The Path of Carbon in Photosynthesis IV: The Identity and Sequence of the Intermediates in Sucrose Synthesis[1]

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THE IDEAL DESIGN OF AN EXPERIMENT to determine the chemical path of carbon from carbon dioxide to the variety of plant constituents is relatively simple and straightforward. It would consist of feeding a photosynthesizing organism radioactive carbon dioxide for various lengths of time and stopping the reaction by killing the plant. By determining those compounds into which the radioactive carbon has been incorporated for each period of illumination and, further, by determining the distribution of radioactivity within each compound, these data could then be used to construct a family of curves depicting the increase in radioactivity in each compound (and in each carbon atom of each compound) as a function of time. From a complete set of such curves it should be possible to draw a map of the path of carbon as it flows into the plant in the form of carbon dioxide and distributes itself among all the plant constituents.

A few such experiments have already been reported (*1–4, 6*). The present paper reports some further experiments toward this end with specific reference to the synthesis of sucrose.

The data are in the form of radioautographs of paper chromatograms made from the extracts of algae which have been photosynthesizing for several different periods of time, as well as one showing the dark fixation after a preliminary period of illumination in the absence of carbon dioxide.

Exposure of algae to $C^{14}O_2$: One-day-old *Chlorella pyrenoidosa* cells were grown under continuous culture conditions (*3*) and harvested immediately before use. A suspension of 1 cc of packed cells in 70 ml of water containing fumarate buffer (3.5 mg fumaric acid plus .032 meq sodium hydroxide) was allowed to photosynthesize for 30 minutes with 4% carbon dioxide in air. This gas mixture was then displaced by rapid flushing with air during 5 minutes. A solution of 40 μc of $NaHC^{14}O_3$ (.0143 mmol) in 0.20 ml was rapidly injected into the suspension. The vessel was shaken vigorously in the light beams (2 × 17,000 lux) until the algae were killed by opening an 8-mm stopcock and allowing the solution to flow into a beaker containing 500 ml of boiling absolute ethanol. The alcohol suspension was filtered with celite and evaporated at room temperature to a volume of 2 cc for convenient application on the filter paper sheet.

Preparation of chromatograms: Fumarate buffer in distilled water was chosen for this work, since inorganic salts, especially phosphates, interfere with movement of compounds on the paper. Alcohol extract of as much as 100 mm^3 of algae may be applied to the filter paper (Whatman No. 1). Development in water-saturated phenol was followed by thorough drying at room temperature. The second solvent was freshly prepared before use from equal volumes of the following solutions:

(A) 1,246 cc *n*-butanol—84 cc water

(B) 620 cc propionic acid—790 cc water[2]

In order to choose a suitable exposure time for the X-ray film (Eastman No-Screen, 14″ × 17″) the activity of the original spot is determined on the paper. With the number of compounds appearing in a 90-second photosynthesis, an activity of 30,000 cpm is sufficient to expose the film in 48 hours.

Although the radioactive fixation products which have been separated in the chromatogram may be eluted and their activity determined accurately, the radiogram serves as a semiquantitative record of the activity fixed in each compound. The relative amounts of each active product may be compared visually in the radioautograph.[3]

An examination of the radiograms reveals that in the very short photosynthetic experiments (30 seconds and 90 seconds) by far the major portion of the newly reduced carbon dioxide is found in the phospho-

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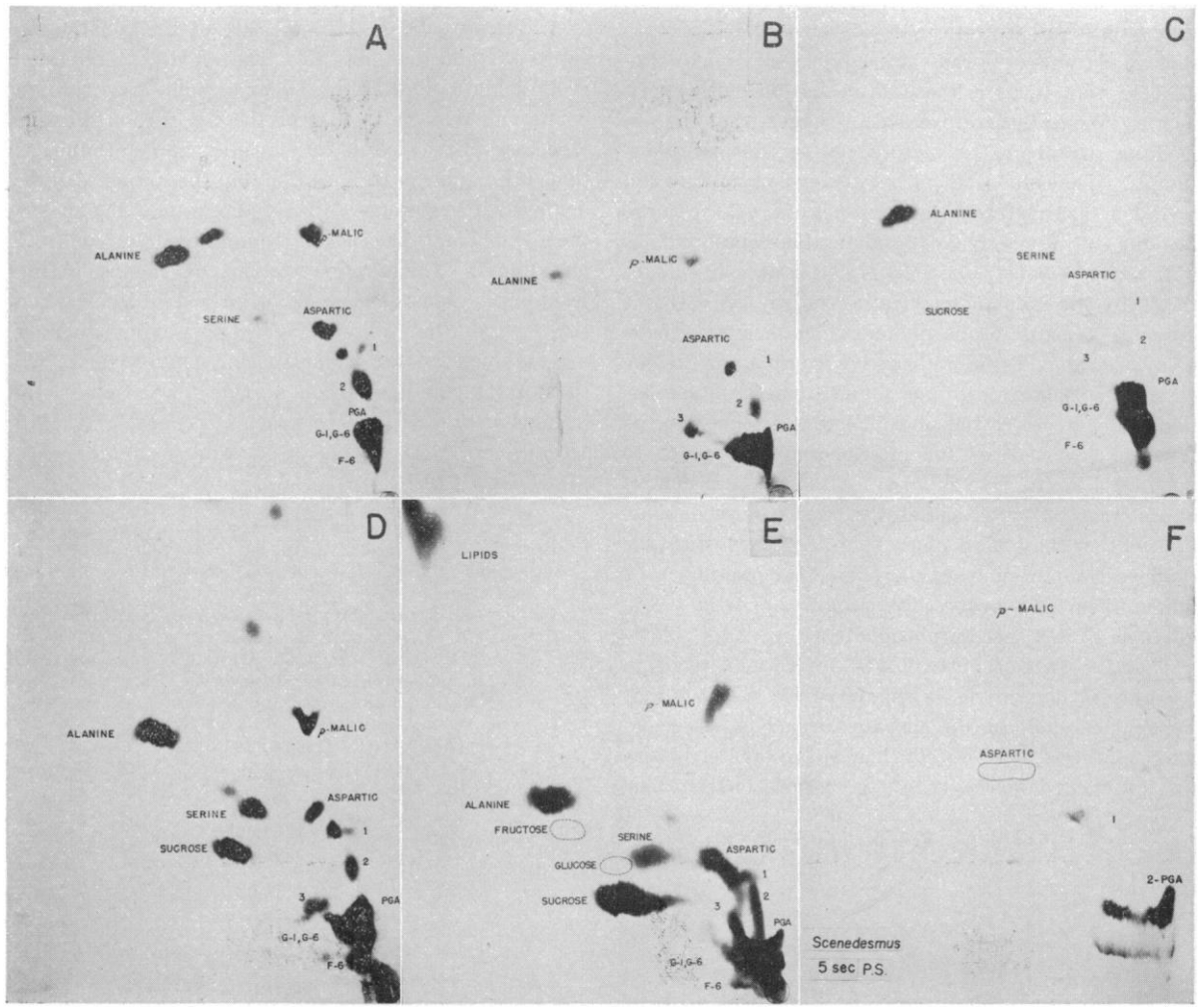
[2] The solvent is adjusted to separate into two phases if cooled two degrees below the temperature at which it is used.

[3] The details of the methods of identification of the spots will be published elsewhere. ("The Path of Carbon in Photosynthesis. V. Paper Chromatography and Radioautography of the Products" by A. A. Benson, J. A. Bassham, M. Calvin, V. A. Haas, and W. Stepka.)

glyceric acids, triose phosphates and the hexose phosphates. This may be taken as additional confirmatory evidence of our previously proposed (*4*) scheme by which the six-carbon hexose skeleton is synthesized through the usual glycolytic intermediates. The details of the path by which the phosphoglyceric acid is formed and the relative rates of the several reactions involved in its conversion to hexose phosphate will be treated in subsequent publications.

What we would like to point out here is the fact that the first free carbohydrate which appears in these plants is sucrose. The positions taken on the chromatogram by free glucose and free fructose are known and they do not contain radioactivity. The nonappearance of radioactivity in a given compound does not necessarily preclude the possibility of its playing a part as an intermediate in a given sequence. For example, the reservoir of this compound in the sequence



C^{14} RADIOGRAMS OF 80% ETHANOL EXTRACTS OF ALGAE[1,2]

A: 15-sec dark fixation by *Chlorella* which had been preilluminated for 15 min in helium. **B**: 5-sec photosynthetic fixation by *Chlorella*. **C**: 30-sec photosynthetic fixation by *Chlorella*. **D**: 90-sec photosynthetic fixation by *Chlorella* (10% of the activity fixed is insoluble in 80% ethanol). **E**: 5-min photosynthetic fixation by *Chlorella*[3] (60% of the activity fixed is insoluble in 80% ethanol). **F**: 5-sec photosynthetic fixation by *Scenedesmus*.[4]

[1] The term "radiogram" is used here to denote the radioautograph of a two-dimensional paper chromatogram.

[2] The abbreviations used in labeling the radiograms indicate the following compounds: PGA, phosphoglyceric acid; G-1, glucose-1-phosphate; G-6, glucose-6-phosphate; F-6, fructose-6-phosphate; p-MALIC, malic acid (position dependent on pH).

[3] Dotted circles indicate the positions of fructose and glucose which are not radioactive.

[4] Aspartic acid, encircled in the radiogram, was identified by ninhydrin spraying and has a small amount of radioactivity.

may be extremely small, or the compound may never exist as a free compound in solution but rather only as an enzyme-substrate complex, so that the amount of radioactivity present in that particular compound may be so small as to be missed. Conversely, the appearance of radioactivity in a particular compound does not necessarily prove its part as an intermediate in a direct sequence. It can be, and often is, the result of a side reaction.

It does not seem likely that, if free glucose or free fructose were intermediates in the synthesis of sucrose, they would fail to appear radioactive either prior to the appearance of radioactive sucrose or simultaneously with it, as is the case in the present experiments. We are, therefore, led to suggest that the immediate precursors to sucrose are two hexose phosphates. That one of them is glucose-1-phosphate can be taken as relatively certain in view of the large amount of radioactivity found in this compound, as well as its demonstrated participation in sucrose synthesis by an isolated enzymatic system (*5*). If the other is fructose-6-phosphate, which has also been identified among the radioactive compounds in the early chromatograms, one might expect a sucrose phosphate in which the phosphorus is attached to the fructose fragment as the intermediate just prior to the formation of free sucrose. Although it is not required that this intermediate be found, since dephosphorylation may take place simultaneously with the condensation to sucrose, there are still a number of unidentified spots in the chromatograms, one of which might well be a sucrose phosphate.

That the fructose phosphates are formed prior to the glucose derivatives is suggested by the fact that the fructose half of the sucrose formed in 30-second photosynthesis by *Chlorella* has approximately twice the specific radioactivity of the glucose half. This was determined by cutting out the sucrose spot from a chromatogram of the total extract, eluting it from the paper, hydrolyzing for 10 minutes in 0.1 N HCl at 80° C and rechromatographing the hydrolyzate after cold evaporation to dryness to remove the HCl. The sucrose formed in 90-second photosynthesis by the same organism is made up of glucose and fructose of equal specific activities (within 5%). This result requires that the functioning reservoirs of precursor hexose phosphates be so small as to achieve equal specific activities in 90 seconds.

Additional evidence for the size of the functioning reservoirs and the speed of turnover may be obtained from a knowledge of the molar specific activity of a compound relative to that of the fed carbon dioxide. One case in which this may readily be determined is that of alanine. Its position on the paper can be defined with reference to the radiogram. The spot is then eluted and the activity determined by counting an aliquot. The alanine content of the remaining solution is then determined colorimetrically. When this is done, the following very approximate values are obtained for the molar specific activity related to that of the starting carbon dioxide for various times of photosynthesis by *Chlorella*: 5 seconds, ~ .04; 15 seconds, ~ .1; 90 seconds, ~ .9; and 5 minutes, ~ 3.

References

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