SOME FACTORS INVOLVED IN MULTIPLE SPOT FORMATION IN THE PAPER CHROMATOGRAPHY OF SYMPATHOMIMETIC AMINES IN THE PRESENCE OF ACIDS*

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The paper chromatographic behaviour of a variety of amines both alone and in the presence of acids in acidic, neutral and basic running solvents is reported. Using a n-butanol: acetic acid: water solvent, multiple spot formation by several pure sympathomimetic amines was observed in the presence of the stronger organic acids. Many weaker acids affected the amines in a neutral solvent. The results emphasise the need for correct controls and the cautious interpretation of chromatograms of biological extracts since the number of spots produced is not necessarily indicative of the number of bases present.

Multiple spot formation in the paper chromatography of pure substances has often been reported[2]. Although satisfactory explanations of the phenomenon in terms of isomerisation, complexing and chemical change for some observations have been given[2,3], the mutual effects of charged ions leading to spot deformation and multiplication in the chromatography of bases have not received detailed attention. However, the effects of acids other than those present in the solvent on the formation of base spots have been noted. For example, Waldron-Edward[4] reported that D-glucosamine and some basic amino-acids and diamines gave two connected base spots in the presence of an equivalent of sulphate, while more than one equivalent resulted in the formation of only the slower base spot. West[5] showed that some pure tissue amines gave two spots when chromatographed in the presence of certain acids. Gore and Adshead[6] found that the R_F values of certain alkaloids were lower when the salts rather than the free bases were used, whereas Resplandy[7] and Büchi and Schumacher[8] reported that the alkaloids and their salts gave identical R_F values in their systems. The use of the same acid in salt and solvent system by Munier[9] resulted in compact spots in the chromatography of diverse bases in accordance with predictions.

In the paper chromatography of biological fluids or extracts, the presence of acids in these materials may alter the R_F values of the bases being examined and give rise to more base spots than there are bases. Quantitative determination of bases by paper chromatography of biological materials may also be seriously jeopardised by the presence of traces of acids. The relative importance of various factors which might be important in the polar interactions involved during the formation of base spots was therefore investigated. In the present paper, sympathomimetic amines only are considered but many of the observations are equally applicable to other amines; qualitative but not quantitative implications in the examination of biological fluids will be presented.

* See reference 1 for a preliminary report of this and related work.

Experimental Methods

Materials. (−)-Noradrenaline acid tartrate was recrystallised from water, m.p. 103° (102–104°[10]). (−)-Adrenaline acid tartrate was recrystallised from water, m.p. 147–148° decomp. (147–154° decomp.[11]). (±)-Isoprenaline sulphate was recrystallised from acetone-methanol, m.p. 178° (180°[11]). Noradrenaline and adrenaline were liberated from solutions of the salts by the addition of dilute ammonium hydroxide solution containing a trace of sodium metabisulphite; the precipitate was filtered off, washed with water, methanol and ether and dried. Noradrenaline gave m.p. 214–216° (216–218°[10]); adrenaline gave m.p. 210° decomp. (211–212°[11]). Isoprenaline was obtained similarly and recrystallised from ethanol, m.p. 155° (155·5°[11]). Tyramine was recrystallised from ethanol, m.p. 163° (164·5°[11]). (−)-Ephedrine hemihydrate was recrystallised from water, m.p. 40° (40°[11]). β-Phenylethylamine and (+)-amphetamine were redistilled; b.p. 196–198° (197–198°[11]) and 200–204° (205°[11]) respectively.

Solvents. (1) n-Butanol: acetic acid: water (4: 1: 5, by volume). The liquids were shaken together and set aside overnight. The upper layer was separated off and used as the running solvent.

(2) n-Butanol: ammonia: water (20: 1: 19, by volume) was prepared similarly and the upper alcoholic layer utilised.

(3) Water-saturated n-butanol.

(4) Liquefied Phenol, B.P.

Spray Reagents. All solutions were aqueous unless otherwise stated. For *adrenaline, noradrenaline* and *isoprenaline*: a solution containing 0·6 per cent w/v potassium ferricyanide and 0·5 per cent w/v sodium hydroxide. For *tyramine, β-phenylethylamine, ephedrine*: ninhydrin (0·2 per cent w/v) in n-butanol; the papers were dried and heated at 80–100° for 2–3 minutes. *p*-Nitroaniline diazo reagent[12] was used to detect *amphetamine.*

The acid radicals were detected on duplicate chromatograms. *Sulphate*: barium chloride (0·1 per cent w/v) followed by sodium rhodizonate (0·2 per cent w/v). *Chloride*: silver nitrate (0·5 per cent w/v) followed by irradiation by ultra-violet light. In the presence of phenolic amines, the silver nitrate sprayed paper was washed twice with dilute nitric acid (5 per cent w/v HNO_3), and exposed to hydrogen sulphide gas. *Polybasic organic acids*: Dragendorff's reagent. *Salicylic acid*: ferric chloride solution (1·0 per cent w/v) or alternatively by observing the fluorescence under ultra-violet light. *Other acids*: Several methods were used but the following proved the most satisfactory: an aqueous solution of bromophenol blue (0·04 per cent w/v) containing 5 per cent w/v ethanol applied after drying the papers at 50–60° for several hours to drive off the acetic acid. Potassium iodide (1 per cent w/v) followed by a potassium iodate (1 per cent w/v) starch solution.

General method. The bases (0·2 μ M) were applied to Whatman No. 1 paper* for chromatography from aqueous or alcoholic solution as

* Whatman No. 4, 20, 54 and 3MM, and Whatman No. 1 paper previously washed with the running solvent and dried were also used with similar results.

either the free bases, the salts or as the salts in solutions containing an excess of the acid (usually 0·5N). Chromatography was carried out by the ascending technique for 18 hours at 19 ± 2°; the solvent front advanced about 25 cm. during this time. The R_F values reported in this paper were calculated from the positions of the advanced edges of the spots and are expressed as percentages. The deviation from the mean R_F value is given for those values calculated from six or more observations.

Results

Results Using the n-Butanol: Acetic Acid: Water Solvent

Amines having low R_F *values* (0–50), *for example, adrenaline* (R_F 42) *and noradrenaline* (R_F 35). The results are summarised in Figure 1.

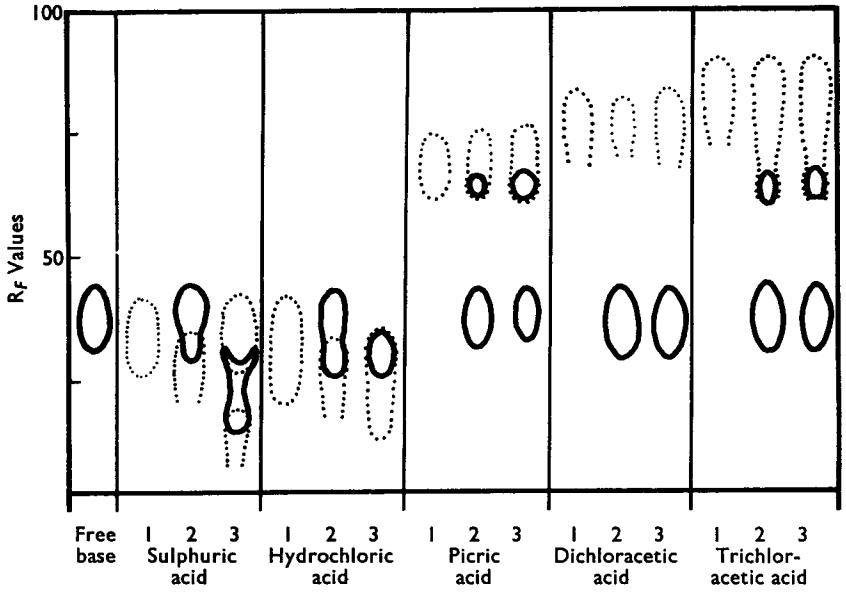

FIG. 1. Chromatograms of adrenaline developed in the butanol: acetic acid: water solvent system when applied to the paper as a suspension (2μl.) of the free base and in solutions (2μl.) containing various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

Both adrenaline (R_F 42 ± 3) and noradrenaline (R_F 35 ± 2) formed elongated amine spots when the hydrochlorides or sulphates were chromatographed. The positions of the leading edges of the amine spots were not significantly different from those obtained when the corresponding free bases were used (see Fig. 1). "Spanner-shaped" amine spots in which part of the acid was present in the cup were produced when either adrenaline or noradrenaline sulphates were applied to the paper with an excess of the acid. The remainder of the acid appeared as an elongated spot below (behind) the amine. This effect was not observed with these

bases in hydrochloric acid when the acid moved in association with the amine as an oval spot having a lower R_F value than that of the free base.

Picrates of adrenaline and noradrenaline yielded two amine spots (R_F 40 and 68 for adrenaline picrate), one of which corresponded with the free base while the other was associated with the picric acid. The R_F value of picric acid is 74 ± 2. Equivalent amounts of weaker organic acids having R_F values similar to that of picric acid, such as maleic acid (R_F 71) and dichloracetic acid (R_F 83) did not affect the movement of adrenaline or noradrenaline although an excess of either acid caused some

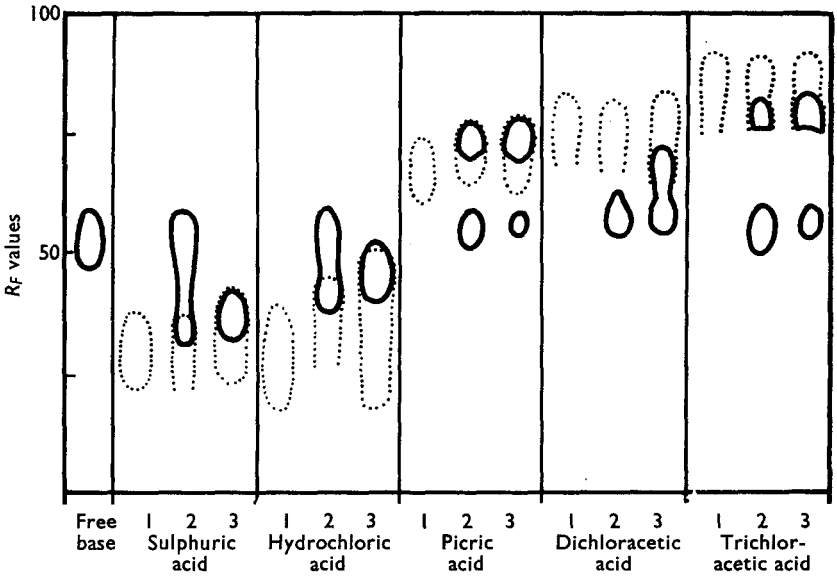

FIG. 2. Chromatograms of isoprenaline developed in the butanol : acetic acid : water solvent system when applied to the paper as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

upward streaking with adrenaline. In contrast, stronger organic acids having high R_F values, such as trichloracetic acid (R_F 90 $\pm$ 3) and trifluoracetic acid (R_F 82 $\pm$ 2), caused formation of two amine spots with both adrenaline and noradrenaline. The slower-moving amine spot corresponded with that of the free base (in equilibrium with the acetic acid of the solvent) while the faster amine spot (R_F of approximately 62) was associated with a halo–acetic acid spot. Increasing the amount of the halo–acetic acid applied with the base resulted in an increasing proportion of the amine in the faster moving spot; the slower amine spot could not be eliminated using up to a twenty molar excess of the acid.

Amines having intermediate R_F *values* (50–70), *for example, isoprenaline* (R_F 59 $\pm$ 2) *and tyramine* (R_F 62 $\pm$ 2). The results for isoprenaline in

the presence of various acids are presented in Figure 2. When either isoprenaline hydrochloride or sulphate was chromatographed, the base appeared as an elongated spot, the leading edge of which corresponded with that of the more compact spot produced when the free base was chromatographed. The R_F value of the amine was reduced when isoprenaline sulphate was applied to the paper in the presence of excess sulphuric acid (0·5N) (see Fig. 2).

Isoprenaline picrate gave two amine spots, R_F 60 and 79, the former being associated with the picric acid. In contrast with adrenaline and

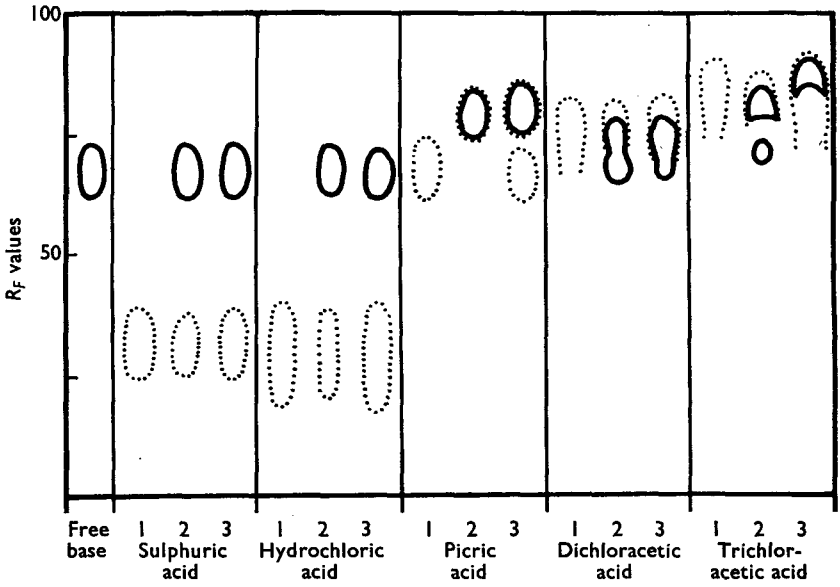

FIG. 3. Chromatograms of β-phenylethylamine developed in the butanol:acetic acid:water solvent system and applied to the paper as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

noradrenaline, dichloracetic acid and maleic acid caused isoprenaline to form two amine spots. The trichloracetate and trifluoracetate of isoprenaline yielded results similar to those obtained for these salts of adrenaline and noradrenaline, except that the R_F value of the isoprenaline spot associated with the acid was higher than for the corresponding spots produced by the slower-running amines. Increasing the relative proportion of the acid again increased the amount of the amine present in the faster spot associated with the acid.

Amines having high R_F *values* (70–100), *for example, β-phenylethylamine* (R_F 73 ± 2), *ephedrine* (R_F 75 ± 3) *and amphetamine* (R_F 76 ± 3). Figure 3 summarises the results for this group of substances. These

amines were unaffected by the presence of mineral acids even when the latter were present at a sixty molar excess; the R_F values of the individual components corresponded with those of the free base and acid.

Single amine spots associated with picric acid resulted from β-phenylethylamine, ephedrine and amphetamine picrates (R_F values of 83, 86 and 88 respectively). The R_F values of these spots were higher than those of either of the components; both ephedrine and amphetamine have higher R_F values than picric acid. Any excess picric acid gives the normal spot for the acid (i.e., one having an R_F value of 74) in addition.

Unlike the picrates, β-phenylethylamine, ephedrine and amphetamine maleates yielded a single amine spot associated with the acid having R_F values of 75, 77 and 76 which were not significantly higher than those of the free acids and bases. Excess maleic acid, if present, gave a slower-running spot (R_F 72) with ephedrine and amphetamine.

Two amine spots resulted when the stronger organic acids were present providing that the R_F values of the components differed sufficiently. The R_F value of the base associated with the trichloracetic or trifluoracetic acids was higher than that for the amines of lower R_F value and a greater proportion of the base was associated with the acid in this case.

The weaker acids, for example, tartaric, citric, benzoic or formic, did not affect the running of any of the amines.

Thus, all the strong acids affected the chromatographic behaviour of the amines unless the R_F values of the individual components differed greatly. The R_F value of the amine spot associated with the strong acids of high R_F value is dependent upon the relative values of the individual components: the higher the normal values, the faster the amine–acid spot and the greater the proportion of the base in this spot. The strength of the acid also affects the latter proportion. Acids having pKa values of greater than 3 did not affect the chromatographic behaviour of the amines under the conditions used. When the amine salt is chromatographed, the quantity applied to the paper is important. For example, elongated amine spots were obtained when increasing quantities (5, 7·5, 10, 15 and 20 μl.) of a 0·05M solution of β-phenylethylamine sulphate were applied to the paper and chromatographed; the lengths of the spots were proportional to the amount added and some overlapping of amine and acid spots occurred. However, β-phenylethylamine and the other faster amines were unaffected when 0·1 μM quantities were applied in solutions of increasing mineral acid concentration. Similarly, the amine spot was identical whether 0·1 μM adrenaline was applied as the free base or as the maleate but upward streaking occurred when larger quantities (0·2 to 0·4 μM) of the salt were chromatographed. Tailing was observed, particularly where the R_F values of the amines spots were not widely separated from those of the acids; the spots became more discrete as the amount of the amine applied was decreased, that is detection of tailing is a function of the reagent sensitivity for the amine.

Two-dimensional chromatograms of both isoprenaline and β-phenylethylamine trichloracetates were prepared using the butanol: acetic acid: water solvent system. The results for the isoprenaline salt are shown in

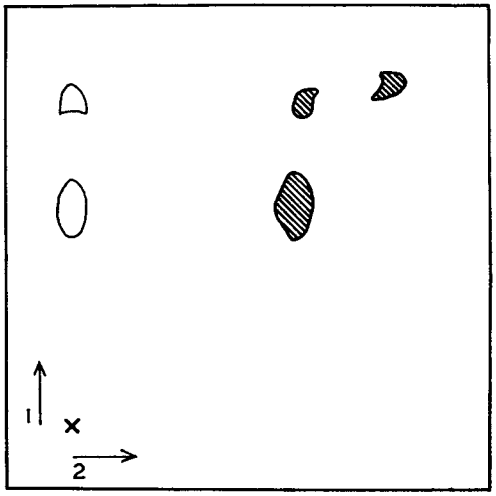

FIG. 4. Two dimensional chromatogram of isoprenaline trichloracetate (4 μl. of 0·05M solution) developed using the butanol : acetic acid : water solvent system. The unshaded spots indicate the positions of the amine after the first run.

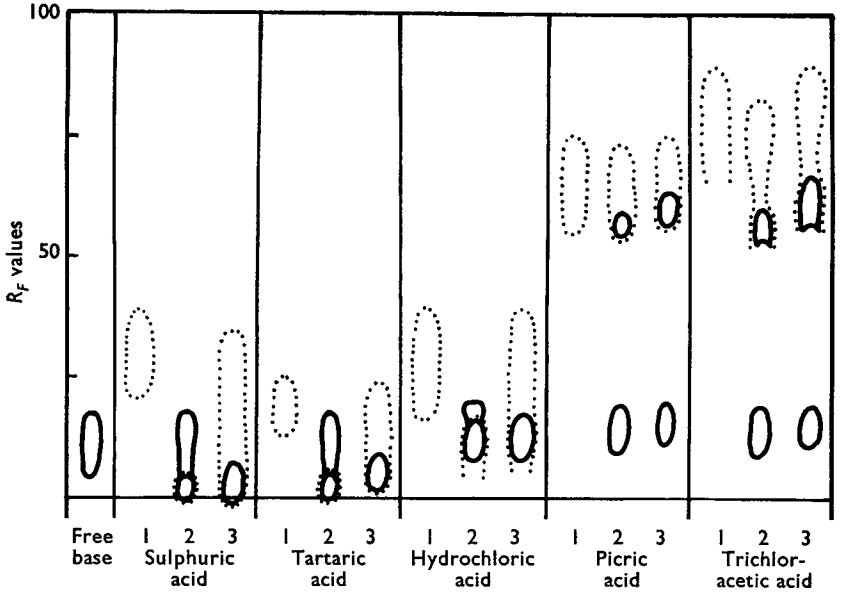

FIG. 5. Chromatograms of adrenaline developed in water-saturated butanol when applied as a suspension (2 μl.) of the free base or as a solution (2 μl.) containing various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

Figure 4 and are similar to those for β-phenylethylamine trichloracetate. The single dimensional chromatogram shows two isoprenaline spots: the faster spot (R_F 82) in which the amine is associated with acid again separates into two on re-chromatographing while the slower free amine spot persists. The shape and relative location of the two new spots in the plane at right angles to the direction of development is characteristic of the location of the base in the parent base–acid spot resulting in the single dimension chromatogram.

Results Using Water-Saturated n-Butanol Solvent

Amines having low R_F *values (less than* 40*), for example, noradrenaline* (R_F 15 ± 2), *adrenaline* (R_F 17 ± 3), *isoprenaline* (R_F 28 ± 4) *and tyramine*

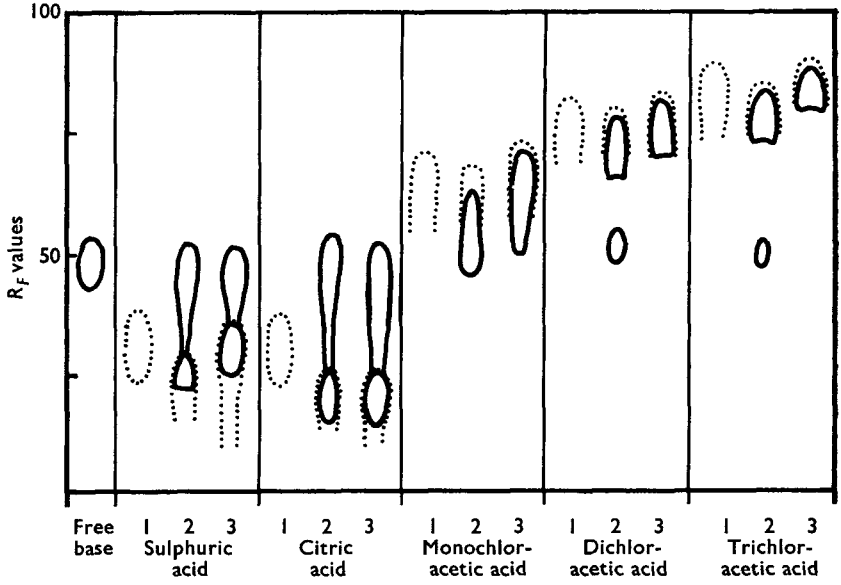

FIG. 6. Chromatograms of β-phenylethylamine developed in water-saturated butanol when applied as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

(R_F 36 ± 3). Both adrenaline and noradrenaline hydrochlorides and sulphates produced two linked spots (see Fig. 5), for example, adrenaline sulphate gave amine spots having R_F values of 4 and 16. An excess of the acid caused localisation of the amine in a single spot having a value of 7. Polybasic organic acids, such as citric and tartaric acids, caused a similar effect. Two amine spots were produced by the *o*-nitro-*p*-hydroxybenzoates, dichloracetates, trichloracetates, trifluoracetates and picrates but not the benzoates or salicylates of the above amines. For example, adrenaline trichloracetate gave amine spots having R_F values of 18 and 60

and adrenaline benzoate gave a single amine spot (R_F 17). Monochloracetic acid only caused elongation of the amine spots.

Amines of moderate R_F *values (greater than* 40*), for example, β-phenylethylamine* (R_F 52 ± 3), *amphetamine* (R_F 60) *and ephedrine* (R_F 56 ± 5). The results are shown in Figure 6. Two linked amine spots were observed

TABLE I

R_F VALUES OF THE AMINES IN THE LINKED AMINE SPOTS OBTAINED WHEN VARIOUS SALTS OF β-PHENYLETHYLAMINE AND EPHEDRINE ARE CHROMATOGRAPHED IN THE WATER: SATURATED BUTANOL SYSTEM

Substance chromatographed	R_F values of the amine spots	
β-Phenylethylamine		
Citrate	23	41
Oxalate	14	51
Hydrochloride	50	57
Sulphate	26	51
Ephedrine		
Citrate	26	50
Sulphate	26	55
Hydrochloride	42	55

when the amines in this group were applied to the paper with an equivalent amount of sulphuric acid or a polybasic organic acid and chromatographed (Table I).

When an excess of any of the acids was used, most of the amine was present in the slower-running compact spot. For example, β-phenylethylamine in excess sulphuric acid gave a dense spot of R_F 38 and a less dense spot of R_F 51.

Multiple spot formation was again observed with the stronger organic acids of high R_F values including dichloracetic, trichloracetic, trifluoracetic, salicylic, 5-nitrosalicylic and *o*-nitrobenzoic acids. Using monochloracetic acid, all the amines gave elongated spots since the values of the

TABLE II

R_F VALUES OF THREE AMINES WHEN APPLIED TO WHATMAN NO. 1 PAPER AS SOLUTIONS IN VARIOUS ACIDS AND DEVELOPED WITH THE n-BUTANOL : AMMONIA : WATER SOLVENT SYSTEM

Acid	β-Phenylethylamine		Ephedrine		Amphetamine	
	Base	Acid	Base	Acid	Base	Acid
Sulphuric	93	4	94	3	94	3
Hydrochloric	94	12	96	11	95	12
Trichloracetic	93	61	95	59	95	60
Picric	93	86	95	78	96	80
Citric	91	4	94	3	—	—
Acetic	93	—	93	—	—	—
Free base	92 ± 2		94 ± 3		95	

acids and bases were not too dissimilar. Figure 6 illustrates the results obtained using β-phenylethylamine and various acids, including the three chloracetic acids, in a neutral solvent system.

Many acids of higher pKa values, except the more volatile formic and acetic acids, affected the chromatographic behaviour of the amines in the neutral solvent system when no effect had been observed with these acids

in the acidic solvent system. Tailing was less extensive. More amine spots were associated with the acid spots in this system unless the R_F values of the components differed greatly.

Results Using Other Solvents

Results using n-butanol: ammonia: water. None of the acids tested affected the R_F values (see Table II) of the stable sympathomimetic amines (i.e., β-phenethylethylamine, ephedrine and amphetamine). All the bases containing the catechol moiety (i.e., adrenaline, noradrenaline and isoprenaline) decomposed under the conditions used.

TABLE III

R_F VALUES OF SOME SYMPATHOMIMETIC AMINES WHEN APPLIED TO WHATMAN NO. 1 PAPER AS SOLUTIONS IN VARIOUS ACIDS AND DEVELOPED USING LIQUEFIED PHENOL AS THE RUNNING SOLVENT

Acid	Adrenaline		Isoprenaline		β-Phenyl-ethylamine		Ephedrine	
	Base	Acid	Base	Acid	Base	Acid	Base	Acid
Sulphuric	44,† 77	47	82	38	91	38	93	37
Hydrochloric	54,† 75	54	83	50	91	48	93	46
Trichloracetic	82	*	89	*	92	*	94	*
Picric	81	81	82	82	93	93	92	78, 92
Salicylic	88	88	87	87	92	92	93	93
Benzoic	86	*	86	*	—	—	—	—
Citric	41,† 74	*	46,† 82	*	92	*	92	*
Tartaric	45,† 75	*	44,† 82	*	91	*	93	*
Free base	76 ± 3		82 ± 1		92 ± 1		93 ± 2	

† Tailing between two spots.
* Not detected.

Results using Liquefied Phenol. Most acids altered the chromatographic behaviour of the amines unless there was a considerable difference between the R_F values of the two components. The results are summarised in Table III. The slower amines, e.g., adrenaline (R_F76) and noradrenaline (R_F71) were affected by several acids, including citric, tartaric, hydrochloric and sulphuric acids.

Discussion

Multiple amine spot formation of sympathomimetic amines on paper chromatograms developed in neutral and acidic solvent systems in the presence of added acids is dependent upon the following major factors:

(1) the relative R_F values of the amines and the added acids;
(2) the relative dissociation constants of the acid present in the running solvent and that applied with the amine;
(3) the relative proportion of the bases and added acids and
(4) the relative local concentrations of the added acid and that present in the solvent system.

Complexing between the acid and base may alter the chromatographic behaviour of the compounds.

When an acid is present in the solution of an amine applied to a paper chromatogram which is then developed with a neutral or acidic solvent system the R_F value of the amine spot may be affected and a pure base may form two spots. If the added acid has a pKa value much less than

that of the solvent system and a normal R_F value greater than that of the free amine, double amine spot formation may be anticipated provided that the R_F values of the two components are neither too close nor too divergent. The faster amine spot will be located as a crescent at the apex of the acid spot and the slower amine spot consists of the free amine in equilbrium with the solvent. The quantity of amine present in the faster spot is dependent upon the strength of the added acid, the relative concentrations of this acid and base and the relative R_F values of the two components: the greater the concentration of acid relative to that of the amine, the greater the proportion of the amine associated with the acid in the faster spot. If sufficient excess of the acid is applied and the R_F values of the acid and base do not differ greatly, all (apparently) of the amine will be associated with acid. When double amine spot formation occurs with organic acids of high R_F values, for example, β-phenylethylamine or tyramine and either trichloracetic or trifluoracetic acids, the R_F value of the amine in the acid spot can be increased by increasing the relative proportion of acid added (see Fig. 3). The stronger the acid the greater will be the proportion of amine held in the faster spot but, with a given acid, the greater the difference between the R_F values of the free base and acid, the smaller will be the proportion of amine associated with the faster amine–acid spot. A single amine spot associated with acid is obtained when the R_F values of the two components are too close and complete separation of amine (to form one spot) and acid spots will occur when their R_F values are too divergent.

Chromatograms of amine salts (acidic solvent) in which the acid component has a lower R_F value than the amine show elongated amine spots associated with the acid, except with fast-running amines when complete separation of the acid and amine occurs. In a neutral solvent system the amine forms two linked spots, the slower of which is associated with acid: the amine–acid spot has a lower R_F value than either of the components (see Fig. 5). The slower-running amines, e.g., noradrenaline and adrenaline, will also form "spanner" or "inverted-crescent" shaped spots in the presence of an excess of a polybasic mineral acid. This may be attributed to the differing R_F values of the mono-, di- and tribasic salts of the acids.

The strength of the added acid is also important. Although the R_F value of free dichloracetic acid is closer to that of adrenaline than is that of trichloracetic acid, the presence of an equivalent amount of the latter acid (pKa 0·65) will cause formation of two amine spots, whereas, the weaker dichloracetic acid (pKa 1·29) does not produce this effect (see Fig. 1). The range of R_F values within which amines are affected by a given acid (the R_F values of the two components being not too dissimilar) is dependent upon the pKa value of that acid and to a lesser extent upon the relative concentrations of acid and base. Amines chromatographed in a neutral solvent system are affected by many weak acids.

Complex formation may also play a role in multiple spot formation of amines since some amine picrates produce an amine–acid spot having a higher R_F value than either of the components (see Fig. 3).

The use of two-dimensional chromatography with amine salts using the same solvent system yields interesting information. If two spots are formed in unidimensional chromatography, for example, isoprenaline trichloracetate (see Fig. 4), then the free amine spot will run as an entity in the second dimension and its centre will be on the line drawn in the direction of development through the centre of the parent spot; the amine associated with the acid will separate into two spots, one of free amine and one of amine associated with acid, and the shape of these spots is predictable. For example, if the acid runs faster than the amine and the amine associated with the acid is localised towards the bottom of the acid spot, then in the second development the new base spot associated with the acid will be beyond and above that of the new base spot (considering the first development to have been in the vertical plane) and will have a lower trailing tail while the new free base spot will have an upper leading horn (see Fig. 4). This situation obtains because, after the first development, the main portion of acid is located as free acid above the base associated with it so that in the second development more facile separation of the base as free base occurs from the lower portion of the base–acid spot than from the upper portion. If the acid has a lower R_F value than that of the base and two spots result, then the upper spot will run as an entity in the second dimension while the lower one will form two spots during the second development, the leading one being free base and located above the slower amine–acid spot. If an amine salt (using unidimensional chromatography) yields one amine spot completely separated from the acid component the second development will again yield a single amine spot. If a single amine–acid spot faster than either component is obtained (a complex), then the second development will again yield a single spot of amine associated with the acid spot.

The practice of applying solutions of known substances in water, acid or any other solvent to produce a reference paper chromatogram may produce misleading information if the reference chromatograms are to be compared with those of biological fluids or extracts. For instance, if during the investigation of the metabolism of an amine drug four amine spots are observed upon chromatography of urine, it does not necessarily indicate that three metabolites of the parent drug are present if one of the spots observed with the urine sample is identical with that obtained from a reference chromatogram prepared by applying a solution of the pure drug in one of the components of the solvent system. For example, pure β-phenylethylamine or pure ephedrine dissolved in urine and chromatographed using the neutral solvent system gave several amine spots; the control solution in water produced a single spot and the urine alone under the conditions used did not give any spots of R_F values similar to those produced on addition of the amines to the urine.

Various methods may be used to overcome this problem. The simplest solution would be to render the biological fluid alkaline and extract the base from the interfering acids and apply the base to the paper as a solution in one of the components of the solvent system used for chromatography. This would not be satisfactory if an acidic grouping were also

present in the molecule or if the materials were unstable to alkali. Difficulty may also occur if trichloracetic acid has been used as a protein precipitant during the preparation of the biological extract for paper chromatography, because in spite of repeated washings of the organic solvent with water, traces of the acid sufficient to affect the chromatographic behaviour of the base may be present; this effect is more serious if the solutions are made alkaline with ammonia solution rather than with sodium hydroxide solution since the partition of the trichloracetic acid into the organic phase is more favoured when ammonia is present. In these circumstances two dimensional chromatography using the same solvent may yield useful information. A spot which consists of free amine will still run as a single spot on the second dimension whereas most amine–acid spots of the first dimension will separate into two spots in the second dimension; one of these spots, and the single free amine spot, will be equi-distant from the solvent front of the second development. The use of different solvent systems is recommended, for example, if the reference compound in the biological fluid after chromatography gave more than one spot in a neutral solvent system the use of an acidic and basic solvent system is recommended. If more than one base spot is obtained using the biological extract, then it is advisable to use a control by adding the substance suspected of being present to an aliquot of the biological fluid. An increased intensity of one or more of the spots will be observed but no additional spot should appear if the added material is present in the biological extract. Further reference chromatograms obtained by adding the suspected compound to an aliquot of the biological fluid from which basic substances have been extracted is also useful: if the extracted biological fluid gives no spots upon chromatography but gives more than one spot upon the addition of the reference substance then the interference of acids may be suspected. The addition of another base substance of slightly differing R_F value to that of the suspected substance to an aliquot of the extracted biological fluid will yield one base spot of slightly different R_F value from that of the suspected compound, but the spots resulting from the association of the base with the acid will be identical with those obtained using the suspected compound in the biological extract, for example, β-phenylethylamine and ephedrine in urine samples. If metabolites of the parent drug are present, then additional spot(s) will be present in the test chromatogram when compared with the reference chromatogram obtained by applying the drug as a solution in the biological fluid tested.

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After Mr. Beaven presented the paper there was a DISCUSSION.