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RIBULOSE AND SEDOHEPTULOSE

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The intermediates of carbon dioxide reduction by plants include phosphorylated derivatives of hydroxy acids and sugars. Their identification became possible when the use of labeled carbon dioxide permitted discrimination between the earliest products and the many other components of photosynthetic tissues. A number of compounds were identified by virtue of the chemical and physical properties of the radioactive compounds in tracer amounts and by direct comparison of these properties with those of suspected known metabolic intermediates.

It became apparent that several labeled compounds found in short exposures to radioactive carbon dioxide were not substances previously identified as metabolic intermediates. Two phosphate esters in particular were observed in the products of the first few seconds of steady-state photosynthesis by all the photosynthetic microorganisms and higher plants

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

examined in this laboratory. These esters have been isolated by paper chromatography in tracer quantities and enzymatically hydrolyzed to give two sugars, ribulose and sedoheptulose. This paper contains a description of the chemical identification of these sugars and some observations and suggestions regarding the function of their esters.

The general importance of these compounds in photosynthesis was surmised before their identification. The products of photosynthesis with $C^{14}O_2$ by each plant included phosphate esters of the same two then unknown compounds in addition to those of the expected glucose, fructose, dihydroxyacetone and glyceric acid. (Figure 1) As the time of steady-state photosynthesis in $C^{14}O_2$ decreased, the fractions of total fixed radiocarbon in the esters of the two unidentified compounds increased.

Normally, the sugars involved in the early stages of photosynthesis are first isolated as phosphate esters.[1] These are eluted and hydrolyzed enzymatically after which chromatography gives the pure sugars. (Figures 2 and 3) In some cases, however, depending on the condition of each plant and the method of killing and extracting leaves, the free sugars were found upon two-dimensional paper chromatography of the original cell extract. (Figure 4)

Rechromatography of the two unidentified phosphorus-free compounds demonstrated their stability. Their positions on the chromatogram, as well as the pH independence of their distribution coefficients, showed them to be neutral. Since their R_f values were much greater with lutidine-water than with acidic solvents like formic acid-glycol dimethylether, their polyhydroxylated nature was indicated. Moreover, acetylation gave polyacetates with very high distribution coefficients compared to the original compounds. Bromine water had no pronounced effect on either of the two compounds since

they were largely recovered upon rechromatography of the reaction mixture. Aldoses, enolic compounds or unsaturated sugars would have given products with solubility characteristics very different from those of the unknowns. These tests led to the suspicion, later confirmed, that the two compounds were ketoses. The following evidence led to the identity of the first compound, sedoheptulose.

Under the conditions of Neuberg and Strauss[2] some 2,4-dinitrophenylhydrazone was formed from the radioactive compound, but the reaction was incomplete at room temperature or when heated for a few hours. Without added carrier, the reaction with the reagent was detected by rechromatography or measurement of the distribution coefficient of the products. With added mannose, the radioactive compound formed an osazone which was difficult to separate by recrystallization from the 2,4-dinitromannosazone.

Sedoheptulose is converted to a bicyclic anhydride when heated in dilute acid.[3,4] The radioactive compound was converted to a new compound under the same conditions. The first clue to the nature of this reaction, easily recognized by the marked change of chromatographic position after treatment with acid, came as a result of the lack of appearance of much of the radioactivity in CO_2, formic acid or formaldehyde upon periodate oxidation of the acid treated product. Analogy with the chromatographic coordinates of fructose and its anhydrides** and the absence of any known sugars in the area occupied by the acid-treated product on the chromatogram, was added evidence for a cyclic anhydride. LaForge and Hudson[3] reported an equilibrium constant of 4.0 for the dehydration of sedoheptulose. The same constant was

(**) We are indebted to Professor L. Sattler for samples of several fructose anhydrides.

observed for the unknown radioactive sugar by counting the chromatographically separated products from the equilibrium mixture. Reversibility of the dehydration was demonstrated by chromatographing the equilibrium mixture of sugar and anhydride obtained by heating the pure radioactive anhydride in acid solution.

Catalytic hydrogenation of the sugar in tracer quantities presented the problem arising from the irreversible adsorption on the catalysts. Raney nickel adsorbed almost all of the product. Adsorption was negligible with small amounts of platinic oxide and added fructose or glucose for carrier. Chromatography of the resulting polyol gave a single radioactive spot.

The carbon skeletons and stereochemical structure of the previously recognized hexoses and three-carbon intermediates of photosynthesis did not suggest the nature of the unknown compound. The first evidence of the carbon structure, aside from chromatographic coordinates which are valuable aids to identification,[5] came from the results of periodate oxidation of the uniformly labeled compound. After five minutes of photosynthesis, hexoses and C_3 compounds have been shown to have uniform C^{14} distribution.[6,7,8] Periodic acid oxidation of fructose gives one mole of glycolic acid, three moles of formic acid and one mole of formaldehyde.[9] The unknown compound, isolated from the products of five minutes photosynthesis in $C^{14}O_2$ by a soy bean leaf, gave 14.8 per cent of the original radioactivity in formaldehyde, 55 per cent in formic acid and 28 per cent in a non-volatile residue which was identified chromatographically as glycolic acid. The unknown anhydride prepared from the same sugar gave 14 per cent of its activity as formic acid but none as formaldehyde. The remaining radioactivity was non-volatile.

Periodate degradation of the chromatographically purified polyol derived from the unknown sugar by hydrogenation gave radioactivity in formic acid and formaldehyde in the molar ratio of 3:1. While the deviation from the theoretical 2.5 for a heptitol was possibly due to experimental error, the great difference from the hexitol ratio, 2.0, was the first clear evidence that the unknown contained more than six carbon atoms.

The final identification of the radioactive sugar and its anhydride was confirmed by two-dimensional co-chromatography with authentic specimens of sedoheptulose and sedoheptulosan respectively. Precise concurrence of the radioactivity and the added sugars was observed by comparison of the radiograms with the colored areas produced by reaction with sprayed resorcinol. (Figure 5)

The second radioactive compound, ribulose, was identified by the following evidence.

The radioactive sugar was reduced in good yields to a compound with R_f values characteristic of the pentitols. The proximity of ribitol and arabitol in chromatograms precluded distinction by R_f measurements alone. The reduced radioactive compound co-chromatographed exactly with added ribitol but not with arabitol.

A prominent property of the sugar diphosphate when present in tracer quantities was the instability toward oxidation, particularly in alkaline solution. When radioactive ribulose diphosphate dissolved in dilute aqueous diethylamine solutions was exposed to the air for four days, the products had the chromatographic coordinates characteristic of phosphoglyceric and phosphoglycolic acids. These phosphates were eluted, treated with phosphatase and rechromatographed to give glyceric and glycolic acids and an acid

suspected to be erythronic acid. These are products to be expected from oxidation of a ketopentose, either between carbon atoms 2 and 3 or between carbon atoms 1 and 2.

Further evidence for the stereostructure of the radioactive sugar was obtained by successful co-chromatography of its epimers, prepared in pyridine, with those obtained by similar treatment of ribose and arabinose. For final identification the radioactive sugar was co-chromatographed with an authentic specimen of ribulose and precise concurrence of the radioactivity with the added sugar observed.

After the phosphate-free compounds had been identified as sugars, the chromatographic position of the heptose phosphate among the known hexose monophosphates indicated that it too was a monophosphate, while the position of the major ribulose phosphate near the origin suggested that it was probably a diphosphate. (Figure 1) These surmises were further confirmed by determining the P/C ratios in chromatographically isolated compounds saturated with P^{32} and C^{14}.***

Experimental Part

Sources of Ribulose and Sedoheptulose. - Both these sugars were observed in radiograms of barley extracts such as that shown in Figure 4. These were eluted for chemical tests. Because of the proximity of alanine to ribulose and of serine and glucose to sedoheptulose in chromatograms, it was difficult to obtain pure samples without re-chromatography. For such specific separation, the solvent is run off the paper until the product approaches the edge of the sheet of Whatman No. 1 paper and has traveled about 50 cm. The

(***) Benson, A. A., In press.

position of the compound on the paper is known from its relationship to dyes (tropeolin, croceine sharlack and ponceau-4-R) placed just beneath the samples at the origin. Chromatographic coordinates of tropeolin (orange II), R_f with phenol, 0.75, R_f with butanol-propionic acid, 0.68, are particularly reproducible.

Larger amounts of these sugars were obtained upon hydrolysis of phosphate esters by appropriate phosphatase preparations. The eluate from a cut-out wedge of the chromatogram (ca. 100-200 μl.) was concentrated by evaporation in a stream of nitrogen gas to about 50 μl. and 100-300 γ of Polidase in 10 μl. of water were added. After incubation under toluene for one to three days at 35° C., the products were chromatographed and the radioactive sugars observed in radiograms such as those in Figures 2 and 3, in which are shown hydrolysates of the compounds eluted from the hexose monophosphate area in chromatograms of all soluble products from four-minutes steady-state photosynthesis by the alga Scenedesmus. Labeled sedoheptulose was obtained most conveniently by hydrolysis of the phosphates formed during photosynthesis by soy bean leaves in which the relative amount of sedoheptulose phosphates is higher than in other plants investigated. After being heated with acid, the eluate containing the sedoheptulose was rechromatographed and the sugar was found to be converted in about 80% yield to sedoheptulosan with R_f phenol 0.69 and R_f butanol-propionic acid of 0.35. Ribulose was obtained in a similar manner by hydrolysis of ribulose diphosphate which is produced in the highest concentrations by very young cultures of Scenedesmus.

Sugar phosphates formed by a variety of organisms and under several conditions were hydrolyzed enzymatically and rechromatographed. The radioactivity in each of the resulting free sugars was counted and the relative

amounts of radioactivity are given in Table I where the quantity of radiocarbon found in each sugar is expressed as a percentage of total radiocarbon found in the four sugars.

Anhydride Formation of Sedoheptulose. - A sample of radioactive sedoheptulose obtained by elution from a chromatogram of the hydrolysis products of phosphate esters formed by photosynthesis in soy bean leaves products was heated thirty minutes in N hydrochloric acid on a steam bath. The solution was evaporated in a stream of air at 40° C. and the products were rechromatographed. The separated products were eluted and counted on 3 cm. discs. The unchanged sugar gave a total of 7,490 cpm. and the anhydride gave 30,300 cpm.; therefore, the equilibrium constant was 4.0 for the conversion of sugar to anhydride.

Hydrogenation of Tracer Amounts of Sugars. - C^{14}-labeled fructose was eluted from chromatograms(****) of photosynthesis products and hydrogenated in a 2 ml. stainless steel bomb with 2 mg. of Raney nickel catalyst at 100° and 150° and 135 atmospheres during ten hours of shaking. When the catalyst was separated by centrifugation, less than ten percent of the radioactivity remained in solution. Exhaustive washing of the catalyst failed to remove an appreciable quantity of the product. The small amount of product was identified by co-chromatography with mannitol.

When the same amount of fructose-C^{14} was hydrogenated at 90° for twenty hours using 10 mg. of Adams catalyst in 1.5 ml. of 50 per cent ethanol, the catalyst adsorbed only 10 per cent of the product. Negligible adsorbtion

(****) The amounts of labeled sugars used in these experiments were of the order of 1-5 μg.

was observed when labeled ribulose and sedoheptulose were reduced using 1-2 mg. Adams catalyst and 100 μg. of glucose for carrier.

The reduction product of ribulose had R_f values corresponding to those of ribitol (phenol, 0.54; butanol-propionic, 0.25) and was located nearer the origin than alanine while the coordinates of ribulose exceeded those for alanine by 10 per cent in both solvents.

Sedoheptitol had chromatographic coordinates closely approximating those of glucose (R_f phenol 0.36; R_f butanol-propionic acid 0.15) while the coordinates of the original heptulose were R_f phenol 0.42 and R_f butanol-propionic acid 0.16.

An authentic specimen of sedoheptitol was prepared from 200 μg. of natural sedoheptulose(*****) by similar hydrogenation. The radioactive heptitol was chromatographed together with 100 μg. of the synthetic product. Exact coincidence of the radioactivity and the authentic heptitol was observed when the radiogram was compared with the black silver deposit produced by spraying the chromatogram with Tollen's reagent. This reagent was prepared by adding concentrated ammonium hydroxide to saturated aqueous silver nitrate in the usual manner and then diluting with methanol to a final concentration of 0.3 M $Ag(NH_3)_2^{++}$. The black spots for sugars or glycols are developed by heating the sprayed chromatogram. It is sometimes desirable to remove phenol and quinone by briefly autoclaving the paper before spraying it with the reagent. Brown silver oxide background is removed by washing the paper with water and a solution of Kodak Liquid X-Ray Fixer.

(*****) Kindly supplied by Dr. E. W. Putman.

Co-crystallization of the 2,4-dinitrophenylhydrazones of the Labeled Ribulose and Arabinose. - A solution of 14.7 mg. D-arabinose and 7,300 cpm. of chromatographically isolated labeled ribulose was heated 24 hours at 100° with 58 mg. of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid according to the method of Neuberg and Strauss.[2] The product was washed with water, dried and recrystallized from ethanol and methyl cellosolve to a constant specific radioactivity of 154 cpm./mg., 4 per cent above the theoretical value.

Periodate Oxidation of Radioactive Sedoheptulose. - To a solution of radioactive sedoheptulose which gave 28,600 cpm. were added 102.5 mg. of barium formate, formaldehyde equivalent to 41 mg. of formaldimedon, 101.5 mg. of barium carbonate, 0.7 ml. of 6 N hydrochloric acid and 0.2 ml. of 0.205 N periodic acid in a closed flask. After 3 days enough sodium hydroxide was added to neutralize the reaction mixture. The formaldehyde was distilled in vacuo into excess dimedon solution which upon acidification gave formaldimedon with a specific activity of 100.6 cpm./mg. after several recrystallizations from aqueous ethanol. Thus, 4,130 cpm. or 14.4 per cent of the radioactivity was present in $-CH_2OH$ groups. The contents of the original flask were acidified and the volatile contents were distilled in vacuo into carbonate-free sodium hydroxide solution. Barium chloride was added and 78 mg. of an expected 115 to 120 mg. of barium carbonate were obtained. Its specific activity was 5.1 cpm./mg. which indicates about 2 per cent of the original activity was converted to carbon dioxide. After the barium carbonate centrifugation, the solution was acidified and steam distilled. Barium formate obtained upon neutralization and concentration of the steam distillate had a specific activity of 153 cpm./mg. after two recrystallizations from water-

alcohol, indicating that 15,650 cpm. or 55 per cent of the starting activity was present originally in -CHOH groups.

Periodate Oxidation of Sedoheptulosan. - A solution of 20,000 cpm. of radioactive sedoheptulosan was oxidized as above with 49.8 mg. barium formate and formaldehyde equivalent to 41 mg. of formaldimedon. The observed specific activity of the formaldimedon was zero. That of the recrystallized barium formate was 59 cpm./mg. indicating 2,940 cpm. or 14.7 per cent of the original radioactivity was present in -CHOH groups. The residue in the flask gave 17,600 cpm. or 88 per cent of the original radioactivity.

Periodate Oxidation of Sedoheptitol. - A chromatographically purified sample of 12,800 cpm. of sedoheptitol was oxidized as above with 100 mg. barium formate and formaldehyde equivalent to 41 mg. of formaldimedon. The specific activity of the barium formate was found to be 100 cpm./mg. so that 10,000 cpm. was contained in -CHOH groups. The formaldimedon gave 80.5 cpm./mg. or 3,300 in CH_2OH groups. Since the sum of radioactivity in these products was only 91-96 per cent of the starting radioactivity, the oxidation may have been incomplete. The ratio of the two radioactivities, 3.0, is perhaps most reliable.

Epimerization of Ribulose. - Authentic ribulose and xylulose were prepared by heating arabinose, ribose and xylose for four hours in pyridine according to the method of Schmidt and Treiber.[10]

Samples of pyridine solutions containing one mg. of sugars were chromatographed two-dimensionally. The yield of ketose was small as demonstrated by spraying with methanolic Tollens reagent. The Roe reagent (resorcinol-hydrochloric acid in ethanol) detected only the ketoses, giving gray-green spots. The solution containing ribulose was co-chromatographed with the radioactive ribulose and gave exact coincidence. Xylulose was found to have

an identical position with a minor radioactive compound separable from but close to ribulose. This radioactive compound was resistant to bromine water. Its accumulation among the products of photosynthesis is much slower than that of ribulose.

Radioactive ribulose was epimerized by heating in pyridine with a small amount of arabinose. The products were chromatographed with 50 μg. each of D-arabinose and D-ribose. The resultant radiogram had three major radioactive areas. One was the original ribulose, the others coincided exactly with the positions of the added pentoses which were observed by spraying the chromatogram with aniline-trichloroacetic acid reagent.

Discussion

Sedoheptulose accumulates during photosynthesis in many of the succulent plants.[11,12,13,******] With the chemical identification of sedoheptulose monophosphate in a wide variety of plants and of free sedoheptulose in some Saxifragaceae[14] and Primulaceae[15], it now becomes apparent that accumulation of the sugar in succulents may represent special cases of the inter-relationships obtained in all photosynthetic organisms.

Synthetic ribulose (adonose) is well known but its general natural occurrence in plant tissues has not been previously recognized. Ribitol (adonitol) was first isolated from Adonis vernalis.[16,17] The general occurrence of D-ribose and D-arabinose, which was predicted by Fischer after his study of ribitol,[18] suggests the probable natural occurrence of their epimer, D-ribulose. Since the yield of ribulose from epimerization of ribose or arabinose is quite low,[10] the equilibrium among the three epimers may strongly favor accumulation of the two aldoses while a low concentration of the ketose might be anticipated in biological systems.

(******) Putman, E. W. and Moran, R., unpublished.

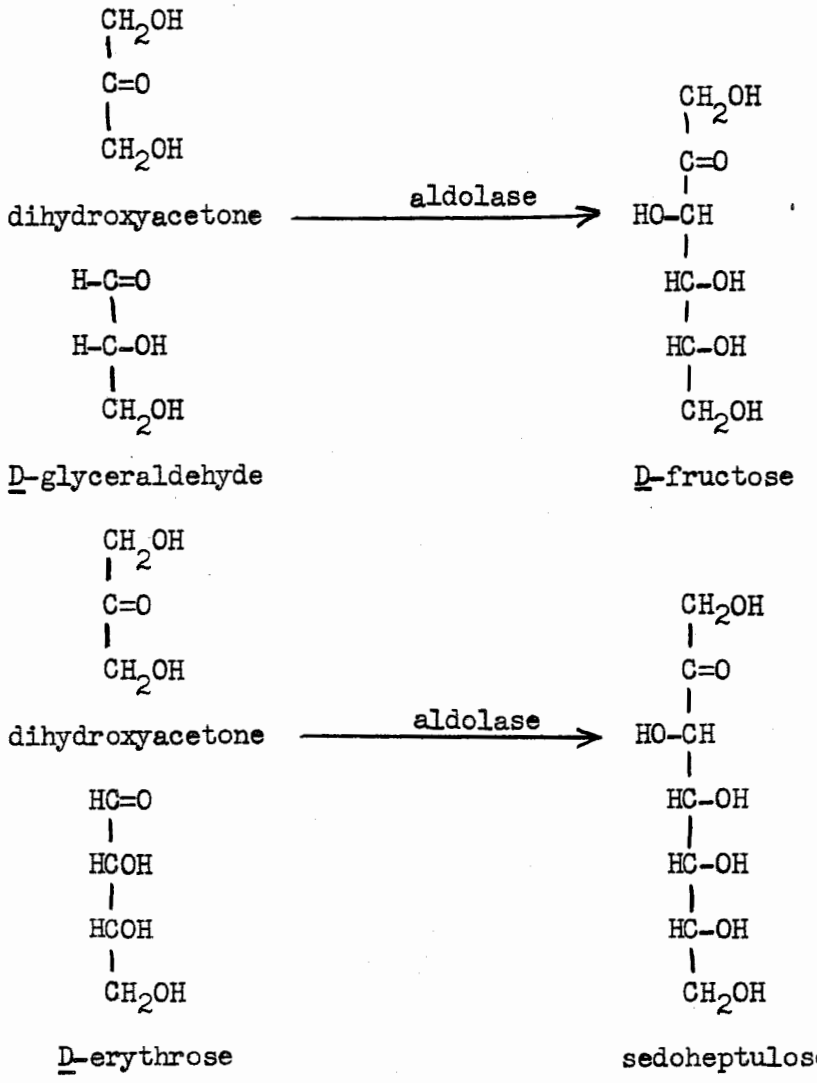
Cohen and Scott[19] obtained from bacteria a pentose phosphate related to ribose and arabinose, and Horecker and Smyrniotis[20] obtained a ketopentose phosphate fraction which they felt was ribulose-5-phosphate. However, ribulose phosphates have not previously been obtained from photosynthetic organisms.

The close relationship between the structure of sedoheptulose and that of D-ribulose strongly suggests a synthetic relationship. The configuration of C-3 and C-4 of ribulose is identical with that of C-5 and C-6 of sedoheptulose. The recent results of Rappoport, Barker and Hassid[21] and Lampen, Gest and Sowden[22] require the ultimate cleavage of an aldose by an acyloin-type reaction. These authors pointed out that such cleavage most likely involves a 2-ketose intermediate. Similar cleavage of sedoheptulose would give glycolaldehyde (diose) and D-ribose which could isomerize to D-ribulose. Subsequent cleavage of the pentose would give diose and a triose. It appears reasonable from the compounds available in plants and the apparently universal aldolase activity in leaves[23] that diose and dihydroxy acetone could condense to form xylulose as discussed by Hough and Jones.[24]

None of these sugars is stereochemically related to glucose by a simple sequence of reactions. One of the functions of these compounds may be to serve as sources of two carbon molecules capable of accepting carbon dioxide to form phosphoglycerate during photosynthesis. The concurrent syntheses of fructose and sedoheptulose may represent steps in carbohydrate synthesis and in regeneration of the required carbon dioxide acceptors respectively.

The fact that the two predominant carboxylations of photosynthesis[6] result in C_3 and C_4 compounds leads one to expect a condensation of the C_3 and C_4 sugars to give sedoheptulose.

Aldolase, acting stereochemically in a manner analagous to that by which it forms fructose, would result in sedoheptulose if erythrose were substituted for glyceraldehyde in the condensation. This configurational relationship is represented by the following pair of schematic reactions.



It is of interest to point out that manncheptulose, the other naturally occurring heptulose, is configurationally related to galactose as sedoheptulose is to glucose. This may indicate a corresponding biosynthetic relationship.[25]

Summary

Sedoheptulose and ribulose phosphates have been identified in a varied group of photosynthetic organisms. These phosphates are among the earliest sugar phosphates formed during photosynthesis.

The biosynthetic relationships of sedoheptulose and ribulose are discussed. It is proposed that they are not directly involved in hexose synthesis but may serve as sources of C_2-carbon dioxide acceptors required during photosynthesis.

Table I

Relative Amounts of Phosphorylated Sugars

Organism	Time of Photosynthesis	Glucose	Fructose	Sedo-heptulose	Ribulose
Rhodospirillum rubrum	20 min.	50%	20%	15%	15%
Scenedesmus D_3 (1 day old	5 min.	40	10	16	34
Scenedesmus D_3 (2 day old)	2 min.	52.5	16.6	29	1.9
Chlorella pyrenoidosa	60 sec.	40	14	40	6
Barley seedling leaves (var. Sacramento)	60 sec.	53	16	17	13
Sugar beet leaf (mature)	5 min.	60	25	5	10
Alfalfa leaf	2 min.	47	25	20	~1
Soy bean leaf	5 min.	39	24	36	~1
Vicia Faba leaf	4 min.	46	34	18	~1
Kalanchoe blossfeldiana	2 min.	57	23	18	--
Crassula arborescens	2 min.	63	14	22	1.5

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Captions to Figures

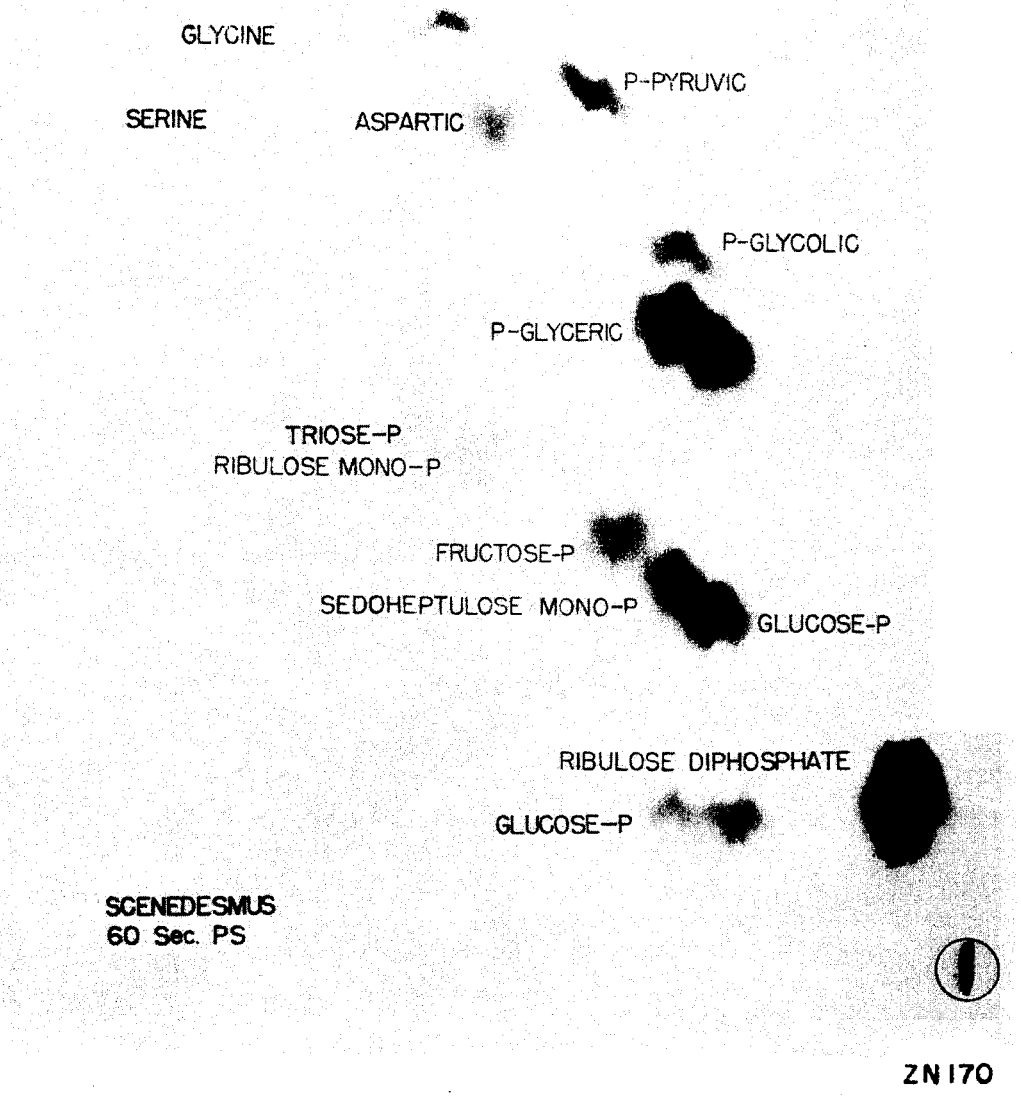
Fig. 1 - C^{14}-labeled phosphate esters formed during 60 seconds photosynthesis by Scenedesmus. The chromatogram was developed until the faster moving phosphates approached the edge of the paper to effect better separation of these esters.

Fig. 2 - Hydrolyzed phosphates from 5 second and 5 minutes photosynthesis by soy bean leaves.

Fig. 3 - Hydrolyzed phosphates from 4 minute photosynthesis by Scenedesmus (monophosphate area).

Fig. 4 - C^{14}-labeled products of 60 second photosynthesis by barley leaves showing the results of phosphatase action during extraction.

Fig. 5 - Left - Sprayed paper containing fifty micrograms each of sedoheptulose and sedoheptulosan. Right - Radiogram of the same paper showing positions of U_S and U_H, the designations given the radioactive sedoheptulose and sedoheptulosan respectively before their identification.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P-GLYCERIC
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FRUCTOSE-P
SEDOHEPTULOSE MONO-P
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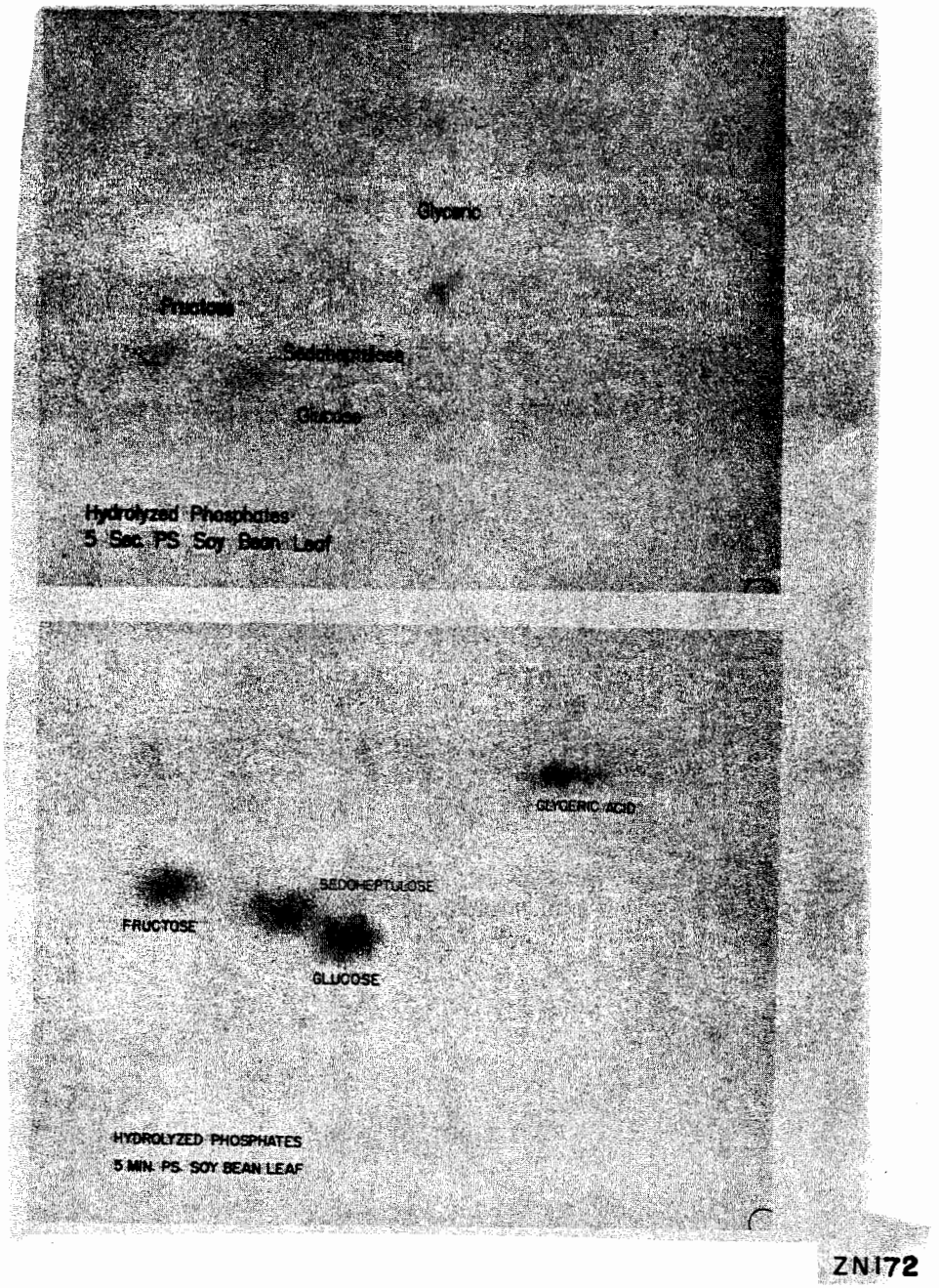
RIBULOSE DIPHOSPHATE
GLUCOSE-P
SCENEDESMUS
60 Sec. PS
ZN 170

Fig. 1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Hydrolyzed Phosphates
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GLYCERIC ACID
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ZN172

Fig. 2

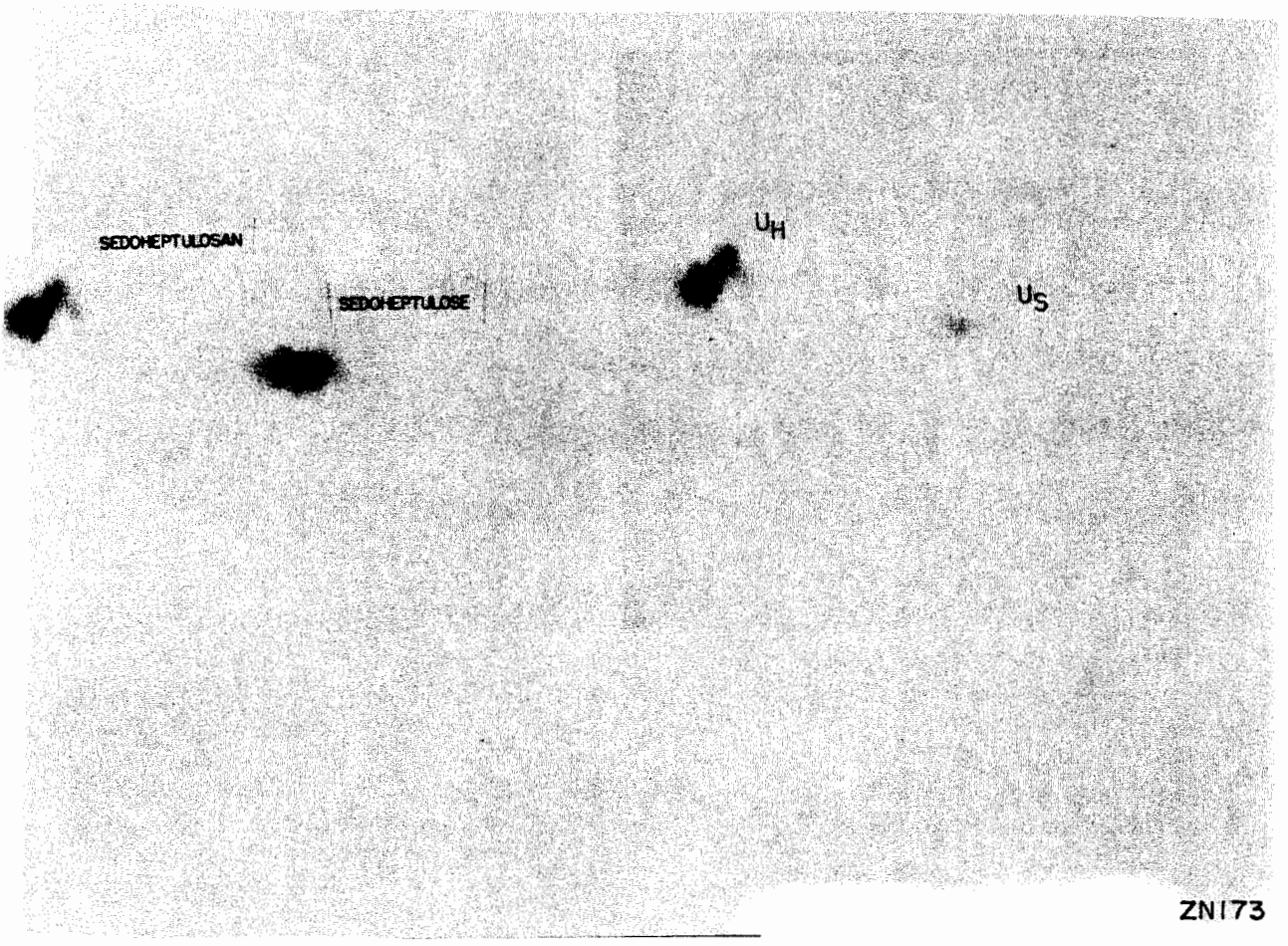
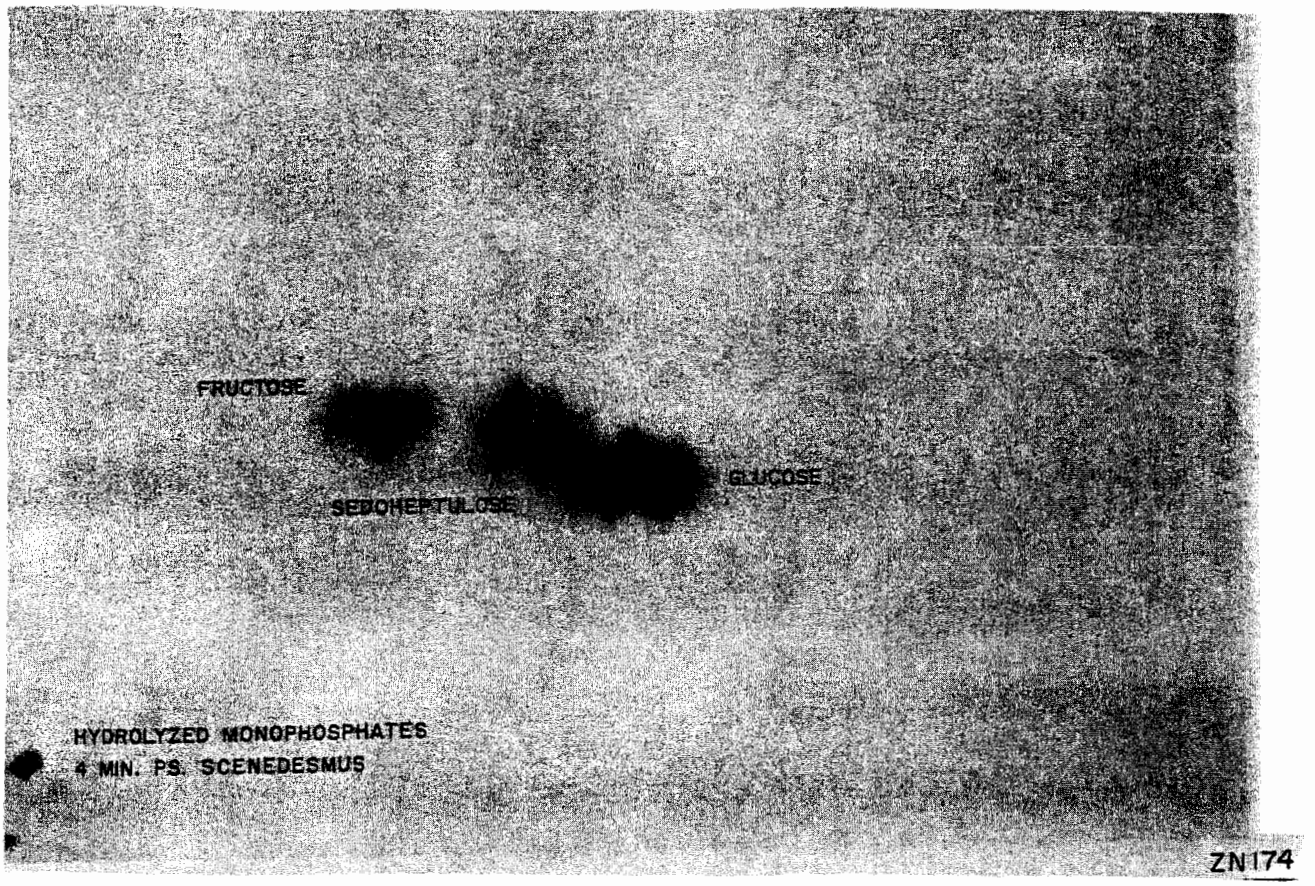


Fig. 5


FRUCTOSE
GLUCOSE
SEDOHEPTULOSE
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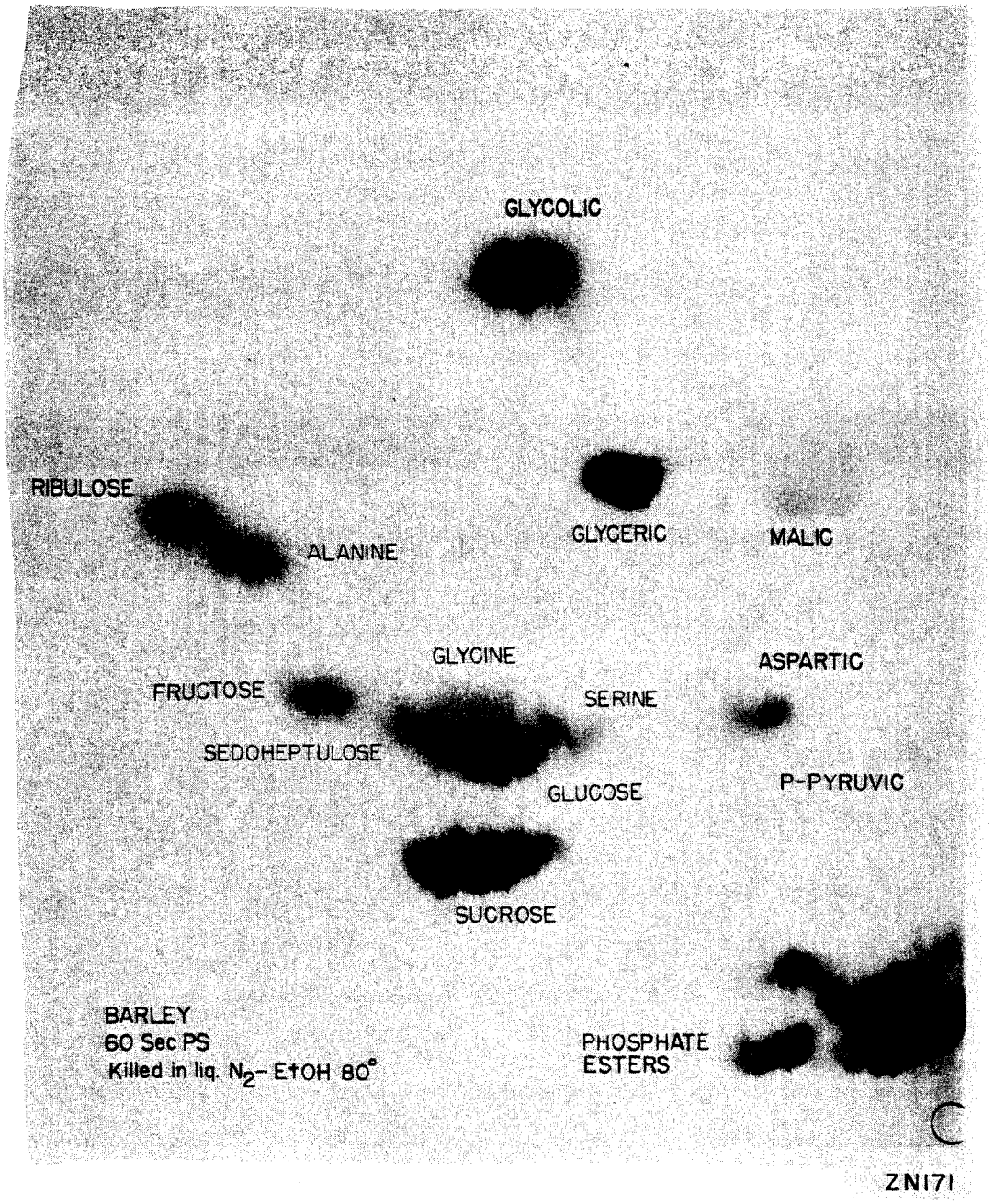
4 MIN. PS. SCENEDESMUS
ZN 174

Fig. 3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RIBULOSE
ALANINE
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P-PYRUVIC
SUCROSE
BARLEY
60 Sec PS
Killed in liq. N_2- EtOH 80°
PHOSPHATE
ESTERS
ZN171

Fig. 4