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FRACTIONATION OF PROTEINS ON SEPHAROSE AT LOW pH AND ON POLYTETRAFLUOROETHYLENE

STELLAN HJERTÉN

Institute of Biochemistry, University of Uppsala, Biomedical Centre, Box 576, S-751 23 Uppsala (Sweden)

CONTENTS

1. INTRODUCTION

Many of the observations and findings presented here on the fractionation of proteins at low pH on columns of Sepharose and polytetrafluoroethylene (PTFE) are several years old, but they have not been presented previously because we have no satisfactory explanation of the mechanism behind the separations obtained, except for the case of Sepharose. Therefore, the author would welcome comments regarding the nature of the interaction between the adsorbents and the proteins.

2. SEPHAROSE EQUILIBRATED IN BUTANOL–ACETIC ACID–WATER

Around 1970, we began our studies on the structure of biological cell membranes[1]. To solubilize the membranes efficiently we used detergents, which interacted strongly with the membrane proteins and brought them into solution (these studies gave us the idea of hydrophobic interaction chromatography, a method which is the subject of several papers presented at this symposium). However, there are some drawbacks to the use of detergents so we also investigated the use of organic solvents for solubilization of the membranes.

We found that a heavily opalescent suspension of erythrocyte membranes became completely transparent upon transfer to a mixture of equal volumes of butanol, acetic acid and water. When this membrane solution was applied to a column of Sepharose 4B equilibrated with the same butanol–acetic acid–water mixture, most of the membrane material was strongly adsorbed and could not be desorbed by a stepwise increase in ionic strength. Similar results were obtained when the acetic acid in the above experiment was replaced with morpholine. The pH of the butanol–acetic acid–water (1:1:1) mixture was 2.2, as measured with a glass electrode, and

that of butanol–morpholine–water (1:1:1) was 11.0. Accordingly, in an aqueous butanol solution there is a strong adsorption of membrane proteins to Sepharose at both low and high pH.

In order to utilize this interaction for the development of a new fractionation method, one must learn more about the separation mechanism. The first obvious question is whether the separations obtained are due to an interaction of an electrostatic nature. To test this we prepared agarose with a very low content of sulphate and carboxylic groups using method 3b in ref. 2 combined with a desulphation step[3,4]. The adsorption was then less, but still strong. This finding seems to indicate that electrostatic interactions contribute to the adsorption, but that additional forces are involved. When we added sodium acetate at a concentration of 0.06 *M* to the butanol–acetic acid–water mixture to suppress the electrostatic interaction (we could not use sodium acetate in a higher concentration than 0.06 *M* as the membrane proteins then started to precipitate) the adsorption was still considerable. This finding supports the view that the main interaction is not electrostatic in nature.

In fact, it is difficult to find a medium that desorbs the membrane proteins completely. The main problem is associated with the requirement that the medium must not precipitate the proteins. After many trial-and-error experiments we found that replacement of butanol with propanol greatly reduced the adsorption (Fig. 1). A glycine–sodium hydroxide buffer, pH 9.8, containing sodium dodecyl sulphate (SDS) appeared to displace at least part of the membrane proteins. There accordingly exist media that permit desorption of the proteins, a fact which opens up the possibility of developing a new separation method. Fractionation in the butanol–acetic acid–

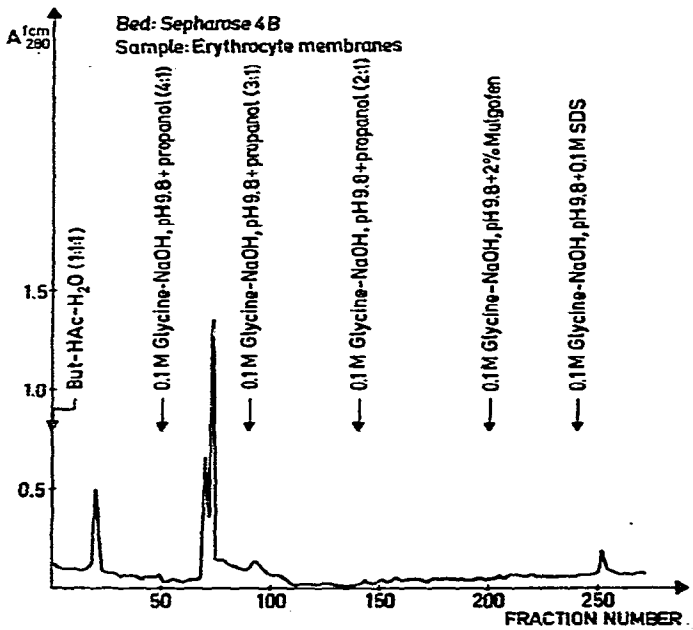


Fig. 1. Chromatographic behaviour of human erythrocytes (17 mg) on a column (28 × 1.4 cm) of Sepharose 4B, equilibrated with butanol–acetic acid–water (1:1:1). A similar experiment with an analysis of the chromatogram is given elsewhere[5].

water system is not discussed further here, but it is considered in the paper on the fractionation of membrane proteins[5].

3. SEPHAROSE EQUILIBRATED IN 0.01 *M* SODIUM ACETATE, pH 4.0

Water-soluble proteins, such as plasma proteins, are not soluble in butanol–acetic acid (morpholine)–water and could not be utilized as model proteins to study the nature of the adsorption of proteins to Sepharose in these media. However, proteins do adsorb to Sepharose at low pH even in the absence of butanol. This is exemplified in Fig. 2, which shows a fractionation of human plasma on Sepharose 4B. The column was equilibrated with 0.01 *M* sodium acetate, pH 4.0. The proteins were desorbed by stepwise increases in pH. When the experiment was performed in 0.06 *M* sodium acetate, pH 4.0, instead of 0.01 *M* sodium acetate, pH 4.0, no proteins were adsorbed. Nor was there any adsorption in 0.01 *M* sodium acetate, pH 4.0, when Sepharose was replaced with an agarose of extremely low sulphate and carboxyl content.

These observations indicate that the charged groups on the agarose are of importance for the adsorption. Agarose contains both sulphate and carboxyl groups. To determine whether either (or both) of these groups in Sepharose might be responsible for the adsorption of proteins at low pH, we studied the behaviour of human plasma on two ion exchangers, one with sulphonate [sulphoethyl(SE)-Sephadex] and one with carboxyl [carboxymethyl(CM)-Sephadex] groups.

From the chromatograms shown in Fig. 3, it is evident that plasma proteins are strongly adsorbed at pH 4 to SE-Sephadex but only slightly to CM-Sephadex. From these experiments one might conclude that the sulphate rather than the carboxyl groups of the Sepharose are mainly responsible for the adsorption. However, the adsorption of plasma proteins to CM-Sepharose (Fig. 4) is considerably stronger than to Sepharose (Fig. 2). Probably both the sulphate and the carboxyl groups therefore contribute to the adsorption to Sepharose at low pH.

The question then arises as to whether the interaction can be mainly ascribed to a true coulombic interaction or to other interactions such as hydrogen bonding. To test this we analysed by agarose gel electrophoresis (Fig. 5) the material corresponding to the different peaks obtained on Sepharose 4B. As shown, the serum proteins are obviously eluted in the order of their electrophoretic mobilities or, in other words, the order of their surface charge densities. The same result was obtained on CM-Sepharose. These findings indicate that the mechanism of adsorption of proteins to Sepharose at low pH is the same as in ion-exchange chromatography, *i.e.*, the interaction is of an electrostatic nature. Our investigations therefore seem to show that one cannot introduce a new separation parameter by performing a chromatographic experiment on Sepharose at low pH, as we had hoped. However, these negative results can also be taken advantage of in the following two ways:

(1) A very inexpensive ion exchanger could be produced based on crude agar. The result of such an experiment on a column prepared from a commercial agar is shown in Fig. 6 (the gel grains were obtained by pressing a 4% gel through a net as described in ref. 6).

(2) The fact that Sepharose acts as an ion exchanger at low pH opens up the possibility of combining ion-exchange chromatography and hydrophobic interaction

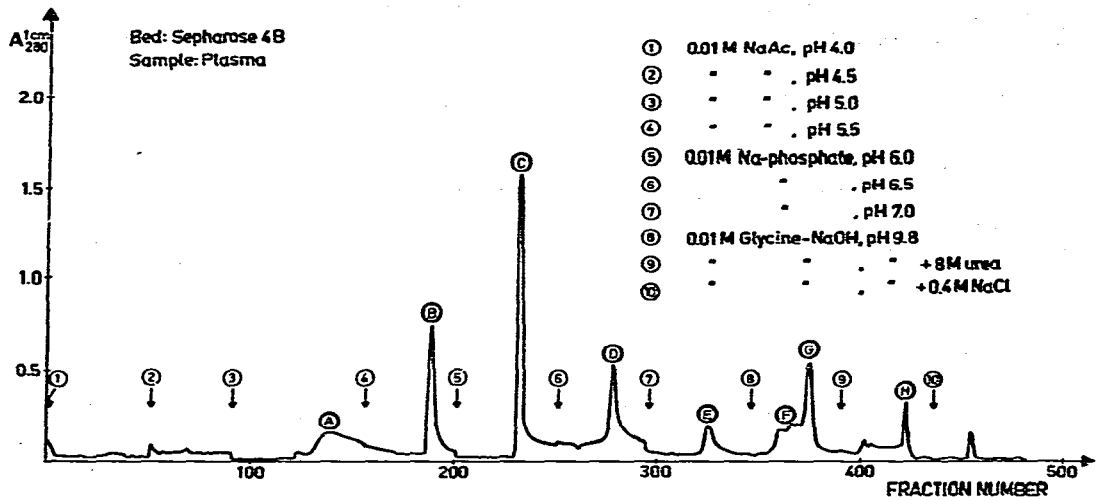


Fig. 2. Chromatographic behaviour of normal human plasma (1 ml) on a column (38 × 1.4 cm) of Sepharose 4B equilibrated with 0.01 *M* sodium acetate, pH 4.0. (The buffers given in this and other figures are those used for desorption. This and similar experiments were run at 20° and a flow-rate of 6 ml/h.)

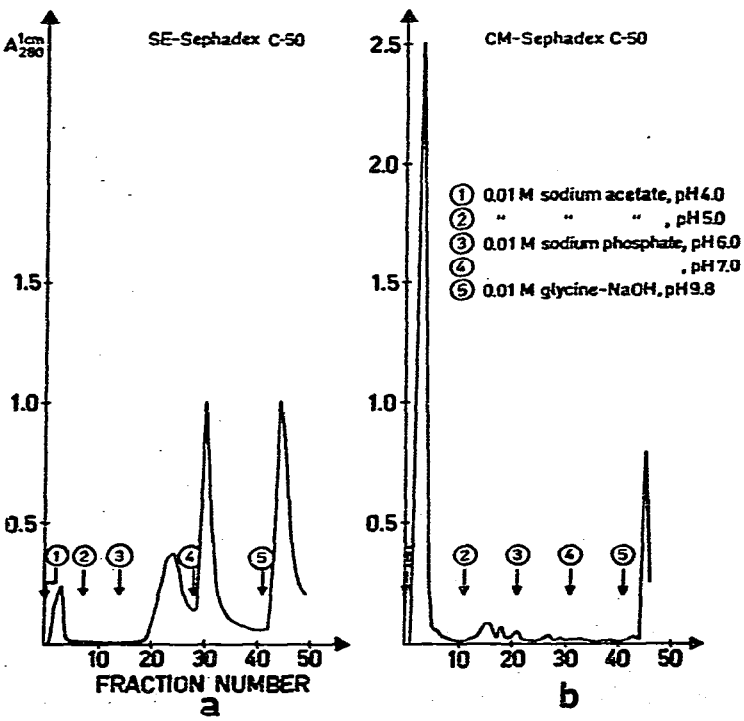


Fig. 3. Chromatographic behaviour of human plasma (0.2 ml) on a column (10 × 0.8 cm) of SE-Sephadex (a) and CM-Sephadex (b), run under identical experimental conditions. The capacities of the ion exchangers were 2.1 and 4.7 mequiv./g, respectively.

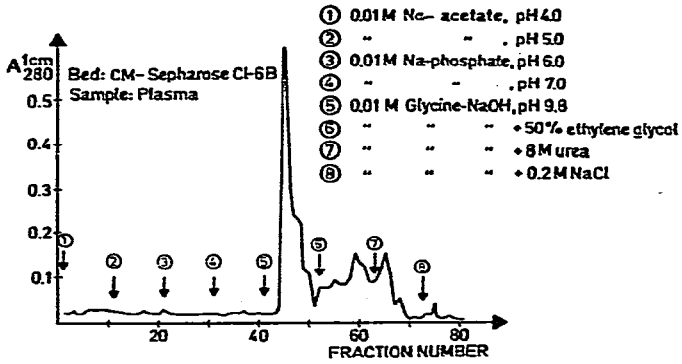


Fig. 4. Behaviour of human plasma (10.2 ml) on a column (10 × 0.8 cm) of CM-Sepharose CL-6B. The capacity of the ion exchanger was 0.12 mequiv./ml gel bed. The adsorption is much stronger than on CM-Sephadex (Fig. 3b) and Sepharose (Fig. 2).

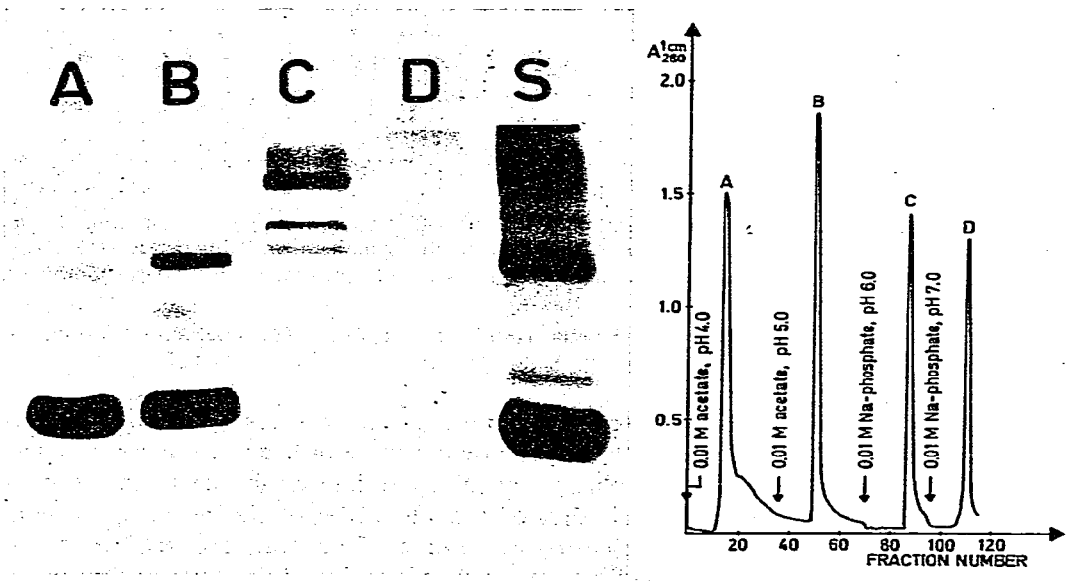
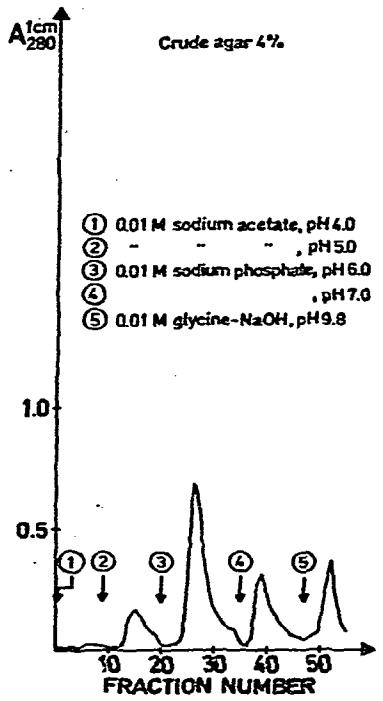


Fig. 5. Analysis by agarose gel electrophoresis of the fractions obtained when human plasma is fractionated on Sepharose 4B equilibrated with 0.01 *M* sodium acetate, pH 4.0 (the chromatogram is on the right). The proteins are eluted in order of decreasing mobilities, *i.e.*, the same order as is obtained in ion-exchange chromatography.

chromatography by running amphiphilic agarose columns at low pH. An example is shown in Fig. 7.

As mentioned, Sepharose behaves as an ion exchanger at low pH. Hydrogen bonds are probably not involved to a great extent. This was supported by the observation that proteins adsorbed to Sepharose in 0.01 *M* acetate, pH 4, were not desorbed when the buffer was supplemented with urea (8 *M*) or ethylene glycol (50%). The hydroxyl groups in the galactose units of the agarose appear not to be good proton donors for hydrogen bond formation. What groups other than hydroxyl groups might



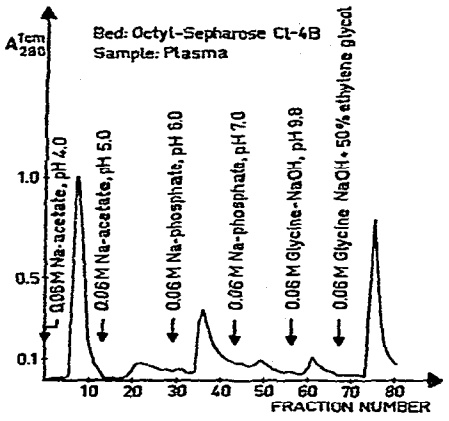


Fig. 6. Behaviour of human plasma (0.2 ml) on a column (10 × 0.8 cm) prepared from commercial agar and equilibrated at pH 4.0. The results is similar to that obtained on SE-Sephadex (Fig. 3a). The capacity of the column was about 0.3 mmole of sulphate per gram of agar (= 0.012 mmole of sulphate per ml gel bed).

Fig. 7. A combination of ion-exchange chromatography and hydrophobic interaction chromatography by performing an experiment on octyl-Sepharose, equilibrated at a low pH.

be suitable for hydrogen-bond chromatography? We have investigated whether allantoin (a urea derivative) coupled to Sepharose could be utilized for such purposes. The chromatogram obtained with plasma on this material was similar to that shown in Fig. 2. Agarose gel electrophoresis showed that the order of elution of the plasma proteins was the same as for Sepharose 4B (Fig. 5). The allantoin groups in the Sepharose derivative thus had no effect on the fractionation, *i.e.*, they did not act as sites for adsorption.

4. POLYTETRAFLUOROETHYLENE EQUILIBRATED AT pH 4 AND HIGHER

We have also investigated the possibility of adsorbing proteins to PTFE at low pH. This plastic powder was obtained from Imperial Chemical Industries, Great Britain, under the Trade-name Fluon, L 169 A.

A column (35 × 1.4 cm), packed with PTFE, was equilibrated with 0.01 *M* sodium acetate, pH 4.0, and 2 ml of human plasma, dialyzed against this buffer, were applied. The adsorbed proteins could not be desorbed by increasing the pH or the ionic strength or by adding urea to the buffer; only SDS displaced the proteins from the column (Fig. 8). The proteins are accordingly very strongly adsorbed to PTFE if

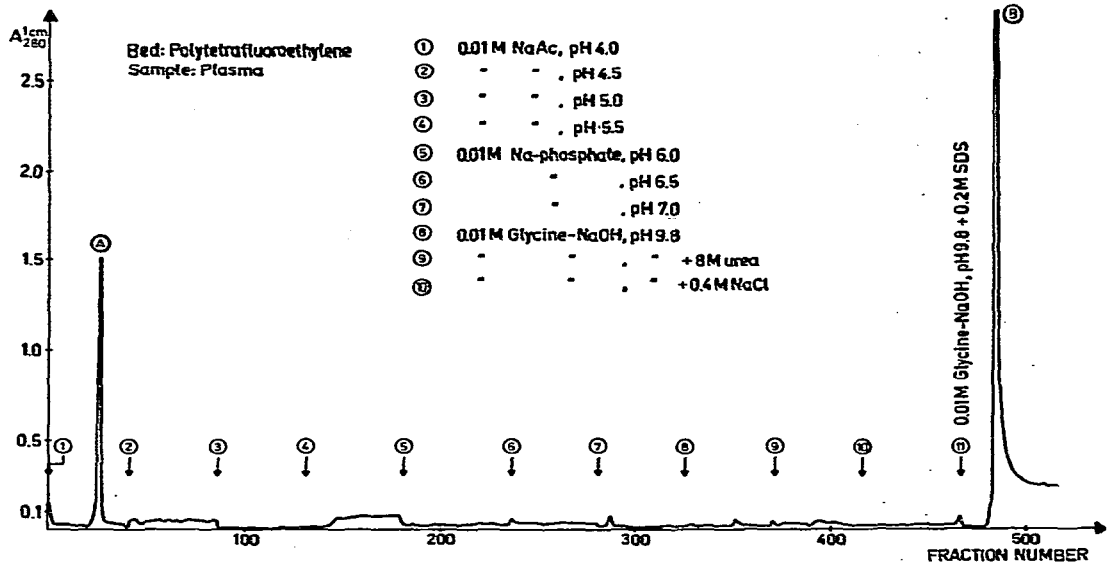


Fig. 8. Chromatogram showing the very strong adsorption of plasma proteins to a column of PTFE equilibrated at pH 4.0. When the column was equilibrated at pH 7.5 only very slight adsorption could be detected, suggesting that some pH between 4 and 7.5 might give an adsorption appropriate for protein fractionation.

the column is equilibrated at pH 4. However, if the column is equilibrated at pH 7.5 (0.01 *M* sodium phosphate) the adsorption is weak. It should therefore be possible to find a pH between 4 and 7.5 that gives a moderate adsorption useful for separation purposes. Such experiments are planned. However, it should be interesting to know the basis for the strong affinity to polytetrafluoroethylene at pH 4. The interaction is probably not electrostatic as the bed should not contain any charged groups. This is supported by the fact that desorption was not favoured by an increase in pH or ionic strength, as Fig. 8 indicates. Another possible adsorption mechanism is hydrophobic interaction, but there are some observations which go against this hypothesis.

(1) Plasma proteins interact very strongly with amphiphilic agarose derivatives (for instance, pentyl- or octyl-Sepharose) when the column has been equilibrated with a buffer of neutral pH containing salts, for instance 4 *M* sodium chloride[7]. Under these conditions only a few plasma proteins are adsorbed to PTFE.

(2) Transfer RNA is not, or is only very weakly, adsorbed to octyl-Sepharose equilibrated with 0.002 *M* sodium phosphate, pH 6.8, containing 2 *M* sodium chloride but is very strongly adsorbed to PTFE (Fig. 9).

(3) Albumin and β-lipoprotein emerge first, before γ-globulin, from a column on PTFE. On pentyl-Sepharose the elution order is reversed[7].

(4) Neither proteins (Fig. 8) nor t-RNA (Fig. 9) could be desorbed by alterations in ionic strength or pH, indicating that hydrophobic or electrostatic interactions are not responsible for the adsorption. Could both of these interactions act simultaneously on the protein molecules? This is theoretically possible, because although the electrostatic interaction is decreased upon increasing the ionic strength,

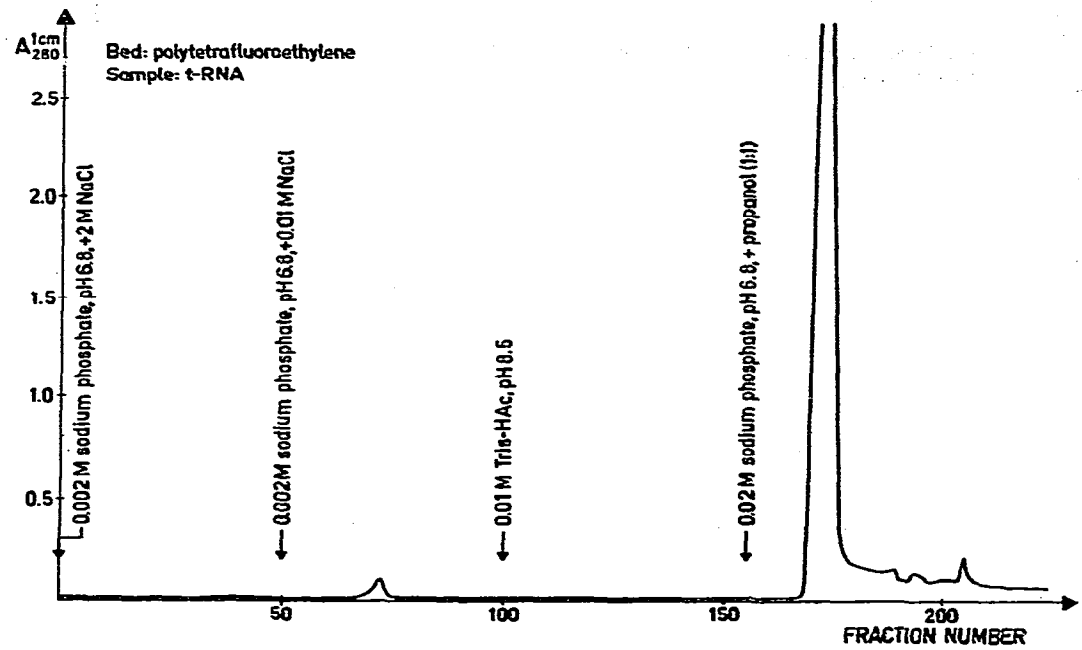


Fig. 9. Chromatogram showing the very strong adsorption of t-RNA to a column of PTFE. Desorption is effected by the addition of propanol, a finding which is being utilized to desalt t-RNA[8].

the hydrophobic interaction increases, which of course may hinder desorption. This seems unlikely, however, because *none* of the plasma proteins could be desorbed by changing the pH and the ionic strength of the eluting medium.

Could hydrogen bonds be responsible for the strong adsorption to PTFE at pH 4? Fluorine is strongly electronegative, and easily forms hydrogen bonds, but probably not in a polymer of this type. Although the nature of the adsorption to PTFE is still obscure, we have had sufficient experience with proteins and nucleic acids on this adsorbent to justify fractionation studies. The above experiments already show that PTFE can be used to desalt t-RNA (suggested by Prof. C. J. O. R. Morris at this symposium[8]).

5. COMMENTS

Although the fractionation of proteins on Sepharose in 0.01 *M* acetate at pH 4.0 appears to be based upon ion exchange, it should be pointed out that the matrix itself often has a pronounced influence on the adsorption. For instance, we have found that plasma proteins show very little adsorption to CM-Sephadex at pH 4 (Fig. 3b), but adsorb strongly to CM-cellulose.

A Sepharose column equilibrated with 0.06 *M* sodium acetate, pH 4.0, shows very little adsorption of plasma proteins, but adsorbs them very strongly when equilibrated with 0.01 *M* sodium acetate, pH 4.0. One might therefore expect that plasma proteins adsorbed to Sepharose equilibrated in the latter buffer should be desorbed by increasing the molarity of the acetate buffer to 0.06 *M*. However, this does not occur, and a considerably higher buffer concentration is required. Similarly,

proteins adsorbed to PTFE at pH 4.0 cannot be desorbed by increasing the pH to 7.5 although very few plasma proteins are adsorbed to a PTFE column equilibrated with a buffer of pH 7.5. Similar observations have been made for hydroxyapatite and amphiphilic derivatives of Sepharose used for hydrophobic interaction chromatography. The phenomenon might be a general one in ion-exchange and adsorption chromatography when polymers are adsorbed and can be expected when we are dealing with multi-point interactions.

6. ACKNOWLEDGEMENTS

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7. SUMMARY

Plasma proteins can be fractionated on columns of Sepharose equilibrated with 0.01 *M* sodium acetate, pH 4.0. The adsorption is probably due mainly to the presence of sulphate and carboxylic groups in the matrix. The use of polytetrafluoroethylene as an adsorbent for the purification of proteins and t-RNA is being explored.

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