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Research Papers

Some possible causes of pharmacological activity in blank eluates following the separation of sympathomimetic catecholamines by paper chromatography

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Eluates prepared from paper chromatograms at Rf values and under experimental conditions that were expected to eliminate the possibility of contamination with catecholamines were shown to inhibit the rat uterus and to cause a fall in the mean arterial blood pressures of anaesthetised rats. These isoprenaline-like actions have been shown to be due to inadequate removal of the phenolic developing solvent and to the use of sodium dihydrogen phosphate solutions as eluants. Attempts to establish qualitative and quantitative relationships between the chromatography blank and isoprenaline have failed, and it is concluded that these factors could not alone be responsible for the isoprenaline-like substance reported to occur naturally in various species of mammal.

METHODS similar to the modification of Vogt (1952) of the recommendations of Crawford & Outschoorn (1951) for the quantitative separation of catecholamines from biological extracts and tissue fluids by paper chromatography gave inconsistent answers. There were variable recoveries, deviation of the results of the biological assay from parallelism, and the presence of pharmacological activity at Rf values other than those of noradrenaline, adrenaline and isoprenaline when estimating concentrations of these amines in rabbit and cat plasma. The inconsistent results were shown to be largely due to displacement of catecholamine Rf values by lipid present in the plasma samples (Roberts, 1963b), but even after prior extraction of this interfering lipid the recoveries of small "physiological" amounts of catecholamine were inconsistent and the biological assays were still not parallel especially when the eluate volumes were in excess of 2 ml.

The results of these recovery experiments (Table 1) indicated that one or more substances were contaminating the eluates resulting in the antagonism of the actions of adrenaline and noradrenaline on the blood pressure of pithed rats. As an additional or alternative feature, the contaminant(s) potentiated the action of isoprenaline on the blood pressure of rats under pentobarbitone sodium anaesthesia. It also seemed possible that the contaminant(s) caused inhibition of the isolated tissue preparations used, with resultant apparent potentiation of the actions of all three amines on these tissues. Furthermore, pharmacological activity was still evident in blank eluates prepared from strips of paper cut at levels corresponding to Rf values other than those of the sympathomimetic catecholamines, and this

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activity (fall in mean arterial blood pressure of the rat and the inhibition of acetylcholine-induced contractions of the isolated rat uterus) seemed likely to be due to the same substance(s) as that which was interfering with the biological assays of the catecholamines.

A modified form of the paper chromatographic techniques of Vogt (1952) was used to demonstrate the presence of an isoprenaline-like substance in the adrenal glands of cat, rabbit, monkey and man (Lockett, 1954), and in cat plasma after electrical stimulation of the sympathetic chains (Lockett, 1957) and after the injection of adrenaline (Eakins & Lockett, 1961). Since the pharmacological activity of the blank eluates noticed in this laboratory on the rat blood pressure and isolated rat uterus was basically isoprenaline-like, there existed the possibility that this chromatographic blank and the isoprenaline-like substance of Lockett and her colleagues was the same substance(s). It was decided to test this possibility and to investigate the source of the activity of the blank eluates.

Methods

Whatman No. 1 papers for chromatography were washed and prepared (Roberts, 1963a, b) and were placed into tanks which were then gassed with carbon dioxide. No amines were applied to the papers. The solvent, phenol containing 15% v/v 0·1 N hydrochloric acid, was allowed to travel 25–30 cm up the paper before "chromatography" was stopped, and the papers were washed with benzene. Eluates were then prepared from strips of paper cut at levels corresponding to the estimated Rf values of noradrenaline, adrenaline and isoprenaline and from strips cut at a variety of other levels, using as eluant a solution containing 0·4 g sodium dihydrogen phosphate and 1 mg ascorbic acid per 100 ml distilled water and employing the techniques described by Crawford & Outschoorn (1952). The activity of these eluates, of initial volume before concentration, 2·2–7·6 ml, was examined on the rat mean arterial blood pressure (pithed or under pentobarbitone sodium anaesthesia) and on the rat uterus responding constantly and submaximally to acetylcholine. The following solutions were also prepared and their activity similarly examined on these biological preparations: (1) 0·01 ml of the phenolic chromatography solvent dissolved in 10 ml 0·9% w/v aqueous sodium chloride; (2) distilled water; (3) 0·9% w/v aqueous sodium chloride; (4) 1·8% w/v aqueous sodium chloride; (5) 3·6% w/v aqueous sodium chloride; (6) 1·0 ml 0·4% w/v aqueous sodium dihydrogen phosphate evaporated to dryness (1 mm Hg, bath temperature 30–35°) and redissolved in 1·0 ml 0·9% w/v aqueous sodium chloride; (7) 5·0 ml, 0·4% w/v aqueous sodium dihydrogen phosphate, dried and redissolved as in 6 above; (8) 10·0 ml 0·4% w/v aqueous sodium dihydrogen phosphate, dried and redissolved as in 6 above; (9) 0·01% w/v ascorbic acid in 0·9% w/v aqueous sodium chloride; and (10) hydrochloric acid (0·01 N).

Recovery experiments were made on 0·1, 0·25 or 0·5 μg quantities of noradrenaline, adrenaline and isoprenaline, each calculated as base, added to cat plasma (5–25 ml) which had been standing at room temperature for

24 hr. Protein and lipid-free extracts of the plasma samples were prepared and chromatographed as 10 cm bands from acetone: ethanol solution (Roberts, 1963b). Eluates were prepared using either a solution containing 0·4 g sodium dihydrogen phosphate and 1 mg ascorbic acid per 100 ml distilled water (Table 1) or distilled water alone (Table 2) as eluants, and elution times varied from 6 to 15 hr. The activity of these eluates was examined as described above.

Drugs. (−)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially.

Results

Marked pharmacological activity was demonstrated in eluates prepared from all areas of the "blank" chromatograms and the activity of eluates prepared from strips of paper representing the Rf values of noradrenaline, adrenaline and isoprenaline was in no way different from that found in eluates obtained from other areas. Solutions prepared from large volumes of eluate were, however, more active than those prepared from smaller volumes. The following results therefore apply to all areas of the paper. The numbers in brackets relate to the solutions described under "Methods" above.

BLOOD PRESSURE OF A PITHED RAT

The blank eluates usually produced an initial rapid rise, which was always immediately followed by a longer lasting fall, in the mean arterial blood pressures of pithed rats when injected intravenously. Both the rise and the fall were increased with increase in dose. Similar rapid rises in blood pressure were observed when the dilute solution of the phenolic solvent (1) was injected but these were not followed by a depressor phase (Fig. 1, left hand record). When the blank eluates and the phenolic

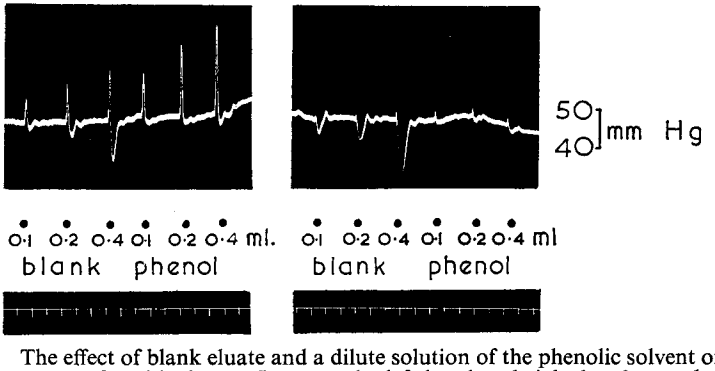

FIG. 1. The effect of blank eluate and a dilute solution of the phenolic solvent on the blood pressure of a pithed rat. Between the left hand and right hand records both solutions were washed with benzene, evaporated to dryness and redissolved. All injections i.v. Time trace in min.

solution were vigorously washed three times with equal volumes of benzene (the benzene layers after separation being sucked off through a capillary pipette), and evaporated to dryness before being redissolved in isotonic saline, the pressor activity was no longer evident in either solution when injected into the rats. The blank eluates, however, still showed marked depressor activity (Fig. 1, right hand record).

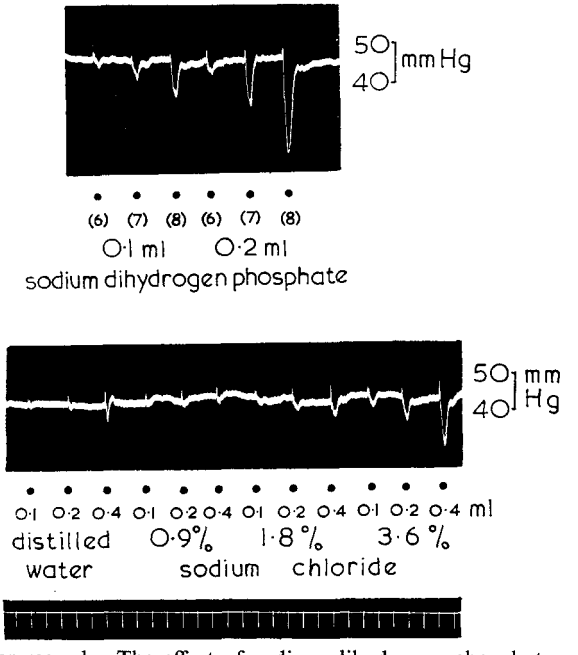

FIG. 2. Upper record. The effect of sodium dihydrogen phosphate on the blood pressure of a pithed rat. (6) = 1 ml, (7) = 5 ml, and (8) = 10 ml. 0·4% w/v $NaH_2PO_4.2H_2O$ evaporated to dryness and redissolved in 1 ml 0·9% w/v aqueous sodium chloride solution (see text).

Lower record. The effect of distilled water, and isotonic and hypertonic sodium chloride solutions on the blood pressure of a pithed rat. All injections i.v. Time trace in min.

Similar falls in blood pressure were observed on injecting solutions (6), (7) and (8), which represented the amount of sodium dihydrogen phosphate expected to be present in eluates of volume 1, 5 and 10 ml respectively. The size of fall increased with the dose volume of any one solution and with increasing concentration of sodium dihydrogen phosphate (Fig. 2, upper record). Depressor activity was also evident when hypertonic sodium chloride solutions were used (4 and 5), the size of the fall again increasing with dose and with increasing hypertonicity (Fig. 2, lower record). Small falls were occasionally seen when distilled water (2) was injected, but isotonic sodium chloride solution (3) was invariably without effect on the blood pressure (Fig. 2, lower record) as were usually ascorbic acid (9) and dilute hydrochloric acid (10).

BLOOD PRESSURE OF RAT UNDER PENTOBARBITONE SODIUM ANAESTHESIA

The blank eluates always produced a fall in blood pressure when injected intravenously into anaesthetised rats, the magnitude of the response increasing with increase in dose volume. No initial rapid rise in blood pressure was seen in these preparations. Depressor activity was also evident when the dilute solution of the phenolic solvent (1), the sodium dihydrogen phosphate solutions (6, 7 and 8) and the hypertonic sodium chloride solutions (4 and 5) were injected. The size of the response to any one solution increased with increase in dose volume and the more concentrated the solution of sodium dihydrogen phosphate or sodium chloride then the greater the fall in blood pressure (Fig. 3). The dose-

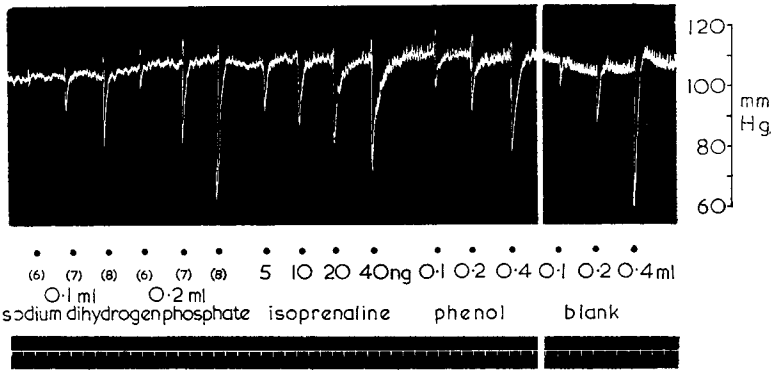

FIG. 3. Comparison of the depressor effects of sodium dihydrogen phosphate, isoprenaline, a dilute solution of the phenolic solvent and blank eluate on the blood pressure of rat anaesthetised with pentobarbitone sodium. (6) = 1 ml, (7) = 5 ml, and (8) = 10 ml 0·4% w/v $NaH_2PO_4.2H_2O$ evaporated to dryness and redissolved in 1 ml 0·9% w/v aqueous sodium chloride solution (see text). All injections i.v. Time trace in min.

response curves obtained with these solutions were steeper than that of isoprenaline and muscle tremors were evident after the administration of the solution of the phenolic solvent (1). Distilled water (2) and dilute hydrochloric acid (10) also caused the blood pressure to fall slightly, while minor fluctuations in blood pressure both above and below the resting level were observed following the administration of the ascorbic acid solution (9) which represented the amount of ascorbic acid that would be present in a 10 ml eluate. No changes in blood pressure were recorded when isotonic sodium chloride solution (3) was injected.

INHIBITION OF THE SUBMAXIMAL RESPONSES OF A QUIESCENT RAT UTERUS TO CONSTANT DOSES OF ACETYLCHOLINE

The blank eluates, the dilute solution of the phenolic solvent (1), the sodium dihydrogen phosphate solutions (6, 7 and 8) and hydrochloric acid (10), all caused inhibition of submaximal responses of a quiescent rat uterus to constant doses of acetylcholine. The degree of inhibition increased with increase in dose volume of all of the solutions and with

increase in the concentration of sodium dihydrogen phosphate. The dose volumes ranged from 0·025 to 0·2 ml and the capacity of the isolated organ bath was 5 ml. The characteristics of the inhibitions produced by these solutions were, however, different from those produced by isoprenaline. Following the initial inhibition produced by isoprenaline, several doses of acetylcholine were required before the responses were back to their original height; after the inhibition produced by the blank or solutions (1), (6), (7), (8) and (10) the responses to acetylcholine returned immediately to their original levels (Fig. 4).

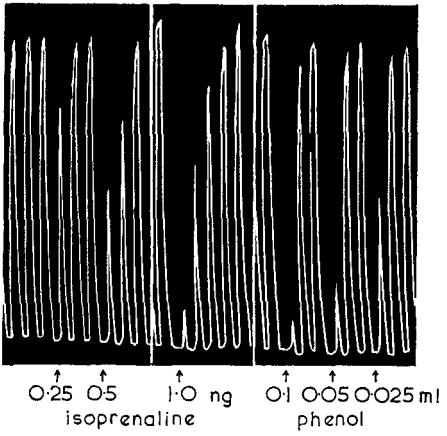

FIG. 4. Comparison of the inhibitory effects of isoprenaline and a dilute solution of the phenolic solvent on the isolated rat uterus responding to 0·5 μg acetylcholine added at 3 min intervals. Arrows mark additions of inhibitory drugs, 30 sec before the next addition of acetylcholine. Volume of organ bath = 5 ml. Contact time of acetylcholine = 45 sec.

Attempts to equate, in terms of (±)-isoprenaline sulphate, the activity of the blank, the dilute solution of the phenolic solvent (1), or the sodium dihydrogen phosphate solutions (6, 7 and 8) on the rat uterus and the rat blood pressure were unsuccessful.

When the sodium dihydrogen phosphate solutions were made up in half calcium rat uterus Ringer (de Jalon's) instead of 0·9% sodium chloride solution, the inhibitions of the rat uterus were much less marked and did not increase with increase in dose or with the amount of sodium dihydrogen phosphate. Also the pH of a 20 ml sample of the Ringer (7·3 as measured on a Pye pH meter) changed rapidly to 6·2 when 1 ml of elution fluid (0·4% w/v sodium dihydrogen phosphate in distilled water, pH 4·6) was added, and then settled down to 6·8. Further additions of elution fluid altered pH only slightly, 5 ml being required to bring the pH down to 6·2.

Distilled water (2), isotonic sodium chloride solution (3), hypertonic sodium chloride solutions (4 and 5) and ascorbic acid (9) were usually without measurable effect on the acetylcholine-induced responses of the rat uterus.

RECOVERY OF CATECHOLAMINES FROM PLASMA

When distilled water only was used as eluant the recoveries of small quantities of the amines were relatively constant (compare Tables 1 and 2) and blank activity was largely eliminated.

TABLE 1. RECOVERIES OF NORADRENALINE, ADRENALINE AND ISOPRENALINE (AS% OF THE AMOUNTS OF EACH AMINE ADDED, 0·1–0·5 μg) OBTAINED AFTER CHROMATOGRAPHIC SEPARATION ON PAPER USING 0·4% W/V AQUEOUS SODIUM DIHYDROGEN PHOSPHATE AS ELUANT

Catecholamine	Noradrenaline		Adrenaline		Isoprenaline	
Assay preparation	Rat colon	Rat blood pressure	Rat uterus	Rat blood pressure	Rat uterus	Rat blood pressure
Number of observations ..	8	8	8	8	7	7
Range of recoveries (%) ..	(47–92)	(19–33)	(48–66)	(18–28)	(51–74)	(76–113)
Mean % recoveries	72·7	23·9	59·7	22·4	61·9	91·4

TABLE 2. RECOVERIES OF NORADRENALINE, ADRENALINE AND ISOPRENALINE (AS % OF THE AMOUNTS OF EACH AMINE ADDED, 0·1–0·5 μg) OBTAINED AFTER CHROMATOGRAPHIC SEPARATION ON PAPER USING DISTILLED WATER ONLY AS ELUANT

Catecholamine	Noradrenaline		Adrenaline		Isoprenaline	
Assay preparation	Rat colon	Rat blood pressure	Rat uterus	Rat blood pressure	Rat uterus	Rat blood pressure
Number of observations ..	5	5	5	5	5	5
Range of recoveries (%) ..	(49–56)	(44–51)	(49–58)	(38–54)	(52–61)	(48–56)
Mean % recoveries	50·9	47·4	53·6	48·8	55·2	51·6

Discussion

Factors such as pH and tonicity of the solutions are shown to be liable to affect the biological assays of catecholamines after paper chromatographic separation. Traces of the phenolic solvent left in the eluates as a result of inadequate washing with benzene, and sodium dihydrogen phosphate used to prepare the elution fluid, cause a fall in the blood pressure of rats anaesthetised with pentobarbitone sodium and inhibition of the submaximal contractions of the rat uterus in response to acetylcholine.

Stimulation of the central nervous system by phenol is manifested in muscle tremors in the anaesthetised rat. The blood pressure falls probably as a result of a direct toxic action of phenol on the myocardium and on the smaller blood vessels, although the absence of such a response in pithed rats suggests that central vasomotor depression might be responsible for the fall in blood pressure in the anaesthetised animals. No explanation is offered of the brief rise in blood pressure caused by phenol in pithed rats. The toxic effects of phenol may also be responsible for the inhibition of the rat uterus.

The falls in blood pressure, in pithed and anaesthetised rats, caused by sodium dihydrogen phosphate are most likely the result of the formation

of non-isotonic solutions, since hypertonic sodium chloride solutions produce similar effects.

The sodium dihydrogen phosphate in the elution fluid remains in the flask after the water has been distilled off and is taken up in the isotonic sodium chloride solution thereby forming an hypertonic solution. It follows, therefore, that the larger the volume of eluate, then the more inherent depressor activity the eluate will possess, and the more erroneous will be the bioassay result.

Since hypertonic solutions of sodium chloride have much less inhibitory effect on the rat uterus than have the solutions of sodium dihydrogen phosphate, hypertonicity cannot be the cause of this particular effect, previously noted by Vogt (1952), of the elution fluid. The fact that the inhibitory effects of the sodium dihydrogen phosphate are markedly diminished when dissolved in rat uterus Ringer before being added to the organ bath suggests that sodium dihydrogen phosphate undergoes a reaction with one or more of the constituents of the Ringer. Solid sodium dihydrogen phosphate added to the Ringer does in fact cause a noticeable evolution of gas (presumably carbon dioxide from the bicarbonate present), and this would alter the pH of the Ringer. Similarly, the first addition of elution fluid to the Ringer causes an immediate increase in acidity followed by a fairly rapid return to near neutrality. The initial rapid reduction in pH is not apparent on further addition of elution fluid to the same sample of Ringer, but under the conditions of the biological assay the Ringer in the organ bath is changed after each dose. Acid solutions (10) do cause reduction in the responses of the rat uterus to acetylcholine, and low pH may also in part be responsible for the inhibition of this tissue caused by the dilute solution of the phenolic solvent (1).

As the depressor and inhibitory actions of the different solutions used were both qualitatively and quantitatively different from those of isoprenaline, it would seem that phenol or sodium dihydrogen phosphate present in the eluates are not by themselves responsible for the "isoprenaline-like substance" reported by Lockett with others (1954, 1957, 1961). They are however responsible for the non-parallel biological assays and inconsistent recoveries of small amounts of amines (Table 1) since the use of distilled water as the eluant prevents these effects (Table 2).

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