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SOME GENERAL ASPECTS OF HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

Characteristic features of hydrophobic interaction chromatography and factors influencing the choice of bed material and eluting agents are discussed in order to facilitate the use of the method.

INTRODUCTION

At the 9th International Congress of Biochemistry in Stockholm in July, 1973, I described hydrophobic interaction chromatography of ordinary water-soluble proteins, non-water-soluble membrane proteins and transfer RNA[1]. The experimental data will be presented in a series of articles. The present article deals with some general features of hydrophobic interaction chromatography based on several years of experimentation with the method.

THE CHOICE OF BED MATERIAL

The bed can either be composed entirely of hydrophobic groups or can be an inert matrix to which hydrophobic groups are attached. The difficult problem is to decide which hydrophobic groups to use. We have mainly employed compounds similar in structure to detergents or hydrophobic probes, two classes of substances that are known to interact hydrophobically with proteins. In fact, the idea of hydrophobic interaction chromatography arose when some years ago we began to study biological membranes and observed how efficiently they could be solubilized by detergents, particularly sodium dodecyl sulphate. We soon realized, however, that the dodecyl group attached to Sepharose interacted too strongly with the proteins, causing denaturation. Therefore we investigated the use of shorter alkyl chains such as butyl and pentyl groups.

It is, of course, very difficult to obtain a bed material that exhibits only hydrophobic interaction (nor is this always necessary or even desirable). Electrostatic, Van der Waals, and hydrogen bonding forces generally play more or less important roles. Therefore, we cannot completely exclude the possibility that the emergence of

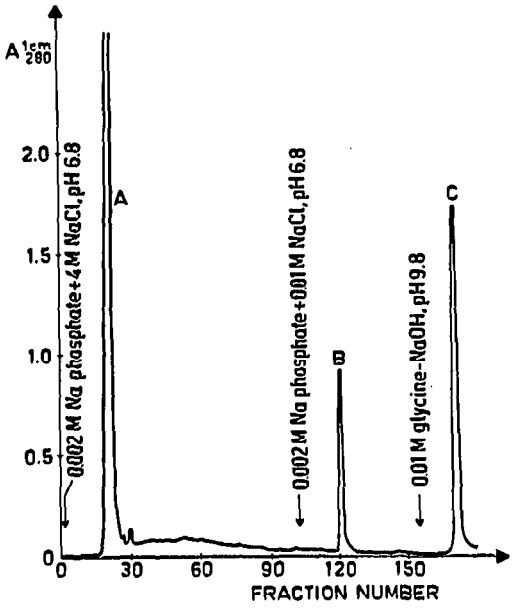

Fig. 1. Chromatogram illustrating that a decrease in ionic strength (peak B) and an increase in pH (peak C) can promote desorption. Column, pentyl-Sepharose 4B (1.1 × 29 cm); sample, 1 ml dialysed serum; flow-rate, 6 ml/h; fraction volume, 2.5 ml.

peak C in Fig. 1 and peak III in Fig. 2, both generated by an increase in pH, might be due to decreases in electrostatic interactions.

Bed material containing both hydrophobic and ionic groups

In ideal hydrophobic interaction chromatography, the partition should be entirely determined by hydrophobic interactions between the bed and the substances to be separated. Probably all supports used so far for this type of chromatography contained charged (for instance carboxylic and amino) as well as hydrophobic groups, and therefore exhibited superimposed electrostatic effects (*cf.* ref. 17).

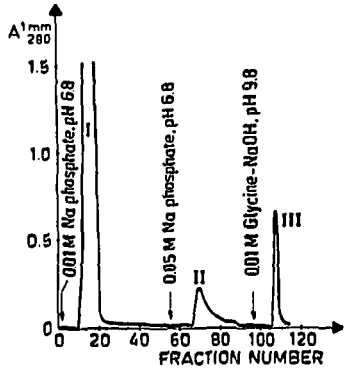

Fig. 2. Chromatogram illustrating that an increase in ionic strength can cause desorption (peak II), probably owing to the presence of ionic groups in the bed material. Column, heptyl-Sepharose 4B (1.4 × 40 cm); sample, 20 ml serum; flow-rate, 10 ml/h; fraction volume, 5 ml.

The support that has been most widely used for this type of chromatography appears to be benzoylated DEAE-cellulose, which was introduced in 1961 by Gillam *et al.*[2] for the fractionation of tRNA. A complement to benzoylated DEAE-cellulose, namely a hydrophobic macroreticular ion exchanger, Amberlyst A-21, has been proposed by Osterman[3]. Weiss and Bücher[4] attached aliphatic amines to a polyacrylic acid resin, which was then used for chromatography of mitochondrial membrane proteins. Yon[5] coupled diaminodecane to Sepharose® by the cyanogen bromide method of Axén *et al.*[6]. He also converted this positively charged aminodecylagarose to a negatively charged product by succinylating the free amino groups. Yon discussed alternative modes of operation for bed material containing both hydrophobic and ionic groups and used these techniques to purify an aspartate transcarbamoylase. Shaltiel and Er-el[7] have coupled aliphatic diamines to Sepharose, and Er-el *et al.*[8] have attached aliphatic monoamines to the same support. They have reported an extensive purification of a glycogen synthetase and a glycogen phophorylase with these beds. One must remember that when a monoamine such as butylamine is coupled to Sepharose by the cyanogen bromide reaction[6], the positive charge of the nitrogen is partly retained[9,10]. This is an obvious advantage when biologically active substances, such as enzymes are to be attached to a matrix, but is of course a nuisance when one wishes to prepare a hydrophobic bed without ion exchange properties. Quite recently, Hofstee[11] has described the use of Sepharose columns substituted with 4-phenylbutylamine or ε-aminocaproyl-D-tryptophan methyl ester. The chromatographic properties of these columns were also ascribed to both ionic and hydrophobic interactions.

We have utilized hydrophobic interaction chromatography for the fractionation of biopolymers using Sepharose to which the following substances have been coupled: alkyl amines of varying chain length, dansyl cadaverine, phenylethylamine, 1-amino-2-naphthol-4-sulphonic acid and phenyl-α-naphthylamine; the amines have been attached by the cyanogen bromide method[6]. All experiments described in this paper refer to columns prepared by this technique.

Bed materials containing only hydrophobic groups

From the above considerations it is evident that amines coupled by the cyanogen bromide method give rise to a charged matrix. When competing and superimposed electrostatic interactions are not desired, completely neutral media must be prepared. Detailed descriptions of such bed materials[1] (for instance poly(chlorotrifluoroethylene) and Sepharose substituted with alcohols) will soon be published.

Marsden[12], Eaker and Porath[13], and particularly Determann and Lampert[14], have shown that low-molecular-weight compounds such as alcohols and organic acids can, under certain conditions, exhibit hydrophobic interaction with neutral, tightly cross-linked dextran gels.

THE CHOICE OF ELUTING AGENTS

For desorption we have utilized some of the following four methods. The basis of the desorbing capability of these elution methods will be discussed only briefly, since the situation is very complex. Any alteration in the environment (for instance the addition of salts and alcohols), can cause unknown changes in the conformation and surface properties of biopolymers such as proteins.

A decrease in ionic strength

This is illustrated in Fig. 1 (peak B). If the hydrophobic beds contain charged groups an increase in ionic strength can cause desorption (see peak II in Fig. 2 and the section The ionic strength dependence). The eluting power of methods based on a decrease in ionic strength or temperature can be appreciated if one recalls that the basis of hydrophobic interaction is the gain in entropy of the system. The desorption of proteins from a hydrophobic bed by lowering the ionic strength has also been demonstrated by Porath *et al.*[17].

A decrease in temperature

Since hydrophobic interaction diminishes with a decrease in temperature one can imagine utilizing this effect to release adsorbed solutes. However, I have not obtained a convincing demonstration of such a temperature dependence; it is in any case too small to be a general method for desorption (see the section The temperature dependence).

A change in pH

Most proteins become more hydrophilic when the pH is increased from 7 up to 9–10, and should thus show less affinity for hydrophobic beds (see Fig. 3 and peak C in Fig. 1). For similar reasons a decrease in pH might also promote desorption

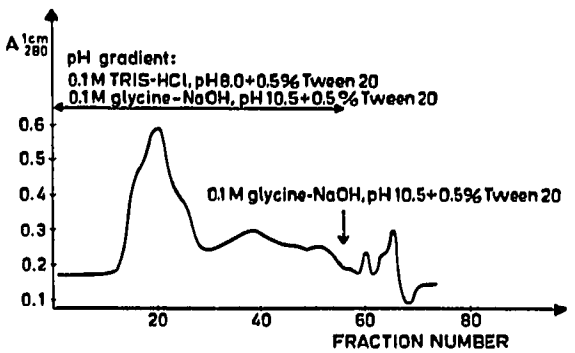

Fig. 3. The chromatographic behaviour of 4 mg of membrane proteins from *Acholeplasma laidlawii* on a column of phenylethyl-Sepharose 4B (1.5 × 26 cm). Flow-rate, 12 ml/h; fraction volume, 4 ml. Fraction 39 contained a protein of a high degree of purity, as judged by crossed immunoelectrophoresis.

(compare the fact that hydrophobic membrane proteins show an increased tendency for solubilization at extreme pH values). However, one must remember that electrostatic interaction with any fixed charges present in the hydrophobic bed might also be affected by a pH change.

Displacement

Elution can be accomplished by adding to the buffer a component that interacts so strongly with the bed that the adsorbed substances are displaced from the column. As displacers we have used aliphatic alcohols (for instance propanol, butanol and ethylene glycol*), aliphatic amines (for instance butylamine), and detergents (for

* Ethylene glycol was proposed by Prof. Morris in connection with the above congress lecture and has been employed by Hofstee[11] who refers to this alcohol as a polarity reducing agent.

instance Tween 20, Triton X-100, sodium dodecyl sulphate). These substances, obviously, interact also with the hydrophobic surfaces of the sample substances making them more hydrophilic, which also facilitates desorption. The latter interactions are sometimes so strong that the displacers cannot be readily removed from the substance(s) of interest. The alcohols and amines mentioned can be regarded as detergents, since they contain both a hydrophobic zone (the hydrocarbon chain) and a hydrophilic zone (the hydroxyl or amino group), and one can expect some denaturing action.

THE IONIC STRENGTH DEPENDENCE

A characteristic feature of all hydrophobic interactions is that they diminish upon decreasing the ionic strength of the medium. In ideal hydrophobic interaction chromatography a decrease in the ionic strength of the buffer might therefore promote desorption. This effect is illustrated in Fig. 1 (peak B) for a column of pentyl-Sepharose. The sample (serum) was dialysed first against 0.002 *M* sodium phosphate (pH 6.8)–4 *M* NaCl, then against 0.002 *M* sodium phosphate (pH 6.8)–0.01 *M* NaCl, and then again against the first buffer. After each dialysis the serum was centrifuged and the precipitate discarded. The emergence of peak B is therefore not accountable to solubilization of material precipitated at the higher ionic strength, which should also have given a much broader peak.

When amines are coupled to Sepharose by the cyanogen bromide reaction[6], positively charged groups are introduced, as mentioned above. These groups (and the negative sulphate and carboxylic groups in the Sepharose) confer ion-exchange properties on the columns. An example is shown in Fig. 2, where a highly purified γ-globulin fraction (see Fig. 4) is displaced by increasing the phosphate concentration from 0.01

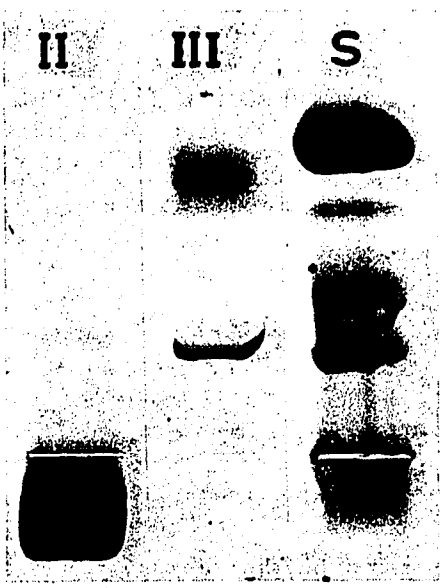

Fig. 4. Agarose gel electrophoresis of fractions II and III in Fig. 2 and of unfractionated serum (S). Buffer: 0.075 *M* veronal buffer, pH 8.6. The agarose was prepared according to method IIIb in ref. 15. As shown, fraction II contained γ-globulin and fraction III β-lipoprotein and an albumin component.

to 0.05 *M*. The presence of charged groups in the hydrophobic beds can thus enhance the separating power of the column (*cf.* ref. 5).

THE TEMPERATURE DEPENDENCE

A hydrophobic interaction should, in contrast to an electrostatic one, diminish when the temperature is decreased. This was tested with the hydrophobic Sepharose columns as follows.

A column (0.5 × 3 cm) of phenylethyl-Sepharose 4B was equilibrated at 20° with 0.075 *M* potassium phosphate (pH 7.5)–0.25 *M* KCl. Phycoerythrin, dialysed against this buffer, was applied until the whole column became red. Excess phycoerythrin was removed by elution with the above buffer. The whole column, thus saturated with phycoerythrin, was immersed in an ice–water bath. After 5 min temperature equilibrium was attained and the elution was continued with the same buffer, at 0°. In this elution some phycoerythrin (about 3%) was released. Since the effect was so small one cannot state with certainty that it was caused by hydrophobic interaction (similar unreliable results were obtained with alkyl-Sepharose columns). Furthermore, the results were not easily reproducible.

When chromatographing organic acids on Sephadex® LH-20, Determann and Lampert[14] have observed a temperature dependence which they ascribe to hydrophobic interaction.

THE RISK OF DENATURATION

In all chromatographic procedures there is a risk that the substances to be separated will undergo denaturation. This risk exists also in hydrophobic interaction chromatography, but is relatively small if the bed material is chosen to give only moderately strong interaction with the solutes of interest. For instance, when working with alkyl-Sepharose one should use comparatively short alkyl chains. This is illustrated in Fig. 5 for a run done on butyl-Sepharose 4B with a crude extract of histidine decarboxylase from mouse mast cell tumour. The activity was found in those fractions

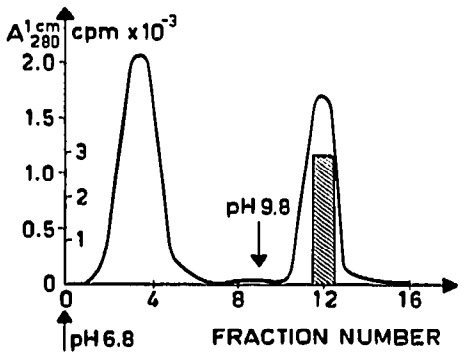

Fig. 5. The chromatographic behaviour of 15 mg of a crude extract of histidine decarboxylase on a column of butyl-Sepharose 4B (0.5 × 6 cm). Flow-rate, 8 ml/h; fraction volume, 0.65 ml. The shadowed area refers to the biological activity (in cpm)[16]. Recovery of biological activity, 66%. The chromatogram was developed with 0.01 *M* potassium phosphate buffer (pH 6.8) and 0.01 *M* glycine–NaOH buffer (pH 9.8).

that had been adsorbed to the column, indicating that the enzyme was not denatured by the interaction with the bed. When the experiment was repeated on columns of propyl-Sepharose and pentyl-Sepharose more material was eluted in the early and late part of the chromatogram, respectively, supporting the hypothesis that the solute–support interaction consisted at least partly of hydrophobic binding.

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