On the Possible Interference of Electrode Reaction Products in Electrophoresis

HARRY SVENSSON

Laboratories of LKB-Produkter Fabriksaktiebolag, Stockholm, Sweden

The relative merits of gassing and non-gassing electrodes for electro-phoresis have been discussed in detail. Special attention has been paid to the buffer volume required between the electrodes and the separation zone. It has been found that platinum electrodes possess consider-able advantages in spite of their gas evolution and that their use in electrophoresis instruments can be recommended, at least if not precise mobility measurements are concerned. It has been shown by theoretical reasoning and by conclusive experiments that the pH boundaries formed at such electrodes migrate towards the separation zone at quite moderate rates which have little to do with the exces-sively high mobilities of free H+ and OH- ions. The boundaries have been found to be very sharp, and the conditions for gravitational stability have been studied experimentally for some commonly used buffers. For ordinary analytical electrophoresis runs, a buffer volume 10 times the volume of the electrophoresis channel has been found to be sufficient. For prolonged runs, this volume can be retained if an acid lock is provided in the cathode vessel and an alkali lock in the anode vessel. The influence of these results on the construction of electrode vessels for an electrophoresis apparatus has been discussed.

T he earliest workers in U-tube electrophoresis used platinum electrodes at the top of the limbs not far from the moving boundaries under observation (see *e.g.* Hardy[1] and Burton[2]). This caused the electrode reaction products to interfere with the substances under investigation.

It was a great advance when Michaelis[3] introduced special electrode ves-sels and an appreciable volume of electrolyte solution between the electrodes and the separation zone. He also introduced reversible electrodes. Svedberg and Jette[4] were still more cautious and used additional safety tubes between the electrode vessels and the U-tube. Tiselius[5] gave an equation for the buffer volume required between the electrodes and the U-tube in a run under specified conditions and of specified duration. Later[6], he pointed out that this volume could be reduced by using an increased buffer salt concentration in the elec-trode tubes, compared to that in the U-tube.

Electrophoresis instruments are now manufactured on a commercial basis in many countries. It is then worth while to reconsider the advantages and disadvantages of different types of electrodes, and to try to get conclusive evidence regarding the necessary buffer volume. A development in this direction has already been initiated by Antweiler [7], who revived the use of simple, irreversible electrodes. He also radically reduced the buffer volume, compared with earlier designs of electrophoresis cells. In paper electrophoresis instruments, which are also produced on a large scale, all constructors seem to have chosen platinum, or even simpler, irreversible electrodes, although the possible interference from electrode reaction products is the same as in the moving boundary method.

RELATIVE MERITS OF DIFFERENT TYPES OF ELECTRODES.

We will distinguish here mainly between gassing and non-gassing electrodes, since this is the most important property. A non-gassing electrode — generally silver-silver chloride electrodes are used — make it possible to use closed electrode vessels, which is regarded as very favourable for making accurate mobility measurements. Such electrodes are, however, not quite safe against gas evolution, unless they are handled very carefully and managed according to a strictly rigid running scheme (cf. Longsworth and MacInnes [8]). Such an electrode must have an appreciable area accessible to the solution in order to keep the risk for gas evolution as small as possible. To achieve that, comparatively much electrode material and a great deal of mechanical workmanship are required. The large dimension of the electrode and the desirability of having closed electrode vessels lead to the use of large and expensive ground joints. The cost of such an electrode together with an appropriate electrode tube must therefore be appreciable, and yet it cannot be managed by technical laboratory personnel without extensive training. In the most accurate mobility measurements a correction for volume changes at the electrodes is required even for non-gassing electrodes.

At a gassing electrode, an uncontrollable amount of gas bubbles sticks to the electrodes and to the adjacent glass walls. These bubbles, as they form and escape to the surface of the liquid, must be expected to give rise to slight back and forth movements of the boundary system and to a corresponding uncertainty in the localization of boundaries in the U-tube in free electrophoresis. In paper electrophoresis, on the other hand, they should be quite harmless. It is, however, always possible to remove such gas bubbles every time a measurement or an exposure is to be taken. Now, most investigators have only a minor interest in the measurement of mobilities and need only rough such values. Although the possibility of making mobility measurements between gassing electrodes has not been proven, the last-mentioned fact of course reduces the possible disadvantage of gas bubble formation.

Gassing electrodes are also connected with radical pH changes — in general, free acid is formed at the anode, and free alkali at the cathode — and with appreciable oxidation-reduction potentials. The former property has always been regarded as their least desirable property, partly because most substances investigated by electrophoresis are very sensitive to pH changes, partly since

the acid and alkali formed at the electrodes are believed, due to extremely high mobilities of the H^+ and OH^- ions, to reach the separation zone much more rapidly than concentration changes occurring at other electrodes. It will be shown later in this article that this argument does not apply in buffered media.

A very favourable property of a gassing electrode is the fact that it can be immersed directly into the electrolytic medium used in the run, whereas a non-gassing one requires a quite special electrolytic surrounding in order to function properly. The latter property necessitates a more complicated, and more expensive, electrode vessel. Further, a gassing electrode need not have a large surface; a short platinum wire is in general sufficient.

With due consideration to all the above points of view, it is quite evident that the difference in cost between the two types of electrodes, together with a properly constructed electrode vessel, is quite appreciable, even if the gassing electrode is made of platinum.

MOVING BOUNDAY SYSTEMS ORIGINATING AT NON-GASSING ELECTRODES.

Tiselius [5] gave the following equation for the volume V of buffer that has to be interposed between the U-tube and the electrolyte solution surrounding the electrode:

$$V = \frac{u\ v}{u_A - u_B}$$

where u_A and u_B are the mobilities of the components to be separated, v the desired volume of the separated components, and u the highest mobility of the ions in the solution round the electrodes. This equation is based on the assumptions that there is no convective transport of ions, and that the foreign ions travel towards the separation zone under the influence of the field strength prevailing in the buffer. In the light of Dole's [9] theory of moving boundary systems, we know now that none of these assumptions is necessarily true. As a matter of fact, the original boundary in the electrode tube between the electrode solution and the buffer will split up into a moving boundary system under the influence of the current in quite the same way as does the starting boundary in the U-tube. Consequently a number of moving boundaries are formed in each electrode vessel, each interboundary layer having its own density, conductance, and field strength. The different densities determine whether the migration will be convection-free or not, while the different conductances determine the absolute velocities of the boundaries.

The nature of the moving boundary systems present in the electrode vessels in the case of silver-silver chloride electrodes has been described by Brattsten [10] in an investigation that in thoroughness and clearness leaves nothing to be desired. She found that the moving boundaries originating from sodium chloride/buffer starting boundaries were, for many commonly used buffers, gravitationally unstable and gave rise to convection as soon as they were formed.

MOVING BOUNDARY SYSTEMS ORIGINATING AT GASSING ELECTRODES.

This discussion will be restricted to electrode/electrolyte systems that give rise to hydrogen gas and alkali at the cathode and to oxygen gas and acid at the anode. The moving boundaries originating from such electrodes can thus be characterized as pH boundaries. Very little is known concerning the behaviour of such boundaries in buffered media. A direct application of eqn. (1), using the mobilities of free H^+ and OH^- ions, would in this case lead to excessively large buffer volumes, due to the extremely high values of these mobilities. There are, however, reasons to believe that acid and alkali boundaries migrate much more slowly in buffered media, because hydrogen and hydroxyl ions entering a buffering zone will be extensively converted into water or into other, charged or uncharged, particles. Some simple experiments which support this view will be reported in the next sections of this article, but a more extensive investigation, including a theoretical treatment of this topic, would be highly desirable.

The pH boundaries, too, may be gravitationally stable or unstable, and it is very important to establish the conditions for convection-free boundary migration and to construct the electrode vessels accordingly. The author has made some simple experiments in order to reveal the most important facts.

Two beakers and a glass tube bridge between them were filled with the buffer solution in question. A trace of an acidimetric indicator, showing colour changes at low as well as at high pH values was added to the buffer. One platinum electrode was put into each beaker, and a direct current was sent through for some time. At the top of the anode beaker, a liquid layer with the acid colour of the indicator was then formed, and at the bottom of the cathode beaker, a layer with the alkaline colour developed (Fig. 1). The result was the same with all different buffers tested: phosphate, mixed phosphate and chloride, acetate, and barbital buffers with sodium as the cation.

These results can of course not be generalized by saying that the electrolytically generated acid is always lighter and the alkali always heavier than the buffer. This has to be tested experimentally in each special case as long as no theoretical treatment is available. Any experiments with ammonium salts
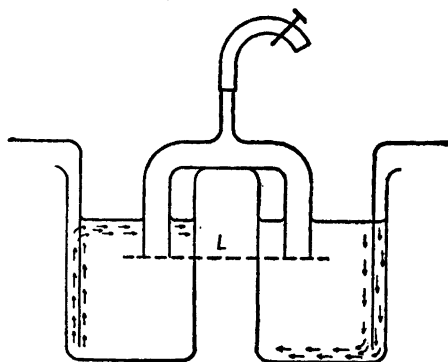

Fig. 1. Showing liquid streamings at platinum electrodes during electrolysis of common buffer solutions with sodium as cation. Electrolytically generated free acid, being lighter than the buffer, collects at the surface of the anode beaker, while the opposite holds for the generated free alkali in the cathode beaker. Acid enters the siphon when the buffer volume above level L has been replaced by acid. Alkali enters the siphon when the volume below level L has been replaced by alkali.
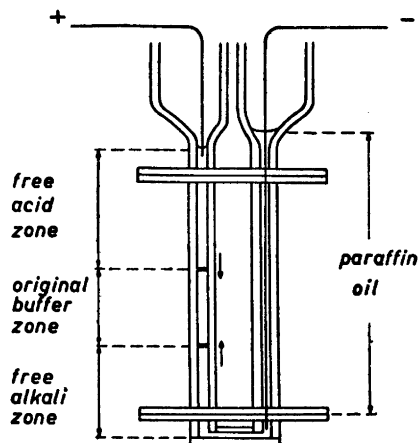
Fig. 2. Experimental arrangement for simultaneous measurement of migration rates of descending acid and ascending alkali boundaries.

have not been carried out, but it is quite certain that they would give the opposite result in the cathode compartment, since free ammonia is even lighter than water. The importance of these simple experiments, as well as of Brattsten's more extensive study, lies in the revealed fact that, in both cases, we have to do with convective ion transport, and that the problem of keeping foreign ions round the electrodes away from the separation zone is not simply a question of a minimum buffer volume in between. A convective ion transport may traverse any volume in a very short time. The bad effects of this convective transport, the ignorance of which has probably caused much trouble in accurate mobility measurements in many laboratories, can only be eliminated by an appropriate design of the electrode vessels. Such designs will be shown later in this article.

Another very interesting and important conclusion from the experiments mentioned above is the fact that the acid and alkali zones were found to grow with sharp fronts in the case of gravitational stability. This means that, with the buffer systems mentioned, an electrolytically generated zone can migrate with a stable, sharp, descending boundary, while an electrolytically generated alkali zone can migrate with a stable, sharp, ascending boundary. This is of course favourable, since sharp fronts do not require a great safety margin to be applied to calculated buffer volumes.

MIGRATION SPEEDS OF ACID AND ALKALI FRONTS IN BUFFERED MEDIA.

For such measurements, the experimental arrangement shown in Fig. 2 was used. In an electrophoresis U-tube according to Tiselius, the left-hand limb of the U-tube, the bottom section, and a small part of the left-hand part of the top section were filled with the buffer solution to be investigated. The right-hand limb of the U-tube and a small portion of the right-hand part of the top section were filled with paraffin oil. The platinum anode was inserted in the left-hand top compartment, while the cathode was put into the bottom sec-

tion, through the paraffin oil layer. After starting the direct current through the U-tube, one descending and one ascending boundary, both exceedingly sharp, could readily be seen in the camera. The migration rates of these boundaries were measured for some different, commonly used buffers.

In order to interpret these measurements, the following working hypothesis was put forward, with reference to Fig. 2. Experimentally it was found that the boundaries migrated at constant rates if the current was kept constant. Moreover, it was assumed that the three liquid phases, the acid phase at the top, the alkali phase at the bottom, and the buffer phase in between, all have constant compositions during the migration of the boundaries towards each other. If this hypothesis were true, both migration rates could be predicted since the buffer cationic radical disappears at the acid boundary, and the buffer anionic radical disappears at the alkali boundary. Consequently, if the field strength in the buffer phase is calculated from the equation:

$$E = \frac{i}{q \, \varkappa} \qquad (2)$$

where $\varkappa$ is the conductance of the original buffer solution, q the cross section area, and i the current, and if the observed migration speeds are divided by this field strength, two mobilities would be obtained which would be expected to check with the mobilities of the two ion constituents of the buffer. Further, if these mobilities were multiplied with the respective equivalent concentrations, then added, and again multiplied with the Faraday constant, then the conductance of the original buffer solution would be obtained, according to the well-known formula:

$$\varkappa = F \sum z_i \, c_i \, u_i \qquad (3)$$

where z_i are valences, with signs, c_i molar concentrations (positive), and u_i mobilities, with signs.

The working hypothesis was thus very easy to control experimentally. In Table 1, the necessary experimental data are collected, together with the conductivity check outlined above. The conclusion to be drawn from this table is that the working hypothesis does not hold quantitatively, since the calculated conductivities only have the correct order of magnitude. Nevertheless, the experiments show that the migration rates of the acid and the alkali fronts have little to do with the mobilities of free hydrogen and hydroxyl ions. They are instead approximately dictated by the mobilities of the ion constituents of the buffer.

The experiments were, for the sake of pure curiosity, continued after the two boundaries had met. It was then found that they united into one boundary (which could be expected) migrating downwards at a lower speed than both original boundaries. The explanation of this may lie in the following mechanism. If, still, the working hypothesis were true, the lower, purely alkaline phase would border directly on the upper, purely acidic phase, and the current would tend to pull the alkali solution further down, to pull the acid solution further up, and to develop a layer of pure water in between. Such a

Table 1. *Showing four experimental tests of the possibility of calculating the conductivity of the buffer from the migration rates of the acid and alkali fronts.*

Measured and calculated conductances ohm⁻¹ cm⁻¹	Ion consti- tuent	Concent- ration c cm⁻³	Valence z	Mobility u cm²/voltsec	Product czu
$2.66 \cdot 10^{-3}$	Na⁺	$68 \cdot 10^{-6}$	$+1$	$+24.5 \cdot 10^{-5}$	$+1\ 666 \cdot 10^{-11}$
	H⁺	$40 \cdot 10^{-6}$	$+1$	$-42.9 \cdot 10^{-5}$	$-1\ 716 \cdot 10^{-11}$
	PO₄⁻⁻⁻	$36 \cdot 10^{-6}$	-3	$-42.9 \cdot 10^{-5}$	$+4\ 633 \cdot 10^{-11}$
$4.42 \cdot 10^{-3}\longleftarrow$			$\times 96\ 496 \longleftarrow$		$+4\ 583 \cdot 10^{-11}$
$2.35 \cdot 10^{-3}$	Na⁺	$1 \cdot 10^{-4}$	$+1$	$+18.2 \cdot 10^{-5}$	$+18.2 \cdot 10^{-9}$
	H⁺	$1 \cdot 10^{-4}$	$+1$	0	0
	Ac⁻	$2 \cdot 10^{-4}$	-1	$-7.03 \cdot 10^{-5}$	$+14.06 \cdot 10^{-9}$
$3.11 \cdot 10^{-3}\longleftarrow$			$\times 96\ 496 \longleftarrow$		$+32.26 \cdot 10^{-9}$
$3.73 \cdot 10^{-3}$	Na⁺	$1 \cdot 10^{-4}$	$+1$	$+19.7 \cdot 10^{-5}$	$+19.\ 7 \cdot 10^{-9}$
	H⁺	$3 \cdot 10^{-4}$	$+1$	0	0
	Ac⁻	$4 \cdot 10^{-4}$	-1	$-6.3 \cdot 10^{-5}$	$+25.\ 2 \cdot 10^{-9}$
$4.33 \cdot 10^{-3}\longleftarrow$			$\times 96\ 496 \longleftarrow$		$+44.9 \cdot 10^{-9}$
$3.78 \cdot 10^{-3}$	Na⁺	$1 \cdot 10^{-4}$	$+1$	$+19.9 \cdot 10^{-5}$	$+19.9 \cdot 10^{-9}$
	H⁺	$3 \cdot 10^{-4}$	$+1$	0	0
	Ac⁻	$4 \cdot 10^{-4}$	-1	$-5.9 \cdot 10^{-5}$	$+23.6 \cdot 10^{-9}$
$4.20 \cdot 10^{-3}\longleftarrow$			$\times 96\ 496 \longleftarrow$		$+43.5 \cdot 10^{-9}$

(In the phosphate experiment, the mobility of the hydrogen ion constituent must be assumed to be equal to that of the phosphate ion constituent, since all hydrogen is bound to the phosphate ions. In the acetate experiments, the mobility of hydrogen must be assumed to be zero, since practically all hydrogen is present in the undissociated species HAc.)

system is apparently gravitationally unstable and causes the acid to fall down again on top of the alkali layer. Although the current tends to concentrate the acid more and more at the anode, the density relationships and convective currents cause the acid solution to become more and more dilute, while in the bottom compartment the alkali can concentrate without interference from convection. The fact that the working hypothesis was found not to be true does not completely invalidate this theory; it is only necessary to modify it.

Next, it was tried to find out what was wrong with the working hypothesis. Since the calculated values of the buffer conductances were consistently higher than those measured, it could be suspected that the buffer ions actually did not disappear completely at the migrating sharp fronts, but were lagging behind. This could be shown to be the case in two ways. By using the newly developed, more sensitive interferometric techniques (Svensson [11],[12]), the sharp fronts were actually found to possess a tail on the side towards the

electrode, indicating a non-constant composition just behind the front. In addition, experiments were performed in a preparative way as follows.

In our research model electrophoresis apparatus, the cathode vessel, the cathode limb of the U-tube, and the bottom section were filled with sodium hydroxide of a density well above that of the buffer. This consisted of a mixture of monochloroacetic acid and its sodium salt, and it was filled in the rest of the apparatus. On closing the current, a very sharp alkali front migrated upwards in the anode limb of the U-tube. Its migration rate was measured. In order to control, however, if chloroacetate ions were lagging behind this boundary, it was allowed to migrate through one whole U-tube cell section, which was then pushed to the side after breaking the current. The contents of said cell section were then tested for chloride ions with silver nitrate and nitric acid, after the chloroacetate had been completely hydrolyzed by strong sodium hydroxyde at 100° C.

It was found that chlorine was consistently present below the ascending alkali boundary. This explains why the working hypothesis presented above could not give quantitative agreement with experiments.

It was also found that the mobility, calculated from the migration velocity of the ascending alkali boundary and the field strength in the buffer layer, varied in a regular manner with the proportion between chloroacetate and free chloroacetic acid. This is shown in the graph in Fig. 3. The mobility of the alkali boundary rises very rapidly as the concentration of excess acid decreases, *i. e.* as the buffering capacity decreases. As a matter of fact, if one tries to
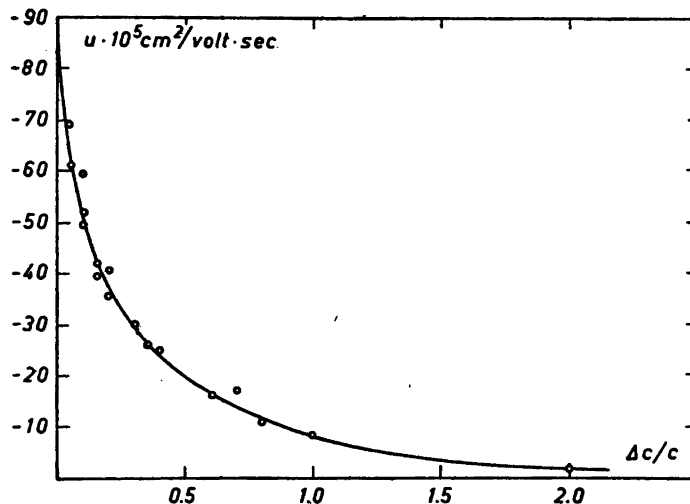

Fig. 3. Migration velocity of alkali front moving into a sodium chloroacetate buffer, per unit field strength in the buffer, as a function of the relative excess of free acid in the buffer, at an ionic strength of 0.1 and a temperature of 0 °C. The value extrapolated to zero acid excess checks with the mobility of free hydroxyl ions (u_{OH} at infinite dilution $= -109 \cdot 10^{-5}$), but already at a relative excess of 0.5, the alkali front migrates more slowly than the buffer anion ($u_{ClAc} = -18.5 \cdot 10^{-8}$).

extrapolate this mobility to zero acid excess, one gets a figure not far from the mobility of free hydroxyl ions. The extrapolation is difficult, however, since the very sharp front characterizing the alkaline zone gradually becomes more diffuse as the excess free acid is decreased.

CONSEQUENCES AS TO THE CONSTRUCTION OF ELECTRODE VESSELS.

The most important result of the above discussion and experiments is the fact that one always has to take into account the density relationships of the moving boundary systems developed by the current in the electrode vessels. Some moving boundaries are gravitationally stable on migration upwards and will give rise to convection if they are forced to migrate downwards, and *vice versa*. An electrode vessel must therefore necessarily be constructed so that the current has a vertical, downward direction in one part of it, while in another part it has an upward direction. By courtesy of Dr. Brattsten, the author was informed of her experimental results at an early stage, which made it possible to incorporate the electrode vessel shown in Fig. 4 into our research model electrophoresis apparatus types LKB 3021 and LKB 3023, which were developed in the years 1948/49. In the central part of this tube, the current flows downwards, while in the outer mantle, it flows upwards, or *vice versa*. A moving boundary gravitationally unstable in one part, is stable in the other. In any case, therefore, there is an appreciable volume of buffer that is effective in delaying the interference of electrode reaction products with substances in the separation zone.

In the paper electrophoresis apparatus described by Valmet and Svensson [13], the electrode vessels are constructed in a similar manner.

ACID AND ALKALI LOCKS.

The experiments reported here have shown that the buffer volume required between electrodes and separation zone is not greater for platinum than for silver-silver chloride electrodes. A volume 10 times as large as that of the separation zone seems to be sufficient under standard experimental conditions. This volume is so small, that there is no need for reducing it any further. If, however, an apparatus constructed for standard conditions has to be used in a prolonged electrophoretic separation, the capacity of the standard electrode vessels is of course insufficient, and the question arises how to circumvent this obstacle without making bigger vessels. Of course it is possible to make use of Tiselius' suggestion of having an increased salt concentration in the electrode vessels, as referred to in the introduction, but, in the case of gassing electrodes generating acid and alkali, there is a much more effective way of delaying the advance of the pH boundaries coming from the electrodes. This consists in the use of acid and alkali locks and has already been described by the author [14] in another connection. Since that publication is not easily available everywhere, however, the principle will be presented here again.

Let us suppose that we are using an acetate buffer, and that we want to delay the advancing alkali front in the cathode tube, which may be assumed to
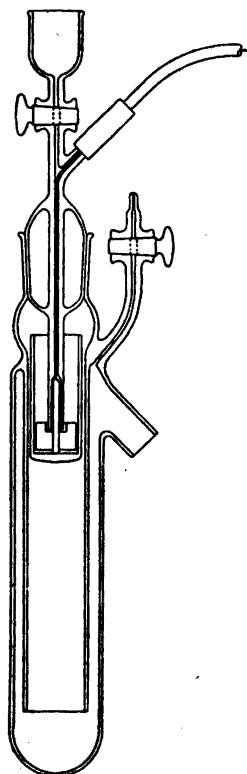
Fig. 4. Electrode vessel belonging to LKB 3021 and 3023 research model electrophoresis apparatus. The volume is subdivided into two parts, which forces the current to pass upwards in one part and downwards in the other. A moving boundary which is gravitationally unstable in one part can always migrate convection-free in the other part.

have the construction according to Fig. 4, but with a platinum electrode instead of the silver one shown in the figure. That can be done by placing a suitable volume of strong acetic acid in the bottom of the tube. This solution must of course be heavier than the buffer. The alkali generated at the electrode will then have to neutralize this acid before it can start to develop an ascending boundary in the outer mantle of the tube. The necessary quantity of acid can easily be calculated by a simple application of Faraday's electrolytic laws. If the acid in question is slightly soluble (barbital), a paste of crystals can be used instead of a concentrated solution.

The new starting boundary between acetic acid and acetate buffer that is introduced in this way will of course develop a moving boundary system during the electrolysis. No boundary migrating towards the U-tube can, however, be expected, since there is only one negative ion constituent in the system (cf. Dole's theory). The only moving boundary developed from this starting boundary is a pH boundary migrating away from the separation zone.

In the anode vessel, the acid boundary can be correspondingly delayed by an alkali lock. In the case of ammonia or other light bases, it must be remembered that the required density must be achieved by the addition of a suitable non-electrolyte, e. g. sugar.

The principle of acid and alkali locks should not be applied in mixed buffers. As an example, let us suppose that we are using a phosphate-chloride buffer and that a mixture of phosphoric and hydrochloric acids is used as an acid lock in the bottom of the electrode tube in Fig. 4. According to Dole's theory, this starting boundary will give rise to one boundary moving in the direction towards the U-tube. This boundary may also be gravitationally unstable and thus give rise to convection. Only if a certain relation between the two acid concentrations is satisfied does this moving boundary disappear, but this relation is very difficult to predict without experiments.

The principle of acid and alkali locks has been used in this laboratory on many occasions and is, according to our experience, both simple to apply and effective.

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