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HIGHLY STABLE pH-GRADIENTS FORMED BY POLYHYDROXYL COMPOUNDS, BORIC ACID AND BORATES

THEORETICAL ASPECTS AND APPLICATION TO ISOELECTRIC FOCUSING OF PROTEINS, PEPTIDES AND AMINO ACIDS

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SUMMARY

Isoelectric focusing of proteins in artificial pH gradients created by polyol concentration gradients in borate buffer solutions were studied. A theory of the method is given. The conditions for obtaining stable pH gradients in electric fields are investigated. Experimental data on focusing of different proteins in narrow and wide[2–12] pH gradients are presented.

INTRODUCTION

Isoelectric focusing is a method of separation of proteins and other substances, by means of their amphoteric properties. The most widespread form of this method is associated with the concept of conducting ampholytes[1–4] as the main means of creating a pH gradient. Recently, along with the great success of this technique, numerous cases of associated artefacts have been described. A variety of chemical compositions and the closeness in the nature of ampholine to that of amino acids and peptides increase the possibility of complex formation between it and proteins, which is sometimes mistaken for fractions of proteins and carbohydrates[5–7].

In view of the great importance of isoelectric focusing, in the study of proteins, it is of interest to consider what variations of this method would lead to increased reliability of the results obtained. For example an artificially created pH gradient may be used[8]. Earlier we described pH gradients formed by boric acid and borates in a concentration gradient of a polyhydroxyl compound (BP-pH gradient)[9,10], which was stable in the acidic region. This method, first developed in 1975, in its present form allows to a stable pH gradient in the range pH 2.0–12. It has now been applied to the fractionation of many proteins.

THEORETICAL

We will first consider some properties of pH gradients of this type. An am-

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pholine pH gradient is formed spontaneously as a result of the arrangement of a set of conducting ampholytes (carrier ampholytes) in the electric field upon electrophoresis, according to the sequence of their isoelectric points. In general such a gradient is an equilibrious system. The studied ampholyte (protein) is found in the sequence of the other ampholytes between more acidic and more basic components of the gradient.

An artificial pH gradient is produced by the simple operation of layering acidic or basic solutions of decreasing concentrations in a vertical glass column. In contrast to an ampholine one, such a gradient is a non-equilibrium system. On applying an electric field, all the constituent ions begin to move and the pH gradient is destroyed. It is important from a practical point of view that the time of its destruction should be greater than the time of protein focusing. A drift in the gradient does not result in an error in pI, if this condition is maintained[4].

Methods of stabilization of artificial pH gradients are based on increasing the buffer pH-gradient capacity. Ions, carried away by the electric current, are replenished by the buffer. The movement of the conventional ionic boundary may easily be described if we assume that the pH gradient is formed by an univalent ion pair between the hydrogen ion and the anion. In this case the rate of migration of the conventional boundary is defined by

$$U = U_1 \alpha E \qquad (1)$$

where U_1 is the absolute rate of ion migration in the dilute solution, α is the degree of dissociation and E is the electric field gradient. It is easy to see that a high degree of dissociation is the cause of the pH-gradient instability, therefore it is advantageous to use concentration gradients comprising weak acids or weak bases.

The pH gradient created by the mixture of boric acid and polyol (BP-pH gradient) is based on the ability of boric acid to increase its acidity in the presence of a polyhydroxyl compound, *e.g.*, glycerol. This property is used in the titration of boric acid. Hence the pK of the acid pK may be changed as desired. This is a unique case and such an acid is very useful for creating a pH gradient.

According to literature sources[13] it is generally supposed that acidification is associated with the formation complexes between polyalcohols and tetraboric acid.

Although, in electrophoresis, polyhydroxyl compounds migrate together with the anions of boric acid, in ion-exchange chromatography they are easily separated from each other[11]. Thus, for the purpose of this work as for the titration it may be supposed that boric acid present in the polyol solutions becomes stronger, *e.g.*, as the result of stabilization of the tetraborate anion through the complex formation:

$$m\mathrm{B} + n\mathrm{P} \rightleftarrows \mathrm{B}_m\,\mathrm{P}_n \qquad (2)$$

$$\mathrm{B}_m\,\mathrm{P}_n = \mathrm{B}_m\,\mathrm{P}_n^- + \mathrm{H}^+ \qquad (3)$$

The acidity increase was studied by Antikainen[13,14]. The dissociation constant of the complex can be calculated from

$$\mathrm{p}K^{++} = \mathrm{p}K_2 - n \log C_\mathrm{p} \qquad (4)$$

where C is the polyol concentration, K^{++} the apparent dissociation constant of the complex B_mP_n and K_2 is the complex formation constant for B_mP_n. However, for the purpose of this work it is more convenient to introduce another dissociation constant of the B_mP_n complex, which is independent of the polyol concentration. From eqns. 2, 3 we obtain $K_3 = [H^2]/C_p$ and then

$$pH = \frac{1}{2}(pK^* - m \log C_b - n \log C_p) \quad (5)$$

where $K^* = K_2 K_3$ is the equilibrium constant of eqn. 3 and C_b is the boric acid contration. Eqn. 4 and 5 are correct only in the presence of an excess of a polyol. Eqn. 4 does not fit the experimental data at low concentrations of polyol, since, in this case, one should take into account the dissociation of H_3BO_3 and B_mP_n which are independent of each other and their constribution to the hydrogen ion concentration are additive. Then, if $C_b > C_p$, the pH gradient can be calculated according to

$$pH = \frac{1}{2}[\log C_b + \log (K_b + K^+C_p^n)] \quad (6)$$

where K_b is the dissociation constant of boric acid. Data, given in Fig. 1, show that both eqns. 5 and 6 give satisfactory fits to the experimental data.

The formation of complexes of polyhydroxyl compounds with boric acid is usually employed in their electrophoresis. In this case the electrophoresis is the cause of the pH-gradient destruction. Apparently, the stability of borate–polyol pH gra-

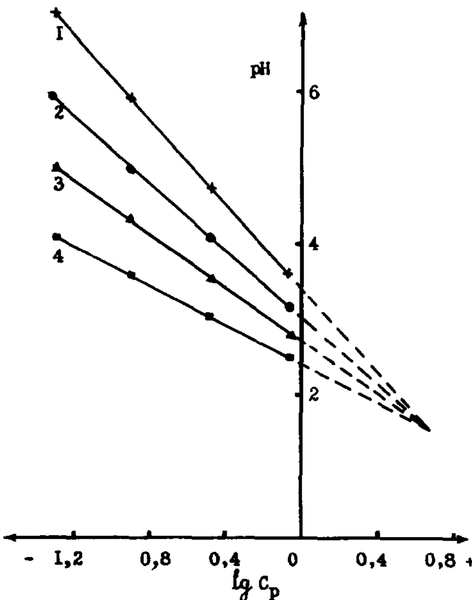

Fig. 1. The pH gradient formed by mannitol in boric acid buffer solution with pH 9.0 (1), 8.5 (2), 7.5 (3), and 4.0 (4).

dients will depend on the mobility of ions creating the gradient and on local changes in the concentration of B_mP_n complex. Changes in this concentration with time will be defined by

$$dC_p/dt = -C_p \alpha U_i E \tag{7}$$

where α is the degree of ionization, U_i is the absolute rate of migration of $B_mP_n^-$ and E is the potential gradient in V/cm. It is apparent also that $\alpha C_p = C_{H^+}$, and this in turn is obtained from eqn. 5 providing C_b is small:

$$C_p \alpha = \sqrt{K^* C_p^n C_b^m} \tag{8}$$

Substituting this expression into eqn. 7 we obtain

$$dC_p/dt = -U_i E C_p^{n/2} \sqrt{K^* C_b^m} \tag{9}$$

and for a particular case, when $n=2$, $m=1$:

$$dC_p/dt = U_i E C_p \sqrt{K^* C_b} \tag{10}$$

The solution of this will be

$$C_p = C_p \exp(-tU_i E) \sqrt{K^* C_b} \tag{11}$$

and on basis of eqn. 5, and that $\Delta pH = -2.303 \log (C_p/C_{p_0})$ we obtain:

$$\Delta pH = 0.43\ U_i t E \sqrt{K^* C_b} \tag{12}$$

K^* is independent of polyol concentration because of the in pH change will be uniform throughout a column. For example, in glycerol ($K^* = 5.36 \times 10^{-7}$) (Table I) in 0.01 M boric acid the pH drift, $\Delta pH/t$ will be 0.00019 units per hour. In principle, this shows the long lifetimes for BP-pH gradients. However, eqns. 11 and 12 are valid only for gradients created by boric acid with borate buffer containing a dissociated salt with anion BP^- the ionization increases several-fold and may defined by:

$$\text{if pH} < 7,\ \alpha = \frac{(1-g)C_s + 10^{-pH}}{C_p} \tag{13}$$

$$\text{if pH} > 7,\ \alpha = \frac{(1-g)C_s + 10^{-pOH}}{C_p}$$

TABLE I

VALUES OF pK^* (FROM EQN. 5) AND U_i FOR DIFFERENT POLYOLS IN BORATE FORM

No.	*Polyol*	*pK**	*$U_i \times 10^{-5}$ ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)*
1	Mannitol	2.75	54.0
2	Sorbitol	3.64	48.0
3	Xylite	3.92	47.0
4	Galactose	5.59	44.0
5	Glucose	5.82	22.0
6	Glycerol	6.54	32.0
7	Saccharose	7.26	7.0
8	Lactose	6.97	16.8
9	Maltose	7.76	14.7

The mobility of the boundary, formed by a polyol in borate buffer, will be described by

$$U = U_i \cdot \frac{(1-g)C_s + 10^{-pH}}{C_p} \qquad (14)$$

or 10^{-pOH} at pH < 7, where U_i the absolute rate of migration of BP^- in $cm^2 \cdot V^{-1} \cdot sec^{-1}$, C_s is the concentration of borate salt and g is the degree of salt hydrolysis.

The comparison shows that the absolute rate of polyol electrophoresis in buffer is far greater in the alkaline medium than in the acidic one, providing C_p is small in the alkaline region where the borate ion concentration is large.

Fig. 2 shows a series of curves demonstrating the distortion of pH-gradient pattern with the course of time. Curves 1 and 2 were obtained in a pH-gradient formed by boric acid and as is seen the pattern is almost unchanged over 24 h of

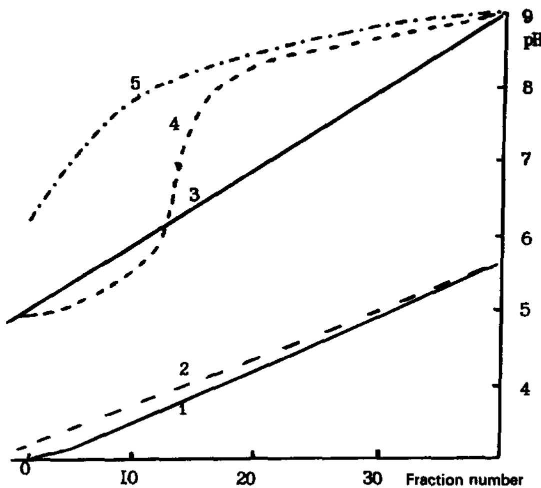

Fig. 2. Deformation of pH gradient created by gradient concentration of xylite in boric buffer solution. Curves: 1, in 0.1 *M* boric acid; 2, the same for 24 h at 20 V/cm; 3, in 0.01 *M* borax solution; 4, the same for 48 h; 5, the same for 46 h.

electrophoresis. Curves 3–5 were obtained in borax solutions. In case 3 the pattern became S-shaped in a short time; however, protein focusing was still possible (of course with lower accuracy) and pH gradient in this case required additional stabilization.

On the basis of the foregoing a few methods of stabilization of the alkaline region of the BP-pH gradient can be described. The first possibility is to use buffer solutions of low ionic strength, resulting in a decrease of the numerator in eqn. 14. The second and most important method is based on the use of buffer solutions, comprising boric acid and a weak base to produce the pH gradient. Finally, one can employ polyhydroxyl compounds with different electrophoretic mobilities. If a polyol having high mobility is placed of the pH gradient where the degree of dissociation is very small, one having an intermediate mobility is placed in the middle and one having a small migration rate is placed, in the upper, more alkaline part, then the pH-gradient stability generally increases significantly in the alkaline part. At the same time, this method allows one to obtain gradients over a wide range of pH[9].

The stability of BP-pH gradients depends not only on the elctrophoresis of polyols but also on the buffer capacity and ionic strength of the buffer solutions used. In this respect such gradients differ from other pH gradients for example those formed from ampholine because the buffer concentration is constant over the whole length of the column because only the pK^+ of the acid is changed, not the salt/acid ratio.

The buffer capacity is determined not only by the local concentration of the buffer, but also by the volume of the electrode vessels. Thus, the local changes in pH of the buffer caused by the concentrating protein are compensated by ionophoresis of iones. This allows on the one hand the use of diluted buffer systems, and on the other hand a much greater protein concentration can be accumulated.

Taking into consideration the fact the glycoside groups of monosaccharides are chemically reactive and may sapontaneously modify proteins, we avoided the reducing saccharides and used in almost all the cases polyalcohols (glycerol, xylite, sucrose) which were unreactive for proteins.

As is seen from Tables II and III, we propose pH gradients of different ranges

TABLE II

PARENT BUFFER SOLUTIONS FOR VARIOUS pH GRADIENTS

No.	*pH*	*Buffer composition (M)*				
		Borax	*Boric acid*	*Tris*	*NH_4OH*	*NaOH*
1	4.45	–	2.0	0	0	0
2	4.45	0	0.5	0	0	0
3	5.15	0	0.1	0	0	0
4	5.60	0	0.01	0	0	0
5	7.00	0	0.05	0.005	0	0
6	8.00	0	0.05	0.024	0	0
7	8.50	0	0.05	0.005	0.001	0
8	9.0	0	0.10	0.005	0.002	0
9	10.00	0	0.10	0.005	0.002	0
10	12.00	0.001	0	0.001	0	0.00075

TABLE III

BP-pH GRADIENTS (pH 2.0–10.0)

The lifetime these pH gradients varies from 240 h in the acidic region to 48 h in the alkaline one.

Buffer solution from Table II	*Polyols*	*Limits of pH gradient*		*ΔpH*	*Lifetime of pH gradient (h)*
		At 1% polyol	*At 60% polyol*		
1	Xylite	2.40	2.00	0.4	240
	Glycerol	3.60	2.70	0.9	240
	Sucrose	4.25	3.35	0.9	240
2	Xylite	2.70	1.80	0.9	240
	Glycerol	4.40	3.50	0.9	240
	Sucrose	4.35	3.65	0.7	240
3	Xylite	3.00	2.15	0.85	240
	Glycerol	4.30	3.35	0.95	240
	Sucrose	4.90	4.00	0.9	240
4	Xylite	3.57	2.65	0.92	240
	Glycerol	4.77	3.85	0.92	240
	Sucrose	5.40	4.50	0.9	240
5	Xylite	6.00	3.00	3.00	240
	Glycerol	6.50	3.65	2.85	240
	Sucrose	6.90	5.65	1.25	240
6	Xylite	6.40	3.45	2.95	240
	Glycerol	7.65	4.95	2.7	72
	Sucrose	7.95	7.10	0.85	72
7	Xylite	8.00	5.75	2.25	72
	Glycerol	8.00	5.85	2.15	72
	Sucrose	8.35	7.45	0.9	72
8	Xylite	8.20	4.30	3.9	72
	Glycerol	8.60	5.80	1.8	72
	Sucrose	8.95	7.35	1.6	72
9	Xylite	8.60	4.80	3.80	48
	Glycerol	9.80	6.10	3.00	48
	Sucrose	9.25	8.50	1.45	48
10	Xylite	11.9	10.9	1.00	48
	Glycerol	11.9	10.78	1.12	48
	Sucrose	11.9	9.60	2.30	48

(1–4 pH units) that are very suitable in practice. However, gradients over the range pH 2.0–10.0 (see Table IV) can also be employed.

EXPERIMENTAL AND RESULTS

Fractionation of proteins

Earlier, we published details of this method, mainly in relation to albumin and actinoxanthine[12]. In addition, it was shown that there is a very good correspondence between the ampholine method and ours[9]. Isoelectric focusing was carried out an electrophoretic glass column of our construction (Fig. 3).

For the creation of the required pH gradient, the buffer solutions given in Table I were used together with a concentration gradient of the indicated polyols.

TABLE IV

GRADIENT FORMATION IN THE RANGE pH 2.0–10.0

Electrode buffer solution: 0.005 *M* boric acid, 0.0018 *M* Tris, 0.01 *M* NaOH, pH = 10.0. pH-gradient created buffer solutions: 1, electrode buffer solution and 5% sucrose, pH = 9.65; 2, 0.3 *M* boric acid, 0.0018 *M* Tris, 0.01 *M* NaOH and 20% glycerol, pH = 6.6; 3, 2.0 *M* boric acid, 0.0018 *M* Tris, 0.0018 *M* Tris, 0.01 *M* NaOH and 40% xylite.

No.	pH	Buffer solution (%)			*n*	Conductivity ($\cdot 10^{-5}$ Ω^{-1})
		1	2	3		
1	9.65	100	0	0	1.340	4.48
2	9.25	99.5	0.5	0	1.3405	4.53
3	8.85	98.5	1.5	0	1.3410	4.58
4	8.45	96.6	3.4	0	1.3420	4.54
5	8.10	94.4	5.6	0	1.3430	4.50
6	7.70	91.3	8.7	0	1.3425	4.46
7	7.35	87.4	12.6	0	1.3440	4.42
8	6.95	81.6	18.4	0	1.3450	4.29
9	6.60	74.0	26.0	0	1.3470	4.15
10	6.20	63.5	36.5	0	1.3490	3.93
11	5.85	51.3	48.7	0	1.3510	3.76
12	5.45	37.8	62.2	0	1.3550	3.35
13	5.10	17.4	82.6	0	1.3590	3.07
14	4.75	0	99.7	0.3	1.3630	2.75
15	4.35	0	98.1	1.9	1.3640	2.77
16	4.00	0	96.0	4.0	1.3650	2.80
17	3.60	0	91.8	8.2	1.3670	3.12
18	3.25	0	85.5	14.5	1.3690	3.50
19	2.85	0	73.6	26.4	1.3740	4.25
20	2.45	0	54.0	46.0	1.3800	5.68
21	2.0	0	0	100.0	1.4000	7.57

Alternatively, all three polyols were used simultaneously for the creation of a wide pH gradient, a routine method for density gradient formation. From eqns. 5–7, if the pH gradient is linear it follows that the concentration gradient of polyol will be logarithmic.

We have shown that the reagents used, particularly the polyols, very often contained contaminating amino acids that focused and distorted the pH gradient, especially in the alkaline zone; therefore we propose their preliminary electrophoretic purification. Fig. 4a shows an isoelectric spectrum of bovine pepsin. This experiment was carried out in a gradient of pH 2–4.5 created with only boric acid and xylite. A distinct separation into three components with p*I* 2.9, 3.1 and 3.4 is evident.

The gradient was not distorted during the time of its formation. Fig. 4b shows an example of a separation of 5 mg of a protein mixture in a gradient of pH 4–6. It is seen that the mixture contained four acidic proteins, ferritin, ovalbumin, β-lactoglobulin and conalbumin with p*I* 4.4, 4.7, 4.9, 5.0, 5.7 and 5.9. As in the previous case, the pH gradient is not distorted in the course of an 18-h experiment at 20 V/cm. Fig. 4c shows an isoelectric spectrum of total and myeloma (dotted line) immunoglobulins, derived from human serum by electrophoresis in agar gel. Myeloma im-

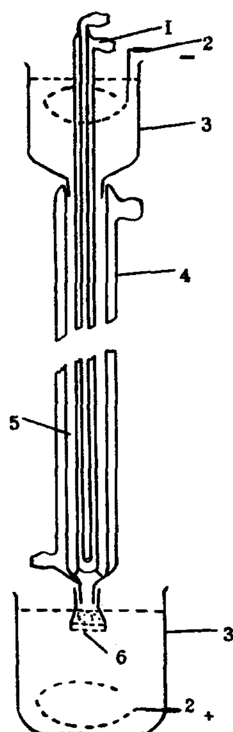

Fig. 3. The apparatus for isoelectric focusing in borate–polyol systems. 1 = Inner cooling; 2 = electrodes; 3 = electrode vessels; 4 = column with cooling jacket; 5 = electrophoretic channel; 6 = gel-cork.

munoglobulin is separated into four components. A component with p*I* 6.9 is distinctly separated from total γ-globulin, while the protein with p*I* from 7.4 to 8.4 is only partially separated, forming a spectrum of superimposed fractions. The high concentration of buffer solution interferes with the aggregation of this protein in the p*I* range; similar results were obtained by us in isoelectric focusing in ampholine. Fig. 4d shows the fractionation of ribonuclease in the gradient pH 7–10 created by a concentration gradient of glycerol. The results are similar to those obtained in ampholines. Three fractions of this protein with p*I* 9.2, 9.5 and 9.8 are revealed. The pH gradient is linear practically throughout the whole range for 20–42 h at 20 V/cm. Fig. 4e shows an isoelectric spectrum of thymus histone, obtained as a result of isofocusing in the pH gradient, formed by a concentration gradient of 50% sucrose. Histone was separated into four fractions with p*I* 10.7, 10.8, 11.1 and 11.5. No distortion of the pH gradient was noted in the course of the experiment which lasted for 18–20 h. Fig. 4f shows the results of fractionation of a mixture comprising pepsin, serum albumin and haemoglobin in the gradient of pH 2–10. The experiment was carried out for 20 h at 20 V/cm. The separation of proteins and the high stability of this pH gradient are evident.

The borate–polyol isoelectrofocusing system has a high buffer capacity because of the possibility to use Tris–borate buffer at concentrations up to 0.5 *M* and the excess of polyol and borate ions migrating along the column provide an additional

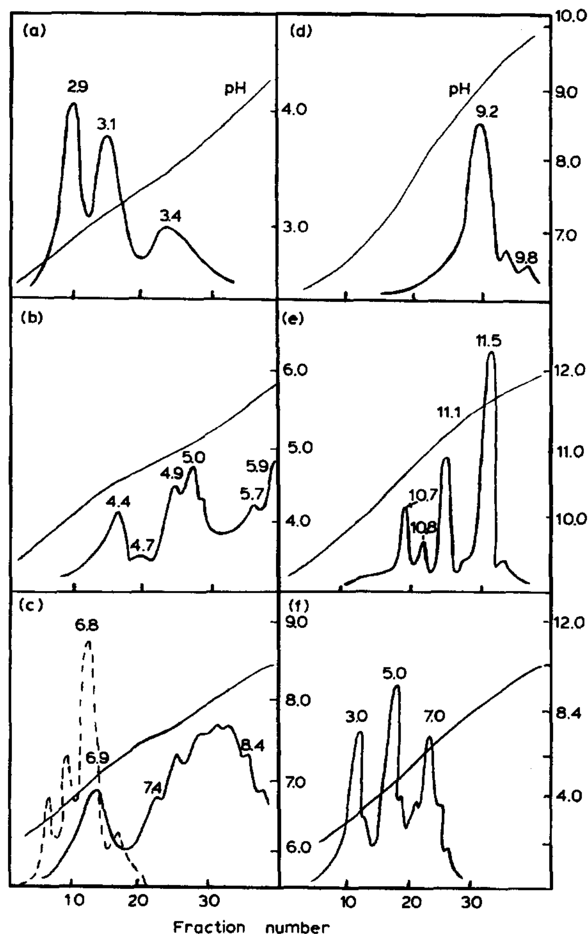

Fig. 4. Examples of separation of proteins in borate–polyol pH gradients: a, bovine pepsin; b, standard proteins from Serva in gradient of pH 3–6; c, blood immunoglobulin (——) and myeloma immunoglobulin (------) in gradient of pH 6–8.5; d, ribonuclease; e, thymus histones; f, pepsin, albumin and haemoglobin mixture in gradient of pH 2–10.

buffer capacity. This makes the system sufficiently flexible for preparative purposes.

Fig. 5. shows the results of isoelectric focusing of human serum albumin under different loads on an analytical column (a, 5 mg/ml; b, 10 mg/ml; c, 15 mg/ml; d, 30 mg/ml). In all the experiments a protein was introduced in region of pH-gradient with pH of the protein solution equal to its isoelectric point. Isoelectric focusing was carried out at 20 V/cm for 20 h the pH gradient being formed by glycerol in a variable concentration Tris–borate buffer, pH = 7.0. From the results it follows that the increase in protein concentration in the column leads to an extension of the isoelectric spectra of albumin to the alkaline zone and the appearance of artefacts at more alkaline p*I* values. This could easily be demonstrated by subjecting one of them to refocusing. The isoelectric spectrum of the artefact with p*I* 6.8 is shown in Fig. 5d. On refocusing of this component we obtained a normal distribution of albumin according to Fig. 5. Thus, the component with p*I* 6.8 appeared as a result of overloading of the column.

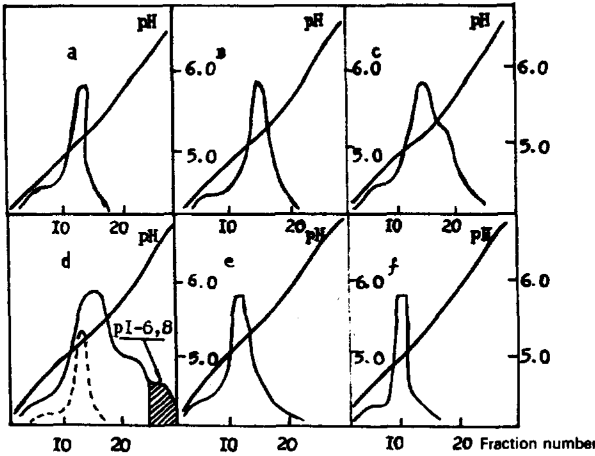

Fig. 5. Isoelectric spectrum of human serum albumin dependence in 0.05 *M* Tris–borate buffer: a, b, c, d, 5, 10, 15, 30 mg of albumin were focused; dotted line in d shows refocusing of fraction with p*I* 6.8; e, focusing of 30 mg of albumin in 0.1 *M* buffer; f, the same in 0.5 *M* buffer.

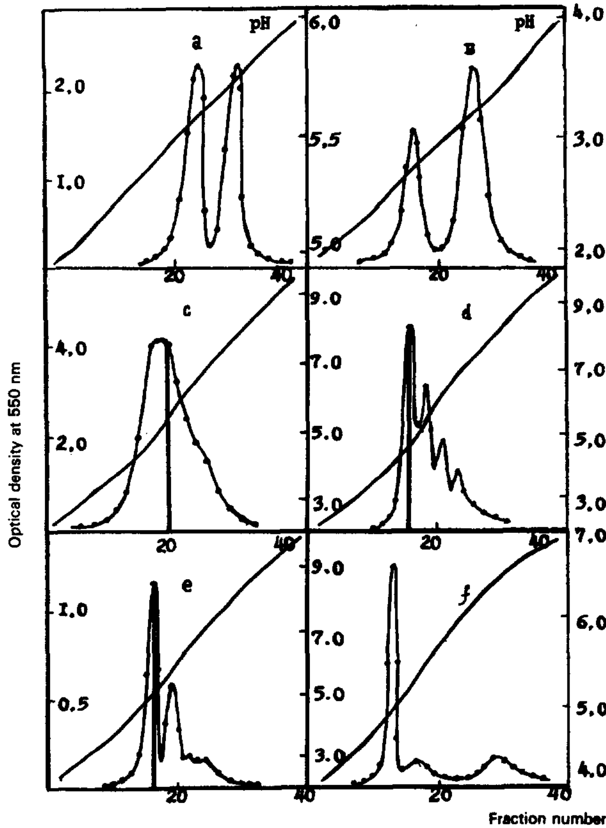

Fig. 6. Separation of amino acids, peptides and carrier ampholine mixtures in borate–polyol systems: a, Gly-Gly and Ala-Ala; b, aspartic and glutamic acids; c, carrier ampholine; d, e, f, refocusing of fraction of ampholines with p*I* 5.

Fractionation of low-molecular-weights ampholytes

A great virtue of the BP-pH gradient method is the opportunity to focus low-molecular-weight substances such as amino acids and peptides since the BP-pH gradient components do not react with ninhydrin and permit an easy preparative separation of amino acids and peptides from boric acid and glycerol.

The main difficulties in the focusing of low-molecular-weight ampholytes is their detection[15,16] as well as the need to use such high voltages that cooling must be applied. We succeeded in obtaining a good separation of amino acids, peptides and ampholines at 50 V/cm. Fig. 6a shows the separation of a peptide mixture (Gly-Gly and Gly-Ala) in 0.1 *M* Tris–borate buffer pH 7.0 in a glass column. A gradient in the range pH 5–7 was formed by a concentration gradient of glycerol. Fig. 6b shows the results of separation of aspartic and glutamic acids. Isoelectric focusing was performed in a gradient of pH 2–4.5 which was created by a concentration gradient of xylite in 0.5 *M* boric acid.

Ampholines may be fractionated in the borate–polyol system as shown in Fig. 5c, d, e, f.

One ml Servalite 4–6 in 1 ml of initial buffer solution was introduced into the column after pH gradient formation. The distribution of ampholines was determined by ninhydrin reaction. A zone at p*I* 5 was successively (Fig. 6d, e) twice refocused in the same pH gradient and then in a gradient of pH 4–7 (Fig. 6d). This resulted in a Servalite with ΔpH = 0.1. It is obvious that this approach will enable more homogeneous ampholines.

Focusing in acrylamide gel

Acrylamide was crystallized twice from benzene and methylene bisacrylamide purified by chloroform extraction. Cyanogum 41 may also be used: gel plates soaked in buffer or photopolymerization of acrylamide in the constituted pH gradient[10,17]. In this case the following solutions were prepared: N1, a buffer for the pH gradient creation in a suitable range; N2, 4 mg riboflavin, 0.4 ml N,N,N′,N′-tetramethylethylenediamine (TEMED) and buffer up to 100 ml; N3, 0.24 ml TEMED, N2, 24 ml N2 solution and buffer up to 500 ml. A series of gradients was formed in ten flasks on the basis of solution N3. According to the pH gradient required, one or another polyol, was added. Then the pH gradient fractions were equilibrated and 6–7% cyanogum was added to each flask. Solutions were kept in the cold without any access of light.

A set of 12–14 standard tubes for disc electrophoresis were placed into a glass or a short cylinder, then a clear buffer solution was pumped with the help of a peristaltic pump through a capillary, lowered to the bottom of the glass. A series of

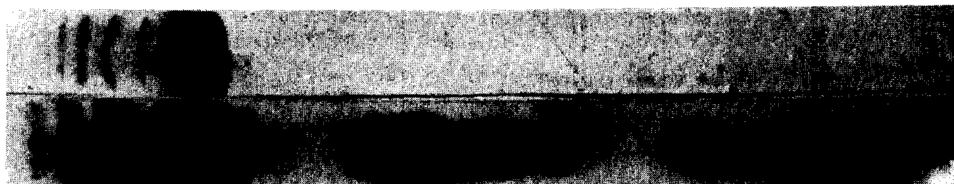

Fig. 7. Isoelectric focusing of serum albumin in polyacrylamide gel: 1, normal albumin; 2, isolated from patients with hepatocirrhosis.

gradient solutions then followed, starting from the lightest one, and which were layered one beneath the other. The filled glass was then placed between two or four fluorescent lamps. Photopolymerization was complete after 14–16 h for the range pH 2–7, after 6–8 h for the range pH 7–12. Then the tubes were taken out of the glass and placed into the apparatus for disc electrophoresis. Solution N1 was used as an electrode buffer solution. The protein sample was introduced into the gel as usual in disc electrophoresis. After focusing (at 200 V for 16–18 h) the gels were taken out and cut into two portions. One portion was stained with 0.1% bromophenol blue or Coomassie-250 in 7% acetic acid for 1 h; the second portion was used for pH measurement by applying surface microelectrodes. Our data on the separation of human serum albumin in a borate–polyol gradient of pH 4.0–7.0, are shown in Fig. 7.

In our work with the above mentioned proteins in the borate–polyol system we did not observe artefact zones.

CONCLUSIONS

(1) Different methods of isoelectric focusing allows conclusions to be drawn about the presence or absence of artefacts.

(2) The chemicals used in the preparation of BP-pH gradients are cheap and hence this method may be used for preparative work.

(3) This method allows the fractionation of low-molecular-weight ampholytes including the carrier ampholine.

(4) Variation of the form and width of the pH gradient possible.

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