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SLOW-CONTRACTING SUBSTANCES IN HUMAN URINE

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A method has been developed for purifying the principle in human urine causing contraction of the rat uterus. The method, based on adsorption on the cation exchange resin Amberlite IRC-50, is better suited for dealing with large quantities of urine than the method of Gomes (1955). The presence of the two active fractions described by Walaszek (1957) has been confirmed by a different method.

The stimulating effect of normal urine on isolated smooth muscles has been investigated by Werle and Erdös (1954), Gomes (1955), and Walaszek (1957), who found evidence of a substance having the properties of a polypeptide closely related to bradykinin, and consequently to the group of substances consisting of kallidin, bradykinin, and substance Z (Gaddum, 1955). Walaszek (1957) purified further the active principle in urine extracts prepared according to Gomes (1955) and found two substances having a contracting effect on the rat uterus.

In the present paper, a method is described for purifying the rat-uterus-stimulating component in human urine and a procedure described by which the active principle could be divided into two fractions. The technique of separation differs from that of Walaszek (1957), because using his method none of his substance Z_1 could be recovered and only small quantities of substance Z_2.

METHODS

Assay.—Uteri from virgin rats weighing 140 to 180 g. were superfused with de Jalon solution (Gaddum, Peart and Vogt, 1949) by the technique described by Gaddum (1953), or were suspended in 3 ml. baths. The rats were injected with stilbœstrol (10 μg./100 g.) on the day before the assay. The period of contact of active solution was for 30 sec. at intervals of 5 min. Atropine sulphate (10^{-6}) was added to the de Jalon solution.

Aliquots of the fractions from the chromatographic columns were generally freeze-dried prior to the assay. Extracts in water, however, were usually diluted with an equal volume of double-strength de Jalon solution; the hydrochloric acid fractions were occasionally titrated with sodium hydroxide solution, and de Jalon solution in double concentration was then added, but with a calculated smaller content of sodium chloride. Organic solvent was removed from fractions containing aqueous methanol or acetone *in vacuo* at room temperature, and the remainder thereafter lyophilized.

Urine Extracts.—Urine extracts were prepared from 24 hr. urine specimens by a butanol extraction method described by Gomes (1955) and later employed by Walaszek (1957). Each preparation yielded from 1.2 to 1.7 g. of extract and the dry extracts appeared to be stable for at least 10 months.

Standard Preparation.—The first urine extract produced was used as standard, one unit having been taken as the activity in 1 mg. of this extract.

Stability Tests at Different pH Values.—The stability of urine extracts was tested within the *p*H range of 0 to 14. For *p*H values from 0 to 3 hydrochloric acid was used and from 12 to 14 sodium hydroxide; from *p*H 3 to 7 the solutions were buffered with citric acid and disodium hydrogen phosphate, from *p*H 7 to 10 with boric acid and sodium hydroxide and from *p*H 10 to 12 with disodium hydrogen phosphate and sodium hydroxide. The *p*H values were determined with a glass electrode.

The *p*H values of the urine extracts in distilled water ranged between 3.3 and 3.8, and the solutions had some buffering capacity. In the stability tests, it was therefore necessary to use added buffers in such amounts as to overcome the buffering capacity of the extracts. Care was taken, however, that the amounts were not so large as to interfere with the uterine contractions after the dilution with de Jalon solution. Extracts containing hydrochloric acid or sodium hydroxide were neutralized with sodium hydroxide, or hydrochloric acid, prior to the assay.

Dialysis.—Cellophane casings (Visking Corp., Chicago, Illinois) were used.

Paper Pulp Chromatography.—Whatman cellulose powder was used. As a rule, the columns were built up with paper pulp suspended in 0.2 N-hydrochloric acid. After the paper had settled down, it was

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usually washed thoroughly with 0.2 N-hydrochloric acid, then washed free of acid with distilled water, and finally washed with the fluid to be used as solvent for the active substance at the succeeding chromatography. In a few experiments the columns were intentionally *not* washed in advance with hydrochloric acid.

Aluminium Oxide Chromatography.—Three types of aluminium oxide (Woelm, Eschwege, Germany) were used, an alkaline, a neutral, and a weakly acid preparation.

Ion Exchange Chromatography.—Amberlite IRC-50 (analytical grade) was used to adsorb the active substances from urine. The resin was prepared in the following way. 100 g. of Amberlite IRC-50 (40 to 60 mesh) in the hydrogen form was stirred with 350 ml. of 1 N-HCl for 30 min. and then washed by decantation with five 500 ml. portions of distilled water. 1 N-NaOH (700 ml.) was then slowly added with stirring, the mixture stirred for 1 hr. and left overnight. Next, the resin was washed by decantation with five 2 l. portions of distilled water and, as a slurry in water, poured into the top of a column filled with water. It was washed with distilled water (backflow) until the *p*H of the effluent reached the range of 8.2 to 8.5. At this stage 0.2 M-sodium phosphate buffer of *p*H 6.0 ± 0.1, with thymol added as a preservative, was passed through the column until the effluent had a *p*H of 5.9 to 6.1. The resin was then kept in the sodium phosphate buffer until use. Amberlite IR4-B (analytical grade) was used to remove the hydrochloric acid in the eluate from the Amberlite IRC-50 columns.

RESULTS

Stability.—There was a difference between the stability of extracts and urine. Two extracts were kept for 27 to 29 hr. at a temperature of 20 to 22° at various *p*H values between 0.3 and 13.5 and then tested for activity. Both extracts gave similar results; no loss was detected in the *p*H range 0.3 to 7.0. At *p*H 7.5 there was some loss and at *p*H 13.5 only 20% of the activity was left.

On the other hand, six urines were adjusted to *p*H 6, and kept at 20°; after 24 hr. only 50 to 70% of the activity was left and, in one specimen tested after 48 hr., only 12% was left. In this specimen kept at 4°, 75% of the activity remained after 48 hr.

Paper Pulp Chromatography.—Attempts to adsorb the active principle on paper pulp from aqueous solutions of extracts revealed that one active fraction passed quickly through the column. In addition, all the extracts analysed contained an active fraction that could not be eluted with water or with 0.1 N-acetic acid, but which was rapidly eluted with 0.2 N-HCl (Table I, Expt. 1). Experiments with increasing amounts of urine extract showed that the capacity of the columns for this active principle was 23 to 27 units/ml.

The urine extracts are buffers with *p*H values in distilled water ranging from 3.3 to 3.8. When the *p*H of the extract solutions was adjusted with sodium bicarbonate to about 6 prior to water chromatography most of the activity was lost (Table I, Expt. 2). When the column was first washed with 0.2 N-HCl and then freed of acid with distilled water, this loss was partially prevented (Table I, Expt. 3), possibly owing to the removal of impurities from the paper. In the method of purification described below the column was washed with 0.2 N-HCl and extracts contained 0.1 N-acetic acid (Table I, Expt. 4).

TABLE I

CHROMATOGRAPHY OF URINE EXTRACT ON PAPER PULP

The total activity in each experiment was 80 units. Column size 8 × 1 cm. (6.3 ml.). Flow 0.3 to 0.35 ml./min. In each experiment the column (a) gives the fraction numbers (each 20 ml.) and the column (b) gives the % recovery.

Expt.	1		2		3		4	
Column washed with	Water		Water		0·2 N-HCl		0·2 N-HCl	
Extract diluted with	Water		$NaHCO_3$		$NaHCO_3$		0·1 N-acetic acid	
*p*H of starting solution	3·8		6·0		6·0		2·9	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Eluted with water	1 2 4–10	65 2·5 0	1–6	0	1 2 3–6	13·5 9·0 0		
0·1 N-acetic acid	11–20	0	7–14	0	7–12	0	1 2 3–6	66·3 1·5 0
0·2 N-HCl	21 22 23–30	8·3 0·6 0	15 16 17–20	3·8 0 5 0	13 14 15–17	8·8 0·5 0	7 8–11	6·5 0
Total	76·4		4·3		31·8		74·3	

Aluminium Oxide Chromatography.—Chromatography on aluminium oxide columns was carried out successfully by Andrade, Diniz, and Rocha e Silva (1953) for purification of bradykinin. Walaszek (1957) could recover no activity when using ordinary alkaline aluminium oxide columns for purification of the urinary constituents; but after washing out with hydrochloric acid, aluminium oxide columns yielded nearly 100% activity, provided the experimental conditions were otherwise favourable. In agreement with the experience of Walaszek (1957) we found that an alkaline aluminium oxide ("Woelm" basic) allowed recovery of very little active substance compared with an HCl-activated type ("Woelm" acid). An almost neutral aluminium oxide

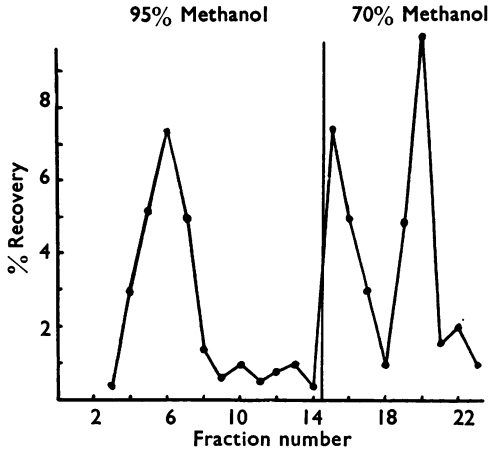

FIG. 1.—Chromatography of urine extract on aluminium oxide. Aluminium oxide (Woelm acid); column size, 135 mm. × 10 mm.; urine extract, 180 units; rate of elution, 0.08 to 0.10 ml./min.; fraction volume, 5 ml.; total recovery, 62.9%.

(" Woelm " neutral) behaved like the alkaline type. Fig. 1 illustrates an experiment in which the active principle was adsorbed on aluminium oxide, " Woelm " acid, from 95% methanol, which was also used as the first eluent. When 13 fractions had been collected, the eluent was changed to 70% methanol. Most of the weight of the urine extract used passed into the fractions 2, 3, and 4, while these contained less than 5% of the original activity. Repeated experiments all showed one peak of activity in the 95% methanol fractions and two peaks of activity in the 70% methanol fractions, thus affording evidence that at least two active principles are present in the urine extract. The total activity recovered in the example in Fig. 1 was considerably higher than that found by Walaszek (1957) with an initial solvent of 95% methanol; it seems, however, to vary greatly with the size of the column. An experiment carried through with a 50% higher column and a 50% larger amount of urine extract, under otherwise equal conditions, gave total activity recovery of 32% against 63% in the above-mentioned experiment.

Ion Exchange Chromatography.—A method better suited for preparations of extracts than that of Gomes (1955), which was based on extraction of an acidified urine with an equal volume of *n*-butanol and precipitation with large volumes of ether, was desirable for isolation of the rat uterus-stimulating substances from urine. Ion exchange resins were tried as adsorbents for the active principles. The cation exchange resin Amberlite IRC-50 proved to be useful when pulverized to 40 to 60 mesh and buffered with sodium ions to *p*H about 6. The capacity was sufficient and the activity could be eluted with relatively small volumes of HCl. This resin was used by Andrade and Rocha e Silva (1956) for the purification of bradykinin.

In a typical experiment, the *p*H of a sample of urine containing 7.0 units/ml. was adjusted to 6 after which it was diluted with an equal volume of water and filtered. (The ion exchange column tended to clog if untreated urine was used, and dilution also increased the capacity of the resin.) The fluid was passed through an Amberlite IRC-50 column (50 by 5 mm.) at a rate of 0.30 to 0.35 ml./min. After 360 ml. (180 ml. of urine) had passed, the activity began to come out, and the adsorption was stopped. Fig. 2 shows how the active principles were eluted with 0.2 N-HCl. Fractions 1+2+3+4 contained in 20 ml. 85% of the eluted activity, and fractions 2+3 in 10 ml. 70%. The total recovery was, however, only about 60%. The shape of the elution curve in Fig. 2 suggested that the loss was unlikely to be due to incomplete elution. The loss may have been due to one or more substances having remained on the column or to destruction of activity on the column. Another possibility was that the activity had been lost in the urine prior to its passage through the column. The instability of untreated urine has already been described.

The anion exchange resin Amberlite IR4-B was found to be suitable for removing excess of HCl in the eluate from the Amberlite IRC-50 columns. 85 to 100% activity was usually recovered, and the passage of the active principles was not delayed by adsorption.

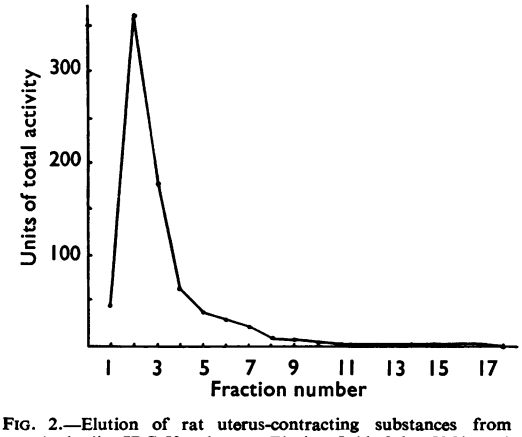

FIG. 2.—Elution of rat uterus-contracting substances from an Amberlite IRC-50 column. Elution fluid, 0.2 N-HCl; column size, 50 mm. × 5 mm.; rate of elution, 0.08 to 0.10 ml./min.; fraction volume, 5 ml.

Purification Procedure.—A typical example of purification procedure of the two rat uterus-stimulating principles in a 24 hr. urine specimen is described below.

To a 1,400 ml. sample of urine (5.3 units/ml.), NaOH was added to a *p*H of 6 and distilled water to produce a volume of 2,800 ml. After filtration, the fluid was passed through an Amberlite IRC-50 column (150 × 10 mm.) at a rate of about 2 ml./min. No activity could be detected in the urine which had passed through the column. The column was washed thoroughly with distilled water. Elution was performed with 0.2 N-HCl at a rate of 0.6 to 0.7 ml./min. and 300 ml. of eluate was collected (plus 300 ml. to control the effectiveness of the elution). The 300 ml. eluate was passed through an Amberlite IR4-B column (290 × 20 mm.) at a rate of 10 to 15 ml./min. This was followed by washing with distilled water to 375 ml. of eluate. The eluate contained 9.5 units/ml. and its *p*H was 8.6. Simple solids were removed by distributing this eluate in 5 dialysis casings ($\frac{5}{8}$ in.) in quantities of 75 ml. and dialysing against 1,200 ml. of distilled water for 3 hr. The outer fluid was changed four times at intervals of 15 min. and then four times at intervals of 30 min. The final *p*H of the inner fluid was 6.8. This process involved the loss of only about 15% of the total activity. Acetic acid to 0.1 M was added immediately after the dialysis.

The two active principles in a small amount of the dialysed acetic acid solution were then separated on a paper pulp column in order to assess the size of column required for the total quantity of active solution. A volume of 10 ml. (81 units) was passed through a column (80 × 10 mm.) at a rate of 0.3 to 0.4 ml./min. and the column was thereafter washed thoroughly with 0.1 N-acetic acid to remove the first active principle completely. Elution was then performed with 0.2 N-HCl and three 20 ml. fractions were collected for freeze-drying and assay. Only 4.3 units were present in the fractions collected, corresponding to 160 units in the total quantity of dialysate (375 ml.).

The amount of acetic acid dialysate left (365 ml.) was then passed through a paper pulp column (200 × 10 mm.) at a rate of about 1 ml./min. and 400 ml. of acetic acid eluate was collected. The volume of this column was about 15.5 ml., which should adsorb over 300 units of activity. The column was thereafter washed thoroughly with acetic acid, and eluted with 0.2 N-HCl. The acetic acid eluate (400 ml.) as well as the HCl acid eluate (110 ml.) were concentrated *in vacuo* at 25° and afterwards freeze-dried.

TABLE II

ISOLATION OF TWO RAT UTERUS-CONTRACTING FRACTIONS IN URINE

Recovery of activity at different stages of the isolation procedure (see text for further explanation of the table).

Fluids	Volume (ml.)	Activity		
		Units Total	%	Units/ml.
Urine	1,400	7,420	100	5·3
HCl eluate from Amberlite IRC-50	a: 300 (b: 300)	3,500 (720)	47 (10)	11.7 (2·4)
Eluate from Amberlite IR4-B	375	3,560	48	9·5
Eluate from Amberlite IR4-B after dialysis	375	3,038	41	8·1
Material not adsorbed on paper pulp	400	1,747	24	4·4
HCl eluate from paper pulp	110	161	2	1·4

Table II shows the activities recovered at the various stages of purification.

Comments on the Purification Procedure.—It is seen in Table II that two steps in the purification method cause a considerable loss of activity, namely the use of the Amberlite IRC-50 column and the adsorption on the paper pulp column. On the other hand, removal of HCl by means of Amberlite IR4-B and the dialysis caused only negligible losses. In the above experiment 47% of the activity was recovered from the Amberlite IRC-50 column (57% if the second eluate was included). In a number of similar experiments on different scales, the recovery varied between 35 and 70%. In these experiments the concentration of active substance in the HCl eluate varied between 10 and 25 units/ml.

The concentration of HCl was not very critical, but the amount of active principle eluted/ml. of HCl decreased if the HCl concentration was too high or too low. Under comparable conditions, eluate using 0.6 N-HCl was seen to contain 6 units/ml.; 0.2 N, 20 units/ml.; 0.1 N, 22 units/ml.; and 0.01 N, only 4 units/ml. In large-scale experiments, it was found expedient to start the elution with a stronger HCl, such as 1 N, to reduce as much as possible the volume required to elute the activity. As soon as the buffer effect of the column had been broken, the concentrated HCl was replaced with 0.2 N-HCl.

While the recovery from Amberlite IRC-50 varied appreciably in different experiments, that involved in separation of the two active fractions on paper pulp remained fairly constantly at 60 to 70%. The principle adsorbed and eluted with HCl generally constituted a small proportion from 2 to 10% of the recovered activity.

The residues after freeze-drying were very hygroscopic. The most active material so prepared contained 100 to 120 units/mg. but proved to be very unstable.

Discussion

The method described for adsorption of the rat uterus-stimulating principles in human urine on the cation exchange resin Amberlite IRC-50 seems to constitute a useful basis for isolation of these principles, though alterations that led to increased recovery from the column would favour its use. Very active fractions were obtained from the extracts by adsorption on paper pulp followed by elution with acid.

The experiments with aluminium oxide (Fig. 1) and with paper pulp (Table I) confirmed the conclusion of Walaszek (1957) that human urine contains more than one substance causing contraction of the rat uterus.

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