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EFFECTS OF TRANSIENT CHANGES OF ACIDITY ON THE ISOLATED RAT'S UTERUS, WITH REFERENCE TO THE ASSAY OF OXYTOCIC ACTIVITY

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For the biological assay of oxytocin, the *British Pharmacopoeia* (1953) recommends that the material to be tested should be washed with " one half as many ml. of a mixture of 0.25 ml. of glacial acetic acid and sufficient water to produce 100 ml. as there are Units present in the quantity of powder." and that it should be diluted so as to obtain responses similar to those from the standard preparation. These recommendations, however, are designed for estimating oxytocic activity in commercial batches of pituitary hormones and cannot be followed when estimating the oxytocic activity of extracts of pituitary glands which contain only a few milli-units of oxytocic activity.

When estimating the oxytocic activity of pituitary glands of frogs, it was found that the addition of acetic acid extracts of pituitary glands to the bath produced marked changes in the sensitivity of the uterus. The aim of the present investigation was to elucidate the causes of these changes and to examine the validity of assays when estimating similar small amounts of oxytocic activity.

METHODS

Pituitary glands of frogs were dried with acetone and each extracted with 1 ml. of a 0.25% acetic acid solution (Levinsky and Sawyer, 1953).

For the assays a strip of the horn of a rat's uterus in dioestrus was suspended in a 3 ml. bath containing a modified Locke solution (García de Jalón, Bayo Bayo, and García de Jalón, 1945) at 29.5° C. and gassed with either O_2 or a mixture of 95% O_2 and 5% CO_2 (Burn, Finney, and Goodwin, 1950). The *p*H of the modified Locke solution, measured with glass electrodes, varied between 7.4 and 7.5.

The assays were of the standard (2 + 2) dose design (*cf.* Holton, 1948); the ratio of high to low dose was the same for standard and unknown. Each dose was given once in each group of four doses and its position within the group was decided at random. Doses were added at intervals of 3 to 5 min., depending on the rate of recovery of the muscle. After the uterus had contracted, the bath was emptied and immediately refilled with the modified Locke solution. Results are given as means with their standard deviations (S.D.) and standard errors (S.E.). Significance of differences between means were determined by Students' *t* test. The fiducial limits of errors were calculated according to the *British Pharmacopoeia* (1953).

To avoid variation of the ionic composition of the bath, all dilutions were made with a 0.9% NaCl solution (van Dyke and Hastings, 1928).

Pitocin (Parke, Davis & Co.) was used as the oxytocin standard.

RESULTS

Preliminary experiments showed that, after *undiluted* doses (ranging from 0.1 to 0.2 ml.) of acetic acid extracts of posterior pituitary glands of frogs, the uterus underwent a series of spontaneous and rapid contractions. Subsequent additions of the undiluted extract resulted eventually in a complete loss of sensitivity of the preparation. This loss of sensitivity was not observed when assaying extracts which had been diluted at least 10 times with a 0.9% NaCl solution. This, however, so reduced the oxytocic activity of the extract that no quantitative estimation was possible.

To see whether both loss of sensitivity and spontaneous contractions produced by the undiluted extract were due to the extract as such or to the presence of acetic acid, two equi-active solutions of oxytocin, one with and the other without acetic acid, were compared. They were made up as follows: (*a*) 1,000 mU. of oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with a solution of 0.25 ml. glacial acetic acid in 100 ml. isotonic NaCl solution; (*b*) 1,000 mU. of oxytocin in 100 ml. of 0.9% NaCl solution. As the two preparations had the same oxytocic activity, it was expected that equal doses of either would produce contractions of comparable magnitude. It was found, however, that when the aqueous solution

was given *after* the acetic acid solution the contraction of the uterine strip was always markedly increased. In other words, the response to a constant amount of oxytocin depended upon whether it was given before or after an equal dose of acetic acid-oxytocin solution. This is illustrated in the experiment of Fig. 1, where, as the doses were given at 4 min. intervals, the sensitizing effect lasted some 30 min. After recording normal contractions of the uterus to standard doses of oxytocin, 0.1 ml. of a 0.25% acetic acid solution diluted 10 times with normal saline had no effect on the uterus (Fig. 2). It was left in contact with the tissue for periods of from 1 to 4 min., and then washed away. Subsequent standard doses of oxytocin produced contractions of the uterus significantly greater and more rapid than those recorded before the acetic acid treatment. The intensity of these changes was correlated with the duration of contact between the uterus and the acetic acid solution (Fig. 2).

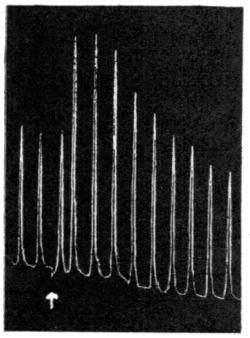

FIG. 1.—Rat uterus in 3.0 ml. bath. Effect of 0.1 ml. acetic acid-oxytocin solution (at ↑) on subsequent doses (0.1 ml. = 1.0 mU.) of standard solution of oxytocin. The acetic acid-oxytocin solution remained in the bath for 2 min. 25 sec.

To find which of the two ions, hydrogen or acetate, was responsible for the sensitization of the uterus, 0.0044 N-sodium acetate and 0.0044 N-hydrochloric acid were tested. Both solutions are equivalent to a 0.025% acetic acid solution (0.025% acetic acid solution = 0.0044 N). The addition of 0.2 ml. of the sodium acetate solution had no effect on the sensitivity of the uterus. When, however, similar doses of the 0.0044 N-HCl had been added to the bath, the uterus became more sensitive to oxytocin (Fig. 3). This showed clearly that the sensitization was the result of a change of the hydrogen ion concentration. This was confirmed by *p*H estimations: 0.1 or 0.2 ml. of either 0.025% acetic acid or 0.0044 N-HCl produced a marked drop of the *p*H to 7.2–7.1 after 2 min. exposure and subsequent removal. The difference between the falls of *p*H when the bath was gassed with O_2 or with a mixture of 95% O_2 and 5% CO_2 was not statistically significant. No significant changes in *p*H were observed when the dilution of either acid solution was increased up to 10 times or when a solution of oxytocin was given alone. Addition of 0.025 ml. 0.1 N-sodium hydroxide in a 0.9% NaCl solution, which increased the *p*H of the bath to between 8.1 and 8.3, had no significant effect on the response of the uterus to subsequent doses of oxytocin.

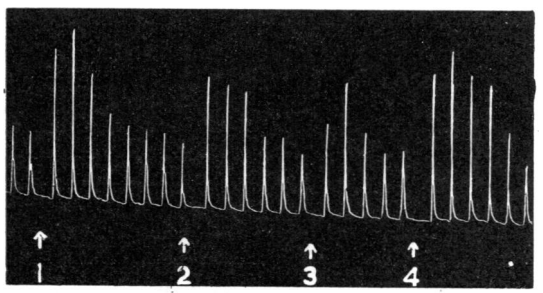

FIG. 2.—Effect of doses of 0.025% (=0.0044 N) acetic acid on subsequent responses of rat uterus (in 3.0 ml. bath) to oxytocin. At 1, 0.2 ml. for 2 min.; at 2 and 3, 0.1 ml. for 2 min.; at 4, 0.1 ml. for 4 min. Addition of acetic acid itself had no effect on the uterus, but sensitized it to subsequent doses of oxytocin. Dose of oxytocin soln. 0.1 ml. = 1.0 mU.

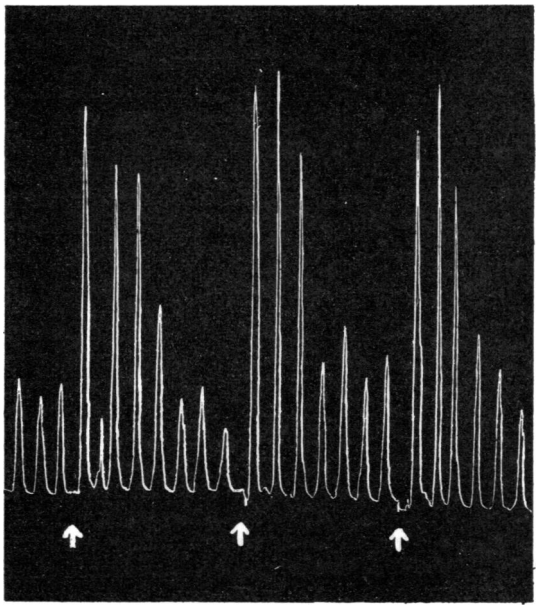

FIG. 3.—Responses of rat uterus, in 3.0 ml. bath, to 0.1 ml. (=1.0 mU.) doses of oxytocin soln. At ↑, 0.1 ml. 0.0044 N-HCl had in itself no effect on the uterus, but sensitized it to the oxytocin.

Changes in the sensitivity of smooth muscle to drugs concomitant with changes of *p*H values have been attributed to variations in the amount of Ca ion available (Evans and Underhill, 1923; Hastings, Murray, and Sendroy, 1927; Kerridge and Winton, 1929). Raising the normal $CaCl_2$ concentration of the bath fluid from 0.06 g./l. to 0.09 and 0.12 g./l.

produced, as a rule, *one* marked spontaneous contraction of the uterus, confirming van Dyke and Hastings' (1928) findings on the guinea-pig uterus. However, as soon as the extra Ca had been washed away, there was no increased sensitivity to oxytocin.

The modified Locke solution, buffered with sodium bicarbonate, was replaced in 6 experiments by a modified Ringer-Evans solution containing a phosphate buffer. Its composition was: NaCl 9.00 g.; KCl 0.42 g.; $CaCl_2$ 0.06 g.; Na_2HPO_4 0.6 g.; 1M-H_3PO_4 0.2 to 0.6 ml.; glucose 0.5 g., made up to 1,000 ml. with distilled water. The presence of a phosphate buffer decreased the sensitivity of the uterus to oxytocin. Addition of 0.2 ml. of 0.25% acetic acid, diluted tenfold with a 0.9% NaCl solution, produced only a transient increase in sensitivity to oxytocin; the response to a second or third dose of oxytocin was always similar to that before the acetic acid solution. Estimations of *p*H values showed that with this Ringer solution the acetic acid produced falls which were not significant; they did not exceed 0.1.

In view of these changes in sensitivity of the uterine preparation, the validity of assays of acid extracts that for practical reasons could not be greatly diluted was examined. Estimations of both "known" and "unknown" solutions of the same concentration of oxytocin were attempted first. The "unknown" solution was divided into halves; one without acetic acid, identical with the known solution, was called "saline unknown"; it contained 750 or 1,000 mU. oxytocin/100 ml. of a 0.9% NaCl solution. The composition of the other half was 750 or 1,000 mU. oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with 0.25% acetic acid solution. This was the "acetic unknown." This concentration of acetic acid is 20 times greater than that recommended by the *British Pharmacopoeia* but 2.5 times less than that used by Levinsky and Sawyer (1953).

TABLE I

(2+2) DOSE ASSAYS OF KNOWN SOLUTIONS OF OXYTOCIN

Description of Unknown Solutions	No. of Assays	Mean Amount of Oxytocic Activity (mU.)	S.E. ±	S.D. ±
1,000 mU. oxytocin in 100 ml. 0·9% NaCl = 1·0 ml. = 10·0 mU. (1)	10	9·9	0·17	0·53
1,000 mU. oxytocin in 90 ml. 0·9% NaCl +10 ml. of 0·25% acetic acid = 1·0 ml. = 10·0 mU. (2)	7	8·6	0·78	2·06
750 mU. oxytocin in 100 ml. 0·9% NaCl = 1·0 ml. = 7·5 mU. (3)	10	7·4	0·26	1·67
750 mU. oxytocin in 90 ml. 0·9% NaCl +10 ml. of 0·25% acetic acid = 1·0 ml. = 7·5 mU. (4)	9	6·0	0·55	0·94

All standard solutions were 1·0 ml. = 10·0 mU. oxytocin.
For (1) and (2), $t = 2{\cdot}017$, $P < 0{\cdot}1 > 0{\cdot}05$ (n=15).
For (3) and (4), $t = 2{\cdot}298$, $P < 0{\cdot}05$ (n=17).

In each assay two doses of "unknown" were compared with two doses of "known"; each estimation consisted of 4 groups of 4 randomized injections. The results of 36 such assays are given in Table I. The two "saline unknowns," containing 7.5 and 10 mU./ml. respectively, assayed at 7.4 ± 0.26 and 9.9 ± 0.7 mU./ml. But the two "acetic unknowns" of identical potency assayed at 6.0 ± 0.55 and 8.6 ± 0.78 respectively. At each concentration there was a significant difference ($P = 0.05$) between the mean estimated potency of "saline unknowns" and "acetic unknowns."

It is not always possible for practical reasons to have the adequate quantity of material necessary for a (2+2) dose assay. In those circumstances, it might be tempting to compare the effect of one dose of "acetic extract" with two different doses of standard in a (2+1) dose assay. It was, however, impossible to estimate the potency of an "acetic unknown" by "matching" its effect with that of two equal doses of the standard, as the reaction of the uterus to the dose of standard given after the "unknown" was significantly greater than that observed for the same dose of standard given before the "unknown." To avoid this difficulty an "acetic unknown" containing 7.5 mU. oxytocin/ml. (750 mU. oxytocin in 90 ml. of 0.9% NaCl solution made up to 100 ml. with 0.25% of acetic acid) was compared with two different doses of standard, 1.0 and 0.5 mU. oxytocin. The "acetic unknown" was bracketed by the two different standards. Theoretically, the reaction of the uterus to 0.1 ml. of the "acetic unknown" should have been equal to the mean of the effects of the two doses of the standard. The results were, however, quite unpredictable. Nine out of eighteen estimations had to be rejected, since the "unknown" failed to fall between the two doses of standard. The range of the remaining 9 estimations, calculated according to Gaddum (1954), varied from 5.3 to 9.1 with a mean of 5.5 ± 0.83 mU. oxytocin/ml. This showed conclusively that no reliable information about the amount of oxytocin activity of a preparation in which the acetic acid was not adequately diluted could be obtained from assays in which the "unknown" was tested at one dose level only.

Discussion

Evans and Underhill (1923) have shown that small amounts of acid enhanced the reactions of the guinea-pig's uterus to posterior pituitary extracts, and this was confirmed on the rat. On the other hand, according to Hemingway (1926), raising the *p*H values of the bath from 7.4 to 8.5 increased the sensitivity of the cat's uterus both to histamine and to posterior pituitary lobe extract. Similar changes in *p*H, however, had no effect on the sensitivity of the rat's uterus.

At *p*H values of 7.0 to 8.0 the buffer capacity of sodium bicarbonate of the modified Locke solution is very poor, since in *in vitro* experiments no regulation of the carbon dioxide levels can obtain (Ling and Smith, 1955). Hence, it was not surprising to find that the addition to the bath of small amounts of a 0.025% acetic acid solution produced significant changes in *p*H. These changes significantly enhanced the response of the rat's uterus to oxytocin; and though the increase of the hydrogen ion concentration was transient, its effect on the uterus lasted up to 30 min. These increased responses did not occur, however, when the acetic acid had been adequately diluted, for example, 100 times.

These findings had a definite bearing on the accuracy of assays of solutions of oxytocin of known concentration made up with 0.25% acetic acid solution tenfold diluted, to imitate a tissue extract of low oxytocic activity. In 16 such assays (Table I), comprising 4 groups of each of 2 unknown and 2 standard, the mean amount of oxytocic activity was found to be about 14% lower than that of a similar solution without acetic acid. From this it would be logical to assume that a transient increase in acidity of the bath may affect the accuracy of assays of posterior pituitary gland extracts, which, on account of their initial low oxytocic activity, cannot be greatly diluted. According to the *British Pharmacopoeia* (1953) the dry powder obtained after extraction of the posterior pituitary gland with acetone should be washed with one half as many ml. of a mixture of 0.25% acetic acid solution as there are *Units* present in the quantity of powder. This can be easily done when dealing with pituitary glands of adult mammals, such as the dog (Dicker and Tyler, 1953a), but not with individual glands of amphibians, young mammals or foetuses, as these contain only a few milli-units of oxytocic activity. The following amounts of 0.25% acetic acid solution have been used; amphibians, 1.0 ml./gland (Heller, 1941; Levinsky and Sawyer, 1953); newborn rats, 0.3 ml./gland (Heller, 1947); newborn infants, 0.5 ml./mg. of dry gland tissue (Heller and Zaimis, 1949); puppies, kittens, newborn rats, newborn guinea-pigs and human foetuses, 1.0 ml./gland (Dicker and Tyler, 1953a and b). When related to their oxytocic activity these amounts were far in excess of those used for adult mammals (Dicker and Tyler, 1953a), or recommended by the *British Pharmacopoeia* (1953). It is clear from the present investigations that unless diluted between 100 to 200 times, the high hydrogen ion concentration of such extracts will increase the normal reactions of the rat's uterus to oxytocin and hence impair the reliability of the assays. Adequate dilutions would, however, be impracticable, as their oxytocic activity would be too small to be estimated. Heller (1947), Heller and Zaimis (1949) and Dicker and Tyler (1953a and b) used extracts which were diluted between 3 and 5 times only, whereas Levinsky and Sawyer (1953) tested undiluted preparations. To overcome this difficulty, it might be advisable to pool as many glands as possible. It would need the pooling of 10 to 15 glands of frogs, puppies, or kittens to have enough oxytocic activity in the extract to allow it to be diluted accurately, i.e., to have enough material to test at an appropriate hydrogen concentration. This, however, would be practically impossible with neurohypophyses of embryos and especially of human foetuses (Dicker and Tyler, 1953b).

Summary

1. Estimations of the oxytocic activity of frogs' neurohypophyses were attempted on an isolated rat's uterus preparation.

2. Addition of dilute acetic acid extracts sensitized the preparation to subsequent doses of aqueous extracts.

3. The sensitization, which lasted some 30 min., was not due to the tissue extract, but was the result of a transient increase of the hydrogen ion concentration.

4. The validity of assays of acetic acid extracts of individual neurohypophyses, which on account of the paucity of their oxytocic activity could not be diluted adequately, is discussed and criticized.

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