PAPER ELECTROPHORESIS: TEMPERATURE, PAPER WETNESS AND SERUM COMPONENT MOBILITY WITH THE KUNKEL SYSTEM*

NELL F. HOLLINGER AND RICHARD K. LANSING

School of Public Health, University of California, Berkeley, Calif. (U.S.A.)

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Paper electrophoresis has been used as a means to separate a heterogenous mixture of charged particles into separate groups, each of more homogeneous nature. When applied to thoroughly studied mixtures, such as human serum proteins, the identity of any of the separated fractions, once established by independent means, is most readily achieved by observation of its position in relation to other fractions of the specimen rather than by measurement of its absolute displacement from the point of application. Measurement of the absolute displacement (or mobility) of a homogeneous group as a means of identification of that group assumes greater importance when the mixtures under study are ones which have not been examined as rigorously as human serum, *e.g.* in the study of proteins of "unknown" plant or animal species.

To extend the usefulness of paper electrophoresis as a basic tool, an investigation was made of some of the factors which control the mobility of serum proteins. McDONALD[1,2] has indicated the importance of solvent movement, moisture content, conductivity and temperature of the paper strip as some of the variables affecting the mobility of the migrating particles. These observations related particularly to a horizontal open system and the observations did not extend to multiple separations on the same strip. Comparisons for qualitative analysis of substances of similar mobilities are conveniently carried out on one sheet of paper[3]. KUNKEL AND TISELIUS[4] have described an electrophoretic apparatus which effects appreciable control of gross movements of solvent and in which multiple samples of a given substance can be run in parallel on a single strip with apparently identical mobilities. The description of this apparatus did not include data concerning reproducibility or the effect of wetness or temperature. This study was undertaken to investigate the effects of temperature and wetness in a rapid Kunkel system and to determine the variability of mobility of multiple separations on the same strip. Comparison is made with separations carried out on a DURRUM type apparatus[5].

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METHODS

The electrophoretic apparatus was similar to the horizontal, closed system described by KUNKEL AND TISELIUS[4]. The lower plate was 4 × 10 × 3/4 in., the upper was 4 × 10 × 1/4 in. All surfaces in contact with the paper were lightly siliconed. Whatman 3MM paper 2 ¼ × 11 in., and barbital buffer, pH 8.6, ionic strength 0.05 were used in all cases. After the paper was positioned between the plates 12 bottles filled with mercury were placed upon the top plate to give a total pressure of 1.25 lbs./sq. in. The level in each reservoir was maintained 1 ½ in. below the horizontal paper and the electrolyte was changed when appropriate.

The voltage gradient was measured by a separate voltmeter by inserting platinum micro electrodes 1–2 mm into the strip 10 cm each side of the origin. All runs were for 60 min at a constant voltage of 15 V/cm as measured in the area in which the proteins were migrating and voltage drops in other portions of the system were ignored.

Heat dissipation from the strip was by conduction to the heavy glass plates which surrounded the paper and thence by convection into the air of a water-cooled, thermostatically controlled cabinet. The glass plates and other portions of the system were temperature equilibrated before each run.

The effects of temperature and wetness were studied at 5°, 16°, and 25°. To obtain measurements of paper wetness, each paper was weighed, then placed in a tared, covered tray made level with a poured layer of paraffin. A known volume of buffer was applied along the paper and allowed to equilibrate 50 min. Three 2 λ samples of serum were applied as spots equispaced along a previously marked line midway between the ends of the strip. After the specimens were applied the strip was immediately transferred to the Kunkel apparatus and the tray was reweighed and the increase in weight due to residual buffer was used to correct the value of buffer applied to the strip. The physical demands of the system limited wetness to values between 1 g buffer/g paper (1/1) and 2 g buffer/g paper (2/1). Less than this amount of buffer would not diffuse uniformly throughout the paper in 50 min and more than 2 g buffer/g paper resulted in an excess which was visibly squeezed out of the paper when pressure was applied. At a lesser pressure, 1.00 lb./sq. in., variability of results is greater, resolution is poorer and increase in mobility with increasing temperature does not show a straight line relationship.

In separate runs, 3 % dextran was applied at the origin, and 5 and 10 cm to each side to measure capillary and endosmotic electrolyte movement.

At the end of the run the paper was dried and stained with bromophenol blue. Movement of the albumin and γ-globulin fractions was measured from the origin to the points of greatest dye concentration. All separations were made on a single pool of human serum stored in 2 ml aliquots at —10°. Fifty-six runs were made, a total of 168 separations.

Comparative data were obtained on a Durrum type apparatus (Spinco model Beckman Instruments), at room temperature (18–21°) with barbital buffer pH 8.6, ionic strength 0.05, on six parallel strips of Whatman 3MM paper at a constant

current of 4.5 mA. The wet strips were equilibrated 30 min and then 10 λ portions of serum were applied at the apex. In separate runs an equal volume of 3 % dextran was applied at the apex and 5 cm to either side.

RESULTS

With the Kunkel system described, the observed (apparent) mobility of albumin and γ-globulin was found to increase with both wetness and temperature. The increase in mobility was in the direction of migration, anodal for albumin and cathodal for globulin. All measurements are based on constant conditions of time and voltage gradient and are presented in terms of actual displacement rather than the equivalent mobility values in terms of cm/sec/V/cm. At a 1/1 wetness value, observed albumin mobility increased 0.063 cm/°C over the entire temperature range investigated and at wetness 2/1 this mobility increased 0.087 cm/°C. At a 1/1 wetness value, observed γ-globulin mobility increased 0.0172 cm/°C and at 2/1 this mobility increased 0.0315 cm/°C (Table I). The data on dextran displacement given in Fig. 1 indicate the net

TABLE I

RELATIONSHIP OF OBSERVED ALBUMIN AND γ-GLOBULIN MOBILITY (y) TO WETNESS (x) AT THREE TEMPERATURES: VARIABILITY WITH KUNKEL TECHNIQUE USING HUMAN SERUM

Component	°C	*Least squares line*	*Standard deviation (S.D.)*	*N**	*Variability of migration*			
					Wetness less than 1.5		*1.5 or greater*	
					***Cm*	****CVx(%)*	**\Cm*	****CVx(%)*
Albumin	5	$y = 0.747 + 0.534x$	0.23	20	0.09	6.4	0.12	7.2
	16	$y = 1.35 + 0.73x$	0.19	12	0.15	6.6	0.22	8.2
	25	$y = 1.7 + 0.95x$	0.21	32	0.15	5.2	0.19	5.6
γ-Globulin	5	$y = 0.153 + 0.108x$	0.10	20	0.06	22.3	0.07	16.8
	16	$y = 0.16 + 0.19x$	0.11	12	0.09	23.4	0.12	24.2
	25	$y = 0.21 + 0.304x$	0.10	20	0.10	16.9	0.07	9.4

* Number of runs.
** Cm = Average difference between most and least mobile on same strip.
*** CVx = (S.D./mean observed mobility) × 100 = coefficient of variation, value as percent.

flow to be toward the center on dry strips and toward the ends on wetter ones. One example of albumin mobility corrected for dextran movement is given in Table II.

On the Durrum apparatus the mean albumin mobility was 6.15 cm, standard deviation (S.D.) 0.65, coefficient of variation (CVx) 10.6 %. The γ-globulin mobility was 0.49 cm, S.D. 0.183, CVx 37.4 % (Table III). Movement of dextran on the Durrum apparatus was in a negative direction and displaced maximally on the positive limb. The dextran movement observed after an 8 h run was the same as that after a run of 16 h (Fig. 2).

With the Kunkel apparatus three specimens were placed in parallel on the same strip. For albumin, the average variability in the distance migrated, from most to

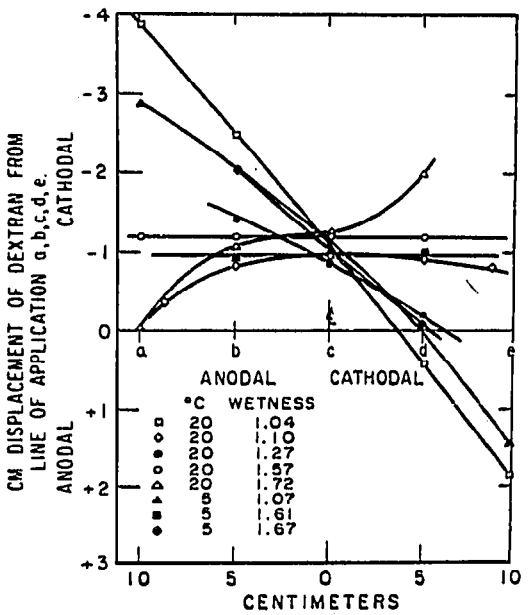

Fig. 1. Dextran displacement from origin, Kunkel type apparatus. The dextran movement is toward the center on dry strips and toward the ends on wetter ones.

TABLE II

OBSERVED AND CORRECTED MOBILITY VALUES (+, —)* ON HUMAN SERUM COMPONENTS, KUNKEL TECHNIQUE

	Albumin				*γ-Globulin*				
°C	5°		20°			5°		20°	
Wetness	1.04	1.72	1.04	1.72		1.04	1.72	1.04	1.72
	Mobility		*Mobility*			*Mobility*		*Mobility*	
Observed**	+1.30	+1.75	+2.68	+3.33	Observed**	—0.26	—0.33	—0.51	—0.73
Marker (dextran)	—1.70	—1.00	—1.70	—1.00	Marker (dextran)	—0.70	—1.50	—0.70	—1.50
Corrected	+3.00	+2.75	+4.38	+4.33	Corrected	+0.44	+1.17	+0.19	+0.77

* + = movement toward anode; — = movement toward cathode.
** From least squares data $y = a + bx$ (*cf.* Table I).

TABLE III

STATISTICAL CONSTANTS DERIVED FROM OBSERVED MOBILITY DATA OBTAINED AT ROOM TEMPERATURE, DURRUM TECHNIQUE, HUMAN SERUM

Component	$\bar{x}$ = *average mobility (cm)*	*Total strips*	*Standard deviation (S.D.)*	*CVx* (%)*	*Average difference in mobility***		
					Cm	*Number of runs*	*CVx(%)*
Albumin	***+6.15	36	0.65	10.6	0.49	6	6.9
γ-Globulin	—0.49	34	0.18	37.4	0.25	6	68.0

* CVx = (S.D./mean observed mobility) × 100 = coefficient of variation, value as percent.
** From greatest to least value for mobility in same run of six strips.
*** + anodal; — cathodal movement.

least mobile on the same strip, was 6.5 % of the distance migrated (range 5.2–8.2 %) (Table I). For the γ-globulin fraction the average difference was 18.8 % (range 9.4–24.2 %) (Table I).

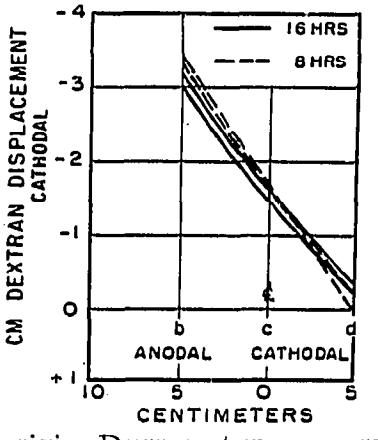

Fig. 2. Dextran displacement from origin, Durrum type apparatus. Movement is always cathodal and more pronounced on the anodal limb.

The average variability in the distance migrated, from most to least mobile among the six strips in the Durrum chamber, was (Table III) for albumin, 6.9 % of the distance migrated (range 5.0–8.6 %). For the γ-globulin fraction for any one run the average difference was 68 % (range 40–100 %).

DISCUSSION

Equilibration

The equilibration time of 50 min was sufficient to allow an apparently even distribution of electrolyte throughout the paper. In order to maintain a constant potential (as measured in the area of the strip in which the specimens were migrating) it was necessary occasionally to make minor readjustments in the voltage during the first 20 min of the run. EDWARD AND CRAWFORD[6] have found equilibration for 20 min adequate in a Kunkel system. Using a modified Kunkel, CRESTFIELD AND ALLEN[7] found an equilibration period of 30–45 min resulted in a constant field strength. MOINAT AND TULLER[8] point out the desirability of measuring voltage on the strip and not only at the power supply.

Temperature

The findings for albumin and γ-globulin show an increase in mobility with an increase in temperature (Table I). Similar increases occur in open strip ionophoresis[9]. FORBES AND TAYLOR[10] found the migration rate of β-lipoprotein but not that of albumin and globulin to increase with increasing temperature. ENGELKE *et al.*[11] concluded from data on migration of markers that temperature changes between 12° and 20° did not affect the migration of the markers. SCHULZ AND WEGNER[12], working with a horizontal type apparatus with no applied pressure, detected a slight increase in apparent mobility at lower temperatures. WHITEHEAD[3] has indicated that heating causes increased electrolyte flow.

This variety of findings suggests that temperature measurements alone are not adequate to account for variations in migrant mobility.

Wetness

McDONALD *et al.*[13] mention that the uncorrected electromigration velocity of a migrant on an open strip decreases to practically zero as the wetness approaches zero, and increases up to the velocity in free solution as the wetness increases.

Choice of a mobility marker and a wetness ratio

The assumption is made that the albumin and the dextran marker are moved equally by the electrolyte flow in the Kunkel system employed. For albumin, a dextran marker probably is correct in size, since the corrected albumin mobility is $6 \cdot 10^{-5}$ cm/sec/V/cm at 5°. This value is in good agreement with free flow mobility measurements of TISELIUS and others. The measured mobility of the dextran marker also is of a magnitude similar to that reported by others, about $2 \cdot 10^{-5}$ cm/sec/V/cm at a wetness of 1.5.

McDONALD *et al.*[9] found paper wetness to stabilize at 1.58/1 in an open system and Whatman No. 1 paper. EDWARD AND CRAWFORD[6] found a value of 1.55/1 with a Kunkel system and Whatman No. 3MM paper. In the Kunkel system described, with evaporation controlled, at a wetness of 1.57/1 the flow is practically uniformly toward the cathode (Fig. 1). This probably represents the minimum flow due to endosmosis upon which siphoning effects are superimposed and is considered to be the wetness ratio of choice by McDONALD[9]. At greater wetness values the flow tends to be from the center outward. The absolute value of the rate of electrolyte flow at various locations on the strip is independent of temperature. Thus, in the Kunkel system at a given pressure, corrected albumin mobility appears to be a function of temperature only and starting wetness merely determines what the rate of electrolyte flow is likely to be. To assess the effect of electrolyte flow will be more complex for the Durrum type apparatus, however, since after a given displacement (which depends upon starting point) the dextran will stop, presumably indicating an equilibrium position between evaporative changes and true endosmosis although buffer equilibrium is never achieved[14]. This was indicated here by the identical dextran displacement on Durrum strips run for 8 or for 16 h (Fig. 2).

If dextran is an acceptable marker for albumin, a different marker may be needed for γ-globulin. This is suggested by the negative temperature coefficient obtained by correcting observed γ-globulin mobility values for dextran movement. McDONALD[15,9] suggests that no single electro-osmotic rate flow indicator is satisfactory for migrants of different molecular volumes. In contrast, little or no variance in flow values has been obtained by others[11] with a variety of markers, including hydrogen peroxide. KUNKEL AND TRAUTMAN[16] express similar disregard for the size and nature of the marker. If the molecular size is unimportant, search must be made for some other factor to account for the variability of apparent mobility values for γ-globulin obtained here with both the Durrum and the Kunkel methods and

elsewhere by McDONALD *et al.*[9] with the open strip method at wetness values giving a cathodal electrolyte flow which is constant throughout the strip.

Distance migrated, variability

Measurement of the distance migrated by albumin and γ-globulin is warranted after a 60 min run in the Kunkel system employed. The elapsed time, including equilibration, staining and drying is less than three hours, the interval designated for rapid ionophoresis by the technique of WERUM, GORDON AND THORNBURG[17].

Findings both for the Durrum and for the Kunkel show an intra-run variation in albumin mobility of about 6.5 %. With the open strip at 15° McDONALD *et al.*[9] have detected equivalent variability. For γ-globulin, the average variability with the Kunkel was 18.8 %, a value of the same magnitude as established by the open strip method[18].

In terms of absolute albumin displacement in the Kunkel system the average variation from most to least mobile on the same strip is 0.15 cm. This variability may represent the sum of the errors inherent in applying the sample, drying the paper and measurement[18–20]. The remaining variability may result from variation in structure of the supporting medium with consequent small, localized variations in paper wetness. Equipment for measurement of voltage and temperature at nine spots on the paper during the run has been designed here and may detect such localized variations. Until such measurements are made it will be difficult to extend the usefulness of paper electrophoresis to a qualitative analysis of components of similar mobilities by a simple characterization in terms of mobility. The described system has been applied in this laboratory to the separation of the whole blood proteins of closely related bird species. Within limits of the variability designated, pattern inspection for specimen purity is satisfactory and in some cases identification of specimens can be made on the basis of mobility alone.

For similar specimens of nearly identical mobility, starch block electrophoresis may yield better differentiation at peak concentration but the yield on elution is low[21]. As a preparative technique none of the methods discussed is likely to be as satisfactory for serum proteins as are continuous flow techniques[22]. The cellulose ion-exchange columns, in use here, offer hope for specific reproducible fractionation in greater volume or in single drop elution aliquots[23].

SUMMARY

A rapid Kunkel type electrophoretic system was used to determine the effect of wetness and temperature upon the mobility of serum components. Comparison is made with a Durrum type apparatus. Variability averaged 6.5 % for albumin with both techniques.

Observed component mobility increases both with wetness and temperature with the Kunkel system. When corrected by use of dextran, observed mobility values are independent of wetness, but increase directly with temperature.

Comment is made on mobility markers and a wetness ratio, on specimen identification by mobility alone and on the usefulness of zone electrophoresis in qualitative analysis.

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