CHROMATOGRAPHIC BEHAVIOUR OF PROTEINS, PEPTIDES AND AMINO ACIDS ON NITROCELLULOSE MEMBRANE FILTERS

T. I. PŘISTOUPIL
Institute of Hematology and Blood Transfusion, Prague (Czechoslovakia)*
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It has been reported recently[1,2] that nitrocellulose ultrafilters (UFS) can be used as a convenient supporting medium for the microelectrophoresis of proteins. Impregnation of the membranes with Tween 60, before electrophoresis, was necessary to prevent a very strong adsorption of the proteins to this support. The pretreatment with the detergent, however, could be omitted during electrophoretic fractionation of protein hydrolysates, since the adsorption of amino acids to nitrocellulose was found to be negligible[3]. The experiments referred to in the present communication were undertaken to obtain more information on the different modes of binding of amino acids, peptides and proteins to nitrocellulose membranes, under various conditions. It was also of interest to determine whether nitrocellulose membranes could possibly be used as a supporting medium for chromatographic separations of the substances under study.

EXPERIMENTAL

Materials

Strips 2–3 cm × 4–5 cm of nitrocellulose membranes VUFS, HUFS, AUFS and RUFS[4] (produced by VCHZ Synthesia, Uhříněves, Czechoslovakia) were washed in boiling distilled water twice for 10 min. After drying at 20–80° the strips were ready for use. Impregnation with 2 % Tween 60 was done as previously described[1] (washing in boiling water was omitted here). Native bovine serum, bovine serum albumin, human γ-globulin, ovalbumin, formolated and heat-denatured bovine serum[5,6], human thyreoglobulin and bovine ribonuclease were the same preparations as used in previous experiments[6].

Crystalline trypsin was a commercial preparation distributed by Laboratorní Potřeby, Prague.

Glutathione (a gift from Dr. J. MÁLEK, VÚPL, Prague) and seryl-glycyl-cysteinyl-vasopressin (a gift from Dr. HLADOVEC, VÚFB, Prague) were used as model peptides. A total acid hydrolysate of bovine serum was taken as a standard mixture of amino acids. Horizontal chromatography was performed in simple humid chambers. Whatman No. 3 paper wicks were used to connect the "starting" end of the membrane with the solvent. The following solutions were used to develop the chromatograms: water, 0.9 % NaCl in water; 0.1 *M* acetate buffer, pH 3.7; acetic acid + formic acid[7],

* Director: Prof. MUDr. J. HOŘEJŠÍ, D.Sc.

pH 1.7; veronal–citrate–oxalate buffer p^{v}I 8.6[1]; 0.01 *M* NaOH. In some experiments urea was added to reach a final concentration of 8 *M* as well as NaCl to a concentration of 1 *M*.

Procedure

The samples (1–5 % solutions), generally dissolved in water, 0.9 % NaCl or in the given buffer, were applied in amounts of the order of 10^{-4} to 10^{-5} ml about 3–10 mm apart from one end of the membrane and 1–10 mm behind the advancing front of the eluant. Amino acids were applied also on dry membranes. When the front of solvent neared the end of the strip the chromatograms were dried at 80° for 10 min. Proteins were detected either by Azocarmine B, Amido Black 10B or Nigrosine[1,8]. A partial decoloration of the background was achieved in 1 % acetic acid in water. A spray of 0.1 % ninhydrin in isopropanol was used to detect the amino acids and peptides. Bromophenol Red or serum albumin stained with Bromophenol Blue were used as convenient indicators of the rate of flow.

RESULTS AND DISCUSSION

The solvent penetrating into the capillary system of the dry membrane usually formed two boundaries which were best seen on the glossy side of the VUFS and HUFS filters. This phenomenon seemed to be connected with the mechanical properties of the microporous membrane structure. The chromatographic behaviour, however, of the samples under study was not influenced significantly by the position of the two boundaries. The chromatography was rapid, taking about 2–5 min on the RUFS and AUFS, about 10–15 min on the HUFS and about 40–60 min on the VUFS membrane filters. The VUFS and especially the HUFS membrane filters were easier to handle and the spots were more regular and reproducible. The latter type was then used in most experiments.

Proteins with molecular weights of the order of 10^5 or with an uncoiled denatured molecular structure, *e.g.* thyreoglobulin, γ-globulin and modified bovine serum, were usually firmly adsorbed to the unimpregnated nitrocellulose membranes under all experimental conditions tested. The samples were not bound strictly to the start line but formed oblong irregular spots which were drawn out slightly from the start towards the front of the chromatogram. The shapes of the spots, characteristic for all firmly adsorbed proteins, are shown in Figs. 1a, 2 and 4a. The addition of urea up to 8 *M* and of NaCl up to 1 *M* to the developing solutions did not change the character of the chromatograms significantly. Proteins with lower molecular weights were adsorbed only in acid buffers whereas in alkaline solutions the binding was much weaker, so that they migrated, partly at least, from the start (*cf.* Fig. 1b). This was observed with serum albumin (mol.wt. 69,000), native serum (containing serum albumin) and especially with ovalbumin (mol. wt. 44,000), trypsin (mol.wt. 24,000) and ribonuclease (mol.wt. 12,700)[9]. A large part of ribonuclease migrated from the start even in acid buffers.

The areas of the compact spots formed in acid solutions were within a certain approximation proportional to the amount and concentration of the given protein sample (*cf.* Fig. 2).

It was observed that the same amounts of proteins formed larger spots on

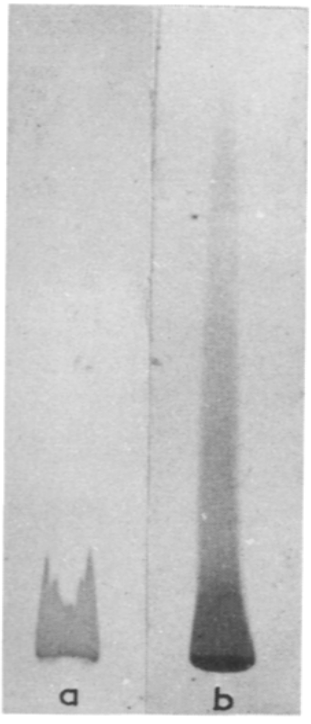


Fig. 1. Chromatogram of native bovine serum on a HUFS membrane. (a) = pH 3.7, acetate buffer; (b) = pH 8.6, veronal–citrate–oxalate buffer. Stained by Nigrosine.

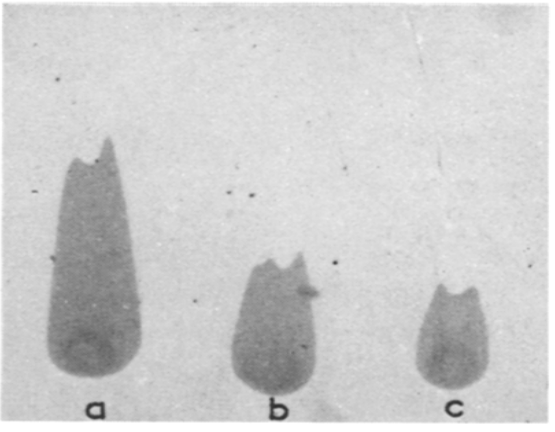


Fig. 2. The dependence of the spot areas on protein concentration. (a) 2%, (b) 1% and (c) 0.5% bovine serum albumin in acetate buffer pH 3.7. Volumes of 1 μl were applied on a HUFS membrane. Developed in acetate buffer, pH 3.7. Stained by Azocarmine B.

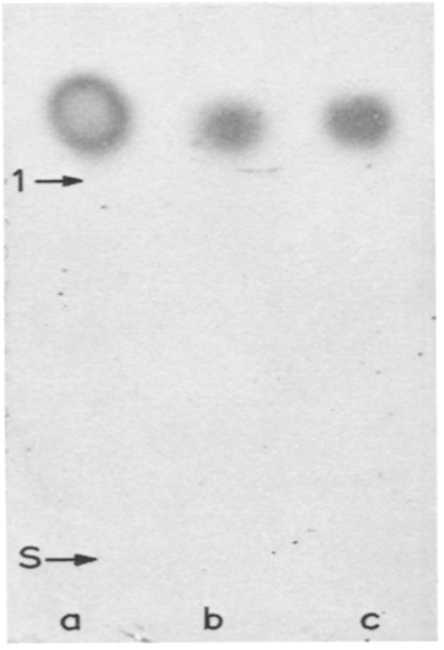
membranes possessing bigger pores. This was most probably in connection with an increased rate of flow of the eluant through the looser membranes and also due to the fact that for the same area of the membrane, the inner surface able to adsorb the proteins was smaller in the looser membranes.

It seems reasonable to assume that the protein molecules coming into a direct contact with the microporous nitrocellulose network[10], are firmly adsorbed, forming a rather homogenous film with a relatively constant thickness. The unbound molecules, however, migrate over this film to be adsorbed on the next free nitrocellulose surface, until all protein molecules applied in the given sample are intercepted. This assumption was further confirmed by other experiments using circular chromatography, where a very accurate relationship was found between the areas of the spots and protein concentration[11].

Assuming that tightly packed serum albumin molecules[9] were lying with their longer sides on the inner surface of the HUFS nitrocellulose membranes, it was possible to calculate that, at a pH 3.7, the albumin film consisted of about 10–40 mono-layers situated one over the other. Data of porosity etc. given by the manufacturer as well as other experimental data[4,10,11] were taken into account here. The above result, however, should be taken only as a rough approximation, since some of the starting data were mean values and since the phenomena of surface-denaturation and unfolding of the molecules must also be considered.

The impregnation of the membranes with Tween 60 caused a fundamental

change in the chromatographic behaviour of proteins. All proteins under study migrated from the start with a R_F of about 1.0, showing neither adsorption nor tailing of the spots in neutral and alkaline buffers (*cf.* Fig. 3). This was in good agreement with the previous findings and interpretations made in connection with the electrophoresis of proteins on nitrocellulose[1,2] impregnated with the detergent. In acid solutions, however, a slight adsorption at the start was sometimes observed, especially when the excess of unbound Tween 60 had been removed thoroughly (*cf.* ref. 2).



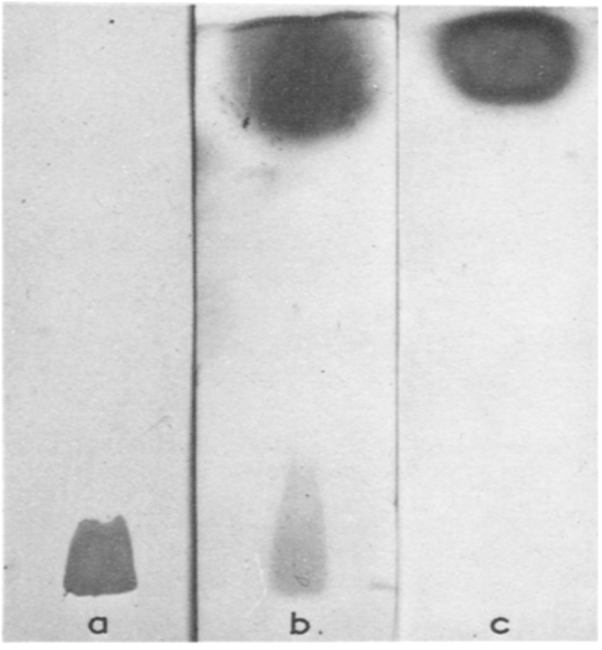


Fig. 3. Chromatography on a VUFS membrane impregnated with Tween 60, (a) = Formolated and heat denatured bovine serum; (b) = human γ-globulin; (c) = bovine serum albumin. The position of the indicator (Bromophenol Red) is shown by arrow No. 1, the start is indicated by S. Developed in veronal–citrate–oxalate buffer, pH 8.6.

Fig. 4. Group separation of amino acids and proteins. (a) = Bovine serum; (b) = bovine serum + protein hydrolysate; (c) = protein hydrolysate. The samples were developed on a HUFS membrane in acetate buffer, pH 3.7. Sample (a) was stained by Azocarmine B, samples (b) and (c) by ninhydrin.

Amino acids present in the acid protein hydrolysate and the two peptides under study migrated in one round spot with an R_F near to 1.0 on nitrocellulose membranes both treated or untreated with the detergent, in alkaline as well as in acidic media (*cf.* Fig. 4c).

Fractionation and separation of individual proteins, peptides or amino acids was not possible under the conditions used here. An excellent group separation, however, of proteins from amino acids and peptides was achieved, on chromatographing a mixture of them on the HUFS membrane in the acetate buffer, pH 3.7 (Fig. 4). This procedure proved to be most convenient for a rapid and gentle deproteinization of various samples especially when working with microamounts of material. The amino acids migrate with the front of the eluent concentrate at the tip of the nitrocellulose strip and are ready for further analysis *e.g.* chromatography, electrophoresis or quantitative determination.

As was found in the orientation experiments, several low-molecular substances behaved in a similar way to amino acids on the HUFS membrane and migrated with the chromatographic front. Thus, unbound glucose, urea, $CuSO_4$ or Bromophenol Red, added previously to serum, could be rapidly separated from the proteins by the deproteinization procedure mentioned above. (The applicability of this technique to certain microanalytical estimations as well as to the study of interactions of proteins with other substances will be referred to later.) Some other low molecular compounds, however, had a lower R_F on the membranes (Bromophenol Blue, Methyl Orange) or remained in the vicinity of the start (Methylene Blue, Crystal Violet, Nigrosine, Azure II) indicating that the chromatographic behaviour of these substances is dependent also on some specific property of their molecules.

From the results of our experiments it may be concluded that both molecular weight and the net charge of the proteins (depending upon the pH of the solution) greatly influence the chromatographic behaviour of the substances under study on unimpregnated nitrocellulose membranes. The role of the molecular weight seemed to be most important here, whereas the pH-dependence was limited to low-molecular proteins, and the effect of several specific qualities of the different proteins was obviously negligible. As for the HUFS membranes, the critical interval between full adsorption and free migration was between a mol.wt. of about 70,000–10,000 at a pH 8.6 and in the vicinity of 10,000 at a pH 3.7. A more detailed interpretation, however, of these findings and of the forces and mechanisms involved in the binding of proteins as well as of other substances (*cf.* ref. 12) will need further investigation.

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SUMMARY

The chromatographic behaviour of soluble proteins, peptides and amino acids was investigated on nitrocellulose membrane filters using different aqueous developing solutions. In general, proteins were rather firmly adsorbed to nitrocellulose especially in acid buffers, whereas amino acids, peptides and some other low-molecular substances were eluted from the start to the front without any significant adsorption. This was found to be most useful for the rapid and gentle microscale deproteinization of various samples. The areas occupied by the adsorbed protein spots were proportional to the protein concentration. On membranes impregnated with Tween 60 the adsorption to nitrocellulose was prevented so that even the high molecular proteins migrated from the start line with an R_F 1.0. Fractionation, however, of mixtures of individual proteins, peptides or amino acids was not achieved under the conditions used.

REFERENCES

1 T. I. PŘISTOUPIL, *Biochim. Biophys. Acta*, 117 (1966) 475.
2 T. I. PŘISTOUPIL, *J. Chromatog.*, 23 (1966) 329.
3 T. I. PŘISTOUPIL AND V. FRIČOVÁ, unpublished experiments.
4 V. HAMPL AND K. SPURNÝ, *Collection Czech. Chem. Commun.*, 21 (1966) 1152.

5 T. I. PŘISTOUPIL, *Experientia*, 21 (1965) 194.
6 T. I. PŘISTOUPIL, *J. Chromatog.*, 19 (1965) 64.
7 L. NAFTALIN AND A. STEPHENS, *Clin. Chim. Acta*, 12 (1965) 365.
8 J. KOHN, *Aerztl. Lab.*, 10 (1964) 233.
9 P. KARLSON, *Základy Biochemie*, Academia, Prague, 1965, pp. 71, 79.
10 K. SPURNÝ AND J. PICH, *Collection Czech. Chem. Commun.*, 30 (1965) 2276.
11 T. I. PŘISTOUPIL, *Nature*, 212 (1966) 75.
12 S. O. WARNAAR AND J. A. COHEN, *Biochem. Biophys. Res. Commun.*, 24 (1966) 554.