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THE PATH OF CARBON IN PHOTOSYNTHESIS

IV. THE IDENTITY AND SEQUENCE OF THE INTERMEDIATES IN SUCROSE SYNTHESIS

By

M. Calvin and A. A. Benson

December 14, 1948

Berkeley, California

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University of California, Berkeley, California

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ABSTRACT

The synthesis of sucrose from $C^{14}O_2$ by green algae has been investigated and the intermediates separated by the method of paper chromatography. It is shown that sucrose is the first free sugar appearing during photosynthesis. It is apparently formed by condensation of the glucose-1-phosphate and a fructose phosphate. A series of radioautographs of paper chromatograms of extracts from plants which have photosynthesized for different periods of time has been prepared. The results indicate that 2-phosphoglyceric acid is the first product synthesized from CO_2 during photosynthesis.

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* This paper is based on work performed under Contract No. W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, California.

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INTRODUCTION

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 would still be a relatively complicated matter to draw a map of the path of carbon as it flows into the plant in the form of earbon and distributes itself among all the plant constituents.

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A few such experiments have already been reported. (1)(2)(3)(4)(5) 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 (5) 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 mmole) in 0.20 ml. was rapidly injected into the suspension. The vessel was shaken vigorously in the light beams (2 x 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.

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(1) A. A. Benson and M. Calvin, Science, 105, 648 (1947).
(2) M. Calvin and A. A. Benson, Science, 107, 476 (1948).
(3) W. Stepka, M. Calvin and A. A. Benson, Science, 108, 304 (1948).
(4) A. A. Benson and M. Calvin, Proceedings of Cold Spring Harbor Symposia in Quantitative Biology, 1948 - in press.
(5) A. A. Benson, M. Calvin, V. A. Haas, S. Aronoff, A. G. Hall, J. A. Bassham and J. W. Weigl, AAAS Monograph on Photosynthesis, in press.

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 extracts 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. 1246 cc. n-butanol - 84 cc. water. B. 620 cc. propionic acid - 790 cc. water. In order to choose a suitable exposure time for the X-ray film (Eastman No-Screen, 14 x 17 in.) 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 radioautograph 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. (6)

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(6) 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.

DISCUSSION

An examination of the chromatograms 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 phosphoglyceric acids, triose phosphates and the hexose phosphates. This may be taken as additional confirmatory evidence of our previously proposed (2) scheme by which the six-carbon hexose sksleton 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 non-appearance 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 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 trapped 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 that 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 the demonstration of its participation in sucrose synthesis by an isolated enzymatic system. (7) 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 chromatogram, one of which might well be sucrose phosphate.

It is of interest to note that the radioactive sucrose formed in 90-second photosynthesis by Chlorella is made up of glucose and fructose of equal specific activities (within 5%). This was determined by cutting out the sucrose spot from a chromatogram of the total extract, eluting it from the paper, hydrolysing for 10 minutes

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(7) W. Z. Hassid, M. Doudoroff and H. A. Barker, J. Am. Chem. Soc., 66, 1416 (1944).

in 1 N HCl at 80° C and rechromatograming the hydrolysate after evaporation to dryness to remove the HCl. The resulting glucose and fructose spots were of equal radioactivity. This result requires that the functioning reservoirs of precursor hexose phosphates be so small as to achieve equal specific activity in the time allowed or that the sucrose be formed from a single hexose phosphate.

Some 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 by refernnce 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.

CAPTIONS FOR THE FIGURES

C^{14} Radiograms of 80% Ethanol Extracts of *Chlorella pyrenoidosa* [1,2]

Fig. 1. 15 seconds in Dark Fixation by *Chlorella* which had been Preilluminated for 15 minutes in Helium.

Fig. 2. 5 seconds Photosynthetic Fixation by *Chlorella*.

Fig. 3. 30 seconds Photosynthetic Fixation by *Chlorella*.

Fig. 4. 90 second Photosynthetic Fixation by *Chlorella*.*

Fig. 5. 5 minute Photosynthetic Fixation by *Chlorella*.**

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Fig. 6. 5 seconds Photosynthetic Fixation by *Scenedesmus*.***

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[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: FDP, fructose-1,6-diphosphate; PGA, phosphoglyceric acid; G-1, glucose-1-phosphate; G-6, glucose-6-phosphate; F-6, fructose-6-phosphate.

* Ten percent of the activity fixed is insoluble in 80% ethanol.

** Sixty percent of the activity fixed is insoluble in 80% ethanol.

*** This chromatogram was run twice in both solvents to increase the separation of the two major compounds. Such treatment produces a distorted chromatogram with increased separation near the origin.

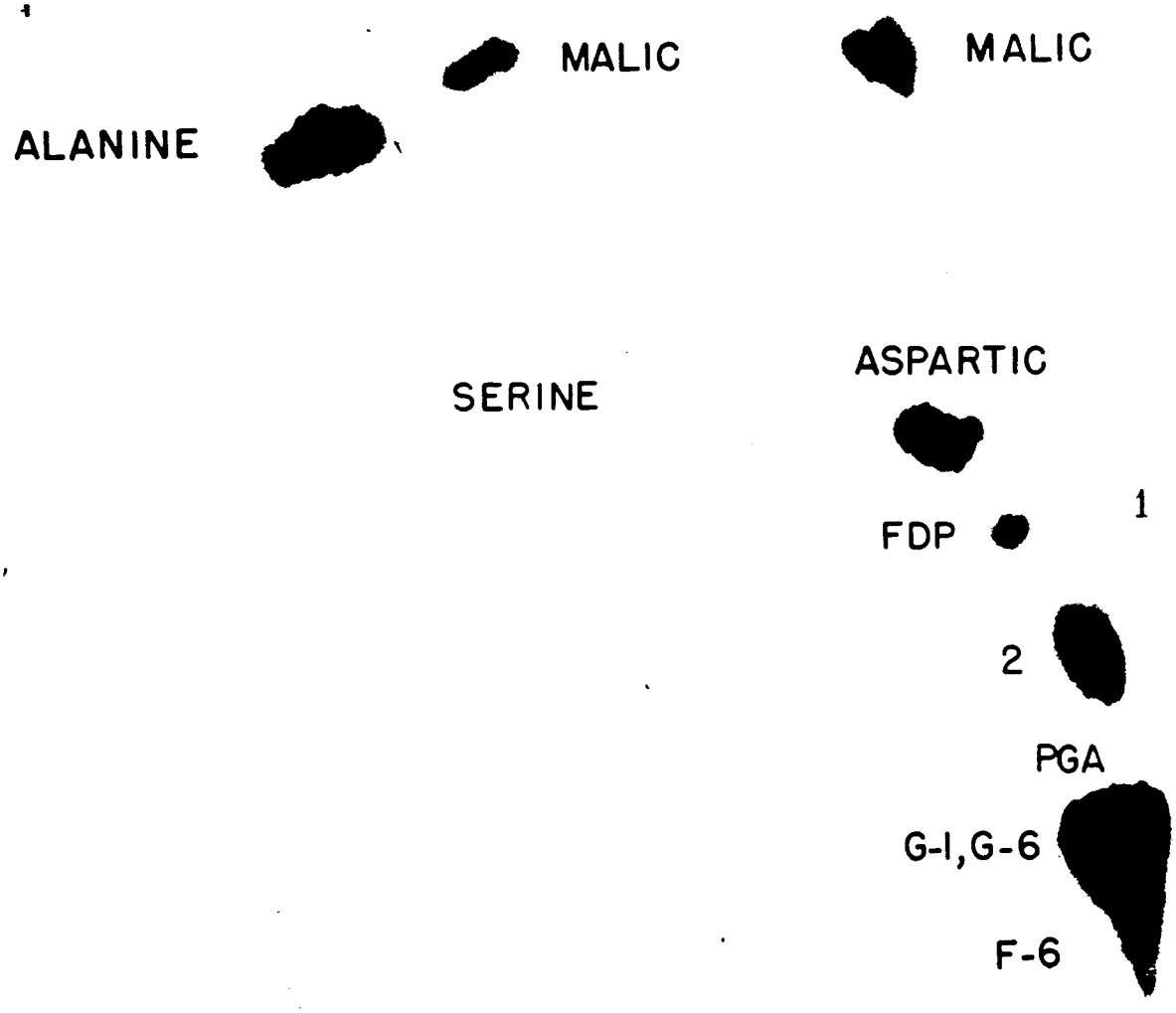


FIG 1

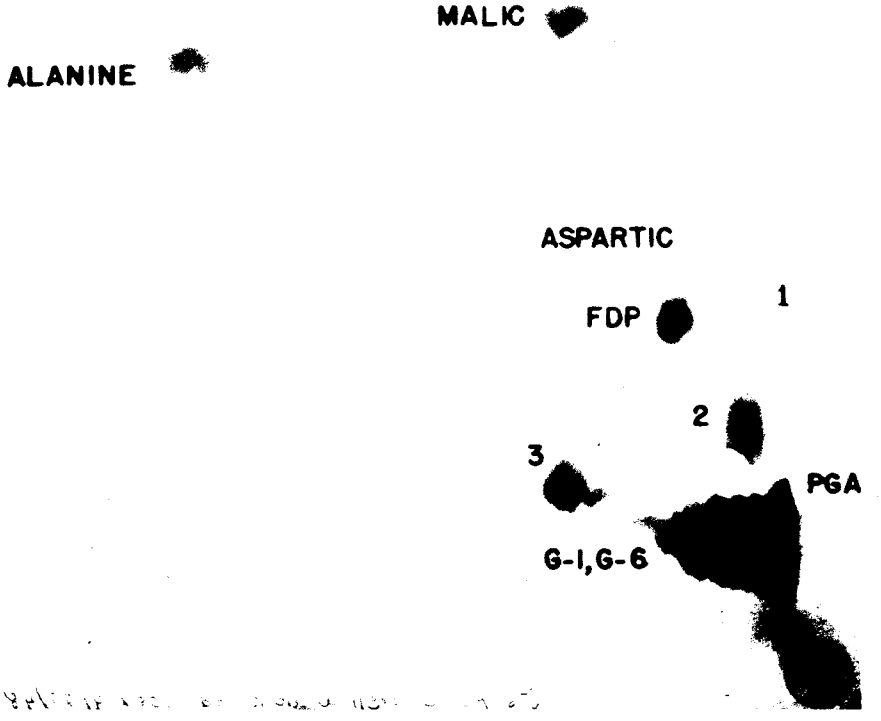


FIG. 2

ALANINE

SERINE

ASPARTIC

FDP 1

SUCROSE

2

3

PGA

G-1,G-6

F-6

FIG 3

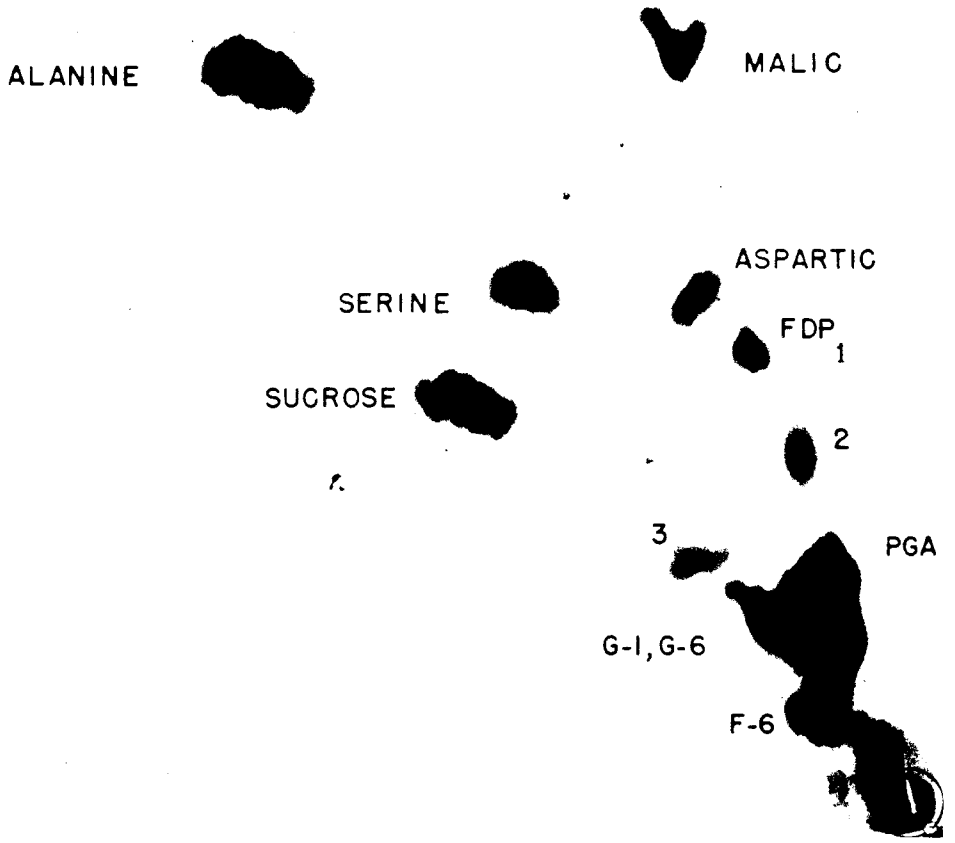


FIG. 4

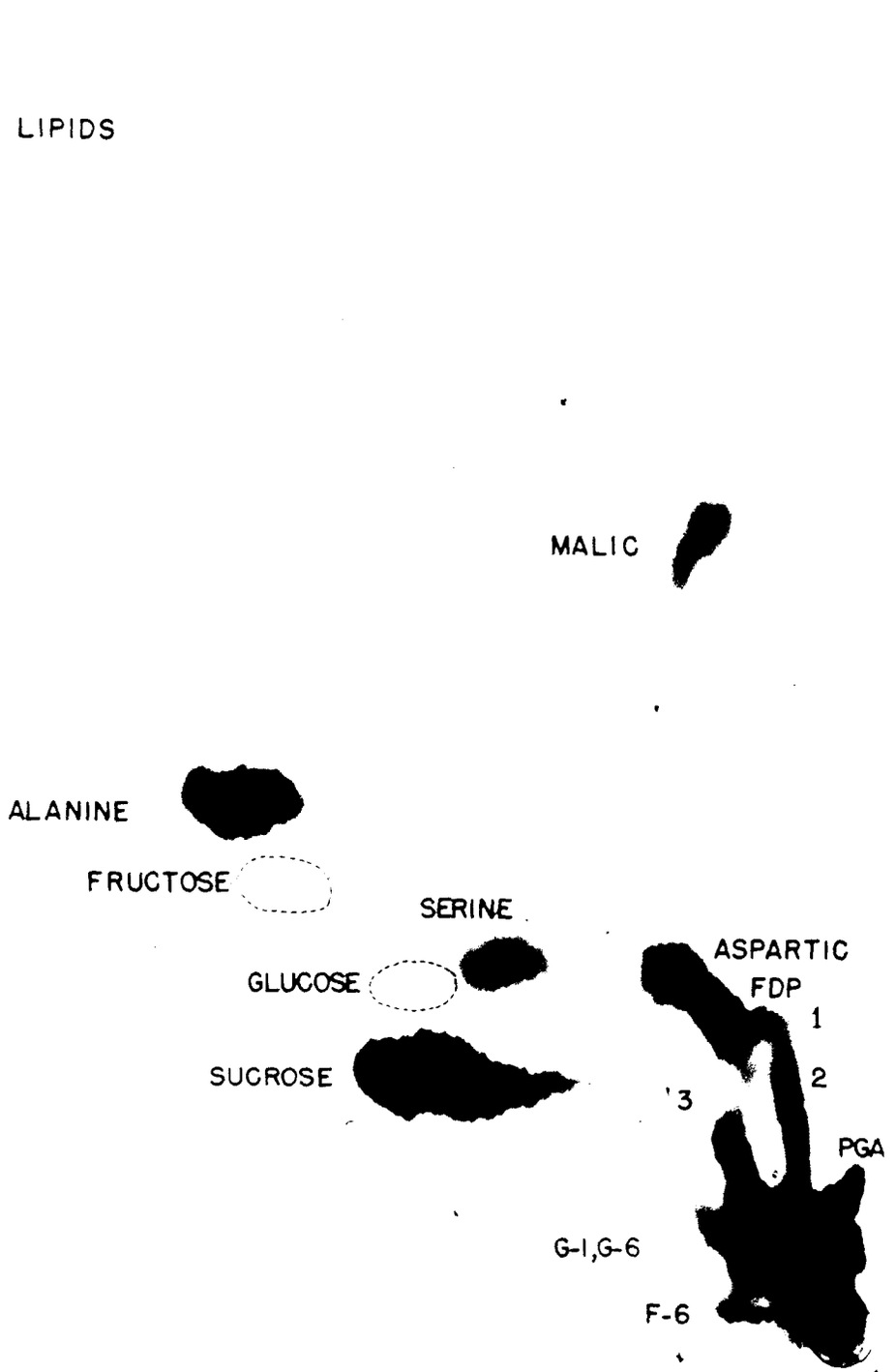
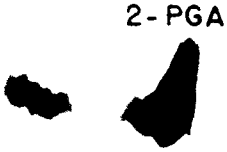

LIPIDS
MALIC
ALANINE
FRUCTOSE
SERINE
GLUCOSE
SUCROSE
ASPARTIC
FDP
1
2
3
PGA
G-1,G-6
F-6

FIG. 5

MALIC

ASPARTIC

2-PGA



Scenedesmus

5 sec P.S.

FIG 6