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PAPER CHROMATOGRAPHY OF PROTEINS BY GRADIENT ELUTION TECHNIQUES

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The chromatographic techniques using paper fibers as adsorbent and several step elution of proteins with salt solutions of increasing concentrations have been developed. The influence of electrolytes on the chromatographic resolution of proteins has been presented.
The chromatographic separation of a series of natural and artificial mixtures has been set forth.

The chambers for ascending and descending paperstrip chromatography procedures with salt concentration gradient have been described.

INTRODUCTION

Twenty-five years have nearly passed since specific adsorption and chromatographic techniques were applied by *Agner* (1) for isolation of enzyme catalase.

Extensive attempts have been made in employing different principles, adsorption, ion-exchange and partition for separation of peptides or proteins from the mixtures.

A. Tiselius used salting out adsorption for separation and isolation of proteins on silica gel (2). *Swingle* and *Tiselius* found that using phosphate buffer and tricalcium phosphate, a displacement elution of proteins could be effected because of preferentially adsorbed phosphate buffer (3). *Polls* and *Schmmukler* (4) published similar experience.

Sober, Kegeles and *Gutter* (5) were the first to use a sulphonic resin to resolve protein mixtures by ionic-exchange resin chromatography.

Hirs, Moore and *Stein* (6), *Talan* and *Stein* (7), *Jolles* and *Fromageot* (8), *Boardman* and *Partridge* (9) have definitively proved the great value of the ion-exchange resin techniques for the chromatography of proteins.

The partition chromatography of proteins was proposed by *Herbert* and *Pinsent* (10) for purification of catalase.

Martin and *Porter* (11) chromatographed ribonuclease using ammonium sulphate, water and ethylcelosolve for producing the phases on Hyflo super-cel as support. Different proteins were isolated and purified by similar methods, e. g. insulin from pancreas extract (*Porter,* 12), chymotripsinogen (*Porter,* 13), and many others.

Paper chromatography of protein mixtures has been the subject of a very great number of publications in the past fifteen years. Different homogeneous liquids of constant composition, mixtures of buffer solutions with organic solvents or neutral salt solutions, have been used as eluents.

The general success of paper chromatography of proteins cannot be claimed satisfactory as yet and further attempts are to be made.

Some essential references are: *Mitchell, Gordon* and *Haskins* (14), *Franklin* and *Quastel* (15), *Jones* and *Michael* (16), *Hall* and *Wewalka* (17), *Boman* (18), *Robinson* and *Fehr* (19), *Tauber* and *Petit* (20).

The need for cellulose adsorbents with different properties for protein chromatography led to the synthesis of cellulose preparations with favourable ion-exchange properties. Diethyl-aminoethyl-, carboxymethyl-, and triethylaminoethyl cellulose were synthetized. Some references are: *Sober* and *Peterson* (21), *Bergmann* and *Rimon* (22), *Conelly* and *Taborsky* (23), *Balley* (24), *Mann* (25), *Sköld* (26), *Ostrowsky* and *Tangita* (27), *Trayser* and *Colowick* (28) and many others.

In this paper we present the retesting of our previously published results (29, 30, 31, 32, 33, 34) on paper chromatography of protein mixtures by selectively eluting the proteins with a series of salt solutions, thus forming a controlled concentration gradient along the paper strip. In addition, a description is given of the construction of original apparatuses for ascending and descending chromatography techniques and preliminary results of proposed chromatographic methods as applied to the protein mixtures of the human serum are presented.

EXPERIMENTAL

Apparatus

The chromatographic chambres for ascending and descending techniques were developed.

For ascending procedure (Fig. 1.a.) a paper strip was fastened with a clip to a support made of plastics and covered with a rectangular glass jar (30 × 16 × 12 cm). A humid atmosphere was created by a wet filter pad disc placed inside the jar.

The essentials of the apparatus for ascending chromatography consist of a movable glass plate provided with 10–20 rectangular ground wells (50 × 3 × 2 mm in size), which are used as receivers for eluents. It is not necessary to open the air tight chamber during procedure because the lower end of the paper strip can be dipped into the next, more concentrated salt solution by merely shifting a furrowed glass plate.

When descending technique (Fig. 1.b.) was applied, the paper strip was inserted through a rectangular hole in the glass cover plate and there it was fastened by means of a heavy glass weight. In regular time intervals (5 minutes), a small volume (circa 0.2 ml) of each serial salt dilution was pipetted on the paper strip end standing out of the cover plate hole.

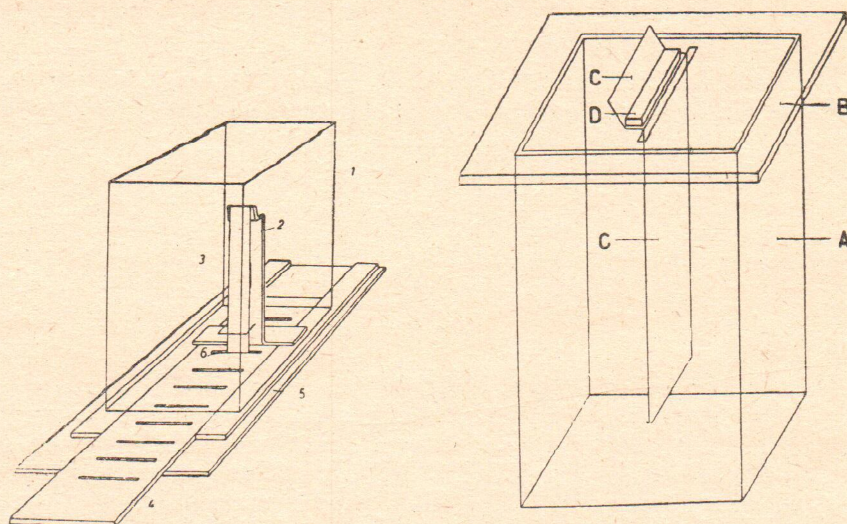

Fig. 1.a Chromatographic chamber for ascending procedure: (1) glass jar, (2) paper strip holder, (3) paper strip, (4) glass plate with 20 ground wells, (5) sliding frame, (6) wells for eluents.

Fig. 1.b Chromatographic chamber for descending procedure: (A) glass jar, (B) glass plate with hole, (C) paper strip, (D) heavy glass weight.

Development of chromatograms

The paper strips with applied protein mixture were developed by ten step serial dilutions of a concentrated buffer or salt solution. The proteins to be tested were dissolved in water or if not soluble, in the salt dilution of the lowest possible ionic strength. The dialysis against water was sometimes carried out previously. Generally, solutions containing 50–100 μg test substance per 0.01 ml were used. This volume was placed by means of a micropipette on the ground edge of a little glass plate ($2 \times 25 \times 25$ mm), fixed on the rubber base. The paper strip was then pressed at the starting line on the glass edge, and the test substance was in this way transferred accurately as a narrow band onto the paper strip.

The chromatograms were developed in such a way that the most diluted solution (of a set of ten) was allowed first to move to the protein

application spot (to the starting line), and then it was exchanged for a more concentrated one, and so on for each centimeter of the paper strip. The development of the chromatogram lasted 50–60 minutes.

The serial dilutions in a progressive scale of concentrations were prepared by exactly pipetting (f-1) ml of water in each of 9 tubes. The tenth tube (C_{10}) contained the most concentrated solution. One milliliter from the C_{10} solution was then passed back from tube to tube, beginning with No. 10 and ending with No. 1.

The individual concentration C_n of a serial dilution set of ten steps including the first, the lowest C_1, and the highest C_{10}, may be computed

by the equation $C_n = C_1 \left[\frac{C_{10}}{C_1}\right]^{1/9}$

The dilution factor $f = \left[\frac{C_{10}}{C_1}\right]^{1/9}$

Paper strips

For a large number of chromatographic experiments *Whatman* No. 1. filter paper strips 25 × 200 mm could be used. Two pencil lines across the paper strip were drawn, one at the distance of 1.2 cm from one end and another 10 cm further off. The intervals of one cm were marked between these two lines. Other filter papers successfully used were *Schleicher–Schüll* Nos. 2043 bMgl, 2043 a, 2043 b, 602 hP, 598 L, several sorts with directed paper fibers 2040 bM, 2045 aM, 2045 bM, 2045; and other kinds of papers for special purposes with anion-exchange properties *Whatman* DE 20 diethylaminoethylcellulose, carboxymethylcellulose, and cellulose citrate.

In order to remove the traces of electrolytes from the filter paper *Whatman* No. 1. paper strips were washed with bidistilled water (24 h) or with 0.2 N HCl and bidistilled water.

For staining developed chromatograms were immersed in a bromophenolblue solution consisting of 6 ml dye stock solution (1 gr of bromophenolblue in 100 ml ethanol, saturated with mercuric chloride), and 200 ml water. The complete safe washing of stained chromatograms can be performed with 0.2 percent aqueous mercuric chloride. Another method can be used succefully by staining the chromatograms in 0.1 per cent methanolic bromophenolblue for 30 minutes and washing the paper strips in 5 per cent aqueous acetic acid.

The photometric evaluation of stained chromatograms can be carried out with Spinco Analytrol model RB, interference filter 500 mμ, or with specially modified Fisher electrophotometer at intervals of 1 mm on the paper strip.

RESULTS

Salt concentration gradient

In order to control the shape and slope of the salt concentration gradient, the blank strips of filter paper were developed. The filter paper was washed previously with distilled water or with hydrochloric acid (0.2 N) and water. As soon as the liquid reached the tenth centimeter, the movement of the solvent was stopped by cutting the strip.

The electrical conductivity of the wet paper strip was measured at various points 0.5 cm apart from each other, by means of a pair of foot-like flat electrodes and the Mullard conductometer. It has been noted (Fig. 2) that some electrolyte pushed ahead nearly in front of the wetting liquid. Because of some reasons not fully explicable, we always failed in obtaining a strictly linear salt concentration gradient on the paper strip. The streaming potential conditions in the process of paper wetting may have caused an increase of the electrolyte concentration in front of the eluent.

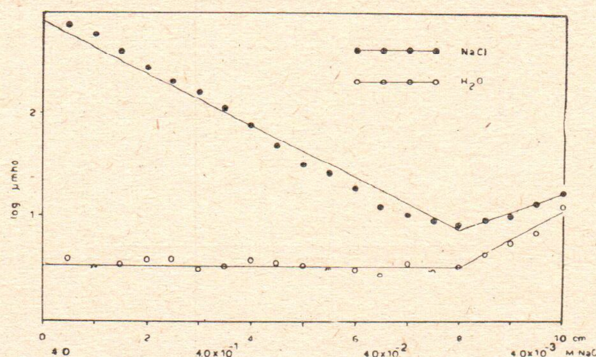

Fig. 2. Diagrams of the logarithmic concentration gradient of sodium chloride 4×10^{-3} to 4 M on the paper strip, and gradient produced on washed paper strip developed only with water, as determined by conductivity measurements. (Asc. techn.)

Influence of salts on protein desorption

In order to acquire some information about the influence of different cations and anions on the desorption of proteins from paper fibers, we investigated the elution of pure human and bovine albumin with chlorides of alkali-metal group. The elution of proteins was accomplished with the same concentration of chloride (2×10^{-3} to 2 M) irrespective of cations Li^+, Na^+, K^+, Rb^+, Cs^+. From the results of testing the effect of lithium salts (Cl^-, J^-, NO_3^-, $SO_4^{=}$) with different anions on the chromatography of nine different pure proteins preparations, we could not notice any regularities.

1. *Ribonuclease*

Several preparations of crystalline beef pancreas ribonuclease of high grade purity (115 K. U./mg) were chromatographed (Fig. 3) on *Whatman* No. 1. filter paper, with NaH_2PO_4 3×10^{-3} to 3 M and H_3PO_4 7.32×10^{-4} to 7.32×10^{-1} M. All the preparations could be separated into a major and a minor component. Both components showed enzymic activity (29). (Ascending technique).

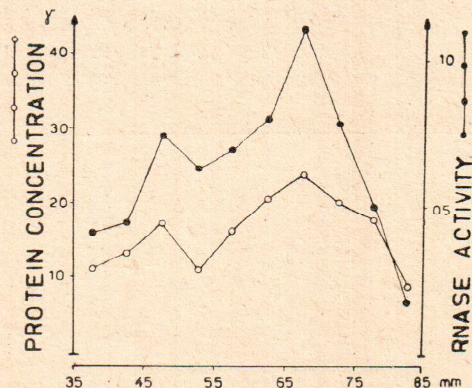

Fig. 3. Four times recrystallized ribonuclease (115 K. U./mg) resolved into two components on Whatman No. 1. filter paper strip with phosphate buffer pH 2.6; protein concentration and Rnase activities were determined in extracts of paper chromatogram cuttings. (Asc. techn.).

2. *Insulin and ribonuclease*

The mixture of recrystallized preparation of insulin and ribonuclease in 10^{-3} M hydrochloric acid was used to simulate the native extract from beef pancreas. The mixture could be separated chromatographically using ten-step serial dilutions of 4×10^{-3} to 4 M sodium chloride (Fig. 4). Under such experimental conditions the enzyme appeared on the chromatograms as a single band. (Ascending technique).

3. *Commercial insulin*

The commercial samples of insulin of lower hormonal potency (22.7 I. U./mg) when chromatographed with ten dilutions of sodium chloride 3.0×10^{-3} to 3.0 M in veronal – veronal sodium buffer (1.84 gr. veronal and 10.3 gr veronal sodium in 1000 ml H_2O) was separated into two protein components. Only one of the components showed hormone activity. The insulin preparation of high hormone potency was chromatographically homogeneous consisting of only one biologically active substance. (Ascending technique).

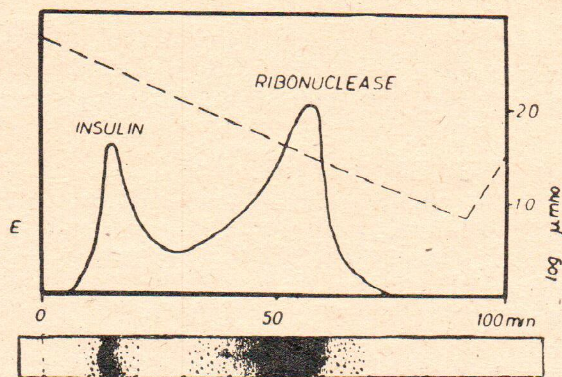

Fig. 4. The photometric evaluation of paper chromatogram of recryst. ribonuclease and insulin. The chromatogram was developed with sodium chloride 4×10^{-3} to 4 M. (Asc. techn.)

4. *Insulin and protamine*

Four times recrystallized insulin and protamine (Mugil cephalus) were separated from a mixture in acid solution (0.01 N HCl) on *Whatman* No. 1. filter paper by sodium chloride solutions; the gradient of concentrations applied was 4×10^{-3} to 4 M (30). (Ascending technique).

5. *Egg albumin and lysozyme*

An artificial mixture containing proteins, egg albumin and lysozyme, otherwise naturally occuring in the egg white was resolved (31) on the paper strip using the same sodium chloride eluents as described above (Fig. 5). (Ascending technique).

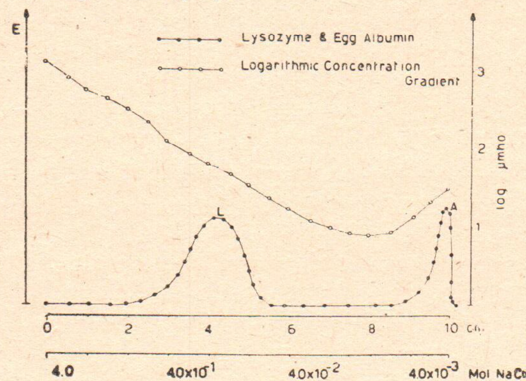

Fig. 5. Photometric evaluation of chromatogram developed with sodium chloride 4×10^{-3} to 4 M; mixture: lysozyme and egg albumin. Logarithmic concentration gradient of sodium chloride on paper strip, as determined by conductivity measurement. (Asc. techn.)

6. *Serum albumin and γ - globulin*

Bovine serum albumin and human γ-globulin (32) could be separated on *Whatman* No. 1. filter paper using sodium citrate buffer pH 10.1. as eluents ($\mu = 3 \times 10^{-3}$ to 3.0) (Fig. 6 b.). (Ascending technique).

7. *Serum albumin, γ-globulin and protamine*

Human γ-globulin, bovine serum albumin and protamine (Mugil cephalus) could be separated from a mixture containing these proteins using ten sodium citrate solutions (pH 10.1., $\mu = 3 \times 10^{-3}$ to 3) for gradient elution chromatography (Fig. 6 c). When electrophoretically pure γ-globulin was chromatographed on *Whatman* No. 1. filter paper with citrate buffer pH 4.6 some albumin and globulins ($\alpha + \beta$) impurities appeared as an additional band (Fig. 6 a). (Ascending technique).

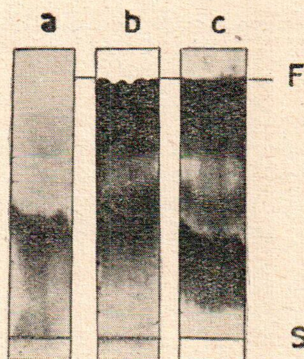

Fig. 6. The chromatograms of protein mixtures developed with sodium citrate buffer pH 10.1, $\mu = 3 \times 10^{-3}$ to 3; (a) bovine serum albumin, (b) bovine serum albumin and human γ-globulin, (c) bovine serum albumin, human γ-globulin and protamine (Mugilin). (Asc. techn.).

8. *Immune serum*

Purified horse immune serum (antitetanus, No. 1847., Immunological Institute, Zagreb) pepsin digested and concentrated, when chromatographed with citrate buffer, pH 5.6, $\mu = 3 \times 10^{-3}$ to 3, showed that it consisted of only one main component and some proteinic impurities originating from the enzymic digestion procedure. The best results were obtained on carboxymethylcellulose paper (*Whatman* CM 50). (Ascending technique).

9. *Human serum*

The human serum dialyzed against water (24 h, 5^{0} C) was chromatographed on *Whatman* No. 1. filter paper with sodium citrate buffer

$\mu = 3 \times 10^{-3}$ to 3, pH 5.2, using descending chromatographic technique. The globulins were well separated from albumin, but reproducibility of the experiment was not quite satisfactory (Fig. 7).

10. *Snake venom*

Ammodytes viper venom, a natural mixture of toxic proteins, was chromatographed on *Whatman* No. 1. filter paper strip (Fig. 8). (Asc. techn.). The sodium chloride solutions of different concentrations ranging from 1.7×10^{-3} to 1.7 M were used as eluents. Seven protein components could be distinguished on bromophenolblue stained chromato-

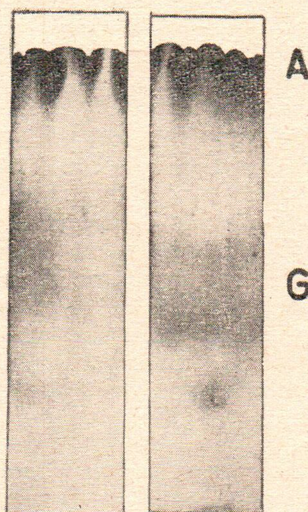

Fig. 7. The chromatograms of human serum developed with sodium citrate buffer, $\mu = 3 \times 10^{-3}$ to 3, pH 5.2. (Desc. techn.).

grams. These results agree with those obtained with electrophoresis. Only double diffusion test with the horse antivenom serum gave better results (34).

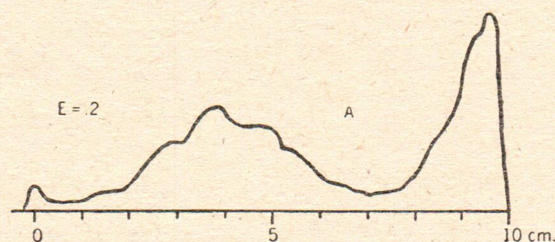

Fig. 8. The photometric evaluation of chromatogram developed with sodium chloride concentration ranging from 1.7×10^{-3} to 1.7 M. *Vipera ammodytes* venom. (Asc. techn.).

DISCUSSION

No type of protein chromatography has achieved success similar to that of the paper chromatography of amino acids or other classes of organic compounds. This could be explained by the fact that proteins represent an enormously large group of compounds showing very different physical and chemical properties, and there is little prospect of working out a general chromatographic method applicable to the resolution of any protein mixture.

The characteristics of the proposed paper chromatography technique are the application of the filter paper as adsorbent and the consecutive elution of proteins with high numbers of salt solutions of increasing concentrations. We have experienced that organic solvents are to be avoided because they may cause denaturation.

The gradient of pH or of salt concentration or of both may be used for separation of protein components, but we have observed that the salt concentration of solvent rather than pH is the controlling factor in the selective desorption of proteins from the paper fibers.

The results of chromatographic experiments presented in this paper demonstrate the usefulness and great potentialities of the proposed paper chromatography technique. The resolution of such artificial mixtures as insulin-protamine, insulin-ribonuclease, egg albumin-lysozyme and others point out the satisfactory reproduction of experiments. Our greatest success was the resolution of crystalline beef pancreatic ribonuclease into two components. These results agree with those previously obtained with partition chromatography by *Martin* and *Porter* (11), and with ion-exchange chromatography by *Hirs, Moor* and *Stein* (6). The preliminary results of descending chromatography of serum proteins can be considered promising, and can serve as a basis for further investigation.

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Sadržaj

KROMATOGRAFIJA PROTEINA NA PAPIRU

Opisani su postupci uzlazne i silazne kromatografije proteina, kod kojih se upotrebljava papir kao adsorbens, a za eluciju niz otopina elektrolita u rastućim koncentracijama. Ispitan je utjecaj elektrolita na kromatografsko razdvajanje proteinskih smjesa. Prikazane su originalne aparature za uzlaznu i silaznu novopredloženu kromatografsku tehniku.

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