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Separation of a Multicomponent Mixture of Proteins by Potential Barrier Chromatography (PBC)

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Abstract

Potential barrier chromatography (PBC) is a high performance liquid chromatographic method which uses an isocratic elution procedure and exploits the high sensitivity of the interaction potentials (between the proteins and the packing) to small differences in their physicochemical properties (isoelectric point, hydrophobicity, molecular size, etc.). The interaction potential is a result of the van der Waals attraction and double-layer, Born, and hydration repulsions. While a sufficiently strong van der Waals attraction has the tendency to generate a deep adsorption energy well, the double-layer repulsion prevents the occurrence of such a deep well; however, the latter repulsion together with the van der Waals attraction also generates a potential barrier to adsorption. Separation is feasible when the above-mentioned interaction potentials between adsorbent and adsorbates produce surmountable energy barriers to adsorption as well as moderately deep adsorption energy wells. The interaction potentials can be controlled by changing the pH and ionic strength of the mobile phase, thus affecting the double-layer repulsions, and by adding a small amount of organic solvent to the mobile phase, thus altering the van der Waals attraction. A separation can be achieved by properly tuning the mobile phase conditions, such as the pH and the ionic strength and/or the organic content of the mobile phase, to produce sufficiently different, but moderate, depths of adsorption energy wells for the individual adsorbates. In the present work an anion-exchange adsorbent and four model proteins (γ-globulin, albumin, bovine serum albumin, and ovalbumin) are employed. The optimum conditions for the best resolution of the four model proteins are identified as pH = 4.35 and no additional electrolytes. As a result of

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double-layer repulsion, the adsorption energy well is not too deep and hence, the processing time is short. Second, the effects of the amount of the protein mixture injected in a single pulse and that of a succession of pulses at short time intervals on the resolution of separation are investigated.

INTRODUCTION

Potential barrier chromatography (PBC) is a liquid chromatographic method based on the physical interactions between the adsorbent and adsorbates (*1*). Its applicability to protein separation, by using a high performance liquid chromatograph, has recently been demonstrated (*2, 3*) by separating two model proteins (ovalbumin and bovine serum albumin). The method was also applied to separate components, such as the immunoglobulins, from a naturally occurring mixture (*4*). PBC employs an isocratic elution procedure and, in general, an inexpensive ion-exchange column, and exploits the possibility to control the interaction potentials between proteins and packing by changing the pH, ionic strength, and/or the organic content of the eluent. Initially, the physical basis of the method is briefly summarized by emphasizing that this kind of chromatographic separation is a result of an adsorption-desorption process governed by physical interactions (such as van der Waals attraction, and double-layer and Born repulsion) between adsorbates and adsorbent. While sufficiently strong van der Waals attractions tend to generate a deep adsorption energy well, the double-layer repulsion moderates the depth of this well.

Subsequently, experiments illustrating the separation of four model proteins are presented. The proteins used were γ-globulin (γ-G), albumin (A), bovine serum albumin (BSA), and ovalbumin (OV). Their physicochemical properties are presented in Table 1. In contrast to the preceding experiments (*2–4*), in which strong cation-exchange adsorbents were employed, a strong anion-exchange adsorbent was used in the present study. Since the adsorbent is positively charged (over the range of pH 2 to 6.5), the isoelectric points of the proteins must be greater than the pH of the mobile phase to achieve the repulsive double-layer interactions necessary to moderate the depth of the adsorption energy well.

The optimum conditions for the best resolution in the separation of the four model proteins were identified. Subsequently, the effects of the amount of proteins injected in a single pulse and that of the periodically injected pulses at short time intervals on the resolution of separation are examined.

TABLE 1
Isoelectric Points of the Proteins

Proteins	Isoelectric points
γ-Globulin	5.8–7.8
Albumin	~4.9
Bovine serum albumin	~4.8
Ovalbumin	~4.6

PHYSICAL INTERACTIONS BETWEEN PROTEINS AND PACKING

Most solid surfaces in contact with aqueous media become electrically charged by one or more possible mechanisms (*5*). Proteins and ion-exchange adsorbents acquire charges through surface-group ionization.

An electrically charged surface attracts ions of opposite charge (counterions) to its vicinity from the bulk liquid. However, opposing this effect, counterions have the tendency to drift away into the bulk liquid due to Brownian motion. As a result of these opposing effects, counterions form a cloudlike distribution near the charged surface. The charged surface together with the diffuse layer of counterions constitutes the electrical double layer. When two entities bearing similar electrical double layers approach each other, the diffuse layers begin to overlap, and the alterations in the distribution of counterions lead, in general, to an increase in the free energy of the system which manifests itself in double-layer repulsion (*6–8*).

Another important interaction is the van der Waals interaction, which is composed of (a) the orientation or Keesom interaction between permanent dipoles, (b) the induction or Debye interaction between the permanent dipole of a molecule and the induced dipole caused by it in another molecule, and (c) the dispersion or London interaction (which is generally the most important among the three) that arises from the existence of instantaneous dipole moments in each atom of any material due to the continuous motion of electrons. Such an instantaneous dipole moment generates an electric field which polarizes another atom, inducing a dipole moment in it. The interaction between these two dipoles generates the dispersion interaction (*5–9*).

Other interactions involved in PBC are the Born and hydration repulsions, which are short range repulsions arising from the overlap of the electron orbitals and/or the difficulty in removing the water molecules from the interfacial region (*5*), respectively.

The sum of all the above-mentioned interaction potentials between

adsorbent and adsorbates constitutes the total interaction potential and, as illustrated in Figs. 1, can have various shapes (*1*). In Fig. 1a the total interaction potential has two minima separated by a maximum. The maximum (the potential barrier) is a result of the combination of double-layer repulsion with van der Waals attraction. The primary minimum (which possesses a finite depth because of the existence of Born and hydration repulsions) constitutes the adsorption energy well which is essentially responsible for the chromatographic behavior of a particular adsorbate. The depth of the energy well and the height of the potential barrier are different for each of the protein species, due to the differences in their physicochemical properties and to the high sensitivity of the interaction potentials to small changes in the adsorbate size and charge (*1*). Thus, adsorbates which have a total interaction potential as shown in Fig. 1b with a deep energy well will reside in the column for a long period of time; on the other hand, proteins which bear the total interaction potential illustrated in Fig. 1c, with a shallow energy well, will elute early near the void volume. The former case arises when the double-layer

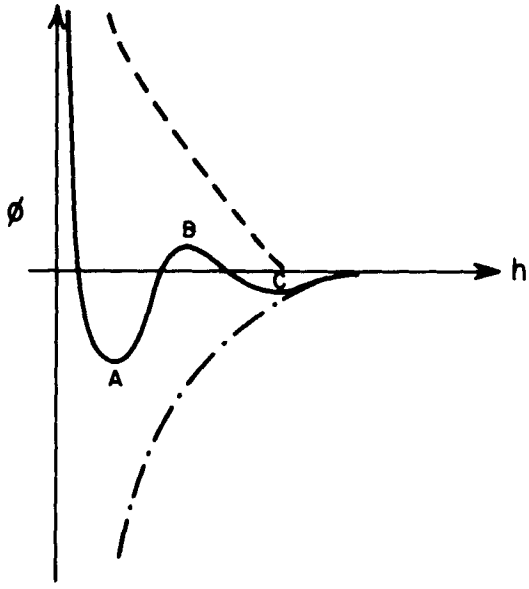

FIG. 1a. The standard shape of the total interaction potential in PBC. (- -) Double-layer repulsion. (– ·) Van der Waals attraction. A: Energy well (primary minimum). B: Potential barrier. C: Secondary minimum. *h*: Minimum distance between adsorbent and adsorbate (assumed spherical for the sake of simplicity).

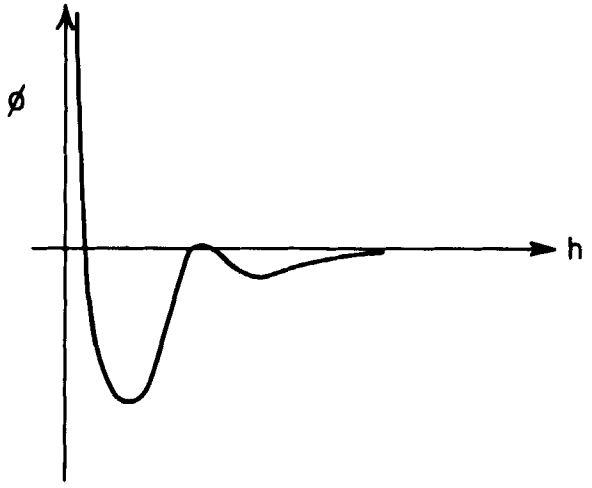

FIG. 1b. The total interaction potential in the case of weak double-layer repulsion.

repulsion is weak, due, for instance, to weak surface charges of the adsorbates, and when, in addition, the van der Waals attractions between the adsorbates and adsorbent are sufficiently strong. This may occur when the pH of the mobile phase is near the isoelectric point of the protein. The latter case (Fig. 1c) occurs when the double-layer repulsion is sufficiently strong compared to the van der Waals attraction. This can

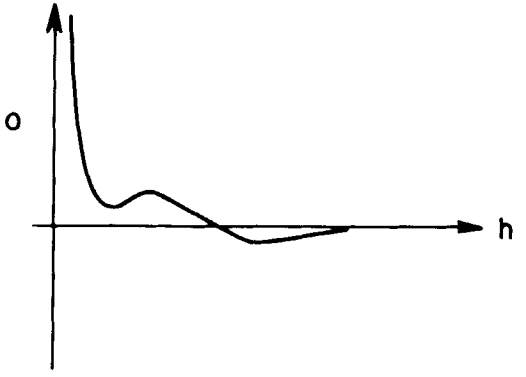

FIG. 1c. The total interaction potential in the case of strong double-layer repulsion.

happen when the pH is far enough from the isoelectric points, leading to sufficiently large electric charges of the proteins (of course, the packing must also have the same kind of charge as the protein does, to ensure repulsive double-layer forces).

Thus, the retention times of the adsorbates can be controlled by changing the pH of the mobile phase. As the pH of the mobile phase gets closer to the isoelectric point of the protein, its retention time increases because the adsorption energy well becomes deeper. However, the pH should be kept lower (higher) than the pIs (the isoelectric points) when the adsorbent is positively (negatively) charged to accomplish the requirement of repulsive double layer (the same kind of charge for both the adsorbent and adsorbates). However, if the isoelectric points of some proteins are too close to the pH of the mobile phase and their van der Waals interactions with the adsorbent are too strong, they can be almost irreversibly adsorbed. Thus, their retention times become exceedingly high. On the other hand, if the differences between the pH and the isoelectric points is too large, the repulsive double layer can become very strong for several proteins and their total interaction potential could be similar to that in Fig. 1c, resulting in their elution near the void volume without any separation.

Thus, there exists an optimum pH at which the separation of the components involved can be accomplished in a reasonable length of time. Similar considerations apply also to the ionic strength of the mobile phase. As the ionic strength of the mobile phase increases, the surface charges of the adsorbent and adsorbates are increasingly screened. This leads to a weaker double-layer repulsion and hence to a deeper adsorption energy well as well as a lower potential barrier to adsorption. Consequently, an increase in the ionic strength increases the retention times of the adsorbates, since it increases the depth of their adsorption energy wells. As in the case of pH, there is an optimum ionic strength at which the proteins involved can be separated in a reasonably short length of time.

Note that, in contrast to ion-exchange chromatography, the retention times of the proteins are enhanced in PBC by increasing the ionic strength and by decreasing the ΔpH (pI $-$ pH of the mobile phase when the surface charges are positive and pH $-$ pI when the surfaces are negatively charged). Additionally, an organic solvent can be employed to alter the van der Waals interactions. However, the present adsorbent does not allow the use of organics and moreover its effect on the resolution is not always very significant (*4*). Further details regarding the conditions of the mobile phase can be found elsewhere (*2–4*).

Thus, the retention times of the proteins can be controlled by changing the pH and the ionic strength of the mobile phase. By properly tuning the conditions of the mobile phase, separation with good resolution can be accomplished in a relatively short processing time. While the van der Waals attractive interaction increases the depth of the adsorption energy well of the total interaction potential, the double-layer repulsion moderates this effect by raising the energy well. However, if the double-layer repulsion is too strong, it can completely impede adsorption.

EXPERIMENT

The experiments were performed with a solvent delivery system (Waters Model 6000A), a nonstop-flow septumless injector (Waters U6K), a UV absorbance detector (Waters UV440), a peak timer and area integrator (Varian CDS 111), and a strip chart recorder (Houston instrument Omniscribe). The column used in these experiments was a Dupont Zipax SAX (2.1 mm i.d. × 100 cm length) containing a strong anion-exchanger adsorbent.

The adsorbent consists of an impermeable glass core onto which quaternary amine polymer-coated silica microbeads are attached, as shown in Fig. 2. This adsorbent allows only limited contact with organic solvents.

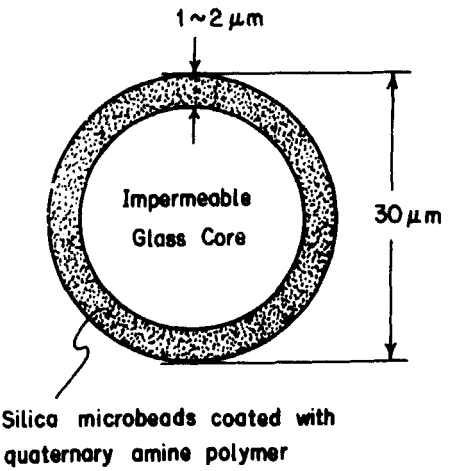

FIG. 2. Schematic of the pellicular Dupont Zipax SAX adsorbent.

The pH of the mobile phase was controlled by titration with solutions of 0.01 *M* citric acid and 0.01 *M* sodium citrate. The total concentration of the buffer in the mobile phase was 0.001 *M*. A digital pH meter (Orion Research 601A) was used for titration.

The mobile phases were prepared fresh daily using demineralized distilled water and analytical grade chemicals, which are degassed subsequently by sonication for 30 min. Mobile phases were pumped through the column at a flow rate of 0.5 mL/min. The column was equilibrated for 2 h before the injections of the model proteins were made.

Proteins were obtained from Sigma Chemical Company and the potassium sulfate (Gold label grade) was obtained from Aldrich Chemical Company. The protein solutions were prepared with the mobile phase for each experiment at a concentration which was 0.5 mg/mL if not otherwise mentioned. They were also sonicated for 1 min to remove air bubbles. The effluent was monitored at a wavelength of 280 nm.

RESULTS AND DISCUSSION

As shown in Table 2, the initial experiment was carried out at a pH of 3.85 in the mobile phase and without any additional electrolyte. At this low pH, the retention times of the four peaks obtained from the independent 20 μL injections of each of the species involved were almost the same, and they were eluted immediately after the void volume peak (Fig. 3a, b, c, d). This is a result of the strong double-layer repulsions experienced by all the adsorbates. Consequently, no separation occurred when the mixture of all four components was injected (Fig. 3e). As the pH of the mobile phase was increased to 4.0, still without additional electrolyte, the retention times of A (albumin), BSA (bovine serum

TABLE 2
Operating Conditions in the Experiments

pH 3.85
pH 4.00
pH 4.20
pH 4.25
pH 4.35 (0.0 *M* K_2SO_4) → (0.001 *M* K_2SO_4) → (0.01 *M* K_2SO_4)
pH 4.45
pH 4.50 (0.01 *M* K_2SO_4)
pH 4.80 (0.01 *M* K_2SO_4)

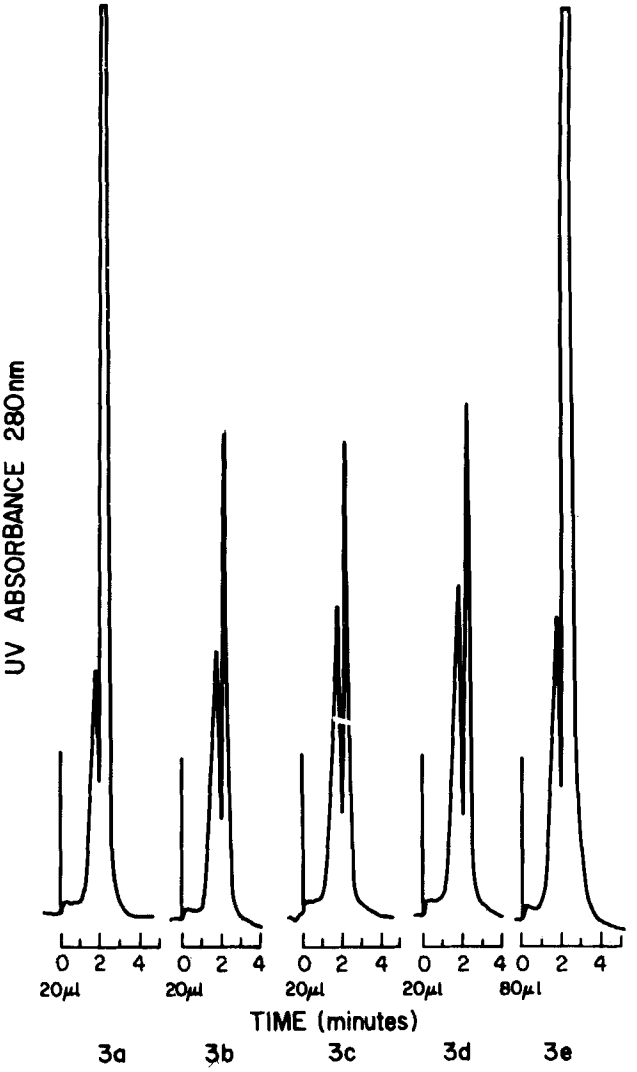

FIG. 3. (a) 20 μL of γ-globulin injected; pH 3.85, no additional electrolyte; wavelength of the detector 280 nm; sensitivity 0.05 AUFS; mobile phase flow rate 0.5 mL/min. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-globulin, albumin, BSA, and OV injected (20 μL of each); as above.

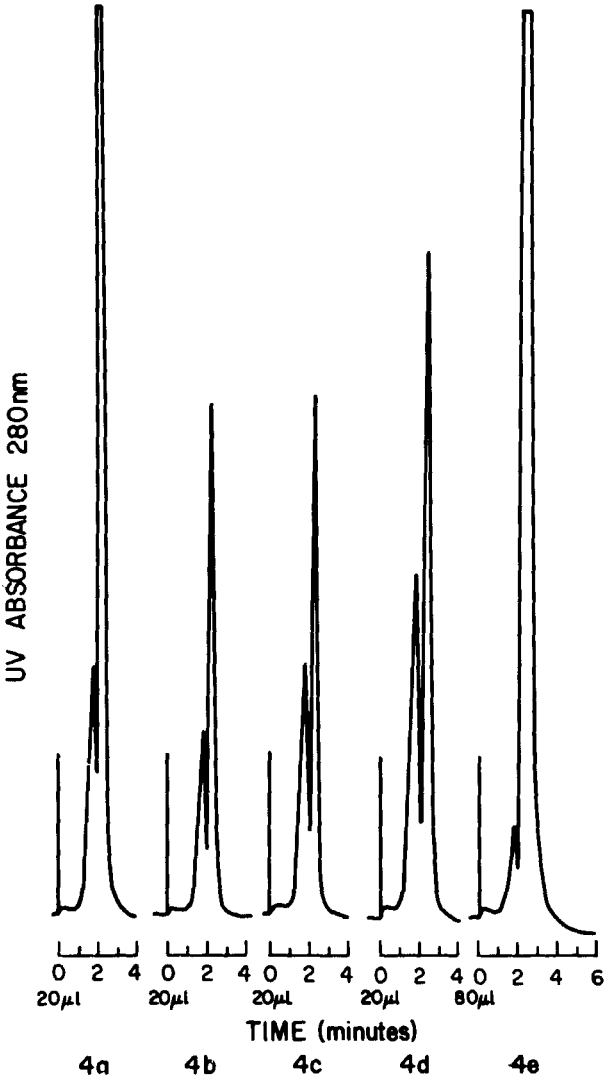

FIG. 4. (a) 20 μL of γ-G injected; pH 4.0; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

albumin), and OV (ovalbumin) have increased marginally (Fig. 4b, c, d), whereas that of γ-G (γ-globulin) remained unchanged. At a pH of 4.2, remarkable changes in the retention times of the A, BSA, and OV, however, took place. For example, the retention times of the A and BSA, which eluted almost simultaneously with γ-G at pH = 4.0 when injected together, have increased to 3.6 and 4.7 min, respectively (Fig. 5b, c). Note that OV has eluted into two peaks (Fig. 5d); a small part of the OV was eluted at about 3 min, while a larger part was eluted the last among the four proteins, between 6 and 7 min. The isoelectric points of the proteins are given in Table 1. However, due to the multiplicity of components constituting each protein, their isoelectric points cover in reality a range of values, especially for the γ-G. The values of the isoelectric points in the range valid for γ-G are greater than those of the other proteins, and hence the difference between the pI of this protein and the pH of the mobile phase is also the largest, leading to high surface charges (i.e., stronger double layers). For this reason, γ-G is eluted first, in a sharp peak, immediately after the peak of the void volume. Albumin is eluted second, because of its second highest isoelectric point. BSA eluted third and OV eluted last. Hence, as the pH of the mobile phase is raised, thus reducing the charge and hence the double-layer repulsion, the somewhat lower isoelectric point (i.e., weaker double-layer interaction) for the BSA results in a greater retention time for the latter than for albumin. Note that the hydrophobicities of the latter two proteins should be comparable for this to occur.

A large portion of the OV eluted last among the four (see Table 3), because of its lowest isoelectric point (i.e., weakest double-layer repulsion), whereas a small portion eluted slightly earlier than albumin. OV consists of three components (*11, 12*), and, as indicated by electrophoresis results (*12*), exhibits charge heterogeneity. Although all the proteins have such heterogeneities, their effect on the retention times will manifest only if the pH of the mobile phase is close to the range of the isoelectric points of the protein. Thus the charge heterogeneity causes a small portion of OV to elute earlier near albumin. A small increase in the pH of the mobile phase, from 4.2 to 4.25 (Fig. 6), also resulted in the increase in the retention times of A, BSA, and OV. However, the peaks of the four proteins still overlap (Fig. 6e) when the proteins are injected together. As shown in Fig. 7, a further increase in pH to 4.35 greatly increased the retention times of A, BSA, and OV (Fig. 7b, c, d), especially that of OV, whereas that of γ-G remained almost unchanged (Fig. 7a). This resulted in almost complete separation of the four proteins (Fig. 7e). Also note that the separation of the four proteins was completed within 15 min by means of the isocratic elution procedure. The peak of OV has broadened

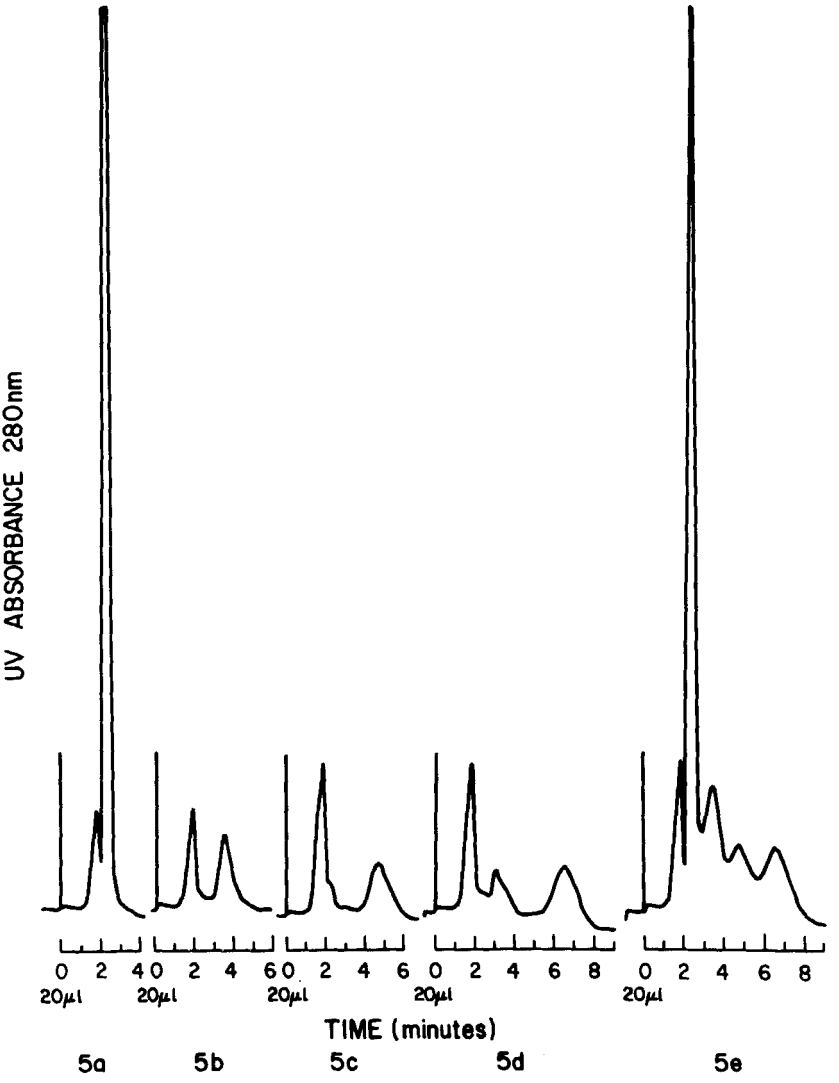

FIG. 5. (a) 20 μL of γ-G injected; pH 4.2; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

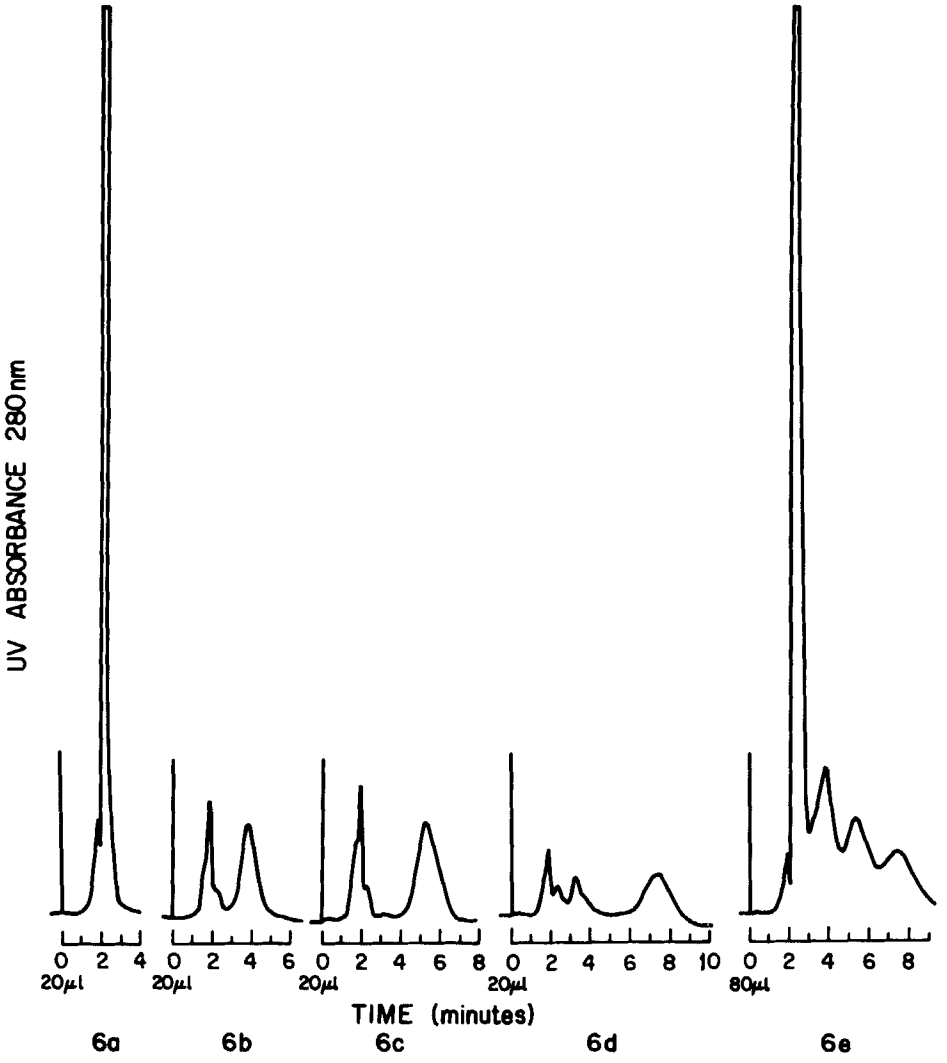

FIG. 6. (a) 20 μL of γ-G injected; pH 4.25; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

TABLE 3
The Order of Elution

(1) γ-Globulin
(2) Albumin
(3) Bovine serum albumin
(4) Ovalbumin

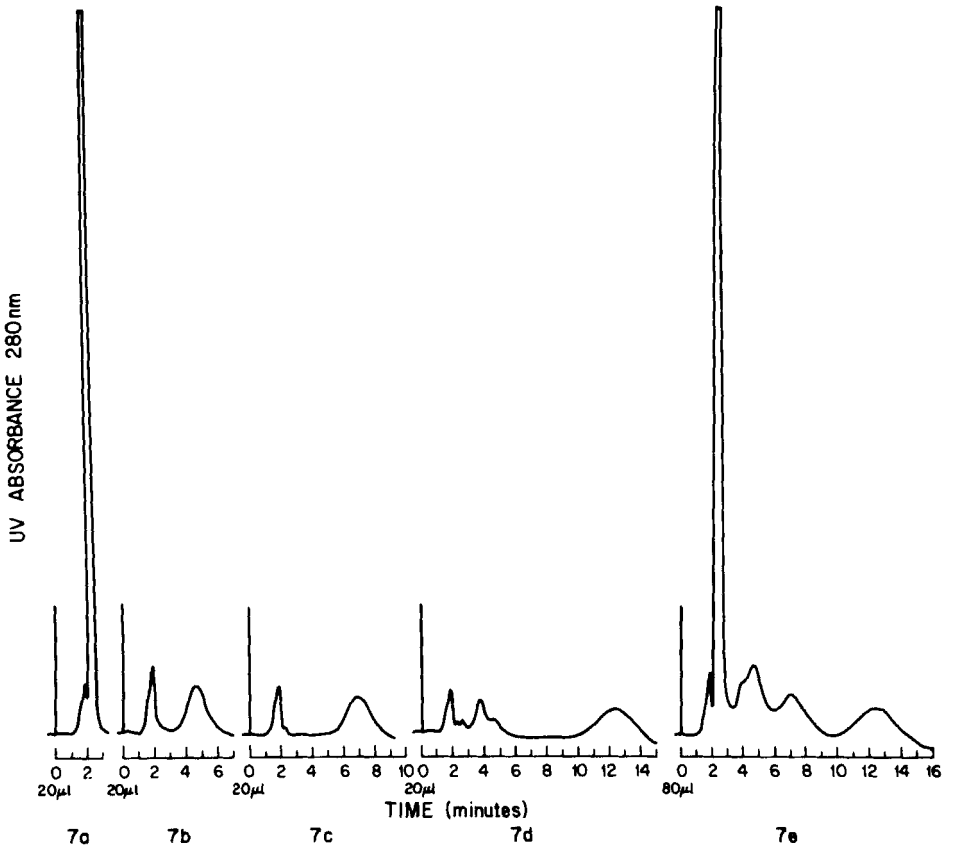

FIG. 7. (a) 20 μL of γ-globulin injected; pH 4.35, no additional electrolyte; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above, (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

significantly due to the longer desorption time. A further broadening of that peak would result in poor resolution.

To further control the double-layer repulsions, an electrolyte (K_2SO_4 in this study) was added to increase the ionic strength of the mobile phase. At the same pH of 4.35, a small increase in the ionic strength, by 0.001 *M* K_2SO_4, decreased the retention times of A, BSA, and OV (Fig. 8b, c, d), and slightly increased that of γ-globulin. This resulted in almost the same retention times for A, BSA, and OV, leading to the separation between γ-G and the rest of the proteins (Fig. 8e). When, at pH = 4.35, the salt concentration was increased to 0.01 *M* K_2SO_4, the retention times of the latter three proteins further decreased (Fig. 9b, c, d), and they eluted simultaneously with γ-G when injected together (Fig. 9e). Thus no separation occurred under such operating conditions. As already noted, one expects an increase in the retention times with increasing ionic strength when the proteins are positively charged. The opposite effect observed for three of the proteins is a result of the fact that the isoelectric points of the A, BSA, and OV are quite close to the operating pH condition of the mobile phase, and hence their surface charges are close to zero. Under such conditions, some positive ions from the bulk could adsorb onto the surface of the adsorbates as the ionic strength of the mobile phase increases. This results in an increase of the double-layer repulsion between adsorbates and adsorbent, and consequently leads to a decrease in their retention times.

The retention time of the γ-globulin increases with increasing ionic strength because its isoelectric point range is far above the pH of the mobile phase. Therefore this protein is strongly positively charged. Thus its retention time increases as the ionic strength of the mobile phase increases.

As shown in Fig. 10, at pH = 4.45 and no additional electrolyte, the retention times of A and BSA increased substantially, as expected, and their peaks broadened to such an extent that they are barely recognizable. Some portion of the proteins might possibly be almost irreversibly adsorbed in the deep energy wells. The retention time of the γ-G remained almost unchanged because of its higher isoelectric point. While the retention time differences between the components are large, the resolution under such operating conditions is not satisfactory because of excessive peak broadening and irreversible adsorption. OV was not injected under these operating conditions for fear of a too strong irreversible adsorption. Note that at a pH of 4.45 and no additional electrolytes, the retention times of the three proteins (A, BSA, and OV) increased substantially and their peaks broadened significantly (Fig. 10). On the other hand, for a mobile phase containing 0.01 *M* K_2SO_4 at the

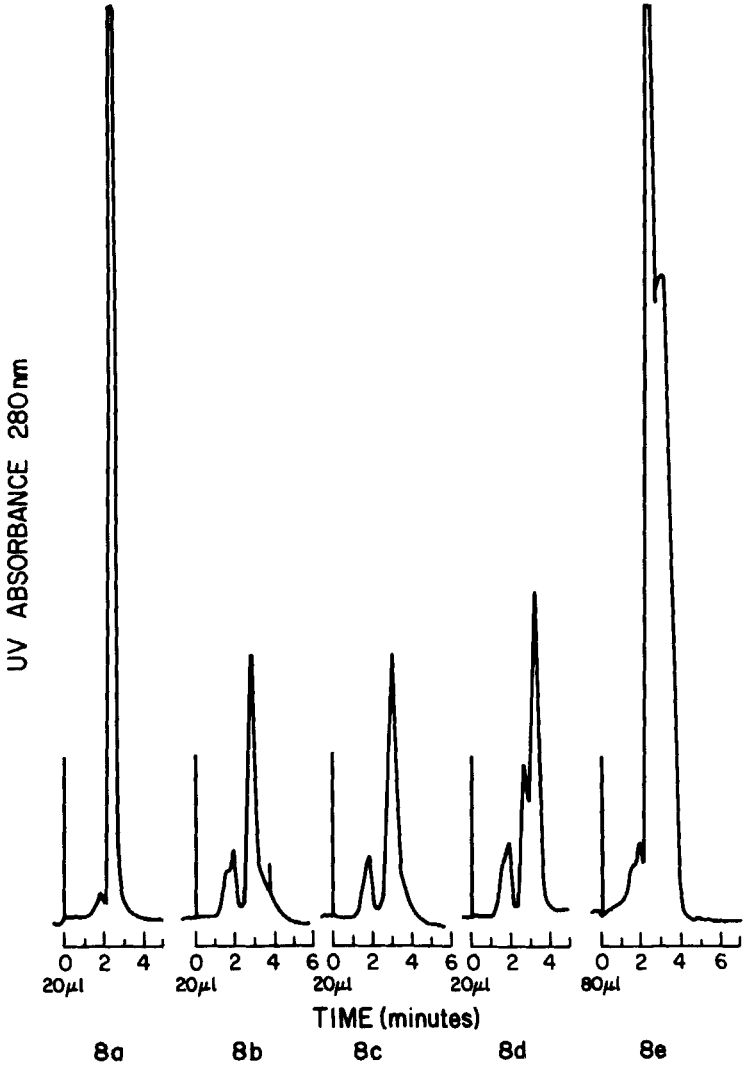

FIG. 8. (a) 20 μL of γ-globulin injected; pH 4.35, 0.001 *M* K_2SO_4; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

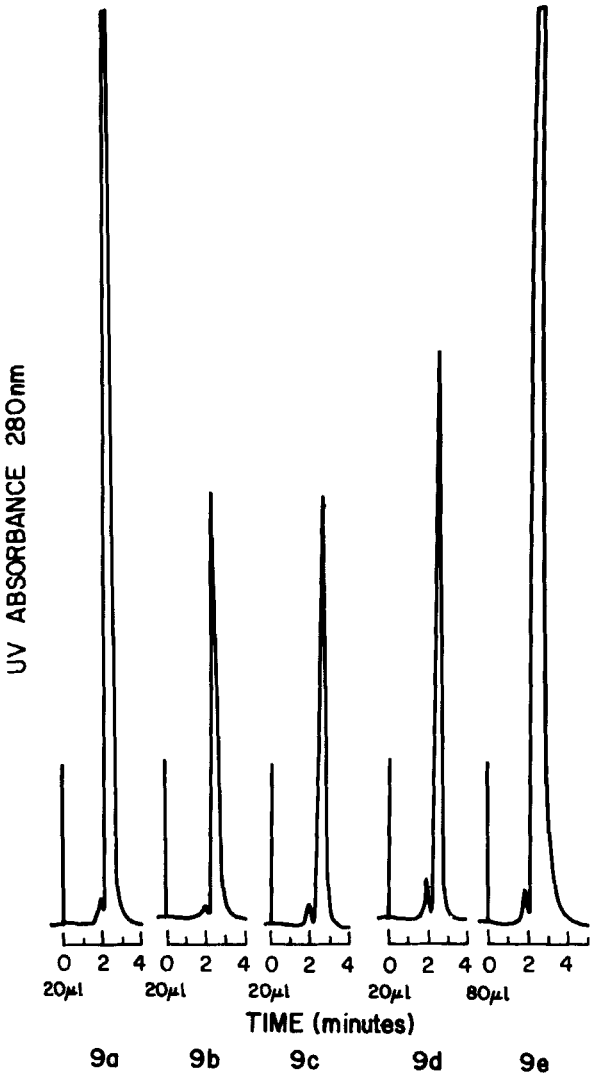

FIG. 9. (a) 20 μL of γ-globulin injected; pH 4.35, 0.01 *M* K_2SO_4; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

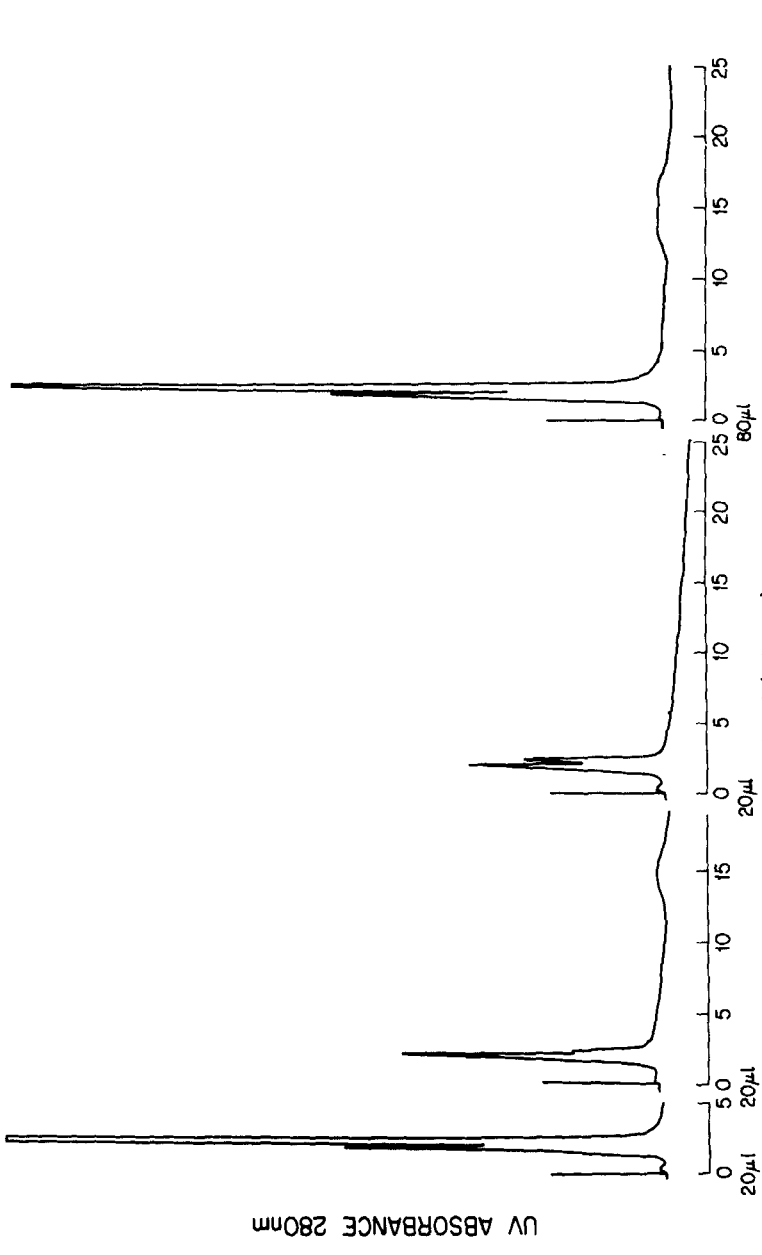

FIG. 10. (a) 20 μL of γ-globulin injected; pH 4.45, no additional electrolyte; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 60 μL of γ-G injected, albumin, and BSA; as above.

somewhat lower pH of 4.35, the retention times of the above three proteins were decreased to become almost the same (Fig. 9). Thus, increasing the pH even closer to the isoelectric points of the proteins increases their retention times, while the increase in the ionic strength decreases their retention times (for the reason mentioned above). As shown in Fig. 11a, b, c, d for 0.01 *M* K_2SO_4 and at a pH increased to 4.5, the four species were eluted at nearly the same time, resulting in no separation. Thus, under these operating conditions, the decrease in the retention times of the A, BSA, and OV, caused by the adsorption of cations on these proteins, dominates over the increase resulting from the higher pH of the mobile phase. As the pH was further increased to 4.8 while keeping the same concentration of K_2SO_4 in the mobile phase, the retention times of A, BSA, and OV showed moderate increases (Fig. 12b, c, d), leading to some separation (Fig. 12e). However, the pH of 4.8 is greater than the isoelectric point of OV (4.6) and equal to that of BSA. Thus OV becomes slightly negatively charged while BSA is almost neutral at this pH. BSA and OV have eluted ahead of albumin, probably because of the adsorption of cations on these molecules. However, the resolution is not as satisfactory as that obtained at pH = 4.35 without any additional electrolytes. Therefore the optimum operating condition for the best resolution of the four model proteins can be considered as pH = 4.35 without any additional electrolytes or organic solvent.

Another group of experiments was carried out to determine: (a) the effect of the amount of proteins injected in one pulse and (b) the effect of injecting a succession of pulses at short time intervals on the resolution of the separation. In order to increase the amounts of proteins separated per unit time, a shorter duration of separation was used (at the expense of a somewhat lower resolution) by choosing pH = 4.2 (instead of 4.35) and also no additional electrolyte. When 80 μL of the protein mixture (20 μL of each) was injected, the separation was completed in 8 min (including the void volume) (Fig. 13a). Since the chromatogram approaches the baseline after 7.5 min and the first protein peak appears 2 min after injection, the time interval between injections could be reduced to 5.5 min. The chromatograms of four successive injections of 80 μL mixtures at 5.5 min time intervals (Fig. 13b) showed that the resolutions between the peaks are not affected by the succession of injections. As the injection interval was reduced to 5 min, the chromatograms still showed good resolution (Fig. 13c). Increasing the amount injected in a single pulse to 120 μL (30 μL of each protein) and performing the injections at time intervals of 5 min, the resolutions still remained unaffected (Fig. 14). When, however, the amount injected was increased to 200 μL (50 μL of each component) while keeping the 5 min injection time intervals, the

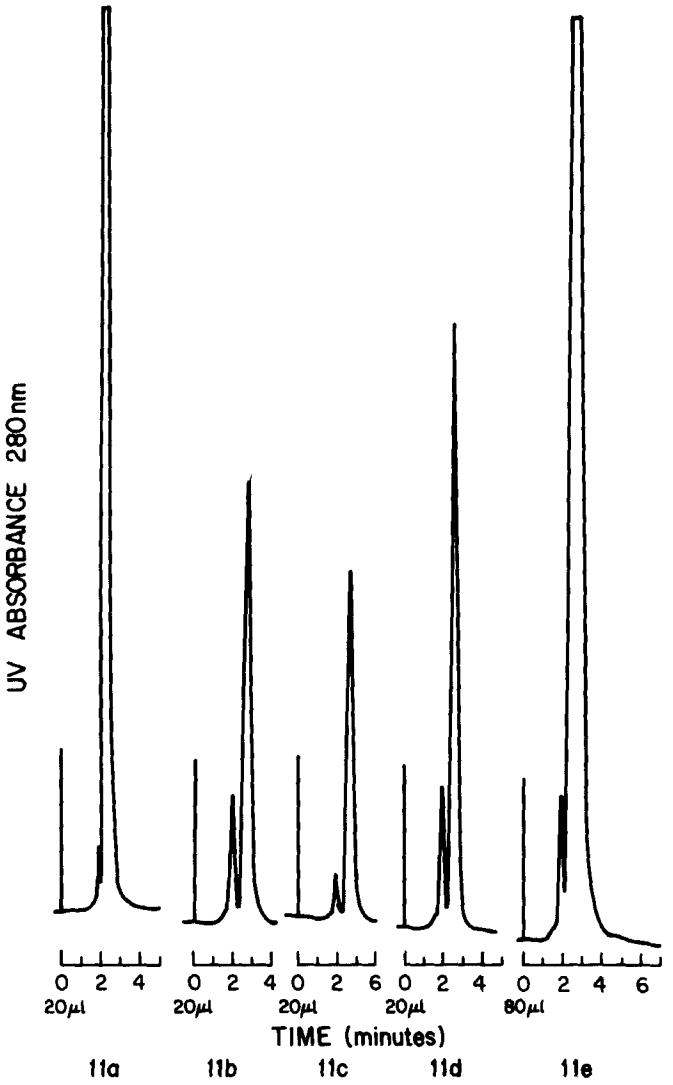

FIG. 11. (a) 20 μL of γ-globulin injected; pH 4.5, 0.01 *M* K_2SO_4; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

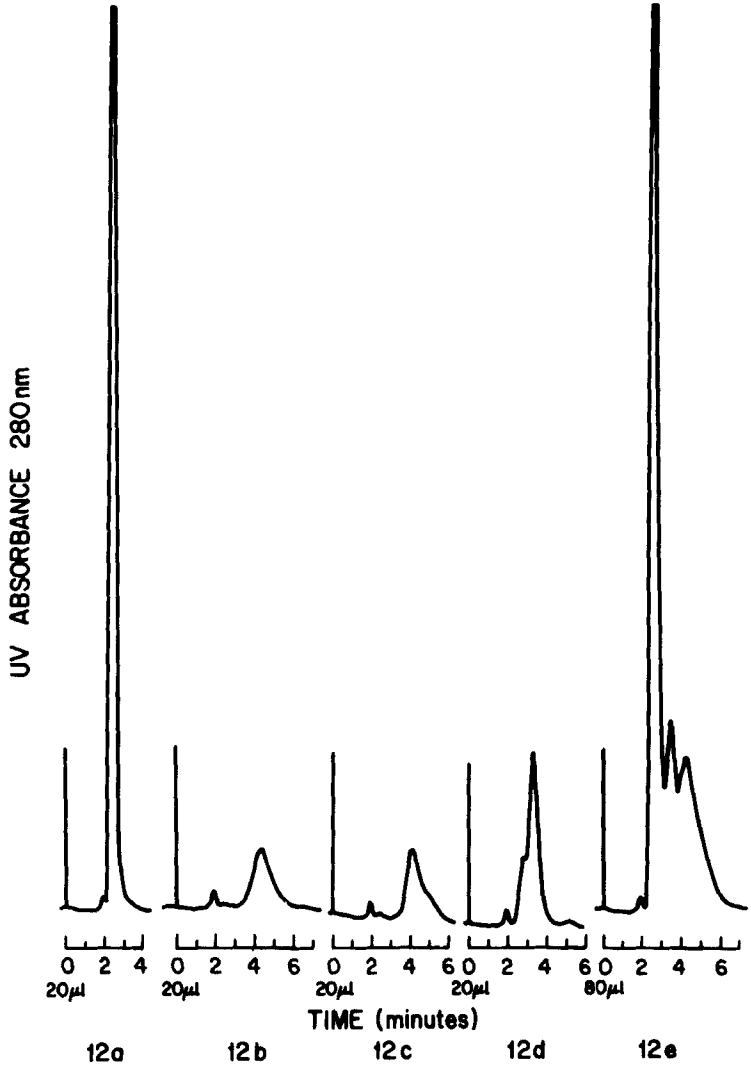

FIG. 12. (a) 20 μL of γ-globulin injected; pH 4.8, 0.01 *M* K_2SO_4; as above. (b) 20 μL of albumin injected; as above. (c) 20 μL of BSA injected; as above. (d) 20 μL of OV injected; as above. (e) 80 μL of γ-G, albumin, BSA, and OV injected (20 μL of each); as above.

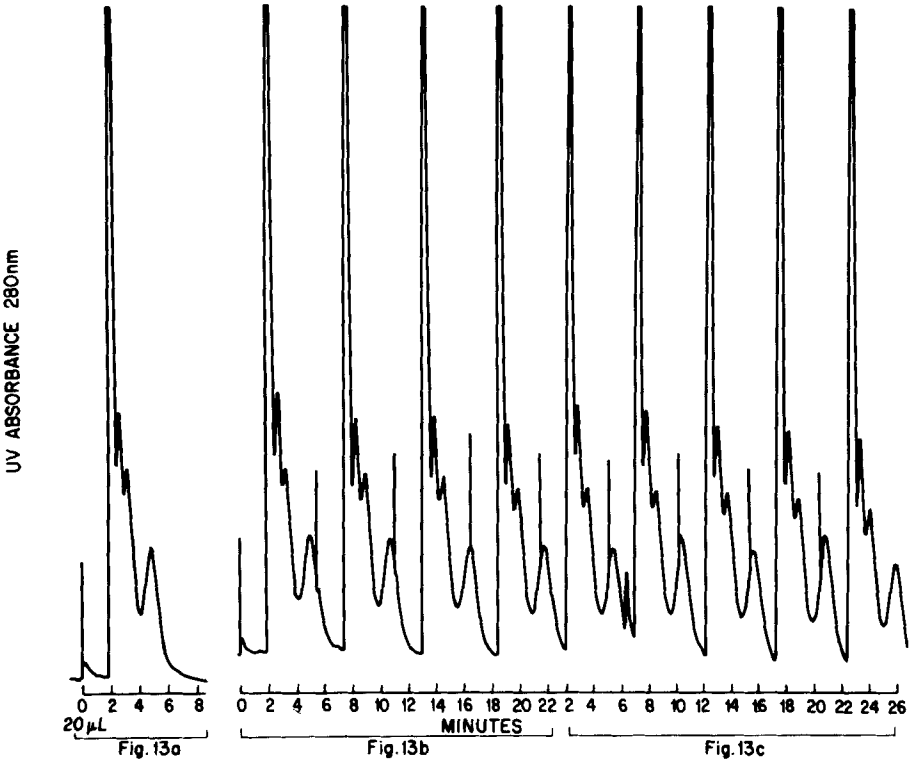

FIG. 13. (a) 80 µL of 4 protein mixture (20 µL of each) injected at the concentration of 0.5 mg protein/mL of mobile phase; pH = 4.2, and no additional electrolyte; mobile phase flow rate 0.5 mL/min. (b) Four pulses at injection intervals of 5.5 min. (c) Five pulses at injection intervals of 5 min.

peaks started to overlap (Fig. 15a). An increase of the injection interval to 6 min for the same amount injected in a single pulse (Fig. 15b) again displayed good resolution. As the amount injected was further increased to 240 µL, at the marginally increased time interval of 6.25 min, the baseline stayed stable during five successive injections (Fig. 16). There is, however, another variable, namely, the concentration of the protein solution itself which can increase the amount of separated proteins in a single pulse experiment, without increasing the injection volume. In the experiments discussed below, this concentration was doubled to 1 mg of protein per milliliter of mobile phase. First, 80 µL of this more concentrated mixture was injected (Fig. 17), and the separation was

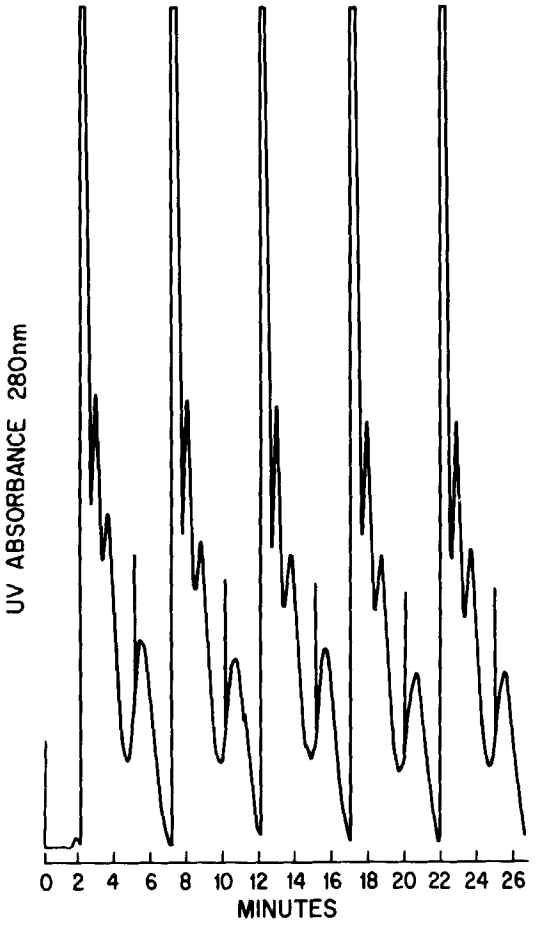

FIG. 14. 120 μL of 4 protein mixture (30 μL of each) at the same conditions as in Fig. 13; five pulses at injection intervals of 5 min.

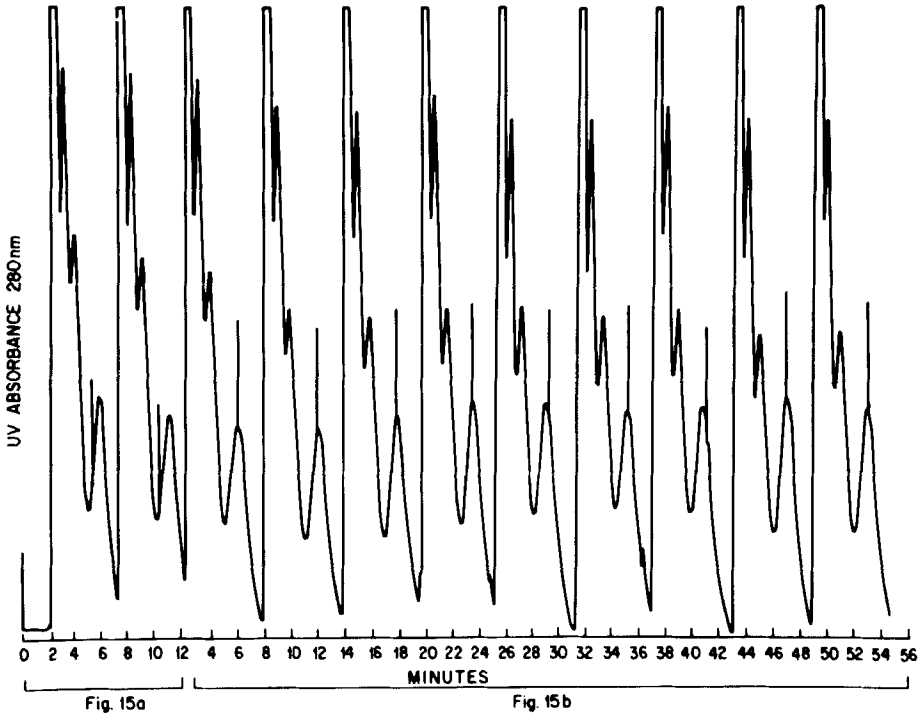

FIG. 15. (a) 200 μL of 4 protein mixture (50 μL of each) at the same conditions as in Fig. 13; two pulses at injection intervals of 5 min. (b) 200 μL of 4 protein mixture (50 μL of each) at the same conditions as in Fig. 13; nine pulses at injection intervals of 6 min.

completed in 9 min. As the amount injected was increased to 160 μL (40 μL of each component), the separation was completed in 10 min (Fig. 18a). For this amount injected and pulses at 7 min intervals (Fig. 18b), as well as pulses at 8 min intervals (Fig. 18c), the resolutions were somewhat decreased compared to the previous chromatograms. However, the amount separated was much larger. It is interesting to note that the chromatograms show great stability over the prolonged succession of pulses. This means that, by controlling the depth of the adsorption energy well by means of the double-layer forces, only a very small amount of protein was permanently retained in the column and thus the adsorption characteristics of the column packing remain largely unaffected.

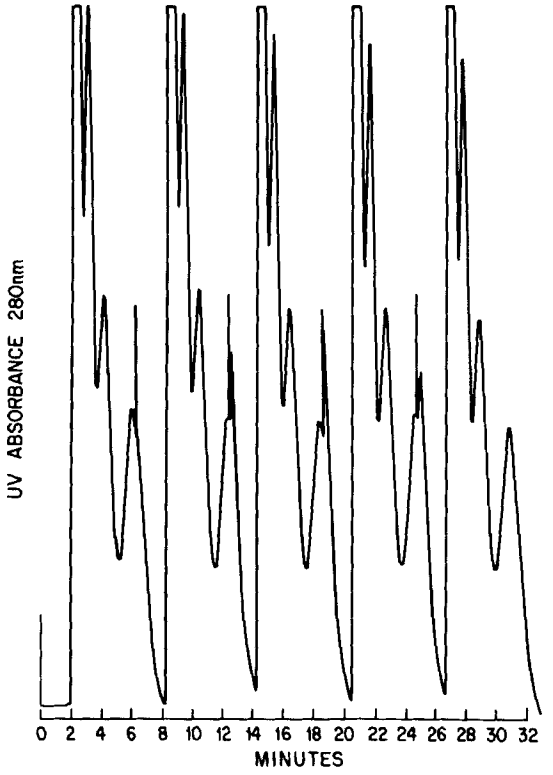

FIG. 16. 240 μL of 4 protein mixture (60 μL of each) at the same conditions as in Fig. 13; 5 pulses at injection intervals of 6.25 min.

SUMMARY

An anion-exchange adsorbent was employed to perform the separation of a multicomponent mixture of proteins (γ-globulin, albumin, bovine serum albumin, and ovalbumin) by properly controlling the physical forces of interactions between the proteins and packing. The physical forces involved are the van der Waals attraction, and double-layer, Born, and hydration repulsions. The double-layer interactions were controlled by changing the pH and the ionic strength of the mobile phase. The best resolution was obtained for a pH = 4.35 and with no additional electrolyte. In addition, the effects of the amount of proteins injected in a

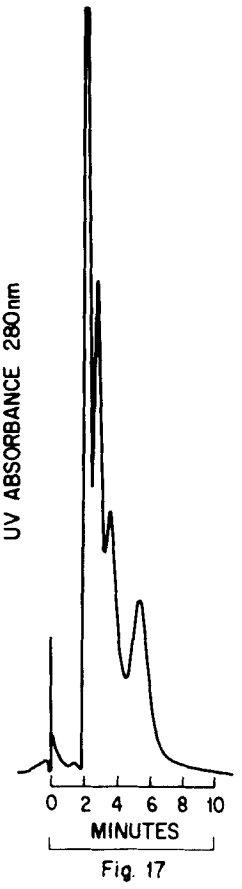

FIG. 17. 80 μL of 4 protein mixture (20 μL of each) injected at the concentration of 1 mg protein/mL mobile phase; pH = 4.2 and no additional electrolyte; mobile phase flow rate 0.5 mL/min.

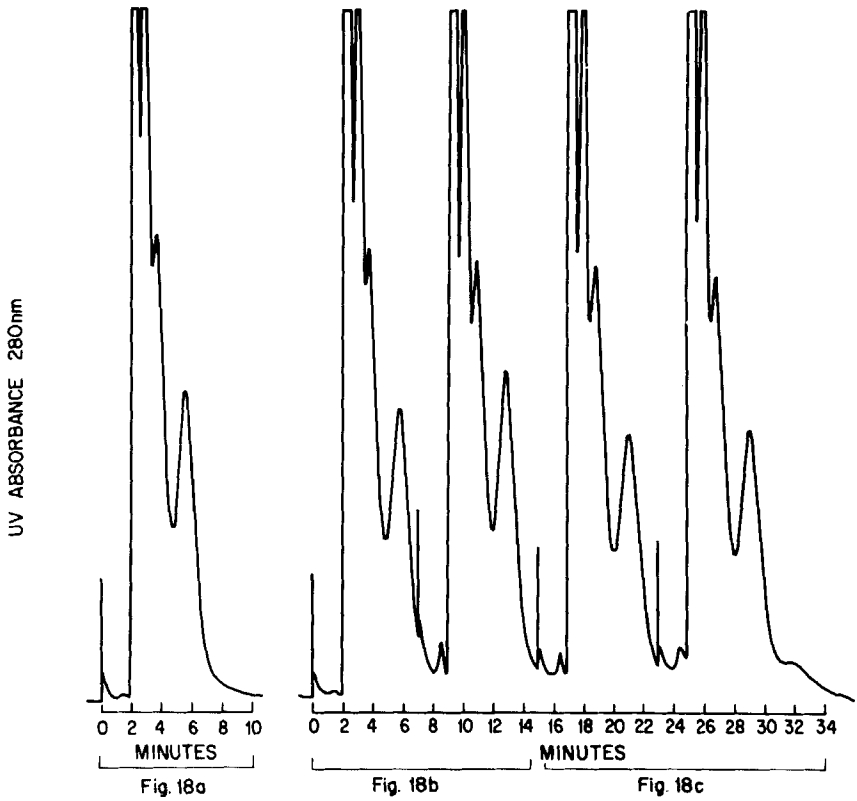

FIG. 18. (a) 160 μL of 4 protein mixture (40 μL of each) at the same conditions as in Fig. 17. (b) 160 μL of 4 protein mixture (40 μL of each); two pulses at injection intervals of 7 min. (c) 160 μL of 4 protein mixture (40 μL of each); two pulses at injection intervals of 8 min.

single pulse as well as that of a succession of pulses on the resolution of the separation were investigated.

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