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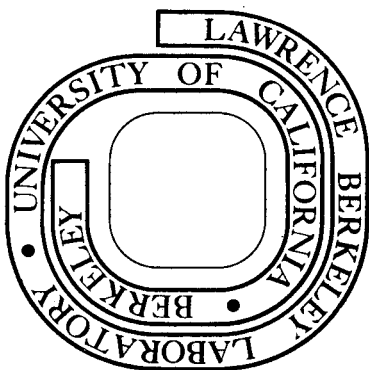
SEPARATION OF ^{14}C -LABELED GLYCOLATE PATHWAY
METABOLITES FROM HIGHER PLANT PHOTOSYNTHATE

Steven G. Platt and James A. Bassham

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SEPARATION OF ^{14}C -LABELED GLYCOLATE PATHWAY METABOLITES
FROM HIGHER PLANT PHOTOSYNTHATE

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In view of the proposed significance of the glycolate pathway in photorespiration in green plants,^{1,2} it is important to analyze the photosynthetic incorporation of tracer carbon into intermediates of that pathway, including glycolate, glycine and serine. We recently developed a method for conducting kinetic studies of ^{14}C flow through many metabolic pathways in whole green leaves during photosynthesis with $^{14}\text{CO}_2$ under controlled conditions³. Our method includes analysis for ^{14}C in a large number of metabolites which are separated by two-dimensional paper chromatography. When we observed that our usual solvents^{3,4,5} were inadequate for isolating and separating glycolate, glycine, and serine from our leaf extracts, we developed alternative paper chromatography systems for the quantitative isolation of those materials. These systems obviate the need for additional procedures such as liquid column chromatography, gas chromatography, and electrophoresis, which have been used for the isolation of glycolate⁶ and glycine and serine⁷⁻⁹ from higher plant photosynthate.

EXPERIMENTAL

Preparation of ^{14}C -Labeled Higher Plant Leaf Extracts

Alfalfa leaflets (Medicago sativa L. var. El Unico) were selected from plants grown as described previously³. Each sample consisted of four leaflets. The leaflets were exposed to $^{14}\text{CO}_2$ in the light essentially as described previously³. Photosynthetic assimilation of $^{14}\text{CO}_2$ (0.026 - 0.20%) in air (21% oxygen) occurred at 3600 ft-c and 16°. The specific radioactivity of the $^{14}\text{CO}_2$ was 25.8 $\mu\text{Ci}/\mu\text{mole}$. Following photosynthesis with labeled gas, the samples were frozen and ground in liquid nitrogen. The leaflet powder was extracted successively with 10 ml 80% aq. ethanol, 4 ml 20% aq. ethanol, and 4 ml water, and the extracts of each sample were then combined.

Glycolate Isolation

A 300 μ l sample of each extract containing 0.5 μ moles of unlabeled calcium glycolate added as carrier was spotted on a sheet of Whatman 1 paper (57 cm x 46 cm). Care was taken not to allow the origins to dry completely before the paper was placed in the chromatography cabinet. Chromatograms were first developed by descending chromatography for 32 h with a solvent made up of 754 ml aq. phenol (ca. 88% by weight, Mallinckrodt Liquefied Phenol, A.R.), 224 ml water, 22.4 ml concentrated NH_4OH , and 2 ml of 0.5 M ethylenediaminetetraacetic acid, disodium salt (EDTA)¹⁰. Although variable small amounts of strong acid were present in the phenol as purchased, the amount of NH_4OH used was sufficient to raise the pH of the solvent to 8-9. After a 16 h drying period, the chromatograms were developed in the second dimension for 51 h with n-butanol - 95% ethanol-water-diethylamine (80:10:20:1)⁶. The papers were dried for 3.5 h and the labeled products were located by radioautography (7-day exposure)⁴. A typical radioautogram is presented in Fig. 1.

The location of glycolate was determined by chromatography of authentic sodium ¹⁴C-glycolate (Amersham/Searle, specific radioactivity 11.36 $\mu\text{Ci}/\mu\text{mole}$) alone as well as in a mixture with labeled alfalfa extract. The locations of other metabolites shown (Fig. 1) were determined by adding them in unlabeled form to the labeled alfalfa extracts prior to chromatography. The unlabeled compounds were detected on the chromatograms by standard chemical means (amino acids : ninhydrin ; sugars and glycerate : AgNO_3 then NaOH).

The glycolate spot was cut from the paper, and labeled glycolate was eluted with 5 ml H_2O , 30°, 2 h, by continuous shaking. An aliquot of the elutant was counted immediately in Aquasol 2 (New England Nuclear, Boston, Mass.) by scintillation counting. The purity of eluted labeled glycolate from alfalfa extract was tested by developing a chromatogram on Whatman 1

paper with n-propanol-concentrated ammonium hydroxide-water (6:3:1)¹⁰ and found to be free of material with mobility other than that of authentic ¹⁴C-glycolate.

Glycine and Serine Isolation

A 400 μ l sample of leaf extract containing 1.3 μ moles of unlabeled glycine and 0.4 μ moles of unlabeled serine added as carriers, was spotted on a sheet of Whatman 1 paper (57 cm x 46 cm). A phenol-water-acetic acid solvent, similar to that described by Pedersen *et al.*⁴ was used, but special treatment was required to insure the correct solvent acidity. In recent years, it has proven impossible for us to purchase phenol consistently free of strong acid. It is therefore, sometimes necessary to treat the phenol in order to neutralize this acid prior to adding acetic acid. The resulting phenol solvent then has a pH determined by the added acetic acid rather than the lower pH due to strong acid.

Seven liters of aq. phenol (ca. 88% by weight), 3 liters of water, and 50 g of Na₂CO₃ were stirred overnight. After the layers separated, 8 liters of the lower phenol phase were siphoned off. Glacial acetic acid was added to it until the pH was in the range 4.1 - 4.4. (The pH of the phenol phase was determined by testing the pH of the upper water layer resulting from mixing a small aliquot of the phenol phase with an equal volume of distilled water). About 400 ml of the acid were required. After pH adjustment, 2 ml of 0.5 M EDTA solution were added per liter of final phenol solution.

Descending development with the phenol solvent for 30 h was followed by drying and then descending development for 7.8 h in the second dimension with ethyl acetate-formic acid (85%)-water (7:2:1)¹¹. Labeled products were again located by radioautography. A typical radioautogram is shown in Fig. 2.

The location of metabolites was determined by adding them in unlabeled form to labeled alfalfa extracts. The unlabeled compounds were then chemically detected as above. Glycine and serine spots were eluted with 5 ml H₂O, 30°, 1 h, by continuous shaking and ¹⁴C content was measured by mixing aliquots of the elutants with Aquasol 2, followed by scintillation counting. The identities of the eluted labeled glycine and serine were confirmed by dilution with unlabeled carriers and chromatography for 21 h with the *n*-butanol-propionic acid-water solvent described by Pedersen, *et al.*⁴.

RESULTS AND DISCUSSION

Glycolate Isolation

The major difficulty in isolation and quantitative analysis of glycolate in labeled plant extracts has been the volatility of glycolic acid^{10,12}. Acidic solvent systems such as those used by Platt *et al.*³, Benson *et al.*⁵, and Pedersen *et al.*⁴, may cause substantial losses of glycolic acid during the chromatographic procedure^{10,12}. Unfortunately, glycolate labeling data have often been accepted from paper chromatograms developed with acidic solvents^{7,8,13-16} although some investigators have attempted to minimize losses after final chromatogram development by spraying the paper with NaHCO₃ in order to form the less volatile glycolate salt^{14,16}. Clearly, it would be preferable to work in basic media throughout chromatography and thereby eliminate losses during the entire procedure. Bassham and Kirk¹⁰ have described such a basic solvent system for the determination of glycolate in labelled Chlorella extracts which did not contain ¹⁴C labeled glycerate. However, we have found that system unsuitable for the analysis of glycolate in labeled alfalfa extracts. The latter contained substantial amounts of labeled sucrose, and more importantly, glycerate, which overlapped with

glycolate when the Bassham and Kirk¹⁰ method was used. Glycerate is a labeled photosynthetic product in numerous species of higher plants^{2,6-8}.

We have obtained excellent glycolate isolation with the basic chromatographic system described in the EXPERIMENTAL. Glycolate is well separated from glycerate and sucrose (Fig. 1). Recovery of authentic ¹⁴C-glycolate whether chromatographed separately or mixed with labeled alfalfa extract prior to chromatography was 88-94%. Thus, isolation and quantitative analysis of glycolate in labeled higher plant extract can be accomplished by a single paper chromatogram.

Glycine and Serine Isolation

Glycine and serine do not separate well in the widely used^{7,13,16-18} solvent systems of Benson et al.⁵, and Pedersen et al.⁴, the latter of which we have also used to isolate many photosynthetic metabolites³. We could not clearly separate glycine and serine from other labeled metabolites in our extracts with the solvent system used by Voskresenskaya, et al.¹⁵. In some investigations concerned, at least in part, with photorespiration and glycolate metabolism, separate analyses of the two amino acids have been obtained only after rechromatographing the overlapping glycine-serine spots^{7,13}. Other investigators have chosen to draw conclusions based upon combined glycine + serine analyses¹⁷⁻¹⁹. This approach may lead to incorrect conclusions about the extent of photorespiration, because glycine and serine can be produced by means other than from glycolate². Our findings^{20,21} that levels of accumulated labeled glycine and serine may change in opposite directions in photosynthesizing alfalfa, underline the need for separate labeling data on the two amino acids.

In the course of our investigation of alfalfa photosynthetic ¹⁴C metabolism^{3,20}, we have observed the presence of significant amounts of labeled free glucose in alfalfa leaf extract, in agreement with earlier

observations with other plant species^{5,15,22}. Free glucose tends to overlap with glycine and serine in the paper chromatographic systems of Benson et al.⁵ and Pedersen et al.⁴. This problem has apparently been obviated previously by a preliminary separation with column chromatography⁷.

Metabolite separation from whole leaf extract by means of our new solvent system is shown in Fig. 2. On a single chromatogram glycine and serine are well separated from each other and from other labeled products of photosynthesis, including glucose. The new procedure thus provides separate data for the two amino acids.

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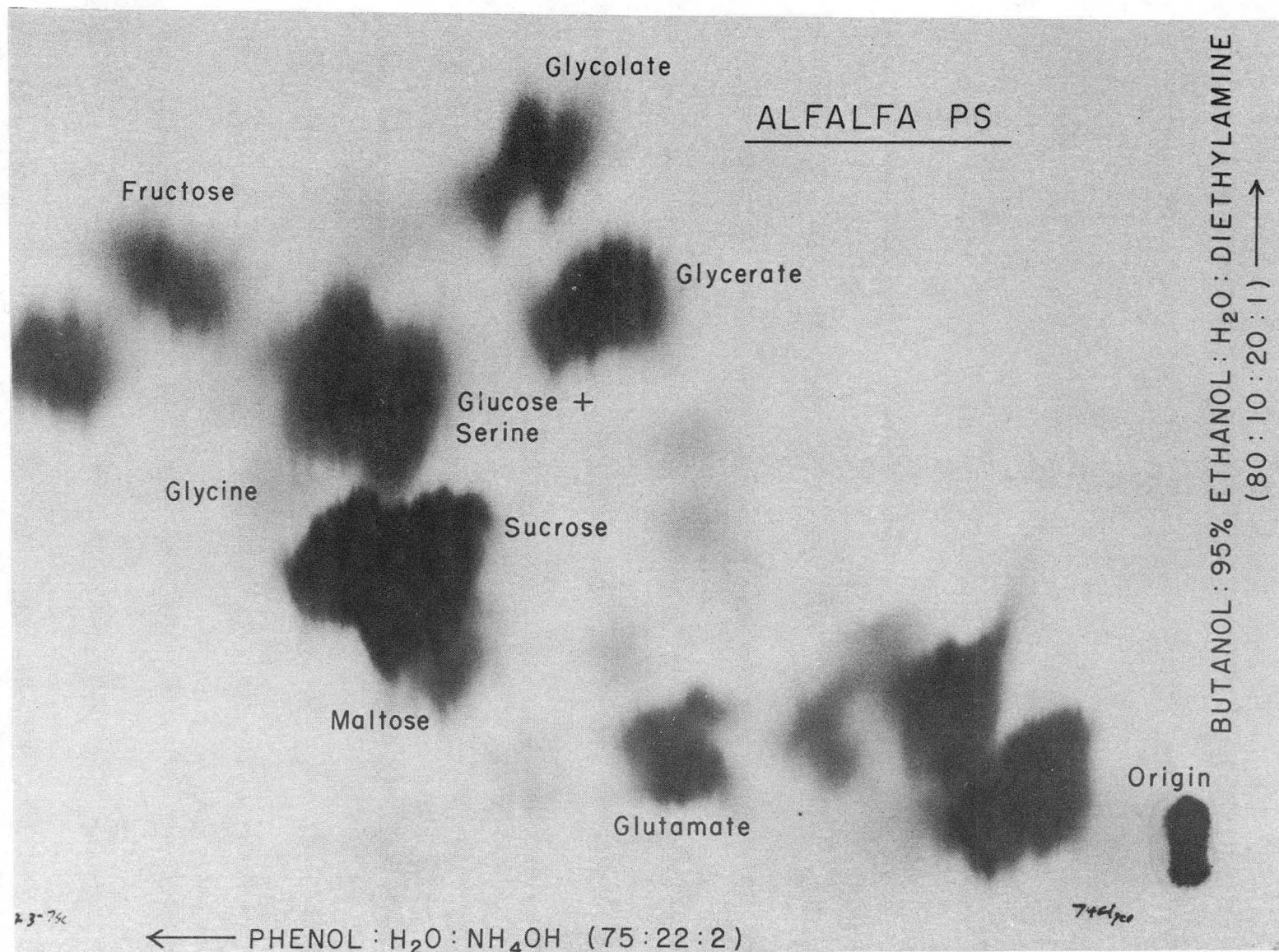
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FIGURE LEGENDS

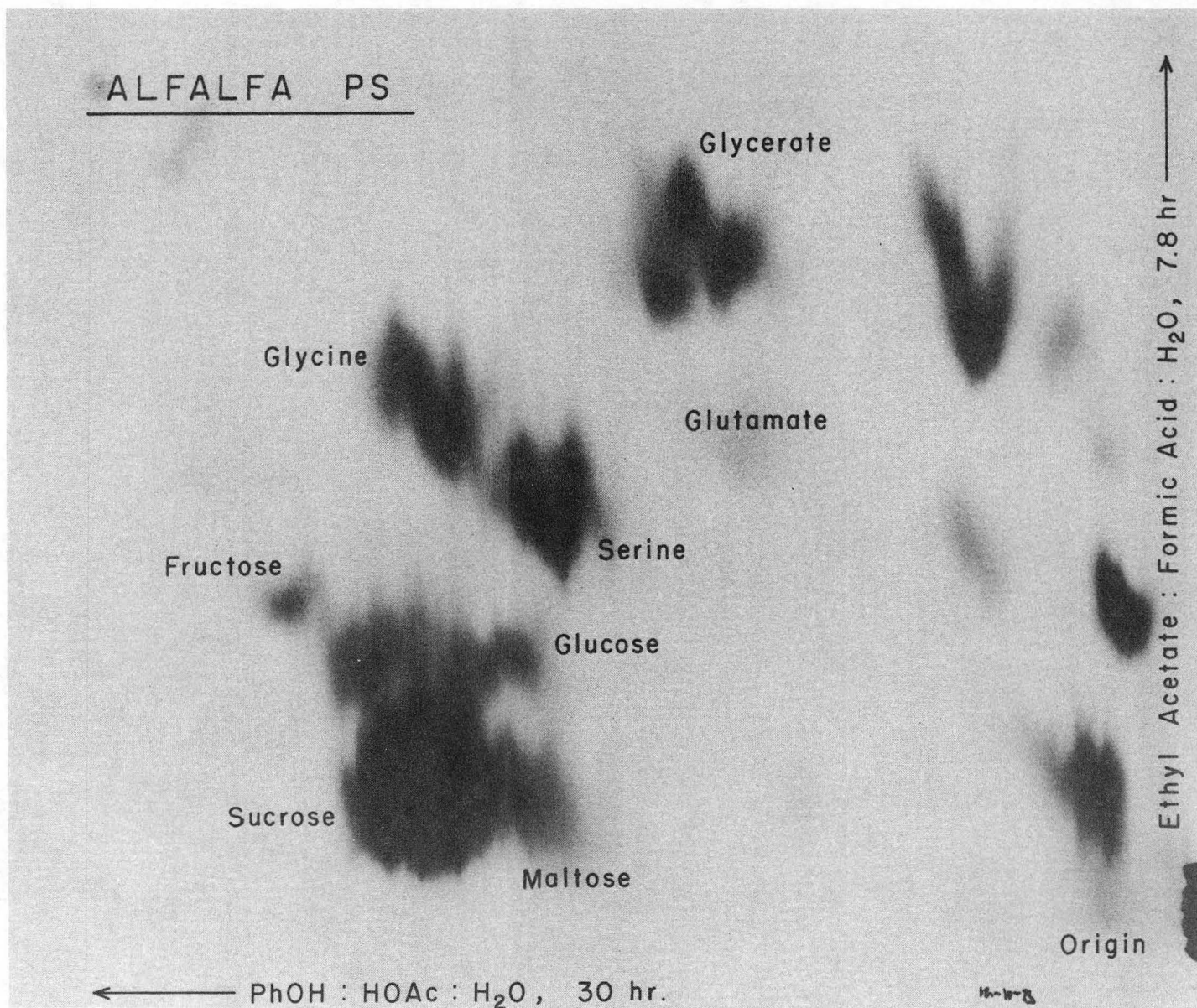
Fig. 1. Glycolate isolation. Radioautograph of two-dimensional paper chromatogram, developed first for 32 h with the phenol-containing solvent and then for 51 h with the butanol containing solvent. Alfalfa photosynthesis with $^{14}\text{CO}_2$. 150,000 DPM of labeled glycolate and 0.5 μmoles of unlabeled calcium glycolate added prior to chromatography.

Fig. 2. Glycine and serine isolation. Radioautograph of two-dimensional paper chromatogram, developed first for 30 h with the phenol containing solvent and then for 7.8 h with the ethyl acetate-containing solvent. Alfalfa photosynthesis with $^{14}\text{CO}_2$. 1.3 μmoles unlabeled glycine, and 0.4 μmoles unlabeled serine added prior to chromatography.



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Fig. 1



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Fig. 2

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