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FATE OF SYNTHETIC OXYTOCIN ANALOGUES IN THE RAT

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The disappearance of phe^3-oxytocin, val^3-oxytocin and oxytocin from the circulation of male rats was shown to be due to qualitatively similar mechanisms, that is, it depends on uptake in the kidneys and organs of the splanchnic vascular area. However, compared to phe^3-oxytocin and oxytocin (whose half-lives were essentially similar) val^3-oxytocin took twice as long to reach half its initial blood concentration. In lactating rats the mammary glands probably participated in the uptake of phe^3-oxytocin, but the rate of disappearance of val^3-oxytocin was not different from that in non-lactating animals. In male nephrectomized rats without splanchnic circulation, phe^3-oxytocin, unlike val^3-oxytocin, was quickly distributed in a volume equal to two-thirds of the total body water. Using oxytocin as the standard, val^3-oxytocin and phe^3-oxytocin were more potent when assayed on a superfused uterus or on a rat uterus *in vivo* than when assayed by the pharmacopoeial method (1958) on the isolated uterus in an organ bath. The difficulties of assaying oxytocin analogues against oxytocin (or the international standard preparation) are discussed.

In a previous investigation (Ginsburg & Smith, 1959) we have shown that, after intravenous injection into rats, oxytocin disappeared rapidly from the circulation, this being due mainly to preferential uptake by the kidneys, organs of the splanchnic vascular area and the lactating mammary gland.

Recently two synthetic analogues of oxytocin, phenylalanyl^3-oxytocin (phe^3-oxytocin) and valyl^3-oxytocin (val^3-oxytocin) were made available to us through the generosity of Sandoz & Co., Basle. The effect of val^3-oxytocin on the uterus *in situ* in rats (Boissonnas, Guttmann, Jaquenoud, Waller, Konzett & Berde, 1956), cats (Berde, Doepfner & Konzett, 1957) and women (Smyth, 1958) is greater than would be expected from the potency estimated *in vitro*, and the present investigation was undertaken in the first place to determine whether some difference in the fate of val^3-oxytocin in the body would account for this apparently enhanced activity *in vitro*. Arising from this investigation, some experiments are described on the relative potency of oxytocin and the oxytocin analogues on rat uteri under different conditions.

METHODS

Adult albino rats of both sexes weighing 180 to 250 g were used.

Assays of oxytocic activity

Rat uterus in vitro. Oxytocins in plasma extracts were assayed on superfused uteri of rats in oestrus or pro-oestrus. The constant temperature of the superfusing fluid was within

the range 30 to 33° C depending upon the reactivity of the uterus; the rate of flow was 3 to 4 ml./min and the composition of the superfusion fluid was that given by Ginsburg & Smith (1959). In some experiments assays were carried out on a uterus bathed in 2 ml. of the rat uterus solution described in the British Pharmacopoeia, 1958.

Rat uterus in vivo. The method of Bell & Robson (1937) for recording uterine movements *in situ* in guinea-pigs was applied to rats. Rats in pro-oestrus or oestrus were anaesthetized with 0.8 ml. of a 25% solution of urethane per 100 g body weight injected subcutaneously. The left horn was isolated from other abdominal viscera and covered with a flattened glass filter funnel; a thread tied in a loose loop around the middle of the uterus was passed through the spout of the funnel and attached to a frontal writing lever. The vaginal end of the uterus was fixed in position with a pin and the ovarian end fixed by a thread passed through a small hole in the funnel. Injections were given into a cannulated vein.

Extraction of oxytocin analogues from plasma

The method described in a previous paper (Ginsburg & Smith, 1959) for the extraction of oxytocin from plasma was used. The mean recoveries of oxytocin analogues added to rat plasma were $83 \pm 2.9\%$ (5) (mean and standard error) for val^3-oxytocin and $96 \pm 7.4\%$ (5) for phe^3-oxytocin.

Standardization of the synthetic oxytocin analogues

1 ml. of the solution of phe^3-oxytocin provided by Sandoz & Co. was stated to possess a vasopressor potency of 1 i.u.; no oxytocic potency was given. Phe^3-oxytocin was therefore assayed on a rat uterus in a 2 ml. organ bath according to the method described in the British Pharmacopoeia (1958). Oxytocin (Pitocin, Parke, Davis & Co.) was used as standard, and doses which gave contractions between 30 and 70% of the maximum were applied. The potency thus estimated was 3.7 ± 0.3 units/ml. (fiducial range at $P = 0.05$). This result was used to express all the doses of phe^3-oxytocin in units of oxytocic activity.

The oxytocic potency of the val^3-oxytocin solution was given as 2.0 i.u./ml. The result of assays using rat uteri in an organ bath was 2.06 ± 0.05 ($P = 0.05$), agreeing with the stated potency.

Estimation of tritium (^3H_2O) in plasma

0.25 ml./100 g body weight of 0.9% sodium chloride solution containing 10 μc/ml. of tritium as tritium oxide was injected intravenously into rats anaesthetized with ether. Sixty minutes after the injection blood was collected from a cannulated carotid artery. Plasma proteins were precipitated by adding to each ml. of plasma 0.93 ml. of 6% trichloracetic acid, so making the ^3H_2O concentration in the supernatant half of that in the original plasma, on the assumption that the water content of plasma was 93%. The supernatant was blended with 2:5-diphenyloxazole in a toluene-ethanol mixture and the radioactivity then measured in a liquid scintillation counter (Ekco type N 612). The ^3H_2O–H_2O mixture used for the preparation of standards also contained 3% trichloracetic acid.

Paper chromatography of oxytocins

The conditions described by Heller & Lederis (1958) for ascending chromatography of oxytocin on paper in an n-butanol:acetic acid:water system were used. The staining method of Reindel & Hoppe (1954) was used to locate the peptides.

RESULTS

Disappearance of injected oxytocins from the circulation

The plan of the experiments followed that described previously for oxytocin (Ginsburg & Smith, 1959): the oxytocin analogue, 200 m-u./100 g, was injected

intravenously into rats anaesthetized with ether. Blood was withdrawn from a carotid artery at intervals, up to 30 min after injection. After extraction from plasma the oxytocin was assayed on the superfused rat uterus. In a preliminary experiment it was found that the rate of disappearance of synthetic oxytocin (Syntocinon, Sandoz) was not different from that for natural oxytocin. It may be concluded that the inert constituents, which in the synthetic preparation are likely to be different from those found in the glandular extracts, do not affect the rate of disappearance of oxytocin from the circulation. It is assumed that inert impurities known to be present in solutions of the oxytocin analogues (Konzett, personal communication) would also be unlikely to affect experimentally determined half-lives.

Fig. 1 shows the half-lives calculated from the rates of disappearance of injected oxytocin and the two synthetic analogues in lactating rats 3 to 14 days post-partum, in intact males, in males without splanchnic circulation (this being arrested by ligation of the coeliac and superior mesenteric arteries and division of the ascending colon and left colic artery between ligatures and ligation of the portal vein) and in male nephrectomized rats. Bilateral nephrectomy significantly slowed the rate of disappearance of all the peptides, the mean half-lives being 1.8, 2.1 and 1.7 times

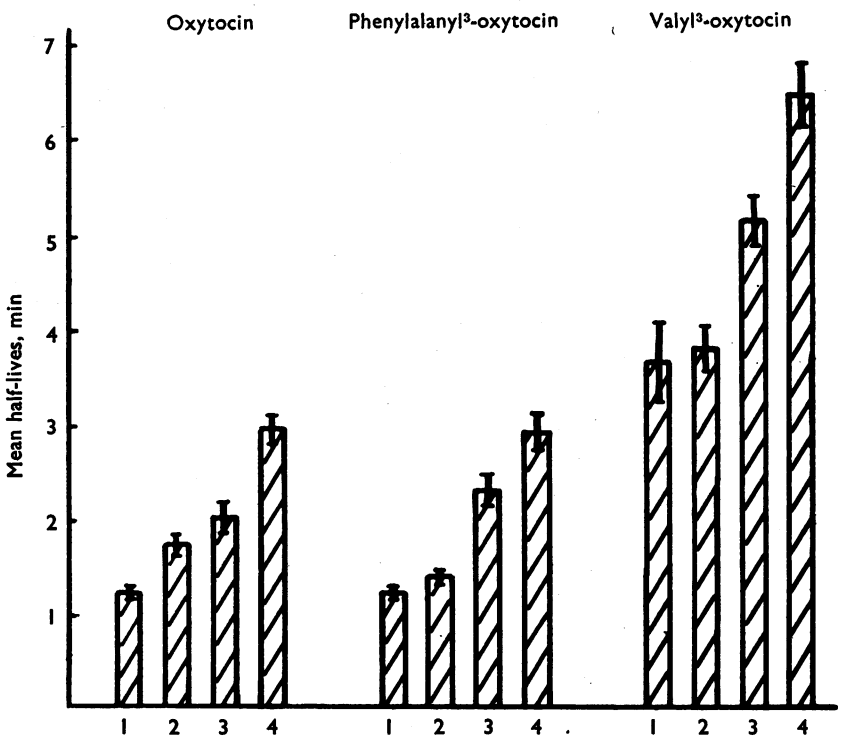

Fig. 1. Half-lives of oxytocin, phe³-oxytocin and val³-oxytocin determined in rats. 1, lactating rats, 3 to 14 days post-partum; 2, intact male rats; 3, male rats without splanchnic circulation; 4, male nephrectomized rats. Each value is the mean of from 5 to 7 experiments ± the standard deviation.

that in intact animals for oxytocin, phe^3-oxytocin and val^3-oxytocin respectively. Arrest of the splanchnic circulation retarded the disappearance of both oxytocin analogues from the circulation, and, in contrast with the results obtained with oxytocin shown in Fig. 1 (see also Ginsburg & Smith, 1958), this retardation was significant at the 5% level of probability. In lactating animals the half-lives of oxytocin and phe^3-oxytocin were significantly shorter than in intact males ($P<0.001$), but the half-life of val^3-oxytocin was unchanged ($P>0.8$). In intact rats, rats with no splanchnic circulation, nephrectomized rats or lactating rats, the mean half-life of phe^3-oxytocin was not significantly different from that of oxytocin. Val^3-oxytocin persisted in the circulation longer than the other peptides, the half-life being approximately double that of oxytocin or phe^3-oxytocin in rats in each of the states investigated.

When oxytocin is injected into a nephrectomized rat without splanchnic circulation, the oxytocin concentration falls for about 10 min when a steady level is reached (Ginsburg & Smith, 1959). Fig. 2B shows the tendency for the concentration of phe^3-oxytocin to reach a steady level at about the same time; in only one experiment out of five, the concentration of phe^3-oxytocin in plasma continued to fall up to 25 min after injection. Thus the preferential uptake of oxytocin and phe^3-oxytocin from the circulation appears to be restricted to the kidneys, the organs of the splanchnic vascular area and the mammary gland. The disappearance of val^3-oxytocin in nephrectomized rats without splanchnic circulation was so slow

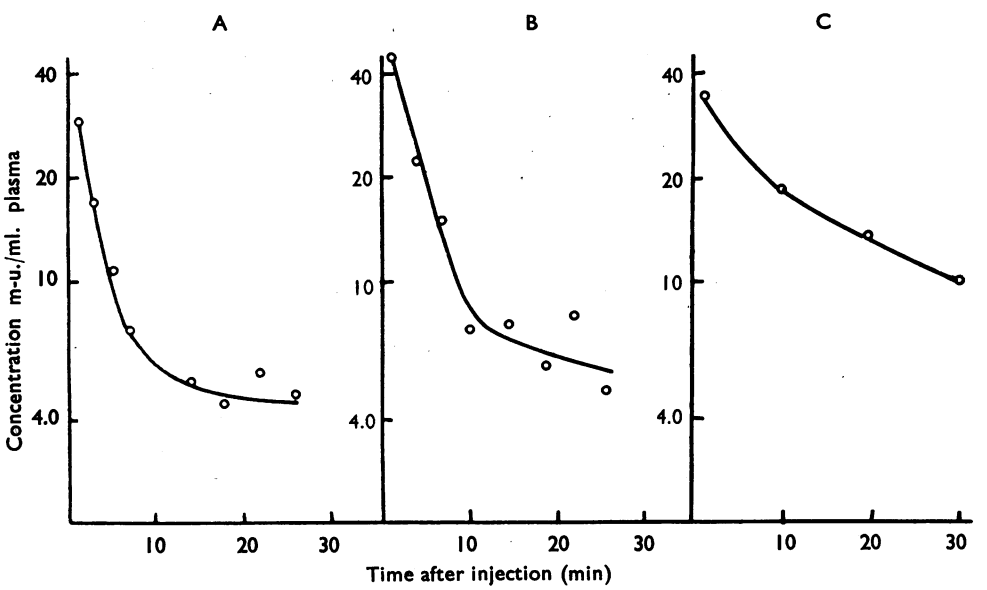

Fig. 2. Plasma concentrations of oxytocin, phe^3-oxytocin and val^3-oxytocin following intravenous injection into male nephrectomized rats without splanchnic circulation. A=oxytocin; B=phe^3-oxytocin; C=val^3-oxytocin. Each point is the mean of from 3 to 5 results.

that even 30 min after injection the mean plasma concentration was over 10 m-u./ml., that is, more than double the equilibrium concentration for the other two peptides (see Fig. 2C).

Tritium oxide space in nephrectomized rats without splanchnic circulation

The equilibrium concentrations of oxytocin and phe^3-oxytocin found in plasma in nephrectomized rats without splanchnic circulation gave volumes of distribution of the order of 40 ml./100 g body weight. The inulin space determined under the same conditions was 12 ml./100 g body weight (Ginsburg & Smith, 1959). For comparison, the tritium oxide space in nephrectomized rats without splanchnic circulation was determined. In four experiments the mean tritium oxide space was 62 ml./100 g original body weight.

Duration of action of oxytocins on the uterus in vivo

If the duration of action of an oxytocin *in vivo* is related to its persistence in the circulation, the action of val^3-oxytocin should be more prolonged than that of the other two peptides. Doses of oxytocin and a synthetic analogue were injected which, judging from preliminary experiments, were expected to be approximately equipotent, that is, to produce similar increases in uterine tonus. The duration of action, taken from the time of injection until the amplitude of the uterine movements returned to the pre-injection level, was compared in 10 pairs of matched injections of oxytocin and val^3-oxytocin. Precise prediction of equipotent doses was not possible; oxytocin produced an excursion of 32.5 mm while the chosen dose of val^3-oxytocin produced an excursion of 30.5 mm (each value is the mean of 8 responses). For each pair of injections the ratio, duration of action of val^3-oxytocin:duration of action of oxytocin, was calculated. The mean ratio for the 10 pairs of injections was 1.4 ± 0.05. Thus, in spite of the slightly greater mean response to oxytocin, the mean duration of action of val^3-oxytocin was significantly longer. In 7 similar experiments the ratio, duration of action of phe^3-oxytocin: duration of action of oxytocin, was 1.0 ± 0.06.

Potency of oxytocin and oxytocin analogues in vivo *and* in vitro

The design of the experiments in which the duration of action of the oxytocins *in vivo* was determined also permitted estimation of their relative potencies *in situ*, increase in tone being used as a parameter for these assays. In four such assays the mean potency of val^3-oxytocin was 3.4 ± 1.5 (fiducial range, $P=0.05$) times that of oxytocin. In three experiments with phe^3-oxytocin the mean potency was 12.5 ± 4.9 ($P=0.05$) times greater than that of oxytocin. Fig. 3 gives an example of one of these experiments. The doses of the oxytocin analogues are expressed in units, according to the assays on isolated rat uteri under the conditions given in the British Pharmacopoeia, 1958, in which there was no internal evidence that the assays from which the potencies of the solutions of synthetic oxytocin analogues were originally obtained were invalid. However, because the doses used were restricted to a narrow range (the ratio high dose/low dose was 1.5), deviation from parallelism might have passed unnoticed. In view of the results obtained on rat

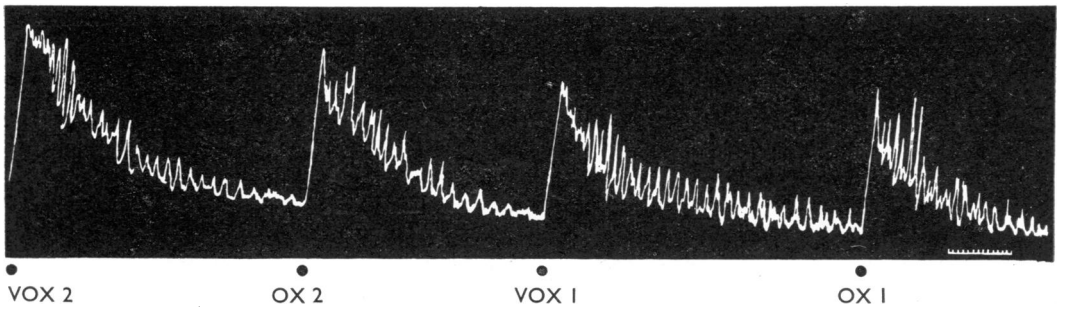

Fig. 3. Assay of val³-oxytocin against oxytocin on an oestrous rat uterus *in situ*. OX 2 and OX 1 are the high and low doses of oxytocin; 200 m-u. and 100 m-u. respectively. VOX 2 and VOX 1 are the high and low doses of val³-oxytocin; 50 m-u. and 25 m-u. respectively. Time, 30 sec.

uteri *in situ*, the potencies of oxytocin and the synthetic analogues were also compared by experiments on the rat uterus *in vitro*.

In each experiment an analogue was compared with oxytocin on an isolated rat uterus upon which 2 assays were performed. In one assay doses were used which gave responses of *less than* 30% of the maximum; in the other the responses were *greater than* 70% of the maximum. The results are shown in Fig. 4. With phe³-oxytocin the estimated potencies at both high and low dose levels agreed with each

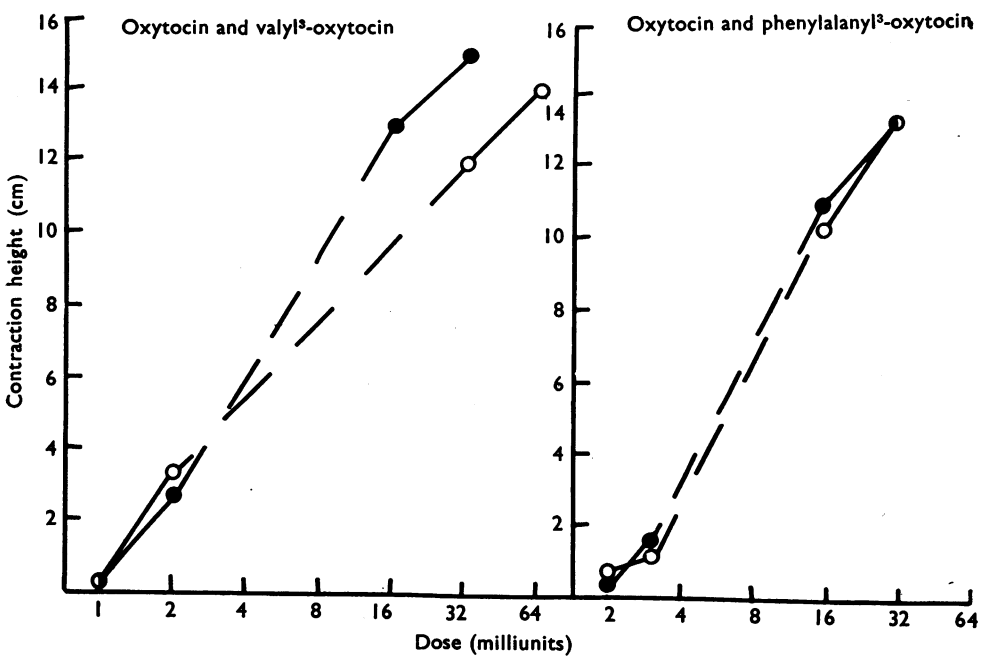

Fig. 4. Log dose-response curves for oxytocin, phe³-oxytocin and val³-oxytocin determined on the isolated rat uterus in an organ bath. ●——●=oxytocin; ○——○=synthetic oxytocin analogue.

other (3.78 and 3.67 i.u./ml. respectively). With val^3-oxytocin the assay using responses below 30% of the maximum agreed well with the potency estimate given by responses between 30% and 70% of the maximum (2.15 i.u./ml. compared with 2.06 i.u./ml.), but at the higher dose level (>70%) the estimate of the potency was 0.96 i.u./ml. Although deviation from parallelism of the log dose-response lines for oxytocin and val^3-oxytocin is not marked enough to be statistically significant over a small range of doses, the potency estimates may differ by a factor of 2, when assays at widely separated dose levels are compared. The estimates of

TABLE 1

POTENCIES OF VAL^3-OXYTOCIN AND PHE^3-OXYTOCIN DETERMINED ON RAT UTERI *IN VITRO* AND AT DIFFERENT DOSE LEVELS

The stated dose of the synthetic analogues is the mean of the high and low dose used in the assay, oxytocin being used as standard

	Val^3-oxytocin		Phe^3-oxytocin	
Assay method used	Dose m-u.	Potency u./ml.	Dose m-u.	Potency u./ml.
Uterus in organ bath	48·0	0·9	24·0	3·7
	1·5	2·0	2·5	3·7
Superfused uterus	0·045	5·3	0·1	6·6

potencies of oxytocin analogues obtained from assays on superfused rat uteri disagreed also with those obtained from experiments on uteri suspended in an organ bath. The potencies estimated for the analogues were greater in the superfusion assays (Table 1).

All the preparations used contained pharmacologically inert material, and there was the possibility that some of these discrepancies in the potency estimates were due to the presence of different impurities. To investigate this possibility, val^3-oxytocin and phe^3-oxytocin were partially purified by ascending paper chromatography. In the case of val^3-oxytocin two spots were revealed ; the oxytocic activity was found in eluates from the faster-moving spot. Phe^3-oxytocin yielded only one spot and the oxytocic activity was found in this region only. To part of the eluate containing the oxytocic activity, eluate from the remainder of the paper strip was added, and this mixture was compared with the eluate from the " active spot " in assays on a rat uterus in an organ bath. The presence of impurities did not affect the oxytocic potency of either of the partially purified oxytocin analogues. Apart from the possibility of impurities which move at the same rate as the oxytocins on the paper, it is unlikely that the discrepancies in the relative potency estimates for the oxytocin analogues were due to any impurities in the solutions.

DISCUSSION

The fate of val^3-oxytocin and phe^3-oxytocin in intact male rats has been shown to be qualitatively similar to that of natural oxytocin, being determined by uptake by the kidneys and organs of the splanchnic vascular area. However, the rate of disappearance of val^3-oxytocin from the circulation was much slower than that of oxytocin or phe^3-oxytocin. This longer persistence of val^3-oxytocin in the circulation was also seen in nephrectomized male rats without splanchnic circulation in which

the distribution of the other two peptides quickly proceeded to equilibrium, suggesting that the slow decline in the blood concentration of val^3-oxytocin may be due to a permeability barrier. The potency per unit weight of val^3-oxytocin is not known, but if its potency was low the amounts injected might have been great enough to exceed the capacity of some part of an inactivating system. However, the weight of the phe^3-oxytocin injected in each clearance experiment was about 20 times that of oxytocin (Boissonnas & Huguenin, 1960), and yet the rate of disappearance was not slower than that of the naturally occurring hormone. If permeability is the critical factor, the barrier which can differentiate between val^3-oxytocin and phe^3-oxytocin or oxytocin must possess a high degree of specificity.

We have confirmed the findings of Boissonnas *et al.* (1956) and Berde, Doepfner & Konzett (1957) that the potency ratio, val^3-oxytocin:oxytocin, is greater when measured *in vivo* than when the assay is performed on a rat uterus in an organ bath. The longer persistence of val^3-oxytocin in the circulation and its longer duration of action cannot account for its greater potency, which is measured by the maximum increase in tonus, that is, an effect which occurs 1 to 2 min after injection. Furthermore, the difference between the potencies of phe^3-oxytocin *in vivo* and *in vitro* is much more marked than those of val^3-oxytocin, although the rate of disappearance and duration of action of the latter compound could not be differentiated from those of oxytocin. Since the potency of val^3-oxytocin *in vitro* is enhanced in the presence of 0.5 mM/l. magnesium, Munsick (1960) has suggested that the difference between the potency of oxytocin and val^3-oxytocin *in vitro* and *in vivo* may only arise when magnesium is absent from the bath solution. However, the potency of phe^3-oxytocin was 12 times greater when measured *in vivo*, but Munsick (1960) found that its *in vitro* activity was only slightly enhanced when the solution had high magnesium content.

The response of an individual uterus under the conditions of the British Pharmacopoeial assay to a given dose of val^3-oxytocin or phe^3-oxytocin can be matched by a dose of oxytocin, but, to match the response of a rat uterus *in situ* to a dose of one of the oxytocin analogues, more oxytocin is needed than could be expected from the *in vitro* results. That is, the responsiveness of the rat uterus *in situ* to the various oxytocins is different from that *in vitro* and the degree of change in response varies from substance to substance. On a weight basis, phe^3-oxytocin is about 20 times less potent than oxytocin *in vitro*, but *in vivo*, in rats, oxytocin is only 1.8 times more potent than the synthetic analogue.

The unit of oxytocic activity is defined in terms of a definite weight of the international standard powder (of which the oxytocic activity is primarily but not solely due to oxytocin), and any assay preparation in which oxytocin is compared with an analogue should give a potency which could be expressed in international units of oxytocic activity. Munsick (1960) has shown that the results of *in vitro* assays comparing oxytocin with an analogue may depend upon the composition of the suspension fluid, particularly with respect to its magnesium content. We have found that the result may also vary with the magnitude of the response used in the assay itself. It would therefore appear that, owing to the fundamental invalidity of all these assays, in that the standard is not identical with the unknown, there is

no definite amount of an oxytocin analogue which could be equivalent to a unit of oxytocic activity. This difficulty is not realized by most workers, who arbitrarily make the assay on the rat uterus *in vitro* the reference assay upon which the unit of oxytocic activity now, tacitly, depends. It would be better, perhaps, if this situation were recognized and the unit redefined.

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