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Authors
Bassham, J.A.
Taylor, S.E.

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Lawrence Berkeley Laboratory
UNIVERSITY OF CALIFORNIA

CHEMICAL BIODYNAMICS DIVISION

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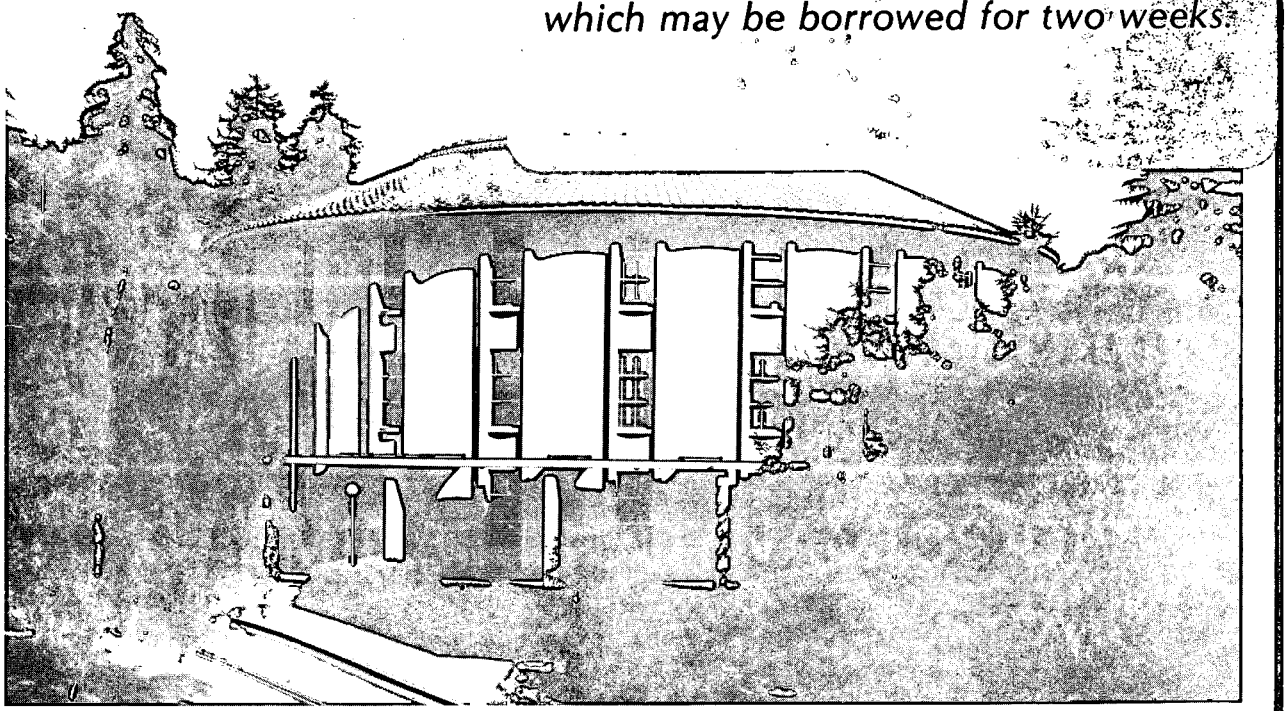

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CARBON METABOLISM

James A. Bassham and Scott E. Taylor

Chemical Biodynamics Division
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

Abstract

Photosynthetic carbon metabolism in cyanobacteria can be studied *in vivo* by exposing cells to ^{14}C and ^{32}P radiolabelled substrates and isolating the products by chromatographic techniques. Experimental designs for the exposure of the cyanobacteria to radioactive compounds in a closed system are discussed. Techniques for product separation by two-dimensional paper chromatography, two-dimensional thin-layer-cellulose chromatography and two-dimensional thin layer electrophoresis/chromatography are described. In addition, a technique utilizing ^{13}C-NMR is discussed.

1. Introduction

Photosynthetic carbon metabolism in cyanobacteria may be studied by exposing photosynthesizing cells to $^{14}CO_2$, subsequently killing the cells, and then separating and identifying the labelled metabolites by a suitable chromatographic technique. Early indications[1] and subsequent detailed studies[2] showing that photosynthetic carbon dioxide incorporation in cyanobacteria occurs via the reductive pentose phosphate cycle (Calvin cycle) were obtained by these methods.

For study of dark metabolism in these organisms, the cells may be first labelled by photosynthesis with $^{14}CO_2$. Alternatively, ^{14}C-labelled compounds such as glucose may be employed as a substrated in such studies. The use of ^{32}P-labelled phosphate, in light and dark, provides additional information about phosphorylated metabolites and levels of ATP and ADP[3,4].

Certain types of kinetic tracer studies are complex, and require specialized equipment. While these requirements will be described, simpler alternatives will be presented for those interested in less complex or less quantitative investigations.

The information obtained from tracer studies <u>in vivo</u> can be used not only to map metabolic pathways but also to study of metabolic regulation, as has been the case with light-dark regulation in green algae[5]. A complete understanding of carbon metabolism requires other kinds of studies such as enzymology and gene expression which are not covered in this article, but will be found in other articles in this volume and in others. In general, techniques used to measure carbon metabolism in other organisms can be applied to cyanobacteria[6].

The present discussion is devoted mostly to the use of labelled substrates with whole cells and to alternative methods for analysis of the resulting labelled metabolites. The classical method of analysis by two-dimensional paper chromatography is still the method of choice for some workers because of its capability of separating a broad range of metabolites: sugar phosphates, sugars, amino acids, carboxylic acids, etc. This method is time-consuming and somewhat tedius, however, and it requires equipment lacking in many laboratories. Variations involving thin-layer chromatography and electrophoresis have been used with success and provide useful alternatives to paper chromatography.

As an alternative to radioactive tracer studies, analysis of some metabolites by ^{13}C-NMR is briefly described.

2. In vivo metabolic studies with radioisotopes.

A. Cell culture. A number of different species of cyanobacteria have been used successfully in tracer metabolic studies[1-4], and it is likely that any photosynthetically-active, single cell species would serve. Multicellular species, such as filamentous cyanobacteria, also can be used, provided they do not represent sampling problems. In general it is desirable to harvest the cells by centrifugation at room temperature, typically 20 min at 20,000 g, followed by resuspension in fresh growth medium. Provided other environmental conditions are kept the same as during growth (temperature, light intensity, CO_2 concentration etc.), the metabolic pattern identified should be representative of that of the growth conditions.

The subsequent analysis of metabolites by chromatography can be adversely affected by high salt concentrations in the media, so that

experiments with marine algae present great difficulty. Desalting methods can be attempted, but often at the cost of loss of quantitative results. It may be possible to grow the cells with a more dilute medium than that prescribed in the literature without serious alteration in the cell metabolism. For some studies, a deliberate change in the suspending medium may be made. For example, the metabolic effects of short-term nitrogen deprivation might be investigated.

After centrifugation, the cells are resuspended in the experimental medium at 1% packed cell volume/suspension volume. Such a concentration usually ensures that when the cells are later killed in 80% alcohol, an aliquot sample of sufficiently small volume can be used for analysis and will contain enough labelled material for quantitative measurement of the labelling of each metabolite, provided other requirements described below are met.

B. Preillumination and gas mixtures. Since it is generally desirable that the experiment be carried out under conditions permitting steady-state metabolism, the cells should be placed immediately in the experimental vessel and be allowed to photosynthesize with unlabelled CO_2 for 30 min to an hour. If the experiment is to be carried out with air levels of CO_2, the cell suspension is provided with a bubbler and air stream during this period. Studies with gas mixtures other than air require some system for bubbling the prescribed gas through the cells during both preillumination and during the exposure to $^{14}CO_2$. While appropriate gas mixtures can be supplied during preillumination by gas tanks and flow metering devices, maintenance of $^{14}CO_2$ at constant specific radioactivity and CO_2 pressure for more than a minute or two

may require a fairly complicated gas handling system, such as that described below.

C. Sources of $^{14}CO_2$. For the simplest, short-term experiments, a solution of radioactive bicarbonate, $H^{14}CO_3^-$, may be injected into a closed, illuminated vessel for a few seconds to a minute and the contents of the flask can then be poured into a killing solution (ethanol)in a fume hood. Such an experiment might serve, for example, to test for the presence of carboxylases in a mutant or wild-type.

For quantitative, kinetic experiments it is necessary to maintain the concentration and specific radioactivity of the carbon dioxide and to control the physiological conditions, either keeping them constant or varying one element (such as light intensity) in a predetermined way. A gas-handling system is required, and usually this will be a closed, gas recirculating system, employing a gas pump, since conservation of $^{14}CO_2$ is necessary for both environmental and economic reasons. Provision must be made for either storing any unused radioactive gas in a closed vessel or absorbing it in alkali at the end of the experiment. If a sufficient quantity is stored in a closed vessel that can be reopened to the system later, it may be possible to conduct several experiments before the concentration or specific radioactivity falls below acceptable levels.

Gas circulation and control require the development of a closed gas recirculating system which meets various requirements. For circulation, a small vibrating diaphragm gas pump capable of circulation rates of several liters per minute may be used. Considerable gas impervious tubing, such as glass or stainless steel, is required to connect the pump with various inlet and outlet valves including those leading to the

cell vessel and others for the inlet of gases such as air and radioactive carbon dioxide, and to gas storage reservoirs, to the sensors of the gas monitoring instruments. This tubing should be kept small enough (on the order of 3 mm I.D.) to keep the overall volume of the minimum system from becoming large compared to the gas storage vessels. In developing a gas handling system one may have to spend some weeks or months adjusting flow rates and pressures with various valves, flow meters, etc. before achieving satisfactory operation. Once developed, however, such a "steady-state" apparatus offers many possibilities for productive studies of metabolism.

The gas should be circulating in the closed gas recirculating system before the end of the preillumination period, separated from the vessel containing the cells only by a four-way stopcock or equivalent valve system. The volume of gas in the tubing leading from these valves to the cell vessel and back should be small compared to the gas flow rate so that once the valves are switched to admit radioactive gas to cells, no more than a few seconds are required to replace ordinary carbon dioxide with labelled gas. If instantaneous change is required, labelled bicarbonate solution of the same specific radioactivity can be injected directly into the vessel at the moment the valves are switched.

A gas cylinder equipped with suitable reducing valves and flow valves may be used for addition of $^{14}CO_2$ to the system. Such a radioactive storage tank should be enclosed in a safety chamber vented through a CO_2-absorbing material (ie, a commercial preparation of soda lime designed for this purpose). Any radioactive gas handling equipment should be approved and monitored by the health safety officers of the institution.

For experiments with lesser requirements, $^{14}CO_2$ can be generated on a vacuum line (again, this should be an enclosure approved for this purpose by safety officers!) by addition of acid to $Ba^{14}CO_3$ and trapping in a loop closed by a four-way stopcock (so the radioactive gas can be flushed out when needed) and equipped with quick-fit joints for attaching to the gas-recirculating system.

D. Light intensity and vessel shape. If a 1% suspension is used, it may be necessary to use a thin vessel and rather strong illumination in order that the cells are exposed to light at an intensity comparable to that of the growth conditions. For experiments at relatively high light intensities, a flat illumination vessel with plastic walls and a space about 6 mm thick and 50-100 ml in total volume has been found useful[2,7,8]. With illumination from both sides, an individual cell is never shaded by more than 3 mm of suspension, and with rapid stirring from the gas bubbling all cells are exposed to an average high light intensity. If strong illumination is used, provision for temperature control, such as a transparent water jacket, is required.

E. Sampling. Sampling requirements vary with the objective. For a relatively slow metabolic conversion, such as the conversion of an applied labelled metabolite to an end product in a secondary biosynthetic pathway, samples probably could be withdrawn by opening a stopcock or by removing the sample with a syringe through a rubber stopper and placing in methanol (final concentration after addition of sample = 80%). For quantitative, kinetic studies of the fast reactions of carbon metabolism in photosynthesis, sampling and quenching (killing) must be accomplished within about 1-2 seconds, since any longer time permits

unacceptable amounts of enzymatic conversions during non-steady-state conditions.

One solution to this problem is to equip the vessel with an outlet valve operated by a pushbutton-controlled solenoid[7]. The stainless steel conical valve, seated on the end of a Teflon outlet tube, is lifted by a wire attached to the tip of the cone and running down through the outlet tube to a lever attached to the solenoid. When activated, the solenoid operates against a compressed spring to raise the valve for a period of about 0.2 sec. As a further refinement, N_2 is injected just at the base of the valve to flush the outlet tube. Sample size is determined by weighing the vial (plus methanol) before and after sampling.

F. Other illumination vessel refinements. For certain types of experiments, the illumination vessel can be fitted with sensing devices, including a pH electrode and a device for monitoring density[7,8]. The outputs from these sensors can be employed to control the automatic addition of acid or base, or of medium to maintain pH, density, or both. For example, kinetic studies of the effects of addition of ammonium ion, as ammonium chloride, required the automatic addition of dilute ammonium hydroxide, since uptake of NH_4^+ caused the green algae used to acidify the medium. Automatic density control is not likely to be needed in most cases since experiments usually will not last long enough for significant density change due to growth to occur.

To insure rapid gas-liquid equilibration, particularly at low CO_2 pressures such as in air, the tip of the bubbler may have to be of sintered glass to provide fine bubbles.

G. Gas monitoring. For experiments requiring addition of $^{14}CO_2$ as a gas and employing a closed gas-recirculating system, instruments to monitor levels of CO_2 and ^{14}C are a necessity. Carbon dioxide can be measured with an instrument which makes use of the absorption of infrared radiation by CO_2. It should be noted that these instruments are somewhat nonlinear in response and require a calibration curve. Moreover, they are much less sensitive to $^{14}CO_2$ than to $^{12}CO_2$, so that total carbon dioxide level can be underestimated when an appreciable fraction is $^{14}CO_2$.

The level of ^{14}C can be monitored with an ionization chamber and a vibrating reed electrometer.

For studies of photosynthesis and respiration, it is also useful to monitor oxygen with an oxygen meter. Solid-state devices are much easier to employ than the older paramagnetism measuring instruments.

A record of the signals from these gas monitoring instruments made with a multichannel recorder allows the specific radioactivity, rates of photosynthesis and respiration, and composition of gas mixture to be calculated during and after the experiments.

H. Specific radioactivity. For quantitative, kinetic tracer experiments, it is usually necessary to start with a high level of radioactivity. The best chromatographic separation is achieved when the quantity of material applied to the chromatogram is small. For the procedure described below, the amount of material applied to the paper chromatogram is derived from about 2 g of wet-packed cells. Only submicrogram amounts of a given intermediate compound may be present. Pure $^{14}CO_2$ would be roughly 60 Ci per mole, and it is useful to employ from 10 to 50 Ci/ mole. This gas should be circulating in the closed

gas recirculating system before the end of the preillumination period, separated from the vessel containing the cells by a four-way stopcock or equivalent valve system.

I. Other labelled substrates. Other labelled substrated such as ^{14}C-labelled glucose (with either uniform labelling or with the label in a specific position), ^{32}P-labelled inorganic phosphate or other phosphorylated compounds, or even tritium(^{3}H)-labelled compounds may be added as solutions injected into the cell vessel through a soft plug with a needle. In the case of ^{14}C-labelled compounds, usually unlabelled carbon dioxide is supplied. High specific radioactivities are required, as discussed above. For ^{32}P-labelled compounds, the radioactivity emissions are much stronger than for ^{14}C, so there is no difficulty in obtaining enough radioactivity, but special precautions must be taken to avoid undue exposure of workers and laboratory contamination. Detailed plans should be discussed with health safety officers.

Dual tracer experiments, employing $^{14}CO_2$ and ^{32}Pi, have been useful in studies of carbon metabolism during photosynthesis and respiration in both green algae[9] and in cyanobacteria[2-4]. The specific radioactivities of the two labelled substrates should be chosen to give comparable film exposures when radioautographs are prepared.

J. The exposure to $^{14}CO_2$. At the conclusion of preillumination to achieve steady-state photosynthesis, the four-way stopcock or valve system is turned, shutting off the flow of unlabelled gas to the vessel and starting the flow of gas containing $^{14}CO_2$. Samples are taken at predetermined intervals. If initial rates of labelling are of interest, samples will be taken every five seconds or so at first, then less frequently up to 10min. By that time, the reductive pentose phosphate

cycle intermediates will be "saturated" with radiocarbon in an actively photosynthesizing culture. The levels of radioactivity in these compounds "at saturation"", when later determined following analysis and radioactivity counting, may be used to calculate the pool sizes, that is, the concentration of the intermediates in the cells, provided the specific radioactivity of the administered carbon dioxide is known[10].

For cell suspensions of 1%, 1 ml samples may be taken directly into 4 ml of ethanol at room temperature in 10 ml vials, preweighed with ethanol and screw caps. The caps are removed briefly for sample taking, then are replaced and the vials reweighed to obtain precise sample sizes.

K. Changes in physiological conditions. When enough time (10 min or more) has passed for metabolites to become fully labelled or "saturated", a physiological condition may be suddenly changed, and samples of the cells taken and killed for analysis to study the most immediate metabolic effects of the change. Some changes that have been studied include light to dark, changes in CO_2 level, O_2 level, and addition of NH_4^+. Since transient changes in the primary photosynthetic pathways and some secondary pathways (for example, synthesis of certain amino acids such as alanine and aspartate) occur within seconds, samples should be taken as rapidly after the environmental change as possible. For example, in one study of light-dark changes in the three species of cyanobacteria[2], labelled 6-phosphogluconate, not detected in the light, rose to its maximum value within a minute after the light was turned off.

3. Separation and identification of labelled metabolites.

A. Two-dimensional paper chromatography. This chromatographic method which played a key role in the discovery of the photosynthetic reductive pentose phosphate cycle (Calvin cycle) in green algae and higher plants[11] was used later, with modifications[12], to examine light-dark regulation and other types of regulation in green algae and carbon metabolism in several species of cyanobacteria[2-4]. In addition to the products of photosynthetic and respiratory metabolism with $^{14}CO_2$, labelled metabolites formed in Aphanacapsa 6714 from ^{14}C-labelled glucose and from ^{32}P-labelled phosphate during dark aerobic and anaerobic metabolism were separated by this method. It should be noted that the specific radioactivity of the uniformly-labelled glucose used as substrate was 20 μCi/μmole. Since this amounts to only about 3.3 μCi/gram-atom of carbon, it is close to the lower limit of specific radioactivity that can be used and still give enough label in individual compounds on the paper chroamtograms to permit their location by radioautography in a reasonable time on the developed paper chromatograms.

As mentioned earlier, in a typical experiment, 1 ml samples of a 1% cell suspension are killed in 4 ml of methanol. A one-fifth aliquot of this algae-water-methanol mixture is applied slowly with an air stream to the origin of Whatman No. 1 chromatographic paper (47 cm x 56 cm).

The chromatograms are developed in two directions, by descending chromatography, in a vapor-tight cabinet. The first solvent, used in the long direction of the paper, is made up of 840 ml "liquified" phenol (about 88% pure phenol and 12% water), 160 ml water, 10 ml glacial acetic acid, and 1 ml 1.0 M ethylenediamine tetraacetic acid adjusted to pH 4.0. The second solvent is made up just before use by mixing equal

volumes of n-butanol-water (370:25 v/v) and of propionic acid-water (180:220 v/v). For a trough with two papers, about 100 ml of chromatographic solvent is needed. For movement of the solvents to the bottom of the paper, the time for development in each direction can range from about 16 to 24 hr, depending on the number of papers, the dimensions of the cabinet, room temperature, etc. Since higher resolution of the phosphorylated compounds may be desired, duplicate sets of chromatograms can be prepared, and one set developed as long as 48 hr in each dimension to obtain greater migration of these compounds, even though this results in other labelled metabolites being lost from the paper Constant room temperature is required for good results. After development in the first direction, the papers must be dried before running in the second direction. A map of a typical chromatogram is provided in Fig. 1.

B. Two-dimensional thin-layer chromatography. Radiolabelled intermediates (both ^{14}C and $32_{P)}$ can also be separated on cellulose-coated plates. To improve separation of ^{14}C-labelled compounds, one-dimensional ion exchange paper chromatography is performed as a first step[11]. Thin strips (2 x 30 mm) of Amberlite SB-2 paper (Serva Co.) are activated with 10 M formic acid and then washed with distilled H_2O. A 10 µl aliquot is applied to one end of the paper strip and the neutral fraction is eluted with 100 µl of 10 M formic acid. These two fractions are then chromatographed separately.

Cellulose plates are prepared by application of a 0.25 mm thick cellulose-water slurry prepared with Cellulose MN 300 (Machery, Nagel and Co.). A 5-20 l sample is applied as a small spot to a corner of the plate, 2.5 cm from each edge. For ^{32}P-labelled compounds, the chromatogram is developed in the first dimension using a solvent system

of isobutyric acid-NH_3-H_2O-EDTA (1000:50:550:0.5, v/v/v/g). The chamber is pre-equilibrated for 5 hr, and the chromatogram then is run for 8 hr. After drying, the TLC is developed twice in the second direction, first for 5 hr (after a 3 hr pre-equilibration) with n-butanol-n-propanol-n-propionic acid-H_2O (400:175:285:373) and then for 5 hr (after a 3 hr pre-equilibration) with n-butanol-acetic acid-H_2O (5:1:4). For ^{14}C-labelled compounds, the chromatogram is developed in the first dimension with isobutyric acid-n-butanol-isopropanol-n-propanol-NH_3-EDTA (1000:30:30:140)40:380:0.5, v/v/v/v/v/v/g). The chamber is pre-equilibrated for 5 hr and the chromatogram is run for 8 hr. The plate is developed twice in the second dimension as described above for the ^{32}P system[12].

C. Thin-layer electrophoresis/chromatography. Labelled intermediates may also be separated by a combination of electrophoresis and chromatography[13]. This technique has been found to be superior to two-dimensional TLC for the separation of polar intermediates[11].

Cellulose thin-layer plates are prepared and spotted as above. The sample spot is covered with a small piece of parafilm and the plate sprayed with the electrophoresis buffer (pyridine-acetic acid-H_2O, 1:3:5:95.5, pH 4.0) until shiny. The parafilm is removed and electrophoresis is performed at 0°C for 1 hr at 900 V on a horizontal electrophoresis apparatus. Whatman No. 1 paper wicks are used to connect the plate to the buffer trays which contain the electrophoresis buffer. The starting point is on the cathode side.

After completion of the electrophoresis run, the plates are dried under cool air and developed in the second dimension using a system of 1-butanol-formic acid-H_2O (6:1:2). The chromatography is stopped when

the front travels 14 cm, the plate is dried and rechromatographed with the same solvent system until the front reaches 17 cm[13].

D. Autoradiography. Location of the labelled compounds on the chromatograms is accomplished by placing the papers in contact with a sheet of medical x-ray film (single emulsion) for 3-7 days (if possible at -70°C), after which the film is developed. If ^{32}P-labelled inorganic phosphate was used as a substrate, it is necessary to avoid fogging the film. This is accomplished by a preliminary autoradiography for 30 min to reveal the center of the inorganic phosphate spot. A somewhat larger area is cut from the paper, which is then replaced on a fresh film for the longer exposure.

E. Identification. A preliminary indication of the identity of a labelled compound can be made from its position. The next step in identification is to obtain an authentic sample of the suspected compound, chromatograph a few micrograms of it, and then convert it to a colored spot on the paper by spraying it with a chemical reagent. Some of the most useful ones are the ninhydrin spray for amino acids[14], the Haynes-Isherwood spray for phosphates[15] and silver sprays for free sugars[16]. Other tests may be obtained from standard work on color reagents[17].

Once the procedure has been tested, the appropriate amount of authentic compound is mixed with enough of the eluted labeled compound to expose an x-ray film in a reasonable time, the mixture is chromatographed and an autoradiograph is prepared, after which the paper is treated to give the colored spot. Coincidence of the spot not only with respect to its location but also the fine details of the spot shape is good evidence for identity. Further chromatographic evidence can be

obtained if the compound can be transformed chemically, rechromatographed to a new location and again sprayed to give a colored spot. For example, a sugar phosphate could be first identified as a phosphate and later, after hydrolysis of a fresh sample, as a sugar. In dual tracer experiments, using ^{14}C and ^{32}P, the first of these two steps (identification as a phosphate) is already accomplished by the tracer. If two films are placed in contact with the paper, the first will register both isotopes, while the second will be exposed only by the more energetic beta particles from the ^{32}P.

F. Counting. Radiolabel incorporation can be determined by slicing the chromatography spots into small pieces, then placing the pieces into a 20 ml scintillation vial, then adding 5 ml of H_2O and shaking for 1-2 hr. About 15 ml of a high quality all purpose scintillation cocktail is added, and the vial is shaken to form a one-phase system (a gel). Samples can then be counted by liquid scintillation counting.

G. Characterization of carbohydrates by ^{13}C-NMR. The most abundant carbohydrate components can be determined using proton-decoupled ^{13}C-NMR. In vivo levels of sucrose, glucosylglycerate, glucosylglyceral and free glutamate have all been measured using NMR[18,19].

To perform this technique, cells are grown in enriched (20-30%) $^{13}CO_2$ environments. Cells are concentrated by centrifugation (5000g for 10 min)[18] and resuspended in growth media to a final concentration of 0.25 g (dry weight) per liter[18]. The solution is made 10% D_2O to provide a lock signal[19]. The sample is placed in a 1 cm diameter NMR tube, containing two 1 mm diameter tubes containing either degassed methyl iodide[19] or tetramethylsilane[18] as line standards. Fourier transformed, proton-decoupled pulsed ^{13}C-NMR spectra are then obtained,

using a line broadening of 20 Hz and 1 sec intervals between pulses[19]. Volume measurements can be obtained by incubating the packed cells with 1 mM Tempone and 90 mM Mn-EDTA to determine the ESR signal of the intercellular probe as described previously[20].

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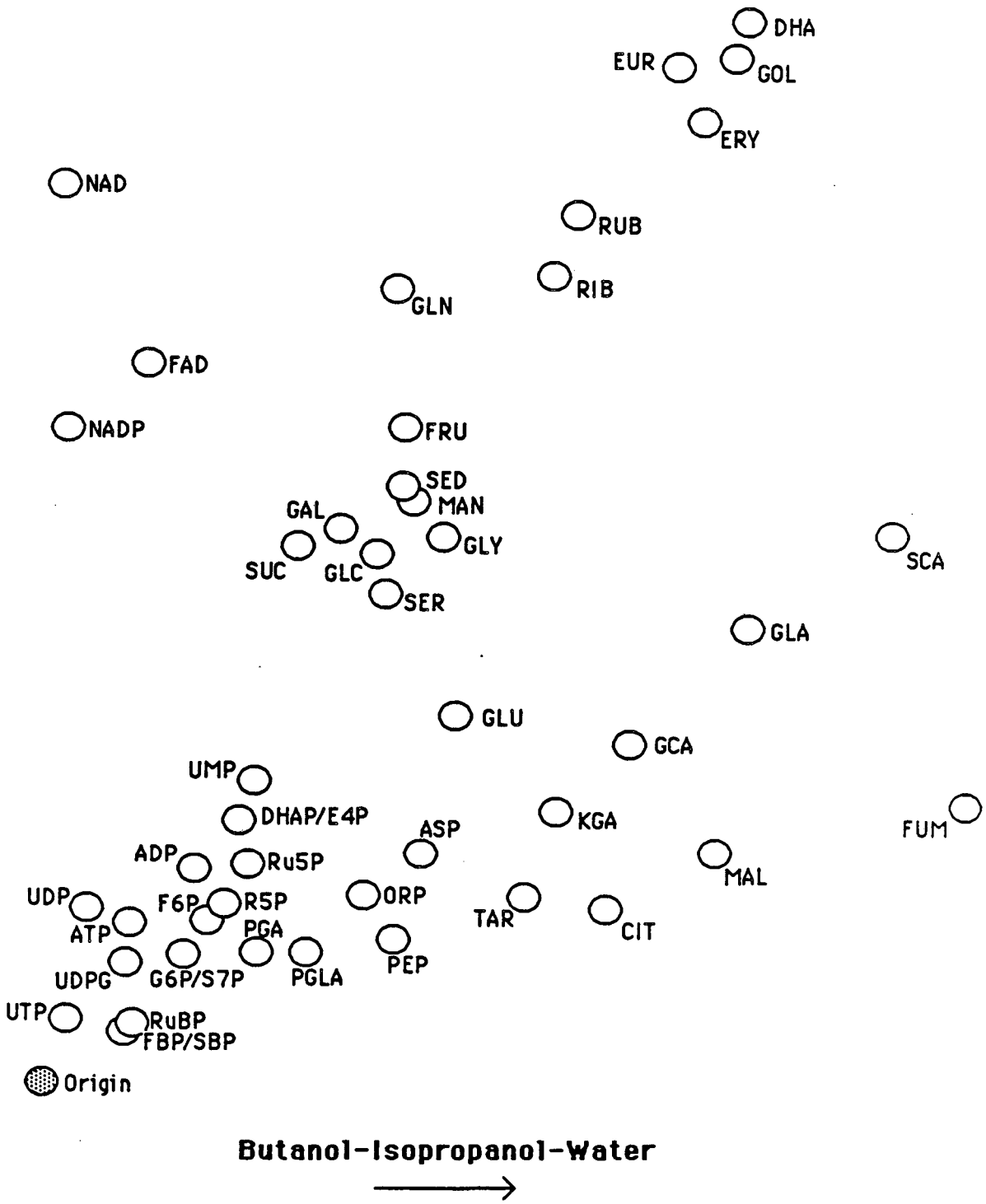
Figure 1. Map of Two-Dimensional Paper Chromatogram. The actual distance travelled may vary from chromatogram to chromatogram, but the relative positions will remain constant. (ASP, aspartic acid; CIT, citric acid; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; ERY, erythrose; EU, erythrulose; E4P, erythrose 4-phosphate; FAD, flavin adenine dinucleotide;FBP, fructose 1,6-bis phosphate; FRU, fructose; FUM, fumaric acid; F6P, fructose 6-phosphate; GAL, galactose; GCA, glyceric acid; GLA, glycolic acid; GLC, glucose; GLN, glutamine; GLU, glutamic acid; GLY, glycine; GOL, glycerol; G6P, glucose 6-phosphate; KGA, alpha-keto glutaric acid; MAL, malic acid; MAN, mannose; ORP, orthophosphate; PEP, phosphoenol pyruvate; PGA, 3-phosphoglyceric acid; PGLA, phosphoglycolic acid; RIB, ribose; RUB, ribulose; RuBP, ribulose 1,5-bis phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; SBP, sedoheptulose 1,7-bis phosphate; SCA, succinic acid; SED, sedoheptulose; SUC, sucrose; S7P, sedoheptulose 7-phosphate; TAR, tartaric acid; UDPG, UDP-glucose).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DHAP/E4P
KGA
FUM
ASP
ADP
Ru5P
MAL
UDP
F6P
R5P
ORP
TAR
CIT
ATP
PGA
PEP
UDPG
G6P/S7P
PGLA
UTP
RuBP
FBP/SBP
Origin
Phenol-Water
Butanol-Isopropanol-Water

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TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720