RAPID PAPER IONOPHORESIS USING ORGANIC BUFFERS IN WATER–FORMAMIDE AND WATER–UREA

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Paper ionophoresis can often separate mixtures not well resolved by paper chromatography, *e.g.*, the 3-methyl and 4-methyl ethers of D-glucose[1]. It can also furnish unique information about the ionizable groups in a molecule, useful in characterizing unknowns. However, it has many limitations. The most serious one is evaporation of water from the paper strip; this can be minimized by using various cooling systems but the apparatus becomes more complex and less convenient to use. Adsorption on the cellulose can distort the ionophoretic movement of spots. Mobility values are usually non-linear with time and not highly reproducible, and ionophoresis is often extremely slow (12 hours or longer).

The work reported in this paper had three main objectives: First, to develop a series of new buffers of various pH values, which would minimize evaporation, adsorption, and non-linear mobility. Second, to develop a series of "tracer dyes", to serve as reference standards for calculation of reproducible relative mobility values. Relative mobility (using glucose as a standard) has been shown to be reproducible and characteristic in ionophoresis of carbohydrates[1]. Third, to reduce the time required for ionophoresis to 3 hours or less, without elaborate apparatus or cooling systems.

APPARATUS

The Misco micro-analytical ionophoresis apparatus (made by Microchemical Specialties Co., Berkeley, Calif.) was used in this work. It is a modification of the WIELAND-FISCHER design[2], and consists of ten small Lucite cells, 6.5 cm long, 3.5 cm wide, and 3.5 cm high. Each cell has a platinum electrode set in a Lucite mount, with male and female couplings. Five cells are usually set side-by-side in a battery, with their electrodes connected into a common electrode, which is connected to one of the terminals of the DC power supply. The other five cells are similarly connected, and to the other terminal. The two sets are placed 15 cm apart, and an indented Lucite plate (33 × 15 cm) set between them to support the paper strips. Five different buffers can be used simultaneously. The buffers have nearly the same resistance, and all five paper strips operate at the same voltage and current, which remain fairly constant throughout the ionophoresis. The Misco cells are designed for small capacity

(5 or 10 ml), since buffers are used only for a few runs (about 25 mA-hours) and then discarded.

For rapid uniform drying of ionograms, a 9 × 9 inch Misco hot-plate is used. This is an aluminum plate with imbedded heating element controlled by a variable transformer. Very uniform, constant surface temperatures can be obtained by setting the voltage to any desired value. This minimizes spot migration and distortion during drying.

The power supply used need not provide more than 300 V at 30 mA, or have very good voltage regulation. The methods described here cause only minor changes in the resistance of the paper during ionophoresis, and these changes are largely compensated for by the use of relative mobility values instead of absolute mobilities.

MATERIALS

All the materials used in this work are obtainable from Microchemical Specialties Company, Berkeley, Calif., who also manufacture the apparatus. Most of the materials are also available from various other sources.

Whatman No. 4 filter paper, 1½ or 2 inches wide, is available in 600-foot rolls. This is cut into 24-cm lengths. Starting-lines are drawn in pencil at 12 cm and edge-marks at 4.5 and at 19.5 cm; from three to six short cross-lines are drawn intersecting the starting-lines at 6 to 9 mm intervals. Spots are later applied to the points of intersection. This allows only three to six spots to one strip, but greater crowding is not desirable. Special Misco ionophoresis cells are available for paper wider than 2 inches, for preparative work or use when many samples are to be run in the same buffer.

It is possible to arrange a battery of three pairs of Misco cells lengthwise and 30 cm apart, and thereby double the path length available for ionophoretic separation. In our experience this system has proved useless. A superior way of gaining a longer ionophoresis path length is to draw two starting lines, one at 7 cm and one at 17 cm. The unknown solution (and also a spot of ABA reference dye solution) is then spotted (on different lanes) on both starting lines. Cations will be separated on one lane, anions on the other.

Buffer solutions

FPF, pH 3.3 buffer. Mix 19 g (16 ml) of 88–90% formic acid and 9 g (8.8 ml) of pyridine. Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2.0 ml of stock buffer, 7.5 ml of formamide, and water to a volume of 25 ml. The dilute buffer is approximately 0.09 *M* in pyridinium ion, 0.09 *M* in formate ion, and 0.2 *M* in formic acid, in 30% (v/v) formamide. If pyridine is objectionable it may be replaced by 10 g of 2-dimethylaminoethanol, or an equimolar amount of some other strong organic base. Anhydrous (98%) formic acid (Eastman 139 or equivalent) may be used instead of the 90% acid. The pH of all buffers used in this work falls within 0.05 unit of the intended value, when the stock is diluted with water and measured with a glass electrode.

DFF, pH 4.0 buffer. Mix 9 g (7.5 ml) of 88–90 % formic acid and 10 g (11.3 ml) of 2-dimethylaminoethanol (Eastman 715 or equivalent). Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2.0 ml of stock buffer, 7.5 ml of formamide, and water to a volume of 25 ml. It is approximately 0.09 *M* in hydroxyethyl-dimethylammonium ion, 0.09 *M* in formate ion, and 0.05 *M* in formic acid.

ADF, pH 4.7 buffer. Mix 10 g of 2-dimethylaminoethanol and 13.5 g of glacial acetic acid. Cool and dilute with water to 100 ml. This stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. It is approximately 0.09 *M* in hydroxyethyl-dimethylammonium ion, 0.09 *M* in acetate ion, and 0.09 *M* in acetic acid. Other strong organic bases may be used to replace 2-dimethylaminoethanol.

IAF, pH 6.0 buffer. Mix 24.6 g β,β'-iminodipropionitrile (Eastman P-6555 or equivalent) and 3.36 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps for at least six months. The dilute buffer is prepared by mixing 4 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.09 *M* in acetate ion, 0.09 *M* in dicyanoethyl-ammonium ion, and 0.23 *M* in iminodipropionitrile.

DPF, pH 7.2 buffer. Mix 19.6 g of 2-dimethylaminopropionitrile (Eastman 6786 or equivalent) and 6 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps for at least six months, but not indefinitely because of slow hydrolysis of the nitrile to the amide and acid. The dilute buffer is prepared by mixing 3 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.12 *M* in dimethylaminopropionitrile, 0.12 *M* in cyanoethyl-dimethylammonium ion, and 0.12 *M* in acetate ion.

EAF, pH 8.0 buffer. Mix 26 g of N-ethylmorpholine (Eastman P-6274 or equivalent), and 6.75 g of glacial acetic acid (plus about 20 ml of water to reduce the violence of the neutralization). Cool and dilute with water to 100 ml. The stock buffer is usually colored, but this does not affect its use. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 100 ml. It is approximately 0.09 *M* in acetate ion, ethylmorpholine, and ethylmorpholinium ion. The similar MAF, pH 7.6 buffer is prepared by replacing the N-ethylmorpholine by 22.8 g of N-methylmorpholine. (The use of 2,2',2''-nitrilotriethanol as a pH 8.0 buffer is not recommended, because it is difficult to remove from the paper strips and interferes with many spray reagents.)

DAF, pH 9.3 buffer. Mix 18 g of 2-dimethylaminoethanol (Eastman 715 or equivalent) and 6.6 g of glacial acetic acid. Cool and dilute with water to 100 ml. The stock buffer keeps indefinitely, but slowly becomes yellow in color. The dilute buffer is prepared by mixing 2 ml of stock buffer, 7.5 ml of formamide, and water to 25 ml. The dilute buffer is approximately 0.09 *M* in acetate, 0.09 *M* in hydroxyethyl-dimethylammonium ion, and 0.075 *M* in dimethylaminoethanol.

DBF, pH 9.3 borate buffer. Mix 27.8 g of 2-dimethylaminoethanol and 21.9 g of boric acid, and dissolve in water (heating if necessary) to a volume of about 450 ml.

Cool and dilute with water to 500 ml. The stock buffer keeps indefinitely. The dilute buffer is prepared by mixing 17.5 ml of the stock buffer and 7.5 ml of formamide. The dilute buffer is approximately 0.25 *M* in hydroxyethyl-dimethylammonium ion, and 0.2 *M* in dimethylaminoethanol. The composition of the borate–boric acid system is uncertain, but the low conductivity suggests that the predominant form is hydrated tetraborate $(H_3BO_3 \cdot H_2BO_3)^{=}_{2}$ ion, at 0.125 *M*, with relatively little H_3BO_3. The dimethylaminoethanol greatly augments the buffering power, but also increases the viscosity of the buffer.

The pH values of the buffers were determined with a glass electrode, at the recommended dilution *in water, without formamide*. For all the buffers except the pH 7.2 DPF, pH 8.0 EAF, and pH 9.3 DAF, the glass electrode reading *in 30 % formamide* is about 0.5 pH unit *higher*. The three exceptional buffers are tertiary nitrogen bases, and give the same pH reading with or without formamide. The other buffers can form strong hydrogen bonds with formamide, and this increases the p*K* values by about 0.5 unit in 30 % formamide. Such p*K* shifts must be considered when (in a subsequent section of this paper) mobility values are used to calculate the p*K* of unknowns.

As new organic nitrogen compounds become available, especially among the 2-hydroxyethyl, 2-cyanoethyl, cyanomethyl, and 3-hydroxypropyl substitution products of ammonia, it will become possible to prepare superior buffers in the range from pH 5 to 7 and from 7.5 to 9. The IAF (pH 5.9) buffer now uses iminodipropionitrile far from its p*K* (about 5.5–5.6), because no organic buffer with a p*K* near 6 is available. It would be preferable to use pure substances in place of the technical grades indicated above, and all substances should be used, where possible, at the p*K* value (*i.e.*, a 1:1 ratio of the ionized and unionized forms). When finer gradations of pH are required, the ratio can be altered to 1.25:1, 1.6:1, or 2:1, to shift the pH by 0.1, 0.2, or 0.3 units. If the concentration of the ionized form must be increased, however, the ionic strength will be too high. The ratio must therefore be altered by decreasing the concentration of the unionized form, which causes some loss in buffering power.

Since many proteins are denatured even in 10% formamide, organic buffers containing 10% urea are recommended for protein ionophoresis, and the ionic strength must be lowered to one-third that of the formamide buffers. To prepare urea buffers, use one-third of the recommended volume of stock buffer, and use 10 ml of a 25% solution of urea in water instead of 7.5 ml of formamide. The most useful urea buffer is DPU (pH 7.2).

Time may be saved in preparation of buffers (except borate) by using a solution of 30% formamide (or 10% urea) to dilute the stock buffers. The final concentration is somewhat lower, but this usually makes little or no difference.

Reference dyes for determination of relative mobility

The use of glucose as a reference standard of mobility, in ionophoresis in borate buffers, is now widespread, since M_G values are relatively reproducible[1,3]. This

principle can be extended to the ionophoresis of all substances by using at least two dyes on each ionogram. One must be a neutral dye having zero charge in all buffers. Another must be an ionized dye whose charge is the same in all buffers. In this work, these two primary standards are the following:

Amaranth (FD&C Red No. 2) is the trisodium salt of 1-(4-sulfo-1-naphthyl-azo)-2-naphthol-3,6-disulfonic acid. Molecular weight of the anion is 523. It is a dark red food coloring, and the commercially available dye is quite pure. At pH values from 3.3 to 9.3, it has three negative charges; the phenolic hydroxyl is undissociated.

Apolon, a new common name proposed for a new, slightly water-soluble dye, 4-[4'-(2-hydroxyethyl)-phenylazo]-2'', 2'''-(phenylimino)-diethanol. The name is derived from the Greek *apolos*, meaning "immovable". The dye is synthesized by diazotizing 4-(2'-hydroxyethyl)-aniline and coupling the diazonium salt with 2,2'-(phenylimino)-diethanol. It is a yellow dye, with zero charge in the pH range from 3.3 to 9.3. In all buffers except the borate buffer (DBF) it has nearly the same mobility as the amino acid, proline. In DBF, proline (like all other neutral amino acids) seems to form a weak complex with borate, and moves as if it had a small negative charge relative to Apolon. Preparations of Apolon may also contain an unidentified purple dye, which has a positive charge in the pH range from 3.3 to 9.3 and is easily separated from Apolon during ionophoresis.

On any ionogram, the distance between Apolon and Amaranth (originally spotted together on the starting line) is defined as 100 *Am* units. The mobility of any other substance can be calculated in *Am* units by measuring its distance from the Apolon spot, dividing by the Amaranth–Apolon distance, and multiplying by 100. The charge on the molecule is indicated by a minus sign, if the molecule has moved in the same direction as Amaranth. For example, the mobility of aspartic acid in pH 3.3 FPF buffer is —21, and that of arginine is 68.

This mobility calculation is valid only if the mobility of every substance at every point on the ionogram is constant during ionophoresis. This is approximately true in the center region of the strip (within 25 mm of the center), but mobilities often show a slight decline in the region 25–50 mm from the center. Since the Amaranth spot usually travels in the 25–50 mm region by the end of one hour of ionophoresis, the *Am* value of a slower-moving spot is often larger in a 60-min run than in a 30-min run, since the slow spot moves at a relatively constant velocity, while the Amaranth spot moves more slowly during the second 30 min of ionophoresis. In order to correct for this effect, a second reference dye, moving about half as fast as Amaranth, is introduced.

Brilliant Blue FCF (FD&C Blue No. 1) is the disodium salt of 4-{[4-(N-ethyl-*p*-sulfobenzylamino)-phenyl]-(2-sulfoniumphenyl)-methylene}-(N-ethyl-N-*p*-sulfobenzyl)-2,5-cyclohexadien-1-imine. Molecular weight of the anion is 746. It is a deep blue food coloring, and a relatively pure commercial dye. At pH values from 3.3 to 9.3 it has two negative charges, and its mobility in the various buffers is from 50 % to 60 % of that of Amaranth. Standard "*Am*" values have been determined for this dye,

using 30-min runs, in all the buffers. For any substance having a mobility equal to or less than that of Brilliant Blue, the *Am* value can be calculated by measuring its distance from the Apolon spot, dividing by the Brilliant Blue–Apolon distance, and multiplying by the standard *Am* value of Brilliant Blue. For substances moving faster than Brilliant Blue, the calculation from the Amaranth–Apolon distance usually gives a more reliable *Am* value.

The reference dyes are combined in one solution (the "ABA" solution) and spotted on each ionogram, and serve as the reference points for calculating the mobility of negatively charged compounds. The purple Apolon-impurity has a relatively low mobility (27 *Am* units) and most positively charged compounds therefore move outside the dye framework. Attempts to find a satisfactory fast-moving, positively charged dye were unsuccessful. Most of the dyes tested lose their charge at pH 9.3, or adsorb very strongly to filter paper, or form insoluble precipitates when mixed with Amaranth. One fairly good dye, used for mobility testing in some of our work, is the well-known antimalarial, quinacrine.

Quinacrine hydrochloride (Atabrin, Mepacrine) is 2-methoxy-6-chloro-9-(1'-methyl-4'-diethylaminobutyl)-aminoacridine dihydrochloride. It has two positive charges at pH 3.3 to 7.2, but apparently only one at pH 9.3. It is adsorbed by filter paper, but not as intensely as acriflavin or other dyes with a quaternary nitrogen atom. It has not been used much in this work, although standard *Am* values have been determined. It is usually simpler to use the Brilliant Blue scale for positively charged substances having a mobility equal to or less than Brilliant Blue, and the Amaranth scale for substances of higher mobility. The mobility lag of substances moving in the 25–50 mm region is about the same, on either the anodal or cathodal side of the strip.

PROCEDURE

(A) Dilute buffers are usually prepared within a few hours before use, by pipetting the proper volume of stock buffer into a 25-ml graduate or volumetric flask, and diluting to the mark with either 30% formamide or 10% urea in water. A 5 ml or a 10 ml aliquot is then pipetted into each of a pair of buffer reservoirs in the Misco apparatus. The apparatus must be properly leveled so that there will be no significant difference in level between the buffer reservoirs; any slight difference merely adds to the electro-osmotic flow, and is corrected for by the neutral dye, Apolon.

(B) A pre-cut 24-cm strip of Whatman No. 4 paper is coated (either with a pipette or a Misco applicator) with the buffer, as uniformly as possible, and slightly undersaturated. The correct volume is about 1.2 ml/100 cm^2, or about 1.0–1.1 ml for a strip 24 cm long and 3.8 cm (1.5 inches) wide. One end of the strip is then inserted in one reservoir, the strip is laid across the supporting plate, and the other end inserted in the opposite buffer reservoir. The edge-marks (at 4.5 and 19.5 cm) should be approximately on the edges of the plate. The strip should never be inserted dry and allowed to become saturated by capillary flow from the reservoirs, as the composition

and pH of the buffer can be drastically altered (by chromatography) by the time the liquid has reached the center of the strip.

(C) Several strips of paper saturated with distilled water are laid along the walls of the chamber, to humidify the air. The cover is closed, and a low voltage gradient (about 2 V/cm) applied to accelerate equilibration of the paper strips. Within 15 min, any unevenness in coating is smoothed out, and the paper becomes nearly saturated with buffer. The voltage is then turned off and the strips are spotted, usually on the center starting line, but in some cases on lines 50 mm nearer the anode or the cathode (if mixtures only of fast-moving cations or anions are being examined). One of the spots must be the mixture of Amaranth, Brilliant Blue and Apolon, that will serve as mobility reference standard.

(D) After spotting, the cover is closed and about 5 min allowed for mixing of the applied spots with the background buffer. A voltage gradient (usually 10 or 20 V/cm) is then applied, for a period from 30 to 180 min, as required to effect separation. Movement of the reference dyes indicates the degree of separation attained. When fast-moving substances are being studied, ionophoresis is stopped before the Amaranth spot reaches the edge of the strip; with very slow-moving substances, the Amaranth spot is allowed to move off the strip and ionophoresis is stopped when the Brilliant Blue spot nears the edge.

(E) Immediately after turning off the voltage, the ends of the strips are cut off with scissors, leaving only the 15-cm central region. Any long delay in isolating the central region from the buffer reservoirs will allow inflow of buffer into the strip and cause spots that have moved far out from the center to move backward toward the center. During ionophoresis, the central region becomes undersaturated because of evaporation of water from the strip, and backward flow from the reservoirs may displace spots by several mm within 5 min. When the highly saturated ends of the strips are cut off, however, backward flow to the center is negligible.

(F) Each strip is then blotted, by pressing between two strips of filter paper, to remove excess buffer. It is then laid flat on the hot plate for a few minutes, until it is almost dry. When thermolabile substances are being studied, strips can be air-dried overnight. The centers of the reference dye spots can be marked with pencil before drying; they should not be displaced during drying by more than 1 mm. The blotted strips can be extracted with acetone, 2-butanone or ether to remove excess buffer, water, and formamide quickly, without heating. However, it is better to dry the paper strips on the hot plate partially, and then extract. Extraction is highly recommended, because the strips can then be detected with almost any spray reagent and with maximum sensitivity.

(G) The buffer in a pair of reservoirs can be re-used several times during one day, with new strips coated with the excess supply of fresh buffer prepared in the morning. When routine separations are being run, buffer can be used for 2 or 3 days, with as many as 12 successive paper strips. In this case, where precise pH maintenance is not needed, only 6 ml of buffer is pipetted into each reservoir, leaving 13 ml (of the original 25) available for coating strips.

Detection of spots on paper ionograms

Many reagents used for detecting spots on paper chromatograms cannot be used directly on paper ionograms because of residual organic buffer, formamide, or urea. It is usually desirable to extract the ionograms with an organic solvent before detecting spots, but this is sometimes unnecessary because some reagents are specific enough to show no interference from the background buffer. The following reagents have been used in this work, either by dipping the ionograms in the reagent, or by pouring the reagent directly from the stock bottle (or with a pipette) over the ionogram. The "pouring" technique is less satisfactory on unextracted ionograms containing residual formamide, because the formamide may form streaks and distort spots; "pouring" should be used primarily on ionograms extracted or completely air-dried.

AD-1 is a 0.1 % solution of ninhydrin in acetone, used to detect amines and amino acids. More sensitive ninhydrin reagents have been described[4], but these usually require spraying. Some amino acids develop a blue color when the ionogram is heated over a boiling water or steam bath; other amino acid spots can be developed by careful heating on a hot plate or over a Bunsen flame.

AD-2 is a 0.1 % solution of alloxan monohydrate in acetone. It is used exactly like AD-1, but is a more sensitive detector for some amino acids.

CD-3 is a periodic acid–benzidine reagent, previously described for detection of many carbohydrates on paper chromatograms[5]. It can be used without prior extraction to remove buffers, and gives brilliant and sensitive detection on borate ionograms. It is a pouring or dipping reagent, but can also be sprayed if desired. It not only detects polyols but also serine and threonine.

Bromphenol Blue (3',3'',5',5''-Tetrabromophenol-sulfonephthalein). This is used for detection of proteins that have been heat-denatured by drying ionograms on the hot plate. The ionogram is washed in 5 % (v/v) acetic acid in water to remove buffer and urea, and then immersed in a 0.2 % solution of Bromphenol Blue in 5 % acetic acid for 5 to 10 min. It is then quickly washed in 5 % acetic acid to remove excess dye, in water to remove excess acid, blotted dry and then completely dried (in air, or on the hot plate). Exposure to ammonia vapor then turns the protein spots deep blue. The ionogram can be de-stained by washing in dilute ammonia water, and then re-stained by the above (or some other) procedure, since the protein spots are permanently fixed to the paper.

RESULTS

Osmotic and evaporative flow rates of various buffers

Since filter paper has some negatively charged groups (probably carboxylic acid) in its structure, application of a voltage gradient to buffer-saturated paper forces the buffer to move as if it were positively charged, toward the anode. All substances in solution are subject to this "electro-osmotic" flow. It can be measured by the displacement of a spot of Apolon applied to the exact center of the paper strip. Values for various buffers and voltage gradients are listed in the column headed "N_0" in

TABLE I

OSMOTIC AND EVAPORATIVE FLOW OF BUFFERS ON WHATMAN NO. 4 PAPER AT 20°–22° AT SEVERAL VOLTAGE GRADIENTS

Buffer			V/cm	mW/cm²	N_0	$-N_{+50}$	$-N_{-50}$
Type	Ionic strength	pH					
FPF	0.2	3.3	20	34	4, 5, —*	32, 19, —*	38, 22, —*
			10	9	4, 3, 4	4, 6, 5	6, 4, 6
			5	3	2, 2, 2	6, 6, 4	6, 5, 5
ADF	0.18	4.7	20	32	6, 5, 6	42, 29, 21	40, 27, 16
			10	8	6, 5, 5	2, 3, 4	4, 4, 5
			5	3	4, 3, 3	2, 3, 2	2, 2, 3
DPF	0.24	7.2	20	32	10, 6, 6	42, 29, 17	44, 29, 18
			10	8	4, 5, 5	6, 6, 6	6, 6, 6
			5	3	4, 3, 3	4, 4, 3	8, 5, 4
DAF	0.18	9.3	20	32	16, 12, 9	38, 27, 15	40, 30, 18
			10	8	6, 6, 5	4, 4, 5	6, 4, 4
			5	3	4, 3, 3	4, 4, 3	6, 4, 4
DBF	0.75	9.3	20	32	14, 10, 8	42, 27, 16	46, 30, 18
			10	8	10, 9, 8	10, 8, 7	10, 10, 8
			5	3	4, 4, 4	4, 3, 2	6, 5, 4

All buffers are the standard 30% formamide systems described under MATERIALS. However, the FPF buffer used in the experiments of Tables I to V had an ionic strength 10% higher than that of the buffer described in the text. This lowered absolute mobility values slightly, but made no other difference. Voltage gradients (V/cm) are approximate, as are values for the electrical heat input in mW/cm².

Spots of the neutral dye (Apolon) were applied in the exact center of the strip (N_0), and 50 mm from the center toward the cathode (N_{-50}), and toward the anode (N_{+50}). All mobilities are in mm/h; three values are given for each spot in sequence. The first value is measured at 30 min, the second at 60, and the third at 120. Low values are not very accurate, since measurements were only to 1 mm. Values for the 50-mm spots have been corrected for osmotic flow by subtracting the corresponding value for N_0; *e.g.*, the observed values for FPF at 10 V/cm were 10 and —1 mm/h, which are altered (by subtracting the osmotic flow rate of 4 mm/h) to 6 and —5 mm/h. All values in column $-N_{+50}$ are negative, because spots move toward the anode, "as if" they had a negative charge.

* No value, since paper charred after 1 hour.

Table I. For the pH 3.3 buffer, which partly suppresses the ionization of carboxyl groups, osmotic flow is about 4 mm/h at 10 V/cm. For the pH 4.7, 7.2, and 9.3 buffers it is about 5–6 mm/h; for the pH 9.3 borate buffer, which augments the negative charge on the cellulose by forming weak borate complexes with its glucose units, the rate is 8–9 mm/h. At lower voltage gradients the rate is proportionately less. At 20 V/cm, however, the N_0 values in Table I are lower than the theoretical because of evaporative flow. Only five of the buffers listed under MATERIALS have been extensively studied and are included in Tables I to V; tests with the other buffers, however, have given very similar results.

Evaporation of water from the buffer cannot be completely suppressed in the Misco apparatus, although humidifying the air reduces the evaporation rate. Strips through which current is flowing never become much warmer than the ambient air. At low values of electrical heat input, most of the heat is lost by convection, and evaporation is very slow. Since heat input increases as the square of the voltage

gradient, there is a critical voltage above which convection cannot dissipate the heat and evaporation then shows a spectacular increase. Data on evaporative flow (in Table I) indicate that this critical voltage gradient is between 10 and 20 V/cm under the conditions used in these experiments. The inflow of buffer from the reservoirs into the center of the strip is very fast at 20 V/cm, of the order of 1 mm/min at a distance of 50 mm from the center. Additional studies (not shown in Table I) indicate that evaporative flow is roughly proportional to distance from the center. At 20 V/cm, it is about 0.5 mm/min at 25 mm from the center, and 2 mm/min at the buffer reservoirs. We can estimate an evaporation rate of about 6 μl/cm^2/h, since the buffer content of saturated Whatman No. 4 paper is about 10 μl/cm^2. This evaporation not only distorts the normal movement of charged substances, but rapidly changes the composition of the buffer. The center of the strip becomes permanently undersaturated, and the buffer in this region may have twice the ionic strength and formamide content of the original buffer. Linearity of voltage gradient is destroyed, and heating of the center may even cause the paper to char.

At 10 V/cm, however, evaporation is less than 1 μl/cm^2/h, and changes in the buffer on the strip are not drastic even after several hours. Nothing is gained by using a lower voltage gradient, since evaporative flow rates are not much less than those at 10 V/cm.

Table II summarizes the results of experiments on varying ionic strength and percent of formamide. Buffers containing 10% urea are similar to those containing 10% formamide. Table II shows that lowering ionic strength to 1/5 of the standard makes it possible to increase the voltage gradient to 20 V/cm without excessive evaporation. This change will seldom be desirable, however, because the lower ionic

TABLE II

EFFECT OF IONIC STRENGTH, FORMAMIDE, AND VOLTAGE ON OSMOTIC FLOW (O.F.) AND EVAPORATIVE FLOW (E.F.)

Buffer	*pH*	*Ionic strength*	*% F or U**	*V/cm*	*mW/cm²*	*O.F. mm/h*	*E.F. mm/h*
DA	9.3	0.18	30% F	10	8	5	4–5
DA	9.3	0.036	30% F	20	11	16	9
DA	9.3	0.036	30% F	50	80	36	130
DA	9.3	0.18	10% F	10	8	4	9
DA	9.3	0.036	10% F*	20	11	14	12
DA	9.3	0.18	0%*	10	8	5	10
DA	9.3	0.036	10% F	10	2	7	8
DA	9.3	0.036	0%*	20	11	10	14
DA	9.3	0.036	0%	5	0.7	3	3
FP	3.3	0.04	30% F	20	13	11	12
AD	4.7	0.036	30% F	20	11	16	10
DP	7.2	0.048	30% F	20	11	15	10
DB	9.3	0.15	30% F	20	11	23	26

* Spots show cometing at 10% F, streaking at 0%. Effect is more severe at low ionic strength and higher voltage gradients. 10% urea gives slightly higher evaporation than 10% formamide. Values of E.F. higher than 10 usually give unsatisfactory ionograms; values of 5 or less give very good ionograms. Values were determined as in Table I by movement of test spots of Apolon.

strength decreases the capacity of the buffer (spots tending to become larger and less symmetrical) and increases the osmotic flow.

Adsorption of substances on the paper strip

Cellulose adsorbs many substances quite strongly, and this has led many workers to abandon paper ionophoresis in favor of ionophoresis in starch gels or other substrates, especially for separation of proteins. Adsorption can be expressed as a coefficient, α, which when multiplied by the "true" or expected mobility of the substance, gives the observed mobility. Adsorption can be measured by saturating a paper strip with a buffer and suspending it from a buffer reservoir so that the liquid flows down the strip by gravity flow. If spots of various substances are applied to a starting line near the top of the strip, all substances should move downward at the same rate unless some are retarded by adsorption. The results of a series of experiments indicate that only the reference dye, Brilliant Blue, is not adsorbed by Whatman No. 4 paper in any of the buffer systems used in this work. Its coefficient may be taken as 1.0 and coefficients for other substances calculated on this basis. Table III gives coefficients for the reference dyes Amaranth and Apolon, and for two smaller molecules (proline and glycerol). The coefficients are altered if the experiment is run in the open air instead of a closed chamber, since evaporation of water lowers the saturation of the paper and increases the actual concentration of buffer and formamide; this usually decreases adsorption.

In all buffers containing 30 % formamide, the coefficients of Apolon, Amaranth, proline, and glycerol are not very different. Omission of the formamide increases the adsorption of the dyes but decreases that of proline and glycerol. Apolon is not a perfect zero-mobility indicator in the aqueous or 10 % urea buffers, if there is any large displacement by evaporative or osmotic flow. This will usually be obvious since the Apolon spot will streak badly. The reduction of strong adsorptive effects by formamide is a very useful characteristic of the buffers. Solvation by formamide may tend to equalize the attractive forces between molecules of different size and chemical structure and molecules of cellulose. Urea has a similar action, but cannot be used at a high concentration.

The most deleterious effect of adsorption is not the reduction in mobility, but the very marked tailing and streaking of spots, which makes it difficult to locate the center of a spot and which may cause adjacent spots to overlap.

The problem of adsorption is discussed in an unusually thorough way by McDONALD[6], who emphasizes the importance of saturation or "wetness" of the paper, of the structure, charge, and concentration of the moving substance, and of the type of paper used. All these factors make the adsorption coefficient unpredictable, and it must be determined empirically.

Mobility of reference dyes

Table IV gives mobilities (in mm/h, relative to Apolon) of Quinacrine (Q), Brilliant Blue (B), and Amaranth (A) in various buffers, at various voltage gradients, and with

TABLE III

ADSORPTION COEFFICIENTS FOR REFERENCE DYES IN VARIOUS BUFFERS ON WHATMAN NO. 4 PAPER
(relative to Brilliant Blue, taken as 1.0)

Substance	*FPF*	*PFU*	*PF*	*ADF*	*ADU*	*AD*	*DPF*	*DPU*	*DP*	*DAF*	*DAU*	*DA*	*DBF*	*DBU*	*DB*	*H_2O*
Apolon	0.83	0.61	0.52	0.87	0.54	0.36	0.88	0.55	0.41	0.87	0.50	0.40	0.81	0.67	0.63	0.70
Amaranth	0.89	0.77	0.67	0.90	0.78	0.65	0.93	0.82	0.64	0.91	0.74	0.64	0.91	0.82	0.82	0.90
Proline	0.91	0.99	1.02	0.87	0.88	1.00	0.88	0.95	0.97	0.86	0.95	0.95	0.86	0.92	0.91	1.03
Glycerol	0.94	1.00	1.05	0.90	0.91	1.00	0.84	0.91	0.97	0.89	0.92	0.95	0.85	0.92	0.90	1.00

TABLE IV

MOVEMENT (mm/h) OF REFERENCE DYES AT SEVERAL VOLTAGE GRADIENTS

Buffer	*V/cm*	*Q*	*Q_{+50}*	*$-B_0$*	*$-B_{-50}$*	*$-A_0$*	*$-A_{-50}$*	*N_0*	*Standard Am values*	
									Q	*B*
FPF (pH 3.3)	20	60, 40, —*	62, 40, —	56, 46, —	50, 47, —	81, 61, —	84, 67, —	5		
	10	28, 33, 35	30, 35, 34	28, 30, 32	28, 31, 29	42, 49, —	42, 47, 48	4	71	63
	5	16, 16, 16	16, 15, 16	14, 14, 14	14, 15, 13	22, 22, 22	20, 22, 21	2		
PFU	10			36, 30, 25		36, 35, 31		5	—	(82)
PF	10			34, 31		28, 28		6	—	—
ADF (pH 4.7)	20	58, 43, —	56, 38, 32	40, 30, 32	44, 31, 31	76, 58, —	78, 59, —	6		
	10	28, 33, 31	28, 34, 32	24, 27, 24	26, 27, 25	44, 47, 43	40, 47, 44	5	70	57
	5	14, 15, 15	17, 18, 18	12, 12, 12	12, 13, 13	22, 22, 22	22, 22, 23	3		
ADU	10			34, 29, 26		40, 39, 37		4	—	(72)
AD	10			36, 27		30, 26		4	—	—

(Continued on p. 137)

TABLE IV *(continued)*

Buffer	*V/cm*	*Q*	Q_{+50}	$-B_0$	$-B_{-50}$	$-A_0$	$-A_{-50}$	N_0	*Standard Am values*	
									Q	*B*
DPF (pH 7.2)	20	56, 45, —	52, 38, 39	42, 32, 32	46, 33, 36	80, 63, —	80, 64, 49	6		
	10	32, 29, 27	28, 29, 27	22, 25, 23	24, 26, 23	40, 43, 44	40, 45, 41	5	66	57
	5	14, 16, 15	16, 14, 14	14, 13, 12	12, 14, 13	24, 23, 21	22, 23, 22	3		
DPU	10			30, 26, 21		40, 37, 34		5	—	(68)
DP	10			34, 25		30, 28		4	—	—
DAF (pH 9.3)	20	34, 26, 30	34, 24, 30	48, 35, 30	50, 34, 29	90, 69, —	88, 65, 55	12		
	10	16, 17, 17	16, 17, 15	28, 29, 26	24, 29, 27	46, 50, —	44, 50, 49	6	34	57
	5	8, 8, 8	10, 9, 8	16, 15, 14	14, 15, 13	26, 26, 25	26, 26, 25	3		
DAU	10			34, 30, 27		44, 42, 43		4	—	(70)
DA	10			36, 30		32, 32		4	—	—
DBF (pH 9.3)	20	16, 16, 19	14, 16, 18	32, 24, 21	32, 23, 21	66, 48, 41	62, 46, 40	12		
	10	10, 8, 10, 10	8, 8, 8, 9	24, 21, 19, 16	24, 21, 19, 18	46, 40, 37, 34	44, 40, 36, 32	8	27	50
	5	4, 4, 4	4, 4, 5	8, 9, 9	10, 11, 9	18, 18, 18	20, 20, 18	4		
DBU	10			24, 17, 10		38, 30, 21		5	—	(55)
DB	10			18, 12		30, 22		7	—	(55)

Dye spots were applied in the exact center of the strip (*i.e.* Q_0), and 50 mm from the center toward the cathode (*i.e.* B_{-50}), and toward the anode (*i.e.* Q_{+50}). First number in column under each dye refers to mm/h movement after first 30 min, second number after 1 h, third number after 2 h, fourth number after 3 h.

Numbers in last two columns refer to calculated *Am* values for Quinacrine (*Q*) and Brilliant Blue (*B*) dyes, and are the ratios of *Q* or *B* movement to Amaranth movement × 100.

* No value, since spot moved off paper.

initial spots applied either at the center (indicated by subscript zero, *e.g.*, Q_0) or 50 mm from the center of the strip (indicated by subscripts of +50 if toward the anode, and −50 if toward the cathode). Values for the first 30 min of ionophoresis are relatively inaccurate because centers of spots were not located to better than ± 1 mm, and movement was relatively small (less than 25 mm in most cases). During the initial period, also, some time is required for establishing a relatively steady state.

During the first 30 min, absolute mobility is nearly proportional to voltage gradient, even at 20 V/cm (except for the very high ionic strength DBF buffer). The location of the initial spot (at 0 or at 50 mm) usually does not affect the mobility value.

After one hour, however, mobilities at 20 V/cm are only from 1.1 to 1.5 times those at 10 V/cm, since the effect of the strong evaporative flow is to drive all spots toward the center of the strip. A gradient of 20 V/cm should never be used when a reliable mobility value (for characterization of a compound) is required. In a few instances, the use of 20 V/cm gives good separation of mixtures in 50 % less time than that required at 10 V/cm, and may therefore be advisable.

At 10 V/cm, ionophoretic mobilities are nearly constant even after 2 h, at all points on the paper strip. There is an approximately 10 % decline from the one-hour value in most cases, but this is largely corrected for if mobilities are expressed in *Am* units rather than in mm/h.

For each of the standard buffers, standard values for mobility (in Amaranth units) can be calculated from the mobilities (at 1 and 2 h) at 10 and 5 V/cm. The value for Brilliant Blue is constant at 57 at pH 4.7 to 9.3, but is somewhat higher at pH 3.3 and somewhat lower in pH 9.3 borate. The small changes probably reflect minor interactions with pyridinium or hydrogen ions and with borate ions. For Quinacrine, the *Am* value is about 70 at pH 3.3 and 4.7, declines slightly at pH 7.2 as the p*K* of the acridinium ion is neared, and falls to half the acid value at pH 9.3, where the acridine ring nitrogen is probably uncharged, while the nitrogen of the dialkylamino group retains its positive charge. There is a further slight decline in pH 9.3 borate, possibly indicating weak complexing with borate ions.

In buffers containing 10 % urea instead of 30 % formamide, the *Am* values for Brilliant Blue are higher. The value is only 10 % higher in 9.3 borate, where the high ionic strength exerts a strong anti-adsorptive effect, but it is 20 % higher at pH 9.3 to 4.7, and 30 % higher at pH 3.3. In buffers containing neither formamide nor urea, Brilliant Blue moves nearly as fast or faster than Amaranth (except in the borate buffer). This effect is largely due to a striking fall in the absolute mobility of Amaranth, since the mobility of Brilliant Blue is almost unaffected by formamide or urea, and is largely explained by the strong adsorption of Amaranth at low formamide (or urea) concentration. It is not necessary to abandon "Amaranth units" as standard mobility scale, however. All that need be done is to use Brilliant Blue as the reference dye (in systems containing less than 30 % formamide), and use the standard *Am* values obtained for Brilliant Blue in 30 % formamide buffers.

All methods of paper ionophoresis other than those described in this paper will

probably give stronger adsorption effects, and mobility values will often differ from those obtained in this work. If other workers wish to use reference dyes with buffers not containing formamide, we recommend that a mixture of Apolon and Brilliant Blue be used, with the distance between the two dyes defined as 60 *Am* units.

Separation and characterization of small organic molecules

Table V gives mobilities (in Amaranth units) for a few amino acids, peptides, and carbohydrates in the standard (30% formamide) buffers at 10 V/cm. All except borate were run for 60 to 120 min; borate runs were 150 to 180 min. Spots were 1 μl, concentrations 0.02 *M* to 0.1 *M*. Carbohydrates and amino acids were run both

TABLE V

MOBILITIES (IN AMARANTH UNITS) OF SOME SMALL ORGANIC MOLECULES*

Substance	*FPF pH 3.3*	*ADF pH 4.7*	*DPF pH 7.2*	*DAF pH 9.3*	*DBF pH 9.3*
Serine	—	—	—	—28	—51
O-Phosphoserine	—60	—66	—74	—95	—90
Glycine	7	0	0	—12	—37
O-Phosphoethanolamine	— 1	—10	—33	—57	—58
Alanine	5	—	—	—10	—22
β-Alanine	61	—	—	— 3	—15
γ-Aminobutyric acid	74	13	2	0	—17
Proline	0	0	0	0	— 8
Aspartic acid	—21	—71	—76	—81	—82
Glutamic acid	— 7	—60	—72	—77	—77
Glycyl-glycine	33	1	— 2	—73	—80
Glycyl-asparagine	22	1	— 2	—58	—65
Glycyl-alanine	—	—	—	—72	—76
Alanyl-glycine	—	—	—	—66	—70
Glycyl-glycyl-glycine	30	—	—	—64	—66
Histidine	68	63	8	—10	—33
Arginine	68	68	62	45	33
Sucrose	—	—	—	0	—16
Lactose	—	—	—	0	—33
Mannose	—	—	—	0	—48
Glucose	—	—	—	0	—64

* Ionophoresis for 1 or 2 h at 10 V/cm, except borate (DBF) buffer which was run for 2 to 3 h. One Amaranth unit corresponds to an absolute mobility (relative to Apolon) of about 0.3 mm/h in DBF, and about 0.45 mm/h in the other buffers. Two spots are clearly resolved if their mobilities differ by about 8 *Am* units.

singly and in mixtures; *Am* values were the same. When a substance was known or expected to have a low mobility in a buffer, it usually was not run; this accounts for many of the blank spaces in the table.

The results for the amino acids are essentially those predictable from the p*K* values of the ionizable groups. The most useful single buffer for characterizing an unknown is probably the FPF pH 3.3 buffer; if *Am* values in this buffer and also in the DAF pH 9.3 buffer are determined, an unknown can often be identified at least as to its class (*i.e.*, as a basic, neutral, or acidic simple peptide or amino acid).

The classic paper of CONSDEN, GORDON AND MARTIN[8] gives a mathematical analysis of the optimal pH for separation of substances whose pK values and ionic mobilities are very similar; the optimal pH lies between the two pK values, and somewhat closer to the pK of the substance having the lower ionic mobility. For aspartic and glutamic acids (pK's 3.65 and 4.25), the optimal pH is about 4.0, since glutamic acid has a slightly lower mobility than aspartic acid. Difficult separations may require very precise control of the buffer pH.

The data in Table V may be used to calculate approximate pK values of ionizing groups. If we assume that the decline in mobility of histidine from pH 3.3 to pH 7.2 is due to loss of one proton, and that the Am value of 68 at pH 3.3 is largely due to this one positive charge, the ratio of the acidic form to the basic form at pH 4.7 is 63/5 and at pH 7.2 it is 8/60. By adding the logarithm of this ratio to the pH of the buffer we obtain estimated pK values of 5.8 and 6.1 (correct value 6.0 for imidazole group of histidine[7]). Analogous calculations for the acidic group of aspartic acid give ratios of 5/71 at pH 4.7 and 55/21 at pH 3.3, and estimated pK is 3.6 and 3.7 (correct value 3.65). For glutamic acid, the ratios are 12/60 and 65/7, and estimated pK is 4.1 and 4.2 (correct value 4.25).

Calculated pK values agree with the correct values when based on ionophoretic mobilities in the acidic buffers, but not in the three alkaline buffers containing tertiary nitrogen bases. For example, values for the alpha-amino group pK, if calculated from mobilities in Table V for the amino acids in the pH 9.3 DAF buffer, are all approximately 0.5 pH unit higher than the correct value. The reason is the "formamide pK-shift" noted in the description of the buffers. All the ionizing groups of the amino acids can form hydrogen bonds, and their pK values are therefore increased by 0.5 pH unit in 30 % formamide. In the acidic buffers (which are "hydrogen-bonding"), the pK of the buffer also shifts and the effects cancel out. In the alkaline (and "non-hydrogen-bonding") buffers, only the amino acids undergo the pK-shift. Therefore, when the pK of an unknown is calculated from mobility values in the "non-hydrogen-bonding" buffers, it will usually be desirable to *subtract* 0.5 pH unit from the estimated pK value.

Table V indicates that one charged group confers a mobility of about 65 to 70 Am units to molecules of molecular weight about 150 (histidine, arginine, aspartic acid, glutamic acid, glycyl-glycine). For γ-aminobutyric acid (molecular weight 103) the maximum Am value per charge is probably 75 to 80, and for β-alanine (molecular weight 89) it is probably 85 to 90. If we assume a value of 90 for β-alanine, the ratio of acidic to basic form at pH 3.3 is about 62/28, and the pK of its carboxyl group is therefore 3.6 (correct value, 3.6). It is clear that a series of Am values at different pH's will sometimes make it possible to estimate either the pK, or the molecular weight, or sometimes both.

Amaranth and Brilliant Blue have molecular weights of 500–700 and an Am value per negative charge of about 33. It is probable that very large molecules (like proteins) have much lower Am values per charge. The empirical value $800/\sqrt{M}$ roughly predicts the Am value per charge as a function of the molecular weight, M.

The data of WEBER[9] (for a large series of amines in pH 3.8 citrate buffer) suggest that the *Am* value per charge would be about 150 for propylamine, 200 for methylamine, and 250 for very small ions like ammonium or potassium.

Mobility of borate complexes

The DBF buffer is a "hydrogen-bonding" buffer, and gives mobilities for most of the amino acids that agree well with those predictable from the known p*K* values of the α-amino groups. This is presumably due to the mutual cancellation of the formamide p*K*-shifts, as in the acidic hydrogen-bonding buffers. However, calculated p*K* values for the α-amino group of aspartic and glutamic acids are 0.3 pH unit too high, and for the imino group of proline 0.3 pH unit too low. It is possible that interactions with borate (or other buffers) may sometimes alter the p*K* values (or the molecular size and shape and charge), so that the p*K* calculated from mobility will not be identical with that obtained in dilute aqueous solution. It is important to note that an increased negative charge on an unknown in the pH 9.3 DBF buffer, compared to that in the pH 9.3 DAF buffer, does not always prove that borate-complexing groups are present in the molecule, especially if the molecule has a hydrogen-bonding ionizing group whose p*K* is in the region from pH 8.5 to 10.5.

In the separation of carbohydrates, DBF gives results similar to those obtained by other workers with borate buffers. Expected *Am* values for sugars can be calculated from the data in Table X of the review by MICHL[3], which gives mobilities relative to glucose. The expected *Am* value for mannose is —47, for lactose —25, and for sucrose —12. The latter two values are about 25 % lower than those in Table V. This may in part reflect a difference between the mobility of Apolon and that of the zero mobility standard used in previous studies (usually proline or a methyl-substituted glucose). It may also be a result of the very high borate concentration of DBF (nearly four times higher than that used by other workers), and the very powerful additional buffering provided by the dimethylaminoethanol in DBF, both of which will intensify complex formation. CONSDEN AND STANIER[10] observed that use of a "strong" borate buffer increased the mobilities of many carbohydrates (but not of ketoses or ribose) by 30 %, when compared to their usual, weak buffer. The absolute mobility of glucose in DBF is only 5×10^{-5} cm²/volt-second, compared to the value of 14.5×10^{-5} reported by CONSDEN AND STANIER. The discrepancy is probably due to the much higher ionic strength and viscosity of DBF. The ionophoretic system used by CONSDEN AND STANIER, however, probably had higher osmotic flow rates and more intense paper adsorption effects than those obtained with DBF. Both relative and absolute mobility values in borate buffers are dependent on borate concentration, pH, buffer viscosity (and therefore temperature), adsorptive power and resistance to liquid flow of the paper used, and even the quantity of the carbohydrate spotted. Relative mobility values in any one ionophoretic system are fairly reproducible, but there will be differences in the values obtained in different systems. Rough tests on many carbohydrates in our system indicate that mobilities relative

to glucose agree fairly closely with the values tabulated by MICHL[3], except that our mobility values below M_G 0.50 are often somewhat higher.

The structure of borate complexes is not definitely known. FOSTER[1] suggests a "monodentate" complex and a "bidentate" complex, in which one negatively charged boron atom forms a chelate ring with one molecule or two molecules of polyol. MICHL[3] suggests that a "tridentate" complex may be possible with certain cyclitols, but the evidence is not conclusive. The data of FOSTER and his co-workers, tabulated by MICHL[3], on the mobility of various substituted glucoses prove that glucose has several reactive configurations. The most active configuration (A) is apparently that of the hydroxyls on carbons 2 and 4 in the open-chain (aldehydo) form. The configuration (B) of the hydroxyls on carbons 1 and 2 in the cyclic form also has a high affinity for borate. Several other configurations react with borate, but much more weakly. We conclude that it is likely that most of the complexed glucose (at pH 9.3 and high borate/glucose ratio) is in a 1:1 borate complex either as A or B, with low concentrations of other types of complexes. The *Am* value of glucose is —65, which is a reasonable value for a complex with one charge and a molecular weight less than 200; a 2:1 complex, with a molecular weight of more than 360, should have a much lower mobility. It is remarkable that no carbohydrate has a higher mobility than glucose; this suggests that nearly 100 % of the glucose in a spot is in a borate complex. Substances with configurations having a lower affinity for borate, such as mannose, are presumably partly uncomplexed and partly in 2:1 complexes, and so have lower mobilities. Most pentitols and hexitols have mobilities near that of glucose, but glycerol has a very low mobility (M_G 0.44, or about 30 *Am* units). This suggests that only linkages between borate and two *secondary* hydroxyl groups in a molecule (especially the *β-cis*-configuration noted by FOSTER[1]) are very strong. Glycerol cannot form such linkages, and is probably only partly complexed (largely as a 2:1 complex). The unusually high mobility of the all-*cis* isomers of inositol and quercitol[3] (M_G 1.60, or about 100 *Am* units) suggests that two borate ions may complex with one molecule.

Mixed complexes between a molecule of carbohydrate and the cellulose of the paper may be largely responsible for the lowering of mobility by adsorption. Presumably, two different carbohydrate molecules in a mixture may similarly interfere with each other, so that mobility values will not be the same as those obtained in the ionophoresis of pure substances. This interference will probably be most severe between substances of relatively low and nearly equal mobility, since the percentages of uncomplexed forms will be high and separation will be slow.

Resolving power in ionophoresis

In the ionophoresis of mixtures, with a starting spot of 1 μl volume, spot radius is 2–3 mm. Mobilities must therefore differ by about 6 mm/h (equal to 12 *Am* units) for clear separation in a 1-h run. In the 2- or 3-h runs required for separation of slow-moving spots, a mobility difference of 4–6 *Am* units should be sufficient, but the spreading of spots by diffusion blurs the separation if the difference is less than 8 *Am*

units. In the DBF borate buffer (which gives lower absolute mobilities), a difference of 10–15 *Am* units may be necessary for complete resolution. In buffers containing less than 30 % formamide, adsorption on cellulose may cause streaking or tailing of spots and lessen the resolving power. If the ionic strength of the solution spotted is much higher than that of the background buffer, spots become very large and even a difference of 20 *Am* units may not give clear separation. If the solution spotted causes a local pH shift in the background buffer, resolution of spots may be lessened. This is why it is desirable to use buffers at a high ionic strength and at a pH near the p*K*, where buffering power is maximal.

Ionophoresis of proteins

When used for separation of human plasma proteins, the 0.06 ionic strength, 10 % urea buffers give very fast separations at gradients of 10 to 15 V/cm, in 30 to 120 min. Four or five protein spots can usually be obtained. Albumin has a mobility of about —45 *Am* units (relative to Brilliant Blue, taken as —57 *Am* units) at pH 9.3, —30 *Am* units at pH 7.2, and 37 *Am* units at pH 3.3. The clearest ionograms are at pH 7.2, where adsorption and denaturation effects seem to be minimal. There seems to be no advantage to the use of lower voltage gradients and longer ionophoresis time, since diffusion and adsorption spread the protein spots. Operation of the apparatus in a cold room, however, would probably be advantageous.

If the 10 % urea is omitted, similar but somewhat less clear ionograms are obtained. Ionograms may be dried by immersion in cold acetone, to preserve enzymes that would be destroyed by the usual hot-plate drying step.

Advantages and disadvantages of the organic buffers

The major reason for using organic ions is that their conductance (at any given ionic strength) is lower than that of the inorganic ions commonly used, and it is possible to use both high ionic strength and high voltage gradients without excessive heating and evaporative flow. High ionic strength is a great advantage in the ionophoresis of unknown solutions containing relatively high salt concentrations. Spots move compactly, without streaking, even when heavy loads (ionic strength 0.2) must be used (either because the method of detection is insensitive, or because the substance being sought is a minor component).

The gain in mobility made possible by higher voltage gradient is partly nullified by the higher viscosity of the buffers, so that absolute mobilities are usually lower than those obtained by other workers. The high ionic strength also depresses absolute mobility; McDONALD[6] has shown that mobility is inversely proportional to the square root of the ionic strength. We have roughly confirmed this rule, since our 30 % formamide buffers used at about 20 % of the standard ionic strength give for Amaranth and Brilliant Blue absolute mobility values that are 2.4 times those at the standard ionic strength. Electro-osmotic flow is also about 2.4 times faster.

When the ionic strength of the solution being spotted is 0.05 or less, it may be

advantageous to lower the ionic strength of the organic buffers by a factor of 2 to 4, so that separation in a given time will be approximately doubled.

All of the organic buffers except borate can be easily removed from the paper by washing with acetone or 2-butanone (which may be acidified with acetic acid), without removing from the paper spots of many polar organic compounds (such as amino acids, peptides, or carbohydrates). This makes it convenient to elute bands from ionograms for further study, after removal of the buffer.

The major disadvantage of the organic buffers is possible interference with the detection of spots on the ionogram. Many spray reagents are specific, and give no background color with the organic buffers. If a non-specific reagent must be used, it may be possible to extract out the organic buffer with acetone before spraying. The use of different organic buffers may make possible the use of some reagents not compatible with those devised for our researches. The present work attempts to keep the number of organic buffers at a minimum. This is why dimethylaminoethanol, which is primarily useful because its pK is 9.3, is also used in the pH 4.7 acetate buffer, although other organic cations might be preferable.

The 30 % formamide in the standard buffers minimizes evaporative flow at a gradient of 10 V/cm, and makes possible fast ionophoresis without the use of cooling systems. It also has a very desirable anti-adsorptive effect, which increases the mobility of many substances and (even more important) prevents spots from tailing and streaking. Its disadvantage is low volatility, which requires high, uniform heating to dry the paper without distortion of the pattern of spots. A minor disadvantage is that buffers must be prepared daily from stock solutions, to minimize the hydrolysis of formamide.

The 10 % urea remains on the paper on drying and may interfere with detection by some spray reagents. It is chiefly useful for protein separations. It can be easily removed from the paper by washing with methanol or ethanol, which does not remove most proteins.

Buffers more acid than pH 3 or more alkaline than pH 10 cannot be used with 30 % formamide, because hydrolysis of the formamide causes the pH to shift rapidly to the 3–10 range. Dimethylformamide is somewhat more stable, and can be used even at pH 2; however, slow hydrolysis causes some pH drift even in 1 h, and such buffers can only be used within an hour after preparation. Dimethylformamide is more volatile than formamide, and is much less effective in preventing evaporation at gradients of 10 V/cm; it must be used at percentages higher than 30 % or at lower voltage gradients. Nevertheless, it may sometimes prove useful because it is a good anti-adsorptive agent and its volatility makes it easy to remove from the paper by air-drying.

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SUMMARY

Organic buffers of high ionic strength but low conductance, in 30 % formamide, are useful for rapid paper ionophoresis in the pH range 3.3 to 9.3. The apparatus is designed for simultaneous use of as many as five different buffers. Charged substances move as compact spots, without adsorption on paper, and with constant mobility in runs lasting 1 to 3 hours. Mobilities are measured relative to a set of reference dyes. From mobility values it is often possible to estimate the molecular weight of an unknown, the pK value of some acidic or basic groups, and the presence of borate-complexing groups. The buffers are useful in separation and characterization of amino acids, peptides, and carbohydrates. If formamide is replaced by 10 % urea, rapid separation of proteins can be effected.

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