SOME PRODUCTS OF $C^{14}O_2$ FIXATION BY *HYDROGENOMONAS FACILIS*[1, 2, 3]

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Until recently almost nothing has been known of pathways of CO_2 assimilation in chemoautotrophs. It is now clear, however, that the major pathway of fixation of CO_2 in *Thiobacillus denitrificans* is identical with or similar to the pathway proposed by Calvin and co-workers and summarized by Calvin (1955). Trudinger (1956) demonstrated several of the enzymes of the photosynthetic cycle in cell-free extracts of *T. denitrificans*, and the operation of the cycle during autotrophic fixation of CO_2 seems likely on the basis of findings by Aubert *et al.* (1957). These workers found the first stable intermediate to be 3-phosphoglyceric acid, and observed the expected labeling pattern in this acid as well as fructose-6-phosphate, sedoheptulose phosphate, and ribulose diphosphate. A similar pathway seems likely in *Thiobacillus thioparus* since cell-free extracts of this organism catalyze the carboxylation of ribulose diphosphate with the formation of phosphoglyceric acid (Santer and Vishniac, 1955). In 1957 Suzuki and Werkman reported that phosphoglyceric acid is also the major early labeled product of autotrophic $C^{14}O_2$ assimilation by *Thiobacillus thiooxidans* as is the case for photoreductive fixation of $C^{14}O_2$ by *Rhodopseudomonas capsulatus* (Stoppani *et al.*, 1955).

Orgel *et al.* (1956) have reported early labeling of formic and acetic acids when the facultative hydrogen autotroph *Hydrogenomonas facilis* was exposed to $C^{14}O_2$ under autotrophic conditions. However, the amount of label in these compounds was not related to the extent of over-all fixation, and no heterotrophic controls (with exposure of cells to $C^{14}O_2$ in the absence of H_2 plus O_2) were reported. Work by Bergmann *et al.* (1958) has indicated that phosphoglyceric acid and a hexose phosphate fraction are the main products of short-term $C^{14}O_2$ fixation by *H. facilis*, but these compounds were not shown to be involved specifically in autotrophic fixation. This paper demonstrates the labeling of fructose-6-phosphate, sedoheptulose-7-phosphate, and ribulose diphosphate as well as phosphoglyceric acid during autotrophic $C^{14}O_2$ fixation by *H. facilis* and reports other products labeled under both autotrophic and heterotrophic conditions.

MATERIALS AND METHODS

Cells were ordinarily cultured autotrophically on the solid medium described previously (Atkinson and McFadden, 1954) but in some cases, which are specified, were grown on the inorganic medium but with agar omitted. In the latter cases the medium and cells were circulated by a pump so that they flowed downward over a column of glass beads through which a gas mixture containing 90 per cent H_2, 5 per cent O_2, and 5 per cent CO_2 was blown upward. This method of culture was developed by Mr. Rimmon C. Fay of the Department of Chemistry, University of California, Los Angeles.

Cells were ordinarily harvested after 40 to 45 hr growth, washed rigorously, and suspended in 0.01 M phosphate buffer, pH 7.0, containing 2 mg of NH_4Cl (McFadden and Atkinson, 1957) and 1 to 1.5 μmoles of $KHCO_3$ per ml. Ten-ml amounts containing 10 to 11 mg (dry weight) of cells were incubated for 20 min under a gas mixture of 0.76 atm of H_2 (or N_2 in the case of heterotrophic controls) and 0.04 atm of air. A solution containing 6 to 13 μmoles of $KHC^{14}O_3$ (50 to 125 μc) at pH 7.0 was then rapidly introduced into

[1] Most of the material of this paper was reported before the Biological Chemistry Division of the American Chemical Society, San Francisco, California, April 1958.

[2] Early portions of the work reported in this paper were performed at the Department of Chemistry, University of California at Los Angeles while the author was a Public Health Service Research Fellow of the National Heart Institute and were supported in part by a grant from the National Science Foundation administered by Dr. D. E. Atkinson.

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the reaction vessel. After the desired period of exposure to $C^{14}O_2$, cells were killed by rapid transfer to boiling 80 per cent ethanol. A $\frac{1}{20}$ portion of the ethanol extract was chromatographed in 2 directions, and labeled compounds were located by radioautography on Eastman Kodak No-Screen X-ray film. It was necessary to cochromatograph the extracts with authentic samples of compounds suspected of containing label, since in most cases the small amounts of material made chemical detection of labeled compounds impossible. In the identification of phosphorylated intermediates, *n*-butanol-propionic acid-water (45:23:32 by volume) was allowed to flow off the end of the paper for 6 hr in the narrow dimension and *t*-butanol-water-picric acid (80 ml:20 ml:4 g) was then used in the long dimension. Organic phosphates were in general located as yellow spots on a mauve-yellow background by the method of Wade and Morgan (1953). Sedoheptulose-7-phosphate was also located by the method of Williams and Bevenue (1953) and fructose-6-phosphate by the method of Partridge (1948). In the identification of amino acid intermediates, the *n*-butanol solvent (see above) was used in the short dimension and phenol saturated with water in the long dimension. Amino acids were located by spraying with ninhydrin. In all cases Whatman no. 1 paper, which had been previously washed with 0.1 M HCl, followed by 0.1 per cent ethylenediaminetetraacetate (Versene), and distilled water, was used.

The amino acids used were C.P. products of the H. M. Chemical Company, Ltd. Phosphoglyceric acid was a product of Nutritional Biochemical Corporation and fructose-6-phosphate was a product of Schwarz Laboratories, Inc. Thanks are due to Drs. B. L. Horecker and W. A. Wood for supplying samples of sedoheptulose-7-phosphate and ribulose diphosphate for use as chromatographic standards. Reagent grade 2,4-dinitrophenol (mp 111 to 113 C) was obtained from Eastman Organic Chemicals Department.

RESULTS AND DISCUSSION

Figures 1*a* and 1*b* are photographs of typical radioautograms obtained from paper chromatograms. Phosphoglyceric acid, fructose-6-phosphate, sedoheptulose-7-phosphate, and ribulose diphosphate are labeled after 45-sec fixation under autotrophic conditions (figure 1*a*). The same compounds are labeled to a lesser extent during heterotrophic fixation (figure 1*b*). These results emphasize the importance of attempts to estimate heterotrophic fixation which may occur simultaneously with autotrophic fixation for a facultative autotroph such as *H. facilis*. The quantitative importance of heterotrophic fixation (i. e., reduction of CO_2 which presumably derives energy and electrons from endogenous oxidations) during autotrophic assimilation cannot be approximated on the basis of these results alone, however, because the presence of H_2 may obviously alter the kinetics of heterotrophic fixation.

Figure 2 demonstrates that autotrophic assimilation of $C^{14}O_2$ for 45 sec in the presence of 2×10^{-4} M 2,4-dinitrophenol, a concentration not affecting the exergonic oxidation of H_2 by O_2 (McFadden and Atkinson, 1957) results in labeled phosphoglyceric acid. Fructose-6-phosphate, sedoheptulose-7-phosphate, and ribulose diphosphate, are not detectably labeled. 2,4-Dinitrophenol completely inhibits heterotrophic fixation. The photosynthetic cycle proposed by Calvin (1955) involves the participation of adenosine triphosphate in the reductive conversion of phosphoglyceric acid to 3-phosphoglyceraldehyde. Thus the labeling patterns obtained in the presence and absence of 2,4-dinitrophenol are consistent with the expected similarity between autotrophic CO_2 assimilation by *H. facilis* and that for photosynthetic species.

After 25-sec assimilation under autotrophic conditions the same intermediates are labeled although ribulose diphosphate is ostensibly labeled to a lesser degree (figure 3*a*). Heterotrophic assimilation for 25 seconds results in diminished over-all fixation and the labeling pattern is qualitatively similar to that for autotrophic fixation although only phosphoglyceric acid was identified as a labeled product (figure 3*b*).

On the basis of these findings it seems likely that *H. facilis* assimilates CO_2 autotrophically by a pathway similar to that which operates in photosynthesis. The volatile labeled products reported by Orgel *et al.* (1956) would have been lost by the methods employed here so that nothing can be said of their relationship to the products herein reported.

Alanine, aspartic acid, glutamic acid, glycine, serine, and threonine are labeled after 1-min exposure of cells to $C^{14}O_2$ autotrophic conditions (figure 4*a*). In this case, 6 mg (dry weight) of cells which had been cultured in a liquid medium

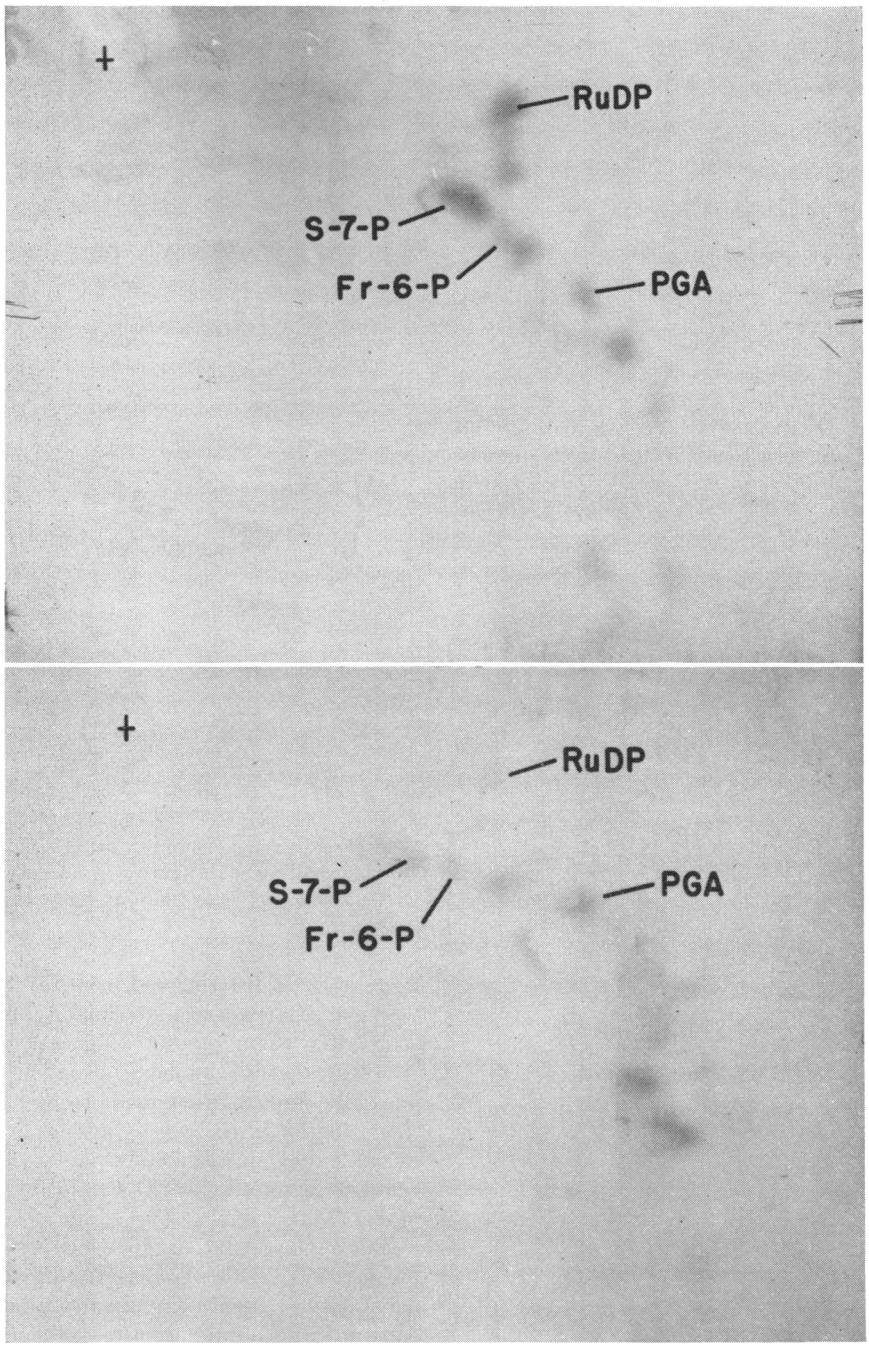


Figure 1a (*top*). Forty-five sec autotrophic fixation. S-7-P, sedoheptulose-7-phosphate; Fr-6-P, fructose-6-phosphate; RuDP, ribulose diphosphate; PGA, phosphoglyceric acid.

Figure 1b (*bottom*). Forty-five sec heterotrophic fixation.

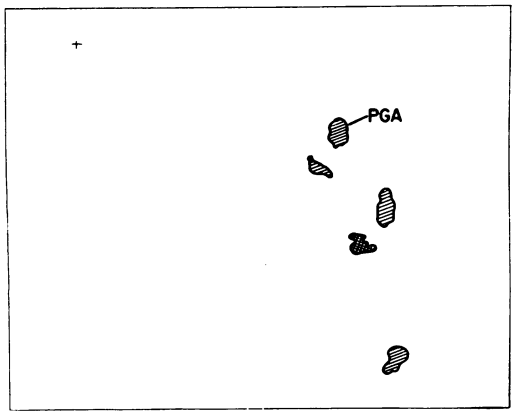
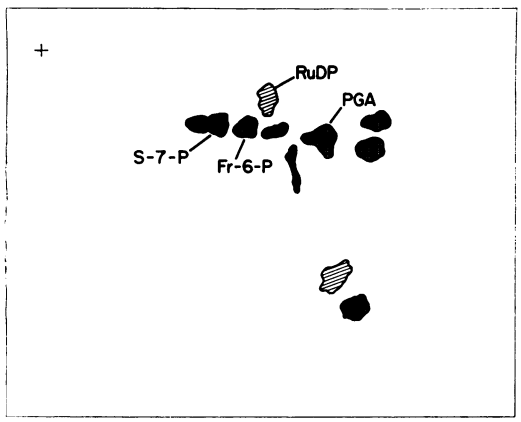


Figure 2. Tracing of the radioautogram representing 45-sec autotrophic fixation in the presence of 2,4-dinitrophenol. ▨ = heavily labeled areas; ▩ = moderately labeled; △ = slightly labeled.



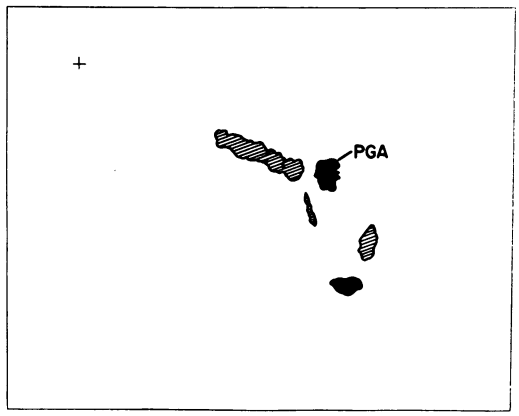
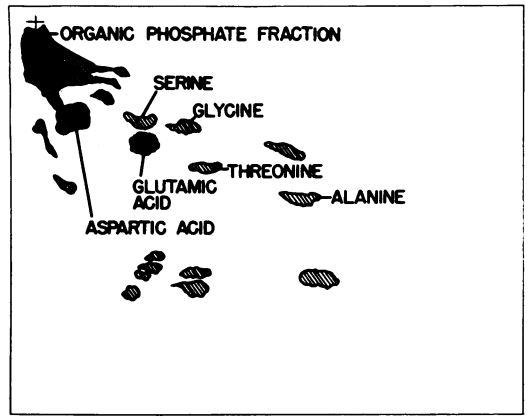


Figure 3a (*top*). Twenty-five sec autotrophic fixation. Key to abbreviations and shading as in figures 1*a* and 2.

Figure 3b (*bottom*). Twenty-five sec autotrophic fixation.



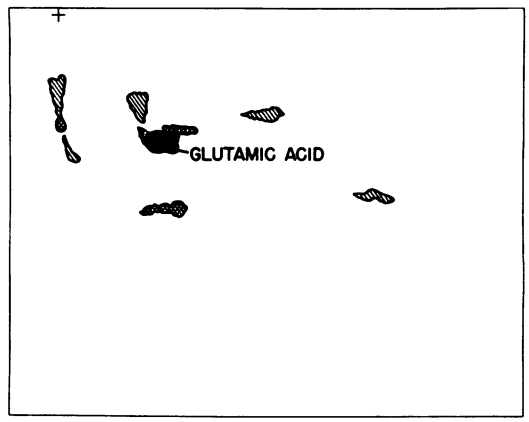


Figure 4a (*top*). One-min autotrophic fixation. Key to shading as in figure 2.

Figure 4b (*bottom*). One-min heterotrophic fixation.

were used. In heterotrophic controls for this experiment the extensive labeling of glutamic acid acid after 1-min exposure (figure 4*b*) suggests, however, that this amino acid may arise at least partially through reactions not dependent upon the oxidation of H_2 by O_2. Glutamic acid is a detectable but quantitatively minor product of short-term autotrophic CO_2 fixation by *T. denitrificans* (Aubert *et al.*, 1957).

Future investigations in which attempts are made to analyze those radioactive products which have not yet been identified and to localize the label in various intermediates of the autotrophic process in *H. facilis* should clarify further the relationships between chemoautotrophic and photoautotrophic CO_2 assimilation.

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SUMMARY

Phosphoglyceric acid, fructose-6-phosphate, sedoheptulose-7-phosphate, and ribulose diphosphate are products of $C^{14}O_2$ assimilation by *Hydrogenomonas facilis* for 45 sec and 25 sec under autotrophic conditions. Under heterotrophic conditions, qualitatively similar labeling patterns are observed but with intermediates labeled to a lesser extent. 2,4-Dinitrophenol (2×10^{-4} M) apparently blocks the formation of these sugar phosphates from phosphoglyceric acid during 45-sec fixation under autotrophic conditions and completely inhibits heterotrophic fixation for this length of exposure. Alanine, aspartic acid, glutamic acid, glycine, serine, and threonine are products of 1-min fixation under autotrophic conditions, whereas glutamic acid is a major product of 1-min heterotrophic fixation.

These findings are discussed in relationship to the pathway of carbon for photosynthetic species.

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