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ELECTROPHORESIS OF HALIDES AND PHOSPHATE ON DEAE-CELLULOSE

APPLICATION TO ELEMENTAL ANALYSIS OF ORGANIC COMPOUNDS BY SODIUM FUSION

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SUMMARY

Electrophoresis on strips of the weakly basic anion exchanger, diethylaminoethyl-cellulose, is used to separate the halides and phosphate in any of four electrolytes spanning a wide range of pH but most effectively in an electrolyte at pH 2 containing a mixture of formic and acetic acids. The anions on pherograms treated with silver nitrate and exposed to ultraviolet light react in different ways depending on the nature of the electrolyte remaining on dried strips and on the concentration of silver ions in the reagent applied.

The method is adapted to the detection and identification of halogens and phosphorus present in organic compounds following sodium fusion on a micro-scale.

The separation also facilitates quantitative estimation of the individual anions present in mixtures, and an example is given of the estimation of residual inorganic bromide present in a chicken feed following fumigation with methyl bromide.

INTRODUCTION

The halides are separable in a variety of ways by chromatographic methods, and reliable procedures using paper[1–3], thin-layer[4–7] and column[7–10] techniques are now available, but it appears that the paper electrophoresis of these ions, with special reference to their separation, has not been as well explored. The results of several electrophoretic studies indicate that the mobilities of chloride, bromide and iodide are too similar to permit their convenient separation from mixtures when ordinary filter paper is used as the supporting medium[11–16], but Tokutomi and Kamiya[17] have shown that, by substituting paper impregnated with anion-exchange resins, such as Amberlite WB-2 paper, satisfactory electrophoretic separations of the halides are possible, using common buffer systems as electrolytes.

It is now shown that strips of the weakly basic exchanger, diethylaminoethyl (DEAE)-cellulose in which the ion-exchange properties are realized by chemical modification of the cellulose itself, may also be used for the rapid separation of mix-

tures of halides using electrolytes spanning a wide range of pH values. The work included an examination of the electrophoretic properties of phosphate on DEAE paper and the results have been adapted to the detection and identification of halogens and phosphorus present in organic compounds following sodium fusion on a microscale.

EXPERIMENTAL

Materials and reagents

Whatman chromatography paper DE-81 (DEAE-cellulose paper) was obtained from H. Reeve Angel (London, Great Britain).

Sodium dihydrogen phosphate and sodium or potassium salts of the halides were commercial samples of analytical-reagent grade, as were the organic compounds used for the sodium fusion tests, with the exception of chloromycetin (chloramphenicol) and betamethasone disodium phosphate, which were pharmaceutical preparations supplied, respectively, by Parke, Davis and Co. (Sydney, Australia) and Glaxo Australia (Melbourne, Australia).

Zirconium(IV) oxychloride and sodium alizarinsulphonate were supplied by E. Merck (Darmstadt, G.F.R.).

Poultry Layer Ration was manufactured by KMM (Melbourne, Australia).

Electrolytes

(1) The electrolyte of choice for the separations was an aqueous solution of approximately pH 2, 1 *M* with respect to acetic acid and 0.75 *M* with respect to formic acid[18]. It is hereinafter referred to as the "acid (pH 2) electrolyte".

The following electrolytes were used for comparative purposes:

(2) Acetate buffer (pH 4.6) containing 6.39 g $CH_3COONa \cdot 3H_2O$ and 3.2 g glacial acetic acid in 1 l of water. The solution was 0.1 *M* with respect to total acetate.

(3) Sodium phosphate buffer (pH 7.0) containing 6.24 g $NaH_2PO_4 \cdot 2H_2O$ and 10.68 g $Na_2HPO_4 \cdot 2H_2O$ per l. The solution was 0.1 *M* with respect to total phosphate.

(4) Sodium borate buffer (pH 9.2) containing 0.2 g-atom of boron per l (ref. 19).

Reagents used for the detection of the anions on pherograms

(1) Silver nitrate (0.3%) in water, (2) silver nitrate (2%) in water, (3) zirconium (IV) oxychloride (0.05 g) and sodium alizarinsulphonate (0.05 g) dissolved in 2 *N* hydrochloric acid (100 ml); *cf.* ref. 4. The reagent deteriorates on storage, even at 5°, and for best results was freshly prepared each 3–4 days.

Apparatus

Paper electrophoresis was conducted in the enclosed strip apparatus described previously[20] using Whatman DE-81 paper or Whatman No. 4 (filter) paper in strips 13.5 × 57 cm with 45 cm under pressure and cooled by the circulation of water at about 20° through the coils of the cooling-plate.

Procedure

DEAE-cellulose paper is especially fragile when wet with the acid (pH 2) electrolyte, but strips were conveniently dipped, blotted free of excess electrolyte and placed in the apparatus by leaving a few cm on each end dry for handling. Impregna-

tion of the strips was rapidly completed by capillary attraction of electrolyte from the electrode compartments at each end.

The strips were equilibrated under pressure for 10 min and samples (0.7 μl) of solutions of the halides and phosphate (each of various concentrations in the range 10^{-1}–10^{-4} M), then transferred to the starting lines by means of a platinum loop. Caffeine was applied as a marker for zero migration.

Electrophoresis on DEAE paper strips was generally allowed to proceed at about 22 V/cm for 20 min and on filter paper strips at 25 V/cm for 10 min, after which the strips were dried in the oven at 100°. When it was required to detect fluoride on acid papers, they were heated no longer than was necessary to just dry them. Traces of fluoride were best detected by applying the appropriate spray reagent to papers still slightly damp with electrolyte.

Caffeine was located on dried papers as a dark blue spot in the light of a "Chromato-vue" UV lamp (Ultraviolet Products, Calif., U.S.A.). DEAE-cellulose should be fully ionised in the acid (pH 2) electrolyte and, as expected for DEAE pherograms run under these conditions, the electroendosmotic flow, as indicated by the movement of caffeine, was strongly anionic — of the order of 4 cm/h·kV of applied potential.

Detection of the anions

Reagents (1) and (2) (silver nitrate solutions) were used for the detection of chloride, bromide, iodide and phosphate. Dried papers were uniformly sprayed with either reagent and exposed, while still wet, to light of the UV lamp (wavelength 2540 Å) for 5 min.

Reagent (3) [zirconium(IV) oxychloride–alizarinsulphonate–hydrochloric acid] was used for the detection of fluoride and phosphate. Sprayed papers were heated in the oven for about 1 min to develop yellow spots for these anions against a purple background.

The mobilities of the anions were calculated in absolute terms as cm/h·kV of applied potential.

Sodium fusion tests

Chloromycetin, α-bromocamphor-π-sulphonic acid (ammonium salt), p-bromomandelic acid, α,β-dibromopropionic acid, iodoacetic acid, 2-chloro-6-fluorobenzaldehyde, p-bromoiodobenzene, glucose-6-phosphate (disodium salt) and betamethasone disodium phosphate were tested. Each compound (3–4 mg) was heated in a Pyrex ignition tube with metallic sodium (*ca.* 10 mg) at red heat for 1 min. The tubes were allowed to cool and water (0.5 ml) added to the contents with which it was triturated with warming. The mixtures were applied without further treatment to DEAE papers impregnated with the acid (pH 2) electrolyte and subjected to electrophoresis under the standard conditions.

Dried pherograms were treated with spray reagents and the results interpreted as described below.

Estimation of inorganic bromide in chicken feed fumigated with methyl bromide

Poultry Layer Ration was treated in bulk with methyl bromide[21] and a sample (10 g) defatted with light petroleum. The pellets were crushed and extracted with 3 N

ammonium hydroxide in three batches of 50 ml each, centrifuging between treatments. The clear supernatants were combined and evaporated to small volume (5–10 ml), an equal volume of ethanol added, and the mixture warmed with stirring. The fine precipitate which formed on cooling was removed by filtration through Celite and the clear filtrate evaporated almost to dryness. The residue was made up to 2 ml with water and this stock solution serially diluted for application to a DEAE-paper impregnated with the acid (pH 2) electrolyte. After electrophoresis under the standard conditions, the pherogram was dried and treated with the 2% silver nitrate reagent. Comparison of size and intensity of the series of "unknown" spots with those of standard bromide solutions applied to the same paper enabled quantitative estimation of the bromide present in the treated feed sample (*cf.* ref. 22).

RESULTS AND DISCUSSION

Effects of pH and supporting medium on mobilities

Absolute mobilities of the anions on both DEAE and Whatman No. 4 (filter) papers are shown in Table I at four levels of pH.

TABLE I

MOBILITIES OF HALIDES AND PHOSPHATE IN FOUR ELECTROLYTES ON DEAE AND FILTER PAPERS

For description of electrolytes 1–4 and conditions of electrophoresis, see Experimental. Mobilities are expressed in cm/h·kV of applied potential.

Anion	*Electrolyte*							
	1, pH 2		*2, pH 4.6*		*3, pH 7*		*4, pH 9.2*	
	DEAE paper	*Filter paper*	*DEAE paper*	*Filter paper*	*DEAE paper*	*Filter paper*	*DEAE paper*	*Filter paper*
Chloride	8.2	25.6	16.6	29.0	25.2	28.6	25.9	29.0
Bromide	5.8	25.8	13.5	30.2	22.0	29.1	22.5	29.0
Iodide	2.2	26.4	9.0	29.5	16.6	28.0	17.4	28.4
Fluoride	4.8	2.4	15.3	20.0	20.3	20.1	20.9	18.8
Fluorosilicate	−0.5	—	—	—	—	—	—	—
Phosphate	2.6	4.4	8.6	11.1	—	—	15.5	16.0

On filter paper at pH 2, chloride, bromide and iodide are almost as highly mobile as at higher pH levels, reflecting the fact that these anions correspond to strongly dissociated acids. Tests using the acid (pH 2) electrolyte as eluent have shown Whatman No. 4 paper to have only a weak adsorptive capacity for iodide and little or none for chloride and bromide (*cf.* ref. 16), hence the finding that these ions are not electrophoretically separable on this supports in spite of large differences in atomic weight is probably almost solely due to counterbalancing effects of differences in their degrees of hydration. Fluoride, however, is easily separable from the other halides as a less mobile ion at all levels of pH on filter paper, apparently because it is especially strongly hydrated. The most rapid separation of fluoride occurs at pH 2 where, as the anion of a relatively weak acid, its ionisation is largely suppressed, augmenting the effects of hydration and resulting in a sharp decline in its mobility.

On DEAE paper, under standard conditions of electrophoresis, mixtures of the halides are resolved cleanly as small, circular or oval spots at all levels of pH, but most effectively at pH 2, where the relative differences in their mobilities are greatest. DEAE-cellulose should be fully protonated at this pH and the fixed centres of positive charge thus created on pherograms most strongly retard the movement of anions by electrostatic attraction. Comparison of the mobilities of the halides at pH 2 with those at higher levels on DEAE paper shows this expectation to be fully realized. With the exception of fluoride, mobilities on DEAE paper are, of course, much lower than those on filter paper in the acid electrolytes, but they are also appreciably lower even in the neutral and alkaline electrolytes, and it is puzzling in this respect that Tokutomi and Kamiya[17] report, conversely, that the migration of these and other anions is larger on Amberlite anion-exchange papers than on filter paper.

The retardation of the halides on DEAE pherograms is partially selective resulting in the separation of chloride, bromide and iodide in that order of decreasing mobilities, and it is possibly significant that this is also the order of numerical decrease of the enthalpies of hydration of the ions[23]. Electrophoresis on DEAE paper thus provides a separation which is complementary to that of most chromatographic systems in that the order in which the ions separate is inverted[1–7].

Under standard conditions on DEAE paper, phosphate migrating as the ion $H_2PO_4^-$ is not completely separated from iodide in the acid (pH 2) electrolyte, but the two ions are easily distinguished within a composite spot using the dilute (0.3%) silver nitrate reagent as described below. Longer runs of an hour or more are necessary to completely separate the ions on DEAE paper, but, if required, complete separation of phosphate from iodide and other halides may be achieved more rapidly using other systems such as the acid (pH 2) or acetate (pH 4.6) electrolytes on filter paper.

Detection of the anions

Chloride, bromide, iodide and phosphate. Halides on pherograms treated with silver nitrate each respond in different ways on exposure to ultraviolet light, depending on several factors which include the nature of the medium itself (whether DEAE or filter paper), the nature of the electrolyte remaining on the dried papers and the concentration of silver ions in the spray reagent applied. Reactions occurring on pherograms run in the acid (pH 2) electrolyte are summarised in Table II.

When DEAE pherograms are treated with the more concentrated (2%) silver nitrate reagent after runs in the acid electrolyte, chloride, bromide, iodide and phosphate are revealed as intense spots (deep brown for chloride and bromide, and blue–black for iodide and phosphate) when more than about 0.5–1 μg of each is present, although smaller quantities down to a limit of about one-tenth of this amount are still visible as weaker spots. The dilute (0.3%) silver nitrate reagent gives the same intense blue–black reaction with phosphate as the more concentrated reagent, but in sharp contrast, iodide is revealed with the dilute reagent as a white or cream-coloured spot against a pale brown background. It seems that iodide, present on pherograms even in traces, protects superimposed silver ions against reduction to metallic silver when these are present in low concentrations. This "protective" action is very powerful and operates even in the presence of large excesses of other anions like phosphate, which normally promote reduction, but the action is then incomplete and results in spots of

TABLE II

REACTIONS OF ANIONS TO SPRAY REAGENTS AFTER ELECTROPHORESIS IN THE ACID (pH 2) ELECTROLYTE ON TWO SUPPORTS

B = brown to black; BlB = blue-black; C = creamy-white; Y = yellow; w = weak; m = medium; s = strong; — = nil.

Anion	*DEAE paper*			*Filter paper*		
	0.3% $AgNO_3$	*2% $AgNO_3$*	*Zirconium-alizarin*	*0.3% $AgNO_3$*	*2% $AgNO_3$*	*Zirconium-alizarin*
Chloride	wB	sB	—	mB	mB	—
Bromide	wC	sB	—	sC	wC	—
Iodide	sC	sBlB	—	sC	mC	—
Fluoride	—	—	Y	—	—	Y
Fluorosilicate	—	—	Y	—	—	Y
Phosphate	sBlB	sBlB	Y	mBlB	mBlB	Y

an intermediate pale blue-grey shade. The effect is usefully exploited in the detection and identification of iodide and phosphate present together in the partially resolved spots obtained by electrophoresis under standard conditions on DEAE paper in the acid (pH 2) electrolyte. Three distinct regions are clearly visible within the oval spots; heads consisting solely of phosphate as it begins to separate from mixtures and which therefore give a characteristic deep blue-black reaction, fully "protective" white tails due to iodide alone, and sharply-defined, lens-shaped intermediate zones consisting of mixtures of the anions which give the mutually modified pale blue-grey reaction.

The dilute silver reagent lacks sensitivity in the detection of chloride and bromide on acid DEAE papers, but it is interesting that bromide also acts protectively with this reagent, though much more weakly than iodide. It is noteworthy that the protective action of iodide (and bromide) is fully and consistently expressed on acid pherograms only. Residues of alkaline electrolytes on pherograms tend to obscure the effect and only higher concentrations of the ions are then detected as pale spots using the dilute silver reagent. Lower concentrations give brown to black reactions using either of the silver reagents.

Iodide and bromide also exert their protective action on acid filter papers when either of the silver reagents is used, but the effect is especially sensitive with the dilute reagent, the limit of detection for iodide then being 22 ng/cm^2 of spot area. When spots remain small, as they do during runs of 10 min under the standard conditions on filter paper, this corresponds to 11 ng of iodide actually applied (0.7 μl of $1.25 \cdot 10^{-4}$ *M* KI solution). Although not specific for iodide, the method therefore compares favourably in terms of sensitivity with the micro-tests of Robbins[24] and of Feigl[25].

If more than about 2 μl of pure water (or any aqueous solution containing other than the halides) is applied to a starting point of a filter paper strip an artefact is likely to appear on the developed pherogram at the position otherwise occupied by the halides. This "water artefact" mimics the protective action of iodide and could therefore be mistaken for a trace reaction due to that ion. Analyses performed during these studies have shown that different batches of No. 4 paper contain, among other background impurities, 6–10 mg/m^2 of surface area of water-soluble chloride. Water ap-

plied to starting points on the paper strips radially displaces this soluble chloride leaving circular spots depleted of their normal content of impurity, and when electrophoresis is subsequently conducted for short periods, the depleted spots are preserved as "holes" in the background of chloride as this moves *en masse* toward the anode with its usual mobility. Background chloride is at least partly responsible for the pale brown ground which develops on pherograms treated with silver reagents and the "holes", necessarily moving at the same rate as chloride, then appear as unreactive paler spots against the ground at a position corresponding with this ion.

The artefact is eliminated by pre-washing the papers with water to remove all soluble chloride, when they prove to have an added advantage in the detection of iodide in that small spots due to trace quantities are even more sharply defined.

Fluoride and phosphate. Fluoride separates narrowly from phosphate as a faster moving ion inder the standard conditions on DEAE paper in the acid (pH 2) electrolyte and the two ions are further distinguished by differences in their reaction to the zirconium–alizarin reagent. Fluoride gives an immediate strong yellow reaction with quantities in excess of about 1 μg, but the spot fades and, within a few hours, weaker ones are no longer visible. Conversely, the yellow spots due to phosphate are relatively weak at first, even for quantities of the order of 10 μg but, on acid papers, they slowly intensify and remain strong for several weeks, at least.

In addition, the ions are well separated on filter paper at pH levels 4.6 and 9.2, but apart from the differences in mobility phosphate is yet further distinguished by its strong reaction with silver nitrate, fluoride giving little or no visible response to this reagent at all.

It was found that fluoride present in sodium fusion mixtures prepared according to directions given in the Experimental section was (very slightly) cationic on acid (pH 2) DEAE papers, and that any phosphate also present in a mixture then separated easily as the leading spot. The effect was traced to the presence of silicate in the fusion mixtures, formed by attack of sodium on the ignition tubes, and to the mixtures being acidified immediately on application to the papers, liberating silicic acid and forming the species HF and HF_2^-. The latter are known to react with silica, even at room temperature, to form fluorosilicic acids, the well-known H_2SiF_6 probably being a major product[26]. The conditions being favourable, it is probable that these complex acids form on pherograms at the moment of application of the fusion mixtures, incorporating all of the free fluoride the latter contain.

In view of the probability that all fluorosilicic acids are strong acids[27], it is surprising that they do not display considerable anionic mobility, even on acid DEAE paper, but their apparent cationic mobility may provide the clue; perhaps they are retarded by partial reversible adsorption on the paper support (see Table I).

Mixtures of sodium fluoride and sodium silicate give rise to the same or similar retarded species when run on acid (pH 2) pherograms, but neither these simple solutions nor appropriate sodium fusion mixtures show the effect at levels of pH 4.6 and higher, probably because of an increasing preponderance of the free F^- ion which is known to be unreactive with silica[26].

Sodium fusion tests

Of the anions commonly present in sodium fusion mixtures of organic compounds[28], only the halides and phosphate are detectable under the standard conditions

of electrophoresis on acid (pH 2) papers. Two other common ions, cyanide and sulphide, are too volatile to be retained on acid pherograms even while heating to dry them partially[29].

The alkaline fusion mixtures are applied directly to papers without pre-acidification because this may result in the formation of gels from the silicate present and make the transfer of samples difficult. The acid electrolyte is relatively concentrated and ensures the required acidification of applied mixtures before electrophoresis begins.

The separated anions corresponding to elements present in the compounds tested were selectively identified by treating each with all three spray reagents, these being applied separately to different pherograms or to different strips of the same pherograms. Except for the mixture from the β-methasone derivative, only one loopful (0.7 μl) of the fusion mixtures, prepared as directed, sufficed for satisfactory results. Fluorine constitutes less than 4% of the disodium phosphate of β-methasone (9α-fluoro-11β-methylpregnisolone) and 3 μl or more of the fusion mixture was required for unequivocal identification of fluoride (as fluorosilicate). The expected strong spot for phosphate separated cleanly as the leading spot.

Inorganic bromide in chicken feed

A further example of the utility of the method is provided by the estimation of ionisable bromide present in a commercially produced chicken feed which is routinely fumigated with methyl bromide by the Division's SPF (specific pathogen free) Poultry Unit (Maribyrnong, Australia) to destroy micro-organisms, insects and mites which may infest it prior to it being fed to experimental birds. Methyl bromide is readily dispelled from the feed by airing it for a few hours, but by its reactions with protein and other feed components, the fumigant leaves a stable and persistent residue in the form of inorganic bromide[21]. An estimate of this potentially toxic ion in treated feeds is required for the assessment of safety limits, and so help define the conditions of fumigation[30]. Residual inorganic bromide following the present fumigation was found by the method outlined in the Experimental section to constitute 0.03% of the feed, the only other soluble constituents detected with the silver nitrate reagent being chloride and phosphate. No iodide was detected. Although chloride and phosphate were present in considerable excess, they separated cleanly from bromide on the DEAE pherogram and so did not interfere with visual comparison of spots of the serially diluted "unknown" sample with those of the standard bromide solutions.

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REFERENCES

1 F. H. Pollard, J. F. W. McOmie and I. I. M. Elbeih, *J. Chem. Soc.*, (1951) 470.
2 A. G. Long, J. R. Quayle and R. J. Stedman, *J. Chem. Soc.*, (1951) 2197.
3 J. A. Broomhead and N. A. Gibson, *Anal. Chim. Acta*, 26 (1962) 265.
4 H. Seiler and T. Kaffenberger, *Helv. Chim. Acta*, 44 (1961) 1282.
5 S. M. Petrovic and V. D. Canic, *Fresenius' Z. Anal. Chem.*, 228 (1967) 339.

6 L S. Bark, R. J. T. Graham and D. McCormick, *Anal. Chim. Acta*, 35 (1966) 268.
7 S. Tustanowski, *J. Chromatogr.*, 31 (1967) 270.
8 S. Tustanowski, *J. Chromatogr.*, 31 (1967) 268.
9 H. Holzapfel and O. Guertler, *J. Prakt. Chem.*, 35 (1967) 113
10 T. Deguchi, *J. Chromatogr.*, 108 (1975) 409.
11 M. Lederer, *Anal. Chim. Acta*, 17 (1957) 606.
12 D. Gross, *Chem. Ind. (London)*, (1957) 1597.
13 G. Grassini and M. Lederer, *J. Chromatogr.*, 2 (1959) 326.
14 G. B. Belling and R. E. Underdown, *Anal. Chim. Acta*, 22 (1960) 203.
15 A. Moghissi, *Anal. Chim. Acta*, 30 (1964) 91.
16 M. Lederer, L. Morpurgo and L. Ossicini, *J. Chromatogr.*, 50 (1970) 475.
17 M. Tokutomi and S. Kamiya, *Bunseki Kagaku (Jap. Anal.)*, 21 (1972) 81; *C.A.*, 76 (1972) 107537b.
18 D. Gross, *Nature (London)*, 176 (1955) 72.
19 J. L. Frahn and J. A. Mills, *Aust. J. Chem.*, 12 (1959) 65.
20 J. L. Frahn and J. A. Mills, *Aust. J Chem.*, 17 (1964) 256.
21 E. G. Harry and W. Burns Brown, *World's Poult. Sci. J.*, 30 (1974) 193.
22 J. L. Frahn, *Aust. J. Chem.*, 22 (1969) 1655.
23 F. A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry: a Comprehensive Text*, Wiley-Interscience, New York, 3rd ed., 1972, p. 645.
24 J. Robbins, *C.R. Trav. Lab. Carlsberg*, 32 (1961) 233; *Anal. Abstr.*, 9 (1962) 1188.
25 F. Feigl, *Spot Tests in Inorganic Analysis*, Elsevier, Amsterdam, 5th ed., 1958, pp. 265–269.
26 J. S. Judge, *J. Electrochem. Soc.*, 118 (1971) 1772.
27 T. Moeller, *Inorganic Chemistry: an Advanced Textbook*, Wiley, New York, 1952, p. 680.
28 A. I. Vogel, *A Text Book of Practical Organic Chemistry (Including Qualitative Organic Analysis)*, Longmans, Green and Co., London, 3rd ed., 1956 (reprinted with corrections and additions, 1961), pp. 1039–1043.
29 J. L. Frahn, *J. Chromatogr.*, 37 (1968) 279.
30 R. M. Leach and M. C. Nesheim, *J Nutr.*, 81 (1963) 193