ISOELECTRIC FOCUSING OF PROTEINS IN GELS

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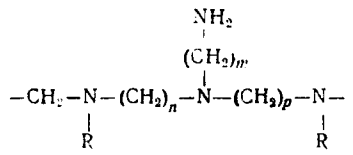
The basis of the method of the isoelectric focusing of proteins is their electrophoretic migration in a pH gradient created by amphoteric "carriers." Since the isoelectric point (pI) corresponds to the pH value at which the total charge of the protein is equal to zero (and, consequently, at which the protein does not move in an electric field), isoelectric focusing stops when the protein arrives in a zone with the pH equal to the pI value of the protein. When a mixture of proteins having different pI values is subjected to isoelectric focusing, the individual components are separated, forming the isoelectric spectrum of the mixture.

The theory of the isoelectric focusing of proteins in a stable pH gradient was developed by Svensson [1, 2]. The method became accessible in practice with the synthesis of ampholytes* forming stable pH gradients [3, 4]. The ampholines comprise a set of aliphatic polyamino polycarboxylic acids possessing various pI values and a considerable buffer capacity.

The general formula of the ampholines is



where R = H or $(CH_2)_X-COOH$, and n, m, p, and $x \leq 5$.

The problem of the stabilization of the protein zones arising as the result of isoelectric focusing has been solved by the creation of a density gradient of sucrose [5] or glycerol [6]. The experimental conditions, the theoretical aspects of this method, and the possibility of using it for separating proteins have been discussed in reviews by Vesterburg [7] and Haglund [8, 9]. This method of isoelectric focusing (in columns with a volume of 110 or 440 ml marketed by the firm LKB) can also be used for preparative purposes, but it is expensive and inconvenient for the analytical separation of proteins which are not infrequently present only in trace amounts. This fact has led to the creation of a micro method consisting in the isoelectric focusing of proteins in acrylamide, Sephadex, or agarose gels. Advantages of the method are the following: the use of simple and inexpensive apparatus, simplicity of preparation and performance of the experiment, short time for an experiment, low consumption of ampholines and possibility of analyzing small amounts of complex protein mixtures, high separating capacity, possibility of using special dyes and also of detecting enzymes directly in the gel from their activities, absence of convective mixing, possibility of separating the proteins appearing at the isoelectric point, possibility of using immunodiffusion or a combination with electrophoresis for increasing separation.

Analytical Isoelectric Focusing in a Polyacrylamide Gel

Isoelectric Focusing in Tubes. This method was developed almost simultaneously in several laboratories by Dale and Latner [10], by Wrigley [11, 12], by Riley and Coleman [13], by Fawcett [14], and by

*At the present time such ampholytes are marketed by the firm LKB (Sweden) under the name of "ampholines." This name is used in the present paper.

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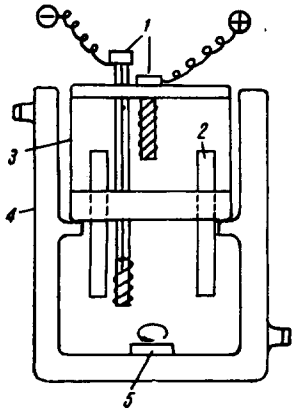

Fig. 1. Basic sketch of the apparatus for isoelectric focusing in a polyacrylamide gel (in tubes) [18]: 1) electrodes; 2) tubes with gel; 3) upper reservoir; 4) lower reservoir with cooling jacket; 5) anchor of a magnetic stirrer.

Catsimpoolas [15]. Its characteristic feature is the possibility of using a polyacrylamide gel as the stabilizing medium and also of using glass tubes and apparatus similar to those used for disc electrophoresis [16]. As in disc electrophoresis, after the completion of the isoelectric focusing and the staining of the gel, the protein bands appear in the form of discs. Consequently, Catsimpoolas and Leuthner [17] have called this modification the method of disc isoelectric focusing in a polyacrylamide gel.

Apparatuses for Isoelectric Focusing. Ordinary apparatuses for disc electrophoresis are most usually used [12, 13, 15]; original instruments have also been made [10, 14]. In the opinion of the majority of workers, the requirements of isoelectric focusing are well satisfied by an apparatus with a cooling jacket covering the greatest possible length of the tubes and by electrodes which in the absence of temperature and concentration gradients are located close to the ends of the tubes. In isoelectric focusing (in contrast to disc electrophoresis), only a small volume of the electrode solutions is required – 50-100 ml (Fig. 1). The most convenient apparatus, in our opinion, is that described by Finlayson and Chrambach [18].

The diameter of the glass tubes in which the gel is polymerized may vary from 3 mm [19-21] to 7.5 mm [11, 22], and their length from 65 mm [12, 13, 15] to 150-200 mm [10, 14, 23]. The best separation of a complex mixture of proteins is achieved by using long tubes.

Composition and Methods of Polymerizing the Polyacrylamide Gel. To obtain the gel it is possible to use a chemical [11, 19, 24] or a photochemical [11, 14] method of polymerization. The working solutions described by Wrigley [11] are most frequently used. The necessary components for preparing the gels are acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), or dimethylaminopropionitrile, ampholines, and either riboflavin (for photopolymerization) or ammonium persulfate (for chemical polymerization).

It is recommended to avoid chemical polymerization if the proteins under investigation are sensitive to persulfate – when free SH groups are present in the protein additional bands may appear (as has been observed in an investigation of yeast enolase) [25, 26]. If the chemical method of polymerization is nevertheless used – for example on working in the pH range from 7 to 10, when photopolymerization is relatively ineffective – before adding the protein solution a preliminary isoelectric-focusing process must be performed for 30-90 min in order to eliminate the ammonium persulfate from the gels completely [19, 27]. After photopolymerization, no preliminary isoelectric focusing is necessary, but, to prevent the oxidation of the protein by the riboflavin, it is recommended to add dithiothreitol or β-mercaptoethanol to the gel to a final concentration of 1 mM [28]. Instead of subjecting the gels to preliminary isoelectric focusing, it is possible to wash them with deionized water to eliminate the impurities that may be present in the acrylamide and the residues of ammonium persulfate or riboflavin. After washing, the gels are equilibrated against 1% solutions of the corresponding ampholines (12-15 ml of a 1% solution of the ampholines in the gel) and are again filled into glass tubes [28]. To fix the gel in the tube, a dialysis membrane or a thin nylon gauze is sometimes drawn over its lower end [18].

When necessary, the gel may contain 6-10 M deionized urea [29-31]. In some cases, sucrose must be added to the gel to a final concentration of 4-25%. As our results and also those of other workers [18, 32] have shown, this increases the sharpness of the protein zones and improves the pH gradient in the gel.

Concentration of the Polyacrylamide Gel. The concentration of the gel is selected in such a way as to obtain a sufficiently dense gel convenient to handle which does not prevent the migration of the proteins. A gel with a concentration of 3.5-5.0% is the optimum for the majority of proteins; the ratio of acrylamide to methylenebisacrylamide may vary from 15/1 to 40/1. Attention must be drawn to the different functions of the gel in isoelectric focusing and in disc electrophoresis [16]. In disc electrophoresis, the concentration of the polyacrylamide gel is determined by the size of the pores of the gel, and the gel plays the role of a "molecular sieve." In isoelectric focusing, the gel is a supporting medium, and in the ideal case the molecular-sieve effect should be absent completely. An increase in the concentration of acrylamide on

TABLE 1. Influence of the Concentration of the Electrode Solutions on the pH Gradient in the Gel

pH Gradient in the gel	Anodic solution			Cathodic solution		
	electrolyte	pH	concn., mM	electrolyte	pH	concn., mM
			Ampholines 3-10			
2— 8	H_3PO_4	1,91	895,0	Ethylenediamine	11,3	572,0
3— 8	H_3PO_4	1,35	244,0	Ethylenediamine	11,2	331,0
3— 7	H_2SO_4	0,70	242,0	Ethanolamine	11,2	331,0
2— 8	H_2SO_4	0,70	242,0	Ethylenediamine	11,0	22,0
3— 8	H_2SO_4	1,33	36,0	Triethanolamine	10,25	33,0
5— 7	H_2SO_4	1,00	73,5	NaOH	12,0	52,5
4— 8	H_2SO_4	2,25	1,44	NaOH	10,6	0,75
3—10	H_2SO_4	2,95	0,36	NaOH	10,05	0,25
			Ampholines 5-8			
3— 7	H_2SO_4	3,80	0,09	NaOH	9,40	0,12
5— 8	H_2SO_4	4,90	0,02	NaOH	8,20	0,05

isoelectric focusing does not lead to an improvement in the results of separation but only increases the time of the experiment. Consequently, for proteins with a high molecular weight (for example, the subunits of myosin) isoelectric focusing is performed in a gel with a reduced concentration of acrylamide — about 2.5% [33].

Peacock and Dingman [34] have described a process for obtaining a dense gel with a low concentration of acrylamide (less than 2%) and with large pores by adding 0.5% of agarose to the gel. The use of such a gel permits the isoelectric focusing of proteins of high molecular weight.

The ampholines are marketed by the firm LKB (Sweden) in the form of 40% aqueous solutions. Several types of ampholines are produced, each of which forms a definite pH gradient (for example, with the pH ranges 3-10, 3-5, 4-6, 5-7, etc.). By combining ampholines of different types it is possible to obtain any gradient in the pH range from 3 to 10. Thus, by mixing equal amounts of ampholines 4-6 and 6-8 the pH range from 4 to 8 will be obtained, and so on. The final concentration of ampholines in the gel should be 1-2%. The addition of a large amount of ampholines to the gel does not improve the separation process [35].

Generally, the ampholines 3-10 are used in the preliminary experiments, and ampholines with a narrower pH range ("width" of two pH units) are selected on the basis of the results obtained. However, in these cases it is not always possible to obtain such sharp protein zones as with the ampholines 3-10 [36].

Working Solutions for the Polymerization of the Gel (according to Wrigley [11] in our modification). A) Photopolymerization. Solution A contains 7 mg of riboflavin and 0.5 ml of TEMED in 100 ml, and solution B 30.0 g of acrylamide and 0.8 g of methylenebisacrylamide in 100 ml. These solutions can be prepared beforehand and be stored in the refrigerator for 2-3 months. Solution C is prepared immediately before an experiment; it consists of 0.8 ml of solution A, 2.0 ml of solution B, 0.3 ml of a 40% aqueous solution of ampholines, and 1.0 ml of water. Solution D (final concentration of acrylamide 5.0% and of ampholines 1.0%) consists of solution C diluted twofold with water or with a solution of the protein in water. Solution D is poured into the tubes and subjected to polymerization. On illumination with a daylight fluorescent lamp, the polymerization of the gels sets in after 10-15 min.

B) Chemical Polymerization. For this method of polymerization, solution A does not contain riboflavin. Solutions B and C are prepared in the same way as for photopolymerization. Solution D, consisting of one part of solution C and two parts of water or a solution of the protein in water, must also contain 0.05% of ammonium persulfate. At room temperature, the polymerization of the gel usually begins after 10-15 min.

It is recommended to eliminate the air from solution D before polymerization by keeping the solution under reduced pressure (10-20 mm Hg) for a few minutes. The solution (D) is filled into tubes for polymerization, and polymerization is performed as for disc electrophoresis [16].

Addition of the Protein Solution. The protein (50-500 μg per gel) is carefully freed from salts, since the presence of salts may cause a distortion of the protein zones and a change in the nature of the migration of the proteins. A small amount of salts is sometimes disregarded [36].

Two methods of adding the solution have been described: direct introduction into the mixture before polymerization (directly into solution D) [37-40], and addition (in a mixture with 10-20% sucrose and

TABLE 2. Conditions for Performing Isoelectric Focusing in a Polyacrylamide Gel

Material investigated	Solutions: anodic	Solutions: cathodic	Ampholines	Current, mA	Voltage, V	Time, h	References
Ovalbumin, plakalbumin, bovine serum albumin	0,2% H_2SO_4	0,4% triethanolamine	3–10	2	350	3	[11]
Nonhistone proteins of liver nuclei	5% H_3PO_4	5% ethylene-diamine	3–10	5	150	5	[29]
L-amino acid oxidase	1% H_2SO_4	2,9% ethylene-diamine	3–10 5–8	Not more than 0.19 W		10	[41]
Immunoglobulins	0,2% H_2SO_4	0,4% triethanolamine	4–9	2	350	3	[43]
Glyceraldehyde phosphate dehydrogenase	3% H_3PO_4	3% ethanolamine	5–8 5–7 4–8	1	180	5–7	[44–46]
Phosphodiesterase	0,2% H_3PO_4	0,4% ethanolamine	3–5	1–2	500	3	[47]
Awamorin (acid proteinase from Aspergillus awamori)	0,01% H_2SO_4	0,02% NaOH	4–6	2	300	2	*
Awamorin	0,001% H_2SO_4	0,002% NaOH	3–5	2	300	2	*

* Our results

and 1-4% ampholines) onto the surface of the gel under a protective layer of 1-4% ampholines (the same ampholines as in the gel) in 5% sucrose [10, 11, 14, 41]. In the second case, the volume of the protein sample must not exceed 0.1-0.2 ml.

The direct addition of the sample is convenient when it is necessary to introduce a large volume of protein solution into the gel by replacing the water with this solution (see above: "Working Solutions for the Polymerization of the Gel"). This method is generally used when the concentration of protein in the initial solution is low and the protein poorly withstands lyophilic drying. However, some authors [18] consider that it is possible to add the protein to the gel mixture only in exceptional cases (for example, when the concentration of protein in the initial solution is very low, which does not permit the use of the other method of adding the sample), since it weakens the intensities of the protein zones. Furthermore, in the polymerization of the gel containing the protein the latter is distributed throughout the volume of the gel and at the beginning of the experiment the contact of some part of the protein with the electrode solutions is not excluded, which is extremely undesirable for labile proteins (particularly proteinases) and may lead to the appearance of artefactual zones [42].

Electrode Solutions. Current. Voltage. Time of Isoelectric Focusing. The following electrode solutions are used in isoelectric focusing in a gel: dilute sulfuric or phosphoric acid (anodic electrolyte) and NaOH or primary, secondary, tertiary, or quaternary amines (cathodic electrolyte). It is recommended to add the optimum concentration of electrode solutions for each type of ampholines [22]. Otherwise a distortion of the pH gradient may take place. Percival et al. [22] have selected conditions for the isoelectric focusing of proteins in the pH ranges from 3 to 10 and from 5 to 8 (Table 1). We have selected conditions for the focusing of acid proteinases from lower fungi in the pH ranges from 3 to 5 and 4 to 6 (Table 2).

The upper electrode reservoir of the apparatus must be hermetically sealed; in no case can the leakage of electrode solutions or their stirring be permitted since, in contrast to the case of disc electrophoresis, this leads to a sharp fall in the voltage and to a distortion of the results [18].

The time of an experiment depends on the sizes of the tubes, the concentration of the ampholines and their type, the concentration of the polyacrylamide gel, the ratio of acrylamide to methylenebisacrylamide, the molecular weights and isoelectric points of the proteins under investigation, the composition of the electrode solutions, the current strength, the voltage, etc., and it may vary from 1 to 14 h. The current strength and voltage are selected so that not more than 0.5-0.75 W is applied to each gel, this being achieved at a voltage of 150-500 V and a current strength of 1-5 mA. The maximum current strength is set at the beginning of the experiment. During isoelectric focusing, the voltage rises and the current strength falls, since the electrical conductivity of the gel decreases. The experiment is usually terminated when the current strength has fallen by a factor of 5-10 in comparison with its initial value. At the same time, in the

investigation of proteins with high molecular weights (for example, phosphorylase B with mol. wt. 180,000), isoelectric focusing must be continued for several hours after the stabilization of the current.

Measurement of the pH Gradient Formed in the Gel. To determine the pH gradient, after being washed the gel is cut into pieces (1-5 mm long) and each piece is immersed in a small volume of water (0.2-1.0 ml) for 0.5-4 h [19, 41, 48], after which the pH of the water is determined by means of microelectrodes. Deionized twice-distilled water must be used, and this can be boiled to eliminate CO_2 immediately before the experiment. The determination of the pH must be performed at the temperature at which the isoelectric focusing was carried out.

The dilution of the ampholines 4-6 and 7-10 with water affects their buffer capacity [49]. Thus, with a lowering of the concentration of the ampholines from 0.12 to 0.06 mg/ml the pH changes by 0.1 unit. Consequently, the volume of water must not exceed the volume of the piece of gel by a factor of more than 7-10 [28]. With such a dilution, the final concentration of the ampholines in the water is not lower than 0.1 mg/ml (at a 1% concentration of the ampholines in the gel).

The presence in the gel of 6-8 M urea may lead to a pH shift, because of which a correction of +0.3-0.4 pH unit is frequently introduced [31, 49, 50].

Chrambach et al. [18, 51] recommended for the determination of the gradient that the pieces of gel should be immersed for 3 h not in water but in a freshly prepared 0.05 M solution of potassium chloride (in a desiccator over KOH) which leads to a more accurate determination of the pH in the gel.

The direct determination of the pH in the gel itself (without elution) is also possible by means of contact microelectrodes [52]. When unknown proteins are being investigated, colored proteins or dyes with known pI values can be used as markers [53].

Determination of the Isoelectric Points of Proteins. After the construction of the pH gradient curve in the gel and the staining and decolorizing of the gels, the isoelectric points of each of the components of the mixture under investigation are found. The possibility must be borne in mind of some decrease in the length of the gel during staining and decoloration with ethanolic solutions. Consequently, for accurate pH measurements the lengths of the gel before staining and decoloration (before the determination of the pH gradient) and after are determined, and a correction factor is introduced into the measurement of the lengths along the gel [48]. It is also possible to use colored "marker" proteins with known pI values (hemoglobins, peroxidases, cytochromes, etc.) or dyes (Patent Blue V – pI 3.00; Rapid Green FCF – pI 3.05 and 3.85; Methylene Blue – pI 3.60; Evans Blue – pI 5.35; Congo Red – pI 5.80; and others) [53].

In studying proteins which precipitate at their pI values, high concentrations of ampholines are used in the gel (up to 3-4%) and urea is added to the gel to a final concentration of 8, 10, or even 12 M in order to prevent precipitation [54]. From our point of view, the use for this purpose of ethylene glycol and some nonionic detergents (such as "Brij 35") is of great interest. These methods of stabilizing proteins at the isoelectric point have been used successfully for the isoelectric focusing in a density gradient of sucrose on LKB columns of the gamma globulins, albumin, etc. [55, 56], and we have used them in investigating some chemical derivatives of pepsin.

The accuracy of the determination of the pI values of proteins may reach hundredths of a pH unit. However, it must not be forgotten that in the determination of pI values the time of the experiment must be fixed, since the pH gradient may change with time (and, consequently, the isoelectric points of the proteins migrating together with the pH gradient will change). On working with high-molecular-weight proteins the gel concentration must be specially selected with the aid of preliminary disc electrophoresis in order to exclude the molecular-sieve effect [18].

Staining and Decoloration of the Gels. As a rule, before the staining of the gels, the ampholines, which form insoluble complexes with the majority of dyes, are eliminated from them completely. To eliminate the ampholines, the gels are immersed in a large volume of 5-12% trichloroacetic acid (TCA), which also fixes the proteins, for several hours with the periodic replacement of the TCA solution, or the ampholines are eliminated electrophoretically [11, 57]. After the ampholines have been removed, the gels can be stained and decolorized in the same way as after disc electrophoresis. Proteins containing several aromatic amino acids are satisfactorily fixed by TCA and can be seen in the form of distinct protein bands on a dark background without staining [58]. The gels are rapidly fixed in the TCA after the end of isoelectric focusing, since after the switching off of the current the protein zones rapidly become diffuse [37].

Coomassie Brilliant Blue, Coomassie Blue, Amido Black, and Ponceau S are usually used for staining [59, 60]. It is convenient to stain the proteins with 0.05% Coomassie Brilliant Blue in 12% TCA [61] which permits the stage of the decoloration of the gel to be omitted: the dye, present in colloidal form, is bound only by proteins. Good results are given by the use of 0.1-0.5% Coomassie in a mixture of 45 parts of ethanol, five parts of glacial acetic acid, and 65 parts of water. Awdeh [62] has developed a simple and rapid method for staining proteins without washing the ampholines out from the gels in TCA. For this purpose the gel is immersed for 1 h in a 0.2% solution of Bromophenol Blue in a mixture of 50 parts of ethanol, five parts of glacial acetic acid, and 45 parts of water. Then the gel is washed with a mixture consisting of 35 parts of ethanol, five parts of glacial acetic acid, and 60 parts of water. The staining of the immunoglobulins has been performed by this method [51]; unfortunately, not all proteins can be detected in this way [36]. According to our results, this method of staining is unsuitable for acid proteinases with a low isoelectric point.

Vesterberg [63] has developed a method of staining proteins which consists in heating the gels to 60°C in a mixture of methanol, acetic acid, and water containing Coomassie for 1-2 h, which permits tenths and hundredths of a microgram of protein to be detected. Frater [64] has proposed a simpler (but also less sensitive) method for staining proteins using a 0.05% solution in 5% acetic acid of Fast Acid Blue B or Coomassie Violet R – dyes which do not form insoluble complexes with the ampholines. Before staining, the gels are fixed in 5% acetic acid, and not in TCA. We have observed that the use of ethanolic solutions of dyes leads to the elution of the low-molecular-weight proteins or peptides from the gel and to a decrease in the intensity of the colored bands. In such cases it is better to stain with Coomassie in 12% TCA [61]. If the gel contains urea, then it is recommended to add sulfosalicylic acid to a final concentration of 5% to the TCA fixing solution and to the dye solution [41, 54].

The decoloration of the gels is generally performed in the same solvent as their staining. After staining and decoloration in solutions containing ethanol or methanol, the dimensions of the gels frequently decrease. To restore the initial dimensions of the gels it is proposed to leave them in 5-7.5% acetic acid for 10-12 h after decoloration.

To eliminate the excess of dye it is also possible to use the electrophoretic decoloration of the gels, for example in a simple instrument [65]. Other methods of staining the proteins are described in reviews on isoelectric focusing and disc electrophoresis [16, 66, 67].

Determination of the Activities of Enzymes in the Gel. Color Reactions for Proteins. If the ampholines do not affect the activities of the enzymes being investigated, the latter can be detected from their enzymatic activity directly in the gel. The method is based on the penetration into the gel of low-molecular-weight substrates of the enzymes and the formation of colored reaction products. This method (without the elution of the ampholines) has been used to identify lipoxidase [68], lactate dehydrogenase [10, 69], procarboxypeptidase A (after activation with trypsin) [70], glyceraldehyde phosphate dehydrogenase [44-46], phosphodiesterase [47], and phosphorylases A and B [71]. It is likely that many of the specific reactions used to reveal the enzymatic activity of proteins in the gel after disc electrophoresis can be applied after isoelectric focusing without the preliminary elimination of the ampholines. However, we have shown that by binding metal ions the ampholines prevent the exhibition of the activity of metal-containing enzymes such as, for example, the leucine aminopeptidase from Aspergillus oryzae.

Proteins can be eluted from the pieces of gel with an appropriate buffer for 10-14 h (generally overnight at 4°C), and then the enzymatic activity in the eluates can be determined. The yield of protein depends on the concentration of the gel and on the molecular weight of the proteins under study, and amounts to 50-75%. Phosphoacetylglucosamine mutase [73] and kininogen [74] have been identified in this way. By this method we have identified the zones in which the two forms of carboxypeptidase C from Aspergillus oryzae were located. Before the determination of the activity of metal-containing enzymes in this way, dialysis is carried out to eliminate the ampholines. Specific color reactions for proteins are also used. Thus, for example, glycoproteins are revealed with the periodic acid–Schiff reagent [17], and ferritin with potassium ferrocyanide [72] without the removal of the ampholines from the gel.

Possible Artefacts of the Isoelectric Focusing and Methods for Their Prevention. The results of isoelectric focusing require attentive and careful interpretation, since heterogeneity is not infrequently found or the determination of the pI value of a protein may not correspond to reality, i.e., the protein may in fact be homogeneous and the true value of the pI may be different. The contact of a protein with the electrode solutions, which are acids and alkalis of fairly high concentration (about 5%) may lead to the appearance of a multiplicity of "artefactual" bands through the decomposition of the protein. Consequently,

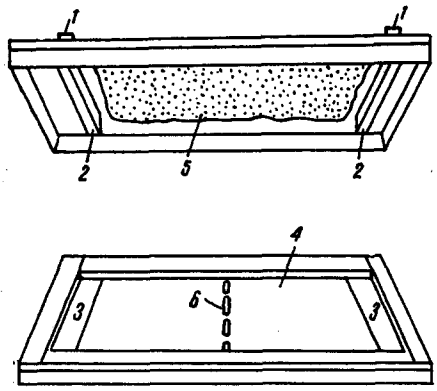

Fig. 2. Apparatus for isoelectric focusing in a flat layer of polyacrylamide gel [77]. 1) Clamps for connecting the source of current; 2) electrodes; 3) spaces for the electrode solutions; 4) glass plate with the gel; 5) moist porous layer; 6) holes for the deposition of the sample.

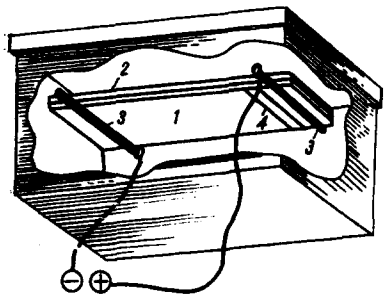

Fig. 3. Apparatus for isoelectric focusing in a flat layer of polyacrylamide gel [78]: 1) layer of polyacrylamide gel; 2) glass plate; 3) graphite electrodes; 4) place for depositing the sample.

the protein is carefully protected from contact with the electrode solutions. Great attention is devoted to the position of the electrode solutions and electrodes in order to exclude the appearance of thermal and concentration gradients and so as not to subject the proteins to the effect of extreme pH values (which may also lead to the appearance of artefacts). We do not recommend the investigation of pH-labile acid proteinases in the pH range from 3 to 10 or the placing of the cathodic solution in the upper electrode reservoir of the instrument on isoelectric focusing in other pH ranges (3-6, 3-5, etc.). Thus, in an investigation of homogeneous awamorin (an acid proteinase from Aspergillus awamori) with pI 3.99 in the pH range from 3 to 10, a multiplicity of artefactual bands with isoelectric points in the pH 6-8 region and higher appears, which shows the degradation of the protein. One must also allow for the fact that the ampholines may sometimes form complexes with certain proteins, which leads to the appearance of additional bands (for example, with the acid protein from wool) [27, 64]. Frater [76] has found bands after the staining of gels containing no protein. Consequently, in the performance of an experiment, control gels to which no protein samples have been added are frequently used.

It must be borne in mind that excessive voltages and current strengths may lead to the heating of the gels, to the formation of bubbles of air in them, to the detachment of the gels from the walls of the tube, to the penetration of the electrode solutions into the space between the gel and the wall of the tube, and to the complete distortion of the pattern of separation. Sometimes, with excessive heating, the gel may even slip out of the tube.

To protect readily oxidized proteins, mercaptoethanol or dithiothreitol is generally added to the gel, preliminary isoelectric focusing is performed, or riboflavin or ammonium persulfate residues are washed out of the gel, etc.

Isoelectric Focusing in a Flat Layer of Polyacrylamide Gel. Isoelectric focusing in a gel may also be performed in a flat layer. One of the advantages of this method is that under identical conditions it is possible to perform comparative investigations of several proteins simultaneously. Vesterberg's review [28] describes isoelectric focusing in a flat layer of polyacrylamide gel.

There is much in common between the methods of isoelectric focusing in tubes and in flat layers. The methods for the polymerization of the gel, its concentration, the types of ampholines, the nature of the electrode solutions, staining, decoloration, the measurement of the pH in the gel, the causes of the appearance of artefacts, etc., remain the same as in isoelectric focusing in tubes. However, in isoelectric focusing in a flat layer apparatuses of a different type are used. Leaback and Rutter [77] have used an apparatus in which the gel lies on a glass support cooled on the bottom; the apparatus has spaces for electrode solutions (Fig. 2). The electrodes are mounted in the lid of the apparatus, and in the working position of the lid they are in contact with the electrode solutions.

Awdeh et al. [78] have used a still simpler apparatus (Fig. 3). The plate with the gel is supported on two platinum or graphite electrodes moistened with solutions of the electrolytes, the gel being on the under side of the plate. The polymerization of the gel is performed between two glass plates (usual dimensions 20 × 10 cm or about this) with a constant thickness of the gap between them of 1-3 mm (Fig. 4). One of the plates is coated with silicone in order to prevent the sticking of the gel. After the end of polymerization, the siliconed plate is carefully removed and the protein solutions are deposited on the gel remaining on the other plate, and the experiment is performed.

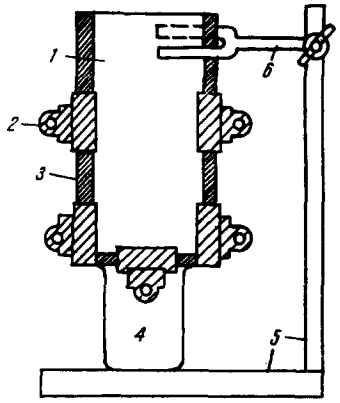

Fig. 4. Sketch of an apparatus for the polymerization of a flat layer of polyacrylamide gel [28]: 1) glass plates; 2) clamps; 3) layer between the plates; 4) beaker; 5) laboratory stand; 6) support with clamp.

To exclude contact with the acrylamide during the polymerization process, the ampholines in the form of a 24% solution (0.5-1.0 ml) or of a 1-2% solution (in an amount equal to the volume of the gel) are poured in a thin layer onto the polymerized gel and are left for 12 h. After this time, the ampholines have diffused into the gel, in which their concentration amounts to 1-2% [77, 79].

The proteins can be added directly to the solution for polymerization (solution D) or be deposited on the finished gel with the aid of pieces of filter paper moistened with the protein solution. Generally from 50 to 500 μg of protein is deposited per band (depending on the heterogeneity of the sample and the sizes of the plates). The whole focusing process usually takes 6-24 h at a voltage of 10-25 V/cm and a maximum current strength of 15-20 mA [60, 77, 78]. It is recommended to perform the experiment in an atmosphere of saturated water vapor or at 2-4°C. With the observance of all the conditions it is possible to perform the separation of proteins the pI values of which differ by 0.06 pH unit and less [48, 49, 77].

Preparative Isolectric Focusing in a Polyacrylamide Gel

In addition to the use of isoelectric focusing in a gel mainly for analytical purposes, preparative variants of this method have also been developed [18, 36]; they consist essentially of a simple "scaling up" of the corresponding analytical methods. For example, the gel mixture (solution D) is poured into a glass tube with a diameter of 1-3 cm and a height of 18-20 cm. After photopolymerization the protein sample (2 mg per zone) is deposited on the surface of the gel beneath a protective layer of 1-2% ampholines. The electrode solutions are 5% phosphoric acid and 5% ethylenediamine. Isoelectric focusing is performed at a voltage of 40 V for 24 h. After the end of the experiment, the gel is extracted from the tubes and is cut into pieces which are suspended in water or the appropriate buffer. After several hours, the proteins have passed from the gel into the water or the buffer and, where necessary, they are separated from the gel residues by centrifuging.

Analytical and Preparative Isoelectric Focusing in a Flat Layer of Sephadex Gel

Sephadex gel can be used instead of acrylamide gel for isoelectric focusing in a flat layer. Isoelectric focusing in Sephadex gel is performed on plates with dimensions of 40 × 20 cm or 20 × 20 cm coated with a layer of Sephadex G-75 Superfine or G-200 Fine, with 1% of the appropriate ampholines [80]. The thickness of the layer of gel is 0.75-1.0 mm. The plates are arranged strictly horizontally on a metal or glass support cooled to 4°C. Contact with the electrode solutions, which contain sulfuric acid and ethylenediamine, is effected by means of Whatman 3 MM chromatographic paper, the ends of which in contact with the gel are surrounded with dialysis bags. Before the deposition of the protein samples the gel is dried somewhat in the air (to a loss of 20% of water). The protein samples (100-250 μg in a 1% solution of ampholines) are deposited on the surface of the gel in the same way as in thin-layer chromatography [81]. The time of isoelectric focusing at a voltage of 10-20 V/cm with 20 × 20-cm plates is 6-8 h, and with 5-10 V/cm and 20 × 40-cm plates it is 18-24 h. The proteins are revealed with the aid of paper replicas (Whatman 3 MM). For this purpose, strips of paper are placed on the surface of the gel and are then removed from it, dried, washed free from ampholines in 5% TCA and sulfosalicylic acid, and are stained with Coomassie solution. It is also possible to determine the enzymatic activity on the paper replicas without first washing them free from ampholines (for example, peroxidase from horse-radish) [80]. The pH gradient in the gel is determined by means of contact microelectrodes directly in the gel or by eluting the ampholines with water [52].

By increasing the thickness of the layer of gel to 3 mm and more it is possible to perform the preparative separation of the proteins in a Sephadex G-200 gel (up to 200 mg of protein on a plate with dimensions of 40 × 20 cm) [52].

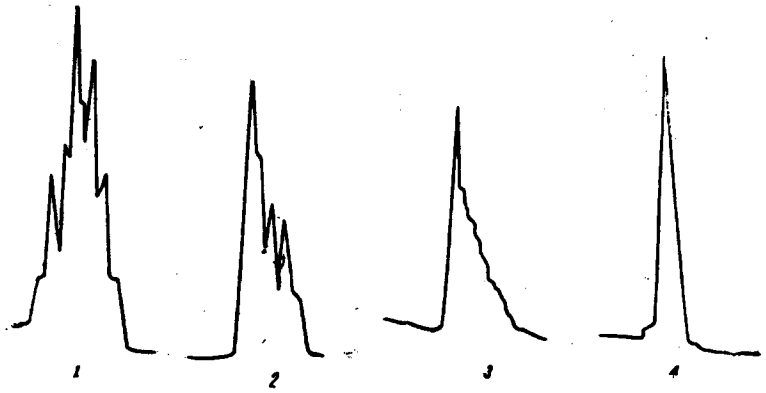

Fig. 5. Densitograms of a preparation of awamorin at various stages of purification [isoelectric focusing in a 5% polyacrylamide gel containing 1% of ampholines 3-5 (tubes with a size of 5 × 65 mm); electrode solutions – NaOH (0.002%) and H_2SO_4 (0.001%); voltage 300 V, maximum current strength 2 mA, time of an experiment 2 2]: 1) initial material; 2-3) intermediate stages of purification; 4) purified material.

Isoelectric Focusing in a Gel in Combination with Electrophoresis

The method consists in the separation of the mixture of proteins by isoelectric focusing in a gel (in tubes) followed by electrophoresis either in a flat layer of polyacrylamide gel [32, 82-84] or in an agarose [85] or a starch [20] gel. In all cases, protein "maps" resembling "peptide maps" are obtained. With this method of separating proteins, the position of a protein spot is a function both of its pI value and of the electrophoretic mobility of each protein (in dependence on the buffer solutions and the gel concentration used). This method has great possibilities, since in electrophoresis it is possible to change the pH values of the electrode buffers and the concentration of the gel. Thus, Wrigley [20], using electrophoresis in a starch gel at pH 3.1 after isoelectric focusing in a gel, separated more than 40 protein components of wheat gliadins.

Immuno Isoelectric Focusing in a Gel

The immunochemical procedure known as immuno isoelectric focusing was developed by Riley and Coleman [13], and also by Catsimpoolas [35, 37, 40]. It consists in the isoelectric focusing of proteins in a gel (in tubes) followed by immunodiffusion or immunoelectrophoresis against the appropriate antisera. Immuno isoelectric focusing in a gel has been described in a review paper by Catsimpoolas [35].

Applications of Isoelectric Focusing

Isoelectric focusing in a gel is generally used for checking the homogeneity of protein preparations and also for determining the isoelectric point of each component. Since in isoelectric focusing in a gel the separation of proteins differing in pI value by only a few hundredths of a pH unit is possible, this method possesses a very high separating power.

The separating power and the possibilities of isoelectric focusing in a gel can be illustrated by the investigation of normal and anomalous hemoglobins [19]. The replacement of the negatively charged glutamic acid in position 6 of the β subunit of normal hemoglobin A_{II} with pI 6.95 by the positively charged lysine leads to the anomalous hemoglobin C with pI 7.40; the replacement of the glutamic acid in the same position by the neutral valine leads to the anomalous hemoglobin S with pI 7.25. Such a change in the isoelectric point is sufficient for the separation of the hemoglobins A_{II}, C, and S, i.e., by gel isoelectric focusing it is possible to detect a change in only one amino acid in a protein with a molecular weight exceeding 60,000. The acetylation of the N-terminal amino groups of the γ chains of hemoglobin F_{II} with pI 7.15 leads to the production of hemoglobin F_I with pI 6.90. The formation by a hexose of a Schiff's base with the N-terminal amino groups of the β chains of normal hemoglobin A_{II} gives hemoglobin A_{IC}, which is well separated from A_{II} by isoelectric focusing.

TABLE 3. Applications of Gel Isoelectric Focusing

Subject of investigation	Literature references
Isoelectric focusing in polyacrylamide gel (in tubes)	
Albumin	[11, 27, 96]
Antibodies against the polysaccharides of type VIII pneumococci	[93]
Antibodies against lysozyme	[94]
Antibodies against steroids	[51]
Soluble H_{-2} antibodies from mice	[95]
Awamorin (acid proteinase from Aspergillus awamori)	*
Serum proteins	[10, 22]
Water-soluble proteins from flour and gluten	[12]
Soya proteins soluble at pH 4.5	[15]
Soluble protein from mouse skin bound to a carcinogenic hydrocarbon	[21]
Nonhistone proteins of liver nuclei	[29]
Cytoplasmatic protein from Neurospora crassa	[98]
Proteins of cerebrospinal fluid	[97]
Proteins of human saliva	[99]
Hydrophobic protein from the cell membrane of Hydrogenomonas facilis	[31]
Cyanogen bromide hydrolyzate of collagen	[18]
Hemoglobins	[13, 14, 19]
Growth hormone	[23]
Soya hemagglutinins	[100]
11 S globulin from soya beans	[101]
Deoxyhemoglobin	[102]
Trypsin inhibitor from soya	[103]
Immunoglobulins	[43]
Insulin	[22, 104]
Cathepsin D	[105]
Kininogen	[74]
Acid carboxypeptidase from Aspergillus oryzae	*
β-Lactoglobulins	[27]
Lyzate of human erythrocytes	[13]
Soya lipoxidase	[68]
Serum lipoproteins	[30, 106]
Mercaptalbumin	[11]
Myoglobins	[13, 19]
Myosin and its chemical derivatives	[33, 54, 91]
Neurophysin	[109, 110]
Nucleoside phosphorylase	[111]
Ovalbumin	[11]
L-Amino acid oxidase	[41]
Oxyhemoglobin	[102]
d-Amino acid oxidase	[108]
Orsellinate hydrolase from Lasallia pustulata	[107]
Plakalbumin	[11]
Prolactin	[23]
Proteinase B from Aspergillus niger	*
Porcine pepsin and its chemical derivatives	*
Subtilisin	*
Triglyceraldehyde phosphate dehydrogenase	[44-46]
Phosphoacetylglucosamine mutase from Neurospora crassa	[73]
Phosphoglucose isomerase	[113]
Human α-fetoprotein	[112]
Phosphodiesterase	[47]

TABLE 3 (continued)

Subject of investigation	Literature references
Ferritin	[72]
Fibrinogen	[86, 114]
Phosphorylases A and B	[71]
Alkaline phosphatases	[115]
IMP: Pyrophosphate phosphoribosyl transferase	[111]
N^{10}-Formyltetrahydrofolate synthetase	[57]
Chymosin (rennin)	*
Chymotrypsinogen A and its carbamoyl derivatives	[58]
Yeast enolase	[88]
Isoelectric focusing in a flat layer of polyacrylamide gel	
Subgroups of antigens from Trypanosoma brucei	[60]
Bovine serum albumin	[48, 49]
Antihaptene antibodies	[116]
Serum proteins	[77]
Myeloma proteins labeled with ^{14}C	[75]
Hemoglobins and myoglobins	[77-79, 117]
Immunoglobulins	[78]
H and L chains of immunoglobulin IgG	[87]
Fab and Fc fragments of immunoglobulin IgG	[59]
Crystallins from the crystalline lens of the eye	[118]
Lactate dehydrogenase	[10, 69]
Ovomucoid	[119, 124]
Proteinase from Serratia marcescens	[120]
Procarboxypeptidase A	[70]
Ribonuclease	[77]
Mouse RNA-ase	[121]
Erythrocyte phosphatase	[77]
Isoelectric focusing in a flat layer of Sephadex gel	
Albumin of bovine serum, myoglobins, hemoglobins, ovalbumin, ferritin, and horse-radish and tobacco peroxidases	[52, 80, 90, 122]
Methylesterase	[123]

* Our own results.

Normal ferrohemoglobin A_{II} with pI 6.95 and ferrihemoglobin A_{II} with pI 7.20, differing only by the charges of the prosthetic groups, are also separated by gel isoelectric focusing. Thus, in certain cases isoelectric focusing permits the separation of proteins differing in the charge of only a single amino acid residue or only by post-translational modifications, which shows the very high separating capacity of the method. In actual fact, as a rule, isoelectric focusing is not inferior in this respect even to such an effective method as disc electrophoresis in a polyacrylamide gel.

Thanks to the use of gel isoelectric focusing the heterogeneity has been shown of highly purified samples (giving a single band on disc electrophoresis) of ferritin [72], fibrinogen [86], L-amino acid oxidase [41], the L and H chains of the globulins from myeloma 5563 [87], and the enolase from yeast [88]. Together with our laboratory colleagues N. M. Azarenkova and T. I. Vaganova, we have shown the molecular heterogeneity of the acid carboxypeptidase from Aspergillus oryzae and have demonstrated the presence of its two active forms [89]. In an investigation of tobacco peroxidase by isoelectric focusing in a flat layer of Sephadex gel, 14 fractions of active isoenzymes were obtained instead of the 5-6 by disc electrophoresis [90]. Fondy et al. [44-46], by using isoelectric focusing in a polyacrylamide gel, traced the appearance of various isoenzymes of glyceraldehyde phosphate dehydrogenase in the liver and skeletal muscles of rats in the process of development of the animals. Two main and three minor isoenzymes of glyceraldehyde phosphate dehydrogenase were found, while no iso forms could be detected by disc electrophoresis. Disc electrophoresis did not succeed in separating a fraction of serum albumins, while the use

of gel isoelectric focusing showed that bovine serum albumin has a considerable heterogeneity [27, 48, 49]. The heterogeneity of the large and small subunits of myosin has also been determined [33, 54, 91], mercaptalbumin and the A and B forms of β-lactoglobulins have been successfully separated [27], etc.

Isoelectric focusing in a gel can be used to investigate not only proteins but also peptides. For example, the heterogeneity of the electrophoretically homogeneous Fab and Fc fragments of IgG has been established [59]. The peptides obtained in the cyanogen bromide hydrolysis of collagen have been studied [18], which is very important in connection with the great increase in the number of papers on the primary structure of proteins.

The method considered has been used successfully for the study of the products of the chemical modification of proteins. It has been possible to separate carbamoylated derivatives of chymotrypsinogen A differing in charge by only one unit [58].

It may be mentioned that gel isoelectric focusing is also used for the separation of nucleic acids such as tRNA [92].

In many cases, the use of gel isoelectric focusing in the stages of the purification of proteins gives very useful information on the physicochemical properties of the materials and enables the most rational scheme of purification to be selected and its progress to be monitored (Fig. 5).

Gel isoelectric focusing has already been used successfully for the investigation of a large number of biopolymers (Table 3).

SUMMARY

The method of isoelectric focusing in a gel has become widely used in spite of the fact that the first papers on this question were published only four years ago. This is due to the advantages of the method: high sensitivity and resolving power, simplicity and rapidity, and possibility of combination with other methods.

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