SEGREGATION OF MEMBRANE COMPONENTS USING ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS

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Received August 7,1972

Summary: A method for efficiently fractionating human erythrocyte membranes into their individual components is presented. This procedure employs isoelectric focusing in polyacrylamide gels after solubilization of the membranes in 8M urea, 0.02M EDTA, 0.2% 2-Me. Electrofocusing of membranes affords higher resolution than previous membrane separation procedures. Also, this method is rapid, requiring 72 hours at most to separate, stain and destain the membrane components in the polyacrylamide gels.

INTRODUCTION

Paramount in examining membrane structure and function are methods capable of separating the membrane components into distinct populations. Two such methods used frequently at the present time are gel chromatography (1) and polyacrylamide gel electrophoresis (2,3), both performed in the presence of the anionic detergent sodium dodecyl sulfate (SDS). These methods do not, however, provide the maximal degree of separation. Since the separation criterion in both cases is subunit size, which is not necessarily an absolute parameter for molecular distinction, adequate resolution necessitates the introduction of other systems of macromolecular segregation.

Svensson and Vesterberg (4-7) introduced the method of isoelectric focusing or electrophoresis in a pH gradient as an effective method of separation of heterogeneous mixtures with respect to charge. Jamieson and Groh (8) applied this technique to whole human erythrocyte and lymphocyte populations. This report describes the extension of isoelectric focusing in polyacrylamide gel support matrices to solubilized membranes and the higher degree of resolution which this technique affords.

MATERIALS

Acrylamide (electrophoresis grade), N,N,N',N'-tetramethylethylenediamine (TEMED) and N,N'-methylenebisacrylamide (BIS) were obtained from Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N.Y. Further recrystallization of BIS from $CHCl_3$ was performed as described (9). Ampholine, pH 3-10, was purchased from LKB, Inc., Rockville, Md. Dialyzed urea was supplied by Heico, Inc., Delaware Water Gap, Pa. Stoddard's solvent was obtained from Fischer Scientific Co., Pittsburgh, Pa. All other reagents were reagent grade.

METHODS

Preparation of Acrylamide Gels: Preparatory to casting gels of desired pore dimensions, the following modified stock solutions were prepared, combining the methods of Finlayson and Chramback (10) and Doerr and Chrambach (11). Acrylamide-BIS monomer was 10% acrylamide (w/v), 0.4% BIS (w/v) and 25% sucrose (w/v) in 8M urea. Sucrose concentrate was 50% sucrose (w/v) in 8M urea. Catalyst was 0.5% $(NH_4)_2\ S_2O_8$ (w/v) in 8M urea. All of the solutions described above were filtered prior to storage in dark containers at 4° C. Chemical stability of these solutions was retained for at least four weeks following preparation. Pyrex gel tubes (length=16.0 cm, O.D.=0.8 cm, I.D.=0.6 cm) were presoaked in concentrated sulfuric acid for 12-18 hours, then rinsed with distilled water, and finally coated with "Tube Rinse" (Canalco, Rockville, Md.). After the gel tubes had been allowed to dry, either at room temperature or in an oven, a sheet of "Parafilm" and a rubber stopper were placed over one end of each tube; they were then placed in a test tube rack in an upright position. A polymer solution, yielding gels whose compositions were 2.5% acrylamide, 0.1% BIS, 12.5% sucrose, 1.0% ampholytes in 8M urea each, was then prepared by mixing the following proportions of stock solutions:

2.50 ml acrylamide-BIS monomer
0.25 ml Ampholine (pH 3-10)
1.25 ml sucrose concentrate
4.75 ml 8M urea
0.01 ml TEMED
1.25 ml catalyst

(To cast gels of different pore dimensions and sucrose concentration, the amounts of acrylamide-BIS monomer, sucrose concentrate and 8M urea were varied accordingly). This mixture was degassed with stirring under a water aspirator and pipetted into the stoppered tubes. Immediately the solution in each tube was overlaid with distilled water and allowed to polymerize for 2 hours at 4° C (initial polymerization occurring after 15-20 minutes). The above volume of polymer solution was sufficient for casting 3 gels of 0.6 cm diameter and 10 cm length.

Preparation of Electrode Buffers: The lower electrode (anodal) buffer was 1.0 liter of 0.2% H_2SO_4. This buffer was placed in the lower buffer vessel of the electrophoresis apparatus at 4° C two hours prior to initiation of electrofocusing. The upper electrode (cathodal) buffer was 1.0 liter of 0.4% ethanolamine. The cathodal buffer was placed in its compartment after the gel tubes were properly oriented (see "Results").

Preparation of Membrane Protein: Human erythrocyte ghosts were prepared by the method of Dodge et al. (12). Lypholized ghosts were solubilized by adding 10 mg to 1.0 ml of 8M urea, 0.2% 2-mercaptoethanol (2-Me), 0.02M Na_2 EDTA. After shaking gently, the solution was placed in a 37° C water bath for 12 hours. Any residual undissolved ghost fragments were removed by centrifugation at 10,000 x g. The resulting solution was cloudy, probably due to the presence of membrane lipids. To each gel 1 mg of solubilized ghost contained in 100 µl was added to 100 µl of sucrose concentrate and carefully pipetted through the cathodal buffer onto the upper face of each gel using a 9 inch Pasteur pipette.

Electrofocusing: After sample application had been completed, electrical connections were made to the power supply. The power was switched on and the current was adjusted to 0.5 mA per gel. Throughout the electrofocusing procedure the current was not allowed to exceed this ratio. After 12 hours at this level

of constant current, the voltage was fixed at 40 V/cm while the current was allowed to diminish and approach zero. This level of voltage was continued for 11-35 hours whereupon it was increased to 80 V/cm. This maximum level of voltage was continued for 1 hour. The power was then shut off and disconnections made. Total electrofocusing time varied from 24 to 48 hours.

Staining for Protein: After removing the gels by rimming with water, protein was stained using either the method of Vesterberg (13) or that of Wrigley (14). Destaining in either case was accomplished by placing each gel in 2 liters of 25% methanol, 5% acetic acid for a period of 24 hours. Destained gels were stored in 5% acetic acid.

Determination of pH: The pH gradient of each gel was determined employing the method of Finlayson and Chrambach (10). Gels were sectioned into 0.2 cm slices; to each slice 0.5 ml of 0.01M KCl was added. After standing for 2 hours with frequent agitation, the pH of each was measured.

RESULTS

During preliminary experiments it was found that bubbles formed within the gel matrix near the region associated with pH 5.0. These bubbles expanded with time and were generated without the application of membrane samples. Because the isoelectric point (pI) observed for the bubbles was very close to that of CO_2, it was conjectured that the bubbles were the result of migration of carbonate and bicarbonate ions to the pI of CO_2. Since the cathodal buffer was the most likely source of these anions, a procedure for preparing anion-free cathodal buffer was developed. One liter of distilled water was stirred at 25° C *in vacuo* for 4 hours prior to electrofocusing. Immediately preceding electrofocusing, 4 ml of ethanolamine was pipetted rapidly into the evacuated distilled water with minimal atmospheric exposure. Then, with the gel tubes properly oriented in the electrophoresis apparatus, the cathodal buffer was gently, but rapidly, poured into the upper buffer reservoir. A thin layer (0.5 cm thick) of Stoddard's solvent was poured over the buffer immediately to curtail further atmospheric exposure of

the buffer during the electrofocusing procedure. Using this technique, bubble formation during electrofocusing was completely eliminated.

It was found that maintenance of a constant voltage of 500 V throughout the electrofocusing procedure produces less distinct bands in comparison with those produced when the voltage is doubled prior to termination. This finding agrees with the theoretical equation for band width formulated by Svensson (4). However, larger increments of potential increase could not be tolerated by the gels; that is, band distortion resulting from the collapse of the pH gradient towards the cathode occurs under more extreme conditions.

The pH gradient generated during the electrofocusing procedure linearly spanned the range of pH 3.0-8.5. This linear gradient was produced regardless of sucrose concentration (fig.1a), duration of electrofocusing or the ion concentration within the application mixture. Also, membrane components such as lipids did not produce any appreciable disfiguration of the pH gradient (fig.1b).

The banding obtained by electrofocusing 1 mg of erythrocyte membrane is illustrated in figure 2. Forty bands can be distinguished within the range from pH 5.90 to 8.25 in this 2.5% acrylamide gel. Identical patterns were observed in either 6.25% or 12.5% sucrose gels. However, diminution of the average pore dimensions restricted markedly the degree of banding achieved. A 5% acrylamide gel was non-permeable to nearly all of the membrane components whereas gels of 2.5% acrylamide composition provided the minimum porosity and maximum mechanical stability possible without reduction of subunit permeability.

DISCUSSION

The above separation technique is relatively rapid and simple, involving at most 48 hours of electrofocusing and 24 hours to stain and destain the gels. Furthermore, this protocol introduces an alternate method for solubilizing membrane subunits prior to electrophoretic separation. This alternate solubilization procedure is necessary since large ions, such as the dodecyl sulfate anion, in considerable concentration would introduce serious difficulties in relative migration during the electrofocusing procedure.

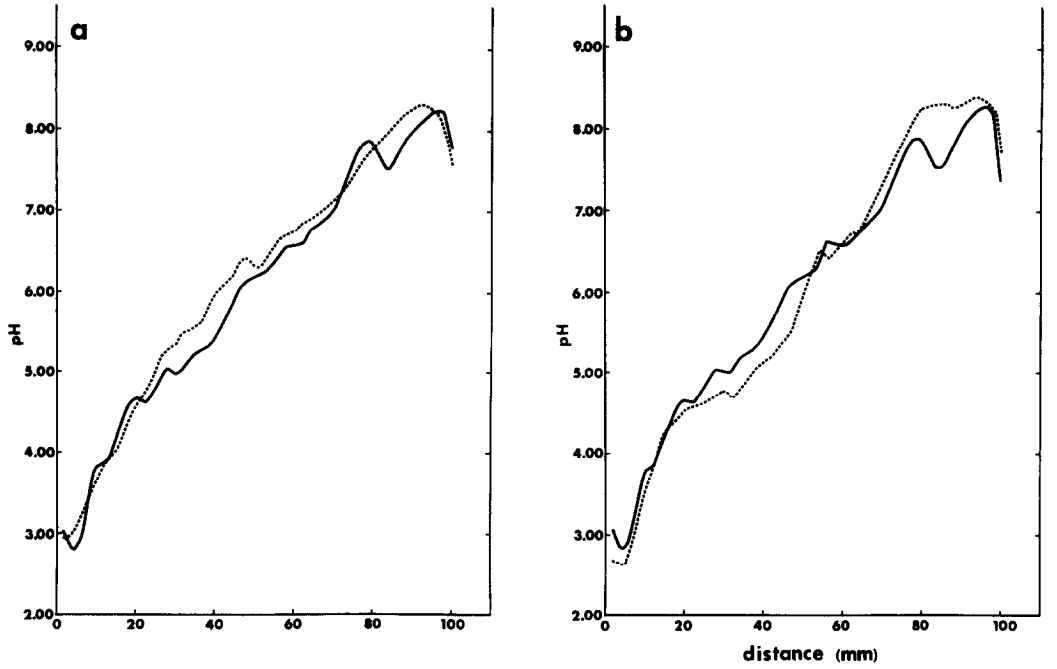

Figure 1a: pH gradients in gels containing 6.25% sucrose w/v (------) and 12.50% sucrose w/v (_____).

Figure 1b: pH gradients in gels in the presence (------) and absence (_____) of membrane subunits. Membrane application is 1 mg. Sucrose concentration is 12.50% (w/v).

The gels (2.5% acrylamide) were electrofocused in each case for 24 hours.

The extension of the high resolution separation technique of isoelectric focusing in polyacrylamide gel matrices to erythrocyte membrane subunits represents a method of fractionation more sensitive than previously employed methods involving anionic detergents such as SDS, since an isoelectric point approximates an absolute parameter for molecular segregation more closely than does subunit size. Indications supporting this can be seen by comparing the number of bands obtained by electrofocusing and those obtained by electrophoretic separation in SDS as reported by Neville and Glossman (15). One sees that electrofocusing produces

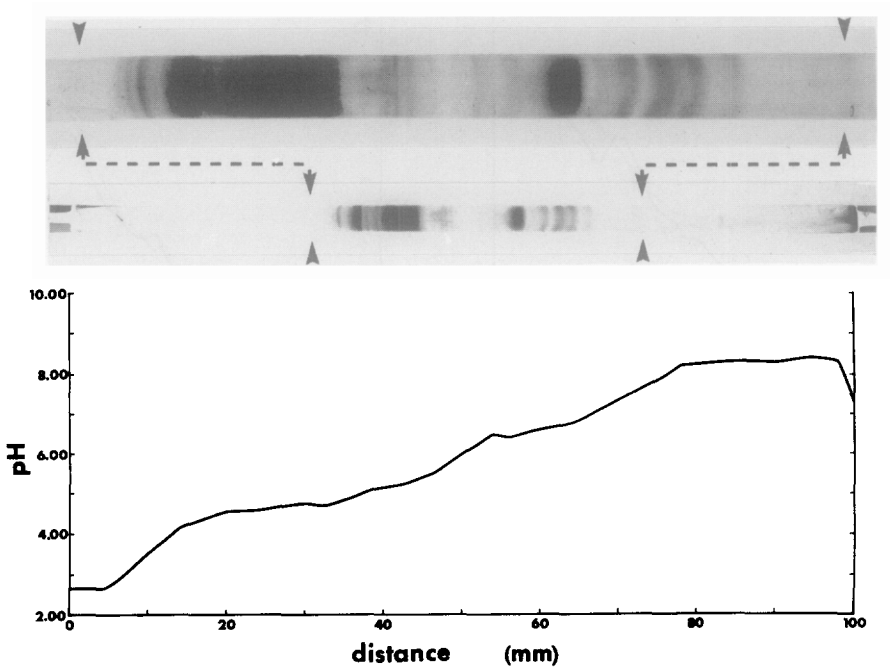

Figure 2: Distribution of membrane components (1 mg) in a 2.5% acrylamide gel containing 12.50% sucrose. Electrofocused for 24 hours.

one and one-half to two times as many bands as does the alternate method. However, because of the difference in concentrations of denaturing agents, the greater number of bands observed in electrofocusing gels does not necessarily represent more effective separation, but rather more sensitive charge distinctions within the subunit population. Combination of both electrophoretic and electrofocusing techniques should enable complete separation of the membrane components.

ACKNOWLEDGEMENTS

We wish to gratefully acknowledge the assistance given us during this research by the following individuals: Dr. Andreas Rosenberg, Mrs. Ronda Litman, Miss Connie L. Finstad and Miss Judy Kempa. This work was supported by USPHS grants AI-08677 and NS-02042, the National Foundation-March of Dimes, the American Cancer Society and the Minnesota Heart Association.

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