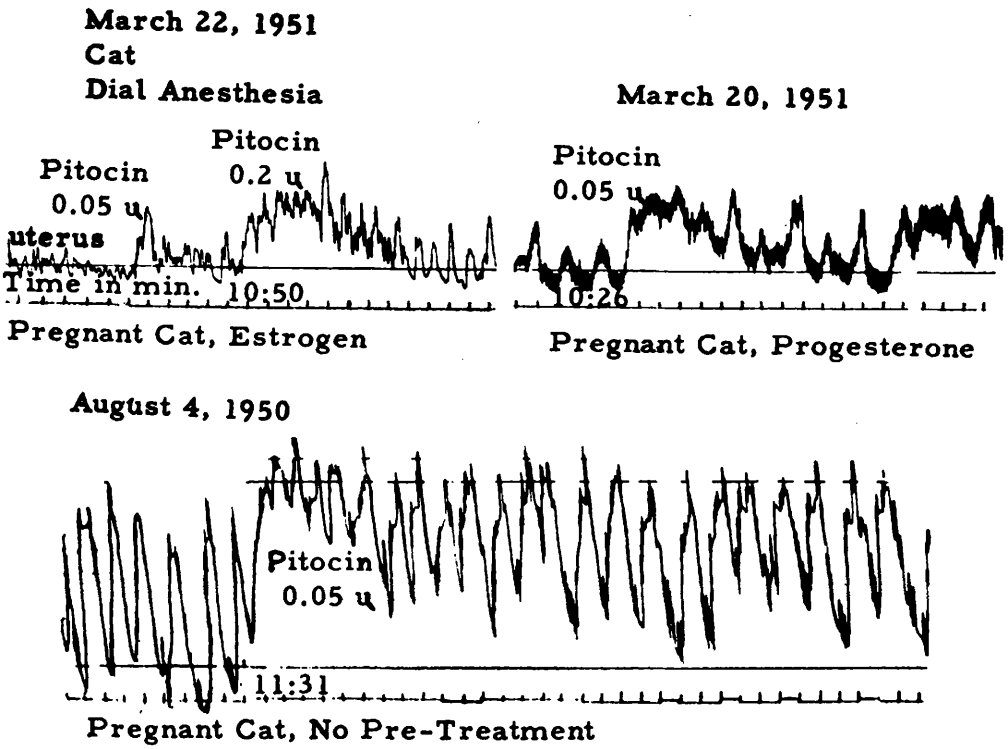

FIG. 4. *Mar. 22.* 1 mg α-estradiol dipropionate (intramusc) on first and third days. Experiment on sixth day. Units of pitocin in total dose. *Mar. 20.* 5 mg of progesterone (intramusc) on 4 consecutive days. Experiment on fifth day. *Aug. 4.* Cat in late stage of pregnancy. Responses of this nature were about as common as those demonstrating a complete lack of reactivity.

hormone would establish some brief uterine responsiveness but that both together were required to give a maximal and prolonged sensitivity to drugs. The two hormones in the cat are synergistic, or at least mutually complementary, and not antagonistic in respect to their effects upon the sensitivity of the cat's uterus *in vivo* to drugs.

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Paper Chromatography of Protein Mixtures and Blood Plasmas. (18926)

A. E. FRANKLIN, J. H. QUASTEL, AND S. F. VAN STRATEN.
From the Montreal General Hospital, Research Institute, Montreal.

A technic for the 2-dimensional chromatography of blood plasmas of protein mixtures on filter paper has been described by Franklin and Quastel(1). It was found that the addition of surface active agents such as the "Tweens" or "Spans" appeared to facilitate separations of the protein constituents and extend the protein "pattern." The technic adopted at present is to add "Tween 85" or "Tween 81" to the plasma and use hemin as the protein marker. A mixture of the benzidine reagent and hydrogen peroxide is used to detect the hemin after employing *M*/10 sucrose solution as developing agent in the

1. Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 803.

first dimension and $M/10$ sodium potassium tartrate solution in the second dimension. This technic has been used in the study of blood plasma composition after heparin administration and diets of fat meals(2), and in investigations of blood plasmas in multiple sclerosis(3). It has also been used in an investigation of the combination between thyroxine and plasma proteins(4).

It is the purpose of this paper to give further details concerning the movements of proteins on filter paper using the above technic, to show how certain proteins may be separated from each other on filter paper, to discuss the nature of the protein pattern and the effects of surface active agents and to present data concerning protein patterns found in various disorders.

Experimental procedure. Details of procedure are given in an earlier paper(1). It is important to note that with blood plasmas or protein mixtures not more than 0.02 ml aliquots should be applied to the paper, and with protein mixtures, aliquots should contain not more than 1 mg total protein. When larger quantities of protein are applied to the paper, protein separations are swamped by the effects of factors operating when a relatively large mass of protein is being slowly irrigated by the solutions used in this work. It is essential that there should be no overloading of the paper with the substances to be separated. When quantities of proteins, under the limits stated, are used, there is generally movement of the entire protein mass from the point of origin to the top of the paper within the experimental period of 1½ hours at room temperature. If a surface active agent is added to the protein mixture, however, the movement of certain proteins is greatly delayed and these may not all leave the point of origin in the first dimension.

Whatman No. 1 filter paper has been used almost exclusively. A variety of other papers has been tried; some papers (*e.g.* Schleicher and Schuell, No. 477 and 507) are quite unsuitable, while others (*e.g.* Whatman Nos. 2, 3, 3MM, 4) give results very similar to those obtained with Whatman No. 1. It is, therefore, important to adhere to one type of paper for the comparison of the protein patterns of plasmas from pathological cases with those of the normal.

It has been found that a convenient size of filter paper is an 8 in. square. Larger papers may give more extended patterns, but do not seem to offer any definite advantages.

Solutions of sucrose and sodium potassium tartrate have been selected after many experiments as the most suitable developing solvents in the first and second dimensions respectively. The former appears to facilitate movement of certain protein-hemin complexes, as well as uncombined hemin, in the first dimension; the latter allows movement of certain protein complexes in the second dimension whilst preventing movement of uncombined hemin. It has been found that in the presence of surface active agents (such as Tween 85) a very slight movement of hemin, when no proteins are present, may appear in the second dimension, but this is negligible compared with the movement of many protein-hemin complexes.

Protein chromatography of cytochrome c *solution.* When a solution of cytochrome *c** is applied to filter paper it is found that the substance moves either feebly or not at all in the first dimension using $M/10$ sucrose solution as developing solution, but is taken into the second dimension by $M/10$ sodium potassium tartrate solution. It is, of course, unnecessary to add hemin as protein marker to a solution of cytochrome *c* as this substance is itself detected with the benzidine reagent. The amount of cytochrome *c* moving in the second dimension is proportional to the amount placed at the origin. Photographs of chromatograms (no surface active agents being present) of various quantities of cytochrome *c* are shown in Fig. 1. The pigment, developed with the benzidine reagent,

2. Swank, R. L., Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 850.

3. Swank, R. L., Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v76, 183.

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

* Kindly presented to us by Wyeth, Inc., Philadelphia, Pa.

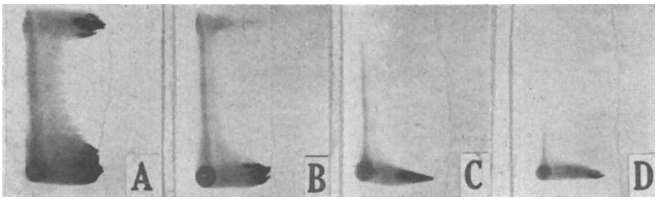

FIG. 1. Cytochrome *c* dilution series: A 133.3 γ, B 66.6 γ, C 33.3 γ, D 12.5 γ.

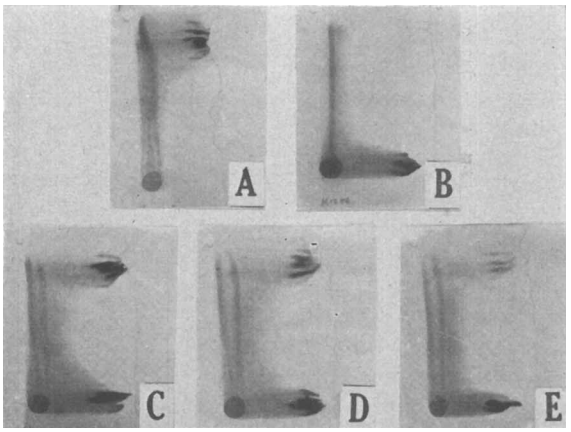

FIG. 2. Albumin and cytochrome *c* separations: A, 200 γ of crystalline human serum albumin (Cohn Fraction V); B, 40 γ of cytochrome *c*; C, Mixture of 200 γ of albumin and 80 γ of cytochrome *c*; D, mixture of 200 γ of albumin and 60 γ of cytochrome *c*; E, mixture of 200 γ of albumin and 40 γ of cytochrome *c*.

may be eluted from the spot in the second dimension with dilute HCl, yielding a yellow solution. The intensity of this color is measured with an electrophotometer. When high concentrations of cytochrome *c* are chromatographed, it is noted that a fraction appears at the top of the paper. This may be an impurity(5).

Paper chromatography of human serum albumin-cytochrome c mixtures. When mixtures of a solution of crystalline human serum albumin and a solution of cytochrome *c* are chromatographed on paper, a sharp separation of the two components appears to take place. There is sufficient hemin present in the cytochrome preparation (see also Holmes and Wynne(5) for evidence of breakdown of cytochrome *c* solution on standing) to form a complex with the albumin and serve as protein marker. Therefore hemin need not be added to the mixture before chromatography. The albumin-hemin complex moves with the advancing front in the first dimension, but the cytochrome is left behind. Both proteins move in the second dimension, albumin at the top of the paper and cytochrome at the bottom. Typical chromatograms of serum albumin-cytochrome *c* mixtures are shown in Fig. 2. It should also be noted that the intensity of color of the albumin fraction decreases with the concentration of cytochrome present, this being due to diminution of the amount of free hemin present.

Paper chromatography of protein mixtures. Preliminary investigations have shown that hemoglobin and cytochrome *c* preparations may also be separated on filter paper. McKerns(6) has reported the separation of the active component of rennin from both pure and crude preparations by the use of our technic, and has also followed changes undergone by casein during hydrolysis by rennin employing the same method.

Paper chromatography of plasma constitu-

5. Holmes, W. L., and Wynne, A. M., *Fed. Proc., Am. Soc. Biol. Chem.*, 1951, v10, 199.

6. McKerns, K. W., *Canad. J. Med. Sc.*, 1951, v29, 59.

ents. (A) *Human serum albumin* (Cohn Fraction V). When a small quantity of a solution of a crystalline human (or bovine) serum albumin (Cohn Fraction V) is chromatographed, using sucrose and tartrate as developing solutions, it is found that the protein ascends in the first dimension with the advancing front as a large spot or a streak. The fact that the protein may ascend as a streak in the first dimension is probably due, largely, to gradual solution of the protein in the spot at the origin. In the second dimension, the streak is split by application of the second developing solution into a number of fractions yielding a "pattern." The extent of fractionation may depend on the length of the streak in the first dimension as well as upon the constituents of the developing solution. The various fractions seen in the second dimension do not necessarily represent different protein species; they may be composed of different molecular aggregations of the same protein, or differently ionized components of the protein, etc. It is, however, of importance that the fractionation observed may be duplicated with considerable regularity, and that the "pattern" observed with one protein differs significantly from that observed with another. A typical chromatogram of human serum albumin is shown in Fig. 3A.

The addition of a surface active agent to a solution of human serum albumin (*e.g.* 0.02 ml Tween 81 to 0.5 ml 6.5% albumin solution) results in a considerable lengthening of the protein spot in the first dimension. Application of the solvent in the second dimension splits the elongated spot into numerous fractions, giving rise to a pattern as shown in Fig. 3B and 3C.

It is known from the work of Putnam and Neurath(7) that albumins combine with surface active agents such as the detergents, to form complexes of various mole ratios. It is, therefore, likely that the elongation of the protein spot in the first dimension is partly due to the different rates of solution of the surface active agent-protein complexes, and

7. Putnam, F. W., and Neurath, H., *J. Amer. Chem. Soc.*, 1944, v66, 1992.

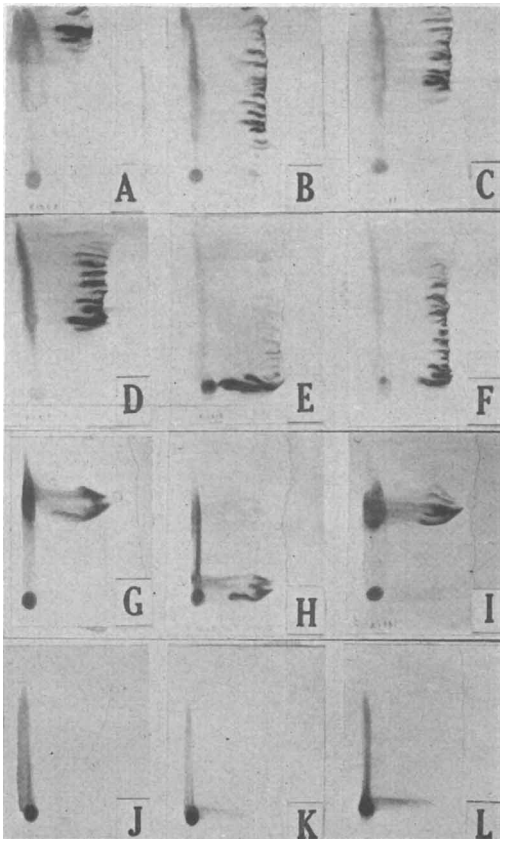

FIG. 3. Influence of Tween 81 and Tween 85 on the chromatograms of various plasma fractions. Cohn Fraction V (65 mg per ml) 95% albumin and 4% α-globulin: A, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; B, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; C, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction IV—4 (65 mg per ml) 16% albumin, 46% α-globulin and 38% β-globulin: D, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; E, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; F, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction II (65 mg per ml) 3% β-globulin and 97% γ-globulin: G, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; H, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; I, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction I (65 mg per ml) 7% albumin, 8% α-globulin, 15% β-globulin, 9% γ-globulin and 61% fibrinogen: J, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; K, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; L, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

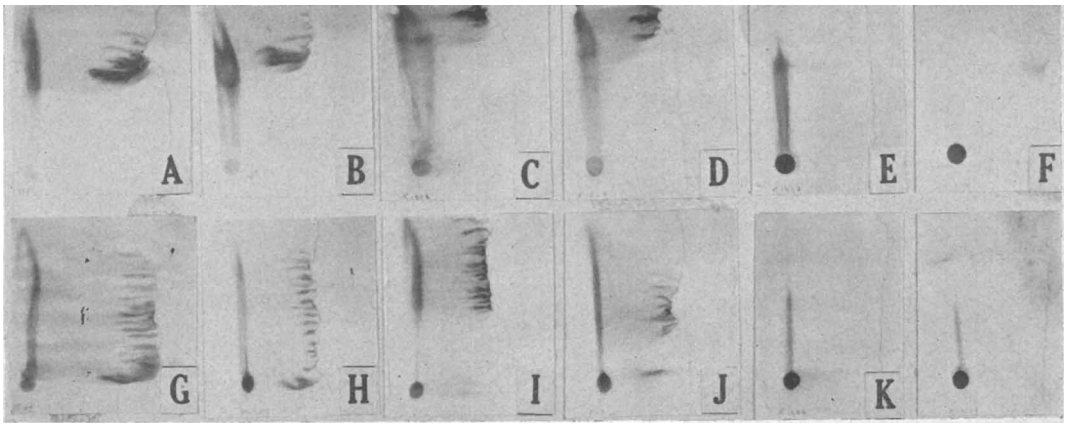

FIG. 4. Influence of Tween 81 on the chromatograms of various plasma fractions (0.02 ml of 0.3% hemin added per 0.5 ml of mixture throughout): A, human plasma; B, reconstituted plasma; C, 3% Cohn Fraction V; D, 1.3% Cohn Fraction IV-4; E, 0.5% Cohn Fraction II; F, 0.6% Cohn Fraction I; G, same as A with 0.02 ml of Tween 81 added per 0.5 ml; H, same as B with 0.02 ml of Tween 81 added per 0.5 ml; I, same as C with 0.02 ml of Tween 81 added per 0.5 ml; J, same as D with 0.02 ml of Tween 81 added per 0.5 ml; K, same as E with 0.02 ml of Tween 81 added per 0.5 ml; L, same as F with 0.02 ml of Tween 81 added per 0.5 ml.

partly due to the varying affinities of these complexes to the cellulose of the paper (see also ref. 8). The increase, therefore, in the number of fractions in the second dimension is probably largely due to the formation of these complexes.

(B) *α and β globulins* (Cohn Fraction IV-4). Paper chromatography of a solution of Cohn Fraction IV-4 containing a mixture of α and β globulins and some albumin shows much higher fractionation in the second dimension in the absence of a surface active agent, than is observed with a solution of the same quantity of serum albumin (see Fig. 3D). The addition of Tween 81 to a solution of these proteins results in a very greatly diminished rate of movement of the proteins in the first dimension, probably due to complex formation. Movement of the complexes takes place in the second dimension as shown in Fig. 3E.

Experiments have shown that the rate of ascent of globulin on the filter paper after addition of a surface active agent, is much more restricted in the first dimension than that of albumin. This may result, when using Tween 81, in little movement of the protein in the first dimension with a consequent heavy streak at the bottom of the paper in the second dimension (Fig. 3E). When a surface active agent such as Tween 85 is used, an elongated spot, commencing at the origin, appears in the first dimension resulting numerous fractions, in the second dimension (Fig. 3F). The inference has been that fractions due to albumin are in the upper half of the chromatogram; those due to globulins are in the lower half.

(C) *γ globulin and fibrinogen.* Movement of γ globulin in the absence of surface active agent takes place in the first dimension with but little movement in the second dimension. This may be contrasted with the ease of movement of the α and β globulins in the second dimension. The presence of a surface active agent, however, causes a reduced rate of movement of the protein in the first dimension, and movement at the bottom, or in the lower half, of the chromatogram in the second dimension. Fibrinogen moves but little in either first or second dimension, with or without surface active agents. Chromatograms illustrating these results are shown in Fig. 3G, H, I, J, K, and L.

Paper chromatography of plasma, and of reconstituted plasma. The chromatogram of a mixture of albumin, α, β, and γ globulins and fibrinogen at the concentrations present

8. Hanes, C. S., and Isherwood, F. A., *Nature*, 1949, v164, 1107.

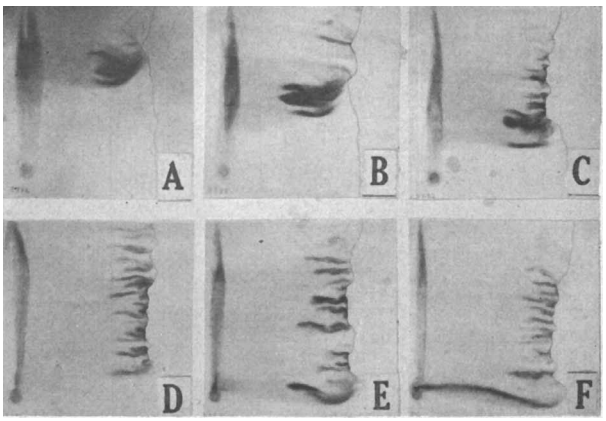

FIG. 5. Influence of Tween 85 on chromatograms of serum albumin, globulin and plasma mixtures: A, human plasma; B, human plasma + 30 mg Cohn Fraction V (95% human serum albumin) added per 0.5 ml of plasma; C, human plasma + 30 mg Cohn Fraction II (97% γ-globulin) added per 0.5 ml of plasma; D, same as A with 0.01 ml of Tween 85 added per 0.5 ml of plasma; E, same as B with 0.01 ml of Tween 85 added per 0.5 ml of plasma; F, same as C with 0.01 ml of Tween 85 added per 0.5 ml of plasma.

in plasma resembles very closely the chromatogram of plasma itself (Fig. 4). It is, therefore, apparent that the chromatogram of a complex mixture such as plasma may be regarded largely as a composite of the chromatograms of the individual proteins. This may not always be the case, for plasma may contain other constituents, such as surface active agents, which may alter the pattern(2) and which may not be present in the isolated protein fractions from plasma.

Effects of dilution of plasma. The addition of water to blood plasma brings about no apparent change in the chromatogram pattern, with or without surface active agents, until 50% dilution has been reached. Further dilution causes significant changes in the pattern, particularly when Tween 85 is used as surface active agent.

Effects of addition of serum albumin or of γ globulin to human plasma. Addition of 30 mg human serum albumin to 0.5 ml human plasma causes little obvious change in the chromatogram pattern (Fig. 5A, B, D, E). On the other hand the addition of 30 mg of γ globulin to 0.5 ml human plasma brings about a marked alteration of the pattern (Fig. 5C, F), a heavy fraction appearing from the point of origin in the second dimension, when Tween 85 is used. This heavy fraction at the bottom of the pattern is typical of the chromatograms of pathological cases in which the globulin/albumin ratio of the plasma has been increased.

Use of dyes as protein markers. We have found that proteins without the addition of hemin may be detected by treating the chromatogram with a dilute alcoholic solution of bromthymol blue, followed by a thorough washing with water. This procedure gives rise to a pattern identical with that obtained using hemin as protein marker, followed by application of the benzidine reagent.

Papastamatis and Wilkinson(9) have also shown that bromthymol blue may be effectively used for the marking of proteins on paper, using a variety of developing solutions. The method was used to detect many classes of proteins (*e.g.* insulin, plasma proteins, egg albumin) subjected to paper chromatography, 20 γ of protein being detectable.

Paper chromatography of blood plasmas of various disorders. It has been found that the paper chromatograms of normal blood plasmas yield patterns, which are surprisingly similar and which duplicate with considerable regularity. Normal patterns have already been published(1-3) and a typical example is shown in Fig. 6A, B.

The patterns exhibited in chromatograms of

9. Papastamatis, S. C., and Wilkinson, J. F., *Nature*, 1951, v167, 724.

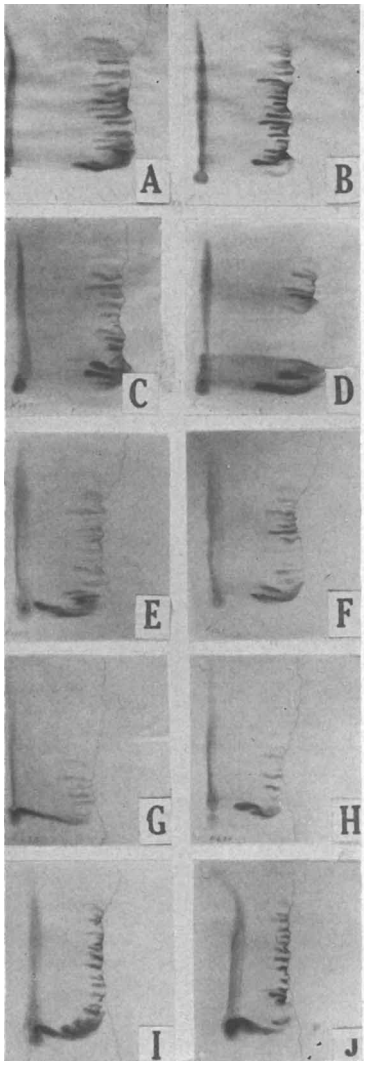

FIG. 6. Comparison of normal and pathological plasma chromatograms employing Tween 81 and 85: A, plasma of normal patient (+ 0.02 ml of Tween 81 added per 0.5 ml of plasma); B, plasma of same patient (+ 0.01 ml of Tween 85 added per 0.5 ml of plasma); C, plasma of a patient with multiple myeloma (+ 0.02 ml of Tween 81); D, plasma of same patient (+ 0.01 ml of Tween 85); E, plasma of a patient with liver cirrhosis (+ 0.02 ml of Tween 81); F, plasma of same patient (+ 0.01 ml of Tween 85); G, plasma of a patient with cancer of the stomach (+ 0.02 ml of Tween 81); H, plasma of same patient (+ 0.01 ml of Tween 85); I, plasma of a patient with lupus erythematosus (+ 0.02 ml of Tween 81); J, plasma of same patient (+ 0.01 ml of Tween 85).

pathological plasmas vary greatly, according to the disease, and usually show distinct changes from the normal.

It would be expected, from the results given above, that plasmas having different albumin-globulin ratios from the normal would exhibit patterns different from the normal. This in fact is the case. Plasmas, such as those from patients with multiple myeloma or liver cirrhosis, where there are large changes in the albumin-globulin ratio, yield chromatograms which are characterized by the absence, or relative weakness, of the upper fractions (due to albumin) and by the presence of heavy basal fractions (due to globulins). (Fig. 6C, D, E, F). Chromatograms are also shown of plasmas of patients suffering from cancer of the stomach and from lupus erythematosis (Fig. 6G, H, I, J,). Whether such chromatograms of plasmas of pathological cases will prove to be of value in diagnosis remains to be seen, but sufficient work has been done to show that the chromatograms duplicate with regularity in the same patient, so long as the clinical condition remains the same. They vary during the course of the disease and may revert to normal with successful medical treatment. More extensive results obtained with pathological cases will be published elsewhere.

Paper chromatography of snake venoms. It was of interest to chromatograph snake venoms of various types, to see if the patterns differ substantially from one another. Using 0.8 mg of dried venom, it has been found that the chromatograms of 2 cobra venoms are almost identical, those of 2 specimens of Russel viper venom are very similar to those of the cobra venom, whilst that of a *Agkistrodon piscivorus* has a pattern differing markedly from those of other snake venoms (Fig. 7). The venoms were chromatographed without the addition of surface active agents.

Summary. Further details are given concerning the movements of proteins on filter paper using $M/10$ solutions of sucrose solution and sodium potassium tartrate as developing solvents in the first and second dimension, respectively. It is shown that mixtures of proteins, such as serum albumin and cytochrome *c*, may be separated from each other on paper, since the former moves in the first dimension, whilst the latter does

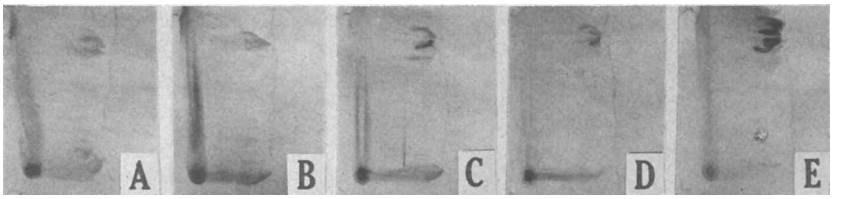

FIG 7. Snake venoms: A, cobra venom supplied by Haffkine Institute, Bombay, India; B, cobra venom supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.; C, Russel viper venom supplied by Haffkine Institute, Bombay, India; D, Russel viper venom supplied by Wellcome Research Labs., Langley Court, Beckenham, England; E, *Agkistrodon piscivorus* supplied by Ross Allen's Reptile Institute, Silver Springs, Fla.

not and both proteins move in the second dimension. The amount of cytochrome *c* moving in the second dimension is proportional to the amount applied to the paper. The chromatographic patterns of purified proteins *e.g.* crystalline human serum albumin, α, β, and γ globulins, and fibrinogen differ from each other in a characteristic manner. The differences are more marked when the proteins are mixed with surface active agents with which they form complexes; and these complexes move at different rates on filter paper. Complexes of surface active agents with albumin move with greater ease along the first dimension than those formed with globulins; the latter tend to remain at the point of origin. Almost all the protein complexes, however, move in the second dimension. The pattern given with a blood plasma is a composite of the patterns of the individual protein fractions, the basal fractions being apparently due to globulin. The patterns of chromatograms of pathological cases (multiple myeloma, liver cirrhosis, cancer of the stomach, lupus erythematosis) differ significantly from the normal, and are often characterized by the presence of heavy basal fractions due probably to increase in the globulin content of the plasma.

The chromatograms of dried snake venoms (cobra, Russel viper, *Agkistrodon piscivorus*) show various distinctive features.

We are greatly indebted to the National Cancer Institute of Canada and to the Rockefeller Foundation for grants which have made this work possible and to the Cutter Laboratories, Berkeley, Cal. for their generous donation of purified plasma protein fractions.

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Effect of Pretreatment with Cysteine on Survival of Mice Exposed to External and Internal Irradiation.* (18927)

HORACE GOLDIE, GADSON J. TARLETON, JR., AND PAUL F. HAHN.

From the Cancer Research Laboratories and Department of Radiology, Meharry Medical College, Nashville, Tenn.

Patt, Tyree, Straube and Smith(1-3) reported that rats injected intravenously with cysteine, shortly before irradiation with lethal

* Carried out under Contract AT-(40-1)-269 with the Division of Biology and Medicine, United States Atomic Energy Commission.

1. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

2. Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 18.

3. Smith, D. E., Patt, H. M., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 198.