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EFFECT OF TYROSINASE PREPARATIONS ON OXYTOCIN, VASOPRESSIN AND BRADYKININ

BY

G. W. BISSET

From the National Institute for Medical Research, Mill Hill, London, N.W.7

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On incubation with a tyrosinase preparation at *p*H 7.5, oxytocin and vasopressin were inactivated. The loss of oxytocic activity did not differ significantly from that of milk-ejecting activity in oxytocin, nor the loss of pressor activity from that of antidiuretic activity in vasopressin. Oxytocin was inactivated less rapidly at *p*H 6.6 than at *p*H 7.5. At *p*H 3.9 neither oxytocin nor vasopressin was inactivated. Analogues of oxytocin and vasopressin, in which tyrosine is replaced by phenylalanine, were not inactivated by the tyrosinase preparation used. On incubation of bradykinin with two different tyrosinase preparations, there was no loss of oxytocic activity at *p*H 7.5 but an almost total loss at *p*H 3.9. In the presence of p-nitrophenol, ascorbic acid, sodium diethyldithiocarbamate and during incubation under anaerobic conditions the inactivation of oxytocin at *p*H 7.5 was inhibited, but not that of bradykinin at *p*H 3.9. It is concluded that the tyrosinase preparations used contain two distinct enzymes or activities, the one inactivating oxytocin and vasopressin at *p*H 7.5 and the other bradykinin at *p*H 3.9.

There are several reports according to which oxytocin and vasopressin are inactivated by tyrosinase (Freudenberg, Weiss & Biller, 1935; de la Maza & Croxatto, 1944; Croxatto & de la Maza, 1945; Fraser, 1950). The hormone preparations available to these workers consisted of posterior pituitary lobe extract or its partially purified oxytocic and pressor fractions. The present paper is concerned with a quantitative study of the action of tyrosinase preparations on synthetic oxytocin and vasopressin. Further, the use of analogues in which tyrosine is replaced by phenylalanine has confirmed that the inactivation of oxytocin and vasopressin by tyrosinase involves a specific action of the enzyme on the tyrosyl residue.

In the course of this investigation, control experiments carried out with another polypeptide, bradykinin, which does not contain tyrosine, revealed that this substance also is inactivated by tyrosinase preparations but under different conditions. Finally, by the use of enzyme inhibitors and by incubation under nitrogen, it could be shown that the enzyme preparations used contain two distinct enzymes or activities, the one inactivating neurohypophysial hormones and the other bradykinin.

METHODS

Incubation of tyrosinase preparations with substrates

Tyrosinase preparations were incubated with samples of oxytocin, vasopressin and bradykinin at 37° C and at *p*H 7.5, 6.6 or 3.9.

Incubations at *p*H 7.5 were carried out in a solution of the following composition, where values are given in g/l. and, in parenthesis, mM: NaCl 6.66 (114), KCl 0.46 (6.2), $CaCl_2$ 0.056 (0.5), $NaHCO_3$ 2.52 (30), NaH_2PO_4 0.12 (1.0), glucose 0.5 (2.8).

The concentrations of salts were calculated to conform with the molar concentrations of ions given in the solution of van Dyke & Hastings as modified by Munsick (1960). Munsick solution, which was introduced for the bioassay of oxytocin on the isolated rat uterus, was considered to be particularly suitable as an incubation medium because of its high buffering capacity at a physiological *p*H and the similarity of its electrolyte composition to that of mammalian plasma. Before use the solution was bubbled with a mixture of 5% carbon dioxide and 95% oxygen. The average *p*H of 10 batches of bubbled solution was 7.52.

Incubations at *p*H 6.6 were carried out in 0.1 M phosphate buffer, and at *p*H 3.9 in Munsick solution acidified by the addition of one-tenth the volume of 10% acetic acid.

In all experiments the reagents were freshly made up in Munsick solution or in phosphate buffer, and a series of incubation tubes was prepared, each containing the required amounts of substrate, enzyme and, in some experiments, inhibitor, in a total volume of 2 ml. The amount of substrate used was 1 u. of oxytocin, 1 u. of vasopressin or 2 μg of bradykinin. The amount of enzyme varied in different experiments.

Samples incubated at *p*H 3.9 were placed in a beaker of boiling water for 5 min at the end of the incubation period. Samples incubated at *p*H 6.6 or 7.5 were first acidified to *p*H 3.9 by the addition of 0.2 ml. of 10% acetic acid and then boiled. Boiling at *p*H 3.9 destroyed all enzyme activity but did not inactivate oxytocin, vasopressin or bradykinin.

Some incubations were carried out under nitrogen in Thunberg tubes. Enzyme and substrate were mixed in the tubes after twice evacuating and re-filling with nitrogen.

The enzyme inhibitors used were p-nitrophenol, sodium diethyldithiocarbamate and ascorbic acid. p-Nitrophenol and sodium diethyldithiocarbamate were incubated with the enzyme preparation for 15 min at 37° C, before the substrate was added, whereas ascorbic acid was added with the substrate.

Bioassay of incubated samples

Immediately before assay the acidified samples were suitably diluted with Munsick solution, and, if necessary, N sodium hydroxide was added to adjust the *p*H to 7.5. In every experiment a standard solution of oxytocin, vasopressin or bradykinin in Munsick solution or in phosphate buffer, acidified to *p*H 3.9, was prepared. This solution was not submitted to boiling and it was kept at room temperature during incubation of the test samples.

A 2 and 2 dose assay design was used as described by Holton (1948) for the standardization of posterior pituitary extract on the isolated rat uterus, or, in some experiments, a 3 and 3 dose assay design.

After incubation with tyrosinase preparations the samples of oxytocin were assayed for oxytocic, and, in some experiments, milk-ejecting activity; the samples of bradykinin for oxytocic activity and the samples of vasopressin for pressor and antidiuretic activity.

Oxytocic activity was assayed on the isolated rat uterus suspended in a 3.5 ml. bath of Munsick solution at 29 to 32° C. A mixture of 5% carbon dioxide and 95% oxygen was bubbled through the solution. The uterus was taken from a virgin rat of 200 to 250 g body weight in which oestrus had been induced by subcutaneous injection of 0.2 mg stilboestrol 18 hr previously.

Milk-ejecting activity was assayed on the lactating guinea-pig, essentially as described by van Dyke, Adamsons & Engel (1955) for the rabbit. A guinea-pig weighing 850 to 1,000 g was taken from its litter about 7 days after parturition and anaesthetized by intraperitoneal injection of 0.5 to 0.7 ml./100 g of 25% urethane in saline. The trachea was cannulated, but artificial respiration was not applied. The external jugular vein was cannulated for injection of the samples to be tested. After excision of the tip of the nipple, nylon tubing size 00, of 0.5 mm internal diameter, was inserted into a milk duct. Milk-ejection pressure

was measured by a Statham strain gauge transducer arranged to write on a potentiometric recorder (Speedomax: Type H: Leeds and Northrup).

Pressor activity was assayed on the blood pressure of the rat, a method introduced by Dekanski (1952). A male rat weighing 250 to 300 g was anaesthetized by intraperitoneal injection of 0.5 to 0.7 ml./100 g of 25% urethane in saline. Atropine sulphate 0.1 mg/100 g was injected intraperitoneally. The left vagus nerve was severed in the neck and the blood pressure recorded from the left carotid artery with a mercury manometer or a Statham strain gauge transducer. The femoral vein was cannulated. The ganglion blocking agent, pentolinium tartrate, 125 μg, was injected intravenously and if necessary the injection was repeated until the blood pressure fell to a constant level. Samples to be tested were injected intravenously and, in order to avoid tachyphylaxis, at intervals of not less than 10 min.

Antidiuretic activity was assayed by a simplification of the methods of Dicker (1953) and Dettelbach (1958). A male rat was selected from a group of 4 animals weighing 250 to 300 g, which, having been allowed free access to food and water overnight, were given 5 ml./100 g of 10% v/v ethyl alcohol by stomach tube and then placed in individual metabolism cages. The rat selected for the assay was one which had excreted 2 ml. or more of urine within 30 min. Such a rat rarely failed to give satisfactory results. It was given 3 ml./100 g of warm tap water by stomach tube and, if necessary, ether was administered to produce surgical anaesthesia. The external jugular vein was cannulated with polyethylene tubing. The urethra was ligated and the bladder cannulated, through an abdominal incision, with polyethylene tubing of 2 mm internal diameter. To replace the fluid lost by diuresis, a fine polyethylene tube was passed into the stomach by mouth and held firmly in place by a skin suture. After the rat had been re-weighed, 2% ethyl alcohol was injected through this tube to restore the water load to 8% of the body weight. The rat was then placed on a pad of cotton-wool on one pan of a pair of laboratory scales and balanced by a beaker of water placed on the other pan. The bladder cannula was connected to two tubes in series of 120 ml. capacity, containing 1% sodium chloride in 50% ethyl alcohol. Fluid was displaced by the flow of urine from the second tube through a hypodermic syringe needle of 0.5 mm internal diameter. This needle served as one electrode and the outflowing drops made contact with a second electrode. The two electrodes were connected to a Thorpe drop counter actuated by a 1 min time clock, and the urine flow was recorded on a smoked drum. Throughout the experiment the weight of the rat was kept constant within 1% by injecting 2 to 3% ethyl alcohol through the polyethylene tube into the stomach until the pointer on the scales was restored to equilibrium. The samples to be tested were injected intravenously.

The height of the trace on the smoked drum is proportional to the rate of urine flow. Several parameters were tested for measuring the antidiuretic effect. The best log dose-response curves were obtained by the use of the following expression, which represents the percentage reabsorption of fluid by the renal tubules during a given period of antidiuresis:

$$\text{Potency} = \frac{100\ (A - B)}{A}$$

where A equals the mean height of the trace in a period of 2 to 10 min immediately preceding the injection, and B equals the mean height in the 5-min period from the second to the sixth minute after the injection, the first minute being ignored. This parameter is essentially the same as that used by Dettelbach.

Materials

Enzyme. Three samples of lyophilized tyrosinase prepared from mushrooms were used. One sample was obtained from L. Light & Co., Colnbrook, Essex, and two samples, both batch No. 570, from Worthington Biochemical Corporation, New Jersey, U.S.A. The Worthington preparation contained 500 u./mg; the unit of activity is an arbitrary one defined in the catalogue.

Substrates. The following substrates were used: Commercially available synthetic oxytocin ("Syntocinon" brand of Injection of Oxytocin, B.P. Sandoz, 10 u./ml.). Highly purified

synthetic oxytocin (Sandoz, 1 mg (450 u.)/ml.). Phenylalanine^3-oxytocin (Bodansky & du Vigneaud, 1959) obtained from Professor du Vigneaud (3.4 to 3.6 avian depressor u./ml.). Synthetic lysine^8-vasopressin (Sandoz, 24 pressor u./ml.). Phenylalanine^3-lysine^8-vasopressin (Boissonnas & Guttmann, 1960). ("Octapressin," Sandoz, 5 pressor u./ml.). Synthetic bradykinin (Parke Davis, Lot No. 4447 X128). Natural bradykinin prepared from ox blood (Elliott, Horton & Lewis, 1961) and estimated to be 80% pure. The nomenclature for analogues of oxytocin and vasopressin is that proposed by Konzett & Berde (1959).

Nitrogen. British Oxygen Co. "white spot" nitrogen was used, containing nitrogen: 99.9% and oxygen: 10 vols./10^6 vols.

RESULTS

Inactivation of oxytocin, vasopressin and bradykinin by tyrosinase preparations

Commercially available synthetic oxytocin

Loss of oxytocic activity. At *p*H values below 5, oxidation of phenols by tyrosinase is known to be inhibited. In the present experiments it was found that at *p*H 7.5 oxytocin was inactivated by tyrosinase preparations whereas at *p*H 3.9 inactivation was inhibited. Three samples of 1 u. of oxytocin were incubated for 4 hr. In the first sample, incubated at *p*H 7.5 with 100 μg of Light's tyrosinase preparation, the loss of activity was >97%. In the second sample, incubated with the same amount of enzyme at *p*H 3.9, the loss was 5%. In the third sample, incubated at *p*H 7.5 without enzyme, the loss was 4%. In another experiment almost complete loss of activity (>98%) occurred on incubation of 1 u. of oxytocin

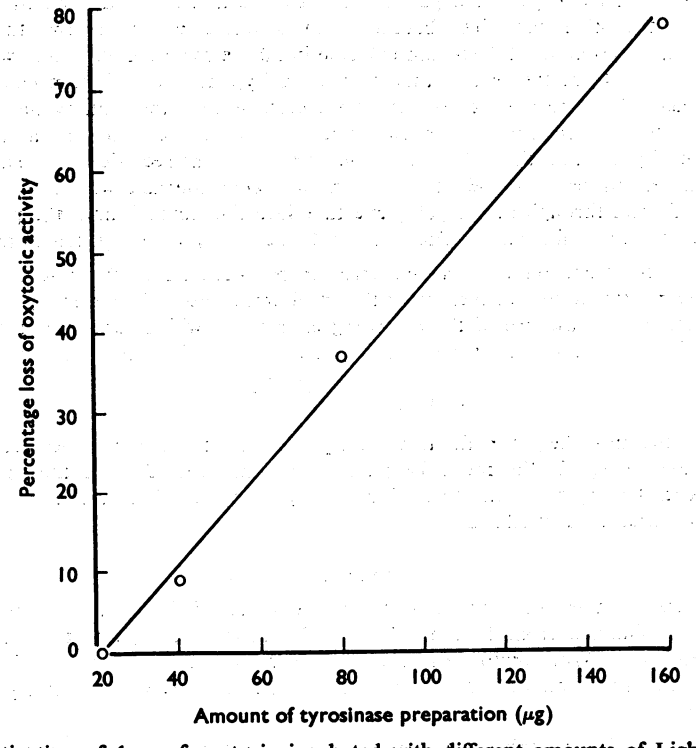

Fig. 1. Inactivation of 1 u. of oxytocin incubated with different amounts of Light's tyrosinase preparation at *p*H 7.5 for 1 hr.

with 1 mg of Light's tyrosinase preparation for 3 hr at *p*H 7.5, but the loss of activity was only 4% when the enzyme solution, before being added to the incubation mixture, had been brought to the boil over a luminous Bunsen flame.

On incubation of 1 u. of oxytocin with 10 μg of Light's tyrosinase preparation at *p*H 7.5, there was no loss of activity after 1 hr and only 20% loss after 5 hr. The results obtained on incubation of 1 u. of oxytocin with larger amounts of enzyme at *p*H 7.5 for 1 hr are shown in Fig. 1. The amount required to produce 50% inactivation was approximately 100 μg. In Fig. 2 is shown the time course of the reaction between this amount of enzyme and 1 u. of oxytocin incubated under the same conditions. Since there was no loss of activity in a control sample incubated at *p*H 3.9 for 150 min, simultaneously with the test samples, it is permissible to extrapolate the graph representing loss of activity at *p*H 7.5 to zero time. The points on the graph are best fitted by a sigmoid curve, but, up to 90 min, there is practically a straight line relationship between percentage loss of activity and time of incubation.

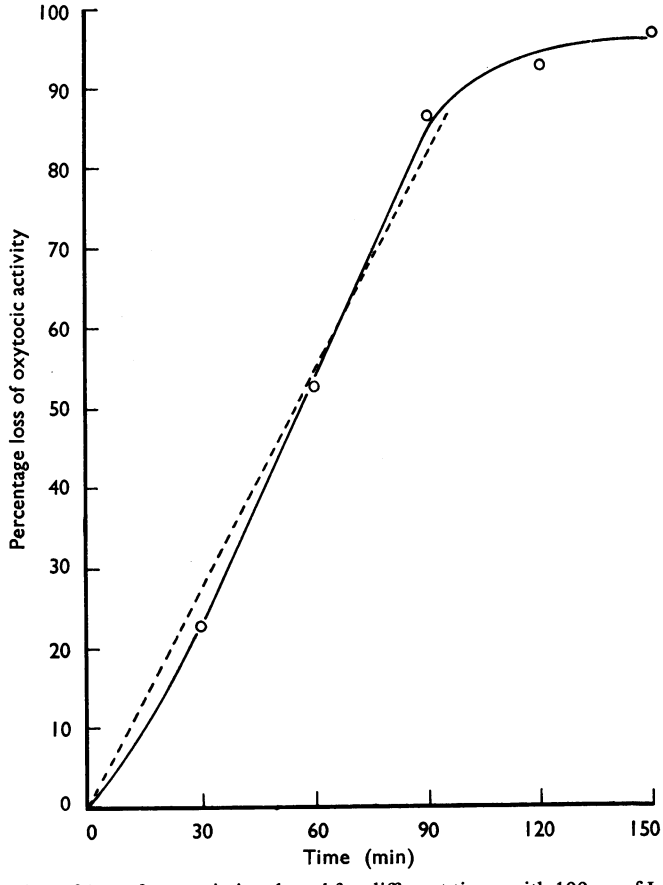

Fig. 2. Inactivation of 1 u. of oxytocin incubated for different times with 100 μg of Light's tyrosinase preparation at *p*H 7.5. The dotted line indicates the practically straight line relationship between percentage loss of oxytocic activity and time of incubation up to 90 min.

Worthington's tyrosinase preparation was found to be about twice as active as the Light's preparation. At *p*H 7.5 the curve for 100 μg of Worthington's tyrosinase was steeper than that for 100 μg of Light's preparation, but this curve was approximately the same as that obtained with 50 μg of the Worthington preparation. This can be seen when the curve in Fig. 2 is compared with the continuous curves in Fig. 3.

Although the optimal *p*H range for oxidation of monophenols by tyrosinase is given as 6 to 7 (Dawson & Tarpley, 1951), it was found that the rate of inactivation of oxytocin by Worthington's tyrosinase preparation was slower at *p*H 6.6 than at *p*H 7.5, and with 50 μg, inactivation not only proceeded more slowly but came to a standstill at 66% after 90 min. This is illustrated in Fig. 3, in which the dotted curves represent the inactivation at *p*H 6.6 and the continuous curves at *p*H 7.5.

Loss of milk-ejecting activity. On incubation of oxytocin with Worthington's tyrosinase preparation, there was a loss not only of oxytocic but also of milk-

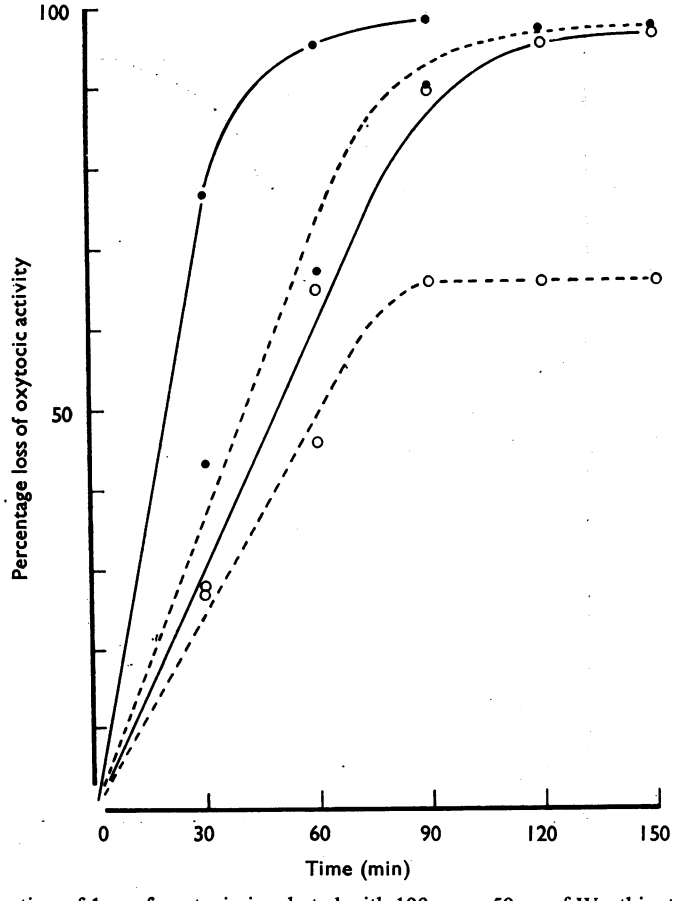

Fig. 3. Inactivation of 1 u. of oxytocin incubated with 100 μg or 50 μg of Worthington's tyrosinase preparation at *p*H 7.5 or *p*H 6.6. ●—● 100 μg at *p*H 7.5; ○—○ 50 μg at *p*H 7.5; ●- - - -● 100 μg at *p*H 6.6; ○- - - -○ 50 μg at *p*H 6.6.

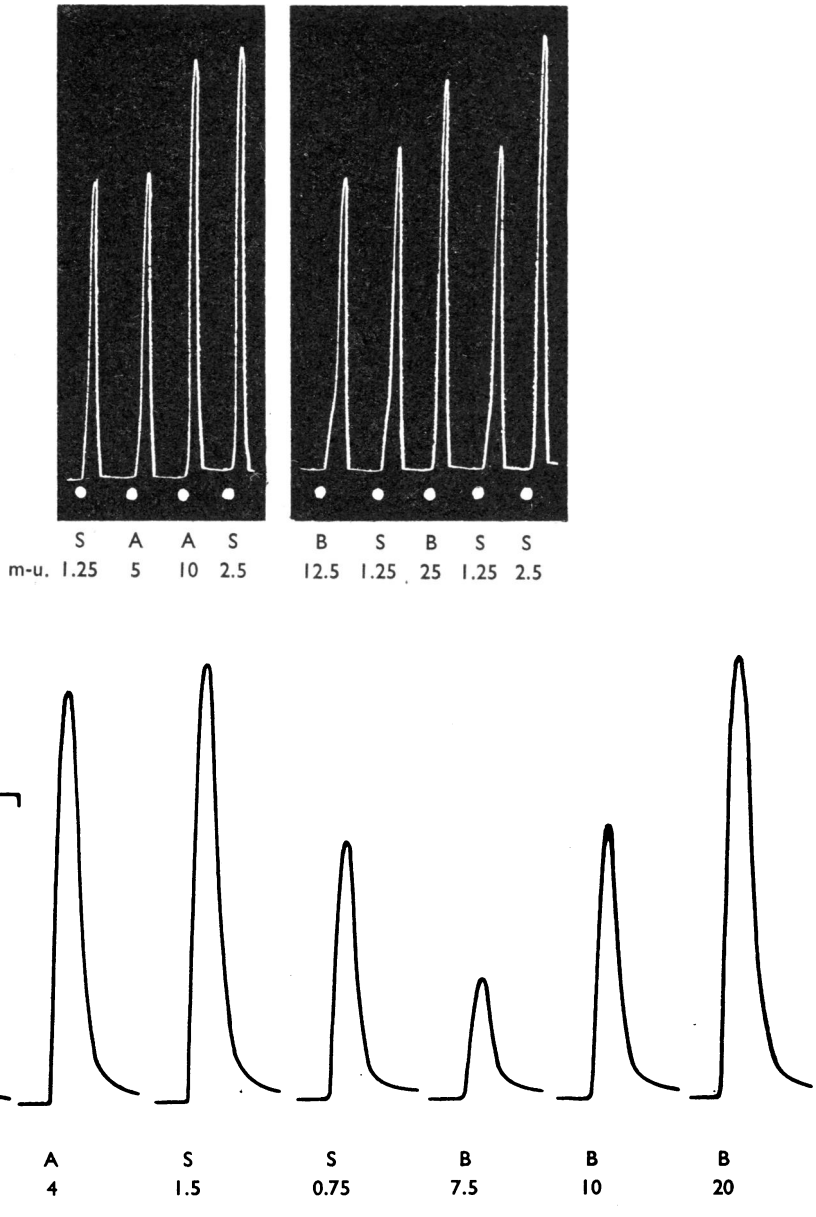

Fig. 4. Upper tracing: isolated rat uterus. Lower tracing: milk-ejection pressure in mammary gland of lactating guinea-pig; intravenous injections at 5-min intervals. Parallel assays of oxytocic and milk-ejecting activities of two samples of 1 u. of oxytocin incubated with 50 μg of Worthington's tyrosinase preparation at *p*H 7.5. Sample *A* incubated for 45, and sample *B* for 90 min. *S*: standard oxytocin. The amounts in each assay are given in m-u., and for the samples they refer to m-u. present before incubation.

ejecting activity. Within the limits of experimental error, there was no significant difference in the loss of the two activities. This was evident when 1 u. of oxytocin was incubated with 50 μg of the enzyme preparation and the incubated samples were assayed for both oxytocic and milk-ejecting activity. On incubation for 90 min at *p*H 3.9 there was no detectable loss of either activity; on incubation for 45 min at *p*H 7.5 the loss of oxytocic activity was 75% and that of milk-ejecting activity 68% ; on incubation for 90 min at *p*H 7.5 the loss of both activities was 92%. Parallel quantitative assays of the samples incubated at *p*H 7.5 for 45 and 90 min are illustrated in Fig. 4.

Highly purified synthetic oxytocin

No difference in the loss of oxytocic activity was detected when commercially available and highly purified synthetic oxytocin were incubated with Worthington's tyrosinase preparation. After incubation for 120 min at *p*H 7.5 with 25 μg of the enzyme preparation, the loss of activity was approximately 20%, and with 50 μg, 88% in both samples.

Phenylalanine^2-oxytocin

Phenylalanine^2-oxytocin was not inactivated by Worthington's tyrosinase preparation. On incubation of this analogue in a quantity equivalent to 1 u. of avian depressor activity with 1 mg of the enzyme preparation at *p*H 7.5 for 120 min, there was no detectable loss of oxytocic activity and only 7% loss of milk-ejecting activity. In the control sample, 1 u. of commercially available oxytocin incubated with 0.1 mg of enzyme, >98% of the oxytocic and 97.5% of the milk-ejecting activity had been lost; on incubation with 1 mg of enzyme, the loss of both activities was >98%. For the incubation with phenylalanine^2-oxytocin, 1 mg of enzyme was used because this analogue is known to have only 60 u./mg of avian depressor activity (Bodansky & du Vigneaud, 1959) compared with 450 u./mg in oxytocin. In order, therefore, to preserve the ratio of enzyme to substrate on a weight basis, 1 u. of the analogue, estimated as avian depressor activity, would require approximately 8 times as much enzyme as 1 u. of oxytocin. When the analogue was assayed against commercially available oxytocin, it was found that 1 u. of avian depressor activity was equivalent to 2.5 u. of milk-ejecting activity and approximately 0.4 u. of oxytocic activity.

Lysine^8-vasopressin

The inactivation of lysine^8-vasopressin by Worthington's tyrosinase preparation is illustrated by 2 experiments in Table 1. A progressive and parallel loss of the pressor and antidiuretic activities occurred when samples of 1 u. of lysine^8-vasopressin were incubated at *p*H 7.5 with 50 μg of the enzyme preparation. On the other hand, on incubation at *p*H 3.9 the loss was only small. The assay of the various samples for antidiuretic activity in the first experiment is shown in Fig. 5. This illustrates the good discrimination between 3 or 4 different doses of lysine^8-vasopressin. The sensitivity was such that residues of activity in the incubated samples of less than 1% could be measured. It is evident from inspection of panel 3 of Fig. 5 that aliquots of the sample E incubated for 120 min at *p*H 7.5, with initial contents of 8 and 16 m-u. had after incubation approximately the same

TABLE 1

INACTIVATION OF LYSINE^8-VASOPRESSIN BY A TYROSINASE PREPARATION

Incubation of 1 u. of lysine^8-vasopressin with 50 μg of Worthington's tyrosinase preparation

Conditions of incubation	Expt.	Loss of activity (%)	
		Pressor	Antidiuretic
150 min at *p*H 3·9	1	12	6
	2	12	4
30 min at *p*H 7.5	1	92	87
	2	91	88
60 min at *p*H 7.5	1	98	98·5
	2	98·8	99·1
90 min at *p*H 7.5	1	99	99
	2	>99	99·7
120 min at *p*H 7.5	1	—	99·6

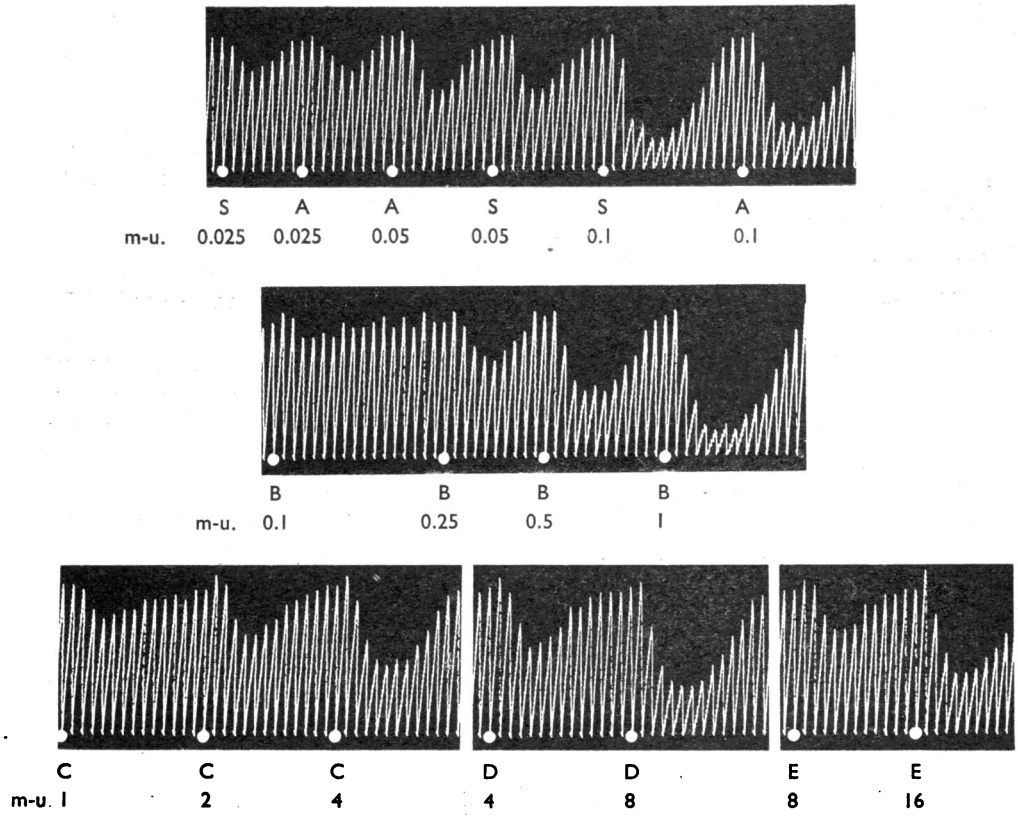

Fig. 5. Urine flow recorded in anaesthetized rat with drop counter actuated by 1-min time clock. Assay of antidiuretic activity of 5 samples (*A* to *E*) of 1 u. of vasopressin incubated with 50 μg of Worthington's tyrosinase preparation at *p*H 3.9 or *p*H 7.5 for different times. Sample *A* incubated at *p*H 3.9; samples *B* to *E* at *p*H 7.5. Time of incubation: *A*, 150 min; *B*, 30 min; *C*, 60 min; *D*, 90 min; *E*, 120 min. *S*: standard vasopressin. The amounts are given in m-u., and for the samples they refer to m-u. present before incubation.

activity as 0.05 and 0.1 m-u., respectively, of the standard S. Calculation according to the parameter described in Methods gave a residual activity of 0.4% in this sample. Similarly, from a 3 and 3 dose assay on the sample A incubated for 150 min at *p*H 3.9 (see panel 1), a small loss could be determined equivalent to 6%. Finally, the progressive loss of activity on incubation at *p*H 7.5 is shown in samples B to E: increasingly large doses had to be given to match the original responses to the standard.

Phenylalanine^2-lysine^8-vasopressin

There was no significant loss of activity when phenylalanine^2-lysine^8-vasopressin was incubated with Worthington's tyrosinase preparation. On incubation of this analogue in a quantity equivalent to 1 u. of pressor activity at *p*H 7.5 for 120 min with 0.5 mg of the enzyme preparation, the recovery of pressor activity was 91% and that of antidiuretic activity 108% ; and, with 1 mg, 81% and 106% respectively. In a control sample of 1 u. of vasopressin incubated with 0.1 mg of enzyme $<1.3\%$ of the pressor activity was recovered and only 0.5% of the antidiuretic activity. Quantities of 0.5 and 1.0 mg of enzyme were used in the incubations with phenylalanine^2-lysine^8-vasopressin, since this analogue has only about one-fifth the pressor activity of vasopressin (Berde, Weidmann & Cerletti, 1961). In order to preserve the ratio of enzyme to substrate on a weight basis, 1 u. of pressor activity in the analogue would therefore require at least 5 times as much enzyme as 1 u. of lysine^8-vasopressin.

Bradykinin

Whereas inactivation of oxytocin by tyrosinase preparations occurred at *p*H 7.5 and was inhibited at *p*H 3.9, the opposite result was obtained in experiments with bradykinin. When 2 μg of natural bradykinin, an amount equipotent in oxytocic activity to 1 u. of oxytocin, was incubated with 100 μg of Light's tyrosinase preparation at *p*H 3.9 for 2 hr, almost complete inactivation of bradykinin occurred ($>98\%$), but at *p*H 7.5 there was no detectable loss of activity. Inactivation at *p*H 3.9 could be prevented by boiling the enzyme preparation before addition to the incubation mixture. The same results were obtained when incubation was carried out with Worthington's tyrosinase preparation and synthetic, instead of natural, bradykinin was used as substrate.

The time-course of inactivation of bradykinin by Worthington's tyrosinase preparation at *p*H 3.9 is illustrated in Fig. 6. The two curves were obtained on incubation of 2 μg of synthetic bradykinin with 10 μg and 50 μg of the enzyme preparation. They show that inactivation of bradykinin at *p*H 3.9 increases progressively with time, which suggests that the effect is enzymatic in nature.

The effect of enzyme inhibitors and incubation under nitrogen

The effect of enzyme inhibitors and of incubation under nitrogen on the inactivation of oxytocin and bradykinin by Worthington's tyrosinase preparation is shown in Table 2. In these experiments 1 u. of commercially available oxytocin was incubated at *p*H 7.5 and 2 μg of synthetic bradykinin at *p*H 3.9. The values given in Table 2 refer to loss of oxytocic activity.

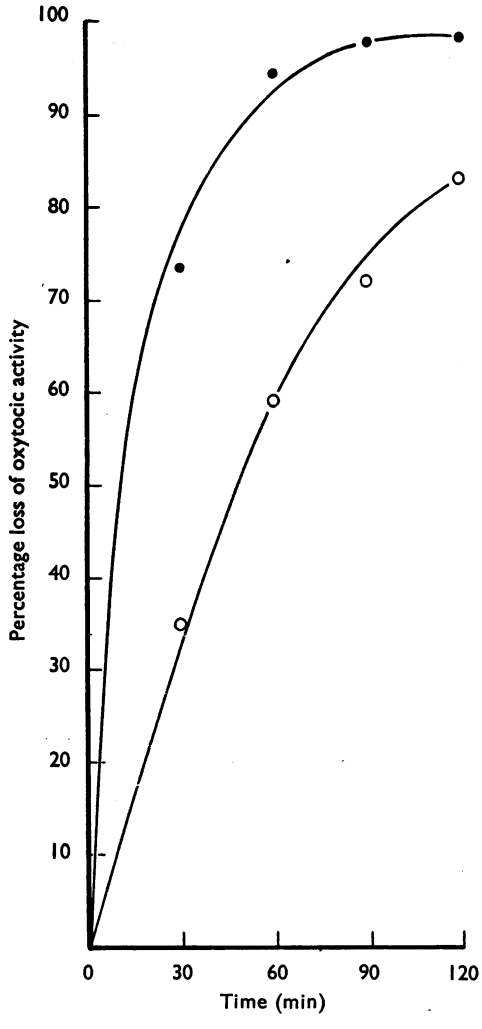

Fig. 6. Inactivation of 2 μg of synthetic bradykinin incubated with 10 μg or 50 μg of Worthington's tyrosinase preparation at *p*H 3.9. ○ —— ○ 10 μg; ● ——— ● 50 μg.

Enzyme inhibitors

p-Nitrophenol. With 10 μ-mole (1.4 mg) of p-nitrophenol, inactivation of oxytocin by 50 μg of the enzyme preparation was completely inhibited. This amount of p-nitrophenol did not significantly reduce the inactivation of bradykinin. With 0.25 to 1 μ-mole of p-nitrophenol, partial inhibition of the inactivation of oxytocin occurred, as is illustrated in Fig. 7. It was observed, however, that at *p*H 7.5 solutions of p-nitrophenol were yellow whereas at *p*H 3.9 they were colourless. This suggests that, at the *p*H at which bradykinin is inactivated by tyrosinase preparations, p-nitrophenol is relatively unionized, and this may affect its capacity to act as an enzyme inhibitor.

TABLE 2

EFFECT OF ENZYME INHIBITORS AND INCUBATION UNDER NITROGEN ON THE INACTIVATION OF OXYTOCIN (O) AND BRADYKININ (B) BY A TYROSINASE PREPARATION

Worthington's tyrosinase preparation was incubated for 120 min either with 1 u. of commercially available oxytocin at *p*H 7.5 or with 2 μg of synthetic bradykinin at *p*H 3.9. Total volume of incubation mixture: 2 ml. If more than one experiment was carried out, a mean value is given with the number of experiments in parentheses

Substrate	Tyrosinase μg	Inhibitor	Loss of oxytocic activity (%)
O	50	—	87 (4)
O	100	—	96 (7)
B	10	—	77 (3)
B	50	—	97 (3)
O	50	p-Nitrophenol 1·4 mg	0
B	50	p-Nitrophenol 1·4 mg	95
O	50	Ascorbic acid 0.1 mg	56
O	100	Ascorbic acid 1·0 mg	38
O	—	Ascorbic acid 1.0 mg	6
B	50	Ascorbic acid 1.0 mg	96
B	—	Ascorbic acid 1.0 mg	17
O	100	Sodium diethyldithiocarbamate 0·002 mg	79
O	100	Sodium diethyldithiocarbamate 0·004 mg	10
O	100	Sodium diethyldithiocarbamate 0·005 mg	0
B	10	Sodium diethyldithiocarbamate 0·0005 mg	77
B	—	Sodium diethyldithiocarbamate 0·0005 mg	0
O	100	Incubation under nitrogen	18
	100	Re-exposure to air after nitrogen	92
B	10	Incubation under nitrogen	64

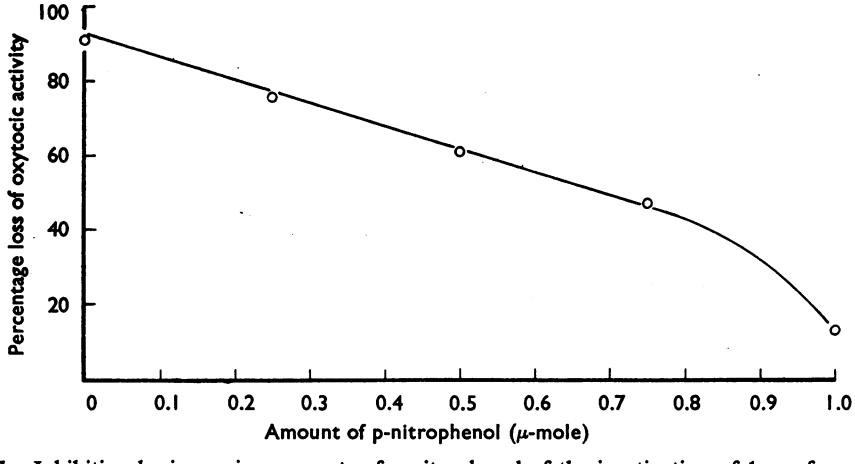

Fig. 7. Inhibition by increasing amounts of p-nitrophenol of the inactivation of 1 u. of oxytocin incubated with 50 μg of Worthington's tyrosinase preparation at *p*H 7.5 for 2 hr.

Ascorbic acid. Only partial inhibition of the inactivation of oxytocin was obtained with ascorbic acid. In the concentrations used in the experiments shown in Table 2, ascorbic acid did not reduce the *p*H of the incubation mixtures. When 1 u. of oxytocin, previously inactivated by incubation for 120 min with 100 μg

of enzyme, was treated with 1 mg of ascorbic acid in order to reduce the oxidized peptide, no reactivation was observed. Ascorbic acid did not affect the inactivation of bradykinin.

Sodium diethyldithiocarbamate. The inactivation of oxytocin was partly inhibited by 2 to 4 μg and completely inhibited by 5 μg of sodium diethyldithiocarbamate. In one experiment the incubated samples were assayed for milk-ejecting activity. With 100 μg of the enzyme preparation the loss of milk-ejecting activity with 2 μg of sodium diethyldithiocarbamate was 69% and, with 4 μg of the inhibitor, 11%. The corresponding values for oxytocic activity were 79% and 10%. In the experiment with bradykinin, only 10 μg of the enzyme preparation was used, to ensure a submaximal effect, and the amount of sodium diethyldithiocarbamate was reduced proportionately to 0.5 μg. Inactivation of bradykinin was not inhibited under these conditions.

Incubation under nitrogen

When oxytocin was incubated under nitrogen for 120 min with 100 μg of the enzyme preparation, the loss of oxytocic activity was only 18%; on re-exposure to air and incubation for a further period of 120 min, 92% of the activity was lost. The loss of bradykinin in the presence of 10 μg of enzyme was 64% under nitrogen compared with 77% under air.

DISCUSSION

Previous workers, using relatively impure preparations of oxytocin and vasopressin, had shown that incubation with tyrosinase caused a loss of the oxytocic activity of oxytocin and of the pressor and antidiuretic activities of vasopressin. With the use of synthetic substrates these observations have been confirmed and it has been found that there is also a loss of milk-ejecting activity when oxytocin is incubated with a tyrosinase preparation. Since the inactivation of oxytocin and vasopressin by tyrosinase could be stopped by acidifying and boiling, it was possible to study the time-course of the reaction.

Within the limits of experimental error, vasopressin lost its pressor and antidiuretic activities at an equal rate after incubation with a tyrosinase preparation. Similarly, oxytocin showed an equal loss of oxytocic and milk-ejecting activity. Treatment with the tyrosinase preparation, therefore, did not dissociate the activities in either substance.

The absence of any significant loss of oxytocic or milk-ejecting activity in phenylalanine^2-oxytocin, and of pressor or antidiuretic activity in phenylalanine^2-lysine^8-vasopressin, on incubation with Worthington's tyrosinase preparation strongly supports the idea that the inactivation of oxytocin and vasopressin by this enzyme preparation is due to a specific action on the tyrosyl residue. Further evidence for this specificity of action is provided by the fact that p-nitrophenol, ascorbic acid and sodium diethyldithiocarbamate, which are known to inhibit the oxidation of phenols by tyrosinase, also inhibited the inactivation of oxytocin by Worthington's tyrosinase preparation. These inhibitors of tyrosinase, which is a copper oxidase, have different mechanisms of action. p-Nitrophenol has been described as a com-

petitive inhibitor (Dawson & Tarpley, 1951). Inhibition by ascorbic acid, which is not itself oxidized by tyrosinase, is probably due either to deprivation of oxygen or to reduction of the oxidation products of the reaction. Sodium diethyldithiocarbamate acts by forming a complex with copper, which is an essential component of the enzyme. The fact that no inactivation of oxytocin occurred under anaerobic conditions is also in accord with the idea that the hormone was inactivated by tyrosinase, since for its action on phenols tyrosinase requires oxygen.

According to Raper's interpretation (Dawson & Tarpley, 1951) the action of tyrosinase on tyrosine is, firstly, to insert a second hydroxyl group in the ortho position to the first one, and, secondly, to oxidize the dihydroxy derivative to an O-quinone. Presumably, the further stage of ring closure of the side-chain to form the indole configuration of melanin cannot take place if the amino and carboxyl groups of tyrosine are involved in peptide linkages with adjacent amino acids in a polypeptide chain, as in oxytocin and vasopressin. Similarly, it has been shown that both N-formyl tyrosine and tyrosine ethyl ester are oxidized by tyrosinase to a dopa analogue, but that neither gives rise to a hallachrome intermediate or to melanin (Sizer, 1953). Since evidence is given that the inactivation of oxytocin and vasopressin by tyrosinase is due to a specific action of the enzyme on the hydroxyphenyl moiety in the tyrosyl residue, the conclusion to be drawn from this work is that the O-quinone derivatives of these peptides produced by oxidation of the tyrosine residue in position 2 are probably inactive.

It is interesting to compare the results obtained with synthetic analogues of oxytocin and vasopressin in which changes in the tyrosyl residue have been introduced. Phenylalanine^2-oxytocin, in which the phenolic hydroxyl group of oxytocin has been removed, was prepared independently by Bodansky & du Vigneaud (1959) and Jaquenoud & Boissonnas (1959). Both groups of workers found an oxytocic potency, measured on the isolated rat uterus, of 30 u./mg. On the other hand, Konzett & Berde (1959) reported that the milk-ejecting potency of the analogue prepared by Jaquenoud & Boissonnas was 140 u./mg. The corresponding analogue of vasopressin, phenylalanine^2-lysine^8-vasopressin, prepared by Boissonnas & Guttmann (1960) has a pressor potency equal to about one-fifth of that of the parent compound (Berde *et al.*, 1961). The conclusion drawn from these studies was that, while the phenolic hydroxyl group contributed to the activity of oxytocin and vasopressin, it was not indispensable. In contrast, in the present experiments, the effect of a tyrosinase preparation on oxytocin and vasopressin was an almost total inactivation. In addition, no dissociation was observed between the oxytocic and milk-ejecting activities of oxytocin, as has been reported for its phenylalanine analogue. Law & du Vigneaud (1960) have synthesized O-methyl-oxytocin, in which the phenolic hydroxyl group has been replaced by a methoxy group. This derivative of oxytocin has an oxytocic potency equivalent to only 1% of the parent compound, and, in addition, it exhibits a weak antagonism towards the pressor action of vasopressin. When the results obtained with synthetic analogues are considered together with the present enzyme studies, it appears that, if the phenolic hydroxyl group is removed from oxytocin or vasopressin, as much as one-third of the original activity may remain, whereas if this group is methylated, or if the tyrosyl

residue is oxidized to an O-quinone derivative, activity is almost completely abolished.

The quinone group is chemically highly active. The action of tyrosinase on oxytocin and vasopressin has been investigated, not only as a contribution to the study of structure-action relationships, but also in the hope that treatment of the quinone derivatives of these peptides with suitable chemical reagents might lead to the formation of specific competitive antagonists, in a manner similar to that in which replacement of the N-methyl group in morphine by an N-allyl group led to the discovery of the potent inhibitor nalorphine. In the present work, simple inactivation of the peptides by tyrosinase was not observed to give rise to products having any inhibitory effect on the response to oxytocin or vasopressin.

The inactivation of bradykinin by tyrosinase preparations at *p*H 3.9 was an incidental observation arising from control experiments which were designed to test the specificity of the tyrosinase preparations for peptides containing tyrosine. The bradykinin inactivation is thought to be enzymatic in nature since boiled enzyme preparations were ineffective, and, with a constant amount of enzyme in the incubation mixture, the inactivation increased progressively with time. Bradykinin is a nonapeptide containing five different amino acids (Elliott *et al.*, 1961). Four of these, glycine, phenylalanine, proline and arginine, are present in oxytocin or arginine-vasopressin. The fifth, serine, contains an aliphatic hydroxyl group, but bradykinin does not contain a phenolic group. The absence of a phenolic group is consistent with the finding that none of the substances which inhibited the inactivation of oxytocin by a tyrosinase preparation at *p*H 7.5 significantly reduced the inactivation of bradykinin at *p*H 3.9. It is concluded that the tyrosinase preparations used in the present experiments contain two distinct enzymes or activities, the one inactivating oxytocin at *p*H 7.5 and the other bradykinin at *p*H 3.9. The mechanism of inactivation of bradykinin has not been elucidated. However, the difference which has been reported between oxytocin and bradykinin in their reaction with tyrosinase preparations may find practical application in bioassay work as a means of identifying, and possibly separating, the two peptides in extracts of body fluids.

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