A NEW METHOD OF ELECTROFOCUSING OF PROTEINS IN A pH GRADIENT FORMED BY THE GRADIENT OF AN ORGANIC SOLVENT

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A buffer solution of low ionic strength produces a pH gradient in a glycerol concentration gradient in which electrofocusing of hemoglobin can be carried out. The results obtained were comparable with those of electrofocusing in ampholyte carriers.

The method of electrofocusing and fractionation of proteins in a pH gradient produced by means of "LKB" ampholyte carriers has recently become very popular. The method has high resolving power, as a result of which the heterogeneity of blood serum albumin with respect to its isoelectric points has been established [8-6], and fractions of antihapten antibodies belonging to one class, to one sub-group, and with the same type of light chain, have been isolated [4, 3]. However, the method is not yet available in all laboratories because it requires the purchase of expensive ampholyte carriers and equipment. In addition, this method has the disadvantage that polyaminopolycarboxylic acids ("Ampholin" – ampholyte carriers) are bound by several proteins [6, 2] and, in some cases, give pseudoheterogeneity. For the reasons given above it is important to seek other methods of producing a stable pH gradient and enabling maximal simplification of the technique. Recently Luner and Kolin [5] described a new method of isoelectric focusing and fractionation of proteins in a pH gradient of buffer solution created by a temperature gradient. The method is distinguished by its simplicity but it is restricted to a fairly narrow pH zone outside which it is difficult to vary the width of the pH gradient.

This paper describes a method of electrofocusing and fractionation of proteins in a pH gradient produced by a concentration gradient of an organic solvent. The method is based on the difference between the dielectric constants of water and the organic solvent. The degree of dissociation of electrolytes is known to be approximately directly proportional to the dielectric constant of the medium. This, in turn, is reflected in the electrical conductivity of the solution and the ionic product of water. A pH gradient of the buffer solution is thus created in the gradient of the organic solvent, which alters the dielectric constant of the medium.

EXPERIMENTAL METHOD

In this investigation glycerol was used to create the pH gradient because of its minimal effect on protein structure. A wider pH interval can be created by means of ethanol and acetone with lower maximal concentrations of solvent, but to prevent the denaturing action of these solvents on the protein the electrofocusing has to be carried out at a low temperature.

A diagram of the simplest apparatus for electrofocusing in a gradient of an organic solvent is shown in Fig. 1. The main parts of the apparatus are two glass tubes (1) of equal diameter, placed vertically and parallel to each other. Their lower ends are joined by a rubber tube (2). Before the apparatus is filled the rubber tube is clamped and an identical gradient of the organic solvent is produced in both column tubes

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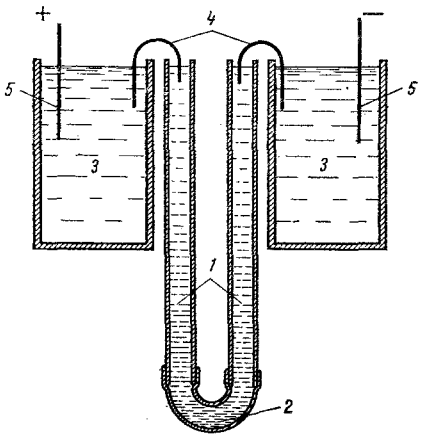

Fig. 1. Diagram of apparatus for electrofocusing in a glycerol concentration gradient (explanation in text).

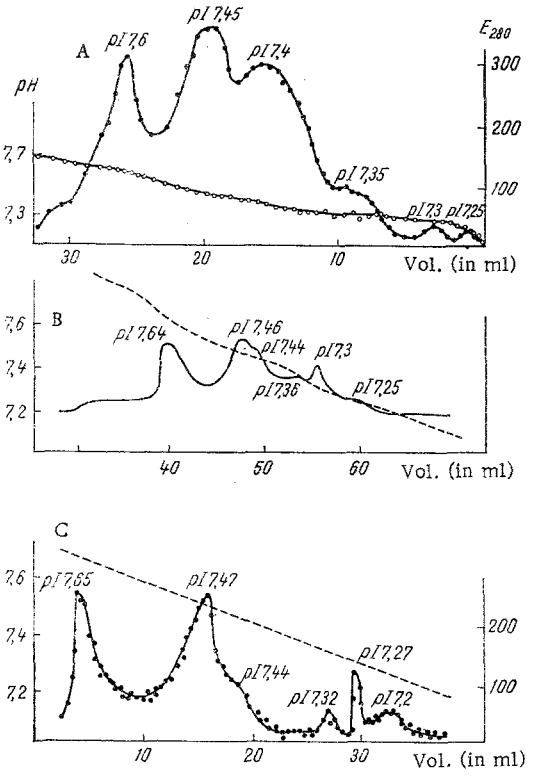

Fig. 2. Curves of electrofocusing of hemoglobin in a glycerol concentration gradient (A), in Ampholin (B), and in a temperature gradient of buffer solution (C).

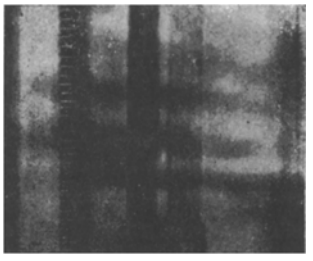

Fig. 3. Fractionation of hemoglobin in column with gradient of glycerol as organic solvent.

by means of a gradient mixer or by layering serial dilutions. The necessary range of the solvent gradient is chosen by sample measurements. For example, a pH gradient from 4.7 to 5.0 was required. A 0.001 M acetate buffer, pH 4.7, was prepared and the minimal concentration of the organic solvent which increased the pH of this buffer to 5.0 was selected. These values are somewhat conventional in character. No data are available for glycerol, but it is known [1] that measurements in ethanol up to 71.9% give results close to the true values.

After the creation of the concentration gradient of the organic solvent in both columns the clamp was removed from the rubber tube. Solution was withdrawn from about the middle of one of the columns, and a certain quantity of protein was dissolved in it. The solution of the protein was then applied in a layer at the same level of the column. Platinum electrodes (5) were placed into large receptacles (3) of buffer, with a capacity of 3 liters or more, connected with the columns by paper bridges (4). The large buffer receptacles or small vessels with a constant flow of liquid are essential in order to maintain the pH of the free buffer constant over a long period. If a pH-stat is available, the volumes of the buffer receptacles can be considerably reduced. Electrofocusing of the proteins was carried out, just as in ampholytes, for 48-70 h using a current of up to 1 mA and a voltage up to 1500 V. At the end of the experiment the rubber tube was clamped and punctured with an injection needle, through which samples of the contents of the columns, one of which was the control, were removed as samples of equal volume. The absorption of the samples from the working column against the control samples from the parallel column was then determined on a spectrophotometer. In this way it is possible to work with solvents which absorb in the same region as the protein. If highly purified glycerol is used, glycerol of any concentration or water can be used as the control. The pH is then determined in each sample and a curve showing the distribution of the protein as a function of its isoelectric points is plotted.

EXPERIMENTAL RESULTS

Comparative results of electrofocusing of bovine hemoglobin in a gradient of organic solvent, in Ampholin, and in a temperature gradient are given below. The curve of electrofocusing of bovine hemoglobin (Reanal) by the method described above is shown in Fig. 2A. The original solution was 0.001 M tris-HCl-buffer, pH 7.8. The pH gradient was produced by serial dilutions of glycerol from 60% to 0. This gave a gradient of pH from 7.0 to 7.8. The hemoglobin solution was layered into the middle part of one of the columns, which was connected by a paper bridge with the negative electrode vessel. Fractionation was carried out for 60 h with a current of 0.2 mA/cm^2 and a voltage of 500 V/cm. During the experiments the pH of the buffer in the electrode vessels was determined periodically and adjusted if necessary to the original values by the addition of HCl or tris. Fractionation of the hemoglobin into four zones was clearly visible at the end of electrofocusing (Fig. 3). Absorption at 280 nm was determined in 0.2-cm cells on a type SF-4A spectrophotometer. The pH was measured with a type LPU-01 pH-meter with glass electrodes. As will be clear from Fig. 3, this specimen of hemoglobin gave six fractions with isoelectric points from 7.25 to 7.65, in close agreement with the results of electrofocusing in a pH gradient produced by ampholin [3] (Fig. 2B) and with the results of electrofocusing obtained by the authors in a pH gradient created by a temperature gradient (Fig. 2C).

The results of electrophoresis in the organic solvent gradient show that the proposed method has a revolving power comparable with that of ampholytes; it is free from some of the disadvantages associated with the latter.

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