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CHROMATOGRAPHIC EVIDENCE FOR THE FORMATION AS AN ARTIFACT OF AN ISOPRENALINE-LIKE SUBSTANCE FROM ADRENALINE

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Abstract—Samples of chromatographically pure adrenaline subjected to extraction techniques designed for biological tissues and fluids produced more than one spot when chromatographed on paper. The formation of these multiple spots was shown to be associated with the use of hydrochloric acid during the extraction procedures and similar spots were demonstrated when the adrenaline was chromatographed from simple solution in this acid. In each case one of the extra spots had an R_f value similar to that of isoprenaline and it is suggested that the isoprenaline-like metabolite of adrenaline reported to be naturally occurring may be an extraction/chromatography artifact. The experimental evidence supporting this suggestion is discussed.

TRACE quantities of a substance indistinguishable from isoprenaline by its colour reactions, by its chromatographic behaviour, or by its pharmacological activity have been found in adrenal glands[1–3] and in blood perfusing cat heart–lung preparations during stimulation of the upper thoracic sympathetic chains.[4] Intravenous administration of adrenaline,[5–7] but not that of noradrenaline,[6] caused the appearance of a similar substance in blood withdrawn from the lower abdominal aortae of anaesthetised cats and rabbits. The formation of this substance was prevented by pyrogallol but not by pretreatment with reserpine, cocaine, phentolamine, dibenamine, harmaline or iproniazid, and it was suggested by Eakins and Lockett[6] that the *O*-methyl transferase system played an essential part in the formation of the isoprenaline-like material as a metabolite of adrenaline. Isoprenaline-like substances have also been demonstrated in extracts of cat blood collected during acute haemorrhages, rabbit heart[9] and human urine.[10] Paper chromatography was used in all of the above investigations but it has been shown that adrenaline and similar compounds can form multiple spots when chromatographed on paper.[11–15] In most cases the multiple spot phenomena were attributable to the use of different acids in the salt and developing solvent systems, but the multiple spots demonstrated by Roberts[15] when sympathomimetic catecholamines were chromatographed from hydrochloric acid (10N) in a phenol–hydrochloric acid solvent system were of different origin. Under these conditions, one of the additional spots formed from adrenaline had an R_f value similar to that of isoprenaline, and it was thought that the practice of evaporating hydrochloric acid–ethanol extracts to small volume might result in sufficient concentration of the

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acid to cause the formation of the substances of higher R_f value discovered by the use of 10N hydrochloric acid.[15]

Experiments have therefore been designed to investigate further the formation of artifact spots from pure adrenaline with special reference to the use of hydrochloric acid during extraction of biological tissues and fluids.

METHODS

The apparatus, materials and techniques used for chromatography have been previously described.[15, 16]. (−)-Noradrenaline acid tartrate, (−)-adrenaline base (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were chromatographed on Whatman No. 1 paper (previously washed with 0·01N HCl and dried) from distilled water, hydrochloric acid or acetone:ethanol (1:1) solution. The developing solvent was phenol containing 15% (v/v) 0·1N HCl unless otherwise stated, and the amines were located by spraying the developed chromatograms with potassium ferricyanide (0·44g) in sodium hydroxide (100 ml, 0·5N). Extracts of cat adrenal glands were prepared as described by Lockett[2] and plasma extracts were made according to the methods of either Vogt[17] or Roberts[18] in all cases reference chromatograms of noradrenaline, adrenaline and isoprenaline (25 μg of each amine in 0·01 ml distilled water) were developed in parallel with the test solutions on the same sheets of paper. Concentrations of adrenaline quoted in the text refer to the amount of amine calculated as base, but in all experiments both base and acid tartrate salt were used indiscriminately with no evident differences in their chromatographic behaviour. Melting point determinations and potentiometric titrations failed to demonstrate any impurities in either of the adrenaline samples used.

RESULTS

In confirmation of the results of Lockett[1, 2] and Subrahmanyan,[3] three spots, having R_f values equivalent to those of noradrenaline, adrenaline and isoprenaline, were obtained when aliquotes of extracts of cat adrenal gland were chromatographed. Quantities of adrenaline (250 μg) equivalent to that which might be expected to be present in the adrenal gland of a cat were also subjected to the extraction process[2] as if they were in fact whole glands. When the developed chromatograms of these 'extracts' were oxidised, two pink spots were obtained from each sample; an intense one with an R_f value equivalent to that of adrenaline and a fainter one with an R_f value similar to that of isoprenaline. Similarly solutions of adrenaline (75 μg in 0·1 ml distilled water) were added to 5 ml samples of cat plasma from which extracts were then prepared[17, 18] and to 50 ml samples of acid–ethanol (0·1 ml N HCl/100 ml ethanol) or of hydrochloric acid (0·01 N) which were then evaporated to dryness. The residues were chromatographed from acetone:ethanol solution. In each case the oxidised chromatograms showed an 'isoprenaline' spot as well as an adrenaline one, although displacement of adrenaline by lipid not removed from the plasma by one of the extraction methods[16] resulted in the two spots merging. On occasions a third spot appeared below adrenaline. In contrast, when hydrochloric acid was omitted from the plasma extraction techniques, and when solutions of adrenaline in ethanol or distilled water alone were evaporated to dryness and chromatographed, only single spots, at the

adrenaline R_f value, were obtained on oxidation. These results are summarised in Fig. 1.

In each case, therefore, hydrochloric acid was somehow responsible for the formation of the extra spots and the question arose as to whether or not this was related to the multiple spot phenomenon seen with adrenaline in the presence of high concentrations (10 N) of this acid.[15] It was readily shown, using a Pye pH meter and a glass

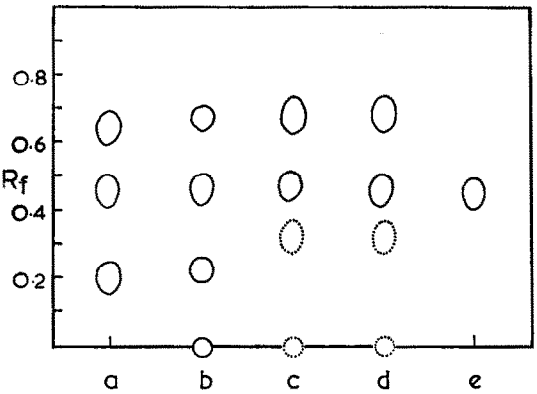

FIG. 1. The influence of extraction procedures on the chromatographic behaviour of adrenaline. From left to right the chromatograms represent a, noradrenaline (lower spot), adrenaline and isoprenaline (upper spot) applied from solution in distilled water; b, cat adrenal gland extract prepared according to the method of Lockett[2]; c, extract of plasma containing 75 μg adrenaline prepared as described by Roberts[18]; d, adrenaline (75 μg) dissolved in acid–ethanol, evaporated to dryness and redissolved in acetone–ethanol; e, adrenaline (75 μg) dissolved in ethanol, evaporated to dryness and redissolved in acetone–ethanol (see text). Dotted outlines indicate that these spots were not always seen. Developing solvent, phenol containing 15% (v/v) 0·1 N HCl.

electrode, that evaporation of dilute hydrochloric acid and acid–ethanol to small volume under reduced pressure resulted in concentration of the acid above normal (N/1), but the formation of constant boiling mixtures prevented concentration to 10 N HCl. Adrenaline (25–200 μg) was therefore chromatographed from solution in distilled water or hydrochloric acid (0·1–10 N) in order to determine the limiting concentration of acid required to produce the extra spots. The number of visible multispots was dependent on both the strength of the hydrochloric acid and the amount of amine chromatographed, but the first and most well defined 'extra' spot formed from adrenaline was usually the one with an R_f value similar to that of isoprenaline. The minimum concentrations of hydrochloric acid required to produce this spot from the different amounts of amine chromatographed were, from 25 μg, not seen at any concentration of acid; from 50 μg, 5 N HCl; from 100 μg, 2·5 N HCl and from 200 μg, N HCl.

In contrast to the results described above, no spot of any description was observed above the adrenaline R_f value when adrenaline (100 μg) was chromatographed from 10 N hydrochloric acid to which pyrogallol (10 mg/ml) had been previously added. Retention at the application point was also prevented. The adrenaline spot itself was masked by the dark brown spot of pyrogallol which had a similar R_f value, and the elongated purple spot below the adrenaline R_f value could not be correlated with any

of the multispots obtained in the absence of pyrogallol; its origin was not investigated. The presence of pyrogallol, therefore, prevented the formation of all of the artifact spots normally seen when adrenaline was chromatographed from 10 N hydrochloric acid alone (Fig. 2).

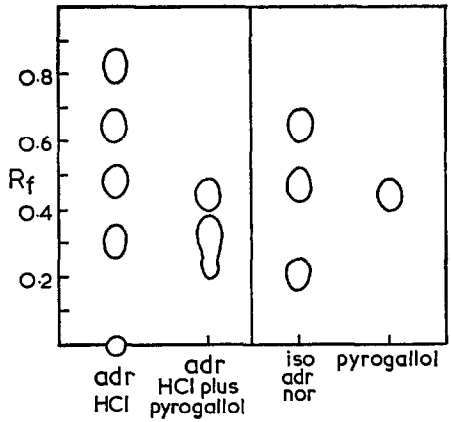

FIG. 2. The influence of pyrogallol on the formation of multispots from adrenaline (adr) in the presence of 10 N hydrochloric acid (left hand panel). The spots obtained with isoprenaline (iso), adrenaline (adr), noradrenaline (nor) and pyrogallol when chromatographed from aqueous solution are shown on the right for comparison. Developing solvent, phenol containing 15% (v/v) 0·1 N HCl.

Further information was obtained by developing two dimensional chromatograms of adrenaline (100 μg) in 10 N hydrochloric acid. Single dimensional chromatograms simultaneously prepared under identical conditions showed four spots (plus retention at the point of application) on oxidation, and the locations of all of these spots when developed in the second direction was characteristic of the locations of the spots obtained in the first direction (Fig. 3).

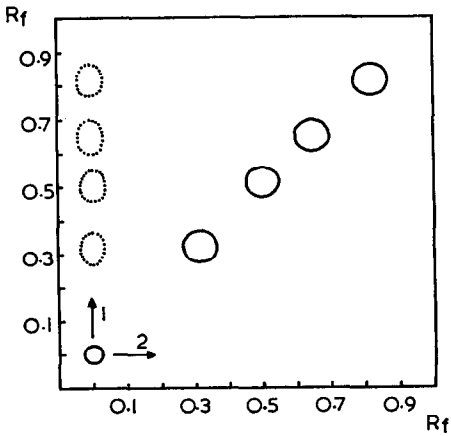

FIG. 3. Two dimensional chromatogram of adrenaline base (100 μg) applied to the paper from solution in 10 N hydrochloric acid. The dotted outlines represent the positions of the spots after development in the first direction. Solvent system phenol containing 15% (v/v) 0·1 N HCl.

Multiple spots were also obtained on chromatograms of adrenaline (100 μg) in 10 N hydrochloric acid when butanol saturated with either 0·5 N hydrochloric acid or 16·7% (v/v) aqueous acetic acid were used as the developing solvents; in each case one of the spots had an R_f value similar to that of isoprenaline (Fig. 4).

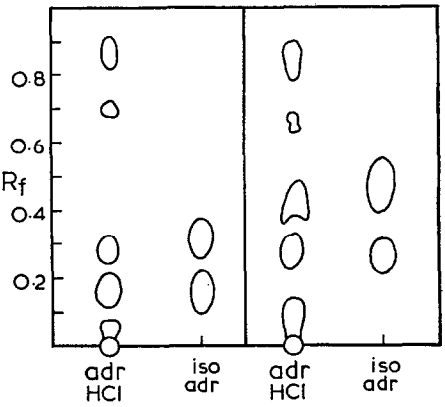

FIG. 4. The influence of hydrochloric acid (10 N) on the chromatographic behavior of adrenaline acid tartrate (200 μg) when developed in butanol saturated with either 0·5 N hydrochloric acid (left hand panel) or 16·7% (v/v) acetic acid (right hand panel). In each case the R_f values of isoprenaline (upper spot and adrenaline (lower spot) obtained following chromatography from aqueous solution are shown on the right for comparison.

DISCUSSION

The results obtained in these and previous experiments indicate that the isoprenaline-like substance(s) which have been demonstrated following chromatography of extracts of biological tissues and fluids could have resulted from the formation of multiple spots as artifacts during the processes of extraction and/or chromatography (Table 1).

Although this present study has indicated that high concentrations of adrenaline are required to produce an 'isoprenaline-like' spot in the concentrations of hydrochloric acid expected to occur during the extraction techniques, the same is true of the 'physiological' isoprenaline-like substances since pink spots at the isoprenaline R_f value have only been satisfactorily demonstrated from adrenal gland extracts. At least 1 μg (as base) of catecholamine is required to show up as a visible pink spot on oxidation with ferricyanide[16] so that trace amounts of isoprenaline-like substances of either physiological or artifact origin could be formed from smaller amounts of adrenaline under more dilute acid conditions and remain undetected by the spray reagent; these trace amounts should, however, be readily detectable by pharmacological means.

The results obtained using two dimensional chromatography suggest that the multiple spots are due to the formation of definite stable chemical structures, and since pyrogallol is a strong reducing agent, the absence of the multiple spot phenomenon in the presence of this polyhydric phenol might indicate that the substances responsible for the spots are oxidation products, perhaps intermediate between adrenaline and adrenochrome. Supporting evidence that this might be so comes from

TABLE 1. A COMPARISON OF THE CHARACTERISTICS OF THE ISOPRENALINE-LIKE SUBSTANCES IN BIOLOGICAL EXTRACTS AND THE ISOPRENALINE-LIKE MULTIPLE SPOTS FORMED *in vitro*

Isoprenaline-like substances	Multiple spots
Pink spot obtained at the isoprenaline R_f value when chromatograms sprayed with alkaline ferricyanide [1, 3, 10].	Pink spot obtained at the isoprenaline R_f value when chromatograms sprayed with alkaline ferricyanide [15], this paper.
Extraction techniques involved the use of hydrochloric acid [1–8, 10].	Multiple spots only demonstrable when hydrochloric acid present [15], this paper.
They are produced as a 'metabolite' of adrenaline but not of noradrenaline.[6]	Both adrenaline and noradrenaline give multiple spots but only adrenaline gives one with an isoprenaline R_f value.[15]
The yield of metabolite following i.v. injection of adrenaline is increased in the presence of monoamine oxidase inhibitors while pyrogallol (*O*-methyl transferase inhibitor) prevents its formation altogether.[6]	The spots increase in intensity and distinction with increase in the amount of adrenaline chromatographed while pyrogallol prevents their formation altogether (this paper).
Metabolite demonstrated in eluates cut at the isoprenaline R_f value when extracts chromatographed in developing solvents other than the usual phenol-HCl system.[4]	Multiple spots demonstrated on chromatograms developed in solvents other than the usual phenol–HCl system, and although none of the spots had R_f values equivalent to those of isoprenaline, in each case one of them is similar enough to make it certain that at least half of the substance responsible for the spot would be eluted from a strip cut at the isoprenaline R_f value (this paper).

the fact that whereas solutions of adrenaline in distilled water or concentrated hydrochloric acid colour on standing and then deposit black insoluble products, similar solutions in more dilute hydrochloric acid remain colourless and form no deposits. Since the black product is probably a melanin formed from adrenochrome,[19] oxidation of adrenaline in the presence of concentrated hydrochloric acid has presumably occurred. In contrast dilute hydrochloric acid is known to inhibit the oxidation of adrenaline. Many different oxidation products of adrenaline (adrenalone, adrenaline quinone, adrenalutin, adrenochrome, oxoadrenochrome, adrenoxine, etc.)[19] have been shown to exist and there are enough of these to account for the munber of multispots seen in the presence of hydrochloric acid. In the absence of confirmatory chemical evidence, however, further speculation at this time is unwise.

The significance of the artifact spots in terms of the natural occurrence of an isoprenaline-like substance is also questionable, for Lockett[4] was able to demonstrate isoprenaline-like activity in blood samples in the absence of any extraction or chromatographic procedures. Furthermore, although it has been possible to demonstrate falls in rat blood pressure with elutates prepared from strips cut at the isoprenaline R_f value from chromatograms of adrenaline in 10 N hydrochloric acid, in the presence of pronethalol the action of these elutes is pressor. In the opinion of the present author, however, the similarities between the isoprenaline-like substances and the chromatographic artifact (Table 1) are too great to be co-incidental, and it is hoped that further experiments will resolve the controversy over the natural occurrence of 'isoprenaline' in mammalian bodies.

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