Journal of Chromatography, 159 (1978) 193–205
Chromatographic Reviews
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHREV. 110T13

STEADY-STATE RHEOELECTROLYSIS

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1. INTRODUCTION

Isoelectric focusing in its modern form dates back to 1961[1], but it did not come into general use until Vesterberg[2] succeeded in synthesizing a useful system of carrier ampholytes and LKB started to supply commercially both columns and ampholytes, from then on called Ampholine[3]. The success of this method has been remarkable, and together with isotachophoresis the method has been the subject of four international symposia[4–7]. However, there are reasons for seeking improvements.

One reason is the expense of Ampholine, which certainly limits the use of isoelectric focusing, especially in preparative work in which large amounts are required. Another reason is the poor solubility of globulins in the isoelectric state in the absence of salts. Isoelectric focusing at an ionic strength much higher than that of focused Ampholine would certainly be able to keep many globulins in solution. A third reason is the insufficient knowledge of the milieu in which each individual protein is brought to rest in its isoelectric state. It is unsatisfactory that the protein in this state is surrounded by an unknown number of ampholytes, each with an unknown structure, conformation, molecular weight and concentration. It is especially unsatisfactory that the conductivity, and hence the field strength, generally assumes a very complicated course from anode to cathode, with several peaks and valleys. Conductivity courses have been recorded by Davies[8], but it should be borne in mind that the conductivity course during focusing is still more complicated as about half an hour of free diffusion during liquid transfer from column to conductolyser preceded Davies's records.

Catsimpoolas and co-workers, who have made considerable contributions to the development of isoelectric focusing, had a special reason to be concerned about the unpredictable conductivity course in isoelectric focusing and about its rapid changes after breaking the current. In a series of papers[9–12] they correctly pointed out that isoelectric focusing has possibilities in the measurement of diffusion coefficients and mobility slopes of focused proteins as these quantities dictate the zone width according to an equation given by Svensson (= Rilbe)[1] and that the rate of free diffusion can be measured by breaking the current. The great difficulties encountered in the interpretation of measurements in transient-state isoelectric focusing (TRANSIF) is intimately connected with the complicated behaviour of the conductivity in focusing, defocusing and refocusing. In a recent paper, Catsimpoolas[13] called for the synthesis of "second generation ampholytes", which hopefully will give not only a stable pH gradient, but also a uniform conductance and concentration distribution course throughout the separation path.

To summarize, there are reasons for requiring electrolytes and buffer systems that allow isoelectric focusing at higher ionic concentrations, that give rise to predictable and more easily controlled conductivity courses and that are cheaper than presently available carrier ampholytes.

Attempts to create stable pH gradients without the use of carrier ampholytes have already been reported. In 1970, Luner and Kolin[14] suggested the use of a temperature gradient as a means of obtaining a stable pH gradient. This method can be used only for very narrow pH regions as a pH span of 1 unit requires a temperature range of about 50 °K. It has not been worked out adequately and has not been taken up by other workers.

Another possibility for obtaining stable pH gradients in ordinary buffer solutions has been suggested by Troitsky *et al.*[15]. They pointed out that the influence of a reduced dielectric constant on the p*K* of a protolytic group is much less for proteins than for small ions as the sum of the ionic radii appears in the denominator of the appropriate equation. Thus, a concentration gradient of a non-electrolyte with a low dielectric constant in an aqueous buffer solution of constant composition must give rise to a stable pH gradient in which proteins can focus isoelectrically as their p*I* values are not much influenced by the varying dielectric constant. A pH range of 0.8–1.0 unit could be obtained with a glycerol gradient, and Troitsky *et al.* demonstrated focusing of haemoglobin and serum albumin in such gradients. This principle appears promising, but it has not been taken up by other workers.

Both the thermal and the dielectric pH gradients require large buffer reservoirs between the electrodes and the separation zone, otherwise acid and alkali will invade the latter and destroy the useful pH gradient. One important advantage of isoelectric focusing with Ampholine has thus been lost, namely the simplicity of an apparatus without electrode vessels.

Even before Vesterberg's[2] synthesis of Ampholine, we started to consider the possibility of creating stable pH gradients by steady-state electrolysis of ordinary buffer solutions in conjunction with superimposed liquid flows between various parts of the electrolysis cell. Such methods will subsequently be called steady-state rheoelectrolysis, in conformity with the Macheboeuf *et al.* term[16,17] "électrorhéophorèse" for paper electrophoresis with superimposed liquid flows. This method also involved a focusing effect giving considerable zone sharpening as the flow of water, which re-

placed evaporated water, increased towards the ends of the paper strips and completely balanced the electric migration.

In steady-state rheoelectrolysis, continuous exchange of liquid between the electrode compartments is especially simple to handle theoretically and will therefore be assumed to take place for the purposes of this paper, irrespective of the possibility that experimental practice may necessitate other arrangements.

2. PRINCIPLE OF OPERATION AND POSTULATES

The principle of rheoelectrolysis is shown schematically in Fig. 1. The electrolyser consists of one convection-free part (7) between the electrode compartments (2) and (5) with the electrodes (1) and (4). The anode (1) has to be made of platinum or carbon. The electrode compartments are constantly homogenized by the stirrers (3) and (6). The pumping device (8) produces a flow of anolyte through the ducts (9) and (10) to the cathode compartment, and a flow of catholyte through the ducts (11) and (12) to the anode compartment. Constant electric and hydrodynamic flows are allowed to continue until a steady state is reached.

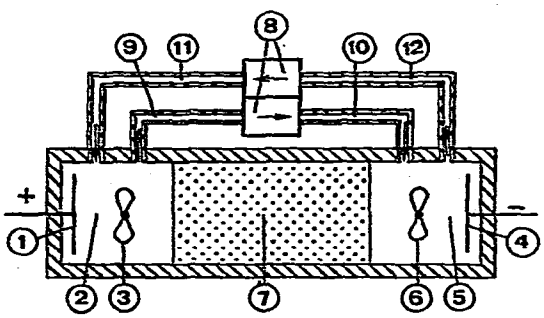

Fig. 1. Principle of steady-state rheoelectrolysis with liquid exchange between anolyte (2) and catholyte (5). The separation takes place in the convection-free portion (7), whereas anolyte and catholyte are constantly homogenized by the stirrers (3) and (6). The double pump (8) transfers equal volumes of liquid in both directions, and in the separation zone there should be no liquid flow.

By electrolysis without superimposed liquid flows, acids are drawn to the anode and bases to the cathode. Somewhere in the central part of the apparatus a complete evacuation of salts occurs and, if ampholytes are absent, a sharp change in pH from very low to very high values will be found at this point. With superimposed liquid flows as indicated in Fig. 1, acid will be transferred to the catholyte and base to the anolyte. If an ordinary buffer solution is being electrolysed, it is obviously possible in this way to retain pH values within the buffer range of the weak protolyte in both electrode vessels and thus to realize a smooth pH gradient within the same range in the convection-free part (7) of the electrolyser. It will be postulated that all electrolytes involved are resistant to anodic oxidation and cathodic reduction and that they suffer no losses due to evaporation or precipitation.

3. STEADY-STATE RHEOELECTROLYSIS WITHOUT INTERNAL LIQUID FLOW

If there is no internal liquid flow, the flow from the anode to the cathode is identical with that in the other direction. In the steady state, the liquid flows through

the pumps in Fig. 1 must balance exactly the added mass transports due to electric migration and diffusion for each ion constituent. This condition is expressed by the differential equation

$$\frac{Ti}{Fz} - Dq \cdot \frac{dC}{dx} = V(C_- - C_+) \tag{1}$$

where the first term on the left-hand side represents the electrical mass transport, the second term is the diffusional mass transport and the term on the right-hand side is the mass transport through the pumps. In eqn. 1, T is the transport number, C the concentration, D the diffusion coefficient, z the valence of the ion constituent in question, i the electric current, F the Faraday constant, q the cross-sectional area, x the coordinate from anode to cathode and V the volume flow-rate through the pumps.

This equation can be easily solved only if T and D are independent of x. As it is possible to choose experimental conditions that are likely to realize constant transport numbers and diffusion coefficients, this solution to the differential equation is of great interest. With constant T and D, dC/dx becomes constant and thus C is a linear function of x. The solution can be written as

$$C(x) - \bar{C} = \frac{Tix}{Fz\,Dq} - \frac{Vx(C_- - C_+)}{Dq} \tag{2}$$

where $\bar{C}$ is the mean concentration prevailing in the centre of the apparatus, where $x = 0$. The concentrations C_+ and C_- in anolyte and catholyte, respectively, are so far unknown, but can be eliminated by putting $x = a/2$, $C(x) = C_-$ and $x = -a/2$, $C(x) = C_+$, and taking the difference. The resulting equation can be solved for the concentration difference between catholyte and anolyte, which gives

$$C_- - C_+ = \frac{Tia}{Fz(Va + Dq)} \tag{3}$$

Insertion of eqn. 3 into eqn. 2 gives the final solution:

$$C(x) - \bar{C} = \frac{Tix}{Fz(Va + Dq)} \tag{4}$$

This equation has to be satisfied by every ion constituent present, but its validity is restricted to ion constituents that have constant transport numbers and diffusion coefficients throughout the electrolyser. In the following discussion, the treatment will be limited to buffers with only two ion constituents, both monovalent. Denoting the cation constituent by the subscript 1 and the anion constituent by the subscript 2, we thus obtain the two concentration courses:

$$C_1(x) = \bar{C}_1 + \frac{T_1ix}{F(Va + D_1q)} \tag{5}$$

and

$$C_2(x) = p\bar{C}_1 - \frac{T_2ix}{F(Va + D_2q)} \tag{6}$$

where $p\bar{C}_1$ replaces $\bar{C}_2$ and the dimensionless parameter p defines the composition of the original buffer solution:

$$p = \frac{\bar{C}_2}{\bar{C}_1} \tag{7}$$

3.1. Rheoelectrolysis of a buffer composed of a weak acid and its salt with a strong base

Constant transport numbers and diffusion coefficients cannot be expected unless both buffer components (salt and excess weak acid) are present everywhere in the apparatus. Consequently, we require $C_1(x)$ to be positive everywhere, even at the anode, and $C_2(x)$ to be larger than $C_1(x)$ everywhere, even at the cathode. These two conditions lead to the inequalities

$$2F\bar{C}_1 (Va + D_1q) > T_1ia \tag{8}$$

and

$$2F(p-1)\bar{C}_1 > i\left(\frac{T_1a}{Va + D_1q} + \frac{T_2a}{Va + D_2q}\right) \tag{9}$$

Both conditions have to be satisfied, and consequently the most restrictive one should be chosen. However, this depends on the starting conditions, and in order to avoid the mathematical inconvenience connected with subdivision of the treatment into two parallel sections, it is worth investigating the sort of starting conditions that make the two inequalities 8 and 9 identical. This can be done by solving the corresponding equations for i and putting the resulting expressions equal. This leads to the equation

$$p = 2 + \frac{T_2 (Va + D_1q)}{T_1 (Va + D_2q)} \tag{10}$$

With a knowledge of the two transport numbers and diffusion coefficients, it is possible to choose an excess of weak acid satisfying this equation. By doing this, rheoelectrolysis has the simplest possible mathematics, as will be shown below.

With the aid of eqn. 10, it is possible to eliminate $Va + D_2q$ in favour of $Va + D_1q$ in eqn. 6, which allows the deduction of the following simple expression for the excess of weak acid:

$$C_2(x) - C_1(x) = (p-1)\left[\bar{C}_1 - \frac{T_1ix}{F(Va + D_1q)}\right] \tag{11}$$

Finally, if the inequality 8 is replaced with the equation

$$T_1ia = 2kF\bar{C}_1 (Va + D_1q) \tag{12}$$

where k is a dimensionless parameter smaller than unity, this equation can be used for elimination of current, transport numbers and diffusion coefficients. Eqns. 5 and 11 then take the forms

$$C_1(x) = \bar{C}_1 (1 + 2kx/a) \tag{13}$$

and

$$C_2(x) - C_1(x) = (p-1)\bar{C}_1 (1 - 2kx/a) \tag{14}$$

These equations are very convenient for deduction of the pH course through the Henderson equation:

$$\mathrm{pH} = \mathrm{p}K - \log\left[\frac{C_2(x) - C_1(x)}{C_1(x)}\right] \tag{15}$$

Insertion of eqns. 13 and 14 gives

$$\mathrm{pH} = \mathrm{p}K - \log(p - 1) - \log\left(\frac{1 - 2kx/a}{1 + 2kx/a}\right) \tag{16}$$

By putting in succession $x = a/2$ and $x = -a/2$, one obtains the total pH range from anode to cathode:

$$\Delta\mathrm{pH} = 2\log\left(\frac{1 + k}{1 - k}\right) \tag{17}$$

Probably the value of k should not exceed 0.9 because a safety margin below the critical value of unity is advisable. One therefore concludes that it is possible to cover a pH range of about 2.6 units by rheoelectrolysis of a simple buffer solution. The minimal pH gradient is found in the centre of the apparatus, where it has the value

$$\frac{\mathrm{d}\,(\mathrm{pH})}{\mathrm{d}x} = 4\,(k/a)\log \mathrm{e} \tag{18}$$

The pH course given by eqn. 16 is illustrated in Fig. 2 for some different k values.

3.2. Rheoelectrolysis of a buffer composed of a weak base and its salt with a strong acid

This case is the converse of that just treated, and the requirements to be formulated are that $C_2(x)$ be positive for $x = a/2$ and that $C_1(x) - C_2(x)$ be positive for $x = -a/2$. These conditions lead to the inequalities

$$2pF\bar{C}_1\,(Va + D_2q) > T_2ia \tag{19}$$

and

$$2\,(1 - p)\,F\bar{C}_1 > ia\left(\frac{T_1}{Va + D_1q} + \frac{T_2}{Va + D_2q}\right) \tag{20}$$

Both of these conditions have to be satisfied, and therefore the simplest mathematics are obtained if they are identical. This occurs for

$$p^{-1} = 2 + \frac{T_1\,(Va + D_2q)}{T_2\,(Va + D_1q)} \tag{21}$$

which is to be compared with eqn. 10. Eqn. 21 can be used for elimination of $Va + D_1q$ in favour of $Va + D_2q$ in eqn. 5, which allows deduction of the following expression for the excess of weak base:

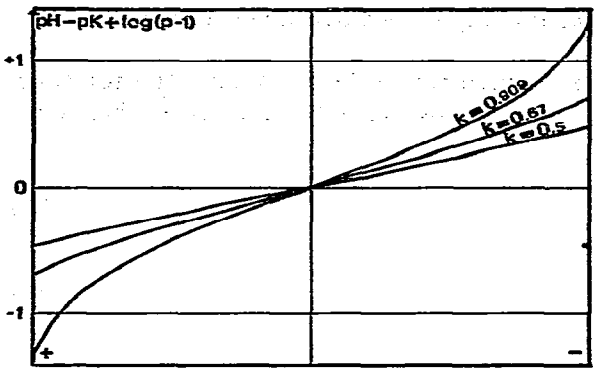

Fig. 2. Theoretical pH courses according to eqn. 16 for rheoelectrolysis of a buffer solution composed of a weak acid and its salt with a strong base. The liquid flow parameter, k, has an upper limit of unity.

$$C_1(x) - C_2(x) = (p^{-1} - 1)\left[\bar{C}_2 + \frac{T_2 ix}{F(Va + D_2 q)}\right] \tag{22}$$

Finally, if the inequality 19 is replaced by the equation

$$T_2 ia = 2kpF\bar{C}_1(Va + D_2 q) \tag{23}$$

where $1 > k > 0$ as before, this equation can be used for elimination of transport numbers and diffusion coefficients. Eqns. 6 and 22 then take the forms

$$C_2(x) = \bar{C}_2(1 - 2kx/a) \tag{24}$$

and

$$C_1(x) - C_2(x) = (p^{-1} - 1)\bar{C}_2(1 + 2kx/a) \tag{25}$$

These equations should be compared with eqns. 13 and 14. Insertion of eqns. 24 and 25 into the Henderson equation for the present case:

$$\mathrm{pH} = \mathrm{p}K + \log\left[\frac{C_1(x) - C_2(x)}{C_2(x)}\right] \tag{26}$$

gives the following pH course for a buffer with a weak base:

$$\mathrm{pH} = \mathrm{p}K + \log(p^{-1} - 1) + \log\left(\frac{1 + 2kx/a}{1 - 2kx/a}\right) \tag{27}$$

which corresponds to the pH course given by eqn. 16.

It should be noted that the pH course in both types of buffer is not symmetrical about the pK of the weak acid or weak base. The point of symmetry in the centre of the electrolyser has a pH more remote from the neutral point. This pH displacement away from neutrality is $\log(p - 1)$ for a weak acid and $\log(p^{-1} - 1)$ for a weak base buffer.

3.3. Rheoelectrolysis of a buffer containing a salt of a weak acid and a weak base

Eqns. 5 and 6 are valid, and the requirements to be formulated are for $C_1(x)$ to be positive in the anolyte and for $C_2(x)$ to be positive in the catholyte. These conditions are

$$2F\bar{C}_1 (Va + D_1q) > T_1ia \tag{28}$$

and

$$2pF\bar{C}_1 (Va + D_2q) > T_2ia \tag{29}$$

They are identical for the p value

$$p = \frac{T_2 (Va + D_1q)}{T_1 (Va + D_2q)} \tag{30}$$

With a knowledge of the relevant data for the two ion constituents, it is possible to choose that buffer composition. If this is done, eqn. 6 can be written in the form

$$C_2(x) = p \left[\bar{C}_1 - \frac{T_1ix}{F(Va + D_1q)}\right] \tag{31}$$

The inequality 28 is further replaced with the equation

$$T_1ia = 2kF\bar{C}_1 (Va + D_1q) \tag{32}$$

where $1 > k > 0$. Insertion of i from this equation into eqns. 5 and 31 yields

$$C_1(x) = \bar{C}_1 (1 + 2kx/a) \tag{33}$$

and

$$C_2(x) = \bar{C}_2 (1 - 2kx/a) \tag{34}$$

Even in this case both concentration courses become linear. The neutral salt prevails where $C_1(x) = C_2(x)$, which occurs at the point

$$\frac{x}{a} = \frac{p - 1}{2k(p + 1)} \tag{35}$$

On the cathodic side of this point, the weak base is in excess, and consequently there is a buffer there with a pH course within the buffer range of the weak base. On the anodic side, the weak acid is in excess, and there we have a buffer with a pH course within the buffer range of the weak acid. Sufficiently far from the point given by eqn. 35, the Henderson equation can be used for calculation of these pH courses, eqns. 26 and 15, respectively, being applicable. In the vicinity of the point given by eqn. 35, however, the Henderson equation cannot be used because in this pH range both ion constituents are incompletely ionized. A correct treatment thus has to deal with dissociation

theory for two simultaneously present weak protolytes, which is too lengthy to be given here. The resulting expression for the pH course is complicated and will not be given here, but its graphical representation is presented in Fig. 3, valid for $p = 1$. As can be seen, the pH gradient has one minimum in each buffer range and a maximum at the location of the pure salt. The total pH range obtainable is greater than twice the pK difference between weak base and weak acid, but the pH course at the location of the pure salt becomes very steep for large values of ΔpK.

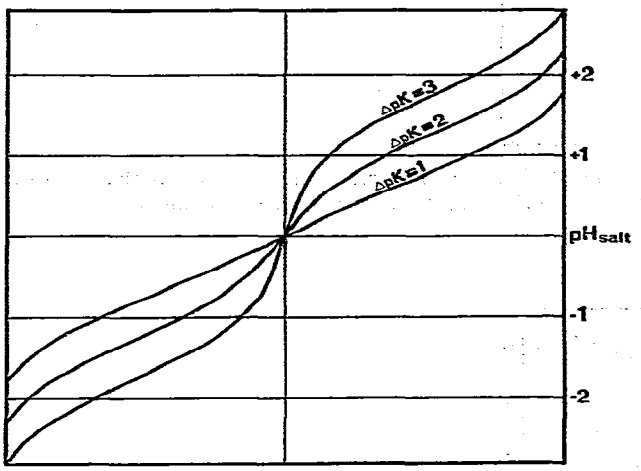

Fig. 3. Theoretical pH courses for rheoelectrolysis of a solution of a salt of a weak base and a weak acid for a liquid flow parameter $k = 0.9$ and for three different values of ΔpK between weak base and weak acid.

4. EXPERIMENTAL

Some attempts at experimental verification of the theory have been made with various forms of multi-compartment apparatus. No protein separations have been performed with this new technique because so far all efforts have been devoted to creation of stable pH gradients with the use of ordinary buffer solutions.

The first attempts were unsuccessful. Thus, one experiment with tris lactate gave the pH course shown in Fig. 4, which has no great resemblance to the theoretical ones in Fig. 3 and is virtually useless. The reason for the large pH plateau at pH 4 was later traced to the fact that the two pumps in Fig. 1 gave unequal rates of flow, resulting in an internal liquid flow from anode to cathode. This will be dealt with in the next section.

After the introduction of arrangements to prevent internal liquid flow, the pH courses shown in Figs. 5 and 6 were obtained, the former with a sodium acetate buffer and the latter with a sodium borate buffer. The pH course in Fig. 5 should be useful although it does not have the fine symmetry possessed by the theoretical curves in Fig. 2. Fig. 5 also includes the conductivity course throughout the electrolyser. The pH course in the borate experiment (Fig. 6) is very good and agrees well with the theoretical shape shown in Fig. 2.

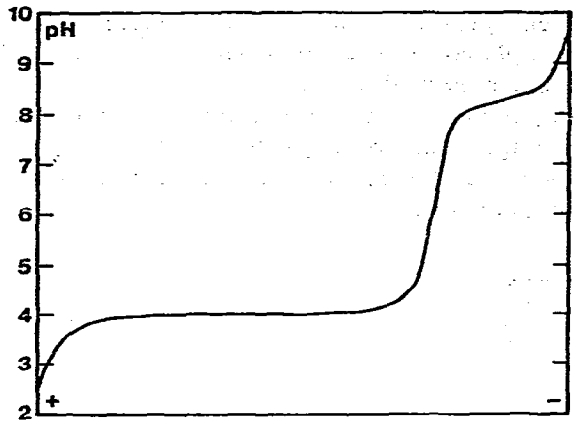

Fig. 4. Experimental pH course on rheoelectrolysis of tris lactate. There is no resemblance to the curves in Fig. 3 owing to an unintentional internal liquid flow from anode to cathode.

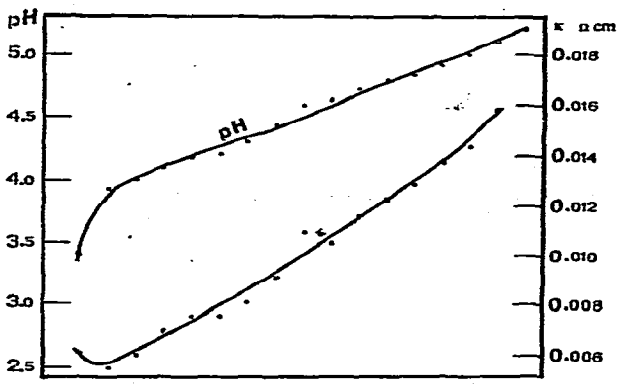

Fig. 5. Experimental pH and conductivity courses on rheoelectrolysis of an acetate buffer solution.

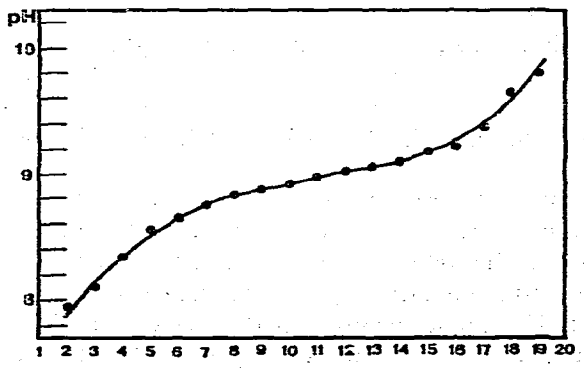

Fig. 6. Experimental pH course on rheoelectrolysis of a borate buffer solution.

5. STEADY-STATE RHEOELECTROLYSIS WITH INTERNAL LIQUID FLOW

If the flow through the cathodic pump is $V + v$ and that through the anodic pump is $V - v$, then the difference, $2v$, gives rise to an internal liquid flow from anode to cathode. The differential equation expressing the balance between the various mass transports then assumes the form

$$\frac{Ti}{Fz} - Dq \cdot \frac{dC}{dx} + 2vC = C_{-}(V + v) - C_{+}(V - v) \tag{36}$$

The solution of this equation is too tedious to be deduced here, and for the present purpose it is sufficient to present the result:

$$C(x) = \bar{C} + \frac{Tia}{Fz} \cdot \frac{2va\, e^{2vx/Dq} - Dq\,(e^{2va/Dq} - 1)}{(2va)^2 + 2v\,(V + v)\,a^2\,(e^{2va/Dq} - 1)} \tag{37}$$

Owing to the presence of the term $2vC$ in eqn. 36, this concentration course is no longer linear, but exponential. Typical concentration courses according to eqn. 37 for cations and anions are shown in Fig. 7. Extended plateau concentrations as illustrated there are obtained when the internal liquid flow is considerable. From the exponential concentration functions, the pH course can also be calculated. The result of such a calculation is demonstrated in Fig. 8, in which eqn. 37 has been applied to rheoelectrolysis of tris lactate. The resemblance between Figs. 4 and 8 is obvious, and thereby it has been proved that the bad result in the tris lactate experiment was due to internal cathodic liquid flow. There is, however, one striking difference between the theoretical curve in Fig. 8 and the experimental curve in Fig. 4, *viz.*, the sharp pH decline in the latter close to the anode. This is explained by the fact that the base tris, contrary to the theoretical postulate, is destroyed by anodic oxidation.

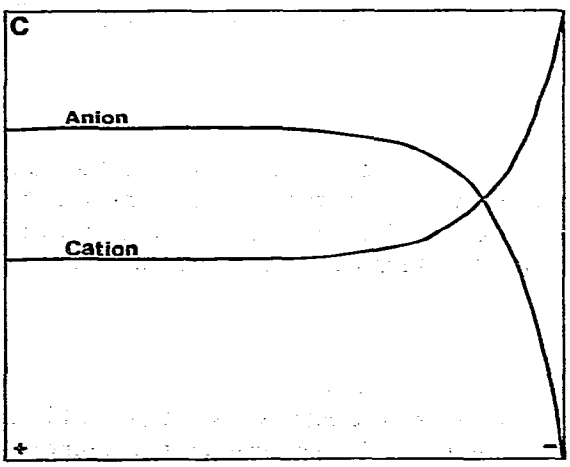

Fig. 7. Theoretical concentration courses for cation and anion according to eqn. 37 on rheoelectrolysis with a considerable internal liquid flow from anode to cathode.

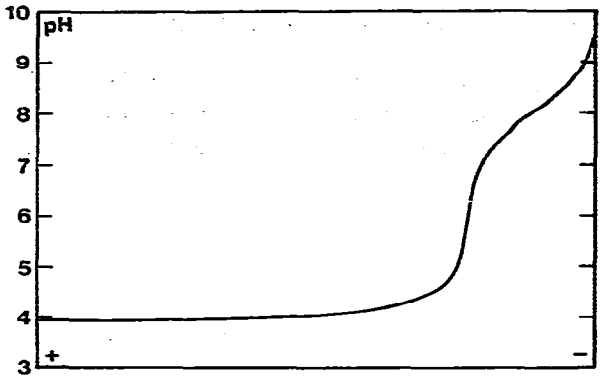

Fig. 8. Theoretical pH course for rheoelectrolysis of tris lactate with a considerable internal liquid flow from anode to cathode. Note the similarity in shape to the experimental curve in Fig. 4. The sharp decline in pH at the anode in Fig. 4 is due to anodic oxidation of tris, not taken into account in the theory behind eqn. 37.

6. DISCUSSION AND CONCLUSIONS

As is evident from the above discussion, this project has not advanced very far experimentally. Owing to a lack of personnel, it has not been possible until recently to begin to study the experimental problems inherent in the project. Various technical difficulties have proved to be more severe than was expected, and the effect of unavoidable small variations in transport numbers and diffusion coefficients have to be investigated both theoretically and practically. At present we are convinced that steady-state rheoelectrolysis is impossible in density gradients and that gels offer special complications not covered by the theory, as they do in conventional isoelectric focusing. Probably, therefore, analytical isoelectric focusing will continue to be dependent on Ampholine or other carrier ampholytes. Preparative work with multi-compartment types of apparatus appears at present to be the most promising application of steady-state rheoelectrolysis, but its degree of utility and versatility is still unknown.

7. ACKNOWLEDGEMENTS

The successful borate experiment was carried out at the Karolinska Institute in Stockholm by Mr. A. Forchheimer and the acetate experiment at this Institute by Dr. M. Almgren, both several years ago. The rheoelectrolysis project is at present financially supported by the Swedish Board for Technical Development, which is gratefully acknowledged.

8. SUMMARY

Rheoelectrolysis is defined as electrolysis of an electrolyte solution within a convection-free portion under simultaneous liquid exchange between two homogenized portions on either side of the convection-free portion of the electrolysis cell. As a special case, the two homogenized portions can be the anolyte and the catholyte, and

this case is particularly simple to treat theoretically. If electric and liquid flows are allowed to proceed at a constant rate for a sufficiently long time, a steady state develops that is characterized by a stable pH gradient in which isoelectric focusing can be carried out, provided that the electrolytes have suitable buffer properties. The tendency of the electric current to separate acids and bases completely is counteracted by pumps which transfer a more acidic solution on the anodic side to the cathodic side and a less acidic solution on the cathodic side to the anodic side of the convection-free portion of the electrolyser. In this way the two ion constituents of an ordinary buffer are made to circulate between the two homogenized portions as they migrate electrically within the convection-free portion and are pumped in the other direction outside the electrolyser.

If the transport numbers and diffusion coefficients of the ion constituents can be treated as constants, the differential equation of steady-state rheoelectrolysis is easily solved. If the two liquid flow-rates are identical, the concentration courses of the ion constituents become linear, and the pH course has a sigmoid shape. At least one of the ion constituents must be a weak protolyte, but if both have buffer action, the pH region that can be covered becomes much greater. The pH gradient decreases as the pumping speed increases.

If there is a net liquid flow within the convection-free portion of the electrolyser, the concentration courses of the ion constituents assume an exponential form, leading to a pH gradient that increases in the direction of this internal flow. Such gradients are not useful for isoelectric focusing.

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