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Note

Distribution of carrier ampholytes in isoelectric focusing

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In 1961, Svensson[1] solved the differential equation for solute concentrations at the steady state and concluded that the concentration distribution of an isoelectrically focused protein in a linear pH gradient of constant conductivity is Gaussian. In 1971, Almgren[2], studying the same problem, concluded that "in a region of an electrolysis column where the pH and conductivity courses are linear and constant, respectively, the concentration distribution of an isoelectrically focused mono-monovalent ampholyte is Gaussian or nearly so". After the synthesis, by Vesterberg[3], of carrier ampholytes, present-day isoelectric focusing (IEF) became a reality. So far, however, it has not been possible to determine either the number of species of carrier ampholytes present in the synthetic mixture or their spatial distribution during IEF. In a recent investigation Brown *et al.*[4] concluded that (1) there are only 62 amphoteric species in the "wide range" Ampholine, (2) upon focusing, each ampholyte component is distributed over a wide area (7–15% of the column length, typically above 10%), and (3) upon focusing, the concentration distribution of carrier ampholytes may be non-Gaussian.

In this paper an attempt is made to gather the information available on the subject, as it is very important for the theory and developments of IEF. The above three conclusions of Brown *et al.* are considered individually below.

(1) Total number of carrier ampholytes. The only conclusion that can be drawn from the experiments of Brown *et al.*[4] is that their ion-exchange column is able to resolve only 62 components. According to Almgren[2], in order to obtain a resolution of 0.02 pH unit in IEF, the system must contain at least 20 different ampholytes per pH unit, which means a minimum of 180 ampholytes species in the "wide pH range" (pH 2–11). According to Vesterberg[5], carrier ampholytes are synthesized from mixtures of polyamines (containing 2–9 nitrogen atoms in the chain) under conditions that lead to a mixture of homologues containing one up to nine carboxyl groups. Thus, "more than 360 isomers and homologues are obtainable. The number of isomers and homologues can be still more increased by adding some methyl or ethyl groups on the amino groups". Recently, Radola *et al.*[6] detected carrier ampholytes, focused in a Sephadex G-75 bed, by means of the paper print technique with formaldehyde, lactose or ninhydrin. In two pH unit ranges, about 150 species were detected by these reactions. In narrow cuts of only 0.5 pH unit, 15–45 peaks were resolved by ion-exchange chromatography. On the basis of these data, more than 500 ampholyte species should be present in the "wide pH range", as opposed to 62.

(2) Wide distribution of carrier ampholytes upon IEF. We were puzzled when Brown *et al.*[4] (broad distribution) and then Radola *et al.*[6] (very sharp zones) presented their data on ampholyte distribution upon IEF at the Hamburg Congress. In the ensuing discussion, Rilbe suggested that Brown *et al.*'s technique was detecting poor carrier ampholytes (focusing broadly) while Radola *et al.*'s technique was revealing good ampholytes (focusing sharply). Catsimpoolas suggested that Brown *et al.* were detecting low-molecular-weight carrier ampholytes (having a high diffusion coefficient, thus focusing broadly) while Radola *et al.* were detecting high-molecular-weight ampholytes (focusing sharply). A third, much simpler, explanation could be that Brown *et al.*'s technique was detecting perhaps only 10–15% of the actual number of amphoteric species.

In fact, on the basis of the detection of focused ampholytes by refractive index gradients, Rilbe[7] has demonstrated an array of sharp zones (distributed over 1–2% of the column length in the basic and acidic regions and over 2–4% in the neutral region where fewer and perhaps "poor" carrier ampholytes are present; see Fig. 3 in ref. 7). Similar results have been obtained by Righetti *et al.*[8] using a similar detection technique in polyacrylamide gels. Catsimpoolas[9], by focusing in sucrose density gradients, and by scanning the tubes *in situ* under voltage, has demonstrated very sharp zones of chromophoric amino acid derivatives (typically distributed over 2–4% of the column length).

The question in fact remains open: how could broad Ampholine zones be compatible with the very sharp sample zones usually obtained in IEF, not only in the "wide pH range", but also in the narrow cuts of 2 pH units? One might argue that this is usually obtained with proteins, which have low diffusion coefficients and often also in polyacrylamide gels, which further restrict diffusion. The experiments described in refs. 7–9 were performed mostly with low-molecular-weight substances and in a liquid support. Earlier experiments[10], published in 1968, also showed that low-molecular weight plant pigments (anthocyanins) in sucrose density gradients focus sharply. In Fig. 6c in ref. 10 there is an incredibly sharp array of red, brown, violet and green pigments from bilberry sap. There, most of the bands have a spatial distribution of less than 1% of the column length. In addition, we have recently revealed Ampholine distribution patterns by focusing dyes on a pre-focused gel slab[11]. The dye gives a highly complex series of zones, each representing a specific Ampholine–dye complex. Their distributions and their widths are very similar to those reported by Johnsson and Pettersson[10].

(3) Non-Gaussian distribution of carrier ampholytes. This is a very important point, which might have far-reaching implications for the theory of IEF and be one of the causes of the cathodic drift[12,13]. Brown *et al.*[4] have demonstrated a skew distribution of TETA ampholytes upon focusing. In view of the fact that TETA ampholytes are very poor carrier ampholytes[8], presenting several conductivity gaps, one would expect asymmetric peaks. Svensson[1] has, in fact, demonstrated a skew concentration distribution for ampholytes when the conductance is not constant. As commercial ampholytes (Ampholine, Servalyt and Biolyte) also exhibit a marked conductivity minimum[14] centred at pH 6.2, it is possible that all ampholytes focusing around neutrality have a skew distribution profile, which might lay at the heart of the plateau phenomenon[13].

However, in focusing histidine (His), Brown *et al.*[4] have also demonstrated

another type of distribution profile, namely a plateau (or truncated peak or square-wave). As this distribution is typical of steady-state stacking (isotachophoresis) (ITP), Brown *et al.* suggest a similarity between IEF and ITP, as previously pointed out by Nguyen and Chrambach[15]. It is considered here, however, that square-wave distributions, with sharp boundaries, typical of ITP, are not compatible with IEF. According to the law of pH monotony[16], there has to be a continuous interdigitation among the ampholyte peaks in IEF, a condition incompatible with ITP. The apparent square-wave distribution of His (Fig. 6 in ref. 4) is quite puzzling. This "plateau" is distributed over 40% of the column length. Thus, one would expect the pH (p*I* 7.47) to remain constant over the same length. This has never been verified experimentally in IEF over the wide pH (3–10) range. Alternatively, if we assume that the pH proceeds linearly, as usual, then one would be faced with the finding of His being isoelectric over a range of *ca.* 3 pH units. This also cannot hold true, as His is a "good" carrier ampholyte ($pI-pK_1 = 1.50$)[17]. Thus, it is considered that the His distribution profile is a "non-focusing" experiment and cannot be used to postulate any similarity between IEF and ITP, and other explanations must be sought.

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