Heard and James (1944).

The triose phosphates are separated in the barium-soluble alcohol-precipitatable fraction. They are readily decomposed in both acid and alkali. Acid hydrolysis gives orthophosphate and methylglyoxal. In alkali, orthophosphate and lactate are formed. The rates of acid hydrolysis of both phosphates

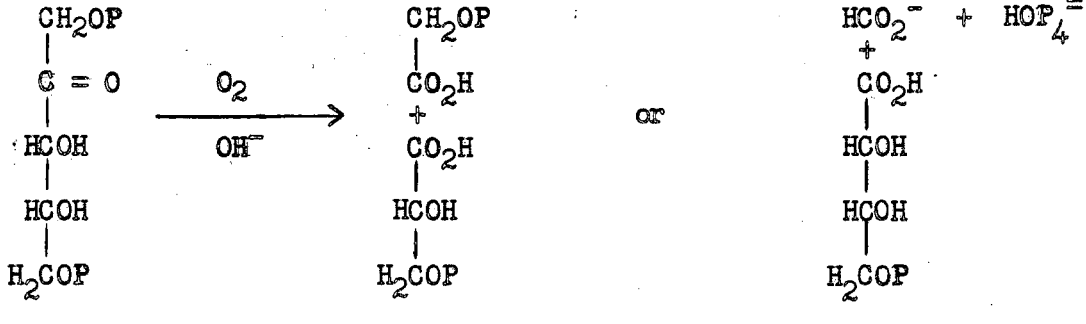
are almost the same; $K = 33.7 \times 10^{-3}$ for DHA-P and $K = 37.5 \times 10^{-3}$ for GA-P. Seven minutes at 100° in N HCl hydrolyzes 44% of the esters and they are 90% hydrolyzed in 30 minutes.

Triose phosphate is completely decomposed in 20 minutes at room temperature in 2 N NaOH. The alkali-labile phosphorus is determined and the lactic acid may be determined by the method of Barker and Summerson (1941) and Umbreit, Burris and Stauffer (1945).

Tetrose Phosphate. - The synthesis of sedoheptulose-7-phosphate probably requires tetrose-4-phosphate as substrate for aldolase condensation with dihydroxyacetone phosphate. This may be derived from C_2-C_4 cleavage of a hexose phosphate. Horecker and Smyrniotis (1953) have reported evidence for tetrose phosphate as a product of sedoheptulose phosphate cleavage. Tolbert and Zill (1954) observed tetrose phosphates as products of sedoheptulose metabolism by barley and sugar beet leaves.

The above tetrose phosphates have been hydrolyzed for the purpose of identification by aniline phthalate spray reagent on paper. Charalampous and Mueller (1953) described an enzymatic synthesis of erythrulose-1-phosphate and its ion exchange and paper chromatographic properties.

Phosphoerythronic acid. - Oxidative degradation of ribulose diphosphate proceeds readily in alkaline solution (aqueous diethyl amine, pyridine, dil. NaOH, etc.) to give almost equimolar amounts of PGA, phosphoglycolate and phosphoerythronate (readily separable paper chromatographically, Benson, et al. (1952).



There is reason to suspect that phosphoerythronic acid may be a true metabolic intermediate (Dickens, 1938) but it is certain that most of that observed in plant extracts is a breakdown product of the copious ribulose diphosphate.

Pentose phosphates. - The concentrations of pentose monophosphates in plant extracts are generally small compared to those of the hexoses. In most photosynthetic tissues ribose and ribulose phosphates preponderate. Ribulose diphosphate accumulates in algae and a large number of higher plants. In soy bean leaf, for example, ribulose normally appears only as a monophosphate. Ribose phosphates, derived from adenosine, are described in the later section on ATP.

Ribose-5-phosphate and Ribose-3-phosphate. - Albaum and Umbreit (1947) used the Mejbaum (1939) test for analysis of ribose phosphates derived from hydrolysis of nucleotides. They determined the optimum time for color development and observed an important difference in rate of color development for R-5-P and R-3-P. The former develops 50% of its color in 5 minutes while R-3-P requires about 13 minutes for developing 50% of its final color density. A detailed study of the influence of glucose and polysaccharides in this determination was presented by Drury (1948).

Mejbaum Orcinol (Bial) Reaction, Modified by Albaum and Umbreit (1947). - To 3.0 ml. sample solution containing 10-30 μg. pentose is added 3.0 ml. of 0.1% $FeCl_3$ (or equivalent $Fe(NH_4)SO_4$) in conc. HCl and 0.3 ml. of 95% alcohol containing 100 mg./ml. of orcinol. The mixture is read in a colorimeter at 660 mμ. and then heated at 100° for 7 minutes, cooled and the color measured once more. Heating is continued for a total of 45 minutes and the final color determined.

The maximum color density is identical for ribose, xylose, arabinose, R-5-P, R-3-P and for nucleotides but the rate of color development varies greatly, being slowest for xylose and arabinose and fastest for ribose, lyxose and R-5-P. At 7 minutes heating 26% of the R-3-P and 65% of the R-5-P color is developed. R-3-P requires 40 minutes to reach the maximum color and R-5-P requires 25 minutes. If the components of the mixture are known it is often possible to take advantage of the different rates of color development in their determination. Presence of polysaccharides in the sample results in decreased rates of color formation and this is often difficult to avoid with samples separated by precipitation methods. However, the final color density is not seriously affected.

Sedoheptulose and mannoheptulose phosphates react with the orcinol reagent and corrections must be made for their interference. Horecker, Smyrniotis and Seegmiller (1951) showed that the chromogenic value of ribulose-5-phosphate is about 12% less than that of aldopentose.

Drury's (1948) examination of the reaction with glucose disclosed that optimum accuracy (~2%) is obtained when the ratio of glucose to pentose does not exceed 10:1 and the amount of pentose does not exceed 25 μg.

Ribulose-5-Phosphate. - Enzymatic decarboxylation of 6-P-G by 6-P-G dehydrogenase was demonstrated in higher plants by Conn and Vennesland (1951) and further studied by Axelrod, Bandurski, Greiner and Jang (1953). Horecker, Smyrniotis and Seegmiller (1951) were able to accumulate the products of this decarboxylation and obtained a mixture containing 25% ribulose-5-phosphate and 75% ribose-5-phosphate. The two pentose phosphates were effectively separated by ion exchange chromatography. Optical rotation of the samples served

to differentiate the products of the elution. Ribose-5-P and ribulose-5-P have specific rotations of +23° and -40° respectively.

The mixture of phosphoric esters was adsorbed on a 15 cm. x 5 $cm.^2$ column of 400 mesh Dowex-1 resin in the formate form. It was eluted with 0.1 M formic acid containing 0.03 M sodium formate (pH 3.1) at the rate of 2.5 ml./min. Optical rotation was measured in 0.2 N HCl on the barium salts precipitated by 80% alcohol. The ribose-5-P and ribulose-5-P peaks occurred at 1.35 l. and 1.7 l. of eluant respectively.

The spectrum of the compound given by ribulose-5-P in the Mejbaum orcinol test (40 minutes heating) is similar to that of aldopentose (maximum, 670 mμ.) except for a small peak at 540 mμ. Its extinction coefficient at 670 mμ. is 20,500 compared to 12,500 for ribulose and 24,000 for arabinose. The extinction coefficient of the 540 mμ. peak, 6,500, is much lower than that of ribulose, 11,000. Hence enzymatic hydrolysis of the phosphate group increases the 540 mμ. absorption thus providing a convenient method for analysis of mixtures of these substances.

Ribulose-1,5-diphosphate. - There is experimental evidence that ribulose-1,5-diphosphate may be the CO_2-acceptor of photosynthetic CO_2 fixation (Bassham, et al., 1954). Ribulose diphosphate is one of the earlier labeled products of $C^{14}O_2$ fixation in most, if not all, plants (Benson, 1951) and often occurs in large concentrations. Its concentration, however, is particularly susceptible to changes brought about by changes in light intensity and CO_2 pressure (see Figure 4). Its chemical instability and its tendency to adsorb readily on polysaccharides and to precipitate with polyvalent cations also suggests that its analysis may be fraught with difficulties.

Ribulose diphosphate was isolated from Scenedesmus by ion exchange chromatography by Goodman (1952). C^{14}-labeled ribulose diphosphate isolated by paper chromatography was used as carrier for detection of the product during the elution of the resin (see Figure 11). The peak containing the carrier radioactivity was precipitated with barium acetate. After removing the barium with Dowex-50 cation resin a sample was hydrolyzed in 0.1 N HCl at 100°. The half time of hydrolysis for the first phosphate was 12.2 minutes ($K = 25 \times 10^{-3}$) compared to a half time of hydrolysis of 70 minutes ($K = 4.3 \times 10^{-3}$) for fructose-1,6-diphosphate under identical conditions. Ribulose diphosphate, therefore, appears to be much more acid-labile than FDP. This property suggests that it can be determined by analysis of the ribulose-5-P obtained by mild acid hydrolysis. It can also be dephosphorylated and determined as free ribulose.

Ribulose diphosphate is oxidized by air in alkaline solutions. The products are phosphoglycolic, phosphoglyceric and phosphoerythronic acids. The reaction proceeds readily in aqueous organic bases like piperidine or diethylamine. This probably precludes the use of alkaline solvents for its chromatographic separation.

D-Xylose-1-phosphate and D-Xylose-5-phosphate. - Xylose monophosphates are known only as synthetic products. The physical constants for X-1-P are reported by Meagher and Hassid (1946) and those for X-5-P are given by Levene and Raymond (1933). The general occurrence of xylose in polysaccharides almost necessarily intimates the occurrence of its phosphates in plant tissues.

Xylulose-1,5-diphosphate. - A compound with the paper chromatographic properties of this diphosphate has been isolated from Scenedesmus in the author's

laboratory. It is likely that unknown esters such as this will first be identified by paper chromatography of the free sugar liberated by phosphatase hydrolysis.

Fructose-1,6-diphosphate. - The Roe analysis for fructose (see section on the Seliwanoff reaction) may be augmented by determination of reducing value (9.5% that of an equal weight of glucose). Since FDP is the only reducing compound in the barium insoluble fraction its reducing value provides a second estimate of its amount.

The hydrolysis of fructose diphosphate has a first order dependence on hydrogen ion concentration. Its two hydrolysis constants at 100° in N HCl are 56.0×10^{-3} and 4.6×10^{-3} (Goodman, 1952); 52×10^{-3} and 4.2×10^{-3} (McLeod and Robison, 1933). The half times of hydrolysis are 5.3 minutes and 55.7 minutes. It is 26.5% hydrolyzed in N HCl in 7 minutes at 100°. In 0.1 N HCl at 100° the half time for the 1-phosphate is 70 minutes.

FDP is often conveniently determined as alkali-labile phosphorus after incubation with muscle aldolase and cyanide (Axelrod, Saltman, Bandurski and Baker (1952). The triose phosphates formed in this specific reaction are readily determined by phosphate analyses.

The Seliwanoff Reaction for Fructose Determination in Fructose Phosphate (Roe, 1934) (Roe, Epstein and Goldstein, 1949). - The sample containing the equivalent of 10-100 μg. fructose was diluted to 2 ml. in a colorimeter tube. Two ml. of resorcinol solution (0.1% in 95% ethanol) and 6 ml. of 30% HCl were added and the solution was heated eight minutes at 80° C. The density of the red color was measured in the Klett colorimeter with a 490 mμ. filter.

Fructose phosphates give a weaker color than pure fructose (Goodman, 1952). Lutwak and Sacks (1952) reported that FMP gave 58% of the theoretical

fructose color density and FDP gave 75%. Upon chromatographic purification by elution from Dowex-1 the color of the eluted FDP rose to 87% of theoretical. (Dische, (1951) reports that the phosphates react with the same intensity as pure fructose.) In each case, therefore, standard solutions of the purest obtainable fructose phosphates must be used for preparation of standard curves.

Ribose-5-phosphate gives a pink color, similar to that of fructose, with a maximum at 529 mμ. Xylulose-5-phosphate gives a green color (ϵ_{max} = 620 mμ.) and ϵ520 only three times less than that of fructose. Ribulose-5-P gives a similar gray-green color and its presence requires a correction in the fructose absorption. Ketopentoses, therefore, are the major obstacles to a satisfactory application of the Seliwanoff reaction (Dische, 1951).

Dische (1951) applied his diphenylamine reaction (1929) to the determination of total fructose esters when ketopentoses were also involved. The ϵ_{max} for fructose at 635 mμ. after 10 minutes heating did not differ from that of F-6-P and FDP and was thirty times as high as that for glucose. A correction for G-6-P and for DHAP was necessary.

Fructose-6-phosphate. - Fructose content of the barium-soluble alcohol-precipitable fraction is a measure of the F-6-P. Its reducing value, 31.6% of that of an equal weight of glucose and can be used to augment fructose determination (see section on Seliwanoff reaction). Its hydrolysis rate in N HCl is low (10% in five hours at 100°). Presumably the fructose moiety is destroyed as the hydrolysis proceeds.

Glucose-6-phosphate. - G-6-P is more acid resistant than F-6-P. Its reducing value is 13.2% of that of an equal weight of glucose (Umbreit, Burris and Stauffer, 1945). Reducing values obtained from barium-soluble

fractions must be corrected for that of F-6-P, pentose-5-phosphates and other possible components.

The availability of G-6-P dehydrogenase offers a very specific spectrophotometric micro method for G-6-P or for TPN (Horecker and Smyrniotis, 1951). The amount of reduced TPN is equal to that of G-6-P in the equation (Conn and Vennesland, 1951).

$$\text{Glucose-6-P} + \text{TPN}^{+} \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-P-gluconate} + \text{TPNH}$$

It can also be used as a manometric method.

Mannose-6-phosphate. - The concentration of M-6-P in a variety of photosynthetic tissues is about half that of F-6-P. It occurs in low concentrations in all plant tissues examined. Its hydrolysis rate in N HCl at 100° is given by $K = 0.33 \times 10^{-3}$ and it is 50% hydrolyzed after about 18 hours. Its paper chromatographic separation from F-6-P is difficult but its ion exchange resin separation from the monophosphates using borate elution should be straight forward.

Glucose-1-phosphate. - The synthesis of starch and a variety of other polysaccharides by phosphorylases utilize G-1-P as the immediate substrate. The energy derived from cleavage of the phosphate group is conserved in the glycosidic bonds of the resultant polysaccharides. Glucose-1-phosphate has been identified in a number of plant tissues. Indeed, its absence in plant tissue would be surprising. The concentration of free G-1-P is generally very low in spite of the very small ΔF° for the phosphorylitic reaction. Any process in the living cell which causes a decrease in the ratio of inorganic phosphate to G-1-P favors polysaccharide synthesis. Growth and photosynthesis result in such a decrease in phosphate concentration which, in turn, results in utilization and diminution of the G-1-P concentration.

G-1-P and G-6-P are found in the "barium-soluble" fraction. They may be well separated by ion-exchange chromatography (Khym and Cohn, 1953). Their great difference in acid hydrolysis rate serves as an excellent means of analysis of a known mixture. G-1-P is completely hydrolyzed in 5 minutes at 100° in N HCl and 50% hydrolyzed in 4 hours at 37° in 0.25 N HCl (Cori, Colowick and Cori, 1937).

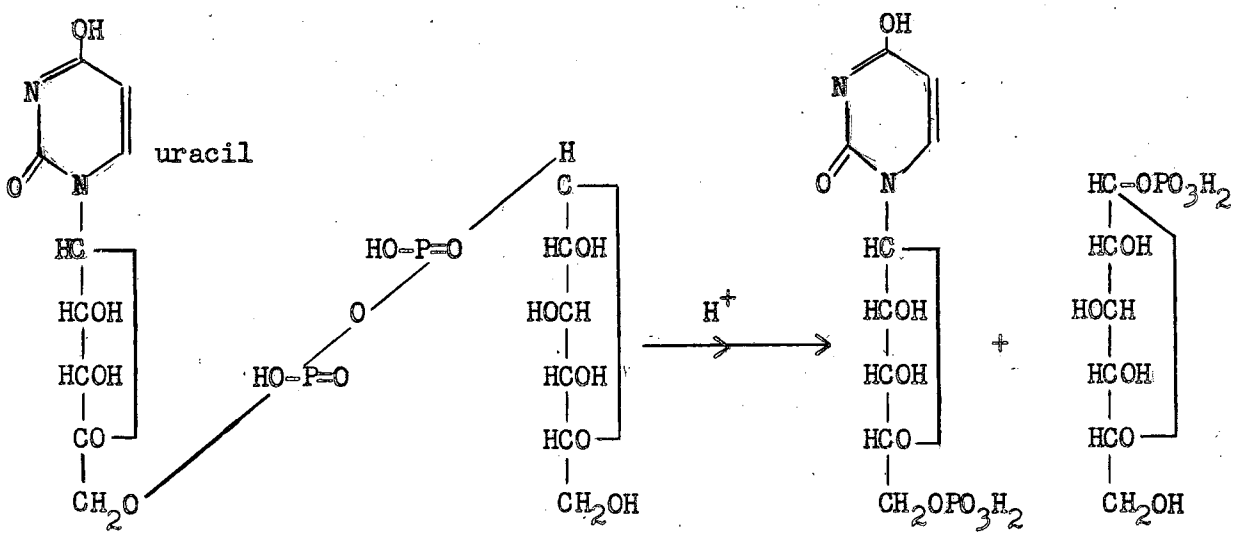
G-1-P is determined as acid labile-phosphorus giving an increase in reducing value with the method of Folin and Malmros (1929) of 66.5% of that of an equal weight of glucose. The large number of compounds which may hydrolyze readily to give increased reducing value renders this type of analysis somewhat uncertain when the components of the mixture are not known. A chromatographic separation followed by hydrolysis and determination of reducing value would constitute a reliable method when other labile compounds are present.

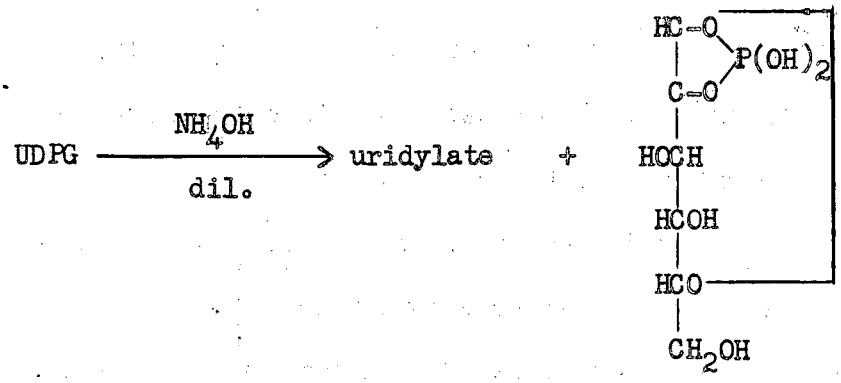
Glucose-1,6-diphosphate. - This diphosphate is readily detected among the C^{14}-labeled products of short photosynthesis in $C^{14}O_2$. It cochromatographs on paper with ribulose diphosphate, xylulose diphosphate, FDP and sedoheptulose diphosphate. In very short times of photosynthesis where fructose phosphates predominate there is very little glucose diphosphate as well. This demonstrates the accepted role of glucose diphosphate as intermediate in the conversion G-6-P to G-1-P (Leloir, et al., 1948).

Leloir, et al. (1949) reported that glucose diphosphate accumulates only to the extent of 0.5% of the concentration of FDP in fermenting yeast. It appears that its concentration in photosynthesizing tissues may be equal that of FDP. The exceedingly sensitive method of Leloir, et al. for determination of this compound involves its use as a coenzyme for phosphoglucomutase.

Uridine diphosphate glucose. - Mention must be made of the chemical properties of this labile nucleotide (Leloir, 1951) which leads to the observation of two glucose monophosphates in plant extracts. Glucose-1-phosphate and glucose in almost equal amounts were obtained upon mild HCl hydrolysis of unidentified UDPG isolated paper chromatographically from plant extracts and led to identification of the unknown as UDPG by Buchanan, Lynch, Benson, Bradley and Calvin (1953). Paper chromatography of UDPG in an acidic solvent such as t-butanol-picric acid-water also gives G-1-P as a decomposition product. This ester appears to contain most of the labile glucose phosphate in many plants and may well be a glucose donor in synthesis of polysaccharides (Leloir, 1951) (Buchanan, et al., 1952).

Under alkaline conditions UDPG forms the cyclic glucose-1,2-phosphate (Paladini and Leloir, 1952). This has been observed in plant extracts which have been allowed to stand at room temperature and must be attributed either to enzyme action or to possibly high pH of the isolation procedure.





α-D-glucose-1,2-phosphate (Leloir)

Due to the susceptibility of UDPG to enzymatic and chemical hydrolysis, care must be taken in developing an analysis method for UDPG in plant extracts. The presence of UDPGalactose and UDPMannose in extracts of Scenedesmus suggests the probable appearance of spurious monophosphates of these hexoses as well as those of glucose.

6-Phosphogluconic acid. - The action of G-6-P dehydrogenase upon G-6-P gives 6-phosphogluconate (6-P-G), and reduced TPN. The apparent ubiquity of this enzyme (Conn and Vennesland, 1951) (Axelrod, Bandurski, Greiner and Jang, 1953) and the present avalanche of evidence that 6-P-G is a primary intermediate of oxidative metabolism places the mechanism of its oxidation among the most important of biochemical problems. The mechanism of 6-P-G dehydrogenase action to give CO_2 and R-5-P is not yet clear.

Barium-6-phosphogluconate should presumably be placed in the category of barium-insoluble compounds. In an experiment with labeled 6-P-G, Axelrod et al. separated the product, R-5-P, in the barium-soluble alcohol-insoluble fraction.

Determination of 6-phosphogluconate can best be effected using purified 6-P-G dehydrogenase (Axelrod, Bandurski, Greiner and Jang, 1953). 2,6-Dichlorophenol indophenol reduction, coupled to TPN oxidation of 6-P-G by peat leaf

enzyme was measured colorimetrically. Horecker and Smyrniotis (1950) demonstrated the stoichiometric relationship between 6-P-G, TPN and pentose in the quantitative oxidation of pure 6-phosphogluconate.

Galactose-6-phosphate. - Ga-6-P occurs in plant extracts but rather little is known of its phytochemical interrelationships.

Galactose-1-phosphate. - The presence of galactose in hydrolysates of a fraction containing uridine diphosphate glucose (Buchanan, Lynch, Benson, Bradley, and Calvin, 1953) demonstrated the presence of uridine diphosphate galactose which could lead to occurrence of galactose-1-phosphate and galactose cyclic 1,2-phosphate in plant extracts.

Sedoheptulose phosphates. - All photosynthetic organisms which have yet been investigated and a wide variety of animal tissue and heterotrophic microorganisms contain sedoheptulose phosphate (Benson, Bassham and Calvin, 1952). It is intimately involved in the synthesis of the CO_2-acceptor of photosynthesis (Benson, et al., 1952). Plants, as well as animal tissues, contain an enzyme system, transketolase, for the transfer of glycolyl groups from sedoheptulose phosphate, ribulose-5-phosphate, hydroxypyruvate and probably other ketoses (Axelrod, Bandurski, Greiner and Jang, 1953; Racker, de la Haba and Leder, 1953) to a variety of acceptor aldehydes.

Robison, MacFarlane and Tazelaar (1938) isolated a heptulose phosphate from yeast which gave a positive orcinol reaction characteristic of mannoheptulose. This preparation has been recently examined in the author's laboratory and found to be sedoheptulose phosphate. Its rate of hydrolysis at 100° in N HCl was 4×10^{-3} (Robison and MacFarlane, 1941). The available evidence places the phosphate group on carbon-7. Sedoheptulose-1-phosphate has been synthesized enzymatically by Horecker (1952), Horecker and Smyrniotis (1953) and found to hydrolyze several times faster than the naturally occurring ester.

Sedoheptulose phosphate can be detected on the paper chromatogram with the orcinol-TCA spray test of Klevstrand and Nordal (1950) by the blue color characteristic of heptuloses. Its color is more blue than that given by mannoheptulose, glucoheptulose or guloheptulose.

Sedoheptulose phosphate has been determined by Horecker, Smyrniotis and Seegmiller (1951) by virtue of its ϵ_{max} at 600 mμ. in the Mejbaum pentose test modified by continuing the heating 40 minutes. Ribulose has orcinol absorption maxima at 670 and 540 mμ. They found it necessary to apply a correction for pentose absorption solving two simultaneous equations for the two components.

It appears that the Dische CyR I analysis (Dische, Shettles and Osnos, 1949) can be modified to give good results for sedoheptulose phosphate (Axelrod, Bandurski, Greiner and Jang, 1952).

4.5 M of a mixture of one part water and six parts H_2SO_4 (reagent grade) are pipetted into a 16 x 150 mm. test tube immersed in ice water. One ml. of the solution to be tested is added with shaking and continued cooling. After a few minutes the tube is placed in tap water a few minutes and then heated for exactly 3 minutes in a boiling water bath and immediately cooled in tap water. To this is added 0.1 ml. of 3% cysteine hydrochloride and the mixture is shaken. In a few minutes a yellow color appears which is measured in the spectrophotometer at 505 mμ. after 18 hours. Figure 12. The determinations are possible in the presence of glucose, fructose, G-6-P, F-6-P, FDP, R-5-P, ribose, ribulose and DHAP. Distinction between mannoheptulose, sedoheptulose and glucoheptulose is not possible.

A variety of colorimetric methods for heptose determination are given by Dische (1953). His primary method is a modification of the Mejbaum test

to give two stable colored products. The specificities of the methods overlap and may be of value in developing colorimetric analyses for heptoses in mixtures of other sugars and phosphate esters.

Sucrose Phosphate. - Free sucrose is liberated from phosphate earlier than the hexose phosphates during photosynthesis by most plants. This implies that sucrose is the result of condensation of hexose phosphates and that a sucrose phosphate is particularly sensitive to phosphatase action immediately after synthesis. The sucrose phosphorylase of Doudoroff, Kaplan and Hassid (1943) catalyzed the breakdown of sucrose in Pseudomonas saccharophila by the following equations:

$$\text{Glucose-1-P} + \text{enzyme} \longrightarrow \text{glucose} - \text{enzyme} + \text{P}$$

$$\text{Glucose-enzyme} + \text{fructose} \longrightarrow \text{D-glucose-1-fructoside}$$

A similar enzyme system has not yet been isolated from tissues of the higher plants. Critical reviews on this subject by Doudoroff (1945, 1953) and by Hassid and Putman (1950) emphasize the type of approaches which have been made to the problem.

The presence of a sucrose phosphate was detected in the sugar monophosphates formed during brief $C^{14}O_2$ photosynthesis by sugar beet leaves by N. E. Tolbert and the author using an invertase-free phosphatase prepared by B. Axelrod. The radiogram of the phosphatase hydrolysate showed fructose, glucose, sedoheptulose and sucrose. Buchanan (1953) isolated the sucrose phosphate from the mixture by paper chromatography of the monophosphates of sugar beet leaf in t-butanol (80 ml.) -water (20 ml.) -picric acid (2 g.) solvent. The R_f of the separated monophosphates decreased in the order: F-6-P, mannose + sedoheptulose-P, G-6-P, sucrose-P. Treatment of the pure sucrose phosphate with invertase-free phosphatase ("Phosphatase," Schwarz

Laboratories, Inc.) gave free sucrose and some glucose and fructose. Acid hydrolysis liberated free glucose, free fructose and what is tentatively identified as fructose-1-phosphate. Since the amount of free fructose corresponded to that expected from its simultaneous acid hydrolysis, Buchanan (1953) proposed that sucrose phosphate is a D-glucosyl-1-phosphofructoside. Its significance in sucrose synthesis is not yet clear. The relatively low concentration of this phosphate ester, even in the sugar beet leaf, does not preclude the necessity of its participation in sucrose synthesis.

Floridiside phosphate. - A phosphate ester of a α-D-galactosyl-2-glycerol (floridiside) has been detected by Bean (1953) in the alga Iridophycus flaccidum. Its chromatographic coordinates in phenol and in butanol-acetic acid solvents resemble that of triose phosphate. It is the precursor of the copious amounts of floridiside accumulated in this plant.

Phosphoshikimic acid. - The phosphorylated shikimic acid identified by Davis and Weiss (1954) is accumulated in the growth medium by an E. coli mutant requiring glucose and aromatic substrates for growth and hence is suspected to be a precursor of shikimic acid and subsequent aromatic compounds. This is a unique example of free excretion of a phosphorylated intermediate.

Phytic acid. - Inositol is completely phosphorylated in most plants. Phytic acid usually occurs as the calcium-magnesium salt, phytin, and as such is the major source of inorganic phosphorus available for cereal seedling. The concentration of phytic acid in seeds and in seedlings is rather high. Increase of inorganic phosphate and concurrent decrease of phytic acid content occur during early growth of a seedling (Albaum and Umbreit, 1943). This observation is the result of increased phytase activity during the first three days of development of the embryo.

The salts of phytic acid with polyvalent cations are very insoluble. Rapoport, Leva and Guest (1941) precipitated phytate with magnesia mixture in ammonia and obtained a reproducible 85% yield. This property is also exploited in the isolation as ferric phytate (Commons, 1939; Singson, 1948).

Ten grams of finely ground tissue was extracted by three hours shaking in 0.5 N HCl. The filtrate was reduced to 25 ml. and neutralized with 25% NaOH. Ten ml. of 0.01% ferric chloride solution in 0.1 N HCl was added and the resulting mixture heated fifteen minutes on a water bath and cooled. The precipitated ferric phytate is filtered, washed with ethanol and ether and then weighed.

An inositol phospholipid was obtained by Fuller (1952) from Neurospora mycelium. It resembled the inositol metadiphosphate-containing lipid isolated by Folch (1949) from brain cephalin. It is likely that such phospholipids may be found in the phospholipids of the higher plants.

Lindenfeld (1934) obtained complete hydrolysis of phytic acid in two hours by heating at 160-170° in 20% formic acid. Ordinarily, however, phytic acid is very acid-stable. Inositol obtained from hydrolysis of its simple phosphates has often been determined by quantitative isolation (Folch, 1949).

A number of inositol phosphates isolated from wheat bran were shown by Anderson (1915) to have been liberated by acid-stable plant phosphatases (phytases) during the extraction procedure. His results, therefore, were complicated by the appearance of partially hydrolyzed derivatives during the isolations.

An enzymatic method, described by Albaum and Umbreit (1943) used a phytase preparation obtained from oat seedlings for quantitative determination of phytic acid. Homogenates of 72 hour-old embryos were adjusted to pH 6.3 and incubated with samples of phytic acid for 6 hours at 37°. Phytic acid was quantitatively hydrolyzed.

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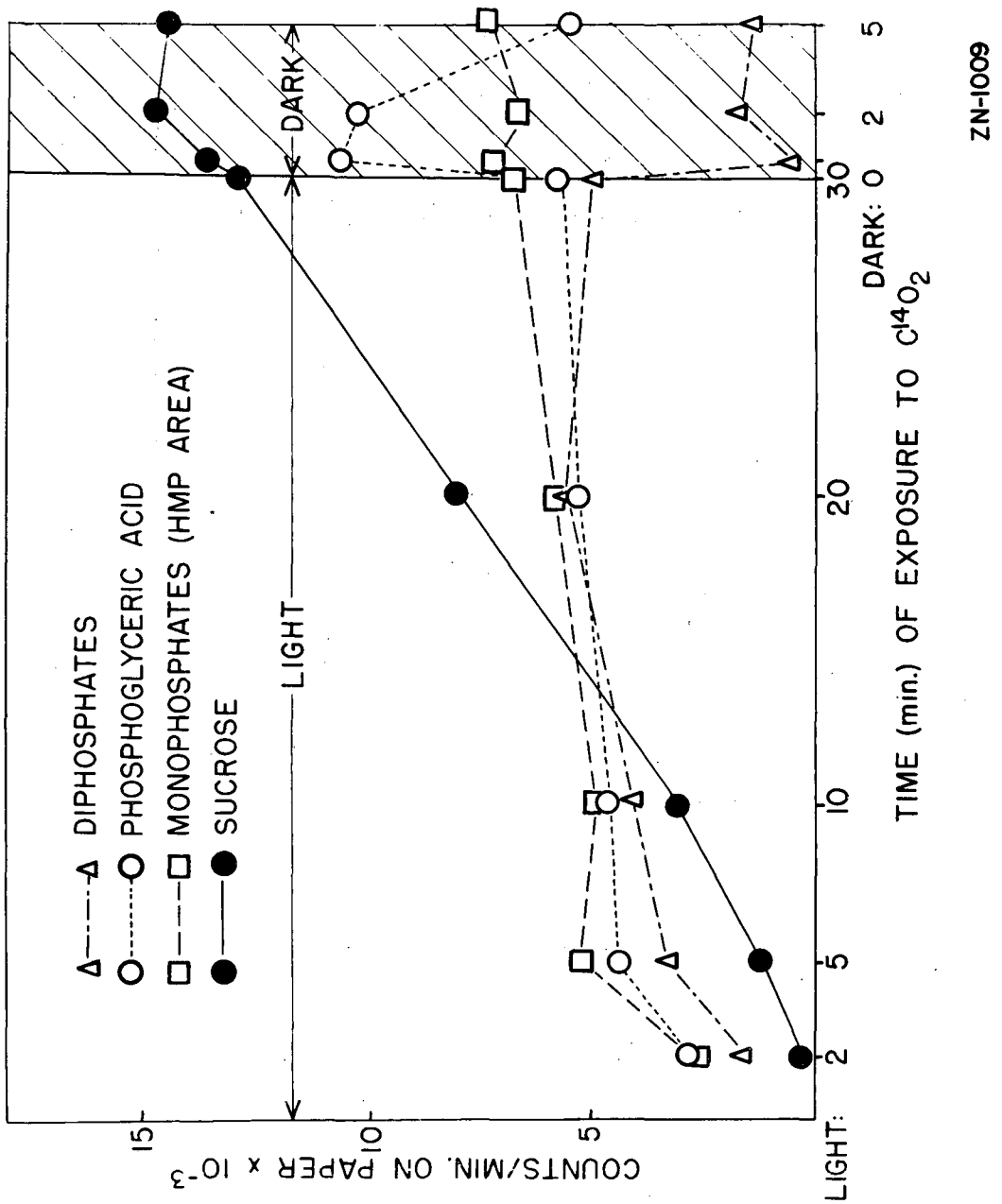


Figure 3. Changes in sugar phosphate concentrations upon darkening in Scenedesmus. (Calvin and Massini, 1952) Algae illuminated in 1 cm-thick vessel, 1% suspension of packed cells in water with light intensity of 700 foot candles from both sides; curve labeled "diphosphate" consists of 90% ribulose diphosphate.

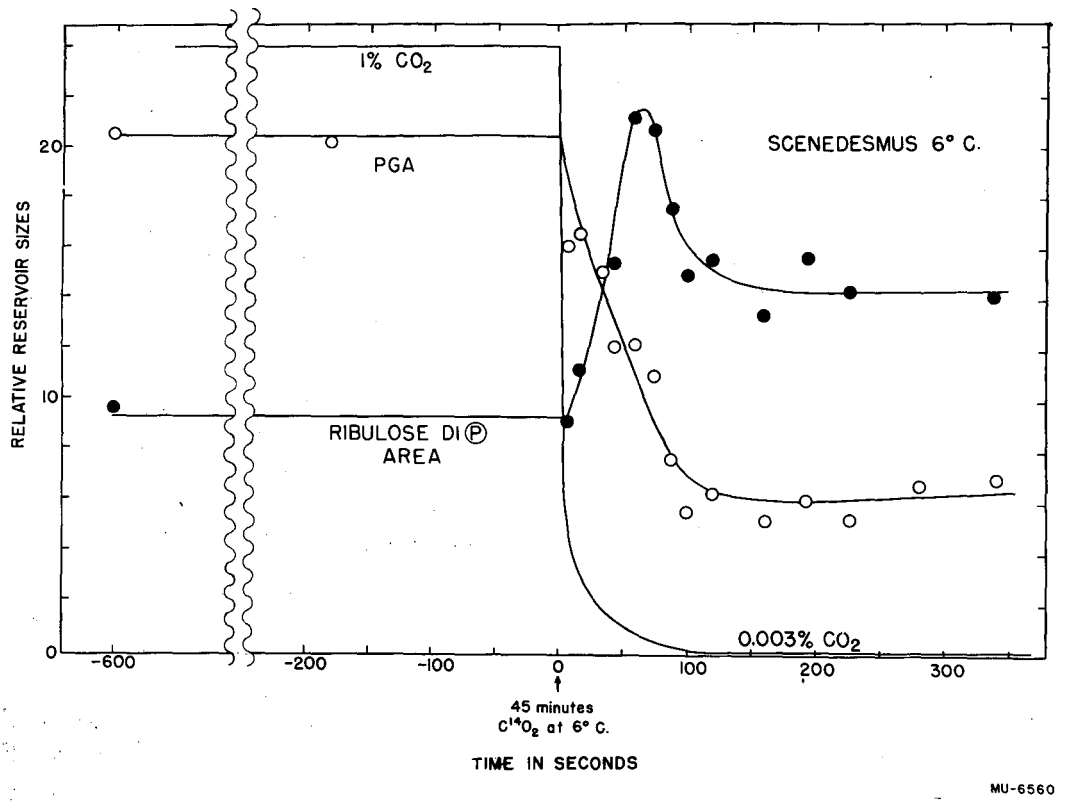


Fig. 4. Effect of reduction of CO_2 pressure upon concentrations of phosphoglycerate and ribulose diphosphate in Scenedesmus. (Wilson, 1954.

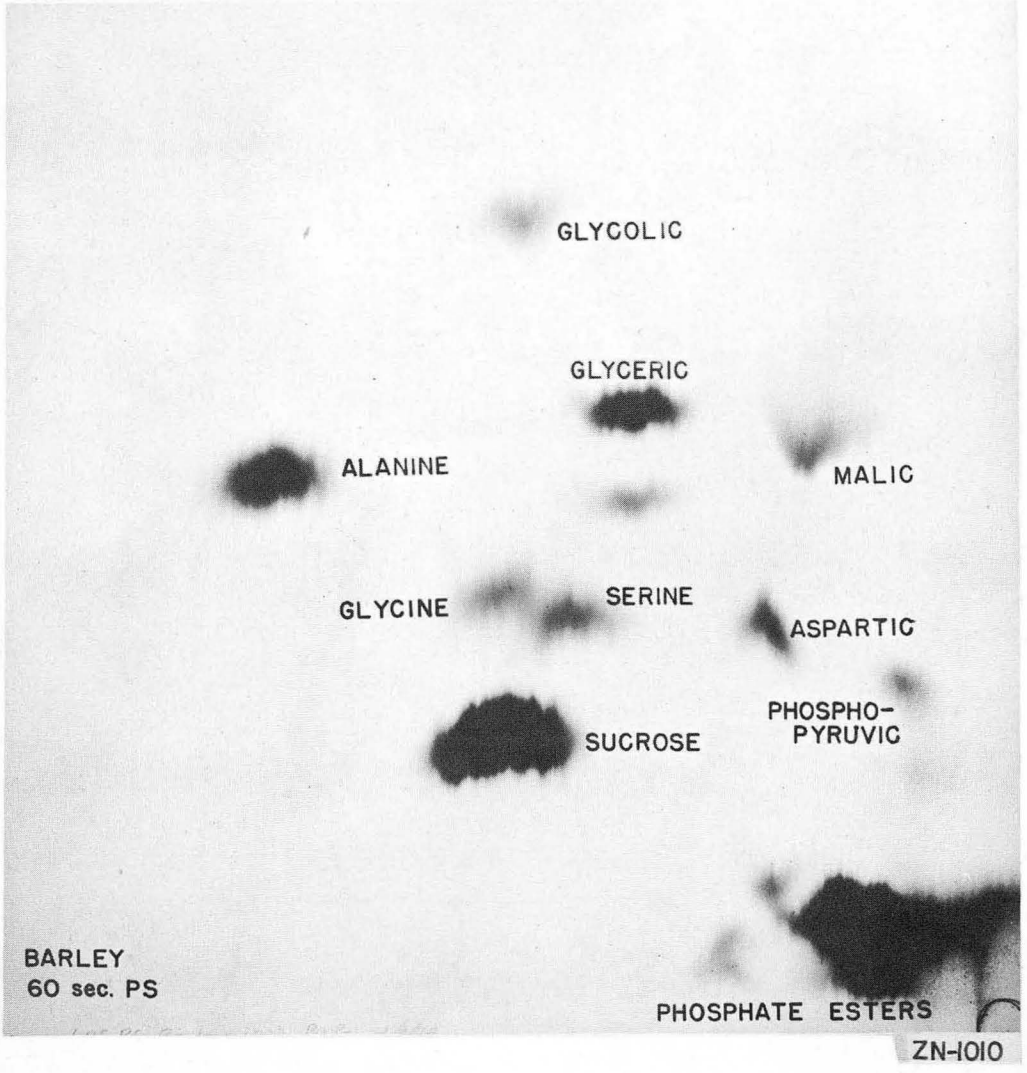


Fig. 5a. Sixty seconds $C^{14}O_2$ fixation by barley seedling leaves: Killed in hot ethanol. Chromatographed on unwashed Whatman No. 1 filter paper (1) phenol, (2) butanol-propionic acid solvent.

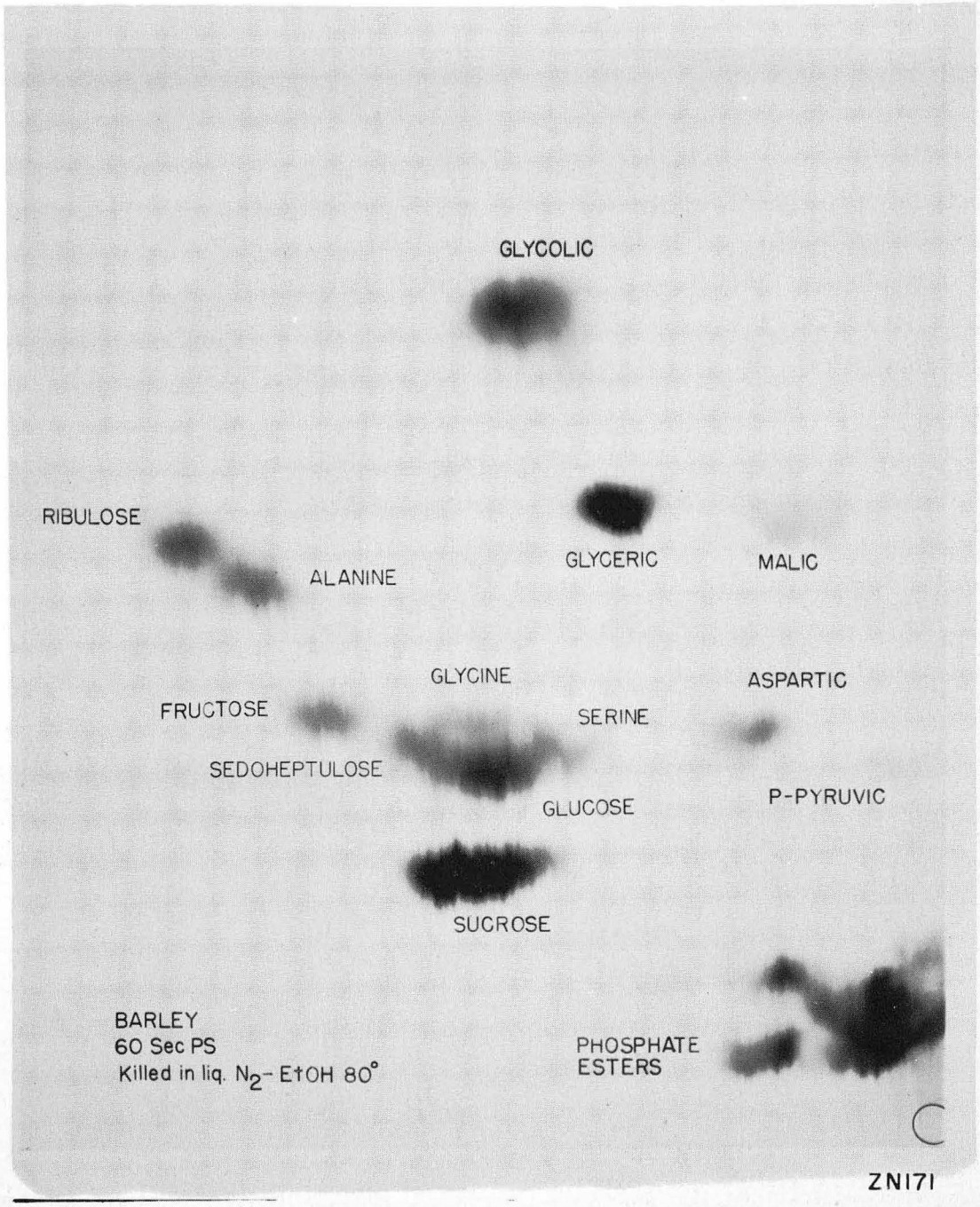


Fig. 5b. Sixty seconds $C^{14}O_2$ fixation by barley seedling leaves: Killed by freezing, grinding and hot extraction.

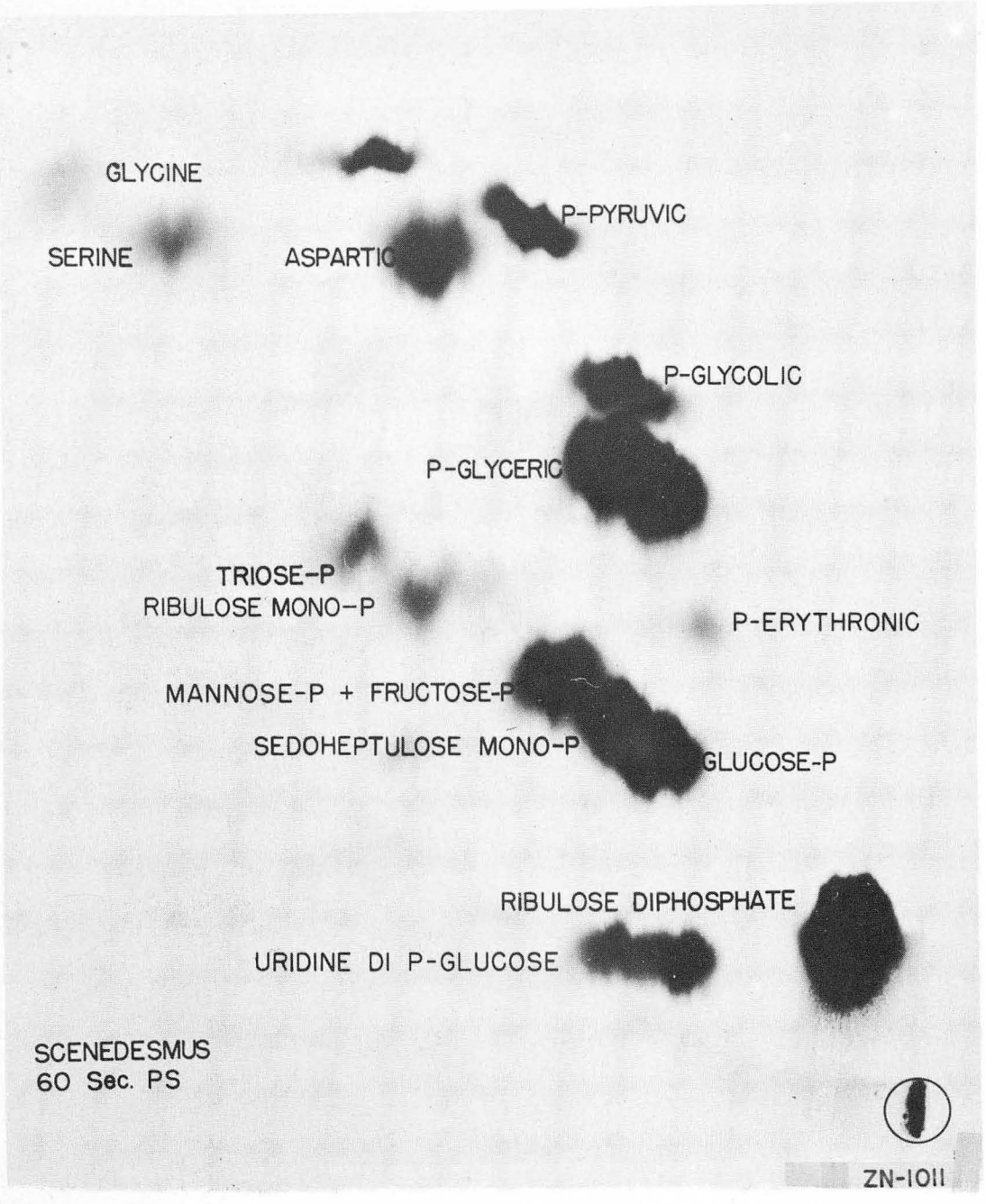


Fig. 6. Radiogram of phosphorylated products of 60 seconds $C^{14}O_2$ photosynthesis by Scenedesmus. The first solvent (right to left) is phenol and second (upwards) is butanol-propionic acid-water. Both solvents are run past the edge of the Whatman No. 1 sheet to obtain optimum separation. Organic dyes, tropeolin, crocein scarlet and Ponceau-4R spotted near the origin serve as indicators of solvent movement after the sheet is totally wet.

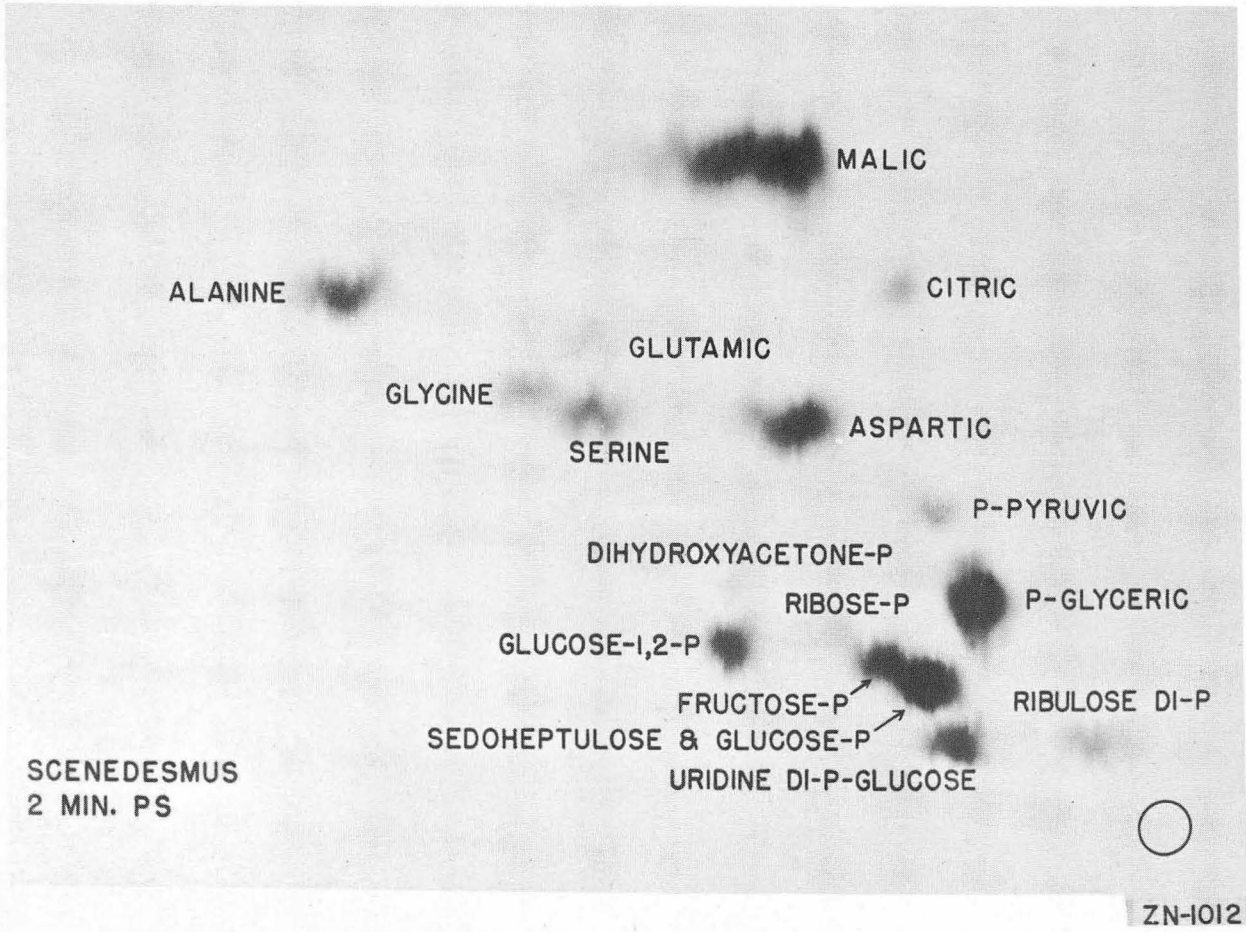
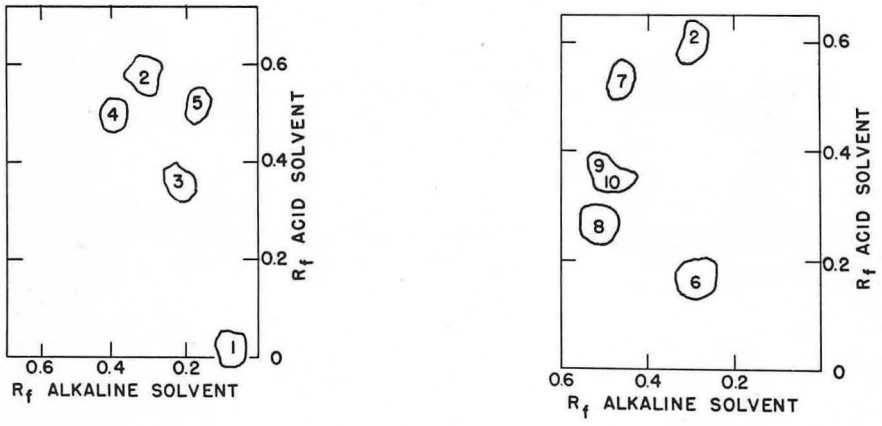


Fig. 7. Separation of photosynthetic products on acid-washed Whatman No. 4 paper. Developed in (1) phenol-water to left; (2) butanol-propionic acid-water upwards.



(A) (B)

Fig. 8. Two-dimensional chromatogram of glycolytic intermediates. Bandurski and Axelrod, 1951.

A. "Barium-insoluble fraction": 1, ATP; 2, orthophosphate; 3, FDP; 4, 3-PGA; 5, 2-PGA.

B. "Barium-soluble fraction": 2, orthophosphate; 6, adeonsine-3-P; 7, phosphopyruvate; 8, G-1-P; 9,F-6-P; 10, G-6-P.

(1) Acid solvent: Methanol-formic acid. 6.5 hours for 28 cm ascending.
(2) Basic solvent: Methanol-ammonium hydroxide, 15 hours for 28 cm ascending.

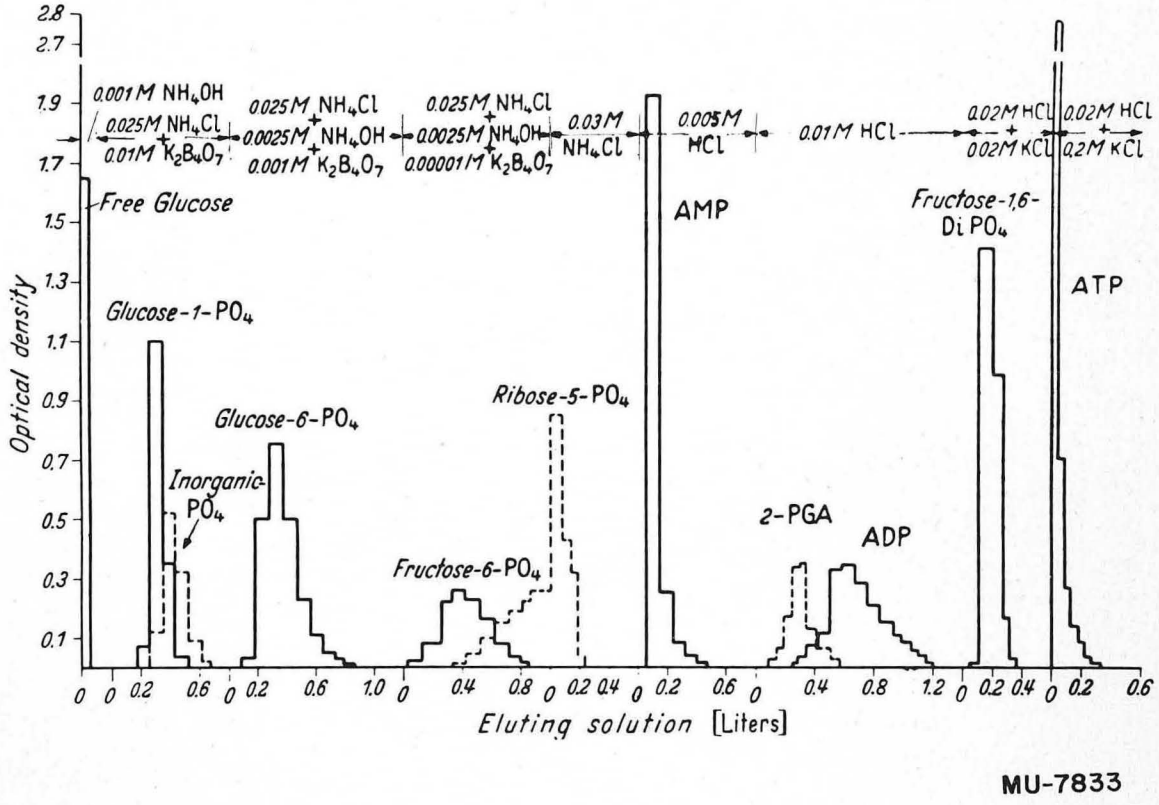


Fig. 9. Ion exchange separation of sugar phosphates, inorganic phosphate, adenosine phosphates and phosphoglyceric acid in the amounts given in Table III. Exchanger Dowex-1 chloride form, ca. 300 mesh, 0.86 cm^2 x 10 cm, flow rate, 3.5 ml/min. (Khym and Cohn, 1953)

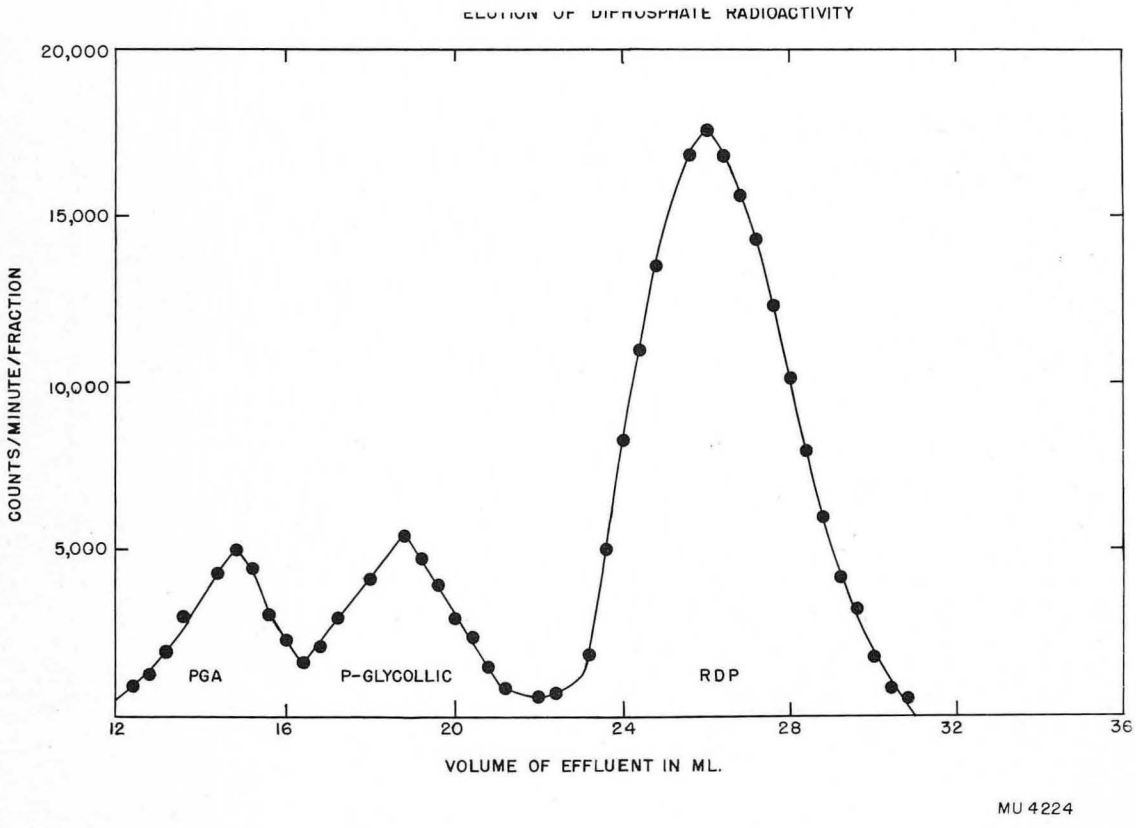


Fig. 10. Acid elution of radioactive ribulose diphosphate from a Dowex-2 chloride column (28 x 0.6 cm). (Goodman, 1952)
Eluting agent: 0.15 N NaCl + 0.05 N HCl; Eluting rate: 0.11 ml/min; Fraction volume: 0.4 ml.

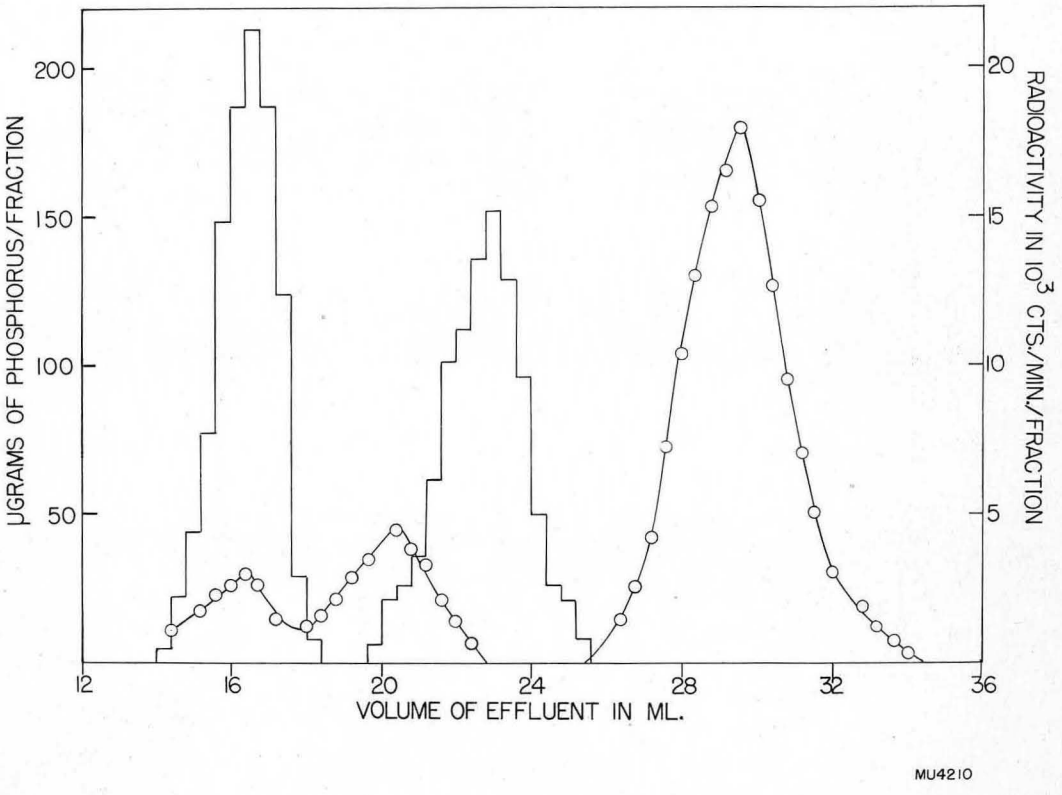


Fig. 11. Acid elution of phosphoglyceric acid, fructose diphosphate and radioactive ribulose diphosphate from a Dowex-2 chloride column (28 x 0.6 cm). (Goodman, 1952) Smooth curve denotes radioactivity. Block curve is obtained from phosphorus analysis.

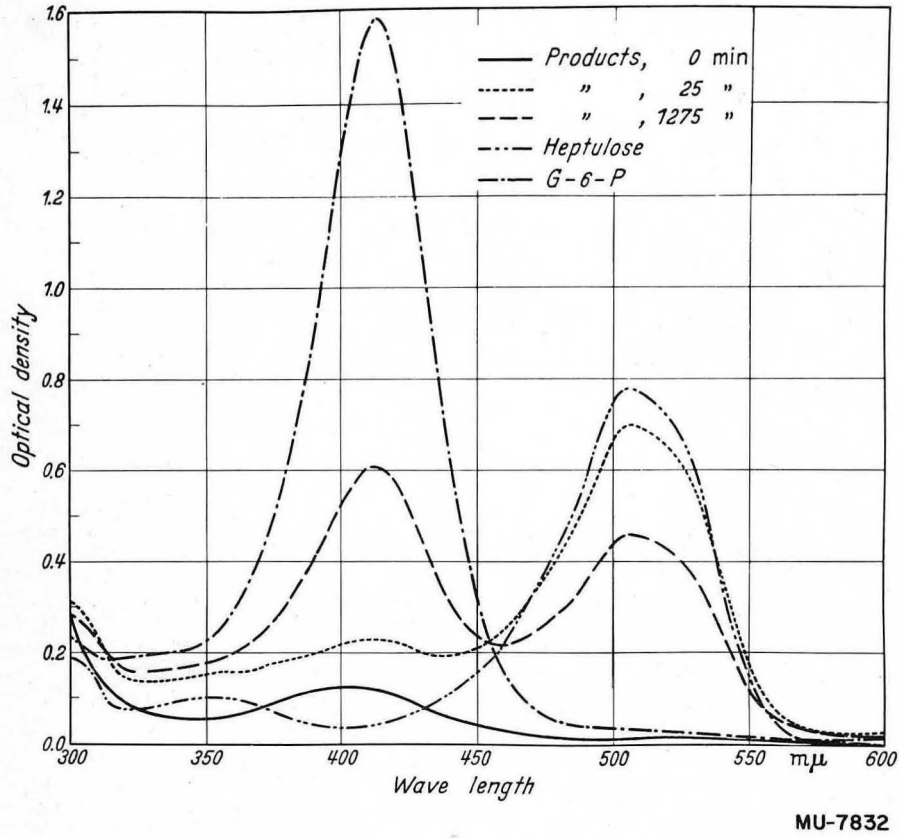


Fig. 12. Adsorption spectra of the reaction products in the Dische test obtained with products of spinach enzyme on ribose-5-phosphate (Axelrod, et al.,1952). Reaction conditions, 0.2 ml of spinach enzyme, 0.2 ml of Tham-HCl pH 7.5, 0.15 M, 0.1 ml of H_2O, and 0.5 ml of R-5-P, 0.26 M. Reaction temperature, 38°. Reaction stopped by adding 10 volumes of 5 percent TCA. 1 ml of a 1:2 dilution of this mixture was employed in the Dische test. The absorption curves were obtained with a Cary recording spectrophotometer in a 2 cm optical path. Spectra obtained with 25 γ of sedoheptulosan monohydrate and 194 γ of Ba G-6-P·$7H_2O$ of 97.4 percent purity were used.