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HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON UNCHARGED SEPHAROSE® DERIVATIVES

EFFECTS OF NEUTRAL SALTS ON THE ADSORPTION OF PROTEINS

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SUMMARY

In the presence of different neutral salts in high concentrations (ionic strength 1–3 *M*), pentyl-Sepharose was saturated with human serum albumin, and decyl-Sepharose with ovalbumin and phycoerythrin; the amount of protein bound to the adsorbent was taken as a measure of the hydrophobic interaction. The effects of the different ions on the adsorption of protein could, with one exception, be arranged according to the Hofmeister series.

As the adsorption might also be influenced by alterations in the protein conformation, caused by the neutral salts, the proteins were studied by circular dichroism. Circular-dichroism spectra showed that 3 *M* sodium bromide and 3 *M* sodium thiocyanate changed the conformation of human serum albumin and ovalbumin, whereas 3 *M* sodium chloride and 1 *M* sodium sulphate did not. The conformational changes observed with sodium bromide and thiocyanate were accompanied by decreasing protein–adsorbent interaction, except for ovalbumin in 3 *M* sodium thiocyanate.

INTRODUCTION

Hydrophobic interaction chromatography has attracted increasing interest over the last few years, and the literature in the field has been reviewed by Shaltiel[1] and Yon and Simmonds[2]. However, the term "hydrophobic (interaction) chromatography" has unfortunately been used for all experimental procedures in which an adsorbent with non-polar ligands is used, irrespective of whether these ligands are

uncharged or charged. The uncharged ligands give an interaction which is chiefly hydrophobic at all ionic strengths[3,4], whereas the charged ones give both electrostatic and hydrophobic interaction at low ionic strength[5–8] and mainly only hydrophobic interaction at high ionic strength. Since a characteristic of hydrophobic adherence is that it decreases with decrease in ionic strength, we shall use the term "hydrophobic interaction chromatography" when desorption is achieved by a decrease in ionic strength, *i.e.*, at all ionic strengths for uncharged adsorbents and, for the charged adsorbents, only at such ionic strengths where electrostatic interactions are negligible.

In hydrophobic interaction, many factors[4] affect the interaction between protein and adsorbent. We have previously tried to quantify the effects of the ionic strength, the hydrophobicity of the substituent, the degree of substitution and the ambient temperature on these interactions[3,4]. In this paper, we describe the effectiveness of neutral salts in promoting protein–adsorbent interaction. Among others, Von Hippel and Schleich[9] and Hatefi and Hanstein[10] have found for many systems that the degree of hydrophobic interaction in free solution differs significantly according to the type of neutral salt used. Nishikawa and Bailon[11] have described some effects of neutral salts in hydrophobic interaction chromatography. Other workers[12,13] have studied these effects at low ionic strength with adsorbents containing both charged and hydrophobic groups.

In this paper we define a neutral salt (as did Von Hippel and Schleich) as "a strong electrolyte which is significantly soluble in water, without bringing about a major change in solution pH" (see ref. 9, p. 423). Ions of such salts have been arranged according to their salting-in and salting-out properties (the Hofmeister or lyotropic series). Salting-in ions, such as Br^-, I^- and SCN^-, are often called "chaotropic"; Hatefi and Hanstein[10] have defined chaotropic ions as "those ions which favour the transfer of apolar groups to water".

Circular dichroism (CD) is widely used for studies of protein conformation, and we have used the technique to investigate whether or not the differences in adsorption to our gels exhibited by a particular protein in various salt solutions can be partly explained by alterations in its conformation.

MATERIALS AND METHODS

Chemicals

Cross-linked Sepharose® CL-4B was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and boron trifluoride ethyl etherate (48%) from Merck-Schuchardt (Hohenbrunn, G.F.R.). The pentyl and decyl glycidyl ethers were prepared according to Ulbrich *et al.*[14]. Charcoal (0.5–1 mm) was from KEBO (Stockholm, Sweden), human serum albumin (HSA) (max. 2% impurities) from AB Kabi (Stockholm, Sweden) and ovalbumin (max. 1% impurities) from the Sigma Chemical Corp. (St. Louis, Mo., U.S.A.); the HSA and ovalbumin were used without further purification. Phycoerythrin was prepared as described by Rosengren *et al.*[4]. All salts and solvents were of analytical grade.

Preparation of gels, buffers and samples

Pentyl and decyl glycidyl ethers were coupled to cross-linked Sepharose CL-4B[3,4], and the degrees of substitution of these gels were determined as described

previously[4]. For 100 ml of gel suspension, corresponding to 50 ml of settled gel, 1 ml of glycidyl ether and 1 ml of boron trifluoride ethyl etherate were used.

All salts were dissolved in the standard buffer (10 m*M* sodium phosphate of pH 6.8), and no correction of pH was made after addition of the salt (or sucrose). Before use, HSA was defatted by treatment with charcoal[15]. The different protein samples were equilibrated by dialysis with the buffered salt solutions and then centrifuged, and protein concentrations were calculated from the absorbance at 279, 280 and 500 nm for HSA, ovalbumin and phycoerythrin, respectively. The physical data used in the concentration and CD calculations are shown in Table I.

TABLE I

PHYSICAL DATA OF THE PROTEINS STUDIED

Protein	*Mol. wt. (dalton)*	$A_{1\,cm}^{1\%}$ *(absorbance units)*	*Literature reference*	
			Mol. wt.	$A_{1\,cm}^{1\%}$
HSA	66,200	$A_{279} = 5.31$	16	16
Ovalbumin	45,000	$A_{280} = 7.50$	17	18
Phycoerythrin	290,000	$A_{500} = 63.3$	19	4

Carbohydrate leakage from adsorbents

Two small columns were each packed with a sedimented bed (volume about 1 ml) of pentyl-Sepharose and equilibrated with four total volumes of buffer in a manner identical with that used in the chromatographic adsorption tests. After equilibration, each gel was washed with 20 ml of the buffer containing either 3 *M* sodium bromide or 3 *M* sodium thiocyanate, respectively. The salt concentration was reduced to avoid interference with the anthrone method by diluting the eluate and then concentrating it to its initial volume in an Amicon Ultrafilter Cell 202 with membrane UM 05; the final salt concentration of each eluate was 30 m*M*. The carbohydrate content was determined by the anthrone method of Burt[20] in an AutoAnalyzer (Technicon Instrument Co., Chertsey, Surrey, Great Britain).

Determination of protein concentrations at high concentrations of neutral salts

The protein concentrations were calculated from spectrophotometric measurements. The absorbance of HSA at 279 nm, ovalbumin at 280 nm and phycoerythrin at 500 nm was examined in the different salts at four protein concentrations, stock protein solutions in the standard buffer being diluted with the appropriate buffered salt solutions. The same dilutions were made in the standard buffer without any extra salt added, thus yielding reference values for the absorbance. The correction factor was expressed as the absorbance value obtained in the presence of a neutral salt divided by the reference value.

Protein binding in the presence of neutral salts

All experiments were performed at 22°. The column size was 1.2 cm × 1.0 cm I.D., the flow-rate was 3.8 ml/h and the amount of gel was 30 mg (dry weight), corresponding approximately to a bed-volume of 1 ml. The gel beds were equilibrated with the standard buffer solution containing different salts, then aliquots of the protein samples (of known concentration) were applied, application being continued

until the gel beds were saturated. After extensive washing, the amount of protein not adsorbed to the gels was determined by spectrophotometry. The amount of adsorbed protein was calculated from the difference in absorbance between the protein solution applied and the unadsorbed protein solution. The proteins were then desorbed with the standard buffer, and the dry weights of the gels were determined[4]. The binding of protein was expressed as mg of protein/30 mg of dry gel. Unsubstituted gels were treated analogously to assess any unspecific adsorption.

To investigate the effect of sucrose on the adsorption, pentyl-Sepharose was equilibrated with the standard buffer solution containing 1 *M* ammonium sulphate and 20% (w/v) of sucrose; the gel was saturated with HSA dissolved in this solution. The same buffer solution without sucrose was then pumped through the column. This procedure was repeated with unused gel, with the exception that the gel and the sample were initially equilibrated with buffer containing only 1 *M* ammonium sulphate before being washed with the same solution containing 20% of sucrose.

Circular dichroism

The CD spectra of HSA and ovalbumin in buffered salt solutions (ionic strength 3 *M*) were recorded at 25° with a JASCO J-20 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The instrument was calibrated with D-10-camphorsulphonic acid, and 1-cm rectangular cells were used. The protein concentrations ranged from 2 to 3 mg/ml for HSA and 0.9 to 1.1 mg/ml for ovalbumin. Because of the absorption of sodium thiocyanate at short wavelengths, CD spectra were recorded in 0.2-cm cells and the protein concentrations were 6 mg/ml for HSA and 3 mg/ml for ovalbumin.

The samples tested in the column experiments were also used for the CD measurements (after dilution to appropriate concentrations). The samples were filtered through a 0.3-μm Millipore filter before determination of the protein concentration and scanning. The molar-ellipticity values[21] were calculated from the mean of at least two scans and the data in Table I.

RESULTS

Carbohydrate leakage from adsorbents

For both the sodium bromide- and the sodium thiocyanate-containing buffers, the concentrations of carbohydrate in the washing solutions (20 ml) were less than 0.5 μg/ml, which represented the sensitivity limit of the AutoAnalyzer. The maximum leakage was therefore equal to 10 μg from a gel bed of 1 ml with a dry weight of about 30 mg. From these figures, it was concluded that the leakage was less than 0.03% of the original carbohydrate content; this figure does not include the contributions from trisaccharides, as they pass through the UM 05 membrane.

Determination of protein concentrations in neutral salts

Table II gives the correction factors for HSA, ovalbumin and phycoerythrin, each result being the mean value from four protein concentrations. As shown, the salting-in ions (Br^- and SCN^-) and the salting-out ions (SO_4^{2-} and Cl^-) had little or no effect on the absorption coefficients of the proteins. The correction factors were used for calculating the protein concentrations in different salt media.

TABLE II

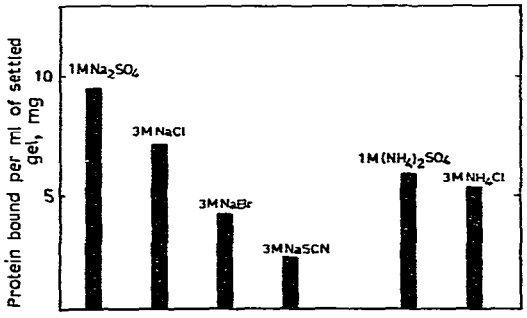
CORRECTION FACTORS FOR DETERMINING PROTEIN CONCENTRATION

Salt	*Concn. (M)*	*Correction factor*		
		HSA at 279 nm	*Ovalbumin at 280 nm*	*Phycoerythrin at 500 nm*
Na_2SO_4	1	1.05	1.05	1.00
NaCl	3	1.01	1.00	1.00
NaBr	3	1.04	1.00	1.00
NaSCN	3	1.01	1.05	—
$(NH_4)_2SO_4$	1	1.02	—	—
NH_4Cl	3	1.04	—	—

Protein binding in presence of neutral salts

The amount of HSA bound to pentyl-Sepharose (degree of substitution 120 mmoles per mole of galactose) in buffered salt solutions of ionic strength 3 *M* is shown in Fig. 1, and Figs. 2 and 3 show the amounts of ovalbumin and phycoerythrin bound to decyl-Sepharose (degree of substitution 20 mmoles/mole of galactose). The ionic strengths of the buffers used for ovalbumin and phycoerythrin were 3 *M* and 1 *M*, respectively. Each salt–protein combination was tested at least twice, and the amount of protein bound was expressed as the mean value. The standard errors in the different tests were less than ±5%. Larger deviations (±20%) were found for both HSA and ovalbumin in thiocyanate solutions. Phycoerythrin concentrations were determined from the absorbance at 500 nm; however, the visible spectrum of phycoerythrin was altered in 1 *M* sodium thiocyanate, and for this reason the adsorption of phycoerythrin to decyl-Sepharose was not tested in this salt.

Ammonium sulphate is commonly used for the salting-out of proteins; we therefore compared the effect of this salt with that of sodium sulphate. As shown in Fig. 1, less HSA was adsorbed in the presence of NH_4^+ than in that of Na^+; this fact



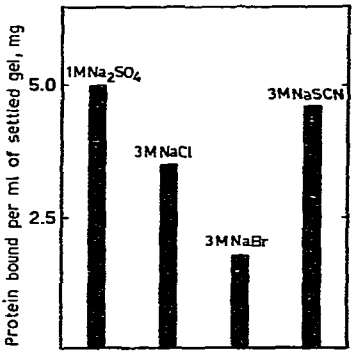


Fig. 1. Adsorption (expressed as mg of adsorbed protein per ml of settled gel equiv. to ≈ 30 mg of dry gel) of HSA to pentyl-Sepharose in different neutral salts at constant ionic strength. All salt solutions were buffered with 10 m*M* sodium phosphate of pH 6.8. Temperature: 22°.

Fig. 2. Adsorption of ovalbumin to decyl-Sepharose in different neutral salts at constant ionic strength. Conditions as for Fig. 1.

was confirmed when ammonium chloride was compared with sodium chloride (Fig. 1). The addition of 20% (w/v) of sucrose to 1 *M* ammonium sulphate had no pronounced effect on the binding of albumin to pentyl-Sepharose, as no measurable protein material was desorbed in either of the two experiments performed (for the experimental details, see "Materials and methods").

When unsubstituted gels were used, there was no adsorption under any of the selected experimental conditions.

Circular-dichroism spectra

The CD spectra of HSA and ovalbumin are shown in Figs. 4 and 5, respectively. Reference spectra recorded for all the salts showed only small differences, except for sodium thiocyanate, which absorbed strongly at wavelengths shorter than 268 nm. Because of the low signal-to-noise ratio below 268 nm, the validity of the spectrum in thiocyanate solution was doubtful. The spectra of HSA in 1 *M* sodium sulphate and 3 *M* sodium chloride (Fig. 4) appeared to be essentially unchanged when compared with the spectrum recorded in the buffer solution. With 3 *M* sodium bromide (Fig. 4), however, there were alterations in the regions of the HSA spectrum that corresponded to tryptophan (280–295 nm) and phenylalanine (255–270 nm)[21]. The same trends were observed in 3 *M* sodium thiocyanate (Fig. 4), although the changes were much greater than in 3 *M* sodium bromide. The ovalbumin spectra in 3 *M* sodium bromide or sodium chloride (Fig. 5) showed slight changes in the phenylalanine region. In 3 *M* sodium thiocyanate, the CD spectrum of ovalbumin showed little similarity with the spectra recorded in the standard buffer alone and in the other buffered salt solutions.

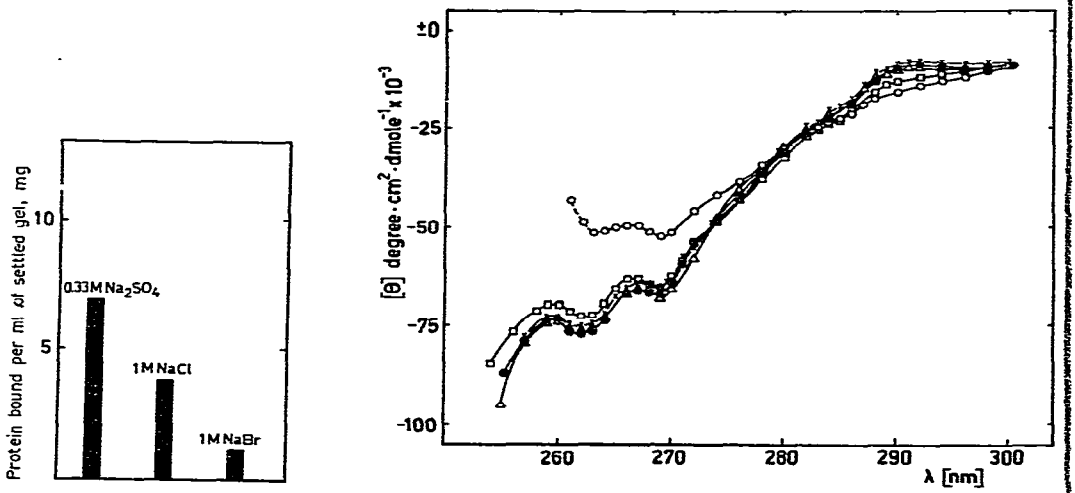


Fig. 3. Adsorption of phycoerythrin to decyl-Sepharose in different neutral salts at constant ionic strength. Conditions as for Fig. 1.

Fig. 4. The CD spectra of albumin in 1 *M* Na_2SO_4 (●—●), 3 *M* NaCl (△—△), 3 *M* NaBr (□—□), 3 *M* NaSCN (○—○) and 10 m*M* sodium phosphate buffer of pH 6.8 (×—×). All salt solutions were buffered with 10 m*M* sodium phosphate of pH 6.8. Temperature: 25°.

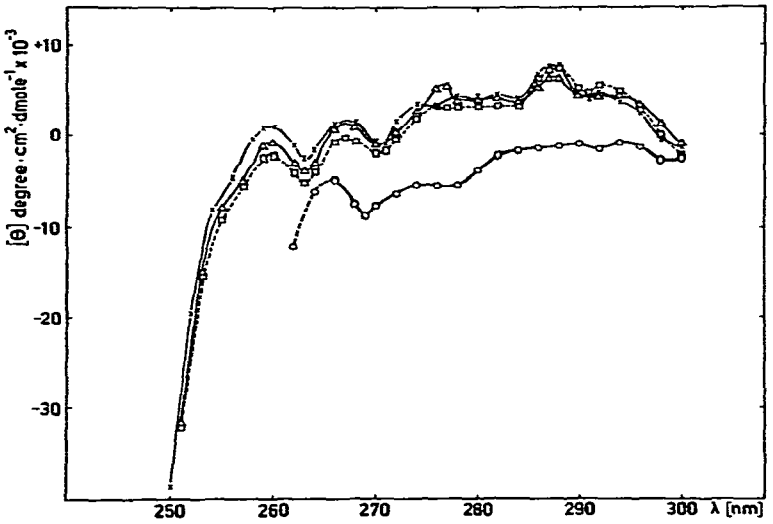


Fig. 5. The CD spectra of ovalbumin in 3 *M* NaCl (△—△), 3 *M* NaBr (□—□), 3 *M* NaSCN (○—○) and 10 m*M* sodium phosphate buffer of pH 6.8 (×—×). Conditions as in Fig. 4.

DISCUSSION

Experimental conditions

In our experiments, we have compared the binding capacities of various gels in solutions of different salts and ionic strengths. The amount of adsorbed protein was calculated from the difference between the weights of applied and unadsorbed protein. This indirect method was used because the proteins could not be completely desorbed simply by decreasing the ionic strength. In both the binding studies and the CD measurements, the need for correct determination of the concentration of the protein solution was important, and we found that spectrophotometry was the best method for our purposes. The method of Lowry *et al.*[22] could not be used, as it gave incorrect values in sodium thiocyanate solutions. Further, owing to the sensitivity of the method, the protein samples had to be diluted several-fold, which led to unacceptable dilution errors.

Thiocyanate and other chaotropic ions are known partly to solubilize agarose[23]. To avoid such solubilization of the gels, we used cross-linked Sepharose. The carbohydrate-leakage tests showed that practically no leakage occurred from the gels in 3 *M* sodium bromide or sodium thiocyanate. These results, together with the determination of the dry weight of each gel, proved that the decreased capacities of the gels in a medium of sodium bromide or thiocyanate were not caused by solubilization of the gel. The degrees of substitution of the gels were not affected by these salts.

Normally, about two fatty acids are bound to each HSA molecule[15]. The removal of the fatty acids ensured that the same number of binding sites for fatty acids was available in all salt solutions, as the binding sites probably interacted with the hydrophobic groups on the gels. The more lipophilic character of HSA compared with ovalbumin was reflected by the fact that the interaction of HSA with pentyl-Sepharose was of the same magnitude as the interaction of ovalbumin with decyl-

Sepharose (see Figs. 1–3). This difference was not due to the high degree of substitution of the pentyl-Sepharose, as ovalbumin did not bind to that gel under the conditions used.

Binding studies in relation to the Hofmeister series

The hydrophobic interaction is considered to be promoted by high concentrations of neutral salts[4,24]. When the effects of different salts (or rather ions) in promoting hydrophobic interaction or affecting protein structure are to be compared, the problem arises of comparing the effects of monovalent and polyvalent ions. Comparison can be made, for example, at constant salt concentration, constant ionic strength or constant space charge density. We kept the ionic strength constant in each series of experiments; if, instead, equimolar salt solutions had been the basis for comparison, the same trend would have been observed, although the difference in effect between the mono- and di-valent ions would have been even more pronounced.

The affinity of proteins for alkyl-Sepharose shows the same salt dependence as the salting-out of macromolecules (*i.e.*, it follows the Hofmeister series). This observation is in good agreement with the work of Schrier and Schrier[25], who have suggested that the salting-out of the ethyl ester of acetyltetraglycine by neutral salts (which also follows the Hofmeister series) is due to interaction with the non-polar part of the molecule. The correlation between the interaction of proteins with amphiphilic Sepharose derivatives and the salting-out of proteins has been suggested by, *e.g.*, Porath *et al.*[26], Rimerman and Hatfield[27] and Memoli and Doellgåst[28]. Yon and Simmonds[2] have proposed that this salt-induced hydrophobic interaction is independent of the nature of the salt, which is contradictory to our results.

Adsorbents with both hydrophobic and charged groups are also affected by neutral salts according to the Hofmeister series in instances when desorption can be achieved by increasing the ionic strength. Although ionic interaction is the major adsorbing force[5–8], chaotropic ions are more effective as desorption agents than, for example, are SO_4^{2-} or Cl^-.

Binding studies in relation to structural changes of the protein

The salting-out of proteins has been shown to be accompanied by changes in their conformational structure[29], and, as we used high concentrations of salts, conformational changes are likely to occur. The CD spectra of HSA and ovalbumin (Figs. 4 and 5) show conformational changes in the presence of high concentrations of sodium bromide and particularly of sodium thiocyanate, but not in solutions of sodium chloride or sodium sulphate. The changes in spectra caused by 3 *M* sodium bromide or sodium thiocyanate show the same trend as the binding studies, *i.e.*, an increasing structural change corresponded to a lower affinity for the gels [although ovalbumin in sodium thiocyanate (Fig. 2) was an exception to this rule]. The difference in adsorption behaviour of proteins in sodium sulphate and sodium chloride could not be explained by changes in protein structure alone, as no conformational changes could be observed by CD.

A change in the tertiary structure of a protein will probably be reflected in a different affinity for hydrophobic ligands. Except for ovalbumin in 3 *M* sodium thiocyanate, the interactions of HSA, ovalbumin and phycoerythrin with alkyl-Sepharose had a salt dependence comparable with the salting-out of proteins. By

contrast, the salt-induced conformational changes were apparently greatest in solutions of salting-in ions (see Figs. 4 and 5). We suggest that the high affinity of ovalbumin for decyl-Sepharose in 3 *M* sodium thiocyanate was due to the major conformational changes (which were detected by CD, as shown in Fig. 5). However, CD spectra could not be used between 250 and 300 nm to quantitate changes in protein structure. Spectra between 200 and 250 nm, where the amount of α-helix and β-structure can be estimated[21], could not be recorded, owing to the strong light absorption of 3 *M* sodium bromide and 3 *M* sodium thiocyanate.

Use of neutral salts and sucrose

The ions chosen by us represent both salting-in (SCN^- and Br^-) and salting-out (Cl^- and SO_4^{2-}) ions of the Hofmeister series. Provided that no pronounced salt-induced conformational changes appear in the proteins, other ions in the series will probably show the same trend in their effect on protein–adsorbent interaction*.

The adsorption of a particular protein to gels substituted with hydrophobic groups will depend on the ionic strength of the medium, the hydrophobicity of the attached groups, the degree of substitution, the temperature and the nature of the salt dissolved. In most experiments, we have changed the ionic strength and the hydrophobicity of the ligand in order to achieve conditions suitable for adsorption and desorption of proteins. Sometimes, one has to choose the salt with care, *e.g.*, when the solubility or the activity of the protein is decreased by the high ionic strength[6]. In other instances, any salt can be used, as long as the interaction is of a suitable magnitude. For uncharged gels, the use of chaotropic ions for enhancing adsorption is of little practical value, as the protein conformation is affected by such salts (which is not normally desirable) and the affinities of the proteins for the gels are lowered without giving better desorption facilities as compared with other salts (see Figs. 1 and 2). On the other hand, if both hydrophobic and electrostatic interactions are present, chaotropic ions may be of great importance, as desorption is more effectively promoted by these ions. From Figs. 1, 2 and 3, it may be concluded that sodium sulphate is the most suitable salt for adsorption, but the solubility of this salt is temperature-dependent. Also, proteins may be salted-out at lower ionic strengths of sodium sulphate than of other salts, *e.g.*, sodium chloride.

The addition of 20% of sucrose to the starting buffer had no effect on the interaction between albumin and pentyl-Sepharose. This does not agree with the results of Simpson and Kauzmann[31], who measured the change in the rate of optical rotation of ovalbumin in urea in the presence of sodium sulphate or sucrose and concluded that both solutes stabilized the protein to a similar degree. Our results are more consistent with those of Lewin (ref. 24, p. 207), who found very small differences in surface tension between 1 *M* monosaccharide solutions and water. The addition of neutral salts, on the other hand, will increase the surface tension, which (according to Lewin) will favour the adherence of hydrophobic groups (ref. 24, p. 208).

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* The trend would also be the same at other high ionic strengths above and below 3 *M*, as the hydrophobic interaction increases continuously with increasing salt concentration (see ref. 30, p. 9).

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