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# Effect of the concentration of ionogenic groups in the sorbent on the separation of protein mixtures

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**Abstract**

Investigations of sequential and simultaneous sorption of protein pairs on ion-exchangers with various contents of ionogenic groups were carried out. The transition from synergism to competition was shown to take place with a decrease in the content of the ionogenic groups. Of significant importance also is the uniformity of ionogenic group distribution in the sorbent volume. An examination of the dynamics of sorption and desorption of the proteins using sorbents with various contents of ionogenic groups allows to conclude, that the effective processes of ion-exchange chromatography can be realized only provided the competitive mechanism of sorption.


*Keywords:* Adsorption; Ion exchangers; Proteins

## 1. Introduction

Low pressure ion-exchange chromatography has a significant place among preparative methods of separation and purification of biologically active substances. This chromatographic method becomes unique in some cases due to its efficiency and ease to scale up the methods designed in laboratory. This method can be successfully used in a number of applications. At the same time the efficiency of protein separation on ion-exchange sorbents does not always meet the requirements concerning the purity of the obtained products in contrast to the separation of rather low-molecular-mass substances (e.g. amino acids and antibiotics).

In carrying out the most economically efficient protein separation and purification, complex multi-component mixtures are passed through the column with the sorbent until the outlet concentration of the target protein becomes comparable to the inlet one. Thus besides the target protein there are also other proteins present as impurities in the sorbent phase. The next step is to separate the target protein and the impurities with the help of various eluting solutions. The ideal chromatographic separation of compounds is achieved when the sorption of each of the mixture components is characterized by a certain sorbate–sorbent interaction energy that is dissimilar for different components. Such requirements are implemented only in the case of competition for sorption centers.

Strong (‘nonreversible’) binding of protein with sorbents of high capacity results in inapplicability of the models based on the ideas of competition of components for free places to processes of multi-component protein sorption. In reversible sorption after the equilibrium between the protein solution and the sorbent takes place, the dilution of the solution should result in partial desorption which in turn results in the correspondence of the sorbed

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protein quantity and the concentration of the equilibrium solution. The hysteresis between ascending and descending isotherm branches indicates that at the given equilibrium concentration the protein can be in two meta-stable quasiequilibrium conditions. The energy barrier preventing the protein transition from one state to another is related either to the change of conformation macromolecule in the sorbed state [1] or to the ability of macromolecule for multipoint interaction with the surface [2]. For protein desorption it is necessary to change the external conditions, for example, pH of the solution, the ionic strength or the organic solvent content.

Strong binding of protein to the sorbent gives the opportunity of intermolecular protein interactions in the sorbent phase. These interactions can significantly affect the chromatographic process as was reported by Xu and Regnier [3]. The conditions for intermolecular protein interactions in the sorbent phase are generated on ion-exchange sorbents with capacity values of protein of about 100 mg/ml. We have shown previously that under experimental conditions close to the requirements of maximal protein binding during sequential and simultaneous sorption on the ion-exchange sorbents with high values of ionogenic groups concentration the synergestic effects are observed [4]. In sequential sorption there is an additional sorption of the first protein after addition of the second protein, in simultaneous sorption the absorbed protein total amount exceeds the sum of sorption capacities of the individual components. These effects are independent of the sorbent morphology such as ionogenic groups type, the sorbent matrix hydrophobicity. The synergism during multicomponent sorption can be based on protein complexation in the solution or protein interaction in the sorbent phase [5].

The protein sorption in the complex form comes into collision with the fact that there is no proportionality between the amounts of the second protein being sorbed and the first protein being sorbed additionally as a result of sequential sorption. It is possible to assume that the protein occupies at first all the accessible sorption centers, thus its interaction with the sorbent is so strong that no replacement by the other protein is possible. Further sorption takes place on the base of protein–protein interactions. The stability (‘nonreversibility’) of protein binding under the given conditions is sustained by hysteresis on the isotherms of individual protein sorption.

The transfer from synergism to competition takes place as the protein–sorbent interaction diminishes. An examination of the solution pH effect on protein binding was carried out on the lysozyme–ribonuclease–carboxylic cation-exchanger system [6]. The transfer from synergism to competition can be achieved by decreasing the ionization degree of sorbent ionogenic groups. Thus the decrease of ionogenic groups concentration should result in the same effect. An examination of the effect of the degree of macroporous sorbent sulphonation on the processes of sequential and simultaneous sorption of insulin and ribonuclease has shown that synergestic phenomena arise due to binding of a protein macromolecule with the several fixed ionogenic groups [7].

It was difficult to investigate the protein separation processes on the sorbents of this series because of the presence of considerable hydrophobic protein interaction with the sorbent matrix (a copolymer of styrene and divinylbenzene). In this connection it would be beneficial to develop ion-exchange sorbents which would be characterized by the following features: (1) the hydrophilicity—the interaction of such sorbents with proteins realizes only via the ion–ion interactions; (2) heteroreticular structure—permits high permeability in relation to protein macromolecules; (3) reduced content of ionogenic groups.

As is noticed in [8], the decrease of ionogenic group concentration results in nonreversible protein sorption. But in this investigation the change of ionogenic group concentration results in significant differences of polyelectrolyte structure because of the change of the relation between ionogenic monomer and crosslinking agent segments. The purpose of our investigation was the maintenance of structural features of polyelectrolyte networks ensuring a sufficient permeability for protein macromolecules and high-speed mass transfer, at decrease of ionogenic group content. It also should ultimately lead to the increasing efficiency of chromatographic separation of protein mixtures.

## 2. Materials and methods

The sorbents used in the examinations were

synthesized in the laboratory of physical chemistry of polyelectrolytes of the Institute of Macromolecular Compounds, Russian Academy of Science. Heteroreticular sorbents are generated as a result of heterophase copolymerization in water and in aqueous solutions of organic solvents, which are precipitants of formed copolymers. The specific feature of these sorbents is that the resulting copolymer separates to form a new phase in a highly hydrated state [9]. As a result, the structure of such sorbents is characterized by a combination of densely crosslinked segments, including microglobules, with lightly crosslinked regions. The swollen sorbents contain a significant amount of solvent. This ensures their high permeability for the protein macromolecules.

The synthesis of sorbent was carried out at 60 °C in an aqueous solution until a gel was formed in the form of a milky block. The specific fraction of the monomers was equal to 0.2, and 2,2′azo-*bis*(isobutironitrile) (AIBN) was used as an initiator. The obtained gel was heated at 80 °C for 45 min. After cooling, the copolymer block was reduced to pieces and successively treated with a 1.0 *M* HCl, demineralized water, 0.5 *M* NaOH, and washed with water until pH 6–7 was achieved. In the investigation we used fractions of heteroreticular sorbents with particle sizes of 100–200 μm.

The potentiometric titration of sorbents was performed using 0.1 *M* HCl or 0.1 *M* NaOH in 0.1 *M* KCl according to the method of separated weighed portions.

All sorbents were characterized with the help of measuring of specific volume in swollen state. The degree of sorbent structural stability has been estimated from the magnitude of relative swelling, i.e. $K_{rel}$ ($K_{rel}$—the ratio of swelling index $K_{sw}$ in the salt form to $K_{sw}$ of the nonionized form).

In dynamic experiments for the acceleration of the protein sorption processes and its approach to the equilibrium conditions besides the granular sorbents the composite sorbents (so-called cellosorbents) on their basis were used [10]. Cellosorbents are ion-exchanger dispersions impregnated in granules of highly porous cellulose. The dispersions of ion-exchanger particles are the sorbing centers, which are accessed by the transport channels system of the composite. The cellosorbents sorption capacities on proteins are one order of magnitude less than the sorption capacities of ion-exchange sorbents, which have formed the basis of the composite. The protein concentration in a grain during the sorption on cellosorbents gives 10–20 mg/ml; however, it is necessary to take into account that this is the concentration averaged over the grain volume, and the protein concentration in the disperse particles is much greater. The sharp improvement of protein sorption kinetics on the composite sorbents (decrease of sorption mean time in 50–200 times) allows to carry out the column processes with flow-rates considerably exceeding those permissible on sorbents serving as the base for composite formation. Cellosorbents were prepared with the sizes of granules equal to 100–315 μm. The properties of composite sorbents used in the investigation are given in Table 1.

Sorption processes were studied under static conditions at 22 °C. Solutions (10 ml) with various concentrations of protein were poured into glass vessels containing weighed portions (10 mg) of the sorbent. After stirring for 48 h sorbent was saturated by protein. Protein concentrations in individual solutions were determined from optical density at a wavelength of 278 nm and protein quantities sorbed by 1 l of the sorbent in the swollen state were calculated.

The multicomponent sorption processes were studied in the model systems including the sorbent and the solutions of two proteins. The processes of sequential and simultaneous sorption were investigated in the static mode. In the case of sequential sorption after the equilibrium in the system of the sorbent–the solution of individual protein was achieved (first stage) the second protein was added to the solution, and when the sorption was terminated

Table 1
Properties of the composite sorbents

| Sorbent | Ionogenic group type | Total exchange capacity[a] (mg equiv./g) |
|---|---|---|
| CS-CMDE-7.5 | –COOH | 0.53 |
| CS-CMDHE-7.5-1 | –COOH | 0.30 |
| CS-CMDHE-7.5-5 | –COOH | 0.08 |
| CS-ADE-10 | $-N(CH_3)_2$ | 0.08 |
| CS-ADHE-10-4 | $-N(CH_3)_2$ | 0.025 |

Specific volume of cellosorbents–10 ml/g. Sorbents were synthesized on the base of heteroreticular sorbents from Table 3.

[a] Complete exchange capacity per 1 g of swollen sorbent.

(second stage) the protein concentrations of binary solution were defined. In the study of the simultaneous sorption the binary protein solution with equal weight concentrations was brought in contact with the sorbent. In order to determine protein concentrations in mixed solutions, RP-HPLC methods were used. Chromatography was carried out on a Milichrom microcolumn liquid chromatograph (Nauchpribor, Orel, Russia). Stainless steel column (2× 65 mm) was packed with LiChrosorb $C_{18}$ (Merck, Darmstadt, Germany). Eluent consumption was 0.1 ml/min, and the wavelength of the spectrometric detector was 210 nm. Effective protein separation was obtained using a stepped gradient elution of a mixture of acetonitrile (Kriochrom, Saint-Petersburg, Russia) and 0.3% aqueous phosphoric acid (Sigma, St. Louis, MO, USA) (acetonitrile content was 0, 10, 20, 30, 40 and 50%). Protein concentrations were determined from calibration plots of concentration vs. peak areas. These plots were constructed for standard protein solutions at concentrations of 0.1–1.5 mg/ml. The error of concentration value determined by this method was about 4%.

To perform dynamic experiments, we placed the sorbent in a steel column (2×62 mm) of the Milichrom chromatograph. The sorption was performed from a protein solution at a concentration of 1 mg/ml and a volumetric flow-rate of 5 μl/min. After the sorbent was saturated with the first protein, a solution of the second protein was passed through the column. To study the sorption of proteins from mixed solutions, a solution containing equal concentration of two proteins (with a total concentration of 2 mg/ml) was used. After the sorbent was saturated with proteins, the column was washed with water, and then proteins were desorbed by 0.3% orthophosphoric acid. It was previously shown that proteins under study should be completely desorbed under these conditions. The concentrations of proteins in the elution peak were determined by RP-HPLC.

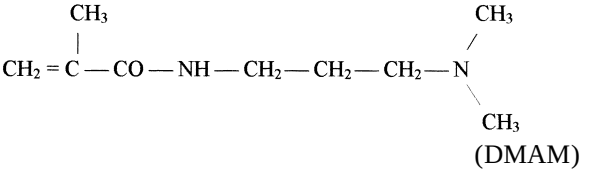
As model proteins we used bovine insulin (Plant of Medical Preparations, Saint-Petersburg, Russia), ribonuclease from bovine pancreas (Biolar, Olaine, Latvia), lysozyme from chicken egg white (Ferein, Moscow, Russia), α-lactalbumin from bovine milk (Sigma). All proteins were purified by additional fractionation on Sephadex-50. Some properties of the proteins are given in Table 2.

Table 2
Properties of the model proteins

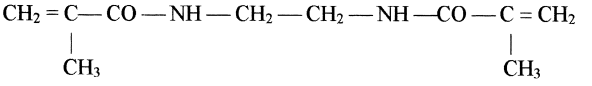
| Molecular mass ($\times 10^{-3}$) | Protein | Isoelectric point | Dimensions (nm) |
|---|---|---|---|
| 34.5 | Insulin-hexamer | 5.4 | |
| 13.7 | Ribonuclease | 9.4 | 3.8×2.8×2.0 |
| 14.2 | α-lactalbumin | 4.3 | 3.7×3.2×2.5 |
| 14.6 | Lysozyme | 11.1 | 4.5×3.0×3.0 |

## 3. Results and discussion

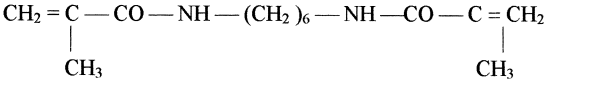
The synthesis of heteroreticular anion-exchangers with various content of ionogenic groups [–N$(CH_3)_2$] was carried out by tertiary copolymerization of *N*-dimethylaminopropylmethacrylamide (DMAM), *N,N′*-ethylenedimethacrylamide (EDMA) and hydrophilic nonionogenic monomer. As a hydrophilic nonionogenic monomer we used *N*-(2-hydroxypropyl) methacrylamid (HPMA). The chemical structure of the monomer is presented below.



(DMAM)



(EDMA)



(HPMA)

The exchange capacity of the anion-exchange sorbents was varied by changing the relation of DMAM and HPMA. The crosslinking agent content was constant and equal to 10 mol.%. Changing the relation between the ionogenic and nonionic monomer permits the control of the quantity of protein–sorbent electrostatic interaction.

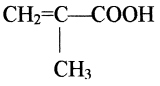
Analogously the synthesis of heteroreticular cation exchangers with various contents of ionogenic groups (–COOH) was carried out by tertiary copolymerization of methacrylic acid (MAA), EDMA and HPMA. The crosslinking agent content of this sorbent series was 7.5 mol.%.

Table 3
Properties of heteroreticular sorbents

| Sorbent[a] | Group type | Specific volume* (ml/g) | $K_{rel}$ | Total exchange capacity (mg equiv./g) | Sorption capacity, m $(M)\cdot 10^3$ |
|---|---|---|---|---|---|
| ADE-10 | $-N(CH_3)_2$ | 10.0 | 1.3 | 5.1 | 3.7** |
| ADHE-10-1 | $-N(CH_3)_2$ | 8.1 | 1.2 | 2.8 | 2.6** |
| ADHE-10-4 | $-N(CH_3)_2$ | 7.7 | 1.2 | 1.2 | 0.65** |
| CMDE-7.5 | –COOH | 7.4 | 1.5 | 9.7 | 8.4*** |
| CMDHE-7.5-1 | –COOH | 7.7 | 1.8 | 4.3 | 5.1*** |
| CMDHE-7.5-5 | –COOH | 8.5 | 1.2 | 1.3 | 1.5*** |

*, Data refer to the swollen ion exchangers in nonionized form; **, data refer to the lactalbumin sorption; ***, data refer to the lysozyme sorption.

[a] In the sorbent abbreviation the first figure refers to the amount of ionogenic monomer (mol%), while the second figure—to the amount of HPMA mol per mole of ionogenic monomer.



(MAA)

The properties of all heteroreticular sorbents used in the investigation are given in Table 3; the values of $K_{rel}$ of all sorbents are $<2$. This indicates the stability of the sorbent structure in the course of ionization.

The relationship between the sorbent capacity of the protein from the pH value for an equilibrium solution is presented in Fig. 1.

The aim of ensuring the maximum selectivity of the sorbent on target protein when developing the preparative separation methods conducts researchers and technologists to search for conditions of maximal protein binding in the elementary system: sorbent–protein solution. The conditions of maximal protein binding on ion-exchange sorbents depend, mainly, on the equilibrium solution pH value. The sorption of lysozyme and ribonuclease on carboxylic cation exchangers was investigated at pH 5.8. The insulin and lactalbumin sorption on anion exchangers was investigated at pH 8.0. The solution ionic strength in all cases gave 0.1 *M*.

It is supposed that the protein binding by sorbents of a given type occurs due to electrostatic interactions, as the experimental data show that the sorbent capacity of protein falls proportionally with a decrease of the ionogenic monomer content. Sorption isotherms for individual proteins are presented in Figs. 2 and 3. The protein binding with copolymers HPMA and EDMA is not observed [11].

An examination of lysozyme and ribonuclease interaction with a series of heteroreticular cation exchangers with various MAA content did not revealed the transfer from synergism to competition with decrease of sorbent ionogenic segments content. The results of protein sorption from the individual solutions and sequential sorption of two proteins are presented in Table 4. At sequential sorption the addition to the system of the second protein not only cannot displace the first one from the sorbent, but causes (being sorbed) an additional sorption of the first protein; i.e. a synergestic sorption is observed. The sorption capacity of cation exchangers decreases with the decrease of MAA content, but the binding still remains strong. Apparently, such effect is the consequence of irregular distribution of MAA in the sorbent bulk. The presence of polymer MAA blocks gives the opportunity for multipoint binding of protein with sorbent matrices.

The similarity of DMAM and HPMA structures presuppose a more uniform distribution of ionogenic segments of heteroreticular anion exchanger matrices. The results of experiments on sequential and simultaneous protein sorption are presented in Table 5.

The protein sorption on ADE-10 occurs according to synergestic mechanism. At sorption on ADHE-10-1 an additional uptake of the first protein after the addition of the second protein is lacking, but at the same time there is no replacement. At the sequential protein sorption on ADHE-10-4 it is apparent that the process is determined by pure competition: the addition of the second protein to the solution results in the replacement of the previously sorbed protein. These data are similar to the results obtained in the

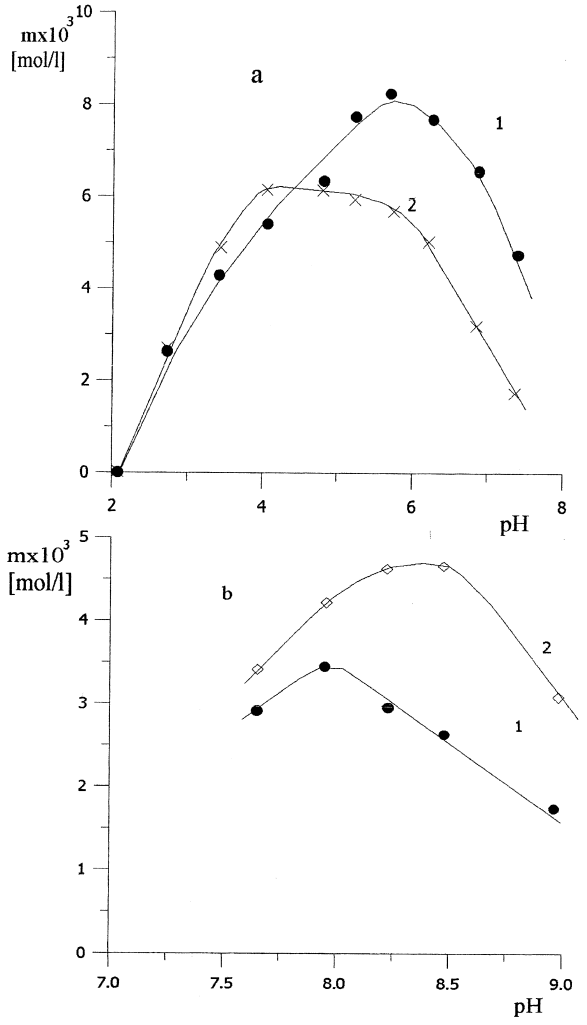



Fig. 1. Sorption of proteins as a function of the pH value of the equilibrium solution: (a) lysozyme (1) and ribonuclease (2) on CMDE-7.5, (b) lactalbumin (1) and insulin (2) on ADE-10.

experiments on insulin and ribonuclease sorption on the sorbents with various sulphonation degrees: with decrease of the ionogenic groups content in the sorbent the transfer from synergism to competition occurs.

There is no replacement of one protein by another in the dynamic experiments at consecutive passing of the protein solutions through the cation exchangers (both through initial heteroreticular sorbent and cellosorbents).

When the proteins are sorbed from a mixed

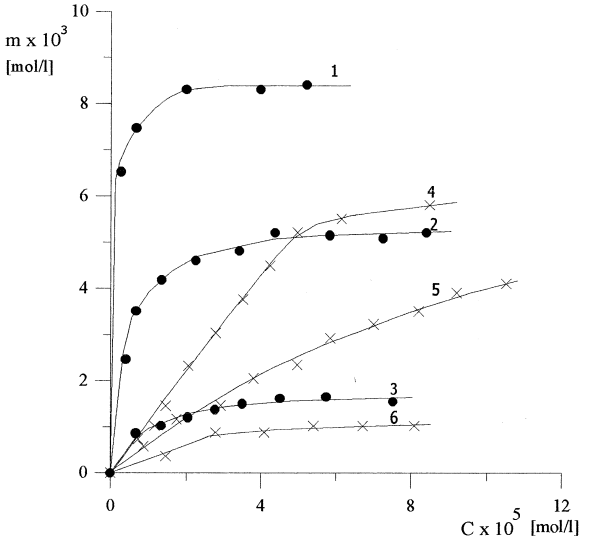



Fig. 2. Sorption isotherms for individual proteins (lysozyme-1,2,3, ribonuclease-4,5,6) on cation exchangers: CMDE-7.5 (1,4); CMDHE-7.5-1 (2,5); CMDHE-7.5-5.

solution, lysozyme–ribonuclease ratio in the sorbent phase approximates that in the solution phase. The similar behavior of the proteins is observed at sorption of insulin and lactalbumin on ADE-10 and ADHE-10-1. When passing the second protein through the column with ADE-10 and ADHE-10-1 sorbents (preliminary saturated by the protein) only a negligible amount of previously sorbed protein is replaced. Apparently, the less bound layer being sorbed due to the protein–protein binding is replaced and the second protein molecules occupy its place. There are two proteins in the sorbed state: the first one bound mainly to the sorbent amino groups and the second bound because of interactions between proteins. During the passing through the column with ADE-10 and ADHE-10-1 the solution of lactalbumin and insulin the sorbent phase composition at saturation appears to be close to the component composition of the solution. As a result the mixture of insulin and lactalbumin comes out in the elution peak on changing the pH value.

At sequential protein sorption on the ADHE-10-4 sorbent the spotting on the column of the second protein results in complete replacement of the first protein. At spotting on the column with such sorbent of the wide band of the mixture of insulin and

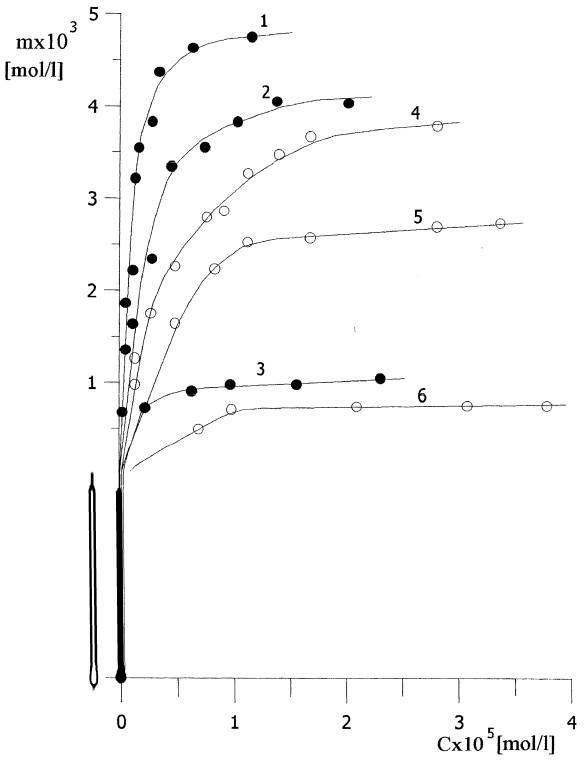



Fig. 3. Sorption isotherms for individual proteins (insulin-1,2,3, lactalbumin-4,5,6) on anion exchangers: ADE-10 (1,4); ADHE-10-1 (2,5); ADHE -10-4.

lactalbumin there is a gradual enrichment of the sorbent by insulin selectively sorbed in the given conditions.

The results of dynamic experiments with cellosorbents are similar to the results obtained for the initial heteroreticular anion exchangers.

The mixed solution of insulin and lactalbumin with equal weight protein concentrations (the total concentration 2 mg/ml or $9.2 \cdot 10^{-5}\,M$) have been passed through the column with cellosorbent. After the sorption up to saturation and washing by water proteins were desorbed. The fractional composition of elution peak from the CS-ADE-10 (a) and CS-ADHE-10-4 (b) cellosorbent after passing through the column 2 ml of the mixture of insulin and lactalbumin is given in Fig. 4. Apparently, the usage of the sorbent with high ionogenic group density for fine separation of proteins appears inefficient. The usage of the sorbent with lower density of ionogenic groups, on which the competitive conditions of multicomponent sorption in requirements of maximal interaction protein–sorbent are provided, allows saturation the sorbent with the selectively sorbed protein in the frontal mode.

## 4. Conclusions

The multicomponent protein sorption modeling in the ion-exchange sorbent–protein pair systems shows, that in conditions close to the maximal binding determined by the pH values of an equilibrium solution, the sorption processes on the ion exchangers with a high content of ionogenic groups are complicated by synergestic effects. The data show that the transfer from synergism to competition

Table 4
Sequential sorption of ribonuclease and lysozyme on cation-exchangers (protein concentrations in the sorbent phase in $M \cdot 10^3$)

| No. | Cation exchangers | KMDE-7.5 | KMDE-7.5-1 | KMDE-7.5-5 |
|---|---|---|---|---|
| 1 | Concentration of ribonuclease in the sorbent phase (after first stage) | 5.8 | 4.1 | 1.1 |
| | Concentration of lysozyme in the sorbent phase (after second stage) | 8.7 | 1.9 | 0.6 |
| | Change of ribonuclease concentration after addition of lysozyme | +3.6 | +0.8 | +0.3 |
| 2 | Concentration of lysozyme in the sorbent phase (after first stage) | 8.4 | 5.2 | 1.45 |
| | Concentration of ribonuclease in the sorbent phase (after second stage) | 7.5 | 2.1 | 0.45 |
| | Change of lysozyme concentration after addition of ribonuclease | +2.3 | +1.3 | +0.5 |

Table 5
Sequential and simultaneous sorption of lactalbumin and insulin on anion-exchangers (protein concentrations in the sorbent phase in $M \cdot 10^3$)

| No. | Anion exchangers | ADE-10 | ADHE-10-1 | ADHE-10-4 |
|---|---|---|---|---|
| | Concentration of lactalbumin in the sorbent phase (after first stage) | 3.6 | 2.7 | 0.65 |
| 1 | Concentration of insulin in the sorbent phase (after second stage) | 2.1 | 1.8 | 0.85 |
| | Change of lactalbumin concentration after addition of insulin | +1.3 | 0.0 | −0.45 |
| | Concentration of insulin in the sorbent phase (after first stage) | 4.75 | 3.95 | 1.05 |
| 2 | Concentration of lactalbumin in the sorbent phase (after second stage) | 1.8 | 1.25 | 0.15 |
| | Change of insulin concentration after addition of lactalbumin | +0.95 | 0.0 | −0.2 |
| 3 | Concentration of lactalbumin in the sorbent phase | 4.2 | 2.3 | 0.2 |
| | Concentration of insulin in the sorbent phase | 5.1 | 3.9 | 0.8 |

1,2, sequential sorption; 3, simultaneous sorption.

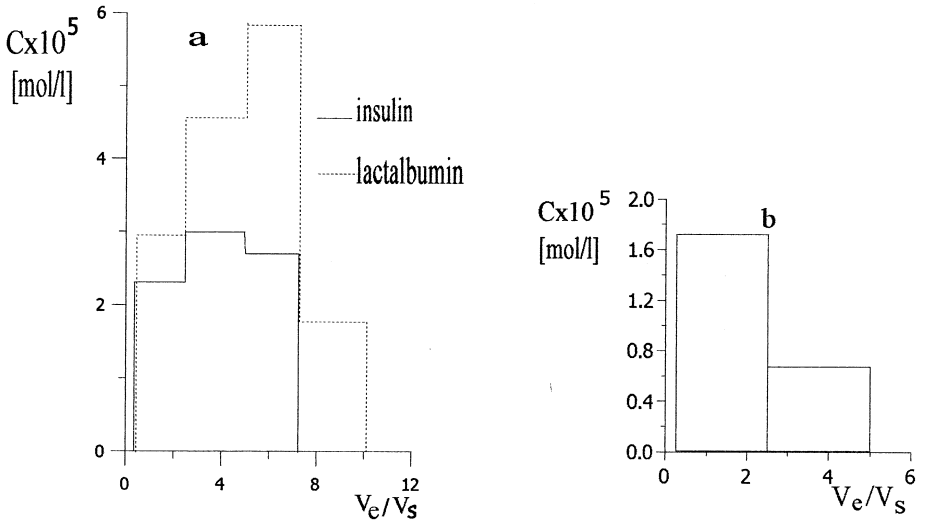



Fig. 4. Elution of proteins after sorption of insulin-α-lactalbumin mixture with equal weight concentrations ($C_{total}$ $9.2 \cdot 10^{-5}$ $M$) (a) CS-ADE-10; (b) CS-ADHE-10-4. $V_e$, eluant volume; $V_s$, sorbent volume.

can be achieved by utilizing the specially synthesized sorbents with decreased concentration of ionogenic groups. Thus the significant value has the uniformity of ionogenic group distribution in the sorbent volume. The maintenance of conditions for competitive mechanism of multicomponent sorption permits improvement in the efficiency of protein separation in ion-exchange chromatography.

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