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Separation of Immunoglobulins by Potential Barrier Chromatography

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Abstract

Potential barrier chromatography is a HPLC method of separating proteins, which is based on the high sensitivity of the interaction potential between adsorbent and adsorbate to factors such as the adsorbate size and adsorbate charge. The interaction potential is composed of van der Waals attraction, and double-layer, Born, and hydration repulsions. Controlling the double-layer repulsion, by changing the pH and the ionic strength, and the van der Waals attraction, by changing the organic content of the eluant, generates sufficiently different adsorption energy wells for the individual proteins to achieve their separation. PBC is exploited in the present paper to separate various immunoglobulins, especially IgA and IgG (which, as it is well known, are difficult to separate), from a naturally occurring mixture of human serum. A fraction more concentrated in immunoglobulins has been first obtained from this mixture by their precipitation with ammonium sulfate. The optimum conditions for the separation of various immunoglobulins from the enriched mixture, by PBC, were: pH = 7.5 and salt concentration $\cong 0.005$ M K_2SO_4. An inexpensive ion-exchange column, conventional HPLC equipment, and an isocratic elution procedure were used. Since this method is based on relatively weak physical interactions between proteins and packing, the duration of separation is small and the likelihood of denaturation is much lower than in other methods.

INTRODUCTION

Potential barrier chromatography (PBC) is a recently developed high performance liquid chromatographic method for protein separation (*1*). It has some similarities with gas chromatography, since (a) it uses an isocratic

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elution procedure and (b) it exploits the differences in the interaction potentials between the adsorbates and adsorbent. Recent work has demonstrated its applicability to protein separation (*2, 3*).

In PBC, the interaction potential is composed of van der Waals attraction, and double-layer, Born, and hydration repulsions between the adsorbate and the adsorbent. In contrast to gas chromatography, however, the interaction potential in PBC can be controlled by changing the pH and/or the ionic strength (both of which affect the double-layer repulsion between the adsorbent and adsorbate), and by introducing small amounts of organic solvents in the eluant (thus affecting the van der Waals attraction). As illustrated in Fig. 1, if the mobile phase conditions are properly chosen, the total interaction profile can have two minima (primary and secondary) and one maximum (which gives rise to a potential barrier). The depth of the energy well (primary minimum), which is essentially responsible for the chromatographic behavior of a particular adsorbate (*2, 3*), and the height of the potential barrier (maximum) are different for each of the adsorbate species. This is a result of the high sensitivity of the interaction potential to even small changes in adsorbate size, charge, and Hamaker constant, the latter quantity being a measure of the magnitude of the van der Waals interactions. Now, consider an adsorbate whose interaction potential has a high potential barrier and a shallow energy well, as shown in Fig. 2. This interaction potential is a consequence of strong double-layer repulsion and/or weak van der Waals attraction. Due to the high potential barrier to

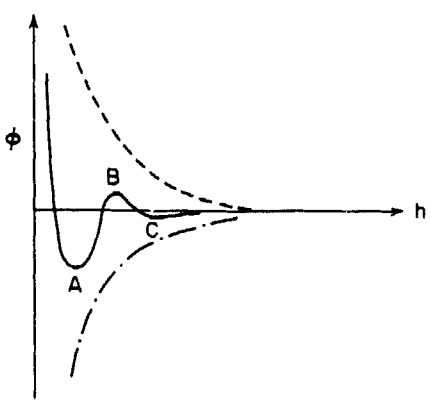

FIG. 1. Standard shape of the total interaction potential for PBC. (A): Adsorption energy well. (B): Potential barrier. (C): Secondary minimum. (- -): Double layer interactions. (. -): Van der Waals interactions. (h): Minimum distance between adsorbent and adsorbate (assumed spherical for the sake of simplicity).

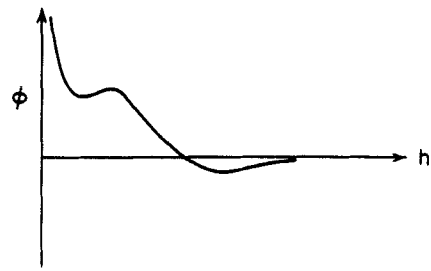

FIG. 2. Total interaction potential in the case of strong double layer repulsion.

adsorption and ease of desorption, such an adsorbate will elute early near the void volume. In contrast, an adsorbate which has the interaction potential such as that of Fig. 3 (with a deep energy well and a low potential barrier) will reside in the column for a long period of time. Thus separation with good resolution can be achieved by controlling the individual interactions so as to obtain sufficiently different depths of the energy wells and heights of the potential barriers for different adsorbates. As a result, different proteins will reside in the column for different periods of time. Since the double-layer interaction is required to be repulsive in PBC, the adsorbate and the adsorbent must carry net charges of the same sign. Thus, if the adsorbent is negatively (positively) charged, the adsorbates also must be negatively (positively) charged. This can be achieved by keeping the pH of the mobile phase above (below) the isoelectric points of the adsorbates. The double-layer repulsion between negatively charged adsorbent and adsorbate will be higher if the difference, ΔpH, between the pH of the mobile phase and the pI (the isoelectric point) of the adsorbate is greater. An increase in the ionic strength of the mobile phase will screen the surface charges of the adsorbent and adsorbates, thus decreasing the double-layer repulsion. Furthermore, the addition of an organic solvent to the mobile phase may be employed to decrease the van der Waals attraction. Further details regarding the mobile phase conditions can be found elsewhere (*2–4*). Since the total interaction potential is the result of the above-mentioned individual interactions, the depth of the energy well, which ultimately determines the residence time of a particular adsorbate, can be controlled by changing the pH, the ionic strength, and the amount of organic solvent in the mobile phase. The depth of the energy well is increased by increasing the ionic strength of the mobile phase and by decreasing ΔpH. Increasing the depth of the energy well results in an increase of the retention time of the adsorbate. Alternatively, the energy well can be raised by the addition of a small amount of an organic solvent to the mobile phase. This obviously decreases the retention time of the

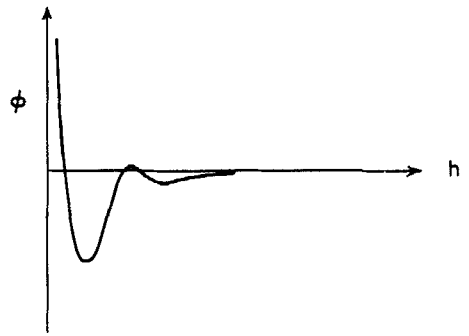

FIG. 3. Total interaction potential in the case of weak double layer repulsion.

adsorbate. By properly selecting the mobile phase conditions so that the interaction potentials have surmountable potential barriers to adsorption and moderately deep adsorption energy wells, separation with good resolution can be obtained in a relatively short time. However, it is important to note that, in order to achieve separation by an isocratic elution procedure, the energy wells of the individual adsorbates should differ sufficiently, while at the same time not becoming too deep. Thus, for a given set of adsorbates and adsorbent, optimum values of ionic strength, pH, and organic solvent content of the mobile phase exist. Therefore, the scope of any investigation concerning the use of PBC is to identify the above optima. In the present study, PBC is employed for the separation of human immunoglobulins, especially IgA and IgG, from a mixture which is primarily composed of immunoglobulins and small amounts of other serum proteins. The latter mixture was obtained from human serum by precipitation with ammonium sulfate. IgA and IgG immunoglobulins, which themselves are mixtures of proteins, are known to be difficult to separate due to close values in their physicochemical properties. For example, as shown in Fig. 4, their isoelectric points are wide and significantly overlapped. In addition, IgA has a tendency to form complexes with other proteins (*5, 6*).

Current methods for the separation of IgA from serum proteins have usually employed immunoadsorption (i.e., affinity chromatography (*7*)). This method uses ligand specificity to bind IgA to the adsorbent and elutes the adsorbed molecules by changing the conditions of the mobile phase. Though this method is useful on a laboratory scale, it is unattractive for large-scale separation. Furthermore, due to the long adsorption times and chemical interactions with the ligand, the activities of the proteins may be compromised. Recently, an attempt was made to separate human IgA by "salt-mediated hydrophobic chromatography" (*8*). This method employs

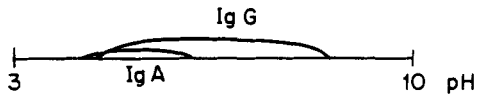

FIG. 4. Isoelectric points of IgA and IgG.

very high salt concentrations to enhance, by "salting out," the binding of IgA to the ligand specific adsorbent, followed by elution of the bound protein with an eluent which has a lower salt concentration. It is important to note that, in addition to changes in the mobile phase conditions, the above method requires rather expensive ligand specific adsorbents. PBC is a promising alternative to the above methods for the separation of IgA because: (a) an isocratic elution procedure is employed, and (b) inexpensive ion-exchange adsorbents can be used in conjunction with the conventional HPLC equipment. Additionally, in contrast to the above procedures which involve specific chemical interactions, PBC is based on relatively weak physical interactions between the adsorbate and adsorbent. As a consequence, the time required to achieve separation is reduced, and therefore the likelihood of protein denaturation is also reduced.

EXPERIMENT

The experimental apparatus consisted of a solvent delivery system (Waters 6000A), a nonstop flow septumless injector (Waters U6K), a UV absorbance detector (Waters UV 440), a peak timer and area integrator (Varian CDS 111), and a strip chart recorder (Houston Instrument, Omniscribe). The column was a 4.6 mm i.d. $\times$ 25 cm length Dupont Zorbax 300 SCX. The column packing is described by the manufacturer as a strong cation exchanger, consisting of 7$\sim$8 μm diameter porous silica microspheres to which aromatic sulfonic acid moieties are strongly bonded. Common organic solvents could be used without the fear of damaging the adsorbent.

The adsorbent is negatively charged for normal operating conditions. Therefore, in order to obtain repulsive double-layer interactions, the pH of the mobile phase must be kept above the isoelectric points of the proteins. However, due to the wide range of their isoelectric points (see Fig. 4), the optimum pH was selected at the expense of having a small portion of IgG positively charged. The column was equilibrated with the mobile phase for 30 to 60 min. The sample was prepared from whole human serum, as shown in Table 1, utilizing the fact that the immunoglobulins become insoluble at much lower concentrations of ammonium sulfate than most other serum

TABLE 1
Sample Preparation Procedure

Human Serum
(undiluted)
↓
Precipitate Globulins
(50 mL of human serum + 25 mL of saturated $(NH_4)_2SO_4$)
↓
Centrifugation
↓
Wash Precipitates
(with solution: 50 mL of saline solution of 0.15 *M* NaCl + 25 mL of saturated $(NH_4)_2SO_4$ solution)
↓
Centrifugation
↓
Dialize Against Distilled Water
↓
Dialize Against PBS
(phosphate buffered saline pH = 7.5, 0.15 *M* NaCl)

proteins. Mobile phases were prepared fresh daily, using demineralized distilled water. The pH of the mobile phase was adjusted by the titration with 0.01 *M* Na_3PO_4 and 0.01 *M* NaH_2PO_4 by monitoring the pH with a digital pH meter (Orion Research 601A). The flow rate of the mobile phase was 0.5 mL/min. The sample pool was stored under refrigeration and shaken well before 10 μL pulses of sample were introduced. The effluent was monitored at 280 nm. Experiments were performed by searching for the optimum conditions for the separation of IgA and IgG, by changing the operating conditions (such as pH and ionic strength) in the direction which showed

improved resolution. Eluate fractions were collected immediately after the detector in individual test tubes for each peak, as the chromatogram was monitored. The collected eluate fractions were then concentrated separately by vaporization through a semipermeable membrane (cellophane tube) for one day under refrigeration. Subsequently, ouchterlony analyses were performed to identify IgA and IgG in each fraction, using IgA and IgG specific antiserums from rabbit and goat, respectively. The immunoelectrophoresis results of the sample mixture show mainly IgG and IgA, but also small amounts of β-lactoglobulin and α_2-macroglobulin fractions and traces of albumin.

RESULTS AND DISCUSSION

The initial experiments were performed at pH = 9.0 with no additional electrolytes. This high pH ensures that all the proteins are negatively charged. As shown in Chromatogram 5a, four major peaks appear under these operating conditions. By decreasing the pH of the mobile phase from 9.0 to 7.5 gradually (Figs. 5a to 5d), the retention times of these four peaks have increased. This is to be expected, since the double-layer repulsion has been decreased by the decrease in the surface charges of the proteins. Also note that the resolution of the four peaks has been improved marginally. Since a further decrease in pH will increase the portion of IgG which is positively charged, thus generating attractive double-layer interactions for that part, a pH of 7.5 was chosen as the optimum pH of the mobile phase. For convenience, let us denote the four peaks by 1 to 4 in the order of elution. The four peaks which appeared in the chromatogram were collected in individual test tubes, from several pulses, in order to obtain sufficient amounts of eluates to perform subsequent analysis. The collected eluates were then concentrated and ouchterlony analyses performed.

From the analysis, IgA and IgG were identified in Peak 1, and only IgG was identified in Peak 2, while neither of them was identified in Peaks 3 and 4. To further control the double-layer interaction, electrolytes have been added to the mobile phase. By increasing the ionic strength of the mobile phase by the addition of salts (K_2SO_4 in the present study), an increase in the retention times of the adsorbates is expected (due to a decrease of the double-layer repulsion). As a result of this, the depth of the energy well is increased. Note that care should be taken in the choice of the electrolyte, since some of them, such as $(NH_4)_2SO_4$, may precipitate the globulins.

As shown in Fig. 6a, a small branch peak appears at 0.005 M K_2SO_4 immediately following Peak 1. Let us denote these peaks by 1a and 1b. Thus, the former Peak 1 separates into two peaks under these operating conditions.

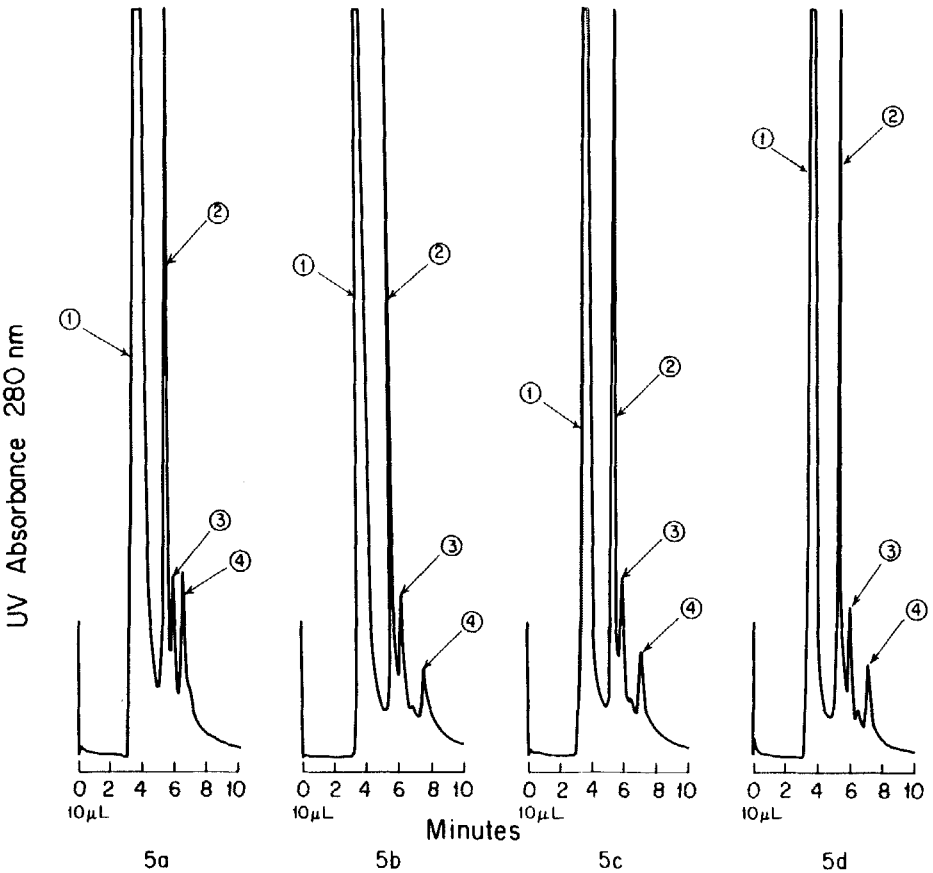

FIG. 5. (a): pH 9.0; no additional electrolyte; 10 μL of sample mixture injected; wavelength of the detector 280 nm; sensitivity 0.05 AUFS; mobile phase flow rate 0.5 mL/min. (b): pH 8.0; as above. (c): pH 7.8; as above. (d): pH 7.5; as above.

The retention times of the former four peaks have also been increased by the increase in the salt concentration as a result of the deeper potential wells. The ouchterlony tests, performed for 0.005 M K_2SO_4 experiments, showed neither IgA nor IgG in Peak 1a; however, both were found in Peak 1b, and IgG was found alone in Peak 2. Thus, Peak 1a, which eluted with almost no retardation during the passage through the column, is most likely to contain serum proteins such as albumin, α_2-macroglobulin, β-lactoglobulin, etc. which have lower isoelectric points than IgA. Because the ΔpH's of these proteins are larger than those of IgA and IgG, they carry higher surface

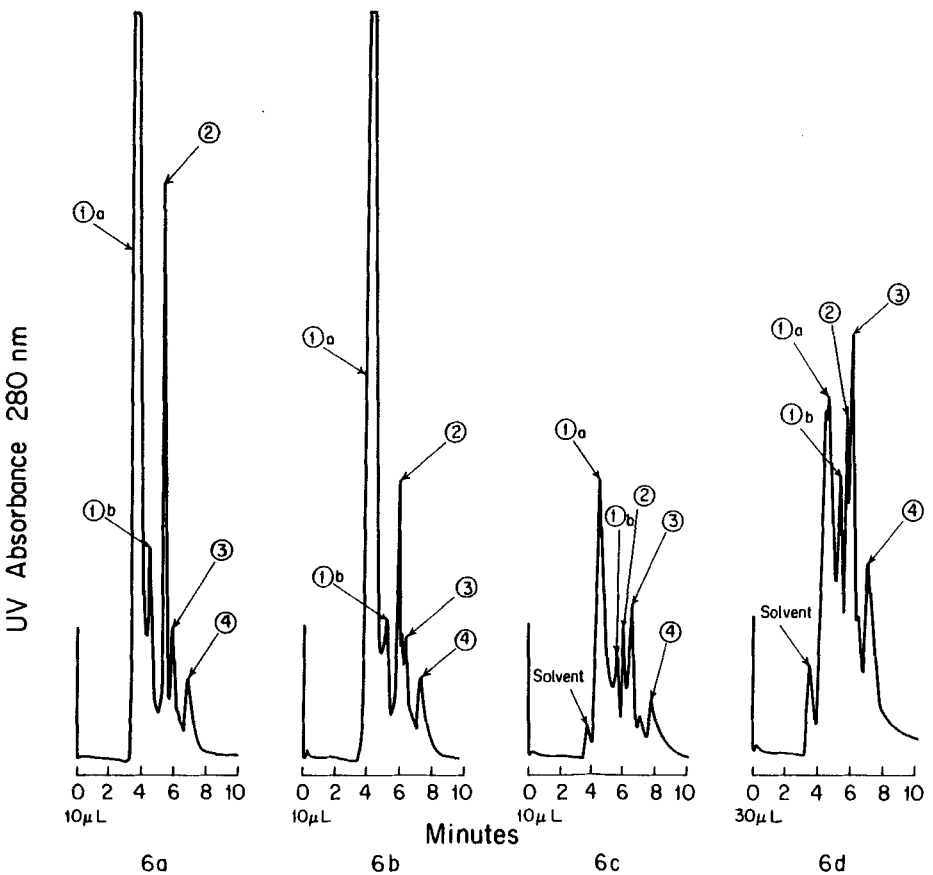

FIG. 6. (a): pH 7.5; 0.005 *M* K_2SO_4; 10 μL of sample mixture injected; wavelength of the detector 280 nm; sensitivity 0.05 AUFS; mobile phase flow rate 0.5 mL/min. (b): pH 7.5; 0.01 *M* K_2SO_4; as above. (c): pH 7.5; 0.03 *M* K_2SO_4; as above. (d): pH 7.5; 0.05 *M* K_2SO_4; as above.

charges. Thus, their retention times are not much affected by this low increase in ionic strength. Therefore, the amounts of the lower isoelectric point proteins that are adsorbed are quite small, and hence they pass through the column with the mobile phase to elute near the void volume. In contrast, the retention times of the less charged IgA and IgG are increased.

It is important to note that, if the double-layer repulsion is too strong, the total interaction potential would look like that shown in Fig. 7, and the adsorbate would elute ahead of its uncharged version or micromolecule

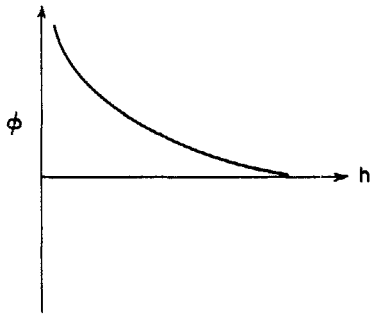

FIG. 7. Total interaction potential in the case of extremely strong double layer repulsion.

tracer, since the latter are not excluded from regions near the surface of the adsorbent, where the mobile phase velocity is smaller than that in the bulk.

Note that, at 0.005 M K_2SO_4, IgA and IgG are separated from the other serum proteins which have lower isoelectric points. Furthermore, as illustrated in Fig. 8, IgA and IgG are distributed between Peaks 1b and 2. Since the isoelectric point range of IgA is lower than that of IgG, IgA will experience stronger double-layer repulsion. In addition, IgA is generally known to be less hydrophobic than IgG. Stronger repulsive interactions and weaker van der Waals interactions result in shorter retention times for IgA than for IgG. Thus, the distribution of IgA and IgG between Peaks 1b and 2 can be explained in the framework of the theory of PBC. Note that a solvent peak (a void volume peak) does not appear because the proteins with lower pI's elute simultaneously with the void volume. At 0.01 M K_2SO_4 (Fig. 6b), the retention times of the five peaks have further increased. A solvent peak still does not appear, indicating that the double-layer repulsion is still high on the low isoelectric point constituents of the mixture. When the concentration of the electrolyte is further increased to 0.03 M K_2SO_4, Chromatogram 6c, a solvent peak appears, indicating surmountable potential barriers for all the species of the mixture. Additionally, the resolution between Peaks 1a and 1b has improved at the expense of increased retention times of the corresponding species. Note that, compared to the chromatograms for lower ionic strengths, the height of Peak 1a is decreased while that of Peak 3 is somewhat increased. However, Peaks 1b and 2, which correspond to IgA + IgG and IgG, remain almost unchanged.

A further increase in electrolyte concentration to 0.05 M K_2SO_4, Chromatogram 6d, adversely affects the resolution between Peaks 1b and 2 which are of prime concern. The increase in electrolyte concentration over

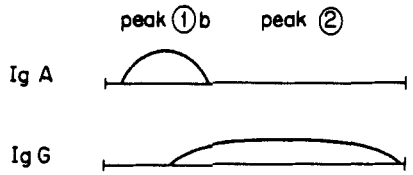

FIG. 8. Distribution of IgA and IgG between Peaks 1b and 2.

0.05 *M* K_2SO_4 decreases the resolution between Peaks 1b and 2. This probably occurs because the decrease in the double-layer repulsions above a certain limit attenuates the differences in the shape of the total interaction potentials of the proteins. Therefore, there is an optimum salt concentration which provides the best resolution.

As shown in Chromatograms 9a to 9d, the addition of small amounts of organic solvent (5 to 10 mL of acetonitrile per 500 mL of mobile phase) slightly decreases the retention times of the peaks. This can also be achieved by a small decrease of the salt concentration. Note that, at 0.01 *M* K_2SO_4, Chromatogram 9c, the addition of 5 mL CH_3CN/500 mL of mobile phase decreases the retention times of the peaks slightly, and also somewhat improves the resolution between Peaks 1a and 1b. However, at 10 mL of CH_3CN/500 mL of mobile phase, Chromatogram 9d, the retention times of the peaks increase again slightly, and the resolution of the Peaks 1a and 1b becomes the same as that without any organic additive. This occurs because the addition of a sufficient amount of organic solvent affects not only the van der Waals interactions, but also the double-layer interactions.

Since the organic solvents are toxic and must be used with care, and, in addition, their effect is less important, the optimum operating conditions in the present study could be considered as pH = 7.5, salt concentration $\cong$ 0.005 *M* K_2SO_4, and no organic solvent.

SUMMARY

A HPLC version of PBC (potential barrier chromatography) was used for the separation of IgA and IgG immunoglobulins from the naturally occurring mixture of human serum. Optimum resolution was obtained for pH = 7.5 and electrolyte concentration $\cong$ 0.005 *M* K_2SO_4.

The changes in operating parameters, such as pH and ionic strength, showed a behavior predictable from the theory of PBC. Finally, let us observe that PBC is based on physical interactions, such as van der Waals

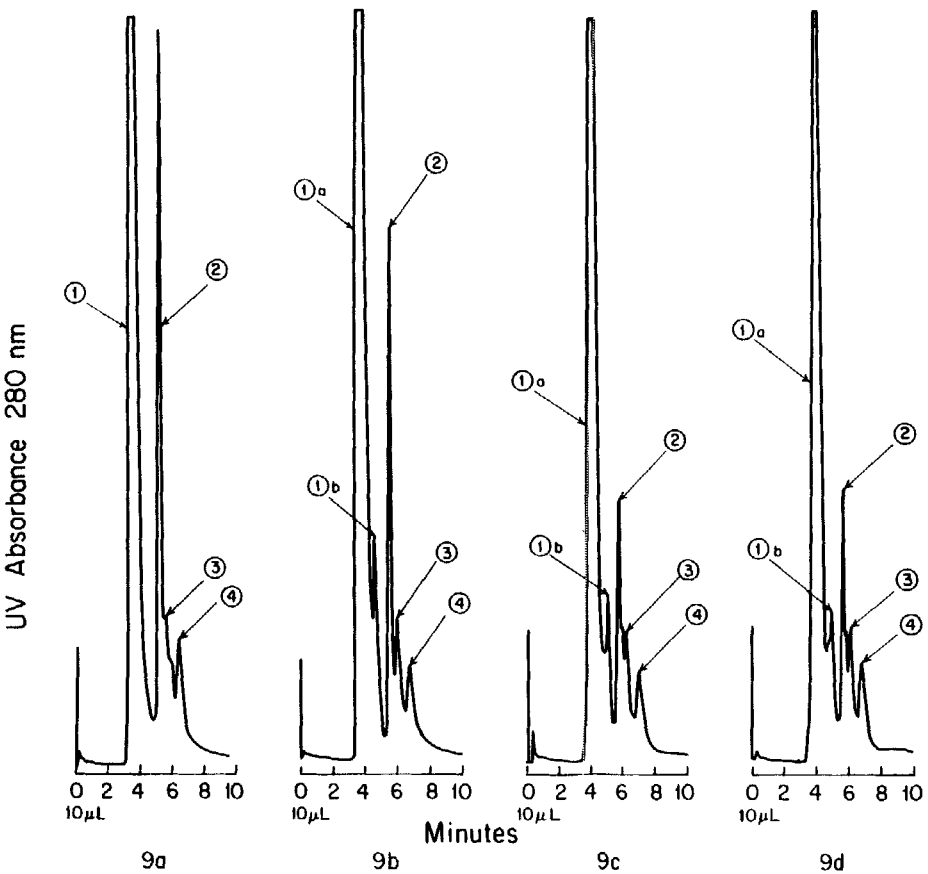

FIG. 9. (a): pH 7.5; 5 mL CH_3CN; 10 μL of sample mixture injected; wavelength of the detector 280 nm; sensitivity 0.05 AUFS; mobile phase flow rate 0.5 mL/min. (b): pH 7.5; 0.005 M K_2SO_4; 5 mL CH_3CN; as above. (c): pH 7.5; 0.01 M K_2SO_4; 5 mL CH_3CN; as above. (d): pH 7.5; 0.01 M K_2SO_4; 10 mL CH_3CN; as above.

attraction, double-layer, and Born repulsions. As a result of this, the elution procedure is isocratic and the elution times are short. In contrast, the other currently used procedures involve chemical interactions between protein and packing and, as a consequence, they are characterized by nonisocratic procedures and large elution times. Therefore, the likelihood of denaturation is much smaller in PBC than in contemporary separation techniques.

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