IN THE LABORATORY

Radiocarbon and Filter-Paper Partition Chromatography[1]

ROBERT M. FINK and KAY FINK[2]

Department of Radiology,
School of Medicine and Dentistry,
University of Rochester

Experiments with radioiodine, I^{131}, (*3*) indicated that filter-paper partition chromatography might be a very useful tool in biological studies with radioisotopes. In order to determine whether the procedure could be used profitably with radioactive elements showing a more general distribution and a less penetrating radiation, an experiment was carried out with radiocarbon, C^{14}.

One mg of *Chlorella* suspended in 0.1 ml of distilled water was exposed to light in the presence of 2 ml of air containing a few micrograms (1 μc) of radioactive carbon dioxide for a period of 4 hrs, and the tube containing the *Chlorella* was then connected with a tube containing solid KOH for an additional hour. The *Chlorella* suspension was divided into two equal portions, one of which was hydrolyzed in 0.1 ml of 6 N HCl for 24 hrs, and the other centrifuged and extracted with 25 μl of hot 80% alcohol for 30 min. A two-dimensional chromatogram of each of the solutions (plus added amino acid markers) was developed with phenol and collidine,[3] and after the filter papers were dry, they were pressed directly against sensitive X-ray film (E.K. "No Screen") for three days. The alcohol extract gave an excellent chromatogram, the radioautograph of which showed exposed areas as follows:

(1) A rather poorly defined very dark spot or spots due to relatively fat-soluble radioactive substances, possibly organic acids of low volatility, which had traveled essentially with the boundary in both phenol and collidine.

(2) A dark spot in approximately the position taken by glucose in routine chromatograms (R_F values of 0.42 and 0.41 in phenol and collidine, respectively).

(3) A moderately dark spot coinciding in position with the glutamic acid added as a marker.

(4) Light spots coinciding in position with the glycine, alanine, arginine, valine, and proline added as markers.

(5) Light spots tentatively identified as aspartic acid, serine, and threonine; two light spots near glycine and two near alanine which could be sugars or peptides; and an unidentified light spot with R_F values of 0.98 and 0.06.

There was no visible exposure in the area occupied by the phenylalanine added as a marker or in the positions ordinarily taken by the amino acids not listed above.

A chromatogram prepared from an 80% alcohol extract of 200 mg of nonradioactive *Chlorella* caused no "chemical exposure" of an X-ray film and, when treated with ninhydrin, showed spots tentatively identified as aspartic acid, glutamic acid, serine, glycine, threonine, alanine, histidine, and arginine, and an unidentified spot with R_F values of 0.10 and 0.05.

Due to a marked temperature change during development, the chromatogram prepared from the *Chlorella* hydrolysate was skewed to such an extent that detailed identifications could not be made. However, it appeared similar to the alcoholic extract chromatogram except for a general increase in the amino acid radioactivity, so that glutamic acid apparently was about as radioactive as the fatty substances, while the other radioactive amino acids present contained amounts of C^{14} similar to that in the spot tentatively identified as glucose.

Reduction in sensitivity of the radioactive measurements due to absorption of the soft C^{14} beta ray in the filter paper appeared to be of moderate degree. A thin-window Geiger counter capable of recording 30% of the beta particles from a thin layer of $BaC^{14}O_3$ counted 10% of the disintegrations from the C^{14} in a filter paper spot. The areas of the filter paper which produced light spots on the radioautograph could hardly be differentiated from the blank areas with an unshielded counter.

These experiments appear to be convincing evidence that an extremely valuable tool for intermediary metabolism studies with radioisotopes is at hand. With the possibility of preparing filter paper chromatograms of moderately water-soluble compounds of low molecular weight, such as amino acids (*1*), sugars (*5*), organic acids (*4*), purines (*6*), peptides (*2*), and a variety of other organic and inorganic substances (*2*), a considerable amount of data concerning the metabolic fate of radioactive compounds may be obtained by a very simple procedure. A particularly valuable feature of the chromatographic technic is its ability, without any deviation from the routine procedure, to isolate and draw attention to unexpected or even unknown compounds involved in a metabolic process under study and, in addition, to aid considerably in their identification.

The lower limit to the quantity of any given compound which may be handled successfully by partition chromatography seems to be determined, in general, by the

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[2] Present address: Birmingham Veterans Administration General Hospital, Van Nuys, California, and the University of California, Los Angeles. The authors wish to thank Albert Frenkel for furnishing the *Chlorella* and W. F. Bale and A. H. Dowdy for their interest and encouragement in this work.

[3] The mixture referred to as collidine in this description was actually the organic phase from a mixture of 1 part 2,4,6-collidine, 1 part 2,4-lutidine, and 2 parts H_2O, as recommended to us by C. E. Dent.

sensitivity of the procedure employed for detecting the spots. With most good visual color reactions this lower limit is in the order of 1 μg (and frequently embarrassingly close to the upper limit), but with radioactive compounds of high specific activity quantities many orders of magnitude smaller may be easily detected, identified, and isolated in a small volume of solvent for further tests. Due to the limited top capacity of the filter paper (upper limit generally in the order of 10^{-4} gm of any single compound) and the consequent desirability of obtaining high specific activity in the radioactive metabolites, microorganisms which are resistant to radiation damage would seem to be the most suitable subjects for study by the procedure outlined. The present results indicate, however, that application of the technic to short-term experiments with small animals may be practical, since radioisotope doses of the order of 10^{-5} c/gm of tissue should be adequate for most purposes. Work is continuing to test the feasibility of obtaining data concerning the mechanisms of biological conversions by preparing a sequence of chromatograms at various periods after administration of an isotopic substance to show the order and rate at which various radioactive spots appear and fade. Preliminary data and calculations also indicate that it may be possible to use the chromatograms as a means of isolating radioactive intermediates for use in further metabolism experiments on a proportionately smaller scale.

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Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent

DANIEL LUZON MORRIS

Department of Nutritional Research, Mead Johnson & Company, Evansville, Indiana

Roman Dreywood recently described a reagent for carbohydrates which is simpler to prepare and use than any of the standard reagents (*1*). Moreover, its specificity for carbohydrates is very high. Dreywood suggested that the reagent might be of value for quantitative determinations and has actually used it for the determination of cellulose and starch.[1]

The specificity that Dreywood claimed for the reagent has been fully confirmed in this laboratory: it has given a positive reaction with all pure mono-, di-, and polysaccharides tested, as well as with all samples of dextrins, dextrans, starches, and plant polysaccharides and gums. Positive reactions were also obtained with pneumococcus polysaccharides of types II and III (but not type I),[2] with all glucosides tested, and with the acetates of mono-, di-, and polysaccharides. No noncarbohydrates tested gave the characteristic blue color; a red color was produced by polyvinyl alcohol and by proteins. The common solvents gave no color, though solutions containing dioxane became fluorescent. The sugar alcohols likewise produced no color.

For a quantitative reagent, 2 gm of anthrone[3] is dissolved in 1 liter of 95% sulfuric acid (prepared by the cautious addition of 1 liter of concentrated sulfuric acid to 50 ml of water, and cooling). Four or 5 ml of the solution to be determined is measured into a test tube of 19- to 25-mm diameter, and 8 or 10 ml of the reagent added. The solutions are at once thoroughly mixed by swirling. After 10 min or more, the color is measured either in an electrophotometer against a blank containing only water and reagent or in a visual colorimeter against a glucose standard. The color varies with the amount of carbohydrate, in accordance with Beer's law, if color filters of 540 mμ (green) or 620 mμ (red) are used. The latter has been found preferable, since it gives higher sensitivity and decreases any errors caused by extraneous colors.

Inasmuch as the reaction is brought about by the heat developed when the reagent and water solutions are mixed, the shape and size of the reaction tube are important. Less color develops with a given amount of sugar in either small (less than 15-mm diameter) or very large tubes, and this error is greater at higher sugar concentrations. This is presumably due to the cooling of the solution before the reaction is finished. Use of small volumes of solution has the same effect. Thus, if a Klett-Summerson colorimeter is to be used, the reaction must be carried out, as described, in 19- to 25-mm tubes, and the solution poured into the Klett tubes just before the readings are made.

Fig. 1 shows absorption curves for glucose, measured with three different filters, in an Evelyn photoelectric colorimeter, with the use of 4 ml of the sugar solution and 8 ml of the reagent. Under these conditions the practical range is from about 8 to 200γ of glucose with the 620 mμ filter, and from 20 to 500γ with the 540 mμ filter. It is clear that the filter at 660 mμ cannot be used. In this laboratory only the 620-mμ filter is used.

[1] Personal communication.

[2] Obtained through the kindness of Dr. Michael Heidelberger.

[3] For convenience, there follows a condensed outline for the preparation of anthrone (*2*). A mixture of 104 gm of anthraquinone, 100 gm of granulated tin, and 750 ml of glacial acetic acid is heated to boiling under reflux. Over a period of 2 hrs, 250 ml of hydrochloric acid (sp. gr., 1.19) is added. The hot solution is then filtered through sintered glass, and 100 ml of water added. The mixture is cooled to 10°, and the precipitated anthrone filtered off with suction and washed with water. The crude product (about 80 gm) is dissolved in warm benzene (8–9 ml/gm), and 1/3 volume of petroleum ether is added. The anthrone that crystallizes is filtered off and air-dried. The yield is about 60 gm.