ACTA CHEMICA SCANDINAVICA 15 (1961) 325–341

Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients. I. The Differential Equation of Solute Concentrations at a Steady State and its Solution for Simple Cases

HARRY SVENSSON

The Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif., USA, and Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden

The properties of natural pH gradients formed by stationary electrolysis are analyzed and compared with those of artificial pH gradients. The pH of a pure ampholyte solution is calculated, and the difference between this and its isoelectric pH is used to prove that a stationary state is characterized by mutually balancing diffusional and electric mass flows. The differential equation of the concentration of a component contributing to a natural pH gradient is formulated. It is shown that such components have to be defined as acids, bases, or ampholytes, and that the mobilities or transference numbers entering the differential equations must be ascribed to these ion constituents of the protolytes which are not H^+ or OH^-. Solutions to the differential equation are presented for a one-component system and for a trace component present in a large excess of other components. The practical significance of the results for fractionation and characterization of ampholytes is discussed. Thus it is proved that the pH at the concentration maximum of an ampholyte is identical with its isoelectric point, which should be of value for direct measurement of isoelectric points. The concentrations of acids and bases or, more generally, of the most acidic and the most basic components present in the system, are shown to increase steadily towards the anode and cathode, respectively, without developing maxima in the mathematical sense. It is shown that the electrokinetic properties of proteins make them especially suitable to isoelectric fractionation, whereas simple ampholytes are extremely useful as "carrier electrolytes" in work with proteins. By a proper choice of simple ampholytes and their concentrations, it is possible in principle to obtain pH gradients of any desired extension and shallowness, but for the present a sufficient number of suitable simple ampholytes is not available.

Isoelectric precipitation is a standard procedure for fractionation and purification of proteins. Isoelectric fractionation by electrical transport has also been used for a very long time, especially for group separations of amino acids and peptides (see the author's [1] review of 1948). However, the resolving power of these methods has stayed far behind that obtainable by moving boundary or zone electrophoresis using differences in electric mobility at a constant pH. A renewed interest in isoelectric methods has now been evoked through a series of articles by Kolin [2-6] and through the experiments described by Hoch and Barr [7] and by MacDonald and Williamson [8].

Isoelectric analysis and fractionation by electric transport is based on sending a direct current through a system of electrolytes such that pH increases gradually from anode to cathode. If the pH gradient can be kept reasonably stable during the time for an experiment, proteins and other ampholytes will be repelled from both electrodes and collect in a region where the local pH is identical with the isoelectric point of the ampholyte. This principle can be applied in a number of different ways depending on the nature of the pH gradient and also on the principle selected for stabilizing the system against remixing by uncontrolled convection.

ARTIFICIAL pH GRADIENTS

The authors just mentioned used artificial pH gradients, that is, they applied their samples in a mixing zone between two buffers of different pH's. Such a method puts great demands on the selection of the two buffers in order to secure a sufficient stability of the pH gradient. Since all buffers are composed of electrolytes, any artificial pH gradient is subject to changes caused by electric migration of the buffer ions. Needless to say, the migration of the pH gradient has to be slower than that of the components to be separated. Since the latter retard to zero mobility in the isoelectric state, it is evident that artificial pH gradients can never be expected to give more than quasi-equilibrium positions of isoelectric compounds. The length of a run may thus often be critical and difficult to predict.

The most favourable buffer systems are pairs of buffers containing the same ion species, *e.g.* two acetate buffers with pH's including the isoelectric points of the proteins. The differences between them is a certain concentration of excess acetic acid, which is very slightly ionized. This constituent is thus almost stationary. According to Kohlrausch's [9] electro-migration laws, the salt concentration also remains constant as long as the buffer reservoir suffices. A pH gradient of this kind is consequently very stable.

Kolin used a mixture of sodium acetate and sodium barbital as the basic, and the same solution mixed with hydrochloric acid as the acidic buffer. Hoch and Barr used a phosphate buffer of pH 7.4 as the basic, and mono-sodium phosphate as the acidic buffer. MacDonald and Williamson applied their samples in a region containing only distilled water between highly conducting anolytes containing hydrochloric or citric acid and catholytes with sodium hydroxyde or sodium citrate.

Artificial pH gradients, if properly selected, have the advantage that the conductance and field strength can be freely chosen and kept constant during the experiment.

NATURAL pH GRADIENTS

The numerous workers who have applied stationary electrolysis have allowed the electric current to create the pH gradient, usually in an apparatus with several compartments separated by membranes. The mechanism of this procedure is the following. Let us suppose that the electrolytic solution does not contain any ions, except the water ions, that can undergo oxidation or reduction at the electrodes. This is essentially the case with sodium sulphate in low concentrations. One then gets evolution of hydrogen at the cathode and of oxygen at the anode (which should be of platinum or carbon), while sodium hydroxyde collects in the catholyte and sulphuric acid in the anolyte. If remixing is prevented in some way or other, the final result is a partial or complete separation of acid and base, with or without a layer of pure water between them, depending on the current density.

If now ampholytes are added to the system, they will acquire a positive charge in the anolyte and a negative charge in the catholyte. Repulsion from both electrodes causes them to migrate towards the center of the apparatus. Between the acid and the alkali, they lose their charges and stop migrating. The final positions of the various ampholytes will be according to the sequence of their isoelectric points, the more basic ones being concentrated near the alkali, the more acidic ones closer to the acid. The ultimate course of pH from anode to cathode will be dictated by the isoelectric points of the ampholytes and by the concentrations and buffering properties of all protolytes in the system.

Subsequently a pH gradient thus formed will be called a natural pH gradient because it is the type of gradient which the current itself has created. For this reason, it is characterized by a complete stability. It is understood, then, that every artificial pH gradient must be reshaped into the natural pH gradient, provided that a sufficient time for electrolysis is allowed and that the system is stabilized against remixing. It is also obvious that an artificial pH gradient made up so as to imitate a natural one can be expected to get a very high stabiltiy.

If the extreme pH's given by sulphuric acid and sodium hydroxyde are not required, there is reason to choose a salt of a weaker acid and a weaker base, *e.g.* ammonium acetate, instead of sodium sulphate, provided that the acid withstands anodic oxidation and the base cathodic reduction. The pH's below 2 and above 12 are then not obtainable, and the whole apparatus can be better utilized for the pH range really needed. If still more restricted pH ranges are sufficient, it may be suitable to exclude all salts and to use only ampholytes and/or acids and bases too weak for mutual salt formation (boric acid, phenols, aromatic amines). For instance, for separations in the range between pH 3 and 4, a mixture of picolinic acid (isoelectric point 3.16), glutamic acid (IP 3.22), nicotinic acid (IP 3.44), anthranilic acid (IP 3.53), and *m*-aminobenzoic acid (IP 3.93) is adequate. In this way restricted pH gradients of any desired

shallowness can be created if suitable ampholytes are available. This is of great importance for high-resolution separations and for handling greater quantities of material.

The unlimited stability of natural pH gradients makes them very attractive, but unfortunately the method is so far hampered by some serious drawbacks. Its most severe limitation lies in the fact that one cannot choose the conductance throughout the system. The conductance generally becomes extremely low round the neutral point, which results in an excessive heating there. This limits the current that can be sent through to such a low value that one gets only a poor separation in other pH regions. Another experimental difficulty of great importance is the scarcity of suitable ampholytes for certain pH regions. Consequently, in spite of the elegance of the method and the simplicity of the equipment required, not much can be expected from natural pH gradients unless one acquires a clear understanding of the reasons for these difficulties and tries to find remedies. In this and following articles it will be shown that it should be possible to increase the versatility and capability of natural pH gradients quite considerably. This relates to refined isoelectric analysis in density gradients as well as to large-scale fractionation in multi-membrane apparatuses.

METHODS FOR STABILIZATION OF ELECTROLYTIC SOLUTION

All workers who have used natural pH gradients hitherto have applied multi-membrane equipment. In such a set-up, the separation occurs in the pores of the membranes, and the compartments between them, which are continuously homogenized by natural convection or mechanical stirring, merely serve as reservoirs for scaling up the capacity. The stabilizing principle is thus a capillary system. The same is the case in the experiments described by Hoch and Barr [7] and MacDonald and Williamson [8], who used ordinary paper electrophoresis equipment.

Kolin applied artificial pH gradients in vertical density gradient columns. The use of such columns in combination with natural pH gradients has recently been successfully explored by the present author and will be the subject of a forthcoming article in this series. It may be mentioned already now that such a procedure, although subject to the difficulties mentioned above, offers great advantages through the extreme simplicity of the equipment. Since the electrode reactions constitute an essential part of the separation process, special electrode vessels with comparatively large volumes of buffer are not to be used. In principle, only a test-tube and two platinum electrodes are required. However, since one electrode has to be at the bottom, it is also necessary to have a gas-escape tube around it. Devices for cooling and for taking fractions also have to be added if the apparatus is to be used for other than demonstration purposes.

THE pH OF A SOLUTION OF A PURE AMPHOLYTE

The pH of a solution of a pure ampholyte is known by experience to be approximately its isoelectric pH. A simple consideration of the electro-neutral-

ity condition reveals, however, that there must be a difference between the actual pH and the isoelectric point, pI. An ampholyte isoelectric at pH 3 gives in solution a hydrogen ion concentration of about 10^{-3} moles per liter. Since the ampholyte is the only possible source of anions, a concentration of the same magnitude must exist in the anionic form, and consequently it is not isoelectric. In the technique under discussion here, it is of interest to know something about the discrepancy between actual and isoelectric pH's, and further how it varies with the properties of the ampholyte.

The difference pH — pI can easily be derived theoretically if only two pK values of the ampholyte exert a measurable influence on its isoelectric point. This is the case for all well-known and easily available low-molecular ampholytes. Actually their isoelectric points can be calculated as the arithmetic mean between two adjacent pK values.

The following notation is convenient, all K quantities having the same dimension of concentration. Known quantities are:

K_1 = first dissociation constant of ampholyte when titrated with acid from isoelectric state;
K_2 = first dissociation constant of ampholyte when titrated with base from isoelectric state;
K^2 = ion product of water = 10^{-14} at 25°C;
K_i = $\sqrt{K_1 K_2}$ = isoelectric constant of ampholyte;
pK_1 = $-\log K_1$;
pK_2 = $-\log K_2$;
pI = $-\log K_i = (pK_1 + pK_2)/2$ = isoelectric point of ampholyte;
C = total concentration of ampholyte in moles per liter.

Unknown quantities are:

h = hydrogen ion concentration;
C_+ = concentration of cationic ampholyte;
C_- = concentration of anionic ampholyte;
C_0 = concentration of undissociated and zwitterionic ampholyte.

Leaving activity coefficients without consideration, one has the following four equations:

$$h\,C_0 = K_1 C_+ \tag{1}$$

$$h\,C_- = K_2 C_0 \tag{2}$$

$$h + C_+ = K^2/h + C_- \tag{3}$$

$$C = C_+ + C_- + C_0 \tag{4}$$

of which (3) expresses the electro-neutrality condition. Elimination of C_+ and C_- between (1), (2), and (4) gives the following relation between C_0 and C:

$$C_0(h^2 + K_1 h + K_1 K_2) = K_1\,h\,C \tag{5}$$

The ion concentrations can then be expressed in h and known quantities:

$$C_+(h^2 + K_1 h + K_1 K_2) = C\,h^2 \tag{6}$$

$$C_-(h^2 + K_1 h + K_1 K_2) = C\,K_1 K_2 \tag{7}$$

They can then be introduced into the electro-neutrality condition, which gives the equation:

$$Ch(K_i^2 - h^2) = (h^2 - K^2)(K_i^2 + K_1 h + h^2) \quad (8)$$

This equation contains h as the only unknown quantity, but it is of the fourth degree.

On consideration of practical examples one finds, however, that the degree falls to three and that the resulting equation is easily solved numerically by successive approximation. Thus ampholytes isoelectric below pH 6 give h^2 values at least 100 times greater than K^2; thus this quantity can be omitted. For all acidic ampholytes, one consequently gets the following third-degree equation:

$$C(K_i^2 - h^2) = K_i^2 h + K_1 h^2 + h^3 \quad (9)$$

On the other hand, for ampholytes isoelectric above *pH* 8, h^2 can be neglected beside K^2. Thus all basic ampholytes obey the equation:

$$Ch(h^2 - K_i^2) = K^2(K_i^2 + K_1 h + h^2) \quad (10)$$

For neutral ampholytes, isoelectric between pH 6 and 8, such simplifications are not possible, but for those no calculation at all is necessary. The pH of such solutions coincides with the isoelectric point to a very high precision at concentrations of practical interest.

Since the pH of the solution is known to be approximately equal to pI, $h = K_i$ is used as a first approximation on the right-hand side of (9) or (10) in order to gain a first approximation of $(K_i^2 - h^2)$. This gives a second approximation of h which, on insertion on the right-hand side, gives a second approximation of $(K_i^2 - h^2)$ and a third approximation of h. In most cases the second approximation of h is accurate enough, and in all cases tested by the author the third one has been correct to less than 0.01 pH unit. Thus, for glutamic acid, with the numerical figures:

$$pK_1 = 2.19 \quad pI = 3.22 \quad C = 0.1$$

one gets a pH of 3.24 in the second, and the same figure in the third approximation. Aspartic acid, with the numerical figures:

$$pK_1 = 1.88 \quad pI = 2.765 \quad C = 0.0563$$

gives pH 2.84 in the second and 2.82 in the third approximation.

On the right-hand side of eqns. (9) and (10), the second term is always the biggest one. However, when K_1 and K_2 both approach K_i, the other two terms also become important. One thus finds the difference pH — pI to be the bigger, the closer together the two pK values of the ampholyte.

The dependence on concentration is easily understood from eqn. (8). The quantity $(K_i^2 - h^2)$ is inversely proportional to concentration, and h approaches K (pH approaches 7) as C approaches zero, which must be expected.

CONSEQUENCES FOR THE THEORY OF THE NATURAL pH GRADIENT

The existence of the difference pH — pI enables one to draw some logical conclusions which are helpful for a proper understanding of a natural pH gradient. As will be shown below, these consequences involve valuable information regarding the concentration distribution of ampholytes and concerning the possibility of direct measurement of isoelectric points.

Hitherto available experimental evidence supports the view that it is possible to reach a steady state by prolonged electrolysis under the conditions defined earlier. A steady state includes the demand of a stationary solvent, that is, absence of electro-osmosis by the use of a density gradient as stabilizing agent, or an electrically inert capillary system, or possibly a hydrodynamic back-flow of solvent balancing the electro-osmotic flow. All these alternatives are experimentally possible. If somebody is worried about the continuous consumption of water in the form of hydrogen and oxygen at the electrodes, he can imagine a hydrogen electrode as the anode and a device conducting the hydrogen formed at the cathode to this anode. In such an idealized stationary state, which is most suited for a theoretical attack, the only thing that happens is a continuous circulatory flow of hydrogen. All other chemical components are stationary, they do not change their concentrations anywhere in the system.

Let us first consider a point in the system where an ampholyte is the only solute. The existence of such a point is evident from the fact that there may be only one component plus the solvent in the whole system; the possibility of complete purification by stationary electrolysis is thus not anticipated. It has been proved in last section that the pH at such a point is a little closer to 7 (in water) than the pI of the ampholyte. Thus the latter must possess a small net charge and take part in the transport of current. The ampholyte is consequently bound to migrate electrically, which seems to be in conflict with the supposition of a steady state. This incongruity can only be solved by assuming a mass flow in the other direction, caused by something else than electric attraction and repulsion. Since convection and other transport of solvent have been ruled out, the only possible cause of such a flow is diffusion. One must consequently conclude that *at any point in a natural pH gradient where there is an ampholyte in a pure state, this ampholyte must necessarily have a concentration gradient directed towards the anode for acidic and towards the cathode for basic ampholytes*, the magnitude of this gradient being such that the mass flow due to diffusion balances the electric migration.

The same reasoning applied to acids and bases leads to a similar conclusion. However, the demand for purity can be omitted since no impurity can reverse the electric charge of a non-amphoteric ion constituent. Thus, *the uninterrupted electric migration of acids towards the anode and of bases towards the cathode is balanced by a diffusion flow away from the electrodes.* The steady state is consequently characterized by acid and alkali concentration gradients directed towards the respective electrodes.

Hydrogen is the only substance that exhibits a net mass flow through the apparatus at a steady state. It should be pointed out that it is erroneous to conclude from this fact that the transference number of the hydrogen ion con-

stituent H^+ is unity throughout the system. The definition of a transference number (Hartley and Moilliet [10]) is based on the mass flow *due to electric migration alone.* The protolytic ion constituents, although with a net mass flow of zero, exhibit electric migration and thus have finite transference numbers.

The impossibility for an ampholyte to have a constant concentration in a pure state leads either to the conclusion that it is nowhere pure (which is in conflict with experimental evidence and with the possibility of a one-component system), or to the consequence that it must have a maximum concentration somewhere, since the amount of the ampholyte is finite. If the ampholyte is an end component collecting at one of the electrodes, its highest concentration is found in contact with that electrode. This is no maximum in the mathematical sense, since the concentration gradient for end components is finite all the way to the electrode. If the ampholyte is an intermediate component collecting in an isoelectric zone, its maximum concentration has a vanishing gradient. It follows that there can be no diffusional mass flow at this point, and hence no electric migration either. It is hereby proved that *the pH at the concentration maximum of an ampholyte in a natural pH gradient is its isoelectric point.* Another corollary is, however, that *the ampholyte is necessarily contaminated by the adjacent component at its point of maximum concentration.*

On the other side of the maximum (the side towards the electrode) the pH is lower than pI for acidic, and higher than pI for basic ampholytes, the reason for this being an increased contamination by the adjacent ampholytes. On each side of the maximum, the electric migration is towards the top, and the diffusion flow away from it.

The steady state concentration distribution of ampholytes is thus characterized by a series of bell-shaped curves, one for each ampholyte that does not collect at an electrode. The pH's at the concentration maxima represent the exact isoelectric points of the ampholytes, but the overlapping of the various curves is so extensive that, going from the electrodes towards the neutral point, the concentration of one ampholyte has never reached zero at the concentration maximum of next ampholyte. The experimental implications of these conclusions will be further considered in the discussion.

DIFFERENTIAL EQUATION OF THE CONCENTRATION DISTRIBUTION OF A PROTOLYTE IN A NATURAL pH GRADIENT

After the qualitative considerations in last section, it is easy to formulate the differential equation of the concentration course of an electrolytic component, regardless whether it is an ampholyte or a non-ampholyte or whether it is an end or intermediate component. This equation is:

$$\frac{Cui}{q\varkappa} = D\,\frac{\mathrm{d}C}{\mathrm{d}x} \tag{11}$$

where:

C = concentration of component and ion constituent in arbitrary mass units per arbitrary volume unit;

u = electric mobility in $cm^2\ volt^{-1}\ sec^{-1}$ of ion constituent except H^+ and OH^-, with positive sign for cationic and negative sign for anionic migration;
i = electric current in amperes;
q = cross-sectional area in cm^2 of electrolytic medium, measured perpendicularly to the direction of current;
$\varkappa$ = conductance of medium in $ohm^{-1}\ cm^{-1}$;
D = diffusion coefficient in $cm^2\ sec^{-1}$ of component corresponding to the ion constituent with mobility u.
x = coordinate along the direction of current, thus x increasing from 0 at the anode towards the cathode.

Each term in eqn. (11) expresses the mass flow per second and cm^2 of the cross-section, that to the left being the electric, that to the right the diffusional mass flow.

It is necessary to stay a little at the concepts of component and ion constituent. The latter term is the concept defined by MacInnes [11]. The left-hand side of (11) necessarily refers to an ion constituent in contrast to a chemical component since the latter, if ionized, has at least two mobilities, one cationic and one anionic. On the right-hand side, there is a diffusion coefficient which must refer to a component since D is not defined as the selfdiffusion coefficient of an ion. The fact that one side of the equation describes the mass flow of an ion constituent, the other the mass flow of a chemical component, necessitates such a definition of the latter that an experimental determination of both mass flows amounts to measurements of mass transports of one and the same ion constituent. In other words, un unambiguous correspondence between component and ion constituent is required.

This condition is only satisfied by regarding every base, every acid, and every ampholyte present in the system as components thereof, and by ascribing the mobility u to that ion constituent which is not H^+ or OH^-. In this way eqn. (11) loses application to the two solvent ions. This is natural concerning H^+ since it exhibits a finite net mass flow. With a hydrogen electrode as anode, OH^- *is* stationary, and eqn. (11) should be valid using the concentration and diffusion coefficient of water. However, this is superfluous since the concentration course of water is unambiguously defined by those of the solute species.

In the case of capillary systems as stabilizing agents, the cross-sectional area q and the conductance $\varkappa$ refer to the whole medium including the solid phase. Now there are no good methods for conductance measurements of such media. Some experimental workers then prefer to measure the conductance of the free liquid and to determine experimentally the cross-sectional area of free liquid in the capillary system. Already this leads to doubtful results, and in addition there is the phenomenon of surface conductance, which is different from conductance in bulk solution, at least for electrically active capillary systems. Capillary systems are thus very difficult to master in quantitative measurements.

Like most differential equations in physics and chemistry, eqn. (11) is rather useless without solutions pertaining to specific experimental conditions.

The impossibility of formulating a simple general solution is evident from the fact that one is concerned with a system of mutually dependent differential equations. The conductance and the mobilities are complicated functions of all solute concentrations. However, because of the ease with which rigorous and approximate solutions can be derived for a couple of simple cases, these will be considered in the next two sections.

SOLUTION OF THE DIFFERENTIAL EQUATION FOR A ONE-COMPONENT SYSTEM. CONCENTRATION DISTRIBUTION OF A PURE END COMPONENT

If the differential equation is written in the form:

$$i \frac{\mathrm{d}x}{q} = \frac{\varkappa D \, \mathrm{d}C}{C u} \tag{12}$$

all concentration-dependent quantities are collected to the right, while q, which may be a function of x due to the construction of the apparatus or due to electro-osmosis, is to the left. Only the current is always independent of both x and C. The general solution is:

$$i \int \frac{\mathrm{d}x}{q} = \int \frac{\varkappa D \, \mathrm{d}C}{C u} \tag{13}$$

The cross-sectional area is either constant or can be measured as a function of x. By suitable experiments, it is also possible to represent, $\varkappa$ D, and u as functions of C graphically or mathematically by curve-fitting procedures. In each case, it is therefore possible to obtain a rigorous numerical integration of the differential equation.

Since the transference number T of an ion constituent is given by the equation:

$$T = \frac{FCzu}{\varkappa} \tag{14}$$

where $F = 96\,500$ coulombs per equivalent is the Faraday constant and z is the valence, with plus or minus sign, of the ion constituent, the solution (13) can also be given in the form:

$$i \int \frac{\mathrm{d}x}{q} = Fz \int \frac{D}{T} \, \mathrm{d}C \tag{15}$$

This is more convenient since it only contains two C-dependent quantities that have to be measured.

Eqn. (15) is still an exact solution, applicable to all experimental conditions. The equation:

$$\frac{ix}{FqzD} = \int \frac{\mathrm{d}C}{T} \tag{16}$$

is restricted to experimental conditions giving a constant cross-section and has an exactness corresponding to the constancy of D. The concentration has to be given in moles per cm^3, a restriction that imposes eqn. (14).

For a detailed discussion of the meaning of a transference number of an ampholyte, reference must be made to a previous article on that subject (Svensson [12]). It may suffice to tell here that, according to the modern perception of ampholytes as protolytes over the entire pH range, it is logical and rational to regard H^+ as the positive ion constituent of every ampholyte. It follows that the ampholytic ion constituent is always negative, even that of arginine. The number z for ampholytes is thus a negative integer equal to the number of acidic groups in the molecule.

The transference number is also a signed quantity, as is evident from eqn. (14). A positive product zu gives a positive transference number, and *vice versa*. Ampholytes have negative transference numbers below their isoelectric points.

To go one step further in approximation and simplification, one can make use of the fact that even transference numbers are in many cases essentially independent of concentration. Then the solution of the differential equation becomes especially simple and is best given in the form:

$$C = \frac{Ti\,(x-b)}{FDzq} \tag{17}$$

where $Tib/FDzq$ is the integration constant. The validity of this equation is of course restricted to the space between the electrodes (the interval between $x = 0$ and $x = a$) and to positive concentrations. The coordinate b may assume any positive and negative values. Negative b values may be obtained for Tz positive and means that the concentration is finite and steadily increasing from anode to cathode. Positive b values greater than a may be obtained for negative Tz values and means that the concentration is finite and steadily increasing from cathode to anode. Positive b values smaller than a, finally, means that the concentration drops to zero somewhere in the apparatus, and eqn. (17) is then restricted to the interval between b and that electrode where the component exists. For the interval between b and the other electrode, one has instead:

$$C = 0 \tag{18}$$

which also satisfies the differential equation (11).

The numerical value of b for given experimental conditions can be calculated in terms of the total mass m of the component present in the apparatus. To that end, C is multiplied by q and integrated over the interval for which eqn. (17) is applicable. For b outside the electrodes, this interval is the whole apparatus, and the integration gives the relation:

$$m = \frac{Tia}{2FDz}\,(a - 2b) \tag{19}$$

Insertion of b from (19) into (17) then gives:

$$C = \bar{C} + \frac{T\,i}{2FDzq}\,(2x - a) \tag{20}$$

where C is the mean concentration. This equation is valid for currents lower than the limiting value:

$$i = \pm \frac{2FDzm}{T\,a^2} \tag{21}$$

for which the concentration drops to zero at one of the electrodes. The sign making i positive is valid.

For currents stronger than (21), the concentration reaches zero between the electrodes, and the integration must consequently be carried out from the anode to b if Tz is negative, and from b to the cathode if Tz is positive. One finds for Tz positive:

$$m = \frac{Ti\ (a-b)^2}{2FDz} \tag{22}$$

$$C = \frac{2m\ (x-b)}{q(a-b)^2} \tag{23}$$

and for Tz negative:

$$m = -\frac{Tib^2}{2FDz} \tag{24}$$

$$C = \frac{2m\ (b-x)}{qb^2} \tag{25}$$

Comparison between the eqns. (21), (22), and (24) now reveals that the current necessary for confining the component to a distance c from the electrode at which it collects, is given by the equation:

$$i = \pm \frac{2FDzm}{T\,c^2} \tag{26}$$

If the current density i/q is squared and then divided by the conductance, one obtains the Joule heat per cm^3 of the medium:

$$w = \frac{i^2}{q^2\varkappa} = \frac{4F^2D^2z^2m^2}{T^2q^2b^4\varkappa} \tag{27}$$

Knowing that the minimum conductance is that of the pure solvent and that evacuation of electrolytes really occurs in this process, this equation may be of value in the design of apparatus, especially its cooling system. The conductance of electrolyzed water may be as low as $5 \cdot 10^{-8}$ ohm^{-1} cm^{-1}.

THE CONCENTRATION DISTRIBUTION OF AN INTERMEDIATE COMPONENT

One single component in a system becomes necessarily an end component, that is, it concentrates at one electrode if it concentrates at all. This is true for ampholytes as well as for acids and bases. Glutamic acid, if present alone, can never reach its isoelectric state and is thus attracted by the anode all the time and everywhere, just like sulphuric acid.

The condensation of an ampholyte into an isoelectric zone thus requires at least two components. The system of two or more differential equations (11) is, however, not solvable by simple mathematical means. The resulting pH and conductivity courses of multi-component systems at a steady state thus cannot be calculated, but they can be roughly predicted from the amounts, isoelectric points, and conductances of the components of the system. For example, if comparatively much glutamic acid is present, one can expect to get an extended region with a very shallow pH gradient around the isoelectric point (3.22) of glutamic acid, and with a conductance not lower than that of pure glutamic acid solutions of concentrations corresponding to that in the apparatus. On the other hand, if no suitable ampholytes isoelectric between pH 5 and 7 are available, then the pH gradient in this region must be expected to be rather steep.

Since it is possible to make experimental records of pH and conductance for each system, it will now be assumed that this has been done and that consequently pH and $\varkappa$ are known functions of x. The differential equation (11) can then be applied to an additional component added in so small amounts that it cannot materially alter the concentration distribution of the other, the "carrier" components. This case has a considerable interest for work with proteins in systems of low-molecular ampholytes.

If eqn. (11) is written in the form:

$$\frac{i}{q}\,\frac{u\mathrm{d}x}{\varkappa} = D\,\frac{\mathrm{d}C}{C} \tag{28}$$

it is seen that it is possible to integrate it if u is known as a function of pH and D as a function of C. Specifically, if the conductance, the diffusion coefficient, and the derivative:

$$p = -\,\frac{\mathrm{d}u}{\mathrm{d}x} = -\,\frac{\mathrm{d}u}{\mathrm{d(pH)}}\,\frac{\mathrm{d(pH)}}{\mathrm{d}x} \tag{29}$$

can be regarded as constant within the protein zone, then $u = -\,px$, and one obtains the following analytical solution:

$$C = C_0 \exp\left(-\,\frac{pix^2}{2q\varkappa D}\right) \tag{30}$$

where x is now defined as being $= 0$ at the concentration maximum C_0. This is a Gaussian concentration distribution with the inflexion points at

$$x_i = \pm\sqrt{\frac{q\,\varkappa\,D}{p\,i}} \tag{31}$$

The relation between the maximum concentration and the total amount of ampholyte is:

$$m = C_0\sqrt{\frac{2\,q^3\,\varkappa\,D}{p\,i}} \tag{32}$$

Eqn. (31) shows that proteins are especially suitable for isoelectric condensation, partly because of their low diffusion coefficients, partly due to to their steep mobility curves in the isoelectric range (large value of the slope $du/d(pH)$). The low conductance generally obtained in stationary electrolysis also contributes to give very narrow protein zones. This in turn increases the exactness of the assumption of constant p and $\varkappa$.

If the conductance is not constant, but a linear or quadratic function of x, it is still possible to integrate the differential equation analytically. For

$$\varkappa = \varkappa_0 + rx \tag{33}$$

the author has found the solution:

$$C = C_0 \exp\left[\frac{pi\varkappa_0}{Dqr^2} \ln\left(1 + \frac{rx}{\varkappa_0}\right) - \frac{pix}{Dqr}\right] \tag{34}$$

which is a skew concentration distribution. For

$$\varkappa = \varkappa_0 + sx^2 \tag{35}$$

the solution is found to be:

$$C = C_0 \left(1 + \frac{sx^2}{\varkappa_0}\right)^{-pi/2sqD} \tag{36}$$

DISCUSSION

In 1941, Tiselius [13] described the steady state in stationary electrolysis as a dynamic equilibrium between diffusion and electric migration. He also gave the differential equation (11) in a slightly different form. This interpretation has now been substantiated, and the differential equation has been thoroughly interpreted and solved for two simple cases. End components, which may be acids, bases, or ampholytes, have concentrations steadily increasing towards the electrodes, while intermediate components, which must be ampholytes, have bell-shaped concentration courses. Thus the stationary state of an end component resembles that in sedimentation equilibrium, while the condensation of ampholytes in zones is much akin to the condensation of macromolecules into isodensity bands in the ultracentrifugal technique developed by Meselson *et al.*[14]

At first glance, it appears as self-evident that the pH's of the ampholyte zones are their isoelectric pH's. However, a closer analysis of the differential equation and of equations for the pH of pure ampholyte solutions reveals that pH varies throughout an ampholyte zone, and that a pure ampholyte in solution is non-isoelectric. It has now been shown that the pH at the concentration maximum of an ampholyte in a natural pH gradient is its exact isoelectric point, which is possible due to a contamination at this point by the adjacent component. This should be of great importance for experimental work. There is no other direct way of measuring isoelectric points than by way of the solubility minimum. The moving boundary method of electrophoresis

offers an indirect way which is rather laborious. At least four experiments at different pH's are required for each ionic strength. Extrapolation to zero ionic strength amounts to about 16 experiments. Mobility measurements in paper or other solid support is hampered by great uncertainty and inaccuracy. Stationary electrolysis seems to offer great advantages in this respect, since a pH measurement at the point of maximum concentration is all that is needed. Moreover, the medium is salt-free, so the isoelectric point obtained refers to essentially zero ionic strength.

The solution of the differential equation given for a one-component system shows that an end component exhibits a linear concentration course if the diffusion coefficient and the transference number as defined in the text are constant, or, more generally, have a constant ratio. For higher electric loads, the concentration drops to zero at a distance from the repelling electrode, leaving a region of pure solvent there. This result of the theory checks completely with measurements on sulphuric acid and sodium hydroxyde performed by Tiselius [13].

The complete understanding of the behaviour of a one-component system facilitates considerably the interpretation of more complicated systems. It should be remembered that the process separates the components to a large extent, and that in the steady state only two or three components coexist. Thus, since a region of pure solvent has been shown to develop at one electrode when only an acid or a base is present, then one must conclude that the same may happen in the center of the apparatus when the electrolysis is started with a salt. This was probably the case in Tiselius' experiment with egg albumin and hemoglobin.

One consequence of the diffusion-electromigration theory is that one ampholyte necessarily extends to and beyond the concentration maximum of the adjacent one. Every ampholyte is therefore more or less contaminated already at the centre of its zone. The ampholyte becomes the more contaminated the closer together two adjacent isoelectric points. A complete separation of ampholytes, with regions of pure solvent between component zones, is a theoretical impossibility unless they are isoelectric on either side of the neutral point.

This fact puts a definite limit to the purity obtainable of low-molecular ampholytes by stationary electrolysis. No important advances in this application over those already described in the literature are to be expected. However, this behaviour of low-molecular ampholytes is very favourable for work with proteins. If a system of simple ampholytes is used as "carrier" electrolytes in the separation of proteins, the overlapping concentration curves of the former result in very smooth changes from one isoelectric pH to the next, and in a helpful buffering capacity even at pH's between the available isoelectric points. In combination with the favourable pH-mobility curves of proteins, this opens possibilities for highresolution isoelectric fractionation of proteins.

Schumacher [15] has recently presented a general theory of "focusing ion exchange", which term includes the condensation of ampholytes in isoelectric zones as a special case. His solution of the differential equation for the case of a constant pH gradient (his eqn. (15)) is rather complex, but the corresponding concentration distribution (his Fig. 3) resembles very much the distribu-

tions according to the simple solutions (30), (34), and (36). The discrepancy between his and the present results seems to depend upon fundamentally different physico-chemical approaches. Thus, while in the present treatment mobilities and diffusion coefficients refer to ion constituents and chemical components, respectively, and are experimentally measurable as functions of pH, Schumacher has introduced separate u and D values for the cationic and anionic subspecies. It is also remarkable that the uncharged subspecies have been left without consideration although they are present in most cases (for protolytes: always), take part in the exchange reactions, and contribute to the diffusional flow.

Another difference in approach lies therein that a constant pH gradient was assumed by Schumacher, whereas a constant mobility gradient $\mathrm{d}u/\mathrm{d}x = p$ underlies the solutions (30), (34), and (36) in the present treatment.

Experimental workers in this field have paid great attention to the electrochemical behaviour of the membranes in equipment where such have been used. Electropositive membranes at the anode and negative ones at the cathode is a recommendation one frequently finds in this literature. In terms of quantities used in this theory, the desirable properties of the membranes can be specified as follows.

If the transference number (14) is introduced directly into the differential equation (11), one obtains:

$$\frac{\mathrm{d}C}{\mathrm{d}x} = \frac{Ti}{FDzq} \tag{37}$$

The higher the concentration gradient, the better is the confinement of an end component to the electrode, and the more effective is the separation as a whole. It is seen that the concentration gradient is directly proportional to the transference number of that ion constituent which is not H^+ or OH^-. Electropositive membranes give increased transference numbers to anions, electronegative ones to cations, which explains the recommendations mentioned above.

It should be of special interest to study the applicability of amphoteric membranes, since they would automatically acquire the selectivity desired. The transference numbers would then be expected to change from membrane to membrane, the concentration course of an end component would no longer be linear, but curved so as to improve the confinement to one electrode.

Acknowledgement. This work was started in 1959 at California Institute of Technology. and the author wishes to express his sincere thanks to Professor Linus Pauling for his kind invitation and for laboratory facilities during a period of six months. It was completed at the Department of Bacteriology, Karolinska Institutet, Stockholm, and the author is indebted to Professor Berndt Malmgren for laboratory space during 1960. Grants covering the traveling costs to California were received from the *Natural Science* and the *Technical Research Councils of Sweden*, and the work in Pasadena was made possible by financial support from California Institute of Technology and from LKB-Produkter, Stockholm. From 1960, this research is supported by the *Natural Science*, the *Technical*, and the *Medical Research Councils of Sweden*, which is gratefully acknowledged.

REFERENCES

1. Svensson, H. *Advances in Protein Chem.* **4** (1948) 251.
2. Kolin, A. *J. Chem. Phys.* **22** (1954) 1628.
3. Kolin, A. *J. Chem. Phys.* **23** (1955) 407.
4. Kolin, A. *Proc. Natl. Acad. Sci. U.S.* **41** (1955) 101.
5. Kolin, A. in Glick, D., *Methods in Biochemical Analysis* **6** (1958) 254.
6. Kolin, A. *Proc. Natl. Biophys. Conf. 1957*, Yale University Press, New Haven, Conn. (1959) p. 125.
7. Hoch, H. and Barr, G. H. *Science* **122** (1955) 243.
8. MacDonald, H. J. and Williamson, M. B. *Naturwiss.* **42** (1955) 461.
9. Kohlrausch, F. *Ann. Physik* **62** (1897) 209.
10. Hartley, G. S. and Moilliet, J. L. *Proc. Roy. Soc. London* **A 140** (1933) 141.
11. MacInnes, D. A. *The Principles of Electrochemistry*, New York 1939.
12. Svensson, H. *Science Tools* **3** (1956) 30.
13. Tiselius, A. *Svensk Kem. Tidskr.* **58** (1941) 305.
14. Meselson, M., Stahl, F. W. and Vinograd, J. *Proc. Natl. Acad. Sci. U. S.* **43** (1957) 581.
15. Schumacher, E. *Helv. Chim. Acta* **40** (1957) 2322.

Received September 10, 1960.