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Note

Artifacts in preparative isoelectric focusing: possible complex formation between Ampholines and alanine

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In isoelectric focusing, the formation of complexes between carrier ampholytes and proteins or polyanions has been reported[1–5], and also a lack of interaction between carrier ampholytes and certain proteins[6]. Originally such complexes were believed to have the same isoelectric point as that of the free protein or to be weak salt complexes dissociated after a certain focusing time. During the course of our experiments aimed at purifying thymic growth factors for lymphocytes cultured *in vitro*, preparative isoelectric focusing was used. We found that material which focused at an acidic pH could be identified as alanine. This suggested the possibility that alanine and other neutral amino acids might form complexes with acidic carrier ampholytes, resulting in an irrelevant isoelectric point. Experiments with [^{3}H]alanine were carried out in order to find a possible explanation for the focusing of alanine at an acidic pH.

MATERIALS AND METHODS

[^{3}H]Alanine

L-[2,3-^{3}H]Alanine (specific activity 37 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, Great Britain); 25–125 μCi of [^{3}H]alanine were used as the sample for isoelectric focusing, which corresponds to 0.7–3.4 nmole of alanine.

Urea

Urea (AnalaR grade), obtained from BDH (Poole, Great Britain), was purified by ion-exchange chromatography on Bio-Deminrolit with indicator (Permutit, Middlesex, Great Britain) at room temperature.

Isoelectric focusing

Preparative isoelectric focusing was carried out in a an LKB 8100 110-ml column (LKB, Stockholm, Sweden) according to Haglund[7]. Convection during focusing was counteracted by a stabilizing sucrose gradient. A pH gradient of 2.5–5 was obtained by mixing equal parts of Ampholine of pH 2.5–4 (20%, w/v) and of pH 3.5–5 (40%, w/v) (LKB) in amounts resulting in a final concentration of 1.5%. A pH gradient of 3.5–10 was obtained with Ampholine of pH 3.5–10 (40%, w/v) used at a final concentration of 2%.

The bottom (anode) solution contained 15 g of sucrose, 4 ml of 1 *M* phosphoric acid and water to 25 ml. The dense gradient solution consisted of 27 g of sucrose (final concentration 50%), 1.5 ml of Ampholine and [^{3}H]alanine dissolved in distilled water to 54 ml. The light solution contained 2.7 g of sucrose (final concentration 5%), 4 ml of Ampholine and water to 54 ml. The top (cathode) solution consisted of 2.5 ml of 1 *M* sodiumhydroxide solution and water to 10 ml. Cooling water (8°) was obtained from a refrigerator (Julabo Paratherm FT 1). The potential was adjusted to give a power of 5 W. During the first 20 h the potential was increased from 200 V to about 1100 V. In different experiments with [^{3}H]alanine the focusing time was 26 h, 3 days and 7 days. In one experiment the [^{3}H]alanine in 0.5 ml of 20% sucrose was introduced in the neutral range of the pH gradient 3.5–10 after an initial focusing of Ampholine for 26 h. The column was emptied by the use of a peristaltic pump at a flow-rate of 60 ml/h. Fraction volumes of 3 ml were collected. The pH curve was determined by measurement of the pH in each fraction immediately after emptying the column.

In some experiments 5 or 6 *M* urea was included in the gradient and electrode solutions and the focusing was run for 3 days at 25°. In one experiment with Ampholine of pH 2.5–5, 0.1 *M* saline was included in the gradient and electrode solutions. This experiment was run for 3 days at 8°.

Liquid scintillation

In some experiments 0.2 ml volumes from three consecutive fractions were pooled and mixed with 3 ml of scintillation fluid (Aqualuma; Biotec, Stockholm, Sweden). Otherwise, 0.6 ml from each fraction was mixed with 3 ml of Aqualuma. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Preparative isoelectric focusing of [^{3}H]alanine with Ampholine of pH 2.5–5 resulted in different apparent p*I* values, depending on the focusing time. With a focusing time of 26 h, maximal radioactivity was found at pH 3.60–3.70. At higher pH the radioactivity gradually declined (Fig. 1A). With a focusing time of 72 h maximal radioactivity occurred in fractions at pH 4.20–4.30 (Fig. 1B). These fractions were refocused in a new focusing experiment run for 7 days. After this prolonged focusing time, maximal radioactivity occurred at the upper border of the pH gradient (pH 5.20). Almost no [^{3}H]alanine was found below pH 4.50 (Fig. 1C).

Preparative isoelectric focusing of [^{3}H]alanine was run for 3 days with Ampholine of pH 3.5–10. Maximal radioactivity occurred at pH 4.3–4.9 and 6.6 (Fig. 2A). Even with a focusing time of 7 days a main radioactivity peak occurred at pH 4.5–4.75 (Fig. 2B).

In a subsequent experiment, Ampholine of pH 3.5–10 was pre-focused for 24 h, then [^{3}H]alanine in a volume of 0.5 ml was introduced in the middle of the column corresponding to a pH of 6–7. The focusing was continued for 6 days. In this experiment the radioactivity had focused around the correct isoelectric point for alanine, pH 6.0, and no radioactivity peak appeared at lower pH (Fig. 2C).

In order to test whether the focusing of alanine at an acidic pH was due

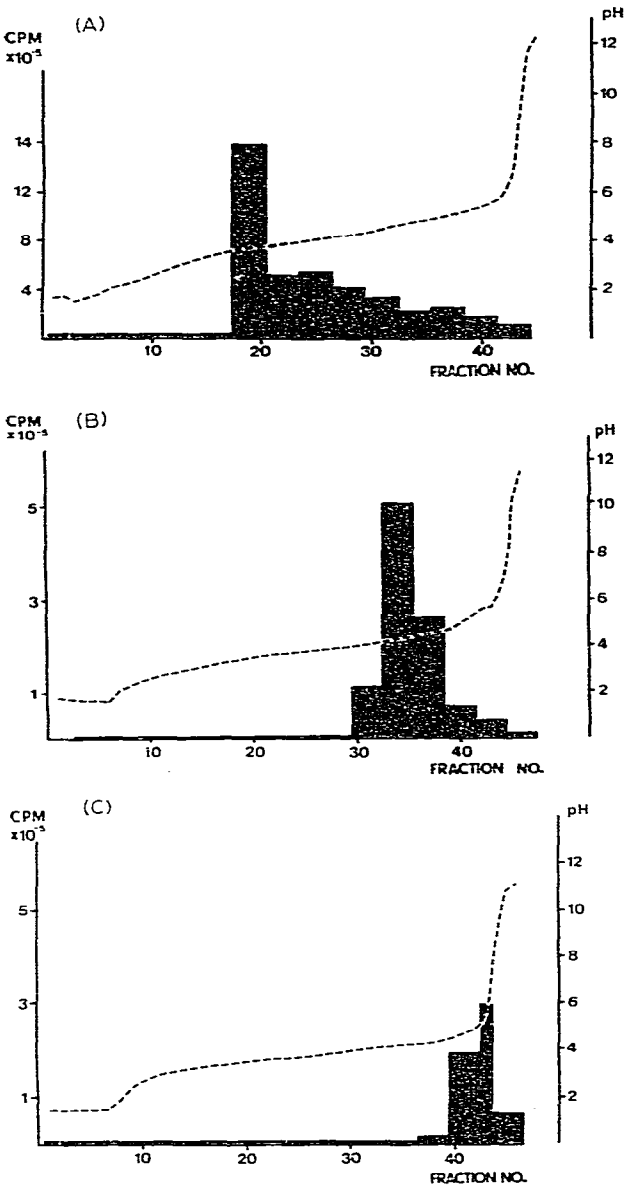

Fig. 1. Preparative isoelectric focusing of [^{3}H]alanine with Ampholine of pH 2.5–5 as carrier ampholyte. Focusing time: (A) 26 h; (B) 72 h; (C) 168 h. In (C) the sample consisted of the radioactive peak from the experiment in (B).

to complex formation between alanine and acidic carrier ampholytes, experiments with urea were performed. In an experiment run for 3 days at 25° with Ampholine of pH 2.5–5 and 6 *M* urea, [^{3}H]alanine appeared in fractions above pH 4.8 without any distinct peak. With Ampholine of pH 3.5–10 and 5 *M* urea, [^{3}H]alanine focused between pH 6.7 and 7.5 with a peak at pH 6.85 (Fig. 3). It has been shown that the presence of urea increases the pH by about 0.4 unit[8]. The pH values observed should

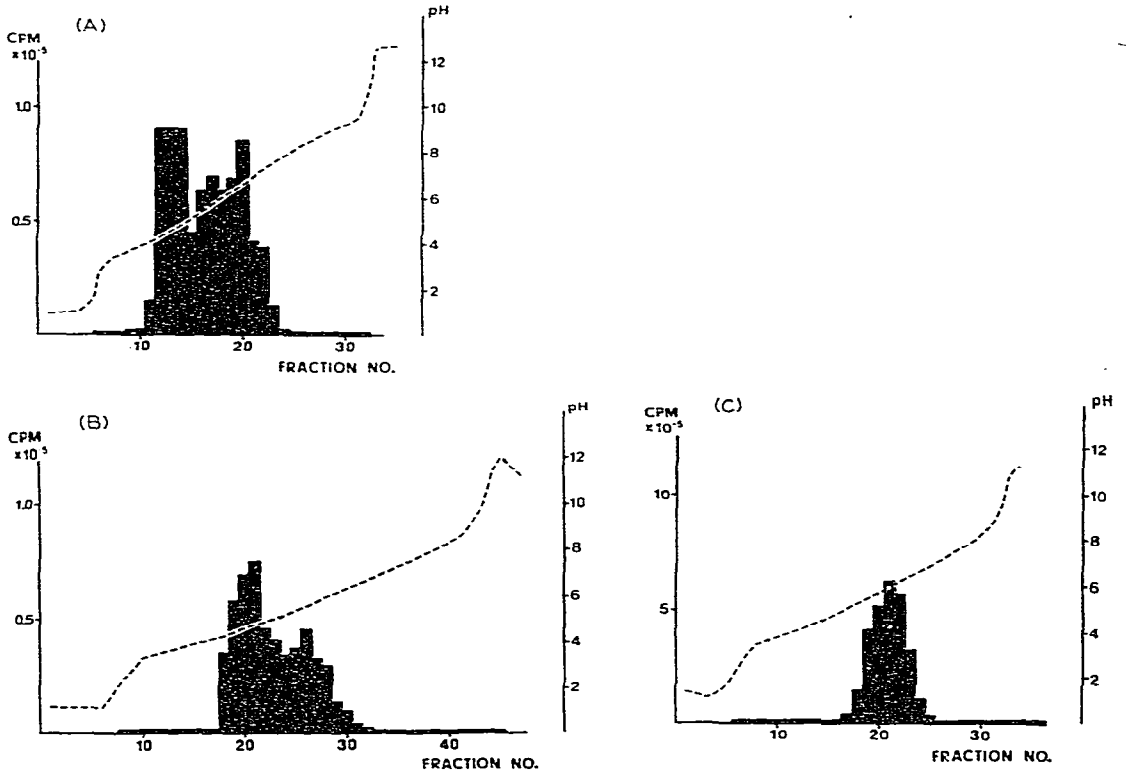

Fig. 2. Preparative isoelectric focusing of [^{3}H]alanine with Ampholine of pH 3.5–10 as carrier ampholyte. Focusing time: (A) 72 h; (B) 168 h. In (C) a pH gradient was pre-formed by focusing of Ampholine for 26 h. Then [^{3}H]alanine in 0.5 ml of 20% sucrose was introduced in the middle of the column, corresponding to pH 6–7 in the gradient. The focusing was continued for 144 h.

therefore be corrected for this effect. Thus, in this experiment alanine focused close to its correct isoelectric point.

In an effort to overcome possible conductivity gaps[9] during focusing of [^{3}H]-alanine, a salt experiment was performed with Ampholine of pH 2.5–5 and 0.1 *M*

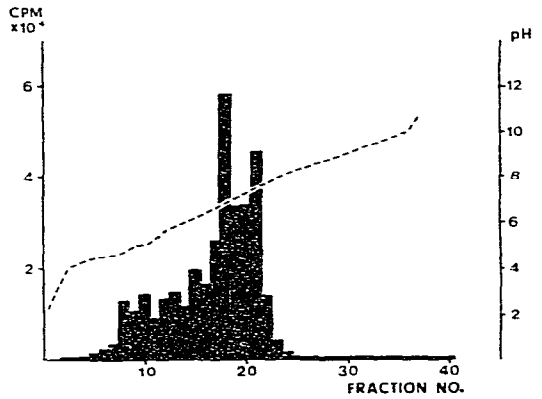

Fig. 3. Preparative isoelectric focusing of [^{3}H]alanine with Ampholine of pH 3.5–10 as carrier ampholyte. The gradient and electrode solutions contained 5 *M* urea. Focusing time: 72 h.

sodium chloride solution. In this experiment and a similar experiment with 0.05 *M* sodium chloride solution radioactivity was found above pH 4.2 without any distinct peak (Fig. 4).

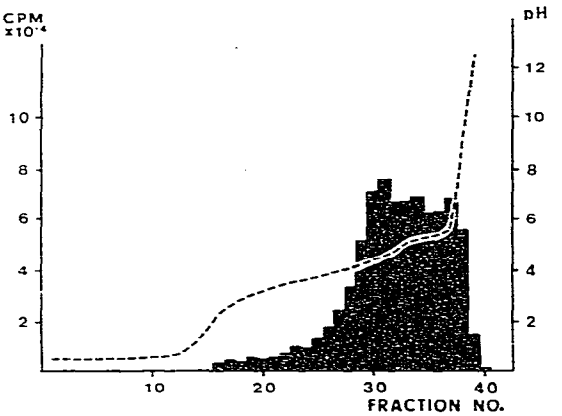

Fig. 4. Preparative isoelectric focusing of [^{3}H]alanine with Ampholine of pH 2.5–5 as carrier ampholyte. The gradient and electrode solutions contained 0.1 *M* NaCl. Focusing time: 72 h.

DISCUSSION

Rilbe[10] and Vesterberg[11] have pointed out that focusing of low-molecular-weight ampholytes is strongly dependent on their titration curves. Only ampholytes isoelectric between two closely spaced pH values are distinctly focused. Thus, alanine would be expected to focus rather diffusely. When trace amounts of [^{3}H]alanine were used for preparative isoelectric focusing, different apparent isoelectric points were found with different Ampholines and different focusing times. Thus, the results could not be explained simply by diffuse focusing.

With Ampholine of pH 2.5–5, the [^{3}H]alanine focused at an acidic pH.When the focusing time was prolonged to 3 and 7 days, the alanine was successively displaced towards the upper border of the pH gradient. Even with Ampholine of pH 3.5–10, with a pH of 7.4 in the gradient solutions, most of the [^{3}H]alanine was found in the acidic part of the gradient. Prolongation of the focusing time did not result in an accumulation of alanine closer to its isoelectric point. Only when [^{3}H]-alanine was introduced in the neutral range of a pre-formed pH gradient of 3.5–10 was focusing at the correct isoelectric point obtained.

Our results may be explained by the existence of conductivity gaps in the gradient formed by the acidic carrier ampholytes, causing a stacking of alanine at the gaps. Such conductivity discontinuities have been reported for acidic Ampholines[9]. Righetti and Chrambach[12] investigated the erroneous focusing of poor carrier amino acids in analytical isoelectric focusing in gels. They reported that 0.1 *M* potassium chloride solution included in the gradient and electrode solutions facilitated the focusing of the amino acids at their correct p*I* values, possibly by bridging conductivity gaps in the gradient. However, our salt experiments did not result in a distinct focusing of alanine.

Alternatively, our findings may depend on the formation of complexes between

alanine and acidic carrier ampholytes. Our urea experiment with a changed distribution of [^{3}H]alanine towards its correct isoelectric point favoured the possibility of complex formation. In previous studies, binding between positively charged Ampholines and acidic dyes[4] or polyanions[5] has been reported, and also complex formation between certain proteins such as serum albumin[3] and Ampholines. Binding seems to occur preferentially when there are multiple binding sites on carrier ampholytes and on the molecules supposed to bind. Although alanine is a small molecule without multiple binding sites, the present findings indicate complex formation.

Irrespective of the cause of the erroneous focusing of amino acids, our results emphasise that findings in preparative isoelectric focusing must be interpreted critically. Experiments must be run under different conditions, including efforts favouring dissociation of complexes and bridging possible conductivity gaps in the gradient of carrier ampholytes, before conclusions of isoelectric points are drawn.

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