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Electrophoretic behaviour of a group of organic anions of biochemical interest in a functionally coherent series of buffers

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Abstract

The electrophoretic behaviour of a test group of organic anions of biochemical interest in a functionally coherent series of buffers has been studied concurrently with the associated electroosmotic flow in silica capillaries. Changes in the electroosmotic flow with pH are correlated with probable changes in the silica surface. The variation of electroosmotic and electrophoretic behaviour with capillary age, buffer molarity and ionic strength is studied and results tested against theory. The properties of 15-crown-5 as an electroosmotic flow modifier are described and theoretical implications discussed. Electroosmotic and electrophoretic velocities are linear with electric field strength and capillary current up to 526 V/cm. Analyte peak areas and heights are linear with analyte concentration.

1. Introduction

Following work by Mikkers *et al.* [1], Jorgenson and Lukacs [2,3] established protocols for conducting electrophoresis in borosilicate glass and silica capillaries using fluorescence or ultraviolet on-column detection systems and described the electrophoresis of urine and the main protein components in human blood serum. Reports of subsequent work on the application of capillary electrophoresis (CE) to the study of naturally occurring biological fluids are notably few in number.

Wallingford and Ewing [4,5] described the use of CE for the separation of neurotransmitter type substances and analysed the fluid from a single nerve cell of the Pond Snail. Guzman *et al.* [6] found ten major peaks from undiluted normal urine after CE. Wildman *et al.* [7] reported on the feasibility of the assay of inorganic anions and the anions citrate and oxalate in diluted urine. Chen *et al.* [8], demonstrated that normal urine produced a complex pattern indicating the presence of many small molecules.

There has been some success in using CE to detect and quantify certain drugs or pharmaceuticals which are not natural constituents of blood or urine. Studies on the assay of methotrexate [9], barbiturates [10], cefpiramide [11] and anti-convulsant drugs [12] are reported and frequently involve the preliminary isolation of the analyte on an octadecyl-bonded phase.

The electrophoresis of the proteins in blood serum by more established techniques involving slabs or sheets of microporous materials like cellulose or agarose is everyday practice in many laboratories. It might have been expected that CE would have provided separations which were notably superior to those by more traditional techniques. This has not been the case. Jorgen-

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son and Lukacs [3] attributed the broad peaks they obtained after the electrophoresis of human serum to microheterogeneity or to slow conformational changes in protein structure. Chen *et al.* [8], obtained results comparable to those obtained by more traditional methods.

Capillary electrophoresis at first sight appears to have the qualities that should make it an ideal tool for the investigation of naturally occurring biological fluids. The requirement of a very small volume of sample, the possibility of separations in columns of very high efficiency and on-line detection of analytes are three such qualities. There is now a range of commercial instruments available but there remain several serious problems to be overcome before CE makes a significant impact on the analysis of charged molecules in natural biological fluids.

The first problem is that of sensitivity which has been reviewed elsewhere [13–15]. For optical detectors this arises primarily from the very short light path across the capillary. The recent availability of laser-induced fluorescence detectors from commercial sources should partly solve the sensitivity problem for those analytes for which a suitable fluorescent tag is available. Previous work, cited above, shows that in urine, for example, many substances are present at concentrations which are well within the sensitivity limits of current UV detectors. There is, however, a long list of substances, including all hormones, which are present in biological fluids at micromolar, nanomolar or picomolar concentrations, well below the sensitivity limits of all current detectors except those based on laser-induced fluorescence.

The second problem in the application of CE to the analysis of biological fluids is that biological fluids such as blood, urine and cellular fluid contain relatively large amounts of ionic material. The majority of this material consists of the anions and cations of strong inorganic electrolytes. The ionic concentrations of the three biological fluids described are high relative to the ionic concentrations of the buffers employed in CE. Optimum peak quality and resolution requires that the resistivity of the sample is greater than the resistivity of the buffer in the capillary column through which electrophoresis is to occur. This subject is discussed elsewhere in detail [14,16]. The high ionic strength of many biological fluids means that peak spreading and loss of quality will occur when analysis of analytes is attempted on untreated biological fluids. When sensitivity is not a limiting factor dilution to give a sample of lower conductivity is possible, but many of the analytes of interest in biological fluids are at concentrations near or below the sensitivity limits of most detectors. Occasionally it may be possible to extract the required analytes using bonded C_{18} phases, but a procedure of general applicability by which the strong inorganic electrolytes could be removed without incurring losses of the organic anions and cations would be useful.

The third problem is the inherent complexity of biological fluids. Biological fluids contain a large number of different known constituents and probably a large number of hitherto unidentified constituents. Success in the application of CE to the analysis of these fluids, or to extracts from them, is likely to depend on running the CE at close to optimum conditions. The problems already described make this difficult. The practical factors involved in the achievement of the theoretically predicted performance in CE have been reviewed by Knox and McCormack [14].

The work to be described in this paper represents the first stages of a broadly based programme the aim of which is to make CE more applicable to the analysis of the endogenous and exogenous charged constituents in biological fluids. These studies have been carried out using a CE instrument with an active capillary temperature control system. The study of the variation of many parameters, *e.g.* molarity, ionic strength, the effect of buffer modifiers, often involve necessary changes in the conductivity of the buffer and it is important to be confident that any observations made are not due to temperature changes in the capillary. The importance of temperature control on column efficiency was recently emphasised by Grushka *et al.* [17].

The CE of nine organic anions and creatinine, all normal or frequently occurring constituents of

human urine, in a functionally coherent series of buffers has been studied. Concurrent studies have been made of the corresponding changes in the electroosmotic flow (EOF) with buffer parameters. The substances selected all had structures which determined that they should be significant absorbers of ultraviolet light and present no problems in detection. Fundamental parameters determining that CE can be used as a quantitative assay for these analytes have been established. The principal variables studied were pH, molarity and ionic strength, ageing of the capillary surface, buffer modifiers and electric field strength. Some aspects of the reduction of the EOF by 15-crown-5 are described. The implications of new information on these topics to current theory is discussed and some proposals for possible modifications are made.

Particular attention has been given to the relationships between EOF, the real electrophoretic motion of analytes relative to the buffer and the net electrophoretic motion of the analytes. The literature contains several reports on the influence of various factors on the EOF in capillaries [18–25] and these same factors might be expected to influence the electrophoretic motion of analytes relative to the buffer in the capillary. Electrophoresis and EOF are converse manifestations of the same underlying physical phenomena [26] yet there are relatively few reports describing the simultaneous investigation of both.

2. Experimental

2.1. Instrumentation

Capillary electrophoresis was performed using the P/ACE 2100 system from Beckman Instruments, Palo Alto, CA, USA. This system may be operated manually but in this work was under the control of an IBM Personal System (PS)/2 Model 55 SX computer running a Microsoft Disc Operating System 5.0 with a Microsoft Windows version 3.0 environment in which Beckman System Gold version 6.0 chromatography and CE software was installed. The software allows complete programming of analytical protocol and subsequent data analysis.

The fused-silica capillary in this instrument is wound on a spiral mandrel and mounted in a sealed self-contained, removable unit, which, when positioned in the instrument, enables the circulation of liquid coolant around the capillary. A Peltier thermostatic device maintains the coolant liquid at a preset temperature within the range 15–50°C but not lower than 5°C below the ambient temperature. The optical detector window is near the cathode end of the capillary in the normal operating mode with a buffer which produces an EOF towards the cathode. Pressure for the hydraulically operated system valves and the pressure-induced rinsing and sample injection functions is supplied by a cylinder of oxygen-free nitrogen.

The ultraviolet detector in this instrument employs a 30 W deuterium lamp as a light source with a range of 190–380 nm and there are, in the usual configuration, four selectable wavelength filters transmitting at 200, 214, 254 and 280 nm. There are three open positions for optional additional filters.

The power dissipated across the length of the capillary is limited by a voltage restriction of 30 kV and a current restriction of 250 μA.

The user output from the IBM PS/2 computer is routed to its Type 8513 visual display unit or to an Epson LX-800 graphics printer. Although the hardware and software described above are alleged to be self sufficient it has been found useful in this work to obtain a true analogue recording of the attenuated signal from the photomultiplier by means of a Hitachi Model 056-1002 50 mV recorder. The chart speed was set at a constant 20 mm/min. The data sampling rate for the digitisation of the analogue output was usually set to 20 Hz.

Several parameters were held constant throughout this work. The temperature of the circulating coolant was constant at 25°C and except for those experiments which specifically required the voltage to vary, the voltage across the length of the capillary was constant at 25 kV. Injection of sample was always pressure induced for a duration of 5 s. Transmission of light

through the optical window of the capillary was measured after passage through the 214-nm filter. Capillaries were cut from the same primary stock of tubing with internal diameter 75 μm, total length 57 cm and a distance between the anode end of the capillary to the optical window of 50 cm.

New capillaries were conditioned before use by pressure induced rinsing with 100 m*M* sodium hydroxide solution for 10 min followed by water for 10 min.

2.2. Reference compounds

Reference preparations of creatinine, free base, anhydrous, crystalline; *trans*-urocanic acid (4-imidazoleacrylic acid); uric acid (2,6,8-trihydroxypurine), monosodium salt; vanillylmandelic acid (VMA, DL-4-hydroxy-3-methoxymandelic acid); hippuric acid (benzoylaminoacetic acid), free acid; *trans*-cinnamic acid (3-phenylpropenoic acid, β-phenylacrylic acid); phenylacetic acid (benzeneacetic acid, α-toluic acid); orotic acid (6-carboxy-2,4-dihydroxypyrimidine, uracil-6-carboxylic acid), free acid, monohydrate; sodium benzoate and sodium salicylate (sodium 2-hydroxybenzoate) were obtained from Sigma, and used without further purification.

2.3. Buffer components

2-(N-Morpholino)ethanesulphonic acid (MES); 3-(N-morpholino)-2-hydroxypropanesulphonic acid (MOPSO); 3-(N-morpholino)propanesulphonic acid (MOPS); N-(2-hydroxyethyl)-piperazine-N″-(2-ethanesulphonic acid) (HEPES); N-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid (TAPS); 3-(cyclohexylamino)-2-hydroxypropanesulphonic acid (CAPSO) and the sodium salts of MES, MOPSO, MOPS, HEPES, TAPS and CAPSO were obtained from Sigma.

2.4. Solvents

Water was purified before use by passage through a reversed-osmosis and ion-exchange procedure in a unit manufactured by Purite, Thame, UK, known as a Model R0200HP water purifier. This water typically had a pH of 5.8. Methanol was obtained from Merck, Poole, UK and was of "HiPerSolv" grade.

2.5. Other chemicals

Sodium chloride, sodium hydroxide, sodium dihydrogenphosphate and disodium hydrogenphosphate were obtained from Merck. The crown ether 15-crown-5 (pentaoxacyclopentadecane) was obtained from Aldrich, Gillingham, UK.

2.6. Preparation of analyte reference solution

A reference solution was prepared from the commercially available preparations described containing creatinine, urocanic acid, uric acid, vanillylmandelic acid, hippuric acid, cinnamic acid, phenylacetic acid, orotic acid, benzoic acid and salicylic acid at a concentration of 200 μM for each component. The solvent was 1% (v/v) methanol in water containing 10 m*M* HEPES and 0.5 m*M* disodium ethylenediaminetetraacetic acid (EDTA). The pH of the reference solution was 7.49. It was stored in 20-ml glass vials at 4°C.

2.7. Preparation of buffer solutions

A common protocol, with the objective of obtaining solutions of well defined composition and the least number of different molecular species, was used in the preparation of all buffers. Buffers throughout this work were used at pH values at or close to their p*K* values.

Stock solutions of buffers were prepared by dissolving 50 mmol of the free acid and 50 mmol of its sodium salt in water and making the volume up to 1 l. The resulting 100 m*M* buffer was then appropriately diluted with water, usually to give buffers of molarity 25, 50 and 75 m*M*, respectively. No other ions were added. The pH of buffers, including those prepared by dilution was always checked at room temperature immediately before use.

Some experiments employed a series of MES

buffers in which the ionic strengths not attributable to zwitterions were equalized. In these cases the stock 100 m*M* MES buffer was diluted with the appropriate volumes of 50 m*M* sodium chloride solution.

MES buffers containing crown ether or methanol were prepared by substituting the appropriate volume of the modifier for the same volume of water when the 50 m*M* MES buffer was prepared by dilution of the 100 m*M* stock MES buffer.

2.8. Electrophoresis protocol

The same routines for the equilibration of the capillary with buffer and the cleansing of the capillary after completion of electrophoresis were followed throughout this work. Solutions were dispensed into the reagent vials from all glass syringes fitted with filter holders in which 0.2-μm membrane filters had been mounted. All analytical parameters were given to the system through the System Gold Software and executed by the instrument under program control. Introduction of liquid to the capillary by the application of high-pressure nitrogen gas to the sealed reagent vials was always in the direction anode to cathode. The individual steps of the procedure are listed below.

Pre-electrophoresis rinse 1. Water for a period of 1.0 min, the effluent from the capillary going to a waste vial.

Pre-electrophoresis rinse 2. Buffer for a period of 3.0 min, the capillary effluent going to waste.

Sample injection. By high pressure for a period of 5.0 s, capillary effluent going to waste.

Electrophoretic separation. This was by the application of a potential of 25 kV at the anode for a period of 10 min. The first 10 s of this period were used to ramp up the voltage from 0 to 25 kV. The cathode end of the capillary was in a vial containing buffer. The temperature of the circulating coolant was thermostatically set at 25°C. During this phase the UV detector monitors the capillary fluid at 214 nm as the fluid passes the optical window. An absorbance autozero switches in after the elapse of 0.5 min.

Post-electrophoresis rinse 1. This was a high-pressure rinse with 100 m*M* sodium hydroxide for a period of 0.5 min.

Post-electrophoresis rinse 2. This was a high-pressure water rinse for a period of 2.0 min.

When, for experimental reasons, the molarity or type of the running buffer was changed, the following run was duplicated and data from the second run analysed. This was to allow the capillary to equilibrate to the changed conditions. As a quality control protocol, the first and last analysis in any working day was always an electropherogram of the 200 μM reference analyte solution with 50 m*M* MES, pH 6.10, as the running buffer and using the analytical protocol given above.

3. Definitions and units

The equation for the electroosmotic mobility due to Smoluchowski [27], in its modern form is:

$$\mu_{eof} = -\epsilon\zeta/\eta \quad (1)$$

where

$$\epsilon = D\epsilon_0 \quad (2)$$

The quantity ϵ is the permittivity of the buffer solution. The quantity ϵ_0 is the electric field constant equal to $8.854 \cdot 10^{-12}$ F m^{-1}. The dielectric constant, D, is a pure dimensionless number. η is the viscosity of the buffer. For water at 25°C. η has a value of $8.95 \cdot 10^{-4}$ N m^{-2} s.

The zeta potential (ζ) is defined as the potential, relative to a bulk fluid potential of 0, at the plane of shear between the mobile electrokinetic unit and the static unit. At the values of pH relevant to the present studies the silica surface is negatively charged. The counter charge in the double layer is positive and by convention ζ is negative and the EOF is towards the negative cathode which is the positive direction.

The electroosmotic velocity (U_{eof}), is the velocity of the EOF when it is driven by a field strength E:

$$U_{eof} = \mu_{eof}E \quad (3)$$

and

$$E = V/L \quad (4)$$

where V is the potential difference in volts across a capillary of length L (cm). U_{eof} is expressed in mm/s in the work reported here.

The value of μ_{eof} is usually expressed as a velocity in μm/s or cm/s per unit field strength in V/cm giving:

$$\mu\text{m cm V}^{-1}\text{ s}^{-1} = \text{cm}^2\text{ V}^{-1}\text{ s}^{-1} \times 10^{-4} \quad (5)$$

The latter unit is used in the present work for the expression of both electroosmotic and electrophoretic mobilities.

Smoluchowski's equation (Eq. 1) for electroosmotic mobilities can be applied to the electrophoresis of large particles with relatively thin double layers. The equation:

$$\mu_{ep} = \epsilon\zeta/\eta \quad (6)$$

is Smoluchowski's equation for the electrophoretic mobility. From Eq. 6 the corresponding equation for electrophoretic velocity is:

$$U_{ep} = \mu_{ep}E \quad (7)$$

Huckel's equation [28] for the electrophoretic mobility of small particles with relatively thick double layers is:

$$\mu_{ep} = (2/3)\epsilon\zeta/\eta \quad (8)$$

The Smoluchowski and Huckel equations were represented at the opposite limits of a particle size continuum by Henry [29].

4. Results and discussion

4.1. Comparative study of buffers

Terabe *et al.* [30] proposed the algorithm (pK − log 2), where pK is the average analyte pK, for giving the optimum buffer pH for the separation of compounds with closely similar pK values. In this work, because no single specific chemical group was being targeted, a more general approach has been adopted, with the aim of providing high-quality separations which might be used as screening procedures for more discriminating techniques, when applied to the analysis of biological fluids, in particular, urine.

Selection of a buffer system

Many of the buffer systems used in CE have been adoptions from those used in gel electrophoresis [31]. A significant fraction of the current theory and analytical practice of CE is founded on work done with phosphate and borate buffers. Good *et al.* [32] listed the disadvantages of phosphate and borate buffers. Borate buffers complex with a range of organic compounds and in particular with *cis*-diols and *ortho*-diols, a property used for the separation of catechols by CE [33]. Phosphate has a poor buffering capacity above pH 7.5. Use of phosphate buffers in the region of pH 7 is accompanied by a significant addition to the ionic strength of the medium.

Good and co-workers [32,34,35] developed a series of organic buffers with a range of pK values from approximately 6 to greater than 10. The organic anions chosen for this study have at least one pK value less than 6. The decision to study the electrophoretic behaviour of these anions in a series of Good's buffers was made for several reasons. Firstly, Good's buffers have a pK range within which the organic anions will be dissociated and negatively charged. Secondly, it is simpler to study the effects of pH and ionic strength when these functions can, as far as possible, be separated. It was decided that the buffer should contribute as few ions as possible and that specific ions or ionic strengths should be achieved by deliberate additions. The use of Good's buffers was expected to result in lower heat generation when compared with equal molarities of phosphate buffers. Borate buffers were not studied in this work. Thirdly it was hoped that Good's buffers would be inert chemically, to both the silica surface and to the organic analytes.

The buffers selected were MES (pK 6.15), MOPSO (pK 6.9), MOPS (pK 7.15), HEPES (pK 7.55), TAPS (pK 8.55) and CAPSO (pK 9.6). All buffers were 50 mM in this set of experiments and each buffer was used at a pH equal to, or close to its pK. The Good's buffers

were therefore of approximately equal ionic strength, with the exception of the 50 m*M* MES buffer at a pH of 7.3.

Capillary current and effective ionic strength

In Table 1 current in the capillary at 25 kV and the EOF mobilities together with other relevant buffer parameters are shown. The 50 m*M* MES buffer at pH 7.3 was produced by dissolving 45.5 mmol of the unprotonated sodium salt of MES and 4.5 mmol of the protonated free acid in water to give 1 l of solution. This pH is outside the normal acceptable buffering range for MES but electrophoresis using this solution was done for two reasons. Firstly, evidence was required that MES buffer at its normal buffering pH in the region of 6.1 was acting as a reasonably inert supplier of a hydrogen ion environment and was not acting in some manner specific to its chemical structure. Comparison of the electropherogram for MES at pH 7.3 in Fig. 1b with that for MOPS at pH 7.23 in Fig. 2b and allowing for the higher ionic strength of MES at pH 7.3 shows that this seems to be a tenable assumption. The second reason for studying MES at pH 7.3 was to confirm the assumption that current measurements provided a valid check on the effective ionic strength of buffers of similar structure and function as represented by the series of Good's buffers used in this work. The term effective ionic strength refers to those ions capable of conducting electric current and excludes zwitterions like the protonated anions of Good's buffers. The current data in Table 1 for the six Good's buffers operating at close to their respective p*K* values show a mean value of 35.2 μA with individual buffers giving currents within 4% of this. From the amounts of the sodium salt used in preparation the effective ionic strength of 50 m*M* MES at pH 7.3 is 1.82 times that of 50 m*M* MES at pH 6.13 giving a predicted capillary current of 61.7 for MES at the higher pH against an observed current of 63.8 μA. It seems therefore that the anions of Good's buffers have virtually identical equivalent conductivities and in situations where the cation is common the measurement of capillary current should provide a good indicator of effective ionic strength.

Phosphate buffer at a concentration of 50 m*M* has 3.0 times the effective ionic strength of 50 m*M* MES buffer when both buffers are at pH values at or near their respective p*K* values and concentrations are expressed as monovalent equivalents. The predicted value of the capillary current for phosphate from that observed for MES is 102 μA against an observed value of 153 μA. As expected when there are considerable qualitative differences in the anions or cations of the buffers the capillary current is not a good predictor of effective ionic strength. The data in Table 1 show that when compared on an equimolar basis Good's buffers provide at least a

Table 1
Table of parameters for the 50 m*M* buffers used in comparative studies for the electrophoresis of reference analytes

Buffer (50 m*M*)	p*K*[a]	pH	Current (μA)[b]	EOF ($cm^2 V^{-1} s^{-1} \times 10^4$)
MES	6.15	6.13	33.9	7.06
MOPSO	6.90	7.02	35.7	7.31
MOPS	7.15	7.23	35.5	7.42
HEPES	7.55	7.65	34.3	7.45
TAPS	8.55	8.65	35.4	7.31
CAPSO	9.60	10.1	36.6	7.36
Phosphate	6.80	6.96	153	6.79
MES	6.15	7.30	63.8	6.79

Conditions: capillary 57 cm × 75 μm I.D., voltage 25 kV, coolant temperature 25°C, monitored at 214 nm.
[a] p*K* values are those in ref. 34 or from the manufacturers' stated values if not available from ref. 34.
[b] Capillary current was measured approximately 3 min after the start of electrophoresis.

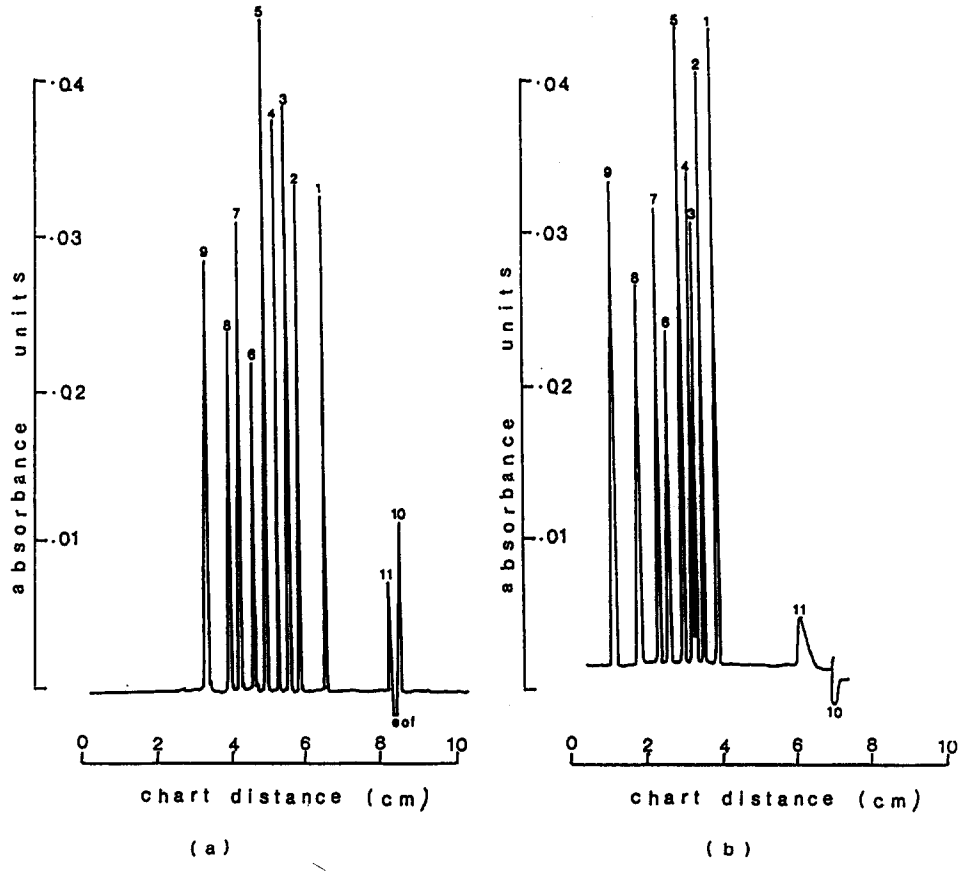

Fig. 1. Electropherograms of analytes in 50 m*M* MES buffer at pH 6.13 and at pH 7.30. (a) 50 m*M* MES at pH 6.13; peaks (retention times in min in parentheses): 1 = urocanate (3.63); 2 = urate (3.97); 3 = VMA (4.11); 4 = hippurate (4.25); 5 = cinnamate (4.41); 6 = phenylacetate (4.57); 7 = orotate (4.75); 8 = benzoate (4.87); 9 = salicylate (5.16); 10 = creatinine (2.64); eof = EOF (2.69); 11 = HEPES from sample buffer (2.80). (b) 50 m*M* MES at pH 7.30; peaks (retention times in min in parentheses): 1 = urocanate (4.43); 2 = urate (4.61); 3 = VMA (4.76); 4 = hippurate (4.79); 5 = cinnamate (4.88); 6 = phenylacetate (5.07); 7 = orotate (5.21); 8 = benzoate (5.45); 9 = salicylate (5.78); 10 = EOF (sample solvent) obscuring creatinine (2.80); 11 = HEPES from sample buffer (3.34). Conditions: 25 kV across capillary, 57 cm × 75 μm I.D., temperature 25°C, sampling time 5 s, wavelength 214 nm, chart speed 2 cm/min.

four-fold reduction in the capillary current produced by the phosphate buffer; all comparisons referring to buffers at or close to their respective p*K* values.

Electropherogram quality in the pH range 6.1 to 10.1

The first objective of these comparative experiments was to ascertain the rules governing the electrophoretic behaviour of a group of organic anions and the electroosmotic movement of buffer in a coherent series of similar buffers of equal molar concentrations, equal ionic strengths, over an appropriate pH range partly governed by the availability of buffers with suitable p*K* values. The second objective was to make comparisons with phosphate buffer from an essentially practical analytical aspect.

The results of this study are shown by the transcripts of recorder tracings in Figs. 1–4 and in Fig. 5 where quantitative mobility data are plotted. Data for the 50 m*M* phosphate buffer and for MES buffer at pH 7.3 have been excluded from Fig. 5 and from the regression analyses listed in Table 2. Good's buffers give a sharp negative peak with the aqueous sample

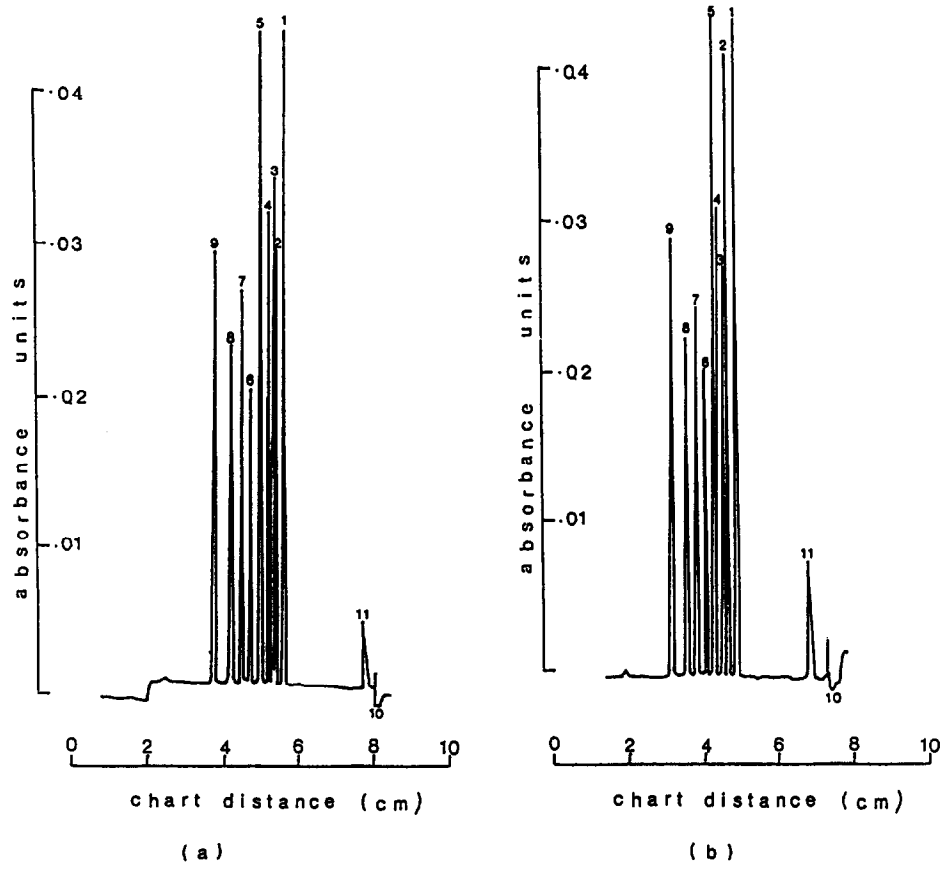

Fig. 2. Electropherograms of analytes in 50 m*M* MOPSO buffer at pH 7.02 and 50 m*M* MOPS buffer at pH 7.23. (a) 50 m*M* MOPSO at pH 7.02; peaks (retention times in min in parentheses): 1 = urocanate (3.88); 2 = urate (4.00); 3 = VMA (4.02); 4 = hippurate (4.08); 5 = cinnamate (4.18); 6 = phenylacetate (4.32); 7 = orotate (4.43); 8 = benzoate (4.57); 9 = salicylate (4.79); 10 = EOF (sample solvent) obscuring creatinine (2.60); 11 = HEPES from sample buffer (2.83). (b) 50 m*M* MOPS at pH 7.23; peak identities as in (a), retention times: 1 = 3.79; 2 = 3.91; 3 = 3.95; 4 = 4.00; 5 = 4.02; 6 = 4.21; 7 = 4.33; 8 = 4.45; 9 = 4.66; 10 = 2.56; 11 = 2.87 min. Conditions as for Fig. 1.

zone when monitored at 214 nm which provides a good measure of the EOF. Phosphate buffers at this wavelength produce a small positive peak for the EOF. These peaks are not visible on all the recorder tracings shown in Figs. 1–4 because they were clipped out by the setting of the recorder zero. Quantitative analysis of electropherogram data was carried out independently by the system software. Recorder tracings, which should be read from right to left, are presented here because they give a more realistic analogue picture of the electrophoretic process than electropherograms produced by the system software which have a digital origin.

Inspection of the electropherograms illustrated in Figs. 1–4 show that two buffers give excellent separations of all the analytes in the reference mixture. These are 50 m*M* phosphate buffer at pH 6.96 (Fig. 4b) and 50 m*M* MES buffer at pH 6.13 (Fig. 1a). Although the phosphate buffer is at a pH which is 0.83 pH units above that of the MES buffer it produces comparable separations due to the slower EOF associated with the higher ionic strength of the phosphate buffer. The two buffers must present useful starting points in many attempts to separate mixtures of similar organic anions. Features perhaps as significant as the resolution obtained are the

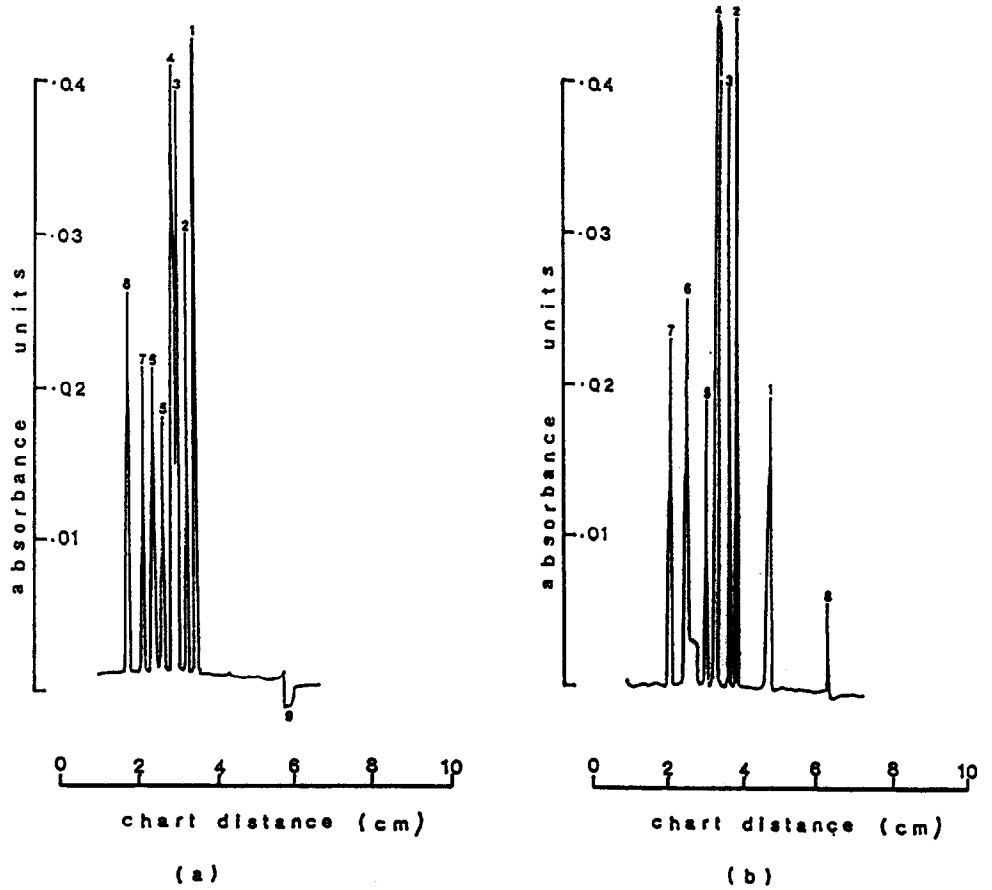

Fig. 3. Electropherograms of analytes in 50 m*M* HEPES buffer at pH 7.65 and 50 m*M* TAPS buffer at pH 8.65. (a) 50 m*M* HEPES at pH 7.65; peaks (retention times in min in parentheses): 1 = urocanate (3.72); 2–4 = specific identities uncertain; 5 = phenylacetate (4.12); 6 = orotate (4.24); 7 = benzoate (4.35); 8 = salicylate (4.53); 9 = EOF (sample solvent) obscuring creatinine (2.55). (b) 50 m*M* TAPS at pH 8.65; peaks (retention times in min in parentheses): 1 = HEPES from sample buffer (3.39); 2–7 = specific identities uncertain; 8 = creatinine (2.64) emerging as anion from sample solvent (EOF, 2.60). Conditions as for Fig. 1.

baseline stability and the peak quality with good sharpness, symmetry and the absence of fronting. The MES buffer has a considerable advantage over the phosphate buffer because of the much smaller capillary current produced by the former (Table 1).

As the pH of the buffer in the capillary is increased from that of MES at 6.13 through 7.02 (MOPSO), 7.23 (MOPS), 7.65 (HEPES), 8.65 (TAPS) to the pH of the CAPSO buffer at 10.1 two distinct types of change occur. Up to the pH of HEPES there is a progressive compression of the earliest peaks (on the right in Figs. 1–3) with failure of resolution in the case of HEPES where the first five components, using the MES buffer as reference, migrate as four peaks (Fig 3a). Peak quality and general electropherogram quality remain satisfactory up to this point in the pH range and it is possible to account for the changes in the electropherograms by changes in the electrophoretic mobilities of certain analytes as the pH of the buffer alters relative to the p*K* value(s) of the analytes. The electropherograms produced by TAPS buffer at pH 8.65 and

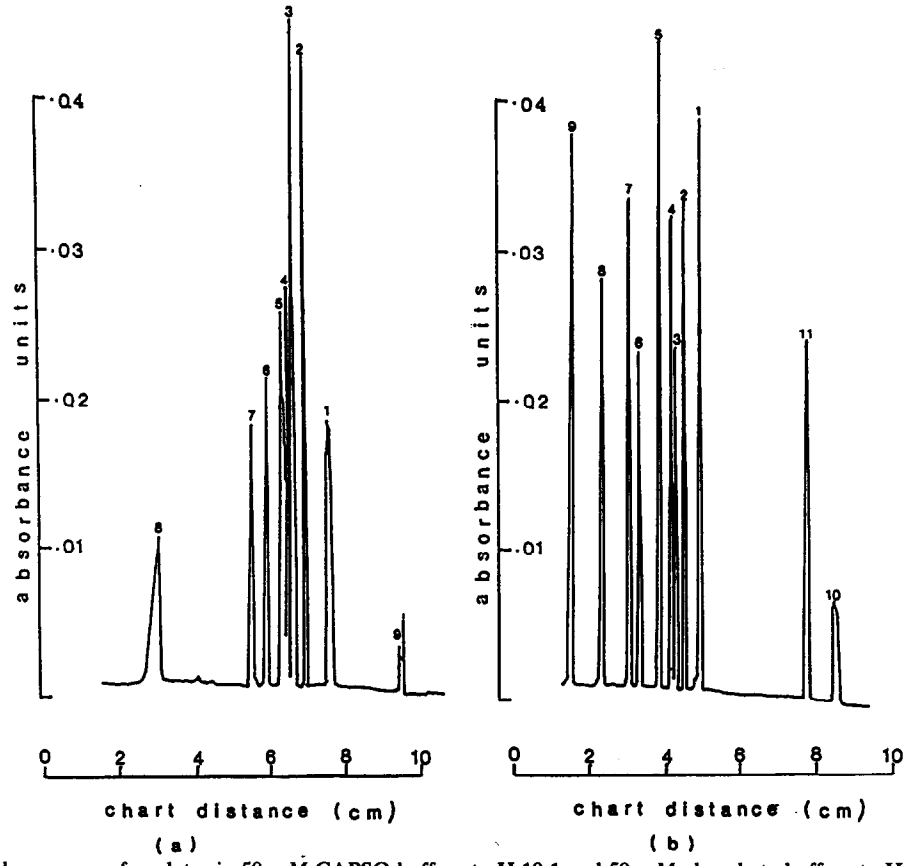

Fig. 4. Electropherograms of analytes in 50 m*M* CAPSO buffer at pH 10.1 and 50 m*M* phosphate buffer at pH 6.96. (a) 50 m*M* CAPSO at pH 10.1; peaks (retention times in min in parentheses): 1 = HEPES from sample buffer (3.60); 2–7 = specific identities uncertain; 8 = orotate as double anion (5.89); 9 = creatinine (2.63) emerging as anion from sample solvent (EOF, 2.58). (b) 50 m*M* Phosphate at pH 6.96; peaks (retention times in min in brackets): 1 = urocanate (4.60); 2 = urate (4.81); 3 = VMA (4.92); 4 = hippurate (4.99); 5 = cinnamate (5.16); 6 = phenylacetate (5.41); 7 = orotate (5.54); 8 = benzoate (5.90); 9 = salicylate (6.30); 10 = sample solvent (EOF) and creatinine (2.80); 11 = HEPES from sample buffer (3.21). Conditions as for Fig. 1.

CAPSO buffer at pH 10.1 in which both resolution and peak quality are evidently corrupted can not be completely explained in this way.

The electroosmotic flow and buffer pH

Further information on the mechanisms by which buffer pH influences the electropherogram obtained by the CE of the reference analyte solution can be derived from the mobility plots in Fig. 5. The mobility coefficients for electroosmotic flow plotted against the pH of the buffers (Fig. 5) show a two-phase relationship. From the pH of MES at 6.13 to that of HEPES at 7.65 the EOF increases with pH in a significantly linear manner ($p < 0.02$, Table 2). From a pH of approximately 8 this linear relationship breaks down and the EOF remains virtually independent of pH through to the pH of CAPSO at 10.1 which is the last observation in the series. Comparison of these results with those in the literature is made difficult by the variety of experimental strategies employed. Tsuda *et al.* [19] examined the variation of EOF with pH by varying the pH of a 50 m*M* phosphate buffer presumably with considerable concurrent variation in ionic strength. They concluded that EOF was "slightly dependent" on pH in the approximate range 5–9. Altria and Simpson [23] measured the EOF by weighing the effluent from the capillary and report two experi-

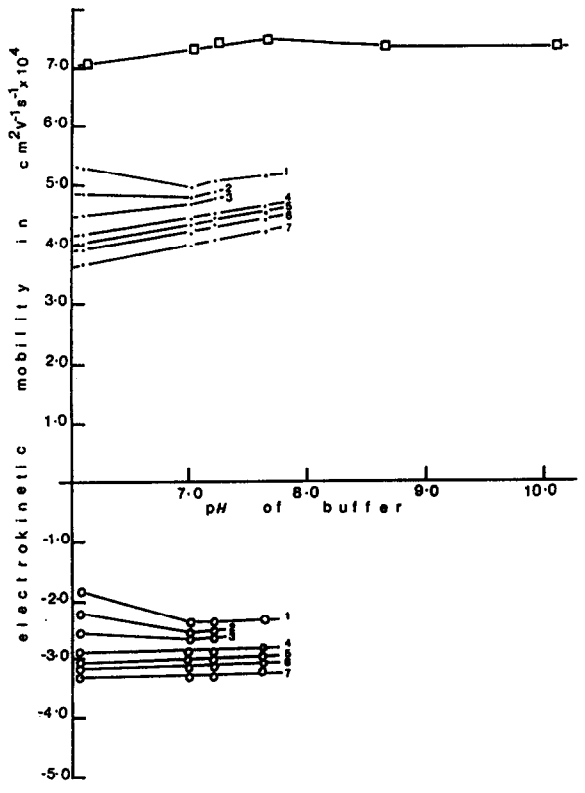

Fig. 5. Plots showing the relationships between electroosmotic mobilities, net electrophoretic mobilities and the real electrophoretic mobilities with pH for the buffers MES, MOPSO, MOPS, HEPES, TAPS and CAPSO at pH values close to their p*K* values (see Table 1). Key: □ = electroosmotic mobilities; ● = net electrophoretic mobilities; ○ = real electrophoretic mobilities; 1 = urocanate; 2 = urate; 3 = hippurate; 4 = phenylacetate; 5 = orotate; 6 = benzoate; 7 = salicylate. Data for VMA and cinnamic acid omitted to preserve clarity. Data from the electropherograms in Figs. 1–4. Regression statistics are given in Table 2. Conditions as in Fig. 1.

ments in which the variation of EOF with pH was studied in the overall pH range 2.8–12.1. The pH range studied in the present work was 6.13–10.1 and within this range Altria and Simpson report a total of three data points. Comparison with the results produced by the current study must therefore be tentative. There is broad agreement between the present results and those of Altria and Simpson for the variation of EOF with pH in the range 6 to approximately pH 8. There is marked, but probably superficial disagreement for pH values greater than 8 where Altria and Simpson report a continuation of the linear relationship obtained at pH values lower than 8.

The change in the relationship between the EOF and pH in the region of pH 8 using buffers which would not be expected to react with silica is attributed in the present studies to ionisation at the silica surface reaching a maximum in the region of pH 8. At this pH there may in addition be concurrent pH-induced conformational changes at the silica surface. This seems a credible hypothesis in view of the known fragility of bonded and unbonded silicas at these pH values in ion-exchange work [36]. These conformational changes could be the precursor stages of the process which leads to the eventual dissolution of silica in alkali. It would seem reasonable to expect that at pH values in the region of 8 the ionisation of silanol groups is at or near its maximum. This would be compatible with the results of Wiese *et al.* [37] who found that the ζ potential of vitreous silica exposed to potassium nitrate solutions at different pH values increased with pH up to a pH of approximately 8 and showed little increase for pH values greater than 8. The chemistry of the silica surface is still a subject of controversy [38,39] and explanations of the variation of EOF with pH must remain, to some extent, hypothetical.

An explanation of the difference between the present results and those of Altria and Simpson may be attributed to their use of borate buffer. In their second experimental series these authors prepared buffers covering the pH range 3.0 to 11.4 by the addition of 20 m*M* sodium hydroxide to a universal buffer solution containing citrate, phosphate, borate and barbiturate. The ability of borate anions to complex with diols has already been mentioned. It is proposed here that borate anions may combine with appropriately configured silanol groups thereby protecting the silica surface at higher pH values and accounting for the difference between the results reported in this work and those of Altria and Simpson. McCormick [40] using phosphate buffers in the region of pH 2 hypothesised the stabilisation of the silica surface by the complexation of an estimated 23 phosphate anions per silanol. It is unknown to what extent phosphate complexes with silanol groups at higher pH values. McCor-

Table 2
Linear regression statistics for the electroosmotic mobility and the net electrophoretic mobilities of anions against the pH of the running buffers calculated from the data of Figs. 1–5

Net mobility (μ) ($cm^2 V^{-1} s^{-1} \times 10^4$)	Regression equation (x = pH)	r^a	p^a
μ_{eof}	$0.271x + 5.412$	0.979	< 0.020
$\mu_{phenylacetate}$	$0.300x + 2.316$	0.995	< 0.005
$\mu_{orotate}$	$0.324x + 2.022$	0.996	< 0.004
$\mu_{benzoate}$	$0.315x + 1.972$	0.996	< 0.004
$\mu_{salicylate}$	$0.341x + 1.590$	0.997	< 0.003

[a] r is the coefficient of linear correlation and p is the probability that it could refer to an uncorrelated universe.

mick found a non-linear relationship between EOF and pH in the range 1.5–6.0 using phosphate buffers of presumably unequal ionic strengths. Schwer and Kenndler [15] report a relatively rapid increase in EOF in the pH range 3–6 followed by a much slower increase up to pH 12 using a phosphate buffer containing potassium chloride. These authors do not state whether the ionic strengths at the various pH levels were equalised, but in a more recent study [41], these workers used 10 m*M* potassium chloride as a swamping electrolyte for a 1 m*M* phosphate buffer, the pH being varied by the addition of potassium hydroxide solution. They report that the silica surface behaves as a weak acid with a p*K* of about 5.3, but unlike a weak acid, without a plateau in the value of U_{eof} at two pH units above 5.3. A plateau would have indicated that ionisation was effectively complete. If these workers had obtained a plateau it would have been in reasonable agreement with the present results. Comparison with the present results is difficult because of uncertainties about the interaction between phosphate anions and the silica surface at different pH values.

The investigation of the variation of EOF with pH in a way which gives meaningful results is not easy. Varying the pH of a given buffer produces concurrent changes in ionic strength in accordance with the Henderson–Hasselbach relationship. Thus Fujiwara and Honda [42] reported a decrease in EOF as the pH of a phosphate buffer was increased by increasing the proportion of disodium hydrogenphosphate. This finding probably represents the relatively small increase in EOF with increasing pH being obscured by the relatively larger decrease in EOF with increasing ionic strength. Any one buffer is strictly only functional as a buffer within a limited pH range but using a series of several different buffers introduces the uncertainty that some buffers may be producing effects specific to their structures. In addition to these difficulties there are the uncertainties about the chemistry at the silica surface [38,39]. Lambert and Middleton [43] demonstrated that EOF was dependent on the way a capillary was conditioned before use, acidic conditions (using hydrochloric acid) producing lower values for the EOF and several days elapsing before stability was achieved. The advantages of alkaline rinsing between injections were emphasised. The strategy employed in the study reported here, using a set of functionally matched buffers, probably represents as good a compromise as possible in an attempt to surmount the problems in a study of this type.

Electrophoretic mobility and buffer pH

The analyte mobility data in Fig. 5 can be interpreted from the p*K* values of the analytes given in Table 3. The data plotted in Fig. 5 comprises two parameters obtained by direct observation, the electroosmotic mobility and the net electrophoretic mobility and one derived parameter, the real electrophoretic mobility. The data were obtained from the quantitative analysis of the electropherograms depicted in Figs. 1–4 by the system software and only data for analytes which were sufficiently resolved to be analysed with confidence are included in Fig. 5.

The differences in the electropherograms of the reference analytes from pH 6.13 (MES) up

Table 3
Molecular masses and pK values of analytes in the reference analyte solution listed in order of increasing retention time on electrophoresis in 50 mM MES buffer at pH 6.10

Analyte	M_r [a]	pK_1	pK_2	pK_3
Creatinine	113	4.83	9.2	–
Urocanic acid (*trans*)	138	3.5	5.8	–
Uric acid	168	5.7	??	??
Vanillylmandelic acid	198	??	??	–
Hippuric acid	179	3.62	–	–
Cinnamic acid (*trans*)	148	4.44	–	–
Phenylacetic acid	136	4.31	–	–
Orotic acid	156	2.4	9.5	> 13
Benzoic acid	122	4.19	–	–
Salicylic acid	138	3.0	??	–

?? Denotes that a pK should theoretically exist but was not found in the literature.
[a] Molecular mass.

to pH 7.65 (HEPES) can be explained by the changes in EOF over this pH range and the expected changes in the real electrophoretic mobilities of analytes as the pH of the buffer varies relative to the pK values of the analytes. Within this pH range the analytes, phenylacetic acid, orotic acid, benzoic and salicylic acids exhibit real electrophoretic mobilities which vary in a linear manner as the pH of the electrophoresis buffer increases (Fig. 5). There appears to be a very small decrease in the real electrophoretic mobilities of these analytes with increasing pH which is of doubtful significance. Vectorial summation with the corresponding values for the EOF gives net mobilities which are significantly linear with increasing pH (Fig. 5 and Table 2). Cinnamic acid (omitted from Fig. 5), for which only three data points were available, appears to follow the same rules. These five analytes have principal acidic pK values sufficiently lower than the lowest pH of the electrophoresis buffers (MES, pH 6.13), that increases in the buffer pH produces no increase in ionisation or real mobility of the analytes. Orotic acid has a second pK at 9.5 corresponding to a weakly acidic hydroxyl group (Table 3). At the pH of the CAPSO buffer of 10.1, orotic acid shows the expected increased mobility of a double anion (peak 8 in Fig. 4a).

The electrophoretic behaviour of the other analytes can be adequately explained by similar methods but there are subtle differences between each analyte. Creatinine has a pK at 9.2 corresponding to a weakly acidic hydroxyl group and a pK at 4.83 corresponding to a weakly basic quaternary nitrogen atom. It is therefore nearly neutral within the pH range 6.13 to 7.65, migrating on the cationic edge of the neutral sample solvent (water) with MES buffer at pH 6.13. With CAPSO buffer at pH 10.1 it moves at the anionic edge of the neutral EOF marker. The real mobility of urocanic acid increases sharply from the pH of MES at 6.13 to that of MOPSO at 7.02 causing a decrease in its net mobility between these pH values (Fig. 5). It has two pK values, one at 3.5 for the carboxylic acid group and one at 5.8 for the weakly basic imidazole nitrogen atom. At the pH of the MES buffer sufficient protonation of this imidazole nitrogen is occurring to limit the full electrophoretic expression of the anionic carboxylic acid group. Thus at pH 6.13 urocanic acid is a partial zwitterion. At the pH of 7.02 (MOPSO) this protonation is much less significant and the mobility of urocanic acid increased. The pK of uric acid at 5.7 is close to the pH of the MES buffer at 6.13. Its effective mobility at this pH is less than maximum because it is only partially ionised and its real mobility at the pH of MOPSO therefore increases with a slight decrease in its net mobility (Fig. 5). No pK is available for VMA but its real and net mobilities

lie between those for uric acid and hippuric acid in Fig. 5 and it seems reasonable to apply arguments similar to those for uric acid. Hippuric acid shows a small increase in real electrophoretic mobility between the pH values of the MES and MOPSO buffers which translates to a small increase in net mobility after vector summation with the increasing EOF.

Inspection of the real mobility curves in Fig. 5 indicates that after the initial changes in the mobilities of some analytes at pH values in the lowest part of the range the real mobility curves of all analytes would form a family of parallel lines showing little change in mobility with increasing pH, except, as in the case of orotic acid, in the region of another p*K*. Vector summation with the EOF appropriate to each pH would give a family of parallel lines for the net mobilities. As the EOF apparently becomes invariate with increasing pH at a pH in the region of 8, the electropherograms of the analytes in buffers with pH values greater than 8 should closely resemble that obtained for HEPES at a pH of 7.65, which, with respect to resolution and peak quality, they do not. It seems possible that at pH values higher than 8 some other effects are operating, perhaps related to, or the same as those producing the anomalous behaviour of the EOF in this pH region. A heterogeneous distribution of conformational changes in the silica surface at pH 8 might for example cause a heterogeneous distribution in the ζ potential with consequent heterogeneity in the EOF. These arguments do not preclude the possibility that practically acceptable electropherograms with Good's buffers at pH values greater than 8 can be achieved, but the majority of reports in the literature for electrophoresis in this pH range involve the use of borate buffers, which, as previously stated, may interact with the silica surface.

4.2. *Buffer concentration and mobilities*

Variable buffer molarity with effective ionic strengths unequal

The molarity of the buffer has a significant effect on the appearance of electropherograms of organic analytes as illustrated in Fig. 6 in which 25 m*M* MES is compared with 100 m*M* MES, the lower-molarity buffer being prepared by dilution of the 100 m*M* buffer with water and without the addition of any other ions. The mobility data obtained when a series of MES buffers is prepared in a similar way and used for the electrophoresis of the organic ions in the reference solution are shown in Fig. 7.

The upper curve in Fig. 7 shows that the EOF decreases with increasing molarity of buffer; the slope of the curve approaching 0 for 100 m*M* buffer. This is in general agreement with the findings in studies using phosphate buffers [18,42] and with those of workers using a variety of buffers including Good's buffers [24,25], with the qualification that there appears to be poor agreement in the absolute values of μ_{eof} between different workers when comparison is possible. The net mobilities of the nine organic anions in the sample form a family of approximately parallel curves although a very slow divergence of urocanic and salicylic acids from the other anions is evident. The most significant feature of the data in Fig. 7 is the linear behaviour of the real mobilities of the organic analytes with increasing molarity of the MES buffer. The corresponding linear regression statistics in Table 4 show that when due allowance is made for experimental error the linearity is at a high level of significance.

The negative slopes of the regression lines relating the real electrophoretic mobilities to the molarity of MES buffer (see Fig. 7 and Table 4) represent a shallow increase in real mobilities of 5–6% over the experimental buffer range. The observed decrease in the electroosmotic mobility between the 25 m*M* buffer and the 100 m*M* buffer is approximately 18%. These results are in broad agreement with those of Salomon *et al.* [44], except that these workers found the real mobilities of tricyclic amines to be invariate with buffer molarity at a pH of 9.55 where these compounds are cationic. Bruin *et al.* [45] obtained apparent linearity between the net mobilities of three proteins with the molarity of a phosphate buffer at pH 3.8 in coated capillaries. Their experimental conditions gave a small EOF

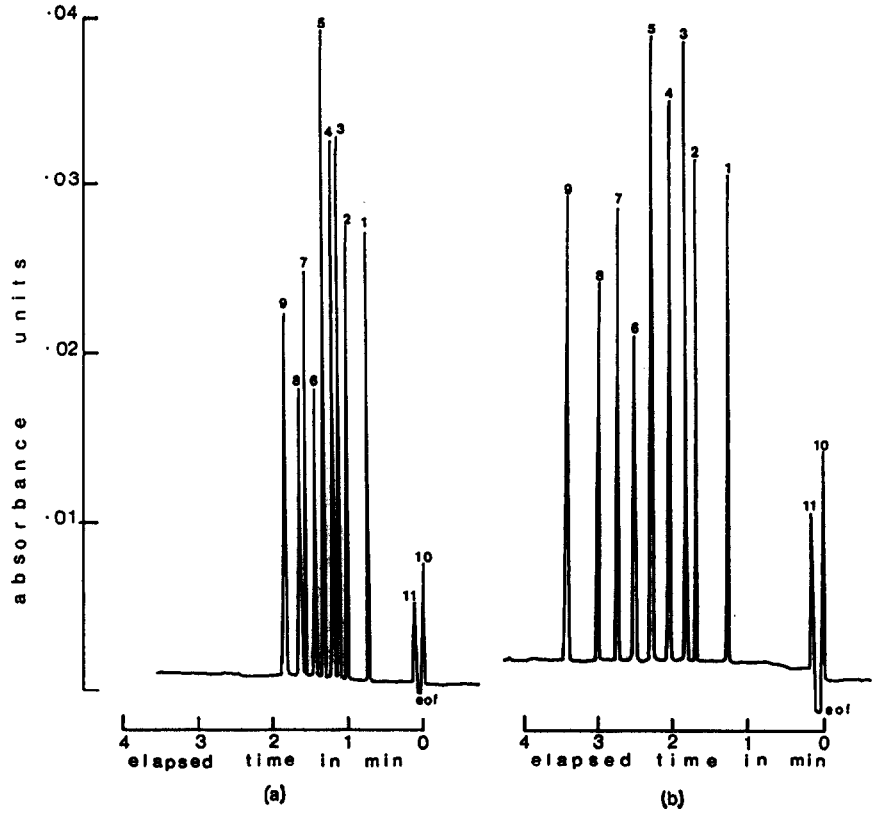

Fig. 6. Electropherograms of analytes after CE in 25 m*M* MES, pH 6.06 and 100 m*M* MES, pH 6.13. The time scale is set with its zero coincident with the retention time of creatinine in both panels. (a) 25 m*M* MES at pH 6.06; peaks (retention times in min in parentheses): 1 = urocanate (3.06); 2 = urate (3.31); 3 = VMA (3.42); 4 = hippurate (3.51); 5 = cinnamate (3.60); 6 = phenylacetate (3.70); 7 = orotate (3.83); 8 = benzoate (3.89); 9 = salicylate (4.08); 10 = creatinine (2.33), sample solvent (EOF, 2.38); 11 = HEPES from sample buffer (2.47). (b) 100 m*M* MES at pH 6.13; peaks (retention times in min in parentheses): 1 = urocanate (4.15); 2 = urate (5.58); 3 = VMA (4.73); 4 = hippurate (4.94); 5 = cinnamate (5.17); 6 = phenylacetate (5.40); 7 = orotate (5.62); 8 = benzoate (5.85); 9 = salicylate (6.29); 10 = creatinine (2.87), sample solvent (EOF, 2.92); 11 = HEPES from sample buffer (3.05). Conditions: capillary 57 cm × 75 μm I.D., 25 kV applied across capillary length, coolant temperature 25°C, wavelength 214 nm, sample was pressure injected for 5 s, chart speed 2 cm/min.

in the same direction as the analyte mobilities and the EOF was related to buffer molarity in a manner similar to that obtained in the present work. In terms of fundamental theory the results obtained here presumably mean that the ζ potential between the silica surface and the bulk fluid decreases significantly with increasing buffer concentration whereas that between the analyte molecules and the bulk fluid may increase to a small extent. This assumes that permittivity and viscosity are constant at the experimental buffer concentrations. The mobility curves illustrated in Fig. 7 show significant quantitative and qualitative differences between the kinetic behaviour of the EOF and the real electrophoretic motion of the analytes when buffer concentration is varied. It will be demonstrated in a later section that these differences virtually disappear when the buffer cation concentration is constant and the effective ionic strengths at each buffer concentration are equal.

The resolution of analytes

A principal reason for studying the variation of mobilities with molarity of buffer is to obtain information about the resolution of analytes. If it

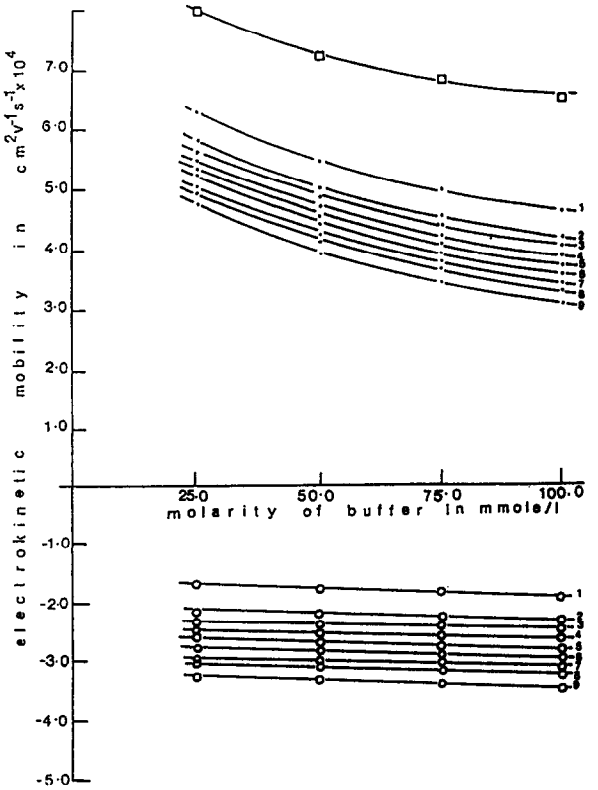

Fig. 7. Mobility plots showing the relationships between the EOF and the real and net mobilities of analytes with varying molarities of MES buffer at pH 6.10. Ionic strengths were not equalised (see text). Key: □ = electroosmotic mobility; ● = net electrophoretic mobility; ○ = real electrophoretic mobility; 1 = urocanate; 2 = urate; 3 = VMA; 4 = hippurate; 5 = cinnamate; 6 = phenylacetate; 7 = orotate; 8 = benzoate; 9 = salicylate. Conditions: capillary 57 cm × 75 μm I.D., 25 kV applied across capillary length, coolant temperature 25°C, wavelength 214 nm, sample was pressure injected for 5 s. Linear regression parameters for real mobilities are given in Table 4.

is assumed that the capillary length to the detector is the same as the length of the capillary, resolution [3,13] may be defined as:

$$R = 0.177(\mu_1 - \mu_2)[V/D(\mu_{eof} + \mu_{mean})]^{1/2} \quad (9)$$

where R is the resolution between two analytes with mobilities μ_1 and μ_2 and D is the (mean) diffusion coefficient. The term μ_{eof} is the electroosmotic mobility under the same conditions and the mean net mobility is equal to $(\mu_{eof} + \mu_{mean})$ when all the analyte mobility terms refer to real mobilities. In this equation V is the voltage applied across the length of the capillary. If all the analyte mobility terms refer to net mobilities the μ_{eof} term in the above equation is set to zero and $(\mu_1 - \mu_2)$ retains the same value.

Application of the above definition of resolution to the mobility plots illustrated in Fig. 7 results in some interesting conclusions. Although the net mobility curves for the nine analytes are essentially parallel, making $\mu_1 - \mu_2$ virtually constant between any pair of analytes, the resolution increases with increasing molarity of buffer because the mean net mobility decreases with the decreasing EOF. This is the algebraic reflection of the fact that decreasing the EOF increases the time available for electrophoresis (assuming there are no significant changes in real mobilities). When the equation for resolution is applied to the real mobilities of the analytes ($\mu_{eof} = 0$), it is apparent from the data in Fig. 7 that a greater value will be obtained for R because the mean real mobility is less than the mean net mobility. The resolution for real mobilities is furthermore decreasing with increasing molarity of buffer because the mean real mobilities increase. The reason for this apparent anomaly is the status of the observer. The analyst is a stationary observer outside the system and sees the resolution for net mobilities. An observer moving with the EOF and at the same speed would see the greater resolution obtained in the calculation using real mobilities. The preceeding arguments apply in particular to the data in Fig. 7, but should be equally applicable to other systems with results depending on the relative magnitudes of the real and net mobilities.

The ageing capillary surface and mobilities

Schwer and Kenndler [15] commented upon the poor reproducibility of the EOF in CE. A variety of factors may be involved including adsorption of trace metals [38,39] or the silica surface requiring long exposure times to electrolyte solutions before equilibration is achieved [26,46]. Van Orman *et al.* [24] were able to show that over relatively short time periods of a few

Table 4
Linear regression statistics for the real electrophoretic mobilities of organic anions in MES buffers of pH 6.10 and molarities ranging from 25 to 100 m*M*

Mobility (μ) ($m^2 V^- s^- 10^4$)	Regression equation (x = m*M*)	r^a	p^a
$\mu_{urocanate}$	$-(0.0021x + 1.72)$	−0.993	0.007
μ_{urate}	$-(0.0016x + 2.20)$	−0.994	0.006
μ_{VMA}	$-(0.0009x + 2.39)$	−0.959	0.041
$\mu_{hippurate}$	$-(0.0012x + 2.54)$	−0.989	0.011
$\mu_{cinnamate}$	$-(0.0017x + 2.66)$	−0.998	0.002
$\mu_{phenylacetate}$	$-(0.0019x + 2.79)$	−0.997	0.003
$\mu_{orotate}$	$-(0.0015x + 2.98)$	−0.984	0.016
$\mu_{benzoate}$	$-(0.0022x + 3.04)$	−0.996	0.004
$\mu_{salicylate}$	$-(0.0022x + 3.26)$	−0.996	0.004

Ionic strengths not equalised. Data from the same experiment as illustrated in Figure 7
[a] r is the coefficient of linear correlation and p the probability that it could belong to an uncorrelated universe.

days reproducible values for the EOF could be obtained with relative standard deviations in the region of 2% but their experiments did not involve any longer-term challenge from possible contaminants. Experience in the course of the present studies would be in agreement with the findings of VanOrman *et al.* [24] on the short-term reproducibility of EOF and experiments have been designed in such a way as not to depend on longer-term reproducibility. The lifetime of a capillary using the equipment described, before it breaks or blocks is variable, ranging from a few days to three months with a mean at approximately six weeks. It was considered important to establish in general terms what effects, if any, capillary ageing might be having on experimental results. The curved line shown in Fig. 7 relating the EOF to molarity of buffer can be linearised by plotting the EOF against the natural logarithm of the molarity [24]. This transformation produces linearity at a high level of statistical significance (see caption to Fig. 8) and is used in this work to enable the comparison of straight lines in preference to the curved lines normally obtained. The theoretical significance of the transformation is at present uncertain and its use in the present context is pragmatic and in the manner of Van Orman *et al.* [24]. The results of a series of such plots for different capillaries at different ages are shown in Fig. 8a for MES buffer and for three different Good's buffers in Fig. 8b. In the intervals between the experiments illustrated in Fig. 8 all capillaries were in normal continuous use running the protocol described in the Experimental section. Although all capillaries are cut from the same stock, each individual capillary will have a different analytical history.

The results illustrated in Fig. 8 show that for MES buffer (Fig. 8a) and three other Good's buffers (Fig. 8b) different ages of capillary give separate lines when the electroosmotic mobility is plotted against the logarithm of the buffer concentration. In Fig. 8a there is a discontinuity in the age related sequence in that capillary B at age 29 days shows slightly greater electroosmotic mobilities than at 16 days. This may be a reflection of the erratic nature of the ageing process in relation to the analytical history of the capillary. The data in Fig. 8b shows that the changes in EOF due to ageing have been sufficient to obscure the relative differences in EOF due to each buffer being at a different pH (Fig. 5). There are insufficient data for statistical rigidity but a tentative hypothesis that EOF declines with capillary age in the longer term can be made. This would be compatible with the findings of Wright [46] who found that the ζ

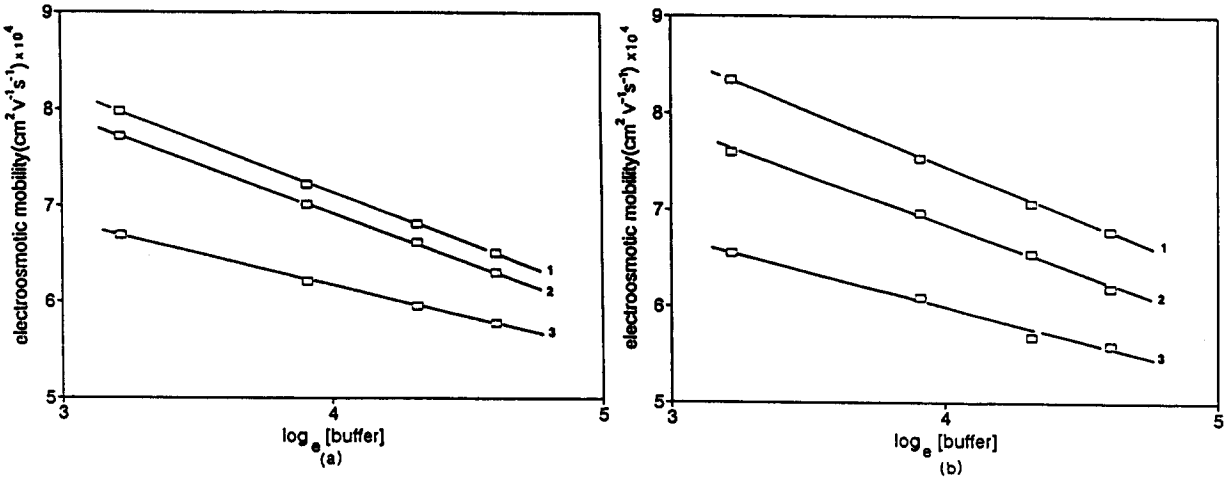

Fig. 8. The effect of long-term ageing of the internal capillary surface on the reproducibilty of EOF in experiments when the molarity of buffer is varied, demonstrated by plotting μ_{eof} against the logarithm of the buffer concentration. Capillary age is counted in days from the time of the initial conditioning. (a) MES buffer, pH 6.10, molarity from 25 to 100 m*M*; curves: 1 = capillary B, 29 days ($r = -0.9998$, $p = 0.00017$); 2 = capillary B, 16 days ($r = -0.9998$, $p = 0.00011$); 3 = capillary A, 39 days ($r = -0.9995$, $p = 0.00044$). (b) TAPS, MOPSO and HEPES buffers, molarity from 25 to 100 m*M*; curves: 1 = TAPS, pH 8.65, capillary C, 8 days ($r = -0.9997$, $p = 0.00028$); 2 = MOPSO, pH 6.95, capillary A, 44 days ($r = -0.9981$, $p = 0.00191$); 3 = HEPES, pH 7.50, capillary A, 65 days ($r = -0.9920$, $p = 0.00801$). Conditions as for Fig. 7.

potential between quartz sand and an electrolyte solution increased to a shallow plateau at 80 h and decreased slowly thereafter.

At the present time it is difficult to make comparisons between mobility coefficients which are quoted by different workers. The range of buffer types, molarities, pH and column temperatures which are used is the cause of this situation. The linear relationship between the real mobilities of analytes and the molarity of MES buffer enables valid extrapolation to give a value for the mobility at zero molarity. The intercepts in the regression equations in Table 4 are the mobilities at zero molarity for the analytes used in the present study. If linearity between real mobilities and molarity of buffer is a general phenomenon, the expression of mobilities at zero molarity would greatly facilitate the comparison of mobility values from different sources.

In Fig. 9 the real mobilities of four analytes from the experiments corresponding to the data illustrated in Fig. 8a for the variation of EOF with capillary age using MES buffer are plotted against the molarity of MES buffer. The real mobilities for the analytes vary to a small extent between the capillaries at different ages for reasons which are at present unknown. The

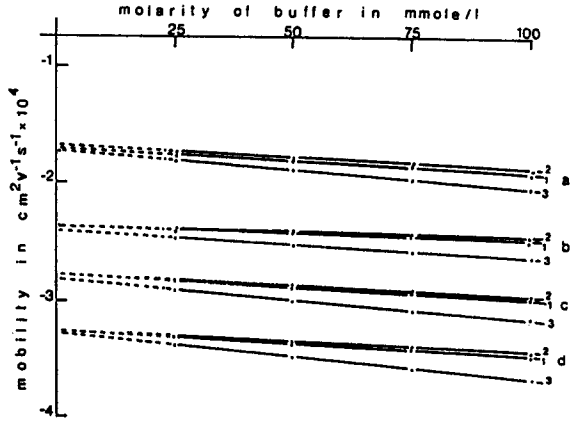

Fig. 9. Convergence of real mobilities of analytes after electrophoresis in capillaries at different ages, when mobility at zero molarity of MES buffer is obtained by extrapolation. Data obtained from experiments illustrated in Fig. 8a. Key: numbers as in Fig. 8a; group a = urocanate; group b = VMA; group c = phenylacetate; group d = salicylate. Conditions as for Fig. 7. The other analytes give similar families of convergent lines but have been omitted to preserve clarity.

interesting feature is the convergence of the mobilities to what may be a common point for each analyte, when the lines are extrapolated to zero molarity. The real mobilities of analytes in other Good's buffers also converge to the same common points as for MES buffer except when the buffer has a pH which produces a different ionised state in the analyte. Thus urocanic acid has completely different mobilities in MOPSO buffer compared to MES buffer. Extrapolation to zero molarity may be useful in compensating for the variations in mobility due to long-term capillary ageing for experiments involving the same buffer with similar experimental conditions. The data in Figs. 8 and 9 also illustrate that although there are long-term changes in the capillary surface which principally affect the EOF, the capillary continues to perform in a consistent manner for the electrophoretic separation of analytes.

Variable buffer molarity with effective ionic strengths equal

It has been known for some time that monovalent cations can be adsorbed by silica surfaces thereby effectively reducing the negative charge density caused by the ionisation of silanol groups [47] and the requirement for positive counter charge in the electrical double layer. Recently the concentration of buffer cation has been proposed as an important determinant in a theoretical explanation of the variation of EOF with buffer concentration [25,44]. The MES buffers used for studying the variation of EOF with buffer molarity (Fig. 7) were prepared by dilution of 100 m*M* stock buffer with water and the concentration of buffer cation (sodium ions) in each buffer was therefore reduced proportionately. An experiment in which the effective ionic strength, as defined previously, in each of the diluted buffers, was held equal to that of the parent 100 m*M* stock buffer was therefore considered an interesting test of recently published theory. The buffers were prepared by diluting the 100 m*M* stock MES buffer with 50 m*M* sodium chloride solution in water and thereafter the experimental procedure was the same as the experiment illustrated in Fig. 7. The variation of the electroosmotic and electrophoretic mobilities when the effective ionic strengths of each buffer are equalised in this way, with the molarity of the MES buffer, are shown in Fig. 10. Because the results of this experiment are of theoretical importance a summary of the linear regression statistics are given in Table 5. The ionic composition of the buffers used in this experiment are summarised in a balance sheet in Table 6.

The data illustrated in Fig. 10 are exactly comparable with the data in Fig. 7 the only difference being that in Fig. 10 the effective ionic strength of each buffer was equalised according to the balance sheet shown in Table 6. The EOF in Fig. 10 decreases in a significantly linear manner ($p < 0.002$, Table 5) as the molarity of

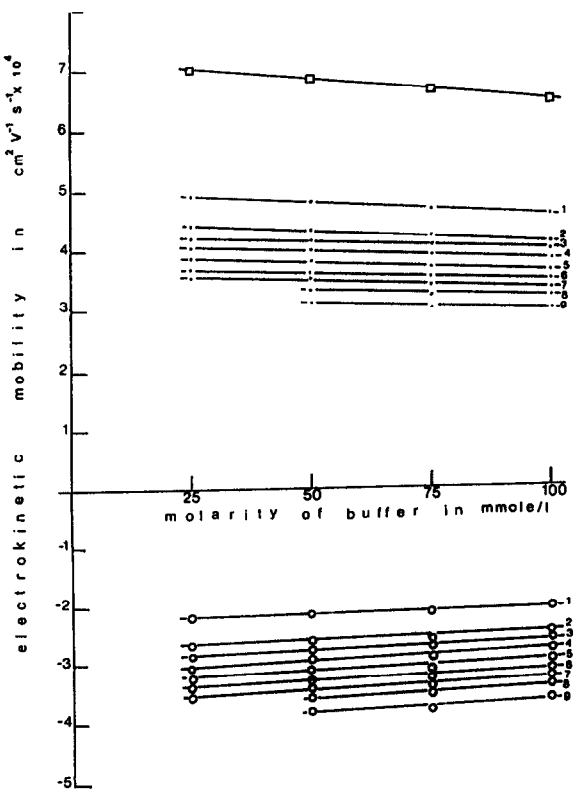

Fig. 10. Mobility plots showing the relationships between the EOF and the real and net mobilities of analytes with varying molarities of MES buffer at pH 6.10. Effective ionic strengths equalised (see text). Key and conditions as for Figure 7. Linear regression parameters are given in Table 5 and the ionic balance sheet for each molarity of MES is shown in Table 6.

Table 5
Linear regression statistics for the variation of mobilities with molarity of MES buffer, pH 6.10 with the effective ionic strength at each molarity equalised (see text)

Mobility ($cm^2 V^{-1} s^{-1} \times 10^4$)	Regression equation ($x = mM$)	r^a	p^a
EOF	$7.27 - 0.0071x$	−0.998	0.002
Net mobilities			
Urocanate	$5.03 - 0.0044x$	−0.999	0.001
Urate	$4.55 - 0.0040x$	−0.997	0.003
VMA	$4.32 - 0.0029x$	−0.995	0.005
Hippurate	$4.15 - 0.0029x$	−0.988	0.012
Cinnamate	$3.96 - 0.0029x$	−0.992	0.008
Phenylacetate	$3.78 - 0.0027x$	−0.986	0.014
Orotate	$3.64 - 0.0027x$	−0.979	0.021
Benzoate	$3.47 - 0.0024x$	−0.961	[b]
Salicylate	$3.21 - 0.0022x$	−0.904	[b]
Real mobilities			
Urocanate	$0.0026x - 2.24$	0.975	0.025
Urate	$0.0031x - 2.72$	0.973	0.027
VMA	$0.0042x - 2.95$	0.990	0.010
Hippurate	$0.0042x - 3.13$	0.985	0.015
Cinnamate	$0.0042x - 3.31$	0.984	0.016
Phenylacetate	$0.0044x - 3.50$	0.983	0.017
Orotate	$0.0044x - 3.64$	0.977	0.023
Benzoate	$0.0048x - 3.81$	0.961	[b]
Salicylate	$0.0050x - 4.08$	0.945	[b]

Data from the experiment illustrated in Fig. 10.
[a] r is the coefficient of linear correlation and p is the probability that it could belong to an uncorrelated universe.
[b] Only three data points available and no meaningful value for p can be calculated.

Table 6
Ionic balance sheet, current and pH data, for the MES buffers of the experiment illustrated in Fig. 10

Buffer parameter	Concentration (mM)			
Total buffer molarity	25	50	75	100
MES Zwitterion	12.5	25	37.5	50
MES Anion	12.5	25	37.5	50
Hydrated sodium cations	50	50	50	50
Chloride anions	37.5	25	12.5	0
Hydrogen ions (μM)	1	1	1	1
Total ion excl. zwitterion	100	100	100	100
Capillary current (μA)	126	107	88	72
Buffer pH	6.02	6.09	6.14	6.19

The series of MES buffers, approximate pH 6.10, were prepared by diluting 100 mM MES buffer with 50 mM sodium chloride solution. CE conditions as in Fig. 7.

MES buffer increases. The net mobilities of the organic anions also decrease with increasing buffer molarity in a linear manner (Table 5). Vectorial subtraction gives real mobilities which decrease with increasing molarity of MES buffer (*cf.* Fig. 7). The data in Table 6 shows that the capillary current appeared to follow the concentration of the highly conductive chloride ion and decreased with increasing buffer concentration which is the reverse of what happens when ionic strengths are left unequalised. The higher chloride concentrations in the lower-molarity buffers were also accompanied by a degradation in baseline stability. Although providing results of considerable theoretical interest the practice of equalising ionic strengths by the addition of sodium chloride could not be justified on analytical grounds.

A theoretical treatment versus experimental results

In 1991 Salomon *et al.* [25,44] published a theoretical treatment describing the variation of EOF with buffer cation concentration. The work described here was at this time nearing completion and seemed to be fortuitously suited to a test of this theory. In this theory the electroosmotic mobility is expressed as:

$$\mu_{eof} = Q(d_0 + \delta)/\eta \quad (10)$$

where Q is the charge per unit area at the internal silica surface of the capillary, d_0 is the thickness of the compact layer as defined by the Stern–Gouy–Chapman model of the electrical double layer, δ the thickness of the diffuse layer and η the viscosity of the buffer. The diffuse layer is treated as a Debye–Huckel thickness inversely proportional to the square root of the ion concentration giving:

$$\mu_{eof} = Q(d_0 + 1/K'[M^+]^{1/2})/\eta \quad (11)$$

where $[M^+]$ is the concentration of a monovalent cation and K' is a constant. The quantity Q is equal to Q_0, the total density of surface ionised silanol groups, reduced by the fraction $1/(1 + K_{wall}[M^+])$ to take account of the ionised silanol groups which are effectively neutralised by adsorbed cations and multiplied by e, the charge of an electron. The parameter e does not appear in the equations published by Salomon *et al.* [25,44]. The quantity Q now becomes:

$$Q_0 e/(1 + K_{wall}[M^+]) \quad (12)$$

It should be noted that K_{wall} is in units of $mmol^{-1} \cdot l$ which requires $[M^+]$ in Eq. 12 to be in mmol/l whereas K' requires $[M^+]$ in Eq. 11 to be a concentration in mol/l. K_{wall} is the equilibrium constant of the adsorption mechanism:

$$M^+ + SiO^-(H_2O) \rightleftharpoons SiO^-M^+ + H_2O$$

The end result of this treatment is an expression for μ_{eof} which is the product of a viscosity term, $1/\eta$, usually regarded as constant for dilute aqueous buffers, a charge density term, $Q_0 e/(1 + K_{wall}[M^+])$ and the electrical double layer thickness term $(d_0 + 1/K'[M^+]^{1/2})$. The mathematical form of the expressions for charge density and the thickness term allow for two types of linear plots if certain assumptions are valid. If the charge density term is assumed to be constant, and is, a plot of μ_{eof} against the reciprocal square root of the cation concentration is expected to be linear if the thickness term is valid. Conversely, if the thickness term is assumed to be constant, and is, a plot of $1/\mu_{eof}$ against the buffer cation concentration will be linear if the charge density term is valid. Both types of partial plot were employed by Salomon *et al.* in the development of their theory and their practice is pragmatically followed here as it appears to reveal some anomalies in the theory.

Salomon *et al.* [25] measured μ_{eof} in a series of MES buffers, pH 6.05, in which the sodium concentration varied from 2 up to 40 m*M*: no other ions than those sustaining the buffer system were present. The authors found that when their values for μ_{eof} were plotted against the reciprocal square root of the buffer sodium concentration there was non-linearity up to a sodium concentration of approximately 7.5 m*M* followed by a linear relationship up to their maximum sodium concentration of 40 m*M*.

The corresponding results in the present studies employ buffer cation concentrations in

the range 12.5 m*M* up to a maximum of 50 m*M* and are illustrated in Figs. 11a and b for MES buffer and 12 for TAPS, MOPSO and HEPES buffers. The data in these figures correspond to the electroosmotic mobility data illustrated in Fig. 8 for three different capillaries with ages ranging from 8 to 65 days and employing four different Good's buffers. In Fig. 11a the electroosmotic mobility data for MES buffer are shown plotted against the reciprocal square roots of the buffer cation concentrations. Fig. 11a shows that a smooth and very shallow curve can be fitted through the experimental data points. In Fig. 11b the experimental points are fitted by straight lines and the linear regression statistics show highly significant linear correlation ($r > 0.99$ and $p < 0.01$) indicating that the assumption of a linear relationship at cation concentrations greater than 12.5 m*M* is at least a fair approximation and supports the hypothesis that for higher cation concentrations the charge density term is constant or nearly constant. Inspection of the data illustrated in Fig. 11a and b suggests that true linearity in the present studies starts at buffer cation concentrations close to but slightly higher than 12.5 m*M* and exists up to the maximum cation concentration studied, namely 50 m*M*. This is to be compared with an estimated linear range from the data of Salomon *et al.* [25] of 7.5 m*M* up to 40 m*M*. It may be noted that the three lines in Fig. 11b for MES buffer in capillaries of different ages extrapolate to a common intercept of approximately 5.0 $cm^2\ V^{-1}\ s^{-1} \times 10^4$ on the mobility axis. This corresponds to a common value for d_0 in the above equations for the experiments using MES buffers.

The data illustrated in Fig. 12 shows that μ_{eof} for the buffers TAPS, MOPSO and HEPES is related to the reciprocal square root of the buffer cation concentration in a manner similar to that described for MES buffer.

Salomon *et al.* [25] corrected for the non-linear behaviour of μ_{eof} when plotted against the reciprocal square root of buffer sodium concentrations in the range 2 to approximately 7.5 m*M* by introducing the adsorption term $1/(1 + K_{wall}[M^+])$. On the assumption that the thickness term was constant they plotted $1/\mu_{eof}$ against the buffer sodium concentration. The authors claimed that linear behaviour was evident up to a buffer sodium concentration of approximately 30 m*M* and non-linear behaviour for concentrations greater than 30 m*M* up to their experimental maximum of 40 m*M*. For the present work Fig. 11c demonstrates that the same strategy gives a non-linear relationship between $1/\mu_{eof}$ and buffer sodium concentration throughout the experimental concentration range of 12.5 to 50 m*M*. This non-linear relationship was expected because of the evidence presented above that the double layer thickness term is not constant but is an important determinant of the value of μ_{eof} when the charge density term is constant or nearly so.

The results obtained by Salomon *et al.* [25] show that between buffer sodium concentrations of approximately 7.5 and 30 m*M* they obtain linearity in both types of plotting regime. This appears to be an anomaly about which the authors make no comment and is particularly difficult to explain because it was not encountered in the studies reported here (Fig. 11c).

Salomon *et al.* were able to obtain a close fit between experimentally obtained μ_{eof} *vs.* sodium concentration curves and those predicted by theory for MES and CAPSO buffers when suitable values for K_{wall}, d_0 and Q_0 were chosen. It may be argued that provided the mathematical form of the equations is correct, obtaining good agreement with experimental data by assigning values to three parameters whose individual values can vary to a considerable extent is of doubtful significance. A comparison of the experimentally determined values of μ_{eof} from the present work with the values predicted by the above equations is shown in Table 7. There is satisfactory agreement between predicted values and those obtained by experiment with maximum discrepancies (mean 3.63% with a range of 0.65–5.40%) occurring with the 100 m*M* buffer. Sodium ions were the only cations present in the buffers of the experiments illustrated in Table 7. The most significant difference between the parameters reported by Salomon *et al.* [25] and the parameters in Table 7 are the much lower values for K_{wall} obtained in the present studies. This could be due to many influences including

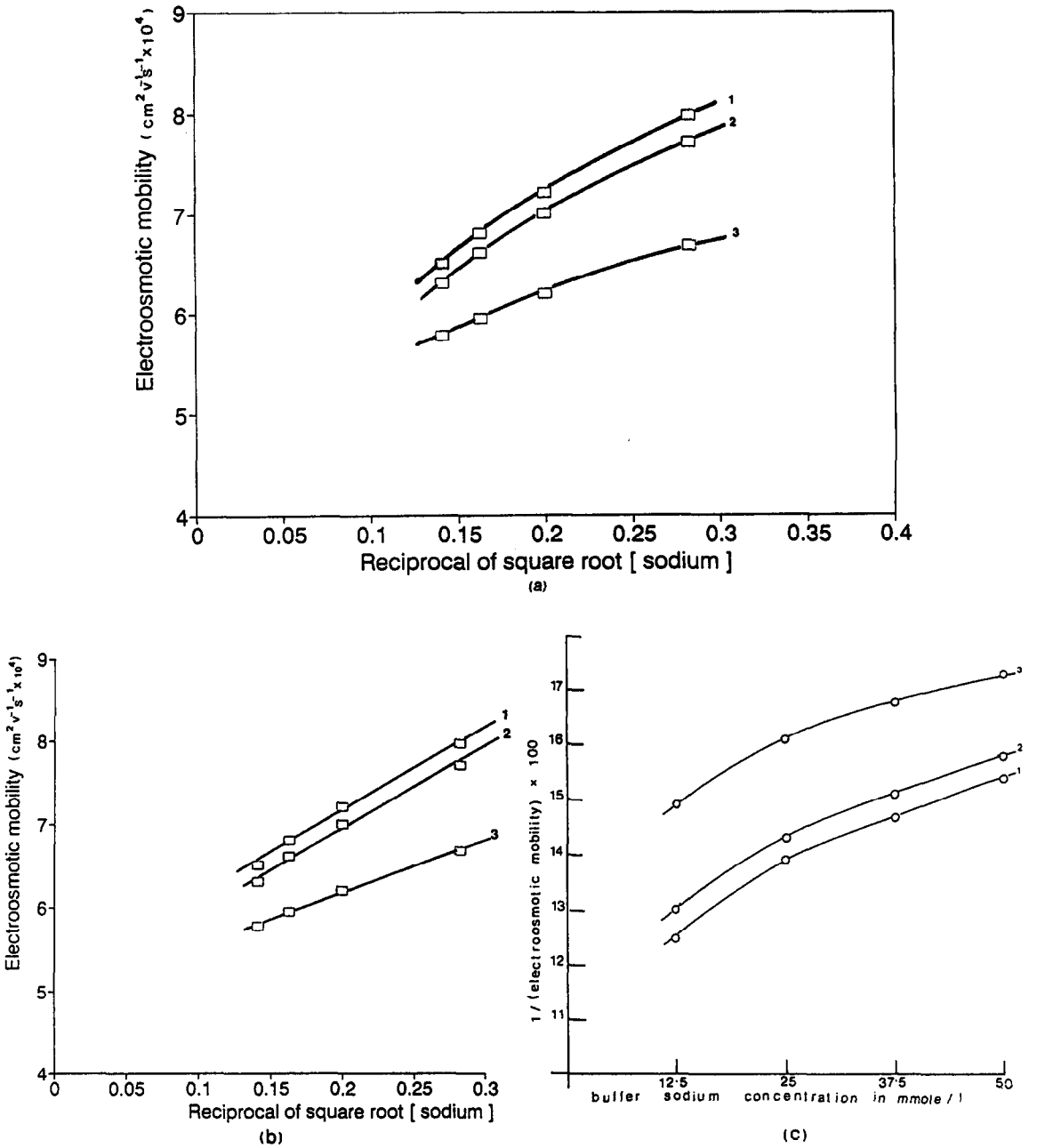

Fig. 11. Electroosmotic mobility data for capillaries of different ages with MES buffer, pH 6.10, plotted after the manner of Salomon *et al.* [25]. The experimental data are from the same experiments as illustrated in Fig. 8a. (a) Electroosmotic mobility data plotted against the reciprocal square root of the buffer sodium concentration illustrating the data fitted by shallow curves; curves: 1 = capillary B, age 29 days; 2 = capillary B, age 16 days; 3 = capillary A, age 39 days. (b) Electroosmotic mobilities plotted against the reciprocal of the square root of the buffer sodium concentration showing the data fitted by straight lines; curves: 1 = capillary B, age 29 days, $r = 0.997$, $p < 0.003$; 2 = capillary B, age 16 days, $r = 0.995$, $p < 0.005$; 3 = capillary A, age 39 days, $r = 0.998$, $p < 0.002$; note: linear correlation coefficients and significance levels calculated using all four data points in each case. (c) Reciprocal of μ_{eof} plotted against the buffer sodium concentration; curves as in (b). Conditions as for Fig. 7.

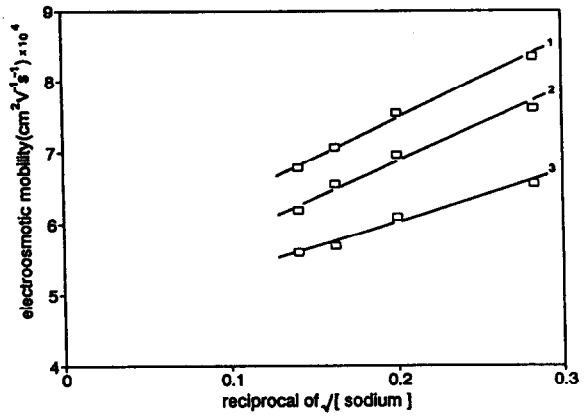

Fig. 12. Linear fitting of the data when μ_{eof} is plotted against the reciprocal square root of the buffer sodium concentration for the buffers TAPS, MOPSO and HEPES with capillaries of different ages. Experimental data from experiments illustrated in Fig. 8b and conditions as for Fig. 7. Key as in Fig. 8b. Linear correlation parameters: 1, $r = 0.9970$, $p = 0.00298$; 2, $r = 0.9881$, $p = 0.01188$; 3, $r = 0.9889$, $p = 0.01109$.

differences in the preparation of the vitreous silica, the conditioning and analytical history of the capillaries and capillary age. It also appears that the lowest values for K_{wall} are associated with the oldest capillaries although there are insufficient data for statistical significance. Mathematically the lower the value of K_{wall} the shallower the slope in plots of μ_{eof} against buffer molarity for the Salomon equation. The data in Table 7 for capillaries containing MES buffer show a mean value for Q_0 of $1.29 \cdot 10^{16}$ for the total number of ionised silanol groups per square meter which compares with an estimated $4 \cdot 10^{18}$ to $5 \cdot 10^{18}$ total silanol groups per square meter [47] and means that approximately 0.3% of the surface silanols are ionised at a pH of 6.10. This is in agreement with the value obtained by Salomon *et al.* but seems a very low degree of ionisation when compared with reported values for the p*I* of silica of approximately 1.0 [48].

Table 7
A comparison of the experimental values for the electroosmotic mobilities from the experiments illustrated in Fig. 8 with the values predicted by the equations of Salomon *et al.* [25] and a summary of the values attributed to constants and capillary parameters for the calculations with current data

Capillary	Age (days)	Buffer	μ_{eof} ($cm^2 V^{-1} s^{-1} \times 10^4$)				Q_0 ($\times 10^{16}$ sites m^{-2})	d_0 ($\times 10^{-8}$ m)	K_{wall} ($mmol^{-1} \cdot l$)
			Concentration of buffer (m*M*)[a]						
			25	50	75	100			
B	28	MES	7.98	7.22	6.81	6.51	1.40	3.2	0.007
			7.99	*7.23*	*6.65*	*6.18*			
B	16	MES	7.72	7.01	6.62	6.31	1.35	3.2	0.007
			7.72	*6.98*	*6.42*	*5.97*			
A	39	MES	6.69	6.21	5.96	5.78	1.12	3.2	0.004
			6.66	*6.21*	*5.87*	*5.59*			
C	8	TAPS	8.34	7.54	7.07	6.78	1.55	3.0	0.007
			8.35	*7.54*	*6.93*	*6.44*			
A	44	MOPSO	7.60	6.96	6.55	6.19	1.37	3.0	0.005
			7.56	*6.97*	*6.52*	*6.15*			
A	65	HEPES	6.55	6.09	5.68	5.59	1.17	3.0	0.004
			6.53	*6.08*	*5.75*	*5.46*			

Values predicted by theory are shown in italics. A value for K' of $3.2 \cdot 10^9$ m^{-1} $(mol/l)^{-1/2}$ is taken from Salomon *et al.* [25]. A value for e, the fundamental electronic charge of $1.60 \cdot 10^{-19}$ C (SI) is used. The viscosity η is set equal to $8.95 \cdot 10^{-4}$ N m^{-2} s (SI) throughout.

[a] The molarity of the buffer cation (sodium) is half the buffer molarity given here.

There are no notable differences between the values obtained for d_0 in the present work and those obtained by Salomon *et al.*

If the variation of μ_{eof} with buffer cation concentration is to be described by a single equation the use of the simple adsorption term $1/(1 + K_{wall}\ [M^+])$ appears doubtful on both theoretical and experimental grounds. Its value varies continuously with the value of $[M^+]$, and it does not, in its present form, provide for a constant charge density term to agree with the experimental findings described above for both Salomon *et al.* [25] at buffer cation concentrations greater than 7.5 m*M* and the present work for concentrations greater than approximately 12.5 m*M*. The treatment proposed by Salomon *et al.* implies that all ionised silanol groups are equivalent and will adsorb cations in the same way. This may be an over-simplification. Nawrocki [38,39] describes the presence of small subpopulations of highly ionised silanol groups possibly caused by the adsorption of trace amounts of heavy metal cations. The results obtained by Salomon *et al.* and the present work suggest that a slightly modified version of their original proposals may be more applicable. If it is hypothesised that only a fraction of the total ionised silanol population is able to adsorb cations like sodium ions, the adsorption of cations will be limited at a certain cation concentration, and thereafter a state of constant charge density will exist at the silica surface. This would be compatible with the experimental results of both Salomon *et al.* and the present work with the adsorption limit occurring in the region of a sodium concentration of 10 m*M*.

The results illustrated in Fig. 10 show conclusively that μ_{eof} decreases with increasing concentration of MES buffer when the buffer cation is held constant at 50 m*M* (Table 6) and furthermore the decrease is linear, in contrast to the variation in μ_{eof} shown in Fig. 7 when the effective ionic strengths were not equalised. The decrease in μ_{eof} in Fig. 10 is approximately 40% of that shown in Fig. 7. These results demonstrate that the buffer cation concentration and the capillary wall parameters cannot be the sole determinants of the value of μ_{eof}. It seems likely that the buffer anion concentration and/or the nature of the buffer anion has some function in determining the structure of the electrical double layer. It is also possible that buffer anions may affect the value of K_{wall} at constant cation concentration [25]. The presence of chloride anions appears to affect the dissociation of acetic acid (note by referee). There is also an approximation in the original theoretical treatment [25,44] in the tacit assumption that $[M^+]$, the cation concentration in the bulk buffer, can be used in place of the cation concentration in the compact layer, where the adsorbed cations must presumably come from. This may not matter in practice when K_{wall} is assigned empirical values to allow the above equations to fit experimental results. As stated earlier the Debye–Huckel treatment of the electrical double layer employed by Salomon *et al.* assumes a simple monovalent cation–anion buffer system. In more complicated buffer systems the variation of μ_{eof} with buffer concentration may behave differently.

The theory presented by Salomon *et al.* requires that both the compact and diffuse layers are electroosmotically mobile. It seems to the present writer that as a consequence a number of theoretical questions arise which require resolution. In particular, what are the physical properties of the compact layer which justify its treatment as an entity distinct from the diffuse layer? Is there still a shear plane and where is it defined? There still has to be a ζ potential but where is it defined? Are the ions in the compact layer subject to thermal diffusion? Salomon *et al.* [25] in defining their compact layer present it as that part of the double layer over which the decay in potential is linear followed by a diffuse layer in which the decay is exponential which in the circumstances appears highly presumptive. Hunter, in his classical treatise [26], regards the electrokinetic unit in electrophoresis as the particle together with its enshrouding compact layer up to the shear plane. If the proposals of Salomon *et al.* are true, under what circumstances does a compact layer behave in a manner which is electrokinetically different from the solid surface with which it is normally associated?

Despite these criticisms, the treatment pro-

posed by Salomon *et al.* represents a useful framework against which to test experimental results. The expression for the thickness term has been particularly applicable in the present studies. It is a first step in the attempt to describe the EOF in terms of the properties of the capillary wall and the buffer system and is open to refinement by subsequent work.

4.3. The properties of 15-crown-5 as an EOF modifier

A variety of organic modifiers have been used as buffer additives in CE, especially methanol and acetonitrile [24,25,41], all of them reducing the value of the EOF with the possible exception of acetone at certain concentrations [41]. At the present time the only practical way of increasing the EOF by altering the system chemistry is by decreasing the molarity of the buffer in which electrophoresis occurs. A chemical additive which increased the EOF would be useful in situations where resolution was not a limiting factor and would allow more control of the electrophoretic process. The results reported here on the properties of 15-crown-5 as an EOF modifier originated in the hope that it might be such an additive. The rationale was that 15-crown-5 in the electrophoretic buffer would compete with the silica surface of the capillary for the buffer cations (sodium ions) and that the 15-crown-5–cation complexes would not enter the adsorbed layer. The 15-crown-5 would act in the manner of a chromatographic pseudo-phase similar to the micelles in micellar electrokinetic chromatography and would remove sodium ions from the surrounding buffer thereby competing with the silica surface. The charge density at the silica surface would be increased with consequent increase in the EOF. In fact 15-crown-5 causes a decrease in the EOF and the original hopes were unrealised.

The crown ether, 15-crown-5, forms clathrate type complexes with monovalent alkali metal cations and is readily soluble in aqueous buffer solutions at the relevant concentrations. The complexes are themselves cations. The effect of incorporating 15-crown-5 in 50 m*M* MES buffer at pH 6.10 on the electroosmotic and electrophoretic mobilities in the electrophoresis of the reference analyte solution is shown in Fig. 13 together with the results of a similar experiment using methanol as a flow modifier for comparison. Table 8 contains a number of parameters which allow a ready comparison between 15-crown-5 and methanol as EOF modifiers.

There are significant differences in the behaviour of 15-crown-5 and methanol as EOF modifiers. In rounded off numbers 1% 15-crown-5 in 50 m*M* MES buffer produces a 14% reduction in the EOF which is the same as the reduction produced by 5% (v/v) methanolic buffer (Table 8). This means that in molar terms 15-crown-5 is 25 times more effective than methanol in reducing the EOF. The electroosmotic mobility curves in Fig. 13 show that increasing the concentration of 15-crown-5 beyond a concentration of 1% (50 m*M*) will produce little further reduction in μ_{eof} with 50 m*M* MES buffer, *i.e.* the effect is limited. As the concentration of 15-crown-5 increases through and beyond 25 m*M*, which is the same as the sodium ion concentration in the buffer, the rate of reduction in μ_{eof} decreases rapidly. In contrast, increasing the methanol concentration beyond 5% will clearly produce further decreases in the EOF. A further difference between the curves in Fig. 13a and b is that the real analyte mobilities (curves in the negative quadrant) are virtually invariate with the concentration of 15-crown-5 whereas there appears to be a significant decrease in real mobilities with increasing methanol concentration.

There have been several studies on the mechanisms involved in the reduction of the EOF by organic modifiers like methanol. VanOrman *et al.* [24] speculated that alcohols interact with the capillary wall and are present in the double layer at higher concentrations than in the bulk buffer. They propose that the local increase in viscosity within the double layer is primarily responsible for the reduction in μ_{eof}. Salomon *et al.* [25] suggested that methanol affects both the silica surface, causing an increase in Q_0, and the thickness of the compact layer, causing an overriding reduction in d_0. Schwer and Kenndler [41] studied the variation of EOF in a series of aqueous solvents containing methanol, ethanol,

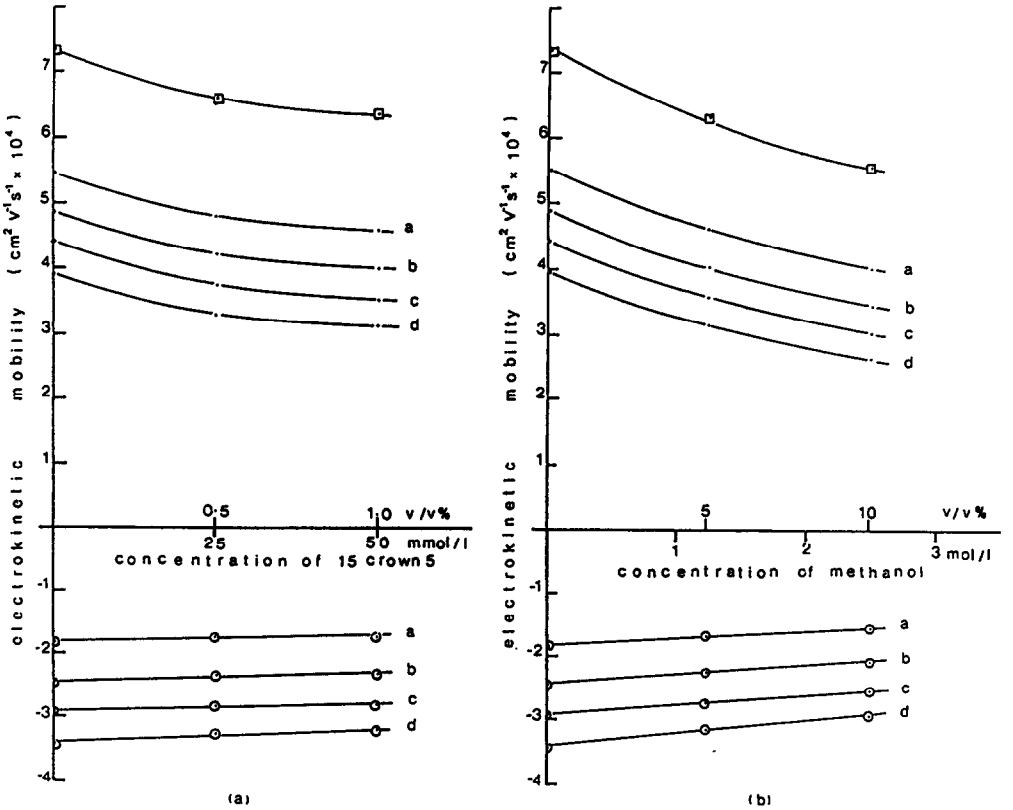

Fig. 13. Comparison of 15-crown-5 and methanol in 50 m*M* MES buffer, pH 6.10 as modifiers of the EOF. Key: □ = electroosmotic mobilities; ● = analyte net mobilities; ○ = analyte real mobilities; a = urocanate; b = VMA; c = phenylacetate; d = salicylate. Conditions: capillary 57 cm × 75 μm I.D., 25 kV applied across capillary length, coolant temperature 25°C, sample pressure injected for 5 s, wavelength 214 nm. Data for other analytes omitted to preserve clarity.

Table 8
Percentage reductions in the value of electroosmotic mobilities and variations in retention times in 50 m*M* MES buffers, pH 6.10, containing 15-crown-5 and methanol as EOF modifiers

Parameter	EOF Modifier				
	None 50 m*M* MES	15-Crown-5 in 50 m*M* MES		Methanol in 50 m*M* MES	
Modifier (%, v/v)	0	0.5	1.0	5.0	10
Concentration of modifier	0	25 m*M*	50 m*M*	1.24 *M*	2.47 *M*
μ_{eof}	7.34	6.57	6.33	6.31	5.57
Reduction in EOF (%)	0	10.5	13.8	14	24
Retention time (min) of salicylic acid	4.84	5.77	6.11	6.05	7.25
Retention time (min) of urocanic acid	3.46	3.95	4.14	4.12	4.76

Data correspond to those illustrated in Fig. 13.

isopropanol, acetonitrile, acetone and dimethylsulphoxide over the pH range 3–11. They accounted for the observed decreases in the EOF by changes in the dielectric properties of the electrical double layer and the ionisation of the silica surface.

The available evidence does not permit more than a speculative explanation of the reduction in the EOF by 15-crown-5. Any explanation must account for 15-crown-5 being 25 times more efficient in molar terms than methanol in producing an initial 14% reduction in the EOF and the reduction in the EOF by 15-crown-5 rapidly approaching a limit when its concentration equals and exceeds that of the sodium ions in the buffer.

One simple explanation, which satisfies the above conditions, is that 15-crown-5 is complexing all cations (sodium ions) in the buffer–capillary surface system resulting in the replacement of sodium ions by the larger cationic crown ether–sodium ion complexes. The resulting reduction in the EOF is then effectively attributed to a change in buffer cation. This cation replacement by crown ether–sodium ion complexes would be expected to produce accompanying changes in the dielectric and viscous properties of the electrical double layer appropriate to the organic crown ether cation.

Apart from the theoretical interest, for the practical analyst the use of 15-crown-5 offers the prospect of producing relatively small reductions in the EOF using lower concentrations of modifier than would be necessary if the same reductions were achieved by the addition of methanol.

4.4. *Electrokinetic velocities and electric field strength*

The Smoluchowski equation for electroosmotic velocities, $U_{eof} = \mu_{eof}E$, requires that if μ_{eof} is constant a graph of U plotted against E should be linear and passing through the origin. The same applies for real and net electrophoretic velocities of analytes where the equation is $U_{ep} = \mu_{ep}E$ with U_{ep} being positive or negative depending on the direction of the electrophoretic motion, *i.e.* the sign of μ_{ep}.

In Fig. 14 the variation in the electroosmotic and electrophoretic velocities (expressed in mm/s) with the electric field strength (in V/cm) when electrophoresis of the reference analyte solution is conducted in 50 m*M* MES buffer, pH 6.12, is illustrated, the voltage across the capillary being varied from 10 to 30 kV in 5-kV steps. Electrophoretic velocity data in Fig. 14 are given for

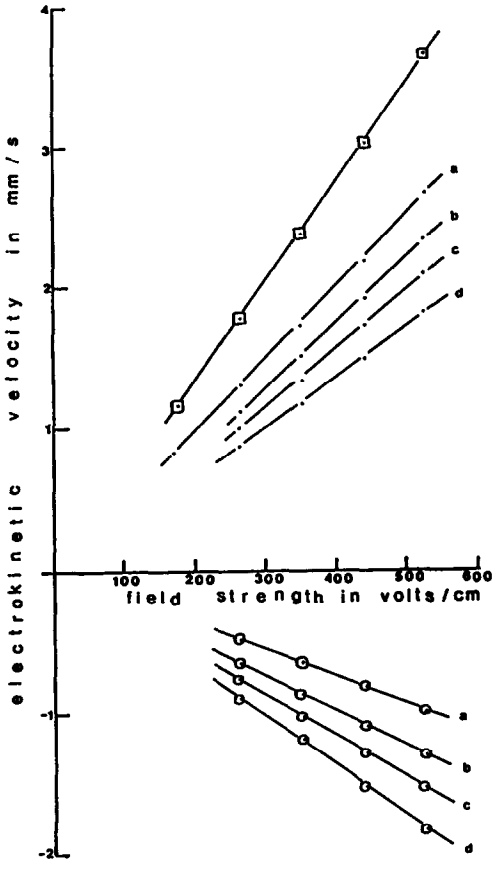

Fig. 14. Linearity between electrokinetic velocities and electric field strength during capillary electrophoresis of the reference analyte solution in 50 m*M* MES buffer, pH 6.12. Key as in Fig. 13. Conditions: voltage across capillary varied from 10 to 30 kV, otherwise as in Fig. 13. EOF regression equation: velocity (mm/s) = 0.0071 × field strength (V/cm) − 0.10309, $r = 0.9999$, $p < 0.000001$. Regression parameters for velocities of analytes [U = net electrophoretic velocity (mm/s), E = V/cm]: a, $U = 0.0051E - 0.0719$, $r = 0.9999$, $p = 0.000002$; b, $U = 0.0046E - 0.1173$, $r = 0.9997$, $p = 0.00032$; c, $U = 0.0041E - 0.1023$, $r = 0.9998$, $p = 0.00025$; d, $U = 0.0036E - 0.0923$, $r = 0.9998$, $p = 0.00017$. Data for other analytes omitted to preserve clarity.

only four of the analytes, the fastest, the slowest and two of intermediate velocity in order to preserve clarity. The remaining analytes behave in a manner similar to those illustrated. The results presented in Fig. 14, together with the accompanying linear regression statistics show that there is a linear relationship between electrokinetic velocities and electric field strength up to a field strength of 526 V/cm (30 kV over a capillary of length 57 cm). The values obtained for the statistic p show that the null hypothesis is discredited beyond reasonable doubt. The regression statistics for the capillary current data, corresponding to the field strength data in Fig. 14, are given in Table 9, show highly significant linear correlation between the capillary current and electroosmotic and electrophoretic velocities. The linearity between electrokinetic velocities and both capillary current and electric field strength is a reflection of the obedience to Ohm's law by the capillary–buffer system at the field strengths used in these experiments.

These results show considerable differences when compared with the results obtained by many workers in similar experiments designed to determine the relationship between electrokinetic velocities and field strength. Jorgenson and Lukacs [49] reported departure from linearity when reciprocal retention times were plotted against applied voltage for voltages greater than 15 kV over a capillary length of 100 cm. Terabe *et al.* [20] using capillaries 65 cm in length commented upon the non-linearity between U_{eof} and applied voltage. Lauer and McManigill [50] were able to demonstrate linearity between field strengths and U_{eof} for relatively low field strengths below 200 V/cm. Altria and Simpson [23] show a curvilinear relationship between the electrophoretic mobilities of quinacrine and quinine up to an applied voltage of 30 kV over a capillary of length 100 cm. Tsuda *et al.* [18,19,21] in a series of studies emphasise the linearity between capillary current density and U_{eof} and Tsuda [21] specifically states that the relationship between U_{eof} and electric field strength is not linear.

It was recognised by early workers in the field that power dissipation within a capillary leading to temperature increases would result in decreased buffer viscosity causing an increase in μ_{eof} and real electrophoretic mobilities, *i.e.* electrokinetic velocities per volt of field strength would not be constant. The theory of heat generation within capillaries has been described elsewhere [13,17,51,52]. In brief, the rate at which heat is generated is proportional to the product of the molar concentration of the buffer, its molar conductivity and the square of the field strength. The temperature difference between the centre of the capillary and the wall is proportional to this product multiplied by the square of the capillary diameter. The reasons for the excellent linearity illustrated in Fig. 14 over a wide range of field strength are twofold. The low molar conductivities of MES buffer and other Good's buffers relative to phosphate buffer have been described in a previous section (Table 1 and accompanying text). This fact alone would

Table 9
Regression statistics for electroosmotic and net electrophoretic velocities and capillary current data corresponding to the field strength data illustrated in Fig. 14

Electrokinetic velocity (mm/s)	Regression equation ($x = \mu A$)	r^a	p^a
U_{eof}	$0.0809x + 0.1303$	0.9989	0.00004
$U_{urocanate}$	$0.0586x + 0.0968$	0.9991	0.00003
U_{VMA}	$0.0506x + 0.1126$	0.9996	0.00039
$U_{phenylacetate}$	$0.0449x + 0.1025$	0.9994	0.00059
$U_{salicylate}$	$0.0393x + 0.0872$	0.9994	0.00065

Electrophoretic conditions as in Fig. 14.
[a] r is the coefficient of linear correlation and p the probability that it could have this value if the null hypothesis were true.

probably not account for the results obtained. In the commercial instrument used throughout this work heat generated in the capillary is actively removed by a circulating inert liquid, the temperature of which is continuously monitored by the system. The importance of careful thermostatic control of capillary temperature on column efficiency is emphasised in a recent theoretical study of temperature gradients within the capillary [17]. The results obtained in the present work for applied voltages up to 30 kV testify for the merits of this strategy.

4.5. Analyte concentrations in relation to peak areas and heights

There are in theory two ways in which the response of an on-line UV detector in CE can be calibrated to enable the concentrations of analytes in samples to be calculated. The first method is to maintain a fixed sampling time and introduce a series of samples containing different concentrations of analyte to the capillary. The second method is to vary the sampling time of a standard solution of the analyte. Throughout these studies sampling has been achieved by high-pressure injection for the fixed time of 5 s. The reasons for this decision were firstly that it is impractical to fully investigate all combinations of all variables and secondly that sampling errors might be expected to become relatively larger as sampling times are decreased.

The statistical parameters which describe the calibration graphs obtained when standard solutions containing different concentrations of analytes are analysed by the protocol described in the experimental section are given in Table 10 for both peak areas and peak heights. Excellent

Table 10
Linear regression statistics for the results obtained by sampling standard analyte solutions containing 40, 80, 120, 160 and 200 μM of each analyte in 10 mM HEPES and measuring the areas of the respective electropherogram peaks

Analyte	Regression equation		r^a	p^a
	x = Peak area, $y = \mu M$	x = Peak height, $y = \mu M$		
Urocanate	$y = 0.626x - 2.091$		0.9985	0.000067
Urate	$y = 0.532x - 4.838$		0.9989	0.000046
VMA	$y = 0.392x - 5.151$		0.9987	0.000054
Hippurate	$y = 0.421x - 4.131$		0.9972	0.000175
Cinnamate	$y = 0.254x - 6.048$		0.9993	0.000022
Phenylacetate	$y = 0.613x - 16.64$		0.9971	0.000188
Orotate	$y = 0.396x - 3.139$		0.9971	0.000189
Benzoate	$y = 0.456x - 2.455$		0.9981	0.000102
Salicylate	$y = 0.298x - 4.502$		0.9997	0.000008
Urocanate		$y = 0.870x - 7.210$	0.9981	0.000097
Urate		$y = 0.841x - 5.877$	0.9985	0.000067
VMA		$y = 0.742x - 2.802$	0.9992	0.000025
Hippurate		$y = 0.757x - 1.433$	0.9989	0.000043
Cinnamate		$y = 0.463x - 1.931$	0.9991	0.000031
Phenylacetate		$y = 1.312x - 7.367$	0.9988	0.000051
Orotate		$y = 0.939x - 9.450$	0.9985	0.000072
Benzoate		$y = 1.252x - 15.15$	0.9969	0.000204
Salicylate		$y = 1.085x - 26.07$	0.9941	0.000547

Analytical conditions: 25 kV across capillary of 57 cm × 75 μm I.D., temperature of coolant 25°C, sample injection high pressure for 5 s, monitored at wavelength 214 nm, buffer 50 mM MES, pH 6.10. Peak areas were expressed in arbitrary units produced by the system software multiplied by 1000. Peak heights in arbitrary units multiplied by 10 000.

[a] r is the coefficient of linear correlation and p is the probability that it could have been obtained from an uncorrelated universe.

values for the linear correlation coefficient are obtained irrespective of whether peak areas or peak heights are used, with parameters calculated using peak areas being marginally superior. The electrophoretic procedure which has been developed is demonstrably capable of giving good quantitative results with the test analytes when they are present in a suitable solution.

5. Conclusions

A functionally coherent set of buffers of equal molarity and ionic strength and operating at or close to their pK values has been used to study the electrophoretic behaviour of a group of organic anions of biological significance as a first step towards developing techniques suitable for the electrophoretic analysis of biological fluids. The studies covered the approximate pH range 6 to 10. Evidence is presented which indicates that changes in the value of the EOF in the region of pH 8 may be correlated with concurrent changes in the silica surface of the capillary. The variation of electroosmotic and electrophoretic mobilities with buffer molarity was studied with particular reference to the effects of long-term ageing of the capillary surface and ionic strength. Unexpected results, possibly of theoretical significance, were obtained when effective ionic strengths in a series of MES buffers of differing molarities were equalised. Agreement with recently reported theoretical work has been tested and the implications of the theory discussed. The use of the crown ether 15-crown-5 as an EOF modifier is reported and discussed. The experimental techniques employed give excellent linearity between electroosmotic and electrophoretic velocities and applied field strengths up to 526 V/cm. Reasons for this are given. Calibration curves for analytes up to a concentration of 200 μM show linearity with respect to both peak areas and peak heights. There remains scope for future fundamental studies on the nature and control of the EOF and the electrophoretic process. Future applications with biological fluids will depend to a large extent on the availability of techniques for sample preparation and sensitive methods for the detection of analytes.

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7. References

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