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PAPER ELECTROPHORESIS OF HYDROXYCARBOXYLIC ACIDS AS THEIR BORATE COMPLEXES WITH SPECIAL REFERENCE TO VIRIDIFLORIC, TRACHELANTHIC, LASIOCARPIC AND HELIOTRIC ACIDS

THE IMPORTANCE OF THE "*gem*-DIALKYL" EFFECT IN COMPLEX FORMATION

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SUMMARY

The electrophoretic mobility of an α-hydroxycarboxylic acid in electrolytes containing boric acid is very dependent on the degree of substitution at the α-carbon atom. A study of eighteen α-hydroxy acids shows clearly that the "*gem*-dialkyl" effect is operating to favour the formation of the strongly acidic boric acid complexes from the more densely substituted acids, the mobilities of which are thus more or less greatly enhanced.

In general, the complexes form best at pH 2, but some are stable at higher levels. Of several that exist at pH 4.6, only the complexes of viridifloric, trachelanthic and lasiocarpic acids also survive paper electrophoresis at pH 9.2. These acids form 1:1 instead of the usual 1:2 complexes and they are further distinguished from the others by an unusually high degree of crowding at their α-carbons, and by containing a β- as well as the α-hydroxyl group.

INTRODUCTION

Many α-hydroxycarboxylic acids form strongly acidic complexes with boric acid[1–3] and it has been concluded that the complexes are spiro-compounds containing the boric and hydroxy acids in 1:2 ratio[3]. Tetracovalent boron is represented as the common atom shared by two identical 5-membered cyclic diester functions, as depicted in Fig. 1.

Classical investigations of the complexes included measurement of increases in electrical conductivity of solutions of the hydroxy acids in the presence of boric acid[3] and, for optically active acids, determination of changes in specific rotatory power at different wavelengths and concentrations of reactants[1].

It is now shown that other properties and reactions of the complexes are conveniently studied by paper electrophoresis utilising the fact that they are more strong-

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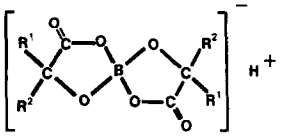


Fig. 1. General structure of 1:2-boro-α-hydroxy carboxylate complexes, R^1 and R^2 representing H or substituent groups.

ly acidic than the parent acids and that their stereochemistry plays an important role in determining their stability.

EXPERIMENTAL

Materials

The compounds for which data are given were pure commercial or research preparations. The α-hydroxy acids were used mainly as 0.1 *M* solutions in water but, for some experiments, in other concentrations up to 0.3 *M*. Aqueous solutions of phenolic acids were 0.025 *M*.

Reaction of heliotric, viridifloric, trachelanthic and lasiocarpic acids with boric acid is relatively slow. The spots that separate as their complexes at pH 4.6 are best seen by pre-dissolving the acids as 0.1 *M* solutions in electrolyte C.

Electrolytes

The following electrolytes were used:

(A) An aqueous solution of formic acid (0.75 *M*), approximately pH 2, made 0.8 *M* with respect to boric acid.

(B) Formic acid solution (0.75 *M*).

(C) Acetate buffer (pH 4.6) made 0.8 *M* with respect to boric acid.

(D) Acetate buffer (pH 4.6)[4].

(E) Sodium borate buffer (0.05 *M*, pH 9.2) containing 0.2 g-atom of boron per l (ref. 5).

(F) Sodium hydrogen carbonate–sodium carbonate solution (pH 9.2) containing 6.72 g $NaHCO_3$ and 1.06 g anhydrous Na_2CO_3 in 1 l of water.

Spray reagents

The following spray reagents were used:

(a) Manganous sulphate–potassium permanganate–sulphuric acid, the preparation and use of which as a spray reagent for papers after electrophoresis has been described by Mills[6]. The reagent reacts with phenolic acids to give immediate yellow spots on a purple background. The spots become white within a few minutes and the background simultaneously changes to brown. The hydroxy acids react more slowly, appearing as white spots on the brown background.

(b) Curcumin reagent for the detection of boric acid on paper strips. Its preparation and use have been described by Frahn and Mills[7].

Paper electrophoresis

Paper electrophoresis was performed on Whatman No. 4 paper in the pre-

viously described apparatus[8] in which the paper is enclosed under pressure and cooled with tap water (at about 20°C). After the paper, wetted with electrolyte, had been equilibrated under pressure for 15 min, solutions (0.5 μl) of the experimental acids were applied by platinum wire loop, and electrophoresis was conducted for 40 min to 1 h at 20–25 V cm^{-1}. The paper strips were then dried at 100°, examined under a Hanovia Chromatolite ultraviolet lamp to reveal the marker compounds and the experimental phenolic acids, and sprayed with the appropriate reagent. Pherograms run in electrolytes A–D should be dried thoroughly before spraying with reagent (a) because residues of formic or acetic acids unduly retard deposition of the brown MnO_2 on the fibres of the paper, this being necessary to provide spot contrast against the background.

Caffeine was used as a marker for zero migration without serious error[9] and the 4-nitrobenzenesulphonate ion as the standard of anionic rate from which the M_N values[5] were calculated. For convenience, $M_N \times 100$ values are used in the text.

Special procedure

Some experimental acids yield two spots on electrophoresis in the acetate electrolyte C at pH 4.6, and some, applied as solutions of higher concentrations (*e.g.*, 0.3 *M*) give two spots in the borate electrolyte E (pH 9.2). To determine which are due to complexes with boric acid, 0.5-μl samples of solutions containing boric and each of the experimental acids in question were subjected to electrophoresis at pH 4.6 or 9.2 in the absence of background boric acid, that is, by using the "indifferent" (non-complexing) electrolytes D and F.

For runs in electrolyte D, 2,3- and 2,5-dihydroxybenzoic, heliotric, viridifloric, trachelanthic and lasiocarpic acids were therefore dissolved as 0.1 *M* solutions in electrolyte C (containing 0.8 *M* boric acid) and the latter three acids were also dissolved as 0.1 *M* solutions in 0.1 *M* boric acid solution for runs in electrolyte F.

Duplicate lanes cut from dried pherograms were sprayed, one with reagent (a) to reveal each experimental acid as two spots and the other with the curcumin reagent which, by reacting with boric acid to give a single spot (apart from that due to free boric acid), showed which of the above two represents the complex.

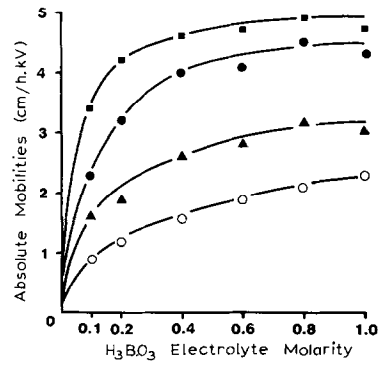


Fig. 2. Relationships between boric acid content in formic acid (0.75 *M*, pH 2) electrolyte and the absolute mobilities of the complexes of four α-hydroxy acids: α-hydroxyvaleric (compound 5, Table I), α-hydroxyisovaleric (8), α-hydroxyisobutyric (13), and α-hydroxy-α-methylbutyric (15).

TABLE I

MOBILITIES OF α-HYDROXY ACIDS IN TWO ELECTROLYTES: (A) FORMIC ACID (0.75 *M*, pH 2) CONTAINING 0.8 *M* BORIC ACID, AND (B) FORMIC ACID (0.75 *M*, pH 2)

Acids were detected after paper electrophoresis in each electrolyte at 20–25 V cm^{-1} and 20°C for 40 min to 1 h. M_N values[5] express mobilities relative to the 4-nitrobenzenesulphonate ion of absolute mobility 8.9 and 9.3 cm/h · kV in electrolytes A and B, respectively.

No.	*α-Hydroxy acid*	R^1 | R^2–C–OH | COOH	R^1	R^2	M_N × 100 *value in electrolytes*		ΔM_N × 100 *value*
					A	*B*	
1	Glycolic		H	H	5	1	4
2	L-Lactic		H	CH_3	18	2	16
3	D-Glyceric		H	CH_2OH	21	2	19
4	L-Malic		H	CH_2COOH	21	4	17
5	α-Hydroxyvaleric*		H	$CH_2CH_2CH_3$	22	1	21
6	L-Mandelic		H	C_6H_5	23	5	18
7	DL-α-Hydroxybutyric		H	CH_2CH_3	27	1	26
8	DL-α-Hydroxyisovaleric		H	$CH(CH_3)_2$	33	1	32
9	D-Tartaric		H	CHOH · COOH	40	9	31
10	Citric		CH_2COOH	CH_2COOH	45–33**	7	38–26
11	Benzilic		C_6H_5	C_6H_5	45	15	30
12	D-Tartronic		H	COOH	45	34	11
13	α-Hydroxyisobutyric		CH_3	CH_3	47	1	46
14	(−)Heliotric		$CH(CH_3)_2$	$CH_3CH \cdot OCH_3$	48	0	48
15	α-Hydroxy-α-methyl butyric*		CH_3	CH_3CH_2	52	1	51
16	(−)Viridifloric		$CH(CH_3)_2$	CH_3CHOH	Streak***	1	–
17	(+)Trachelanthic		$CH(CH_3)_2$	CH_3CHOH	Streak***	2	–
18	(+)Lasiocarpic		$HOC(CH_3)_2$	$CH_3CH \cdot OCH_3$	Streak***	3	–

* Of unspecified configuration.
** Elongated spot.
*** The heads of almost undifferentiated streaks are at $M_N \times 100 = 58$ (for acid 16), 57 (17) and 51 (18).

RESULTS AND DISCUSSION

The effect of electrolyte content of boric acid on complex formation at pH 2

The weakly ionising boric acid applied to papers impregnated with the acid (pH 2) electrolyte B is non-migrating, as expected, and it was conveniently used in some experiments as an accurate marker of electroendosmotic flow. With some exceptions, the ionisation of the α-hydroxy acids studied is similarly suppressed at pH 2 and they also have zero or low mobilities in this electrolyte, but in the presence of boric acid (dissolved in the electrolyte) all the acids become anionically mobile, demonstrating their interaction with boric acid to form species more strongly acidic than themselves. In electrolytes of low boric acid content the anionic spots tend to be somewhat diffuse with more or less long comet-like tails, but in the presence of increasing concentrations of boric acid, the spots generally become more compact, have shorter, less intense tails and are also more highly mobile.

Data presented in Fig. 2 for a selection of four typical α-hydroxy acids show how their anionic mobilities increase with increasing concentrations of boric acid (in the range 0.1 to 1 *M*) dissolved in formic acid electrolyte at pH 2*. As the electrophoretic mobilities depend on the positions of the respective equilibria and therefore provide a guide to the extent of formation of the charged complexes, it follows from the above data that these are favoured by high concentrations of boric acid. Early workers[3,10] showed conversely, however, that an increase in boric acid content of solutions of polyols favours the formation of neutral compounds, because although 1:1 complexes containing tetra-covalent (charged) boron form initially, such complexes are unstable and pass to others containing tri-covalent boron which is uncharged. Clearly, this conclusion regarding the effect of boric acid concentration on polyol complexing does not apply to the hydroxy acids. Nor does the conclusion regarding the stability of 1:1 complexes hold for all hydroxy acids because some studied in the present work that are strongly substituted on their α-carbon atoms form highly stable, charged 1:1 complexes as the preferred species, even in solutions of low boric acid content.

The electrolyte containing 1 *M* boric acid was supersaturated with respect to that compound and the excess often crystallized out on papers during runs, causing some distortion of the pherograms. The electrolyte of choice for this study was therefore the one containing 0.8 *M* boric acid as it provides optimum conditions with respect to compactness and mobility of the complex spots and thus for characterisation of the hydroxy acids, as well as for separations of some of their mixtures.

The effect of substitution on the α-carbon atoms of the experimental acids

In Table I, 18 α-hydroxy acids are listed in increasing order of the mobilities of their complexes in the 0.8 *M* boric acid electrolyte A (pH 2), and it can be seen that the more highly mobile are those carrying the bulkier groups on their α-carbon atoms. The $\Delta M_N \times 100$ values included in Table I provide a truer measure of the effects of complex-formation in each case because they represent the differences ob-

* The complex-forming phenolic (β-hydroxy) acids discussed below display similar trends of increasing anionic mobility with increasing boric acid content in formic acid electrolyte at pH 2.

served in the relative mobilities ($M_N \times 100$ values) before and after the addition of boric acid to the electrolyte.

As reference to the data shows, substitution of glycolic acid (compound 1) with, for example, one methyl group to form lactic acid (2) results in a four-fold increase in the $\Delta M_N \times 100$ value of the complex, and α-hydroxyisobutyric acid (13), formed by substitution with a second methyl group is seen to have a Δ value almost 12 times that of the unsubstituted acid. Again, comparison of the values for the similar series, glycolic (1), mandelic (6) and benzilic (11) acids show similar progressive increases. The Δ value for benzilic acid is 66% greater than that for mandelic acid, the enhancement occurring in spite of an increase of 50% in molecular weight due to the extra phenyl group.

The Δ value of α-hydroxyisobutyric acid (13) is more than 75% greater than that of the isomeric α-hydroxybutyric acid (7), and its α-carbon is more crowded. The large difference in mobilities permits rapid separation of these isomers from mixtures. Similarly, the three structurally isomeric acids, α-hydroxyvaleric (5), α-hydroxyisovaleric (8) and α-hydroxy-α-methylbutyric (15), differently substituted on their α-carbons, are easily separable because of the relatively large differences in their mobilities. Such separations are not possible in non-complexing electrolytes, however. Reference to Table III shows that the mobilities of these isomeric acids in acetate electrolyte D (pH 4.6), for example, are almost identical.

It is clear that for acids more densely substituted on their α-carbons, the "*gem*-dimethyl" or "Thorpe–Ingold" effect[11] is operating to augment the effect of boric acid concentration and force the equilibria more in favour of the 5-membered cyclic boric acid complexes, it by now being well-established that the steric properties of *gem*-dimethyl and similar groupings cause remarkable stabilisation of small rings, and also greatly facilitate their formation[11,12].

Some of the most densely substituted of all the acids studied did not conform, however, in the way expected. Instead of forming discrete spots, viridifloric (16), trachelanthic (17) and lasiocarpic (18) acids (Fig. 3a–c) were detected after runs in electrolyte A as almost undifferentiated anionic streaks leading to weak comet-shaped heads. This anomalous behaviour is discussed in a later section.

Boric acid complexes of aromatic hydroxy acids

Böeseken[3] reported that boric acid causes no increase in the electrical conductivities of non-cyclic β-hydroxy acids and, in the present work, it was similarly found that no enhancement of the electrophoretic mobilities of, for example, β-hydroxybutyric and β-hydroxy-β-methylglutaric acids occurs when these are run in the presence of boric acid. In the aromatic series, however, complex formation with acids substituted with a phenolic group *ortho* to the carboxyl is well-known, and increases in the conductivities of salicylic, 2,4-dihydroxybenzoic and 2,4,5-trihydroxybenzoic acids have been shown to occur in the presence of boric acid, even though they are β-hydroxy acids[3].

Likewise, with one exception among those studied, the electrophoretic mobilities of *ortho* (2−)-substituted phenolic acids are enhanced when run in the presence of boric acid, as reference to Table II indicates for acids 19, 23 and 24. Evidence of complex formation for 2,6-dihydroxybenzoic acid (25), doubly substituted in the *ortho* positions, is obscured by fact that the free acid is very strong ($pK_a = 1.3$). Its

TABLE II

MOBILITIES OF PHENOLIC ACIDS IN TWO ELECTROLYTES: (A) FORMIC ACID (0.75 *M*, pH 2) CONTAINING 0.8 *M* BORIC ACID, AND (B) FORMIC ACID (0.75 *M*, pH 2)

The standard conditions of electrophoresis given in Table I were used.

No.	*Phenolic acid*	$M_N \times 100$ *value in electrolytes*		$\Delta M_N \times 100$ *value*
		A	*B*	
19	Salicylic	43	10	33
20	3-Hydroxybenzoic	2	2	0
21	4-Hydroxybenzoic	2	3	−1
22	4-Hydroxy-3-methoxy benzoic (vanillic)	2	3	−1
23	2,3-Dihydroxybenzoic	38	11	27
24	2,5 Dihydroxybenzoic (gentisic)	33	11	22
25	2,6-Dihydroxybenzoic (γ-resorcylic)	60	71	−11
26	3,4-Dihydroxybenzoic (protocatechuic)	2	4	−2
27	3,5-Dihydroxybenzoic (α-resorcylic)	2	4	−2
28	3,4,5-Trihydroxybenzoic (gallic)	2	5	−3

ionisation is therefore by no means completely suppressed at pH 2 and its carboxylate group probably competes with the complex to form the anionic centre of the ion.

Complex formation with the *ortho* phenolic acids is no doubt facilitated by the functional groups being held rigidly in one plane, that of the aromatic nucleus. As expected from steric considerations, and as demonstrated by the electrophoretic data, phenolic acids otherwise substituted do not form complexes. The method may therefore be used either diagnostically to detect the presence of *ortho* substituted hydroxyl groups in phenolic acids or, analytically, for the rapid separation of some mixtures, including isomers. It is useful to note that the phenolic acids are readily distinguishable from the series of aliphatic α-hydroxy acids by their strong fluorescence on pherograms exposed to UV light, as well as by the rapidity with which they react with spray reagent (a).

The effect of pH on the stability of the complexes

The ionisation of the hydroxy acids at pH 2 is largely or almost completely suppressed and the boric acid reacts with uncharged hydroxy acid molecules to an extent determined by their stereochemistry and by the electrolyte content of boric acid, as described above. In electrolytes of higher pH, however, the hydroxy acids become more strongly ionised and the resulting symmetrical carboxylate groups, stabilised by resonance energy, then become the preferred centres of anionic charge, generally opposing the tendency of the acids to form their complexes.

The effect at pH 4.6. With the exception of α-hydroxy acids 10, 14, 16, 17 and 18, and phenolic acids 19, 23, 24, 25, 26 and 28, the data of Table III giving mobilities

TABLE III

MOBILITIES OF HYDROXY ACIDS IN TWO ELECTROLYTES: (C) ACETATE BUFFER (pH 4.6) CONTAINING 0.8 *M* BORIC ACID, AND (D) ACETATE BUFFER (pH 4.6)

The standard conditions of electrophoresis given in Table I were used.

No.	*Hydroxy acid*	$M_N \times 100$ *value* in electrolytes*		$\Delta M_N \times 100$ *value*
		C	*D*	
1	Glycolic	105	109	−4
2	Lactic	94	96	−2
3	Glyceric	94	95	−1
4	Malic	110	112	−2
5	α-Hydroxyvaleric	80	80	0
6	Mandelic	81	85	−4
7	α-Hydroxybutyric	86	89	−3
8	α-Hydroxyisovaleric	80	81	−1
9	Tartaric	122	128	−6
10	Citric	120	102	+18
11	Benzilic	62	69	−7
12	Tartronic	131	133	−2
13	α-Hydroxyisobutyric	83	85	−2
14	Heliotric	52, *69***	70	−18, −1
15	α-Hydroxy-α-methylbutyric	76	78	−2
16	Viridifloric	*58*, 69**	74	−16, −5
17	Trachelanthic	*58*, 71 **	75	−17, −4
18	Lasiocarpic	*53*, 68**	72	−19, −4
19	Salicylic	77	92	−15
20	3-Hydroxybenzoic	62	64	−2
21	4-Hydroxybenzoic	43	44	−1
22	4-Hydroxy-3-methoxybenzoic	39	39	0
23	2,3-Dihydroxybenzoic	43, *69****	77	−34, −8
24	2,5-Dihydroxybenzoic	35, *68****	78	−43, −10
25	2,6-Dihydroxybenzoic	75	89	−14
26	3,4-Dihydroxybenzoic	52	37	+15
27	3,5-Dihydroxybenzoic	53	53	0
28	3,4,5-Trihydroxybenzoic	48	32	+16

* Absolute mobility of 4-nitrobenzenesulphonate in electrolyte C is 10.5 cm/h · kV; in electrolyte D, 11.0 cm/h · kV.

** These acids separate into two spots; the mobility of the major spot is shown in italics.

*** These acids separate into two spots connected by a streak. The faster spot (in italics) is the major one in each case.

of the acids in electrolytes C and D (both at pH 4.6) do not provide evidence of complex formation. The mobilities are, in general, similar or identical in both electrolytes, that is, with and without added boric acid.

The exceptions posed by citric (10), 3,4-dihydroxybenzoic (26) and 3,4,5-trihydroxybenzoic (28) acids are conveniently considered together because their mobilities are all enhanced at pH 4.6 in the presence of boric acid. The complexes evidently survive at this higher level of pH in equilibrium with non-complexed ions and appear to contribute in an additive way to the total charge on the respective ions. Ionisation

of only the third carboxyl of the tribasic citric acid, corresponding to pK_a 6.4, would be effectively suppressed at pH 4.6, but its suppression allows the relatively strongly acidic complex to form, thus adding its charge to those already present due to ionisation of the other carboxyls.

The hydroxyl groups of hydroxy benzoic acids 26 and 28 are too distant to enter into complex formation with the carboxyls which are then free to ionise at pH 4.6 as simple carboxylates. The hydroxyl groups, however, constitute vicinal diol and triol systems, respectively, and as such form boric acid complexes, contributing in this way to the overall charge on the ions. Catechol (1,2-dihydroxybenzene) was used to confirm that simple vicinal diols of this type do form boric acid complexes at pH 4.6. Catechol has appreciable anionic mobility ($M_N \times 100 = 18$) in electrolyte C, but does not migrate in electrolyte D. By contrast, its non-vicinal isomers, resorcinol (1:3-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) do not migrate in either electrolyte. In addition, the ethyl ester of phenolic acid 28 (ethyl gallate), immobile in electrolyte D, has $M_N \times 100 = 28$ in electrolyte C.

The data for salicylic (2-hydroxybenzoic) acid (19) and 2,6-dihydroxybenzoic acid (25) show that their boric acid complexes compete successfully at pH 4.6 with ionisation of their carboxyl groups as centres of anionic charge. The complexes exist, however, in rapid dynamic equilibrium with the respective free acid anions, and the samples thus migrate in the presence of boric acid as compact, single spots of somewhat lower mobility than the corresponding free acid anions in electrolyte D.

The dihydroxybenzoic acids, 2,3- (23) and 2,5- (24), each migrate as single spots in electrolyte D, but separate into two spots connected by a streak in the presence of boric acid. The faster-moving, heavier spots in each case represent free acid anions, and it was shown, using the special procedure described under Experimental, that the slower, weaker spots were due to the respective complexes.

It appears that, like the above hydroxy benzoic acids 19 and 25, the isomeric acids 23 and 24 form complexes in electrolyte C which are in equilibrium with the corresponding free acid anions. Equilibration between complexed and uncomplexed species for acids 23 and 24 is relatively slow, however, allowing the observed separation of the two species during the subsequent electrophoresis. Slow breakdown of complex in the slow-moving spot and its re-formation in the faster spot account for the inter-connecting streak in each case. Because equilibration is so slow, the mobilities of the slower spots should at least approximate those of the complexes moving alone as pure species, and it should therefore be possible to determine their composition. If the mobility of the free acid anions (mol. wt. 153) is 78 (Table III), a mean value of 39 for the mobility of the complexes would correlate with a mol. wt. of 306, corresponding closely with the calculated mol. wt. of 315 for 1:2 complexes of boric with the phenolic acids. The conclusion that 1:2 complexes form under the present conditions for at least these two acids is thus consistent with the conclusion of early workers using different methods with respect to salicylic and to α-hydroxy acids, generally[3].

The above phenolic acids 19, 23, 24 and especially 25, being relatively strong acids, ionise strongly at pH 4.6. The formation of their complexes clearly competes effectively with that of the respective carboxylate ions, however, showing again how very favourable the rigid disposition of hydroxyls *ortho* to carboxyls in benzene nuclei is for complexing.

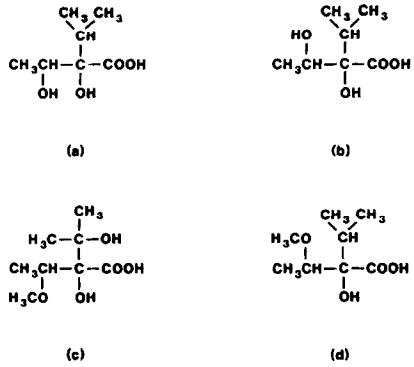


Fig. 3. Formulae of (a) viridifloric (16), (b) trachelanthic (17), (c) lasiocarpic (18) and (d) heliotric (14) acids.

The more densely substituted viridifloric (16), trachelanthic (17), lasiocarpic (18) and heliotric (14) acids (Fig. 3a–d) each yield two discrete spots in electrolyte C containing boric acid. No interconnecting streaks are seen. The faster-moving spots correspond with the respective free acid anions, as indicated by comparison of their mobilities with those of the single spots formed in electrolyte D at the same pH but in the absence of boric acid. The slower-moving spots are the major ones separating from viridifloric (16), trachelanthic (17) and lasiocarpic (18) acids and the minor one from heliotric acid (14), and it was shown by the special procedure (Experimental) that these slower spots correspond with the respective boric acid complexes. The mobilities of these spots, by comparison with those of the respective acid anions, indicate that they represent 1:1 complexes, in contrast to the 1:2-spiro type complexes that form with other hydroxy acids[3].

As noted in the Experimental section, complex formation with these acids is comparatively slow. Only small spots for the respective complexes separate if aqueous solutions of the acids are applied to pherograms immediately prior to electrophoresis in electrolyte C. Large spots representing the complexes are best seen after pre-dissolving acids 16–18 in the electrolyte and allowing a few minutes to pass before conducting the electrophoresis. The fact that the complexes then separate cleanly as discrete spots without equilibrating with free acid anions shows that, once formed, they are extremely stable species, persisting unchanged for at least the duration of an electrophoretic run. Their stability is further demonstrated by their persistence as separate entities even when run in the indifferent electrolyte D. Relatively few other such stable borate complexes are known[13]. Among those that are, the tridentate diborate complexes of *scyllo*-inositol and aminodeoxy-*scyllo*-inositol also migrate without significant decomposition during paper electrophoresis in indifferent electrolytes[7], and it has been reported that they are also slow to form[7,13–15].

The effect at pH 9.2. As in electrolyte C at pH 4.6, the dihydroxy carboxylic acids, 16–18, each yield two compact, well-separated spots, unconnected by streaks, on electrophoresis in borate electrolyte E at pH 9.2. The effect is observed in this electrolyte, however, only after applying the acids to pherograms in more concentrated solution of the order of 0.3 *M* (Table IV). Applied as weaker solutions (*e.g.*, 0.1 *M* or less) the acids run as single spots the mobilities of which correspond, re-

TABLE IV

MOBILITIES OF SOME ACIDS IN TWO ELECTROLYTES: (E) BORATE BUFFER (pH 9.2), AND (F) CARBONATE–BICARBONATE BUFFER (pH 9.2)

The standard conditions of electrophoresis given in Table I were used.

No.	*Acid*	$M_N \times 100$ *value* in electrolytes*		*% retardation in E*
		E	*F*	
1	Glycolic	120	139	14
3	Glyceric	99	115	14
9	Tartaric	135	165	18
10	Citric	133	162	18
14	Heliotric	68	79	14
16	Viridifloric (0.1 *M*)	69	82	16
	Viridifloric (0.3 *M*)	53, 69	84	37, 18
17	Trachelanthic (0.1 *M*)	72	85	15
	Trachelanthic (0.3 *M*)	51, 72	85	40, 15
18	Lasiocarpic (0.1 *M*)	65	77	15
	Lasiocarpic (0.3 *M*)	43, 66	79	45, 16
	Formic	156	183	15
	Acetic	117	138	15
	Malonic	155	180	14
	Succinic	132	159	17

* Absolute mobility of 4-nitrobenzenesulphonate in electrolyte E is 9.5 cm/h · kV; in electrolyte F, 9.6 cm/h · kV.

spectively, with the faster of the above pairs. Using the special procedure (Experimental) it was shown that these (faster) spots represent the free acid anions and that the slower of the paired spots, by reacting with curcumin, are again due to the complexes. All other hydroxy acids tried as solutions of various concentrations in borate electrolyte run as single spots, though some in higher concentrations give the large, elongated or comet-shaped spots characteristics of an overloaded paper.

When subjected to electrophoresis in the absence of borate using the carbonate electrolyte F (pH 9.2) acids 16–18 give single (free acid anion) spots irrespective of the concentration of the applied solutions. As reference to the data of Table IV shows, however, their free acid anions separating in borate migrate about 15% more slowly than in the carbonate electrolyte* and it was found also that all other aliphatic hydroxy acids used in this study, as well as some randomly chosen non-hydroxylated fatty and dicarboxylic acids, are similarly retarded in borate electrolyte. As typical examples, data for glycolic (1), glyceric (3), tartaric (9), citric (10), formic, acetic, malonic and succinic acids are included in Table IV for comparison. Evidently, retardation of the order of 14–18% in the presence of borate (pH 9.2) does not involve the OH groups of the hydroxy acids but is due to some more general interaction with their carboxylate groups. Complex formation with acids 16, 17 and 18, on the other

* Relative mobilities measured in "Tris" and glycine electrolytes, each at pH 9.2, closely match those in the carbonate electrolyte confirming that the latter is an indifferent or non-interacting electrolyte against which to compare the borate mobilities.

hand, results in significantly higher retardations of 37, 40 and 45%, respectively (Table IV).

In considering the mechanism of formation of these complex spots, it is of interest that they separate on borate pherograms only if the parent acids are applied as un-neutralised solutions. A second (complex) spot does not separate if solutions of the sodium salts are used, even in high concentrations —only single spots corresponding to the free acid anions are then seen.

It is of further interest that heliotric acid (14, Fig. 3d), closely related to the above three, does not form a detectable second (slower) spot under any conditions at pH 9.2 although, as noted in the previous section, it does show a tendency, albeit weakly, to do so at pH 4.6 in electrolyte C (Table III).

The nature of the 1:1 complexes of heliotric (14), viridifloric (16), trachelanthic (17) and lasiocarpic (18) acids

Glycol systems are well-known to form charged complexes in borate buffer (pH 9.2)[5,16], but it is unlikely for the isomeric viridifloric and trachelanthic acids (Fig. 3a and b) that the present complexes are of the glycol type. The following reasoning applies. The glycol groups must alone be responsible for any complexing that occurs with these acids in ester form, and it has been found that their methyl esters have mobilities ($M_N \times 100$ values) in borate buffer of 45 and 18, respectively. The large difference in mobilities reflects the fact that the *erythro* configuration of the viridiflorate hydroxyls is much more favourable for complexing than the *threo* configuration in methyl trachelanthate, and similar differences have been reported for the mobilities of pyrrolizidine ester alkaloids containing these acids[4,17]. The mobilities of the complexes of viridifloric and trachelanthic acids formed under present conditions are almost identical, however, and are different from either of those of their methyl esters in the same electrolyte, strongly indicating that the complexes are not formed by direct interaction with the glycol groups. This conclusion is supported by the lack of evidence of complex formation at pH 9.2 for the less hindered acids, glyceric and tartaric, which each also contain a glycol grouping (Table IV). Evidently, the strong ionisation of the carboxyls of these acids suppresses the tendency to form glycol complexes as a second, contiguous centre of anionic charge.

Complex-formation with viridifloric and trachelanthic acid and, by analogy, with lasiocarpic acid also, must therefore involve the α-hydroxyxcarboxyl systems, as it does with heliotric and the simpler α-hydroxy acids at lower pH, and it is proposed that the charged 1:1 complexes of acids 14, 16–18 that form at any pH level are represented by Fig. 4. The substituent groups, CH_3CHOR^1 and $CH_3CR^2CH_3$, on the α-carbons of these acids are especially large and, extrapolating from the above examples (Table I) demonstrating the influence of smaller groups on complex-for-

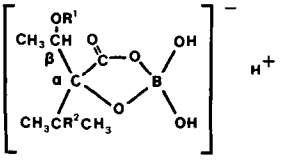


Fig. 4. Boric acid complexes (1:1) of: heliotric acid (14), $R^1 = CH_3$, $R^2 = H$; viridifloric acid (16), $R^1 = R^2 = H$; trachelanthic acid (17), $R^1 = R^2 = H$; and lasiocarpic acid (18), $R^1 = CH_3$, $R^2 = OH$.

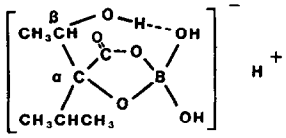


Fig. 5. Boric acid complex (1:1) of viridifloric (16) or trachelanthic (17) acid showing how H-bonding could stabilise the complex.

mation, would be expected to stabilise a complex to a marked degree. Exceptional widening of the angle between the two bonds of the α-carbons by the bulky substituents brings about a corresponding decrease in the angle enclosed by the remaining two bonds that form part of the complex ring[12]. This effect, relayed to the boron atom, results in a widening of the angle between the two free B–OH bonds. This widening could explain why a second acid molecule is not added to form the usual 1:2 complexes.

The exceptional stability of the complexes at pH 4.6 was noted in a previous section. The case of the heliotric acid complex appears to be anomalous, however, in that its formation is not as highly favoured as the others (at pH 4.6), and it does not appear at all on borate pherograms at pH 9.2. Heliotric acid differs from the other three in lacking a free β-OH group, and it is postulated that the presence of this OH group in acids 16–18 augments the already large effect due to the *gem*-dialkyls, stabilising the complexes yet more, by intramolecular hydrogen bonding to the oxygen atom bonded to boron, as indicated in Fig. 5 for the complex of viridifloric (or trachelanthic) acid. Models indicate that the relevant oxygen atoms, separated by about 2.5–2.8 Å, are appropriately spaced for normal H-bonding to occur[18]. It is unlikely, because of the strain involved, that the third ester bond of a tridentate complex would form across this space by elimination of H_2O, but the H-bonded species could be regarded structurally as an intermediate form of complex between the bi- and tridentate types, sharing with the latter the same order of stability[7,13–15].

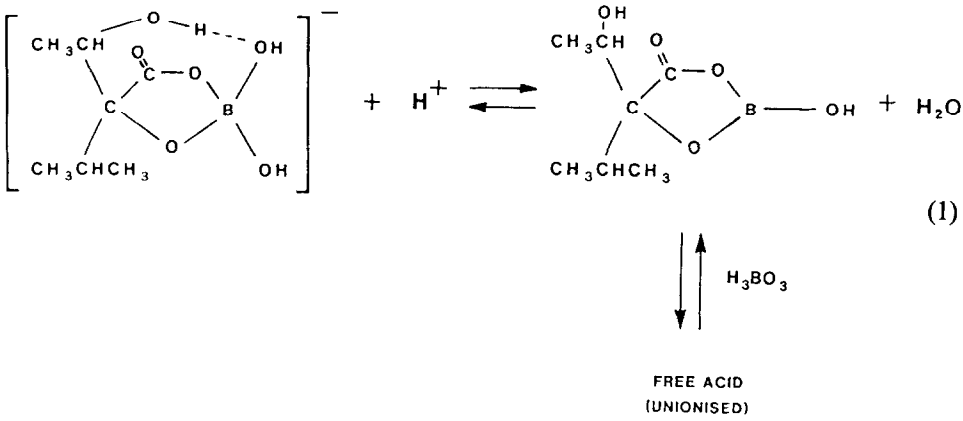
When acids 16–18 are applied as dilute solutions to borate papers at pH 9.2, they are converted completely to the corresponding salts, and the charged carboxylate groups effectively suppress complex formation. The acids then migrate as single spots. The more concentrated (0.3 *M*) solutions, however, are only partially neutralised by the (0.05 *M*) borate buffer to form carboxylates, and the pH at the point of application of the sample solutions to pherograms falls sharply, liberating boric acid. Local conditions thus being rendered favourable, residual unionised hydroxy acids then form their respective complexes. Reaction is again relatively slow*, but once formed, the complexes are so stable that they survive intact without equilibration to free acid anions, even when the pH within their migrating spots rises to the paper background of 9.2 during subsequent electrophoresis.

It appears, therefore, that the formation of the competing anions, carboxylate and complex, are mutually exclusive reactions. If the carboxylate forms first, complex formation is completely suppressed, this being convincingly demonstrated by the use

* A delay of a few minutes between applying the sample solutions to the starting line of pherograms and beginning the electrophoresis results in the appearance of a somewhat larger complex spot at the expense of that of the carboxylate, indicating that reaction is slow.

of pre-neutralised 0.3 *M* solutions of acids 16–18. Only large, single (faster) spots are then seen. The prior formation of the complexes, on the other hand, precludes simple ionisation, even under conditions where the latter might be expected to compete exclusively, as it does with heliotric and the simpler α-hydroxy acids at pH 9.2.

Because of the way in which the complexes are formed on borate papers, the "double-spotting" phenomenon could be classified as an artefact, but it does serve to emphasise the remarkable stability of such complexes. The phenomenon is also reported here in detail to alert others to what could be the misleading electrophoretic behaviour of pure compounds of this kind.



(1)

The streaks (Table I) that acids 16–18 give on boric acid pherograms at pH 2 are probably a result of the comparative slowness with which these particular complexes form. On application of the sample solutions to the pherograms, it is postulated that equilibria represented by eqn. 1 are set up between free (unionised) acid and the two 1:1 complexes shown, one being the stable, charged complex described above and the other, analogous to the neutral polyol complexes of classical theory[3], being uncharged and containing tricovalent boron with its coplanar bonds. The angle between the B–O bonds in this trigonal form is 120°, too large to be accommodated without considerable strain in the 5-membered ring of the neutral complex[19] which, therefore, should be relatively unstable. It may exist only fleetingly as an intermediate between free acid and the charged complex. The hydrogen ion concentration of the background (pH 2) electrolyte being very high, equilibrium should be forced at least partially to the right to form the uncharged species–neutral complex and free (unionised) acid. These are left at the origin during the subsequent electrophoresis while the charged complex migrates anionically. Equilibrium is then re-established at the origin, but only slowly for these acids, and the continuous release of the slowly formed complex anions results in the undifferentiated streaks observed.

The highly crowded nature of the α-carbons of acids 14, 16–18, responsible in large part for the stability of their complexes, also has biological significance. The acids occur in nature as esters of amino alcohols in hepatotoxic pyrrolizidine alkaloids which are elaborated by plants that are distributed widely throughout the world[20]. The plants are often poisonous to livestock grazing them but their alkaloids *per se* are not directly responsible. The amino alcohol parts are metabolically con-

verted in the liver, as esters but not in the free state, to pyrrolic compounds and these are the actual cytotoxic agents[21]. The bulky α-substitution of the acids confers resistance to hydrolysis by esterases and so leads to a much higher degree of conversion to toxic metabolites than would otherwise occur.

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