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SEPARATION OF SERUM ALBUMIN BY RADIAL CHROMATOGRAPHY ON NITROCELLULOSE MEMBRANES

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SUMMARY

Radial chromatography of serum proteins was investigated under various experimental conditions on nitrocellulose membranes. Development of about 15 μg/0.5 μl samples on Sartorius 11002 filters at pH 8.0–9.0, and with a salt concentration of up to 5 % NaCl at laboratory temperature (18–25°) was found most suitable for the small scale separation of serum albumin from the majority of other native or denatured serum proteins, within about 1 minute.

As reported earlier[1], serum proteins form two zones during radial chromatography on suitable nitrocellulose membranes at neutral and slightly alkaline pH. The inner zone seemed to correspond to the globulins, the outer to the albumin fraction.

The present communication deals in more detail with the finding mentioned above, by investigating the various conditions which have been assumed to enhance the desorption of proteins and by characterizing immunochemically the main protein zones formed during radial chromatography of serum. Some practical aspects, *e.g.*, a rapid separation of serum albumin and estimation of the albumin–globulin quotient, were also examined.

MATERIALS AND METHODS

Native human serum, gamma globulin and serum albumin from the Cohn's ethanolic fractionation (prepared in our Institute) as well as serum albumin denatur- by heating a 1 % solution in Michaelis buffer[3] pH 8.5 at 100° (no precipitation occured) red were used as standard samples. Before radial chromatography the samples were diluted 1:1 by the given buffer and about 0.5 μl were applied to the origin wetted with buffer[1,5]. In some experiments serum and albumin were stained with bromophenol blue before application, to permit the convenient observation of the movement of the albumin fraction. Radial chromatography was performed on nitrocellulose membranes Synpor 3 and 6 (Chemapol, Prague) and Sartorius 11001, 11002, 11006 and 11011

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(Göttingen) as described earlier[1]. Strips 2 × 2.5 cm were used. The samples were developed continuously, the buffer being applied by a suitable capillary[1]. A series of isotonic Michaelis buffers[3] from pH 3.8 to pH 9.3 and veronal buffer pH 8.6[2], $\mu = 0.05$ were used to develop the chromatograms.

Both the outer and the inner protein zones were identified by micro-immunoelectrophoresis in agar gel[2]. Rabbit antihuman serum SEVAC-Prague (1:10,000) was used for immunoprecipitation. The outer zone of the chromatogram (presumably albumin stained by bromophenol blue) was cut out immediately after chromatography, divided into several pieces and placed at the start of the agar gel. A similar procedure was used to identify the inner zone on a parallel chromatogram. In that case, however, it was convenient to elute the proteins of the inner zone with 2 % Tween 60 in the Michaelis buffer, pH 8.5, containing 5 % NaCl, immediately after the first procedure mentioned above. The front of the second developing solution was visible without staining, so that it was also possible to cut out this zone immediately after developing, place it while still wet on the start of the agar gel and wet it with another drop of 2 % Tween 60 in Michaelis buffer, pH 8.5.

The chromatograms were stained in solutions of 0.01 % nigrosine or 0.05 % amidoblack 10 B in 3 % trichloroacetic acid for 10 min at laboratory temperature. Immunoelectrophoretic patterns were stained with a solution of 0.1 % amidoblack 10 B in an acetate buffer pH 3.5. Decolorisation of the background was carried out in 3 % acetic acid.

The determination of the albumin–globulin quotient A/G was attempted by radial chromatography of diluted human serum using the Michaelis buffer, pH 8.5. After staining the chromatogram in amidoblack 10 B for 10 min and decolorising the background, both zones were cut out close behind the colour edge and added to 1.5 ml of 0.1 *N* NaOH. The absorbancy of the solutions was measured at 630 nm using the Specol photocolorimeter.

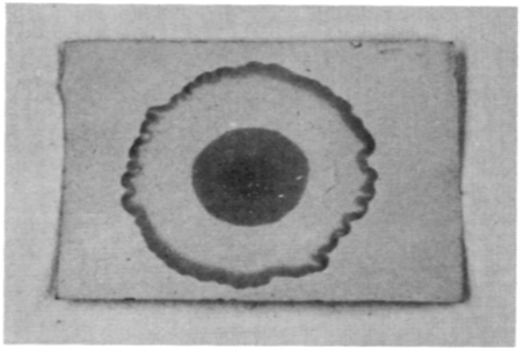
RESULTS AND DISCUSSION

The adsorption of serum proteins and especially of serum albumin on intact nitrocellulose membranes was found to change gradually at different pH values (*cf.* ref. 4). At pH 3.7 the serum proteins formed one uniform spot, with an area proportional to the protein concentration[1,5]. When chromatographed in a series of buffers with the pH value increasing between 3.8 and 9.3, the stepwise formation of two zones was observed beginning at about pH 6.5. The first fraction moved with the front of the developing buffer, while the second fraction remained adsorbed in the center of the circle (Fig. 1) even at pH 9.3. The best separations were achieved on Sartorius 11002 membranes at pH 8–9, preferably at 8.5.

As proved by immunoelectrophoresis, the outer area corresponded predominantly to serum albumin (Fig. 2), while the globulins remained mainly in the inner zone. However, a slight precipitation zone adhering to but not crossing the albumin zone was also observed (Fig. 2). Minute amounts of albumin in the inner zone as well as in the "free" area between the two zones were also found immunoelectrophoretically. Nevertheless, the chromatographic technique mentioned above permits a simple and rapid single-step micropreparation of crude serum albumin.

Comparative chromatographic experiments were also performed at pH 8.5

using isolated human gamma globulin and serum albumin instead of whole human serum. Albumin formed one main zone at the front but a slightly diffuse spot at the center was also observed. Gamma globulin only formed a single circular spot at the center of the chromatogram.



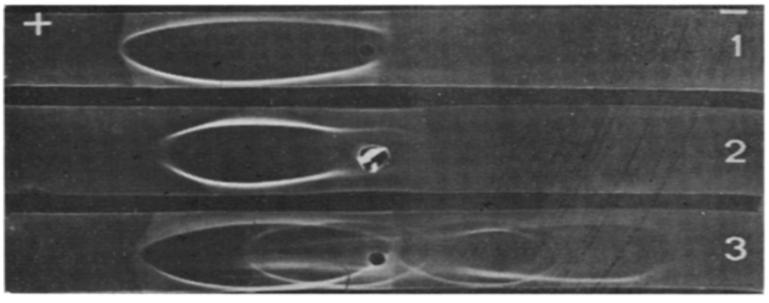


Fig. 1. Radial chromatography of human serum on Sartorius 11002 nitrocellulose membrane. Developed in Michaelis buffer[3] pH 8.5 for about 1 min. Outer diameter about 18 mm. The outer zone corresponds predominantly to serum albumin, the inner zone to the globulins.

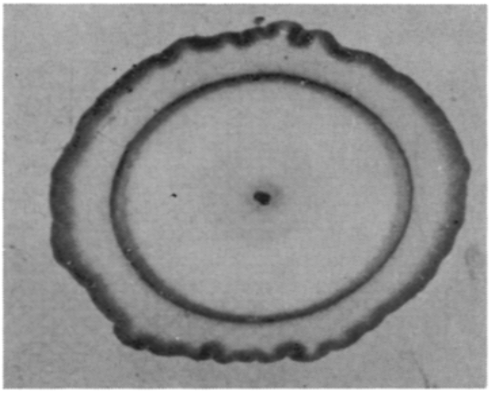
Fig. 2. Immunoelectrophoretical characterization of the "albumin" zone (see Fig. 1) in agar gel. 1 = Comparative run of human serum albumin; 2 = proteins of the outer zone (*cf.* Fig. 1); 3 = comparative run of human serum. Veronal buffer pH 8.6[2].

A similar result to that with gamma globulin was achieved with denatured serum albumin, and likewise with denatured and modified whole serum and globin[1]. The areas of the spots, formed as a result of circular chromatography of equal amounts of native and denatured serum, were significantly different: 100 % for native serum at pH 3.7, 30 % for the denatured one at pH 3.7 and 28 % for the denatured at pH 8.5. Apparently, denatured molecules and their aggregates are more tightly packed at the nitrocellulose surface or form higher multilayers. With regards to the firm, adsorption of denatured proteins and the globulins, even at pH's above 7, we are inclined to believe that it may be partly explained by the formation of nonpolar bonds between the lipophilic groups in the uncoiled parts of the protein molecules and the lipophilic groups of nitrocellulose. Changes in the conformation and molecular weight of the particles also seem to play an important role here[4]. Experimental work dealing with the above question is under investigation.

It seems to us that the inner zone observed on the chromatograms of isolated serum albumin probably consists of small amounts of "impurities", *e.g.* denatured albumin, globulins and also albumin polymers and aggregates. This was confirmed in separate experiments where the advancing front-zone was allowed to spread on to another nitrocellulose membrane (after having arranged a direct contact between them). No central spot corresponding to "impurities" was observed here. Consequently, membrane chromatography on nitrocellulose might be useful as a simple means for a rapid check on the quality of various albumin preparations.

Practically all firmly adsorbed proteins could be washed from the center by 2 % Tween 20, 40, 60 or 80 over the whole pH range tested (3.8–9.3) (Fig. 3). However, marked differences were observed depending on whether the 2 % Tween 60 had been dissolved in water or in Michaelis buffer, pH 8.5. When an unbuffered aqueous Tween

solution was used for the second elution, after chromatography of serum at pH 8.5, a part of the proteins still remained adsorbed at the central spot. It was possible to desorb them by adding 5 % NaCl to the solution of Tween (Fig. 4). Elution by different concentrations of Tween was not suitable for fractionation, since it has the property of replacing chromatography with an "all or none" elution effect on proteins. A series of various reagents and conditions, *e.g.* development by 8 *M* urea in water or with increasing concentrations of 0.9–20 % NaCl, as well as the use of extreme pH values on the acid and alkaline sides, were not successful in attempts to desorb the proteins or to achieve their further fractionation. This fact was probably due to the denaturation of the proteins under those conditions.



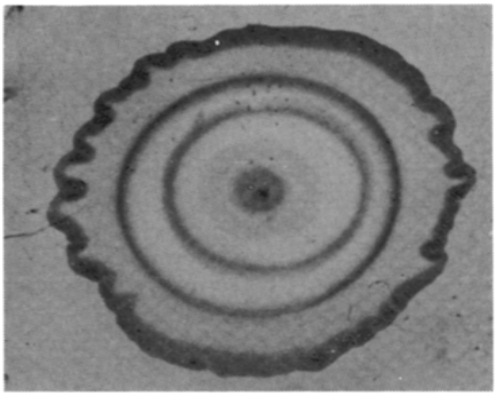


Fig. 3. Double development of human serum. First development was identical to that in Fig. 1. For the second development 2 % Tween 60 in Michaelis buffer, pH 8.5, was used.

Fig. 4. Triple development of human serum. First development as in Fig. 1; the second was made by 2% Tween 60 in water, the third by 2% Tween in 5% NaCl.

However, it was found possible to use the separation of albumin from other serum proteins on nitrocellulose membranes, at slightly alkaline pH, for a rapid and simple determination of the albumin–globulin quotient in a clinical laboratory. In preliminary experiments we found the A/G quotient of normal human serum to be 2.0, which is in good agreement with frequently encountered data.

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