MICROCHROMATOGRAPHY, IMMUNOCHROMATOGRAPHY AND DETECTION OF PROTEOLYTIC ACTIVITY ON NITROCELLULOSE MEMBRANES IMPREGNATED WITH PROTEINS

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In the course of experiments with electrophoresis[1] and chromatography[2] of proteins on nitrocellulose membrane filters, it was found that various proteins were firmly adsorbed on intact nitrocellulose. Experimental results indicated the formation of a relatively homogeneous stationary film of proteins covering the nitrocellulose network of the membrane[3]. However, amino acids, peptides and some other low-molecular weight substances were not adsorbed and were easily eluted from the start during chromatography[2]. On membranes impregnated with a nonionic detergent, *e.g.* Tween 60, even the adsorption of proteins was prevented[1,2,12].

It was decided to investigate whether protein-impregnated membranes could be used as a supporting medium for chromatography and electrophoresis of various substances. We assumed that this technique would be a simple way for the rapid detection and investigation of a series of interactions, including immunochemical and enzymatic reactions.

EXPERIMENTAL

Materials and methods

Strips 1 × 4 cm or 2 × 4 cm of nitrocellulose membrane filters VUFS, HUFS, AUFS and RUFS with pore sizes 0.1–0.3 μ, 0.3–0.5 μ, 0.6–0.9 μ and 1.2 μ respectively (produced by VCHZ Synthesia, Uhříněves, Czechoslovakia) were washed in boiling water twice for 10 min and dried at below 80°. The strips were then placed on the surface of the impregnating protein solution and after the solution had been soaked up from below, the membranes were immersed thoroughly for at least 5 min. Undiluted bovine, fowl and rabbit sera, 1–2 % native and heat-denatured bovine serum albumin and serum (heated for 10 min to 100° at pH 8.6–9.0) were used in these experiments. The excess of unbound proteins, occluded in the pores of the membranes, was washed off on a Büchner funnel using the same buffer as in the corresponding subsequent procedure.

For chromatography, wet strips were slightly blotted with filter paper, placed horizontally as a bridge on glass supports in a moist chamber and connected at one end with a filter paper wick of appropriate width, to supply the strip with the developing buffer at a given rate from a reservoir. The other end was laid on a dry

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Whatman No. 1 filter paper wick to ensure a regular suction of the buffer through the membrane. Veronal–acetate buffers[4] in the pH range 3.7–7.1, and the veronal–citrate–oxalate buffer[1], pH 8.6, were used in the chromatographic experiments. The samples to be chromatographed were applied either with a capillary or preferably with a filter paper wick[1].

The following substances were chromatographed: phenolsulphophthalein, dibromophenolsulphophthalein, tetrabromophenolsulphophthalein (reagent grade, Lachema, Czechoslovakia), bovine serum albumin (prepared by ethanolic fractionation in our Institute), bovine serum, rabbit serum and rabbit antibovine serum (Institute of Sera and Vaccines, Prague). After drying at 80° for 10 min the protein spots were stained with Amido Black 10 B (0.1 % solution in 2 % aqueous acetic acid).

For the spot test detection of proteolytic activity, trypsin (in veronal–citrate–oxalate buffer, pH 8.6), papain (in veronal–acetate buffer, pH 5.0) and pepsin (in acetic acid–formic acid buffer, pH 2.0) were used in concentrations below 1 %.

Usually 1 μl of the enzyme solutions was applied on to the glossy side of the protein-impregnated membranes which had been laid on filter paper strips of the same dimensions as the membranes and had been wetted with the appropriate buffer. After the application of the samples, the strips were incubated in a moist chamber at 37° for 10–60 min. Then the membranes were rinsed by dipping them into 0.9 % NaCl for 5 min, dried at 80° for 10 min and stained either with 0.1 % Amido Black 10 B or 0.04 % Bromophenol Blue in 2 % aqueous acetic acid.

RESULTS AND DISCUSSION

Chromatography of dyes

The investigation of the chromatographic behaviour of phenolsulphophthalein and its dibromo- and tetrabromo-derivatives on nitrocellulose membranes has shown that the results were dependent on the type of the membrane used, on the impregnation or nonimpregnation of the membranes, as well as on the pH of the developing buffer.

In general, the greater the mean pore size of the membrane, the faster was the flow of the buffer and the migration of the samples. In this sense the RUFS-membranes were too "fast" and the VUFS too "slow" for practical use so that the AUFS and, especially, HUFS membranes were found most suitable for our experiments.

The role of impregnation with proteins is shown schematically in Fig. 1.

There was no marked difference between chromatograms run on membranes impregnated with albumin or whole serum or (at alkaline pH values) with heat denatured albumin. The rate of flow of the buffer through impregnated membranes was substantially less than on intact membranes, possibly because the pores were partially blocked by adsorbed proteins.

The most marked differences, which could be ascribed to the interaction of the dye with the stationary protein layer, were found in the behaviour of bromophenol blue and bromophenol red (tetrabromo- and dibromophenolsulphophthalein, respectively). The formation of a compact zone of bromophenol blue at the start on impregnated membranes (contrasting with the diffuse spot on intact membranes) seems to be in accordance with the well known binding of this dye to proteins. The formation of four distinct spots of bromophenol red might be due to some inhomo-

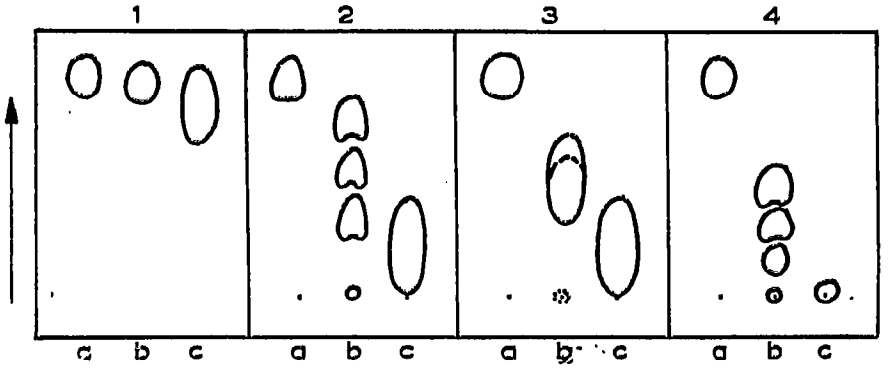

Fig. 1. Chromatography of dyes on intact and on protein-impregnated nitrocellulose membranes. (1) AUFS membrane, not impregnated; (2) AUFS membrane, impregnated with bovine serum; (3) HUFS membrane, not impregnated; (4) HUFS membrane, impregnated with bovine serum. a = Phenolsulphophthalein; b = dibromophenolsulphophthalein; c = tetrabromophenolsulphophthalein. Developed in veronal–acetate buffer, pH 3.7 for 20–30 min (AUFS) and for 60 min (HUFS). Length of the strips 4 cm.

geneity of the given preparation or to the presence of different forms of the dye, but in any case, this result may be taken as an example of the changed resolving abilities of impregnated nitrocellulose membranes as compared to the nonimpregnated ones. The chromatographic behaviour of phenol red (phenolsulphophthalein) was similar on impregnated as well as on intact membranes.

Chromatography of the dyes at pH 5.4, 7.1 and 8.6 led to similar results as at pH 3.7. However, in buffers with higher pH values a greater shift of all the spots from the start was observed, in general.

Immunochromatography

Interesting results were achieved during experiments with immunochemically reactive systems *e.g.* bovine serum and rabbit antibovine serum. The antigen (bovine serum or bovine serum albumin) was adsorbed to the HUFS membrane to form the stationary protein layer as usual. The corresponding undiluted rabbit antiserum as well as normal inactive rabbit serum (control) were applied in amounts of the order of 10^{-4}–10^{-5} ml to this immunosorbent strip and chromatographed either immediately or after 10 min incubation on the start at a pH 7.1. This incubation led in some experiments to better results.

As can be seen in Fig. 2 a distinct spot of the antigen–antibody complex was formed on the start whereas inactive proteins migrated from the start completely. The immunospecifity of this reaction was further confirmed by chromatographing the same rabbit antibovine serum on membranes impregnated with rabbit and fowl sera. No adsorption was found on the start here. Negative results were also achieved when chromatography of antibovine serum was made on bovine serum-impregnated membranes at an acid pH (3.7), known to favour the splitting of the antigen–antibody complex[5].

With regard to these results it may be stated that the antigen-impregnated nitrocellulose membranes seem to be a very suitable supporting medium for a rapid immunochromatographic detection, removal or absorption of specific antibodies in ultramicroamounts of material. It seems to us that this simple method could in many cases replace more laborious and time-consuming methods used for similar immuno-

chemical purposes. Working with nitrocellulose membrane strips was found to be simpler and more convenient, than the use of various solid (mostly powdered) immunosorbents[6–10] which are sometimes difficult to prepare and to handle.

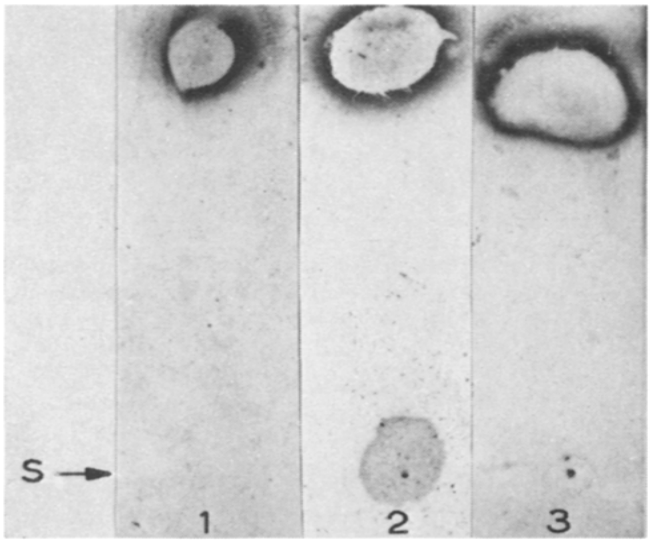

Fig. 2. Immunochromatography on protein-impregnated HUFS nitrocellulose membranes. (1) Membrane impregnated with fowl serum, rabbit antibovine serum applied; (2) membrane impregnated with bovine serum, rabbit antibovine serum applied; (3) membrane impregnated with bovine serum, normal rabbit serum applied. Impregnation and chromatography was made in a veronal–acetate buffer, pH 7.1. Chromatographed for 60 min. Sample volumes were of the order of 10^{-4} ml. Stained with Amido Black 10 B. (Irregular coloration of the migrating protein spots was caused by overloading the membranes with superfluous protein which was split off as a crust during staining.)

However, in orientation experiments where antiserum was adsorbed to the membranes and the antigen was chromatographed, there was found only a negligible adsorption of the antigen–antibody complex on the start. This "negative" result could be caused, among other reasons, by a partial destruction of the specific architecture of the antibody molecules by surface denaturation during their adsorption to the membranes.

Further investigations of a nitrocellulose membrane immunosorbent and its use for quantitative immunochemical estimations are under way.

Detection of proteolytic activity

Protein impregnated nitrocellulose membranes were also found very suitable for the simple and convenient detection of ultramicroamounts of proteolytic enzymes. A characteristic result of a test for trypsin diluted from 50 mg % to 0.05 mg % is shown in Fig. 3. (Similar results were also achieved with pepsin and papain.) In the place where the enzyme had been applied to the impregnated membrane and where an enzymatic cleavage of the stationary protein layer took place, light "negative" spots with sharp contours were formed on a dark background within a few minutes. Membranes impregnated with heat denatured serum were most suitable for this purpose allowing 0.0001 μg of trypsin to be detected and even less in 1 μl after 20 min incubation. When smaller volumes of the order of 10^{-5} ml were applied[1,11], picogram amounts (10^{-12} g) of trypsin could still be detected easily. Sharper spot formation on

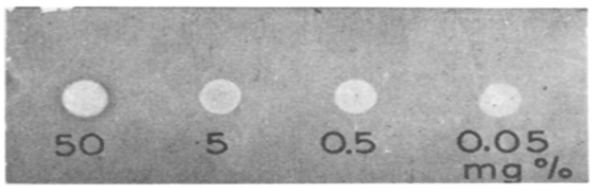

Fig. 3. Detection of trypsin on nitrocellulose membranes impregnated with heat denatured bovine serum. Trypsin samples diluted by a veronal–citrate–oxalate buffer, pH 8.6, to the indicated concentrations were applied in 1 μl volumes and incubated at 37° for 20 min. Stained with bromophenol blue.

this medium (as compared to the results on membranes impregnated with native proteins) is apparently due to the fact that heat denatured proteins are usually more readily split by enzymes and also their binding capacity towards dyes is generally greater than in the case of native proteins.

It was also possible to perform a microelectrophoretic fractionation (*cf.* ref. 1) of the enzymatically active preparations directly on the protein-impregnated membrane before incubation. However, care must be taken to use a pH region during electrophoresis, such that the activity of the enzyme is minimal. Thus, a veronal buffer, pH 8.6 was used for pepsin and after electrophoresis (*cf.* ref. 1) the membrane was incubated for 1 h in a moist chamber containing vapours of HCl, to acidify the medium.

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SUMMARY

Nitrocellulose membrane filters impregnated with proteins were found to be very suitable supporting media for microchromatography and detection of various substances, especially those interacting with the stationary protein layer. This was demonstrated in experiments with phenolsulphophthalein dyes and immunochemically active sera, as well as with proteolytic enzymes. A simple detection of specific antibodies was achieved within about 60 min by chromatographing 0.1 μl aliquots of rabbit antibovine serum on membranes impregnated with the antigen, *i.e.* bovine serum or serum albumin. Nitrocellulose strips impregnated with heat denatured serum were used for the rapid detection of proteolytic activity. Picogram amounts of trypsin were thus detected within about 30 min.

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