478. *The Separation of Acids by Paper Partition Chromatography.*

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The use of various basic solvent systems for the paper partition chromatography of certain organic acids has been investigated, and a method has been evolved for their separation.

The study has been extended to include anions of amino-acids and of inorganic, partly esterified phosphoric and sulphonic acids, as well as phenols and metallic cations. The application of the method to oxidative degradation studies has been indicated.

During structural studies on complex naturally occurring substances it is frequently necessary to work with very small quantities of material; oxidative degradations involve the separation and identification of acidic compounds on such a small scale. The technique of paper partition chromatography is peculiarly suited to the separation of substances on a micro-scale, and its application to acids has now been investigated with a view to evolving a method suitable for general use.

The dimerisation and ionic dissociation of many organic acids render their partition between solvents dependent on concentration, and complicate any separation based on distribution. Furthermore, the volatility of lower fatty acids and phenols precludes the possibility of any chromatography of the unmodified compounds on paper sheets. The various methods hitherto tried with a view to overcoming these difficulties (for reviews, see Elsden, *Biochem. Soc. Symposia*, 1949, No. 3, p. 74; Martin, *Ann. Reports*, 1948, **45**, 282; *Ann. Rev. Biochem.*, 1950, **19**, 531) can be classified under two headings: (*a*) methods involving prior modification of the acidic function, *e.g.*, by esterification (Boldingh, *Rec. Trav. chim.*, 1950, **69**, 247) or by formation of the derived hydroxamates (Fink and Fink, *Proc. Soc. Exp. Biol.*, N.Y., 1949, **70**, 654); and (*b*) methods depending on the control of pH by the presence of excess of volatile acids (Lugg and Overell, *Nature*, 1947, **160**, 87; cf. Synge, *Biochem. Soc. Symposia*, 1949, No. 3, p. 90) or bases (Brown and Hall, *Nature*, 1950, **166**, 66; Hiscox and Berridge, *ibid.*, p. 522; Brown, *Biochem. J.*, 1950

47, 598; *Nature*, 1951, **167**, 441). Of these alternatives, methods of the second type, entailing the simplest operating conditions, were preferred for the present work. The choice of basic in preference to acidic solvent systems was governed by the more effective variations possible in their composition, and by the fact that volatile acidic compounds are not lost during the separation. Hence, in all experiments the solvent was a solution of a base in an aqueous alcohol, usually a single-phase system.

By observing standard experimental procedure it is possible to obtain a series of reproducible R_F values. On Whatman No. 54 paper with ethanolic ammonia as the solvent, the various types of acids separate into the following broad zones, defined by their R_F values: aliphatic polycarboxylic acids and aromatic carboxylic acids containing three or more carboxyl groups, 0—0·25; aliphatic dicarboxylic acids, 0·3—0·6; aromatic dicarboxylic acids, 0·4—0·5; aliphatic monocarboxylic acids, 0·7—0·8. Furthermore, inorganic, sulphonic, and partly esterified phosphoric acids, amino-acids, and certain phenols may be separated in this manner. The R_F values obtained for the homologous series of aliphatic mono- and di-carboxylic acids were used to calculate the corresponding series of R_M values (Bate-Smith and Westall, *Biochim. Biophys. Acta*, 1950, **4**, 427). An approximately linear relationship was found to exist between this value and the number of methylene groups in the acids (Figs. 1 and 2).

The R_F value of a particular ion depends on three independently variable factors, namely, texture of paper and nature of base and of alcohol. Whatman No. 54 paper gives lower R_F

FIG. 1.

Monocarboxylic acids, $H\cdot[CH_2]_n\cdot CO_2H$.

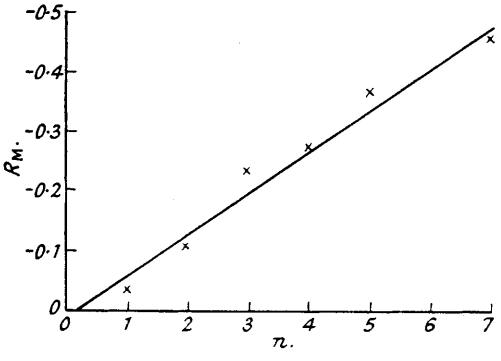
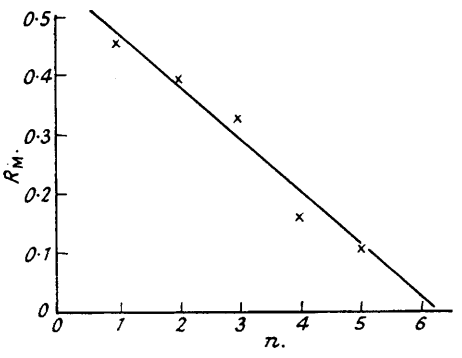


FIG. 2.

Dicarboxylic acids, $[CH_2]_n(CO_2H)_2$.



values than No. 1; previous washing of the paper has no effect on the chromatogram beyond accelerating the movement of the solvent. The organic bases investigated give higher R_F values than does ammonia; *e.g.*, oxalic acid moved at R_F 0·31 in ethanolic ethylamine, whereas in ethanolic ammonia it exhibited a characteristic streak from the starting line. The use of higher alcohols than ethanol reduced the R_F values. Considerable adjustment can be effected by attention to these various factors; for instance, the R_F value of the acetate ion can be increased from 0·28, when *tert.*-butanol–ammonia is used, to 0·81 with ethanol–morpholine. The presence of a metal cation causes no variation in the R_F value of the associated anion, and its presence does not affect the detection of the anion, except in a few cases where the ions separate incompletely. Under standard conditions the R_F value of a given anion is accurately reproducible, a fact attributable perhaps to the ready equilibration of the volatile solvent system.

For the detection of the acidic compounds, B.D.H. Universal Indicator is very satisfactory and was preferred to the indicators recommended by Brown and Hall, and by Hiscox and Berridge (*locc. cit.*). The spots fade in air, but the colours may be restored by further spraying.

Ninhydrin has been used extensively to detect amino-acids, the development of the colour being due to initial reduction of the ninhydrin to hydrindantin by the amino-acid, followed by the formation of the intensely coloured diketoindanylideneaminoindanedione (Ruhemann, *J.*, 1911, **99**, 792, 1486; Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367). This reaction proceeds to some extent with amides or ammonium salts, and hence its applicability to the detection of the acid salts on paper was investigated. In many cases its sensitivity was found to be greater than that obtained by spraying with indicator, and the resulting spots were permanent. The method is not as sensitive for the ammonium salts as it is for amino-acids, a fact attributable to the lack of hydrindantin in the former case, and therefore some source of this compound was

sought. Ene-diols cause the desired reduction of ninhydrin to hydrindantin (West and Rinehart, *ibid.*, 1942, **146**, 105), and in fact, the inclusion of ascorbic acid in the reagent solution increases its sensitivity (cf. Tetzner, *Mikrochem.*, 1940, **28**, 141). However, the background colour, probably due to traces of the base strongly associated with the paper, is likewise more intense; this modification therefore does not greatly faciliate the detection of ammonium salts on paper, and has not been studied further (cf. Fowden, *Biochem. J.*, 1951, **48**, 327).

The oxidation of iodide by iodate depends on the presence of hydrogen ions, and this furnishes a further method for the detection of regions of acidity on the paper (cf. Buchanan, Dekker, and Long, *J.*, 1950, 3162). In the present instance, the free acids may be generated by thermal decomposition of their ammonium salts, and iodine is then liberated at the sites of the various fractions when a mixture of sodium iodate and sodium iodide is applied as a spray; starch increases the sensitivity. The method was originally used successfully for the detection of acylamido-acids (G. W. Kenner, private communication), but is unreliable for the simpler acids. This may be due to the volatility of the acids themselves, or to the predominance of amide formation (cf. Degering, "An Outline of Organic Nitrogen Compounds," University Lithoprinters, Michigan, 1945, p. 400).

Several other methods of detection were tried with limited success (see Experimental section). The sensitivity of the various methods varies from compound to compound: 20—100 μg. of material is a safe working range, although particular compounds can be detected at concentrations considerably below this. Fractions may be differentiated from each other not only on the basis of R_F values, but also, in some cases, by the application of specific spraying reagents (see Experimental section).

The applicability of the method to the separation of products from micro-oxidations was tested with the following compounds, alkaline permanganate or hydrogen peroxide being used as oxidising agents: formaldehyde to formic acid; benzaldehyde to benzoic acid; propane-1 : 2-diol to acetic and formic acids; benzyl alcohol to benzoic acid. The oxidation products were identified by the methods described above. On a larger scale a mixture (100 mg.) of benzoic and malonic acids was quantitatively separated on a column of powdered cellulose, ethanolic ammonia being used as the developing solvent.

The method has been applied to structural studies, and further examples of its use will be found in subsequent communications on several natural products.

Experimental.

The method of paper partition chromatography employing a descending solvent system as described by Consden, Gordon, and Martin (*Biochem. J.*, 1944, **38**, 224) was used. The solvent front was allowed to move a distance of about 30 cm., which, in the case of ethanolic solvents, took approximately 8 hours on Whatman No. 1 paper at 19—20°. The inclusion of *n*-butanol in the solvent mixture retarded the progress of the solvent front, and with *tert.*-butanol it was necessary to extend the time of irrigation to 36 hours. The solvents percolated through No. 54 paper more rapidly than through No. 1 paper. Commercial 95% ethanol, 70% ethylamine, and ammonia solution (AnalaR; *d* 0·88) were used throughout. The other bases and alcohols employed were distilled before use. Whatman No. 1 and No. 54 papers only were employed, and, for experiments with washed paper, the sheets were treated successively with 2N-acetic acid (A.R.; 6 times), distilled water (3 times), 2N-ammonia solution (A.R.; 6 times), and finally with distilled water until neutral. They were dried at 100°.

The chromatograms were run with 20—100 μg. of material, applied in the form of a 0·2—1·0% aqueous solution of the free acid or its ammonium or alkali-metal salt.

Location of Spots.—(a) *Ninhydrin method.* The papers were dried at 80° for 15 minutes and sprayed with a 0·2% solution of ninhydrin in ethanol containing formic acid (5% v/v), then heated for a few minutes at 100—120°. The ammonium salts appeared as bluish spots on a paler background of bluish-pink. No. 1 paper was found to be more suitable when ninhydrin was used, as poor contrast between the spots and the background was obtained with No. 54 paper. The sensitivity of the method varied; *e.g.*, the spot from 20 μg. of acetic acid was of equal intensity to that given by 100 μg. of benzoic acid. The utility of this method of locating spots was limited to chromatography using ammonia solution as the base. In the case of ethylamine systems, a strong background colour was given even by papers which had been heated for 2 hours at 120° before being sprayed with ninhydrin solution. The sensitised ninhydrin reagent was prepared by dissolving ninhydrin (200 mg.) and ascorbic acid (50 mg.) in 95% ethanol (100 c.c.); it was used either in this state or mixed with formic acid (5 c.c.).

(b) *Indicator method.* The papers were dried at 80° for 15 minutes and sprayed with B.D.H. Universal Indicator, the pH of which was previously adjusted to 9—10 with sodium hydroxide solution. When ammonia solution was used in the running solvent, the zones appeared immediately and slowly faded, whereas with an organic base the paper turned a uniform greenish colour; on exposure to air the spots gradually appeared. The appearance of acidic bands was observed with some papers. Cations appeared as blue spots.

(c) *Iodate–iodide method.* The papers were heated at 130° for 30 minutes and sprayed with a mixture (1 : 1) of aqueous sodium iodate (0·2%) and iodide–starch (0·4% and 0·5%, respectively). Blue spots appeared within 10 minutes. This method, which gave good results with acylamido-acids, benzoic, oxalic, and toluenesulphonic acids, failed with formic, acetic, cinnamic, and salicylic acids. It was necessary to use No. 1 papers owing to the background colour given by No. 54 papers.

(d) *Miscellaneous methods.* The following reagents were investigated for the detection of ammonium salts, but were found inferior to the above: Nessler's reagent (cf. Libermann, Zaffaroni, and Stotz, *J. Amer. Chem. Soc.*, 1951, **73**, 1387), and phenol and sodium hypochlorite solution (Snell and Snell, "Colorimetric Methods of Analysis," van Nostrand, New York, 1937, p. 345). Unsuccessful attempts were made to convert the acids on the paper into benziminazoles (Hickinbottom, "Reactions of Organic Compounds," Longmans, Green and Co., London, 1948, p. 326), which would be detectable in ultra-violet light (cf. Buchanan, Johnson, Mills, and Todd, *J.*, 1950, 2845), and to detect them by decomposition of their silver salts in ultra-violet light.

(e) *Methods for specific compounds.* Additional information concerning the nature of some of the fractions was obtained by the use of the following selective spraying reagents: ammoniacal silver nitrate for differentiating between formic and acetic acids; a solution (2%) of iodine in ethanol for phenols and certain acids (cf. Brante, *Nature*, 1949, **163**, 651; Marini-Bettolo-Marconi and Guarino, *Experientia*, 1950, **6**, 309); alkaline permanganate solution for unsaturated acids (Pacsu, Mora, and Kent, *Science*, 1949, **110**, 446); diazonium salt solutions (Bray, Thorpe, and White, *Biochem. J.*, 1950, **46**, 271) and ammonium phosphomolybdate (Riley, *J. Amer. Chem. Soc.*, 1950, **72**, 5782) for phenols. Simple inspection of the paper under ultra-violet light revealed fluorescent (or dark) spots in the case of such aromatic compounds as salicylic acid and α- and β-naphthols.

Limits of Resolution.—It is generally possible to resolve a mixture of two substances having R_F values differing by not less than 0·05 (*i.e.*, a separation of 1·5 cm. in a normal chromatogram).

Characteristics of Individual Acids.—The R_F values obtained for a variety of acids on Whatman No. 54 paper when ethanol (80), ammonia solution (4; *d* 0·88), and water (16) were used as the solvent system are listed in Table I. B.D.H. Universal Indicator was used for detection, unless otherwise stated.

TABLE I.

Aliphatic monocarboxylic acids. Formic (0·50), acetic (0·52), propionic (0·56), butyric (0·64), valeric (0·65), hexanoic (0·70), octanoic (0·74), *iso*butyric (0·64), *iso*valeric (0·67), phenylacetic acid (0·60).

Aliphatic halogeno-acids. Trifluoroacetic (0·75), chloroacetic (0·52), dichloroacetic (0·60), trichloroacetic (0·70), bromoacetic (0·54), β-chloropropionic (0·60).

Aliphatic hydroxy-acids. Citric (0·11), lactic (0·49), malic (0·25), tartaric (0·19).

Aliphatic and other amino-acids.[1] Alanine (0·43), arginine (0·20), aspartic acid (0·18), cystine (0·10), glutamic acid (0·22), glycine (0·26), lysine (0·60), phenylalanine (0·68), proline (0·44), serine (0·32), threonine (0·40), tyrosine (0·48), valine (0·61), carbobenzyloxyphenylalanine (0·83), carbobenzyloxyphenylalanylglycine (0·83).

Aromatic monocarboxylic acids. Benzoic (0·58), *o*-bromobenzoic (0·76), *p*-bromobenzoic (0·76), *m*-chlorobenzoic (0·65), cinnamic (0·68), 2 : 4-dinitrobenzoic (0·60), 3 : 5-dinitrobenzoic (0·60), α-naphthoic (0·69), β-naphthoic (0·69), *m*-nitrocinnamic (0·83), protocatechuic (0·39), salicylic (0·73), *o*-toluic (0·66), *m*-toluic (0·63), *p*-toluic (0·63).

Dicarboxylic acids. Oxalic (0—0·15),[2] malonic (0·26), succinic (0·29), glutaric (0·32), adipic (0·41), pimelic (0·44), maleic (0·31), phthalic (0·39), *iso*phthalic (0·45), terephthalic (0·45).

Other acids. Aconitic (0·16), trimesic (0·00).

Phosphoric acids. Adenosine-5′ benzyl hydrogen phosphate (0·48), adenosine-5′ phosphate (muscle adenylic acid) (0·03).

Sulphonic acids. Sulphanilic (0·53), 1-hydroxynaphthalene-5-sulphonic (0·67), 2-hydroxynaphthalene-6-sulphonic (0·58), toluene-*p*-sulphonic (0·73).

Phenols. Catechol (0·76), 2 : 4-dinitrophenol (0·76), *m*-hydroxybenzaldehyde (0·85), *p*-hydroxybenzaldehyde (0·69), α-naphthol (0·84), β-naphthol (0·82), *m*-nitrophenol (0·83), *p*-nitrophenol (0·66), phloroglucinol (0·61), picric acid (0·71), resorcinol (0·77).

Salts[3] (the italicized figures are values for cations). Sodium salts: fluoride (*0—0·13*, 0·27), chloride (*0·26*, 0·43), bromide (*0·25*, 0·48), iodide (*0·24*, 0·53), acetate (*0·26*, 0·52), azide (*0·26*, 0·54), chromate (0·09), nitrate (*0·28*, 0·48), nitrite (*0·27*, 0·47), phosphate (*0·21*, 0·02), sulphate (*0·20*, 0·09), sulphite (*0·20*, 0·04). Lithium salts: chloride (0·48), nitrate (0·55), phosphate (0·02), sulphate (*0·38*, 0·11). Potassium acetate (*0·19*, 0·52); cæsium alum (*0·10*); magnesium acetate (*0·00*, 0·52); calcium acetate (*0·00*, 0·52).

[1] Amino-acids were run on Whatman No. 1 paper; ninhydrin was used for detection. [2] Oxalic acid forms a characteristic streak in this solvent. More satisfactory results can be obtained in other solvent systems (see below). [3] Cf. Pollard, McOmie, and Elbeih, *J.*, 1951, 470.

The R_F values of some acids under various conditions are listed in Table II. Unless otherwise stated, Whatman No. 1 paper was used.

TABLE II.

Anion.	Solvent system (see below). A.†	B.†	C.	D.	E.	F.	G.	H.†	J.	K.†	L.†
Acetic	0·69	0·81	0·66	0·28	0·44	—	—	0·53	0·67	0·64	0·52
Benzoic	—	—	0·74	0·51	0·67	0·64	0·80	—	0·72	—	0·58
Citric	—	—	0·07	—	—	0·00	*	—	—	—	0·11
Formic	0·70	0·80	0·65	0·33	0·44	—	—	0·53	0·67	0·64	0·50
Oxalic	—	0·53	*	—	—	—	*	0·18	0·35	0·31	*
Phthalic	0·68	0·70	0·38	—	—	0·15	0·67	0·37	0·57	0·52	0·39
Resorcinol	0·77	—	—	—	—	—	—	0·70	—	—	0·77
Salicylic	—	—	—	—	—	0·73	0·69	—	—	—	0·73
Succinic	0·56	0·64	0·42	—	—	0·00	0·64	0·28	0·50	0·42	0·29
Sulphanilic	0·78	—	0·57	—	—	0·18	—	0·61	0·65	0·64	0·53
Tartaric	0·85	—	0·22	—	—	—	*	0·17	0·36	0·29	0·19
Toluene-*p*-sulphonic	0·80	0·73	0·70	—	—	—	0·67	—	—	—	0·73

* These acids give streaks on the paper. † Whatman No. 54 paper.

Solvent systems : (A) Ethanol (32), water (8), *cyclo*hexylamine (3·3); (B) ethanol (32), water (8), morpholine (2·6); (C) ethanol (80), water (16), ammonia (4; *d* 0·88); (D) *tert.*-butanol (80), water (16), ammonia (*d* 0·88) (4); (E) *n*-butanol (40), ethanol (40), water (16), ammonia (4; *d* 0·88); (F) *n*-butanol saturated with 0·75N-aqueous ethylamine; (G) ethanol (80), pyridine (12), water (8); (H) ethanol (80), 33% aqueous ethylamine (20); (J) ethanol (80); 16% aqueous ethylamine (20); (K) as (J); (L) as (C).

Identification of Acids produced in Oxidation Experiments.—Unless otherwise stated, each compound (see Table below) was allowed to react in aqueous solution (1 c.c.; 1%) with the oxidising agent. The resulting solution, clarified if necessary by being centrifuged, was applied to a Whatman No. 1 paper strip in such a way that each drop spread to no more than 0·7 cm. diameter. Satisfactory results were obtained if this was done 3 times, the paper being dried after each addition. The chromatograms were developed with ethanolic ammonia (see Table I), and the fractions located with ninhydrin. In each case behaviour corresponding with that of the expected acidic products, which were run alongside, was observed.

Substance.	Oxidising agent.	Reaction time.	R_F Values of products.
Formaldehyde	$KMnO_4$ (1 c.c.) [1]	4 hrs.	0·60 (formic acid)
Benzaldehyde	$KMnO_4$ (1 c.c.)	4 hrs.	0·63 (acetic acid) [2] 0·73 (benzoic acid)
Propane-1 : 2-diol	$KMnO_4$ (1 c.c.)	3 hrs.	0·65 (formic + acetic acids) [3]
Benzyl alcohol [4]	$KMnO_4$ (2 c.c.)	12 hrs.	0·58 (benzoic acid) [5]
Formaldehyde	H_2O_2 (1 c.c.; 20-vol.), Na_2CO_3 (0·5 c.c.; 3N.)	4 hrs.	0·60 (formic acid)
Benzaldehyde	do.	4 hrs.	0·63 (acetic acid) [2] 0·73 (benzoic acid)
Propane-1 : 2-diol	H_2O_2 (0·5 c.c.; 100-vol.), aq. NH_3 (0·5 c.c.; *d* 0·88); 100°	5 mins. [6]	0·65 (formic + acetic acids) [3]

[1] The alkaline permanganate solution was prepared as described by Pacsu, Mora, and Kent (*loc. cit.*). [2] Oxidation was carried out in aqueous-ethanolic solution, which was shown by blank experiments to yield some acetic acid under these conditions. [3] It was impossible to distinguish by their R_F values these two acids, both of which would be expected as oxidation products. [4] A 0·3% aqueous solution was used. [5] Whatman No. 54 paper. [6] The reagents were evaporated under reduced pressure, and the oxidation repeated. Finally, the products were dissolved for chromatography in 0·5N-ammonia solution (1 c.c.).

Separation of Benzoic and Malonic Acids on a Cellulose Column.—Cellulose (34 g.), prepared by shredding Whatman "Ashless" tablets through an 80-mesh sieve, was packed into a column (16 × 4 cm.) and a solution of 8-hydroxyquinoline (200 mg.) was washed through with the usual ethanol–ammonia solvent in order to remove metallic cations. Further solvent (300 c.c.) was run through after complete elution of the hydroxyquinoline. A mixture of benzoic (50 mg.) and malonic acids (50 mg.) was put on the column, and fractions (5 c.c.) of the eluate were collected. The samples were tested by spotting on to a filter-paper and developing this with ninhydrin as previously described. The benzoic acid was eluted in fractions 16—19, and the malonic in fractions 39—44; the acids were recovered quantitatively from these fractions.

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