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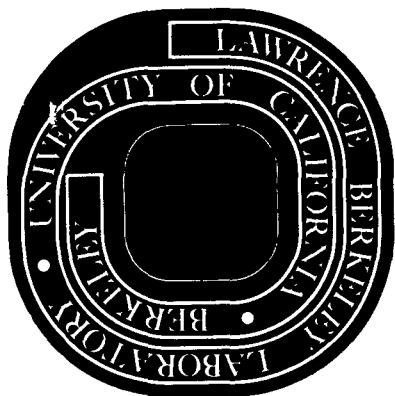"Experimental Plant Physiology,"
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^{14}C-LABELLING AND TWO-DIMENSIONAL PAPER CHROMATOGRAPHY OF PLANT METABOLITES

J. A. Bassham

Advantages of Paper Chromatographic Analysis and Radioautography for Studies in Intermediary Metabolism

A great many biochemical reactions occur simultaneously within a living cell. Many classical methods of study of intermediary metabolism are able to examine only a few of the substances involved in these reactions in any single experiment. In contrast, analysis by two-dimensional paper chromatography of labeled compounds formed from labeled substrates permits an examination of a great many different compounds at once. Kinetic studies using this method provide a dynamic picture of the flow of material through the metabolic pathways.

Metabolic intermediates in green plant cells are readily labeled by the introduction of radioactive tracers such as carbon-14, phosphorus-32, or both. Carbon-14 may be administered as $^{14}CO_2$ to leaves of plants or as $H^{14}CO_3^-$ in solution to aquatic plants. ^{32}P-labeled phosphorus can be administered as inorganic phosphate in solution directly in the medium of aquatic plants or to the roots or by injection into the veins of leaves of plants. With either or both of these labeled compounds, green plants very quickly incorporate the radioactive tracer into a large number of metabolic intermediates. In photosynthesizing cells these metabolites include 3-phosphoglycerate, a number of sugar phosphates and sugar diphosphates, amino acids, carboxylic acids, and other compounds of small molecular weight. Some of the radioactivity finds its way into macromolecules in relatively short times.

Two-dimensional paper chromatography has proved to be extremely use-ful as a method of separating a large number of diverse intermediary meta-bolites. Other chromatographic methods, such as thin-layer chromatography, column chromatography with ion exchange resins, vapor phase chromatography, etc., are also suitable for separations of some of these classes of com-pounds formed in green plant cells. However, for a one-step separation of many labeled compounds, two-dimensional paper chromatography is probably the most useful method. One reason for this is that two-dimensional paper chromatography provides a high degree of resolution of a broad spectrum of intermediary metabolites. A second reason is that the shape and fine structure of the areas of paper covered by individual compounds are highly distinctive, permitting a kind of fingerprinting and nearly absolute iden-tification when radioactive compounds are found to coincide with carrier unlabeled compounds. A third reason is that when radioactive compounds are being analyzed, extremely small quantities may be detected by paper chromatography followed by radioautography with medical X-ray film.

The use of paper chromatography in amino acid separation by Consden, et al.[1] in 1944 led to the application of this technique by Calvin and co-workers to the analysis of products of photosynthesis with $^{14}CO_2$ in green plants.[2] This method, described more fully by Benson et al. (1950),[3] was of key importance in the elucidation of the path of carbon fixation in photosynthesis via the reductive pentose phosphate cycle.[4,5] Since that time the method has been used extensively to study not only the path-ways of carbon fixation in photosynthetic plants, but also the mechanisms of the regulation of metabolism in green plants.[6,7] Over the years, im-provements have been made in the method which result in chromatograms superior in resolution to those obtained in the early work.[6]

Principles of Paper Chromatography

As with many other types of chromatography, paper chromatography depends upon the partition of the compounds to be separated between two different phases. In the case of paper chromatography, these two phases are liquid phases. One liquid phase is stationary and absorbed on the cellulose fibers in the paper. In general, the stationary phase is predominantly aqueous but contains organic solvents mixed into it. The mobile phase is predominantly organic but contains water dissolved in it. If α represents the distribution coefficient of a particular solvent between the two phases, the relative rate of movement of that solute is given by the equation $[R_F = A_L/(A_L + \alpha A_S)]$. A_L is the fraction of the cross-sectional area of the chromatogram occupied by the mobile phase, and A_S is the fraction of the cross-sectional area occupied by the stationary phase. R_F is defined as the distance from origin to solute divided by the distance from origin to solvent front.

This theory was tested[3] with a variety of carboxylic acids and sugars from green plant cells by distributing these compounds between an organic and an aqueous phase in separatory funnels and comparing the calculated R_F with the measured R_F. Fairly good agreement with the theory was obtained. However, it should be noted that with some compounds additional effects were seen which might be attributed to absorption of compound by the cellulose fibers.

With this theory in mind, the selection of suitable chromatographic solvents becomes a problem of finding solvents in which the majority of the solutes to be separated will be distributed to some extent between the two phases but with a variety of distribution coefficients depending upon the physical properties of the particular compound. For two-dimensional

paper chromatography, the two chromatographic solvents should have not too similar properties, since if they were exactly the same, a diagonal row of compounds would be produced by equivalent resolution in the two directions. One way to achieve two different types of resolution for the two dimensions of chromatography would be to make one solvent acidic and the other basic. However, most of the compounds of interest in intermediary metabolism of photosynthetic green cells are either neutral or acidic--that is, they are either neutral amino acids, acidic amino acids, carboxylic acids, sugars or sugar phosphates, or sugar nucleotide phosphates. For such compounds, basic solvents are generally not very effective, since many substances tend to be held back and run together. Therefore, it has been found that most products of photosynthesis and intermediary metabolism in green cells are best separated by two acidic solvents with rather different solvent properties.* The first solvent used is phenol with a nearly saturating amount of water. Sufficiently pure phenol can now be purchased in the form of liquified phenol which already contains some water. To this is added enough water to bring it up to the desired condition of just under saturation. Since such phenol commonly is acidic as purchased due to impurities, it is desirable to maintain a constant acidity by adding additional acid so as to reduce the variability from one lot of chromatographic solvent to another. This is achieved by adding glacial acetic

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* An exception to this is glycolic acid, which is volatile and thus is partly lost from papers developed in acidic solvents. A basic solvent (for example, one containing added NH_4OH) can be used for the separation of glycolate from other metabolites. When the chromatogram is dried, the ammonium salt of glycolic acid is not lost.

acid. Another addition is made of a small amount of a 1-molar solution of ethylene diamine tetraacetic acid (EDTA). The purpose of this addition is to form a complex with divalent metallic ions and carry them from the paper. This in turn greatly improves the chromatography of sugar phosphates and other phosphorylated compounds which would tend to react with the divalent ions in the paper.

A second solvent commonly used is a mixture of butanol, propionic acid and water. The solvent properties of propionic acid are not greatly different from those of acetic acid, but the solvent properties of butanol which constitutes a major part of the second solvent, are very different from the solvent properties of phenol which constitutes the major part of the first solvent. Thus, a different kind of separation is achieved in the two directions, even though both solvents are acidic.

Two-dimensional paper chromatograms are prepared in research laboratories by applying a suspension or solution of the biochemical mixture to be separated to a small area near one corner of a large sheet of suitable chromatographic filter paper. This point of applicatijn is called the origin. The one edge of the paper next to the origin is then folded and placed in a dry chromatographic trough. A second paper is usually placed in the same trough so that the folds of the two papers overlap and the papers hang down from the trough on opposite sides. Usually each paper hangs over an anti-siphoning rod which is horizontal and slightly above and separated from the top edge of the trough by a small space (about 5 mm) so that siphoning of solvent between paper and trough edge does not occur. A bar weight is then placed on top of the folds of the papers.

Papers and trough are then placed in a chromatographic cabinet which is vapor tight, and the chromatographic solvent is added to the trough.

The solvent travels by capillarity through the paper, moving at a uniform rate down the paper and across the origin. As it passes the origin it dissolves the compounds which were applied there, and carries them along with the solvent at some rate which is less than the rate of travel of the solvent front. In some cases the solvent front is allowed to reach the far edge of the paper, whereas in other cases it is allowed to drip from the far edge of the paper in descending chromatography. This depends upon how rapidly the compounds to be separated are moving--that is, their R_F values. When the separation is deemed complete, the paper is dried either by removing the paper from the box and hanging it in a hood, or, preferably, if the equipment is available, by applying a suction to the box to draw a stream of air through the box into an exhaust system. The time for development of the paper in the first solvent may be anywhere from 6 hr to 48 hr or more, depending upon the solvents used and the R_F's of the compounds to be separated.

After the drying from the first direction, the paper is rotated 90° and the other edge of the paper is placed in another trough for chromatography in the second dimension. The weight is again added to the paper fold, the trough and papers are placed in the box, the solvent is added, and development proceeds as just described. When this second development is completed, the paper is again dried, at which point it is ready for radioautography.

The paper is then removed from the box and folded around a large sheet of medical X-ray film. This is then placed in a light-tight cassette and kept for a few days until the radioactive emanations from the labeled compounds have had time to expose the X-ray film. The film is then developed, and dark spots appear wherever there were radioactive compounds on

the paper in contact with the film.

Exposure of Plant Tissue to Radioactivity

For studies of metabolites of photosynthesis, leaves, suspensions of unicellular algae, or suspensions of isolated chloroplasts may be used. For either suspensions of algae or of chloroplasts, the biological material is suspended in a suitable buffer and placed in a small flask. A solution of ^{14}C-labeled bicarbonate and/or a solution of ^{32}P-labeled phosphate is then added to this flask. The flask is illuminated with gentle swirling for a minute or so, after which the biological material is killed by the addition of methanol to a final concentration of 80% at room temperature. The methanol quickly dissolves the lipid membranes of the cell and denatures the protein enzymes stopping the biochemical reactions at the time of killing. If a kinetic experiment is to be run, samples of the algae or chloroplasts may be removed from time to time, and separately killed.

For experiments with leaves, the leaves of a small plant are placed in a chamber with transparent walls or windows, through which they can be illuminated. Gaseous $^{14}CO_2$ is added, or is generated within the chamber by addition of acid to $Ba^{14}CO_3$. After a short period of photosynthesis, the leaves are removed from the chamber and killed. After killing, an aliquot sample of the entire suspension of dead biological material is applied to the origin of a paper chromatogram. An air current from a hair dryer may be used to facilitate drying of the methanol and water, leaving the biological material on the origin. If a hair dryer is used, the temperature should be set fairly low so that the compounds, some of which are chemically labile, are not destroyed. Alternatively to a hair dryer, a stream of air or nitrogen from a tank may be applied through a suitable

piece of tubing held in a clamp.

Laboratory Experiments

Several variations of the techniques used in research studies will be adapted for use in student laboratories. Many such laboratories will not be equipped with the large chromatographic boxes required to run paper chromatograms measuring 46 cm or so by 57 cm. However, the principles may be adequately tested by preparing much smaller chromatograms of the order of 25 cm x 25 cm. Also, experiments will be described for the use of unicellular algae, or leaves. Either of these biological experiments may be combined with analysis by either the large or the small chromatograms.

Materials

Plants. For experiments with land plants, healthy leaves of plants capable of high rates of photosynthesis, and of such a texture as to be easily extractable with organic solvents, are required. Among plants which have been successfully studied are spinach, soy bean, pea, alfalfa. Plants which exhibit the additional pyruvate-malate pathway of CO_2 fixation, such as maize or sugar cane may also be used, but are often more difficult to extract due to the fibrous nature of their leaves.

In order to permit each student to do an experiment, a single alfalfa leaf about 2 cm long (or other leaf of similar size) will be used. Whatever leaves are chosen, they should be harvested and kept on ice, and should be used the same day as picked if possible.

Among fresh water aquatic plants, unicellular green algae such as Chlorella pyrenoidosa have been widely used in research and are very suitable. Marine algae present a serious chromatographic problem because of the high salt content in their medium.

Radiocarbon. For leaves and higher plants, $^{14}CO_2$ mixed with $^{12}CO_2$ is used. For algae and chloroplasts suspensions, a solution of $NaH^{14}CO_3$ mixed with unlabeled bicarbonate is used. In either case, a specific radioactivity of at least 10 microcuries per micromole should be used so that the small amounts of materials on the paper chromatograms will contain enough ^{14}C for easy detection. For class use, one ml of a solution of $Na^{14}HCO_3$ and $NaH^{12}CO_3$ with a specific radioactivity of 20 ucuries per umole, 0.05 M (50 umoles) is recommended. This solution, containing 1 millicurie of ^{14}C, is placed in a 2 ml serum bottle with a tight-fitting serum cap. For all experiments, each student may withdraw 20 ul (20 ucuries) with a 0.1 ml graduated microsyringe equipped with a 2-1/2 inch, no. 20 gauge hyperdermic needle. All ^{14}C contaminated glassware and needles should be rinsed with dilute HCL in a fume hood.

Chromatographic solvents. The solvent for the first dimension is made up of from 840 ml "liquified" phenol (Malinckrodt, about 88% phenol and 12% water), 160 ml water, 10 ml glacial acetic acid, and 1 ml 1.0 M ethylene diamine tetraacetic acid.[6]

The solvent for the second dimension is made up by mixing together just prior to chromatography equal volumes of the two following mixtures: A. n-butanol-water (370:25 v/v), and B. propionic acid:water (180:220 v/v).[6]

Chromatographic paper. For paper chromatography, a fine-grained, chromatographic grade filter paper is required for best results. One paper often used in research work is Whatman #1. Care should be taken to insure that the first solvent (phenol-water) is run with the "grain" of the paper. This is difficult to see in a fine-grained paper, but is usually specified in a chromatographic-grade paper. With Whatman #1, it is the long direction of the paper.

Formerly, the paper was often washed with a solution of oxalic acid
or of EDTA to remove divalent cations which could interfere with the move-
ment of phosphate esters on the paper. This laborious paper-washing is
now eliminated by addition of the EDTA to the phenol solvent.

Radioactive ink. For exact matching-up of X-ray films with paper
chromatograms after development, it is desirable to label the corners of
the chromatographic paper with radioactive ink. Thus, when the film is
developed, the ink on the paper can be exactly matched with the correspon-
ding exposed mark on the paper, thus guaranteeing that all radioactive
spots on the paper will be precisely located with respect to dark spots
on the film. The ink can be prepared by the addition of nonvolatile radio-
active compounds (such as ^{14}C-labeled glucose) to ordinary black ink.

X-Ray film. Single coated, blue sensitive medical x-ray film (such
as Kodak SB-54) is used. Double coated film could be used but would have
twice the background (due to cosmic radiation) and only one side would
be exposed by the weak beta rays from the ^{14}C.

Other materials. Among other materials required are methanol for
killing the plants, liquid N_2, and dry ice if leaves are to be killed and
extracted, microsyringes and needles, and various standard biochemical
compounds, such as the amino acids alanine, aspartate and glutamate; sugars,
carboxylic acids, etc., for identification purposes. Ninhydrin may be
employed as a color reagent spray for amino acids.

Equipment

Chambers for plants or leaves. A chamber for an experiment with
a whole small plant or with excised whole leaves must be transparent or
have windows to provide for illumination of the plant material and must

either have inlet and outlet valves for introducing CO_2, or must provide for generation of $^{14}CO_2$ within the chamber. The actual design will depend on the size and shape of the plants and materials available.

For a small leaf such as a single alfalfa leaf, a 4 ml widemouth vial (14 mm OD, 45 mm high) stoppered with a Size 15 rubber serum stopper, sleeve type, may be used. The $^{14}CO_2$ will be generated inside this bottle with the leaf in place. After one minute the stopper and leaf will be removed from the vial in a dry box which is exhausted to a fume hood through a NaOH trap to remove the small amount of $^{14}CO_2$ remaining.

Exposures of algae and isolated chloroplasts. For this purpose, small (about 10 ml), round-bottom glass flasks with tightly fitting serum caps may be used. Since the green material to be used has a high coefficient for light absorption, a thin layer of biological material on the bottom of the flask is desirable. This is achieved by using 0.5 to 0.1 ml of the liquid suspension of cells or chloroplasts, and providing a gently swirling motion (by hand or mechanical shaker) which distributes the material in a thin layer on the bottom of the flask.[8] Illumination may be provided from the bottom, preferably through a water bath with a transparent bottom to control temperature in the flasks.

For the exposure of larger amounts of algae suspension, a flat-sided, disc-shaped vessel with stopcock-equipped inlets and outlet at top and bottom may be used. The inside thickness of the vessel should be no more than 5 mm. Radioactive $H^{14}CO_3^-$ solution may be added through the top stopcock, and periodically, samples may be taken from time to time by opening first the top stopcock, and then briefly, the bottom stopcock. The vessel is held in a vertical position and illuminated from the sides, usually with incandescent lamps shining through infrared absorbing filters immersed

in baths of cooling water. Much more elaborate systems for conducting
kinetic experiments with algae exposed to radioactive tracers have been
described.[9] Such systems have provision for recirculating the mixtures of
$^{14}CO_2$ in air through the algae and gas-monitoring instruments in a closed
system, using a gas pump. Solenoid-operated valves permit taking samples
of uniform size rapidly by push button.

Chromatographic Equipment. The simplest and cheapest equipment for
two-dimensional paper chromatography consists of a wide-mouth glass jar
such as is used in home fruit canning, equipped with a screw-on lid.
Ascending chromatograms can be run in such jars by placing solvent in the
bottom to a depth of about 2 cm, and setting a paper cylinder in the sol-
vent, capping the jar and allowing the solvent to travel up the paper
by capillarity. To prepare the cylinder, the mixture to be analyzed is
first dried on the 20 cm x 20 cm paper about 3 cm in from one corner, and
the paper is rolled into a cylinder and the edges stapled together. After
the first solvent reaches the top of the paper, the paper is dried, unsta-
pled, and rolled in the other direction, stapled, and the cylinder is
placed in a second solvent for the second dimension. Unfortunately, this
method does not give reproducibly good resolution of compounds compared to
the resolution obtainable with descending chromatography and somewhat
larger papers.

For small two-dimensional paper chromatograms (23 cm x 23 cm), a
bench top chromatography jar unit, square, about 30 x 30 cm square and
60 cm high, may be purchased. This unit is supplied with a glass lid,
adjustable stainless steel rack and four solvent trough assemblies, each
consisting of one glass solvent trough, one trough-holder, two glass anti-
siphon rods, and one glass anchor rod, for about $125. This assembly is

satisfactory, provided the experiments can be run in a room with no more than 3° C variation in temperature. For rooms with larger temperature variation, more expensive, insulated assemblies are required.

For large two-dimensional paper chromatography (which gives the best results, and is used in research), formica-lined cabinets with inside dimensions about 40 x 65 cm and 60 cm deep may be purchased. Equipped with solvent assemblies, these cost about $500. These boxes may be easily modified for drying chromatographic papers in situ after development. Two 10 cm diameter round holes are made in the cover on the centerline of the long direction, with the edges of the holes about 6 cm apart. Each hole is equipped with a 10 cm diameter air pipe flush with the inside of the lid and projecting 5 cm on the outside. On the bottom of the lid, a 4 cm x 4 cm board, 30 cm long, is attached between the holes and running across the short direction of the lid. To this board is attached a thin plate of stainless steel, or other resistant material, 30 x 30 cm.

When chromatograms are being developed, both pipes are closed with large plugs. When the chromatograms are to be dried, the plugs are removed, a flexible vent hose is attached to one of the pipes, and led to an exhaust system. If the suction by the exhaust system is not sufficient, a closed air blower is placed in the exhaust line to move air from the box chromatographic cabinet to the exhaust system. Air enters the cabinet through the other pipe, is distributed through the cabinet by means of the plate attached to the bottom of the lid, and leaves by the exhaust system, drying the paper chromatograms in place. In this way, people are protected from excessive inhalation of the fumes of the volatile chromatographic solvents, and the papers do not have to be transferred while wet, thus minimizing the possibility of dropping and tearing.

Experimental Procedures

Either of the experimental procedures for exposing plants to $^{14}CO_2$ may be combined with either of the chromatographic procedures.

Photosynthesis with leaves. Place a few crystals (about 10 mg) of citric acid in the bottom of a 4 ml widemouth vial. Cover the crystals with a thin layer (about 3 mm) of glass wool. Cut off a leaf from the alfalfa branch, weigh to 1 mg, and place the leaf in the vial, standing on its stem on the glass wool. Stopper the vial with the serum cap, and place in "dry" box.

Carry out the following operations in a dry box vented to a fume hood through Ascarite. In this way, when the leaf is removed from the vial, the residual $^{14}CO_2$ will be trapped in the Ascarite (NaOH on asbestos) and will not escape to the atmosphere. Federal and State regulations now place very low limits (0.1 ucurie per cubic meter of air) on $^{14}CO_2$ that can be legally released to the atmosphere.

Insert a 2.5 inch, no. 20 hyperdermic needle attached to a 0.1 ml graduated microsyringe into the stock solution of $NaH^{14}CO_3$ and draw 20 ul of the solution up into the microsyringe. Then suck in a few ul of air and carefully lay aside the syringe and needle on a piece of tissue in a tilted position (needle point up) so that the solution will not be spilled until needed.

Hold the vial containing the leaf by the stopper and illuminate from the side with an incandescent lamp for one minute; then remove from the light temporarily and insert the hyperdermic needle through the serum cap until the tip touches the crystals of citric acid at the bottom of the vial. Be very careful during the insertion of the needle so as not to allow the solution in the syringe to flow out through the needle prematurely.

It is recommended that this technique be practiced several times without radioactive solution. When forcing the needle through the serum cap, take care not to push on the plunger of the syringe!

When the needle is in place with the tip touching the citric acid, hold the vial in the light and inject the bicarbonate solution into the citric acid by pushing in the plunger. Withdraw the needle and place it in a beaker containing dilute HCL. Continue the exposure of the leaf for one minute. (If the instructor wishes, the time can be varied from one experiment to another to demonstrate the increase in incorporation of ^{14}C with time in different compounds.) At the end of this period, remove the serum cap, remove the leaf with a tweezers and place it into a small dewar of liquid nitrogen. When frozen, transfer leaf to a 12 ml graduated conical centrifuge tube containing 2 ml of 80% methanol-20% water, pre-cooled to about -15° C. If liquid N_2 is not available, the leaf may be placed directly into this cold methanol-water mixture. After 30 minutes remove leaf from dry box. Using a small glass stirring rod, break or mash the leaf in the methanol-water mixture. Stopper and store the test tube in the freezing compartment of a refrigerator for three days. Occasionally stir with the stirring rod.

Centrifuge the leaf material by spinning the centrifuge tube in a small centrifuge at low speed. Remove the solution with a pipette to a second graduated tube. Add 1.0 ml of 20% methanol-water (room temperature) to the leaf residue, stir and centrifuge. Add the supernatant solution to the 80% extract. Measure the total volume after mixing. For chromatography, use a one-fiftieth aliquot portion of the solution.

Photosynthesis with algae. Withdraw and centrifuge enough algae solution from the culture flask to contain at least 1 cm^3 of packed algae

after centrifugation. Resuspend the algae in a 1.0×10^{-3} M phosphate buffer adjusted to pH 6, to give a 1% v/v suspension.

Each student should withdraw 0.25 ml of this suspension and place it in a 10 ml round bottom flask. Stopper the flask with a serum cap. Place in dry box, equipped with exhaust through Ascarite. In the dry box, draw up 1 ml of methanol through a hyperdermic needle (20 gauge) into a 2 ml syringe. Set aside for later use.

In dry box, withdraw 20 ul $NaH^{14}CO_3$ solution through a hyperdermic needle into 0.1 ml microsyringe. (See precautions under Photosynthesis with leaves experiment.) Holding flask by stopper, illuminate for one minute. Insert hyperdermic needle through serum cap until tip of needle is in algae suspension. Inject bicarbonate solution and withdraw needle. Illuminate for one minute, giving the flask a gentle swirling motion to spread out suspension on the bottom of the flask.

At the end of this period, insert the needle attached to the 2 ml syringe and inject the 1 ml of methanol. Withdraw the needle, and swirl the flask for a few seconds. Remove the stopper, and allow at least a half hour for $^{14}CO_2$ to escape before removing the flask from the dry box.

Use 50 ul of the killed suspension for the small chromatograms, and 200 ul for the large chromatograms, as described below.

Chromatography in Small Cabinets. Cut sheets 23 cm square from large sheets (46 x 57 cm) of Whatman no. 1 filter paper, marking the small sheets to show the long direction of the original paper. A pencil may be used for making this and other identifying marks on the paper without inter- ference with the chromatography. Mark the spot for the origin at a point near one corner and 5 cm from each edge.

Using a micropipette and syringe apply the plant material slowly to

this origin, keeping the wetted area as small as possible, and in any case not larger than one cm in diameter. If marker compounds such as the amino acids alanine and glutamate are to be cochromatographed with the radioactive compounds, they should be added to the plant material before it is applied to the chromatogram. Enough of each should be added to the suspension of plant material in methanol-water that the aliquot sample applied to the paper will contain 25 ug of each. An air dryer (such as a hair dryer) or a stream of air or nitrogen may be used to speed the drying process. The spot need not be absolutely dry before it is placed in the chromatography cabinet - excessive drying may in fact lead to some sticking of compounds to the origin during development.

Make a one cm fold along the edge of the paper which is near the origin and at a right angle to the "long" direction of the paper (see above). Place two such papers in each chromatography trough so that the folded edges overlap, but the remaining parts of the two papers will hang on opposite sides of the trough. Place the weight on the crease in the papers to hold them in the trough.

Pour about 20 ml of the phenol-water solvent into a small, flat dish in the bottom of the cabinet. Place the trough with papers in the cabinet. When all of the troughs are in place, carefully add 25 ml of the phenol-water solvent to each trough, taking care that the solvent does not splash over and run down the paper. Close the cabinet or chromatography jar tightly. Any leak which allows the solvent vapors to escape or air to enter may cause the chromatograms to run unevenly.

The chromatograms are normally allowed to develop until the solvent reaches the bottom of the paper, 7 or 8 hours. Then open the cabinet and remove excess solvent from the troughs with a pipette.

The phenol solvent can cause severe skin burns. Using rubber gloves, remove the troughs with the papers still in place and place them on a suitable rack or stand in a fume hood for drying.

When the papers have dried, straighten out the folded edge as much as possible, and make a new fold one cm from the second edge near the origin. This fold will be at a right angle to the first fold. Place about 25 ml of butanol-propionic acid solvent in bottom of cabinet. Place papers in trough as before, and place troughs in cabinet. Add 25 ml of butanol-propionic acid solvent to each trough, close the cabinet and allow the chromatograms to develop until the solvent reaches the bottom of the papers, about 5 hours. Again remove excess solvent, hang troughs with papers in fume hood and dry for about two hours. The chromatograms are now ready for radioautography with X-ray film.

Chromatography with Large Papers. Larger chromatograms made with full size sheets of chromatographic paper (46 x 57 cm) have the advantage that larger amounts of material may be analyzed while at the same time better resolution of compounds is obtained.

Mark the origin at a point 7.5 cm in from each of two edges. If marker compounds are to be added, for example alanine and glutamic acid, add to suspension of biological material before applying to chromatograms. In the case of amino acids, about 100 ug of each acid can be applied to a chromatogram. Apply the prescribed amount (see previous sections) of biological material on the origin, using an air or nitrogen stream (a commercial hair dryer may be used for this purpose). Apply biological material at such a rate that wetted area remains small, not exceeding 2 cm in diameter.

Make a fold in the paper 2 cm in from edge near the origin and across

the short direction of the paper. When a second chromatogram has been prepared, fold it similarly, and place the two papers in the chromatographic trough, with folds overlapped and the bar weight on top of the fold. The papers hang from opposite sides of the trough, over the antisiphoning rods.

In order to insure rapid equilibration of vapor and solvent when the cabinet is later closed, about 50 ml of phenol solvent plus 50 ml of warm water may be placed in the bottom of the cabinet before the papers are put in place. Then place the chromatographic troughs and papers in the cabinet. Add 100 ml of phenol solvent to each trough, taking care that the solvent does not splash onto paper outside of trough. Close cabinet tightly. If air ducts have been added to the cabinets as described above, they must be tightly stoppered. Any loss of vapor from the cabinet will interfere with obtaining good chromatograms.

From 24 to 28 hours will be required for the phenol solvent to reach the bottom of the paper. Then open the cabinet, remove excess phenol solvent from the troughs, and dry the papers, either by removing and hanging in a fume hood, or by attaching the air duct (see chromatographic equipment) if the cabinets have been equipped for drying papers in place. Wear rubber gloves if the wet papers are to be handled, as phenol can cause severe skin burns. If phenol does come in contact with skin, the affected area should be immediately rinsed with a few ml of ethyl alcohol, followed by flushing water. Alcohol is much more effective than water for removing phenol from the skin surface layer.

It is necessary to allow overnight for drying the phenol papers. Then fold a second edge next to the origin and along the long direction of the paper. Again place a second paper in the trough and add the bar weight. Place about 100 ml of the butanol-propionic acid solvent in the bottom of

the cabinet. Place the troughs with papers in the cabinet and close tightly as before.

Although the butanol-propionic acid solvent will reach the bottom of the papers in about 16 hours, it is recommended that the papers be allowed to develop for a total of 24 hours, with the solvent dripping off the bottom of the papers during the last part of this period. Very few compounds of biochemical interest will be lost during this period, and much better resolution of the remaining compounds on the paper will be obtained.

If the best resolution of all compounds is required, a second set of chromatograms is prepared and the papers are developed for 48 hours in each direction. In this case, many metabolites will be lost from the paper, but excellent resolution of sugar phosphate and diphosphates, and of nucleotide phosphates can be obtained.[6]

When the second solvent has run for the prescribed time, open the cabinet, remove excess solvent from the troughs and dry the papers as before.

Radioautography. When the paper chromatograms are dry, radioautographs may be prepared by placing the papers in contact with X-ray film. Trim the edges of the papers next to the origin with a straightedge and scissors to one cm from the origin. In order to insure that papers and film can later be precisely matched, mark the papers with radioactive ink at the corners on either side of the corner next to the origin (since no compounds will be likely to travel there). The marks used may also serve to identify the papers and their films.

Two of the small papers or one of the large papers will be placed in contact with a piece of 35 x 42.5 cm medical X-ray film, but for the larger papers, the outer edges will have to be folded around the film. Place the

papers on the film with the inked sides next to the emulsion. If single-coated film is used, be sure the paper is in contact with the emulsion side. A Kodak Wratten 6B filter may be used to provide light in the dark room.

The small chromatograms may be held in place with small pieces of masking tape at the corners. Place the paper and film in the paper cover supplied with the film, and then place this in some light-tight container in which the film and paper will lie flat and undisturbed during the exposure. Commercial X-ray film cassettes, an old film box, or any other light-tight box the right size may be used for this purpose. Since beta radiation from ^{14}C does not penetrate the X-ray film, a number of papers and films in the film wrappers may be piled flat together in a single old film box, without any danger of cross exposure.

Allow the films to be exposed for about five days. Develop the films with medical x-ray film developer and fixer. Dry the films. The radio-autographs may now be examined for the pattern of labeled metabolites formed during the experiment.

Compare the pattern of radioactive compounds with a chromatographic map for these solvents. Identify as many compounds as possible from the map and the relative positions. If a thin window Gieger-Muller tube and counter is available, determine the relative radioactivity of the strongly labeled compounds. For this purpose, it is helpful to first outline with pencil the positions of the compounds on paper by holding film and paper to a window on a light table, using the radioactive ink marks to line up paper and films.
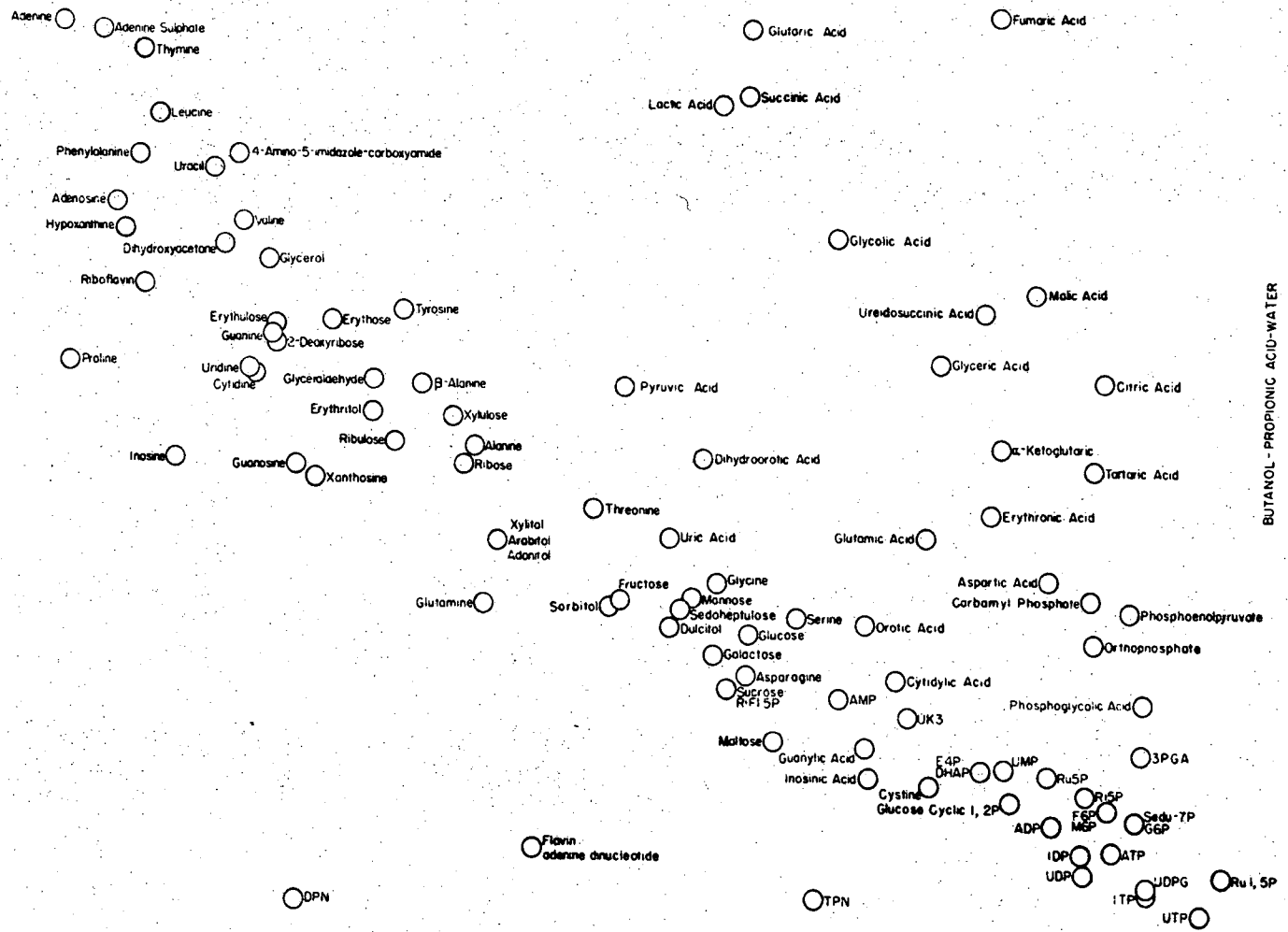
After the compounds have been tentatively identified and counted, the papers may be sprayed with a chemical to develop colored spots if

marker compounds have been added. If amino acids have been added, spray with ninhydrin solution (0.1% in 95% ethanol) and heat the papers in an oven at 90° C for about 5 min., or until the blue-purple spots appear. Other reagents are available for developing color with phosphates or sugars.

Prepare a table of labeled compounds. List compounds tentatively identified (from chromatographic map), compounds identified by co-chromatography, and unknown compounds (as A, B, C, D, etc.). Make a sketch of your radioautograph showing relative positions of all appreciably labeled compounds. In table, give the amount of radioactivity (in counts per minute) for each compound.

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Adenine

Adenine Sulphate

Thymine

Leucine

Phenylalanine

Uracil

4-Amino-5-imidazole-carboxyamide

Adenosine

Hypoxanthine

Valine

Dihydroxyacetone

Glycerol

Riboflavin

Erythulose

Erythose

Tyrosine

Guanine

2-Deoxyribose

Proline

Uridine

Cytidine

Glyceraldehyde

β-Alanine

Pyruvic Acid

Erythritol

Ribulose

Xylulose

Inosine

Alanine

Guanosine

Xanthosine

Ribose

Dihydroorotic Acid

Threonine

Xylitol

Arabitol

Adonitol

Uric Acid

Glutamic Acid

Glutamine

Sorbitol

Fructose

Glycine

Mannose

Sedoheptulose

Serine

Orotic Acid

Dulcitol

Glucose

Galactose

Asparagine

Cytidylic Acid

Sucrose

R·F15P

AMP

UK3

Maltose

Guanylic Acid

Inosinic Acid

E4P

UMP

3PGA

DHAP

Cystine

Ru5P

Glucose Cyclic 1, 2P

ADP

R5P

F6P

Sedu-7P

M6P

G6P

Flavin

adenine dinucleotide

IDP

ATP

UDP

DPN

TPN

UDPG

Rul, 5P

ITP

UTP

Glutaric Acid

Fumaric Acid

Lactic Acid

Succinic Acid

Glycolic Acid

Malic Acid

Ureidosuccinic Acid

Glyceric Acid

Citric Acid

α-Ketoglutaric

Tartaric Acid

Erythronic Acid

Aspartic Acid

Carbamyl Phosphate

Phosphoenolpyruvate

Orthophosphate

Phosphoglycolic Acid

PHENOL - WATER

BUTANOL - PROPIONIC ACID-WATER