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ON THE MECHANISM OF ADSORPTION OF PROTEINS TO NITROCELLULOSE IN MEMBRANE CHROMATOGRAPHY

T. I. PŘISTOUPIL, M. KRAMLOVÁ AND J. ŠTĚRBÍKOVÁ
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)*
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SUMMARY

Nitrocellulose membranes were found to have a general negative electric charge in neutral and alkaline aqueous solutions, and to display a cation-exchange capacity of about 0.1 mequiv. NaOH/g. The adsorption of proteins seems to be caused mainly by (1) multiple electrostatic attraction between the numerous permanent dipoles (the nitrate groups) of the carrier and the dissociated polar and polarizable groups of the protein molecule, (2) hydrogen bonds, (3) a general trend of the organized water molecules to force hydrophobic groups to the interface. However, in alkaline solutions those forces are either potentiated or weakened through ionic interactions between the negatively charged groups of the carrier and dissociated groups of the proteins.

It was found that polyethylene glycol 6000 was firmly adsorbed to nitrocellulose in a way similar to polyoxyethylene sorbitan monoesters (the Tweens) and proteins.

The relatively small adsorption of the above macromolecules on acetylcellulose membranes and on cellulose (contrasting with the high adsorption on nitrocellulose) was explained by the high dipole moment of the nitrate group as compared with that of the acetate and hydroxyl groups, and by steric factors influencing the accessibility of the dipoles.

INTRODUCTION

Nitrocellulose membrane filters are known to adsorb most proteins firmly and somewhat irreversibly, while low molecular weight hydrophilic substances and even some high molecular weight nucleic acids are generally not adsorbed[1–3]. The adsorption of proteins is usually enhanced by slightly acidic conditions[4,5] and also to some extent by some metal ions (*e.g.* Na, Mg) present in about 0.1–0.2 *M* concentrations[6,7]. These empirical facts have been used recently for the simple and rapid estimation of ultra micro amounts of proteins either by chromatography[4,5] or filtration[6,7] technique and also for simple chromatographic microdeproteinization[1], the separation of serum albumin from other serum proteins[8], the separation of proteins from nucleic acids[3], etc.

* Managing director: Prof. J. HOŘEJŠÍ, M.D., D.Sc.

The adsorption of proteins can be decreased or even prevented by impregnating the membranes with other proteins or with suitable detergents[7,9], especially the neutral ones such as polyoxyethylene sorbitan esters (the Tweens) which are bound to the membranes more firmly than proteins[1,9]. Compared with nitrocellulose, the adsorption of proteins and Tweens to acetylcellulose[10] or cellulose is negligible.

However, an explanation of the above facts has not yet been given, and the main factors and forces participating in the binding of proteins to nitrocellulose membranes remain obscure.

It is known that the chromatographic behaviour of a given substance is generally the result of its distribution between the mobile and stationary phases and may be defined by the sum of intermolecular binding forces between individual functional groups of the molecules under the given conditions[11]. The individual attractive and repulsive forces are also influenced and modified by various factors such as intramolecular binding, steric hindrance and polarization.

The interpretation of chromatographic behaviour on such a level is very difficult even with well-defined low molecular weight substances in defined systems and on known carriers. Nevertheless, we attempted to formulate and to verify experimentally an interpretation of the firm adsorption of proteins and the Tweens to nitrocellulose. In the present work, we were concerned with the following points.

(1) Examination of the general electrical charge of the membranes in aqueous media and their possible ion-exchange properties.

(2) Examination of the abilities of nitrocellulose, acetylcellulose and cellulose carriers to bind substances by non-ionic physical interactions, with regard to the characteristic structural differences between the nitrate, acetate and hydroxyl groups.

(3) Examination of the chromatographic behaviour of selected proteins and of suitably defined model substances, especially of those simulating the behaviour of proteins.

EXPERIMENTAL

Material and methods

Nitrocellulose membranes. Synpor 6 (Chemapol, Prague, Czechoslovakia), Sartorius 11002, 11302 (Membranfiltergesellschaft Göttingen, G.F.R.), Selectron BA 83, BA 85 (Schleicher & Schuell, Dassel, G.F.R.), Millipore HA (Millipore, Malakoff, France) were used and washed in boiling water[1] before use unless stated otherwise.

Acetylcellulose membranes. Oxoid electrophoresis strips (Oxoid, London, Great Britain), Sartorius 11106 and Celotate (Millipore) were used.

Partial denitration. The Synpor 6 membranes were partially denitrated[12] in about 0.1 *N* NaHS for 1 h at room temperature.

Detection of the electrical charges. The electrical charges of the membranes in aqueous solutions were detected by observing the electroendoosmotic flow indicated by the passive migration of glucose from the start during microelectrophoresis. Electrophoresis was performed at 25 V/cm, 0.3 mA for 3 min on strips usually 5–10 × 30 mm in appropriate electrolytes (0.025 *N* HCl, 0.025 *N* NaOH or citrate buffer, pH 7.0, ionic strength = 0.1) with 10% glycerine. Parallel runs were performed simultaneously on different types of membranes.

Ion-exchange properties. These were observed qualitatively on wet membranes in the H-cycle, stained with saturated aqueous Bromophenol Blue adjusted with NaOH to a greenish colour. When the reaction was positive, application of neutral 1 *M* NaCl by means of a capillary led to the formation of a yellow ring due to the protons freed by Na ions. The ion-exchange capacity was estimated quantitatively as usual by titration in 1 *M* NaCl with 0.1 *N* NaOH up to pH 10.0 (ref. 13).

One-dimensional or radial microchromatography was performed as described previously[1,5,8,16]. The substances were dissolved in water, 0.1 *N* and 0.01 *N* NaOH or HCl, resp., or in the appropriate Michaelis buffers (*cf.* ref. 8) and developed in the same solutions. The adsorption or nonadsorption to the strips was easy to observe, since the substances either remained in the vicinity of the origin or migrated (*cf.* refs. 1, 3, 8).

The following low molecular weight substances were chromatographed: methylene blue, toluidine blue, crystalline violet, methyl orange, eosine, fluoresceine, phenol, trinitrophenol, glucose.

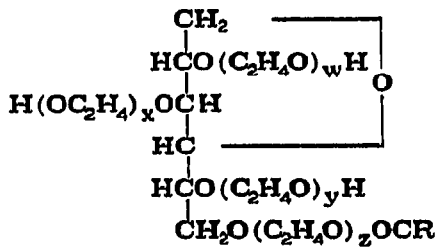

Fig. 1. General formula of polyoxyethylene sorbitan monoesters (the Tweens). OCR = fatty acid residue; $w+x+y+z$ = number of ethyleneoxide residues (n).

The detergents, Tween 20, 40, 60 and 80 (Atlas), polyoxyethylene sorbitan monoesters of lauric, palmitic, stearic and oleic acids, resp. (Fig. 1), were used as 2% solutions in water or in the given buffer. Ethylene glycol, triethylene glycol and polyethylene glycol 6000 (Lachema) were used as 0.1–10% solutions in buffers at pH 3.8 and 7. Dextran (Léčiva-Modřany) was used as a 5% solution. The chemicals were of reagent grade.

The following proteins were chromatographed: horse heart cytochrome *c* (Sigma), trypsin and pepsin (Lachema), bovine serum albumin and gammaglobulin (prepared in our Institute), and dolphin myoglobin (a gift from Dr. P. NEDKOV).

Proteins were detected with 0.1% Amido Black 10B or with 0.01% nigrosine in 5% trichloroacetic acid. Nigrosine is generally a much more sensitive stain, which can detect a significant adsorption of proteins even on acetylcellulose strips. Amino acids were stained with 0.1% ninhydrin in isopropanol. The Tweens and polyethylene glycol 6000 were both stained with Dragendorff's reagent[14]. Dextran, ethylene glycol and triethylene glycol were detected as brown spots on a pink background after moistening the dry chromatograms with 1% $KMnO_4$ in 5% H_2SO_4 and heating carefully to 60–80° for a few minutes. Glucose was detected with $AgNO_3$ in ammonia[11].

RESULTS AND DISCUSSION

Electric charge and ion-exchange properties of nitrocellulose membranes

The results achieved during the investigation of the electroendosmotic flow

under different conditions are presented schematically in Fig. 2 and can be summarized as follows: There was a marked flow of the electrolyte towards the cathode in alkaline and neutral pH on all types of nitrocellulose membranes tested. The magnitude of the negative charge was differed according to the type of membrane and was evidently dependent on the number of accessible negatively charged groups and, indirectly, on the porosity of the membrane. Carboxylic groups are most probably the carriers of the negative charge here. Their presence in cellulose[11] and in nitrocellulose[12] is known to originate from glucose residues as oxidative by-products during preparation and cleaning of the crude product.

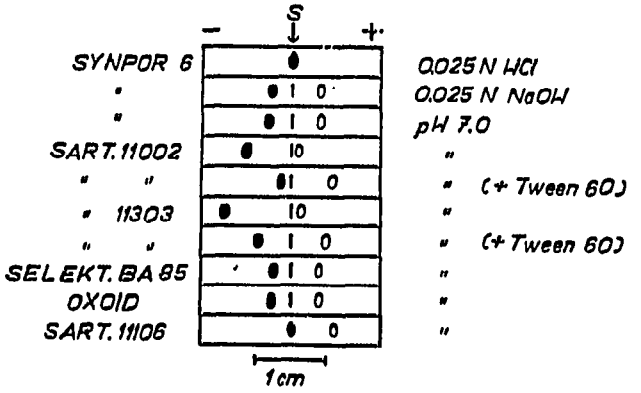

Fig. 2. Electroendosmotic flow on various membranes. Black spot = glucose; White spot = Bromophenol Blue; S = start. For details see *Materials and methods*.

The negative charge of the membranes was markedly suppressed in 0.025 *N* HCl and at neutral pH on membranes impregnated with Tween 60. However, in 0.025 *N* NaOH, even impregnated membranes were negatively charged.

A negative charge was also observed with the acetylcellulose membrane Oxoid, whereas acetylcellulose Sartorius 11106 and Celotate were practically without electrokinetic charge.

The qualitative spot-test for the ability of membranes to exchange cations was positive with all nitrocellulose membranes tested, either untreated or impregnated with Tween 60. For the acetylcellulose membranes, the test was positive only with Oxoid, while with Sartorius 11106 and Celotate the tests were negative.

Titration of the ion-exchange capacity led to 0.1 mequiv. NaOH/g with Synpor 6 and 0.05 mequiv. NaOH/g with Oxoid (± 6%). These capacities are relatively low compared with ordinary modified celluloses[13] and correspond instead to the values found with filter paper[11]. Neutralization titration curve of the Synpor 6 membrane is presented in Fig. 3 and shows a marked buffering region at pH 6–7.

Attempts to use the ion-exchange properties of the nitrocellulose membranes for the separation of some model mixtures, *e.g.* amino acids, inorganic ions and proteins, under conditions usual for work with cellulose ion-exchangers[13], gave unsatisfactory results (*cf.* ref. 1).

This was probably due to the low exchange capacity of the membranes and to

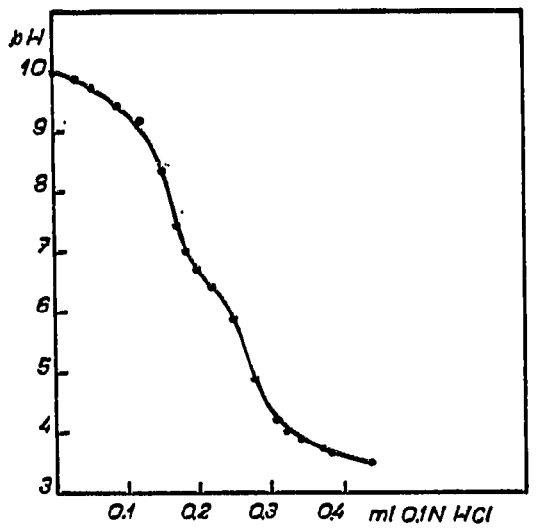

Fig. 3. Neutralization titration curve of Synpor 6 membrane in 1 *M* NaCl.

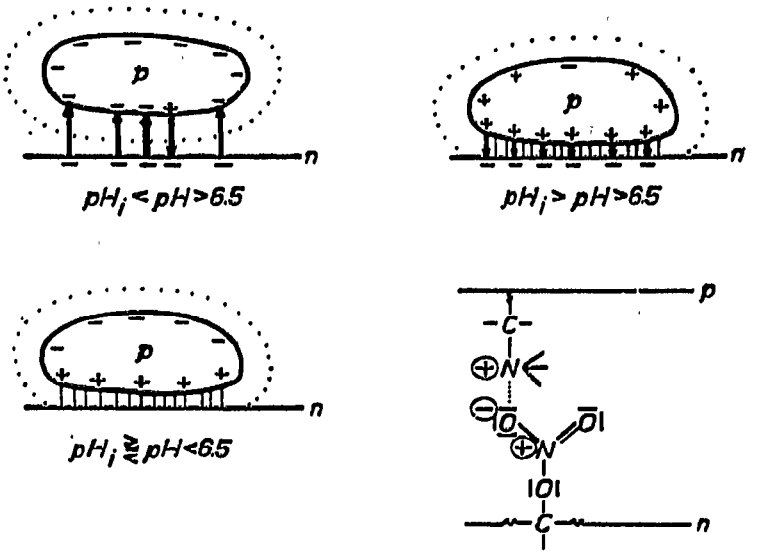

Fig. 4. Scheme of binding between proteins and nitrocellulose under different conditions. p = protein molecule; n = nitrocellulose; pH_i = isoelectric point of the protein. The arrows indicate interactions between dissociated groups. Thin lines indicate ion–dipole, dipole–dipole and other short-range interactions. Dotted lines indicate the aqueous and ionic envelope of the protein molecule. The formulas illustrate in detail the attraction of a lysine residue to the fixed nitrate dipole at pH<6.5.

steric hindrance. As for the proteins, they seem to be bound to a great extent by non-ionic forces like non-ionic detergents (see below), especially at pH values below 6 where the negative charge of nitrocellulose is decreased (*cf.* Figs. 2 and 4). However, at a given alkaline pH, electrostatic repulsion occurs between negatively charged groups of the carrier and of the proteins which have a lower isoelectric point, pH_i, while other proteins having a higher pH_i and hence a positive charge will be attracted to the membrane by strong ionic forces. This may explain the low adsorption of serum albumin at pH 8–9 (*cf.* ref. 8) and the high adsorption of basic proteins such as cytochrome *c* and trypsin under the same conditions (*cf.* also ref. 2).

The ability of nitrocellulose membranes to bind substances by non-ionic physical interactions. Characterization of the carriers

In order to shed more light on this problem and keeping in mind the marked difference between the firm adsorption of proteins and the Tweens to cellulose nitrate as well as their relative lack of adsorption on cellulose acetate and cellulose, we considered some important data on the chemical properties of different membranes and of the nitrate, acetate and hydroxyl groups.

Nitrocellulose membrane filters[15,16] are usually prepared from cellulose nitrate which has about 11–13.5% nitrogen (as compared with 14.15% of fully nitrated cellulose trinitrate), *i.e.* about 78–96% esterification. The difference from 100% is made up of nonesterified hydroxyls and carboxyl groups which are probably involved in lowering the hydrophobic character of the membranes, causing the spontaneous suction and flow of water through the capillary structure.

A similar situation occurs with the acetylcellulose membranes which consist of

a mixture of more highly acetylated esters[10], presumably di- and triacetates as deduced from their total solubility in chloroform and acetone. However, nitrocellulose membranes, being more polar, are dissolved only in acetone and not in the less polar chloroform. Both nitrate and acetate membrane filters are not strictly hydrophobic carriers and can bind about 1 % water from the atmosphere at room temperature and 65–75 % relative humidity[15]. (At 100 % relative humidity we found that Synpor 6 membranes bound up to 16 % water, which was caused presumably by microcondensation in the capillaries.)

Another important factor should also be mentioned here, namely the questionable chemical homogeneity of some commercial membranes used in filtration and chromatography. It is known that the manufacturers often add small amounts of additives such as higher alcohols, glycerine and detergents to the membranes in order to improve the mechanical, filtering or flow characteristics of the carriers[17]. Some of the additives can be easily removed by washing (*e.g.* from Synpor[1,15] or Sartorius series 110 nitrocellulose membranes), while others may be strongly adsorbed to or even built into the solid structure of the membranes and can influence the experimental results and their interpretation.

According to the literature[18,19] and taking the dipole moments, μ, of the respective methyl derivatives for comparison, it can be stated that the nitrate group ONO_2 ($\mu = 2.85$ D) has a greater ability to interact by dipole–dipole or dipole–ion[22] association and to induce new dipoles than the acetate group $OCOCH_3$ ($\mu = 1.67$ D) which is in this respect comparable to the hydroxyl group ($\mu = 1.69$ D). The dipole moments of the esters are substantially less than those of the corresponding NO_2 ($\mu = 3.54$ D) and $COCH_3$ ($\mu = 2.85$ D) compounds. Both ester groups can participate in hydrogen bonds as donors of paired electrons. The acetate has the ability to interact by dispersion forces through its methyl group, which in turn considerably limits accessibility to the dipole.

It seems reasonable to assume that the active surface of the nitrocellulose membranes is formed by a great quantity of permanent and easily accessible dipoles, ONO_2, oriented with the negative pole outward, and a small number of carboxyl and hydroxyl groups which immobilize only a limited amount of water molecules. A similar assumption can be made for the acetylcellulose membranes; however, their binding force will be substantially lower than that of nitrocellulose for the reasons mentioned above. Compared with the cellulose esters, the surface of intact cellulose is generally more hydrophilic due to the numerous hydroxyl groups bound by intramolecular hydrogen bonds which attract a protecting multiple layer of water molecules. It also contains a small amount of carboxyl groups[11].

Chromatography of model substances and of proteins

In order to verify the above assumptions about the character of the membranes and the type of binding in aqueous solutions, the behaviour of several substances (*cf. Materials and methods* and refs. 1, 9 and 20) has been tested in orientation chromatographic runs especially on nitrocellulose membranes. The results of these and other similar experiments[1,20] may be summarized as follows.

Low molecular weight substances having a hydrophilic nature (*e.g.* soluble salts, saccharides, urea, amino acids) were not adsorbed on nitrocellulose under the given conditions, while substances possessing significant hydrophobic structures with

polar but not fully dissociated substituents (*e.g.* some aromatic dyes, detergents) tend to be adsorbed. The presence in the molecules of dissociated groups, especially of negatively charged ones, enhanced the desorption from the start in neutral and especially in alkaline pH, indicating the importance of electrostatic repulsion between the ionized groups of the carrier and of the given substance. This is probably the reason why the basic dyes were bound more firmly than the acid ones in neutral and alkaline pH. On the other hand, dipole association and hydrogen bonds seem to be very important binding factors.

Of the substances tested, only the Tweens[1] and polyethylene glycol 6000 significantly simulated the behaviour of proteins, *i.e.* the firm adsorption on nitrocellulose and the lesser adsorption on acetylcellulose and cellulose. Both substances were able to prevent the adsorption of proteins to nitrocellulose (*cf.* ref. 1). These results indicate that polyoxyethylene chains, and not the hydrophobic ester substituents in polyoxyethylene sorbitan esters (*cf.* Fig. 1), play an essential role in the active binding to nitrocellulose membranes. In contrast to polyethylene glycol 6000, the monomeric ethylene glycol and triethylene glycol were not adsorbed nor did they prevent the adsorption of proteins, thus indicating the importance of multiple intermolecular dipole and H-bond interactions. An analogy may be made with the adsorption of proteins and the nonadsorption of amino acids and peptides[1] or with the firmer adsorption of polymers in ion-exchange chromatography[13].

It is important to state here that the high molecular weight polyglucan dextran did not prevent the firm binding of proteins to nitrocellulose, nor was it adsorbed itself. This is probably due to the large number of intramolecular H-bonds between the hydroxyl groups and the high degree of hydration of the dextran molecules. On the other hand, polyethylene glycol chains contain periodically repeating hydrophobic methylene groups which probably not only lessen the formation of intramolecular hydrogen bonds but can improve intermolecular interactions with nitrocellulose either through more convenient steric configuration and distance of the corresponding dipoles or through a preferred exclusion from the aqueous solution to the interface.

Further investigation of this question and of the use of polyethylene glycols in membrane chromatography of proteins and other substances is currently being studied.

Chromatography of proteins confirmed both earlier findings[1,20] and the assumptions summarized in Fig. 4, and also revealed some new facts. Trypsin, which tends to stay at the origin in water or dilute salt solutions, migrated in 8 *M* urea—this was in contrast to the behaviour of most other proteins. A plausible explanation can be given by considering the dissociation of trypsin into 3 components in urea which are not adsorbed due to their low molecular weight[21].

The results achieved with partially denitrated[12] Synpor 6 membranes were also interesting. Compared with intact Synpor 6 membranes the adsorption of trypsine, cytochrome *c* and of myoglobin was significantly decreased here, indicating the important role of the nitrate groups in adsorption.

CONCLUSION

In conclusion, the following general interpretation of the adsorption of proteins to nitrocellulose membranes can be proposed (*cf.* Fig. 4).

The protein macromolecules containing many dissociated and dissociable groups as well as permanent dipoles, polarizable groups and a certain number of hydrophobic structures on the surface, are stabilized in solution by a large multilayer of immobilized water and by the ionic atmosphere[22]. They approach the surface of the membrane which is formed predominantly by permanent dipoles of the nitrate groups and by a small number of hydroxyl and carboxyl groups, and which is covered by a relatively thin layer of immobilized water.

Simultaneous intermolecular electrostatic attraction and association of a sufficiently large amount of dipoles and charged groups distorts the solvation layer, bringing both partners closer and anchoring the protein molecule on the carrier. The binding, which also depends on molecular weight, is possibly tightened by hydrogen bonds and by a general trend of the cooperating water molecules to force hydrophobic structures out of the solution toward the interface. This interpretation seems plausible for solutions below pH 6.5. However, in neutral and alkaline solutions at a given pH, the dissociated carboxyl groups of the carrier can exert either a strong interference through electrostatic repulsion, which hinders the negatively charged protein molecules having a lower pH_i in the nearer approach to the carrier, or a potentiation of the binding through electrostatic attraction of positively charged proteins having a higher pH_i.

The effect of cations partially enhancing the adsorption of proteins[7] or phages[23] might be explained by a nonspecific 'salting out' phenomenon due to the partial destruction and weakening of the protecting layer of immobilized water on both surfaces, and by a partial compensation of the mutual electrostatic repulsion between the negatively charged groups.

The type of binding of the Tweens and of polyethylene glycol to nitrocellulose seems to be based on principles generally similar to those mentioned above. However, their firmer binding and their desorbing effect towards proteins can be explained by the fact that there are no dissociable groups in their molecules which would favour their greater solvation and repulsion through strong ionic forces as is the case with proteins or with cationic and anionic detergents (*cf.* ref. 9). The bound molecules of the Tweens and polyethylene glycol 6000 evidently 'saturate' the nitrate dipoles, and their chains presumably fold on the surface creating a flexible steric hindrance to protein molecules.

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