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Determination of Free Protein Mobilities by Paper Electrophoresis with Evaporation *

II. Evaluation of Temperature and Concentration Increases, Influence of the Carrier Medium, and Measurement of Serum Protein Mobilities

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After the determination of the joint effect of evaporation and electroosmosis by means of an uncharged substance had been established, the final equation for the calculation of free solution mobilities was obtained by introduction of correction factors for the influence of the carrier medium.

A direct quantitation of *protein adsorption* was achieved on basis of the actual electrophoretic experiment. The presence of evaporation is indispensable for this evaluation. This emphasizes the value of evaporation for accurate mobility determinations, in contrast to hitherto prevailing opinions. Differing from γ-globulin, serumalbumin was not adsorbed in the media employed.

The *path-length increase* owing to the paper structure was calculated by means of the paper/buffer conductivity ratio and found to be a paper constant. The final, uniform *salt concentration* of the migration zone was evaluated by the migration of an uncharged substance or by the voltage drop. The *temperature increase* on the strip was determined through conductivity measurements at several time intervals and appears as a function of the watt input.

42 samples of human serumalbumin and γ-globulin were run in sodium acetate and barbital buffers of 0.10 ionic strength from pH 4.0 to 8.8, and the free mobilities calculated according to the derived equation. The results were converted to their values at 1°C and compared to moving-boundary data. Absolute agreement between our values and the free electrophoresis mobilities is found.

In view of the advantages inherent to paper electrophoresis, the development of the open-strip technique into an accurate method for mobility determinations may open new perspectives for enzymological, clinical and interaction studies.

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In spite of an immense amount of work on paper electrophoresis, very few attempts have been made to adapt this method for the estimation of protein mobilities. Presumably this is due to the prevailing opinion that any such attempt has to fail, because of the complicated effects produced by evaporation. Even when evaporation was suppressed, adsorption of the proteins to the filter paper seemed to prevent mobility determinations.

In the preceding paper [1] it was shown that the buffer flow caused by evaporation and electroosmosis can be calculated and the results experimentally confirmed. It is one of the objects of the present communication to describe how the temperature increase and the changes in the medium, inherent to the open-strip technique with free evaporation, can be evaluated and taken into account in mobility determinations.

However, the sole presence of a carrier medium produces some complications which have to be dealt with in the first instance. The migration rate of a given substance, moving on a semisolid support such as moist filter paper, would obviously differ from that found in free solution even if all other conditions could be kept identical. The rate must be lower due to the retarding effect connected with the intricate geometrical structure of such a supporting medium, and to a possible adsorption of the proteins to the carrier medium.

The results presented here demonstrate that both the geometrical factor and the adsorption can be evaluated, and that mobilities identical with those obtained in free solution — subsequently called *free mobilities* — can be calculated when corrections are introduced for these effects.

MATERIALS AND METHODS

Apparatus. As described [1].

Buffers. Sodium acetate buffers of $\Gamma/2$ 0.08 were used over the whole acid range in order to maintain a constant buffer composition. Above pH 5.3 barbital was added to 0.025 M. Experiments carried out at pH 8.6 – 8.8 were performed in Longsworth's sodium barbital buffer of the same ionic strength.

Proteins and dextran. Normal human serum albumin and γ-globulin prepared by ethanol fractionation were obtained by kindness of Dr. Albert Hansen, Statens Serum Institut, Copenhagen. 5 % (w/v) solutions in the respective buffers were prepared at 4°C and dialyzed for 24 h in the refrigerator. γ-globulin solutions were prepared once a week. The albumin was electrophoretically homogeneous, whereas a small albumin impurity was present in the γ-globulin preparation.

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

Procedure. As described [1], except for specimen apposition: Two transversal lines were drawn on the paper at 7 and 8 cm from the center of the strip toward the anode and the cathode, respectively. After immersion in buffer and blotting off excess fluid from the strip, the paper was placed in the apparatus. After equilibration, albumin, γ-globulin and dextran solutions were applied, each as one individual 5 μl aliquot to both of these lines, the distance between the 3 spots on a line being 2.5 cm.

For photometric evaluation of the protein migration, the paper was kept overnight in liquid petrolatum and scanned with an EEL (Harlow, Essex) Densitometer, employing a green glass filter (OGR-1). The apex of the absorption peak was taken as the end point of protein migration.

pH Measurements. The pH values were measured potentiometrically with a glass electrode, using a calomel reference electrode (Radiometer, Copenhagen), after calibration with standard phosphate. Controls were performed immediately after the run by cutting a 1×15 cm segment from the strip, which then was thoroughly eluted with boiled

distilled water, the pH measured, and compared to the same dilution of the original buffer solution. The average value, corrected for dilution, was taken as the experimental pH. Experiments showing an increase larger than 0.30 units were discarded.

Temperature. The starting and final, as well as the maximum and minimum ambient temperatures were recorded. Experiments where the maximum variation exceeded 2.5°C were discarded. The temperature on the paper was obtained by adding to the mean ambient temperature the increase (ΔT) taken from Fig. 2.

Determination of the conductivity ratio. The *electrical resistance of the moist paper* strip was found by measuring current (D.C.) and voltage after 3 h equilibration in the apparatus, at constant temperature. The *buffer content* per cm length of paper was obtained by weighing the horizontal part of the strip immediately afterwards. After drying, the difference – corrected for buffer salt content – was divided by the specific gravity and the strip length. The *conductivity* of the free solution was determined for the same temperature by means of an alternating current bridge (Danbridge, LB-1). The conductivity ratio, q, was then calculated according to eqn. II, 8.

RESULTS

A. Adsorption

Adsorption of proteins to filter paper, manifesting itself through "protein-tailing", has been considered one of the main obstacles in the determination of mobilities by paper electrophoresis. However, this problem has apparently not always been critically analysed, since it is necessary to distinguish between the effect on migration rates of reversible adsorption on one hand, and of irreversible adsorption on the other. Depending upon medium and pH, most proteins will probably undergo both processes. The "tail" left by the irreversibly adsorbed proteins does not necessarily signify a retardation of the migration rate, and Durrum's idea [2] that it might be compared to the "unrolling of a carpet" appears to give an adequate picture of the process. Provided that a sufficient amount of substance is available for migration, as it was the case in the present experiments, the "head" will reach its final position, unhindered by a possible irreversible adsorption of excess protein, and enough material will assemble at the end point as to grant the measurement of the migration distance.

On the other hand, protein migration rates would be decreased by reversible adsorption processes occurring during paper electrophoresis. Since chemical pretreatment of the paper made in order to eliminate adsorption might interfere with the protein mobilities, we have suggested the use of chromatographic R_F-values as correction factors [3]. However, a critical reappraisal of this idea showed that, although the chromatographic R_F-values possess general interest as protein-constants, the conditions in chromatography are too different from those existing in electrophoresis to allow the use of these values for correction. In ascending chromatography 19.8 μl buffer were absorbed per cm^2 paper, which is about 30 % less than the amount of fluid present during electrophoresis under our experimental conditions. Another factor which undoubtedly plays a major role is the difference between chromatographic and electrophoretic migration rates. In chromatography [3] the proteins moved at 15 cm/h, whereas the range in the electrophoretic experiments here described varied from 0.05 to 0.30 cm/h.

It was thus fundamental to evaluate the adsorption directly from the actual paper electrophoretic experiment. The following considerations demonstrate the feasibility of such a determination. The close correspondence of the "dextran shrinkage" with the ratio of initial to final voltage between the secondary electrodes in all our electrophoretic experiments [1] demonstrates that dextran is not significantly adsorbed to filter paper in the conventional buffers. This has also been substantiated by chromatographic experiments [3,4].

On this basis, a preliminary approach to the evaluation of protein adsorption was made by submitting albumin, γ-globulin and dextran to *alternating current* (50 cycles/sec.) in an electrophoresis set. Under these conditions, the migration of both protein and dextran is caused exclusively by the buffer displacement produced by evaporation. Accordingly, for an unadsorbed protein we find that the migration distances of this specimen (s_p) and of dextran (s_d) are identical:

$$s_p = s_d \qquad \text{(II,1)}$$

When adsorption occurs, the migration of the protein is delayed and a factor ($\varrho \leqq 1$) has therefore to be introduced:

$$s_p = \varrho \; s_d \qquad \text{(II,2)}$$

Thus, in alternating current experiments, the adsorption factor (ϱ) can be determined by means of one protein-dextran pair. A number of such experiments are summarized in Table 1, where it is shown that albumin and dextran migrated invariably almost equal distances, while γ-globulin was significantly adsorbed to the filter paper. A schematic presentation of an alternating current experiment is given in Fig. 1(a).

Table 1. Protein adsorption in alternating current experiments. s_p protein migration distance; s_d dextran migration distance; adsorption factor $\varrho = s_p/s_d$; σ_m standard deviation of the mean.

Buffer	Ionic strength	pH	Albumin			γ-Globulin		
			Nr. expt.	ϱ	σ_m	Nr. expt.	ϱ	σ_m
Sodium acetate	0.025—0.30	5.20	8	1.01	0.02	8	0.95	0.02
Zinc acetate	0.025—0.30	6.00	8	1.02	0.01	6	0.95	0.04

ϱ-Values corresponding to the specific experimental conditions, however, are obtained directly from the actual *electrophoretic experiment* (*i. e. direct current*), by the use of two protein dextran pairs per strip (cf. Materials and Methods). According to eqn. 13 of the preceding communication [1], which gives an expression of the mobility of an unadsorbed substance:

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

For an adsorbed protein, the equation becomes:

$$\frac{s_p}{\varrho} - s_d = m\ V_t t \qquad \text{(II,3)}$$

As eqn. II, 3 is valid irrespective of the point of application of the samples, we find that

$$\frac{s_{p1}}{\varrho} - s_{d1} = \frac{s_{p2}}{\varrho} - s_{d2} \qquad \text{(II,4)}$$

p_1d_1 and p_2d_2 indicating the two protein-dextran pairs. This relation is schematically presented in Fig. 1(b).

In consequence,

$$\varrho = \frac{s_{p1} - s_{p2}}{s_{d1} - s_{d2}} \qquad \text{(II,5)}$$

In mobility experiments described below (Fig. 3) it was seen that albumin has a mean ϱ-value of 1.025, as compared to a value of 0.903 found for γ-globulin. It is interesting to note the close correspondence of these results with those obtained in the alternating current experiments (Table 1). The scattering observed cannot be attributed to the pH, as no clear correlation between pH and ϱ is found. On the other hand, later experiments indicate that the adsorption increases parallel to the buffer concentration. The results of these investigations will be published elsewhere.

It has thus been shown how the adsorption factor can be determined both in a system based upon evaporation alone (A.C.), and in "open-strip" electrophoresis (D.C.), where evaporation, electroosmosis and protein charge contribute to the migration. The latter, direct, determination has to be preferred, as it corresponds to the specific conditions of the experiment.

In *electrophoretic systems devoid of evaporation*, however, the adsorption factor cannot be evaluated, as shown by the following considerations. Evaporation produces a liquid flow converging from both ends toward the center of the strip and diminishing in intensity as this point is approached. This flow adds to the electroosmotic flow and to the electrophoretic migration, thus giving rise to a marked asymmetry between the anodic and the cathodic side of the strip [Fig. 1(b)]. When this asymmetry is lacking, identical specimens will obviously migrate the same distances, irrespective of their starting positions on the paper [Fig. 1(c)]. In consequence, for experiments where evaporation is suppressed, it is seen that

$$s_{p1} = s_{p2} \qquad \text{and} \qquad s_{d1} = s_{d2} \qquad \text{(II,6)}$$

and, in accordance with eqn. II,5,

$$\varrho = \frac{0}{0}$$

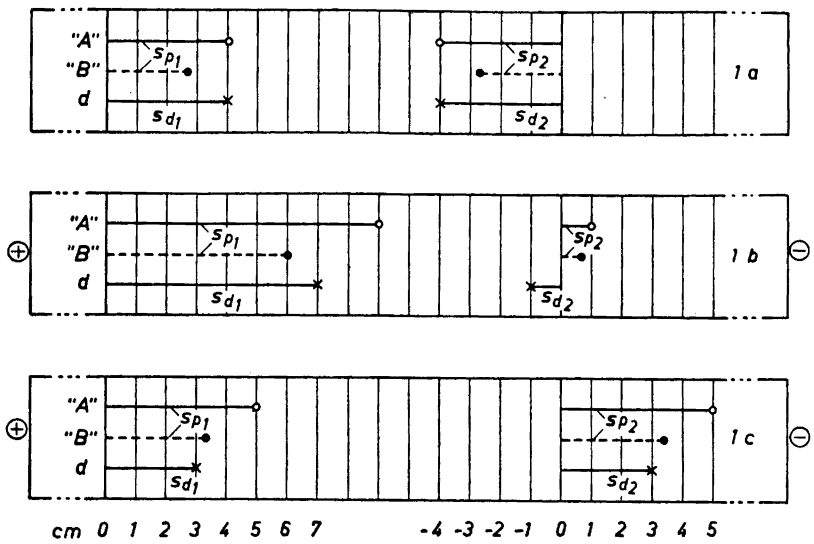

Fig. 1. Schematic illustration of migration relationships with protein adsorption. —○ Unadsorbed "A"-protein. ———● Adsorbed "B"-protein, adsorption factor $\varrho = 0.666$. —× Uncharged substance (dextran). – Assumed migration distances valid for the 3 experiments: Owing to evaporation ± 4 cm, – to electroosmosis 3 cm, and to protein charge 2 cm. (*cf.* eqn. I, 11). For simplicity, "A" and "B"-proteins are given the same charge. The points of application appear as zero. Migration to the cathode is considered positive.

1 a. Alternating current experiment. (Substances subject to evaporation only.) "A"-protein: $s_p = s_d$. "B"-protein: $s_p/\varrho = s_d$. Both sides yield the same result.
1 b. Open-strip electrophoresis. (Evaporation, electroosmosis and electrophoretic migration taking place.) "A"-protein: $s_{p_1} - s_{d_1} = s_{p_2} - s_{d_2}$. "B"-protein: $s_{p_1}/\varrho - s_{d_1} = s_{p_2}/\varrho - s_{d_2}$.
1 c. Electrophoresis devoid of evaporation. (Substances under the influence of electroosmosis and electrophoretic migration.) "A" and "B"-proteins: $s_{p_1} = s_{d_1}$. ϱ cannot be calculated.

Hence, this direct determination of the adsorption factor is not feasible in systems devoid of evaporation. From the schematic illustration in Fig. 1(c), the same conclusions can be drawn by geometrical considerations.

The result is of fundamental significance, since it is generally assumed that a possible determination of free mobilities by paper electrophoresis can be carried out only when evaporation is suppressed [2,5a]. In view of our results, it becomes clear that the presence of evaporation is even of advantage, as it allows the direct estimation of the adsorption factor. Moreover, the accuracy of the ϱ-values will increase in proportion to the migration distance due to evaporation. In order to introduce the adsorption factor into results obtained from systems devoid of evaporation, parallel alternating current experiments would have to be performed. On this ground it can be concluded that for an accurate calculation of mobilities, the open-strip method is to be preferred.

Attention should finally be drawn to the adsorption problem when protein mixtures, such as sera, are analyzed. If it is assumed that some protein is irreversibly held by the paper, the foremost specimen would saturate a number of paper centers and hereby possibly "accelerate" the fractions following behind, the ϱ-values of which thus would become higher than those of their isolated counterparts. Observations in this sense were made in our chromatographic investigations [3]. In practice, however, it is noted that the ϱ-values of albumin and γ-globulin do not differ by more than 10 % in the conventional buffers. Moreover, when — as described — two serum spots are applied, and if the order in which the fractions migrate is identical for both spots, ϱ-values different from the isolated fractions will be found, but the calculated free mobilities will be the same.

B. The influence of paper-structure

The concept of a "tortuous path" was first envisaged by Kunkel and Tiselius [6]. According to their idea, the distance covered by the migrating substance is in reality longer than that measured on the paper because of the intricate channel system proper to the filter paper texture. The observed migration distance has thus to be divided by a factor smaller than 1.0 to get the real path-length. Due to the increased path length, the measured voltage gradient has to be multiplied by the same factor in order to obtain the voltage acting on the migrating substance.

The problem is considered by McDonald *et al.*[7] under a somewhat different viewpoint in their "barrier theory". The supporting medium is supposed to act as a mechanical barrier slowing down the velocity of the migrating substance through repeated collisions. Such an effect should be more pronounced with increasing molecular weight of the migrating substance. In view of the R_F-values of approximately 1.0 found for dextran [3,4] and the correspondence between "dextran shrinkage" and the V_o/V_t ratio [1], it is apparent that the influence of the geometrical factor is the same in the case of the buffer solution as with dextran. The concept of a "tortuous path" in the sense of Kunkel and Tiselius would therefore seem more adequate than the barrier theory by McDonald *et al.*

Kunkel and Tiselius determined the real path length by comparing the conductivity of the moist paper with that of a buffer column of the same length and same amount of buffer. According to their calculations the conductivity ratio represents the ratio of the measured to the true length. This value was squared to get the correction factor for the calculation of the free mobilities. We have used the same experimental approach but applied the conductivity ratio directly to the conversion of the measured mobilities. Kunkel and Tiselius, in their paper [6], overlooked the fact that an increased path length with constant buffer content involves a corresponding reduction of the cross-sectional area, causing the resistance to increase with the square of the path length. The conductivity ratio thus gives the square of the ratio between the measured and the true length, and should be applied directly.

It is also evident that, as far as factors of identical influence, such as path-length, are concerned, the reduction in the mobility of a protein migrating on paper will be equal to that of the buffer ions and thereby to the conductivity. The free mobility, u, is thus obtained by dividing the mobility, m by the conductivity ratio, q.

$$u = \frac{m}{q} \tag{II,7}$$

The conductivity ratio q, which was used in our calculations, was determined according to the following equation:

$$q = \frac{\text{Paper conductivity}}{\text{Buffer conductivity}} = \frac{\frac{i}{V/l \cdot w}}{\varkappa} = \frac{i \cdot l}{V \cdot w \cdot \varkappa} \tag{II,8}$$

i = current employed, after equilibration of the strip (A)
l = distance between the secondary electrodes (cm)
V = voltage over distance l at current i, corrected for polarization and voltmeter resistance (V)
w = buffer per cm length of paper (ml . cm $^{-1}$)
$\varkappa$ = buffer conductivity (Ω^{-1} cm^{-1})

The value obtained from 22 experiments was 0.740 (S.D.M. $\pm$ 0.0047). Since q is equal to the square of the ratio of measured to true length, the latter is found to be 16.3 % longer than the distance measured. As observed from Table 2, q remains constant for a given brand of paper, in spite of varying buffer type, pH and ionic strength. This is in full agreement with Kunkel and Tiselius' results [6] and shows that this factor depends solely on the type of paper used. A standard error below 1 % (Table 2) indicated that small differences between different batches [5b] are without significance in the present case.

It was mentioned [1] that the "wetness" w, on which special emphasis is laid by McDonald *et al.*[7], remains constant during an experiment. That is further evidenced by the fact that the different buffer concentrations, given in Table 2, resulted in practically identical w values, and shows that no variations in q owing to wetness changes [8] occur during the experiment. Temperature increases within the observed range do not appear to influence the outcome of the q-value.

Since the different types of buffer used yielded the same q values, it can be concluded from Table 2 that reversible ion adsorption does not take place during the process. The absence of an irreversible ion adsorption was independently demonstrated by eluting a known amount if buffer from a paper strip and comparing the conductivity of the eluate with a solution of the same ion concentration as directly prepared. The difference was within the experimental error (<1 %) in the sodium and zinc buffers employed.

The q value of 0.740 (Table 2) has been applied to all subsequent mobility calculations in the following way. According to eqn. II,3:

$$m = \frac{\frac{s_p}{\varrho} - s_d}{V_t t} \tag{II,3}$$

Table 2. Conductivity ratio, q, calculated according to eqn. II,8. Muncktell paper No. 20/150 employed.

No.	Buffer	$\Gamma/2$	pH	q
1	Sodium acetate	0.051	5.25	0.732
2	id. id.	0.051	5.25	0.747
3	id. id.	0.080	4.05	0.734
4	id. id.	0.080	4.44	0.730
5	id. id.	0.080	4.90	0.723
6	id. id.	0.080	5.32	0.761
7	id. id.	0.150	5.37	0.730
8	id. id.	0.150	5.37	0.730
9	id. id.	0.300	5.32	0.718
10	id. id.	0.300	5.32	0.730
11	id. id. + 0.02 M barbital	0.080	5.87	0.734
12	id. id.	0.082	6.80	0.773
13	id. id.	0.083	6.20	0.726
14	Phosphate	0.050	7.60	0.723
15	id.	0.100	7.60	0.709
16	Sodium barbital	0.075	8.45	0.797
17	id. id.	0.080	8.80	0.752
18	id. id. + 0.06 M sodium chloride	0.150	8.45	0.783
19	id. id.	0.200	8.55	0.747
20	id. id.	0.250	8.55	0.752
21	id. id.	0.350	8.50	0.723
22	id. id.	0.400	8.50	0.722
			MEAN	0.740
			σ_m	0.005

By introducing the conductivity quotient according to eqn. II,7, it is found that

$$u^{T°C} = \frac{m}{q} = \frac{\frac{s_p}{\varrho} - s_d}{q \cdot V_t t} \qquad \text{(II,9)}$$

$u^{T°C}$ = free mobility at experimental temperature T°C ($cm^2V^{-1}sec^{-1}$)
s_p = protein migration distance measured (cm)
s_d = dextran migration distance measured (cm)
ϱ = adsorption factor (eqn. II,5)
q = conductivity ratio (eqn. II,8)
V_t = voltage/cm at the conclusion of the experiment, corrected for polarization and voltmeter resistance ($V \cdot cm^{-1}$)
t = duration of the experiment (sec)

Eqn. II,9 is the final formula employed in the calculation of free mobilities.

The correction factors for adsorption and increased path length may also be introduced into the equation I, 11 of the previous communication [1], resulting in

$$u = \frac{1}{q}\left(\frac{s}{\varrho V_i t} - e - afi\right) \qquad \text{(II, 10)}$$

From this equation it would be possible to determine free mobilities on paper without the use of an uncharged reference substance provided that the constants e, f, and ϱ had been determined beforehand. Because of the obvious uncertainty of such a procedure, the use of dextran cannot be dispensed with, and the present method has to be preferred.

C. Temperature determination

Since the use of thermocouples during an experiment would produce considerable technical difficulties, the determination of the actual temperature on the strip indicated the convenience of an evaluation of the temperature increase (ΔT) as function of the watt input.

It has been shown in the preceding communication [1] that in experiments with constant current the reciprocal voltage plotted against time gives a straight line (Fig. 6 in Ref.[1]). By extrapolation to the ordinate an expression of the initial conductivity at the temperature prevailing during the experiment is obtained ($1/V'$). The difference between $1/V'$ and $1/V_0$ is directly proportional to the conductivity increase caused by the temperature rise.

The viscosity of the buffer solution used is nearly identical with that of pure water, and the temperature function of the latter was therefore employed to convert the conductivity increase to temperature. This conversion is performed as follows: The reciprocal viscosity of water corresponding to the mean ambient temperature during such an experiment, is taken from a table. This value is increased by the percentage of conductivity increase found as described above, and the corresponding temperature read from the table. ΔT is then obtained by subtracting the mean ambient temperature from this value.

In Fig. 2 the temperature increase found in 16 buffers of widely varying ionic strengths and different composition by plotting the reciprocal voltage against time, is correlated with the initial watt input ($V_0^{(15cm)} \times$ mA per strip). In spite of some scattering, it appears justified to accept a ΔT-value of 3°C per $W_0^{(15cm)}$/strip.

In the experiments which laid the basis for Fig. 2 no substances were applied to the strips after equilibration, as it was the case in the mobility determinations. Since the equilibrium undoubtedly will be disturbed by the opening of the covering plates, as required with our apparatus, the voltage before and after protein and dextran application was measured. It was seen that the difference between the two values is of no significance at all, as even 5 min opening, together with the application of six 5 μl-aliquots, does not produce a voltage decrease greater than 1 %, which in the present series would mean a maximum error of 5×10^{-3} °C.

The $\Delta T/W_0$ correlation will obviously vary with the apparatus used and is the only function here described which will have to be determined at each laboratory.

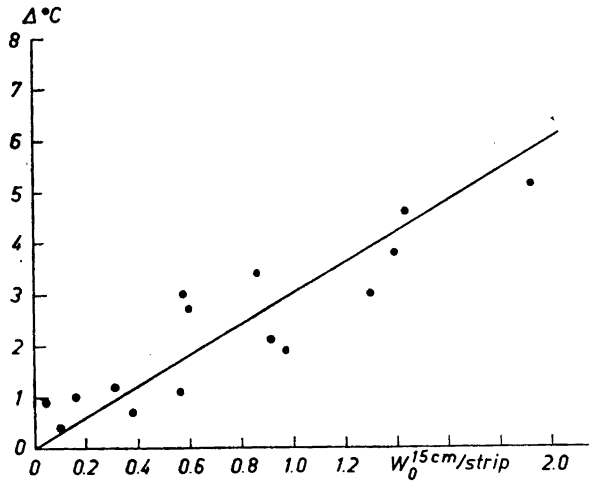

Fig. 2. The temperature increase as function of the initial watt input. $W^{15\ cm}$: Watt per 15 cm strip length (120 cm^2). Sodium acetate, barbital and zinc acetate buffers, ranging from $\Gamma/2 = 0.025$ to 0.30, used. Experimental points obtained by extrapolation procedure (see p. 22).

When highly concentrated or viscous buffers are employed, the conductivity should be measured as function of the temperature, and the resulting curve used as reference for exact temperature conversions. In the ordinary concentration range, however, the difference would be negligible.

From the described experiments it has been seen that the temperature increase occurs within the first hour, and it appears therefore justified to add ΔT, taken from the $\Delta T/W_0$ plot, directly to the mean ambient temperature. The resulting value is then used for the conversion of the mobilities to their values at 1°C. The viscosity ratio was used to this end. This is in accordance with Tiselius' procedure [9] and it has been shown by McDonald *et al.*[7] that the free mobility as function of the temperature is, in fact, proportional to the reciprocal viscosity. Deviations from this relation, which have been mentioned for certain inorganic ions [10], do apparently not apply to serum proteins. We used 1°C for comparison, since, although not always clearly stated, most free electrophoresis data are obtained at a temperature of about 1°C within the cell. A variation of 1°C in this range changes the mobility value by about 2 %.

D. The concentration increase and its effects

Open-strip electrophoresis suffers from the characteristic inherent to all procedures with evaporation, that the salt concentration on the strip increases during the run. The final ionic strength can easily be calculated by multiplying the initial concentration by the "dextran shrinkage" (distance between the starting positions divided by the distance between the final positions). The mean ionic strength ($\Gamma/2_m$) is then considered valid for the whole experiment.

In our mobility determinations an initial ionic strength of $\Gamma/2 = 0.080$ to 0.084 was used, which increased to about 0.12, thus resulting in a mean concentration of $\Gamma/2_m = 0.102$ (S.D. = ± 0.0036).

The error involved by taking the average ionic strength is rather insignificant, since the mobility changes only slightly with the ionic strength in monovalent ion buffers [11]. According to our own determinations, the mobilities of both serum albumin and γ-globulin in sodium acetate buffer of $\Gamma/2_m = 0.08$ differ by about 5—7 % from the values found in the same buffer of $\Gamma/2_m = 0.12$, the change becoming less pronounced in higher concentrations. These determinations were made at pH 4.75 and 5.65. It is evident that the average concentration ($\Gamma/2_m$) would differ even much less from the real value.

pH changes measured on the strip could not be caused by a concentration increase of the magnitude observed. Since the buffer capacity was sufficient and electrolysis products were prevented from diffusing into the paper, the pH variations were produced by the evaporation of volatile buffer components. Routine controls were therefore especially required in sodium acetate buffers. No pH gradients can be supposed to occur within the uniform concentration zone.

E. The mobilities of human serum albumin and γ-globulin from pH 4.0 to 8.8. Determination and calculation

The crucial test for the validity of the considerations set forth above is the determination of a number of mobilities and their comparison with results obtained by free electrophoresis.

As shown in the preceding chapters, the final eqn. II,9 for the computation of mobilities has been derived from eqn. 13 in Ref.[1] by gradual introduction of correction factors for adsorption and paper structure. The migration distance of the protein is divided by the corresponding ϱ-value, the dextran migration then subtracted, and the difference divided by the conductivity ratio, final voltage and time.

The temperature conversion to 1°C is then performed as described above.

It will be noted that two protein-dextran pairs were applied per strip in order to evaluate the adsorption factor ϱ. Once this factor has been calculated, it is seen from eqn. II,4 that either pair can be used for the mobility calculation according to eqn. II,9.

The mobilities of human serum albumin and γ-globulin were determined over a pH range from 4.0 to 8.8, and the results calculated according to eqn. II,9.

In Fig. 3 the mobilities are presented as function of the pH, together with previous findings by other authors working with free electrophoresis (moving boundary). Values obtained from media other than those employed in this investigation were disregarded.

A full accordance between free and paper electrophoretic results is observed. Isoelectric points of pH 4.68 for albumin and of pH 6.83 for the γ-globulin preparation were obtained by our method, the scattering of the experimental points being less than to be expected from a corresponding study employing free electrophoresis.

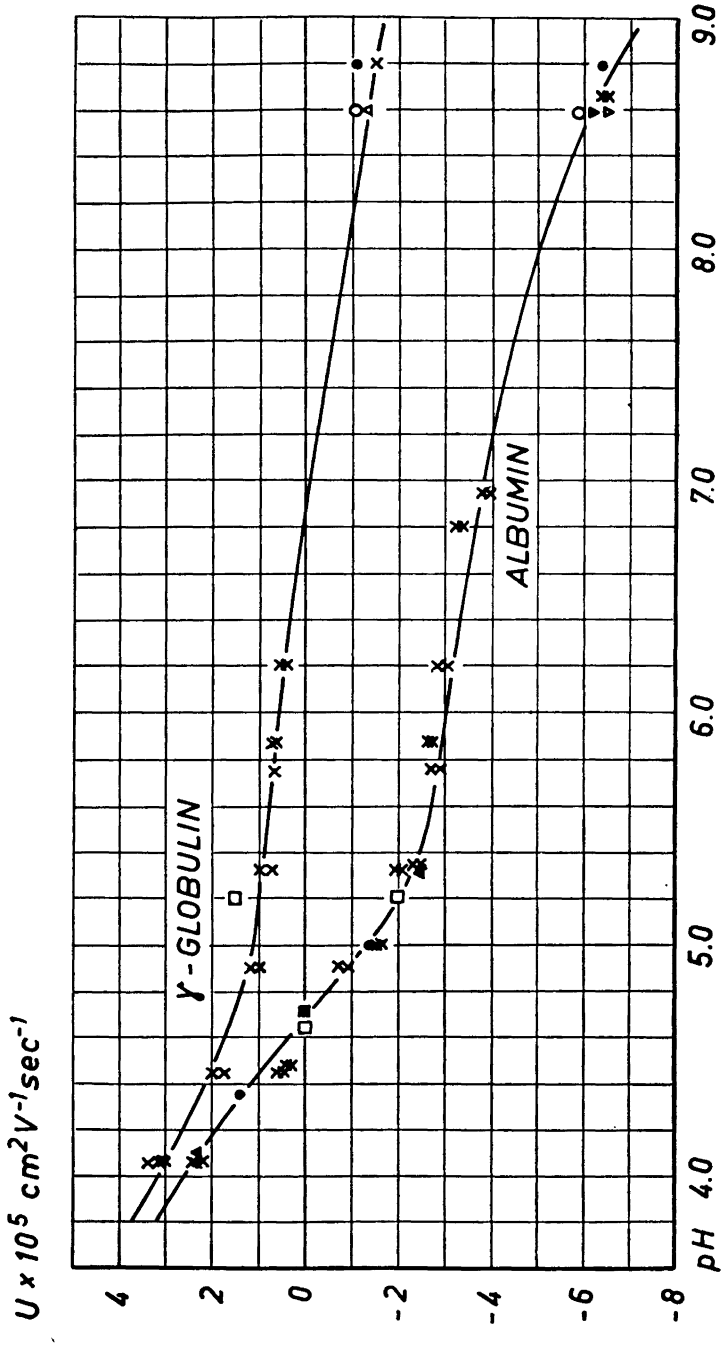

Fig. 3. The electrophoretic mobilities of human serumalbumin and γ-globulin at 1°C in 0.10 ionic strength buffers. pH 4.0—5.5 sodium acetate; pH 5.5—7.0 sodium acetate + barbital; pH 8.6—8.8 barbital.

× *RESULTS OBTAINED BY THE PRESENT PAPER ELECTROPHORESES.*

Moving boundary electrophoresis:
● Kunkel and Tiselius [6]
◀ ■ ▼ Schilling [20], Baldwin [19], Pigman *et al.* [22]
□ Stenhagen [18]
○ Dole and Braun [21]
▽ Woods and Gillespie [12]
△ own determination

The excellent agreement of the paper electrophoretic results with those obtained in free solution establishes the value of the present procedure.

DISCUSSION

In view of the reported results, previous work by other authors on the determination of free mobilities by means of paper electrophoresis is discussed first.

Procedures working without evaporation were described by Kunkel and Tiselius [6] and McDonald *et al.*[7] In experiments by Woods and Gillespie [12] a correction was introduced. The only method working with uninhibited evaporation has been published by Macheboeuf *et al.*[13,14] who even take advantage of this factor.

In the first group mentioned, the sole complications are the electroosmotic flow and the influence of the supporting medium. In the fundamental study of Kunkel and Tiselius [6] — the only publication giving a comparison with moving boundary data for more than one pH value — the uncharged dextran was used as indicator of the electroosmotic flow. Correct mobilities for serumalbumin were obtained at three pH values, in spite of the fact that the square of the conductivity ratio was erroneously applied as tortuous path factor. It might be presumed that this was compensated by a retarding of the protein migration owing to the silicone employed in Kunkel and Tiselius' closed-strip system. No correction for adsorption effects was introduced. In this specific case, however, this does not influence the final mobility values, since — as shown above — serumalbumin remains unadsorbed in the conventional media.

McDonald *et al.*[7] describe several approaches to the attainment of free mobilities, all of them obligatorily working at low temperatures. In a method working with slight evaporation the mobility of serumalbumin at pH 8.6 was obtained by extrapolation of mobility *vs.* time to zero time on the basis of about 50 experiments, whereas γ-globulin could not be estimated because of its low mobility. In a "direct method" requiring 24—36 h per determination, the maximum voltage applicable is 0.5 V/cm. Apparently, no account is taken of the electroosmosis nor of the "barrier effect", so strongly emphasized by the authors. Since it is furthermore shown that a change in the type of paper may change the results by a factor of about 2.0, the agreement with free mobility values appears rather fortuitous. In a third approach, where ionic strengths of 0.0125 may not be exceeded, a reference substance of known mobility is required to correct for the effects of electroosmosis and paper interaction. In an empirical equation various constants are determined using results obtained with the reference protein. When the migration velocity of an unknown substance is then introduced into this equation, it is claimed to yield the free mobility. The different interaction with the paper is estimated separately in conductivity measurements which require a considerable amount of the substance tested. The procedure presents appreciable technical difficulties, and the results reported for γ-globulin, showing 20 % deviation from the free mobility value after determining the parameters by means of albumin, cannot be considered to establish the validity of the proposed equation.

According to McDonald [5c] mobilities may be defined as specific for a given support. This was actually done in a later publication [15], and means — in

our view — a virtual abandonment of the attempts to determine free mobilities by paper electrophoresis, since the mobility is only significant when considered as a physical constant independent of the carrier employed.

Mobility determinations in the presence of evaporation, reported by Woods and Gillespie [12], contain an empirical correction for evaporation effects. It should be noted that the comparison with free mobilities is based on an erroneous temperature correction, since a 2 % change of the mobility value per °C, as derived from the viscosity, is only valid when converting from 25°C to 0°C, and not in the opposite sense. Their value would thus be 27.2 % lower than that found by free electrophoresis. The authors do not consider any corrections for the paper structure. If this is done, the discrepancy would be reduced.

Important investigations have been carried out by Macheboeuf *et al.* [13,14]. The procedure called "electrorheophoresis" has been discussed in the preceding communication. An example of its practical application has been published by Meulemans [23].

Both in Macheboeuf *et al.*'s method and in McDonald *et al.*'s third approach, proteins of known mobility are used as reference substances, in order to correct for buffer flow and carrier medium effects. However, the fact is neglected that, as shown above, the adsorption depends on the specific protein, and some doubts concerning results thus obtained must be expressed.

In this connection, it should be pointed out that the use of dextran, too, involves a series of problems which remain to be solved. Our calculations are based on the assumption that no interaction between the dextran indicator and neither the carrier nor the buffer takes place. While this holds true in the present experiments, conditions may be conceived where dextran is adsorbed or acquires a significant charge. In experiments aiming beyond the conventional media it may therefore be necessary to gain a previous knowledge of the electrochemical behavior of dextran under these conditions. An approach to this problem is at present being worked out and will be published. It does not appear probable that dextran can be easily replaced by a more appropriate indicator, but a final solution might possibly be found by resorting to labelled molecules.

It is evident that paper electrophoresis shares with the moving boundary method certain problems, inherent to the migration of colloidal particles in the electric field, which await further elucidation. Preliminary observations indicate that the mobility of albumin, at a given pH, decreases with increasing protein concentration, which is in agreement with Svensson's results in moving-boundary electrophoresis [16]. Nevertheless, extrapolation to zero protein concentration is not usual in routine mobility determinations and the protein concentration is simply stated together with the other parameters.

One of the most significant advantages of paper electrophoresis being the minute quantity of specimen required, the present results open new ways for studies on the electrochemical properties of enzymes and pathological samples only available in scanty amounts.

An interesting application of the new procedure lies in the field of ion interaction studies. As stressed by Steinberg and Mihalyi [17], some of the subfractions observed in the moving-boundary method may be artifacts produced by

incomplete ion binding resulting from the high protein/free ion ratio. In paper electrophoresis, on the other hand, a considerable and constant excess of the free ion assures the displacement of the equilibrium toward protein-ion binding. By computation of the changes produced in the mobility through addition of increasing ion concentrations to the buffer, binding constants and charge of the different fractions can be evaluated. When working with ions whose activity coefficients change appreciably with the concentration increase caused by evaporation, corrections would have to be introduced. The open-strip technique offers the possibility of further implementing such studies by the introduction of labelled ions.

On this ground it is hoped that the present results, aside from providing an exact micromethod for mobility determinations, may contribute to the attainment of new insights into protein binding and structure.

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