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SALT-PROMOTED ADSORPTION: RECENT DEVELOPMENTS*

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SUMMARY

Efficient fractionation of human serum proteins is accomplished by use of a series of tandem-coupled beds of group-affinity adsorbents. The general fractionation strategy for group fractionation of a complex protein mixture is discussed.

INTRODUCTION

The total number of different proteins present in the human organism has been estimated at 30 000—50 000 [1]. The number is even greater if we additionally consider all of the protein complexes present in our bodies. All other organisms, likewise, are composed of different proteins, nucleic acids and their complexes in enormous numbers. Obviously, for protein indexing and isolation of trace proteins, there is an urgent need not only to improve present separation techniques but also for the introduction of other methods based on, as yet, unexploited separation parameters.

Development of adsorption-based large-scale methods is lagging behind progress in analytical chromatography, especially fast techniques, which are now in vogue. Such developments and the need for a more rational approach to larger-scale separation strategies are also necessary prerequisites for acceptance of separation science as a reputable enterprise among our "consumers" and economic supporters. These arguments provide fuel for our present engagements in the field.

STRATEGY IN THE DEVELOPMENT AND APPLICATION OF PROTEIN ADSORBENTS

Protein indexing of a mixture, say human serum or brain extract, and subsequent purifications must be based on given coordinates. Since fractionation methods are seldom based on a single physically or chemically well defined

*This article is dedicated to the memory of my friend, Professor Nobuo Ui.

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separation parameter (such as charge or size), it may be practical to base the indexing on coordinates of operation variables (such as relative retention values, mobilities, sedimentation constants, etc.).

Since mapping and isolating protein components in complex biological samples nearly always necessitates the utilization of multi-dimensional fractionation, we must be able to efficiently design the appropriate number of consecutive fractionation steps, taking advantage of different separation parameters. Individual "dimension" refers to a given separation parameter and N-dimensional column chromatography refers to the use of N different separation parameters.

Variations in gel electrophoresis allow separations by net surface charge and molecular size. Utilizing these parameters in sequence, a protein mixture may be fractionated to give a two-dimensional electrophoretogram [2]. The efficiency in electrophoretic fractionation may be further increased by the addition of protein complex-formers to the buffer system [3, 4]. However, adsorption techniques are better suited than electrophoresis for large-scale fractionation and, frequently, also for the initial stages of a complex multi-step fractionation scheme. Protein mixtures may now be resolved more efficiently and in much shorter times, thanks to the impressive advances with rigid, highly permeable supports in column chromatography, especially high-performance liquid chromatography (HPLC). However, unless new separation principles are also introduced, I fear that we may soon reach a mechanistically limited stage in the development of HPLC methods and little will be gained by further refinements of the existing methods.

Suppose, in a hypothetical model experiment, we want to isolate 200 different proteins from a certain extract. It is doubtful whether any single method would ever be adequate for such a task. Even if it were possible, such a method would surely be complicated and thereby pose enormous obstacles during larger-scale implementation. The problems should be simplified greatly by application of a multi-dimensional fractionation scheme. Preliminary fractionation may conveniently be made by simple adsorption—desorption steps in a tandem column comprised of many beds with different adsorbents.

SOME PROBLEMS ASSOCIATED WITH GROUP-SELECTIVE AFFINITY ADSORBENTS

Three fundamental problems must be solved in the design of an ideal adsorbent based on a single separation function: (1) finding a matrix material of desirable physical properties with no affinity for any of the substances present in the sample to be fractionated; (2) finding a ligand substance to form the adsorption centre such that only one separation parameter will be operative (I doubt that this is strictly possible with any ligand!); (3) finding a method to introduce the ligand into the matrix without sacrificing those properties that define ligand specificity and affinity for the acceptor.

An adsorbent that is perfect in every respect will never be synthesized, but we may come rather close to the ideal. We, and others, have spent more than two decades of research on problems 1 and 3 outlined above. We are presently focusing on problem 2, with particular emphasis on the production of adsorbents for salt-promoted protein adsorption chromatography.

In my opinion, matrix-bound ligands should be of the simplest possible structure, with a predominant single-affinity function. However, there are several difficulties to overcome in the selection of even simple ligands. The connector or spacer arm will also influence the adsorption properties. The effect of an ionogenic group introduced by alkylamine has been much discussed [5—7]. A non-ionogenic type of linkage was used first by us to circumvent mixed-function effects of hydrophobic ligands [5]. However, we have recently discovered that O- and S-bridged octyl-agarose have slightly different adsorption properties, the latter gel being superior with respect to selectivity for serum albumin [8].

We are faced with further complications with phenyl-agarose, with its mixed-function ligand accounting for weak π-complexation superimposed on the comparatively much stronger hydrophobic interaction. We may produce very useful adsorbents by reinforcing the tendency to form π-complexes, e.g. by nitration of the ligand-forming substance [9], and without unduly increasing the hydrophobicity or introducing charge into the ligand.

Sulphonyl and thioether sulphur interact with some, as yet not identified, regions on the molecular surface of proteins. These regions are called thiophilic and the corresponding adsorbents with the thioether sulphonyl ligands are called thiophilic adsorbents [10] (T-adsorbents, Fig. 1). Other similar adsorbents with the structure R—S—, where R is a π-electron-rich aromatic or heteroaromatic group, are called thioaromatic adsorbents [11, 12]. Adsorbents with the structure R—SO_2—CH_2— or R—S—CH_2—CH_2—SO_2— are directed towards the thiophilic protein structures and are therefore thioaromatic T-adsorbents. With these notations, we may also use the expressions thiophilic and thioaromatic adsorption and affinities.

Salting-out adsorbents also include immobilized metal ion affinity (IMA) gels [13—16] among which iminodiacetate (IDA) gel and triscarboxymethylethylenediamine (TED) gel are most commonly used.

In contrast to protein interaction with ion exchangers, adsorption of proteins by the immobilized ligands listed in Fig. 1 increases with buffers containing a high concentration of water-structure-forming salts. Fractionation

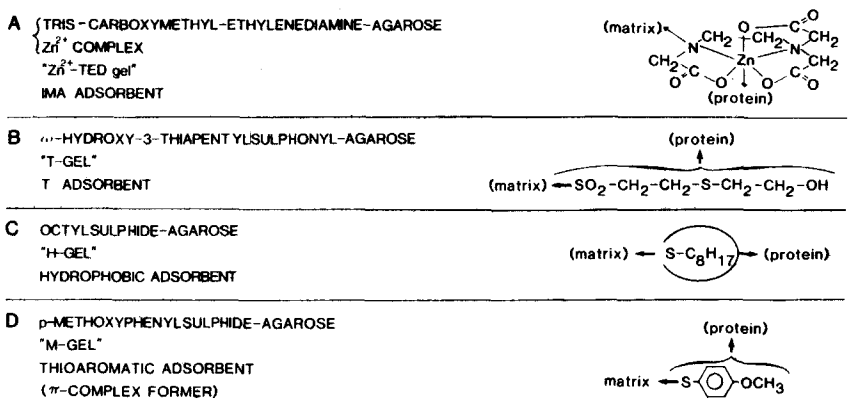

Fig. 1. Ligand structures of some representative salting-out adsorbents. The protein binding site is indicated.

at high ionic strength in such buffer systems offer some advantages. For example, microbial growth is prevented or suppressed and proteins are frequently stabilized against denaturation.

SALT-PROMOTED ADSORPTION

We discovered in 1973 [5] that hydrophobic adsorption is reinforced by adding sodium chloride or sodium phosphate to the buffer system. Furthermore, subsequent elution could be effected simply by deleting the salt. Further material was eluted by including ethylene glycol in the eluent. The paper described, probably for the first time, salting-out adsorption of proteins by the use of a well defined separation parameter: hydrophobic interaction.

About 40 years ago, Tiselius [17] separated proteins and dye components in ink by paper chromatography in strong sulphate and phosphate solutions. He did not give an explanation, but he coined the term salting-out chromatography to indicate a parallelism to precipitation. Since then, salting-out chromatography has been used and studied in various connections.

Zone precipitation [18] can be regarded as a form of salting-out chromatography. In this case, the protein components in the sample are thought to be repeatedly or continuously precipitated and redissolved upon migration through a gradient of suitable precipitating agent. This principle was first demonstrated for serum proteins chromatographed on a Sephadex column using a gradient of ammonium sulphate.

Permeable, hydroxyl-group-containing supports other than cellulose and Sephadex are also suitable for salting-out chromatography. The reader may consult the monograph of Scopes [19] for further references. Salt-promoted adsorption of proteins on different adsorbents is likely to be the rule rather than the exception. Failure to appreciate this fact earlier is not surprising in view of the prevalence of ion-exchange chromatography for proteins.

Lyotropic salts may be arranged in the Hofmeister series to indicate their relative abilities to salt-out or salt-in proteins from or into aqueous solutions. Such a series runs from precipitating (water-structure-forming) salts such as phosphates or sulphates to dissolving salts (chaotropics) such as iodides and thiocyanates. The salting-out behaviour can be quantitatively described by the empirical Setchenow equation: log (solubility) = $A - k \cdot$ (concentration of salt), where k is the so-called Setchenow constant, which is characteristic for the protein and the salt in question, and A is an integration constant.

A direct relationship between precipitation and adsorption has not yet been demonstrated. This open question might possibly be answered if a correlation can be found between the constant k and some suitable retention parameter for the same system.

Consider two possible extremes, schematically illustrated in Fig. 2. According to case A, each ligand is able to bind a single ligate to form a stoichiometric 1:1 complex. Assume further that the 1-protein has a molecular weight of 50 kD and consider the fact that most of the gels have a ligand concentration in the range 10—100 μmol/ml of swollen gel. These assumptions lead to an estimated maximum adsorption capacity of the order of 1 g of protein per ml of swollen gel. The experimentally found capacity is less by

two orders of magnitude, i.e. ca. 10 mg/ml or 10—20% protein per g of matrix. There is no capacity limit for case B, where the L. . . l complex serves as a nucleation centre for precipitation. We cannot decide definitely from such a simple argument which mechanism, A or B, might explain salt-promoted adsorption.

The relative adsorption-promoting efficiencies of potassium sulphate and sodium chloride for protein adsorption on H- and T-gels are reflected in the capacities of these adsorbents, shown in Table I. The H-gel binds more serum proteins (in this case, almost exclusively albumin) in 4 *M* sodium chloride than in 0.5 *M* potassium sulphate, whereas the opposite is true for the T-gel (which binds the immunoglobulins and α_2-macroglobulin). This behaviour is important for the selection of adsorbents and for planning an efficient strategy for the group fractionation of serum.

For the method to be practicable, recoveries in salt-promoted chromatography must be almost quantitative and the adsorption capacity must be easy to restore. Jennisen [20] has discussed in detail the adsorption hysteresis of hydrophobic agarose, and we have mentioned another phenomenon called delayed desorption [13]. The desorption apparently depends on the preced-

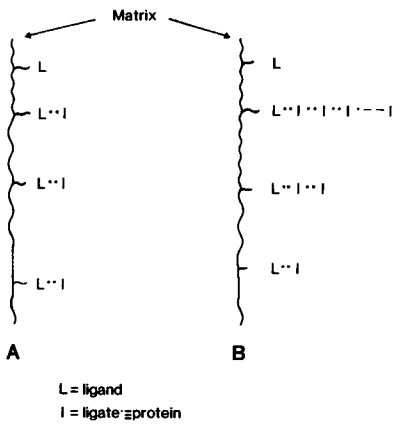

Fig. 2. Schematic representation of (A) ligand—ligate 1:1 complex formation and (B) multiple adsorption from ligand—ligate nuclei.

TABLE I

ADSORPTION CAPACITIES OF THE H- AND T-GELS FOR SERUM PROTEINS

Each column was equilibrated with 0.1 *M* triethanolamine—hydrochloric acid buffer (pH 7.6) containing the indicated concentration of salts. Column volume 10 ml; serum volume 2 ml.

Type and concentration of additional salt	Amount of protein adsorbed (% of applied)	
	H-gel	T-gel
None	10	8
4 *M* Sodium chloride	58	5
0.5 *M* Potassium sulphate	42	44

ing history. Each species of protein molecules is distributed on the adsorbent in states of varying strengths of interaction. Water-structure-forming salts strengthen the protein bonding and probably promote multi-point attachment. The distribution of adsorption complexes among different energy states broadens, which in turn counteracts separation by making it necessary to use stronger elution conditions. Incomplete desorption and broad protein distribution indicate that adsorption hysteresis is most severe for high-molecular-weight substances (as might be expected) and for hydrophobic adsorbents. It is not so much of a problem in IMA adsorption. Long contact times tend to increase the relative proportions of the more strongly adsorbed proteins. A further decrease in eluent polarity may thus be required to effect a more complete desorption of bound proteins. High concentrations of ethylene glycol or ethanol, or even extensive washing with strong alkali, may be necessary for regeneration of the adsorbent. A drastic but mild procedure for IMA adsorbents consists simply in removal of the adsorption site, i.e. the metal ion itself, by a strong chelator such as EDTA.

Proteins can be eluted in an ionic-strength-dependent manner from ion columns directly onto these types of gels. The fact that adsorption capacity can be manipulated easily by the concentration of salt, keeping other parameters constant, makes salt-promoted adsorption easily reproducible on any scale.

PROSPECTS FOR INDUSTRIAL APPLICATIONS

Industrial application of protein chromatography is still in its infancy, but I take the liberty to report a recent exploratory experiment that may illustrate the present state of the art in our hands.

Fig. 3 shows the tandem arrangement of salting-out adsorbents. Potassium sulphate (0.5 *M*) was added to human serum. A sample of 395 ml was applied to the tandem column, which had been pre-washed with starting buffer, i.e. 0.03 *M* Tris—HCl (pH 7.6) containing 0.5 *M* potassium sulphate. The tandem column was washed with this buffer at a flow-rate of 1.25 l/h until all non-adsorbed proteins have been eluted. The tandem column was then dismantled and each bed was eluted separately to give a series of chromatograms as shown in Fig. 4.

At this stage, there are several possible ways to further fractionate the material adsorbed to the beds. For example, the beds may be assembled into simpler tandem arrangements for further chromatography of adsorbed proteins. In the experiment referred to here, each section was separately eluted in only two steps (see legend to Fig. 3 for details). Selective, and therefore more efficient, desorption may also be tried.

The protein distributions in the eluates are shown in Fig. 4 and the gradient gel electrophoretograms in Fig. 5.

The order of beds usually dictates the outcome of the experiment, since there is a certain overlapping of the adsorbed species. For example, the T-gel adsorbs α_2-macroglobulin as well as immunoglobulins (the major components of human serum with thiophilic affinity). The zinc gel serves as a trap for α_2-macroglobulin and also adsorbs haemopexin. If the T-gel is con-

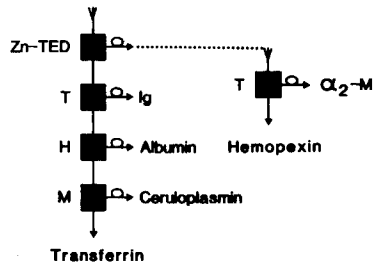

Fig. 3. Fractionation scheme for human serum. A dialysed serum sample (395 ml) is introduced at a flow-rate of 1.25 l/h into a Zn—TED bed at the top of a tandem column (gel type, see Fig. 1; total volume 5.2 l; prewashed in 0.03 *M* Tris—HCl buffer (pH 7.6), 0.5 *M* in K_2SO_4) followed by sulphate-containing buffer until the effluent shows negligible absorption at 280 nm. The passed material (see Figs. 4 and 5) contains transferrin as the major component. The tandem column is dismantled and each section is separately eluted in two steps. Step I: deleting the sulphate from the buffer causes a release of the major portion of adsorbed proteins. Step II: remaining proteins are desorbed by a solvent containing 30% isopropanol and 70% 0.03 *M* Tris—HCl buffer (v/v). The chromatograms are shown in Fig. 4. The excellent group fractionation is demonstrated in Fig. 5. The major components in the eluted fractions are indicated in the scheme. It is also indicated how further fractionation of the adsorbed material in the Zn—TED bed is possible by attaching a "fresh" T-bed equilibrated with the sulphate—Tris buffer. Washing this with equilibrium buffer containing a competing ligand, imidazole for example, elutes proteins from the Zn-gel which in part will be adsorbed on the T-gel. Haemopexin will pass the T-gel and the α_2-macroglobulin, and minor components can be eluted from the T-gel by washing with sulphate-free Tris buffer. → Indicates passage of non-adsorbed material; ⇝ indicates adsorbed and subsequently eluted material.

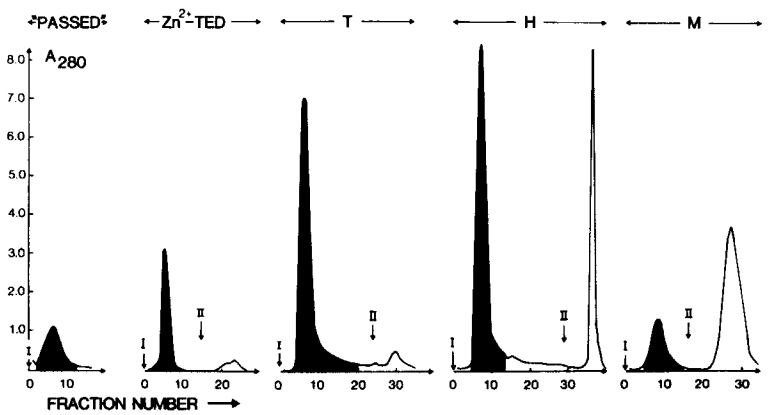

Fig. 4. Chromatograms obtained from the tandem column (left peak) and the separately eluted beds referred to in the text and Fig. 3. The arrows indicate the positions where the sulphate-free buffer (I) and the isopropanol-containing buffer (II) are introduced. Each fraction contains 0.2 l. The material in the black peaks was subjected to electrophoretic analysis (see Fig. 5).

nected after the Zn-gel, as was done in the above experiment, the desorbed fraction from the T-gel will thus consist of immunoglobulins that are eluted in at least 95% purity.

The adsorbed material on the Zn-gel may be further fractionated on a

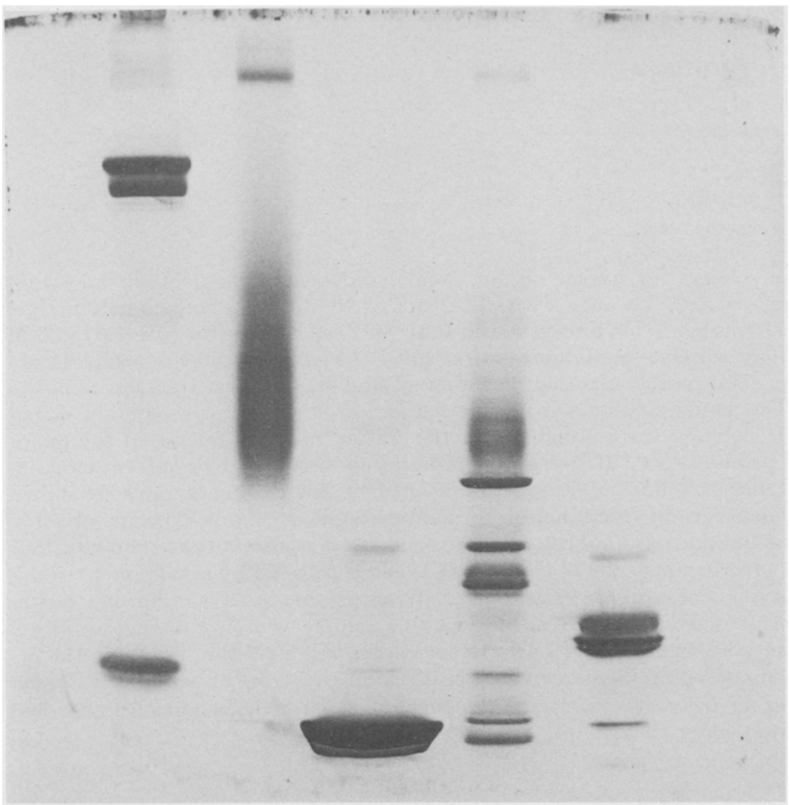

Fig. 5. Density-gradient gel electrophoretogram of fractions eluted with sulphate-free buffer referred to in Fig. 4 and the text. From left to right: TED-Zn, T, H, M and "passed".

T-gel by eluting the Zn-gel with imidazole dissolved in the sulphate-containing buffer and applying the eluate directly onto a T-gel. Since imidazole has no effect on thiophilic adsorption, α_2-macroglobulin will thus be adsorbed by the T-gel whereas haemopexin will elute from the column unadsorbed (Fig. 3).

The chromatography takes ca. 24 h and regeneration of the adsorbents can be made overnight. We are now trying to increase the capacity, shorten the time of the chromatographic cycle and make the process fully automatic.

In salt-promoted protein chromatography, the proteins partition between the adsorbent and solvent with the extreme distribution constants, either $K_D = 0$ or $K_D = \infty$. For one-sided partitioning, batch-wise adsorption—desorption procedures are perhaps more practical on a tonne or cubic metre scale than is chromatography. At the other extreme end of the scale (μl—μg or less), batch methods might again be simpler than column chromatography: a solution of the sample may conveniently be contacted with single particles or thin fibres of affinity adsorbents.

By using a column packed with M-gel of higher capacity than that shown in Fig. 3, one can obtain transferrin as the only major component in the unadsorbed, passed fraction. With two very simple bed arrangements, it is thus

possible to isolate from human serum five of the major components in a purity better than 90%; namely, albumin, immunoglobulins, α_2-macroglobulin, haemopexin and transferrin.

Within each group fraction a more efficient resolution and separation can be achieved by carrier displacement, gradient or affinity elution. IMA chromatography is particularly well suited for such modified fractionation programmes. Since molecular dimensions and electric charge have not been utilized in the experiments discussed here, further fractionation can be based on molecular sieving, ion-exchange chromatography or electrophoresis.

INTERACTING PROTEIN SIDE-CHAINS INVOLVED IN ADSORPTION

IMA adsorbents

Together with Sulkowski and Zhao, we have shown [13—16, 21, 22] that IMA adsorption on Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} is well suited for selective fractionation of proteins according to their content of exposed histidine side-chains. IMA chromatography can in fact be used to study the surface topology of a macromolecule with respect to those side-chains that interact specifically with metal ions (see also the report by Muszyńska and Porath [23]).

At neutral pH, the same IMA adsorbents also show a strong affinity for cysteine and a weaker affinity for tryptophan. Very weak but still significant affinities between immobilized Ni^{2+} and side-chains other than those mentioned have been revealed by model experiments [24].

Adsorption owing to coordinative complexation is strengthened by high concentrations of water-structure-formers. IMA adsorbents based on hard metal acids behave differently. Depending on the pH of the medium, Fe^{3+} chelate bonded to iminodiacetic (IDA) ligands behave either as a cation or an anion exchanger [25]. I should like to report exciting discoveries based on the fact that Fe^{3+} adsorbents interact with significant selectivity towards certain side-chains in the proteins. Certain proteins can then be specifically eluted by affinity displacement. Glycoproteins with terminal neuraminic acid can be selectively displaced from the Fe^{3+} adsorbent by neuraminic acid or pyruvic acid (which, in hydrated form, is similar to the C_1-surroundings of neuraminic acid) [26].

Andersson and Porath [27] have recently demonstrated the specificity of Fe^{3+}—IDA—agarose for phosphoproteins. Also, certain lanthanide gels interact specifically with phosphoproteins. Different components of ovalbumin can be adsorbed to Fe^{3+}—agarose at pH 3.1 and 5.0 and are eluted by applying a gradient of increasing pH. Elution occurs in the order of the number of phosphate groups per mole of protein (0, 1 or 2). The specificity of the Fe^{3+}—phosphate ester interaction was corroborated using phosphoserine, phosphothreonine and phosphotyrosine as models. In acid medium, these amino acids were strongly adsorbed even at high concentrations of salts.

Other adsorbents

The hydrophobic gels are thought to interact with hydrophobic patches on the molecular surface of proteins. In contrast, in the case of the thiophilic gels, nothing is known about the surface-located interaction sites on the pro-

teins. Significant adsorption of immunoglobulin G occurs only in strong water-structure-forming salt solutions. The importance of a more-or-less intact chain folding is revealed by the fact that the Fc fragment of IgG is the only peptide in a papain digest to show any detectable affinity for the T-gel. The Fc fragment is only slightly retarded under conditions where immunoglobulin G is strongly adsorbed. It should be mentioned that the T-gel is an excellent tool for the isolation of immunoglobulins from other species besides humans and for the purification of monoclonal antibodies [28]. Thioaromatic adsorbents may be considered as belonging to a sub-division of charge-transfer adsorbents [29].

IS THE SALT-PROMOTED ADSORPTION ENTROPICALLY DRIVEN?

The role of water in hydrophobic adsorption has been much discussed, with emphasis on the importance of the entropy gain accompanying the release of bound or structured water. Salt-promoted adsorption is a common denominator in all techniques discussed here. May we therefore indeed, by inference, conclude that an entropy increase of the same kind in all cases plays a similar or the same leading role? I believe that a discussion of the thermodynamics of salt-promoted adsorption is premature. May it suffice to point out that salting-out in the sense of expulsion of proteins out of the aqueous bulk phase into the vicinity of the matrix, is a prerequisite for strong adsorption. For example, a necessary condition for extensive protein adsorption to a T-gel is the presence of high concentrations of a water-structure-forming salt. Interaction leading to adsorption is facilitated by mass action and by the evocation of short-range forces governed by charge—dipole, dipole—dipole potentials, etc.

Salting-out and facilitated short-range-force-dependent interactions thus, together, seem to account for the common as well as the diversified salt effects observed in salt-promoted protein chromatography.

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REFERENCES

1 N.L. Andersson, J.J. Edwards, C.S. Giornetti, K.E. Willard, S.L. Tollaksen, S.L. Nance, B.J. Hickman, J. Taylor, B. Coulter, A. Scandora and N.G. Anderson, in B.J. Radola (Editor), Electrophoresis '79, Advanced Methods, Biochemical and Clinical Applications, Walter de Gruyter, Berlin, New York, 1980, p. 313.
2 P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007.
3 T.C. Bøg-Hansen, Anal. Biochem., 56 (1973) 480.
4 V. Horejsi, Anal. Biochem., 112 (1981) 1.

5 J. Porath, L. Sundberg, N. Fornstedt and I. Olsson, Nature (London), 245 (1973) 465.
6 S. Hjertén, J. Chromatogr., 87 (1973) 325.
7 M. Wilchek and T. Miron, Biochem. Biophys. Res. Commun., 72 (1976) 108.
8 F. Maisano, M. Belew and J. Porath, J. Chromatogr., 321 (1985) 305.
9 J. Porath and B. Larsson, J. Chromatogr., 155 (1978) 47.
10 J. Porath, F. Maisano and M. Belew, FEBS Lett., 185 (1985) 306.
11 J. Porath, F. Maisano and M. Belew, in B. Rånby (Editor), The Svedberg Symposium on the Physical Chemistry of Colloids and Macromolecules, IUPAC, Blackwell Scientific Publications, Oxford, 1986, Ch. 20, in press.
12 J. Porath, to be published.
13 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, Nature (London), 258 (1975) 598.
14 E. Sulkowski, Trends Biotechnol., 3 (1985) 1.
15 J. Porath and B. Olin, Biochemistry, 22 (1983) 1621.
16 J. Porath and M. Belew, in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983, p. 173.
17 A. Tiselius, Ark. Kem. Min. Geol., 26B (1948).
18 J. Porath, Nature (London), 196 (1962) 47.
19 R. Scopes, Protein Purification, Principles and Practice, Springer, New York, 1982.
20 H.P. Jennisen, J. Chromatogr., 159 (1978) 71.
21 E. Sulkowski, Y.-J. Zhao and J. Porath, to be published.
22 E.S. Hemdan, Acta Univ. Ups., Abstr. Uppsala Diss. Fac. Sci., No. 759 (1984).
23 G. Muszynska and J. Porath, Abstracts of the 6th International Symposium on Bioaffinity Chromatography and Related Techniques, Prague, Sept. 1—6, 1985, p. P43.
24 E.S. Hemdan and J. Porath, J. Chromatogr., 323 (1985) 255.
25 N. Ramadan and J. Porath, J. Chromatogr., 321 (1985) 93.
26 N. Ramadan, Dissertation, Acta University Upsaliensis 1984, Abstract No. 760.
27 L. Andersson and J. Porath, J. Chromatogr., in press.
28 N. Jontii, A. Larsson, M. Belew and J. Porath, to be published.
29 J. Porath, J. Chromatogr., 159 (1978) 13.