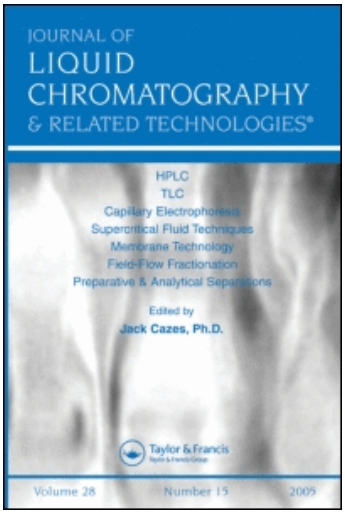

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Thin-Layer Chromatographic Separation of ^{14}C-Labeled Metabolites from Photosynthate

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THIN-LAYER CHROMATOGRAPHIC SEPARATION OF ^{14}C-LABELED METABOLITES FROM PHOTOSYNTHATE

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ABSTRACT

A method for separating ^{14}C-labeled metabolites (including key intermediates of the glycolate pathway) from plant extracts by thin-layer chromatography is reported. The method provides for rapid and convenient determination of the ^{14}C-content of metabolites in leaf samples obtained during kinetic investigations of photosynthesis with $^{14}CO_2$.

INTRODUCTION

A recent publication from our laboratories described paper chromatographic (PC) solvent systems for separating glycolate pathway intermediates from leaf extracts (1). These systems,

along with an additional PC solvent system, have been used in several investigations to determine the extent of ^{14}C-accumulation in various plant metabolites during photosynthesis with $^{14}CO_2$ (2-4). We have now adapted the solvent systems to thin-layer chromatography (TLC). The TLC procedure utilizes a recently developed device for collection and transfer of TLC zones into scintillation vials for counting (5) and has several advantages over PC. For many investigations, it may obviate the need for additional separation procedures (thin-layer electrophoresis, gas chromatography, and liquid column chromatography) which have been used in the isolation of labeled metabolites from photosynthate (1, 6-9). The technique used is advantageous relative to previously described photosynthate fractionation methods (1,9,10) as it separates glycine from serine and isolates glycolate under basic conditions without losses due to its volatilization.

MATERIALS AND METHODS

Radioactive Standards

^{14}C-labeled glycolate, glycine, serine, malate and citrate were purchased from New England Nuclear (Boston, Mass.), Biochemical and Nuclear Corp. (Burbank, Calif.) and Amersham (Oakville, Ont., Canada).

Preparation of ^{14}C-labeled Plant Extracts

Alfalfa plants (Medicago sativa L., var. El Unico) were grown, leaflets selected, leaf discs prepared and exposed to

$^{14}CO_2$ as described previously (4). Photosynthetic assimilation by the leaf discs of $^{14}CO_2$ (0.038%, specific radioactivity 30.2 μCi/μmole) in air (20% O_2) occurred at 2400 ft-c and 27°C Following photosynthesis the samples were frozen and ground in liquid nitrogen. The leaflet powder was extracted with 80% aq. ethanol, 20% ethanol, and water, and the extracts were combined as described previously (4).

Chromatography Equipment and General Procedures

TLC plates (20 cm x 20 cm) coated with a 250 μm layer of MN 300 cellulose, were purchased from Analtech Inc. (Newark, Del.). A band of cellulose, ca. 3 mm wide, was scraped from the left and right edge of the plates in order to make the layer uniform. A leaf disc extract (20-μl aliquot plus added carrier solution, as needed) was applied to a corner (1.7 cm from both edges) of the plate using a disposable micropipette. The extract was dried in an air stream at room temperature.

Plates were developed in a multi-plate Desaga TLC developing tank (Brinkmann Inst., Westbury, N.Y.). As many as seven plates can be developed simultaneously in a single tank. Solvents were placed in the tanks approx. 15 min prior to insertion of the plates, and development was allowed to continue until the solvent had reached the top of the plate. Following development, plates were dried overnight on plastic racks (Analabs Inc., North Haven, Conn.) in a hood. Shorter drying periods are sufficient if the solvents do not contain phenol. When dry, the corners of the

plate were marked with radioactive ink (ink plus any available ^{14}C-labeled compound). The plate was covered with a thin film of plastic (Saran Wrap) and labeled products were located by radioautography (4 to 5-day exposure) (11).

TLC Zone Radioactivity Determination

The chromatogram was placed on a light box and overlaid with the radioautogram, using the corner marks for alignment. The outlines of zones to be collected were traced through the radioautogram onto the TLC layer by pressure with a ballpoint pen (12). The zone outline material was scraped loose from the plate using a needle, collected by suction (13), and this material was discarded.

The cellulose adsorbent inside the outlines was quantitatively collected and transferred into scintillation vials using the device shown in Fig. 1 (5). A disk of Whatman GF/A glass-fiber filter paper was held on the fritted disk by suction. A zone was scraped loose from the plate with a scalpel blade and the collector was held over the loosened adsorbent. The air flow through the glass-fiber filter was strong enough to cause the adsorbent to adhere to the filter. The bottom of the collector was inserted into the mouth of a liquid-scintillation vial and its valve was closed. With the suction shut off, the adsorbent and filter fell to the bottom of the vial. The collector was now ready for use with the next sample, no cleaning being required.

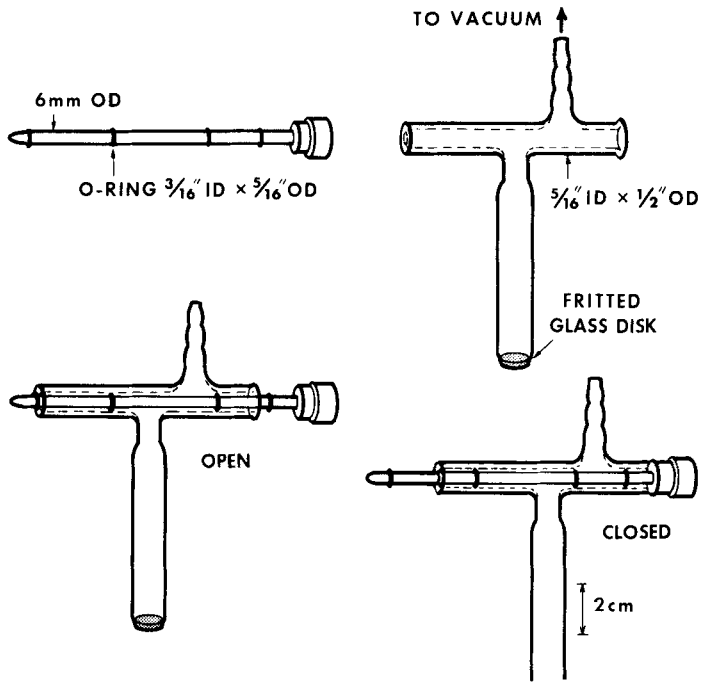


FIGURE 1

Zone collector and transfer device for thin-layer chromatography.

The samples were eluted from the sorbent by adding 5 ml H_2O to each vial and then shaking them in a water bath at 30°C for 1.5 hr. This was followed by addition of 15 ml Aquasol 2 (New England Nuclear, Boston, Mass.,), thorough shaking, and scintillation counting. Alternatively, an aliquot of eluent could be counted and the remainder of the sample used for other purposes. The counting efficiency (ca. 70%, determined by adding a ^{14}C-toluene standard) was not significantly changed by the presence of the filter and adsorbent in the vials.

Solvent System I

Most metabolites of interest were separated in a single two-dimensional thin-layer chromatogram. In the first dimension, we used a modified version of the phenol-water-acetic acid-ethylenediaminetetraacetic acid (EDTA) solvent (11,14,15) previously described for the PC analysis of photosynthate. The modified solvent was prepared as follows (1): Seven liters of aq. phenol (ca. 88%,w/w), 3 l of water, and 50 g of Na_2CO_3 were stirred overnight. After the layers had separated, 8 l 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.

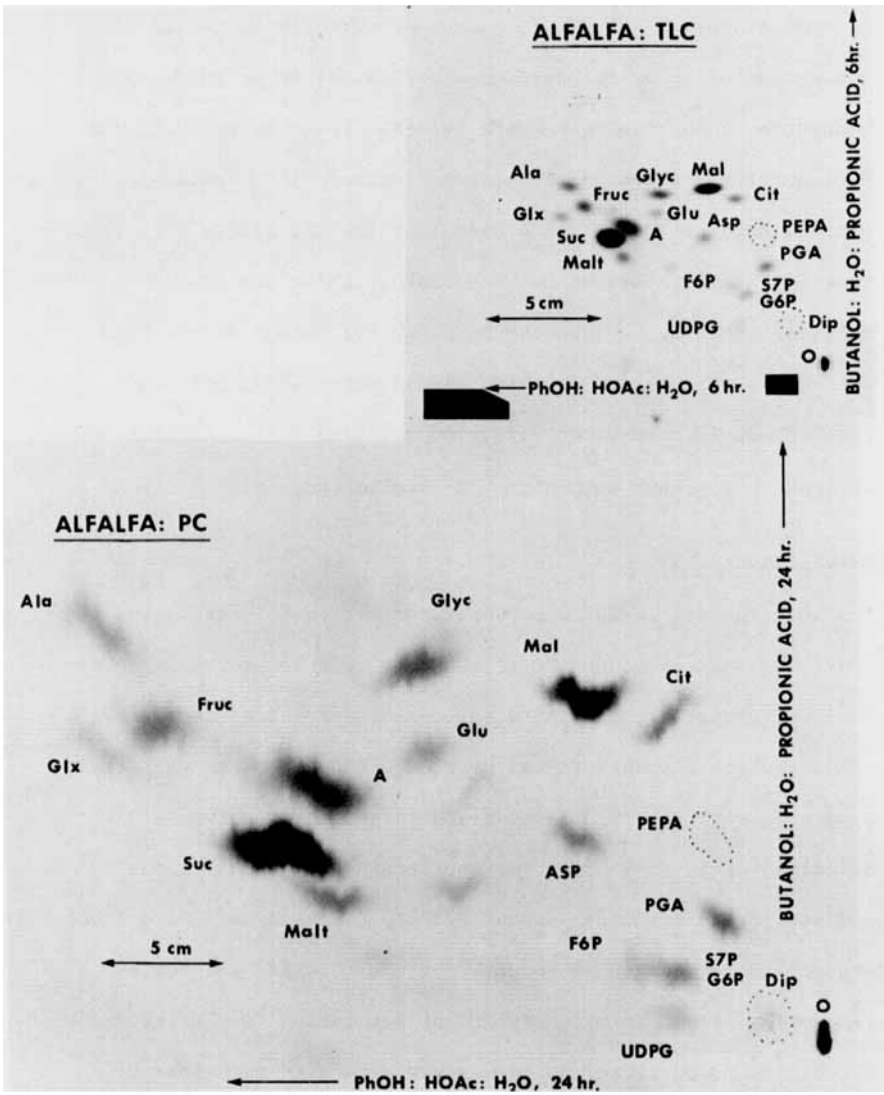
In the second dimension, we used a solvent prepared by mixing equal volumes of n-butanol-water (370:25) and of propionic acid-water (180:220) (14).

Development with each solvent required from 5-7 hr. A typical radioautogram, prepared from a thin-layer chromatogram developed with this solvent system is presented in Fig. 2. For comparison, a paper chromatogram (Whatman 1 paper, 57 cm x 46 cm, 100 µl aliquot of the same extract) developed for 24 hr in each direction with the same solvents is also shown.

The locations of most metabolites were ascertained by adding them in unlabeled form to the alfalfa extracts prior to chromatography. The carrier compounds were then detected on the chromatogram by standard chemical tests: amino acids by ninhydrin, sugars and glycerate by $AgNO_3$ followed by NaOH, and phosphorylated compounds by Benson's method (16). Malate and citrate were collected and eluted from the TLC plates. Their identity was established by rechromatographing the eluted materials in three solvent systems (pH 4.1 phenol-acetic acid-water-EDTA (1), butanol-propionic acid-water (14), and ethyl acetate-formic acid-water (17)) and checking their mobility against that of the authentic ^{14}C-labeled compounds.

Solvent System II

Glycine and serine were separated with a solvent system previously described for PC (1). Prior to chromatography, 0.67 µmol of carrier glycine and 0.48 µmol of carrier serine were added to each aliquot of leaf extract. The TLC plate was first developed with the phenol-water-acetic acid-EDTA solvent described above (5-7 hr). For the second dimension, ethyl acetate-formic acid (85%)-water (7:2:1) (17) was used (ca. 2 hr). Products were located as described above. Glycine and serine recoveries were determined by TLC of authentic ^{14}C-glycine and ^{14}C-serine, both alone and in mixture with alfalfa extract. A typical radioautogram is shown in Fig. 3.



Solvent System III

Glycolate was separated from the plant extracts as follows: Carrier glycolic acid (0.013 μmol) was added to the aliquots of extract before they were applied to the TLC plates. Chromatograms were first developed (6-8 hr) with a solvent prepared by mixing 754 ml of aq. phenol, 224 ml of water, 22.4 ml of concentrated NH_4OH, and 2 ml of 0.5 M EDTA (18). When dry, the adsorbent in a dark band, ca. 5 cm wide near the solvent front, was scraped off the plate and discarded. The plates were then developed in the second dimension with n-butanol-95% ethanol-water-diethylamine (80:10:20:1) for 4-6 hr (19). A typical radioautogram is shown in Fig. 4. Glycerate was located as described above. Glycolate location was determined by chromatography of authentic ^{14}C-glycolate alone as well as in a mixture with alfalfa extract. In order to determine recovery, glycolate TLC zones were collected, and their radioactivity determined as described above.

FIGURE 2

Radioautograms of a two-dimensional thin-layer chromatogram (TLC) and a paper chromatogram (PC), prepared by using Solvent System I. Photosynthesis in alfalfa leaflets with $^{14}CO_2$. Abbreviations: Ala, alanine; Glyc, glycerate; Mal, malate; Cit, citrate; Fruc, fructose; Glu, glutamate; A, serine + Glycine + Glucose; Asp, aspartate; PEPA, phosphoenolpyruvate; Glx, glutamine; Suc, sucrose; Malt, maltose; F6P, fructose 6-phosphate; S7P, sedoheptulose 7-phosphate; G6P, glucose 6-phosphate; PGA, 3-phosphoglycerate; UDPG, uridine diphosphoglucose; Dip, sugar diphosphate; O, origin; PhOH, phenol.

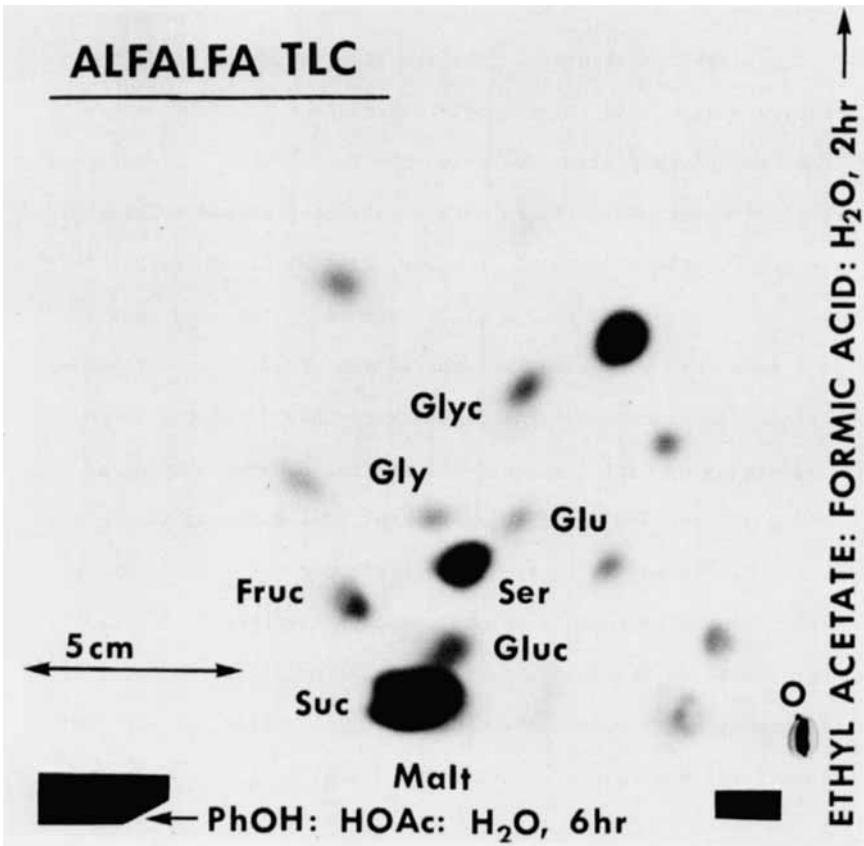


FIGURE 3

Separation of glycine and serine by TLC with Solvent System II. Radioautogram of two-dimensional thin-layer chromatogram. Photosynthesis in alfalfa leaflets with $^{14}CO_2$. Carrier glycine (0.67 µmol) and carrier serine (0.48 µmol) added prior to chromatography. Abbreviations: Gly, glycine; Ser, serine; gluc, glucose; remainder as in Fig. 2.

RESULTS AND DISCUSSION

The TLC technique described produces excellent separation (Figs. 2-4) of a wide range of metabolites. It is capable of

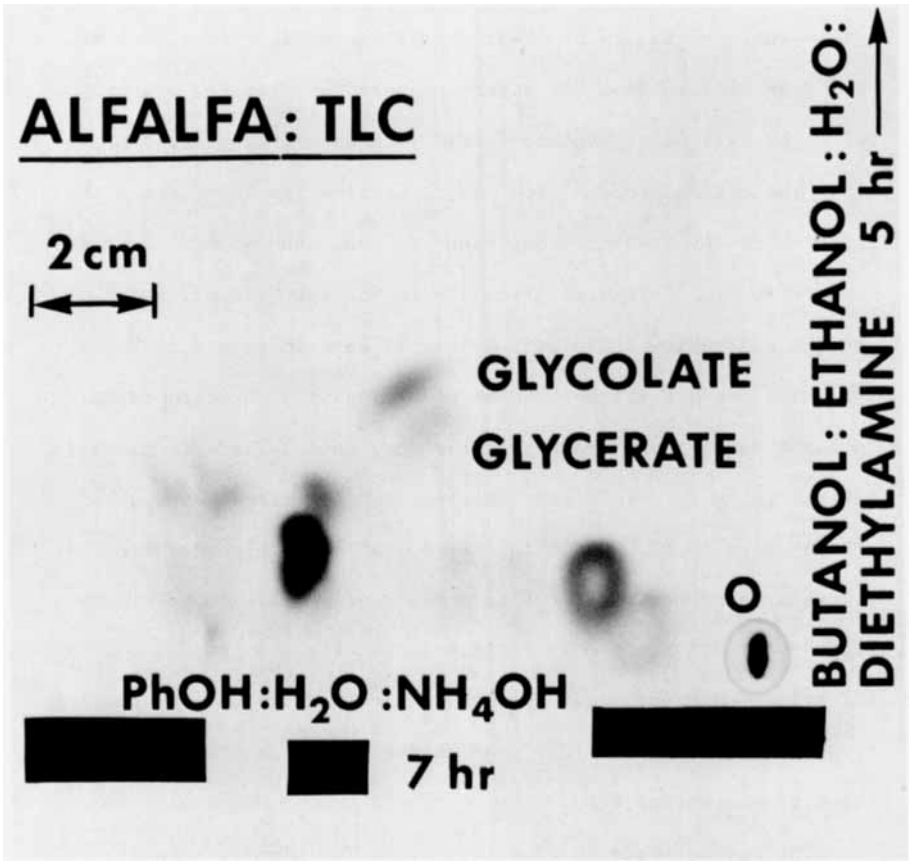


FIGURE 4

Glycolate isolation by TLC with Solvent System III. Radioautogram of two-dimensional thin-layer chromatogram. Photosynthesis in alfalfa leaflets with $^{14}CO_2$. Carrier glycolic acid (0.013 µmole) and labeled glycolate (18570 dpm; 11.36 µci/µmol) added prior to chromatography.

providing superior resolution and significantly more compact zones than PC (Fig. 2). Developing time is reduced to approximately one-fourth of that required for the same solvent in PC: phenol-acetic acid-water-EDTA, 5-7 hr vs. 24-30 hr (1,14); butanol-propionic

acid-water, 5-7 vs. 24 hr (14); phenol-water-NH_4OH-EDTA, 6-8 hr vs. 32 hr (1); butanol-95% ethanol-water-diethylamine, 4-6 hr vs. 51 hr (1); ethyl acetate-formic acid-water, 2 hr vs. 8 hr (1). The entire process from sample application to starting the radioautography can be accomplished in less than 48 hr. Average recoveries of ^{14}C-labeled glycolate in Solvent System III and glycine and serine in Solvent System II were in excess of 90%.

Many extract samples can be simultaneously chromatographed in small tanks in an ordinary laboratory hood. There is no need for the bulky PC development chambers and the large amounts of solvent used in PC. The aliquots of leaf extract needed for the TLC separation are smaller than those required for PC. This is because the zones are more compact in TLC (Fig. 2) and also because scintillation counting is more efficient than the Geiger counting technique (ca. 70% vs. 10%) used in our laboratory for evaluating paper chromatograms (20).

The device shown in Fig. 1 provides for simple, quantitative collection and transfer of large numbers of TLC fractions to liquid scintillation vials (5). We have not found it necessary to resort to the cellulose acetate layer stripping technique (21,22), the time-consuming process of direct counting of zones on the TLC with a Geiger counter, or the elaborate devices that have been designed for the analysis of radioactive TLC or PC zones (20,23).

In conclusion, it may be mentioned that we have successfully applied the procedures described in this paper to a full kinetic

study of plant metabolism, involving the separation of a large number of extract samples and the determination of the radioactivity of a wide range of metabolites (24).

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