Title

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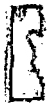
May 1961



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PREPARATION OF BIOLOGICAL MATERIAL FOR RADIOAUTOGRAPHIC STUDIES OF INTERMEDIARY METABOLISM*

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ABSTRACT

The intermediary metabolism of many kinds of biological material may be studied by supplying the tissue with radioactive substrates and analyzing the reaction products by paper chromatography and radioautography. The preparation of the tissue in a form suitable for such study frequently presents difficulty; the present communication discusses several experimental approaches to this problem which have been applied successfully to the metabolism of tissues from widely differing organisms.

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* The preparation of this paper was sponsored by the U.S. Atomic Energy Commission.

The use of the combined techniques of radioactive tracers and paper chromatography has led to a development of a method for the investigation of wide areas of tissue metabolism in a single system. Analysis of the results of incubating biological material with labeled compounds can demonstrate beyond reasonable doubt the formation of products many steps removed from the original substrate. Owing to the wide variety of possible products derived from such common starting materials as sugars, amino acids, etc., and the necessity, when dealing with radioactive substances, of physically separating from each other all those substances containing the labeled isotope, a chromatographic system must be used for analysis which is capable of achieving the required degree of separation for the particular products found in the experiment.

For studies of simple systems in which a limited number of products of one type are expected, it is usually possible to utilize standard biochemical techniques for the isolation and concentration of the products for subsequent analysis by chromatography. Thus, a preliminary fractionation with ion-exchange resins can purify amino acids, carboxylic acids, or sugars, and satisfactory chromatograms of these fractions can be run without trouble arising from inorganic salts present in the incubation medium. With more complex situations, such as those found in the studies by Calvin and his co-workers[1] on the path of carbon in photosynthesis, a preliminary fractionation by

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1. J. A. Bassham and M. Calvin, The Path of Carbon in Photosynthesis. Prentice-Hall, Inc., Englewood Cliffs, N.J., 1957.

ion-exchange resins of the products of photosynthesis is complex, due to the different ionic states of these products. Yet chromatography on a single two-dimensional sheet of paper in the appropriate solvent system is sufficient to separate almost all the compounds incorporating major quantities of C^{14} from labeled carbon dioxide. Such methods may be used with many different types of biological material, provided due consideration is given to the incubation conditions, the nature of the suspending medium, and the manner in which the metabolic products are extracted from the material and prepared for chromatography. The present communication is a discussion of various methods for handling the cellular material in studies fundamentally similar to those carried out on photosynthesis. These may be applied to the investigation of the metabolism of sugars, amino acids, phosphate esters, and other small molecules by plant, animal and microbial tissues, as well as by subcellular fractions such as chloroplasts and mitochondria.

One may start from two basic considerations: the quantity of cell extract and inorganic salts which can be carried by a sheet of chromatogram paper, and the price of radioisotopes. The less the amount of material spotted onto a chromatogram, the more satisfactory is the separation of the compounds contained in the mixture. It has been found experimentally that the most a standard 46 cm x 57 cm sheet of Whatman No. 4 filter paper can hold and still produce a reasonably acceptable chromatogram is the extract from about 100 mg wet weight of cells (equivalent to 10-20 mg dry weight for most tissues), together with 300-600 μg of inorganic salts, or about 9 mg of sucrose as might be used in media for subcellular particles. Quantities above these

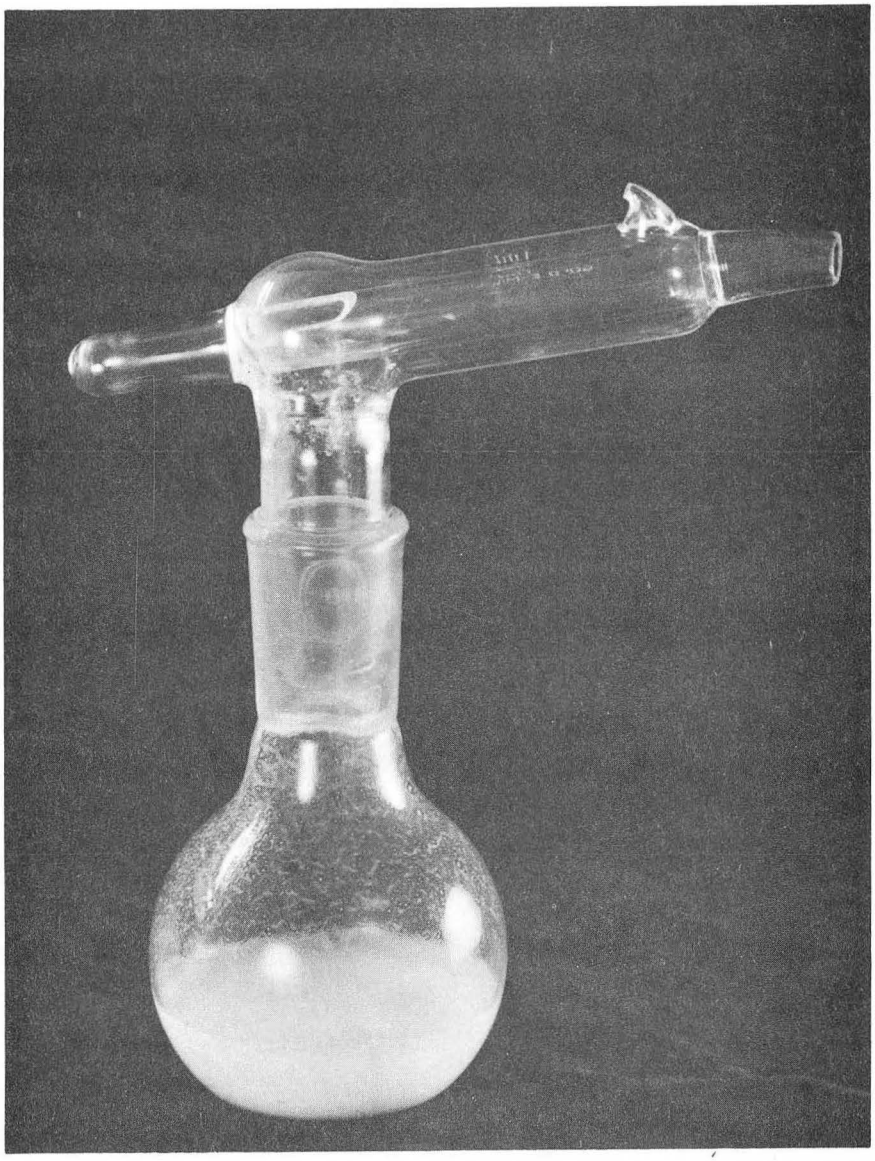
give increasingly poor chromatograms as a result of streaking, salt-front effects, and so on. Thus, from the point of view of analysis of a reaction mixture on one chromatogram only, no more than the extract from 100 mg of cells is required. As radioisotopes are expensive, it is most economic to supply the desired amount of isotopically-labeled compound to such an amount of biological material that the whole of the extract from the sample may be applied to the chromatogram. This will result in the shortest exposure time to prepare a radioautogram, a high counting rate for assaying the amount of radioactivity in each product substance, and a minimum expenditure on the purchase of the labeled substrate. It may not always be possible to use the extract from as much as 100 mg of cells due to the high salt concentration necessary in the incubation medium. For many systems, very small amounts of tissue suspended in a small fraction of a milliliter of liquid are sufficient for the preparation of each chromatogram.

Studies of intermediary metabolism with labeled substrates are usually of two main types: firstly, a kinetic study of the metabolism of one substance, involving the taking of samples under constant experimental conditions from the reaction mixture at a number of timed intervals after the start of the experiment; and secondly, investigation of the metabolism of different substances by one tissue, or of one substance under different environmental conditions. In all these situations it is important to be able to dispense replicate samples of the tissue preparation into various incubation vessels, or to remove replicates from a single reaction mixture at various times. It is therefore highly desirable that the tissue should be in a form in which it may be treated as a liquid suspension either in a pipette

or other liquid-flow system.

Of the different biological systems, micro-organisms are among the most convenient to use. Many of them are unicellular, and even the multi-cellular types can often be grown in the form of short filaments of pellets, and can readily be dispensed from a pipette. Their preparation for experimental purposes does not involve removal from their normal in vivo locations and cutting them into small pieces. They can usually be suspended in distilled water or in very weak salt solutions without showing ill effects. Two further characteristics are important: micro-organisms are often very active metabolically when compared with the tissues of higher plants and animals, and they usually do not permit large quantities of intermediary substances to leak out of the cells into the medium. On the other hand, they suffer from the disadvantage of impermeability to many substances which it would be interesting to use as substrates. For experiments using a number of replicate samples, each reaction might consist of 0.5 ml of distilled water or weak buffer containing about 25 mg wet weight of cells supplied with 1-5 μc of labelled substrate. The cells can be killed and extracted by adding 1 ml of boiling ethanol, and the cell debris removed by centrifugation after extraction is complete. The residues are usually extracted again with 0.5 ml or less of boiling 20% ethanol, the extracts pooled, concentrated in vacuo at 40° C., and chromatographed. For kinetic experiments the simple automatic tilt pipette shown in Fig. 1 may be used to remove samples at various times after addition of the labeled substrate without disturbing the rest of the suspension.[2] With a 1 ml

2. V. Moses and M.J.H. Smith, Biochem. J., 76, 585 (1960).



ZN-2773

Fig. 1. Automatic tilt pipette for sampling incubation mixtures.

pipette the variation in the volume of sample actually removed is ± 5%, and it is possible to kill the first sample about 5 sec. after adding the substrate by pouring the cell suspension in four volumes of ethanol.

For very short incubations, of the order of a second or less, Bassham et al.[3] have used a flow system as shown in Fig. 2. Labeled substrate is injected with a syringe into a tube containing the cell suspension flowing at a predetermined rate into a beaker of boiling methanol. The length of the incubation period may be varied by making the injection at various points along the tube.

With photosynthetic micro-organisms standard conditions of lighting must be employed and Fig. 3 shows the flat-sided "lollipop" for performing photosynthetic experiments. A suspension of algae is placed in the incubation vessel, which is kept at constant temperature by a circulating stream of water, while light is shone onto both flat surfaces. The substrate is injected into the top of the vessel and at the appropriate time the algae are run out from the bottom into the killing agent. Fig. 3 also shows the filter which can be used to separate the cells from the medium if a non-volatile substrate is being employed. The sintered-glass filter bears a thin layer of Celite filter aid, and a vacuum is applied to one flask with the two-way stopcock open through to that flask. The algal suspension is run onto the filter disk and the medium sucked through into the flask. The stopcock is then shut and boiling alcohol poured onto the cells. After a suitable period for extraction, the alcohol is allowed to run into the second flask, and the cells may be extracted with a further quantity of alcohol. With practice, it takes about 2 sec. to filter and kill the cells.

3. J.A. Bassham, A.A. Benson, L.D. Kay, A.Z. Harris, A.T. Wilson and M. Calvin, J. Am. Chem. Soc., 76, 1710 (1954).

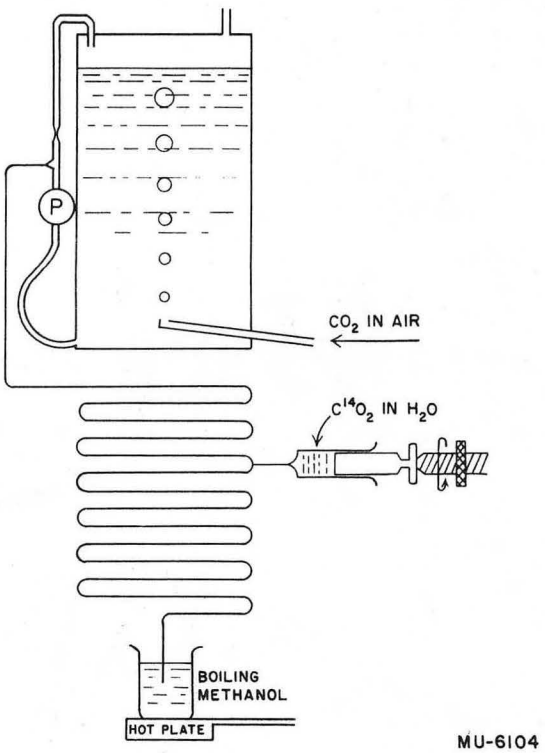
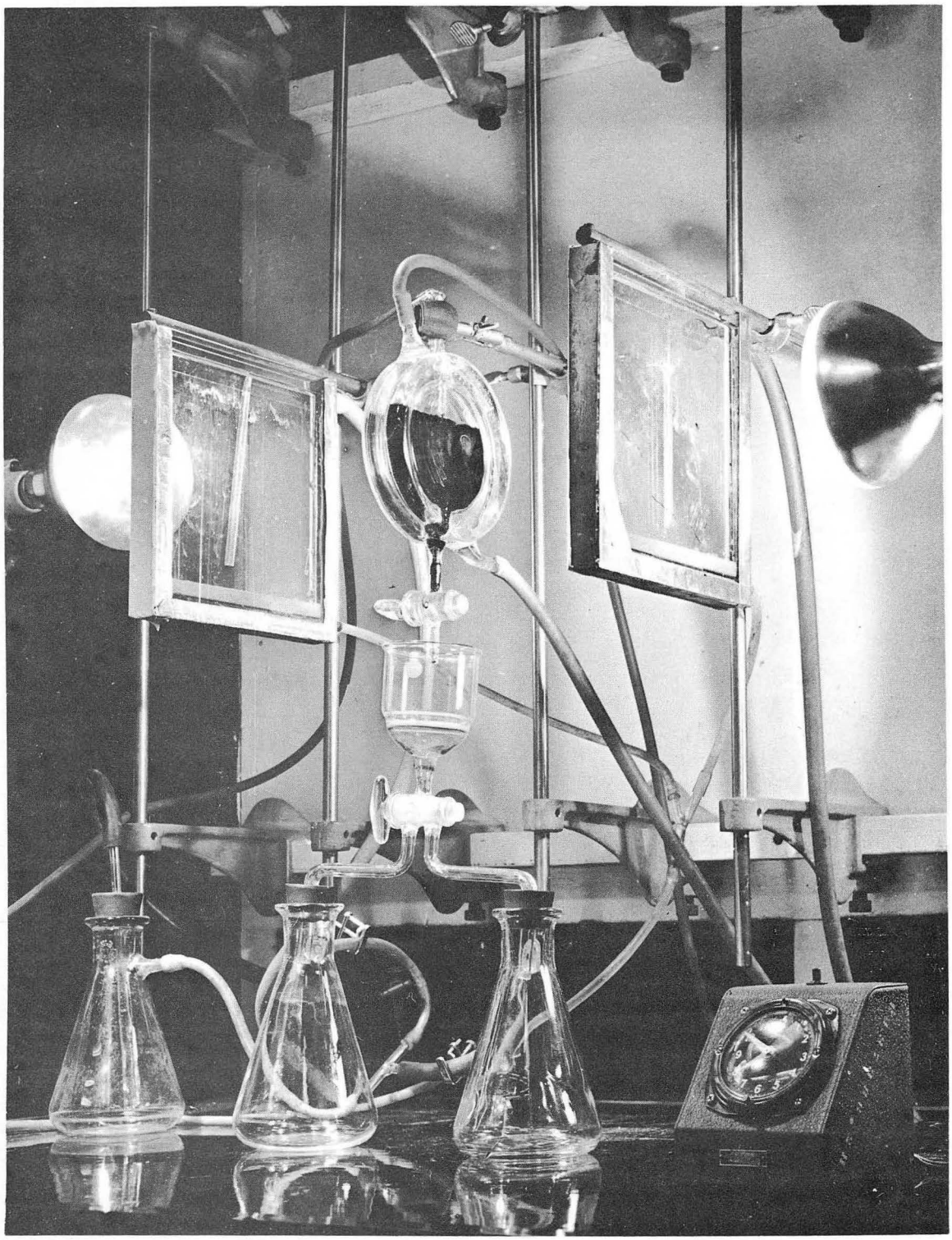


Fig. 2. Schematic diagram of flow system for short exposure of biological organisms to radioctive substrates.[3]



ZN-415

Fig. 3. Lollipop arrangement for photosynthesis experiments with algae.[4]

For kinetic studies of photosynthesis, a "steady-state" machine was described by Wilson and Calvin,[5] the principle of which is shown in Fig. 4.[4] The system allows samples of the algal suspension to be removed at intervals from a reservoir of cells kept under constant environmental conditions. The gas is recirculated in a closed system by a pump, and is continuously bubbled through the illuminated algal suspension. In the system are an ion chamber connected to a vibrating reed electrometer to permit continuous monitoring of the C^{14} content of the gas phase, as well as an infra-red carbon dioxide analyzer and an oxygen analyzer which works by measuring the paramagnetism of the circulating gas. A loop in the system contains a known amount of radioactive carbon dioxide which is admitted to the system when desired. A large gas bulb is provided to contain sufficient supplies of gas to ensure a constant gas environment for a long experiment, but this can be by-passed and the volume of the closed system reduced for measurements of the rate of gas exchange during short periods. Apart from its value in photosynthesis, the steady-state machine can be used for respiratory and fermentative studies, and in fact in any system in which it is desired to record continuously the changes in gas composition as well as to remove samples of the cells from the reaction vessel for analysis.

Photosynthesis experiments with higher plant organs can also be performed in a lollipop or steady-state machine. Fig. 5 shows a leaf arranged for short-term studies of the incorporation of labeled carbon dioxide.[4] The chamber is equipped with two tubes, the upper

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4. J. A. Bassham, University of California Lawrence Radiation Laboratory Report, UCRL-8676, 1959.
5. A. T. Wilson and M. Calvin, J. Am. Chem. Soc., 77, 5948 (1955).

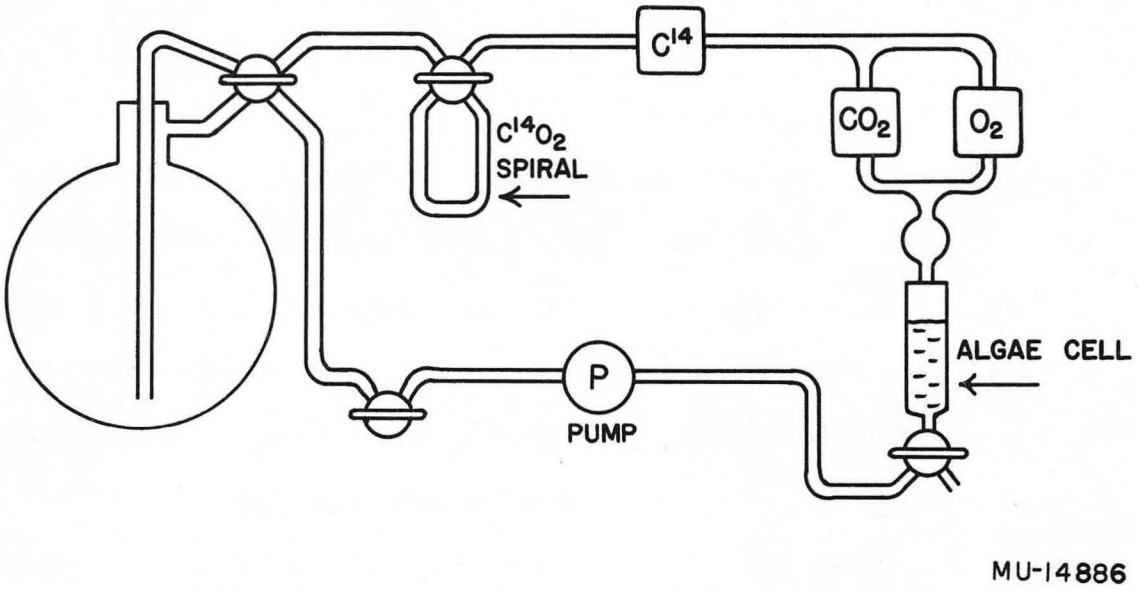
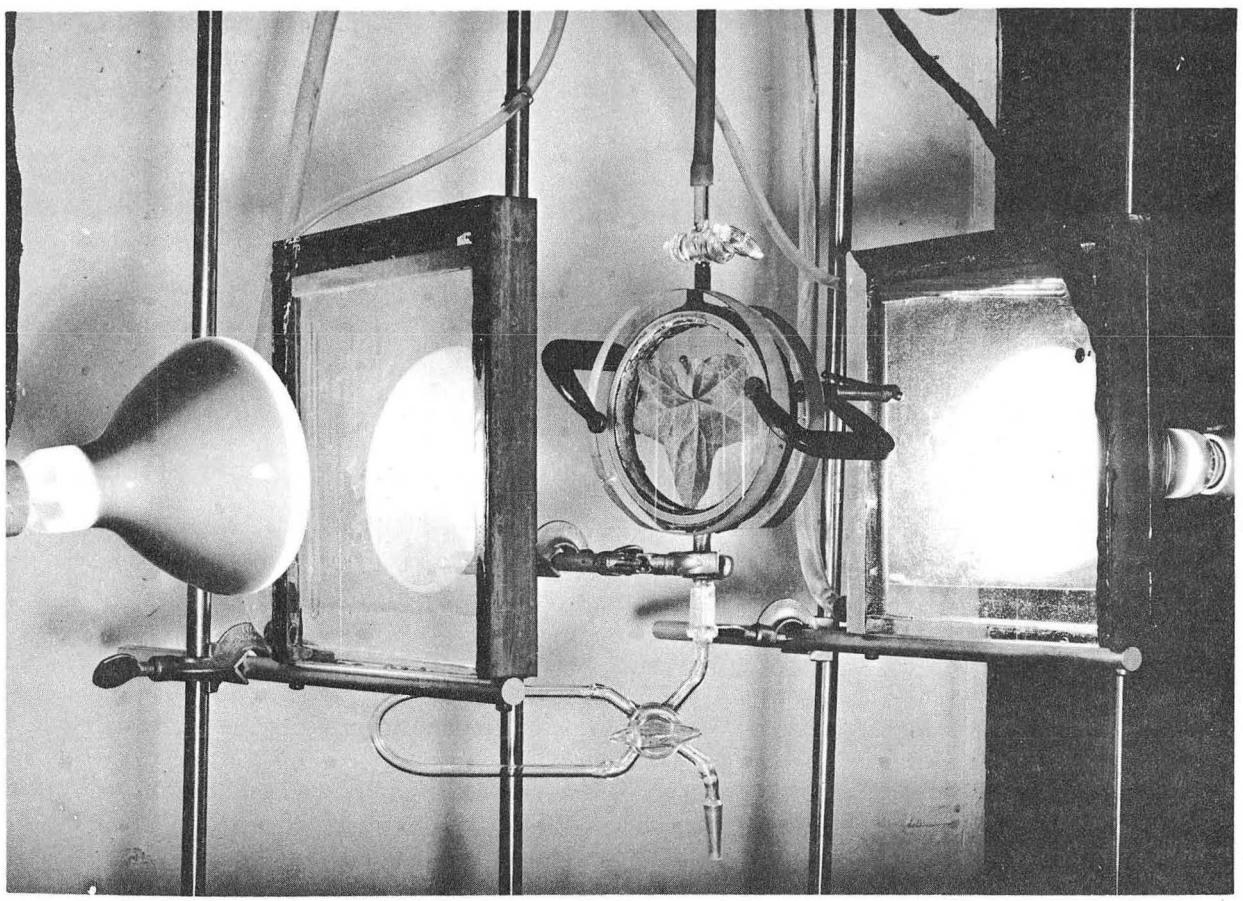


Fig. 4. Diagrammatic representation of the "steady-state" machine. The boxes labeled "C^{14}", "CO_2" and "O_2" symbolize the positions of the analytical instruments for measuring these parameters.[4]



ZN-952

Fig. 5. Lollipop arrangement for photosynthesis experiments with a leaf.[4]

one leading through a two-way stopcock to a looped tube containing labeled carbon dioxide. A loosely tied thread leads from the leaf stem under the detachable face gasket and then through a bath of boiling ethanol and a glass tube to a weight. The chamber is partially evacuated and the clamps holding the face of the chamber are removed, the face remaining in position by atmospheric pressure. When the upper stopcock is opened, air enters the chamber via the loop, sweeping radioactive carbon dioxide before it. The detachable face falls off and the leaf is pulled into the ethanol; it is estimated that the leaf is exposed to the carbon dioxide for about 0.4 sec.

Animal tissue preparations present the greatest variety of problems. The tissue must almost invariably be suspended in a salt-rich medium, and most tissues have to be cut from a whole animal. Experiments have been successfully performed with human erythrocytes[6] and with HeLa cancer cells grown in tissue culture.[7] The cells are suspended in the appropriate liquid medium and each experimental sample is used in a total volume of 0.05 ml. The reaction is started by the addition of 1-5 μc of labeled substrate, and the cells killed by adding 0.2 ml of ethanol. The whole ethanolic suspension can then be spotted onto paper with no preliminary extraction.

Diaphragm muscle from rats may be removed from the animal, cut into quarters, and each piece, weighing about 50 mg., incubated in a small beaker in 0.5 ml of medium, using the apparatus shown in Fig. 6.

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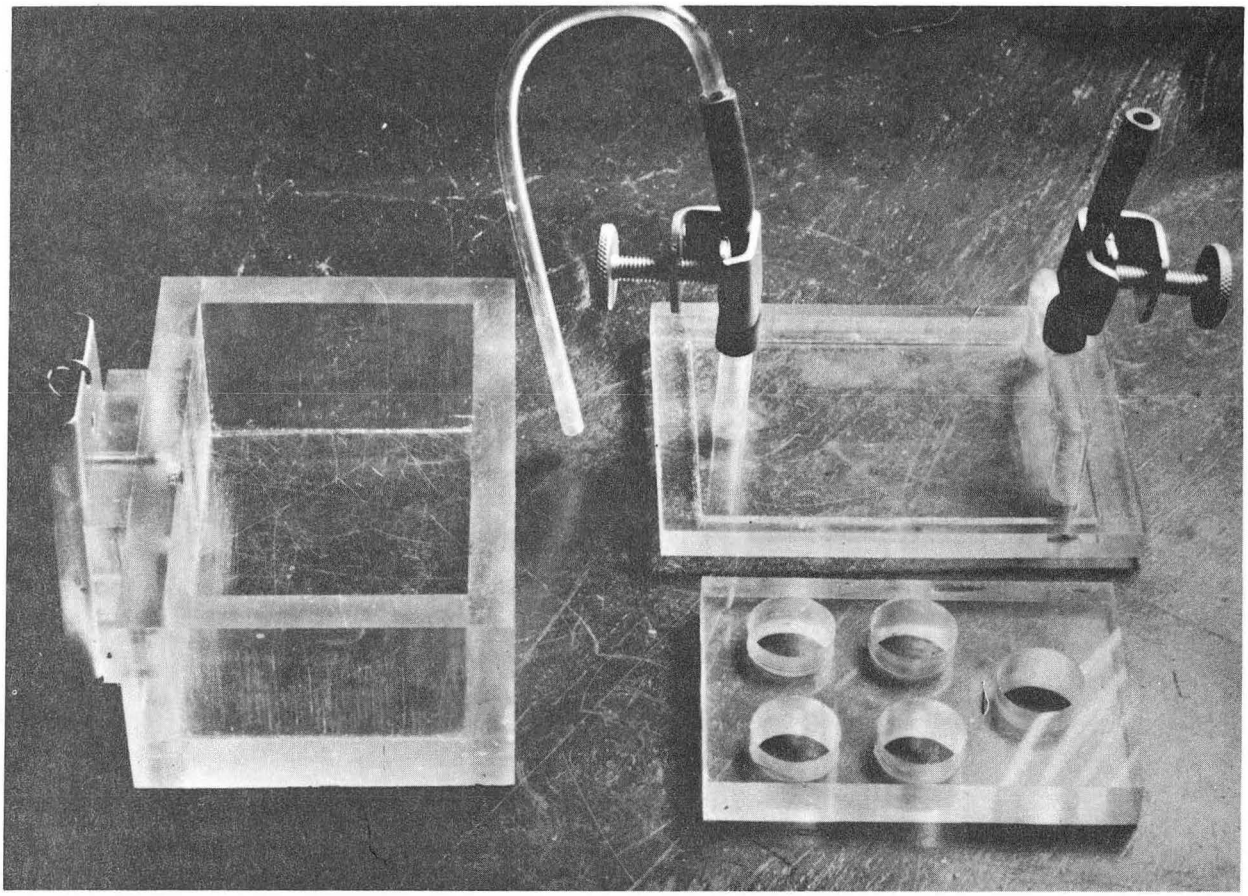
6. V. Marks and V. Moses, unpublished observations, 1960.

7. C. Bryant and V. Moses, unpublished observations, 1960.



ZN-2774

Fig. 6. Incubation vessel for diaphragm studies. The individual beakers are placed in the holes in the perspex plate which lies at the bottom of the box. The vessel is lined with moist filter paper to prevent evaporation from the experimental sample.[8]

The apparatus is fitted with tubes for flushing with the desired gas mixture, and the whole box is shaken in a bath at 37° C.[8] At the end of the experiment each piece of diaphragm is simply removed from its beaker, washed, and dropped into ethanol.[9]

With other animal tissues, slices can be used if there is no alternative, but to achieve randomization of sliced material, much more tissue must be used than would be required from chromatography. There are, however, two types of tissue preparation which can readily be dispensed in small quantities. One of these, obtained by homogenization, will contain a high proportion of damaged cells and reactions dependent on an intact cell architecture may be lost. Another preparation is produced with a tissue chopper designed by McIlwain and Buddle[10] and is illustrated in Fig. 7. The tissue is laid on a pad of moist filter paper on the turntable and cut into slices by repeated blows from a razor blade clamped to an arm, the turntable moving a preset distance between each cutting stroke. The slices are next laid flat on another pad of filter paper and cut in two directions, producing small blocks of tissue. These blocks (Fig. 8) can be made small enough to be dispensed with a wide-mouthed pipette, and have a large surface area in proportion to their bulk. After chopping, the tissue may be washed free from debris by centrifugation and the blocks from each gram of tissue before chopping suspended in

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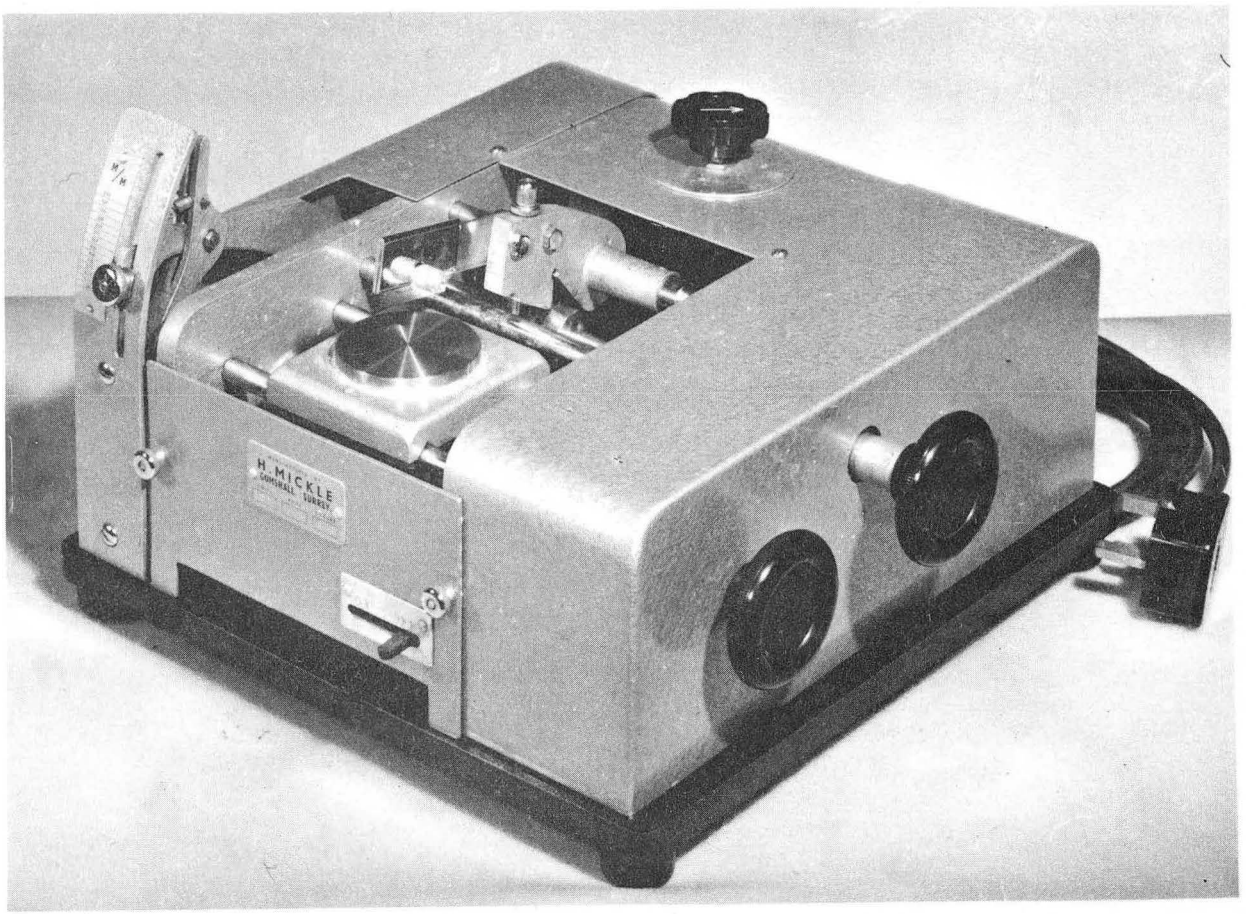
8. A. K. Huggins, M.Sc. Thesis, University of London, 1959.

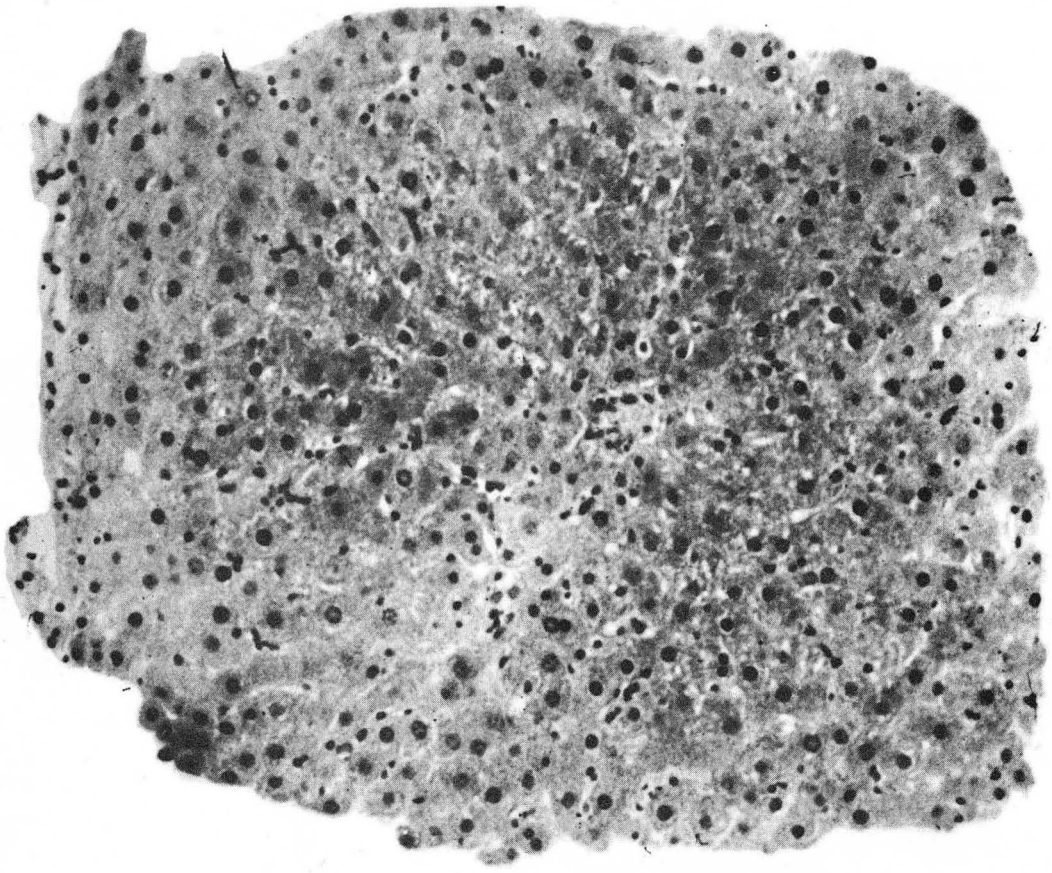
9. A. K. Huggins, unpublished observations, 1960.

10. H. McIlwain and H.L. Buddle, Biochem. J., 53, 412 (1953).



ZN-2775

Fig. 7. Tissue chopper. The sample is placed on a pad of moist filter paper on the turntable, and razor blade is clamped to the arm which is raised and lowered by a cam and eccentric. The scale on the left indicates the thickness of the slices being cut.



ZN-2777

Fig. 8. Rat liver preparation obtained with the tissue chopper shown in Fig. 7. Each small division is 1 mm.

about 2.5 ml of medium; 0.25 ml aliquots are dispensed into tubes for each experimental sample. The standard error of pipetting samples in this way is only 2-3%. After killing and extracting each sample of tissue with 1 ml of ethanol, about 20% of the extract can be spotted onto each chromatogram without exceeding the salt-carrying capacity of the paper. In spite of the waste, it has not been found possible to perform the experiment in a volume less than 0.25 ml owing to the difficulties of sample replication.[11] Fig. 9, which illustrates a section through a block of tissue from rat liver, shows that apart from damage along the actual cut edges of the block, the tissue appears quite normal.

Sub-cellular particles can be treated in the same way as erythrocytes and tissue culture cells, and the experiment performed in a total volume of 0.05 ml. With the usual type of incubation medium used for particles, containing about 0.25 M-sucrose, and thus about 4 mg of sugar in 0.05 ml of medium, the whole sample may be run on one chromatogram.

Figs. 10-19 show the results of incubating various biological tissues and sub-cellular particles with a number of C^{14}-labeled substrates. The basic method of incubating biological preparations with radioactive substrates, and analyzing the products by paper chromatography and radioautography has a wide application, and by suitable experimental techniques most types of tissue and cellular fractions can be investigated in this way.

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11. V. Moses and M.J.H. Smith, Biochem. J., 78, 424 (1961).

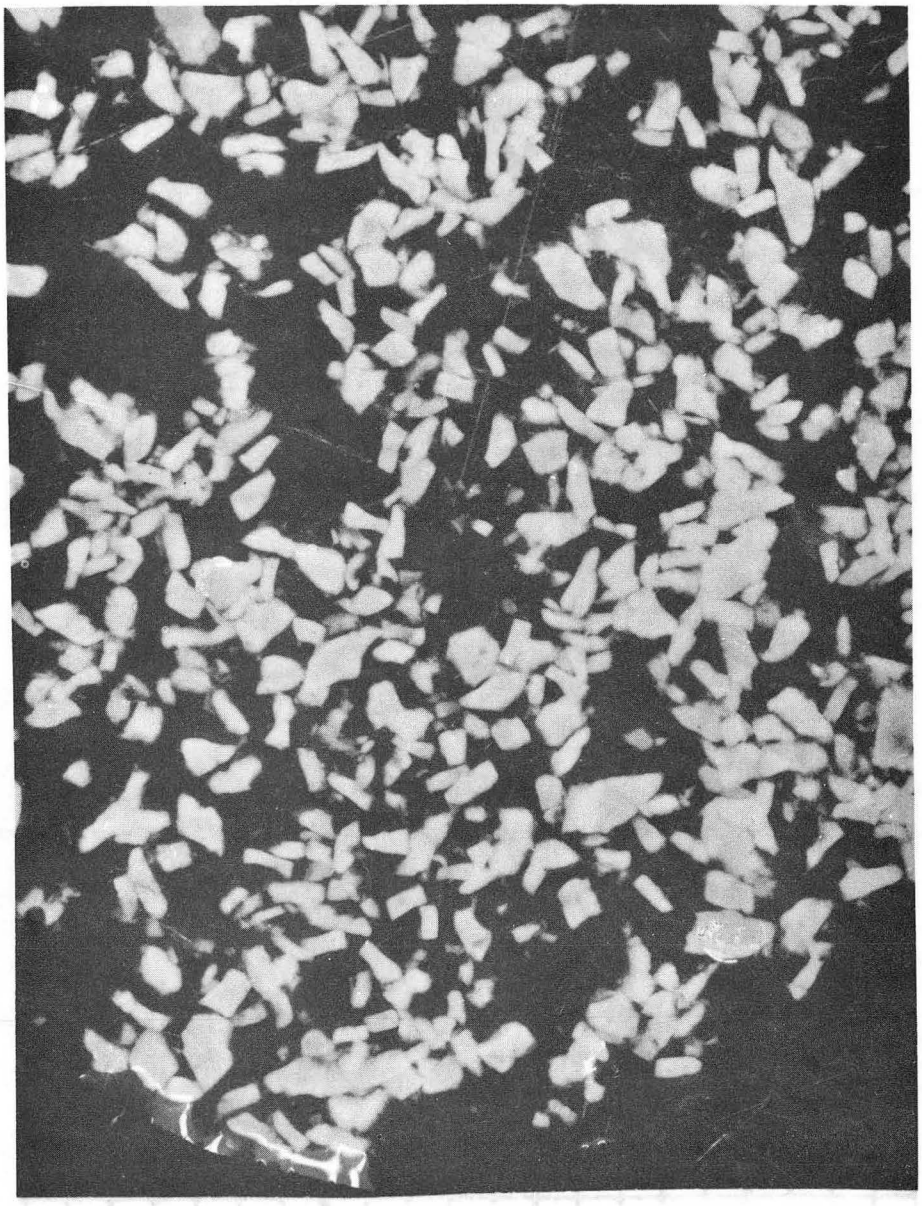

Fig. 9. Histologic section through a piece of chopped liver shown in Fig. 8.

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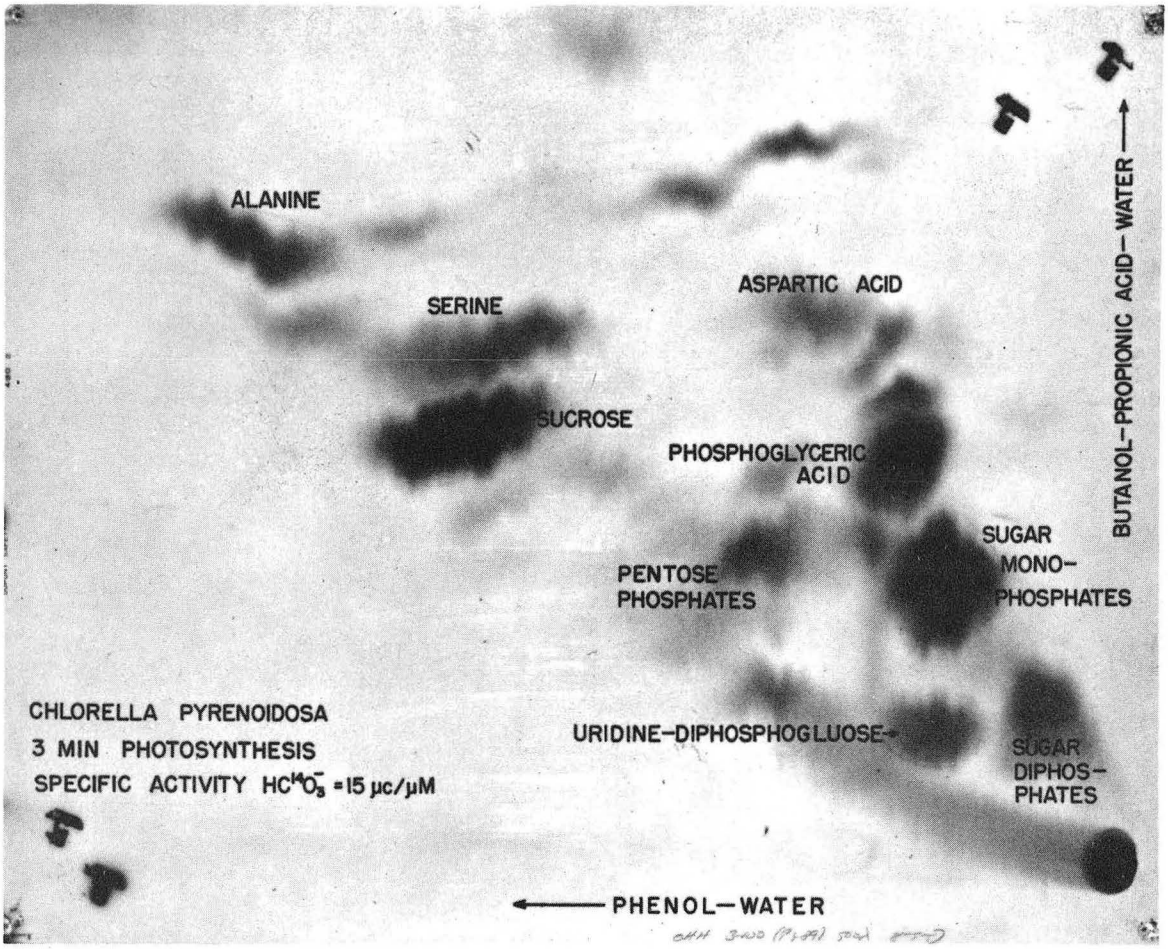

ZN-2781

Fig. 10. Radioautogram of chromatogram of extract of the green alga Chlorella exposed to $C^{14}O_2$ for 3 min. in the light.[12]

12. O. Holm-Hansen, V. Moses, C. F. van Sumere and M. Calvin, Biochem. Biophys. Acta, 28, 587 (1958).

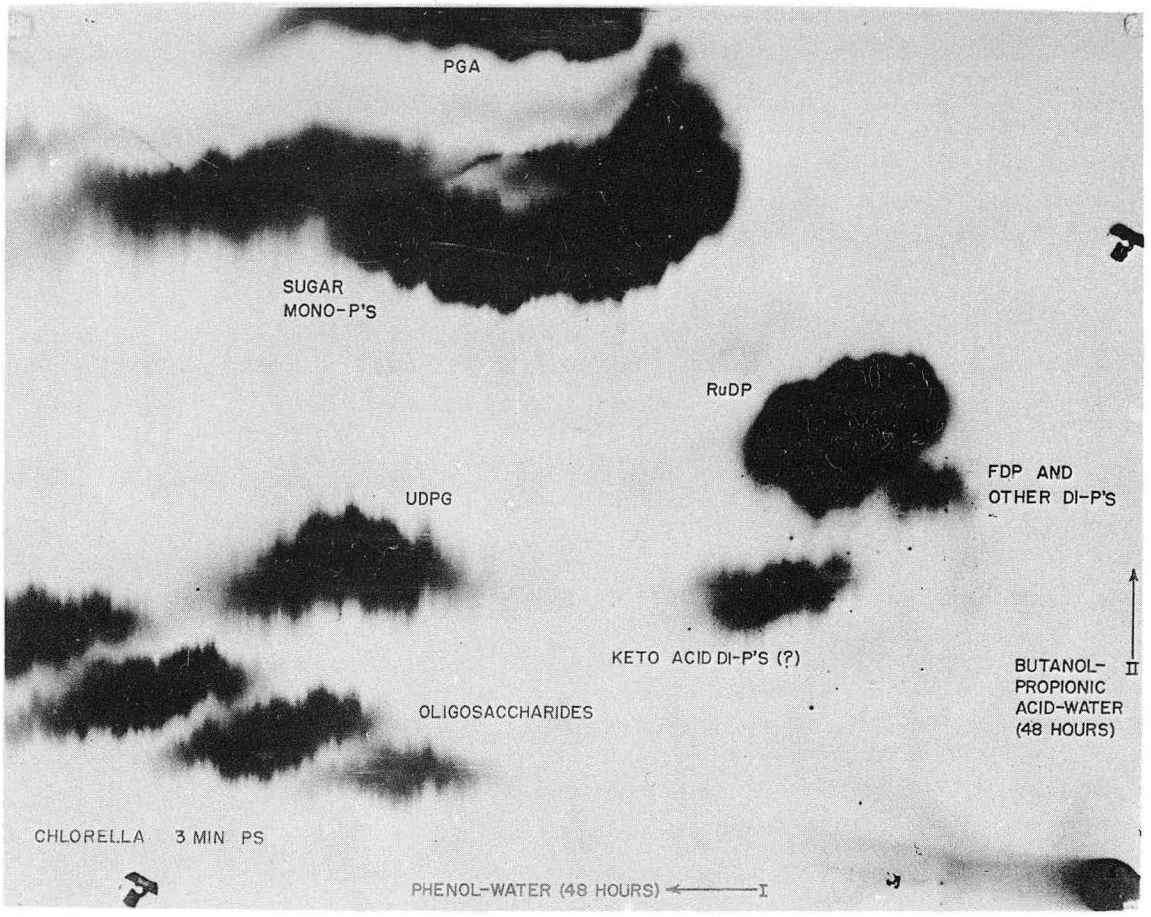

Fig. 11. Radioautogram of extract similar to that in Fig. 10, after much longer chromatographic development to achieve separation of the phosphate esters.[13]

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13. V. Moses and M. Calvin, Proc. Natl. Acad. Sci., 44, 260 (1958).

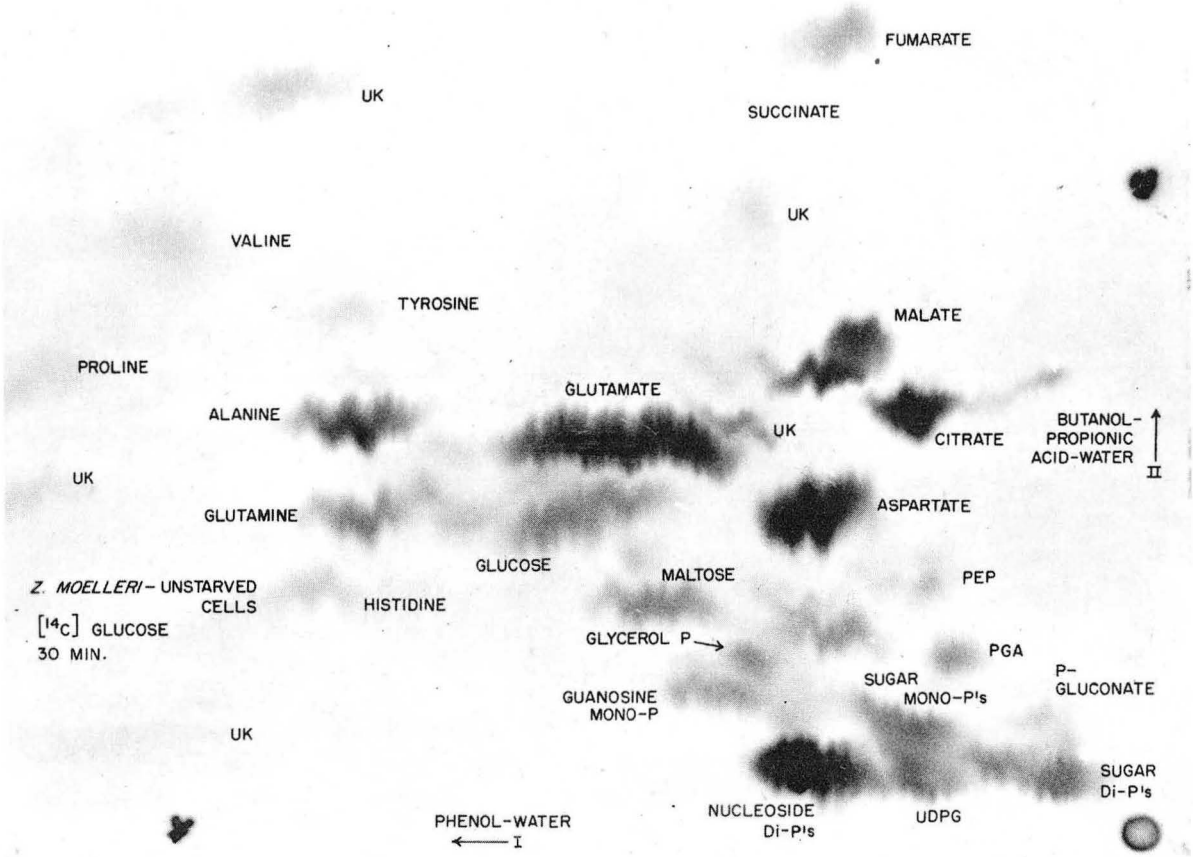

Fig. 12. Radioautogram of chromatogram of an extract of the filamentous fungus Zygorrhynchus moelleri incubated with glucose-U-C^{14} for 30 min.[14]

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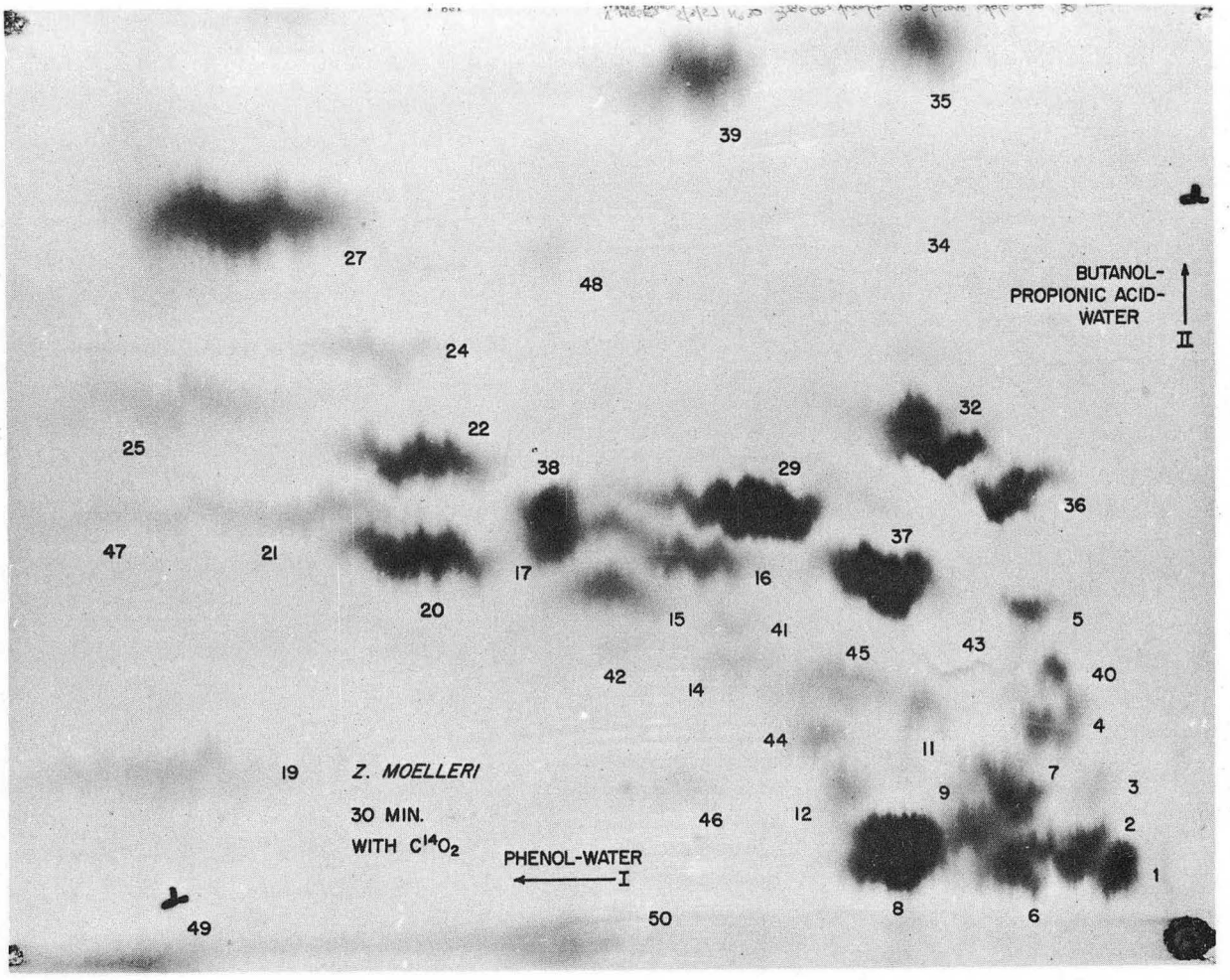
14. V. Moses, J. Gen. Microbiol., 20, 184 (1959).



ZN-1981

Fig. 13. Similar to Fig. 12, using $C^{14}O_2$ as the substrate. Key to numbers: 1, uridine triphosphate; 2, uridine diphosphate plus inosine triphosphate; 3, phosphogluconic acid; 4, phosphoglyceric acid; 5, phosphoenolpyrivic acid; 6, uridinediphosphoglucose; 7, hexose monophosphates; 8, nucleoside diphosphates; 9, adenosine triphosphate plus uridine diphosphate; 14, maltose; 15, sucrose; 16, serine; 17, glycine; 20, glutamine; 21, citrulline; 22, alanine; 24, tyrosine; 25, proline; 27, valine; 29, glutamic acid; 32, malic acid; 35, fumaric acid; 36, citric acid; 37, aspartic acid; 38, threonine; 39, succinic acid; 40, phosphoglycollic acid, rest unidentified.[15]

15. V. Moses, O. Holm-Hansen and M. Calvin, J. Bact., 77, 70 (1959).

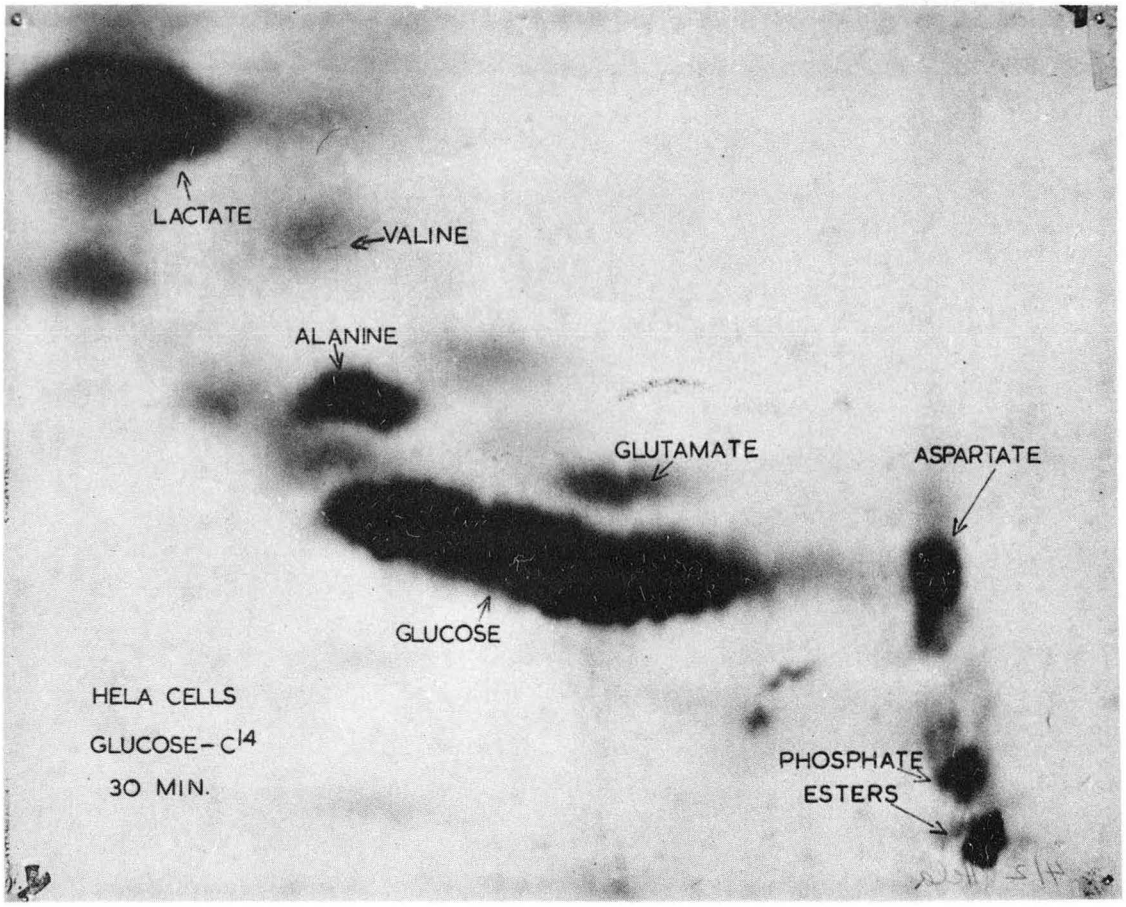
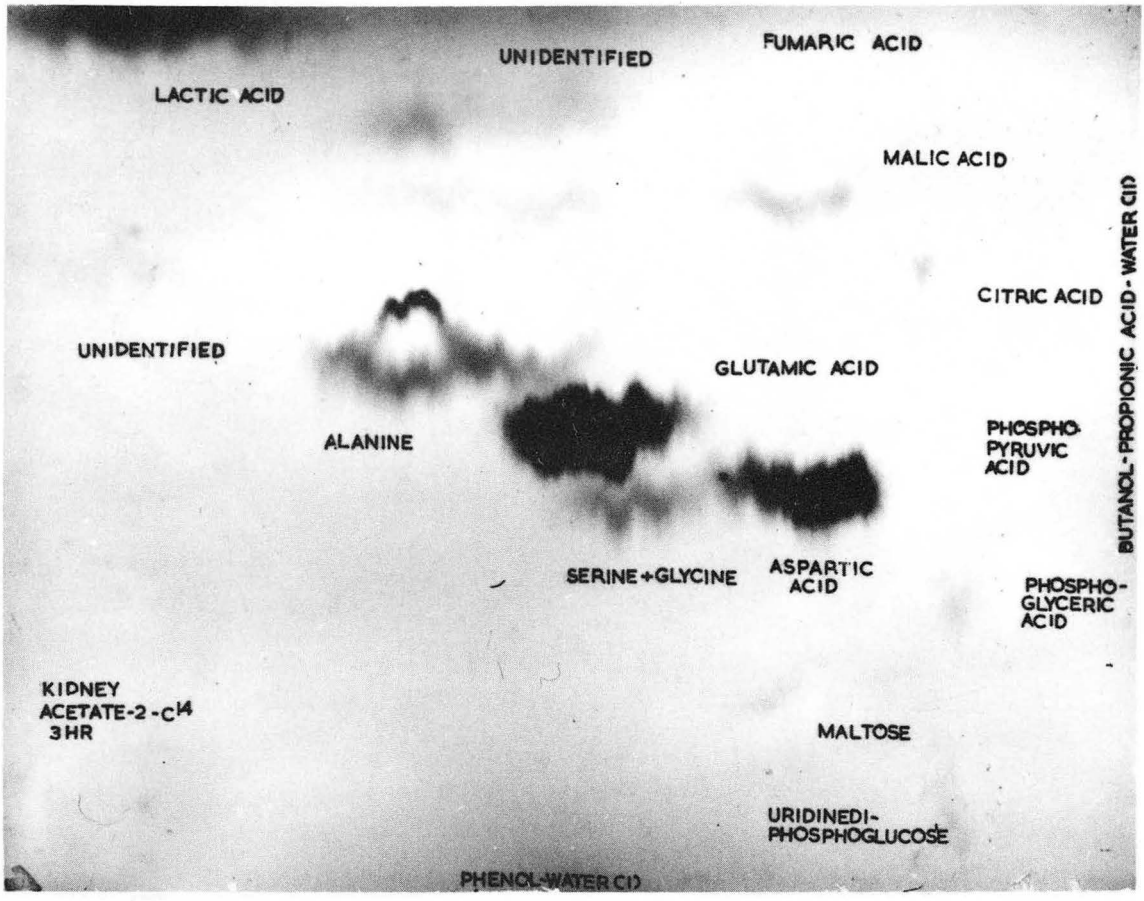


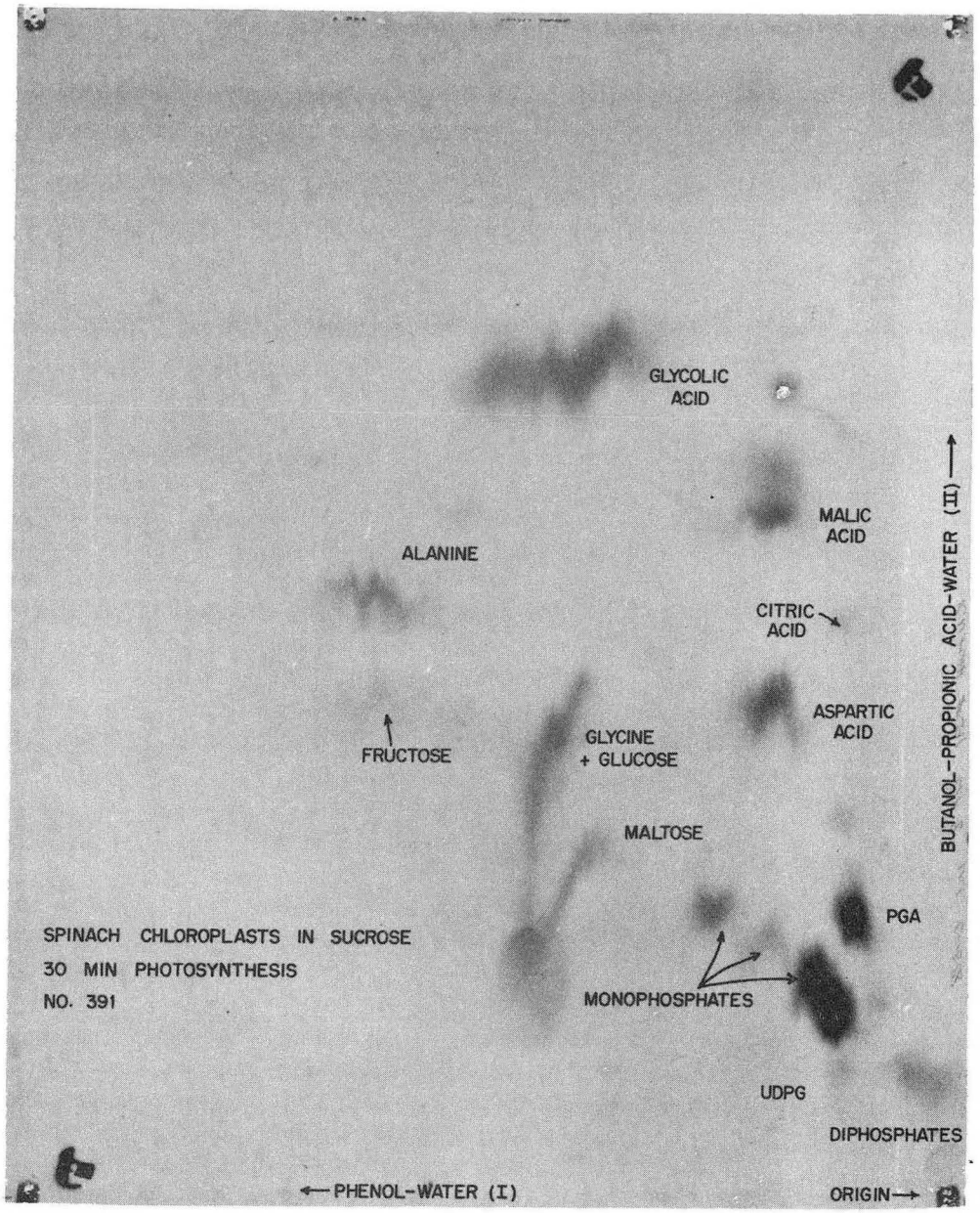
Fig. 14. Radioautogram of chromatogram of HeLa tumour cells grown in tissue culture and incubated with glucose-U-C^{14} for 30 min.[7]



ZN-2779

Fig. 15. Radioautogram of chromatogram of extract rat kidney incubated with acetate-2-C^{14} for 3 hr.[16, 17]

16. M. J. H. Smith and V. Moses, Biochem. J., 74, 17p (1960).
17. M. J. H. Smith and V. Moses, Biochem. J., 76, 579 (1960).



ZN-2161

Fig. 16. Radioautogram of chromatogram of extract of spinach chloroplasts exposed to $C^{14}O_2$ for 30 min in the light.[18]

18. O. Holm-Hansen, N.G. Pon, K. Nishida, V. Moses and M. Calvin, Physiol. Plant., 12, 475 (1959).

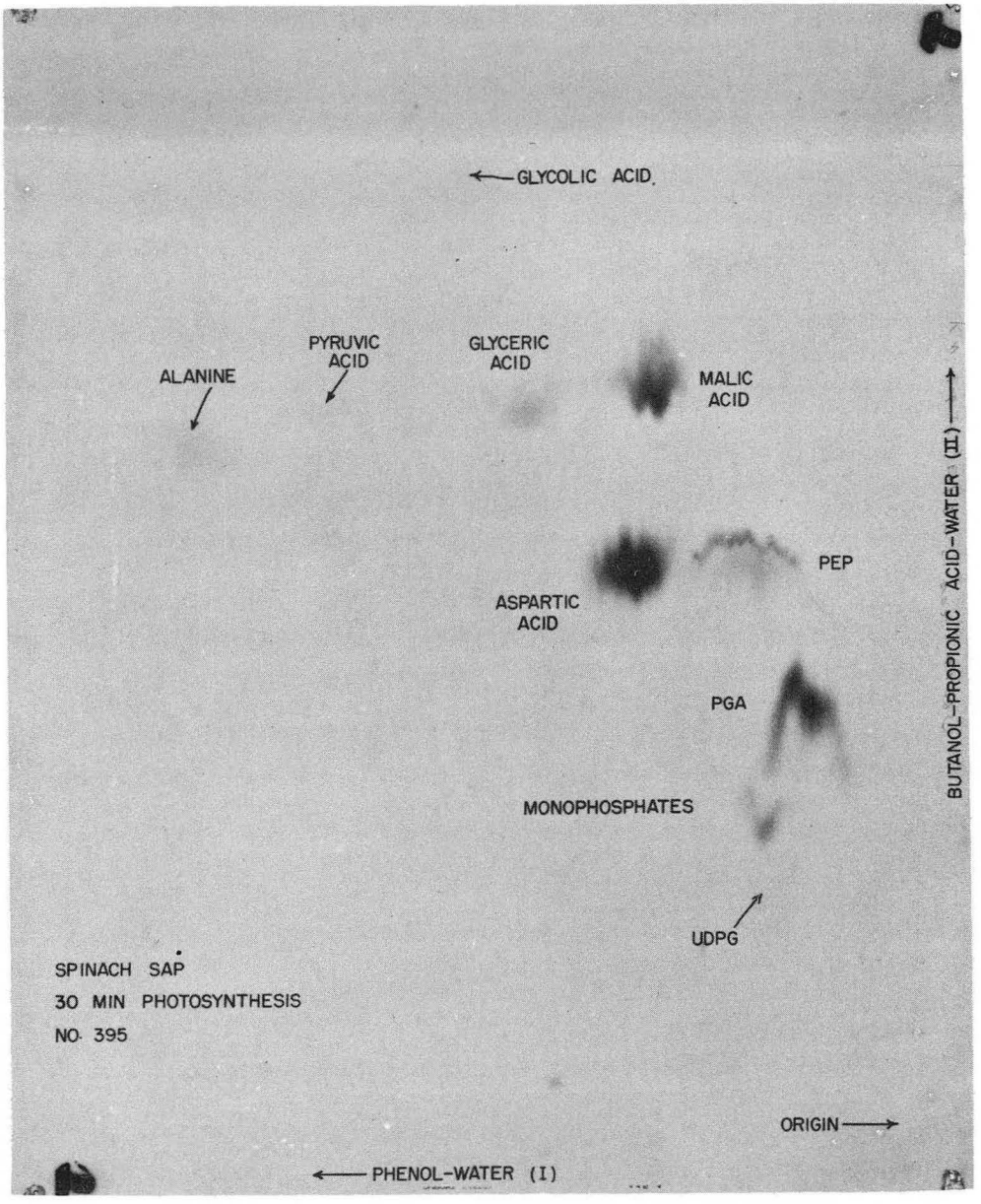


Fig. 17. Radioautogram from spinach sap exposed to $C^{14}O_2$ for 30 min in the light.[18]

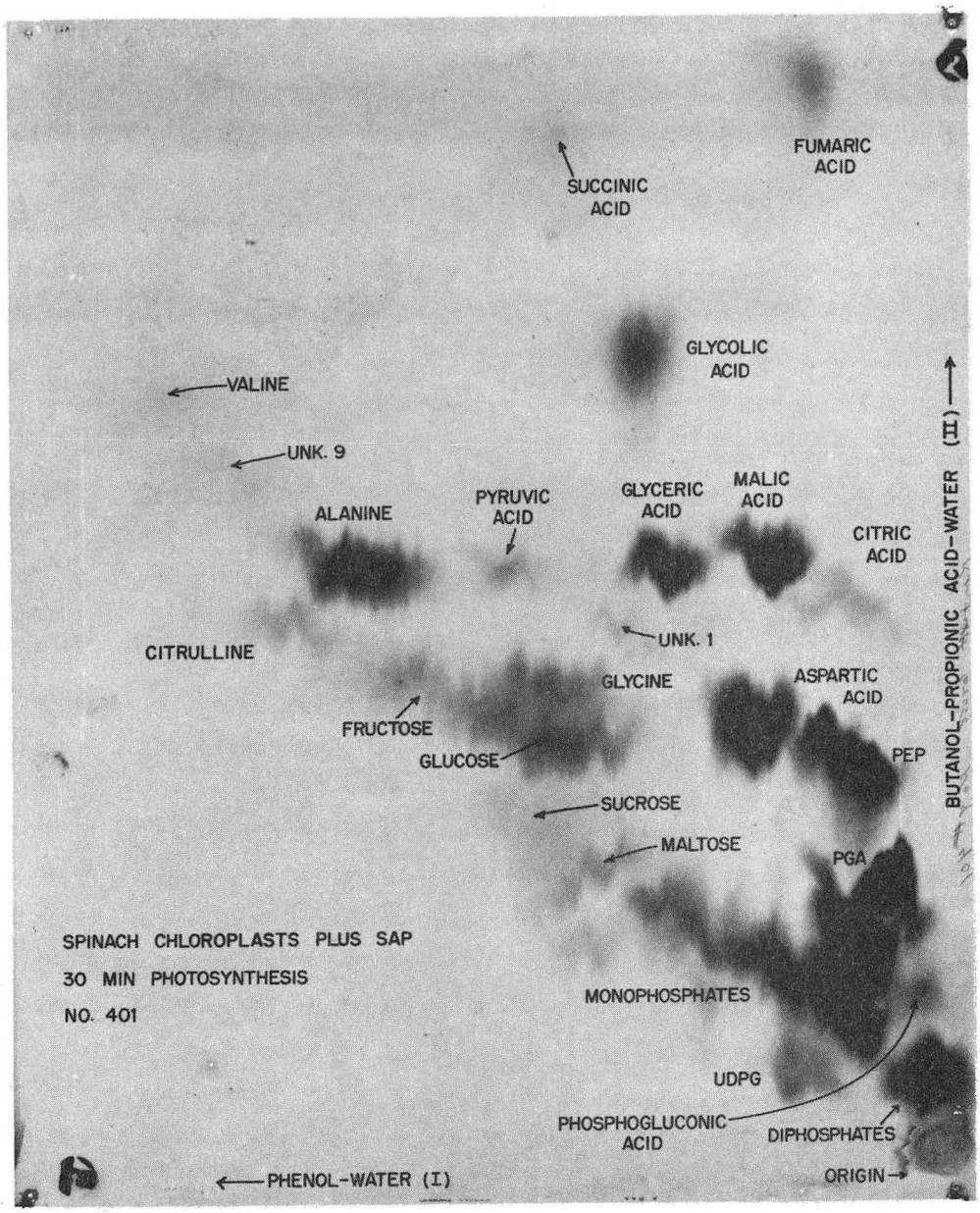


Fig. 18. Radioautogram showing the effect of mixing spinach chloroplasts with sap.[18]

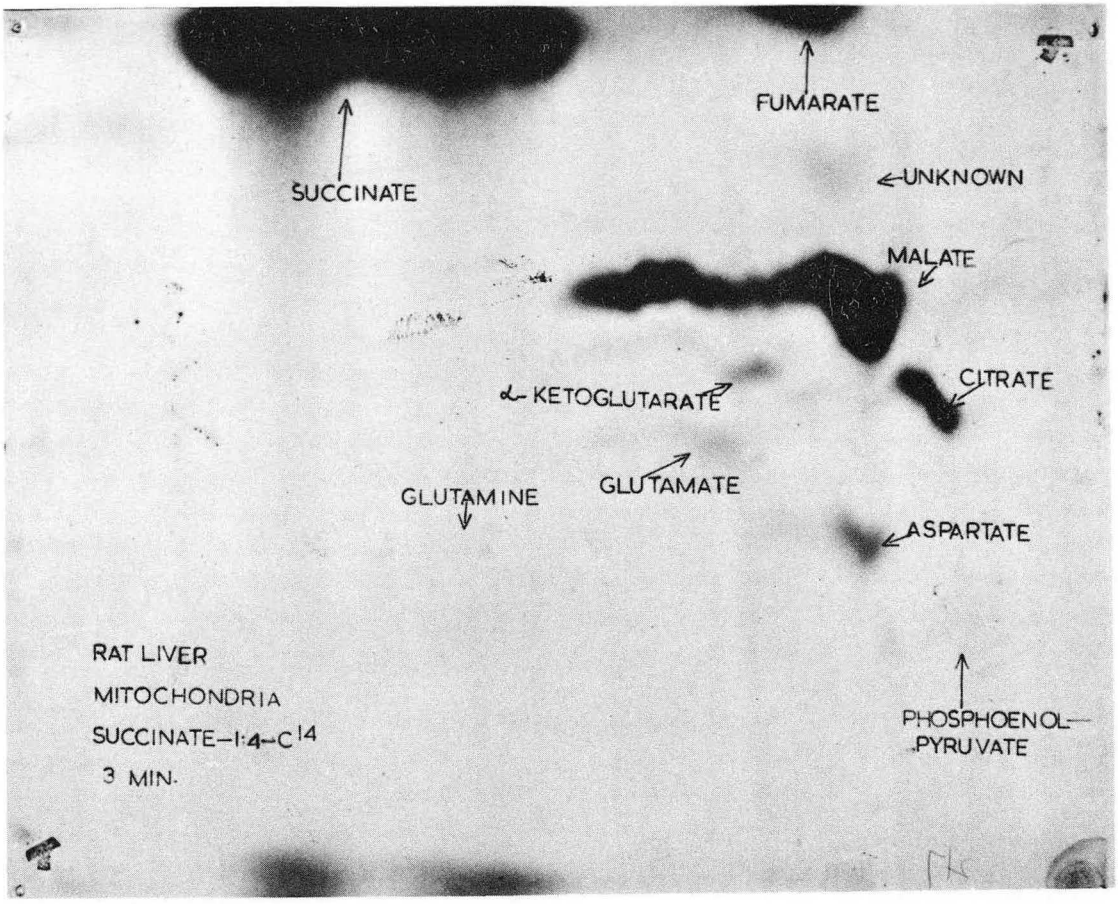


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Fig. 19. Radioautogram showing results of incubating rat liver mitochondria with succinate-1:4-C^{14} for 3 min.[7]