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MEASUREMENT OF PHOSPHOROUS--CARBON RATIOS IN SOME PHOTOSYNTHESIS INTERMEDIATES

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August 1951

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The majority of the early intermediates of carbon dioxide fixation by plants and microorganisms now appear to be phosphorylated derivatives of hydroxy acids and sugars. The earliest intermediate which is observed in steady state photosynthesis was identified as phosphoglyceric acid by its adsorbtion properties on ion exchange resins and isolated as the crystalline barium salt by Calvin and co-workers. Subsequent compounds in the synthetic sequence leading to sucrose were shown to have the chromatographic properties of several of the hexose phosphates involved in glycolysis.[1]

Hydrolysis of chromatographically isolated phosphate esters by phosphatase preparations and chromatography of the products indicated that two major phosphates were not derivatives of expected trioses or hexoses. The experiments reported in this paper provided part of the evidence used in the identification of these as ribulose 1,5-diphosphate and sedoheptulose monophosphate.

Since the sugars were unidentified, it was suspected that a determination of the ratio of carbon to phosphorous in their phosphates should give clues to their nature. The close relationship of the chromatographic

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

coordinates of sedoheptulose monophosphate with those of known monophosphates and of ribulose diphosphate with the known diphosphates practically established the number of phosphate groups per molecule. It should be pointed out that the method used is particularly applicable for unknown phosphate esters isolated in microgram quantities or less by two-dimensional paper chromatography.

When the photosynthetic intermediates of a plant are equilibrated with both radiophosphate and radiocarbon, the ratios of C^{14} to P^{32} in the separated phosphate esters provide evidence for their composition. Such ratios were determined in Scenedesmus and compared with that of phosphoglyceric acid from the same experiment for which the ratio is three. The ratio for any simple compound should be a simple fraction of that for phosphoglycerate. Before complete C^{14} equilibration with a reservoir of an intermediate, the ratio is a measure of the completeness of such equilibration. A summary of the results of preliminary experiments of this type described below provides information required for more precise determinations.

Experimental Part

The green alga, Scenedesmus (1 gram of cells in 100 ml. of suspension), was equilibrated with 1-2 mc. of radiophosphate during 20 hours at moderate light intensity with adequate carbon dioxide. The light intensity was raised to 2,000 foot candles for twenty minutes. The suspension was quickly centrifuged and resuspended in water with the same illumination. $C^{14}O_2$ was introduced and circulated vigorously through the the solution with a rubber tubing pump of small volume[2] for periods of five and thirty-five minutes after which the algae were rapidly killed in five volumes of boiling ethanol. The cells were filtered from the solution and re-extracted with 10% ethanol.

The total extract was concentrated and the extract of 10-25 mm.^{3} of fresh cells was chromatographed[1] on oxalic acid-washed Whatman No. 1 filter paper. The phosphate esters are readily separated into three major areas, phosphoglycerate, hexose monophosphates and the compounds with chromatographic properties of the hexose diphosphates. It was known from the C^{14} labeled hydrolysis products that the phosphoglycerate, glucose monophosphate and the diphosphate compound were quite pure while fructose monophosphate is difficultly separated from the phosphate of a sugar with chromatographic properties almost identical to those of sorbose.

The doubly labeled compounds whose positions the radiograms defined were eluted and appropriate activities were counted on 3 cm. discs. The P^{32} radioactivity was determined by superimposing a 12.5 mg./cm^2 aluminum absorber upon the sample. The absorber was calibrated with pure radiophosphate and found to absorb 3% of the P^{32} radiation and 97% of the C^{14} radiation. Hence, the P^{32} radiation could be calculated and subtracted from the total C^{14} plus P^{32} activities to give C^{14} radioactivity alone.

Since the P^{32} activity in the chromatograms was initially too high, it was allowed to decay until P^{32} and C^{14} in phosphoglycerate were approximately equal. At this time the measurements which involve differences between similar numbers are most accurate. The results given in Table I make it clear that although precision of the measurements could be improved, the results serve to demonstrate the rate of equilibration of $C^{14}O_2$ photosynthesis intermediates with the unlabeled metabolic pools as well as the nature of the unknown sugar phosphates.

Table I

Compound	Concentration in Scenedesmus	C^{14}/P^{32} Theoretical	5 minute Photosynthesis C^{14}/P^{32} Measured	5 minute Photosynthesis C^{14}/P^{32} Standardized	35 minute Photosynthesis C^{14}/P^{32} Measured	35 minute Photosynthesis C^{14}/P^{32} Standardized
Phospho-glycerate	5.7×10^{-3}M	3.0	1.60	3.0	1.35	3.0
Glucose monophos-phate	1.2×10^{-3}M	6.0	1.12	2.1	2.35 2.24	5.1
Fructose plus sedoheptulose monophosphates	*5.9×10^{-4}M	6.55	1.3	2.4	2.62 2.63	5.8
Ribulose diphosphate	$\sim 1 \times 10^{-3}$M	2.5	1.05 0.90	1.8	0.84 0.92	2.0

(*) Scenedesmus has been observed in several experiments to have 55% sedoheptulose phosphate in this particular phosphate area.

Discussion

The results of phosphoglycerate and hexoses degradations in this laboratory have shown that both approach uniformity of labeling in Scenedesmus after about five minutes of photosynthesis at high light intensity with no great differences in rates. The data for five minutes of steady state photosynthesis given in Table I, however, demonstrate that although the large phosphoglycerate reservoir rapidly approached saturation with C^{14}, this is not at all true for the hexose monophosphate reservoirs which are only about one third as saturated. It is clear from degradation data[3] that the uniformity of labeling within C_3 units extends into the C_6 reservoirs even though the latter are in rapid equilibrium with large reservoirs such as those of dextrins and starch. It is reasonable to expect that glucose phosphate radioactivity will be the most thoroughly diluted by exchange with glucose from

polysaccharide reservoirs. In a kinetic analysis of the incorporation of C^{14} into the intermediates of sucrose synthesis, there exists a competition between influx of C^{14}-labeled compounds and the dilution of this radioactivity by dilution or exchange with unlabeled reservoirs.

The results of the thirty-five minute $C^{14}O_2$ photosynthesis suggest that the glucose monophosphate reservoir was nearly equilibrated with C^{14} and that the C^{14}/P^{32} ratios probably could be used with some reliance. If one accepts the system as only an approximation to equilibration, the glucose monophosphate ratio could have been assumed 6.0 instead of choosing phosphoglycerate for a standard. Then one obtains a ratio of over seven for sedoheptulose monophosphate and 2.4 for the ribulose diphosphate.

Determination of Concentrations of Phosphorylated Metabolites in Scenedesmus.- A Scenedesmus suspension in nutrient solution (C^{14} in PS) per 100 ml. was equilibrated with 2 mc. radio phosphate during 20 hours photosynthesis with 4% CO_2 in air at 3,000 f.-c. The cells were killed in the light in boiling ethanol and thoroughly extracted with hot dilute ethanol and water. Aliquot portions of the concentrated extract were chromatographed. The specific activity of the phosphorous in the cell extract determined by total phosphorous determination and P^{32} measurement was 6.7×10^{12} cpm./gram atom. The radioactivity of the phosphoglycerate, for example, was determined by direct counting of the chromatogram of extract from 1.5 $mm.^3$ of cells and found to be 57,000 cpm. Hence, there was 8.5×10^{-9} moles of phosphoglycerate and the concentration in the original algae was 5.7×10^{-3} M. Since several P^{32}-labeled compounds have chromatographic coordinates close to those of ribulose diphosphate, its calculated concentration may be somewhat less accurate. Since the concentration of these esters is known to vary with condition of the culture, pH light intensity, etc., these results may only be considered as typical.

Summary

The ratios of carbon to phosphorous in several phosphatylated intermediates of photosynthetic carbon dioxide fixation have been determined by measuring carbon and phosphorous radioactivity when they are saturated with C^{14} and P^{32}. The data obtained in these preliminary experiments with Scenedesmus serve as evidence for the identifications for sedoheptulose monophosphate and ribulose diphosphate and provide information regarding the degree of saturation with C^{14} for a number of phosphorylated compounds. The concentration of these compounds has been measured.

The methods used are particularly applicable for phosphate esters isolble by paper chromatography.

References

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