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OXYTOCIC POLYPEPTIDES IN HUMAN URINE

BY

E. J. WALASZEK*

From the Department of Pharmacology, University of Edinburgh

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The work of Gomes on the polypeptide in human urine which causes contraction of the rat uterus has been extended. Concentrates were prepared by means of paper pulp columns similar to those used by Rocha e Silva and his colleagues for the purification of bradykinin, and evidence was obtained for the presence of two different active substances not only in human urine but also in a preparation of bradykinin. The effect of human urine on the rat uterus is probably due to bradykinin itself or a mixture of similar substances.

The substance responsible for the stimulating effect of crude urine and urinary extracts on the superfused rat uterus has been shown to have the properties of a polypeptide (Gomes, 1955). Evidence has been presented that the active principle closely resembled bradykinin and appeared to be related to that group of polypeptides which is represented by kallidin, bradykinin, substance U and substance Z (Gaddum, 1955; Werle and Erdös, 1954).

This report deals primarily with the purification of the substance. Some observations on bradykinin and its similarity with this substance are also included.

METHODS

Rat Uterus.—Uteri from virgin rats weighing 190 g. were superfused with de Jalon's solution (Gaddum, Peart and Vogt, 1949) by the technique described by Gaddum (1953).

Preparation of Alumina.—Aluminium oxide which is standardized (Savory and Moore) was found to absorb the active principle irreversibly. An acid washed alumina, as used by Pernow (1953) for the purification of substance P, was found to be an excellent medium for the purification of our principle. The aluminium oxide was washed twice with normal HCl for two hours. Then it was washed with distilled water until the *p*H of the washings was 6, dried at 90° C. and stored in a desiccator. The aluminium oxide columns were prepared as a slurry in 80% methanol which was allowed to settle by gravity. The material for purification was put on the column with 80% methanol and elution was effected with decreasing alcohol concentration. The solution was passed through the column by gravity or under slight pressure when necessary to maintain a flow of 1 ml./min. A 1.1×24 cm. column was used for 100 mg. of crude material, but a 1.1×8 cm. column is sufficient.

Paper Pulp Column.—The method of Andrade, Diniz and Rocha e Silva (1953), employing Whatman Cellulose Powder, which had proved successful in the purification of bradykinin, was used. The column was prepared by suspending the paper pulp in a 7% phenol solution and allowing the paper to settle by gravity until the desired height was reached. A 2.2×9 cm. column was found to be ideal for the purification of about 200 to 300 mg. of dry crude powder. The column was washed with additional 7% phenol, which removed a yellowish impurity from the paper pulp. The active principle was adsorbed from small volumes (5 to 10 ml.) of 7% phenol, which was also used as the developing solvent. Elution was effected with 0.4% acetic acid v/v and 0.1 N-HCl. The rate of elution was 4 ml./min. with the 7% phenol and 1 ml./min. with 0.4% acetic acid and 0.1 N-HCl. Of each 10 ml. fraction collected, 2 ml. was placed in a small test tube and lyophilized. The 2 ml. aliquots, after being lyophilized, were reconstituted with de Jalon's solution and assayed on the superfused rat uterus.

Paper Chromatography.—Ascending chromatography with Whatman No. 1 filter paper was used. The chromatograms were developed at room temperature (15 to 18° C.) for 18 to 24 hr. The solvents were acetone-N-butanol-HCl (10-4-2), and the organic phase of N-butanol, acetic acid and water (4-1-5) mixture.

The active substance was localized on the paper strips by biological assay on the superfused rat uterus. The simplest procedure was to cut the strip in 1 cm. portions, place in test tubes with 2 ml. of de Jalon's solution, allowing 5 to 10 min. for elution, and testing these solutions on the rat uterus.

Lyophilization.—Most of the purification operations were carried out in aqueous solutions. Because of the lability of the active principle, care had to be taken in reducing the volume of these solutions or in producing a dry powder by evaporation of the water solution. This

* Present address, Department of Pharmacology, University of Kansas.

was accomplished by lyophilization. No loss in activity occurred during the process. The dry powder was then directly reconstituted with de Jalon's solution and an assay performed.

When using the paper pulp column a few samples had phenol present. This was removed before lyophilization by ethyl ether extractions. The remaining ether in the solution was removed by freezing the sample and pouring off the small amount of ether.

The alcoholic solutions from the aluminium oxide columns were reduced in volume by vacuum distillation at 30 to 35° C. The remaining aqueous solution could then be frozen and lyophilized.

Preparation of a Standard Crude Powder.—The method of extraction was one described by Gomes (1955), and previously used by Clark, Winckler, Gollan and Fox (1954) for the extraction of hypertensin. The urine was acidified with HCl to *p*H 1.5, saturated with NaCl and extracted with an equal volume of N-butanol. The butanol was separated, filtered over anhydrous sodium sulphate and 5 volumes of ethyl ether (dry, distilled over sodium) added. This produced a flocculent precipitate which was allowed to settle overnight at room temperature. The ether was then decanted and the precipitate washed twice with fresh ether, collected on filter paper and stored in a desiccator. Approximately 1 g. of pinkish white powder was obtained from 1 l. of urine.

When this powder was prepared from samples of individual urines, it was found that the most potent preparation was three times more active than the weakest preparation obtained (Table I). This was probably due

TABLE I

VARIATION IN POWDERED EXTRACTS PREPARED FROM INDIVIDUAL URINES WHEN ASSAYED AGAINST A STANDARD PREPARATION FROM POOLED URINE

The unit is an arbitrary one (see text).

Preparation	Units/mg.
Standard	1·0
M.K.P.	2·4
E.J.W.	1·4
R.B.	0·95
D.S.	0·85
I.D.	1·0

to differences in salt concentration and not to any specific individual variation. In order to facilitate the evaluation of potency, a large sample of pooled urine (10 individuals) was used to prepare a standard preparation. This preparation was arbitrarily given the valuation of one unit of oxytocic activity per mg. of dry powder.

A partial purification of approximately 2 to 3-fold could be obtained by dissolving the crude dry powder described above in absolute methanol, filtering and adding 5 volumes of dry ethyl ether to the clear filtrate. A flocculent white precipitate resulted which was collected and stored in a desiccator. This preparation was not as stable as the crude dry powder, which was chosen as the standard preparation.

Chemicals.—The preparation of bradykinin used was kindly presented by M. Rocha e Silva. Its activity was given as 5 × Pool I (Rocha e Silva, 1955).

RESULTS

Hypertensin, which may be obtained by the extraction procedure used here (Clark *et al.*, 1954), has been further purified by using the cation exchanger IR-100H to remove salts. When this procedure was applied to our crude powder, the desalting was complete, but the recovery of pharmacological activity ranged from 20 to 40%.

Aluminium Oxide Columns.—Aluminium oxide, when prepared as described above, became a useful tool for the purification of the active principle. The results of two experiments are reported in Table II.

TABLE II

FRACTIONATION OF THE ACTIVE POLYPEPTIDE ON ALUMINIUM OXIDE COLUMNS

In Fractionation A the column measured 1·1 × 8·0 cm., and in Fractionation B a column 1·1 × 24 cm. was used.

Fraction	Solvent: % Methyl Alcohol	Solvent: Vol. (ml.)	Dry Material (mg.)	Activity Units/mg.	Total Activity in Units
A Original	95	50	100	1·0	100·0
1	95	50	56	0·02	1·2
2	70	50	4	4·5	18·0
3	50	100	2	7·0	14·0
4	Dist. water	35	25	0·28	7·0
				Sum	40·2
				Yield	40·2%
B Original	80	80	75	1·0	75·0
1	80	80	46·5	0·9	42·0
2	70	80	3·0	7·9	23·8
3	50	80	2·1	1·0	2·1
4	50	120	4·0	0·88	3·5
5	50	200	—	—	0·6
				Sum	72·0
				Yield	96%

The first experiment, using 95% methanol as the first solvent, showed a low recovery. Fraction 3 gave a 7-fold purification, but only a 14% recovery. The total recovery, when using high concentrations of methanol as the initial solvent, was only 40.2%. Starting with high concentrations of methanol offered the advantage of removing a great deal of weight with very little activity. The method using lower concentrations of methanol as the initial solvent necessitated careful cutting of fractions in order to achieve an increase in purification. Since the recovery was high, the remainder could always be rechromatographed for the fraction with the highest potency. Satisfactory results were obtained by the method using 80% methanol as initial solvent and 70% methanol as eluting solvent. This method consistently gave recoveries exceeding 85%. The method using absolute or 95% methanol as the initial solvent always gave recoveries of less than 50%. The longer column (1.1 × 24 cm.) was of some value in cutting the most active fractions, since, with a water-alcohol solution, the bands were very wide and tended to overlap on a smaller column (Table II).

Paper Pulp Column.—Andrade, Diniz and Rocha e Silva (1953) have used a paper pulp column successfully for the purification of bradykinin. Since the active substance in urine was similar to bradykinin in pharmacological activity (Gomes, 1955), the same method of purification was attempted.

The active principle is adsorbed on the paper pulp column from a 7% phenol solution and thus differs from hypertensin which is not adsorbed (Helmer, 1950). This is additional evidence that the activity is not due to hypertensin. Elution of an active fraction was effected with 0.4% acetic acid. Rocha e Silva used 0.2% acetic acid. Concentrations of 0.1%, 0.2%, 0.4%, 0.7% and 1% acetic acid were tried and 0.4% was found to be the most satisfactory. With this method, the recoveries of activity as assayed on the superfused rat uterus were from 30 to 60%.

The paper pulp column gave excellent results in purifying the crude powder preparation. With adsorption from the 7% phenol solution and subsequent development with this solvent, 90% of the weight of the original powder was found in the eluate. This powder was completely inactive on the superfused rat uterus in a concentration of 6.5 mg./ml. while the standard preparation was active in a concentration of 0.05 mg./ml. The original potency adsorbed on the column was equivalent to 250 mg. of the standard preparation.

The powder obtained from the acetic acid eluates of the paper pulp columns had activities ranging from 6 to 9 units/mg. where the starting material was 1 unit/mg.

In several purification experiments, when large volumes of the 0.4% acetic acid were passed through the column, a second output of activity was noticed. This was usually seen after 150 to 250 ml. of solution had been collected. Upon continued washing of the column (up to 650 ml.), this material continued to be detectable on the superfused rat uterus.

The active polypeptide preparation under these conditions thus showed the presence of two active substances. Various methods were tried to elute the remaining activity from the column and the most efficient eluting solvent found was 0.1 N-HCl. This brought down from 15 to 30% of the original activity. If combined with the acetic acid eluates the recoveries of the original activity ranged from 60 to 92% (Table III).

Provisionally, the substance eluted with acetic acid has been called Z_1 and that eluted with HCl, Z_2 (Fig. 1).

The technique used in the separation of Z_1 and Z_2 by the paper pulp column was as follows: About 400 mg. of the dry crude powder was dissolved in a small volume of distilled water, and a concentrated phenol solution added until the concentration of phenol was 7%. This solution was allowed to filter through the paper pulp column. About 150 to 250 ml. of the 7% phenol removed approximately 90% of the original weight. This was seen as a yellow band moving down the column. The eluate was a deep yellow colour. When the eluate became colourless, the 0.4% acetic acid was started. Usually 150 to 250 ml. was collected. Activity was detected in the first 10 ml. of the 0.4% acetic acid eluate, the peak being between

TABLE III

FRACTIONATION OF Z_1 AND Z_2 ON A PAPER PULP COLUMN

Fraction	Solvent	Vol. of Fraction (ml.)	Units/ml.	Total Units
Original	7% phenol	—	—	216
1	,,	250	0·0	0
2	0·4% acetic acid	50	1·2	60
3	,,	25	0·8	20
4	,,	25	0·4	10
5	,,	25	—	< 1·5
6	,,	25	—	< 1·5
7	,,	25	0·04	1
8	,,	25	0·2	5
9	,,	50	0·12	6
10	,,	50	0·1	5
11	0·1 N-HCl	75	0·4	30
12	,,	75	0·03	2
			Sum	140
			Yield	65%

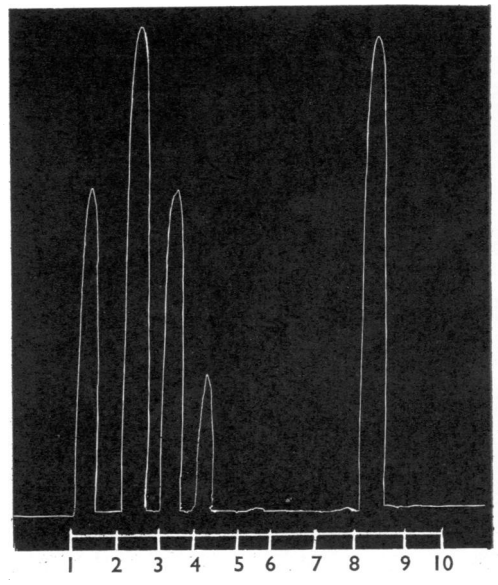

FIG. 1.—Rat uterus. Superfusion. Fractionation of Z_1 and Z_2 on the paper pulp column. Each response is due to a 1 : 5 dilution of an aliquot of 50 ml. fractions. Eluting fluids, 1 to 7 were 0.4% acetic acid, and 8 to 10 were 0.1 N-HCl.

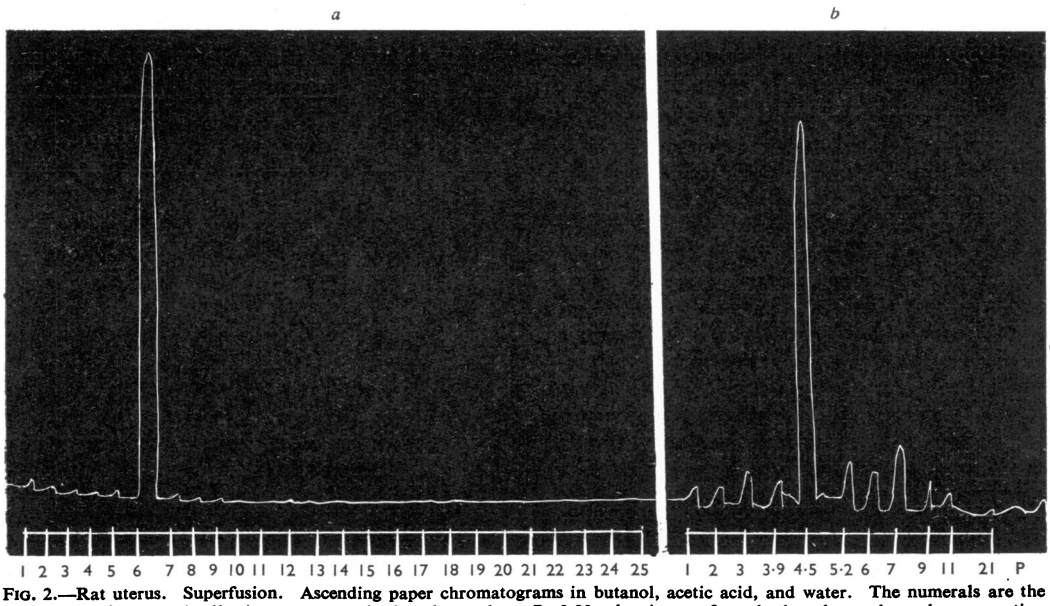

FIG. 2.—Rat uterus. Superfusion. Ascending paper chromatograms in butanol, acetic acid, and water. The numerals are the distance from starting line in cm. *a*, standard crude powder. R_F 0.22. *b*, mixture of standard crude powder and a preparation of bradykinin in equipotent amounts. R_F 0.2. P = paper only.

50 and 100 ml. The second eluting solution was 0.1 N-HCl. A pink band was then seen on the column. The first few ml. of the 0.1 N-HCl eluate contain almost all of the activity. It was best to collect the first few fractions in small volumes, as the later ones had less activity on a weight basis. The active fraction preceded the pink band. When the pink band was collected and dried, a red solid was obtained which had less activity than the preceding fraction. The first 50 ml. of eluate has 95 to 99% of Z_2, the next 50 ml. held the remainder and the red solid.

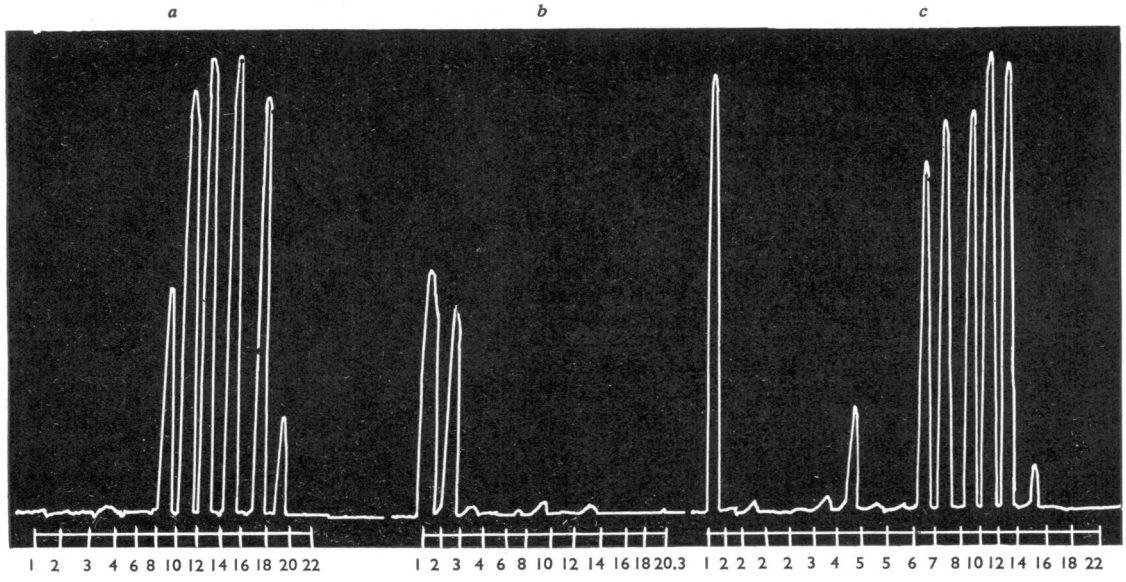

FIG. 3.—Rat uterus. Superfusion. Effects of eluates from ascending paper chromatograms in acetone-butanol-HCl (10 : 4 : 2). The numerals are the distance from starting line in cm. *a*, Z_1, R_F 0.6: *b*, Z_2, R_F 0.0. *c*, A preparation of bradykinin showing both fractions. Small activity shown at 4 cm. was always present, even in preparations of Z_1 from urine.

Combined Columns.—In an attempt to prepare a more purified preparation, the paper pulp and the aluminium oxide columns were used. Andrade *et al.* (1953) have shown that a combination of both columns was very successful in the purification of bradykinin. In these experiments, the crude powder was chromatographed on the paper pulp column and then chromatographed on the aluminium oxide column by the methods described above. Only Z_1 was purified in this way. The activity was 35 to 68 (35–44–68) times that of the original crude powder.

Paper Chromatography.—When an extract of the crude powder was chromatographed on paper using N-butanol-acetic-acid-water, the R_F of the activity was 0.2. It was lower when urine was used directly. Upon colour development with ninhydrin there are 5 spots on the chromatogram when the crude powder was chromatographed. In ultra-violet light, two spots were observed, one at the baseline and the other near the advancing front. The 5 spots with ninhydrin correspond to R_F values of 0, 0.1, 0.15, 0.2 and 0.35. The spot associated with the activity was pale violet in colour. If the strip was slightly overloaded with a larger amount of crude powder, a brownish spot was observed at R_F of 0.2. It seemed that the activity was always associated with this brownish spot which could be seen in daylight without any colour development.

The crude powder showed a spread of activity over the first 5 to 7 cm. of paper when chromatographed in the acetone, butanol and HCl system (Fig. 2). At times, the assay of the paper strip showed two peaks of activity, one at 0 cm. and another at 5 cm., but clearly defined results were never obtained. However, when Z_1 was chromatographed alone (Fig. 3*a*), a rather diffuse band was obtained with an R_F of 0.6. Z_2, in this system, gave an R_F of 0 (Fig. 3*b*).

A peculiarity noticed was that the R_F values of both Z_1 and Z_2 were 0 in the butanol, acetic acid and water (4 : 1 : 5) mixture, while the activity in the crude powder containing both Z_1 and Z_2 had an R_F of 0.2.

Comparison with Bradykinin

The similarity of this principle to bradykinin prompted a comparison of these two substances by paper chromatography. In the butanol-acetic-acid-water (4 : 1 : 5) system, the bradykinin had an R_F of 0.22, and the urine principle had an R_F of 0.20. When a mixed chromatogram was run (Fig. 2*b*), only a single peak of activity was noted.

When bradykinin was subjected to the chromatographic procedure for the separation of Z_1 and Z_2, it was found that it also gave two peaks, corresponding to Z_1 and Z_2. The preparation of bradykinin used in these experiments contained two active substances and the fraction corresponding to Z_2 in three different experiments contained 19, 21 and 25% of the total activity. The urinary extracts always contained a larger proportion of Z_2 than this, the average being 30% of the total activity. Bradykinin was also shown to contain two substances when chromatographed on paper in the acetone-butanol-HCl system (Fig. 3*c*). With this system, two active substances were found at R_F values corresponding roughly to those of the active substances in the urinary extract.

Discussion

The two active polypeptides found in urine seem to correspond to the two active polypeptides found in bradykinin. Gomes (1955) has previously shown that the active polypeptide found in urine resembled bradykinin when subjected to parallel assays on the rat uterus, guinea-pig ileum and hen rectal caecum. In our experiments, bradykinin was shown to have the same chromatographic properties as the urinary substance, and, furthermore, the preparation of bradykinin used contained two active polypeptides similar to those found in the purification of the urinary substance. The possibility exists that this bradykinin-like substance is a naturally occurring compound which is manufactured in the human body and partly destroyed by enzymes in blood and kidney cortex, and partly excreted in the urine (Hamberg and Rocha e Silva, 1954; Rocha e Silva, 1955).

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