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The effect of chemical structure on chromatographic multiple spot formation by sympathomimetic amines in the presence of hydrochloric acid

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Of 22 sympathomimetic amines chromatographed on paper from solution in distilled water or concentrated hydrochloric acid, nine in the presence of hydrochloric acid produce multispots which are not apparent when they are chromatographed from distilled water. The presence or absence of the multiple spot phenomenon depends upon the chemical structure of each amine.

SYMPATHOMIMETIC catecholamines were recently shown by Roberts (1964) to form multiple spots when they were chromatographed on paper from solution in 10N hydrochloric acid using a phenol-hydrochloric acid solvent system. Since the amines must have been present as hydrochlorides under these conditions, the same acid was being used in the salt and the developing solvent, and the multispots must therefore have been different in origin from the spots previously reported (Shepherd & West, 1952; West, 1959; Beckett, Beavan & Robinson, 1960a,b). Because this phenomenon may occur with the use of hydrochloric acid during the preparation of biological extracts for chromatography (Roberts, 1966) we have examined the chromatographic behaviour of 22 sympathomimetic amines in detail.

Experimental

MATERIALS AND METHODS

The apparatus, materials and techniques used have been previously described (Roberts, 1963; 1964). The amines (50, 100, 200 μg) were chromatographed on Whatman No. 1 paper (washed with 0·01N hydrochloric acid) from freshly prepared solution (10 mg/ml) in distilled water or hydrochloric acid (10N). Each solution was then subjected to further chromatography at intervals until discrete spots were no longer obtained. The developing solvent was phenol containing 15% v/v 0·1N hydrochloric acid and chromatography was at 25–30° by the ascending technique until the solvent front had advanced at least 25 cm. Potassium ferricyanide (0·5 g) in sodium hydroxide solution (100 ml, 0·5N) or ninhydrin (0·25 g) in butanol (100 ml) followed by heating (120° for 3 min), were used to locate the amines. The Rf values were measured from the centre of each spot.

DRUGS

Phenethylamine hydrochloride, (–)-noradrenaline acid tartrate, dopamine hydrochloride, metanephrine (*m*-*O*-methyladrenaline), (±)-amphetamine

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sulphate, (−)-phenylpropanolamine (norephedrine) sulphate, (±)-phenylpropanolamine (*dl*-norephedrine), (+)-norpseudoephedrine hydrochloride (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate, (±)-isoprenaline sulphate (Burroughs Wellcome & Co.), L-dopa, tyramine hydrochloride (B.D.H. Ltd.), and phenylethanolamine (Aldrich Chem. Co.) were obtained commercially. Noradrenalone acid tartrate, (±)-*N*-ethylnoradrenaline, (±)-*N*-butylnoradrenaline, (±)-*N*-isobutylnoradrenaline (Prof. A. S. V. Burgen), nordefrin (α-methylnoradrenaline; Cobefrine) hydrochloride, oxedrine (1-*p*-hydroxyphenyl-2-methylaminoethanol) tartrate, phenylephrine hydrochloride (Bayer Prod. Ltd.), metaraminol acid tartrate (Merck Sharpe & Dohme Ltd.), and Epinine (3,4-dihydroxy-*N*-methylphenethylamine) hydrochloride (Dr. H. T. Openshaw) were all gifts for which we are most grateful.

Quantities quoted in the text refer to the amount of each amine calculated as base.

Results

All the results obtained are summarised in Table 1. In the presence of hydrochloric acid the Rf value of every amine (spot d, Table 1) was always significantly higher than that obtained when chromatography was from distilled water, but only 9 of the amines showed the multiple spot phenomenon; of these, adrenaline produced the maximum number of discrete multispots over a seven day period (Table 1, Fig. 1). Thereafter all spots gradually disappeared so that chromatograms of solution 8 weeks old showed nothing more than a streak from the point of application to very near the solvent front. The same sequence of events was observed when the developed chromatograms were sprayed with ninhydrin, except that all the spots now showed pink. Over the same 8-week period of time solutions of adrenaline in distilled water produced only one spot on chromatography.

Of the other 21 amines investigated, noradrenaline, *N*-ethylnoradrenaline, isoprenaline, *N*-butylnoradrenaline, *N*-isobutylnoradrenaline, as well as nordefrin, oxedrine and metanephrine all produced four or more multispots. In each instance the phenomenon was always associated with retention of amine at the application point (spot a) and a colouring of the solutions on standing. Despite differences in Rf values, colour reactions and the number of multispots produced, it was possible to correlate each spot seen with these amines with one or other of the spots seen with adrenaline under the same conditions (a–g, Fig. 1 and Table 1). All other amines giving spot c did so after only 24 hr contact with the hydrochloric acid, in contrast to the 7 days required with adrenaline. The remaining 13 amines did not show baseline retention, did not form coloured solutions on standing and, with the exception of phenylephrine which showed spot f after 24 hr, did not produce multispots relatable to those described for adrenaline.

Epinine, metaraminol, phenylethanolamine, (−)-phenylpropanolamine and amphetamine did, however, produce two spots when chromatographed

TABLE 1. THE INFLUENCE OF HYDROCHLORIC ACID (10 N) ON THE PAPER CHROMATOGRAPHIC BEHAVIOUR OF β-PHENETHYLAMINE DERIVATIVES WHEN DEVELOPED IN PHENOL CONTAINING 15% v/v 0·1N HYDROCHLORIC ACID. LETTERS a–g CORRESPOND TO THE SPOTS AS DESCRIBED IN THE TEXT AND AS ILLUSTRATED IN FIG. 1. THE ABSENCE OF Rf VALUES IN SOME COLUMNS INDICATES THAT THE RELATIVE SPOTS WERE NOT OBTAINED

Amine	Spray reagent	Rf values in distilled water	Soln colour (24 hr)		Rf values and colours of multispots in 10N hydrochloric acid						
			Water	10N HCl	a	b	c	d	e	f	g
Adrenaline acid tartrate	F	0·44 ± 0·006 Pink	Pink	Brown	0 Pink	0·30 ± 0·018 Grey	0·36 ± 0·005 Scarlet	0·48 ± 0·011 Pink	0·64 ± 0·011 Blue	0·71 ± 0·011 Turquoise	0·77 ± 0·009 Pink
Noradrenaline acid tartrate	F	0·22 ± 0·006 Pink	Pink	Blue	0 Pink	0·02 ± 0·001 Brown	0·13 ± 0·007 Scarlet	0·21 ± 0·008 Pink	0·40 ± 0·012 Blue		0·60 ± 0·008 Pink
N-Ethylnoradrenaline hydrochloride	F	0·57 ± 0·011 Pink	Pink	Brown	0 Pink	0·53 ± 0·009 Blue	0·51 ± 0·013 Scarlet	0·60 ± 0·010 Pink	0·70 ± 0·008 Blue	0·73 ± 0·009 Turquoise	0·81 ± 0·008 Pink
Isoprenaline sulphate	F	0·63 ± 0·005 Pink	Pink	Brown	0 Pink		0·61 ± 0·007 Scarlet	0·66 ± 0·008 Pink	0·75 ± 0·006 Pink	0·73 ± 0·006 Turquoise	0·84 ± 0·011 Pink
N-Butylnoradrenaline hydrochloride	F	0·63 ± 0·007 Pink	Pink	Brown	0 Pink		0·63 ± 0·009 Scarlet	0·66 ± 0·009 Pink	0·75 ± 0·011 Blue	0·75 ± 0·004 Turquoise	0·83 ± 0·012 Pink
N-Isobutylnor-adrenaline hydrochloride	F	0·64 ± 0·005 Pink	Pink	Brown	0 Pink		0·64 ± 0·012 Scarlet	0·70 ± 0·004 Pink	0·78 ± 0·006 Blue	0·76 ± 0·003 Turquoise	0·85 ± 0·006 Pink
Nordefrin hydrochloride	F	0·36 ± 0·004 Orange	Pink	Brown	0 Pink	0·08 ± 0·012 Pink		0·34 ± 0·014 Orange	0·49 ± 0·020 Pink		0·69 ± 0·011 Mauve
Dopamine hydrochloride	F	0·40 ± 0·003 Dark brown	Brown	—				0·44 ± 0·008 Dark brown			
Epinine hydrochloride	F	0·38 ± 0·004 Orange 0·63 ± 0·005 Crimson	Brown	—				0·42 ± 0·004 Orange 0·66 ± 0·005 Crimson			
Noradrenalone acid tartrate	F	0·19 ± 0·007 Brown	Brown	—				0·24 ± 0·020 Brown			
L-Dopa	N	0·13 ± 0·008 Orange 0·26 ± 0·006 Yellow	Brown	—				0·15 ± 0·010 Orange			
Oxedrine acid tartrate	N	0·15 ± 0·006 Orange 0·73 ± 0·015 Mustard	—	Green	0 Pink	0·58 ± 0·008 Mauve		0·14 ± 0·008 Orange 0·75 ± 0·008 Mustard	0·83 ± 0·008 Pink		0·91 ± 0·007 Pink
Metanephrine	N	0·78 ± 0·011 Mauve	—	Brown	0 Pink	0·55 ± 0·009 Pink		0·78 ± 0·006 Mauve			0·87 ± 0·009 Pink
Tyramine hydrochloride	N	0·64 ± 0·008 Grey	—	—				0·64 ± 0·015 Grey			
Phenylephrine hydrochloride	N	0·67 ± 0·008 Mauve	—	—				0·67 ± 0·005 Mauve		0·78 ± 0·007 Orange	
Metaraminol acid tartrate	N	0·10 ± 0·002 Orange 0·40 ± 0·005 Mauve	—	—				0·16 ± 0·005 Orange 0·52 ± 0·014 Mauve			
Phenylethanolamine	N	0·59 ± 0·007 Pink 0·79 ± 0·003 Yellow	—	—				0·64 ± 0·006 Pink 0·74 ± 0·003 Yellow			
Phenethylamine hydrochloride	N	0·79 ± 0·007 Purple	—	—				0·80 ± 0·007 Purple			
(−)-Phenylpropanol-amine sulphate	N	0·70 ± 0·006 Mauve	—	—				0·09 ± 0·002 Black* 0·70 ± 0·010 Mauve			
(±)-Phenylpropanol-amine sulphate	N	0·68 ± 0·004 Mauve	—	—				0·08 ± 0·002 Black* 0·70 ± 0·007 Mauve			
(+)-Norpseudo-ephedrine hydrochloride	N	0·69 ± 0·005 Mauve	—	—				0·70 ± 0·007 Mauve			
Amphetamine sulphate	N	0·81 ± 0·013 Pink	—	—				0·10 ± 0·002 Black* 0·82 ± 0·007 Pink			

F = Ferricyanide; N = Ninhydrin; * = Char spot due to sulphate

in the presence of 10N hydrochloric acid. Of these, the first three showed similar double spots when chromatographed from solution in distilled water and no further explanation of the phenomenon was sought. The remaining three amines showed only single spots when chromatographed from distilled water, but the extra spots obtained in the presence of hydrochloric acid had identical Rf values. These spots were characterised by a charring of the paper, and were subsequently shown to be associated with the use of amine sulphates when ninhydrin followed by heating was used to detect the amines. The hydrochloric acid presumably displaces the sulphate which then undergoes chromatographic separation as sulphuric acid.

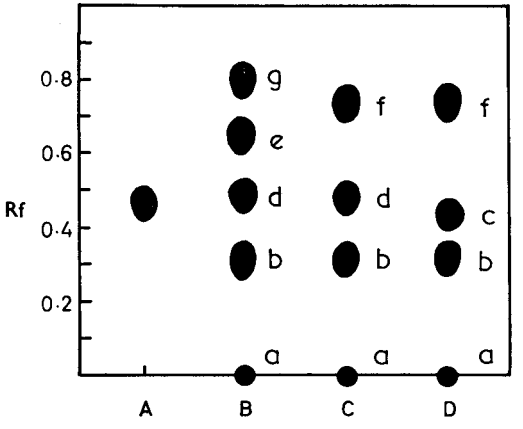

FIG. 1. Multiple spot phenomena exhibited by adrenaline acid tartrate (200 μg) when chromatographed from hydrochloric acid (10 N) immediately after (B), 24 hr after (C) and 7 days after (D), preparing the solution 10 mg/ml. At A, adrenaline acid tartrate chromatographed from solution in distilled water. Developing solvent, phenol containing 15% v/v 0·1 N HCl. The spots are labelled a - g to correspond with the text and Table 1.

Conversely, L-dopa formed two spots when chromatographed from solution in distilled water, but only one when 10N hydrochloric acid was used. A possible explanation of this phenomenon is that the dilute acid conditions prevailing in the developing solvent cause only partial conversion of the dopa in aqueous solution to the zwitterion form, in contrast to the complete conversion expected in the presence of 10N hydrochloric acid.

Discussion

Adrenaline and phenethylamine both form discrete single spots when chromatographed on paper from solution in distilled water, but whereas chromatography from 10N hydrochloric acid again results in the formation of one spot (d) only from phenethylamine, under these conditions the production of six additional spots is demonstrated with adrenaline. An

examination of Table 1 indicates that this difference in the chromatographic behaviour of the two amines results from substitution of the phenethylamine molecule.

The substituent common to all the amines showing multiple spot formation is an alcoholic β-hydroxyl group in the side-chain. When this group is removed (adrenaline to Epinine, noradrenaline to dopamine) or replaced by a ketone oxygen (noradrenaline to noradrenalone) the multiple spot phenomenon is not observed. However, the presence of an alcoholic β-hydroxyl group alone [phenylethanolamine, (–)-phenylpropanolamine] is not in itself sufficient to result in the formation of multispots, for extra spots in the presence of 10N hydrochloric acid are only seen when the benzene ring also contains an hydroxyl substituent. The position of this phenolic hydroxyl group is important. Its presence in the *para*-position (oxedrine) is associated with the production of a coloured solution in 10N hydrochloric acid, and with the formation of spots a (retention at the point of application), b, e and g. In contrast, *meta*-substitution of a phenolic hydroxyl group (phenylephrine) is associated solely with the formation of spot f from acid solutions of amine 24 hr old. The presence of spot c, on the other hand, is only evident when the alcoholic β-hydroxyl group is accompanied by both *meta*- and *para*-phenolic hydroxyl groups.

Catecholethanolamines are therefore expected to produce a total of seven spots (a–g) following chromatography from solution in 10N hydrochloric acid. Of the eight amines investigated, however, adrenaline and *N*-ethylnoradrenaline are the only ones which fulfil this expectation. The results with the other amines indicate a further set of structural requirements for the formation of individual spots which influence their formation even when phenolic and alcoholic β-hydroxyl groups are present. Spot f, for example, is not evident in the absence of substitution on the primary amino nitrogen (metaraminol, noradrenaline, nordefrin) or when the *meta*-hydroxyl group is *O*-methylated (metanephrine). On the other hand, when the *N*-alkyl substituent is greater than ethyl (isoprenaline, *N*-butylnoradrenaline, *N*-isobutylnoradrenaline) spot b is not formed. In addition, the substitution of a methyl group on the α-carbon atom, as in nordefrin, prevents it forming a spot equivalent to c although it is a catecholethanolamine with a primary amine nitrogen.

Several points of interest arise from our findings. When hydrochloric acid is used during the production of concentrated extracts of biological material for chromatography, the number of spots obtained need not necessarily indicate the number of amines present in the starting material (Roberts, 1966). This could have special significance during the investigation of the metabolism of a pure amine. Furthermore, we have found that the substances responsible for the spots corresponding to c are dibenzocycloheptatrienes of the type described by Kawazu (1958a,b). The close structural resemblances of these compounds to the antidepressive iminodibenzyl derivatives prompted us to postulate an *in vivo* synthetic route and a depressive pathological function for noradnamine, the noradrenaline dibenzocycloheptatriene (Roberts & Broadley, 1965). It may be significant that following the intracisternal administration to cats some

sympathomimetic amines caused arousal while others caused stupor (Leimdorfer, 1950); those amines which resulted in stupor are those which in our experiments formed multispots in the presence of hydrochloric acid.

References

Beckett, A. H., Beaven, M. A. & Robinson, A. E. (1960a). *Nature, Lond.*, **186**, 775–776.
Beckett, A. H., Beaven, M. A. & Robinson, A. E. (1960b). *J. Pharm. Pharmac.*, **12**, *Suppl.*, 203*T*–216*T*.
Kawazu, M. (1958a). *J. pharm. Soc. Japan*, **78**, 399–410.
Kawazu, M. (1958b). *Ibid.*, **78**, 974–982.
Leimdorfer, A. (1950). *J. Pharmac. exp. Ther.*, **98**, 62–71.
Roberts, D. J. (1963). *J. Pharm. Pharmac.*, **15**, 532–537.
Roberts, D. J. (1964). *Ibid.*, **16**, 549–556.
Roberts, D. J. (1966). *Biochem. Pharmac.*, in the press.
Roberts, D. J. & Broadley, K. J. (1965). *Lancet*, **1**, 1219–1220.
Shepherd, D. M. & West, G. B. (1952). *Nature, Lond.*, **169**, 797.
West, G. B. (1959). *J. Pharm. Pharmac.*, **11**, 595–599.