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The use of the rabbit ear artery in the bioassay of catecholamines in urine

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Urine causes transient constriction of the isolated central artery of the rabbit ear. The substance responsible is qualitatively indistinguishable from noradrenaline when assessed by α-adrenergic blockade, sensitivity to oxidation by ferricyanide, behaviour on alumina, and comparison of dose response curves. The artery is equally sensitive to noradrenaline and adrenaline and approximately one hundred times less sensitive to dopamine. The results of bioassay, and chemical assay using the trihydroxyindole method, indicate that the constrictor response of the artery provides a useful guide to the urinary content of noradrenaline plus adrenaline.

A method for the bioassay of catecholamines on the isolated artery of the rabbit ear was described by de la Lande & Harvey (1965). The following experiments were made to assess the application of this method to the assay of catecholamines in urine.

Experimental

METHODS

Bioassay. The only modifications to the method of perfusing the artery described by de la Lande & Harvey (1965) were: (i) the use of short arterial segments (1 to 4 cm) which were cleaned of adhering tissue, and (ii) cannulation and mounting of the artery in the organ bath so that the direction of flow was upwards (de la Lande, Cannell & Waterson, 1966). The cannula must be of sufficient diameter to contribute little if any resistance to the perfusion. The sensitivity is reduced and the responses become erratic if the cannula itself increases the perfusion pressure by more than 10 to 20 mm Hg.

After perfusion of the artery was commenced (approximately 8 ml/min) test doses of noradrenaline were injected at 2–5 min intervals for approximately 1 hr, after which 5-hydroxytryptamine (5-HT), 10–20 μg/litre was added to the perfusion fluid. When there was no further change in sensitivity to noradrenaline, mepyramine 10 μg/litre was added to the perfusion fluid. Control solutions of noradrenaline bitartrate (10 ng/ml) were prepared in 0·9% saline containing ascorbic acid 0·005% (ascorbic saline). Urine was filtered, adjusted to a pH of between 5·5 and 7·0, and diluted with ascorbic saline. Solutions for assay (0·1–0·3 ml) were injected into the intraluminal perfusion medium at 2 min intervals and their vasoconstrictor actions recorded by the increases in perfusion pressure.

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Chemical assay. The noradrenaline and adrenaline contents of the urine were measured by the trihydroxyindole procedure.

A 25 ml sample of filtered urine at pH 4·0 containing ascorbic acid (0·01%) and sodium edetate 1·0 g (2%) was agitated with 1–1·5 g of activated alumina by means of nitrogen bubbles while the pH was brought to, and maintained at 8·4 (±0·1) with M sodium hydroxide. After 2½ min the alumina was separated by centrifugation at 4°, and washed twice by shaking and subsequent centrifugation, with 10 ml lots of deionized water. Elution was by 0·2M acetic acid, the alumina being shaken for 4 min periods with two 10 ml portions of the acid and the supernatants collected after centrifugation. After the addition of 2 ml of 3% sodium edetate, 10 ml of the combined supernatant was adjusted to pH 6·0 with 0·5M Na_2HPO_4, centrifuged, and diluted to 20 ml (referred to subsequently as eluate).

For oxidation, 0·1 ml of $K_3Fe(CN_6)$ (0·25%) was added to a solution of 1·0 ml of eluate, 0·5 ml of sodium phosphate buffer (0·2M) at pH 6·5, and 2·0 ml of distilled water. Oxidation was terminated after 3 min by the addition of 1·0 ml of a solution of ascorbic acid, sodium hydroxide and β-thiopropionic acid. (This solution was prepared 15 sec earlier by adding 0·4 ml of 10% β-thiopropionic acid to 2·0 ml of a solution of 0·2% ascorbic acid in 5M sodium hydroxide prepared 5 min previously.) Fluorescence was measured after 10 and 15 min using filter sets I and II respectively on a Turner model 110 fluorometer fitted with the high sensitivity kit and blue lamp (Palmer, 1964). Blanks were prepared by reversing the order of addition of the alkali-ascorbic-thiopropionic mixture, and potassium ferricyanide (Price & Price, 1957). Each assay was made in duplicate with internal controls for noradrenaline and adrenaline.

The above method is that of Euler & Floding (1955) modified by (i) the use of a batch instead of a column procedure for alumina adsorption (Crout, 1961); (ii) the differential wavelength procedure for discriminating between noradrenaline and adrenaline (Bertler, Carlsson & Rosengren, 1959); (iii) the use of β-thiopropionic acid to stabilize the lutines (Palmer, 1964). The filter set combinations were those described in the "Manual of Clinical Laboratory Procedures" by G. K. Turner Associates. Filter set I gives excitation at 405 mμ and fluorescence at 510 mμ, and yields nearly equal readings for noradrenaline and adrenaline. Filter set II gives peak excitation at 436 mμ and fluorescence at and above 520 mμ, and yields approximately four times greater fluorescence for adrenaline than for noradrenaline. The fluorescent spectra of urine samples were checked periodically on a Farrand spectrophotofluorimeter.

In 16 urine samples assayed by the above method, 15 parallel assays were made on the same urine samples containing added noradrenaline 100 ng/ml (12 samples) or adrenaline 100 ng/ml (three samples). The mean recovery of added noradrenaline was 84·5%. The recovery was low in three samples (40, 53 and 58%), and in the remainder recoveries ranged between 77 and 123%. Recoveries of added adrenaline were 73, 82 and 87%.

Results

An injection of urine into the artery always caused a transient rise in perfusion pressure qualitatively similar to that produced by noradrenaline or histamine (Fig. 1). The following tests indicated that the response was probably due to catecholamines.

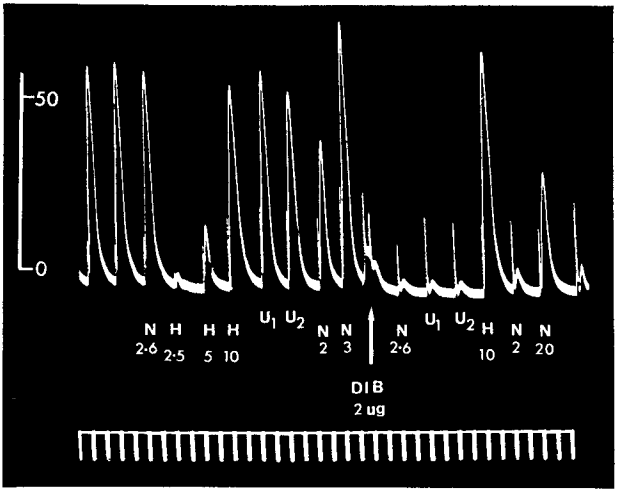

FIG. 1. Constrictor responses of an artery, in mm Hg, to injections of noradrenaline (N), histamine (H), and two diluted urine samples (U_1 and U_2), before and after phenoxybenzamine HCl (DIB) in the dose shown. The doses of N are in ng, of H are in μg; the volumes of U_1 and U_2 are each 0·2 ml, corresponding to 0·04 ml of the undiluted urines. Mepyramine (10 ng/ml) and 5-HT (20 ng/ml) present throughout. Time scale in min. Ordinate, increase in perfusion pressure in mm Hg.

Phenoxybenzamine abolished the response to urine and to noradrenaline (17 urine samples). The response to histamine was either unaffected or enhanced to a small extent, indicating that the effect of phenoxybenzamine on the urine response was not due to non-specific depression of the artery. A typical result is included in Fig. 1.

The responses to 16 urine samples were unaffected or changed in magnitude by less than 20% by mepyramine in concentrations (10 μg/litre) which abolished equivalent responses to histamine. However, the response to one urine sample was abolished by mepyramine. The sample was from a patient with suspected sympathetic nerve degeneration; subsequent analysis on the guinea-pig ileum revealed that this urine contained an abnormally large amount of histamine (28 mg/24 hr) compared with the normal daily output of 17–90 μg (Gowenlock & Platt, 1963).

In each of 10 urine samples the response was abolished by pretreating the urine with potassium ferricyanide at pH 6·0. Potassium ferricyanide oxidizes both noradrenaline and adrenaline at this pH (Euler & Floding, 1955). Potassium ferricyanide pretreatment was as follows. The pH

of the urine was adjusted to approximately 6·5, and 0·5 ml of 0·2M phosphate buffer (pH 6·5) added to a 5 ml sample. To this was added 1·0 ml of 0·25% zinc sulphate followed by 2·0 ml of 0·25% potassium ferricyanide. After at least 3 min the latter was converted to ferrocyanide with 0·5 ml of 2% ascorbic acid, and the sample brought to the same dilution with ascorbic saline as the untreated urine. Injections of 0·05–0·4 ml of this "oxidized urine" produced no response and caused little alteration in the sensitivity of the preparation to noradrenaline given simultaneously or subsequently.

The constrictor material in the urine was adsorbed onto alumina, and eluted from alumina in an identical fashion to catecholamines. The experimental conditions of adsorption and elution were those employed for chemical assay (see methods), with the minor modification that 0·2M hydrochloric acid was used for elution instead of acetic acid. The activity remaining in a 10 ml sample of urine after shaking with 1 g of alumina, and the activity in the (neutralized) hydrochloric acid eluate of the alumina, were estimated by the volume required to elicit the same contractile activity as the untreated urine. In each of 5 urines, the treated urine contained less than 10% of the activity of the untreated control. The activities recovered in the acid eluates were between 70 and 80% of that originally present in three urines. In two urines the activities recovered were approximately 124 and 140% of that originally present.

On the basis of the above tests, it was assumed that the constrictor activity of the urine was due to catecholamines.

Quantitative estimations of the catecholamine content of the urine sample were made by comparing the responses of an artery to each of the following: (1) a urine sample; (2) the urine sample plus noradrenaline; (3) noradrenaline added to the urine sample after the constrictor action of the urine had been abolished with ferricyanide; (4) noradrenaline added to ascorbic saline. Each solution was tested in duplicate or triplicate at three dose levels. An example of the curves obtained on one artery for one of the urine samples (No. 6, Table 1) is shown in Fig. 2. The main feature is that the shapes of the curves are similar and departures in slope and curvature are relatively minor. The results of eight experiments are summarized in Table 1. It will be noted that the estimates of noradrenaline content based on the two types of noradrenaline control solutions (ascorbic saline, and oxidized urine) showed reasonable agreement, with the exception of experiment 3 where the separate estimates were 105 and 67 ng/ml. In this experiment there was also a large discrepancy between the measured and calculated contents of noradrenaline in the urine to which noradrenaline was added (the recovery of added noradrenaline being 152% instead of 100%). The difference between the noradrenaline assays probably reflected the presence of sensitizing substances in the urine. A number of urine samples were screened during this study by the less rigorous procedures of dose matching or comparison at two dose levels with noradrenaline in ascorbic saline. The mean noradrenaline-equivalent content of the total of 48 urines from 31 subjects (including those in Table 1) was 56·3 $\pm$ 29·3 ng/ml (s.d.). In Table 2

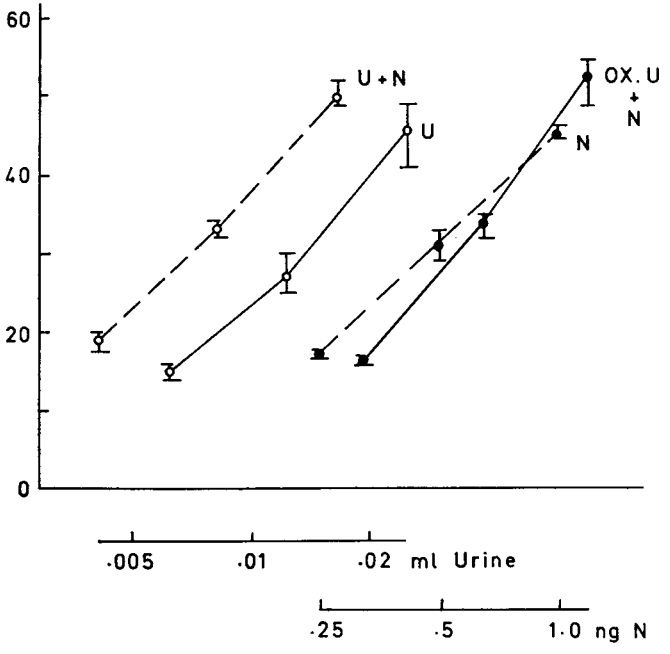

FIG. 2. Dose-response curves of an artery to urine (U), urine + noradrenaline 40 ng/ml (U + N), noradrenaline in ascorbic saline (N), and urine whose contractile activity had been abolished with ferricyanide and to which noradrenaline 40 ng/ml was subsequently added (OX.U + N). The extent of the variation of the responses to repeated doses is shown by a vertical bar. Ordinate: increase in perfusion pressure in mm Hg. The abscissa, for U and U + N is ml of urine, and for OX.U + N, and N, is ng of noradrenaline. The urine sample is No. 6 of Table 1.

TABLE 1. NORADRENALINE-EQUIVALENT CONTENTS OF URINE SAMPLES

	Compared with noradrenaline in ascorbic saline			Compared with noradrenaline in oxidized urine		
Sample No.	Mean (ng/ml)	Range (ng/ml)	% recovery of added noradrenaline	Mean (ng/ml)	Range (ng/ml)	% recovery of added noradrenaline
1	38	35–41	81	47	42–50	88
2	54	48–65	105	54	48–58	101
3	105	93–120	152	67	57–79	103
4	72	60–85	—	87	72–101	—
5	46	43–49	93	54	52–55	111
6	42	38–44	81	44	40–51	79
7	51	49–54	107	53	52–55	109
8	44	40–51	122	59	52–70	148
Means	56		105	58		105

The noradrenaline-equivalent contents of the urine samples were calculated from the dose response curves at the lowest, middle, and highest levels of the responses common to noradrenaline and to the urine. Hence the mean in each case is based on three estimates of which the minimum and maximum values are included under "range".

TABLE 2. NORADRENALINE-EQUIVALENT CONTENTS OF URINE SAMPLES

Origin of sample	Noradrenaline equivalent ng/ml	No. of samples	% recovery of added noradrenaline	No. of samples	Ratio* of activity of adrenaline to noradrenaline	No. of samples	Comments
31 subjects 3 to 24 hr samples	56·3 ± 29·3 (s.d.)	48	112 ± 47 (s.d.)	24	0·9 ± 0·06 (s.d.)	12	The recoveries of noradrenaline in 21 of the samples were between 63 and 133%. However, in three samples the recoveries were 200, 224 and 250%.
V	(i) 275 (ii) 12·5	1 1	100 100	1 1	1·0 0·6	1 1	Samples (i) and (ii) taken in each case before, and after, removal of adrenal tumours.
M	(i) 800 (ii) 85	1 1	Not est'd. ,,		1·0	1 1	
P	(i) 790 (ii) 417	1 1	130 Not est'd.	1 1	1·2	1	Samples (i) and (ii) taken at an interval of 3 months from a patient with tumours of extra-adrenal chromaffin tissue.

* Estimated by dose matching

this level is compared with those of two patients with phaeochromocytoma of the adrenal medulla, and one with recurrent phaeochromocytomas of extra-adrenal chromaffin tissue. The method revealed high levels of noradrenaline in the urine of each of the patients, and low levels after the removal of the medullary tumours. Complete removal of tumours was not possible in the third patient.

CHEMICAL ASSAY

The results of bioassay were compared with those of chemical assay on 12 urines, comprising 4–24 hr samples from 11 colleagues or patients. The bioassay was based on comparison with noradrenaline in ascorbic saline, using two dose levels of each. The results are shown in Table 3. The bioassay was within ±25% of the chemical assay in nine of the

TABLE 3. COMPARISON OF BIOASSAY AND CHEMICAL ASSAY FOR NORADRENALINE AND ADRENALINE

Subject	Chemical assay (ng/ml)		Bioassay (ng/ml)	Ratio of results $\frac{\text{Bioassay}}{\text{Chemical assay}}$
	Noradrenaline	Noradrenaline + Adrenaline	Noradr. equiv. (= noradr. + adren.)	
P	740	740	790	1·07
V	124	239	275	1·15
RH (ii)	164	164	125	0·76
NP	57	77	42, 73	0·58, 0·95
AB	35·5	59	60	1·02
JW	48	53	40	0·76
JMc	21	50	41, 50	0·82, 1·0
ID	40	46	46, 48	1·0, 1·04
MT	30	45	43	0·96
DF	33·2	44	27–40 (51)	0·77
RH (i)	17·8	36	66	1·83
JM	24	34	17–27 (28)	0·66

Note (1) The chemical assay data are corrected for a mean recovery of noradrenaline + adrenaline of 85%.

(2) The mean ratio $\frac{\text{bioassay}}{\text{chemical assay}}$ is 0·95.

comparisons, but gave values which were 0·67 and 1·82 times those obtained by chemical assay in two samples (JM and RH (i) respectively). The cause of discrepancy between chemical and bioassay was not analysed systematically, but in three of the comparisons it may be attributed largely to the bioassay. Thus the dose response curves to urine samples JM and DF were much flatter than those to their noradrenaline control solutions and for this reason ranges rather than mean values are quoted in Table 3. The difference between the curves was consistent with an inhibitory effect of the urine on the sensitivity of the artery to noradrenaline, and this was confirmed by the finding that after treating the urines with alumina, the noradrenaline-equivalent contents of the hydrochloric acid eluates of the alumina (shown in brackets in Table 3) were greater than those of the untreated urines. An inhibitory effect of urine may also have accounted for an unusual discrepancy in sample NP. This sample, when assayed a second time on the same artery after an interval of approximately 2 hr, yielded a noradrenaline-equivalent content of 73 ng/ml compared with the initial value of 42 ng/ml.

ADRENALINE AND DOPAMINE

The ratio of the sensitivity of the artery to adrenaline compared with that of noradrenaline in 12 arteries was $0{\cdot}9 \pm 0{\cdot}06$ (s.d.). The corresponding mean ratio for dopamine was approximately 0·01. This value is based on ratios of 0·01, 0·01, 0·01, 0·004 and 0·0025 observed in the present study, and ratios of 0·03, 0·03, 0·015 and 0·01 described previously (de la Lande & Harvey, 1965).

Discussion

The vasoconstrictor substance(s) in urine, and noradrenaline behaved in a similar fashion when assessed by (i) sensitivity to blockade by phenoxybenzamine; (ii) sensitivity to oxidation by ferricyanide; (iii) adsorption onto and elution from alumina; (iv) comparison of dose response curves. Each of these tests is not conclusive in itself, but together they provide strong evidence that catecholamines are solely responsible for the constrictor activity on the artery of most urine samples. The catecholamines may be assumed to be mainly noradrenaline and adrenaline, since the constrictor potencies of these amines are approximately equal and are 30 to 300 times greater than that of dopamine. The normal urinary output of dopamine is 100–200 μg/day (Udenfriend, 1962), i.e. approximately two to six times that of noradrenaline plus adrenaline, assuming mean normal daily outputs of the latter amines of either 36 μg (Crout, 1961) or 68 μg (Jacobs, Sobel & Henry, 1961).

The comparisons between biological and chemical assay indicate that the noradrenaline-equivalence of the constrictor response to urine corresponds reasonably well with the content of noradrenaline plus adrenaline. The agreement is of a similar order to that obtained by Euler & Floding (1955) on urine samples assayed by the trihydroxyindole method, and by biological assay on the cat blood pressure, and rat uterus.

If it is assumed that the discrepancy between chemical and bioassay is due largely to error in the bioassay, the error appears to be somewhat less than $\pm 40\%$ for most urines. An error of this magnitude is probably unimportant in phaeochromocytoma, where one of the contributory aids to diagnosis is a urinary catecholamine output of 200 μg/day or more, i.e. at least twice the upper limit of the normal range (Udenfriend, 1962).

Although the method cannot be used as a quantitative substitute for the chemical method particularly where urinary levels are in the lower range of normal, by providing a semi-quantitative guide to these levels it should prove useful in screening programmes to aid the selection of urines for subsequent chemical assay. Its major advantage over other bioassay methods including the aortic strip method of Helmer (1961) is that it combines the virtues of high sensitivity to noradrenaline and to adrenaline, with speed of assay, and ease of preparation of the artery and the urine.

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