Journal of Chromatography, 131 (1977) 223–232
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CHROM. 9435

CARRIER AMPHOLYTE DISTRIBUTION IN ISOELECTRIC FOCUSING

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(First received December 29th, 1975; revised manuscript received June 2nd, 1976)

SUMMARY

The spatial distributions of individual components of carrier ampholyte mixtures obtained by isoelectric focusing in cylindrical (6 mm × 75 mm) polyacrylamide gels (PAG) and in a thin-layer Sephadex gel (TLSG) were determined by ion-exchange chromatography of the eluates of gel slices. A *ca.* 1-mm slice of PAG separating the A and B forms of β-lactoglobulin (isoelectric points of 5.21 and 5.34, respectively) was found to contain at least 12–14 ampholyte components out of a total of at least sixty in the ampholyte mixture (LKB Ampholine, pH 3.5–10, Lot No. 17). Fifteen to twenty components were found in each 2-mm slice of a sequence of six slices of PAG subsequent to isoelectric focusing of sperm whale myoglobin with the same ampholyte mixture.

An ampholyte mixture prepared by the copolymerization of triethylenetetramine with acrylic acid was focused in TLSG on 20 cm × 5 cm plates and the eluates obtained from *ca.* 5-mm slices were analyzed by ion-exchange chromatography. The results were compared with the caramelization pattern obtained by the Felgenhauer technique. The spatial distributions of individual ampholyte components were broad with half-band widths of *ca.* 1 cm.

The effects of sample load, ampholyte concentration, and duration of focusing on spatial distribution were investigated in the PAG focusing of [^{14}C]histidine. The narrowest and most symmetrical distributions were obtained with small sample load and high ampholyte concentration.

INTRODUCTION

Isoelectric focusing is a high-resolution electrophoretic method for the fractionation of proteins and other macromolecules in a pH gradient according to their isoelectric points[1–3]. The pH gradient is formed by the application of an electric field to a mixture of low-molecular-weight carrier ampholytes and stabilized through the use of a sucrose gradient or a gel, *e.g.*, polyacrylamide or Sephadex. The ampholytes employed have generally been aliphatic polyamine–polycarboxylic acids prepared by the copolymerization of acrylic acid with oligoethyleneimines[4–6]. Recently comparable

preparations have been obtained by the reaction of oligoethyleneimines with sultones[7] and with propyl sulfone or vinyl sulfonate[8].

These complex mixtures of carrier ampholytes can be analyzed quantitatively by ion-exchange chromatography using a pH and ionic strength gradient[9]. However, their distribution in the pH gradient formed during the isoelectric focusing has not been investigated. Staining procedures result in the visualization of a limited number of ampholyte bands[10]. A glucose caramelization procedure on paper devised by Felgenhauer and Pak[11] allowed the observation of neutral and alkaline as well as acidic ampholytes. Recently, Righetti *et al.*[6] have examined the ampholyte distribution obtained by focusing in thin-layer polyacrylamide gels by scanning photographs of the gels.

Svensson[12] has considered the concentration distribution of an electrolyte as an equilibrium between electrophoretic and diffusional mass flow. Assuming a constant pH gradient and a constant conductivity throughout a focused zone, an analytical solution can be obtained which describes the concentration distribution of the ampholyte as Gaussian. Almgren[13] has investigated an artificial system comprising a sequence of ampholytes with identical Gaussian concentration distributions and has shown that, to obtain a resolution of 0.02 pH units, the system must contain at least 20 different ampholytes per pH unit.

We present below the results of experiments performed to obtain an estimate of the number of carrier ampholyte components in individual slices of cylindrical polyacrylamide gels (PAG), to find the distribution of individual ampholyte components in Sephadex thin-layer gel (STLG) focusing and to determine the effect of sample load, ampholyte concentration, and duration of focusing on the concentration distribution of a focused ampholyte in PAG.

MATERIALS AND METHODS

Isoelectric focusing was performed in 6 mm × 75 mm PAG cross-linked with methylenebisacrylamide (6% T, 3.0% C) and polymerized using 0.1% (w/v) N,N,N′,N′-tetramethylethylenediamine and 0.075% (w/v) ammonium persulfate. The gels were prefocused for 1.5 h. An Ampholine concentration of 2% (w/v) was used unless otherwise stated. The Ampholine used in the gel experiments was LKB Lot No. 17 (pH 3.5–10). After the samples had been applied the current was maintained at 1 mA per tube until a voltage of 150 V was reached. Focusing was carried out for 20 h at 4°. In a few experiments the voltage was increased until a potential gradient of 100 V/cm was obtained. This was maintained for 1 h. There was little change in current during this hour. The anolyte and catholyte were 0.01 H_3PO_4 and 0.02 *M* NaOH, respectively.

Two types of experiments were performed. In one, sperm whale myoglobin (Mann Labs., New York, N.Y., U.S.A.) and β-lactoglobulin (Pentex, Lot No. 34) were focused separately, employing loads of 15 and 60 μg per gel, respectively. Upon completion of focusing, the gels were removed from their tubes and frozen immediately in liquid nitrogen. This process required about 45 sec to 1 min. The frozen gels from the β-lactoglobulin experiment were then sliced manually. In the case of β-lactoglobulin one slice of about 1 mm was cut from between the two precipitated components. In the case of myoglobin, six slices 2.34 ± 0.29 mm wide were cut, starting with the protein band and proceeding in the direction of the anode. The slices were extracted in

1 ml of water for two days at room temperature. In the case of β-lactoglobulin, the slices from three gels were extracted together. The extract was flash evaporated, brought up in 0.25 ml of pH 2.7 citrate buffer, and analyzed by ion-exchange chromatography using an automated ninhydrin procedure[9].

In another set of experiments different amounts of histidine containing about 0.04 μg of [^{14}C]histidine were focused on PAG as described above for various times. The gels were frozen immediately after removal from the tubes with powdered dry ice and sliced into 1 ± 0.11 mm slices with a Mickle gel slicer (Brinkman). The gel slices were incubated with 0.25 ml of a 5% v/v water–Protosol mixture for 3 h at 60°. After this 10 ml of scintillation solution (16 g PPO, 0.4 g POPOP diluted to 1900 ml with toluene) was added to each gel slice and they were counted in a Packard Model 3375 liquid scintillation spectrometer. The counts obtained from two or more 5-min periods were averaged. The [^{14}C]histidine was obtained from New England Nuclear (Boston, Mass., U.S.A.) and had a specific activity of 2 mCi/mg.

Isoelectric focusing was also done on TLSG. The gels were prepared by spreading a slurry of 3.25 g of dry superfine Sephadex G-75 in 50 ml of solution containing 0.4 g of carrier ampholyte on a 20 cm × 5 cm glass plate. The separation was carried out at 200 V for 3 h, at 300 V for 3 h, and finished with 500 V for 1 h. A caramelization pattern[11] was obtained and the gel was cut into 5-mm sections. For ninhydrin analysis the Sephadex fractions were eluted with 5 ml of water. An aliquot of 0.3 ml was analyzed by ion-exchange chromatography using an automated ninhydrin procedure[9].

RESULTS

Location of ampholytes in PAG focusing

The two genetic variants of β-lactoglobulin, A and B, possess isoelectric points at 5.21 and 5.34, respectively[14]. They are completely resolved by PAG isoelectric focusing. The gel slice, about 1 mm thick between the two precipitated bands, was cut out, extracted with water, and analyzed (Fig. 1). Although a major component was present, appreciable amounts of other Ampholine components were found. A total of 12–14 components were observed. These cover a fairly wide area of the chromatogram of the complete Ampholine mixture.

In another series of experiments using PAG focusing, we analyzed the Ampholine focused in six sequential slices, each 2 mm wide, using myoglobin as a marker protein. Analysis of the slices (Fig. 2) showed that each of the slices contained 15–20 components. Gels frozen 2 and 5 min after completion had identical analyses with those frozen within 45 sec. Components with identical chromatographic behavior appeared in several slices. The width occupied by a given Ampholine component was wider than one would expect from the sharpness of the protein bands.

Spatial distribution of ampholytes in STLG focusing

Because the ion-exchange chromatographic elution patterns obtained with the commercial Ampholine mixture are complex due to the large number (60–500) of components present, it is not possible to readily follow the concentration of a given component in successive slices. To avoid this problem an ampholyte prepared by the copolymerization of acrylic acid with triethylenetetramine (TETA) was used. Our preparation possessed a relatively small number of well resolved components which

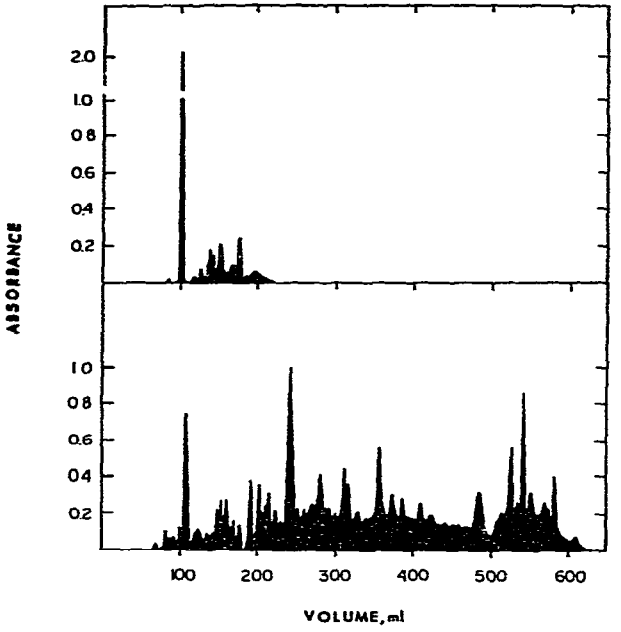

Fig. 1. Ion-exchange chromatography with automated ninhydrin analysis[9] of the eluate from the PAG slice between the β-lactoglobulins A and B and of the LKB Ampholine (Lot No. 17, pH 3.5–10). Absorbance is plotted against elution volume. The baseline obtained in a blank run was subtracted in both cases.

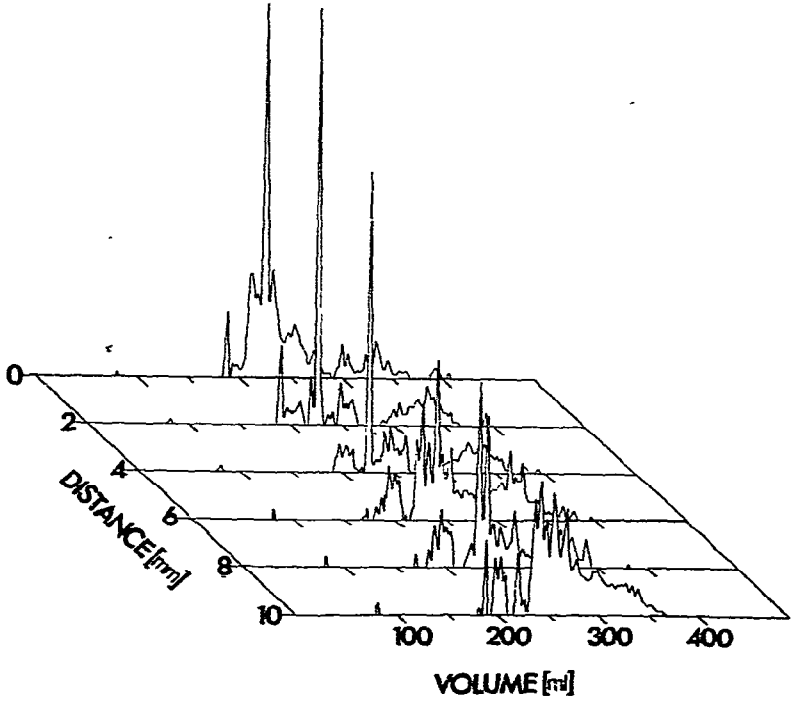

Fig. 2. Ion-exchange chromatography with automated ninhydrin analysis of the eluate from six sequential, 2 mm wide, PAG slices cut towards the anode, starting with the myoglobin band. Absorbance is plotted against elution volume.

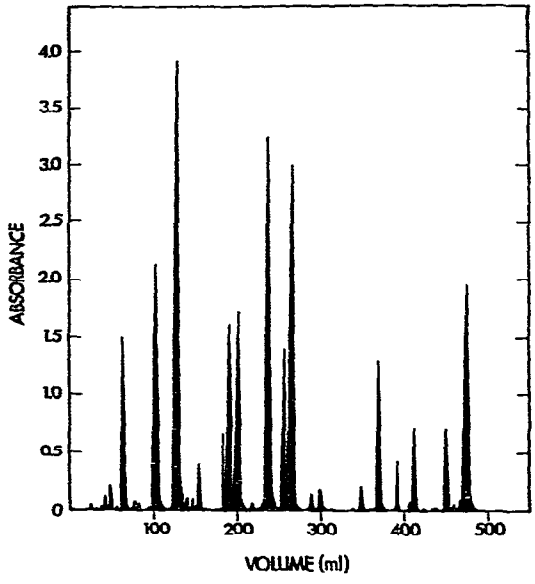

Fig. 3. Ion-exchange chromatography with automated ninhydrin analysis of an ampholyte mixture prepared by the copolymerization of TETA with acrylic acid. Absorbance is plotted against elution volume. The baseline obtained in a blank run was subtracted.

could be identified by their ion-exchange chromatographic behavior (Fig. 3): out of a total of about forty components, about fifteen components were major. This ampholyte mixture was focused in a thin layer of Sephadex G-75. The resultant pH gradient and caramelization pattern obtained by the procedure of Felgenhauer and Pak[11] is shown in Fig. 4. Ion-exchange chromatography and ninhydrin analysis of fifteen of the thirty-eight fractions obtained by TLSG isoelectric focusing are given in Table I and the concentration of certain components is plotted in Fig. 4. The results obtained permit a direct comparison with the caramelization pattern. The individual components are again distributed into several fractions. Although any given component was found in only four to six of the 5-mm fractions, the distribution of some is quite asymmetric. It is also evident that the caramelization reaction does not correlate well with the ninhydrin color.

Spatial distribution of histidine in PAG focusing

Isoelectric focusing of ^{14}C-labeled histidine was used to obtain information about the behavior of a single low-molecular-weight amphoteric substance. Mixtures consisting of various amounts of carrier histidine added to a constant amount of [^{14}C]histidine were focused and the distribution of radioactivity was determined. When labeled histidine alone was focused, a single, sharp, symmetric peak was obtained (Fig. 5). This peak became flatter and more asymmetric with increase in histidine concentration. A few experiments were done at a potential gradient of 100 V/cm. The histidine distribution was not appreciably sharpened under these conditions.

The duration of isoelectric focusing was varied in order to determine whether it affected the distribution observed at high histidine concentrations. The results

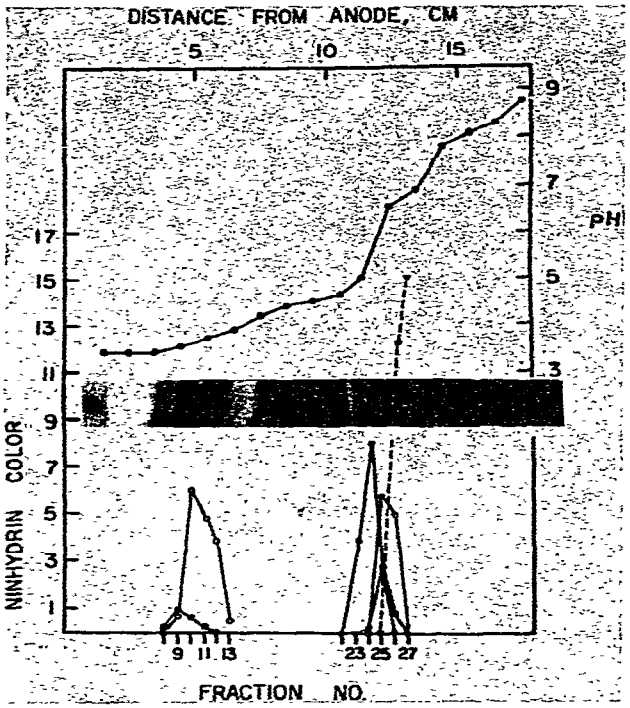

Fig. 4. pH gradient, caramelization pattern, and distribution of some of the ampholyte components after isoelectric focusing of the TETA ampholyte preparation in a thin layer of Sephadex G-75. Individual ampholyte components are identified by their elution volume in the ion-exchange chromatography: ● = 48 ml; ○ = 63 ml; ■ = 101 ml; □ = 184 ml; ★ = 201 ml; ▼ = 235 ml. Each fraction corresponds to about 5 mm of gel layer. Each point in the lower portion represents the sum of the absorbances under each peak of the foregoing ampholyte components in the analyses of the individual fractions.

(Fig. 6) showed that there was little difference, other than a slight cathodal migration, between focusing for 18 and 24 h. After 42 h the histidine peak was further flattened and shifted towards the cathode.

The effect of carrier ampholyte concentration upon the distribution of histidine was examined by running a constant amount of histidine (0.04 μg [^{14}C]-histidine plus 0.8 mg carrier histidine) in the presence of 4, 2, and 0.2% ampholyte (Fig. 7). Under the conditions used the histidine focused most sharply at the highest ampholyte concentration.

DISCUSSION

Following isoelectric focusing each ampholyte component is distributed over a wide area. This was shown in three different experiments: PAG focusing of Ampholine or [^{14}C]histidine and TLSG focusing of TETA ampholytes. An individual component extends along 7–15% of the gel length. Histidine, even when used in small amounts (40 ng), was distributed over 14% of the gel length in PAG focusing. Diffusion subsequent to the termination of the run is one of the more important factors which might broaden the concentration distribution of a carrier ampholyte. To

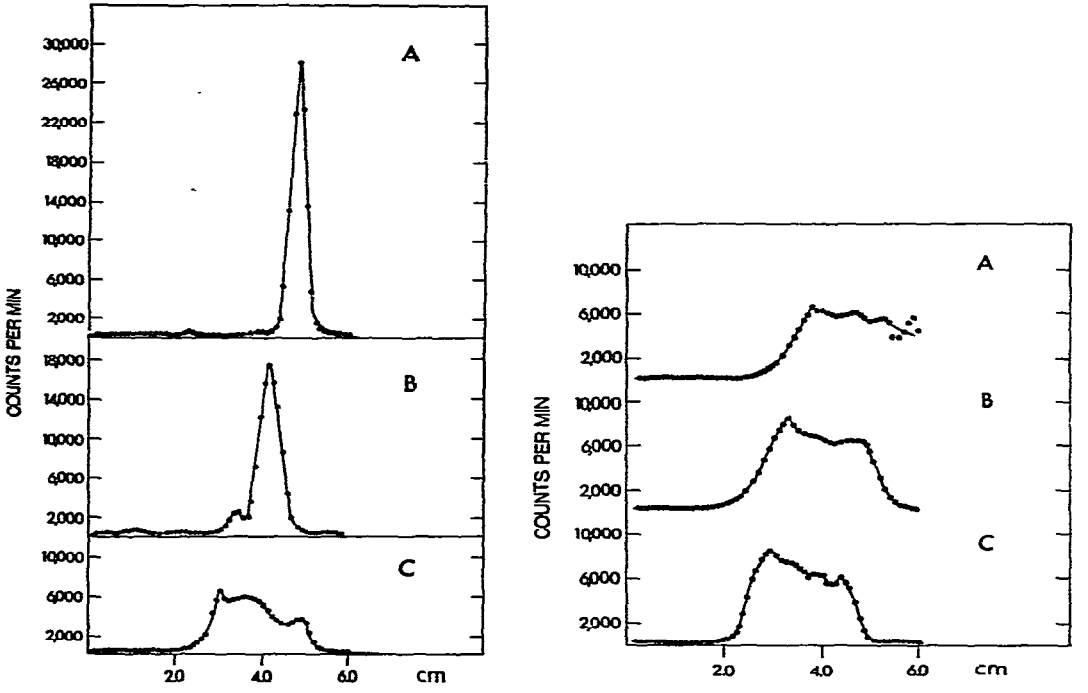

Fig. 5. Effect of total histidine concentration on the distribution of 0.04 μg of [^{14}C]histidine during isoelectric focusing in PAG. Radioactivity is plotted against distance from the anode. The total amount of histidine used per ml of gel was 0.04 μg (A), 0.8 mg (B), and 6.1 mg (C).

Fig. 6. Effect of duration of focusing in PAG upon the distribution of [^{14}C]histidine. Histidine, 6.1 mg/ml, was focused in 2% LKB Ampholine (Lot No. 17) for (A) 42, (B) 24, and (C) 18 h.

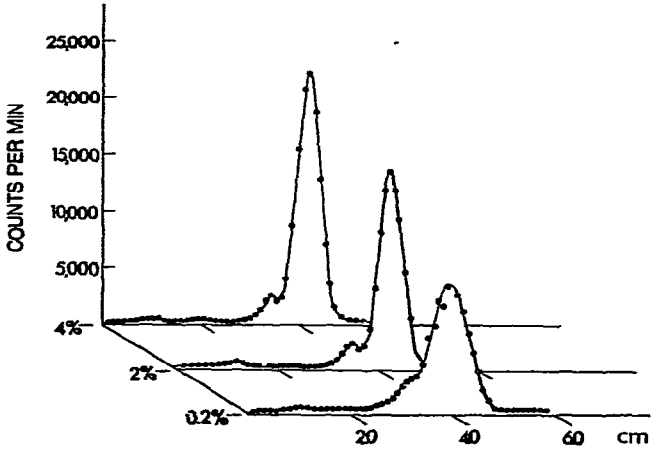

Fig. 7. Effect of the ampholyte concentration 4, 2, and 0.2% (w/v) upon the distribution of 0.04 μg of [^{14}C]histidine plus 0.8 mg carrier histidine per ml of gel during isoelectric focusing in PAG.

TABLE I

ION-EXCHANGE CHROMATOGRAPHIC ANALYSIS OF FRACTIONS OBTAINED BY TLSG ISOELECTRIC FOCUSING OF TETA AMPHOLYTES

Elution volume (ml)	*Per cent of total ninhydrin color*	*Per cent of total ninhydrin color of each ampholyte component applied to TLSG recovered in each fraction**														
		8	*9*	*10*	*11*	*12*	*13*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*34*	*37*
48	0.54	0.03	0.18	0.12	0.03											
63	4.01		0.12	1.08	0.85	0.71	0.09									
76	0.33					0.06										
101 } 114 }	6.88							0.45	0.45	0.69	1.38	0.42				
130	16.07							0.16								
152	1.63							0.06	0.09	0.16						
184	1.99										0.04	0.49	0.13			
190 } 196 }	6.55					0.02	0.06		0.05	1.04**	1.46					
201	6.55										0.75	0.89	0.77	0.08	0.42	
235	14.05											0.06	2.16	2.70	0.36	
256	6.53															
265	10.60													1.71		
296	0.70														0.08	0.18
367	5.01														2.21	
391	1.17															
411	2.37															0.04
449	2.40															0.85
473	7.48															0.78

* Each fraction corresponds to about 5 mm of thin-layer gel.

** The assignment of this peak is uncertain.

minimize diffusion, gels were frozen as quickly as possible after the termination of a run. Controls were performed where gels were left 2 or 5 min before freezing, slicing, and analysis. No differences were found between the controls and the determinations done after 45 sec. These findings suggest that diffusion after the termination of the run did not cause the broadening. This is consistent with the report of Lunney *et al.*, who found that the dispersion coefficient of transferrin in polyacrylamide gel electrophoresis was seven times greater than the diffusion coefficient[15]. Incomplete focusing in the gels is ruled out by time studies. There is little change in band width for gels focused for periods between 18 and 42 h. The shift in the location of the ^{14}C distribution is probably associated with the instability of the pH gradient. This phenomenon has been carefully studied by Chrambach and co-workers[16,17].

Our experiments with ampholytes are in agreement with those of Catsimpoolas and co-workers[18,19] who examined the distribution of amino acid derivatives by spectrometry during the course of isoelectric focusing. They used a 10-cm cell in a liquid system stabilized by a sucrose density gradient. Various derivatives possessed band widths of 3.5–6.4 mm. This is in reasonable agreement with our findings.

Our observations stand in contrast with those of Righetti *et al.*[6]. They focused ampholytes in PAG sheets and photographed them against a black background using side illumination. The print was scanned and the resulting peaks were interpreted to represent carrier ampholyte concentration distributions. Well resolved components had widths of 1–2 mm. An alternative explanation, which would be consistent with our results, is that each peak in the tracing represents a refractive index gradient caused by a change in concentration of a component or components. If this were so, the method would give no direct information about the band width of a given component.

Isoelectric focusing is often portrayed as giving an array of sharply distributed components. If the distribution curve of a given component were very sharp and the components were evenly distributed along the 65-mm gel length, one would expect to find only one of the 62 Ampholine components in a 1-mm slice of PAG. Instead 12–14 components were found in a slice of this size (Fig. 1). This is a much larger number than expected and might indicate that the components are unevenly distributed. Rilbe[20] suggests that groups of carrier ampholytes form during focusing, causing an uneven distribution. If the distribution were uneven there should be slices with few components. To clarify this, the ampholytes in six sequential slices were analyzed. Approximately 16–25 components were found in each slice. There were no slices with a small number of components.

While 62 components were found in the Ampholine used in the experiment, there is no evidence that all of the components were resolved or that each gives a color with ninhydrin. If 62 components were evenly distributed along the gel and if each had a width of 6.5 mm one would expect to find only seven or eight components in a 2-mm slice. It is likely that the large number of components per slice is in part the result of further fractionation of the Ampholine by the focusing process.

Increasing the amount of [^{14}C]histidine broadens its focusing pattern. When large amounts of histidine are used its distribution is clearly non-Gaussian. Incomplete focusing was not responsible for the shape of the histidine distribution curve. Focusing at a higher potential gradient (100 V/cm) or for a longer time did not alter the shape of the histidine curve or make it appreciably narrower. At constant histidine concen-

trations, the distribution was broader at low Ampholine concentrations. Likewise, the distribution of TETA ampholytes in TLSG focusing is often non-Gaussian although fewer fractions were analyzed.

Nguyen and Chrambach[21] have suggested that steady-state stacking may be the mechanism by which electrolytes orient themselves to provide the pH gradient in isoelectric focusing. The distribution of histidine found when large concentrations are employed appears to be consistent with their idea. The rapid changes in concentration would represent the boundaries of the stack and the plateau would be the concentration of the stacked zone. The failure of the zones to narrow appreciably at higher potential gradients would also be expected.

The information given by the caramelization pattern and that given by the automated ninhydrin analysis are expectedly dissimilar. The chemistry of ampholytes is not well understood nor are the factors influencing either of the analytical reactions entirely clear. Accordingly differences between the procedures are not unexpected. The advantages of the ninhydrin method are that it allows one to follow a given ampholyte component and to resolve a complex mixture of carrier ampholytes.

ACKNOWLEDGEMENT

The authors acknowledge with gratitude several helpful discussions with Dr. Andreas Chrambach.

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