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DETERMINATION OF SERUM AND BODY FLUID PROTEINS BY MEMBRANE CHROMATOGRAPHY ON NITROCELLULOSE FILTERS: SYNPOR, SARTORIUS AND MILLIPORE

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SUMMARY

Different types of nitrocellulose membrane filters, *viz.* Synpor, Sartorius and Millipore, were tested as a carrier medium for chromatography and electrophoresis of proteins. All the membranes displayed a characteristic strong adsorptivity towards proteins which could be prevented by an impregnation with neutral detergents (*e.g.* the Tweens). Some important differences in chromatography on various types of membranes were also found and discussed.

Rapid quantitative protein determinations could be obtained by radial membrane chromatography of solutions containing concentrations of 0.001–6 % of various proteins.

In a previous paper[1] we described a simple technique for the rapid quantitative determination of very small amounts of protein (*e.g.* 1 μg/1 μl) by radial chromatography on nitrocellulose membranes (Synpor 6) (former name: HUFS) at pH 3.7. The areas, which were well formed, sharp circular spots of the adsorbed proteins, were proportional to the protein concentration. In the experimental programme it was further observed that Millipore HA membranes were also suitable for the same purpose[2], and with Sartorius 11011 membranes (former name: MF 10) it seemed possible to determine serum proteins even at a neutral pH[3].

The present investigation was undertaken to compare the applicability of the various types of nitrocellulose membranes to protein determinations in native serum, in cerebrospinal fluid, various preparations of modified serum[4] and modified globin[5]. Special attention was given to the analysis of very low protein concentrations, down to 1 mg %. Some other properties of the different membranes were also studied, *e.g.* their impregnation with detergents preventing the adsorption of proteins.

MATERIALS AND METHODS

Native human and rabbit serum, human cerebrospinal fluid, bovine serum modified by heat and formaldehyde[4] and carboxymethylated human globin[5] were used as standard model samples.

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The following membranes were used: Synpor 1-9 (Chemapol, Prague), Millipore RA, HA, VC (Millipore s.a., Paris) and Sartorius 11001-11011 (Göttingen). It was usually necessary to wash the membranes in boiling water before use to permit regular development of the spots[1]. This operation could be omitted in most cases with Sartorius membranes 11001-11006, and some batches of 11011. Acetate buffer, pH 3.7[1], and citrate buffer, pH 7.0, were used to develop the chromatograms. Radial chromatography of the more concentrated (*e.g.* 1–6 %) protein solutions, application of 1 μl volumes of the samples, rinsing of the capillary, staining with 0.01 % nigrosine or 0.05 % amidoblack 10 B in 3 % trichloracetic acid and quantitative evaluation of the protein spots was carried out as described earlier[1,3]. Self-filling capillaries calibrated up to volumes of 50 μl were used for the analysis of the very dilute protein solutions. The simple arrangement shown schematically in Fig. 1 was found most convenient for the automatic application and development of protein samples on the membranes. The development was performed by application of 1–2 volumes of the buffer using another capillary. Dry strips (about 3 × 5 cm) of Whatman No. 1 filter paper were used to allow further spreading of the developing buffer from the 2 × 2 cm strips of the nitrocellulose membrane. It was advantageous to slightly wet the contact between the membrane and the paper (*cf.* Fig. 1) with the buffer just before chromatography. No closed chromatographic chamber was necessary for these experiments.

Separate control determinations of the protein concentrations of the standard solutions were done by the Kjeldahl method, the Folin method and by refractometry[6].

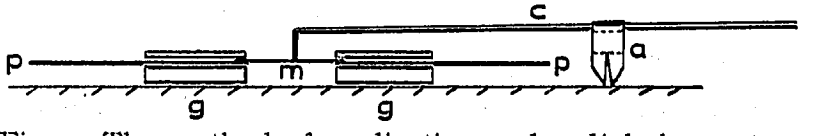

Fig. 1. The method of application and radial chromatography of diluted protein solutions. c = Self-filling glass capillary; a = adhesive paper tape stabilizing the vertical orientation of the capillary; m = membrane; p = filter paper; g = glass slides.

RESULTS AND DISCUSSION

It was found, generally, that all the different types of intact nitrocellulose membranes under investigation adsorbed the proteins investigated in a similar way, However, the areas of the spots formed by the same amounts of the proteins were different on different membranes (Fig. 2). Generally, larger spots were formed at a greater speed on membranes having larger mean pore sizes[1]. The quality of the spots (their symmetry, sharpness of the contours, reproducibility) differed, too. Some types of membrane were not of any use for accurate determinations (*e.g.* Synpor 1 and 2).

When analyzing undiluted or slightly diluted native serum, very good results were achieved at pH 3.7 on Synpor 5,6,8, and Millipore RA, HA, VC membranes and on all the Sartorius membranes. The deviation of the mean was between 3–5 % on different membranes. Modified serum and modified globin (which precipitate at pH 3.7) could be analyzed accurately at pH 7.0 on the same types of membranes as mentioned above. Native serum could only be determined quantitatively, at pH 7.0, on Sartorius 11011 filters; however, in this case the area of the spot was not quite homogeneous. On the other types of nitrocellulose membrane, chromatography of serum at neutral and slightly alkaline pH led to the formation of at least two sharp

circular zones, the inner corresponding to the globulins, the outer to the albumin fraction. The latter phenomenon, which seems to be of both theoretical and practical interest, is being investigated in detail and will be referred to later.

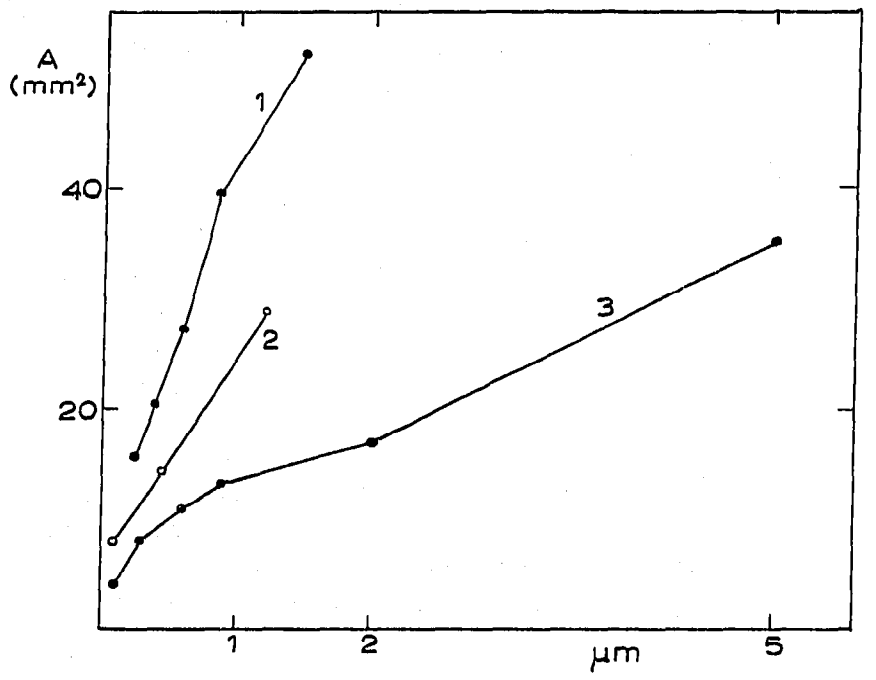

Fig. 2. Areas of protein spots on different types of nitrocellulose membranes. 1 = Synpor; 2 = Millipore; 3 = Sartorius. About 1 μl of 1% human serum (diluted by acetate buffer, pH 3.7) was applied by the same capillary (*cf.* ref. 1). A = Areas of the spots; μm = mean pore sizes of the membranes, according to the manufacturer's data.

The calibration curves shown in Figs. 3 and 4 indicate that the proposed simple technique permits accurate and rapid estimations of proteins also in the very low concentrations which are encountered, for example in cerebrospinal fluid (liquor), in pathological urine, or in contaminated water. Centrifugation or filtration to remove small inhomogeneities in those fluids was not necessary here—they remained fixed at the start. The volume of the solution to be analyzed may be chosen for a given series of samples and a given type of membrane over a wide range in order to achieve protein spots of a convenient size (*i.e.* with a diameter of about 5–15 mm). The samples applied in larger volumes (*i.e.* of the order 10^{-2} ml) were adjusted to pH 3.7 by adding the acetate buffer before chromatography to ensure better reproducibility. When this step was omitted and samples were applied without previous adjustment of the pH, the protein spots sometimes had two well formed concentric areas, the inner being distinctly darker after staining. It was assumed that the inner area probably corresponded to a second protein layer deposited over the first one which was fixed directly to the membrane. This assumption seemed to be confirmed by the fact that a linear calibration curve was only obtained by adding both areas for each concentration.

The areas of the protein spots were generally greater when measured on the wet membranes than on the dry ones. The difference (about 5–10%) was due to a regular

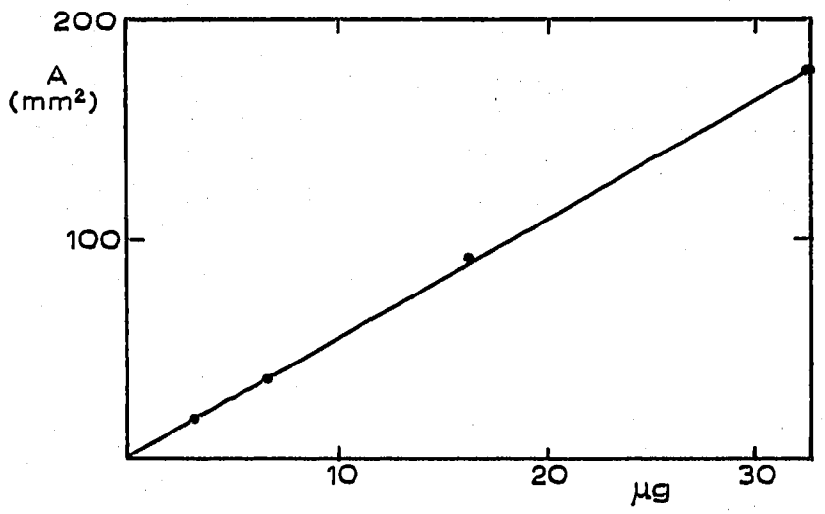

Fig. 3. Calibration curve of very dilute rabbit serum. Rabbit serum diluted by the acetate buffer, pH 3.7, was applied in 50 µl volumes on Sartorius 11001 membranes (mean pore size 5 µm). Chromatography took about 6 min.

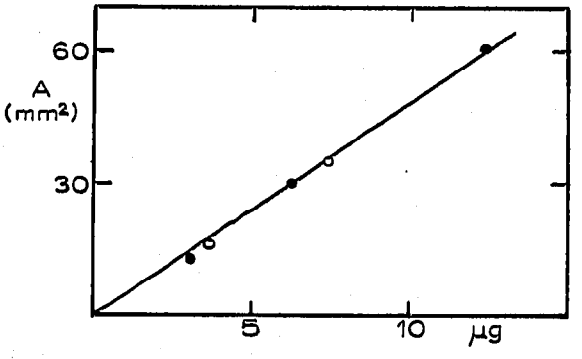

Fig. 4. Calibration curve of human cerebrospinal fluid. Cerebrospinal fluid diluted 2 and 4 times respectively, was applied in 20 µl and 40 µl amounts, respectively, on Sartorius 11001 membranes. ● and ○ correspond to two different patients. Chromatography took about 3 min.

shrinkage of the membranes during drying. The change was "reversible" on wetting the dry chromatogram repeatedly. Thus, two different linear calibration curves can be obtained, one for wet and one for dry membranes.

Furthermore, there were great differences (up to 30 %) when the same spots were measured on the front and the back of the chromatogram. These differences were negligible on Sartorius 11001. On those membranes, where the front and back are easily recognized (*e.g.* Synpor 6 membranes have a glossy surface on one side) it was convenient to use only one side for the calibration and protein determination (*cf.* ref. 1). However, on other membranes, the most accurate results were obtained by calculating the average value from the areas measured on both sides of the dry membrane.

In the studies on the impregnation of the membranes with neutral detergents, it was found that Tween 20, 40, 60 and 80 all had an equal effect in preventing the adsorption of proteins on all types of nitrocellulose membranes tested. Microelectrophoresis of proteins was carried out on the impregnated membranes as was described earlier[7]. Good results were obtained on membranes having pore sizes about 0.2–0.4 µm.

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