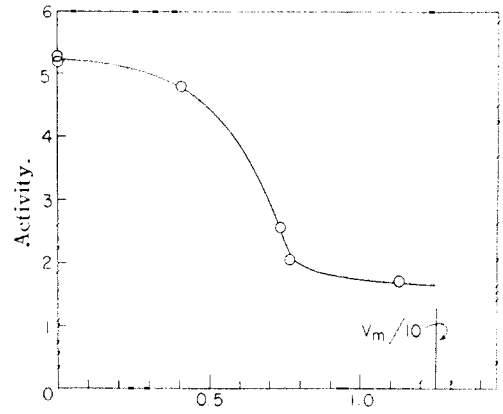

Fig. 2.—Activity as a function of the volume of hydrogen pumped off.

We at first supposed that the data of Fig. 2 could be explained by the diffusion of oxygen and water from the interior of the catalyst. However, the experiment of Fig. 3 shows that this is not so. In the pumping-off experiments the absence of hydrogen during the pumping and the varying temperatures of pumping indeed would allow differing concentrations of oxygen to accumulate on the surface. However in the quenching experiments the same high pressure of hydrogen is always present to remove oxygen. The short temperature range over which the loss of activity is encountered also indicates an activated process other than diffusion.

This second experiment also indicates that the hydrogen actively involved is not in ordinary bulk solution. The endothermic solution of hydrogen in copper would require more hydrogen to be dissolved at high temperature and thus quenching, if anything, would cause more hydrogen to be retained than would slow cooling. The experiments show the opposite to be true.

Conclusion

It appears from these data that hydrogen chemisorbed in the neighborhood of 120° has a marked effect on the activity of a reduced copper catalyst.

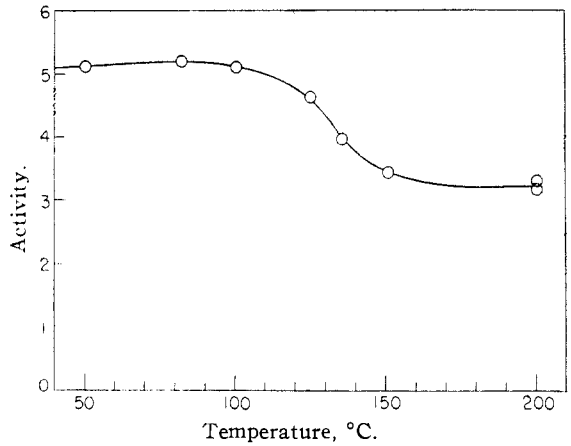

Fig. 3.—Activity as a function of quenching temperature following slow cooling from 200°.

The adsorption of this critical hydrogen does not take place at an appreciable rate at 0°; it is therefore activated adsorption.

Since, once this hydrogen is adsorbed, activity can be maintained by treating the catalyst with hydrogen at 0°, the chemisorbed hydrogen is not removed by reaction; it thus constitutes part of the catalyst, and is not a reactant.

Acknowledgments.—We wish to thank Dr. A. Wheeler for discussing some of the points in this paper with us, and Mr. William Schaeffer of Godfrey L. Cabot, Inc., for determining the surface area of the catalyst.

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Chromatography of Proteins.[1] Frontal Analysis on a Cation Exchange Resin

BY HERBERT A. SOBER, GERSON KEGELES AND FREDERICK J. GUTTER

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Chromatographic frontal analysis on a column of a sulfonic acid cation exchange resin has been applied to a mixture of proteins from an egg white albumin fraction and to artificial mixtures of the purified proteins, bovine plasma albumin and human carbon monoxide hemoglobin. The effluent from the column was examined continuously by refractometric methods. Correct quantitative analyses have been obtained in the *p*H range of 5.6–8.6 when the protein mixtures were equilibrated with 0.01–0.10 ionic strength buffers. Cation exchange is not an essential prerequisite for adsorption and resolution of these protein mixtures although specific cation effects have been observed. Adsorption of protein by this cation exchange resin is small and concentration dependent. The effluent from frontal analysis of these protein mixtures through this cation exchange resin showed no change in charge or weight distribution. Quantitative elution analysis shows no irreversible binding of bovine plasma albumin. It is proposed that homogeneity in chromatographic analysis be considered one of the required criteria of protein purity.

Introduction

Chromatography, a technique of considerable versatility, has had little application to the study of proteins, probably because they are easily denatured and must be handled with very mild techniques. Enzyme chemists, following the lead of Willstätter, however, have been using adsorption and elution in batch processes[2] for some time. Although occasional reports have appeared on the use of chromatography in the purification of crude solutions of proteins,[3] it is only in recent years, since the appearance of the optical methods of Tiselius[4,5] and Claesson,[6,7] the partition chromatography of

(1) Presented in part at the 117th National Meeting of the American Chemical Society, Philadelphia, Penna., April, 1950.

(2) E. Bamann and K. Myrbäck, "Die Methoden der Enzymforschung," G. Thieme, Leipzig, 1940, pp. 1452-1466.

(3) L. Zechmeister, "Progress in Chromatography, 1938-1947," Chapman and Hall, London, 1950.

(4) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(5) A. Tiselius, *Arkiv. Kemi, Mineral., Geol.*, **14B**, No. 22 (1940).

(6) S. Claesson, *ibid.*, **23A**, No. 1 (1946).

(7) S. Claesson, *Ann. N. Y. Acad. Sci.*, **49**, 183 (1948).

Martin and co-workers,[8] and the concept of salting out adsorption,[9] that reports of the analysis and fractionation of proteins have begun to appear in the literature.[3,9-23] Even the advent of the highly selective ion exchange resins[24] did not result in any published reports although their use had been suggested by Tiselius.[12]

In 1949, we reported the analysis of a mixture of proteins from egg white with a cation exchange resin as adsorbent,[15] using the method of frontal analysis.[5-7] Since that time, several other reports of the use of ion exchange resins in protein chromatography have appeared.[17-21]

The present communication contains the result of a systematic investigation into the effect of several variables, namely, *p*H, ionic strength, ion exchange and protein concentration on the resolution of certain protein mixtures, in an attempt to gain some understanding of the fundamental nature of the chromatographic process as it concerns proteins. The protein mixtures used were an egg white "albumin fraction"[25] consisting essentially of ovalbumin, conalbumin and ovomucoid,[26] and various mixtures of bovine plasma albumin (BPA) and human carbon monoxide hemoglobin (COHb).

Experimental

The egg white albumin fraction was prepared from hen's egg white by the addition of an equal volume of saturated ammonium sulfate and the subsequent removal of the precipitate formed.[25,26] Further treatment of this preparation, the arrangement for taking intermittent schlieren scanning photographs,[27] and sampling and electrophoretic identification of the chromatographically resolved fractions have already been described.[15]

Two preparations of bovine plasma albumin (BPA) were used in this study, the first, Armour's crystalline control No. G4802, and the second, Armour's Fraction V, refractionated with ammonium sulfate and then exhaustively dialyzed against distilled water in the cold until a negative Nessler test was obtained. This albumin solution was then lyophilized and the dry white powder was stored in a vacuum desiccator over phosphorus pentoxide in a refrigerator.

The human carbon monoxide hemoglobin (COHb) was fractionated with ammonium sulfate[28] and the ovalbumin was prepared by the method of Sørensen and Høyrup[29] and stored under saturated ammonium sulfate in the cold. Both preparations used had been studied extensively in the ultracentrifuge.[28] For the present study, the COHb was lyophilized and stored under carbon monoxide in the cold.

The buffer anions were phosphate, acetate and veronal. When used with the COHb, the buffers were saturated with carbon monoxide.

The cation exchange resin employed, Dowex 50 (Dow Chemical Co.), sold under the name of Ion-X[30] (200–500 mesh), is a stable sulfonated hydrocarbon with the sulfonic acid residue as the only active exchange group. The resin was conditioned as follows: washed with water and then treated alternately with 4% ammonium hydroxide and 4% sulfuric acid, washing to neutrality with water after each reagent. This cycle, beginning with the ammonium hydroxide wash, was repeated at least twice. In most instances, the resins were given a final treatment with 4% ammonium hydroxide and then washed with boiled, carbon monoxide-saturated water until the effluent was neutral or only weakly positive to Nessler reagent. Unless otherwise stated, the resin was used in the ammonium form. When the resin was used in the sodium or hydrogen form, the final reagent prior to the water-wash was sodium hydroxide or hydrochloric acid, respectively.

The use of the schlieren scanning system[27] for chromatographic analysis[15] permits the application of the frontal analysis method of Tiselius and Claesson,[6,7,31] where each component, since it enters the cell from below, forms a layer of increased total solute concentration, thus forming a density-stable system.

For the determination of the effect of protein concentration on the resolution of protein mixtures and for the construction of adsorption isotherms, it became necessary to measure accurately the volume at which the protein first appears. In addition, because of the slow flow rates used (0.2–2 ml./hr.) to ensure equilibrium conditions on the resin, the experiments frequently required 12–36 hours observation to obtain intermittent records with the arrangement described previously.[15] Furthermore, unless the investigator was in constant attendance, scanning photographs of the protein fronts were usually obtained some time after the first boundary had already entered the optical cell and started to broaden due to diffusion.

Accordingly, an instrument was constructed[32] to record automatically and continuously, as a function of time, the volume percolated through the column, as well as the refractive index at the point of emergence from the adsorption column. The instrument is adaptable interchangeably as an interferometer or prism cell recorder, as well as a continuous refractive index gradient recorder, while simultaneously recording the volume of effluent liquid in each arrangement. When the concentration gradient or prism cell was used with this instrument the protein solution was fed into the adsorption column (1 × 12 cm. containing 4–6 g. of dry resin) from below, as with the earlier arrangement,[15] so as to maintain a density-stable system. Since communicating tubes of small diameter (1 mm.) are used throughout the interferometric adaptation, density-stable systems were not essential.[6,32] Therefore the adsorption column (1 x 25 cm. containing 10 g. of dry resin) for the interferometer arrangement was mounted above the optical cell to remove the limitation on column length imposed by the bath dimensions in our other arrangements. The reservoir and column above the interferometer cell which extended above the water-bath were cooled to bath temperature (0.76°) by thermostat liquid circulated with a water-pump. The

(8) A. J. P. Martin and R. L. M. Synge, "Advances in Protein Chemistry," Vol. 2, Academic Press, Inc., New York, N. Y., 1945; A. J. P. Martin and R. R. Porter, *Biochem. J.*, **49**, 215 (1951).

(9) A. Tiselius, *Arkiv. Kemi, Mineral., Geol.*, **26B**, No. 1 (1948).

(10) I. Moring-Claesson, *Biochim. Biophys. Acta*, **2**, 389 (1948).

(11) V. T. Riley, *Science*, **107**, 573 (1948); *ibid.*, **109**, 361 (1949).

(12) A. Tiselius, *Chem. Eng. News*, **27**, 1041 (1949).

(13) H. K. Mitchell, M. Gordon and F. A. Haskins, *J. Biol. Chem.* **180**, 1071 (1949).

(14) A. E. Franklin and J. H. Quastel, *Science*, **110**, 447 (1949).

(15) H. A. Sober, G. Kegeles and F. J. Gutter, *ibid.*, **110**, 564 (1949).

(16) C. C. Shepard and A. Tiselius, *Discussions Faraday Soc.*, **7**, 275 (1949).

(17) S. Paléus and J. B. Neilands, *Acta Chem. Scand.*, **4**, 1024 (1950).

(18) H. A. Sober and G. Kegeles, Abstracts 117th National Meeting, Amer. Chem. Soc., 33C, April, 1950.

(19) H. A. Sober and G. Kegeles, *Federation Proc.*, **10**, 249 (1951).

(20) C. W. H. Hirs, W. H. Stein and S. Moore, THIS JOURNAL, **73**, 1893 (1951).

(21) H. H. Tallan and W. H. Stein, *ibid.*, **73**, 2976 (1951).

(22) S. M. Swingle and A. Tiselius, *Biochem. J.*, **48**, 171 (1951).

(23) L. Zechmeister and M. Rohdewald, *Enzymologia*, **13**, 388 (1949).

(24) B. A. Adams and E. L. Holmes, *J. Soc. Chem. Ind. (London)*, **54**, 1 (1935).

(25) J. LaRosa, *Chemist-Analyst*, **16**, No. 2, 3 (1927).

(26) L. G. Longsworth, R. K. Cannan and D. A. MacInnes, THIS JOURNAL, **62**, 2580 (1940).

(27) L. G. Longsworth, *ibid.*, **61**, 529 (1939).

(28) G. Kegeles and F. J. Gutter, *ibid.*, **73**, 3770 (1951).

(29) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1917).

(30) Microchemical Specialties Company, Berkeley 3, California. A batch of Dowex 50 obtained directly from the Dow Chemical Company was unsatisfactory, as reported by us in *Science*, **111**, 68 (1950). Subsequently three different shipments of Ion-X were essentially equally active. The probable explanation for the unsatisfactory sample appears to be due to the larger size particles in the unscreened Dowex-50 sample.

(31) A. Tiselius, "Advances in Protein Chemistry," Vol. 3, Academic Press, Inc., New York, N. Y., 1947.

(32) G. Kegeles and H. A. Sober, *Anal. Chem.*, **24**, 654 (1952).

automatic refractometer was employed to obtain most of the data reported in the present study.

The components of the BPA–COHb system were easily identified because of the cherry-red color of the COHb. Using the mercury arc with a red filter and red sensitive plates,[33] the COHb position could also be identified on a properly exposed photographic plate, by a decrease in density which occurred as the COHb front appeared.

For the BPA–COHb system, the mixtures were prepared by weight, dialyzed against the desired solution, and then made up to volume. The evaluation of component concentrations from chromatographic analysis diagrams obtained with the automatic recording refractometer is described in detail elsewhere.[32] By the use of the interferometric arrangement of this instrument, the specific refractive index of crystalline BPA, chromatographically freed of solvent of crystallization, was determined to be 1865×10^{-6} at 5461 Å. The specific refractive index of BPA at 6720 Å. was determined to be 184×10^{-5} and the specific refractive index for COHb was determined to be 192×10^{-5} for the red lines of mercury[33] from our prism cell deflection.

The retention volumes of the individual protein components were always determined from the peak centers of a concentration gradient plot even when the original data was obtained as a direct concentration function in the prism or interferometer cell.[32] Calculation of the corrected retention volume required the accurate determination of the void volume, *i.e.*, the volume of interstitial liquid between the resin particles in the adsorption column. This was accomplished by subtracting the volume of the wet resin from the volume contained in each empty glass column up to the scanned level of the optical cell. The volume of wet resin in each loaded column was calculated from the dry weight of resin, obtained after each run, using an independently determined ratio of wet volume to dry weight of resin. This ratio was determined separately for each form of the resin used, in the following manner. The wet resin was allowed to drain by gravity until no more liquid appeared. This gravity-drained resin then constitutes our operational definition of the wet resin, and the difference between the volume of an empty column and a column packed with resin in this form is taken as the void volume. An aliquot was weighed in a pycnometer and the density, d_2, of the wet resin was determined after filling the pycnometer with light mineral oil of independently determined density. The density, d_1, of the dry resin was determined in a similar manner for resin desiccated overnight at 110°. Assuming that the density of bound water, d_0, is equal to that of pure water at the same temperature, the volume of wet resin per gram of dry resin, V, was calculated from equation (1) and was found for the ammonium form of the resin to be 1.38 ml./g. of dry resin.

$$V = (d_1 - d_0)/d_1(d_2 - d_0) \qquad (1)$$

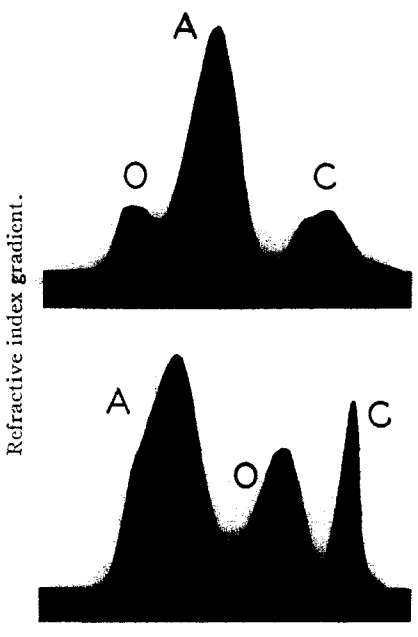

Fig. 1.—Resolution of egg white albumin fraction: O, ovomucoid; A, ovalbumin; C, conalbumin; a, upper diagram, 2.22% protein equilibrated with 0.01 μ *p*H 7.3 buffer; b, lower diagram, 3.46% protein equilibrated with 0.01 μ *p*H 8.4 buffer.

(33) G. Kegeles and F. J. Gutter, THIS JOURNAL, **73**, 3539 (1951).

Results and Discussion

The Egg White Albumin System.—The egg white albumin fraction was initially chosen as a source of an easily prepared reproducible mixture of proteins whose composition was already in the literature.[26] In Fig. 1 are presented two examples of the concentration gradient diagrams obtained of the material emerging from the column during chromatographic frontal analysis. The separation into three major components parallels the electrophoretic results of Longsworth, Cannan and MacInnes.[26] The correlation between the relative proportions of each component obtained by the chromatographic analysis and by electrophoretic analysis has already been discussed.[15]

In an attempt to define the conditions required for protein separation, a study of the effects of *p*H and ionic strength on the resolution of the egg white system was attempted and somewhat erratic results were obtained. The results obtained, however, indicate that resolution of the components of the protein mixture can be obtained if the protein solution is equilibrated with buffers of ionic strength between 0.01–0.1 and in the investigated *p*H range of 6.5 to 8.5.

Occasionally, two minor components were observed in addition to the usual three major components of ovomucoid, ovalbumin and conalbumin. As the study progressed, using the same sample which had been stored over a period of 278 days, it became impossible to achieve the original separation into three components as in Fig. 1. Usually, only two well-defined major components were obtained, one much larger than the other, even though conditions were used presumably identical to those employed in successful experiments. When the first component, the larger, was withdrawn from the cell and subjected to electrophoretic analysis, it was found to consist of both ovomucoid and ovalbumin in the correct relative proportions. However, when the egg white albumin fraction was prepared anew from a fresh batch of egg white, successful analyses were obtained once again. The result of a chromatographic frontal analysis performed with the fresh protein buffered at *p*H 8.4 with 0.01 ionic buffer is shown in Fig. 1b. It should be noted that the order of components is changed. Ovalbumin appears first, followed by ovomucoid and finally by conalbumin. It was at first believed that the shift in the order of retention of the components by the adsorbent was due to a *p*H effect, but additional runs at other *p*H values indicated that the ovalbumin in this second protein preparation was always the least adsorbed. Since the experiments with the second preparation of the egg white albumin fraction were all performed at higher total protein concentrations than the early

runs, it appeared likely that the retention of the several components of this system was quite concentration-dependent. An experimental verification of this conclusion would require precise retention volume data obtained with the individual components of the mixture.

Such a study was not undertaken for this protein system, since the investigation of the effect of *p*H and ionic strength was complicated by the change in the analysis due to the aging of the starting material. Instead, a more reproducible protein system consisting of synthetic mixtures of bovine plasma albumin (BPA) and human carbon monoxide hemoglobin (COHb) was selected for the remainder of this study. This system, the components of which were available in the dry form, permitted precise manipulation of the relative as well as absolute protein concentrations, and allowed a quantitative evaluation of the errors involved in the frontal analysis of proteins.

The Effect of *p*H and Ionic Strength.—The study of the effects of *p*H and ionic strength on the resolution of mixtures of BPA and COHb with the ammonium form of the cation exchange resin indicated that separation of COHb and BPA (0.1–3.0% total protein concentration) could be accomplished with a water-washed resin when the protein was buffered at *p*H values from 4.1–8.5 and when the BPA/COHb ratio was above unity. Lack of resolution at *p*H 7.1 occurred at a BPA/COHb ratio of 0.63 and is an example of the dependence of adsorption on the relative concentration of the protein components to be discussed later. At *p*H 4.1, BPA was separated into two components, as at the other *p*H values, but COHb emerged very gradually over a considerable volume. Variation in ionic strength from 0.01–0.10 at *p*H 7.0 did not affect the separation of these two proteins. If, in addition to buffering of the protein, the resin had also been washed with the same buffer prior to the chromatographic procedure, the *p*H range, 4.1–8.6, over which resolution had been achieved was then reduced to 6.0–7.4.

Ion Exchange.—Since washing the resin with *p*H 8.6 sodium barbital buffer, to achieve an effluent *p*H of 8.3, replaced all the ammonium ion of the resin with sodium ion and since no resolution resulted on passage of sodium barbital-buffered protein through this column, it appeared that cation exchange might be a necessary condition for resolution. This seemed substantiated by a failure to obtain separation of BPA and COHb at *p*H 7.0 when both the protein and the resin contained the same cation, *i.e.*, when ammonium resin and ammonium phosphate buffered protein, or sodium resin and sodium phosphate buffered protein were used. Supporting evidence of the simultaneous occurrence of cation exchange with protein adsorption was obtained in the following manner. Successive fractions were collected during the passage of sodium-buffered protein through ammonium resin at *p*H 7.0 in the automatic recording refractometer, and these samples were analyzed for sodium ion, ammonium ion and total nitrogen. It was found (Fig. 2) that the increase in ammonium ion and in total nitrogen coincided with the appearance

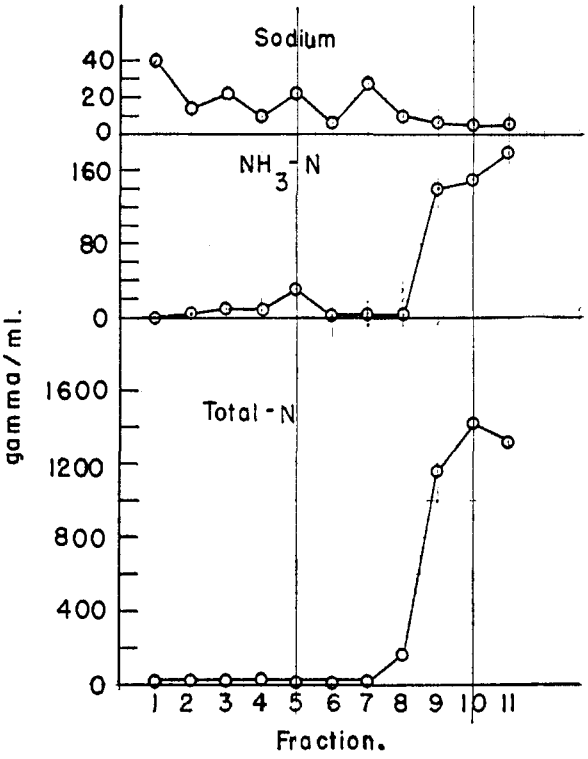

Fig. 2.—Analysis of successive 1.1-ml. fractions of eluate during frontal analysis; 0.85% carbon monoxide hemoglobin equilibrated with 0.01 μ sodium phosphate buffer at *p*H 7.1; 4.99 g. of dry resin in the ammonium form.

of the protein front, without a concomitant rise in sodium ion. This indicated that sodium ion was replacing the ammonium cation of the resin and the protein was emerging with ammonium ion as the gegenion although it had entered the column as sodium proteinate. It should be pointed out that the emergence of the buffer in these experiments, as distinguished from the appearance of the gegenion, is much delayed and does not occur within our diagrams.

In order to obtain data on the cation influence on adsorption, the specific retention volume of BPA was determined in the presence of various cation pairs. The data in Table I indicate that there is a considerable cation influence on the specific retention volume of BPA. In conflict, however, with the thesis that ion exchange is required for protein adsorption, BPA was most strongly adsorbed in the ammonium–ammonium system (last line in Table I), and Fig. 3 shows that resolution of BPA into two components did in fact occur under these conditions. Therefore it must be concluded that although cation exchange may

TABLE I

CATION INFLUENCE ON RETENTION OF BOVINE PLASMA ALBUMIN

BPA, %	Buffer cation	Resin cation	Specific retention volume, ml./g.
0.638	K^+	H^+	0.42
.637	Na^+	H^+	.31
.644	Na^+	NH_4^+	.20
.630	K^+	NH_4^+	.57
.618	NH_4^+	NH_4^+	.66

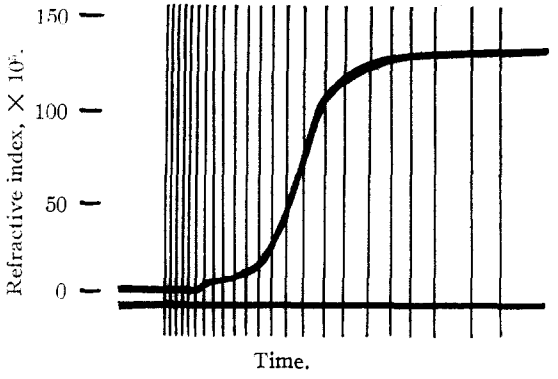

Fig. 3.—Resolution of 0.61% bovine plasma albumin. Both protein and 3.78 g. dry resin in the ammonium form, equilibrated with 0.01 μ *p*H 7.0 ammonium phosphate. First vertical line = 4.22 ml. Succeeding vertical lines represent volume increments of 0.088 ml.

occur during protein adsorption on this resin, this exchange is not an essential prerequisite to either adsorption or protein resolution.

In addition to the effect of different cations on retention by the resin, listed in Table I, a striking example of a specific cation effect on a particular protein is shown in Fig. 4. These figures are direct concentration *vs.* time recordings obtained with the prism cell adaptation of the automatic refractometer[32] with the vertical lines representing volume increments of 0.176 ml. The two diagrams represent frontal analyses with an ammonium resin column of mixtures of BPA and COHb differing only in that for the upper diagram the protein mixture was equilibrated with *p*H 7.0, 0.01 ionic strength *potassium* phosphate buffer and for the lower diagram, with *p*H 7.0, 0.01 ionic strength *sodium* phosphate buffer. The first two concentration steps in the diagrams are due to BPA and the third step is due to COHb. The COHb front in the lower diagram obtained with sodium as the protein gegenion is a normally sharp step. On the other hand, when the protein gegenion was potassium, in the upper diagram, the concentration step is not at all well defined and occurs over a relatively large volume, indicating very tight binding of this protein under these conditions. Thus individual cations apparently may modify the adsorption characteristics of specific proteins.

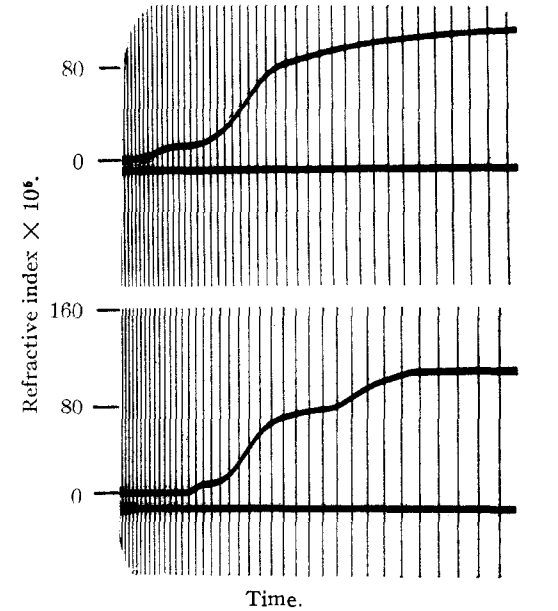

Fig. 4.—Effect of cations on the resolution of 0.40% bovine plasma albumin and 0.16% carbon monoxide hemoglobin. Upper-protein equilibrated with 0.01 μ, *p*H 7.0 *potassium* phosphate buffer, lower-protein equilibrated with 0.01 μ *p*H 7.0 *sodium* phosphate buffer; 4.30 and 4.24 g. dry resin in the ammonium form used. Vertical lines represent volume increments of 0.18 ml.

Accuracy of the Method.—Claesson[6] has discussed and indicated a general method for evaluating the error involved in frontal analysis due to displacement of the more weakly adsorbed component by the following component. In evaluating the magnitude of the displacement error with the present system, *i.e.*, BPA and COHb, the two components of BPA were considered as a single one, since experimentally it was difficult to be certain of the amount of small BPA component present in any given experiment. Reference to Table II, where both the known concentration of the two components, BPA and COHb, and that found as a result of frontal analysis on the cation exchange resin are compared, indicates that when sufficient resolution was achieved for analysis, uniformly good recoveries were obtained with no consistent deviation in either direction. Therefore, a systematic error due to mutual displacement need not be considered with the protein system investigated. Furthermore, the adsorption of a resolved component in these mixtures can be determined directly from the corrected retention volume and the observed concentration increment at the component front.

The Role of Protein Concentration.—Compared to the influence of the absolute and relative concentration of the components of the protein mixture to be studied, the effects of *p*H and ionic strength are of relatively small importance. The latter quantities may be varied considerably, maintaining a given protein concentration, without materially affecting resolution. On the other hand, even under optimal conditions of *p*H and ionic strength, resolution of these proteins may not occur under certain concentration conditions. The data and calculations presented in Table III are used for the construction of adsorption isotherms (Fig. 5) from which these facts can be deduced. These data were obtained from a series of frontal analyses at 0.76° at different concentrations of BPA and COHb equilibrated at *p*H 7.1 with 0.01 ionic strength sodium phosphate. The retention volume of each component may be represented by the slope of a line drawn from the origin to a point on the curve at the concentration selected.[34] Thus, since mutual displacement does not take place, it can be predicted that solutions containing ratios of BPA/COHb greater than or approximately equal to unity, will be resolved, but that solutions with ratios of BPA/COHb below one will probably not be resolved. This is the explanation for the lack of resolution mentioned

(34) A. Tiselius, *Arkiv Kemi, Mineral., Geol.*, **16A**, No. 18 (1943).

TABLE II

CHROMATOGRAPHIC ANALYSIS OF SYNTHETIC MIXTURES OF PROTEINS[a]

	BPA, %	CoHb, %	Total, %
Known	0.080	0.54	0.62
Found	No Resolution		.66
Known	.29	.32	.61
Found	No Resolution		.69
Known	.33	.22	.55
Found	.34	.21	.55
Known	.36	.10	.46
Found	.39	.11	.50
Known	.37	.16	.53
Found	.41	.18	.59
Known	.60	.11	.71
Found	.64	.12	.76
Known	1.12	.40	1.52
Found[b]	1.01	.50	1.51
Known	1.51	.32	1.82
Found[b]	1.49	.31	1.80
Known	1.89	.30	2.19
Found[b]	1.95	.32	2.27

[a] These analyses were performed at 0.76° with the ammonium form of the resin washed with distilled water. All protein solutions were equilibrated with 0.01 ionic strength *p*H 7.1 sodium phosphate buffer. [b] These analyses were performed in the continuous refractive index gradient adaptation. The remaining analyses were done in the prism cell arrangement.

before in the *p*H studies and that shown in Table II at *p*H 7.1 and 0.01 ionic strength, conditions under which different relative proportions of these two components were resolved. The concentration

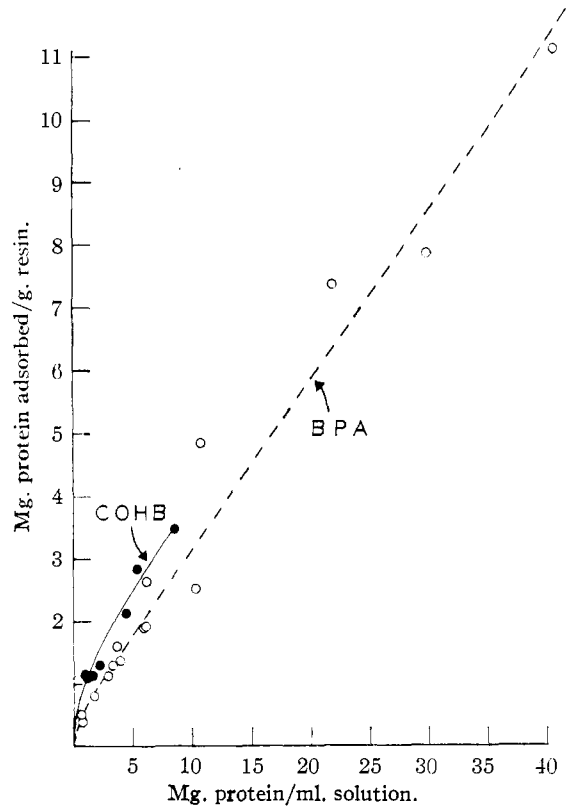

Fig. 5.—Adsorption isotherms at 0.76° and *p*H 7.1 of bovine plasma albumin (major component) and carbon monoxide hemoglobin. Resin in ammonium form and proteins equilibrated with 0.01 μ sodium phosphate buffer.

TABLE III

DATA AND CALCULATIONS FOR THE ADSORPTION ISOTHERMS

A Protein concn., mg./ml.	*B* Dry resin weight, g.	*C* Wet resin volume ($B \times 1.38$),[a] ml.	*D* Empty column volume, ml.	*E* Void volume g. dry resin ($(D - C)/B$), ml./g.	*F* Obsd. retention volume,[b] ml.	*G* Obsd. specific retention volume (F/B), ml./g.	*H* Cor. specific retention volume ($G - E$), ml./g.	*I* Mg. protein adsorbed g. dry resin (ΔH), mg./g.
Bovine plasma albumin (major component)								
0.62	4.17	5.75	8.28	0.607	5.94	1.426	0.819	0.51
0.74	4.32	5.98	8.28	.532	4.58	1.060	.528	0.39
1.66	3.84	5.31	8.28	.774	4,83	1.259	.485	0.81
2.70	4.16	5.76	8.28	.605	4.28	1.028	.423	1.14
3.09	4.22	5.85	8.28	.575	4.22	1.000	.425	1.31
3.37	4.69	6.49	8.28	.382	4.05	0.864	.482	1.62
3.66	4.16	5.76	8.28	.605	4.09	.983	.377	1.38
5.56	4.16	5.76	8.28	.606	3.96	.952	.346	1.92
5.67	3.70	5.12	8.28	.854	4.88	1.319	.465	2.64
5.71	4.63	6.40	8.98	.558	4.14	0.895	.337	1.92
9.55	5.08	7.04	9.75	.539	4.09	.804	.265	2.53
9.93	5.13	7.09	9.61	.491	5.03	.981	.490	4.87
20.17	4.88	6.75	9.61	.587	4.65	.954	.367	7.40
27.45	4.97	6.88	9.75	.577	4.30	.865	.288	7.91
37.29	5.07	7.01	9.75	.547	4.30	.849	.302	11.26
Carbon monoxide hemoglobin								
0.93	4.69	6.49	8.28	.382	7.57	1.615	1.233	1.15
1.09	4.16	5.76	8.28	.606	6.69	1.608	1.002	1.10
1.62	4.24	5.86	8.28	.571	5.37	1.268	0.697	1.13
2.18	4.22	5.85	8.28	.575	4.97	1.177	.602	1.31
3.21	4.16	5.76	8.28	.606	4.28	1.028	.423	1.36
4.46	4.67	6.47	8.98	.537	4.75	1.016	.479	2.14
5.38	4.32	5.98	8.28	.532	4.58	1.060	.528	2.84
8.46	4.99	6.91	9.75	.557	4.84	0.970	.413	3.49

[a] See text above for the determination of the factor 1.38 (V). [b] The observed retention volume was interpolated from the volume record at the peak centers of a concentration gradient plot.

effect may also be considered the probable explanation for the change of order in the components of the egg white system shown in Fig. 1. Resolution of BPA into two components is again dependent on concentration. The small component of BPA (Figs. 3, 4) amounting to 7–10% of the total, is retained less by the adsorption column than the major component when run at or below 1% total protein concentration. On the other hand, above approximately 2.5% total concentration, the small component is the more strongly adsorbed while at intermediate concentrations only one component can be recognized. The small BPA component remains to be studied, but it may be analogous to the heavy component seen in the ultracentrifuge,[28] and in light scattering measurements.[35] This heavy component apparently increases with aging.[35]

From the lower portion of the isotherm curves, Fig. 5, it might be predicted that resolution of BPA and COHb would be possible, regardless of their relative concentrations if the sample were diluted to a sufficiently low concentration.

Capacity of Adsorbent.—The adsorption of these two proteins from one per cent. solutions by the resin is small (3–4 mg./g. resin) when compared to the amino acids. For example, at the 1% level the capacity of the resin for glycine is considerably more than three times that for protein. On the other hand, sucrose at 1% is weakly adsorbed (3.3 mg./g. resin). Swingle and Tiselius[22] reported that bovine plasma albumin (0.05%) is adsorbed by hydrated calcium phosphate at *p*H 5 to the extent of about 310 mg./g. while silica gel showed an adsorption of 110 mg./g. At the same level of protein concentration, 0.06%, BPA is adsorbed to the extent of 0.5 mg./g. under the conditions described here. In addition to the low order of adsorption exhibited, the differences in adsorption between the two proteins are small enough (BPA 3.0 mg./g. and COHb 4.0 mg./g.) at the 1% protein level and with a 6–10-g. column to require the high resolving power of the optical system used. Non-recording schlieren optical systems can be used, but do not provide records of satisfactory sharpness. Collection of fractions and adsorption measurements at 268 mμ would require either a continuous arrangement or fractions of exceedingly small volume (0.1 ml.). With the interferometric adaptation of the automatic refractometer,[32] protein concentrations of the order of 0.5–15 mg./ml. can be measured to an absolute accuracy of 0.001% in a 2.5-cm. cell and therefore chromatographic frontal analyses can be successfully performed on small amounts of protein.

The Effect on the Protein of Passage through the Resin.—To investigate the possible effect of the resin on the protein as a result of passage through the column during frontal analysis, electrophoretic and ultracentrifugal analyses were performed on material before it entered the adsorbent column and on effluent material collected after no further chromatographic resolution was indicated by the optical system. The quantitative agreement between the electrophoretic analyses of the egg white albumin fraction and its effluent from the ion-exchange column has already been reported.[15] Passage of five times recrystallized ovalbumin (a notoriously sensitive protein) through the adsorbent column also resulted in no change in the ultracentrifuge analysis. At 1.87%, the sedimentation constant of the effluent protein was 3.24 Svedberg units, as compared with a value of 3.16 Svedberg units interpolated from the data[28] for a four times recrystallized preparation. Preliminary experiments on the elution analysis of known amounts of BPA (3.45 mg.) resulted in the recovery, as determined from the interferometric diagram,[32] of all of the BPA (3.53 mg.). The unchanged sedimentation constant of this eluted material of 4.34 Svedberg units at 0.30% concentration as compared with a value of 4.28 Svedberg units interpolated from the data of Kegeles and Gutter[28] indicate that irreversible adsorption on the resin does not take place with *this* protein. The use of another cation exchange resin with several enzymes[17,20,21] and the recovery of biological activity, further substantiates the thesis that the chromatographic study of proteins with ion exchange resin is feasible and offers great promise.

From our results, it may be concluded that fractionation and analysis of proteins on this cation exchange resin appears practical and the possibility of application to other protein systems must be investigated. Moreover, still another criterion of the homogeneity of proteins now exists, since resolution of BPA into two components was achieved over a wide range of conditions, and a change in the egg white albumin fraction on aging was detected by chromatographic analysis which was not observed by electrophoretic analysis. It is proposed that homogeneity in chromatographic analysis be considered one of the required criteria of protein purity.

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(35) M. Halwer, G. C. Nutting and B. A. Brice, THIS JOURNAL, **73**, 2786 (1951).