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Determination of Free Protein Mobilities by Paper Electrophoresis with Evaporation *

I. Evaluation of the Buffer Flow Due to Evaporation and Electroosmosis

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A procedure was devised for the determination of electrophoretic mobilities by means of open-strip paper electrophoresis.

An equation covering evaporation and electroosmosis was derived. The fundamental premise is the constancy of the electric current during the experiment. Evaporation and electroosmosis are represented by the parameters f and e, respectively. The determination of these parameters may be omitted in the calculation of electrophoretic mobilities, since a correction for both evaporation and electroosmosis is also obtained by running a single aliquot of an uncharged substance parallel to the sample.

The equation was tested in an experimental series where current and concentration were systematically varied. It was thus confirmed that the migration takes place in a zone of increasing, but uniform, salt concentration. This was further substantiated by independent procedures. The migration of dextran spots placed evenly along the strip was found to be in agreement with the theoretical basis. Straight lines were invariably obtained when the migration distance was plotted against the position of the application points. When the migration distance was first divided by time and voltage, all the lines intersected the ordinate at the same point, and the slopes were proportional to the electric current, thus demonstrating the constancy of the parameters f and e.

Problems arising from the presence of evaporation in paper electrophoretic mobility determinations are discussed. It is emphasized that a satisfactory method is obtained by using constant electric current, instead of the constant voltage hitherto considered essential.

Since the appearance in 1937 of the first report on zone electrophoresis [1] numerous technical modifications, aiming at the achievement of greater reproducibility and precision, have been introduced by various authors in order

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to raise the reliability of the method to the level attained by free electrophoresis.

Undoubtedly this object has been accomplished as far as fractionation and clinical analysis of proteins are concerned, where zone electrophoresis, especially on paper strips, has even proved superior in several aspects to the classical Tiselius technique. However, serious difficulties are encountered in determinations of the electrophoretic mobility since interfering factors unknown from the Tiselius method, *i. e.* the electroosmotic flow, the evaporation of the buffer due to heat development and the influence of the carrier medium, have to be taken into account.

On the other hand, paper electrophoresis offers so many advantages, one of them being the minimal amount of substance required, that it may be considered a matter of great interest to arrive at a satisfactory method for the determination of mobilities and isoelectric points by means of this technique.

The mobility obtained in free solution at a defined pH and temperature, although in some degree dependent on the type and concentration of the buffer used, may be considered a characteristic property of a protein and is referred to as *free mobility* in the following. In order to have any practical value, a method using paper electrophoresis would have to yield the same free mobilities.

Previous approaches are scarce and seem in several respects open to criticism. In the present study the effects of the interfering factors have been evaluated quantitatively and a procedure developed yielding results identical with those obtained by free electrophoresis. In the first part of this investigation the buffer flow caused by the combined effects of evaporation and electroosmosis has been studied, and it is shown that, when a constant current is used throughout the experiment, a simple expression can be derived for the mobility of a migrating substance.

MATERIALS AND METHODS

Apparatus. Horizontal open strip electrophoresis sets with buffer cells of about 1 000 ml capacity were employed, each holding two paper strips. The height of the buffer levels was equalized by means of leveling glass tubes prior to equilibration of the strip. The paper rested upon nylon threads between two 29 × 8.5 cm glass plates, kept 6 mm apart and tightened at the edges by plastic borders which held the nylon threads. At all times the strip was in strictly horizontal position. The length of the vertical paper section to the buffer surface was 4.5 cm. The voltage gradient over the paper was measured by secondary platinum electrodes fixed to two of the nylon threads at 15.0 cm distance from each other. The power supply was a valve rectifier in conjunction with an adjustable electronic current stabilizer, giving a constancy of about 0.1 %.

Solutions. Sodium acetate buffers of pH 5.10 and of 0.045 and 0.09 ionic strength.

4 % solutions of dextran (Pharmacia, Uppsala) in the buffer, prepared by gentle heating.

Procedure. Two 8 × 39 cm strips of Munktell paper No. 20/150 were used per cell unit. The strips were immersed in buffer for a few seconds and blotted on crepe-paper before being placed into the apparatus. After a minimum of 3 h equilibration, six 10 μl spots of dextran solution were applied at 3 cm distance from each other measured along the longitudinal axis of the paper. In order to avoid interference, they were placed alternately to the right and the left at 2.5 cm distance from the paper edge.

The current was switched on immediately after the application of the solution to the paper, and the experiments were run for 17 to 20 h at room temperature ranging from 20.8 to 21.5°C. Current and voltage were recorded at the beginning and conclusion of the experiment. Corrections for voltmeter resistance were introduced and a correction of 0.8 V (0.053 V · cm^{-1}), due to the polarization of the secondary platinum electrodes was added to all readings. The polarization was estimated by measuring the voltage between 3 platinum electrodes A, B, and C on the paper with constant current. The difference between the voltage readings AB + BC and AC would be equal to the polarization potential. The same value was obtained from rapid reversal of the electrode polarization.

After conclusion of the experiment, the vertical paper ends were cut off, and the strips dried horizontally in the air at room temperature, after which they were placed for 30 min in a hot air oven at 110°C and then stained for 25 min with a 1 % bromo phenolblue (Merck) solution in 95 % ethanol containing about 10 % (w/v) $HgCl_2$. Since the dextran spots appeared most clearly during the first few minutes of staining, their centers were marked with a pencil during the process. Finally, the strips were washed several times with methanol until the appearance of a white background, and the migration distance measured.

THEORY

The equations derived below are based on a few simple assumptions which, as well as the conclusions drawn from them, have been experimentally tested, as described in the next paragraph.

The symbols used will be defined below in connection with the explanations. Distances and velocities are considered positive in the direction anode to cathode, according to the usual convention.

It is assumed that the migration velocity ds/dt is made up of three separate components in the following way:

$$\frac{ds}{dt} = m \cdot V + e \cdot V + (a - s)\ \mathrm{L} \qquad \text{(I,1)}$$

The first term gives the migration rate due to the influence of the electric field on the migrating particle and is proportional to the potential gradient V. It appears as $m \cdot V$ in equation I,1. The factor m used for the mobility differs from the free mobility u on account of the retarding effects of the carrier medium. This problem will be treated in the subsequent communication [2].

The two other terms express the migration velocity caused by the buffer flow. $e \cdot V$ represents the electroosmotic flow, which too is approximately proportional to V. The last term of the equation gives the migration velocity due to evaporation. Assuming uniform evaporation along the strip and that the capillary forces keep the amount of solution in the paper constant, this component of the migration velocity will be proportional to the distance

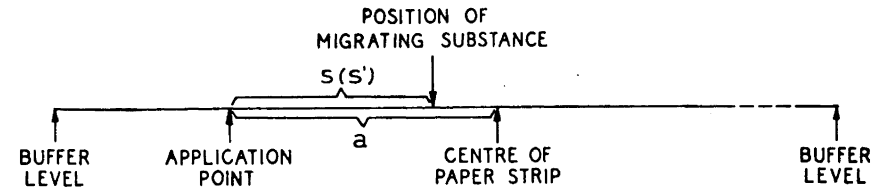

Fig. 1. Schematic presentation of the experimental arrangement.

between the sample and the center of the paper strip, as expressed by $(a—s)$, where a is the distance from the point of application to the paper center and s the distance of migration (Fig. 1).

L denotes the intensity of evaporation and may be assumed to be proportional to the wattage dissipation $(i \cdot V)$ in the paper:

$$L = f \cdot i \cdot V \tag{I,2}$$

The increase of the salt concentration caused by the evaporation from the strip produces a corresponding increase of the buffer conductivity. Working with constant current this results in a steadily decreasing voltage V. To obtain the correct time function we again consider Fig. 1. s' now represents the distance which the buffer has migrated by evaporation alone. The concentration has increased by the factor $\frac{a}{a—s'}$, and, assuming that the activity coefficients remain practically constant, as in the case of monovalent ions in the concentration range usual in electrophoresis, the voltage has dropped correspondingly from its initial value V_0:

$$V = V_0 \frac{a—s'}{a} \tag{I,3}$$

The migration velocity ds'/dt of the buffer as caused by evaporation will, in analogy to the third term of eqn. 1, be determined by

$$\frac{ds'}{dt} = (a—s')\ L \tag{I,4}$$

and substituting eqns. 2 and 3 we obtain

$$\frac{ds'}{dt} = fiV_0 \frac{(a—s')^2}{a} \tag{I,5}$$

Integration and combination with eqns. 2 and 3 result in

$$\frac{a}{a—s'} = 1 + fiV_0t \tag{I,6}$$

$$V = \frac{V_0}{1 + fiV_0t} \tag{I,7}$$

$$L = \frac{fiV_0}{1 + fiV_0t} \tag{I,8}$$

Substituting eqns. 7 and 8 in eqn. 1 we arrive at

$$\frac{ds}{dt} = \frac{m \cdot V_0}{1 + fiV_0t} + \frac{eV_0}{1 + fiV_0t} + \frac{(a—s)fiV_0}{1 + fiV_0t} \tag{I,9}$$

which after integration gives

$$s = (m+e+afi)\ \frac{V_0 t}{1 + fiV_0 t} \qquad \text{(I,10)}$$

A more convenient expression is obtained by substituting eqn. 7, resulting in

$$s = (m+e+afi)V_t t \qquad \text{(I,11)}$$

where the voltage V_t at the time t has to be used in the calculation.

When an uncharged substance such as dextran is used, eqn. 11 reduces to

$$s_d = (e+afi)V_t t \qquad \text{(I,12)}$$

When protein and dextran are run under identical conditions and applied at the same distance from the center, the difference in migration distance is given by

$$s_p - s_d = mV_t t \qquad \text{(I,13)}$$

obtained by subtracting eqn. 12 from eqn. 11.

It appears from this fundamental equation that by running an uncharged substance parallel to the protein, not only is it possible to correct for electroosmosis but also for the more complicated effects of evaporation. The voltage referred to in eqn. 13 is that measured at the end of the experiment, and a necessary condition for the validity of this equation is the use of a constant current during the whole run.

Once the parameters e and f have been determined, it should be possible to obtain mobility values corrected for evaporation and electroosmosis from eqn. 11, without the use of an uncharged indicator substance. However, since e and f may vary with the experimental conditions, we have preferred to apply dextran spots in each mobility determination, and to use eqn. 13 in the calculations.

EXPERIMENTAL VERIFICATION

The validity of the propounded equations was tested in a number of experiments in sodium acetate buffer of pH 5.1. To visualize the movement of the buffer solution, dextran spots were placed at various positions. In chromatographic experiments with the same buffer and the same paper [3] dextran yielded a R_F-value of approximately 1.0. It may thus be concluded that dextran follows the surrounding fluid and that its displacement closely reflects the movement of the buffer solution. This is further substantiated by voltage measurements described below. The slight electrophoretic mobility of dextran can be neglected, since it amounts to only $-0.16 \times 10^{-5} \cdot \text{cm}^2 \cdot \text{V}^{-1} \cdot \text{sec}^{-1}$ in pH 8.8 barbital buffer [4] and may be expected to be even less at pH 5.1.

When the migration distance of the dextran spots was plotted as function of their starting positions, straight lines were obtained (Fig. 4). Similar experiments have been performed by other authors [5,6,12] and they prove that the salt distribution remains uniform within the dextran zone. This was controlled in

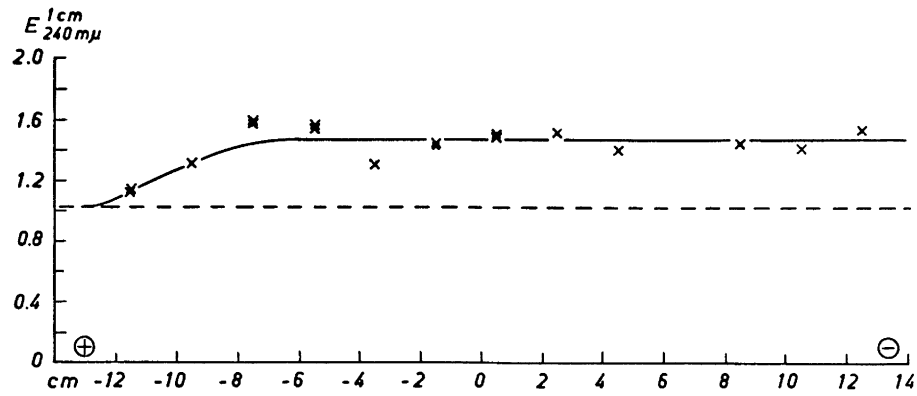

Fig. 2. The concentration increase caused by evaporation. The dotted line represents the original concentration. Determinations by U.V. absorption. pH 8.8 barbital buffer of $\Gamma/2$ 0.08 employed.

an experiment with 5 secondary platinum electrodes placed at various positions along the paper. The conductivity was measured at several time intervals. It rose twofold during the run, but was at any time uniform along the strip. Moreover, it was found by weighing that the amount of buffer in the paper — 27.5 μl/cm^2 — did not change during the experiment.

In an experiment with pH 8.6 barbital buffer, the paper strip was cut in sections perpendicular to the electric field after the current had passed for 19 h. The paper sections were eluted and the barbital concentration determined by measuring the extinction at 240 mμ. The results are shown in Fig. 2, and it is evident that there is a broad zone of increased, but uniform, concentration.

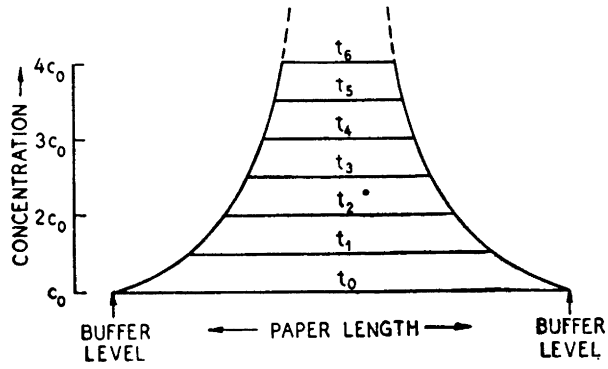

Fig. 3. Schematic presentation of the uniform concentration zone according to theory showing the shrinkage with time and the concentration increase owing to evaporation. The additional effect of electroosmosis will produce a certain skewness varying with the experimental conditions. The t values recorded are arbitrary.

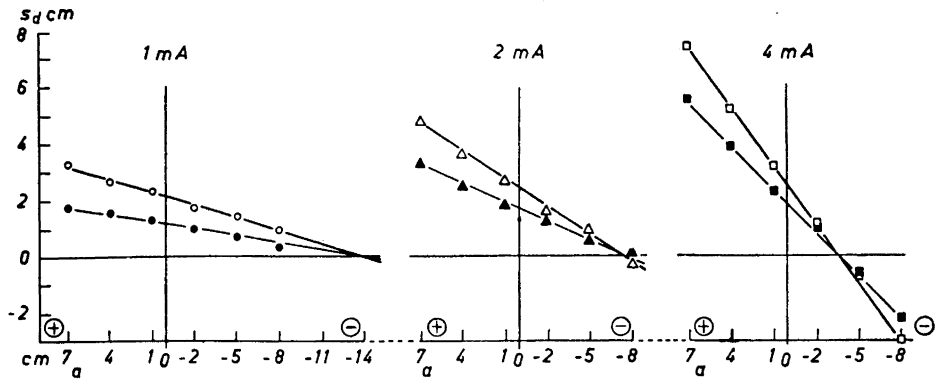

Fig. 4. Dextran migration with different currents and buffer concentrations. Migration distance (s_d) plotted against the position of the application points (a). The scale of the abscissa of this and the following figure reads from right to left because a is measured from application point to paper center.

	Sodium acetate pH 5.10	
mA	$\Gamma/2$ 0.045	$\Gamma/2$ 0.090
1.0	○	●
2.0	△	▲
4.0	□	■

The only possible explanation of all these findings is a homogeneous evaporation from the strip together with a fluid displacement resulting in uniform buffer distribution.

It is clear that toward each end of the paper we come to a point where the concentration begins to fall off until, at the buffer level, we reach the original concentration. This is seen in Fig. 2 and schematically presented in Fig. 3, where it is shown how the zone of uniform concentration shrinks with time.

It is essential that the substance, the mobility of which is to be determined, as well as the electrodes used for voltage measurements are within this zone. This condition can easily be fulfilled. In our mobility measurements [2] performed over 16—19 h with 2.5 mA and 2—3 V/cm, the evaporation amounted to about 30 % of the buffer solution in the paper with a corresponding reduction in length of the uniform concentration zone. In recent experiments even 10 mA/strip have been routinely employed for the same length of time. By increase of the strip length still wider ranges of current and time could be used.

Uniform buffer distribution and straight lines when plotting dextran migration against starting positions will be obtained regardless of any variations in voltage and current during the experiment. Under defined conditions, however, it is possible to calculate the position of the spots at a certain time. The assumptions made in the theoretical part thus led to eqn. 12. In order to test this equation, experiments were performed at different currents and buffer concentrations. Representative results are shown in Fig. 4.

The point of intersection of the dextran lines with the abscissa gives the position on the paper termed π by Macheboeuf *et al.*[7,8] At this point the buffer displacement caused by evaporation is exactly counterbalanced by the electro-osmotic flow. It is apparent that the lines obtained at different ionic strengths but identical current intersect the abscissa at the same point. This is in accordance with eqn. 12. With $s_d = 0$, also $(e+afi)$ must be equal to zero, and therefore the corresponding application distance

$$a_0 = -\frac{e}{fi} \qquad \text{(I,14)}$$

As e and f are constants, a_0 will be constant with constant current, irrespective of variations in the other conditions. It follows further from eqn. 14 that a_0 will be inversely proportional to i. Comparison of the values of a_0 obtained from Fig. 4 at 1.0, 2.0 and 4.0 mA demonstrates that this is indeed the case.

To evaluate the slopes of the various dextran lines, we write eqn. 12 in the form

$$\frac{s_d}{V_t t} = e+afi \qquad \text{(I,12a)}$$

In Fig. 5, $\frac{s_d}{V_t t}$ *viz.* the migration distance divided by time and final voltage, is plotted against a.

As to be expected from eqn. 12a, lines from experiments with the same current coincide, and the slopes are proportional to the current applied. Further, the intersection with the ordinate is very nearly the same with all currents, indicating the constancy of e, and thereby the validity of the second term of eqn. 1.

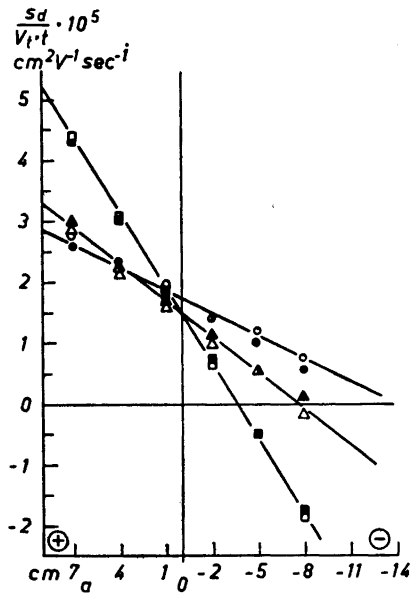

Fig. 5. Dextran migration distance divided by time and final voltage as function of the position of the application points.

	Sodium acetate pH 5.10	
mA	$\Gamma/2$ 0.045	$\Gamma/2$ 0.090
1.0	○	●
2.0	△	▲
4.0	□	■

The value of e can be obtained from Fig. 5. It is found to be about 1.6 $cm^2 \cdot sec^{-1} \cdot V^{-1}$ and shows the magnitude of the electroosmotic flow under the experimental conditions. The small deviations observed in these and similar experiments indicate that the electroosmotic flow is rather sensitive to minor experimental variations.

It has been claimed that the electroosmotic flow may vary along the length of the paper strip. With uniform evaporation and buffer distribution, as demonstrated by our experiments, it follows from obvious reasons that the electroosmotic flow must be uniform even if the driving force varies along the paper. The constant amount of buffer held in the paper by capillary forces behaves like a fluid in a tube with a uniform cross sectional area.

The concentration being uniform in the central part of the strip, the evaporation can be followed by the voltage decrease as well as through the slope of the dextran lines. Comparisons made according to eqn. 3 revealed a scattering of a few per cent, but on an average the agreement between V_0/V_t and the "dextran shrinkage" (ratio of distance between starting positions to distance between final positions) was excellent.

The fact that the slopes in Fig. 5 are indeed proportional to the current demonstrates that the factor f in eqn. 2 is constant with different currents. Experiments covering a 50 fold variation of the initial watt input showed only minor deviations in the value of f.

It follows from eqn. 2 that f indicates the fraction of the watt input consumed by evaporation. When the buffer has moved the distance s' (Fig. 1), an amount of water equal to $w \cdot s'$ has evaporated, w being the amount of water present in 1 cm length of paper. $585\ w \cdot s'$ cal or $585 \times 4.18\ w \cdot s'$ W sec. are used for evaporation, and the dissipation of electrical energy is determined by $\int (a - s')\ iV dt$. After substituting eqn. 6 and integrating, the ratio is found to be $585 \times 4.18\ w \cdot f$. w was determined separately by weighing in a number of experiments and found to be 0.22 g at the employed paper width of 8 cm.

Taking $f = 1.1 \times 10^{-3}$ cm/watt · sec. from the dextran experiments, we find that about 60 % of the electrical input is consumed for evaporation within wide limits of current and voltage. This value probably will vary considerably with the type of apparatus and the ambient temperature. It was estimated at about 70 % by Macheboeuf *et al.* in their experiments [8].

According to eqn. 7, the reciprocal voltage plotted against time should result in a straight line. A typical example is shown in Fig. 6 where a perfectly straight line is obtained. Only in the beginning a greater increase in $1/V$ is noted. This undoubtedly is caused by an initial temperature increase. Extrapolation of the straight line to the ordinate gives the initial conductivity value at equilibrium temperature and the difference from the measured value indicates the temperature increase which here amounts to $\Delta T = 1.75$°C. It is apparent that temperature equilibrium is established within one hour after starting. The straight line of Fig. 6 proves the time relationship expressed in eqn. 7, which was used in the derivation of the final equations.

DISCUSSION

In developing a procedure for the determination of free mobilities on paper strips, the most important requirement is to establish a quantitative relation which makes it possible to correct for the effects of evaporation. The evapora-

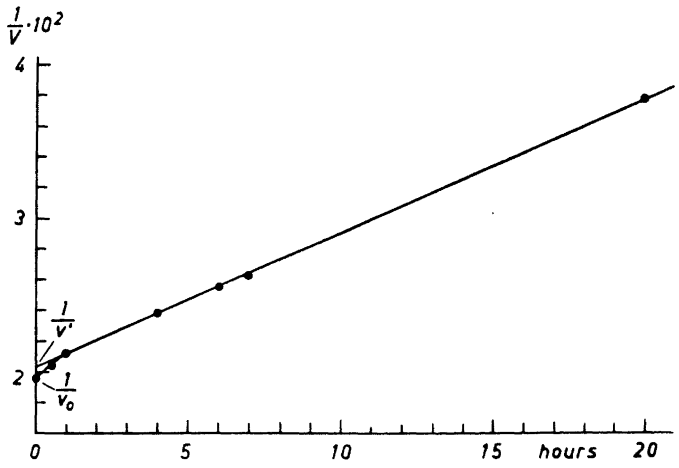

Fig. 6. Voltage decrease due to evaporation as function of time. Experiment carried out in $\Gamma/2$ 0.09 sodium acetate, pH 5.10. Current 3.0 mA; V: Voltage over 15 cm.

tion from the paper causes a continuous change in the salt concentration on the strip and thereby of the voltage gradient acting on the migrating substance, even if the voltage on the apparatus as a whole is kept constant. Furthermore, evaporation causes a flow toward the center of the paper. Since the velocity of this flow increases with the distance from the center its effect on the migration distance cannot be simply corrected for by comparison with that of an uncharged substance, because specimens differing in their migration velocities will be under the influence of different flow rates. Finally, the intensity of evaporation will change during the run because of variations in voltage and current.

In fact, most authors are of the opinion that a reliable method can only be worked out by suppressing evaporation. Low temperatures and very low currents or the addition of substances able to reduce the vapor pressure, such as glycerol [9] have been used to this effect. Even thus it is difficult to eliminate the evaporation to a degree where it may be fully neglected. The closed strip apparatus operated at low temperatures [4], in which the paper is compressed between glass plates and, in most cases, made air-tight by chemical seals, seems to be the best way to avoid distillation from the paper surface.

On the other hand, the open strip technique with free evaporation is claimed to give a much clearer resolution of the protein pattern [11] aside from preventing contact of the specimens with glass plates or alien substances used as seals, which might modify their migration rates. Also in experiments which employ the tracer technique, a contact with the enclosing plates should preferably be avoided. Any method which makes it possible to determine free mobilities using an open strip in the presence of evaporation would therefore be of great value.

An attempt to correct for the effects of evaporation was made by Woods and Gillespie [6] who, although working with a closed strip apparatus, found evaporation to be present in their experiments. The correction applied, however, is empirical and does not appear convincing. Moreover, as shown in the following communication [2], their mobility value for albumin in pH 8.6 barbital buffer is about 25 % lower than the corresponding free mobility.

An interesting approach to the problem of mobility determination with open paper strips was made by Macheboeuf *et al.*[7,8] This method even takes advantage of evaporation for the calculation of free mobility values. It had previously been pointed out by Durrum [10] that in the presence of evaporation a substance will finally reach an equilibrium position where the migration in the electric field is counterbalanced by the buffer flow caused by evaporation and electroosmosis. A necessary condition is that the evaporation remains constant with time. The time for the attainment of this equilibrium position is theoretically infinite and, as the duration of the experiment is limited on account of the increasing buffer concentration, satisfactory results cannot always be obtained. In Macheboeuf's method the substance is placed as a diagonal streak across the paper and the point is noted where it does not move to either side, this being the equilibrium position which can be discerned after a few hours. By applying an uncharged substance on the same paper, correction can be made for the effect of electroosmosis. The equilibrium position is, of course, dependent on the intensity of evaporation and, in order to obtain the electrophoretic mobility, a reference substance of known mobility is required.

In their calculations Macheboeuf *et al.* postulate a constant evaporation as well as a constant voltage gradient. On account of the increasing buffer concentration on the paper these two assumptions are incompatible. The theoretical basis of this approach is therefore more complex than apparent from Macheboeuf's equations. The equilibrium position will thus generally vary in the course of an experiment. Although the conditions are not defined as a function of time, in the determination of mobility the deviation will cancel out due to the use of a reference substance and correct values should be obtained. It should be pointed out that to ensure a uniform voltage gradient, also in Macheboeuf's method, all readings must be performed within the central zone of uniform concentration shown in Fig. 3.

In these, as in all other previous procedures aiming at the determination of free mobilities, a constant voltage was applied, and it seems generally accepted that a reliable determination of electrophoretic mobilities can only be achieved by the maintenance of a constant voltage throughout the experiment. It must be emphasized that this is not the case. The only necessary condition is a knowledge of the voltage as a function of time. Moreover, the maintenance of a constant voltage in experiments with evaporation presents difficulties of practical as well as of theoretical nature. In spite of a constant voltage between the electrode vessels, the concentration changes along the paper result in a continuous decrease of the voltage gradient in the central part where the measurements are performed. Even if this voltage should be kept constant by a suitable regulation, very complicated functions would determine the migration of a substance. A thorough calculation with experimental verification has as yet not appeared.

In the present paper it is demonstrated that all the described difficulties can be overcome by application of a constant current during the whole experiment. This has previously been recommended in order to minimize the influence of the temperature on the migration distance [11]. The application of a constant current in the present method differs fundamentally from this, since its object is the determination of the free mobility of the migrating substance. Under these conditions it has been possible to arrive at a simple expression for the mobility, taking into account the effects of both evaporation and electroosmosis.

In addition to the effect of the buffer flow thus established, the migration velocity of a protein depends on the properties of the carrier medium. It should, however, be stressed that the equations derived above for a medium where retarding factors do not exist, are still valid when these factors come into play as far as dextran is concerned. The influence of the carrier medium as well as of the temperature increase and change in ionic strength will be evaluated in the subsequent communication [2], together with the actual determination of serum protein mobilities.

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