and back-titrated with hydrochloric acid, using brom thymol blue as indicator: equiv. wt., 125.

The debenzylated polymer was dialyzed and treated as in section a; yield 4.6 g. The multichain polyglutamic acid was hydrolyzed and the hydrolysate analyzed chromatographically analogously to the multichain polytyrosine. A molar ratio of glutamic acid to lysine, 16 to 1, was obtained. The intact multichain polyglutamic acid gave amino N, 0.60 (Van Slyke), and Na, 2.9, determined with a flame spectrophotometer. The multichain polyglutamic acid was desaminated analogously to the desamination of multichain poly-DL-alanine, and the product hydrolyzed as usual. The hydrolysate was analyzed chromatographically, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase. The chromatogram contained spots of lysine and glutamic acid, but no spots corresponding to the products appearing in the hydrolysate of desaminated polylysine.

Poly-L-lysine, Free Base.—A solution of poly-L-lysine hydrobromide, DP 18 (78 mg.), in water (5 ml.) was passed through a column of Amberlite IRA 400, and the fraction giving a negative Volhard and a positive ninhydrin test (20 ml.) was collected and dried *in vacuo*; yield, 45 mg. of a white powder.

Anal. Calcd. for poly-L-lysine (DP 18): neut. equiv., 128. Found: neut. equiv., 134, determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using thymol blue as indicator.

The polylysine free base (DP 18) obtained could not be redissolved in water. It is soluble in glacial acetic acid, in phenol and in aqueous acids. It is insoluble in ether, dioxane, methanol, pyridine, triethylamine, tributylamine and boiling dimethylformamide. It may be precipitated from its solution in glacial acetic acid by dioxane.

Physical Measurements.—The sedimentation measurements were carried out in a Spinco ultracentrifuge Model E with a Klett optical system. The sedimentation constant was calculated as an average of the values $S = 2(x_2 - x_1)/(x_2 + x_1)\omega^2(t_2 - t_1)$, where x_1 and x_2 are the distances from the axis of rotation to the boundary at the times t_1 and t_2, respectively, and ω is the angular velocity. Final corrections were made in the customary manner to a solvent with the density and viscosity of water at 20°. For this purpose a partial specific volume of 0.72 was employed.

Diffusion measurements were carried out in a Claesson diffusion cell,[25] at 20°. A Tiselius–Longsworth apparatus[26] with a schlieren-scanning optical system was used. In the measurements made the diffusion curves were found to be practically normal. The average diffusion coefficients were derived using the formula $D = \sigma/2t$, where σ denotes the standard deviation and t the time of diffusion. The standard deviation was calculated from the first and second moments measured with an Amsler Integrator.

The refractive index of multi-poly-DL-alanyl-poly-L-lysine (*b* 34, *p* 22) in glycine buffer, *p*H 9.1 and ionic strength 0.1, at 20°, was measured by means of a dipping refractometer compensated to sodium light. The refractive index increment, $dn/dw = 0.182$, was calculated from the slope of linear relation between refractive index and concentration found experimentally (w is the ratio between the weight of solute and the weight of solution).

Viscosity measurements were carried out in an Ostwald viscosimeter at 20°.

(25) S. Claesson, *Nature*, **156**, 824 (1946).

(26) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

REHOVOT, ISRAEL

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

Chromatography of Proteins. I. Cellulose Ion-exchange Adsorbents

BY ELBERT A. PETERSON AND HERBERT A. SOBER

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Ion-exchange adsorbents have been prepared from cellulose under conditions such that physical properties suitable for column chromatography are maintained. These adsorbents possess high capacity for the adsorption of proteins, yet permit elution under mild conditions. Titration curves are presented.

The remarkable success of chromatography in the separation of amino acids, lipids, sugars and many other substances has been an important factor in recent advances in biochemistry. The application of chromatography to the separation of proteins, however, has not met with the general success obtained with smaller, simpler molecules. The large size of the protein molecule prevents its penetration into the adsorbent particle, therefore only the adsorbing sites located on the exterior can be utilized. An enormous surface, as provided by very fine division, then becomes necessary for the attainment of reasonably high adsorption capacity. The instability of the protein molecule is also a factor, not only because of the severe limitations it imposes upon the choice of solvents, but also because profound configurational changes may accompany the adsorptive and desorptive processes if binding sites are sufficiently numerous and strong to compete with the multitude of weak bonds which maintain the native configuration of the protein. Even where this does not occur, too high a density of binding sites may result in adsorption so firm that the conditions required for simultaneous dissociation of all the adsorptive bonds are destructive to the integrity of the molecule.

However, progress has been made in recent years, particularly in the field of ion-exchange chromatography. Relatively stable proteins of rather low molecular weight and high isoelectric point have been chromatographed on columns of finely divided Amberlite IRC-50, a weak cation exchanger.[1] The chromatography of hemoglobin, a neutral molecule of more typical size, has been demonstrated on the same resin, but the conditions required were such as to preclude ion exchange as the adsorption mechanism.[2] Very recently the separation of four kinds of human carbon monoxide hemoglobin with IRC-50 has been reported.[3] Early work in this Laboratory achieved the frontal analysis of artificial mixtures of typical proteins on the strong cation exchanger, Dowex-50, but the capacity was very low.[4] The same resin has been used for the chroma-

(1) C. A. Zittle, *Advances in Enzymology*, **14**, 319 (1953); E. Margoliash, *Nature*, **170**, 1014 (1952); *Biochem. J.*, **56**, 529, 535 (1953); C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953); C. H. W. Hirs, *ibid.*, **205**, 93 (1953); C. F. Crampton, S. Moore and W. H. Stein, *ibid.*, **215**, 787 (1955).

(2) N. K. Boardman and S. M. Partridge, *Nature*, **171**, 208 (1953); *Biochem. J.*, **59**, 543 (1955).

(3) H. K. Prins and T. H. J. Huisman, *Nature*, **175**, 903 (1955).

(4) H. A. Sober, G. Kegeles and F. J. Gutter, THIS JOURNAL, **74**, 2734 (1952); *Science*, **110**, 564 (1949).

tography of prostatic phosphatase,[5] and the application of an anion exchanger, Dowex-2, to the fractionation of human serum proteins has very recently been reported.[6] The use of inorganic gels such as calcium phosphate has been promising in the chromatography of typical proteins,[7] but adsorbents with greater chemical and physical stability, allowing a wider selection of eluents, and with better defined ionizing groups are to be desired.

In the following pages the preparation and properties of new adsorbents derived from cellulose, having these characteristics as well as relatively large capacities for the adsorption of proteins, will be described. The effectiveness of such adsorbents containing carboxymethyl (CM–), diethylaminoethyl (DEAE–) and phosphate (P–) groups in the chromatographic purification of enzymes and other proteins has been reported in preliminary communications from this Laboratory.[8] A companion paper demonstrates the resolution achieved in the separation of serum proteins on DEAE–cellulose, an anion exchanger.[9] Further information concerning the use of these adsorbents in the chromatography of enzyme preparations will be published at a later date.

Another adsorbent prepared in this Laboratory, ECTEOLA–cellulose,[10] containing basic groups derived from triethanolamine bound to cellulose through reaction with epichlorohydrin, was found to be less promising in protein adsorption experiments but will be described here because of its exceptional merit in the chromatography of nucleic acids, as shown by work carried out in other laboratories.[11]

A number of cellulose ion exchangers have appeared in the literature, including oxycellulose,[12] cellulose succinic half ester,[13] and a variety of treated cotton fabrics.[14] Although oxycellulose has been used for the purification of ACTH oligopeptide preparations by batch adsorption,[15] no attempt to employ materials of this type for the adsorption of proteins has been reported. In several instances, however, the adsorption of enzymes by cellulose, itself, has been observed and utilized for purification.[16] Such adsorption can probably be attributed to a very small carboxyl content normally present in cellulose.

General Considerations.—Alpha cellulose offered particular promise as a support for ionizing groups intended to bind proteins because of its hydrophilic nature and enormous surface. It was readily available at low cost, and simple modifications of a reaction extensively used in the industrial manufacture of cellulose derivatives provided the means of attaching a variety of ionizable groups. The preparation of ion exchangers of this type is, however, subject to a limitation arising from the nature of the cross-linking bonds in cellulose. Hydrogen bonding between the hydroxyl groups is responsible for the insolubility of that material, and the attachment of small amounts of any substituent to the chain tends to increase swelling in water by interfering with hydrogen bonding. A larger number of groups will cause the cellulose derivative to become water-soluble, like the carboxymethylcellulose of industry. Although a certain amount of swelling is advantageous in protein chromatography in that greater accessibility to the binding sites is afforded, there is a concomitant increase in resistance to the flow of aqueous solutions which is of considerable importance when the material is to be used in adsorption columns. In the procedures to be described the degree of incorporation is controlled to keep the physical properties of the product within the range useful for chromatographic purposes. It has been regarded as essential to avoid conditions which might solubilize or disperse the product, even temporarily, for in those cases where reprecipitation was possible a gelatinous material having extremely high resistance to the flow of aqueous solutions was obtained. Such an adsorbent may be useful in batch operations when sedimentation can be employed for separation of the phases, but it is ill-suited to column chromatography or batch filtration. Although insoluble preparations of both CM– and DEAE–cellulose containing 2 meq. per gram have been obtained, products containing much more than 1 meq. per gram had a tendency to be gelatinous, with a high resistance to the flow of water. This limitation on the number of groups incorporated proved to be not serious, for the capacity for protein adsorption was very high, on a mass basis, even when the acid–base capacity of the adsorbent was of the order of 1 meq. per gram. Since multiple-site binding is undoubtedly involved in the protein–adsorbent combination, a low density of binding sites on the adsorbent is advantageous (provided that the capacity is adequate) in that it must contribute toward ease of desorption of polyelectrolytes. A low buffering capacity (desirable in gradient elution chromatography) and a low capacity for the adsorption of small electrolytes are additional favorable consequences.

The cellulose ion exchangers described here have all been prepared by the reaction of a chloro compound with cellulose which has been allowed to swell in strong alkali. In general, the number of

(5) H. G. Boman, *Biochim. Biophys. Acta*, **16**, 245 (1955).

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

(7) K. Agner, *Biochem. J.*, **32**, 1702 (1938); S. M. Swingle and A. Tiselius, *ibid.*, **48**, 171 (1951); A. Tiselius, *Arkiv Kemi*, **7**, 443 (1954).

(8) H. A. Sober and E. A. Peterson, THIS JOURNAL, **76**, 1711 (1954); E. A. Peterson and H. A. Sober, *Federation Proc.*, **13**, 278 (1954); H. A. Sober and E. A. Peterson, Abstracts 126th National Meeting, Am. Chem. Soc., 90-C, Sept., 1954.

(9) H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, THIS JOURNAL, **78**, 756 (1956).

(10) The intended reactions involved ether formation between the hydroxyl groups of triethanolamine and the epoxide ring of epichlorohydrin, as well as between the halogen function of the latter and the NaO–groups of the sodium cellulose. However, since other reactions are possible (including polymerization) the structure of the attached groups cannot be specified, and the adsorbent has been designated ECTEOLA–cellulose to identify the reactants.

(11) A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser, THIS JOURNAL, **77**, 3671 (1955); D. F. Bradley and A. Rich, Abstracts 128th National Meeting, Am. Chem. Soc., 3-C, Sept., 1955.

(12) E. C. Yackel and W. O. Kenyon, THIS JOURNAL, **64**, 121 (1942).

(13) F. C. McIntire and J. R. Schenck, *ibid.*, **70**, 1193 (1948).

(14) C. L. Hoffpauir and J. D. Guthrie, *Textile Research J.*, **20**, 617 (1950); W. A. Reeves and J. D. Guthrie, *ibid.*, **23**, 522 (1953); L. M. Soffer and E. Carpenter, *ibid.*, **24**, 847 (1955).

(15) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1951).

(16) H. Tauber, *J. Biol. Chem.*, **113**, 753 (1936); R. W. Payne, M. S. Raben and E. B. Astwood, *ibid.*, **187**, 719 (1950); R. O. Hurst and G. C. Butler, *ibid.*, **193**, 91 (1951).

groups attached per gram of dry product was approximately a linear function of the amount of chloro compound used, provided sufficient alkali was present, and so long as the incorporation did not greatly exceed 1 mmole per gram. The degree of reaction appeared to be largely independent of the particle size of the cellulose employed, at least over the range of 100 mesh to finer than 325 mesh. Good reproducibility with respect to number of groups attached and gross physical properties was obtained under the conditions described.[17] If, however, the incorporation were carried to a point approaching that at which gels were obtained, poor reproducibility of both incorporation and physical properties was observed.

Experimental

Cellulose.—The cation exchangers, CM- and P-cellulose, were prepared from Whatman ashless standard cellulose powder,[18] which has a cotton origin. In preparations of this type, wood cellulose offered little advantage with respect to reactivity, whereas the white color of the Whatman product was highly desirable. The anion exchanger, DEAE-cellulose, was preferably made from wood cellulose, for the reactivity of the latter was about twice that of cotton cellulose in this reaction. Polycel,[19] a wood cellulose, was initially employed in preparations of DEAE-cellulose. These products possessed excellent properties for the column chromatography of proteins, and considerable use was made of them[5,8] before it became known that Polycel was no longer being manufactured. Subsequent experiments have employed Solka-Floc, another wood cellulose of high purity.[20] Of the several grades available, SW-A, SW-B, and BW have been tested in the preparation of DEAE-cellulose. All were comparable to Polycel in reactivity. Products made from the SW-A and BW grades, however, exhibited a greater tendency to shed "fines" on washing and a somewhat greater resistance to the flow of aqueous solutions which was particularly marked in strong alkali. Products made from the SW-B grade were intermediate in these respects. Adequate amounts of all the particle sizes used in these experiments were obtained by sieving commercial 40 or 100 mesh material.

2-Chlorotriethylamine Hydrochloride.—The commercially available material (Eastman Kodak, P6436) was recrystallized from absolute methanol to remove highly colored impurities.

Other Materials.—Chloroacetic acid, epichlorohydrin and triethanolamine (2,2',2''-nitrilotriethanol) were white label products of the Eastman Kodak Company and were used without further purification.

Preparation of DEAE-SF.—A solution of 40 g. of NaOH in 170 ml. of water was stirred[21] into 60 g. of Solka-Floc. The mixture, dry in appearance, was allowed to stand in an ice-bath for 30 minutes,[22] with occasional stirring. A solution of 35 g. of recrystallized 2-chlorotriethylamine hydrochloride in 45 ml. of water was added in several portions. After thorough blending, the mixture was immersed in an 80–85° oil-bath for 35 minutes and stirred occasionally. The product was cooled in an ice-bath and 250 ml. of 2 *M* NaCl[23] was added in several portions, care being taken to achieve complete mixing.

The resulting thick suspension was filtered with gentle suction in a 5-inch coarse sintered glass funnel, and the filter cake was washed with 1 *N* NaOH until the emerging filtrate was no longer deeply colored. The product was then resuspended on the filter in sufficient 1 *N* HCl (about 350 ml.) to make a strongly acid suspension, and this was immediately filtered. Successive washing with 250-ml. portions of 1 *N* NaOH, 1 *N* HCl and 1 *N* NaOH followed. The cake was tamped down and pulled dry to the cracking point between washings but was not rinsed with water. Finally, the cake was again suspended in 250 ml. of 1 *N* NaOH, then transferred to a large vessel and diluted with water to 3 liters. After standing overnight, the cloudy supernatant liquid was decanted, and the sediment was washed 5 or 6 times by decantation to remove particles that would not settle.[24] The final supernatant liquid was almost clear and was free of alkali. The sediment was filtered to remove as much water as possible, then washed with about 500 ml. of ethanol in 3 portions, the last one absolute ethanol. As much of the alcohol as possible was drawn off on the filter, then the partially dried product was abraded to a powder. The remaining alcohol was evaporated *in vacuo*,[25] with a sintered glass disc inserted at the mouth of the flask to prevent the escape of the light powder into the cold trap and pump. When cellulose finer than 325 mesh was used, about 45 g. of adsorbent containing approximately 1.4% nitrogen (1.0 meq. per gram) was obtained. With coarser cellulose the yields were somewhat greater.

Preparation of CM-W.—A solution of 90 g. of NaOH in 200 ml. of water was stirred into 60 g. of Whatman cellulose powder (sieved to remove material coarser than 325 mesh) to form a stiff, friable mass.[21] The flask was then immersed in an ice-bath for 30 minutes,[22] and the contents were stirred occasionally. A solution of 30 g. of chloroacetic acid in 40 ml. of water was added in 3 or 4 portions and mixed well after each addition. The soft, doughy mass was then heated in a 70° oil-bath for 20 minutes, with occasional stirring. After removal from the oil-bath, the bright yellow product was cooled in an ice-bath, and 500 ml. of 10% acetic acid (or an equivalent amount of dilute HCl) was added in several portions. The suspension, which was still strongly alkaline, was then diluted to 2 liters with water and allowed to settle. After careful decantation of the cloudy yellow supernatant fluid, the sediment was washed repeatedly in a similar manner to remove particles which would not sediment, as well as color and alkali.[24] About 10 ml. of glacial acetic acid was then added to the diluted suspension to make it just acid to congo red, and after settling and decantation of the supernatant liquid, a slurry of the sediment was poured into a 5 inch coarse sintered glass funnel and washed with water until the filtrate was no longer acid. The cake was pulled as dry as possible on the filter, then washed with ethanol and dried in the manner described for the preparation of DEAE-SF. About 45 g. of white powder containing 0.7 meq. of acidic groups per gram was obtained.

Preparation of P-W.—A solution of 75 g. of NaOH in 185 ml. of water was stirred into 50 g. of Whatman cellu-

(17) In six preparations of DEAE-SF, following the same procedure, the average deviation from the mean nitrogen content was 2%.

(18) H. Reeve Angel and Company, Inc., 52 Duane Street, New York 7, New York.

(19) The Polycel was a gift of the Industrial Chemical Sales Division of the West Virginia Pulp and Paper Company, Covington, Virginia.

(20) The Solka-Floc was generously provided by the Brown Company, 500 Fifth Avenue, New York 36, N. Y.

(21) Since the volume of the added liquid was restricted to increase the efficiency of the reaction with cellulose, uniform distribution of the alkali among the particles was difficult. Therefore, the alkali was added in several small portions and the mixture was stirred well after each addition to break up the wet lumps and coat the fragments with relatively dry material.

(22) Because the mercerization of cellulose proceeds at lower concentrations of alkali when the temperature is low, the swelling process was carried out in an ice-bath to promote penetration of the particles while at the same time diminishing reaction of the strongly alkaline cellulose with oxygen.

(23) The presence of NaCl controlled swelling and facilitated filtration, which was otherwise sometimes extremely difficult under the strongly alkaline conditions required for the removal of colored side products from the anion exchangers.

(24) Because of variations in settling rate under different conditions of pH and ionic strength, as well as variations among the different kinds of cellulose used, a fixed settling time cannot be specified. Usually 1–2 hours was sufficient. At some point in the procedure the small amount of coarse, aggregated material present in the suspension was eliminated by careful decantation of the dilute suspension after it had been permitted to settle for a few minutes.

(25) All of the adsorbents prepared in this study were dried before use in order to facilitate characterization and storage. It is only reasonable to assume that the wet products were suitable for use at any stage after the washing process, and storage as the alcohol-damp powder may be most convenient, at least in the case of the anion exchangers. Partial esterification might be expected if the CM- and P-celluloses are stored for long periods in alcohol. The vacuum method of removing the alcohol was adopted when initial attempts to air-dry CM-cellulose preparations on a filter, after washing with alcohol and ether, yielded hard, lumpy material.

lose and the well mixed,[21] friable mass was allowed to stand in an ice-bath for 30 minutes.[22] Then 330 ml. of water was added slowly. The suspension was stirred mechanically at a slow rate, with the bottom of the flask immersed in an ice-bath. After about 15 minutes the dropwise addition of 80 ml. of an ether solution containing 40 ml. of reagent grade $POCl_3$ was begun. Throughout the addition, which was completed in 50 minutes, the temperature of the suspension was maintained at 25–30°. Its final *p*H was approximately 6.

After a few minutes the suspension was diluted with water to 2 liters in a large precipitation jar and allowed to settle overnight. The sediment was washed repeatedly by decantation[24] until an almost clear supernatant liquid was obtained. It was then filtered, washed with alcohol and dried as described for the preparation of DEAE–SF. The product was a white powder weighing 38 g. and containing 1.9% phosphorus (0.6 mmole phosphate per gram).

Preparation of ECTEOLA–SF.—A solution of 60 g. of NaOH in 150 ml. of water was stirred into 60 g. of Solka-Floc and the well blended mixture[21] was immersed in an ice-bath for 30 minutes.[22] Then a mixture of 35 ml. of triethanolamine and 60 ml. of epichlorohydrin was added in several portions. The friable mass was stirred for several minutes to assure thorough mixing and penetration. It was then immersed in a 75–80° oil-bath for 30 minutes and stirred at intervals.

Approximately 10 minutes after immersion an exothermic reaction suddenly became manifest by a sharp rise in internal temperature to well over 100°, accompanied by a rapid evolution of vapor. This reaction subsided very quickly, leaving a gummy amber mass. At the end of the heating period the contents of the flask were cooled in an ice-bath, and 250 ml. of 2 *M* NaCl[23] was added in portions, stirring well after each addition to obtain a smooth suspension. The product was then filtered, washed and dried in the manner described for the preparation of DEAE–SF. About 60 g. of adsorbent containing 0.25 to 0.30 meq. of basic nitrogen per gram was obtained.

In one experiment the mixture of triethanolamine, epichlorohydrin and NaOH–cellulose was allowed to stand overnight at room temperature before heating. A very slow and moderate rise in temperature occurred spontaneously during the first two hours of standing, and there was no evidence of further reaction when a portion of the mixture was heated the next day. After washing and drying, identical nitrogen contents (0.42 meq. basic nitrogen per gram) and titration curves were obtained from the heated and unheated products. Their adsorption behavior has not yet been evaluated.

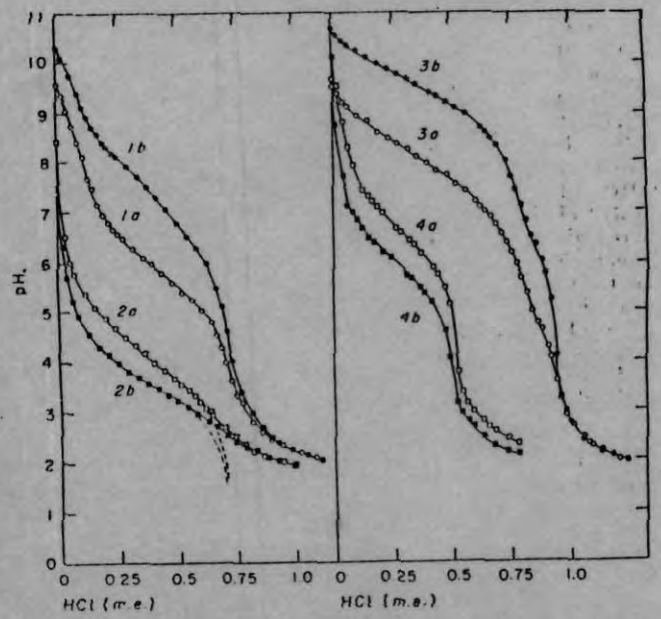

Figure 1.—Titration curves of four cellulose ion exchangers: 1, ECTEOLA–SF; 2, CM–W; 3, DEAE–SF; 4, P–W. (a) Curves obtained in water alone, (b) those obtained in 0.5 *M* NaCl. Broken lines are corrected for water blank. The small shoulder on the acid side of the DEAE–SF was apparently due to bicarbonate formation during the washing procedure. It was reduced but not eliminated by alkali treatment followed by washing with boiled water. Estimations of the pK' in this case were based upon the symmetrical portion of the rest of the curve.

Acid or Base Content.—The extent of incorporation of ionizing groups was determined readily in the DEAE–, ECTEOLA– and P–celluloses by nitrogen or phosphorus analysis, as well as by potentiometric titration.[25] Where it was available, the elemental analysis was taken as the basis for calculating the content of such groups, but agreement between the two methods was good. The carboxyl content of the CM–celluloses was obtained solely by titration with HCl after the addition of a small excess of NaOH. The magnetically stirred suspensions reached a steady *p*H value very rapidly after each addition of acid, permitting the titrations to be carried out as for homogeneous systems. This was further substantiated by the fact that points obtained after a 20-hour equilibration of CM–W or DEAE–SF with measured amounts of acid fell upon curves obtained by rapid titration. An ordinary glass electrode was employed without applying a sodium correction, but the procedure provided quickly a valid estimate of the ionizable groups attached to the cellulose and a close approximation of the apparent dissociation constant.

Titration Curves.—Representative titration curves appear in Fig. 1. In every case, titration in water alone yielded

TABLE I

EFFECTS OF TYPE OF CELLULOSE AND NUMBER OF GROUPS ON THE pK' OF CELLULOSE ION EXCHANGERS

Cellulose[a]	Ionizing groups, mmole/g.	pK' in water[b]	pK' in 0.5 *M* NaCl
DEAE–cellulose			
Solka-Floc SW-A, 325–	0.45	7.5	9.5
Solka-Floc SW-A, 325–	0.99	8.0	9.5
Solka-Floc SW-A, 325–	1.34	8.5	9.5
Solka-Floc BW, 230–325	0.99	8.0	9.5
Solka-Floc BW, 100–230	.97	8.1	9.5
Solka-Floc SW-B, 325	.94	8.0	9.5
Solka-Floc SW-B, 100–230	.90	7.6	9.5
Polycel, 270–325	.78	7.5	..
Polycel, 325–	.92	7.5	9.5
Polycel, 325–	1.03	7.5	..
Polycel, 325–	1.98	8.0	9.4
Whatman, 325–	0.18	6.5	..
Whatman, 325–	.31	6.5	..
ECTEOLA–cellulose			
Solka-Floc SW-A, 325–	.29	5.9	7.4
Solka-Floc BW, 325–	.25	6.0	7.6
Solka-Floc BW, 325–	.42	5.8	7.5
CM–cellulose			
Whatman, 325–	.75	4.2	3.5
Whatman, 325–	1.10	3.8	..
Whatman, 325–	1.57	3.8	..
Solka-Floc BW, 325–	0.59	4.3	..
Solka-Floc BW, 325–	.67	4.3	3.7
P–cellulose			
Whatman, 325–	.60	6.7	6.0
Whatman, 325–	.50	6.8	..

[a] Figures indicate mesh size. Finer than 325 mesh is designated by 325–. [b] Since NaCl was formed during the titration of CM– and P–celluloses, those titrations were not carried out in salt-free water. The concentration did not exceed 0.03 *M* at the mid-point of the titration in most cases, however.

(26) We are indebted to Mr. R. J. Koegel and his staff for the determinations of nitrogen and phosphorus and to Mrs. Mary M. Wyckoff for technical assistance in titrating the adsorbents.

lose and the well mixed,[21] friable mass was allowed to stand in an ice-bath for 30 minutes.[22] Then 330 ml. of water was added slowly. The suspension was stirred mechanically at a slow rate, with the bottom of the flask immersed in an ice-bath. After about 15 minutes the dropwise addition of 80 ml. of an ether solution containing 40 ml. of reagent grade $POCl_3$ was begun. Throughout the addition, which was completed in 50 minutes, the temperature of the suspension was maintained at 25–30°. Its final *p*H was approximately 6.

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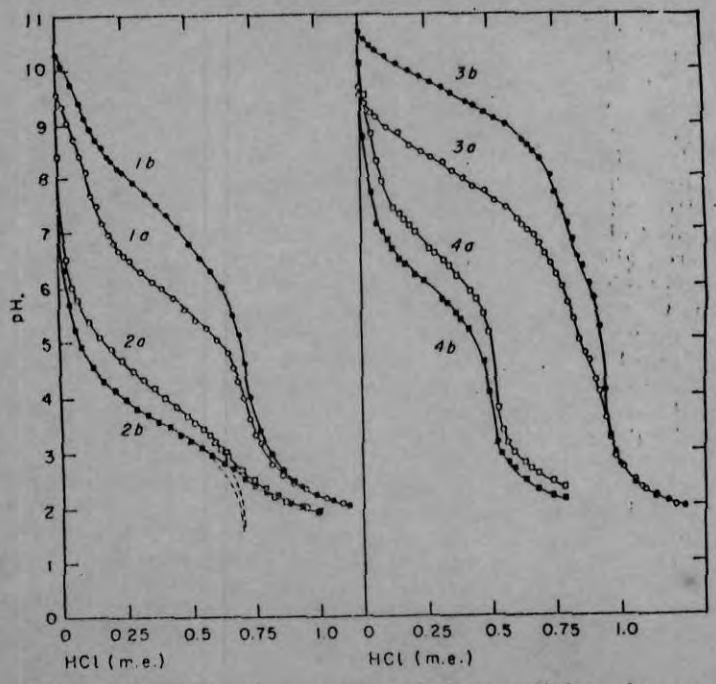

Figure 1.—Titration curves of four cellulose ion exchangers: 1, ECTEOLA–SF; 2, CM–W; 3, DEAE–SF; 4, P–W. (a) Curves obtained in water alone, (b) those obtained in 0.5 *M* NaCl. Broken lines are corrected for water blank. The small shoulder on the acid side of the DEAE–SF was apparently due to bicarbonate formation during the washing procedure. It [illegible]ced but not eliminated by alkali treatment fol[illegible]ashing with boiled water.

Acid or Base Content.—The extent of incorporation of ionizing groups was determined readily in the DEAE–, ECTEOLA– and P–celluloses by nitrogen or phosphorus analysis, as well as by potentiometric titration.[26] Where it was available, the elemental analysis was taken as the basis for calculating the content of such groups, but agreement between the two methods was good. The carboxyl content of the CM–celluloses was obtained solely by titration with HCl after the addition of a small excess of NaOH. The magnetically stirred suspensions reached a steady *p*H value very rapidly after each addition of acid, permitting the titrations to be carried out as for homogeneous systems. This was further substantiated by the fact that points obtained after a 20-hour equilibration of CM–W or DEAE–SF with measured amounts of acid fell upon curves obtained by rapid titration. An ordinary glass electrode was employed without applying a sodium correction, but the procedure provided quickly a valid estimate of the ionizable groups attached to the cellulose and a close approximation of the apparent dissociation constant.

Titration Curves.—Representative titration curves appear in Fig. 1. In every case, titration in water alone yielded

TABLE I

EFFECTS OF TYPE OF CELLULOSE AND NUMBER OF GROUPS ON THE pK' OF CELLULOSE ION EXCHANGERS

Cellulose[a]	Ionizing groups, mmole/g.	pK' in water[b]	pK' in 0.5 *M* NaCl
DEAE–cellulose			
Solka-Floc SW-A, 325–	0.45	7.5	9.5
Solka-Floc SW-A, 325–	0.99	8.0	9.5
Solka-Floc SW-A, 325–	1.34	8.5	9.5
Solka-Floc BW, 230–325	0.99	8.0	9.5
Solka-Floc BW, 100–230	.97	8.1	9.5
Solka-Floc SW-B, 325	.94	8.0	9.5
Solka-Floc SW-B, 100–230	.90	7.6	9.5
Polycel, 270–325	.78	7.5	..
Polycel, 325–	.92	7.5	9.5
Polycel, 325–	1.03	7.5	..
Polycel, 325–	1.98	8.0	9.4
Whatman, 325–	0.18	6.5	..
Whatman, 325–	.31	6.5	..
ECTEOLA–cellulose			
Solka-Floc SW-A, 325–	.29	5.9	7.4
Solka-Floc BW, 325–	.25	6.0	7.6
Solka-Floc BW, 325–	.42	5.8	7.5
CM–cellulose			
Whatman, 325–	.75	4.2	3.5
Whatman, 325–	1.10	3.8	..
Whatman, 325–	1.57	3.8	..
Solka-Floc BW, 325–	0.59	4.3	..
Solka-Floc BW, 325–	.67	4.3	3.7
P–cellulose			
Whatman, 325–	.60	6.7	6.0
Whatman, 325–	.50	6.8	..

[a] Figures indicate mesh size. Finer than 325 mesh is designated by 325–. [b] Since NaCl was formed during the titration of CM– and P–celluloses, those titrations were not carried out in salt-free water. The concentration did not exceed 0.03 *M* at the mid-point of the titration in most cases, however.

(26) We are indebted to Mr. R. J. Koegel and his staff for the d[illegible]

a pK' corresponding to much weaker acidity or basicity than that exhibited by similar groups in free solution.[27] However, the addition of NaCl at the start of the titration resulted in pK' values which were closer to those expected. This phenomenon is characteristic of insoluble polyelectrolytes and appears to be a reflection of the relative inaccessibility of interior groups to the titrating agent.[28] The data of Table I demonstrate this effect and also differences in pK' arising from the kind of cellulose used and the number of groups incorporated. Thus, among the DEAE-celluloses, values obtained in water alone ranged from 6.5 (cotton cellulose, 0.18 meq. per g.) to 8.5 (wood cellulose, 1.34 meq. per g.), whereas with a given cellulose and a given number of attached groups reproducibility within 0.2 pH unit was obtained. The pK' values obtained by titration in the presence of 0.5 M NaCl, however, were characteristic only of the groups involved and were not affected by the kind of cellulose and the number of groups attached. Presumably, the accessibility of the ionizing sites to hydrogen ions is dependent to some extent upon the factors mentioned. That accessibility of the sites to protein is similarly affected is suggested by the following adsorption experiments.

Protein Adsorption Capacity.—The specific adsorption values listed in Table II represent the amount of protein adsorbed by 100 mg. of adsorbent suspended in a buffer in which the protein concentration was gradually increased to 2 mg. per ml. over a period of about two hours by the addition of a concentrated solution at a constant rate. At higher concentrations of protein additional adsorption occurred, but in this region the slope of the isotherm was much lower.[31] Since a measure of the adsorption capacity was desired, conditions of pH and salt concentration were selected under which desorption of the protein was negligible. The table demonstrates the very high capacity of these adsorbents for bovine plasma albumin and for carbon monoxide hemoglobin of the horse, as well as the influence of the kind of cellulose and the number of incorporated groups on the adsorption capacity. DEAE-cellulose prepared from Polycel possessed an adsorption capacity for crystalline bovine plasma albumin significantly lower than that of a similar adsorbent prepared from Solka-Floc SW-A or BW, whereas Solka-Floc SW-B yielded a material with intermediate adsorption capacity. The capacity increased rapidly with increasing incorporation of ionizing groups beyond the range compatible with physical properties suitable for column chromatography, and it is reasonable to believe that gelatinous adsorbents having much higher capacities could be prepared for special purposes. The experiments with DEAE-cellulose indicated that adsorbents made from 100–230 mesh cellulose possessed the same adsorption capacity as those made from material finer than 325 mesh.

TABLE II

ADSORPTION CAPACITIES OF CELLULOSE ION EXCHANGERS

Adsorbent	Cellulose[a]	Ionizing groups meq./g.	Specific adsorption[b]
Anion exchangers[c]			
DEAE-SF	Solka-Floc SW-A, 325–	0.45	16
DEAE-SF	Solka-Floc SW-A, 325–	0.96	75
DEAE-SF	Solka-Floc SW-A, 325–	1.34	122
DEAE-SF	Solka-Floc BW, 100–230	0.97	76
DEAE-SF	Solka-Floc SW-B, 325–	.94	66
DEAE-Pol.	Polycel, 270–325	.10	7
DEAE-Pol.	Polycel, 270–325	.78	36
DEAE-Pol.	Polycel, 325–	.91	54
ECTEOLA-SF	Solka-Floc SW-A, 325–	.29	5
Cation exchangers[d]			
CM-W	Whatman, 325–	.73	98
CM-SF	Solka-Floc BW, 325–	.67	98
P-W	Whatman, 325–	2 × 0.60	93

[a] Figures indicate mesh size. Finer than 325 mesh is designated by 325–. [b] Expressed as mg. protein adsorbed per 100 mg. adsorbent when final protein concentration is 2 mg. per ml. [c] Tested with crystalline bovine plasma albumin[29] in 0.01 M sodium phosphate at pH 7.0. [d] Tested with horse carbon monoxide hemoglobin[30] in 0.01 M sodium phosphate at pH 6.0.

Physical Characteristics and Stability.—Columns were prepared from slurries of these adsorbents, adjusted to the proper pH and molarity of buffer, by allowing the material to settle in glass tubes fitted with coarse sintered glass bottoms under flow conditions induced by gravity alone, then applying air pressure (10 p.s.i.) to compact the adsorbent. Columns prepared in this manner possessed the ability to hold the buffer level at the top of the adsorbent against the pull of gravity. Flow rates suitable for chromatographic purposes were attainable in columns as long as 40 cm. by means of an adjustable hydrostatic head. In the case of CM- and p-celluloses prepared from Whatman powder the head required for a flow rate of 5 ml. per cm.² per hour was generally less than the height of the column itself. DEAE-cellulose prepared from Solka-Floc finer than 325 mesh required a head equal to 2–3 times the column height to attain a similar rate. Since the particle size did not appear to affect the adsorption capacity markedly, the use of coarser cellulose to obtain higher flow rates is suggested. There was little or no change in the dimensions of the columns in passing through a 4–8 pH range, but very high salt concentrations caused some shrinkage in the cation exchangers. These comments are based upon experience with sodium phosphate and chloride solutions. Other ions may alter the properties of these adsorbents materially.

These exchangers could be used repeatedly and suffered no apparent deterioration upon months of storage in a cold room in contact with aqueous solutions ranging from 0.05 M NaH_2PO_4 to 1 N NaOH.[33] Prolonged exposure to solutions more acid than pH 4 was avoided, although it is known that brief contact with 1 N HCl is not significantly harmful. After each use the anion exchangers were treated with an equal volume of 1 N NaOH to remove any residual protein, then washed with water to remove the alkali. A mixture of 0.5 M NaCl–0.5 N NaOH was used in the case of CM-cellulose to control the swelling effect of strong alkali on this adsorbent.

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(27) Glycolic acid, diethylaminoethanol, and triethanolamine were titrated in water in the same manner, giving pK' values of 3.7, 9.9 and 7.8, respectively. The corresponding values obtained in 0.5 M NaCl were 3.6, 10.0 and 8.0.

(28) H. P. Gregor and J. I. Bregman, THIS JOURNAL, 70, 2370 (1948); R. Kunin and R. J. Myers, *ibid.*, 69, 2874 (1947); P. Alexander and J. A. Kitchener, *Textile Research J.*, 20, 203 (1950).

(29) Armour Laboratories, Chicago, Illinois.

(30) G. Kegeles and F. J. Gutter, THIS JOURNAL, 73, 3770 (1951).

(31) A more complete report on adsorption isotherms will be published at a later date.

(32) Unpublished work.

(33) Phosphorylated cellulose is a probable exception. It was not subjected to the conditions described, but susceptibility of its ester bond to acid or alkaline hydrolysis can be expected.

[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

BY HERBERT A. SOBER, FREDERICK J. GUTTER, MARY M. WYCKOFF AND ELBERT A. PETERSON

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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 *p*H 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 *p*H, 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 blood[1] 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 resins[2] or by the careful and controlled addition of ammonium sulfate[3] 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 convection[4] and zone electrophoresis in supportive media[5] 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] 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 collected[10] 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 *p*H 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-

(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 Biochemistry*, 14, 319 (1953).

(7) H. G. Boman, *Nature*, 176, 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-SF[8] were used. After the exchange had been adjusted to the desired *p*H[11] by the addition of NaH_2PO_4 solution, they were washed with buffer of the chosen *p*H 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 *p*H and temperature equilibrium. Approximately 28 g. of adsorbent was used to prepare packed columns of 40–47 × 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 *p*H—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 established[12] 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 mμ. Occasionally, other appropriate wave lengths were employed when colored components were eluted. Volume and *p*H 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 Friederick 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 *p*H 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 combinat[illegible] fractions were examined by paper electrophore[illegible] samples are designated in the figures and the text [illegible] case letters and numbers in italics and represent 3 [illegible] 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[5,15] in 0.075 μ veronal at *p*H 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% albumin[18] (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.

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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 × 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 *p*H.

(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**, 872 (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.

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.

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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[5,15] in 0.075 μ veronal at *p*H 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% albumin[18] (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 *p*H 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 × 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 3/4 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 *p*H.

(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**, 872 (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 *p*H 7.0, was continued to point II, after which decreasing *p*H 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.

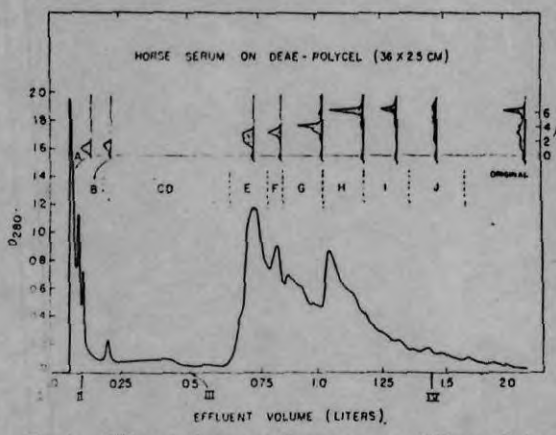

Fig. 1.—Effluent diagram of horse serum: 670 mg. dialyzed and lyophilized protein (79 mg. N) in 6 ml. applied to 25 g. of adsorbent; effluent collected in 5–6 ml. fractions. Buffers: I, 0.005 *M* Na phosphate, *p*H 7.0; II, gradient to 0.02 *M* Na phosphate, *p*H 5.9; III, gradient to 0.1 *M* NaCl–0.05 *M* NaH_2PO_4; IV, gradient to 0.5 *M* NaCl–0.1 *M* NaH_2PO_4. 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^{-5} 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, *p*H 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* NaH_2PO_4 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.

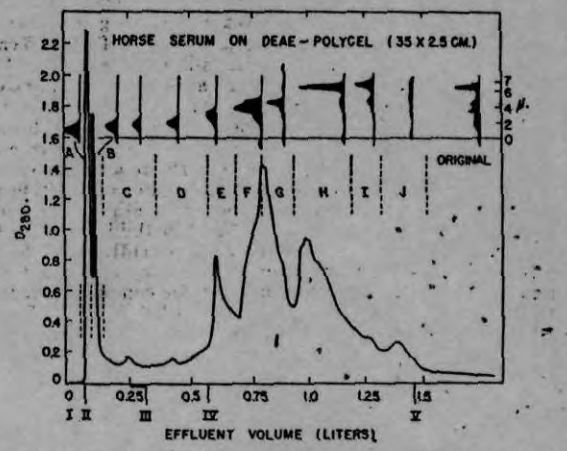

Fig. 2.—Effluent diagram of horse serum with altered elution schedule: 880 mg. dialyzed and lyophilized protein in 6.1 ml. applied to 24 g. of adsorbent; effluent collected in 6–7 ml. fractions. Buffers: I, 0.005 *M* Na phosphate, *p*H 7.0; II, gradient to 0.02 *M* Na phospate, *p*H 6.0; III, gradient to 0.05 *M* NaH_2PO_4; IV, gradient to 0.1 *M* NaCl–0.05 *M* NaH_2PO_4; V, gradient to 0.5 *M* NaCl–0.1 *M* NaH_2PO_4. 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, *p*H 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 comparable[19] 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 *p*H 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]

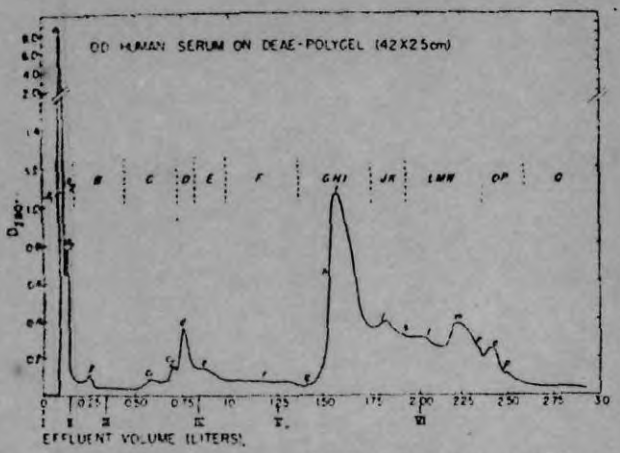

Fig. 3.—Effluent diagram of OD human serum: 970 mg. dialyzed and lyophilized protein (128 mg. N) in 7.5 ml. applied to 29 g. of adsorbent; effluent collected in 6-ml. fractions; flow rate, 15–16 ml./hr. Buffers: I, 0.005 *M* Na phosphate, *p*H 7.0; II, gradient to 0.02 *M* Na phosphate, *p*H 6.0; III, gradient to 0.05 *M* NaH_2PO_4; IV, gradient to 0.02 *M* NaCl–0.05 *M* NaH_2PO_4; V, gradient to 0.05 *M* NaCl–0.05 *M* NaH_2PO_4; VI, gradient to 0.1 *M* NaCl–0.05 *M* NaH_2PO_4. Mixing chamber volume 2.50 ml. See text for explanation of symbols.

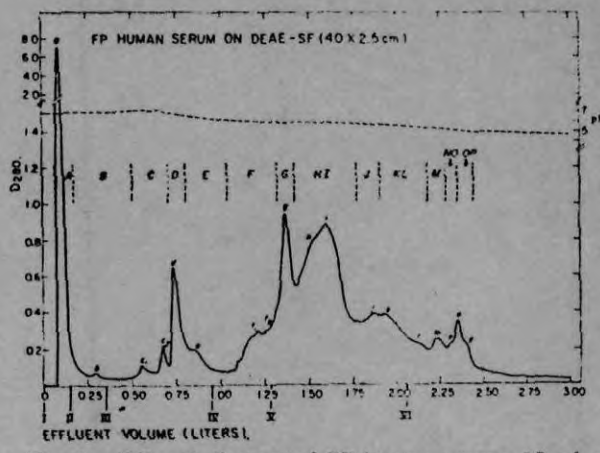

Fig. 4.—Effluent diagram of FP human serum: 20 ml. of dialyzed fresh FP serum (156 mg. N) applied to 28 g. of adsorbent; effluent collected in 6–7 ml. fractions; flow rate, 11 ml./hr. Broken line and scale at right represent *p*H of effluent. Remainder as in Fig. 3.

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 *1*, *8*, 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 ± 12%, which in view of the large number of subfractions collected (as many as 400) and the many manipulations involved, can be considered quantitative.

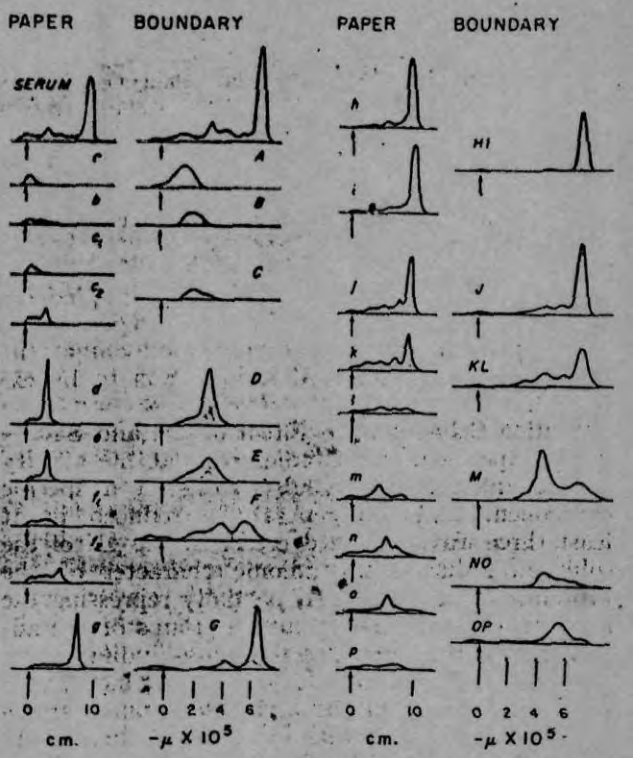

Fig. 5.—Comparison of paper and boundary electrophoresis patterns of chromatographic fractions from Fig. 4. See text for symbols and electrophoretic conditions.

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-

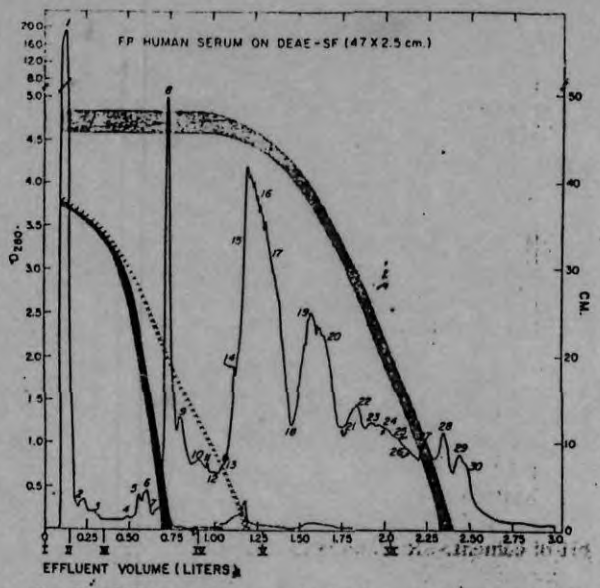

Fig. 6.—Effluent diagram of 5-fold amounts of FP human serum: 5.0 g. of dialyzed and lyophilized protein (775 mg. N) in 39.5 ml. applied to 30 g. of adsorbent; effluent collected in 7-ml. fractions; flow rate, 12 ml./hr. The diagonal, crossed diagonal and stippled areas represent the red, tan and blue 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_{435} mμ, dashed-dotted line, D_{406} mμ. Remainder as in Fig. 3.

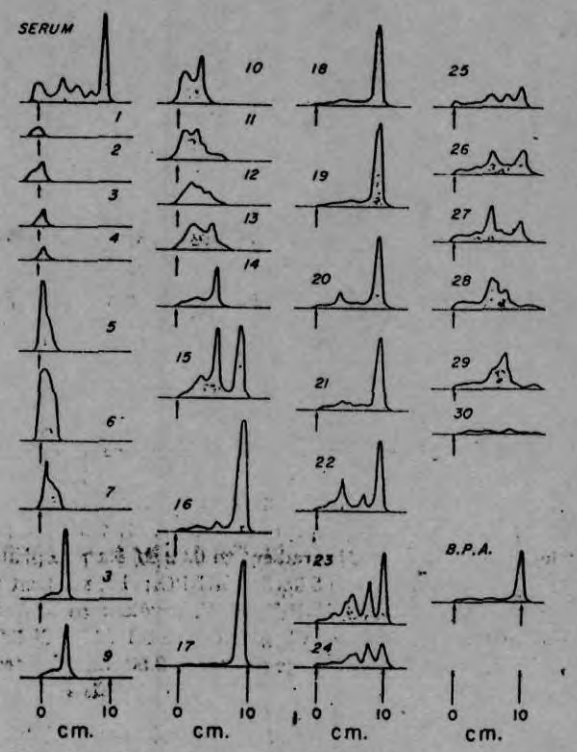

Fig. 7.—Paper electrophoretic patterns of chromatographic fractions from Fig. 6. See text for symbols and electrophoretic conditions.

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 *p*H 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 *p*H of the solution, reducing the anionic character of the protein; (2) a rise in *p*H, partially repressing the ionization of the tertiary amine groups of the adsorbent[8] 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 *p*H 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 *p*H 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 *p*H 7.0, accomplishing elution by the gradual reduction of *p*H 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.

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 *p*H and salt gradients.

As an illustration of the effect of the adsorbent and the protein in modifying the eluting solution, the *p*H of the effluent (broken line) is included in Fig. 4. The *p*H remained at the initial value of 7.0 for about 250 ml. A gradual rise to a maximum of *p*H 7.27 at peak c_2 occurred, followed by a slow decrease. The *p*H was 6.40 at IV, 5.90 at V, and 5.44 at VI. At the end of peak *p*, the *p*H 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 *p*H 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 *1–16* 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 *p*H 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 (μ = −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-globulin[20] have provided fractions which emerged from the anion exchanger with a decreasing *p*H and increasing salt gradient in the order of increasing mobilities. On the other hand, elution from a cation exchanger, CM-cellulose,[1] with a gradient of increasing *p*H 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 *16* 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 (*p*H 7.0–4.8) but unstable at *p*H 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 (μ = −7.7) emerged after albumin (μ = −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 (μ = −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 *p*H 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 differences 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 *1–4* (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

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

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

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 mμ) and the tan color (406 mμ) 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 *p*H 8.6 (Fig. 7), and material with this mobility accounted for 90% of the sample. The solution showed maxima at 278 and 462 mμ.[22] Its properties agreed with those of the β_1-metal-combining protein which was first described by Schade and Caroline[23] and later named siderophilin[24] or transferrin.[25] It was concentrated by Surgenor, *et al.*,[26] and crystallized by Koechlin[27] 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 mμ with a 278/407 ratio of about 10. This colored component resembled in absorption spectrum and electrophoretic mobility the "methemalbumin" prepared by Rosenfeld and Surgenor[28] 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 *p*H 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 electrophoresis (Fig. 7). This fraction showed absorption maxima at 278 and at 602 mμ. The blue color and the absorption at 602 mμ 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 reported[32] 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 localized[34] in fraction *29* (Fig. 7). None was detected in fractions *28* or *30*. Since caeruloplasmin preparations have always shown thrombin activity[35] 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 examination[36] of effluent fractions (Fig. 6) for albumin by the Oudin[37] 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 examined[38] 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

(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 mμ/470 mμ and 470 mμ/408 mμ 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 mμ/462 mμ and 462 mμ/408 mμ, 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. Koechlin and L. E. Strong, *J. Clin. Invest.*, 28, 73 (1949).

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

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

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

(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.

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(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-orosmucoid.

(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 "pervaporation" 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 *p*H 5.0, followed by displacement in a sharp band with a strong eluting agent such as 0.05 *M* NaCl–0.05 *M* NaH_2PO_4.

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 *p*H 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 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 *p*H 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 adsorbents[8] 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-globulin[20] have indicated that immunological properties have been retained and fractionated. Studies with crystalline bovine serum albumin[18] have shown that the sedimentation constant and the electrophoretic mobility at *p*H 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.

BETHESDA, MD.

(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).

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