

Dans un premier temps, il est préférable de faire migrer de l'eau distillée sur la plaque pour vérifier l'absence de nucléosides, qui ne sont pas fixés sur la PEI-cellulose et sont entraînés par l'eau et pour éliminer les cations qui peuvent gêner la chromatographie.

Cette technique a été mise au point sur l'ADN de thymus de veau; son application aux ADN des virus et des ADN des mitochondries de foie de cobaye est actuellement en cours d'étude.

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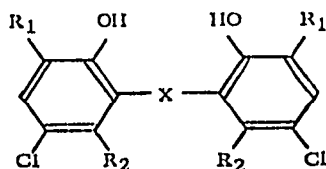
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**The use of acid dissociation constants in selecting buffers to effect the electrophoretic separation of bithionol, fenticlor, hexachlorophene, bromchlorophene, dichlorophene, tetrachlorophene and 4-hexylresorcinol**

Halogenated *o,o'*-dihydroxydiphenyl sulphides and methanes are important compounds pharmaceutically, and hexachlorophene in particular has found widespread use in medicated soaps, shampoos and toothpastes. Other compounds, including bithionol and fenticlor, are available commercially for use as bacteriostats, fungicides, spermicides and anthelmintics.



Dichlorophene	X = CH <sub>2</sub> ;	R <sub>1</sub> , R <sub>2</sub> = H
Tetrachlorophene	X = CH <sub>2</sub> ;	R <sub>1</sub> = Cl, R <sub>2</sub> = H
Bromchlorophene	X = CH <sub>2</sub> ;	R <sub>1</sub> = Br, R <sub>2</sub> = H
Hexachlorophene	X = CH <sub>2</sub> ;	R <sub>1</sub> , R <sub>2</sub> = Cl
Fenticlor	X = S;	R <sub>1</sub> , R <sub>2</sub> = H
Bithionol	X = S,	R <sub>1</sub> = Cl; R <sub>2</sub> = H

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The present authors have been investigating methods of determining these compounds and their use as analytical reagents for metals. Procedures for the determination of iron(III) with bithionol have been described<sup>1,2</sup>. As part of this investigation the proton-ligand and ligand-metal formation curves and stability constants of bithionol, fenticlor and hexachlorophene, and certain of their metal complexes, have been determined potentiometrically<sup>3</sup>.

¶ Many procedures for the determination of halogenated *o,o'*-dihydroxydiphenyl sulphides and methanes have been published. These include potentiometric pH titrations with sodium hydroxide<sup>4</sup>, direct UV spectrophotometric determination<sup>5-7</sup> and colorimetric determination with 4-amino-antipyrine<sup>8</sup>. In each case, however, difficulty is experienced in distinguishing between individual compounds of this type and in avoiding interference from similar compounds.

Chromatographic procedures for the separation of certain of these compounds have been described. Hexachlorophene and dichlorophene have been separated by ascending paper chromatography and by thin-layer chromatography prior to their UV spectrophotometric determination<sup>9</sup>.  $R_F$  values for several compounds of this type have been given<sup>10</sup>, and mixtures of 4-hexylresorcinol, bithionol, hexachlorophene and dichlorophene have been separated by two-dimensional thin-layer chromatography<sup>11</sup>.

In the present communication, an investigation of the electrophoretic separation of several *o,o'*-dihydroxydiphenyl sulphides and methanes and 4-hexylresorcinol, which is found in certain similar pharmaceutical preparations, is described. As these compounds are dibasic acids, the movement achieved electrophoretically will increase with their degree of ionisation. A knowledge of their acid dissociation constants ( $K_a$ ), therefore, is of value in selecting suitable buffers for effecting separations. In our earlier stability constant work<sup>3</sup> and in the electrophoretic work described here ethanol-water (3:1) solutions have been used, because of the water-insolubility of these compounds. The procedure for determining acid dissociation constants described previously<sup>3</sup> involved the use of a constant ionic background (1 *M* sodium perchlorate) and careful temperature control, titration technique and measurement of pH. Dissociation constants of sufficient accuracy for the present purpose may be obtained by the simple procedure described below.

The value of the dissociation constant is affected quite considerably by the ionic strength of the solution. In the present electrophoretic work buffer solutions of about 0.1 *M* concentration were used, and therefore the dissociation constants used were determined at an ionic background of 0.1 *M* sodium perchlorate.

### *Experimental*

**Reagents.** The sample solution, 0.0065 *M* solution in ethanol, was prepared as follows: dissolve  $6.5 \times 10^{-4}$  mole of the halogenated *o,o'*-dihydroxydiphenyl sulphide or methane in ethanol (B.P. grade) and dilute the solution to 100 ml in a volumetric flask with ethanol. The standard sodium hydroxide solution, 10<sup>-1</sup> *M* in ethanol-water (3:1), was prepared using a B.D.H. concentrated volumetric solution. Sodium perchlorate was used in the form of a 0.4 *M* solution in water.

**Procedure.** Add from a burette 37.5 ml of sample solution in ethanol to a 100-ml glass titration vessel or beaker, and add 12.5 ml of sodium perchlorate solution. Titrate the resulting solution with the standard sodium hydroxide solution (added from a

10-ml burette), following the titration potentiometrically using a pH responsive glass electrode and a calomel reference electrode.

All pH values quoted in this paper are strictly pH meter readings, and the values of dissociation constants refer to the ethanol-water (3:1) medium at the particular ionic strength indicated. The pH meter and electrode system was standardised using a 0.05 *M* aqueous solution of potassium hydrogen phthalate (pH 4.008 at 25°).

*Estimation of acid dissociation constants.* The two protons in the compounds studied react with hydroxyl ion in two quite separate pH regions, and it may be assumed that in the region where the first proton reacts only H<sub>2</sub>A and HA<sup>-</sup> are present, and in the region where the second proton reacts only HA<sup>-</sup> and A<sup>2-</sup> are present.

The first dissociation constant,

$$K_{a1} = \frac{[\text{HA}^-][\text{H}^+]}{[\text{H}_2\text{A}]}$$

Thus at the half neutralised point  $[\text{HA}^-] = [\text{H}_2\text{A}]$ ,

and

$$\text{p}K_{a1} = (\text{pH})_{[\text{HA}^-]=[\text{H}_2\text{A}]}$$

Similarly, at the half-neutralised point of the second proton,

$$\text{p}K_{a2} = (\text{pH})_{[\text{A}^{2-}]=[\text{HA}^-]}$$

Values of  $\text{p}K_{a1}$  and  $\text{p}K_{a2}$  for the *o,o'*-dihydroxydiphenyl sulphides and methanes studied here and for 4-hexylresorcinol are given in Table I. The effect of increasing ionic strength in lowering the values of  $\text{p}K_a$  is clearly illustrated.

*Electrophoresis.* Cellulose acetate membranes (Sartorius Membranfilter GmbH, 17 × 5 or 2.5 cm) were used in conjunction with a Gelman electrophoresis tank having 10 cm between supports. A Shandon Vokam stabilised power supply was operated in the constant current mode at 0.1–0.2 mA per cm width and 400 V. Approximately 0.1 *M* buffer solutions in ethanol-water (3:1) were prepared from molar aqueous solutions of sodium acetate and acetic acid, and ammonium acetate and ammonia.

TABLE I

ACID DISSOCIATION CONSTANTS IN ETHANOL-WATER (3:1)

	<i>In 0.1 M sodium perchlorate<sup>a</sup></i>		<i>In 1 M sodium perchlorate<sup>a</sup></i>		<i>In 1 M sodium perchlorate<sup>b</sup></i>	
	$\text{p}K_{a1}$	$\text{p}K_{a2}$	$\text{p}K_{a1}$	$\text{p}K_{a2}$	$\text{p}K_{a1}$	$\text{p}K_{a2}$
Hexachlorophene	5.6	12.4	5.4	11.8	5.10 ± 0.03	≈ 11.5
Bithionol <sup>c</sup>	5.9	10.5 <sub>n</sub>	5.7	9.6	5.33 ± 0.02	9.05 ± 0.03
Bromchlorophene	6.4	12.3	6.1	11.6	5.75 ± 0.02	≈ 11.1
Tetrachlorophene	6.7 <sub>6</sub>	12.2	6.5	11.5	6.10 ± 0.03	≈ 11.0
Fenticlor	8.4	11.8	8.2	11.0	7.68 ± 0.02	10.38 ± 0.03
Dichlorophene	9.1	12.5	8.8	11.9	8.26 ± 0.03	≈ 12.1
4-Hexylresorcinol	11.6 <sub>6</sub>	12.6	11.2	12.0	10.53 ± 0.05	≈ 12.6

<sup>a</sup> Obtained by the method described in this paper;  $\text{p}K_a$  values based on pH meter readings.

<sup>b</sup> Obtained by the method described previously;  $\text{p}K_a$  values based on  $-\log [\text{H}^+]$

<sup>c</sup> The values of  $\text{p}K_{a1}$  and  $\text{p}K_{a2}$  obtained for bithionol without the addition of sodium perchlorate were 6.2 and 11.5.

TABLE II

## RESULTS OF ELECTROPHORESIS

	<i>Migration (cm) in 2.5 h</i>	
	<i>pH 6.4</i>	<i>pH 8.9</i>
Hexachlorophene	5.3	6.3
Bithionol	4.4	6.4
Bromchlorophene	2.7	5.9
Tetrachlorophene	1.9	6.2
Fenticlor	0.7	4.6
Dichlorophene	0.7	2.5
4-Hexylresorcinol	0.7	0.5

The pH of each buffer was measured with a Pye 290 pH meter and a combined glass-calomel electrode. Minor adjustments to the pH were made where necessary by the addition of small amounts of strong acid or base.

The membranes did not stretch in these buffer solutions.

Aliquots of the compounds studied were applied to the membranes by the microscope cover slip technique 5 cm from the cathode end of the membrane. During the electrophoresis period—up to a maximum of 2.5 h—the voltage dropped slightly

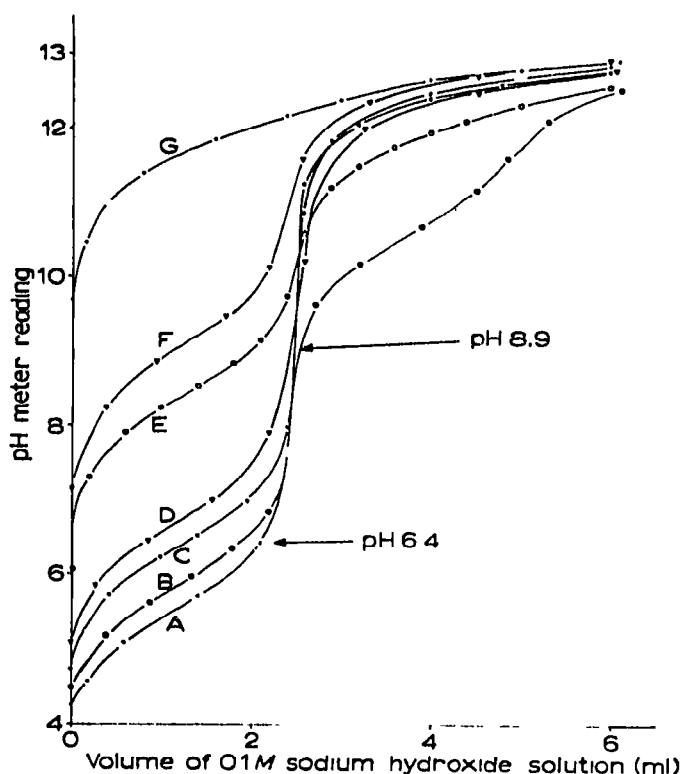


Fig. 1. pH titration curves. A = hexachlorophene, B = bithionol; C = bromchlorophene, D = tetrachlorophene, E = fenticlor, F = dichlorophene; and G = 4-hexylresorcinol.

to about 390 V. When the potential was limited to 400 V evaporation of solvent from the membranes did not exceed an acceptable level.

After electrophoresis the compounds were located by immersing the membrane in a freshly prepared aqueous solution of ferric nitrate and potassium ferricyanide<sup>9</sup>; the membrane was finally washed with water. After drying the membrane at room temperature the compounds appeared as various shades of blue.

### Results and discussion

The use of dissociation constants in selecting a buffer suitable for effecting electrophoretic separation of particular compounds is clearly illustrated by reference to the  $pK_a$  values given in Table I and to the results of the electrophoretic runs given in Table II. When the titration data are available the selection of buffers can be made more conveniently directly from the titration curves (Fig. 1). Alternatively, the formation curves,  $\bar{n}_A$  (= combined proton concentration/total reagent concentration) *versus* pH, can be calculated from the values of the dissociation constants determined at the correct ionic strength. As the two protons in each compound are ionised in quite separate pH regions, the following approximate formulae are valid.

$$\bar{n}_A = \frac{1 + 2 \text{ antilog}(pK_{a1} - \text{pH})}{1 + \text{antilog}(pK_{a1} - \text{pH})} \quad \text{for } \text{pH} = pK_{a1} \pm 1$$

and

$$\bar{n}_A = \frac{\text{antilog}(pK_{a2} - \text{pH})}{1 + \text{antilog}(pK_{a2} - \text{pH})} \quad \text{for } \text{pH} = pK_{a2} \pm 1$$

From examination of the titration curves shown in Fig. 1, it was considered that a buffer of pH 6.2 would be most suitable for the separation of hexachlorophene, bithionol, bromchlorophene and tetrachlorophene from each other and from the other compounds. Good electrophoretic separation was effected at this pH, but from an examination of the developed membrane and the titration curves, it seemed possible that the optimum separation could be effected at the slightly higher pH of 6.4. Optimum separation was in fact obtained at this pH.

Similarly, for the separation of fenticlor, dichlorophene and 4-hexylresorcinol, a buffer of pH 8.9 was selected. This proved to give optimum separation.

At all pH values studied in the present work the electrophoresis zones were well defined, with no evidence of adsorption.

In the above separations, the optimum pH in each case proved to be about 0.2 higher than the mean of the  $pK_{a1}$  values of the particular group of compounds. Thus, for hexachlorophene, bithionol, bromchlorophene and tetrachlorophene, the mean  $pK_{a1}$  value is 6.2 and the optimum pH for separation is 6.4. Similarly, the mean  $pK_{a1}$  value of fenticlor and dichlorophene is 8.75 and the optimum pH for separation is 8.9. Electrophoresis at these slightly higher pH values was necessary in order to obtain slightly greater movement of the less ionised compounds.

At pH values below 6.2 bromchlorophene and tetrachlorophene are unresolved, and at pH values just above 6.4 hexachlorophene and bithionol are unresolved. At pH 8.7 dichlorophene is not so well separated from 4-hexylresorcinol as at the optimum pH 8.9, but fenticlor is slightly better separated from the four compounds with  $pK_{a1}$  less than 7. At pH values above 9.1 there is less separation of fenticlor from the latter

compounds. All these results are closely similar to those expected from a consideration of the pH titration curves.

For routine use in electrophoretic work, the prior determination of dissociation constants (if these are not already known) can in many cases effect a considerable saving of time, and, because the pH data can be used in conjunction with the electrophoretic data, identification is made more certain. Indeed, the use of pH data makes the electrophoresis less empirical. By consulting a library of titration curves (or formation curves) not only can the optimum pH for electrophoresis be selected readily, but possible ambiguities of identity can be anticipated. It is assumed throughout that adsorption effects and strong ion association with the buffer ions are absent; this was clearly the case in the present work.

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