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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON ALUMINA

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SUMMARY

Proteins can be separated on alumina with an aqueous mobile phase by ion-exchange and size exclusion chromatography and by a combination of both techniques. The pH dependence of the ion-exchange retention mechanism is explained on the basis of two similar concepts: the isoelectric point of the protein and the zero point of charge of the alumina surface. The possibility of influencing the surface properties of alumina by the choice of the buffer anion lends great flexibility to the system. However, the size exclusion selectivity is limited owing to the small choice of presently available aluminas. Examples are given that demonstrate the advantages of alumina for the separation of strongly basic proteins at high pH.

INTRODUCTION

The separation of proteins is one of the most challenging areas in modern liquid chromatography and is currently receiving great attention. Separations on soft gels[1] and classical ion exchangers[2] cannot be adapted to the high flow-rates used in high-performance liquid chromatography (HPLC). However, owing to the size of the molecules, diffusion rates are low and even with rigid supports acceptable efficiency can only be obtained at relatively low flow-rates. The complexity of the molecules gives rise to mixed retention mechanisms, which also have a negative influence on peak shape.

Various materials have been developed during the last 10 years, mostly directed to ion-exchange and size exclusion mechanisms. Pure silica is only partly successful, and has been replaced by modified silicas to avoid electrostatic interactions with the remaining silanol groups[3,4]. Great effort has been put into the development of silica-based materials with a large pore size range and efficient coverage of the active sites with hydrophilic layers. Such materials can be used in a gel permeation mode. For a review, see ref. 5.

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According to Regnier[6], high-performance ion-exchange materials can be divided into three classes: rigid organic resins, composite organic–inorganic packings and surface-modified inorganic materials. Regnier[6] cites numerous practical applications on such stationary phases.

In this context, alumina presents an attractive alternative. It has earlier been used for the separation and purification of enzymes and proteins by Zechmeister and Rohdewald[7] and Von Euler and Fonó[8]. Sato and co-workers utilized its size exclusion properties for the separation of polymers[9,10]. In earlier work[11,12], we demonstrated the different qualities of alumina with respect to ion-exchange capacities and pH stability. The purpose of this work was to investigate the possibilities of using alumina as a stationary phase for the separation of proteins.

THEORETICAL

Like many other metal oxides, alumina has amphoteric properties arising from the amphoteric nature of the AlOH groups present at the surface[13]. Depending on the pH of the surrounding solvent and its position at the crystal surface, the metal–oxygen bond may remain intact to liberate a proton or it may dissociate to yield a hydroxyl group. As a result, alumina retains cationic and anionic exchange properties over fairly broad and overlapping pH ranges. Nevertheless, it is possible to define a pH where the net charge of the surface is zero (zero point of charge, ZPC). At lower pH the net charge is positive and at higher pH it is negative.

Various methods have been employed to determine the ZPC, electroosmosis[14], streaming potential[15] and electrophoresis[16] being the most popular ones. Generally, the ZPC of an aqueous suspension of the oxide does not differ much from that of the solid metal hydroxide. However, the structure of the oxide and the presence of other ions coprecipitated during its preparation exert a large influence on the ZPC. Abramson *et al.*[17] stressed that the determination should be made in the absence of all ions other than hydrogen, hydroxyl or those inherent in the solid. Notably, specific adsorption of anions tends to increase the negative charge of the surface and shifts the ZPC to lower pH. The Al–anion–H sites are generally more acidic than the original AlOH groups.

For example, Clearfield[18] has shown that the presence of 10^{-5} *M* $H_2PO_4^-$ shifts the ZPC of dry γ-alumina quoted by Parks[19] as 9.2 by three pH units to a value of 6.2. This observation is confirmed by the results presented in Fig. 1. Over the pH range considered (4–6.5) brucine and dihydromorphine are positively charged, whereas the benzoate ions are negatively charged. The high retention of anions and the low retention of cations observed in the presence of an acetate buffer demonstrates that alumina is positively charged in this instance. Above pH 6 the retention of the anions drops rapidly and the ZPC is about 6.5 in the presence of acetate ions. Conversely, in the presence of a citrate buffer of similar pH, the anions are unretained and the high retentions of the cations show that alumina is now negatively charged. The ZPC is now shifted to a value as low as 3.5. In both instances the sodium concentration was kept constant.

The anion from the weak acid is responsible for the acidic shift. The more effective the ionic form is, the more the ZPC is shifted to a value where this form ceases to be present. Clearly the ZPC must be confined to a pH range where the

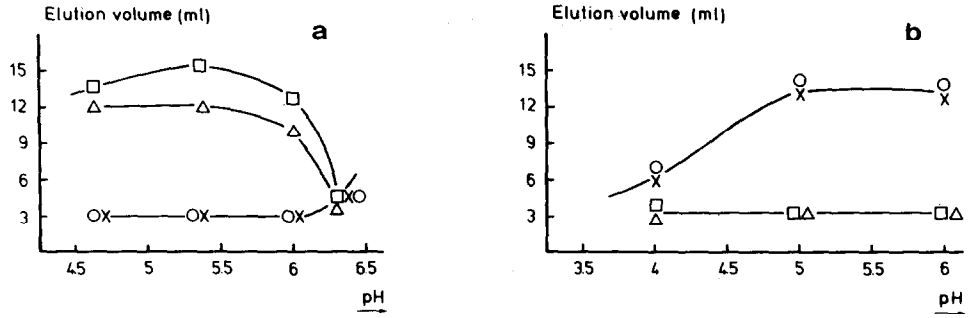

Fig. 1. Retention behaviour *vs.* pH of *m*-hydroxybenzoate (□), benzoate (△), brucine (×) and dihydromorphine (○). (a) Acetate buffer (0.10 *M* Na^+); (b) citrate buffer (0.02 *M* Na^+).

anion still exists. Remarkably, for all buffers used, the ZPC is localized at a pH value where the first ionized form is the most abundant. This is true not only for acetate ions (ZPC = 6.5) and for citrate ions (3.5) but also for phosphate (6.5), borate (8.3) and carbonate (9.2). As a result, the ZPC is invariably shifted to a pH where the buffer used has a low buffer capacity.

For the separation of proteins the concept of ZPC has a close parallel in the definition of the isoelectric point (p*I*), where the net charge of the polyelectrolytic protein is zero. For pH > p*I* the net charge of the protein is negative and for pH < p*I* it is positive. Consequently, the relative positions of the ZPC of alumina and the p*I* of a protein are of paramount importance for the ion-exchange retention observed. Two situations can be distinguished. If ZPC < pH < p*I*, then the positively charged protein is in contact with negatively charged alumina and will be retained by cation exchange. If, on the other hand, p*I* < pH < ZPC, the negatively charged protein experiences the presence of a positive alumina surface and will be retained by anion exchange. In either case, no ion-exchange retention should be observed for pH values outside this range. We may also expect the largest variation in retention when the pH is varied around either the ZPC of alumina or the p*I* of the protein.

So far, the argument has focused on the ion-exchange properties of alumina. Obviously this is not the only retention mechanism possible for proteins. The pore size of commercial alumina (6 nm for Alox T, 13 nm for Spherisorb) leads to size exclusion effects for proteins with molecular masses between 1000 and 20,000, provided that electrostatic attraction of the charged proteins is overcome by the addition of high concentrations of salts to the eluent. A calibration graph on Spherisorb A5Y can be obtained with polyethylene glycols with water as the mobile phase (see Figs. 2 and 3). This is impossible on bare silica. For details, see Experimental.

The combined application of size exclusion and ion exchange and both mechanisms separately provide an attractive increase in separation power. Finally, the possibility of varying the pH over a broad range, extending up to pH 12[11], makes alumina an attractive stationary phase for the separation of strongly basic proteins.

EXPERIMENTAL

The chromatographic equipment consisted of an M 6000 pump (Waters Assoc., Milford, MA, U.S.A.) and a Model 7125 injector (Rheodyne) with a 50-μl loop.

The proteins were detected with a UV detector (a Pye LC UV3 or a Waters Assoc. 450) at 220 or 280 nm depending on the UV transparency of the buffer. An R 401 differential refractometer (Waters Assoc.) was also used.

Columns of standard dimensions (25 cm × 4.6 mm I.D.) filled with 5-μm Spherisorb A5Y were obtained from Chrompack. According to Holdoway[20], the surface area measured by the BET nitrogen adsorption technique is 100 m^2/g. The mean pore diameter is 13 nm and the pore volume 0.36 $cm^3\ g^{-1}$.

Water was treated with ion-exchange resins and carbon filters after distillation. The buffers used were of the highest quality available. Standard proteins were purchased from Sigma (St. Louis, MO, U.S.A.). Standard polyethylene glycols were purchased from BDH (Poole, U.K.).

The molecular weight calibration graph of Spherisorb A5Y was made by plotting the logarithm of the weight-average molecular weight ($\bar{M}_w$) of standard polyethylene glycol against the distribution coefficient, K, defined as

$$K = (V_R - V_0)/(V_t - V_0)$$

where V_R is the retention volume of the solute, V_0 the retention volume of a totally excluded substance (PEG 40,000) and V_t the retention volume of the totally permeating glycol. Water was used as the eluent. A chromatogram and the calibration graph are given in Figs. 2 and 3, respectively.

RESULTS AND DISCUSSION

A few examples will demonstrate the effect, discussed under Theoretical, of small pH variations around either the pI value of a protein or the ZPC value of alumina. For the former effect we use basic proteins and take pH $\approx$ pI > ZPC, so that alumina is negatively charged and acts as a cation exchanger.

When the pH is now decreased from a value slightly above to a value slightly below pI, the cation-exchange retention of the protein is greatly enhanced. This is observed for cytochrome C (pI = 10.6) and lysosyme (pI = 10.7) in a carbonate buffer (ZPC = 9.2), for trypsinogen (pI = 9.3) in a borate buffer (ZPC = 8.3) and for myoglobin (pI = 7.0) in a phosphate buffer (ZPC = 6.5). Obviously, this effect is protein specific in the sense that each protein has its own sensitive pH, whereas the charge of the alumina surface remains well defined.

Alternatively, when pH $\approx$ ZPC > pI, we observe an increase in anion-exchange retention for all acidic proteins when the pH is allowed to pass below the ZPC value of alumina. This is observed for bovine serum albumin (pI = 4.3) and ovalbumin (pI = 4.6) in a phosphate buffer around ZPC = 6.5. Similarly, these proteins and myoglobin (pI = 7.0) are strongly retained when the pH decreases below a ZPC value of 9.2 in a carbonate buffer solution.

The large variation in the ZPC of alumina with the nature of the buffer anion, from 9.2 (carbonate), through 6.5 (phosphate and acetate) to 3.5 (citrate), seems to offer attractive flexibility in manipulating the retention of proteins. Unfortunately, unlike the situation where the pH is varied around the pI, poor peak shapes are observed when the pH passes below the ZPC. This is illustrated by the example of myoglobin in Fig. 4. The unretained, sharp peak observed at pH 10.3 is not only

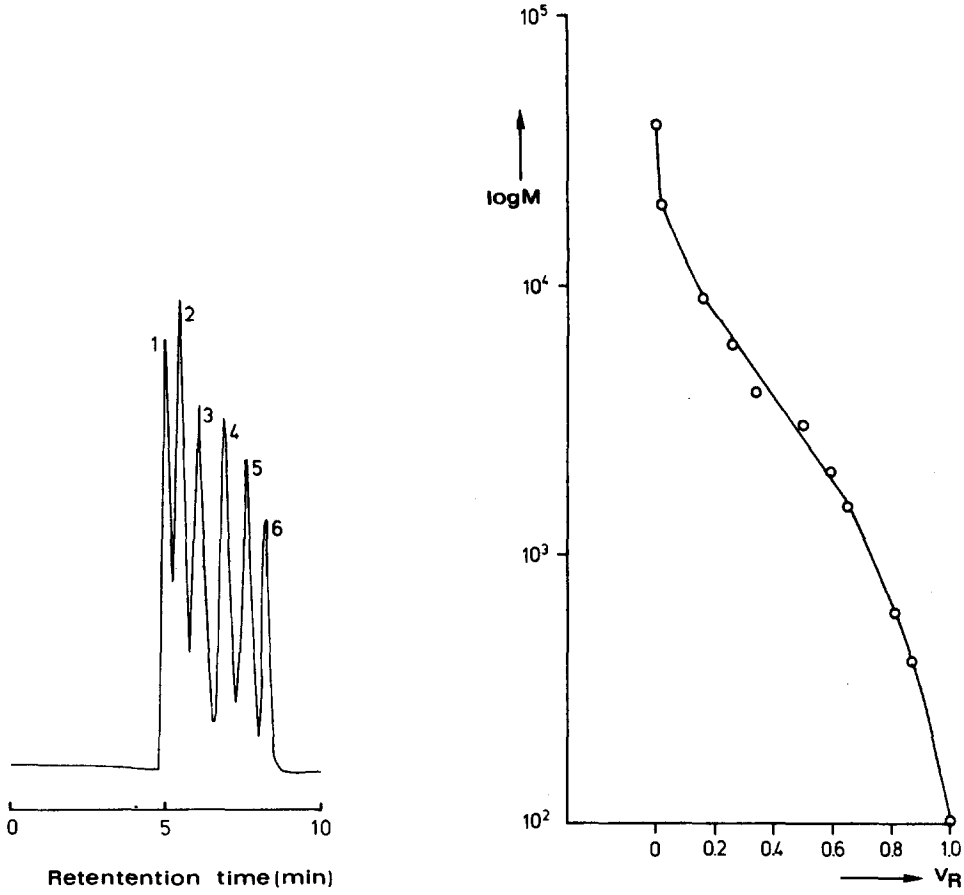

Fig. 2. Chromatogram of polyethylene glycol standards on a Spherisorb A5Y column (25 cm × 4.6 mm I.D.) with water as eluent. Molecular masses of 40,000 (peak 1), 9000 (peak 2), 4000 (peak 3), 2000 (peak 4), 600 (peak 5) and ethylene glycol (peak 6). Refractive index detection; flow-rate 0.4 ml/min.

Fig. 3. Calibration of polyethylene glycols on Spherisorb 5AY alumina. For conditions see Fig. 2.

retarded, but also severely broadened when the pH is gradually decreased to a ZPC value of 9.2 in a carbonate buffer. This behaviour is not unique for myoglobin but was observed for all protein retentions when the pH was varied around the ZPC.

One reason for this phenomenon may be that each buffer has a low buffer capacity at the pH of its characteristic ZPC. As a result, the concentrated zone of protein moving down the column may influence the pH at precisely the position of interaction with the alumina. Although this effect may occur, it was not always apparent in model experiments. We consider it more probable, therefore, that the alumina surface is inherently ill-defined at pH values around the ZPC and displays both cation- and anion-exchange properties. This may be the cause of the simultaneous presence of a sharp unretained and a broad retained peak in the chromatogram of myoglobin at pH 9.3 (Fig. 4).

We conclude from these initial experiments that variation of the pH around

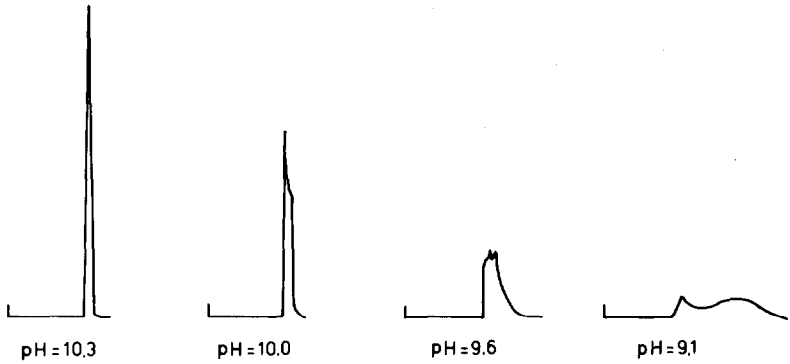

Fig. 4. Influence of pH on the peak shape in the retention of myoglobin. Carbonate buffer (Na^+ = 0.5 *M*).

the ZPC of alumina is of little, if any, chromatographic value. It is much more interesting to use a pH well above the ZPC, when proteins with p*I* < pH can be separated in the size exclusion (SE) domain, whereas proteins with p*I* > pH will be retained by cation exchange. A typical example is shown in Fig. 5, various proteins being separated at pH 9, obtained with a phosphate buffer that renders the ZPC of alumina equal to 6.5.

Indeed, the acidic proteins (1–6) with p*I* < 9 are eluted in reverse order of their molecular mass within the SE domain of the column, which is typical of the gel permeation chromatographic mechanism. The more basic proteins with p*I* > 9 are

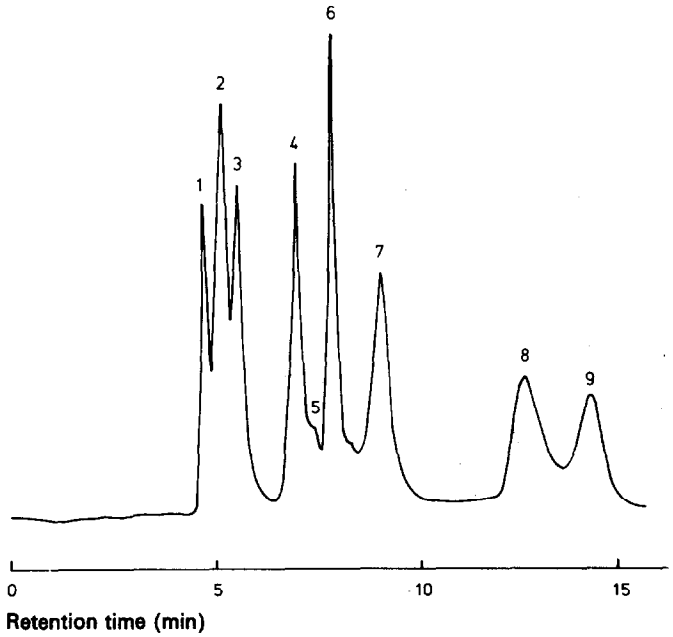

Fig. 5. Chromatogram of some standard proteins. Mobile phase: 0.25 *M* Na_2HPO_4 (pH 9); flow-rate, 0.4 ml/min. Solutes: 1 and 2 = bovine albumin; 3 = ovalbumin; 4 = myoglobin; 5 = unknown; 6 = arginine vasopressin (AVP); 7 = trypsinogen; 8 = lysozyme; 9 = chymotrypsinogen.

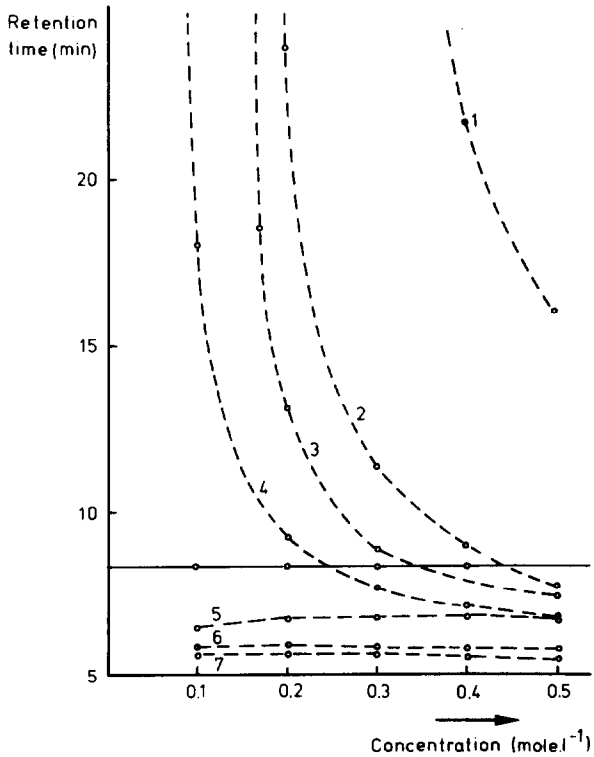

Fig. 6. Relationship protein retention and buffer concentration. Phosphate buffer (pH 9). 1 = Cytochrome *c*; 2 = adrenocorticotropic hormone (ACTH); 3 = lysozyme; 4 = trypsinogen; 5 = myoglobin; 6 = ovalbumin; 7 = pepsin.

eluted roughly in order of increasing p*I* on the basis of cation exchange. The retention of these proteins may be manipulated by appropriate choice of the pH provided that the pH exceeds the ZPC. In practice, the freedom of manipulation is somewhat restricted, because most buffers can only be used over two pH units and a change in the nature of buffer usually implies a change in the ZPC. In this respect, phosphate is highly advantageous, because its successive stages of ionization allow coverage of the pH range from 6.5 (the ZPC of aluminia in phosphate) to 11.

Fig. 6 shows the influence of the buffer concentration on protein retention at constant pH well over the ZPC. Whereas the negatively charged proteins with p*I* < 9 experience no influence, the retention of the positively charged proteins (p*I* $>$ 9) decreases dramatically with increasing buffer concentration. In fact, of the proteins shown in Fig. 6, three pass over from the cation-exchange domain into the SE domain when the phosphate concentration is increased from 0.25 to 0.5 *M*. Acceptable retention of cytochrome *c* (p*I* = 10.6) is obtained only at very high phosphate concentrations, close to the limit of its solubility. This is illustrated in Fig. 7. This effect of ionic strength is well known in protein separations and may be accomplished with any salt. For obvious reasons of instrument protection, it is advisable to use highly soluble salts, such as sodium chloride.

Gradient techniques are widely used in protein separations. Whereas pH gra-

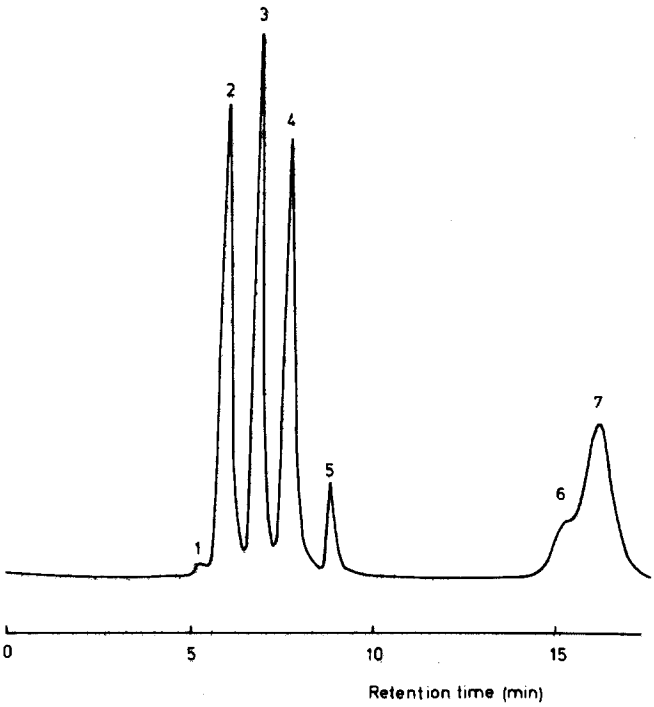

Fig. 7. Chromatogram of some standard proteins. Mobile phase, 0.5 *M* Na_2HPO_4 (pH 9); flow-rate, 0.4 ml/min. 1 = γ-Globulin; 2 = ovalbumin; 3 = myoglobin; 4 = lysozyme; 5 = solvent peak; 6 = unknown; 7 = cytochrome.

dients have only limited possibilities with aluminia, as argued above, salt gradients provide a simple opportunity of improving the separation power of the system. An illustrative example is presented in Fig. 8.

CONCLUSION

Aluminium oxide is an interesting stationary phase for protein separations, especially in the case of basic proteins. The combination of the size exclusion and the ion-exchange mechanisms allows excellent separations to be obtained by simple means.

The possibility of influecing the surface properties of alumina by the choice of the buffer anion lends great flexibility to the system. For basic proteins it was shown to be profitable to use a buffer such that the zero point of charge of the alumina surface is well below the isoelectric point of the proteins. However, this conclusion has wide applicability. For example, in a previous paper[12] we reported the separation of basic drugs (with p*K* values around 7) through cation exchange at pH 5. This is realised with a citrate buffer, for which the ZPC of alumina is 3.5.

With the alumina presently available the size exclusion selectivity is rather limited. It would obviously be advantageous to develop a material with wider pores and a broader range of pore size. The extreme stability of alumina in alkaline solu-

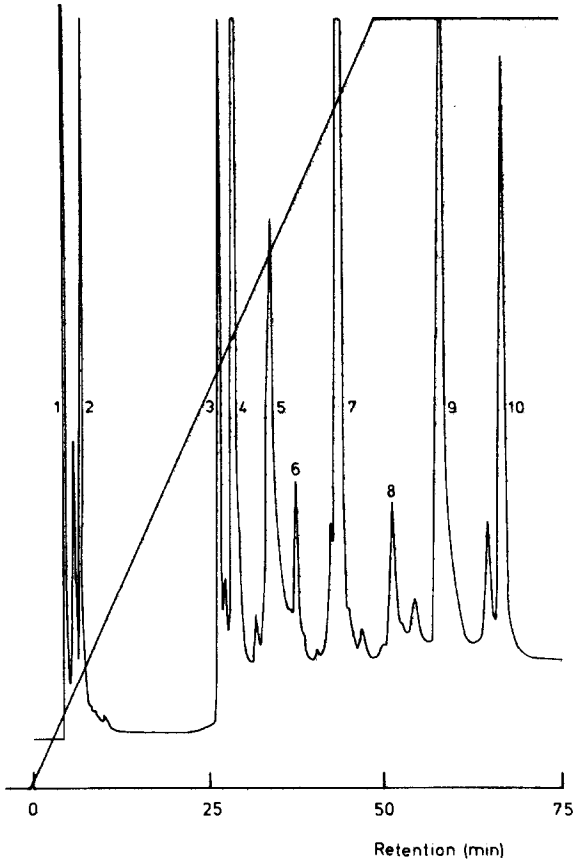

Fig. 8. Separation of standard proteins using a salt gradient at pH 9; 0–0.5 *M* Na_2HPO_4 in 45 min. 1 = Catalase; 2 = oxytocin; 3 = AVP; 4 = myoglobin; 5 = haemoglobin; 6 = trypsinogen; 7 = lysozyme; 8 = ACTH; 9 = tetracosactide; 10 = cytochrome *c*.

tions permits suppression of electrostatic interactions, so that the proteins are compressed in the SE domain.

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