Paper Chromatography of Protein Mixtures and Blood Plasmas. (18055)

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It has been shown by Franklin and Quastel (1) that it is possible, by means of paper chromatography, to effect separations of proteins on filter paper, using the capillary ascent technic (Williams & Kirby)(2) and hemin as a 'marker' for the proteins with which it combines. Buffer and aqueous salt solutions, instead of nonaqueous solvents, are employed as developing agents. Working with solutions of pure (crystallized) proteins these workers have shown that the results of two dimensional paper chromatography are in accordance with the findings of electrophoretic analyses, those proteins exhibiting homogeneity or heterogeneity under the conditions of electrophoresis giving substantially the same patterns on filter paper. Moreover, they have shown that a protein such as egg albumin, does not lose its serological properties (precipitin formation with the specific antiserum) on its ascent on filter paper, and that an enzyme such as urease, may ascend filter paper to a particular position without loss of its enzyme properties. It is evident, therefore, that the technic may be applied to the separation, and possible identification, of proteins and enzymes. It is intended, in a series of forthcoming papers, to show how the technic, modified from that originally described by Franklin and Quastel, may be applied to the study of animal sera and plasmas under different pathological conditions and to the investigations of various problems relating to the interaction of proteins, enzymes and a variety of organic substances.

It has already been demonstrated by Gross, Leblond, Franklin and Quastel(3) that the technic of paper chromatography may be used for showing the combinations between thyroxine and plasma proteins.

It is of interest, in connection with this work, that Cochran(4) has already shown that the protein of tobacco mosaic virus will ascend filter paper and still retain its infectivity; incidentally, its position on paper was marked by the application of the Sakaguchi test for arginine. More recently, Mitchell, Gordon and Haskins(5) have succeeded in separating constituents of takadiastase by the use of a chromatopile.

It is intended, in this paper, to give a brief account of the chromatography of protein mixtures with particular reference to animal plasmas.

Procedure. Whatman No. 1 filter paper is used almost exclusively throughout this work, although other filter papers have been tried. The paper is cut into 8″ x 8″ squares, and the aliquot of protein solution is placed in the lower left hand corner. Plasmas are taken with a heparinized syringe (1/10 ml of 1% heparin-Na salt for 10 ml blood), and centrifuged for about 20 minutes. The plasma is placed in culture tubes in 0.5 ml quantities, and 0.02 ml 0.3% hemin is added. If a surface active agent is to be used, it is generally added to the tube first to facilitate its solution. The contents of the tube are now well mixed, and 0.02 ml aliquots are applied to the paper, each sample usually being tested in duplicate. After the aliquot has dried at room temperature, the paper is formed into a cylinder in such a manner that the edges do not touch. The cylinders are placed upright in a pyrex plate, which contains 100 ml of the developing solution. It is covered with a suitable container (a large

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1. Franklin, A. E., and Quastel, J. H., *Science*, 1949, v110, 447.

2. Williams, R. J., and Kirby, H., *Science*, 1948, v107, 481.

3. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H., *Science*, 1950, v111, 605.

4. Cochran, G. S., *J. Phytopathology*, 1947, v37, 850.

5. Mitchell, H. K., Gordon, M., and Haskins, F. A., *J. Biol. Chem.*, 1949, v180, 1071.

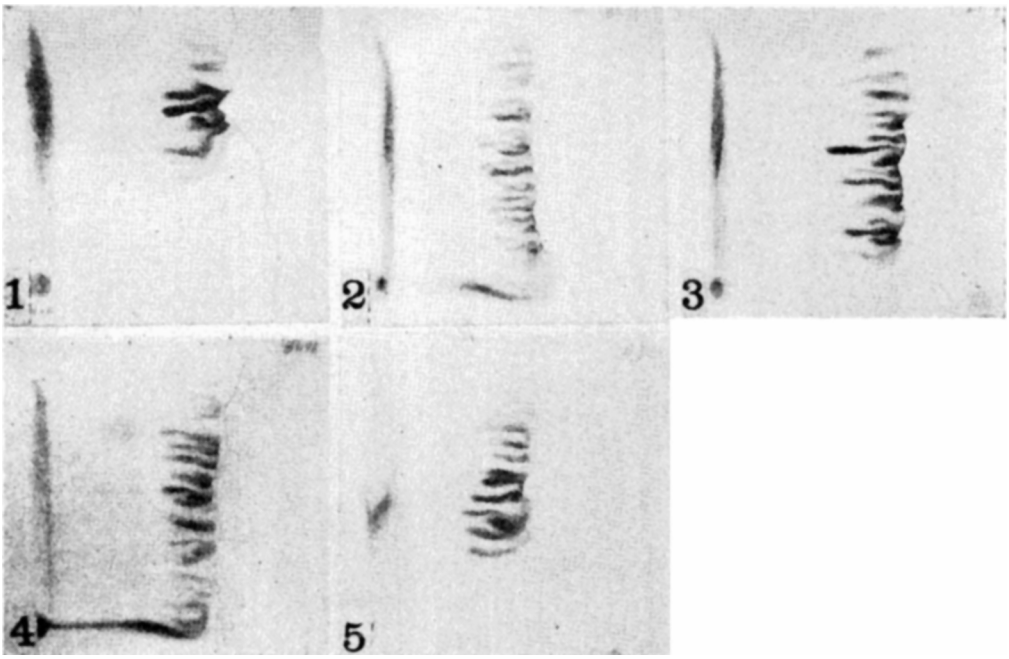

FIG. 1. Human plasma.
FIG. 2. Human plasma with 0.03 ml "Span 20" added per 0.5 ml plasma.
FIG. 3. Human plasma with 0.03 ml "NNO" added per 0.5 ml plasma.
FIG. 4. Human plasma with 0.05 ml "BRIJ-30" per 0.5 ml plasma.
FIG. 5. Human plasma with 0.04 ml "Tween 20" per 0.5 ml plasma.

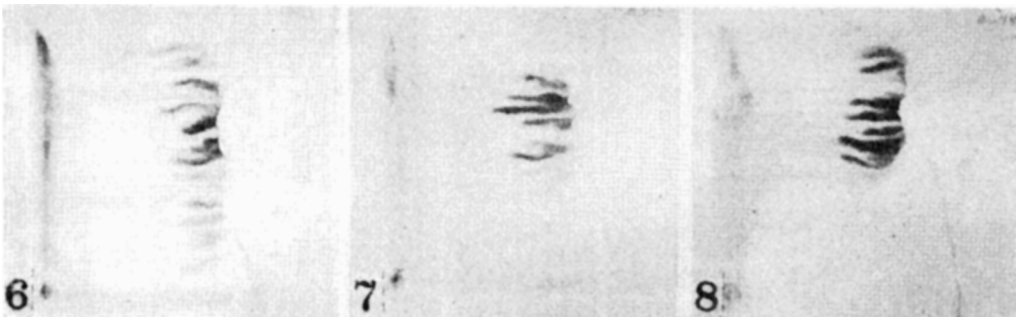

FIG. 6. Human plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 7. Rat plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 8. Guinea pig plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.

cylinder) to maintain constant humidity. After the solution has reached the top of the cylinder, which usually requires about 90 minutes, the cylinder is taken out and allowed to dry at room temperature. After thorough drying, the cylinder is re-formed and stapled again at right angles to the original direction and placed in the pyrex dish which contains the developing solution required for the second dimension. It is not necessary to allow the solution to reach the top of the filter paper in the second dimension, but it may be stopped about two thirds of the way up. The cylinder is again dried, and the filter paper is streaked with freshly prepared benzidine reagent. The chromatogram is photographed immediately due to the rather rapid background color development. The entire process may be easily carried out in 5-6 hours.

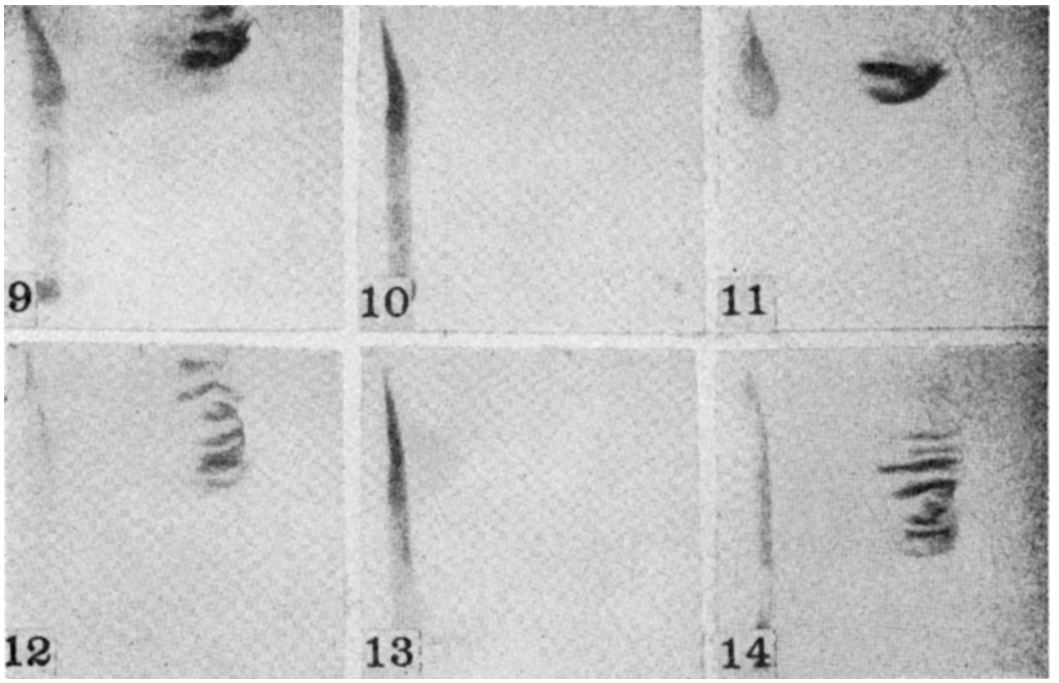

FIG. 9. Protein (Cohn) Fraction V (16 mg) dissolved in 0.5 ml 0.85% saline.
FIG. 10. Protein (Cohn) Fraction III (8 mg) dissolved in 1.0 ml 0.85% saline.
FIG. 11. Mixture of Protein (Cohn) Fractions; 22 mg V + 4 mg II + 5 mg IV—3,4, dissolved in 0.5 ml 0.85% saline.
FIG. 12. As in Fig. 9 with 0.01 ml "Tween 85" per 0.5 ml solution.
FIG. 13. As in Fig. 10 with 0.01 ml "Tween 85" per 0.5 ml solution.
Fig. 14. As in Fig. 11 with 0.01 ml "Tween 85" per 0.5 ml solution.

Hemin. The hemin solution is prepared by dissolving 30 mg crystalline hemin in 10 ml of distilled water, to which has been added a small quantity of sodium bicarbonate. Hemin solution is generally added in the ratio of 0.02 ml hemin to 0.5 ml of the protein solution.

Benzidine solution. This solution must be prepared daily, by mixing equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide. It is made acid with glacial acetic acid. The solution is applied with a paint brush, and the chromatogram is photographed immediately.

Developing solutions. A large number of solutions of organic salts and buffers at different concentrations have been investigated for their suitability as developing agents. It has been found that M/10 sucrose solution is very efficient for use in the first dimension, when this is followed by M/10 sodium potassium tartrate solution in the second dimension. This combination is used in all the chromatograms given in this paper (Fig. 1-18). Other combinations also give very good results, as for example, dextrose, lactose or maltose in the first dimension, and nicotinate, malate, succinate, fumarate, mandelate, acetate or citrate in the second dimension. Certain amino-acids also give good results when employed in developing solutions, for example, M/10 methionine solution followed by M/10 acetyl glycine solution, or M/10 cysteine solution followed by M/10 sodium glutamate solution.

Effects of various filter papers. The choice of filter papers seems to be very important, as it appears that not all animal plasmas give the best results with the same filter paper. Human and rat plasmas give the best chromatograms with Whatman No. 1 but guinea pig plasma gives the best results when

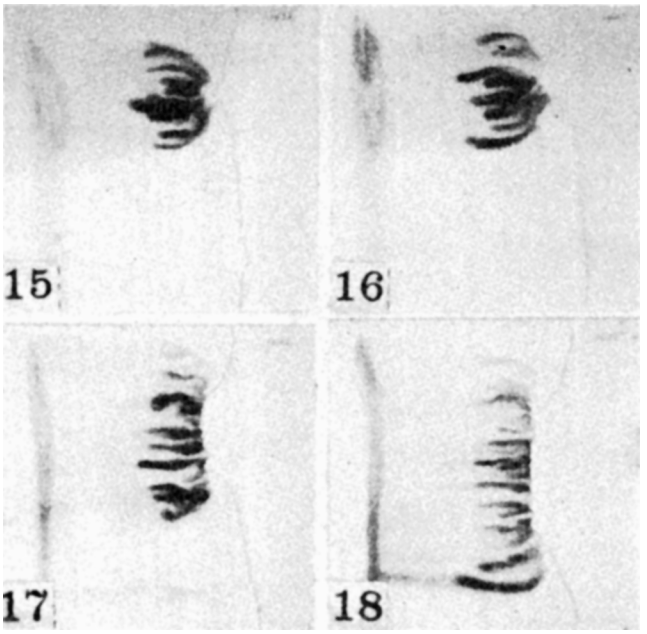

FIG. 15. Normal horse serum.
FIG. 16. Horse serum immunized against diphtheria toxin.
FIG. 17. As in Fig. 15 with 0.01 ml "Tween 85" per 0.5 ml serum.
FIG. 18. As in Fig. 16 with 0.01 ml "Tween 85" per 0.5 ml serum.

using S and S 589. When comparing the plasmas of various species, however, Whatman No. 1 has been consistently used.

Effects of the addition of surface active substances. When a plasma is chromatographed in the manner that has been described above, the various protein components do not separate very well and tend to remain bunched together. This is illustrated in Fig. 1. It is found that if certain surface active substances are added to the plasma before chromatography, good separations of the protein fractions take place. The following substances yield excellent results: sodium alginate, the "Elvanols," "Spans," "Tweens," "BRIJ" and "NNO."* Results with sodium tauroglycholate are relatively poor. The various surface active substances do not give identical results, both their structures and their concentrations being important in determining the pattern of the protein chromatogram. It has been made a matter of practice to make chromatograms of a blood plasma, or a protein mixture, in presence of different concentrations of a selected surface active substance. Usually 0.01 ml to 0.06 ml of the solution of the surface active agent is added to 0.5 ml plasma before addition of the hemin, and subsequent chromatography. The separation of the plasma constituents, as facilitated by the presence of surface active agents, are shown in Fig. 2, 3, 4 and 5. High concentrations of surface active agents must not be added to the plasma; in their presence, the movement of the proteins on filter paper appears to be greatly suppressed.

Effects of dialysis of blood plasma. Dialysis of blood plasma against running water seems not to disturb the pattern of the

*"Elvanols": A group of polyvinyl alcohols produced by hydrolysis of polyvinyl acetates of varying degrees of polymerization (Dupont).
Span 20: Sorbitan monolaurate.
NNO: Glycerol mannitan laurate.
BRIJ 30: Polyoxyethylene lauryl alcohol.
Tween 20: Polyoxyethylene sorbitan monolaurate.
Tween 85: Polyoxyethylene sorbitan trioleate.

plasma chromatogram. It is concluded, therefore, that the dialysable material of plasma has little or no influence on the movement of the proteins on filter paper.

Consistency of results. With any given plasma, or protein mixture, excellent duplication of results has been obtained. It is necessary to be strictly accurate in the measurement of the surface active material added to the plasma, but if due care is taken, there is no difficulty in obtaining good duplication with any sample of a protein mixture.

Applications of the technic. Comparison of human, rat and guinea pig plasmas. Heparinized plasmas of different animals show different patterns on the chromatogram when they are chromatographed under similar conditions.

With rat and guinea pig, the blood is removed with a heparinized syringe from the ether anaesthetized animal, and centrifuged for about 20 minutes. 0.5 ml of plasma is mixed with 0.02 ml 0.3% hemin solution, and various amounts of a surface active agent is added to a number of such samples. Using 5 mg "Elvanol 31-31" as surface active agent and two dimensional chromatography with M/10 sucrose solution in the first dimension followed by M/10 sodium potassium tartrate solution in the second, evident differences between the plasma chromatograms appear (Fig. 6, 7, and 8). On the other hand, the presence of "Elvanol 51-05" fails to show any obvious differences. "Elvanol 54-22" yields a much better separation of constituents of human plasma than is obtained with either rat or guinea pig plasma. "Span 20" gives excellent separations of the protein components, but, whilst failing to show a marked difference between human and rat plasma, it gives a distinctive pattern with guinea pig plasma. "NNO" gives a good separation of the constituents of each of the plasmas, but differences between the plasma patterns are not very evident. "Tween 20," "Tween 81" and "Tween 85" are the best surface active agents to use when examining various human plasmas.

As a matter of practice when chromatographing human plasmas, from different pathological conditions, "Tween 81" and "Tween 85" are being used regularly. They seem to give the most satisfactory separations so far. It is obvious, however, that there is great room for improvement, and new surface active agents are being continually tested.

It has already been found that the pattern of the chromatogram of a plasma, depends on the nutrition of the animal and the results of our investigation into this matter will form the subject of a separate communication. It is desirable, in comparing the plasmas of patients, to keep dietary conditions as constant as possible. Preliminary work with the plasmas of dogs, maintained on a selected diet, has shown little individual variation, so long as a proper control of the diet is maintained.

Chromatography of pure proteins and protein mixtures. A number of chromatograms has been made of preparations of purified proteins and protein fractions isolated from blood. These preparations have been kindly presented by the courtesy of the Cutter Laboratories and consist of the fractions of blood plasma isolated according to the procedures of Dr. Cohn and his colleagues(6). Electrophoretic analyses have shown that fraction III consists of a mixture of α, β, and γ globulins and that of fraction V is largely albumin. Chromatograms of fraction V are shown in Fig. 9 (without Tween) and Fig. 12 (with Tween) and those of fraction of III are shown in Fig. 10 (without Tween) and in Fig. 13 (with Tween). It will be noted that the chromatograms of fraction V indicate the presence of more than one protein, whilst those of fraction III show but little movement in the second dimension. The chromatograms of the separate fractions do not resemble that of blood plasma. When mixtures of the fractions are taken in such proportions and in such quantities as to constitute the proportions and amounts of albumin and globulin in blood plasma, chromatograms are obtained which resemble those of normal blood plasma.

6. Cohn, E. J., Strong, L. E., Hughes, W. L.,, Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

This is shown in Fig. 11 (without Tween) and Fig. 14 (with Tween). The evidence from a number of chromatograms of protein mixtures indicates that an interaction between proteins occurs, whereby the chromatogram of a mixture of proteins is not necessarily that to be expected from a simple addition of the chromatograms of the proteins taken separately.

This fact is also illustrated by the results of some preliminary experiments carried out with mixtures of proteins with hemoglobin and with cytochrome *C*. Chromatograms indicate that both these substances may form associations with other proteins, but further experiments are required to decide whether the conditions of the chromatographic technic may result in sufficient dissociation of the hemin from the hemoglobin or from the cytochrome to produce hemin complexes with the other proteins present.

Comparison of normal and hyper-immunized horse serum. Through the courtesy of the Department of Microbiology and Hygiene of the University of Montreal, specimens of normal horse sera and the sera of hyperimmunized horses (immunized against diphtheria toxin) have been obtained. Chromatograms of the two sera both with and without the addition of a surface active agent are shown in Fig. 15, 16, 17, and 18. For experiment, 0.01 ml "Tween 85" was added to a mixture of 0.02 ml 0.3% hemin solution and 0.5 ml serum. Chromatography was carried out on Whatman No. 1 filter paper using M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. It will be seen that the chromatograms of the sera (both with and without the addition of the Tween) show differences from each other. That of the hyperimmunized serum indicates the existence of fractions not present in the normal serum.

Summary. A technic for the two dimensional chromatography of blood plasma and protein mixtures on filter paper is described. Separations of protein constituents are greatly facilitated by the addition of surface active substances such as the "Tweens" or "Spans," etc. The technic at present adopted is to add "Tween 85" or "Tween 81" and, using hemin as protein marker, to employ M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. Differences between the protein patterns of chromatograms of human, rat and guinea pig plasma are noted. With protein mixtures, associations of proteins may occur that may be detected by chromatography. The sera of hyperimmunized horses (as against diphtheria toxin) yield chromatograms which differ from, and exhibit fractions not present in, chromatograms of normal horse sera.

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