ture was heated on a steam-bath and the liberated oil extracted from the cooled solution at intervals. Trimethylamine was evolved during this decomposition. The resultant neutral product was dissolved in acetone, a drop of water added, and oxidized with potassium permanganate until the color of the added reagent was permanent for 30 min. Water was added, the acetone boiled out, and the mixture was filtered after a little calcium chloride had been added. The clear filtrate after acidification yielded to ether extraction a small amount of residue which was boiled with a little water and cooled. The sparingly soluble acid proved to be *p*-anisic acid. The more soluble fraction was treated with methylamine, evaporated to dryness, and the residue sublimed *in vacuo* (approx. 180° (0.5 mm.)). The sublimate was extracted with ether which left a small amount of insoluble material. The soluble portion was then recrystallized from hot water (m.p. 86°). It was again sublimed *in vacuo*, washed with pentane, and recrystallized again from water. It then melted at 89° and in admixture with an authentic specimen of 3-ethoxy-4,5-dimethoxy-N-methylphthalimide (m.p. 92°)[2] it melted at 90–91°. Various fractions of slightly lower melting points were obtained and all of these had their melting points raised when admixed with an authentic specimen.

GUELPH, ONTARIO, CANADA

[CONTRIBUTION FROM VENEREAL DISEASE EXPERIMENTAL LABORATORY, U. S. PUBLIC HEALTH SERVICE, UNIVERSITY OF NORTH CAROLINA, SCHOOL OF PUBLIC HEALTH]

Two Dimensional Paper Chromatography of Proteins

BY HENRY TAUBER AND EDWARD L. PETIT

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A protein staining reagent, containing the fluorescent dye eosin, and methyl orange has been developed for locating the movement of proteins in two-dimensional filter paper chromatography. Improvements in the two-dimensional technique have been described.

Introduction

Franklin and Quastel[1] have claimed that some proteins are separable by two dimensional filter paper chromatography using buffer solutions and the ascent principle.[2] These investigators converted the proteins into protein–hemin complexes and identified the position of each protein by streaking the paper with benzidine and hydrogen peroxide. Their method, however, has limited use since some proteins do not react with hemin, and when applicable, the color formation is so intense that a background develops which obscures the color given by the protein–hemin complex. To overcome this interference the patterns must be immediately photographed.

In 1937 Feigl and Anger,[3] and more recently others[4,5] found that dyes may be used for the detection of proteins on filter paper. There is, however, no detailed procedure available concerning the applicability of this principle to two dimensional paper chromatography. We have developed a convenient staining reagent containing the fluorescent dye, eosin, and methyl orange. Using this reagent the outlines of the patterns on the paper may be checked while damp, with the aid of ultraviolet light (Mineralight). The stained chromatograms offer a permanent record. A few two dimensional protein chromatograms are presented.

Experimental

Protein Solutions.—Twenty mg. of protein is dissolved in 1 ml. of sodium chloride–phosphate buffer of *p*H 7.5.[6] Five 0.01-ml. aliquots (total weight of protein 1000 γ) are applied to the paper for chromatography unless otherwise stated. When blood serum is used, two 0.01-ml. aliquots suffice.

Staining Solution.—Citric acid (1.5 g.) is dissolved in 1263 ml. of distilled water. Glycerol (51 ml.) and 3 l. of acetone are added. After mixing, 0.6 g. of methyl orange (Eastman Kodak 432) and 0.6 g. Eosin Y (Harleco, water and acetone soluble) are dissolved.

Application and Developing.—Whatman No. 1 filter paper is cut into 26.5 × 26.5 cm. squares (23 × 23 cm. may also be used). Border lines are drawn in pencil 2.5 cm. from the edge of the paper. The protein solutions are applied with a 0.1 ml. pipet 2.5 cm. inside the lines in the lower left-hand corner. The papers are dried at 37° in a tissue drier with circulating air supply after each application. After the last drying a hole is punched in each of the upper corners of the papers and then the papers are attached with strings to glass rods in a tank.

Two aquarium tanks (52 × 31 × 26 cm.), one for the first and one for the second dimension, are employed. Each tank contains 5 l. of the specific aqueous developing solution. One-half inch of Plasticine is placed around the top of each tank. Five (eight if the smaller paper is used) glass rods, spaced evenly, are embedded in the Plasticine at each end of the tank. Each chromatogram is immersed 2.5 cm. into the developing solution. The tanks are covered with glass plates, pressed firmly into place and sealed, to maintain constant humidity within the tanks. The chromatograms are removed when the developing solution reaches the line 2.5 cm. from the top. Then the papers are dried and returned in the tanks at 90° angle. Our experiments were carried out in a constant temperature room at 24°. Each dimension requires about 90 minutes and each series of chromatograms is completed in about 5 hours.

Staining.—The staining solution is placed in an appropriate pan. In another pan is placed 8 l. of tap water, of about 45°, acidified with 0.5 ml. of concentrated sulfuric acid. After drying, the filter paper sheets are stained for 5 minutes, then transferred into the acidified water to remove excess stain. This requires about 5 minutes. Then the sheets are placed between filter paper for partial drying. Final drying is carried out at 37°. When very dilute protein solutions (less than 100 γ) are employed, the protein outlines may temporarily disappear during drying due to the darkening of the background. The outlines reappear immediately, however, if immersed in the acidified water.

Filter Paper Chromatography of a Few Proteins.—Cytochrome c (Wyeth) in a concentration of 110 γ was subjected to two dimensional filter paper chromatography. 0.1 *M* sucrose solution of *p*H 6.6[7] was the developing medium in the first dimension and 0.1 *M* sodium potassium tartrate of *p*H 7.0[7] was employed in the second dimension (Pattern A).

Pattern B shows the chromatogram given by once recrystallized, but highly active (Kat. f. value 32,000)[8] cow

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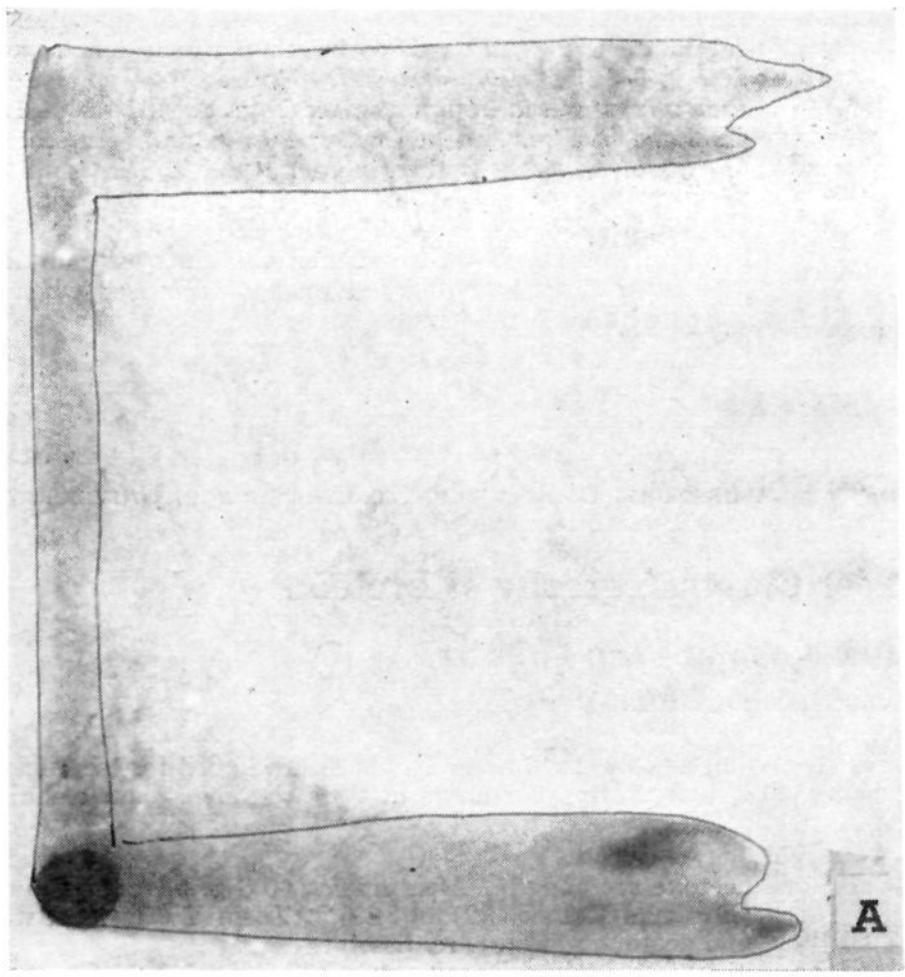

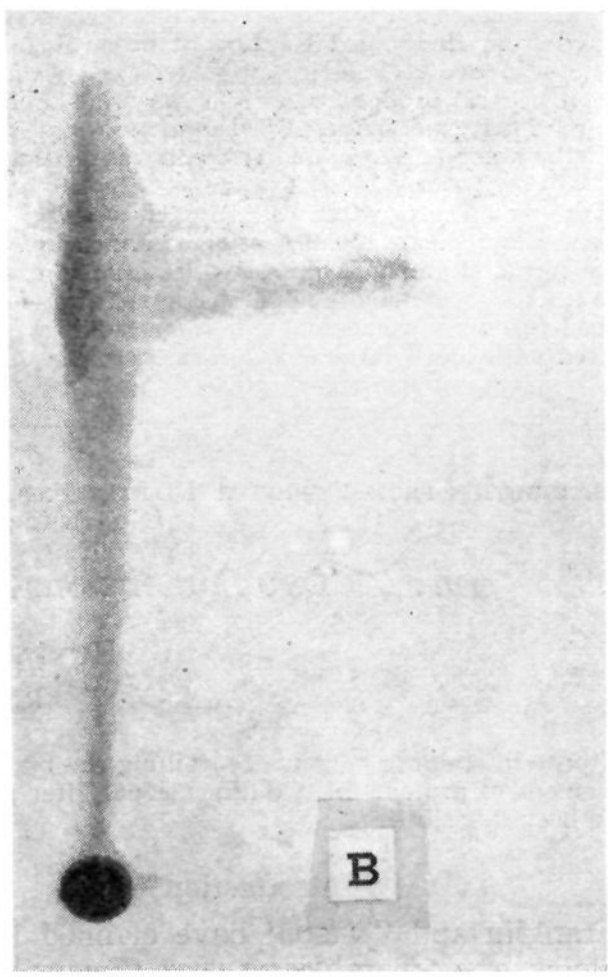

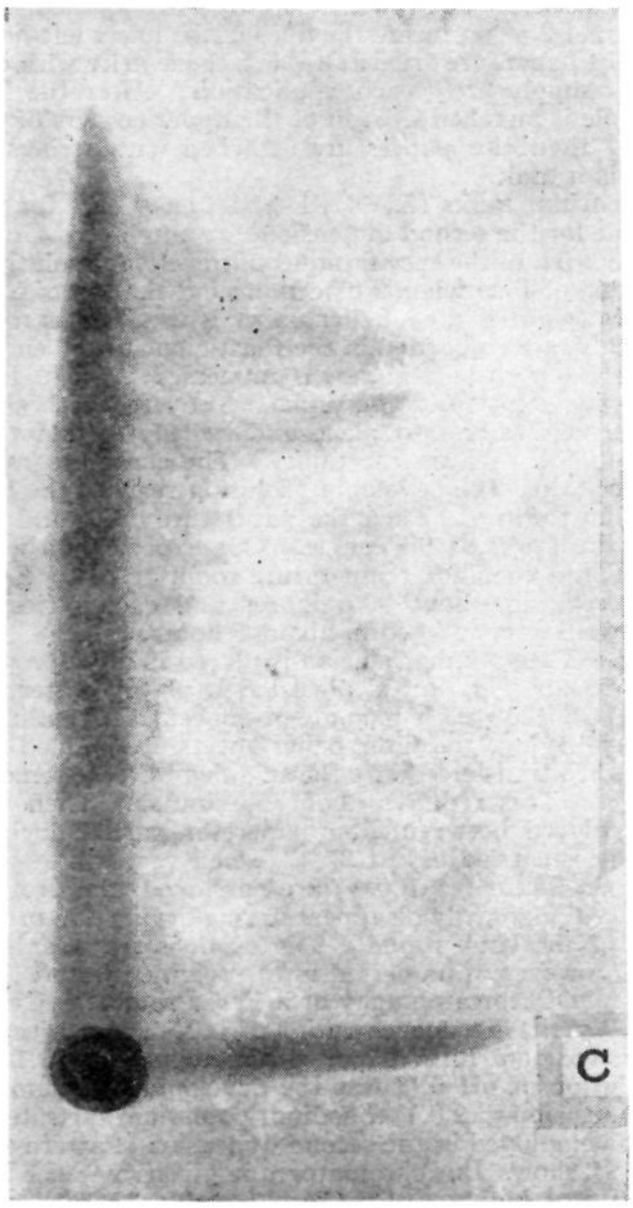

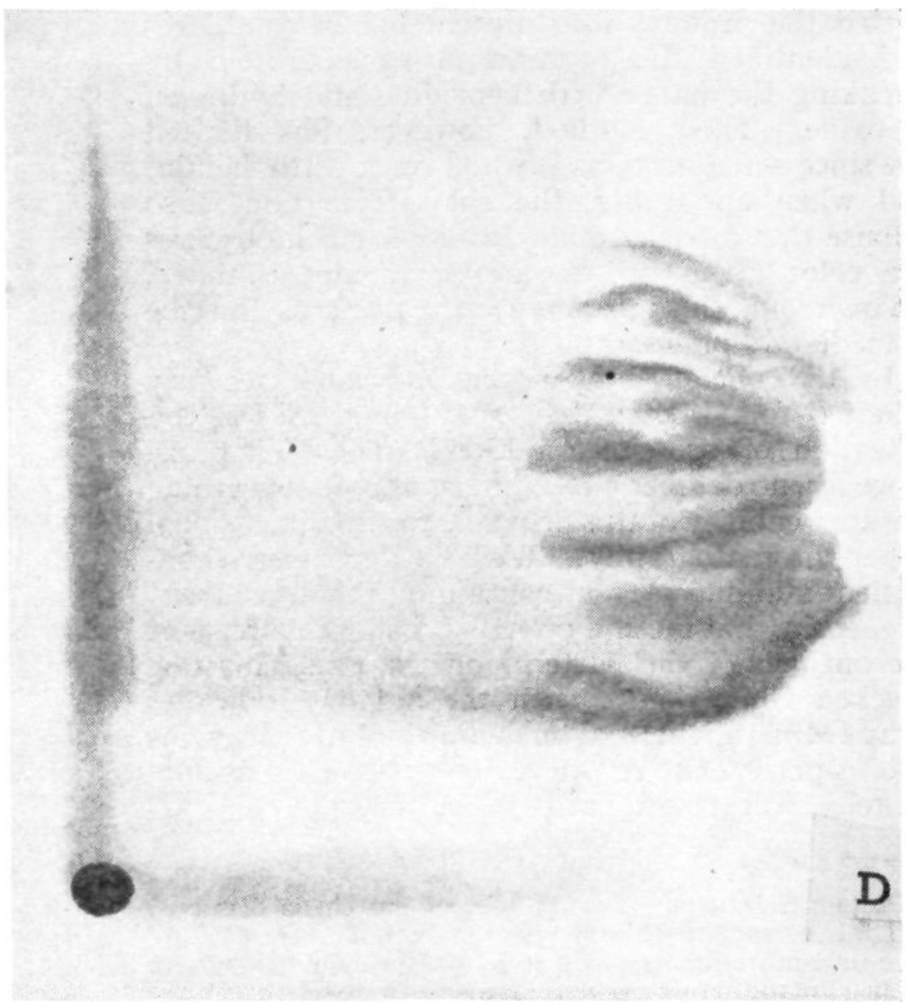

Fig. 1.—A, chromatogram of cytochrome c; B, chromatogram of cow liver catalase; C, chromatogram of gamma globulins; D, chromatogram of human blood serum.

liver catalase. 110 γ of the enzyme was employed. Sucrose and sodium potassium tartrate were the developing agents. The pattern obtained with the hemin-containing enzyme catalase is quite different from the pattern given by the

hemin-containing protein cytochrome c. The catalase experiment was run in triplicates. All parts of the pattern showed catalase activity.

Pattern C shows the chromatogram obtained with human plasma "gamma globulins" fraction prepared according to method 10 of Cohn and associates.[9] Trisodium citrate buffer (0.02 *M*) of *p*H 6.0 containing 9.25 ml. (2 *N*) of hydrochloric acid and 50 g. of sodium chloride per 5 l. was used in the first dimension and 2% tartaric acid in the second dimension.

It has been shown by Franklin, *et al.*,[7] that normal human serum gives a typical pattern when subjected to two dimensional filter paper chromatography and in various disorders specific patterns are found. Pattern D shows the pattern which we obtained with normal human serum (total application 0.02 ml.) using 0.1 *M* sucrose solution in the first dimension and 0.1 *M* sodium potassium tartrate solution in the second dimension. Our staining procedure was then applied. This pattern of course cannot be similar to the normal pattern obtained by Franklin, *et al.*,[7] since in their method some proteins do not combine with hemin.

Discussion

It is generally assumed (with low molecular compounds) that multiplicity of spots indicates a multiplicity of molecular species. Franklin and associates speak of "patterns" when serum or snake venum is concerned and of "separations" when less complex protein mixtures are considered.

Recently Hall and Wewalka[10] subjected the procedure of Franklin and co-workers to serious criticism but concluded that the separation of proteins of quite dissimilar nature by this method is perfectly practicable but not of complex mixtures such as serum.

The proteins which we studied consist probably of more than one molecular species. In view of this, at present, we cannot support the claim of Franklin, *et al.*, that separation of proteins takes place at any time under the conditions of two dimensional paper chromatography. It has been shown by Tauber,[11] however, in a paper entitled "The Selective Adsorption of Enzymes by Cellulose," long before filter paper chromatography was introduced, that enzymes may be separated from other proteins by adsorption on filter paper. The enzymes were eluted with salt solutions.

It is apparent that definite developing solutions must be found for each type of protein. An important question that is still unanswered: Why do most proteins leave on the filter paper, at the application spot, a firmly bound concentric ring? This occurs even when the protein is dried at low temperatures.

While it is safer, at the present, to speak of patterns rather than separation of proteins, it is hoped that our simple procedure, as described in this report, will contribute toward further development of the technique of paper chromatography of proteins.

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CHAPEL HILL, N. C.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

A New Di-D-fructose Dianhydride

BY M. L. WOLFROM, H. W. HILTON[1,2] AND W. W. BINKLEY[1]

Concentrated hydrochloric acid at −5° dehydrates D-fructose to yield, in addition to the previously reported di-D-fructopyranose 1,2′:2,1′-dianhydride and D-fructopyranose-D-fructofuranose 1,2′:2,1′-dianhydride, the difructose anhydrides I (di-D-fructofuranose 1,2′:2,1′-dianhydride) and II of Jackson and co-workers as well as a new crystalline dianhydride characterized as a hexaacetate and designated diheterolevulosan III. Periodate oxidation of the latter compound indicates that it is either di-D-fructopyranose 1,2′:2,3′-dianhydride or an anomeric form of D-fructopyranose D-fructofuranose 1,2′:2,1′-dianhydride (diheterolevulosan II). Analysis of the optical rotatory data in the light of the Hudson isorotation rules makes it probable that diheterolevulosans II and III and difructose anhydrides II and III contain the dioxane ring and differ only in the anomerism of one of the two asymmetric centers in such a ring.

Pictet and Chavan[3] studied the dehydrating action of concentrated hydrochloric acid solutions upon D-fructose and isolated a crystalline substance designated by them diheterolevulosan (herein termed diheterolevulosan I) later[4−6] shown to be di-D-fructopyranose 1,2′:2,1′-dianhydride. An isomer of this, isolated from such a reaction mixture by chromatographic methods,[6] was designated diheteroleuvulosan II, and was proved to be D-fructopyranose-D-fructofuranose 1,2′:2,1′-dianhydride.[7] The relative quantities of these two compounds so formed was studied[7] by chromatographic methods and there remained a sirupy mother liquor product. We report herein a further and rather exhaustive chromatographic fractionation of this residual material (Fraction D) upon clay[8] with the results shown diagrammatically in Fig. 1. The characterization of Fractions A, B and C has been detailed previously.[7] Further quantities of diheterolevulosans I and II were found in zones 1b and 3a (Fig. 1), respectively. Zone 2b yielded a new difructose dianhydride (m.p. 255–258°, $[\alpha]^{26}D$ −179° in water) further characterized as a crystalline hexaacetate. This substance, designated diheterolevulosan III, consumes three moles (per mole of reductant) of periodate with the formation of one mole of formic acid and no formaldehyde, this behavior being identical with that of D-fructopyranose-D-fructofuranose 1,2′:2,1′-dianhydride (diheterolevulosan II).

(1) Sugar Research Foundation Fellow (to July 1, 1951) and Research Associate (W. W. B.) of The Ohio State University Research Foundation (Project 190).

(2) The Visking Corporation Fellow (from July 1, 1951).

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