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CHROMATOGRAPHY OF LABELLED CELL EXTRACTS

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V. Moses

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CHAPTER

CHROMATOGRAPHY OF LABELLED CELL EXTRACTS

Introduction. The pioneer work of Calvin and his collaborators[1, 2] at the University of California on the pathway of carbon in photosynthesis, made extensive use of the techniques of chromatography and radioautography following the feeding of plant material with labelled carbon dioxide. By killing and extracting the cells after various short incubation periods, and analysing the products of carbon dioxide metabolism chromatographically, they were able to work out eventually the whole metabolic path by which the carbon in carbon dioxide becomes converted to carbohydrates, carboxylic acids, amino acids, etc. In spite of the power of the method for studies in intermediary metabolism, it has not been used extensively, though studies have been made of the dark fixation of carbon dioxide and the metabolism of some carbohydrate substances in certain tissues.[3, 4, 5, 6] The lack of use of these techniques is probably due to the technical complications, which appear formidable unless personal experience in their use has been gained. It is the object of this chapter to describe in detail the various manipulations involved in experiments of this type together with some remarks on modifications necessary when different biological tissues are used, and the interpretations of the findings, in the hope that more use will be made of these methods once it is realized that the difficulties involved are not insuperable.

Applicability and Limitations of the Method. The chromatographic system to be described is capable of separating satisfactorily on one or two chromatograms most of the substances present in the soluble

extracts of cells which are included by the following classes of compounds: free sugars, sugar acids, phosphate esters of sugars and sugar acids, carboxylic acids (especially di- and tri-carboxylic acids), amino acids, nucleosides and nucleotides, and certain purine and pyrimidine bases. It must be stressed that the solvent systems to be described below are not necessarily the best for separating compounds within each of these classes, when only such substances are present in the mixture; for this purpose reference should be made to the appropriate chapter elsewhere in this book. However, the solvents are very suitable for achieving a good separation of most of these compounds when they are present together in one and the same cell extract. The system is designed to be used only for the analysis of radioactive compounds, owing to the very great sensitivity of the detection techniques (e.g. X-ray film radioautography: see p.) when the compounds contain sufficient amounts of radioactivity. It cannot be used when chemical detection methods have to be employed, (a) because of the number of different colour tests which would have to be applied to the same paper, and (b) because of the limited amount of cell extract which can be accommodated on one sheet of paper, and the variety of compounds which it contains: many substances would be present in concentrations much too low to be detected colorimetrically.

The chromatographic maps reproduced in Figs. 1, 2, and 3[2] show the positions of over 100 different compounds: not all of these can be satisfactorily separated together, but when a biological system is incubated with a labelled substrate, label is hardly likely to be found in the same extract in all 100 mapped substances. In practice, there is little difficulty in separating the 30 or 40 compounds which may be produced from a labelled starting material in even a fairly long incubation period.

These techniques are not directly applicable to the ethanol-water insoluble material of cells, and to the lipid components: thus fats, polysaccharides, proteins, peptides, nucleic acids and other large molecules cannot be investigated unless they are first separated from each other and then broken down into smaller units. The separation of the large molecules from each other in a complex mixture is a very difficult problem, as with labelled substances the separations must be virtually complete, and will not be discussed further.(7)

Tissue Preparation

The metabolism of labelled substances by animal, plant and microbiological tissues, as well as by sub-cellular particles, can be investigated by these techniques, though different practical considerations are necessary depending on the source of the tissue.

Experiments will probably be of two basic types: (a) a kinetic study of the metabolism of one substance by one tissue, involving the taking of samples from the reaction mixture at various time intervals after addition of the labelled substrate; and (b) studies of the metabolism of different labelled substrates by one tissue, or of one substrate under different experimental conditions. (Experiments involving the metabolism of one or more substrates by different tissues are not considered here as they may be regarded for our purpose as a series of different experiments.) In both of these types of experiment it is necessary, or at least desirable, to be able to take replicate samples from the incubation mixture for analysis. For reasons which will become clearer later, experiments of the sort described here need small amounts of tissue, and it is thus advantageous to be able to pipette the tissue as a suspension.

The nature of the medium in which the tissue is suspended is of great importance. Excessive quantities of salt must be avoided on chromatograms. Once a tissue is killed and extracted, it becomes very difficult to separate the inorganic salts of the medium from materials extracted from the cells. The usual desalting techniques (electrolytic desalting, or removal of salt with ion-exchange resins) can normally not be used. With the exception of free sugars, most of the substances of importance in intermediary metabolism are themselves ionized at one pH or another.(8) In practice, salt must either be nearly or completely absent from the incubation medium, or else the tissue must be effectively separated from the medium before it is killed and extracted.

Micro-organisms. These organisms are among the most satisfactory to use. They are often unicellular, and thus present no problems of sample replication. They can be used as whole organisms, and do not suffer the damage sustained by the tissues of a higher organism when these are removed from their in situ locations and cut into small pieces, or otherwise mutilated. Many micro-organisms can be suspended in distilled water without showing deleterious effects. Even when salt is necessary to provide essential elements, or as buffers, the salt concentration for short experiments can often be kept very low, between 0.01 M and 0.001 M. Micro-organisms can readily be washed free from salt present in their growth medium, and are frequently very active metabolically when compared with the tissues of higher plants and animals. For this reason very small quantities of cellular material are all that are required, with a resultant simplification of chromatography.

The amount of living material in a suspension of micro-organisms is conveniently measured by determining the turbidity of the suspension and

comparing this with a standard curve of turbidity versus dry weight or total protein, or by centrifuging the cells out of suspension at a suitable speed in haematocrit or graduated conical centrifuge tubes, and finding the volume of the wet packed cells. If desired, the dry weight of a pellet of wet packed cells of known volume can be found in the usual way. The amount of material to be used in each experimental sample will depend on a number of factors, chiefly the number of chromatograms to be run with each sample, the amount of cell extract which can be spotted onto each chromatogram, and whether operations other than chromatography are to be performed with each sample. The amount of material which can be spotted onto a paper will be mentioned again below.

Filamentous micro-organisms should, if possible, be prepared in the form of pellets, or as suspensions of very short filaments, so that the tissue can be dispensed in a pipette.

Animal Tissues. For reasons of sample replication mentioned earlier, it is desirable to obtain animal tissue in a form which can be dispensed in a pipette. Slices can be used if there is no alternative, but to achieve randomization of sliced material several slices must be used, and these constitute a relatively large amount of material, only a small proportion of which may be required for chromatography; the rest thus represents a surplus. Owing to the high cost of radioactive materials, large excesses of the incubation material above chromatographic requirements are best avoided.

Apart from cells grown in tissue culture, there are two sorts of animal tissue preparations which are convenient for the experimental procedure under discussion. One is a homogenate of the tissue. Since

this contains a high proportion of damaged cells, many metabolic reactions which depend upon the presence of an integrated cell architecture may be lost. The other preparation is tissue which has been sliced or chopped in three dimensions to produce small cubes or prisms. While the outermost layer of cells may have been damaged, there is a considerable residue of more or less intact cells in the body of each prism. A suitable chopper has been designed by McIlwain and Buddle,[9] and is marketed by H. Mickle, Gomshall, Surrey, England. Briefly, the machine consists of a turntable on which the tissue is placed on several layers of moist filter paper, while an arm holding a razor blade (Wilkinson-Sword) is brought down sharply on the tissue and cuts it through. Between each blow with the razor blade, the turntable moves a pre-determined (and adjustable) distance, resulting in a series of cuts through the tissue which is thus sliced. The slices are next laid flat onto another pad of filter paper, and the chopping operation repeated again, this time in two directions at right angles to each other. The result is a large number of small cubes or prisms of fairly constant size. The prisms can be made small enough to pass through the mouth of a pipette, and have a large surface area in proportion to their bulk, enabling rapid penetration of labelled substrate to take place to the cells in the centre. The distance between cuts is adjustable between 0.052 mm. and 0.728 mm., and the machine is operated electrically. It has been found convenient with several rat tissues (liver, brain, and kidney) first to slice the organ with the cuts at 0.728 mm. intervals, followed by cutting the slices in two directions at 0.312 mm. intervals. Some tissues, such as testis, are so soft when the limiting capsule is removed, that preliminary slicing at 0.728 mm. intervals is not necessary, and two dimensional chopping at 0.312 mm. of

the whole organ after removal of the capsule is adequate. The chopper is easy to use after a little practice, and should be employed in a cold room, the tissue being kept chilled until just before the incubation period commences.

After chopping, the tissue may be centrifuged at low speed and the debris from the damaged cells removed by washing. The tissue prisms are then suspended in the incubation medium at an appropriate concentration, best found by experience, bearing in mind the amount of cell extract which can be placed on chromatography paper.

Animal tissue must be suspended in a salt mixture, often containing glucose. The salt concentration is usually quite high (of the order of 12 mg./ml.), and as much as possible of this salt must be removed before the tissue is killed. In some circumstances it may be permissible to centrifuge the tissue and wash away the remaining salt before killing it, though changes in the tissue are likely to occur once the salt has been removed, and before it is killed. It would be difficult to wash the tissue free of salt by centrifugation in less than several minutes, and this may represent a significant fraction of the total incubation time.

A technique which has been found suitable for animal tissues is as follows. Sintered glass funnels of No. 2 porosity (pore size 40-90 μ.) are fitted with rubber stoppers on their stems. A suitable size of funnel when small volumes of incubation mixture are being used (1-5 ml.) is one of 30 ml. capacity, with a disk 30 mm. in diameter. "Celite" filter aid is slurried in distilled water, poured into the funnel, and the liquid removed with a water pump leaving a layer of Celite on the sintered disk. The funnel is then placed in the neck of a vacuum flask,

but instead of a seal being made between the rubber stopper on the funnel and the neck of the flask, a rubber ring is interposed between the stopper and the flask. This ring should be made of fairly thick rubber (so that it will not collapse under vacuum), should be large enough to cover the opening of the vacuum flask leaving several mm. all round as a margin, and should contain a central hole large enough for the glass stem of the sintered funnel to pass through easily, but small enough for the rubber stopper to remain entirely on top of the ring. The ring is placed on top of the vacuum flask, the stem of the funnel inserted loosely into the hole, and a vacuum applied to the side tube of the vacuum flask. It will be found that a good seal is obtained between the funnel and the ring.

At the end of the incubation period, the tissue suspension is poured in toto onto the sintered disk. The liquid is rapidly (less than 1 sec. for small volumes) sucked through the disk into the flask by vacuum, leaving the tissue (prisms, slices, etc.) on the Celite. A few ml. of washing water may be passed through (another 1 sec.). The vacuum is broken by turning up the edge of the rubber sealing ring, the funnel is removed, its stem inserted into a centrifuge tube so that the rubber stopper forms a seal, and 5 ml. of boiling 80 per cent ethanol are poured onto the tissue on the disk. The rubber sealing ring enables the vacuum to be broken instantaneously: it is sometimes very difficult to extract a well-fitting stopper from a system under vacuum. The whole operation of filtering and killing the tissue can be performed, with practice, in a few seconds. Using 1 ml. of incubation mixture, followed by 5 ml. of washing water, and 5 ml. of 80 per cent ethanol for killing, over 80 per cent of the medium can be removed before the cells are killed.

Fairly large porosity sintered funnels are desirable to allow rapid filtration to take place. However, the sintered disks tend to be clogged rapidly owing to the softness of the tissue, and this is avoided with Celite.

After allowing extraction to take place for an hour or more, the stopper of the funnel is loosened in the centrifuge tube so that the ethanol may run through, assisted by pressure on top of the funnel from a compressed air line or nitrogen cylinder. The stopper is seated in the tube again, and the cells extracted with 2 ml. of boiling 20 per cent ethanol. The process is repeated with 2 ml. of boiling water. The extracts are pooled.

The amount of salt remaining in the extract should be too small to affect chromatography. It has been found that standard 10 in. squares of Whatman No. 4 paper can carry about 150 µg. of salt, or 300 µg. if the chromatograms are over-run for twice the normal distance (see below).

Plant Tissues. Plant tissues are often somewhat easier to handle than animal tissues as less damage is caused by removal of the tissue from the plant, and incubation media usually contain less salt. Unicellular plants are treated in the same way as other micro-organisms. Higher plants may be used whole if they are small enough (mosses, simple multicellular algae, fern prothalli, etc.), or organs may be removed more or less intact (seeds, small leaves, roots, etc.). When larger organs are to be dealt with they may be cut into small pieces (leaves, roots and stems), or disks may be used in the conventional manner. Whole leaves or roots can be stood with the cut ends in a solution of the active material. If a salt medium is being used the tissue must be separated from the medium in the same way as with animal tissues, using

the Celite and sintered funnel technique if the tissues have been chopped; with larger pieces of thin tissue, such as leaves, the Celite pad may be omitted provided the sintered disks do not become clogged.

Large pieces of tissue (whole plants, whole leaves and roots) may conveniently be killed by dropping them straight into boiling 80 per cent ethanol, though if they are thick it is sometimes useful to stop the reaction by plunging the organ or plant directly into liquid nitrogen. The frozen tissue is ground to a fine powder which is sprinkled into boiling ethanol.

Sub-cellular Particles. These are treated in the same way as micro-organisms. However, sub-cellular particles are usually suspended in media containing large amounts of salts or sugars, and unless the particles can be removed from the medium by centrifugation before they are extracted, the salts and sugars will be present in the final extract. In this case it is necessary to ascertain for each experimental system the capacity of the filter paper to hold these substances without the final chromatogram being affected.

As a guide the following example may be of some value.[10] An incubation mixture contained the following substances (mg./ml.): sucrose, 171; various salts and coenzymes, 3. 1 ml. of incubation mixture, after various extractions with ethanol and water, was made up to 9 ml. Of this only about 125 µl. (or 1/72 of the total) could be spotted onto a sheet of paper 10 in. square; more could be used if the paper was overrun.

Purity and Specific Radioactivity of Substrates

Mention has already been made (p.) of the necessity of purifying substances which contain radiochemical contaminants.[11] This is liable to be of particular importance when the substances have been prepared

biosynthetically. Generally labelled glucose, for example, is prepared from plants allowed to carry on photosynthesis in the presence of labelled carbon dioxide. Although radiochemical contamination may not exceed 1 per cent, the contaminants often run chromatographically close to fructose, sucrose and aspartic acid, and may obscure these important substances in subsequent analysis if they are not first removed.

Unless there are contra-indications, high-specific activity substrates are most useful, as the biological system is then forced to use relatively more of the labelled material than it would if much unlabelled material were also present. Hence, more activity can be spotted onto chromatograms, films need a shorter exposure period, and assay of activity in the spots is more accurate. There are, however, dangers in using high-specific activity substances, of which investigators should be aware. There might be a relatively higher degree of radiation decomposition, i.e. breakdown of the substance under the influence of its own radiation (p.). If large quantities of radioactive materials are used, the tissue might suffer deleterious effects due to the radiation, though reports of this in short period experiments are somewhat contradictory.[12,13,14,15] Certainly, whole viable organisms suffer damage when exposed to high levels of radiation, varying for different species. The use of very small amounts (by weight) of high activity substrates may result in the system being depleted of substrate before the end of the experiment, with various consequences, desirable in some cases but not in others.

Incubation of Tissue with Labelled Substrates

The tissue should be incubated in as small a volume of medium as possible. This speeds the subsequent evaporation of superfluous liquid and avoids waste of tissue and radioactive materials. Sufficient

materials should be used to provide reasonable levels of radioactivity on the chromatograms, but as the capacity to carry cell extracts is, of course, limited, only sufficient for the number of chromatograms to be run should be used, plus a small margin for manipulation and to repeat chromatography in the event of accident or unsatisfactory spot separation; this inevitably occurs occasionally. The experiment may be carried out in one vessel from which samples are withdrawn at various intervals, or a number of separate incubation flasks may be set up, each for one particular set of conditions and time. At the appropriate time, the radioactive substrate is added and the incubation carried on for as long as desired.

The experiment is terminated, in the case of individual flasks, in one of four ways: (i) by filtering off the tissue and killing and extracting it with ethanol (as described in the section on Animal tissues above); (ii) by centrifuging off the tissue and killing with 80 per cent ethanol; (iii) by pouring the incubation mixture directly into four volumes of 100 per cent ethanol; (iv) by adding four volumes of 100 per cent ethanol to the tissue in suspension. Rapid mixing should be promoted both when the substrate is added to the tissue, and when the tissue is mixed with ethanol.

When one incubation vessel is being used from which samples are to be taken at intervals, the withdrawal of samples by mouth pipettes should be avoided. Samples of approximately equal volume may be removed without disturbing the rest of the suspension by using an incubation flask fitted with an E-Mil Kipps Automatic Tilt Measure of appropriate volume: the tilt measure is best fitted with a male standard glass joint which is fitted into a corresponding female joint on the incubation flask. If it is necessary to know accurately the amount of tissue removed, the samples may be run into tared flasks of ethanol, which can be weighed again later

and the amount of sample withdrawn determined. The samples should be run into four volumes of 100 per cent ethanol. Accurate replicate samples of small volumes of cell suspension may often conveniently be taken with a hand-operated automatic pipette; several patterns of these are available commercially.

Particularly with micro-organisms, rapid filtration can often be achieved with membrane filters (Millipore Filter Corp., Bedford, Mass.; Oxoid Ltd., Southwark Bridge Road, London, S.E.1). A standard aliquot of the incubation mixture is pipetted onto the filter, excess liquid is rapidly sucked through, and after disassembling the filter funnel the filter membrane may be removed and rapidly placed in the killing solution. All this may be done within a few seconds. Some filter membranes disintegrate and dissolve on contact with methanol and ethanol, but are resistant to such higher alcohols as isopropanol which is miscible with water and may be used as the killing solution.

While boiling ethanol is desirable for killing and extracting the cells, as extraction takes place more rapidly at elevated temperatures, there may be occasions when it is preferred that temperatures higher than ambient be avoided due to heat-labile substances in the extracts. There is probably very little difference in the rate of killing of unicellular organisms, or small pieces of tissue, between hot and room temperature ethanol. If it is not convenient to use boiling ethanol to kill the cells, but there is no other objection to heat, the cells in ethanolic suspension may be brought to the boil momentarily at a later stage. Ethanol is best heated on a hot plate, or in a bath of hot water, and tends to bump unless kept moving.

Extraction and Evaporation

The cells in 80 per cent ethanol are allowed to stand for at least an hour, and are then removed by centrifugation. The supernatant is retained, two volumes of boiling 20 per cent ethanol are mixed with the residues, and allowed to stand for another hour. The process is repeated with two volumes of boiling water. The supernatants are pooled and the residues discarded. Some substances, particularly sugar diphosphates, are not very soluble in 80 per cent ethanol, but are readily brought into solution in 20 per cent ethanol or in water.(16)

The pooled extracts from each sample are concentrated to a small volume (less than 1 ml.) in vacuo below 40°C. The extracts must not be evaporated to dryness as phosphates stick to the glass and are not easily removed.

Vacuum evaporation may be carried out with a Craig Type rotary vacuum evaporator (J. W. Towers & Co. Ltd., Widnes, England) connected to a water pump, or better, with a rotating high vacuum-type evaporator (Rinco Instrument Co. Inc., Greenville, Illinois, U.S.A.) connected through a trap containing acetone and dry ice to a high vacuum pump. Very suitable flasks for the concentration of extracts are made by sealing a very small tube (2 cm. long and 1 cm. diam.) onto the bottom of a 500 ml. round-bottom flask fitted with a standard joint. The small tube acts as a reservoir into which the small volume of liquid remaining after the evaporation can drain. The bottom of the flask should be blown out a little so that liquid can drain evenly into the tube without being obstructed by any sort of lip.

As centrifuges are not usually available for 500 ml. round-bottom flasks it is not always possible to get the last traces of liquid into

the tube. A makeshift centrifuge can be simulated by the investigator swinging the flask in vertical circles at arm's length through ten or twelve revolutions. It is important to finish this operation with both the centrifuge and flask in an upright position. The concentrated extract is removed from the small tube with a teat-operated capillary pipette, and made up to volume with ethanol (which evaporates rapidly when spotted onto paper, and is thus better than water).

Chromatography of Cell Extracts

Paper. Whatman No. 1 or 4 are the commonly used papers. The former takes longer to develop and often produces more compact spots, but the improved quality of the chromatogram is often not great enough to warrant the extra development time. An extremely good paper which produces more compact spots than any of the Whatman papers, and with less tailing, is Ederol No. 202 (J. C. Binzer Papierfabrik, 3550 Hatzfeld/Eder, West Germany).

Spotting. Appropriate aliquots of the extract are spotted onto the filter paper. It is essential to use air at room temperature for drying the spots, or sticking of the phosphates to the origin will occur.

The amount of cell extract which can be spotted onto one piece of chromatogram paper must be determined for each separate system. Some guide may be provided by the following values obtained with baker's yeast: for chromatograms which are not over-run, the extract from about 10 µl. of yeast (measured by centrifugation at 1,600 g.) equal to about 10 mg. wet wt. of cells, may be spotted onto each paper. More can be spotted onto over-run papers (p.). If too little material is placed on the paper, the chromatograms must be left in contact with

X-ray film for an unreasonably long period, and the counting rate from each spot will be correspondingly low. Too much extract on each paper results in a poorer separation of spots, and some background fogging. Fig. 4 illustrates radioautograms from several chromatograms of the same extract, using increasing quantities of extract on successive chromatograms.

Useful landmarks on the chromatogram may be obtained by applying a small spot of "Skrip" Washable Black Ink (W. A. Sheaffer Pen Co.), or a mixture of the three organic dyes tropeolin, crocein scarlet and Ponceau-4R, to the origin before the chromatogram is run (p.). The dyes can also be used to indicate the extent of over-running (p.).

Apparatus. The all-polythene (or stainless steel) apparatus must be used, as the solvents are corrosive.

Solvents

Phenol-water. This is prepared by mixing 740 g. of phenol with 260 ml. of distilled water, 10 ml. of glacial acetic acid and 1 ml. of M-ethylenediaminetetraacetic acid (potassium salt). The mixture is monophasic above about 16°C. Distillation of the phenol is unnecessary.

n-Butanol-Propionic Acid-Water. Two solutions are prepared. Solution A contains 919 ml. of n-butanol and 81 ml. of distilled water. Solution B contains 469 ml. of propionic acid and 531 ml. of distilled water. Equal volumes of solutions A and B are mixed just before use. The mixture is monophasic above 9°C. Neither the butanol nor the propionic acid need to be distilled.

The paper is developed first with the phenol-water solvent. This is essential, as it is impossible to remove all traces of propionic acid

by drying the papers at room temperature. The first solvent is almost neutral, and the separation thus depends on running first in a neutral solvent, followed by an acid solvent. If phenol-water is used second, the effect is that of running in two acid solvents owing to propionic acid remaining on the paper.

It is important to dry the papers thoroughly after phenol-water. Drying should take place in a current of air at room temperature: heating may dehydrate the paper, and result in phosphates sticking and not running in the second solvent. Pre-equilibration of the papers in the phenol-water tank is desirable but not essential: pre-equilibration with butanol-propionic acid-water is detrimental to the final separation of compounds on the chromatogram. An overnight, or shorter, run is sufficient with both solvents (200 ml. in the tray).

Alternative solvent to phenol-water. Although phenol-water produces a very satisfactory distribution of compounds along the paper there are some disadvantages in its use; the most serious of these is the breakdown of some amino acids during chromatography in phenol.[17,18] A new solvent was therefore developed to replace phenol-water; the distribution of R_f values is not quite so good as in phenol-water, but little or no breakdown of amino acids occurs. The solvent contains the following components:[19] ethylenediaminetetraacetic acid, 1.2 g.; 17 N-ammonia solution, 100 ml.; water, 950 ml.; n-propanol, 350 ml.; isopropanol, 75 ml.; n-butanol, 75 ml.; isobutyric acid, 2500 ml. The constituents are mixed in the above order and are allowed to stand for 24 hr. before use; the solvent does not deteriorate, at least for several weeks. The running time is about the same as that for phenol-water, and papers should be pre-equilibrated.

Radioautography

Radioactive ink marks are stamped in the corners of the dried chromatogram, which is then placed in contact with X-ray film as described in Chapter . The length of time necessary to expose the film will depend on a variety of factors and should be found by trial and error. This has been discussed on page . The positions of the radioactive spots are marked out on the chromatogram, and these are counted, if desired, with the Scott tube, or other equipment (Chapter).

Spot Identification

The whole success of the procedure depends on the ability of the experimenter to identify the active spots on the chromatogram. The ultimate confirmation of identity usually relies on co-chromatography of the unknown substance, or a derivative, with an authentic marker, followed by "fingerprinting" (p.). However, the rapidity with which the worker decides on the correct authentic marker depends on his shrewdness and experience. Instructions cannot be given for all the possible spots which may be met under a wide variety of experimental conditions, but a number of useful practical suggestions can be made which should help the investigator to hit upon the correct nature of the spot.

Use of Chromatogram Maps and Landmarks. The R_f values of compounds originally present in cell extracts is of only limited value in deciding upon their nature. This is because there exists interaction between different compounds in a mixture which may alter their R_f values to a significant extent. The overall pattern presented by the spots, together with an intelligent use of the maps shown in Figs. 1, 2, and 3, will prove much more rewarding than slavish devotion to R_f numbers.

The first thing to do is to try to identify some of the expected products of the incubation reaction. These are very often prominent, and provide valuable reference points in the identification of other compounds. As examples of the way in which this may be done, mention may be made of the probable formation of such compounds as malic, citric, aspartic, glutamic acids, and other substances associated with the tricarboxylic acid cycle, when tissue is provided with labelled acetate, pyruvate, or carbon dioxide; the formation, in addition to Krebs cycle intermediates of several sugar phosphates from labelled carbohydrate sources; the products of transamination when amino acids or keto acids are supplied; sugar phosphates and nucleoside phosphates from inorganic P^{32}; methionine and cystine from S^{35}, etc. Use may also be made of the substrate spot, if any remains in the extract. The characteristic positions occupied by the dyes in "Skrip" ink should be determined, and these provide additional landmarks. Help may be obtained from Figs. 5, 6, 7, 8, and 9, and from published photographs of chromatograms (and radioautograms) which have been developed in the same solvent systems.[1,2,3,4,7,10,15,20,21,22,23,24] It should be noted when using photographs of radioautograms published here and elsewhere that photographic reproduction sometimes loses some of the weaker spots on the X-ray film.

Although R_f values may change from one experiment to another, the overall patterns remain very constant. The distances which some acidic and basic compounds run in the first dimension depend on the acidity of the origin, and may vary somewhat in different experiments. But apart from this, the pattern remains similar; a little expanded here, perhaps, and constricted there, but still recognizable.

Having decided upon the nature of a few landmark substances, further presumptive identifications may be made from the maps and photographs. A spot which runs, for instance, a little further than alanine in phenol-water, and the same distance in butanol-propionic acid-water may well be ribulose, or one lying roughly midway between phosphoglyceric acid and phosphoenolpyruvic acid could similarly be phosphoglycollic acid.

The chromatogram maps have been reproduced here in three sections for convenience and legibility. It is recommended that workers should make their own large scale composite maps from these three. Reference points appear on the maps printed here so that they may be aligned together: sucrose, dihydroxyacetone, and alanine (or ribose, which runs next to it) appear on all three maps; others (several phosphates) appear on two maps. Additional compounds of particular interest to individual workers may be added as required. They should first be run as pure compounds in the solvent system to get some idea of their position on the map, and then an unlabelled sample of the compound to be added to the map is chromatographed together with a labelled cell extract containing substances already identified. The position of the new compound may be determined by a colour reaction, and its position found in relation to other substances in the extract.

Over-running to Separate Phosphates. It will be noticed from the chromatogram maps that all the phosphates are crowded together near the origin. If these compounds are important to the experimenter's purpose, a better separation of them can be achieved by over-running the chromatogram (p.). One sheet of 10 in. square paper is sufficient as a pad for over-running one ascending chromatogram. For

descending chromatograms, two papers in one trough should be supplied with about 43 ml. of solvent, and allowed to run until the solvent has all been used. An extra guide may be obtained from the position of the "Skrip" ink dyes: the red dye should be run off the paper in both solvents. As it will already have been lost after the first solvent, another "Skrip" ink spot can be applied to the paper after drying off the phenol-water for indicating development in the butanol-propionic acid-water.

Over-running as described above will spread the phosphate spots about twice as far as usual, and should give a greatly improved separation. About twice as much extract and salt can be accommodated on over-run papers. However, the size of the origin should be kept small (1 cm. diam.) even though twice as much material as usual is applied to the chromatogram.

Examples of over-run chromatograms are shown in Figs. 6 and 7.

Use of Large Size Chromatograms. Although quite satisfactory chromatograms can often be produced with cell extracts on standard 10 in. square papers, it has been the author's experience that separations are frequently much better when larger papers are used (Figs. 8 and 9). One of the main troubles with small papers is that the amounts of material on them must be kept minimal (p.), and the resultant small spots do not always produce clear film images and may have very low counting rates. Keeping origins small is tedious when volumes of liquid over 0.05-0.1 ml. must be spotted. It is therefore proposed to give a few instructions for the use of larger sized chromatograms.

Standard 18.25 in. x 22.5 in. Whatman No. 4 papers are used. The

origins are spotted 3 in. from two edges at one corner, using air at room temperature. The origins can be made as large as 2 cm. diam. for papers developed with the solvents to the edges, and about 3 cm. diam. for over-run papers. Chromatograms are run by the descending technique in the solvents described above (p.). All apparatus coming into contact with the papers and solvents should be constructed of the type 316 stainless steel; a suitable type of trough, 24 in. long, and fitted with anti-syphon bars, is described by Benson, Bassham, Calvin, Goodale, Haas and Stepka.[25] Adjacent troughs are placed 1.5-2 in. apart in the tanks. Although not mentioned by these authors, before the papers are put in the trough a U-shaped sleeve of thin stainless steel, 23.5 in. long, is placed over the anti-syphon bar, with the prongs of the U pointing downwards. The paper is then placed in the trough in the usual way, curving downwards over the sleeve, and weighted in position with a stainless steel anchor rod. The sleeves have the advantage that the papers need not be touched when wet. Before removing the papers from the trough after running, one or two stainless steel or polythene-coated "bulldog" clips are used to clip the paper to the sleeve, the sleeve and paper are removed together, and the paper dried by suspending the sleeve at each end on a pin in a suitable frame.

For running solvents to the edges of the paper only, 100 ml. of solvent is added to each trough containing two papers. At 22°C the phenol-water reaches the bottom in 8-10 hr., and the butanol-propionic acid-water in about 7 hr., the phenol-water being run first along the long direction of the paper. As 10 hr. is often inconvenient for a solvent run, the papers may be run overnight in phenol-water, the

trough containing 60 ml. of solvent for two papers. When the papers are to be over-run, the trough contains 200 ml. for two papers, and the solvents are allowed to run for 24 hr. with phenol-water, and 20 hr. with butanol-propionic acid-water. If a constant temperature cannot be maintained, the trough should contain about 170 ml. of solvent for two papers, and development continued until no solvent remains in the trough. Individual workers may wish to vary the amount of solvent and the time allowed for over-running to suit their own purpose.

For the preparation of radioautograms 14 x 17 in. sheets of X-ray film are used: this is a standard size. It is necessary to wrap the edges of the paper around the film (or to cut them off): see discussion on p. .

Confirmation of Spot Identity. Having made a preliminary decision as to the identity of an active spot from its position on the chromatogram, confirmation may be obtained by co-chromatographing the unknown spot with an authentic marker substance. The spot should be cut out from the chromatogram in such a manner that one edge of the piece of paper cut out is straight, and the opposite edge is trimmed to a point. The material may then be eluted off the paper with water in the apparatus shown in Fig. 10. A paper wick, the length and width of which depends on the size of the spot to be eluted, is hung from a trough containing water. The wick is thoroughly wetted with water and sufficient surplus moisture is added to allow a drop to form along the bottom edge. The straight edge of the spot is applied to the bottom edge of the wick to give 1-2 mm. of overlap, and the spot adheres to the wick by surface tension. A small test tube is placed below the spot, with the point of the latter just fitting into the opening of

the tube, the glass chromatography tank is placed upside down over the apparatus, and elution is allowed to proceed. For a spot of average size about 0.25 ml. of water is sufficient for quantitative elution. A guide to the progress of the elution is provided by the use of "Skrip" ink. Two or three small spots of the ink are placed near the straight edge of the spot to be eluted before it is hung on the paper wick. The ink contains three dyes: red, yellow, and blue. The blue dye is eluted from the paper first, followed by a mixture of the red and yellow dyes. By the time the red and yellow dyes are almost eluted from the paper, all the radioactivity will have been eluted, except possibly with some diphosphate esters. The completeness of elution may be checked at a later stage by removing the spot from the wick, drying it, and then counting it with a Scott tube, paying particular attention to the pointed end. The presence of the dyes in the eluate will not interfere with any subsequent chemical manipulations, and will provide useful markers on the chromatogram to be run for co-chromatography.

The eluted unknown substance is mixed with authentic marker (see the appropriate chapter for quantities) and chromatographed. It may be desirable, if there are several possible identities for the unknown spot, to subdivide the eluate and co-chromatograph the unknown spot with more than one marker on separate chromatograms. Generally, hot air may be used for spotting at this stage, with a resultant saving of time.

In many cases it is advisable to delay co-chromatography until after a preliminary investigation has been made into the nature of the unidentified substance. The preparation of derivates of certain compounds (e.g. dinitrophenylhydrazones of keto-acids) may assist

identification, particularly if the substances are volatile (e.g. pyruvic and glyoxylic acids). Keto acids may also be reduced to the corresponding hydroxy acids by potassium boroxide. 0.05 ml. of freshly prepared potassium borohydride solution (4 mg./ml.) is added to the unidentified compound and an authentic marker contained in about 0.25 ml. of water. The pH should be above 7, and provided no acid is otherwise present in the solution, a high enough pH will be provided by the borohydride itself. The unknown substance is allowed to stand at room temperature for 1 hr. and is then co-chromatographed with the marker.

Treatment of Phosphates with Phosphatase. Even on over-run chromatograms the phosphates run fairly close together, and it is sometimes difficult to decide the probable nature of a particular spot from its chromatographic position alone. However, hydrolysis of the phosphate ester results in a sugar, sugar acid or nucleoside, and these are much easier to identify than their corresponding phosphate esters.

The phosphates are eluted from the paper. To a solution of the unknown phosphate contained in about 0.25 ml. of water is added 0.025 ml. of 0.2 M-acetate buffer, pH 5.0, containing 0.01 M-$MgCl_2$. Authentic marker is next added, followed by phosphatase solution (see below): the mixture is incubated at 37°C for 2-20 hr. depending on the phosphatase used, and is then chromatographed.

Two acid phosphatase preparations can be used. The most satisfactory is the supernatant liquid from human semen after centrifugation. Semen can be obtained from the fertility clinics of hospitals; aged semen, of no further clinical use, works quite well. The phosphatase in this preparation is very active, and when 5-10 µl. is added to about 0.25 ml. of solution containing the unknown phosphate, hydrolysis is complete in 2 hr. at 37°C.[26]

Polidase S (Schwarz Laboratories, Inc.) is a crude enzyme preparation from which an acid phosphatase can be purified as follows.[27] 1 g. of polidase S is suspended in 10 ml. of distilled water. 5.66 g. of ammonium sulphate are added giving 83 per cent saturation. The mixture is held at 0°C for 10 min., and then centrifuged at 25,000 g. for 5 min. at 0°C. The residue is discarded, and to the supernatant is added 1.20 g. of ammonium sulphate. The solution is cooled to 0°C for 30 min., and again centrifuged for 5 min. at 25,000 g. The supernatant is discarded, the precipitate dissolved in 0.6 ml. of 0.05 M-acetate buffer, pH 5.0, containing 0.01 M-$MgCl_2$, and the solution dialysed to remove excess ammonium sulphate. About 30 µl. of the final solution is added to 0.25 ml. of solution containing the unknown phosphate, and the mixture is incubated overnight at 37°C. It is advisable to add a few drops of toluene to the mixture to prevent bacterial growth. Solutions of both phosphatases should be stored in the deep freeze.

Some nucleoside polyphosphates are incompletely phosphatased with the enzymes, and monophosphates may be formed. However, these can be well separated chromatographically from the di- and tri-phosphates.

Having identified the sugar, sugar acid, or nucleoside component of the phosphate, the number of phosphate groups on the original substance is inferred from its chromatographic position using the maps. Isomeric forms of phosphate esters (e.g. glucose-1-phosphate and glucose-6-phosphate; 3-phosphoglyceric acid and 2-phosphoglyceric acid) cannot usually be separated in this solvent system.

Chromatograms of cell extracts incubated with labelled carbohydrates and other substances often show two prominent spots, one of

mixed sugar monophosphates, and the other of sugar diphosphates. These are likely to contain appreciable amounts of fructose and glucose phosphates, and even after the spots have been treated with phosphatase, separation of other sugars (e.g. sedoheptulose) lying chromatographically between fructose and glucose is difficult in the phenol-water, butanol-propionic acid-water system. It may be necessary to use another solvent system designed specifically for sugar separations (p.), though some improvement of the separation in the present system can be obtained by heating the solution, after treatment with phosphatase, with N-HCl at 100°C for 10 min. This converts over 80 per cent of the sedoheptulose to sedoheptulosan, which runs well in advance of fructose in phenol-water, and about the same distance in butanol-propionic acid-water.(28)

For chromatography of substances after treatment with phosphatase it is not necessary to remove the enzyme, and hot air may be used in spotting.

Carboxylic Acids. Co-chromatograms for identification of carboxylic acids should not be run with butanol-propionic acid-water as the second solvent, if the positions of the marker carboxylic acids are to be detected with indicators, because the background is liable to be too acid. A second useful solvent in this case is the organic (bottom) layer of a mixture of 8 parts of tert-amyl alcohol, 8 parts of chloroform, 8 parts of water, and 3 parts of 98 per cent (v/v) formic acid.(29) The formic acid may be completely removed by drying for 3-4 hr. in a current of air at room temperature, and the paper then gives a neutral reaction to indicator.

Care should be taken to ensure that marker organic acids are in the same ionic state as the active substances to be identified, since salts run somewhat differently from free acids in certain solvents and incorrect results may be obtained.

Identification when Markers are not Available. Authentic markers may not be available for all substances encountered on the chromatograms. In such cases definite confirmation of identity may be impossible, though a number of chemical tests may be made with the active substance which can give clues as to its nature. Each test is followed by chromatography of the products in an attempt to pick up an easily identifiable substance. As the amount of material on chromatograms is very small in terms of weight, the chemical testing of unknown compounds must depend on following the radioactivity through various analytical treatments.

It is, of course, impossible to cover all such compounds liable to show up in cell extracts. However, a few simple suggestions can be made to illustrate the type of procedure which is often successful.

If the spot appears in the phosphate area of the chromatogram, its susceptibility to hydrolysis by phosphatase can be investigated. Sometimes removal of phosphate groups from polyphosphates is not complete (see above).

Many acids can exist in acid or lactone forms. Such substances can be spotted onto paper from acid or alkaline solutions to observe possible differences in chromatographic behaviour under these conditions. To promote lactone formation heat the solution at 100°C for a few min. with 0.1 N-HCl; to promote formation of the acid form, heat at 100°C with 0.1 N-NaOH.

Keto acids are reduced to hydroxy acids with potassium borohydride (see above), or 2:4-dinitrophenylhydrazone derivatives can be formed which are then reduced to amino acids.[30] Certain acids can be decarboxylated by heating with HCl.

Oligosaccharides usually form a crescent of spots starting from the origin and curving up towards the disaccharides. Spots suspected of being small oligosaccharides (or peptides) can be hydrolysed and re-run with markers. Larger polysaccharides and peptides do not move from the origin.

Some substances (glycollic and other acids) are quite volatile, and even though film blackening may be obtained from them there is apparently no radioactivity present when the chromatograms come to be counted.[31]

Some conclusions can be drawn from the chromatographic parameters of an unknown substance. In the solvent system described in this chapter, neutral substances lie more or less on the diagonal line from the origin to the opposite corner. Acid substances usually move further than the neutrals in butanol-propionic acid-water, while basic substances do not move so far. Homologous series of compounds, and their close relatives, usually lie on a straight line or shallow curve on the chromatogram map (see p.). Glycine, alanine and valine lie on such a curve, and another spot lying on the curve between alanine and valine might be α-amino butyric acid. Hydroxy acids generally run further in both solvents than their corresponding keto acids, while the amino acids usually run further in phenol-water than the keto acids, but not so far in butanol-propionic acid-water. Ketoses run somewhat further in phenol-water than the corresponding aldoses (e.g. glucose and fructose; ribose and ribulose). Sugars run much further in phenol-water than sugar acids,

and about the same distance in butanol-propionic acid-water (e.g. glyceraldehyde and glyceric acid; glucose and gluconic acid, the latter runs close to aspartic acid), while sugar alcohols sometimes run further than sugars in both solvents (e.g. glycerol and glyceraldehyde; sorbitol and glucose), and sometimes run behind the sugars (e.g. erythritol and erythrose; adonitol and ribose).

Overlapping. In a few instances trouble is likely due to overlapping of two spots of common substances when both are present in the same cell extract. Valine runs together with methionine, and is separated after elution by oxidizing the methionine with hydrogen peroxide to methionine sulphone and sulphoxide, both of which run well clear of valine (p.). Serine and glycine overlap with glucose, and asparagine with sucrose: these mixtures can be separated by other solvent systems, or by electrophoresis.

Interpretation of Results

By feeding a system with a labelled substrate and taking samples for analysis at intervals thereafter, it is possible to follow the pathways by which the substrate is metabolized. The activity in all the spots on the chromatogram (except the spot of substrate, if it is at all present) is counted, and the activity in each spot is expressed as a percentage of the sum of the activities in all the spots. The percentage is plotted against time for each substance.

In the shortest time, only the first product of metabolism of the substrate will contain activity, provided the first sample is taken after a sufficiently brief incubation period. Thus, 100 per cent of the total activity is present in the first product. Thereafter the per-

centage in the first product declines, and that in the second product, next in line along the metabolic pathway, rises. This reaches a peak, and declines in turn as the percentage in the third compound rises, and so on. In this way the order of compounds along the metabolic chain can be found.

Three main difficulties are likely to arise. Firstly, it may be difficult to achieve incubation times short enough to obtain 100 per cent of the activity in the first compound. For example, Calvin, Bassham and Benson[32] found in photosynthesis experiments with $C^{14}O_2$, that after 5 sec. of incubation with the labelled substrate, 87 per cent of the product activity was in 3-phosphoglyceric acid, the first product of carbon dioxide fixation. Incubation periods of less than 1 sec. resulted in over 90 per cent of the activity being found in phosphoglyceric acid. In other systems, however, the rates of metabolism may be much lower: in the fixation of $C^{14}O_2$ by a blue-green alga in the dark, aspartic acid contained 100 per cent of the fixed activity after 30 sec., and 95 per cent after 60 sec.[3] There is thus a large range in activities between different systems. If the incubation period cannot be made short enough at room temperature or thereabouts, the experiment might be repeated more successfully near 0°C. Very short incubation periods can be achieved by injecting the labelled substrate with a syringe-controlled pipette held in one hand, and immediately afterwards adding ethanol from a second syringe held in the other hand. The incubation vessel should be shaken continuously for rapid mixing. A second person may work a stopwatch on instruction from the first. Incubation periods as short as 0.5 sec. can be obtained and measured fairly accurately, but it is not always possible in this way to hit exactly upon the desired period.

Secondly, difficulties arise when the metabolic pathway branches, or the labelled substrate is taken up at more than one point in the metabolism of the cell. Cyclic pathways offer additional complications. It may become necessary in the event of cycles and branches to degrade the metabolic products to find which atoms have become labelled, and then to draw the conclusions.[33] Thirdly, as the labelled isotope passes further and further along the metabolic chain, the peak of percentage activity as it passes through each intermediate becomes successively lower and lower, until it becomes difficult to distinguish individual peaks from chance irregularities in the curves. With a pathway uncomplicated by branches and cycles it might be possible to follow the first six or seven intermediates along the chain, depending on the speed of the reactions and the number of samples taken.

In experiments in which tissues have been incubated with substrates for long periods, the end products of metabolism rather than intermediates are likely to be observed. It must be borne in mind that labelled atoms may enter various substances by enzymic exchange reactions, without net synthesis taking place. For instance, label may be found in amino acids as a result of rapid transamination reactions with the corresponding keto acids. This is an indication that the reactions can take place, but not necessarily that they play a significant role in the experimental system being studied. Substrates may be oxidized by the tissue to labelled carbon dioxide, which is then fixed elsewhere and results in two superimposed patterns. This is of great importance with green plants incubated in the light.[34,35] Exchange reactions may take place by pairs of enzymes, or through non-specific chemical exchange: P^{32} supplied in a nucleotide may be hydrolysed enzymically

to inorganic P^{32}, and refixed into another nucleotide by a process such as oxidative phosphorylation. P^{32} is thus transferred from one nucleotide to another, but not via a simply enzyme system. In studies with tritiated water care must be taken to avoid non-specific chemical exchange, and any interpretation of the results must take this possibility into account.(31) These examples could be greatly multiplied, and just a few are given here to illustrate some of the pitfalls which must be avoided.

Simultaneous Use of Two Different Isotopes

For certain experimental purposes it is possible to use two isotopes simultaneously, provided that there is some way of distinguishing between them. The isotopes must differ to a considerable extent either in the penetrating power of the radiation they emit, or in the lengths of their half-lives.

Use of Isotopes with Radiations of Different Penetrating Power. A good example of the use of such pairs of isotopes is provided by the combination of C^{14} with H^3, or of C^{14} with P^{32}. The penetrating power of the β-radiation from H^3 is so weak that the particles will not penetrate through a sheet of Melinex (p.), while the β-particles from C^{14} suffer an attenuation of only about 10 per cent. Hence, a chromatogram of a mixture of substances containing both these isotopes may be exposed to X-ray film twice, once with a sheet of Melinex placed between the paper and the film, and again without Melinex. The second film will receive an image due to both isotopes, and the first from C^{14} only. Spots containing only H^3 can be distinguished in this way from those containing C^{14}. The activities in the spots due to each of the isotopes can be determined by counting the spots once with a Scott tube fitted

with a Melinex window, to detect only C^{14}, and once with a windowless Scott tube, when both isotopes will be counted (p.). The attenuation factor for C^{14} due to passage of the particles through Melinex can be found, and the activity in the C^{14}-containing spots counted through Melinex corrected for the attenuation. The activity due to C^{14} when the spots are counted without a window is deducted from the total, and the activity due only to H^3 calculated.

If a scintillation counter is available the two isotopes may be counted simultaneously in scintillation liquid either after elution or by putting the whole excised spot into the vial (p.).

The same principle can be applied to experiments using C^{14} and P^{32} together. In this case C^{14} represents the weaker isotope, and the chromatogram is exposed to two sheets of film simultaneously, one above the other. The film nearest the chromatogram will receive an image from both isotopes, and the one furthest from the paper only from P^{32}. By differential counting of the spots, with and without an index card placed between the paper and the counter tube, the activities of both nuclides can be found. Scintillation counting can be used here as well.

Use of Isotopes of Different Half-lives. When two isotopes of greatly differing half-life are used together, one film is exposed to determine the positions of all the labelled substances on the chromatogram. Each spot is counted to find the activities due to both isotopes. The shorter-lived isotope is then allowed to decay for 8-10 half-lives, and the paper counted again. After 10 half-lives only about 0.1 per cent of the original activity of the shorter-lived isotope will have survived, and the longer-lived isotope can then be assayed with a minimum of interference.

Suitable pairs of isotopes for use by this method are C^{14}, Cl^{36}, or H^3 used together with Br^{82}, I^{131} or P^{32}. Unfortunately C^{14} is difficult to use in combination with S^{35} as the β-radiations from both isotopes have similar powers of penetration, and the half-life of S^{35} is so long that the chromatogram would need to be counted twice at an interval of 2-3 years.

Use of Two Isotopes for Identification Purposes. In some cases it is desirable to use a second isotope for the identification of spots containing the nuclide in which the experimenter is mainly interested. When incorporation studies are made with P^{32}, the phosphate spots lie fairly close together on the chromatogram, and good separation may not always be obtained even when the chromatograms are over-run. The spots cannot be identified by treatment with phosphatase (p.) as the P^{32} will be liberated as inorganic phosphate, and the non-phosphate moiety of the molecule will remain unlabelled. This difficulty can be overcome by labelling the whole system with C^{14} before P^{32} is supplied. The P^{32}-containing spots are distinguished from those also labelled with C^{14} by exposing two films to the chromatogram (see above), and the non-phosphate part of the molecule identified after treatment with phosphatase by virtue of the C^{14} present in it.

REFERENCES

1. Calvin, M. J. chem. Soc., 1956, 1895.
2. Bassham, J. A., and Calvin, M. The Path of Carbon in Photosynthesis, 1957, Prentice-Hall, Inc., Englewood Cliffs, N. J.
3. Moses, V., Holm-Hansen, O., and Calvin, M. J. Bact. 1959, 77, 70.
4. Moses, V. J. gen. Microbiol. 1959, 20, 184.
5. Beloff-Chain, A., Catanzaro, R., Chain, E. B., Masi, I., Pocchiari, F., and Rossi, C. Proc. roy. Soc., 1955, 143B, 481.
6. Beloff-Chain, A., Catanzaro, R., Chain, E. B., Masi, I., and Pocchiari, F. Proc. roy. Soc., 1955, 144B, 22.
7. Stange, L., Moses, V., and Calvin, M. Biochim. Biophys. Acta, 1960, 42, 272.
8. Davis, B. D. Arch. Biochem. Biophys., 1958, 78, 497.
9. McIlwain, H., and Buddle, H. L. Biochem. J., 1953, 53, 412.
10. Holm-Hansen, O., Pon, N. G., Nishida, K., Moses, V., and Calvin, M. Physiol. Plant., 1959, 12, 475.
11. Baker, N., Gibbons, A. P., and Shipley, R. A. Biochim. Biophys. Acta, 1958, 28, 579.
12. Emerson, R. J., and van Bruggen, J. T. Arch. Biochem. Biophys., 1958, 77, 467.
13. Boichenko, E. A., and Zakharova, N. I. Conference of the Academy of Sciences of the U.S.S.R. on the Peaceful Uses of Atomic Energy, July 1-5, 1955, 121.
14. Boichenko, E. A., and Zakharova, N. I. Biochimia, 1956, 21, 377.
15. Holm-Hansen, O., Moses, V., van Sumere, C. F., and Calvin, M. Biochim. Biophys. Acta, 1958, 28, 587.

16. Wilson, A. T., and Calvin, M. J. Amer. chem. Soc., 1955, 77, 5948.

17. Huggins, A. K., and Moses, V. Nature, 1961, 191, 668.

18. Moses, V. J. Chromatog., 1962, 9, 241.

19. Crowley, G. J., Moses, V., and Ullrich, J. J. Chromatog., 1963, 12, 219.

20. Bassham, J. A., Shibata, K., Steenberg, K., Bourdon, J., and Calvin, M. J. Amer. chem. Soc., 1956, 78, 4120.

21. Benson, A. A. Modern Methods of Plant Analysis, 1955, 20, 113.

22. Bidwell, R. G. S. Canad. J. Bot., 1957, 35, 945.

23. Poel, L. W. J. exp. Bot., 1953, 4, 157.

24. Moses, V., and Calvin, M. Proc. nat. Acad. Sci., Wash., 1958, 44, 260.

25. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W. J. Amer. chem. Soc., 1950, 72, 1710.

26. Schmidt. G. Methods in Enzymology, 1955, 2, 523.

27. Cohen, S. S. J. biol. Chem., 1953, 201, 71.

28. Calvin, M. Harvey Lect., 1950-51, 46, 218.

29. Bentley, L. E., personal communication.

30. Towers, G. H. N., Thompson, J. F., and Steward, F. C. J. Amer. chem. Soc., 1954, 76, 2392.

31. Moses, V., and Calvin, M. Biochim. Biophys. Acta, 1959, 33, 297.

32. Calvin, M., Bassham, J. A., and Benson, A. A. Fed. Proc., 1950, 9, 524.

33. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J. Studies of Biosynthesis in Escherichia coli, 1955, Carnegie Instution of Washington Publication No. 607, Washington, D. C.

34. Ryther, J. H. Nature, Lond., 1956, 178, 861.

35. Moses, V., Holm-Hansen, O., Bassham, J. A., and Calvin, M. J. mol. Biol., 1959, 1, 21.

Captions for Figures

Fig. 1. Chromatogram map 1.[2] Reproduced by permission of Prentice-Hall International Inc.

Fig. 2. Chromatogram map 2.[2] Reproduced by permission of Prentice-Hall International Inc.

Fig. 3. Chromatogram map 3.[2] Reproduced by permission of Prentice-Hall International Inc.

Fig. 4. Effect of amount of cell extract applied to 10 in. square chromatograms on the resultant separation. 200 µl. of baker's yeast were suspended in 2 ml. of distilled water and incubed with 10 µC. (0.4 µ moles) of glucose-U-C^{14} for 3 min. at 24°C. The cells were killed and extracted as described in the text. The pooled extracts after evaporation were made up to 0.9 ml. Varying amounts were applied to the chromatograms as follows: (a) 10 µl. (equivalent to 2.22 µl. of extracted cells); (b) 25 µl. (5.55 µl. cells); (c) 50 µl. (11.11 µl. cells); 75 µl. (16.67 µl. cells). X-ray film was exposed to each chromatogram for 13 days. The radioautograms cover the whole area of the chromatograms.

Fig. 5. Extract of baker's yeast incubated for 3 min. with glucose-U-C^{14} (Fig. 4 (c) labelled). Key to numbers: 1, sugar diphosphates; 2, hexose and heptose monophosphates; 3, uridine-diphosphoglucose; 4, nucleoside diphosphates; 5, phosphogluconic acid; 6, phosphoglyceric acid; 7, phosphoenolpyruvic acid; 8, aspartic acid; 9, glutamic

acid; 10, citric acid; 11, malic acid; 12, fumaric acid; 13, succinic acid; 14, glucose; 15, disaccharide (probably trehalose); 16, threonine; 17, alanine; 18, valine; 19, leucine + iso-leucine + phenylalanine; 20, probably histidine; 21, unidentified; 22, unidentified; 23, lipids, etc.

Fig. 6. Effect of over-running (I). Same extract as appears in Figs. 4 and 5 over-run in the ascending direction with one sheet of 10 in. square filter paper as a pad. 50 µl. of cell extract (equivalent to 11.11 µl. of cells) applied to the chromatogram. Film exposed for 13 days. Key to numbers: 24, pentose phosphate; rest as for Fig. 5.

Fig. 7. Effect of over-running (II). Same extract as appears in Figs. 4, 5, and 6, over-run in the descending direction. 150 µl. of cell extract (equivalent to 33.3 µl. of cells) applied to the chromatogram. Film exposed for 13 days. Key to numbers: as for Figs. 5 and 6.

Fig. 8. Advantages of large-sized chromatograms. 100 µl. of baker's yeast suspended in 1 ml. of 0.002 M-phosphate buffer pH 5.0, incubated for 3 min. at 24°C with 2.5 µC. (0.1 µ moles) of glucose-U-C^{14}. Cells killed and extracted. The pooled extracts, after evaporation, were made up to 0.9 ml., of which 0.3 ml. (equivalent to 33.3 µl. of cells) was applied to a sheet of paper 18.25 in. x 22.5 in. A sheet of X-ray film (14 in. x 17 in.) was exposed to the chromatogram for 27 days. Key to numbers: 25, glyceric acid; 26, glycolic

acid; 27, glutamine; 28, probably lactic acid; 29, tyrosine; 30, proline; rest as for Figs. 5 and 6.

Fig. 9. Effect of over-running (III). Same extract as appears in Fig. 8. 0.6 ml. of cell extract (equivalent to 66.7 µl. of cells) applied to one sheet of paper 18.25 in. x 22.5 in. which was over-run in the descending direction as described in the text. A sheet of X-ray film (14 in. x 17 in.) was exposed to the chromatogram for 27 days. Key to numbers: 31, uridine triphosphate; 32, disaccharide (probably trehalose) phosphate; 33, unidentified; 34, unidentified; rest as for Figs. 5, 6, and 8.

Fig. 10. Apparatus for elution of chromatogram spots. The spot adheres to the wick by the surface tension of a water film.

Figures

Figs. 1-10 correspond exactly to Figs. 26.1-26.10 of the 2nd edition, and the same blocks should be used.