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A SLOW CONTRACTING SUBSTANCE IN NORMAL HUMAN URINE

BY

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Crude urine of man and other animals as well as urinary extracts have repeatedly been shown to have a stimulating effect on isolated plain muscle. However, few attempts have been made to characterize and purify the active principles.

As it had been observed that superfused rat's uterus showed great sensitivity to diluted human urine (Gaddum, unpublished), a study of the properties of the substance causing this effect was undertaken. Besides being extremely sensitive, this preparation has the advantage of requiring only small volumes of active solutions.

METHODS

Rat's Uterus.—Uteri from virgin rats weighing 160–190 g. were superfused with Jalon's solution (Gaddum, Peart, and Vogt, 1949) by the technique described by Gaddum (1953a). At first they were injected the day before the experiment with stilboestrol (10 μg./100 g. wt.), but later the use of stilboestrol was abandoned because the uterus in artificial oestrus, though usually more sensitive, also frequently gave irregular responses.

Only freshly voided urine of normal people of both sexes was used and no considerable individual variations were observed. The preparation responds usually to a concentration of 1% (v/v) urine, though, sometimes, depending on the sensitivity of the organ and the activity of urine, responses are elicited with dilutions as low as 0.1%. Only exceptionally was it necessary to use concentrations higher than 2% to get suitable contractions.

Dilutions of urine and its extracts were made in the superfusion fluid, to which atropine sulphate (1 mg./l.) was routinely added. Of these dilutions 5 drops were applied to the uterus, after stopping the flow, and allowed to act for 30 sec. before being washed away. The contractions show a characteristic latent period variable with the dose, and greater in the less active samples. They are quite different from those induced by acetylcholine or 5-hydroxytryptamine and similar to those obtained by bradykinin, angiotonin, or substance P. They begin usually 20–40 sec. after the addition of the solutions and reach the maximum after 60–100 sec.

The administrations were made at intervals of 4 or 5 min., the latter interval generally giving more uniform responses.

A good preparation discriminates between doses differing by 20%. Tachyphylaxis for the active substance studied has not been observed; on the contrary, as a rule, there is a progressive sensitization during the first stages of the experiment. That the organ in superfusion is maintained in a good condition is shown by the fact that it was often possible to work with the same uterine horn for more than 8 hr.; sometimes the same horn was even used for 2 consecutive days, being kept in the refrigerator overnight.

Experiments done with the urine of different animals (rat, cat, dog, rabbit, guinea-pig), withdrawn from the bladder immediately after death, showed the same property of stimulating the rat's uterus, in the characteristic slow-reacting way. No attempt was made to identify the responsible principle (or principles) with that of human urine, which has been exclusively used in the present experiments.

Guinea-pig's Ileum.—From animals weighing 200–250 g., fasted for 20 hr., a piece of terminal ileum was removed and superfused with Tyrode's solution containing atropine sulphate (10^{-6}) and mepyramine maleate (10^{-6}). In this preparation, as pointed out by Gaddum (1953a), it was necessary to shorten the stoppage of flow to 15 sec.

Hen's Rectal Caecum.—This organ was also superfused with Tyrode's solution containing atropine and mepyramine as above. Owing to slow relaxation the intervals between application were 6–7 min. Stoppage of the flow for 30 sec. did not cause contraction.

Blood Pressure in the Rat.—The technique as described by Dekanski (1954) for the assay of angiotonin was followed. All the drugs were tested before and after dibenamine treatment.

Blood Pressure in the Rabbit.—Rabbits (1.8–2.3 kg.) under urethane anaesthesia (8 ml. of a 20% sol. i.v.) were used. Heparin (1,000 units of heparin "Boots") was injected routinely. Arterial blood pressure was recorded from the carotid artery by a Hg manometer and drugs were injected in the external jugular vein at 5–10 min. intervals. Assays were made both before and after section of the vagus.

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Chemicals.—The substance P was a preparation made by A. H. Amin and T. B. B. Crawford and estimated to contain 13.8 Euler units/mg. The bradykinin was a preparation obtained from M. Rocha e Silva through H. O. Schild. The angiotonin was "solution angiotonin" containing 10 cat units/ml., made by Eli Lilly and Co., and kindly supplied by J. Dekanski.

Lysergic acid diethylamide was kindly presented by Sandoz Products and 5-hydroxytryptamine creatinine sulphate by Messrs. Upjohn.

Crystalline preparations of trypsin, chrymotrypsin, and pepsin were obtained from Armour and Co., Ltd.

RESULTS

Extraction

It was found that both activated charcoal (B.D.H. 10 g./l.) and fuller's earth (2 g./l.) adsorbed the active principle of urine. In these concentrations they retained at least 90% of the activity when shaken with urine for 2 to 4 hours.

Method A.—A first method of extraction was developed using charcoal and eluting with glacial acetic acid (5 ml./g. of charcoal). It was found that the recovery in this stage was increased by bringing the urine to *p*H 9. The addition of 10 volumes of ether to the eluate led to the quantitative precipitation of the active material. A further purification consisted in the addition of ethanol with 20% water to the precipitate, in which the active principle dissolved, leaving a considerable amount of insoluble and inactive material. The supernatant fluid was reduced in volume in a vacuum at 40° C. and afterwards completely dried in high vacuum in the presence of P_2O_5. The resulting product was a very hygroscopic yellow powder. The final recovery, with this method, did not exceed 30% owing to difficulties in eluting the active substance from the charcoal, even when acetic acid was repeatedly added.

Method B.—A modification of the method employed by Clark, Winckler, Gollan, and Fox (1954) for the extraction of angiotonin was found to be simpler and more advantageous. Urine is acidified with HCl to *p*H 1.5, saturated with NaCl, and extracted by shaking with *n*-butanol. With Clark's procedure (extraction three times with 0.25 vol.) only about 60% was recovered (30% in the first extract). When an equal volume of butanol was used in a single extraction the recovery of the oxytocic activity was practically 100%. The final product obtained in this way was more active and more soluble in water than that obtained by repeated extractions with smaller volumes. Both conditions—acidification and saline saturation—were seen to be indispensable for the passage of the active principle to the butanol.

The butanol layer was separated by centrifugation. The addition of 5 vol. of ethyl ether to this precipitated the active material. This precipitation was as complete at room temperature as at −20° C., which those authors consider necessary for angiotonin. After drying *in vacuo* at 40° C. a pinkish white powder was obtained, 1 litre of urine yielding about 1 g. This preparation is stable and appears to contain all the active substance originally present. Further purification has not yet been attempted.

Dialysis

The active principle is slowly dialysable through cellophane. In experiments with urine against distilled water, using dialysers similar to those described by Verney (1926) and mounted in a shaker, equilibrium was slowly reached. After 4 hr. the activity in the water was practically nil and there was no appreciable loss in the urine. After 20 hr. the activity was equally distributed on both sides.

Stability

The stability of the substance in the urine and of the extracts in aqueous solutions depends on the *p*H. After 24 hr. at room temperature there was a loss of activity of about 50% in different urines at *p*H 7. In one sample of urine brought to *p*H 5 with 4N-HCl, and kept in the same conditions, the destruction did not exceed 10–20%, whereas in another sample of the same urine at *p*H 9 (4N-NaOH was added) more than 75% was destroyed in the same period of time. Aqueous solutions of extracts gave similar results.

A solution of a butanol extract kept in the refrigerator (4°) at *p*H 5 showed, after a fortnight, a 10 to 20% loss of activity.

The thermostability was studied with extracts made by the second method described. Samples of solutions in 0.03N, 0.3N, and 1N-HCl and 0.1N-NaOH were placed in boiling water in test-tubes closed with rubber dam and specimens for assay taken at different intervals. In 0.03N-HCl practically all the original activity was present after 6 hr. In 0.3N-HCl 50% was destroyed at the end of 1 hr. and 75% after 4 hr. boiling. More than 80% was inactivated after 30 min., when N-HCl was used as solvent. In alkaline solutions the substance is much less stable. There was a loss of 50% after 15 min. and complete destruction after 30 min. when it was boiled in 0.1N-NaOH.

At a *p*H near neutrality urine stands boiling for periods as long as 4 hr. without apparent loss of activity.

Solubility

The active principle is soluble in water, glacial acetic acid, aqueous ethanol (80%), aqueous acetone (80%), phenol, *n*-butanol and methanol. It is completely insoluble in ether, chloroform, benzene and petroleum ether, scarcely soluble in absolute ethanol, and practically insoluble in pure acetone.

Action of Proteolytic Enzymes

Both urine and solutions of butanol extracts were completely inactivated by chymotrypsin after incubation at *p*H 8 and 38° C. for 60 min. (Fig. 1*a*). Neither trypsin nor pepsin caused any change in the activity when incubated for 4 hr. at *p*H 8 and 2, respectively (Fig. 1*b* and 1*c*). The powerful stimulating activity of trypsin for rat's uterus made it difficult to study the action of this enzyme. Contrary to what had been described for guinea-pig's ileum (Rocha e Silva, 1951) the organ was not desensitized after repeated administrations of the enzyme. Making use of the greater thermostability of the urinary principle it was possible to destroy trypsin almost completely, by boiling for 30 min., and prove the preservation of that principle in the incubate.

Paper Chromatography

Extracts obtained by both methods were studied by paper chromatography, using the ascending method in Whatman No. 1 filter paper, washed previously with 0.01N-HCl. The solvents were the organic phase of the mixture *n*-butanol-acetic acid-water (4:1:5) (Partridge, 1948) and phenol saturated with 0.01N-HCl. The chromatograms were developed at room temperature (15–18° C.) for 20–24 hr., in most of the experiments in air, in only a few in nitrogen which proved to be unnecessary.

The localization of the active substance was detected by biological assay. Strips of paper were cut at variable intervals from the starting line to the solvent front, and eluted by descending chromatography, overnight, in small individual troughs (7 ml. capacity) with 0.01N-HCl in an airtight chamber. The eluates were evaporated to dryness in a vacuum (40° C.), taken up in Jalon's solution and assayed on the superfused uterus.

With the purest extracts obtained by the first method there were always at least three spots stained by ninhydrin (0.2% solution in acetone with 5% acetic acid). These bore no relation to the active principle, since their R_F was much lower

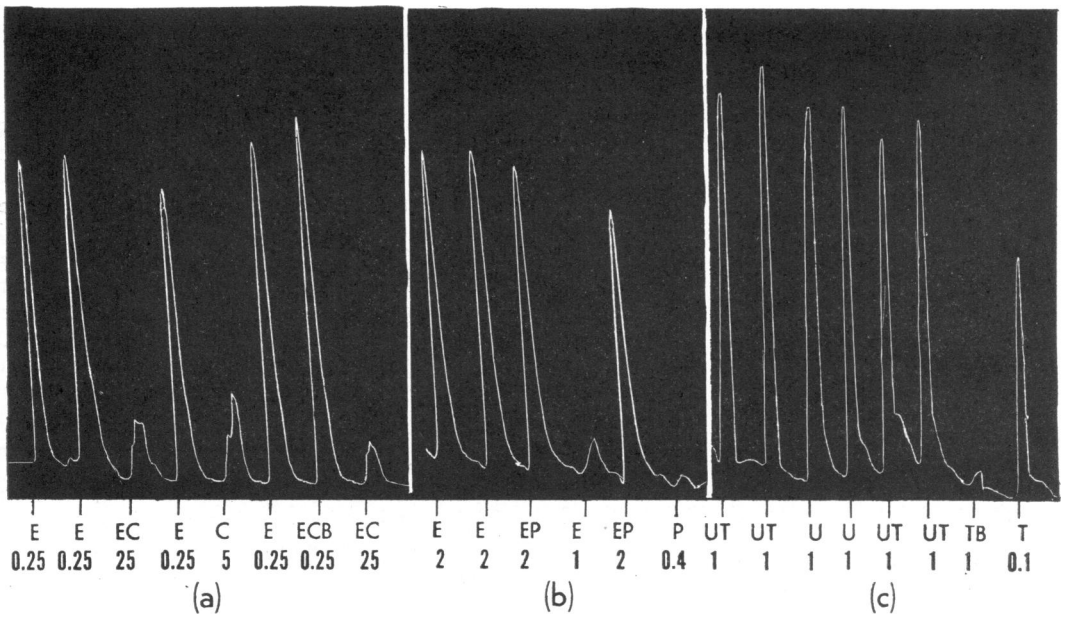

FIG. 1.—Rat's uterus. Superfusion. Active substance destroyed by chymotrypsin, but not by pepsin or trypsin. Concentrations mg./100 ml. (solids); ml./100 ml. (urine). E, dried extract of urine. C, chymotrypsin. P, pepsin. T, trypsin. TB, trypsin boiled 30 min. EC, E incubated 1 hr. with chymotrypsin (0.2 mg./mg. E). ECB, control with boiled chymotrypsin. EP, E incubated 4 hr.with pepsin (0.2 mg./mg. E). U, urine boiled 30 min. UT,urine incubated 4 hr.with trypsin (10 mg./ml.) and then boiled 30 min.

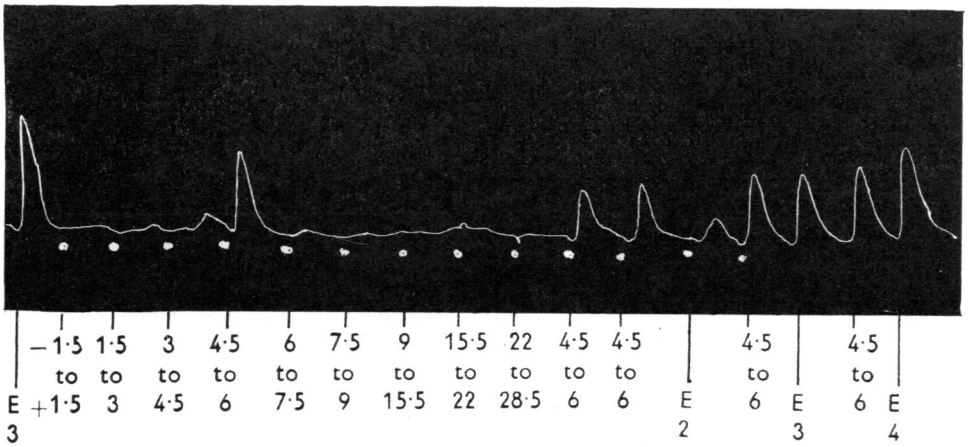

FIG. 2.—Rat's uterus. Superfusion. Effects of eluates from ascending paper chromatogram developed with butanol-acetic acid-water for 24 hr. Distances from starting line in cm. Solvent front 28 cm. E, butanol extract of urine (mg./100 ml.). Weight of E used, 0.4 mg. Volume of eluates 4 ml. R_F, 0.18. Total recovery 30%.

than that of the biologically active strip in chromatograms developed with phenol.

The butanol extracts, in the amounts used (spots containing 0.2 mg. of extract), did not give any coloured spot with ninhydrin or with Pauly's reagent.

The R_F values obtained by biological assay showed small variations with the purity of the product and were about 0.2 in the butanol-acetic acid-water mixture and 0.9 in phenol. With both solvents the total recovery from the paper chromatogram was about 30%, all the activity being always confined to a narrow zone (Fig. 2).

One of the butanol extracts was submitted to acid hydrolysis for chromatographic study. 0.5 ml. of 4N-HCl was added to 50 mg. of extract in an ampoule that, after being sealed, was put in an oven at 100° C. for 24 hr. The contents of the ampoule were afterwards dried on a boiling-water bath *in vacuo* and redissolved in 5 ml. of distilled

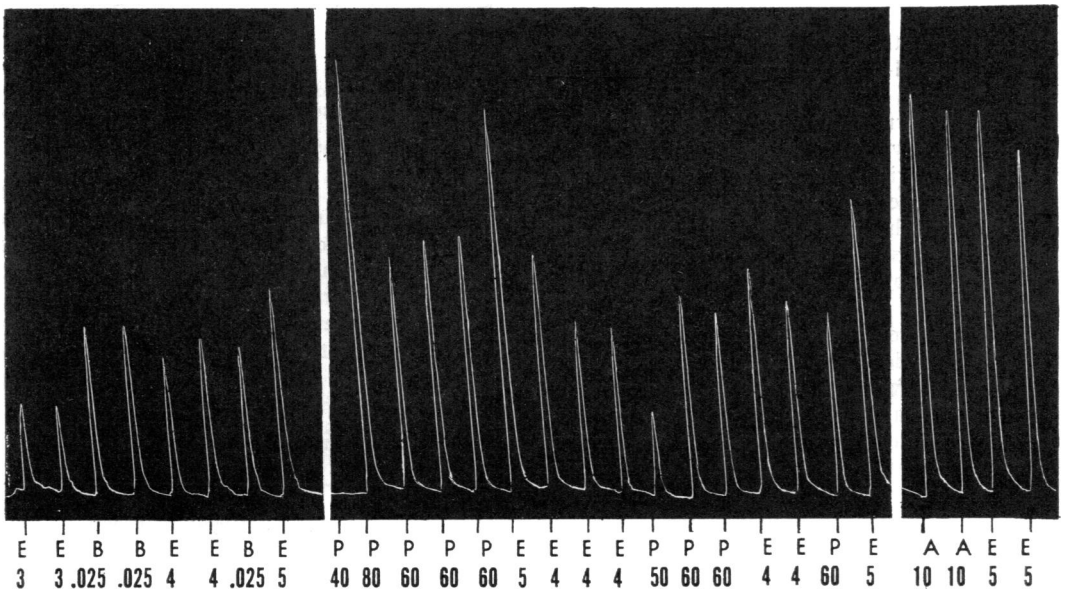

FIG. 3.—Rat's uterus. Superfusion. Comparison between a butanol extract of urine (E), bradykinin (B), substance P (P), and angiotonin (A). Concentrations in mg./100 ml. (E and B) and units/100 ml. (P and A).

water. After filtration the hydrolysate was applied to filter paper (spots of 0.02 ml.) and developed by ascending chromatography with both of the above mentioned solvents. In these conditions there were at least 6 ninhydrin positive spots which have not yet been identified. A solution of the original extract, in the same concentration and run simultaneously, failed to show any spot.

Other Pharmacodynamic Properties

Besides superfused rat's uterus (Fig. 3) a few other preparations have been used to distinguish the active principle under study from other known substances with similar properties. Parallel assays were made using the guinea-pig's ileum, hen's rectal caecum and rat's or rabbit's blood pressure with substance P, bradykinin and angiotonin. Bradykinin could not be used for blood-pressure assays, as it was not available in sufficient amount.

Both urine and urinary extracts were less active on the guinea-pig's ileum than on the rat's uterus. In some experiments concentrations as high as 10% of urine only elicited small contractions in the ileum. Furthermore, this organ did not give, as did rat's uterus, regular responses consistent with an accurate estimation of activity. As a rule the contractions were in a "staircase," very different from those in the uterus, with a latent period of 40–60 sec.

No contraction was obtained in the hen's rectal caecum, though very high concentrations of urinary extract were used. Bradykinin, substance P and angiotonin elicited contractions of this preparation as reported by others.

The rat's blood-pressure preparation, in the conditions used, was very sensitive to angiotonin, but gave weak and irregular responses to substance P, as was found by Amin, Crawford and Gaddum (1954). The urinary extracts elicited irregular responses, and clear hypotensive effects were only observed after the injection of large doses, and were then accompanied by symptoms of general intoxication followed after a short period by the animal's death.

In the rabbit too it was necessary to administer relatively high doses to obtain depressor effects and these were far from constant. Some animals seemed particularly resistant, and in the same animal an originally hypotensive dose produced no effect when repeated some time later. Comparison with substance P (Fig. 4) in doses equiactive on the uterus, or even on the ileum, showed that the depressor action of the urinary extracts was feeble.

In a rabbit that gave clear hypotensive responses the comparison was made of a butanol extract and the urine used in its preparation. It was found (Fig. 4) that the hypotension produced by the extract, in a dose corresponding to 100 times that of urine, was of a much shorter duration.

Since the oxytocic substance was quantitatively extracted in other experiments by butanol, the depressor effect of the urine must be mainly due to other substances.

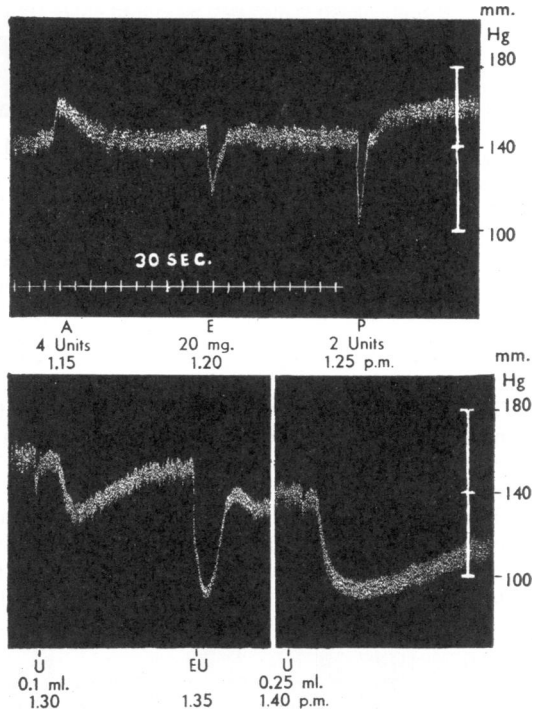

FIG. 4.—Rabbit. 2.3 kg. Arterial pressure. Urethane. Vagi cut. Artificial respiration. Intravenous injections. A, angiotonin. E, same extract as in Fig. 3. P, substance P. U, urine. EU, butanol extract from 25 ml. of the same urine.

Table I shows the results of assays of a urinary extract against substance P, angiotonin and bradykinin by several different methods. It will be seen that when substance P or angiotonin was used as a standard the urinary extract showed high activity on the rat's uterus compared with much lower activity on the other tissues used. The active sub-

TABLE I

PARALLEL ASSAYS OF THE AMOUNTS OF OTHER POLYPEPTIDES EQUIVALENT TO 1 MG. OF A PREPARATION OBTAINED FROM URINE

		Substance P (Units)	Angiotonin (Units)	Bradykinin (mg.)
Rat ..	Uterus	15	2	0·006
Guinea-pig	Ileum	0 6	0·16	0·008
Hen ..	Rectal caecum	<0·08	<0·08	<0·08
Rabbit ..	Blood pressure	<0·1	Pressor	Not done

stance cannot therefore be substance P or angiotonin. On the other hand, when bradykinin was used as a standard the results obtained with rat's uterus and guinea-pig's ileum agreed well with one another, and the results obtained with hen's rectal caecum were consistent with the theory that the active substance is identical with bradykinin. The urinary extract had no effect on this preparation in the doses used, but the other preparations all had some effect, and it was found that roughly 1 unit of substance P, 1 unit of angiotonin and 1 mg. of the preparation of bradykinin were equivalent to one another in their actions on the hen's rectal caecum.

Discussion

The inactivation by chymotrypsin, the slow dialysis, and the chromatographic behaviour suggest that the active principle under study is a polypeptide. The fact that it can be extracted by methods used for the extraction and purification of substances of established polypeptide nature supports this view.

The complete suppression of the stimulating activity of urine by chymotrypsin and by boiling in strong acid or alkaline media, and the localization of all the activity in a single zone of chromatograms developed in two different solvents, suggest that a single substance is responsible for the effect on rat's uterus.

However, other substances present in the urine could contribute to the stimulating action. Therefore some experiments were made in order to clarify this point.

Urea and potassium have no action on the uterus in concentrations much higher than those likely to be present in the diluted urines used in these experiments.

Nicotine, which has been found in the urine of smokers (Helmer, Kohlstaedt, and Page, 1939), had no action on the uterus in concentrations from 0.05 mg. to 100 mg./ml. of the tartrate, nor did it modify the response to extracts of urine if simultaneously added.

Work done by Twarog and Page (1953) with the isolated heart of *Venus mercenaria* led to the conclusion that 5-hydroxytryptamine (HT) is normally present in human urine. Though the rat's uterus in superfusion is very sensitive to this substance, the concentrations mentioned by those authors are below the threshold of the preparation. Making use of the inhibitory action of the lysergic acid diethylamide (LSD) (Gaddum, 1953b) it was possible to demonstrate persistence of the response of the organ after the addition of urine and complete abolition of the effects of a solution of HT which was equipotent before the addition of that antagonist (Fig. 5).

Darmstoff was originally assumed by Kuck and Vogt (1950) to be identical with substance P, but is distinguished from this and the urinary principle by its solubility in chloroform.

Kallikrein (Frey, Kraut, and Werle, 1950) is easily distinguished because it is thermolabile and undialysable, and does not contract the rat's uterus or the guinea-pig's ileum.

Wollheim's Depressan is a substance which, according to the author (Wollheim, 1937), is present in the urine of normotensive individuals and absent in those suffering from essential hypertension. It is a potent depressor and it has, in its purified preparations, practically no stimulating action on the intestine or uterus. Other properties add to its distinction: it is not dialysable, it is not adsorbed by charcoal, it is destroyed by pepsin and is less thermostable than the principle discussed here.

A similar if not identical depressor substance was described by Little, Green, and Bumgarner (1948). Stewart (1953) reported the stimulating effect of extracts of human urine on the isolated uterus of the guinea-pig or mouse, and on the guinea-pig's ileum, the latter organ being less sensitive. This active principle is stated to be undialysable through cellophane.

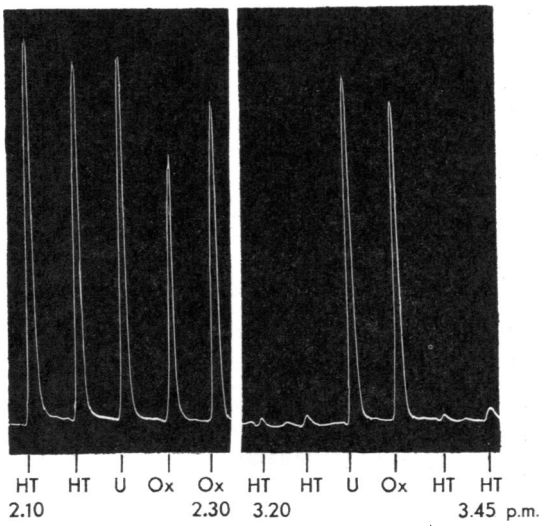

FIG. 5.—Rat's uterus. Superfusion. HT, 5-hydroxytryptamine 10 μg./l. U, urine 1%. Ox, oxytocin 0.3 units/l. Lysergic acid diethylamide (10^{-7}) in the superfusion fluid from 2.35 onwards. The effect of HT was abolished, but not that of U or of Ox.

Although the same activity has been found in female urine, the possibility of contamination of male urine, which was mostly employed, with semen must be considered. A depressor and plain muscle stimulating substance in human semen has been reported independently by Goldblatt (1935) and v. Euler (1936a). This secretion was shown to induce marked contraction of the uterus of different animal species, including the rat. The active principle, according to Euler, is easily distinguishable from the urinary substance by its solubility in organic solvents (ether, chloroform) and its strong and lasting hypotensive effect.

It remains to discuss the relation of the substance described here to a group of natural polypeptides which stimulate plain muscle and are described in a book edited by Gaddum (1955).

There is evidence that posterior pituitary hormones are excreted in the urine. It will be seen that the oxytocic action of the urine used in the experiment shown in Fig. 6 was equivalent to that of 40 units of oxytocin/l. This effect was not, however, due to the presence of these large amounts of oxytocin, since treatment of the urine with thioglycollate by the method of Ames, Moore, and van Dyke (1950) did not inactivate it.

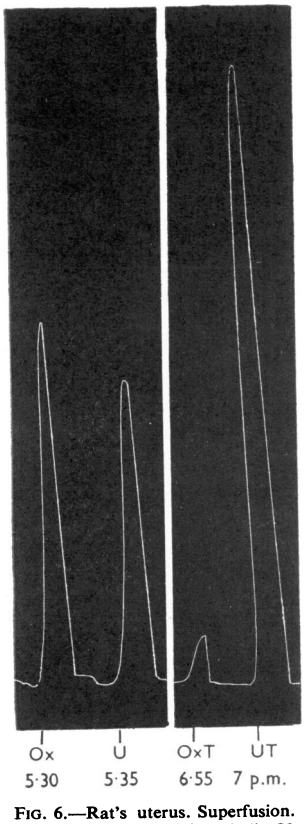

FIG. 6.—Rat's uterus. Superfusion. Ox, oxytocin 0.3 units/l. U, urine (1%). OxT and UT, after treatment with thioglycollate which inactivated Ox but not U.

Table I shows that the active substance is not substance P (Euler and Gaddum, 1931; Gaddum and Schild, 1934). The fact that substance P is inactivated by trypsin (Euler, 1936b) is also evidence against this identification.

The fact that the rat's uterus is, according to Ludueña (1940), particularly sensitive to angiotonin (hypertensin) as well as many of its physical and chemical properties suggested that this might be the substance in question. It was for this reason that Clark's method of extraction was used. The results shown in Table I, together with the fact that angiotonin is inactivated by pepsin and trypsin, seemed to disprove this view. None of these urinary extracts had a pressor action.

Bradykinin (Rocha e Silva, 1951) shows many similarities with the urinary principle (dialysis, stability at different *p*H's, solubility, action of proteolytic enzymes, etc.). The results shown in Table I are in favour of their identity, but more evidence is required.

The effects described here seem to be due to a substance, or group of substances, which have been studied by various groups of workers. Kallidin was described by Werle (1937) and given its name by Werle and Berek (1950). It is formed by the action of an enzyme in urine on blood. According to Werle, Kehl, and Koebke (1950) it is identical with bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949), which is formed by the action of snake venom or trypsin. Substance U (Beraldo, 1952, 1955) is formed by urine in much the same way that kallidin is formed, and substance Z (Werle and Erdös, 1954) is naturally present in urine. All these substances share with the substance described here the property of being destroyed by chymotrypsin, but not by trypsin.

The main value of the present work is thought to lie in the fact that it provides a simple method of assay which measures activity of this kind and which does not appear to be significantly affected by any of the other known active principles which may be present in urine. It also provides a method of making a dry standard preparation.

SUMMARY

1. Normal urine diluted 1:100 has been found to cause contraction of superfused rat uterus.

2. The active principle is stable in weak acids, dialysable, and soluble in 80% aqueous acetone or ethanol, but not in ether or chloroform. It was destroyed by a preparation of chymotrypsin, but not by trypsin or pepsin, and is probably a polypeptide. Two methods are described by which active dry preparations of the substance may be made.

3. Evidence is presented that this effect is not due to potassium, urea, nicotine, 5-hydroxytryptamine, kallikrein, depressan, oxytocin, substance P or angiotonin.

4. The active principle is closely allied to the substance, or substances, known as kallidin, bradykinin, substance U and substance Z.

This research was carried out at the suggestion of Professor J. H. Gaddum, to whom I wish to express my gratitude for advice and criticism throughout it.

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