

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Chromatography of Proteins. II. Fractionation of Serum Protein on Anion-exchange Cellulose

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Chromatographic analysis on a cellulose anion-exchange adsorbent of the natural mixture of typical proteins found in serum has been described. Gradients of pH and salt concentration were employed for elution. Ion exchange appeared to play a primary role in that most of the protein components emerged in the order of decreasing isoelectric point in the decreasing pH , increasing salt gradient. Effluent fractions were examined by ultraviolet spectrophotometry and characterized by electrophoretic techniques. Practically quantitative protein nitrogen recovery was obtained. The chromatographic patterns were reproducible, and loads as high as 170 mg. protein per gram of adsorbent could be chromatographed with good resolution. Several chromatographic components were obtained in each of the globulin subdivisions. Bands of colored protein components could be followed down the column and characterized in the effluent. Other specific components were detected by chemical and immunological techniques.

Most protein fractionation procedures have been designed for the purpose of purification of specific components of complex mixtures. Relatively few attempts have been made in the direction of inclusive schemes of fractionation which would simultaneously separate as many components of a mixture as possible. These have relied, in general, on solubility differences or differences in migration velocity induced by various force fields. Particularly for the fractionation of the proteins of blood serum, does it appear necessary that inclusive procedures of great resolving power be developed for preparative or analytical purposes.

Several groups of investigators have attempted this with varying degrees of success. The elegant methods based on solubility phenomena developed by Cohn and his group for the fractionation of blood¹ have provided ample evidence of the valuable results to be obtained by such procedures. Other investigators, by the systematic removal of salts by ion-exchange resins² or by the careful and controlled addition of ammonium sulfate³ have provided the basis for still other inclusive fractionation schemes based on solubility.

The physico-chemical methods such as electrophoresis and ultracentrifugation have, in general, been used as analytical rather than fractionation procedures although separations into groups of similar charge or weight distribution have long been practiced. The more recent developments in electrophoretic convection⁴ and zone electrophoresis in supportive media⁵ offer considerable promise for the simultaneous and possibly continuous separation of various components of protein mixtures.

The chromatographic method with ion-exchange adsorbents, which has been so successful with simple and well-defined organic structures, has just begun to find application in the separation of the more complex, high molecular weight compounds.⁶

(1) "Blood Cells and Plasma Proteins," J. L. Tullis, ed., Academic Press, Inc., New York, N. Y., 1953, and W. L. Hughes in "The Proteins II," B. H. Neurath and K. Bailey, eds., Academic Press, Inc., New York, N. Y., 1953, p. 741-753.

(2) A. F. Reid and F. Jones, *Ind. Eng. Chem.*, **43**, 1074 (1951).

(3) Y. Derrien, *Biochim. Biophys. Acta*, **8**, 631 (1952); E. P. Steyn-Parve and A. J. Van Den Hout, *ibid.*, **10**, 320 (1953).

(4) J. R. Cann and J. G. Kirkwood, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 9 (1950).

(5) A. Tiselius and P. Flodin, *Advances in Protein Chem.*, **8**, 461 (1953); R. J. Block, E. L. Durrum and G. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1955.

(6) C. A. Zittle, *Advances in Enzymology*, **14**, 319 (1953).

Here again, however, except for a recent report,⁷ the emphasis has been on the study of specific rather than general components of a protein mixture.

The development in this Laboratory of cellulose ion-exchange adsorbents of relatively high protein capacity and low irreversible adsorption, and the promising preliminary results achieved in a variety of protein systems,^{8,9} prompted an investigation of the use of these adsorbents in analytical and preparative fractionation schemes for the protein components of serum. The experience to date affords evidence for the feasibility of the chromatographic procedure employing cellulose ion exchangers and for the resolving power and reproducibility of the technique, as well as for the tentative identification of specific components in some areas of the chromatogram. The procedures to be described are not presented as established methods, but rather as a basis for further development along the diverse lines undoubtedly required for the application of this principle to the highly ramified studies of serum proteins.

Experimental

Preparation of the Sample.—Three different serum preparations were used. Horse serum was prepared from the fresh whipped blood of a single donor. OD human serum was prepared from the outdated plasma samples of 3 normal donors.¹⁰ The plasma was clotted by the addition of thrombin, centrifuged, dialyzed against tap water for 4 days, and finally, after dialysis against distilled water, it was lyophilized and stored in a refrigerator. FP human serum was collected¹⁰ from another group of 3 normal volunteers and allowed to clot without the addition of thrombin. The serum, after centrifugation was orange-yellow and quite cloudy. Some was used immediately, and the remainder was dialyzed for 24 hours against distilled water before lyophilization and storage in a refrigerator.

The fresh or lyophilized protein samples to be chromatographed were dialyzed in the cold against the starting buffer, 0.005 *M* sodium phosphate at pH 7.0, the same buffer with which the columns were equilibrated. The small amount of white flocculent precipitate (globulin) which formed was separated by centrifugation, and the clear amber supernatant liquid was applied to the column and allowed to enter the adsorbent under flow conditions induced by gravity. Samples containing 100-150 mg. of N (600-900 mg. pro-

(7) H. G. Boman, *Nature*, **175**, 898 (1955).

(8) E. A. Peterson and H. A. Sober, *THIS JOURNAL*, **78**, 751 (1956).

(9) H. A. Sober and E. A. Peterson, *ibid.*, **76**, 1711 (1954); E. A. Peterson and H. A. Sober, *Federation Proc.*, **13**, 273 (1954); H. A. Sober and E. A. Peterson, Abstracts 126th National Meeting, Amer. Chem. Soc., 90c, New York, Sept., 1954.

(10) We are indebted to the National Institutes of Health Blood Bank for the outdated blood samples and for the collection of blood.

tein) in 8–16 ml. were used, except in the experiment shown in Fig. 6, where 775 mg. N (about 5 g. protein) in 39.5 ml. was the column-charge.

Preparation of the Adsorbent Column.—The anion-exchange cellulose adsorbents DEAE-Polycel or DEAE-SF⁶ were used. After the exchange had been adjusted to the desired pH^{11} by the addition of NaH_2PO_4 solution, they were washed with buffer of the chosen pH and ionic strength several times on a filter. Non-sedimenting material was then removed by decantation. The adsorbent was poured as a slurry into a glass tube fitted at the bottom with a coarse fritted disc and was allowed to settle under flow conditions induced by gravity. After gravity settling was nearly complete, the adsorbent was further compacted by the application of air pressure at 10 p.s.i. until a constant column height was achieved. After such preparation, the column would not run dry under gravity flow.

The packed adsorbent column was then mounted above a fraction collector in a cold room (5°) and washed with several column volumes of the same buffer to ensure pH and temperature equilibrium. Approximately 28 g. of adsorbent was used to prepare packed columns of $40\text{--}47 \times 2.5$ cm. The exact height depended on the adsorbent employed.⁸

Development of the Chromatogram.—The equilibrated protein samples were washed into the column with several 1-ml. portions of buffer before the continuous flow of buffer was begun. Gradients of salt or pH —or both—were employed for elution in order to achieve the required range of eluting power without sharp changes in the composition of the eluant. Sodium phosphate buffers containing toluene were used, with the addition of $NaCl$ in the later parts of the elution schedule to raise the salt concentration. Flow rates of 10 to 12 ml. per hour were achieved with a total hydrostatic head of about 40 in. At the completion of the run, the adsorbents were regenerated with 1 *N* $NaOH$.⁸

Gradients were established¹² in the usual way by introducing, from a Mariotte-tube controlled separatory funnel, buffer having the composition of the gradient limit into a constant volume (250 ml.) mixing chamber. In the following discussion gradient solutions will be designated in the text and in the legends of the figures by their limit concentrations, although these were never reached in these experiments.

All dialysis and column operations were performed in the cold room and effluent tubes were covered and stored in a refrigerator. Individual tube collections of about 6 ml. were determined by a drop counter but the volume of each tube was measured directly.

Examination of the Effluent.—The effluent fractions were routinely examined in a Beckman DU spectrophotometer at 260 and 280 $m\mu$. Occasionally, other appropriate wave lengths were employed when colored components were eluted. Volume and pH determinations were performed at room temperature on the contents of individual tubes. Nitrogen was determined on aliquots from pooled fractions by a modification of the Friedrick method.¹³

Electrophoretic Examination.—Effluent fractions from the column were also routinely examined by electrophoretic techniques. Rather large volumes of effluent, indicated by the dotted vertical lines in the figures and designated by upper case letters (in block capitals and in italics for horse and human serum, respectively) were concentrated by "pervaporation"¹⁴ and subjected to boundary electrophoretic analysis at pH 8.6 in 0.1 μ veronal at 1° in the standard 11 ml. cell of the Klett apparatus. Mobilities and relative concentrations were calculated from the descending boundaries. The gross sampling was necessary in order to provide sufficient protein for each determination.

In order to utilize more fully the resolving power of the

chromatographic column, smaller combinations of effluent fractions were examined by paper electrophoresis. These samples are designated in the figures and the text by lower case letters and numbers in italics and represent 3 to 5 tubes (18–30 ml.) at positions immediately above or below the appropriate symbol. The solutions were concentrated by ultrafiltration in the cold room to 0.3–0.5 ml. in the manner described below.

The paper electrophoretic determinations were made on 0.01–0.03 ml. of such concentrated effluent in a hanging-strip cell^{6,15} in 0.075 μ veronal at pH 8.6. Eight Whatman 3 MM strips were run simultaneously in one cell for 18 hours at room temperature under constant current conditions of 6 milliamp. per cell and under a potential of 68–72 volts. The strips were stained in brom phenol blue under the conditions recommended by Jencks, *et al.*¹⁶ The paper strip patterns were evaluated by transmission densitometry with the Analytrol,¹⁷ using a cam which provided linear response for albumin dye-binding. Each electrophoretic run in a given cell included at least 1 strip which contained 0.01 ml. of a standard 3% albumin¹⁸ (BPA). Migration distances or "paper mobilities" were calculated relative to the movement of this standard.

The boundary electrophoresis patterns (upper case letters) were traced from a projected image and have been adjusted along the mobility axis so that the various patterns can be compared directly for mobilities, although not for total concentration. The peak at the zero mobility point is due to the starting salt boundary and is not a protein component. The paper electrophoresis patterns (lower case designations) were symmetrically reduced with the aid of a pantograph and have not been adjusted with regard to concentration or mobility.

In the discussion that follows, a chromatographic component will be classified, in the absence of data pertaining to a specific characteristic, on the basis of its mobility in an electrophoretic field at pH 8.6. The values expressed should be multiplied by 10^{-5} $cm.^2$ $sec.^{-1}$ $volt^{-1}$. Components with mobilities less than -2.1 will be designated γ -globulins; between -2.1 and -3.4 , β -globulins; between -3.4 and -4.6 , α_2 -globulins; between -4.6 and -5.5 , α_1 -globulins; and between -5.5 and -7.0 , albumins. It will become apparent that these designations are arbitrary and will have to be modified in the future since several distinct chromatographic components have appeared in most of these categories.

Ultrafiltration Apparatus.—A 20 cm. disc was cut from $1/2$ in. brass stock and equipped with a $1/8$ in. rubber gasket to fit the ground surface of the lower part of a small desiccator (Corning 3078). Seventeen $5/8$ in. holes were drilled through the disc and also a small hole connecting the edge with the bottom surface. A short brass tube fastened at the side opening permitted connection to a vacuum line. After lubrication of the gasket with silicone grease, the disc was mounted on the desiccator. Reservoirs of 40 ml. capacity were formed by sealing 5 cm. lengths of selected 6–7 mm. o.d. glass tubing to 12 cm. lengths of 20 mm. tubing. The tip of each reservoir was inserted into a No. 1 rubber stopper, and a cellophane dialysis sac, $1/4 \times 2 1/2$ in. and knotted at one end, was slipped over the protruding end until the knot was 4 cm. from the end of the glass tubing. It was then bound tightly to the glass with a well-stretched rubber band. After the addition of the sample and the removal of bubbles from the sac, the unit was inserted into one of the holes in the brass disc-desiccator assembly. When all the holes were filled, the internal space was evacuated, and a cylindrical battery jar of suitable size was inverted over the reservoirs to prevent evaporation and contamination from dust. With a filtering sac 4 cm. long and a pressure difference of $2/3$ of an atmosphere, about 6 ml. of liquid passed through each membrane per day. Usually the ultrafiltrate was collected in vials mounted below the sacs in a plastic tray. Protein solution concentrated at the

(11) The clear supernatant was always used for the final determination of pH .

(12) K. O. Donaldson, V. J. Tulane and L. M. Marshall, *Anal. Chem.*, **24**, 185 (1952); R. S. Alm, R. J. P. William and A. Tiselius, *Acta Chem. Scand.*, **6**, 826 (1952); A. Cherkin, F. E. Martinez and M. S. Dunn, *THIS JOURNAL*, **75**, 1244 (1953).

(13) We are indebted to R. J. Koegel and his staff for the ultraviolet spectra obtained on a Cary spectrophotometer and for the nitrogen analyses which were performed by the method of G. E. Secor, M. C. Long, M. D. Kilpatrick and L. M. White, *J. Assoc. Offic. Agr. Chemists*, **33**, 372 (1950).

(14) P. A. Kober, *THIS JOURNAL*, **39**, 944 (1917); L. Farber, *Science*, **82**, 158 (1935).

(15) F. G. Williams, Jr., E. G. Pickels and E. L. Durrum, *ibid.*, **121**, 829 (1955).

(16) W. P. Jencks, M. R. Jetton and E. L. Durrum, *Biochem. J.*, **60**, 205 (1955).

(17) The electrophoretic cell, power pack and Analytrol were obtained from the Spinco Division, Beckman Instruments, Inc., Belmont, California.

(18) Crystallized bovine plasma albumin, Armour Labs., Chicago, Illinois.

wall sank to the bottom of the sac and in several cases brightly colored layers were formed. Losses due to the drying of protein on the wall of the sac were much smaller than those encountered when "pervaporation" was used, because a very small area of cellophane was employed and only a fraction of that was allowed to become dry in the course of concentrating the sample to 0.3-0.5 ml. Upon completion of the ultrafiltration the sacs and their contents were inserted into small vials and cut away from the reservoir tips with a razor blade, then stored in a refrigerator until used.

Results

Horse Serum.—In Fig. 1 is shown the elution diagram obtained when dialyzed and lyophilized horse serum protein was chromatographed on the anion exchanger, DEAE-Polycel. The starting buffer, 0.005 *M* sodium phosphate at *pH* 7.0, was continued to point II, after which decreasing *pH* and increasing salt gradients were instituted at points indicated in the figure. The first three chromatographic components (in A and B, Fig. 1) had mobilities in the γ -globulin range as represented by the shaded areas at the top of the figure. Observations made in other experiments indicate that all the protein emerging before point III belonged in that category. In the subsequent fractions there was a general trend toward increasing mobility, but none of the combinations of fractions examined electrophoretically were homogeneous. In this chromatogram, material with albumin mobilities did not begin to appear until fraction G, after about 900 ml. of effluent had emerged.

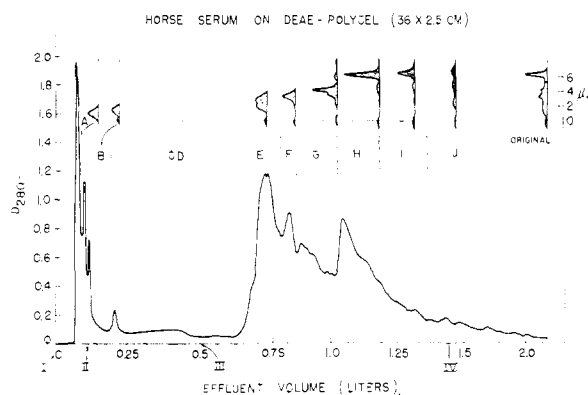


Fig. 1.—Effluent diagram of horse serum: 670 mg. dialyzed and lyophilized protein (79 mg. N) in 6 ml. applied to 25 g. of adsorbent; effluent collected in 5-6 ml. fractions. Buffers: I, 0.005 *M* Na phosphate, *pH* 7.0; II, gradient to 0.02 *M* Na phosphate, *pH* 5.9; III, gradient to 0.1 *M* NaCl-0.05 *M* NaH₂PO₄; IV, gradient to 0.5 *M* NaCl-0.1 *M* NaH₂PO₄. Mixing chamber volume, 250 ml. Shaded areas are boundary electrophoretic patterns of the fractions included between the broken vertical lines. Peaks at the zero mobility point are due to the starting salt boundary and are not protein components. Mobility scale units = -1×10^{-6} cm.² sec.⁻¹ volt⁻¹.

Another chromatogram of horse serum protein which differs from that just mentioned only in regard to the elution schedule is shown in Fig. 2. In this case, a gradient to 0.02 *M* sodium phosphate, *pH* 6.0, was introduced at about 72 ml. (II), at the beginning of peak A rather than at the end of the

peak as in the previous figure. As a result, fewer chromatographic peaks appeared in fractions A and B. Another change in elution schedule, the introduction of a gradient to 0.05 *M* NaH₂PO₄ at 250 ml. (III, Fig. 2) resulted in the emergence of a new, well resolved peak, E. This component appeared in Fig. 1 as a small hump on the leading edge of the large peak, E. The remainder of the chromatogram was less well resolved than in Fig. 1. It is noteworthy that in both of these chromatograms a component which moved faster than albumin in the electrophoretic field became apparent only after chromatographic separation (see original and G in Figs. 1, 2) and emerged before the bulk of the albumin.

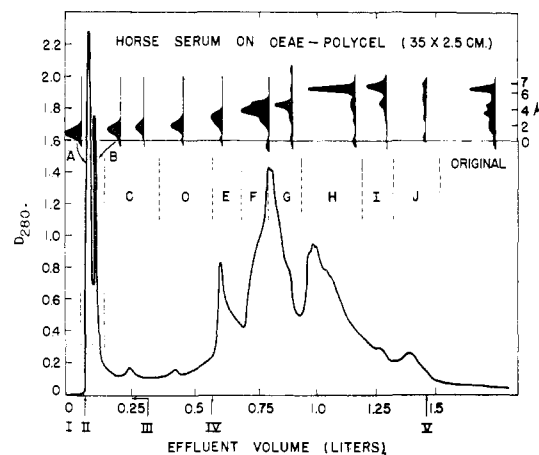


Fig. 2.—Effluent diagram of horse serum with altered elution schedule: 880 mg. dialyzed and lyophilized protein in 6.1 ml. applied to 24 g. of adsorbent; effluent collected in 6-7 ml. fractions. Buffers: I, 0.005 *M* Na phosphate, *pH* 7.0; II, gradient to 0.02 *M* Na phosphate, *pH* 6.0; III, gradient to 0.05 *M* NaH₂PO₄; IV, gradient to 0.1 *M* NaCl-0.05 *M* NaH₂PO₄; V, gradient to 0.5 *M* NaCl-0.1 *M* NaH₂PO₄. Remainder as in Fig. 1.

Human Serum.—A standardized elution schedule derived from our experience with horse serum has been applied to two different samples of human serum, and the effluent diagrams that have resulted are shown in Figs. 3, 4 and 6. The protein was applied to the adsorbent in 0.005 *M* sodium phosphate, *pH* 7.0, at I, and gradients were instituted as indicated in the figures.

The effluent pattern obtained from dialyzed and lyophilized outdated human serum (OD) is shown in Fig. 3, and a comparable¹⁹ chromatogram obtained from the fresh serum of other donors (FP) is given in Fig. 4. Comparison of these elution diagrams showed that an additional sharp peak, g, (Fig. 4) was obtained from the fresh serum, although absent from the OD serum chromatogram. Chromatography of lyophilized FP serum resulted in an elution diagram essentially identical to that shown in Fig. 4. However, dialysis of the lyophilized FP serum against the starting buffer, 0.005 *M* sodium phosphate at *pH* 7.0, for 55 hours rather than 18 hours, produced a chromatogram in which

(19) No difference in resolution has been encountered between DEAE-Polycel and DEAE-SF although the latter showed a higher resistance to the flow of buffer.⁸

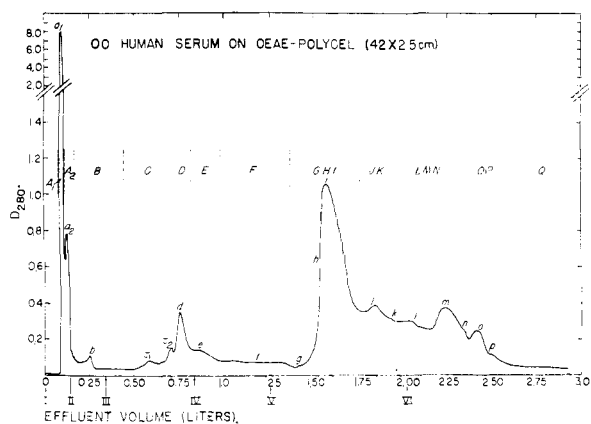


Fig. 3.—Effluent diagram of OD human serum: 970 mg. dialyzed and lyophilized protein (128 mg. N) in 7.5 ml. applied to 29 g. of adsorbent; effluent collected in 6-ml. fractions; flow rate, 15–16 ml./hr. Buffers: I, 0.005 *M* Na phosphate, pH 7.0; II, gradient to 0.02 *M* Na phosphate, pH 6.0; III, gradient to 0.05 *M* NaH₂PO₄; IV, gradient to 0.02 *M* NaCl–0.05 *M* NaH₂PO₄; V, gradient to 0.05 *M* NaCl–0.05 *M* NaH₂PO₄; VI, gradient to 0.1 *M* NaCl–0.05 *M* NaH₂PO₄. Mixing chamber volume 2.50 ml. See text for explanation of symbols.

peak *g* was absent. Perhaps prolonged dialysis after lyophilization removed a small molecule from an “albumin complex” so that the chromatographic behavior of the residual albumin was no longer different from that of the bulk of the albumin in serum. The apparent absence of an *a*₂ peak in Fig. 4 is a consequence of the broadening of the bands of slightly retarded protein as a result of the application of the sample in twice the usual volume (unconcentrated serum). Chromatography of lyophilized FP serum, applied in a smaller volume, demonstrated the presence of this component. However, other differences in the elution diagrams appear to reflect real variation in the composition of the two samples.

Load, Recovery and Reproducibility.—The usual column charge in the human serum fractionation studies was 130 to 150 mg. of protein N for 28 to 29 g. of adsorbent, about 5 mg. N per g. (Figs. 3 and 4). This has been increased 5-fold in Fig. 6, while maintaining good resolving power, and it became easy to follow the migration of colored bands along the column. The effluent pattern of Fig. 6 is generally similar to that of Fig. 4 in that corresponding components emerged at approximately the same effluent volumes (compare fractions *a*, *d*, and *o* with *l*, *s*, and *28*). Additional detail in the form of small peaks has appeared in Fig. 6, but the components labeled *f*₁, *f*₂ and *g* in Fig. 4 are poorly resolved in the corresponding region of Fig. 6 (*13*–*18*).

Recovery of protein from the columns was determined in 27 different chromatographic experiments dealing with serum or serum fractions. With column loads of 3 to 23 mg. of protein N per g. of adsorbent, the nitrogen recovery averaged $87 \pm 12\%$, which in view of the large number of sub-fractions collected (as many as 400) and the many manipulations involved, can be considered quantitative.

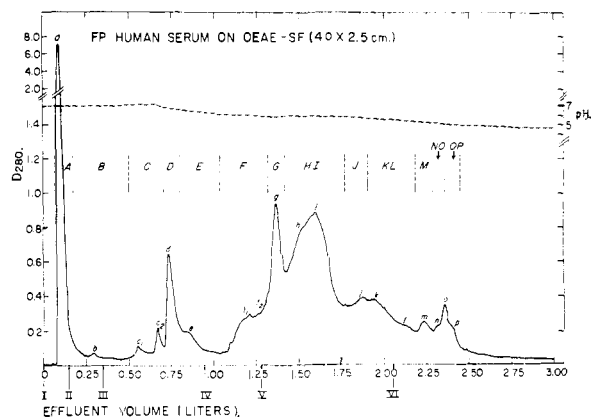


Fig. 4.—Effluent diagram of FP human serum: 20 ml. of dialyzed fresh FP serum (156 mg. N) applied to 28 g. of adsorbent; effluent collected in 6–7 ml. fractions; flow rate, 11 ml./hr. Broken line and scale at right represent pH of effluent. Remainder as in Fig. 3.

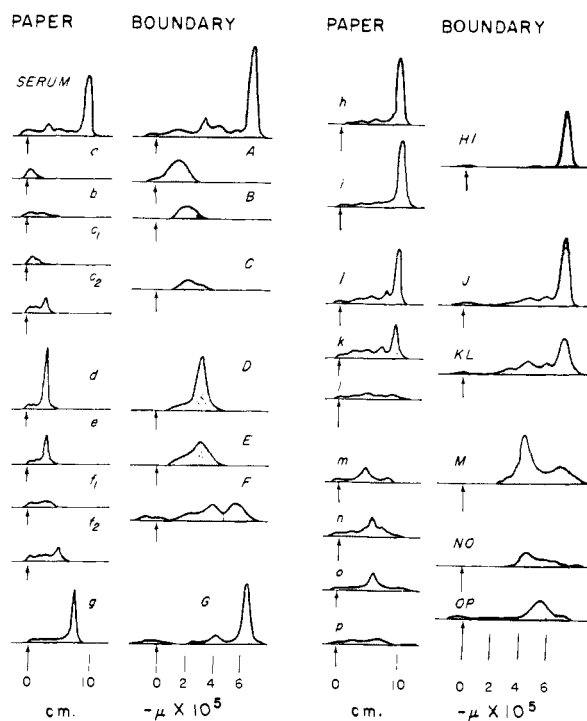


Fig. 5.—Comparison of paper and boundary electrophoresis patterns of chromatographic fractions from Fig. 4. See text for symbols and electrophoretic conditions.

With a given elution schedule and a given serum the reproducibility of the positions and magnitudes of the chromatographic peaks was excellent. When serums from different donors were chromatographed the peaks varied in size, but corresponding components emerged at the same points. A pooled fraction similar to that encompassed by *GHI* of Fig. 3 was concentrated by “pervaporation” and rechromatographed on the same adsorbent column (regenerated) with the original elution schedule. The center of the resulting peak emerged at 1680 ml., closely matching its appearance in the original chromatogram at 1670 ml. A small second peak appeared at the position occupied by the succeed-

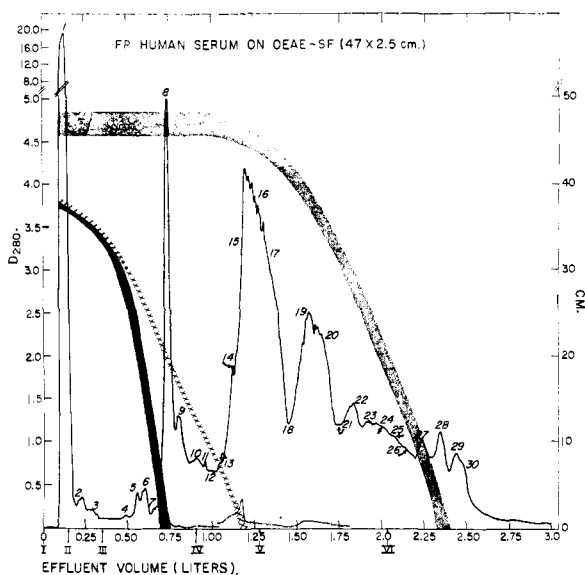


Fig. 6.—Effluent diagram of 5-fold amounts of FP human serum: 5.0 g. of dialyzed and lyophilized protein (775 mg. N) in 39.5 ml. applied to 30 g. of adsorbent; effluent collected in 7-ml. fractions; flow rate, 12 ml./hr. The diagonal, crossed diagonal and stippled areas represent the red, tan and blue bands, respectively, with the distance of the band from the bottom of the column indicated by the scale at the right. Broken line represents D_{468} m μ , dashed-dotted line, D_{406} m μ . Remainder as in Fig. 3.

ing component of the original chromatograph (corresponding to j in Fig. 3). Since the original peaks overlapped, the presence of the second component in the rechromatographed sample was to be expected.

Elution Schedule, the Effect of pH and Salt.—Early experiments had indicated that the affinity of the anion exchange adsorbents for a specific component could, in general, be reduced in at least three ways: (1) a decrease in the pH of the solution, reducing the anionic character of the protein; (2) a rise in pH , partially repressing the ionization of the tertiary amine groups of the adsorbent⁸ and thus curtailing the anion-binding capacity of the exchanger itself; and (3) an increase in the ionic strength of the buffer. Chromatograms have been developed with increasing or decreasing pH gradients and with salt gradients alone. Each of these procedures resulted in some degree of resolution.

Because the isoelectric points of the recognized components in serum range from pH 3 to 8,¹ and they are generally stable in the slightly acid range, the serum protein mixtures have been applied to the adsorbent at pH 7.0, accomplishing elution by the gradual reduction of pH and the simultaneous increase in salt concentration. Relatively little work has been done on the improvement of elution schedules. Probably no single scheme can be expected to suit all purposes. The schedules employed here have given a fair degree of resolution, particularly with the serum proteins of high isoelectric points, but exploration in this area has been deferred in favor of developmental work on the characterization of a multiplicity of small fractions.

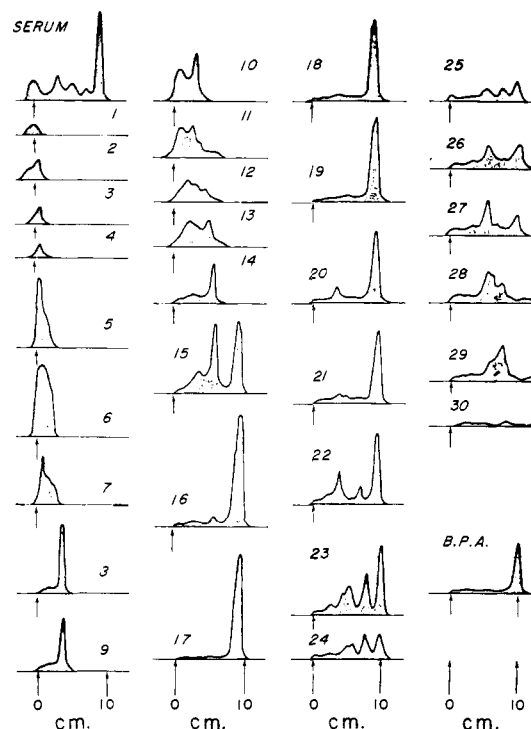


Fig. 7.—Paper electrophoretic patterns of chromatographic fractions from Fig. 6. See text for symbols and electrophoretic conditions.

It should be emphasized that while the elution schedules in the legends to the figures are designated in terms of the gradient limit, protein eluted from the column during the period in which that gradient was being used was never in contact with solution of that composition since the gradients were not permitted to run long enough, and the buffering action of the adsorbent, as well as that of the protein, tended to modify the pH and salt gradients.

As an illustration of the effect of the adsorbent and the protein in modifying the eluting solution, the pH of the effluent (broken line) is included in Fig. 4. The pH remained at the initial value of 7.0 for about 250 ml. A gradual rise to a maximum of pH 7.27 at peak c_2 occurred, followed by a slow decrease. The pH was 6.40 at IV, 5.90 at V, and 5.44 at VI. At the end of peak p , the pH had only dropped to a value of 4.80, although NaH_2PO_4 had been entering the column since 350 ml. (III). Although $NaCl$ was introduced at 900 ml. of effluent (IV) chloride did not emerge from the column until about 1500 ml. had appeared. Since the maximum mechanical hold-up of the column was about 73 ml. (the volume at which the first peak appeared), retardation of chloride by the adsorbent is evident. The addition of $NaCl$ was a convenient means of increasing the salt concentration of the eluent without contributing additional buffer components (e.g., NaH_2PO_4) which would undergo acid-base reaction with the incompletely titrated adsorbent. The observed binding of chloride by the adsorbent was the result of exchange with phosphate already bound.

Electrophoretic Characterization.—An inspection of the electrophoretic patterns in Figs. 1, 2, 5

and 7 shows that there was a general trend toward higher electrophoretic mobilities in successive fractions, as one would expect if ion exchange were playing a dominant role. The proteins with high isoelectric points lost their anionic character earlier in the declining pH gradient than those with lower ones. This generalization holds for fractions A through F in Figs. 1 and 2, for samples *a* through *g* in Figs. 3, 4 and 5; and for samples *I-16* in Figs. 6 and 7. It can be most easily demonstrated from Fig. 2 in which the first four peaks, A, B, C and D were electrophoretically homogeneous in veronal at pH 8.6, with mean mobilities of -0.86 , -1.32 , -1.53 and -1.84×10^{-5} cm.² sec.⁻¹ volt⁻¹, respectively. A combination of peak A and B ($\mu = -0.86$ and -1.32) resulted in a single peak in the electrophoretic field with a mean mobility of -1.13 . Fraction E had 2 components with mobilities of -2.29 and -3.00 and fraction F contained components with mobilities of -2.73 , -3.59 and -4.33×10^{-5} cm.² sec.⁻¹ volt⁻¹.

Preliminary chromatographic studies with Red Cross γ -globulin²⁰ have provided fractions which emerged from the anion exchanger with a decreasing pH and increasing salt gradient in the order of increasing mobilities. On the other hand, elution from a cation exchanger, CM-cellulose,⁸ with a gradient of increasing pH and salt concentration provided fractions which emerged in the order of decreasing mobilities.

Electrophoretic examination of the fractions emerging from the anion exchanger beyond F in Figs. 1 and 2, *g* in Figs. 3 and 4, and *I6* in Fig. 7 reveal several components which do not appear to follow the generalization just presented. These anomalies may result from (1) a difference in the relative order of decreasing electrostatic charge on the proteins within the buffer-column milieu, as compared with that obtaining in the veronal buffer used for electrophoresis; (2) the formation of protein complexes which are stable under the chromatographic conditions employed (pH 7.0-4.8) but unstable at pH 8.6 in veronal; or (3) the existence of specific affinities of a non-electrostatic nature between the adsorbent and the protein.

The constituent mentioned above which moves faster than albumin in the electrophoretic field would be expected, on a purely ion-exchange basis, to move more slowly on the anion exchanger than albumin itself. This was actually the case for both lots of human serum, where the faster-than-albumin component ($\mu = -7.7$) emerged after albumin ($\mu = -6.4$ to -6.9) at the end of the chromatogram (see *NO* and *o*, Fig. 5; and *28-30*, Fig. 7). However, in the horse serum chromatograms the corresponding component ($\mu = -8.2$) emerged before albumin in fraction G (Figs. 1 and 2). A component in normal undialyzed plasma with similar electrophoretic properties in 0.1 M NaCl at pH 8 has been reported.²¹

A comparison of the diagrams obtained from specific areas of a serum chromatogram (Fig. 4) by paper and boundary electrophoresis is given in Fig. 5. General agreement is apparent. The differ-

(20) Obtained through the courtesy of Dr. J. N. Ashworth of the American Red Cross.

(21) H. Hoch and A. Chanutin, *J. Biol. Chem.*, **200**, 241 (1953).

ences that do occur are a reflection of the fact that the paper electrophoresis was performed on smaller combinations of effluent tubes than the boundary electrophoresis. A careful examination of Fig. 5 as well as Fig. 7, which contains the patterns obtained from specific areas of the chromatogram shown in Fig. 6, indicates the difficulty encountered in attempting to classify components solely on the basis of electrophoretic mobility. Samples *a-c*₁ (Fig. 5) and *I-4* (Fig. 7) showed only mobilities in the γ range, whereas the effluent diagrams (Fig. 4 and 6, respectively) clearly indicated several components. Protein with mobilities in the β range appeared first in samples *c*₂ and *5* and continued through *f*₂ and *15*. The heterogeneity of this β -protein, apparent in the chromatograms, was substantiated by sharp differences in the color of the fractions.

Mobilities in the α_2 range were present to a considerable extent in samples *f* and *g* and *12-16* but were practically absent in samples *h*, *i* and *17-19*. More than 95% of the latter samples consisted of material with the mobility of albumin. In fraction *20*, a peak in the β -region was again discernible and α_2 - and α_1 -mobilities began to appear. Fractions *21-23* showed a progressive increase in relative content of a "fast" α_2 - and an α_1 -component, concomitantly with a relative decrease in material in the albumin range. Samples *26-29* showed a progressive redistribution of material having α_2 - and α_1 -mobilities, the first decreasing while the latter increased. In fractions *28-30* (Fig. 7) the faster-than-albumin component appeared. The same general trends could be observed in other serum runs (e.g., Fig. 5).

Specific identification of each of the components indicated by this combination of chromatographic and electrophoretic techniques requires further separation or the use of other criteria.

Colored Components.—The application of the yellow serum protein solution to the top of the adsorbent column quickly produced a series of distinct colored bands. In the order of increasing adsorption, they appeared buff, red, tan, yellow and blue. During elution in the usual chromatograms, where 1 g. of protein was added to a 30-g. adsorbent column, the buff band was visible for only a short time. The red and tan bands moved down the column at different rates and could be followed for some distance. The yellow band broadened and moved away from the top of the column leaving a narrow blue band which was the last to move. In the usual runs (Figs. 3 and 4) none of the bands could be followed out of the column although the blue band was discernible for about 80% of the column length. Concentration of the effluent fractions by ultrafiltration revealed a deep red in fraction *d* and a deep blue in fraction *o* (Fig. 4). The intervening fractions were very faintly yellow.

In the chromatographic run shown in Fig. 6 where the initial serum load was 5 times the usual amount, the colored bands were so intense that some of them could be followed all the way down the column into the effluent. The movement of the red, tan and blue bands is shown in Fig. 6 by

the diagonal, crossed diagonal and stippled areas, respectively. The yellow band between the tan and blue was not plotted since it became very broad, with diffuse boundaries, and appeared to contain three maxima which could not be defined reliably. The distribution of the red color (458 $m\mu$) and the tan color (406 $m\mu$) is shown in the figure by broken and dashed-dotted lines, respectively. The blue color was followed to the bottom of the column but unlike the red and tan components could not be seen in the unconcentrated effluent.

Concentration of the effluent tube combinations some 50-fold by ultrafiltration produced colorless solutions in fractions 1-7, deep red in 8, orange in 9, yellow in 10-13, yellow-amber in 14, and deep tan in 15. Fractions 16-27 were yellow with maxima at fractions 16-17 and 19-20. Thereafter the intensity of the yellow color decreased. Fraction 28 was a deep blue and 29-30 were colorless.

The red component in fraction 8 had the electrophoretic mobility of a β -globulin at pH 8.6 (Fig. 7), and material with this mobility accounted for 90% of the sample. The solution showed maxima at 278 and 462 $m\mu$.²² Its properties agreed with those of the β_1 -metal-combining protein which was first described by Schade and Caroline²³ and later named siderophilin²⁴ or transferrin.²⁵ It was concentrated by Surgenor, *et al.*,²⁶ and crystallized by Koehlin²⁷ and Laurell.²²

The tan band emerged in fraction 15 (Fig. 6), the point at which albumin first appeared (Fig. 7). It showed maxima at 278 and 407 $m\mu$ with a 278/407 ratio of about 10. This colored component resembled in absorption spectrum and electrophoretic mobility the "methemalbumin" prepared by Rosenfeld and Surgenor²⁸ and reported to occur as an abnormal component of blood plasma in certain diseases associated with excessive hemolysis.²⁹

The yellow fractions, 16-17 and 19-20 which contained primarily albumin-type mobilities may well be complexes of albumin and various small molecules stable under chromatographic conditions but dissociated in veronal at pH 8.6. However, it has been observed, in these studies, that the mobility of the albumin component in successive fractions varied in electrophoretic mobility from -6.40 to -6.85×10^{-5} cm.² sec.⁻¹ volt⁻¹.

The appearance of the blue component at the end of the chromatogram in fraction 28 coincided with the appearance of a "fast" α_2 -component in paper

(22) C. B. Laurell (*Acta Chem. Scand.*, **7**, 1407 (1953)) has described a highly purified and recrystallized preparation of "transferrin" from pig's plasma. He suggested that the absorption ratios 278 $m\mu$ /470 $m\mu$ and 470 $m\mu$ /408 $m\mu$ be used as criteria of purity and reported 25 and 1.4 as the respective values obtained from 4-5 times crystallized material. Our fraction *d* (Fig. 4) from human serum gave values of 21 and 1.3 for 278 $m\mu$ /462 $m\mu$ and 462 $m\mu$ /408 $m\mu$, respectively, without further purification.

(23) A. L. Schade and L. Caroline, *Science*, **104**, 340 (1946).

(24) A. L. Schade, R. W. Reinhart and H. Levy, *Arch. Biochem.*, **20**, 170 (1949).

(25) C. G. Holmberg and C. B. Laurell, *Acta Chem. Scand.*, **1**, 944 (1947).

(26) D. M. Surgenor, B. A. Koehlin and L. E. Strong, *J. Clin. Invest.*, **28**, 73 (1949).

(27) B. A. Koehlin, *THIS JOURNAL*, **74**, 2649 (1952).

(28) M. Rosenfeld and D. M. Surgenor, *J. Biol. Chem.*, **183**, 663 (1950).

(29) M. Rosenfeld, C. G. Zibroi, W. D. Blake and J. A. Shannon, *J. Clin. Invest.*, **27**, 138 (1948).

electrophoresis (Fig. 7). This fraction showed absorption maxima at 278 and at 602 $m\mu$. The blue color and the absorption at 602 $m\mu$ disappeared in the presence of ascorbic acid and was regenerated by exposure to atmospheric oxygen. The blue component exhibited the properties attributed to caeruloplasmin^{25,30,31} and comprised about 30% of this fraction, on the basis of spectrophotometric data. A deficiency of caeruloplasmin has been reported³² in the serum of patients with hepatolenticular degeneration (Wilson's disease) by Scheinberg and Gitlin, who suggested that this disease was another example of a pathologic condition related to a congenital deficiency of specific plasma protein.

Other Specific Components.—The distribution of hexosamine in the column effluent was examined.³³ It was found, in a run similar to that shown in Fig. 3, that 2 maxima of hexosamine concentration occurred: one at fraction *d* (1.8% hexosamine in protein) and one at *o* (4%). Fractions *a* and *h, i* contained 0.4% hexosamine, a value as low as that obtained for crystalline albumin.¹⁸ This distribution of hexosamine is in general accord with the reported distribution among the electrophoretically characterized proteins.¹ However, as much as 7.25% hexosamine has been found in a terminal peak of a chromatogram of pathological serum.

Thrombin activity, as measured by the ability to form a clot with fibrinogen, was localized³⁴ in fraction 29 (Fig. 7). None was detected in fractions 28 or 30. Since caeruloplasmin preparations have always shown thrombin activity³⁵ and since fraction 28 contained the bulk of the caeruloplasmin but no thrombin activity, it can be concluded that thrombin was a contaminant in previous caeruloplasmin preparations.

Immunological examination³⁶ of effluent fractions (Fig. 6) for albumin by the Oudin³⁷ technique showed that albumin was just detectable in fraction 9 but absent in earlier ones. Albumin reaction rose to a maximum in the area encompassed by fractions 16-22 and then decreased. Slight albumin activity was detected beyond fraction *p*.

Effluent fractions were also examined³⁸ for orosomucoid.³⁹ Nearly all of the orosomucoid was found in an area corresponding to *n, o* and *p* of Fig. 3, which is also the area with the highest

(30) K. Laki, Abstract Communications, XVII, *Intern. Physiol. Congress, Oxford*, 373 (1947); C. G. Holmberg and C. B. Laurell, *Acta Chem. Scand.*, **2**, 550 (1948).

(31) We are indebted to Dr. D. R. Kominz for unpublished results which he kindly made available to us.

(32) I. H. Scheinberg and D. Gitlin, *Science*, **116**, 484 (1952).

(33) We are indebted to Dr. N. F. Boas and Mr. J. B. Foley for the hexosamine determinations (*J. Biol. Chem.*, **204**, 553 (1953)).

(34) We are indebted to Dr. K. Laki for these determinations.

(35) Personal communication from Dr. K. Laki.

(36) We are grateful to Dr. E. L. Becker of the Army Medical Service Graduate School, Washington, D. C., who performed this examination with sheep anti-mercaptalbumin.

(37) J. Oudin, *Compt. rend. Acad. Sci.*, **222**, 115 (1946).

(38) We are indebted to Dr. R. J. Winzler of the University of Illinois College of Medicine, Chicago, Illinois, for the examination of the chromatographic fractions by a quantitative immunological method with chicken anti-orosomucoid.

(39) R. J. Winzler, A. W. Devor, J. W. Mehl and I. M. Smyth, *J. Clin. Invest.*, **27**, 609 (1948); H. E. Weimar, J. W. Mehl, and R. J. Winzler, *J. Biol. Chem.*, **185**, 561 (1950); K. Schmid, *THIS JOURNAL*, **75**, 60 (1953).

hexosamine content. A sub-fraction contained 74% orosomucoid. Orosomucoid, with its low isoelectric point of 2.8 emerged from the column at a position in accord with the assumption that ion exchange was primarily responsible for the chromatographic separation.

Discussion

The procedures described were time consuming (8-9 days), and the protein emerged from the column considerably diluted. Concentration by "per-vaporation" involves the risk of losses by drying on the cellophane walls and ultrafiltration is relatively slow. However, preliminary experiments have indicated the feasibility of concentrating large volumes of dilute protein solution of low ionic strength by adsorption on very short cation-exchange columns at pH 5.0, followed by displacement in a sharp band with a strong eluting agent such as 0.05 M NaCl-0.05 M NaH₂PO₄.

Work in progress has indicated that the complex mixture of proteins in serum can be separated into groups by stepwise elution from short columns in one day. Further resolution of these groups can then be attempted on separate columns of the same type under different elution conditions or with other cellulose ion-exchangers.

The chromatograms presented here demonstrate the separation of γ -globulins from whole serum into electrophoretically homogeneous fractions having different mobilities at pH 8.6. As indicated in the discussion of the horse serum chromatograms in Figs. 1 and 2, γ -globulin fractions with distinctly different individual mobilities may appear as a single electrophoretic peak when combined. These observations support the concept of a more or less continuous distribution of mobilities within the γ -globulin range which is not resolved by conventional electrophoretic techniques but has been indicated by results obtained by electrophoretic convection and immunological methods.⁴⁰ Preliminary studies with Red Cross γ -globulin on cellulose ion-exchangers have also resulted in the separation of several components.⁹

Protein with the mobility and immunological

(40) S. M. Timasheff, J. G. Kirkwood and A. W. Moyer, *Federation Proc.*, **13**, 310 (1954); J. R. Cann, R. A. Brown, J. G. Kirkwood and J. H. Hink, *J. Biol. Chem.*, **185**, 663 (1950); J. R. Colvin, D. B. Smith and W. H. Cook, *Chem. Revs.*, **54**, 687 (1954).

properties of albumin was found throughout the latter half of each serum chromatogram. It is unlikely that this was merely a consequence of poor desorption of albumin and mercaptalbumin or an artifact of the chromatographic procedure. Evidence for the multiple nature of the albumins, either as distinct molecular species or as complexes with small molecules, is afforded by the distribution of color, the differences in the electrophoretic mobility of successive fractions, and the chromatographic integrity of an albumin fraction rechromatographed under the original conditions. Moreover, preliminary results obtained in the chromatography of Red Cross serum albumin (Fraction V)²⁰ on a cellulose anion-exchanger demonstrated the presence of at least three components which differed in electrophoretic properties at pH 4.0, in sugar content, in tyrosine-tryptophan ratios, and in immunological behavior.⁴¹

It is unfortunate that the poor separation in the albumin and α -globulin regions tends to obscure the rather good separation of the specific proteins examined. Recourse to the use of other elution schedules or rechromatography on other adsorbents⁸ will be necessary.

Results obtained to date have shown that the chromatographic procedure with the cellulose exchangers can become the basis for the separation of a variety of protein mixtures. Preliminary studies with Red Cross γ -globulin²⁰ have indicated that immunological properties have been retained and fractionated. Studies with crystalline bovine serum albumin¹⁸ have shown that the sedimentation constant and the electrophoretic mobility at pH 8.6 have remained unchanged after the protein has been chromatographed three times on the anion-exchange adsorbent. Effluent fractions subjected to ultracentrifugation before concentration or storage have shown a great reduction, if not complete removal, of the heavy component usually present in this crystalline protein.

These preliminary observations as well as more extensive chemical, immunological and enzymatic characterization of the serum fractions are being further developed and the isolation of specific components on a preparative scale is contemplated.

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(41) Unpublished work in collaboration with Dr. E. L. Becker.