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DETECTION OF ESTERS AS HYDROXAMATES AFTER SEPARATION BY PAPER ELECTROPHORESIS

J. L. FRAHN
Division of Nutritional Biochemistry, CSIRO, Kintore Avenue, Adelaide, South Australia, 5000 (Australia)
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SUMMARY

Paper electrophoresis lends itself to the study of several classes of compound containing a carboxylic ester function. These compounds can be located on pherograms after reaction with alkaline hydroxylamine, the resulting hydroxamates being revealed as coloured ferric iron complexes using a ferric chloride spray reagent. Wide variation is evident in the response of esters to the hydroxylamine reagent, and the differences in reactivity could be diagnostically useful. Although acetates and lactones react in mildly alkaline medium at room temperature, many esters such as acid phthalates, benzoates, and some tropane and pyrrolizidine alkaloids require a strongly alkaline reagent. Other pyrrolizidine alkaloids and many esters of *o*-sulphobenzoic acid require forcing conditions — elevated temperatures (up to 110°) in strongly alkaline medium — to form the corresponding hydroxamates. Some esters are not detectable using even more energetic conditions.

INTRODUCTION

The hydroxamate test for esters[1,2] has been successfully adapted to the detection of carbohydrate acetates and lactones on paper and thin-layer chromatograms[3–8], but the method has seldom been used outside the field of carbohydrate chemistry, or in chromatographic studies of esters other than acetates. There are no examples of its application in the presence of aqueous electrolytes used for paper electrophoresis, presumably because esters as a class are not normally suitable for electrophoretic study. We have found that paper electrophoresis can conveniently be used with several classes of compound containing a carboxylic ester function. Examples include separation according to molecular weight of homologous lower alkanols in the form of acidic esters (hydrogen phthalates and *o*-sulphobenzoates), separation of the lower members of the series of linear polymers derived from lactic acid, and separation of various alkaloids containing ester or lactone groups (in the pyrrolizidine, tropane and yohimbine groups). When attempts were made to locate such compounds on pherograms by reaction of their ester groups, it became obvious that wide variations occur in the response of esters to the hydroxylamine reagent.

Published descriptions of the hydroxamate test as used in paper and thin-layer chromatography include a wide range of reaction conditions without clear evidence that optimum conditions were determined in any particular case. SMITH and co-workers[3,4] found that sugar acetates and aldonolactones formed hydroxamates under mild conditions (reaction for 10 min at room temperature after chromatograms had been sprayed with a methanolic solution of hydroxylamine containing 0.05 *N* free KOH), and the hydroxamate spots were then located as the ferric complexes by spraying with a solution of ferric chloride (1–2 %) in hydrochloric acid (1 %). Later workers successfully used the same reagents for aldonolactones on paper[5] and thin-layer[6] chromatograms, but others have recommended strongly alkaline hydroxylamine solutions (up to about 1.8 *N* NaOH) for carbohydrate acetates, with either reaction at room temperature[7] or heating[8] to temperatures up to 110°. In general, use of a strongly alkaline hydroxylamine reagent, with or without heating, has been recommended for the formation of hydroxamates from non-carbohydrate esters, including lactones[9], acetylated phenols[7] and palmitoyl lysolecithins[10].

The present survey of the hydroxamate reaction under conditions of paper electrophoresis has been supplemented by spot tests on esters that could not be subjected to electrophoresis. Acetates and lactones tested were readily detected under the mild conditions described by SMITH *et al.*[3,4] but increasingly vigorous conditions were required for various other classes of ester, and several were not detectable. The differences in reactivity could be diagnostically useful.

EXPERIMENTAL

Materials

Samples of carbohydrate esters were provided by Dr. J. A. MILLS* and the pyrrolizidine alkaloids by Dr. C. C. J. CULVENOR**. Other compounds, including reagents, were either laboratory preparations or commercial samples of reagent or B.P. grade.

Monoalkyl phthalates were prepared according to published procedures[11,12], and neutral solutions of the esters of sulphobenzoic acid were prepared from *o*-sulphobenzoic anhydride and appropriate alcohols according to the directions of IYER AND MATHUR[13], except that pyridine was substituted for dioxane as the reaction medium. (*o*-Sulphobenzoic anhydride was readily purified by recrystallisation from acetic anhydride to give thin plates melting sharply at 128°.)

Mixtures containing lactide, lactic acid, lactyllactic acid and higher polymeric esters were obtained by heating *dl*-lactic acid at 70° under reduced pressure (20 mm Hg). Water liberated from the self-esterification of the lactic acid distilled off continuously as it formed during a period of 2 h. The temperature of the mixture was then raised (maintaining the pressure at 20 mm) to remove most of the residual free lactic acid which boiled at about 130°.

In paper electrophoresis, samples were applied as aqueous solutions, generally 0.1–0.2 *M*, or saturated for sparingly soluble compounds. The pyrrolizidine alkaloids were applied as 0.05 *M* solutions in 0.05 *M* acetic acid. In spot tests with carbohydrate esters insoluble in water, 0.1 *M* solutions in ethanol were used.

* Of this laboratory.

** Division of Applied Chemistry, CSIRO Chemical Research Laboratories, Melbourne, Australia.

Electrolytes

The following electrolytes were used: (a) sodium sesquicarbonate (0.05 *M*; pH ≃ 10), (b) sodium borate buffer (pH 9.2) containing 0.2 g-atom of boron per litre[14], (c) sodium acetate electrolyte prepared by adjusting a 0.1 *M* solution to pH 6 by the addition of glacial acetic acid, (d) hydrochloric acid (0.1 *N*).

Reagents

Hydroxylamine reagents

Reagent 1. A freshly prepared solution made by mixing equal volumes of 1 *N* methanolic $NH_2OH \cdot HCl$ and 1.1 *N* methanolic KOH and removing the precipitated KCl by filtration. This reagent was prescribed by ABDEL-AKHER AND SMITH[3] for the detection of carbohydrate acetates and lactones. It was 0.05 *N* with respect to free KOH.

Reagent 2. Equal volumes of 1 *N* methanolic $NH_2OH \cdot HCl$ and 3.6 *N* methanolic KOH were mixed and the solution filtered. The reagent was 1.3 *N* with respect to free KOH.

Other reagents were sometimes used containing intermediate concentrations of free KOH.

Reagent 3. A saturated methanolic solution of $NH_2OH \cdot HCl$ (ref. 15).

Ferric chloride reagent

An aqueous solution of $FeCl_3 \cdot 6H_2O$ (6 %) mixed with an equal volume of formic acid (98–100 %).

Chromium trioxide–permanganate–sulphuric acid reagent

The preparation and use of this reagent have been described[14].

Apparatus and procedure

Paper electrophoresis was conducted in the enclosed-strip apparatus described previously[14,16]. Whatman No. 4 paper was used, and solutions of the esters were applied in line across the middle of paper strips by means of a platinum loop delivering approx. 0.5 μl. Caffeine was used as marker for zero migration.

Electrophoresis was allowed to proceed at about 21 V/cm in electrolytes (a), (b) and (c), and at 7 V/cm in electrolyte (d), for periods of 30 min to 1 h. For the separation of the polymeric esters of lactic acid in the acetate buffer (c), longer periods of about 2 h were required.

The papers were dried in the oven at 110° and caffeine and some esters then located as dark blue spots under a Hanovia "Chromatolite" ultraviolet lamp.

Tests to compare the ease with which the various esters undergo hydroxamation were conducted by spraying the dried pherograms evenly on both sides with one of the hydroxylamine reagents. The papers were then placed between pieces of glass (1/4 in. plate), cut to a convenient size, where reaction was allowed to proceed for 15 min at room temperature, or at any required temperature up to 110°. For treatment of papers at elevated temperatures it was necessary to preheat the glass plates for 1 h or more at the required temperature, and to return them to the oven immediately after enclosure of the pherograms.

The treated papers were subsequently sprayed with the ferric chloride reagent and the hydroxamates located as pink or rust-coloured spots on a creamy-yellow background. It was necessary to spray both sides of more strongly alkaline papers

because a one-sided application caused a red-brown coloration (due to precipitated ferric hydroxide) to appear on the reverse side. This was quickly and easily dispelled, however, when the reverse side was sprayed after an interval of a few seconds.

Spot tests

Solutions (0.5 μl) of carbohydrate esters (acetates and benzoates) were applied to strips of Whatman No. 4 paper over which borate buffer was then sprayed to bring about some lateral diffusion of the ester spots. The strips were dried and treated with the reagents under various conditions as described above for the pherograms.

Esters subjected to the spot tests included the following: hexa-O-acetyl-D-mannitol, hexa-O-acetyl-*myo*-inositol, penta-O-acetyl-β-D-galactopyranose, phenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside, 1,2:5,6-di-O-isopropylidene-3-O-acetyl-α-D-glucofuranose, penta-O-benzoyl-β-D-glucopyranose, 1,2,5,6-tetra-O-benzoyl-D-mannitol, 1,2-O-isopropylidene-3,5,6-tri-O-benzoyl-α-D-glucofuranose, 1,6-di-O-benzoyl-D-mannitol, and 1,2-O-cyclohexylidene-6-O-benzoyl-α-D-glucofuranose. Other esters tested in this way were methyl-*p*-nitrobenzoate, diethyl oxalate and diethyl succinate. The latter two esters (boiling points 185° and 217°, respectively) were applied undiluted to papers to which ethanol was then applied as a spray. The papers, dried at room temperature for 5–10 min, retained sufficient of the volatile esters for the tests.

RESULTS AND DISCUSSION

Separation and detection of o-sulphobenzoate esters

The lower members of the series of *n*-alkyl *o*-sulphobenzoates (up to *n*-heptyl) are easily separable in either of the electrolytes (b) or (c) described in the EXPERIMENTAL section. Methyl sulphobenzoate has an absolute anionic mobility in the acetate buffer (c) of 82 mm/h/kV of applied potential, and the numerical values of the mobilities of the higher esters form a series which regresses in proportion to the decreasing charge/mol. wt. ratios of the esters.

The sulphobenzoates reacted satisfactorily only on papers sprayed with reagent 2 — the more strongly alkaline hydroxylamine solution — and heated between glass plates maintained at 110° for 15 min. Reagent 1 was ineffective, and the use of reagent 2 at lower temperatures (*e.g.* 70°) or of a less strongly alkaline reagent (*e.g.* 0.4 *N* KOH) at 110° gave only very weak spots after treatment of the papers with ferric chloride. Some of the sulphobenzoates were similarly unreactive when papers treated with reagent 2 were heated at 110° not between glass plates. And, further, no advantage was gained when water was partially or wholly substituted for methanol as the solvent for the hydroxylamine reagent. The spots tended, if anything, to be more diffuse and were more easily displaced from their true positions on the pherograms.

Although the sulphobenzhydroxamate formed from the sulphobenzoates by use of reagent 2 was accompanied by excess strong base, it was readily detected by using the ferric chloride spray with a high concentration of formic acid, described in the EXPERIMENTAL section. The volume of spray required was fairly low, and this avoided the risk of overwetting the papers in the process of bringing them to optimum values of pH for the formation of the coloured ferric iron–hydroxamic acid complex (known to be pH 1–1.4 for acetohydroxamic acid[17]). It was undesirable to acidify the ferric chloride by using correspondingly high concentrations of hydrochloric acid in place

of formic acid, because there was then a high risk, by inadvertently overspraying papers, of reaching HCl concentrations where the yellow complex chlorides of ferric iron[18] form in competition with the required hydroxamic acid complex.

A variety of non-reducing carbohydrates and polyols were subjected to electrophoresis as poly-esters of sulphobenzoic acid, and the same vigorous conditions of hydroxamation were also necessary for their detection on pherograms. It is of interest, in this connection, that random, incomplete reaction of a given polyol with sulphobenzoic anhydride results in the formation of a mixture of its mono-, di-, triesters, and so on, each with its own characteristic electrophoretic mobility. Electrophoresis of the mixture and treatment of the resulting pherogram with the hydroxylamine and ferric chloride reagents yields a series of spots corresponding to the separated esters, the number of which equals the number of free hydroxyl groups in the parent polyol. This affords an alternative, therefore, to the method of DITTRICH[7] for enumerating the hydroxyl groups in a polyhydroxy compound without the necessity of referring to its molecular weight. The procedure is analogous to the demonstration of the number of reactive groups in a polyamine by electrophoresis following formation of the carbamates[16].

Polymers from lactic acid

Paper electrophoresis in the acetate buffer (c) separated the partially dehydrated preparation from lactic acid into a series of spots, which seemed to represent lactic acid, the bimolecular cyclic ester (lactide) and all linear oligomers, $Me \cdot CHOH \cdot CO(O \cdot CHMe \cdot CO)_nO \cdot CHMe \cdot CO_2H$, from $n = 0$ (lactyllactic acid) to $n = 6$. All components (except lactic acid itself) reacted with hydroxylamine at room temperature and, although reagent 2 gave best results, especially for lactyllactic acid, most polylactic acids were also detectable with less alkaline reagents. Lactide was readily detected and, being neutral, provided a convenient measure of electroendosmotic flow.

Free lactic acid reacts strongly with the chromium trioxide–permanganate–sulphuric acid reagent. The polymers did not react with this reagent, but it was possible to hydrolyse them to lactic acid by spraying the pherogram with dilute alkali (0.1 *N*) and heating it to dryness in the oven, and then reveal them by a subsequent spraying with the chromium trioxide–permanganate reagent as a series of spots corresponding to the ester spots located by the hydroxamate method.

Other acidic esters

Monomethyl- and monoethyl phthalate are separable in the borate buffer and have absolute anionic mobilities of 91 and 86 mm/h/kV, respectively. They were more easily detected on pherograms than the sulphobenzoates in that they reacted with reagent 2 at room temperature. Methyl salicylate reacts well under the same conditions, but is also detectable using the weakly alkaline reagent 1. It has an anionic mobility of 72 mm/h/kV in the sesquicarbonate electrolyte (a).

Esters of carbohydrates and other neutral esters

The electrophoretic properties of numerous carbohydrates and their derivatives have been recorded for a variety of electrolytes[19,20], but very few esters of carbohydrates have been tested, probably because many are either insoluble or difficultly soluble in aqueous media. But it is possible that some partially esterified carbo-

hydrates are sufficiently soluble and contain the free hydroxyl groups necessary for the formation of anionic complexes in electrolytes such as borate buffer. It is also possible that the electrophoresis of some less soluble esters may be facilitated by conducting experiments at elevated temperatures under controlled conditions[21]. Hydrolysis, or migration of acyl groups, could be a problem in the alkaline electrolytes. However, 1:6-di-O-benzoyl-D-mannitol was successfully run in borate buffer maintained at 50°, and had an anionic mobility of 65 mm/h/kV. This ester was subsequently detected after reaction with hydroxylamine at room temperature, but only by using the strongly alkaline reagent 2, and it appears to be generally true that the benzoates of carbohydrates do not react well in more weakly alkaline media. This fact was demonstrated, not on pherograms, but by means of the spot tests described in the EXPERIMENTAL section, because most of the carbohydrate esters which were available to us are not electrophoretically mobile. It was also shown that no improvement in the reactivities of the benzoates occurred (using reagent 2) when the reaction temperature was raised to 110°. The benzoates were all quite unreactive when the weakly alkaline reagent 1 was used, even at elevated temperatures. On the other hand, spot tests of carbohydrate acetates, and also of diethyl oxalate, gave good results and were equally successful using either reagent 1 or reagent 2 at room temperature. This is consistent with the relative ease with which acetates and oxalates are known to saponify, and it is probable that formyl esters, too, would undergo hydroxamation on paper under similarly mild conditions. The reactivity of diethyl succinate resembles that of the carbohydrate benzoates — it undergoes hydroxamation at room temperature only in strongly alkaline medium. Methyl *p*-nitrobenzoate requires more vigorous conditions, giving a strong reaction only on treatment with reagent 2 at 110°.

Basic esters including alkaloids

Esters of *p*-aminobenzoic acid are cationically mobile in dilute mineral acid electrolytes, the ethyl ester (benzocaine) and the methyl ester having mobilities of 97 and 105 mm/h/kV, respectively, in 0.1 *N* HCl. They are as resistant to hydroxamation as methyl *p*-nitrobenzoate and the sulphobenzoates, reacting well on pherograms only in strongly alkaline medium at 110°. Procaine (2-diethylaminoethyl-*p*-aminobenzoate; mobility 140 mm/h/kV) requires strongly alkaline conditions for hydroxamation but is sufficiently reactive at room temperature. The *p*-aminobenzoates are also detectable with chromium trioxide–permanganate–sulphuric acid with which, being aromatic amines, they react strongly.

Many members of the tropane and pyrrolizidine groups of alkaloids contain ester functions, and most of them are electrophoretically mobile over a wide range of pH. Variations in the degree of protonation of their tertiary nitrogen atoms permit separations of some mixtures by choosing electrolytes of suitable pH. Many pyrrolizidine alkaloids also contain vicinal glycol groups which form anionic complexes in sodium borate and sodium arsenite electrolytes, thus extending the possibilities for their separation. Electrophoretic data for a number of pyrrolizidines in seven electrolytes have already been recorded[22], but only a limited number of methods is available for their detection on pherograms. It is now found that most alkaloidal esters respond well to treatment with hydroxylamine but under a variety of conditions depending upon the precise nature of their ester linkages. Spectabiline[23] contains three ester functions one of which is an acetate, and it reacts strongly at room temperature with

reagent 2 but is also easily detectable using reagent 1. Latifoline[24], containing a lactone ring, reacts moderately well under the same conditions, but responds better to reagent 2 at higher temperatures. Seneciphylline[25], a macrocyclic di-ester, is as easily detectable as spectabiline. However, other pyrrolizidine alkaloids, *e.g.*, lasiocarpine, supinine, crispatine, senecionine, fulvine, jacobine, jaconine, 7-angelyl heliotridine, monocrotaline, echinatine and supinidine viridiflorate[26] were best detected after exposure to reagent 2 at 110°, although for several of these, milder conditions sometimes sufficed. Esters of heliotric acid are known to be highly resistant to hydrolysis[26] and it was found that the methyl ester and the alkaloidal esters, heleurine and heliotrine were not hydroxamated even after prolonged treatment with reagent 2 at 150°. They therefore remained undetected on pherograms by the present method.

Yohimbine and the tropane alkaloids, atropine, cocaine and hyoscine were each detected after electrophoresis in the acetate electrolyte (pH 6) using reagent 2 at room temperature. They had cationic mobilities of 43, 77, 72 and 76 mm/h/kV, respectively. Acetyl choline had a mobility of 114 mm/h/kV in the same electrolyte and, being an acetate, it responded readily to reagent 1 at room temperature.

Lactones and amides

Derivatives of carboxylic acids other than esters undergo hydroxamation under conditions outlined here (*cf.* ref. 27), but of these, acid anhydrides and acid chlorides, being susceptible to hydrolysis in aqueous medium, would probably not survive an electrophoretic separation. The work of SMITH[3] and others[5,6,9,27] indicates that lactones, as a class, should be hydroxamated fairly readily at room temperature, if not with reagent 1, then with reagent 2. Many lactones, however, would be more easily decomposed than esters during electrophoresis in some alkaline electrolytes, but they should be stable under mildly alkaline and acid conditions (*e.g.* latifoline[22]).

Amides are also converted to hydroxamates with hydroxylamine, but it is well known that the reaction occurs only at elevated temperatures[28–31]. Spot tests with a selection of amides (acetamide, benzamide, acetanilide, asparagine and glutamine) showed that they reacted well with reagent 2 at 110°, but not at all at room temperature. ROBERTSON AND BUTLER[15] detected amides on paper chromatograms after converting them to hydroxamic acids by treatment with unneutralized $NH_2OH \cdot HCl$ at 100° for 20 min. We tested a number of esters under these acid conditions (using reagent 3, EXPERIMENTAL section) but none gave a positive result. It seems, then, that although amides react on paper under the same conditions as the more resistant esters, they can be distinguished by their reactivity with acidic NH_2OH. We found, however, that for our selection of amides, the alkaline reagent 2 was the more sensitive. ROBERTSON AND BUTLER[15] reported that the limit of detectability of some amides, *e.g.* asparagine, on paper chromatograms was about 50 μg. Under the more favourable conditions of the spot tests, we found that about 25 μg of asparagine was easily detected using the acid reagent but that, with reagent 2, less than 10 μg was sufficient for a good response.

CONCLUSIONS

Of compounds capable of conversion to hydroxamates, only those containing ester, lactone and amide linkages are likely to be sufficiently stable in the aqueous

electrolytes used in paper electrophoresis to enable their subsequent detection by this means. It appears that, in many cases, the proper choice of the conditions of hydroxamation may help to distinguish between these types and even to aid the identification of specific compounds.

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