A Sensitive Hydroosmotic Toad Bladder Assay

Affinity and intrinsic activity of neurohypophyseal peptides

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ABSTRACT A sensitive and precise method for assaying the water permeability response evoked by neurohypophyseal hormones and their synthetic analogues on the isolated urinary bladder of the toad (*Bufo marinus* L.) is described. The method permits detection of 8-arginine-vasotocin at concentrations as low as 10^{-12}M. This sensitivity, not achieved heretofore with this tissue, results largely from minimizing interference of inhibitory substances by means of an "in vitro circulation assembly." The precision of the method derives from a direct comparison between the cumulative dose-response curve of an agonist of unknown potency acting on one hemibladder and that of a reference compound acting on the contralateral hemibladder. Crystalline deamino-oxytocin is used as the reference standard in this assay. The intrinsic activity of 2-(*O*-methyltyrosine)-oxytocin, as defined by the maximal response, is 12% lower than that of deamino-oxytocin. All other hormonal peptides investigated have the same intrinsic activity as deamino-oxytocin, even 5-valine-oxytocin, in spite of its extremely low affinity. A comparison of the potencies of 8-arginine-vasotocin vs. 8-arginine-vasopressin, 8-ornithine-vasotocin vs. 8-ornithine-vasopressin, 8-alanine-oxytocin vs. 8-alanine-oxypressin, and deamino-8-alanine-oxytocin vs. deamino-8-alanine-oxypressin suggests that an isoleucine residue in position 3 imparts a higher specificity for binding of the hormonal peptide molecule to the bladder receptor than a phenylalanine residue in this locus.

INTRODUCTION

The mammalian antidiuretic hormone, vasopressin, modifies the permeability to water of the distal nephron and thereby accelerates osmotic equilibration of the forming urine with the renal interstitial fluid (4). Similarly, neurohypophyseal peptides increase the rate of water movement along an osmotic gradient across the skins and bladders from frogs and toads. In these target tissues the hormone interacts with a receptor moiety in the epithelial cell

which in turn modifies the selective permeability of the cell membrane. One approach towards elucidating the mechanism of the initial hormone-receptor interaction is to examine the contribution of individual molecular features of agonists to biological function. Studies of this type, however, have met with great difficulties in toad bladder preparations.

This publication describes a sensitive bioassay procedure for determining the specific activities of neurohypophyseal hormones and synthetic analogues on the isolated urinary bladder of the toad, *Bufo marinus*. The extension of this method to the study not only of drug-receptor reaction kinetics but also of cumulative dose-response relationships permits evaluation of pharmacological concepts such as affinity, intrinsic activity, and antagonism.

MATERIALS AND METHODS

Male and female toads (*Bufo marinus*) weighing 180 to 250 g used in these experiments were kept on moist peat moss. After the animals had been double pithed, the urinary bladders were immediately dissected and placed in Ringer's solution of the following

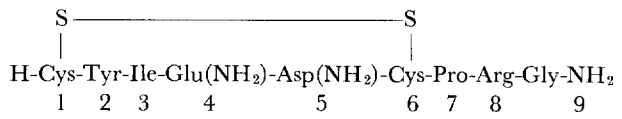

FIGURE 1. Structure of 8-arginine-vasotocin. Numbers indicate the positions of the individual amino acid residues.

composition: NaCl 110 mM, KCl 3 mM, $CaCl_2$ 1 mM, dextrose 1 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 3 mM; tris(hydroxymethyl)aminomethane, HCl (Trizma®) 10 mM was employed as a buffer and the pH was adjusted with NaOH to 8.4 unless otherwise indicated in the text; the osmotic activity was 242 milliosmols/liter.

The following hormones and analogues were employed in this study: 8-arginine-vasotocin (Fig. 1) (26), 8-arginine-vasopressin (7), 8-ornithine-vasotocin (17), oxytocin (8, 9), deamino-oxytocin (10, 16, 22), 2-(*O*-methyltyrosine)-oxytocin (23, 28), 8-alanine-oxytocin (18, 37), deamino-8-alanine-oxytocin (37), 8-alanine-oxypressin (37), deamino-8-alanine-oxypressin (37), 5-valine-oxytocin (39), 8-ornithine-vasopressin (17). These synthetic hormonal peptides were prepared in our own laboratories and those of the Academy of Science in Czechoslovakia and of Sandoz S.A. in Switzerland.

The compounds were assayed for avian vasodepressor activity according to the method of Munsick, Sawyer, and Van Dyke (29). In the fowl bioassay a four point design was used with the USP Posterior Pituitary Standard as a reference. The values obtained for the avian vasodepressor activity for the analogues enumerated in Table II were in accordance with those previously published. The only exception was 2-(*O*-methyltyrosine-oxytocin, which possessed only half the avian vasodepressor activity reported earlier (28), perhaps due to inactivation during storage. The potency value of 2-(*O*-methyltyrosine)-oxytocin obtained for the water flux on the toad bladder was therefore corrected by a factor of 2. Biological activities of the analogues on the toad

bladder preparation were determined by employing crystalline deamino-oxytocin as a reference standard.

Preparation of the Toad Bladder

The isolated hemibladder is gently stretched over a hollow glass rod of 0.5 cm diameter so that the mucosal bladder surface is in contact with the glass. A square knot with surgical silk is loosely placed 4.3 cm from the base of the rod. Subsequently the bladder is pulled downward and secured with a second knot just above the broadened base.

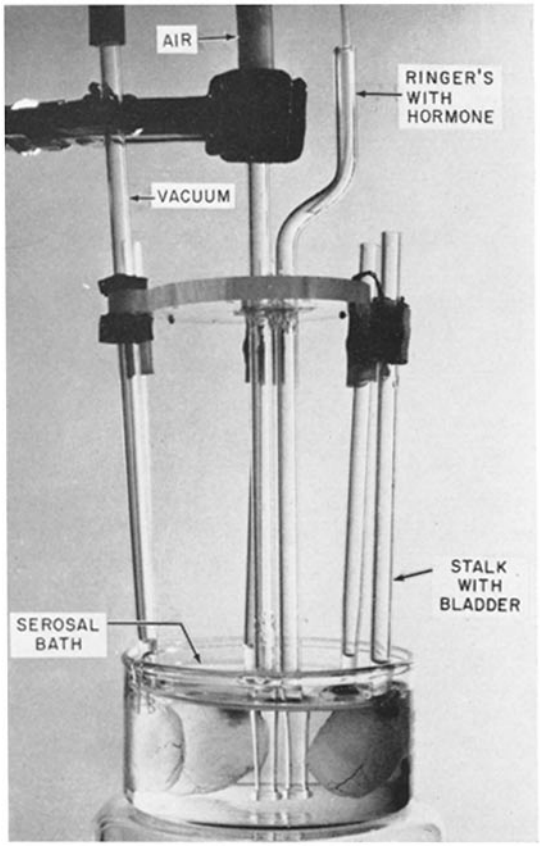

FIGURE 2. Experimental preparation for measuring the permeability to water of the isolated toad bladder. The serosal fluid is continuously circulated by siphoning hormone-containing Ringer's fluid from a reservoir at a controlled rate into the bottom of a bath and by suctioning fluid in excess of 100 ml from the surface of the bath. Bladders, mounted onto hollow glass tubes and filled with hypotonic fluid (mucosal fluid), are placed in a bath of isotonic fluid, vigorously stirred by a jet of air saturated with water.

After removal of excess tissue, the bladder is filled with 7 ml of Ringer's fluid which had been diluted to $\frac{1}{5}$th strength (mucosal fluid). This "sac" preparation of the bladder is similar to that described by Bentley (3) and it guarantees uniformity of membrane surface area. Four to six bladders thus prepared were suspended in a bath of full strength Ringer's solution as detailed in Fig. 2.

Assay Assembly

For the water movement studies the following assembly was designed to assure constancy of the various components in the serosal bathing media, including hormone

concentration. As illustrated in Fig. 2 the fluid bathing the serosal (outside) surface of the bladder is replenished at a rate of 6 ml/min with fresh full strength Ringer's fluid containing a given hormone concentration. This serosal bath is vigorously stirred with air saturated with water and is maintained at a constant volume of 100 ml by removing bathing fluid at a rate equal to the input. The net increase in transmembrane water flow along an osmotic gradient was determined every 30 min by weighing the individual bladder assemblies on a Sartorius microbalance (type 2303). Following each 30 min weighing period, the mucosal fluid was replaced with 7 ml of fresh fluid.

Protocol for Dose-Response Studies

10 hemibladders were filled with 7 ml of $\frac{1}{5}$th strength Ringer's solution (mucosal fluid) and incubated for 120 min in 400 ml of hormone-free full strength Ringer's solution stirred vigorously with air. Meanwhile two series of serosal solutions (500 ml for each hormone concentration) were prepared, containing our standard deamino-oxytocin and the analogue to be tested, respectively. The hormonal dosages were increased geometrically by a factor of 2 starting from a threshold concentration of the standard and analogue. One set of five hemibladders (control) was removed from the hormone-free incubation medium and placed into a 100 ml aliquot of fresh full strength Ringer's solution containing the lowest concentration of deamino-oxytocin. The contralateral set of five hemibladders (experimental) was also placed into a 100 ml bath of fresh full strength Ringer's solution but containing the lowest dose of analogue instead of standard. The hormone solutions in both control and experimental serosal baths were replenished at a flow rate of 6 ml/min from their respective 360 ml reservoirs. After the second weighing period (60 min) control and experimental hemibladders were transferred directly to fresh serosal baths with the next higher concentration of standard and analogue. Here again, the flow rate of the fresh hormone solutions from the reservoirs was set at 6 ml/min. This transferring procedure was repeated until a further geometrical increase in hormone concentration failed to yield a higher response. At this point both sets of hemibladders were challenged with a supramaximal dose (approximately 20 times the ED_{50}) to test whether the maximal response had indeed been reached during the cumulative dose-response experiment.

RESULTS

Effect of a "Circulating" Serosal Solution on the Osmotic Flux of Water

The response of one set of hemibladders to 5×10^{-9} M oxytocin in a serosal bath with a flow rate of 6 ml/min (control) is compared with the response of the contralateral set of hemibladders to the same dose of hormone but in a serosal bath with a flow rate of 0 ml/min (experimental) (Fig. 3). For the first 30 min period the responses of the paired hemibladders were of comparable magnitude, but in subsequent periods they diverged increasingly, i.e. the control bladders maintained a high level of response throughout the time course of the experiment, in contrast to the experimental bladders which showed a rapid decline in response following the first 30 min period.

Effect of Hormone Concentration on the Time Course of the Response

The response of bladders to various concentrations of oxytocin (1×10^{-7} M, 5×10^{-9} M, and 1×10^{-9} M) was followed for 300 min (Fig. 4) without prior incubation in hormone-free Ringer's fluid. In the course of the experiment the flow rate of the hormone-containing serosal solution was maintained at 6 ml/min. It should be noted that the optimum response to each dose of hormone varied not only in magnitude, but also in the rates at which these optima were reached. Thus with 1×10^{-7} M oxytocin the optimum of 55.4 mg/min

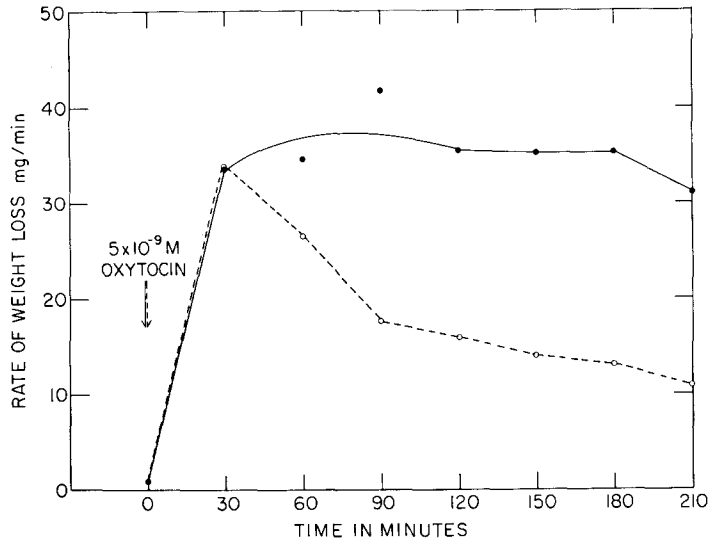

FIGURE 3. Curve A (filled circles), average rate of weight loss of 10 control hemibladders exposed to 5×10^{-9} M oxytocin; the serosal bath had a circulation rate of 6 ml/min. Curve B (open circles), average rate of weight loss of 10 experimental contralateral hemibladders, exposed to an identical concentration of hormone; the serosal bath had a circulation rate of 0 ml/min.

had already been obtained during the first 30 min interval; with 5×10^{-9} M oxytocin the optimum of 33.2 mg/min was recorded during the second 30 min interval, and finally, with 1×10^{-9} M oxytocin the optimum of 25.0 mg/min was not reached until the fourth 30 min interval. Furthermore, a correlation in the rate of fading of the response to these various doses of hormone is apparent from Fig. 4. While the response of the bladder to 1×10^{-7} M oxytocin declines rapidly after its maximum to approximately 50%, the further decline is an extremely gradual one. In contrast, the rate of fading of the response to 5×10^{-9} M and 1×10^{-9} M oxytocin is minimal.

Effect of Incubation on Bladder Reactivity

Bladders were kept in hormone-free Ringer's solution vigorously agitated with moist air for increasing periods of time. Subsequently, the bladders were challenged for 30 min by transferring them into a fresh bath of Ringer's solution containing 2.5 × 10^{-9} M oxytocin. The effect of incubation on the reactivity of paired hemibladders was ascertained for 2 hr intervals by com-

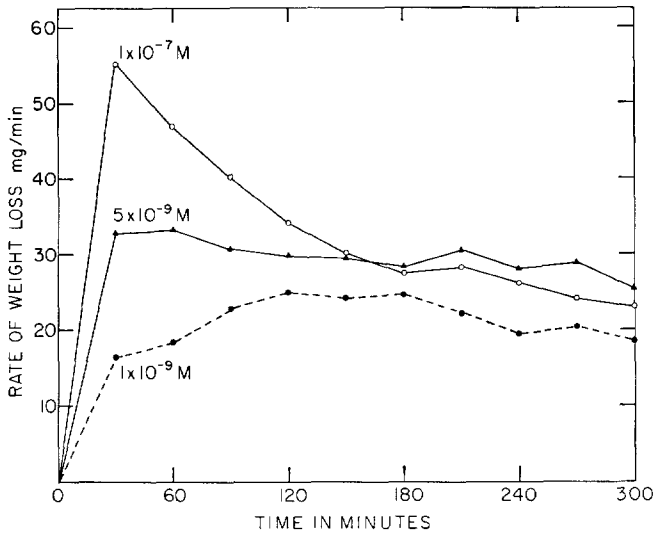

FIGURE 4. Hemibladders exposed to serosal Ringer's fluid circulating at a rate of 6 ml/min but containing different doses of oxytocin. Curve A (open circles) shows average rate of weight loss of 16 control hemibladders challenged with 1 × 10^{-7} M oxytocin. Curve B (filled triangles) and curve C (filled circles) show average rate of weight loss of experimental contralateral hemibladders challenged with 5 × 10^{-9} M and 1 × 10^{-9} M oxytocin, respectively.

paring bladders which were kept in hormone-free Ringer's solution for: 0 vs. 120 min; 120 vs. 240 min; and 240 vs. 360 min. The response following the 360 min incubation period was taken as 100%. It is apparent from Table I that the reactivity of the bladders increases with time of incubation.

Time-Dose-Response Studies

A modified cumulative dose-response curve obtained on the toad bladder is illustrated in Fig. 5. Bladders were challenged with increasing doses of deamino-oxytocin as outlined under the Protocol for Dose-Response Studies. The response of the bladders during exposure to each hormone level was followed for two consecutive 30 min periods. The study revealed a stepwise increase of response with increasing dosage of the agonist. The magnitude of the response during the first and second weighing periods was similar in

the lower dosage range; however, in the higher dosage range, the response observed during the second weighing period was considerably less than that observed during the first weighing period.

TABLE I

EFFECT OF INCUBATION TIME ON RESPONSIVENESS OF BLADDER TISSUE TO AN IDENTICAL DOSE OF OXYTOCIN

Time of incubation in hormone-free Ringer's solution	Number of bladders	Response to oxytocin (2.5×10^{-9} M, pH 8.0) as % of maximum
min		
0	3	32
120	6	47
240	7	83
360	4	100

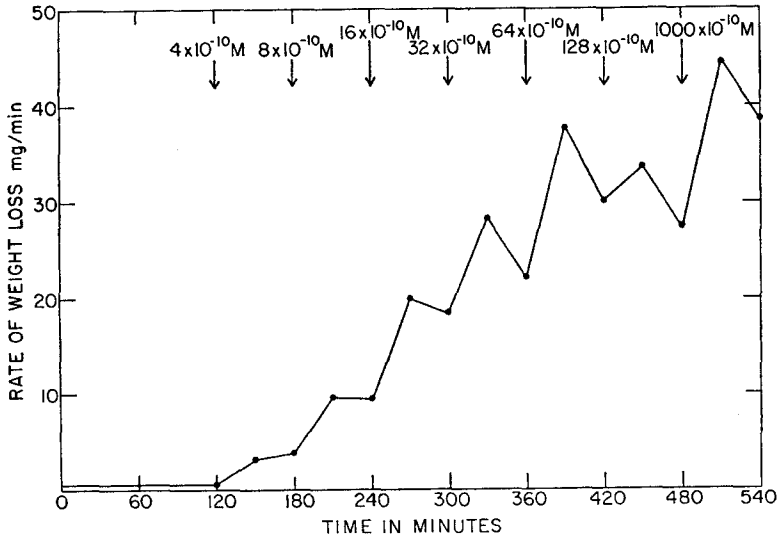

FIGURE 5. Average rate of weight loss of 16 hemibladders incubated in hormone-free Ringer's fluid for 120 min. The bladders were then transferred to a serosal bath containing deamino-oxytocin with a circulation rate of 6 ml/min. The response to each concentration of agonist was determined for two successive 30 min weighing periods. Bladders were then placed directly into a fresh serosal bath containing the next higher concentration of deamino-oxytocin, etc.

Effect of Theophylline on the Response to Oxytocin

With a set of hemibladders a cumulative dose-response curve was obtained for oxytocin in the presence of a 0.5 mM concentration of theophylline—a concentration below the level capable of inducing hydroosmotic flux; with the contralateral set of hemibladders a cumulative dose-response curve was

obtained for oxytocin alone. The concentration of oxytocin required for half-maximal response (ED_{50}) was 1.29×10^{-9} M in the absence of theophylline, and 1.92×10^{-10} M in the presence of theophylline.

Effect of Hydrogen Ion Concentration on the Response to Oxytocin

Following a 2 hr incubation period, bladders were exposed to a constant concentration of hormone but to different hydrogen ion concentrations in a serosal bath circulating at 6 ml/min. At 60 min intervals the pH of the serosal bath was raised from an initial pH of 6.10, to 6.85, to 7.15, to 7.77, to 8.40.

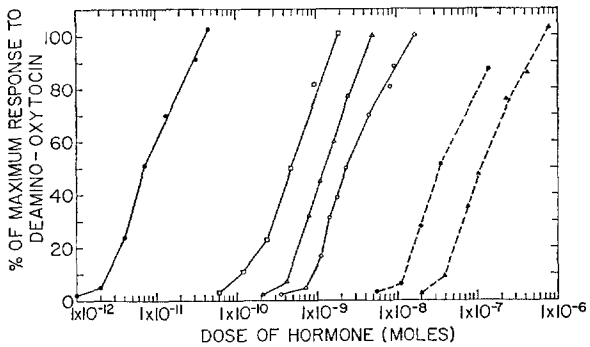

FIGURE 6. Cumulative log-dose-response curves for crystalline deamino-oxytocin (open circles), oxytocin (open triangles), 8-arginine-vasotocin (filled circles), 2-(*O*-methyltyrosine)-oxytocin (filled circles connected by broken lines), 8-deamino-alanine-oxypressin (filled triangles connected by broken lines), 8-ornithine-vasotocin (open squares). Deamino-oxytocin served as a standard for control hemibladders. Each analogue was tested in contralateral experimental hemibladders against the standard at various response levels.

Throughout the experiment the oxytocin concentration was kept constant at 5×10^{-9} M. It was found that with this stepwise increase in pH from 6.10 to 8.40, the response increased from 3.4 to 42.4%, 52.5%, 89.8%, and 100%, respectively.

Dose-Response Plots of Neurohypophyseal Hormones and Analogues

Time-dose-response curves of individual analogues and of the standard, deamino-oxytocin, were obtained simultaneously on contralateral hemibladders. The dose-response curves were derived from such modified cumulative dose-response studies as illustrated in Fig. 5. The optimal response for any given dose of hormone during the 60 min challenge period was selected for graphing the log-dose-response plots. The maximum response was determined with a final dose amounting to 20 times the ED_{50}. For some analogues this value was checked by challenging fresh contralateral hemibladders with supramaximal doses of reference standard and unknown. Fig. 6 shows such

TABLE II

RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY OF NEUROHYPOPHYSEAL HORMONES AND SELECTED ANALOGUES

Compound.	Depressor* (Fowl)	Uterus* (Rat)	Pressor* (Rat)	Antidiuretic* (Rat)	Milk-ejecting* (Rabbit)	Water flux‡ (Toad bladder)	Water flux, ED_{50} (Toad bladder)
Deamino-oxytocin§	975±24	803±36	1.14±0.6	19.0±1.0	400±8	100	2.35×10^{-9} M
Oxytocin	507±15‖	546±18¶	3.1±0.1‖	2.7±0.2‖	410±16‖	182±9	1.29×10^{-9} M
2-(*O*-methyltyrosine)-oxytocin	~5** 8‡‡	~5** 2§§	Inhibits AVP**	0.01‡‡	—	6.80±0.48	3.45×10^{-8} M
5-Valine-oxytocin‖‖	~0.1	~0.3	—	—	—	0.95±0.1	2.47×10^{-7} M
8-Alanine-oxytocin	240±25¶¶	166±6¶¶	13±1¶¶	2.38***	353±20‡‡‡	170±11	1.38×10^{-9} M
Deamino-8-alanine-oxytocin	453±6¶¶	314±10¶¶	8±0.4¶¶	1.45***	393±10‡‡‡	70.0±6	3.37×10^{-9} M
8-Alanine-oxypressin	38±2¶¶	15±0.6¶¶	21±1¶¶	45.2±3.3‡‡‡	~65‡‡‡	2.94±0.26	8.00×10^{-8} M
Deamino-8-alanine-oxypressin	47±2¶¶	25±0.6¶¶	4.6±0.1¶¶	37.9±2.1‡‡‡	~135‡‡‡	2.10±0.27	1.12×10^{-7} M
8-Arginine-vasotocin§§§· ‖‖‖‖	150 285	~75 155	125 245	74 250	~100 210	33,200±910	7.10×10^{-12} M
8-Ornithine-vasotocin¶¶¶	90±3	42±5	103±10	2.5±0.3	95±6	486±16	4.83×10^{-10} M
8-Arginine-vasopressin	~60****	12‡‡‡‡	~400‡‡‡‡	~400****, ‡‡‡‡	~70‡‡‡‡	1,375±120	1.70×10^{-10} M
8-Ornithine-vasopressin¶¶¶	21±1	10±2	360±26	88±17	~50	14.3±0.8	1.64×10^{-8} M

* Expressed in USP units/mg.

‡ As percentage of crystalline deamino-oxytocin on a molar basis.

§ Ferrier, B. M., D. Jarvis, and V. du Vigneaud. 1965. *J. Biol. Chem.* **240**:4264.

‖ See reference 6.

¶ Chan, W. Y., M. O'Connell, and S. R. Pomeroy. 1963. *Endocrinology.* **72**:279.

** See reference 28.

‡‡ Jošt, K., J. Rudinger, and F. Sǒrm. 1963. *Collection Czech. Chem. Commun.* **28**:1706.

§§ Zhuze, A. L., K. Jošt, E. Kasafírek, and J. Rudinger. 1964. *Collection Czech. Chem. Commun.* **29**: 2648.

‖‖ See reference 39.

¶¶ See reference 37.

*** Walter, R., and I. L. Schwartz. 1968. *Life Sci.* **7**:545.

‡‡‡ Chan, W. Y. Personal communication.

§§§ See reference 26 and also Katsoyannis, P. G., and V. du Vigneaud. 1959. *Nature.* **184**:1465.

‖‖‖ Huguenin, R. L., and R. A. Boissonnas. 1962. *Helv. Chim. Acta.* **45**:1629.

¶¶¶ See reference 17.

**** See reference 7.

‡‡‡‡ Van Dyke, H. B., W. H. Sawyer, and N. I. A. Overweg. 1963. *Endocrinology.* **73**:637.

dose-response curves obtained for 8-arginine-vasotocin, 8-ornithine-vasotocin, oxytocin, deamino-oxytocin, deamino-8-alanine-oxypressin, and 2-(*O*-methyltyrosine)-oxytocin. The dose-response curves of all analogues studied paralleled that of the standard deamino-oxytocin. The curves for several analogues were shifted variously to the left of the standard curve: 8-arginine-vasotocin dramatically, 8-ornithine-vasotocin and 8-arginine-vasopressin moderately, and both oxytocin and 8-alanine-oxytocin slightly. The following dose-response curves were displaced to the right of the standard curve: the oxypressins (deamino-8-alanine-oxypressin and 8-alanine-oxypressin) and 5-valine-oxytocin markedly, both 8-ornithine-vasopressin and 2-(*O*-methyltyrosine)-oxytocin moderately, and deamino-8-alanine-oxytocin slightly. Differences in the maximum response for all analogues were insignificant with the exception of 2-(*O*-methyltyrosine)-oxytocin, which exhibited a maximum of only 88% of that of the standard.

The activities of these peptidyl hormones in the toad bladder hydroosmotic assay are compared in Table II with the activities of these compounds in the fowl vasodepressor, rat uterotonic, rat antidiuretic, rat pressor, and rabbit milk ejection bioassays. The toad bladder hydroosmotic activity of the compounds is expressed in two ways: (*a*) in terms of the concentration of analogue required to evoke a half-maximal response, i.e. the ED_{50}, which reflects an average sensitivity of the particular bladder preparation; (*b*) in terms of the potency (expressed as a per cent of the potency of the deamino-oxytocin standard) as derived from the ED_{50} value measured on one hemibladder and compared directly to the ED_{50} value measured for the standard on the contralateral hemibladder, which makes the potencies independent of variations in sensitivity of bladders from different toads.

DISCUSSION

Several procedures have been employed for determining the relative potencies of neurohypophyseal hormones and analogues with respect to the effect of these agents on the permeability to water of the isolated urinary bladder of the toad, *Bufo marinus* (3, 11, 32). However, it is germane to an understanding of structure-activity relationships, that parameters, such as intrinsic activity, affinity, synergism, and antagonism, be evaluated by means of dose-response studies. In addition, such investigations should be complemented by a kinetic analysis of factors influencing the response pattern of the receptor tissues to a given challenge. It is apparent that the study of the potency, type of action, and pharmacokinetic properties of hormones requires not only a specific and sensitive preparation, but also one in which the response can be readily quantitated, and in which several doses of an unknown can be tested against a standard. The widely used sac preparation of the isolated toad bladder (3) falls short of meeting several of these requirements. A particular deficiency

emerges from the observation that the sensitivity of this membrane changes with time (11, 13, 35) and with the history of transmembrane hydroosmotic flux (12), circumstances which have made dose-response studies (especially cumulative dose-response studies) unrewarding in the past.

In this context it may be recalled that the bladder tissue releases into the serosal solution an inhibitory substance of unknown structure (13, 24) and that this antagonist was subsequently isolated in increased yields from whole bladder homogenates (25). In addition, a peptidase, which completely abolishes the activity of 8-lysine-vasopressin, was obtained from such homogenates (5). It has been an arduous task to discern the interdependence of these inhibitory substances and to determine their relative contribution at various loci—intracellular and extracellular—to alterations in membrane permeability.

In approaching this problem we devised methods which entail the physical removal of inhibitory substances which constantly escape from the tissue into the serosal bath. This was accomplished with an assay assembly (Fig. 2) consisting in essence of an in vitro circulation which continuously bathes the membrane in fresh serosal solution and maintains a constant concentration of hormone. Such a method made it possible to retain the response to 5×10^{-9} M oxytocin at a near constant level for a prolonged period of time (Fig. 3, curve A). The experiment shows that inhibitory factors which diminish the response in the uncirculated preparation (Fig. 3, curve B) can be physically removed.

The finding that a constant response plateau could be maintained by a circulating serosal bath (Fig. 3, curve A) suggested that the self-limiting character of the response to 5×10^{-9} M oxytocin in the uncirculated preparation (Fig. 3, curve B) was due to the accumulation of inhibitory substances. In an effort to discern factors other than inhibitory substances affecting bladder reactivity, we studied with the circulation assembly the time course of the response to grossly different doses of hormone. Curve A in Fig. 4 shows that the initial response following a supramaximal dose of oxytocin cannot be sustained; in fact, the immediate rise to a maximum is followed by a rapid decline. In this particular experiment the response diminished by approximately 50% and slowly faded thereafter. The fact that an increase in the circulation rate of the bath from 6 to 25 ml/min did not influence the nature of the response militates against the possibility that the decline in tissue sensitivity was caused by an increased rate in the release of inhibitory substances into the serosal medium. When the bladders were challenged with an intermediate dose of hormone (Fig. 4, curve B), the pattern of response was characterized by a slightly delayed optimum followed by a gradual decline similar to that shown in curve A after the half-maximum permeability level had been reached. If the bladders were stimulated by an even lower dose of

hormone (Fig. 4, curve C), the most striking difference in the response pattern was not the phase of decline, but rather the rate at which the optimum was reached. Thus the sensitivity of the bladder is affected by opposing factors and their relative influence will determine the reactivity of the bladder. The magnitude of the factor which tends to diminish reactivity is related to the intensity and duration of prior stimulation. The magnitude of the other factor—the tendency to enhance tissue sensitivity—is dependent upon the duration of exposure of bladders to hormone-free Ringer's fluid (Table I). These considerations proved useful in following the change in bladder reactivity during the course of a cumulative dose-response study in that only those responses in which the tissue sensitivity did not change appreciably during two successive 30 min periods of exposure to a given hormone concentration were considered reliable (e.g., the first four concentration levels of hormone in Fig. 5.).[1] We found usually that changes in bladder reactivity were minimal below the 70% response level—a response range that is well within the limits required for affinity determinations. Above the 70% response level the reactivity to a given dose of hormone was less consistent; thus it was not always possible to ascertain the minimal dose required to evoke the maximal response. In order to test whether the maximum response had indeed been reached during the cumulative dose-response experiment, the bladders were stimulated with a final dose at least 20 times greater than the ED_{50}. In instances in which a discrepancy in sensitivity between contralateral hemibladders were suspected because of differences in duration and extent of previous hydroosmotic fluxes, the maximal responses were tested by challenging fresh contralateral hemibladders with supramaximal doses of reference standard and unknown; identity of the maximal response to the analogue with the maximal response to the standard signified identical intrinsic activity.

In order to enhance tissue sensitivity prior to challenge with the lowest hormone concentration, bladders were incubated in hormone-free Ringer's solution for a period of 2 hr. This time interval was chosen for practical purposes to assure the completion of a cumulative dose-response study within a reasonable time span—although a longer incubation period was found to further enhance bladder sensitivity. Moreover, we established that a stepwise increase in serosal pH from 6.1–8.4 was paralleled by a graded increase in the response of the tissue to a constant concentration of oxytocin. The finding of a pH optimum (8.4) of membrane reactivity to oxytocin confirms previous observations by Gulyassy and Edelman (14, 15). In addition, we recorded a

[1] In instances when only limited amounts of hormone are available, a cumulative dose-response curve may also be obtained, although the change in tissue sensitivity cannot be ascertained, by following the response to each dose for only one 30 min period. It should be noted that the cumulative dose-response curve in Fig. 5 bears striking resemblance to the theoretical cumulative dose-response curve calculated by Ariëns (2) on the basis of Paton's rate theory (31).

6.7-fold increase in the reactivity of the bladder to oxytocin in the presence of a subthreshold concentration of theophylline—an effect reported qualitatively by Orloff and Handler (30). These pH and theophylline experiments show that this assay method can quantitate such parameters as synergism and antagonism.

The sensitivity of the toad bladder preparation is reflected in the dose required to evoke a half-maximal response (ED_{50}). However, the accuracy of this bioassay method hinges upon a direct comparison of the ED_{50} value of an analogue, measured with one hemibladder, with the ED_{50} value of a standard, measured with the contralateral hemibladder. The precision of this method, therefore, depends upon sensitivity differences between contralateral hemibladders of the same toad, and not—as in previous toad bladder bioassays (see reference 32)—upon substantial fluctuations in sensitivity between bladders from different toads. It should be pointed out that the expression of hydroosmotic activity of a hormone analogue in terms of concentration (ED_{50} values from *noncontralateral* hemibladders) is less precise than in terms of potency (derived from ED_{50} values from *contralateral* hemibladders). This difference is illustrated in the following example: The potency of 8-ornithine-vasotocin is 486 with a standard error of the mean (SEM) of ± 16 (Table II). This value was obtained by comparing the ED_{50} of 8-ornithine-vasotocin with the ED_{50} of deamino-oxytocin in contralateral hemibladders. Subsequently the results from several such determinations were averaged. If, on the other hand, the potency of 8-ornithine-vasotocin was calculated from the same ED_{50} values, but from randomly chosen sets of noncontralateral hemibladders, a potency value of 920 ± 80 was obtained.

In a preliminary study with this new assay procedure we have gathered data to assess the effect upon affinity and intrinsic activity of structural changes in neurohypophyseal hormones. In particular we investigated a series of selected hormone peptides exemplifying such varied groups as the oxytocins (with an isoleucine residue in position 3 and a neutral amino acid residue in position 8), vasotocins (with an isoleucine residue in position 3 and a basic amino acid residue in position 8), oxypressins (with a phenylalanine residue in position 3 and a neutral amino acid residue in position 8), and vasopressins (with a phenylalanine residue in position 3 and a basic amino acid residue in position 8). The biological activities of the neurohypophyseal hormones and their analogues were compared with that of crystalline deamino-oxytocin following an earlier suggestion of Jarvis and du Vigneaud (22). The use of deamino-oxytocin as a reference for the toad bladder hydroosmotic assay offers several advantages: (*a*) it is a homogeneous material of known structure readily prepared by solid phase synthesis (36); (*b*) it is obtained in crystalline form and hence yields material with reproducible biological activity irrespective of the mode of preparation; (*c*) it is stable upon storage in the solid state

and less readily inactivated in solution than 8-arginine-vasopressin, 8-lysine-vasopressin, or 8-arginine-vasotocin; (*d*) it occupies an intermediate position in the potency spectrum of neurohypophyseal peptides in the toad bladder.

As shown in Fig. 6 and Table II all compounds investigated, except for 2-(*O*-methyltyrosine)-oxytocin, possessed essentially the same intrinsic activity. However, the affinity for the toad bladder receptor varied markedly with different analogues. For example 8-arginine-vasotocin, a natural amphibian neurohypophyseal hormone, possessed the highest affinity, 332-fold greater than that of our reference standard (crystalline deamino-oxytocin). In this context it may be recalled that Jard and Morel (19–21) in their elegant study of agonism and antagonism of neurohypophyseal hormones in the frog kidney (*Rana esculenta* L.) were able to elicit an antidiuretic response with 10^{-11} M 8-arginine-vasotocin, a dosage which approximates that found to be effective in our present in vitro study with the toad bladder.

In an attempt to correlate structural modifications of neurohypophyseal hormones with changes in membrane permeability to water, we focused on amino acid residue substitutions in positions 3 and 8—loci which were particularly susceptible to mutational alterations in the course of evolution (1, 33). The conversion of 8-arginine-vasotocin to 8-arginine-vasopressin brings about a 24-fold decrease in hormone affinity. An analogous replacement in 8-ornithine-vasotocin resulted in a 35-fold decrease in activity. We next explored the effect on transmembrane hydroosmotic flux of replacing the basic amino acid residue in 8-arginine-vasotocin by neutral instead of basic amino acid residues. Thus we determined the potencies of oxytocin and of 8-alanine-oxytocin, an analogue in which the leucine moiety of oxytocin is replaced with an alanine moiety. Both compounds exhibited an activity of comparable magnitude. Furthermore, modification in position 3 of 8-alanine-oxytocin by replacing the isoleucine moiety with a phenylalanine moiety—an alteration analogous to that described for the 8-arginine and 8-ornithine analogues (see above)—results in 8-alanine-oxypressin, a molecule exhibiting a low affinity for the bladder receptor.

The structure-activity relationship of this series of analogues with amino acid replacements in position 3 presents a pattern which shows that an isoleucine side chain in position 3 (as compared with a phenylalanine side chain in this locus) imparts a higher specificity for binding of the hormone molecule to the bladder receptor. A comparison of our results with those of Rasmussen et al. (32), the most extensive attempt to date to define the contribution of a particular group in the natural hormone to biological activity in a toad bladder system, reveals that our present assay method is 100 times as sensitive as that employed in their study.

Previously it was shown (38) that structural alterations in position 5 of neurohypophyseal hormones drastically diminished their biological activities

in the avian vasodepressor, rat pressor, rat uterus, and rat antidiuretic assays. A representative of this group is 5-valine-oxytocin (39), an analogue in which a valine residue replaces the asparagine residue of oxytocin. The potency of this compound in all assays studied was so low that a test for intrinsic activity has been heretofore precluded by excessive material requirements. Hence it appeared desirable to study 5-valine-oxytocin with respect to its affinity and intrinsic activity in the toad bladder assay. It is of interest, that this analogue possesses, in spite of an extremely low affinity, an intrinsic activity equal to that of deamino-oxytocin.

In view of the difficulties previously encountered in characterizing the amphibian hydroosmotic activity of 2-(*O*-methyltyrosine)-oxytocin (32), we reinvestigated this problem and established that the intrinsic activity of this analogue was only 88% and its affinity 6.8% that of the standard. Also in other assay preparations, viz. uterotonic assays (27), a diminished maximal response in cumulative dose studies was reported previously for this compound under a variety of experimental conditions. Furthermore, a comparison of the hormonal octapeptides with their corresponding deamino-analogues indicates that the replacement of the free amino group in position 1 by hydrogen lowers the potency of these analogues in the toad bladder preparation. The opposite effect of this replacement—namely, enhancement of potency—has been abundantly demonstrated in the rat antidiuretic, rat uterotonic, and fowl vasodepressor assay systems (6, 34).

In summary, a systematic investigation of factors influencing bladder reactivity has led to the development of a convenient, sensitive, and precise assay method for measuring transmembrane hydroosmotic flux. This method was used to gain insight into drug-receptor interactions by evaluating dose-response and time-response relationships. In this study we have probed physiological properties of the receptor elements of a neurohypophyseal hormonal target tissue employing as agonists a series of analogues, with chemical modifications in nonpolar, polar, and formally charged groupings.

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