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Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients

IX. A Method for Obtaining pH Gradients in the Region below pH 3, Stable Enough to Permit Isoelectric Focusing of Ampholytes

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Natural pH gradients in the region between 1 and 3, for which commercial carrier ampholytes are not available, have been created by electrolysis of a system of acids and a couple of acidic, commercially available ampholytes. The high electric current required in this pH region in order to get a sufficiently strong electric field has been attained by using a layer of constant pH and density, within which free convection is allowed, as a catholyte. In this solution, a well-conducting basic ampholyte (histidine) is present at an appreciable concentration.

As has been shown by Svensson,[1–3] a stable pH gradient can be produced by stationary electrolysis of a mixture of so-called carrier ampholytes, which are defined as ampholytes of low molecular weight possessing a considerable buffering capacity and conductivity in the isoelectric state. The carrier ampholytes, a system of aliphatic, saturated poly-amino-poly-carboxylic acids, are now commercially available through LKB-Produkter AB, Bromma, Sweden, under the trade name "Ampholine". Vesterberg [4] and others have shown that they are very useful for isoelectric analysis of proteins within the pH range between 3 and 10.

The problem of creating stable pH gradients in the region below 3 arose in connection with isoelectric focusing of the pigments of red beet. When using pH gradients obtained with Ampholine carrier ampholytes, many coloured components were found to migrate to the anode, where a pH of about 1 generally prevails, but the pH change from 3 to 1 occurred over a very small part of the column (steep pH course). Since the resolving power increases as the pH gradient diminishes (Vesterberg and Svensson [5]), it proved to be an important task to design electrolysis experiments capable of giving extended regions in the column with low and slowly changing pH values.

PRINCIPLE

Carrier ampholytes isoelectric below 3 are extremely scarce in commercial catalogues and other possibilities have therefore been examined. It is evidently possible to build up an artificial pH gradient by stratification of layers of rather concentrated solutions of acids in the order of decreasing strength and by allowing these layers to interdiffuse. The principle in the field of isoelectric focusing has, however, been the use of natural pH gradients created by the electric current itself, since only such gradients are perfectly stable in the course of time.

The problem of the concentration distributions of acids of various strengths that is to be expected in the steady state after a sufficiently long convection-free electrolysis is very complicated. Following general features can, however, be anticipated. The strongest acid, the ionization of which is largely uninfluenced by the others, will collect in a restricted layer close to the anode, and its concentration course will be expected to decline in the direction of the current. The next strongest acid, the ionization of which is extensively depressed in the layer occupied by the strongest one, will thus show a very slow anodic migration in this layer. Consequently, by virtue of the differential equation for the steady state (Svensson [1]), the concentration gradient must be small, that is, the concentration must be almost constant in this layer. Outside the layer occupied by the strongest acid, the next strongest one takes over the role of the strongest one relative to the weaker acids, its ionization becomes appreciable, its anodic migration increases and, as a consequence thereof, its concentration declines rather rapidly to zero. The same qualitative reasoning regarding the third strongest, the fourth strongest, *etc.*, acid reveals that each acid in the steady state will have essentially constant concentrations from the anode to the zone where the acid is the strongest one and within which its concentration declines to zero. Each acid is essentially active in creating and defining the pH gradient only within the zone where it is the strongest acid present. The quantities closer to the anode are ionized to a very little extent and merely play the role of components of a mixed solvent. These quantities are, however, necessary for the differential equation of the steady state to be satisfied. The conclusion to be drawn is that a steady state concentration distribution requires the larger amounts, the weaker the acid.

The time required for weak acids to reach their steady state concentration courses can be expected to be much longer than for ampholytes. This is due to the exceedingly slow electric migration of the almost uncharged acids in the anodic region. The attainment of the final steady state thereby becomes almost exclusively diffusion-controlled. This may be suspected to require electrolysis times measurable in weeks. However, electrolysis times longer than a few days cannot be considered practical. Fortunately, however, it is not necessary to have a real steady state. A quasi-equilibrium changing so slowly that ampholytes have enough time for focusing is good enough for practical purposes. The concentration distribution of every acid, then, would be more like the distribution obtained by stratifying the acids in the order of decreasing strength. If the acids are introduced in the column simultaneously with the density gradient, such a distribution would be favoured by placing

the strongest acids in the dense and the weakest ones in the less dense solution. This is the method chosen in this work.

Between the weakest acid, migrating towards the anode, and the base added at the cathode and attracted by it, a portion depleted of electrolytes will develop. This means that the current will be quenched untill uncontrolled convection starts. A method of keeping the electric current at a level high enough to get a sufficiently strong electric field is the following. The density gradient is limited to 95 ml (normally 110 ml) and starts with a density corresponding to 30 g sucrose/l. On top of the density gradient a solution of histidine (pI 7.47) is placed. The histidine migrates towards the cathode, concentrates there and falls downwards due to gravity. Due to this circulation of histidine a good conductance is maintained in this part of the column.

EXPERIMENTAL

Material and methods. The electrolysis runs were performed essentially as described by Vesterberg and Svensson.[5] For creating the density gradients we used, however, a special device, giving a constant density gradient.[6] The strongest acids were always added to the dense solution (the anode was always at the bottom of the column). This implied that a pH gradient automatically was formed simultaneously with the density gradient on filling the column. The ampholytes used were glutamic acid (pI 3.22) and aspartic acid (pI 2.98). The acids used were dichloroacetic acid (pK 1.48), phosphoric acid (pK_1 2.12), monochloroacetic acid (pK 2.85), citric acid (pK_1 3.08), formic acid (pK 3.75), and acetic acid (pK 4.75). Normally, 0.10 g of each one of the ampholytes and 0.20 g of each one of the acids were used. pH was measured continuously at +25°C by using a flow cell, Beckman 97633 capillary pH electrode assembly, connected to a Beckman Expandomatic pH meter. The pH course was recorded on a Beckman ten-inch linear, potentiometric recorder (Jonsson *et al.*[7]).

Experiments. In order to examine the formation of electrolyte-free regions, parallel experiments were performed with two columns in which the dense and the less dense solutions, respectively, contained the same amounts of electrolytes. In the upper part of one column, just below the cathode, a solution of 0.25 g histidine in 15 ml water was introduced on top of a "shelf" in the density course with a density corresponding to 30 g sucrose/l, whereas in the other column the density gradient extended all the way to the cathode. The columns were connected in parallel to the same power supply. The current was during the whole run larger in the column with the gradient-free histidine region. Moreover, the difference between the currents in the two columns increased at the higher electric loads. At the end of the run (normally after 4–5 days with a final voltage of 350–450 V) the current in the column without histidine was only 40 % of that in the column with histidine.

The sharp boundary which from the start existed between the density gradient and the histidine region moved downwards during the run, that is, an increasing region of constant pH and density was formed due to convection. In the column without histidine there was soon formed a boundary, too, above which there was constant pH and density due to convection. This boundary also moved downwards. Experiments which were performed with the same electric load over both columns showed that the region of constant pH and density increased considerably more rapidly when no histidine was present.

Fig. 1 shows the pH gradient from an experiment in which acetic acid, formic acid, and citric acid were placed in the less dense solution together with glutamic acid and aspartic acid, whereas monochloroacetic acid, phosphoric acid, and dichloroacetic acid were placed in the dense solution. The initial pH gradient is also illustrated.

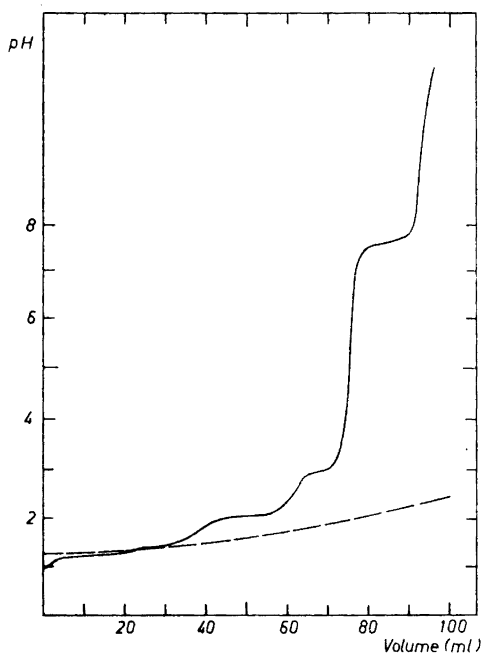

Fig. 1. (– – –) pH gradient obtained when filling the column, due to the distribution of the acids between the dense and less dense solutions (Less dense: Aspartic acid, glutamic acid, acetic acid, formic acid, and citric acid. Dense: Monochloroacetic acid, phosphoric acid, and dichloroacetic acid).
(———) The pH gradient at the end of the run (Electrolysis time 96 h).

DISCUSSION

The pH gradient from 1 to 2.1 occupies about 60 ml, that is, 55 % of the column volume. In this region there is a rather shallow pH course, but a steep pH course remains between 2.1 and 2.9. Attempts to achieve a levelling in this region have not been successful. The amount of acetic acid has, for instance, been increased considerably without any levelling effect. The plateau at about pH 7.5 is the region of constant pH and density, and pH in this region is close to the isoelectric point of histidine. The plateau at about pH 2.9 originates from aspartic acid. Still another plateau, at about 3.2, originating from glutamic acid, is usually present, but is missing in Fig. 1. By introducing sulphuric acid one can extend the pH gradient down to pH 0.

Recently, the isoelectric point of pepsin has been determined in a pH gradient of this type. Earlier, plant pigments were successfully focused in acid pH gradients.[8] In bilberry sap two red components were found, isoelectric at pH 1.4 and 2.3. In red beet sap as many as seven couloured components, isoelectric between pH 1.3 and 3.1, were separated.

The focusing is, however, slow in the most acid parts due to the very high conductance. This can be serious at such low pH values. The most acid components in the red beet sap, for instance, grew pale during the run. It is also unfavourable to have the anode at the bottom of the column in the high sucrose concentration. The sucrose hydrolyzes under these conditions and gives rise to products which are strongly UV absorbing [9] (anodic oxidation

of fructose). In some of the experiments with pepsin sorbitol was used instead of sucrose for the density gradient. Even then UV absorbing products were formed, though not as much as with sucrose. Still lower UV absorption has been obtained by using glycerol for the density gradient.

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