Structural Requirements for the Action of Neurohypophyseal Hormones upon the Isolated Amphibian Urinary Bladder

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ABSTRACT The response of the isolated amphibian urinary bladder to thirty-four structural analogs of arginine vasotocin was determined in an effort to define the physiological significance of specific structural groups on the hormone molecule. All but one of the analogs tested possessed full intrinsic activity in this system but varied greatly in their affinity for the receptor site. An analysis of the effect of changes in hydrogen ion concentration upon the response of the bladder to oxytocin was performed in order to determine the number and nature of the ionizable groups involved in hormone receptor interaction. Two ionizable groups with apparent pK's of 7.1 and 7.75 were found to be important in determining the magnitude of the hormonal response. On the basis of the results it was postulated that hormone-receptor interaction can be considered a two-step process: (*a*) The binding or attachment of hormone to receptor site through ionic, hydrogen, and hydrophobic bonds and (*b*) a disulfide interchange reaction between hormonal disulfide and receptor sulfhydryl. The latter step is considered to be the reaction which initiates the chain of events leading to the observed change in permeability.

Although the distinction between the affinity and intrinsic activity of a drug for its receptor site has received theoretical consideration and application in pharmacology (1), little usefulness has been found for these concepts in the study of hormone-receptor interactions. However, because of the recent developments in the isolation, characterization, and synthesis of polypeptide hormones, they may become of practical value in the study of hormone-receptor interactions. The classic work of du Vigneaud and his colleagues on the neurohypophyseal hormone (2–7) has evoked new and exciting possibilities in the study of the correlation between structure and activity of

polypeptide hormones. A great deal of effort has already been expended in the preparation and biological testing of synthetic analogs of these hormones (2–14). In general these studies have been confined to an investigation of the comparative activities of a new analog in a number of different biological test systems. From work of this kind it has been possible to conclude that a particular amino acid in a given position is more important for one type of biological activity than for another. However, there have been few studies concerning the effect of a given structural change upon the affinity and intrinsic activity of the hormone for a single receptor site. Undoubtedly this can be attributed in part to the fact that a number of the biological assay methods for these hormones employ intact organisms and as such are unsuitable (14). However, in recent years several *in vitro* assay systems have been developed which should be suitable (14). Obviously to be most useful the response of the given tissue should be specific, reproducible, and readily quantitated. Perhaps of all the isolated systems which respond to these hormones, the amphibian urinary bladder most nearly approaches the ideal for a system amenable to this type of analysis. From the work of Leaf (15), Bentley (16), Sawyer (17), and Schwartz *et al.* (18), it is apparent that these hormones produce two characteristic responses in this tissue and that relatively few other agents mimic these responses. Of the two responses the most reproducible and more easily quantitated is the change in bulk flow of water produced when the two surfaces of the bladder are exposed to solutions of different total solute concentrations. This response has been employed by a number of investigators. It has the advantage of accuracy, simplicity, and, if certain conditions are controlled, of reproducibility. It was selected therefore as particularly suitable for a study of the effect of alterations in hormone structure upon the affinity and intrinsic activity of the hormone molecule.

In considering the interaction of a hormone molecule with its receptor it is axiomatic that some type of chemical interaction must occur. The five natural neurohypophyseal hormones (oxytocin, arginine and lysine vasopressin, isotocin, and arginine vasotocin) all possess three striking structural features: (*a*) a 20-membered ring closed by a disulfide bond; (*b*) charged groups consisting of an amino group on the amino acid in position 1, a phenolic hydroxyl on the amino acid side chain in position 2, and in all but oxytocin and isotocin, a basic group on the amino acid in position 8; and (*c*) a hydrophobic or non-polar region in position 3, 4, and 5. In a previous study (18), these hormones were labeled with tritium and then employed to determine the nature of binding between hormone and receptor. In brief, evidence was found for the formation of a covalent chemical bond which was considered to be disulfide in nature.

Granting that such a bond is actually formed, it could function in one of two ways: either to attach the hormone to receptor or to initiate hormonal

action. Thus one could envision two possible sequences of the chemical events involved in hormone-receptor interaction. On the one hand, the initial fixation of hormone to receptor could be brought about by linkages other than disulfide bonds, specifically ionic, hydrogen, and hydrophobic bonds; and the initiation of hormone action then induced by disulfide bond formation. On the other hand, disulfide bond formation might account only for initial fixation with some other type of chemical interaction being responsible for the initiation of hormone action. Obviously, a given change in hormone structure might effect one or the other of these two steps, but it might be possible on the basis of a quantitative analysis of hormone-receptor interaction to decide between the two alternatives.

It is the purpose of this report to describe the results of a study on the response of the isolated amphibian urinary bladder to thirty-four different natural and synthetic neurohypophyseal analogs, and to changes in hydrogen ion concentration.

Theoretical Considerations

Before discussing the experimental techniques, the theoretical foundations upon which this study was initiated will be summarized.

One current model of hormone action is that the interaction of a hormone (h) with its receptor (R) leads to the formation of a complex (Rh) which in turn initiates a chain of reactions leading to the observable physiologic activity (ap).

$$h + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Rh \longrightarrow ap$$

It must be assumed that the response is some function of the concentration of the hormone-receptor complex and furthermore that the concentration of this complex is the rate-limiting factor in the sequence of reactions from hormone-receptor interaction to physiological response. This latter is undoubtedly true for small concentrations of hormone but it is not yet possible to prove its validity at high hormone levels. In addition the assumption is made that the receptors represent a homogeneous population.

With these few assumptions the situation is analogous to that of enzyme-substrate interaction or drug-receptor interaction where

R = free receptor concentration
r = total receptor concentration
Rh = concentration of complex
h = concentration of hormone
k_1 = rate of association of R and h
k_2 = rate of dissociation of Rh

α = proportionality constant defined by $\frac{ap}{Rh}$

ap = physiological activity, that is, the measured response

Ap = maximal physiological activity

$K_h = \frac{k_2}{k_1}$

$K_H = \frac{1}{K_h}$ and is a measure of the affinity of the particular analog for the receptor.

Then it can be shown that

$$ap = \frac{\alpha(r)(h)}{\frac{k_2}{k_1} + h}$$

and $Ap = \alpha r$ so that

$$ap = \frac{A_p}{\frac{K_h}{(h)} + 1} \tag{1}$$

an expression analogous to the Michaelis-Menten expression for enzyme-substrate interaction. In this particular formulation the hormone-receptor complex is not considered as breaking down into an active complex, but if (Rh) did break down then the situation would be completely analogous to the enzyme-substrate interaction and α would be replaced by k_3. In either case the type of response curve would be similar, but in the latter case a continuous supply of hormone would be required.

In a study in which there is a wide range of affinities for the receptor site, it is most useful to plot the log-dose hormone concentration against physiological response. The log expression of equation (1) rearranged gives:

$$\log (h) = \log K_h + \log \frac{ap}{Ap - ap} \tag{2}$$

which is an expression of the usual log-dose response (Fig. 1).

Comparison of the response of two hormone analogs should aid in distinguishing between a change in affinity or intrinsic activity produced by a given structural alteration. Affinity is indicated by the reciprocal of K_h, K_H, which is a measure of how much hormone-receptor complex will be formed for a given analog at a given concentration; and intrinsic activity is indicated by α which signifies the effectiveness of a given concentration of hormone-receptor complex, and thus determines the maximal physiological activity (Ap) obtainable with a given hormonal analog. The effects expected from structurally induced changes in affinity or intrinsic activity are illustrated in

Fig. 1. A change in affinity shifts the curve along the dose axis without altering the maximal obtainable response whereas a change in intrinsic activity alters the maximal response.

This approach to hormone-receptor interaction can be extended to a consideration of competitive and non-competitive inhibition and other special circumstances such as the influence of pH change upon hormone action.

It remains only to point out that a change in affinity implies that the catalytic function or active center of the hormone molecule has not been influenced by the given structural change, whereas a change in intrinsic activity suggests an alteration in the active center of catalytic groups of the hormone molecule. However, it is possible to envision situations in which structural alterations not involving the active center could lead to a decrease of intrinsic activity by changing the orientation of the hormone with its receptor site. Hence the

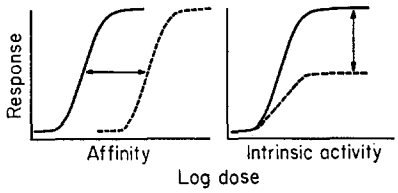


FIGURE 1. The left hand portion of the figure illustrates the hypothetical effect of altering the affinity of a hormone for its receptor site without modifying its intrinsic activity. Conversely, on the right is illustrated the effect of so modifying hormone structure as to alter its intrinsic activity.

interpretations of changes in intrinsic activity are open to more ambiguity than are those in affinity.

MATERIALS AND METHODS

The urinary bladders obtained from toads, *Bufo marinus*, weighing 120 to 220 gm were employed for assessing hormonal activity by the technique of Bentley (16), with only minor modifications. The toad bladder was mounted, serosal side out, in 20 ml of amphibian Ringer's (220 mOsM/liter) and contained 3 ml of hypotonic Ringer's (50 mOsM/liter). Weight loss was measured every 20 minutes. At the end of each 20 minute period the inside and outside solutions were changed. For the study of the various hormonal analogs the pH was maintained at 7.4. For each dosage level of each analog, six separate half-bladders were used. Observations with the same doses of hormone were repeated with a second batch of bladders. After two 20 minute control periods, the appropriate dose of analog was added to the serosal bath. Weight loss was measured for three more 20 minute periods. The rates of weight loss (milligrams per minute) for the second and third 20 minute periods were recorded, and the mean of the twenty-four values obtained (two values from each of twelve half-bladders). The usual variation calculated as the standard error of the mean was 6 to 8 per cent at

low response values, and 12 to 15 per cent at high response values. The mean values for different dosages of each analog were used to plot the log-dose response. To normalize the values obtained on different bladders at different times of the year, the response of a given set of bladders to a standard preparation of oxytocin was used. The maximal response of various bladder sets to oxytocin varied from 32 to 38 mg/min. under the conditions of these experiments. The value of 35 mg/min. was chosen as the standard and all data normalized to this value in order to have a more precise comparison of the response of analogs tested at widely different times. The concentration required to produce a half-maximal effect (K_h) was used to compare the effectiveness of the various analogs.

In the case in which the bladders did not respond to large doses of analog, oxytocin was added at the end of the second or third experimental period, and the response followed for an additional one or two periods to determine whether the inactive analog had any inhibitory effect.

The effect of changes in hydrogen ion concentration upon the response of the bladder to a large range of concentrations of oxytocin was studied employing Ringer bicarbonate buffers containing 112 mM Na^+, 4.2 mM K^+, 2 mM Ca^{++}, 2.2 mM phosphate, and 118 mM Cl^- plus HCO_3^-; O_2–5 per cent CO_2 was employed as the gas phase. The pH of a particular buffer was produced by varying the ratio of Cl^- to HCO_3^- without changing their total concentration. A dose-response curve for the hormone at pH's 6.2, 6.4, 6.65, 6.9, 7.2, 7.45, 7.8, and 8.0 was determined in a manner similar to that employed in the study of each analog. As an additional means of comparing responsiveness as a function of pH, paired half-bladders were run at two different pH's in each experiment, and in some experiments the response of the same bladders at two different pH's was determined. The response of a given set of bladders at pH 7.45 was employed as the standard and these responses normalized. The responses of bladders studied at other pH values were corrected to this normalized value. These particular studies were carried out before those with the various analogs and were done on bladders obtained from smaller toads (120 to 150 gm as compared to 200 to 250 gm). Only 2 ml of hypotonic Ringer's was added to the bladder rather than 3 ml as in the analog studies. Thus in these experiments the maximal response was 21 to 27 mg/min. rather than 32 to 38 mg/min. as was the case in the analog studies.

In addition to this complete study of the dose response as a function of pH for oxytocin, a less extensive study of the effect of variations in hydrogen ion concentration upon the response of the bladder to a single concentration of the desamino analog of oxytocin was performed.

To determine the effect of reduction of the hormonal disulfide bond upon hormonal activity, 10^{-2} M cysteine was added to the bath 20 minutes before a standard dose of oxytocin. The effects of 10^{-2} M glycine and 10^{-2} M oxidized glutathione were also studied in the same manner. The effect of 10^{-2} M cysteine upon the action of cyclic AMP[1] (3′,5′-adenine mononucleotide) and theophylline was also determined.

A number of other hormones which mimic the action of neurohypophyseal hor-

[1] Abbreviations, cyclic AMP, 3′,5′-adenine mononucleotide; AVT, arginine vasotocin; LVP, lysine vasopressin; AVP, arginine vasopressin.

mones upon certain receptor tissues, such as the uterus or small blood vessels, were tested for their effect on the bladder. These were angiotensin, serotonin, epinephrine, insulin, acetylcholine, bradykinin, glucagon, and parathyroid hormone.

The natural and synthetic analogs were obtained from several sources. Professor Vincent du Vigneaud of Cornell University supplied us with synthetic arginine vasotocin, lysine vasotocin, the ring amide of oxytocin, sarcosine oxytocin, 2-phenylalanine oxytocin, desamino oxytocin, desamino-desoxy-oxytocin, 2-*O*-methyl oxytocin, desamino lysine vasopressin, 1-(hemi-*d*-cystine)-oxytocin, and 1-(hemi-homocystine)-oxytocin. Natural arginine vasotocin, oxytocin, arginine vasopressin, and lysine vasopressin were prepared in our laboratories. Dr. R. Boissonnas and Dr. H. Schwarz of Sandoz Inc. supplied synthetic oxytocin, synthetic lysine vasopressin, synthetic arginine vasopressin, 2-phenylalanine lysine vasopressin, 3-phenylalanine oxytocin, 8-isoleucine oxytocin, 3-valine oxytocin, isotocin, 4-asparagine oxytocin, and bradykinin. 2-Serine lysine vasopressin and the tyrosine homologs of lysine vasopressin

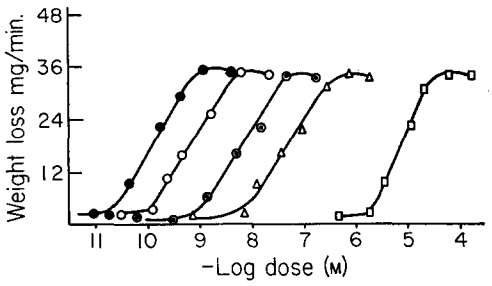


FIGURE 2. The log-dose response of the amphibian urinary bladder to five different neurohypophyseal analogs: argine vasotocin (●), lysine vasotocin (○), oxytocin (⊙), lysine vasopressin (△), and *N*-sarcosyl oxytocin (□).

were obtained from Professor Beyerman of Delft and N. V. Organon of Oss, Holland. Professor Joseph Rudinger of the Czechoslovak Academy of Science prepared the 3-leucine- , 3-alloisoleucine- , 3-norleucine- , 3-valine- , 1-(hemi-*d*-cystine)- , 3-norvaline- , 1-*N* methyl- , 2-*O*-methyl- , 2-leucine- , 1-*N*-sarcosyl- , 1-*N*-leucyl-glycyl-glycyl- and 1-*N*-glycyl oxytocin. Dr. Joseph Freid of E. R. Squibb and Sons donated the 8-citrulline oxytocin and 8-citrulline vasopressin. Angiotensin II was supplied by Dr. F. Merlin Bumpus, and serotonin by Dr. D. W. Woolley.

RESULTS

The usual weight loss during the control periods was 0.5 to 1.0 mg/min., and the maximal response of individual bladders ranged from 30 to 40 mg/min. Between these extremes there was a linear relationship between the log-dose of active analog and the response. The log-dose responses for five different analogs are shown in Fig. 2. It is apparent that the response obtained with the different analogs differed only with respect to the dose required to obtain a given level of response. All the active analogs gave a comparable qualitative response indicating that none of the structural alterations produced a loss of intrinsic activity but influenced only the affinity of a particular analog for the receptor. The effectiveness of the various analogs is compared in Table I

TABLE I

RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND BIOLOGIC ACTIVITY OF ANALOGS OF ARGININE VASOTOCIN UPON THE AMPHIBIAN URINARY BLADDER

Cy-S — Tyr — Ileu — Glu(NH_2) — Asp(NH_2) — CyS — Pro — Arg — Gly(NH_2)
1 2 3 4 5 6 7 8 9

Preparation	Substitution	Concentration for half-maximal effect (10^{-9} M)	K_H	AVT*
				per cent
Arginine vasotocin‡	None	0.04	2.5×10^9	100
Lysine vasotocin	8-lys	0.16	6.2×10^8	25
8-Isoleucine oxytocin	8-ileu	0.50	2×10^8	8
8-Citrulline oxytocin	8-citr	0.60	1.7×10^8	6.6
Oxytocin	8-leu	1.00	1×10^8	4.0
4-Asparagine oxytocin	4-asp(NH_2), 8-leu	1.00	1×10^8	4.0
Sarcosine oxytocin	8-leu, 9-sar	1.60	6.3×10^7	2.5
Isotocin	4-ser, 8-ileu	2.50	4×10^7	1.6
Arginine vasopressin	3-phe	3.15	3.2×10^7	1.3
Desamino oxytocin	(-1, NH_2), 8-leu	8.80	1.1×10^7	0.45
Lysine vasopressin	3-phe, 8-lys	10.00	1×10^7	0.40
3-Alloisoleucine oxytocin	3-alloileu, 8-leu	12.00	8.3×10^6	0.34
Oxytocin ring amide	6-NH_2 (-7, 8, 9)	14.00	7.2×10^6	0.28
3-Leucine oxytocin	3-leu, 8-leu	17.00	6×10^6	0.24
8-Citrulline vasopressin	3-phe, 8-citr	25.00	4×10^6	0.16
3-Valine oxytocin§	3-val, 8-leu	33.50	3×10^6	0.12
2-Phenylalanine oxytocin	2-phe, 8-leu	41.60	2.4×10^6	0.098
2-*O*-Methyl oxytocin‖	2-*O*-Me, 8-leu	47.00	2.1×10^6	0.085
3-Norvaline oxytocin	3-norval, 8-leu	83.00	1.2×10^6	0.048
Desamino lysine vasopressin¶	(-1, NH_2), 3-phe, 8-lys	118.00	8.5×10^5	0.036
1-*N*-Methyl oxytocin	1-*N*-Me, 8-leu	121.00	8.3×10^5	0.083
Desamino-desoxy-oxytocin	(-1, NH_2), 2-phe, 8-leu	200.00	5×10^5	0.020
1-(hemi-*d*-cystine) oxytocin‖	1-*d*-cysteine, 8-leu	237.00	4.2×10^5	0.017
1-(hemi-*d*-cystine) oxytocin**	1-*d*-cysteine, 8-leu	250.00	4×10^5	0.016
1-(hemi-homocystine) oxytocin	1-homocysteine, 8-leu	270.00	3.6×10^5	0.015

* K_H is the reciprocal of the concentration of hormone needed to produce a half-maximal response and is a measure of the affinity. Per cent AVT refers to the potency of the particular analog in reference to that of arginine vasotocin.

‡ Synthetic and natural preparations of arginine vasotocin, arginine vasopressin, lysine vasopressin, and oxytocin gave comparable activities.

§ Samples of this analog obtained from Sandoz, Inc., and from Professor Rudinger had similar potencies.

‖ Obtained from Professor Rudinger.

¶ Relative estimate because of insufficient material to do a complete analysis.

** Obtained from Professor du Vigneaud.

‡‡ Substitution of an additional tyrosine in the ring between positions 1 and 2.

TABLE I—*Concluded*

Preparation	Substitution	Concentration for half-maximal effect (10^{-6} M)	K_H	AVT*
				per cent
3-Norleucine oxytocin	3-norleu, 8-leu	307.00	3.2×10^5	0.013
3-Phenylalanine oxytocin	3-phe, 8-leu	315.00	3.1×10^5	0.012
2-Phenylalanine lysine vasopressin	2-phe, 3-phe, 8-lys	500.00	2×10^5	0.0080
1-*N*-Glycyl oxytocin	1-*N*-gly, 8-leu	968.00	1×10^5	0.0043
1-*N*-Sarcosyl oxytocin	1-*N*-sar, 8-leu	1,000.00	1×10^5	0.0040
1*a*-Tyrosine lysine vasopressin‡‡	1*a*-tyr, 3-phe, 8-lys	1,000.00	1×10^5	0.0040
1-*N*-Leu-cylglycylglycyl oxytocin	1-*N*-(leu-gly-gly), 8-leu	1,380.00	7.2×10^4	0.0031
2-Leucine oxytocin	2-leu, 8-leu	5,440.00	1.8×10^4	0.00074
2-Serine lysine vasopressin	2-ser, 3-phe, 8-lys	—	—	Inactive

where (K_H) for each analog is recorded. The potency of the analog is compared to that of arginine vasotocin (AVT). All but one of the analogs possessed biological activity. The inactive analog was 2-serine lysine vasopressin which had neither direct nor inhibitory activity at the highest dose tested (5×10^{-5} M). Its lack of activity may have been due to the lack of sufficient material to test at higher doses.

One other analog, 2-*O*-methyl oxytocin prepared by du Vigneaud, was found on repeated testing to exhibit an unusual activity. It was inactive at low doses, gave a small effect at moderate doses (1×10^{-7} M) but no effect at higher doses. The higher doses did not act as an inhibitor of oxytocin. However, the same analog prepared by Rudinger and coworkers did possess full activity at high dosage. There is at present no explanation for this difference.

A number of substances besides oxytocin have an "oxytocic" effect upon the isolated uterus. Some of these were tested for their effect upon the isolated bladder. As seen in Table II, angiotensin II, which on a weight basis has one-eigth of the oxytocic activity of oxytocin on the rat uterus (19), had no effect on the isolated bladder even at a dose 2,500 times that of oxytocin (Table I) required to produce a half-maximal effect. Similarly, serotonin, bradykinin, epinephrine, acetylcholine, and insulin had no action on the bladder (Table II) whereas theophylline and 3′,5′ cyclic AMP did possess activity, as previously demonstrated by Orloff and Handler (20).

The results shown in Fig. 2 indicate that in all likelihood the various analogs are acting in a similar manner upon the same receptor sites. To test this supposition further, the effects of submaximal and just maximal doses of two different analogs were studied. The results with the submaximal doses were additive, and the response with just maximal doses of two analogs was no greater than with either alone.

The effect of alteration of the disulfide bond upon hormonal activity was determined by adding 10^{-2} M cysteine (pH 7.4) to the serosal bath. Under these circumstances, a dose of either oxytocin, lysine vasopressin, valyl oxytocin, or desamino oxytocin, five times greater than that sufficient to produce a maximal response under normal conditions, caused no change in permeability (Table III). Furthermore, if cysteine were added after the hormone began to exert its action, the hormonal effect was reversed immediately, the bladder returning to its original state of relative impermeability. This effect

TABLE II

EFFECT OF NON-NEUROHYPOPHYSEAL AGENTS UPON THE PERMEABILITY OF THE AMPHIBIAN URINARY BLADDER

Preparation	Concentration 10^{-8} M	Response
		mg/min.
Angiotensin II	2.5	0
Insulin	100.0	0
Parathyroid hormone	50.0	0
Glucagon	10.0	0
Bradykinin	10.0	0
Serotonin	100.0	0
Epinephrine	100.0	0
Acetylcholine	100.0	0
Theophylline	1,000.0	15.2
Caffeine	1,000.0	14.7
3′,5′-AMP	400.0	18.2

TABLE III

THE EFFECT OF CYSTEINE AND GLYCINE 10^{-2} M UPON THE RESPONSE OF THE AMPHIBIAN URINARY BLADDER TO VARIOUS AGENTS

Agent	Concentration	Glycine 10^{-2} M	Cysteine 10^{-2} M	Rate of weight loss
				mg/min.
Oxytocin	1×10^{-8} M	−	−	22
Oxytocin	1×10^{-8} M	+	−	19
Oxytocin	5×10^{-8} M	−	+	1.3
Cyclic AMP	5×10^{-3} M	−	−	16
Cyclic AMP	5×10^{-3} M	+	−	13
Cyclic AMP	5×10^{-3} M	−	+	15
Theophylline	5×10^{-2} M	−	−	23
Theophylline	5×10^{-2} M	+	−	26
Theophylline	5×10^{-2} M	−	+	21
None	—	−	−	1.5
None	—	+	−	0.9
None	—	−	+	1.2

of cysteine was completely reversible. When the cysteine was washed out of the serosal bath, the bladder would then respond normally to hormone. Glycine and oxidized glutathione at the same concentrations had no effect upon hormonal action. Both 5×10^{-3} M 3′,5′ cyclic AMP and 5×10^{-2} M theophylline (which mimic hormone action) induced a characteristic permeability change in the presence of 10^{-2} M cysteine (Table III) indicating that the cysteine had not inhibited cellular processes responsible for change in membrane permeability.

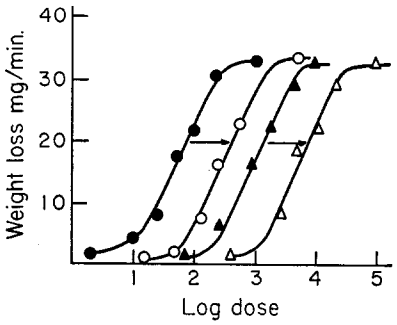
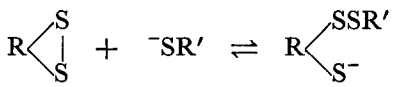


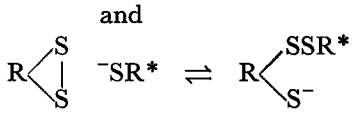
FIGURE 3. The effect of 5×10^{-3} M cysteine upon the log-dose response to two different analogs: 8-citrulline oxytocin and lysine vasopressin. The closed circles (●) and the closed triangles (▲) show the log-dose response to citrulline oxytocin and lysine vasopressin, respectively, in the absence of cysteine, and the open circles (○) and open triangles (△) in its presence.

The effect of 5×10^{-3} M cysteine upon the log-dose response to two different analogs with different affinities, lysine vasopressin and 8-citrulline oxytocin, was studied. The results are shown in Fig. 3. This dose of cysteine shifted the log-dose response of each analog to a comparable degree along the dose axis. These findings do not exclude the disulfide bridge as the active center because they may be explained in terms of the equilibrium conditions of the reaction between the hormone analog and cysteine. In fact, these findings are to be expected if it is assumed (*a*) that the equilibrium constant of the reaction between hormone (analog) and receptor is the same in the presence or absence of cysteine, (*b*) that only hormone molecules in the disulfide form are active, (*c*) that reaction of cysteine with hormone reaches equilibrium rapidly under these conditions, and (*d*) that the concentration of disulfide-containing (native) analog is an inverse function of the cysteine concentration. In other words if in the case of either analog, there are two competing reactions in the presence of cysteine:



Hormone Receptor

and



Cysteine

At these low concentrations of hormone there would not appear to be significant further sulfhydryl-disulfide interchange reactions between hormone and hormone-cysteine complexes.

The rate of weight loss *vs.* concentration of oxytocin as a function of pH is plotted in Fig. 4. It is apparent that there is a decreased response with increasing hydrogen ion concentration, and the most striking effect is upon the maximal obtainable response. This is more clearly shown in Fig. 5 where the

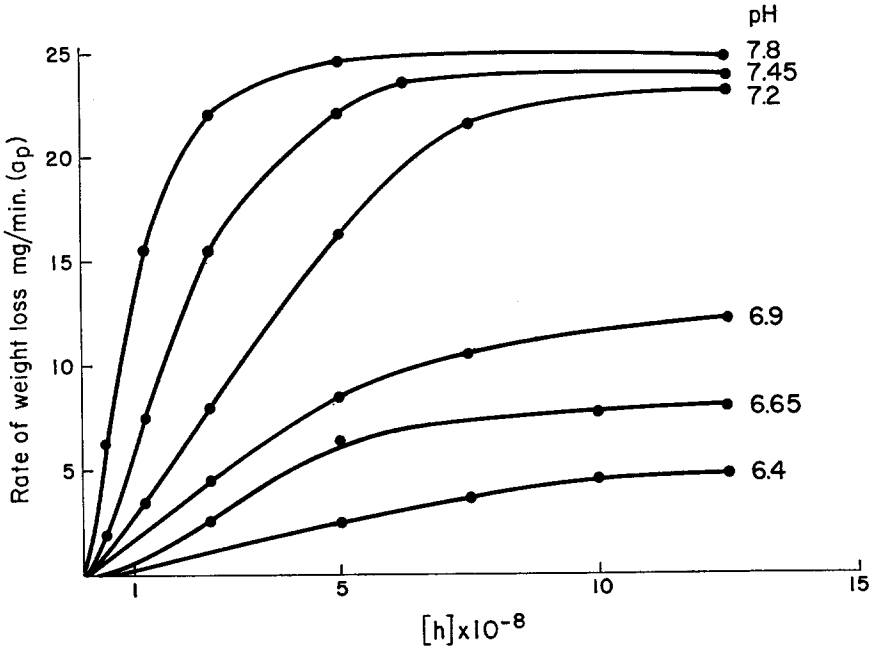


FIGURE 4. The rate of weight loss *vs.* oxytocin concentration as a function of pH. At pH 6.4, 6.65, and 6.9 the response was also measured at higher hormone concentrations to ensure that the maximal response was obtained. These data are not plotted in this figure, nor are the results obtained at pH 8.0 which were nearly identical to those at pH 7.8.

log of the maximal physiological activity (Ap) is plotted as a function of pH. From the data recorded in Fig. 4 the negative log of hormone concentration (ph) was plotted against the rate of weight loss; and from this plot, the apparent pK_h at each pH determined graphically. The pK_h was plotted as a function of pH as was the log of the response (a_p) produced by a small dose (1×10^{-8} M) of hormone (Fig. 5). It was apparent that there are two ionizable groups of importance in determining the bladder response to hormone. Applying the rules worked out for enzyme-substrate interactions (21), the group with the apparent pK of 7.75 is either on the free hormone or free receptor whereas the one with an apparent pK of 7.1 is either on the hormone-receptor

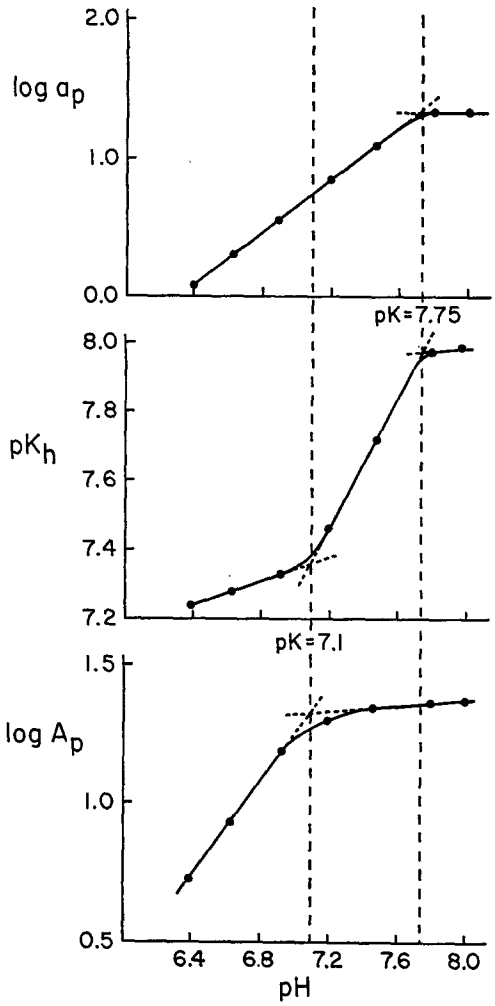


FIGURE 5. A plot of log ap, pK_h, and log Ap as a function of pH for the response of the bladder to oxytocin.

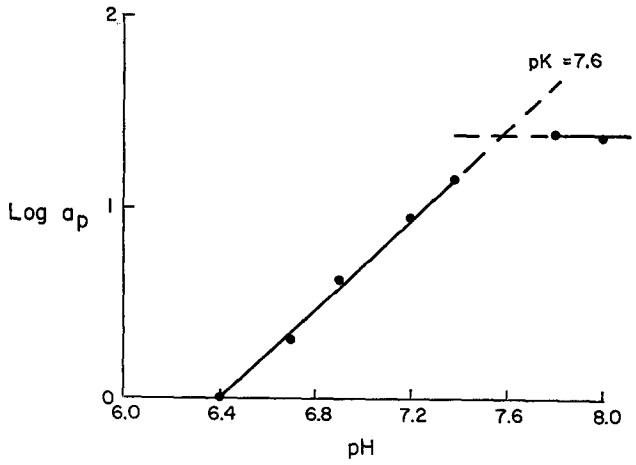


FIGURE 6. A plot of log ap *vs.* pH for 8×10^{-8} M desamino oxytocin. Compare with the upper portion of Fig. 5.

complex or is involved at some subsequent step in the sequence of reactions leading to the physiologic response.

The only group on the free hormone which could have an apparent pK of 7.75 is the α-amino group although the ionization constant for this group is usually somewhat lower. It seems unlikely that this is the group involved because it is not essential for the activity of the hormone (Table I) and because a plot of the log of the physiological response (ap) against pH for the desamino analog is nearly identical (Fig. 6) with that for oxytocin (Fig. 5). This indicates that the group is a part of the free receptor.

DISCUSSION

This work extends the observations of Sawyer (17) and Jard, Maetz, and Morel (22) who have studied the relative potencies of some of these analogs upon the isolated frog bladder. There are slight differences in some of their relative values as compared to ours, but no major discrepancy except for the activity of lysine vasotocin. The explanation for our observation of a much higher activity for this analog than that of Jard *et al.* may be the fact that different species of amphibia were employed and/or that the samples of lysine vasotocin were prepared in different laboratories and may not have been comparable. All data on comparative biological activities recorded here and elsewhere must be considered only relative because all these agents are unstable in solution or as dry powder. For instance, we have noted unexplained losses of activity of some analogs following lyophilization or storage. Because of these difficulties, the data on the biological activities of these agents obtained at different times with different hormone preparations in different laboratories cannot be compared in a strict sense.

From the data in Table I it is possible to determine the effect of substitutions in various positions upon hormonal affinity.

Changes in Position 1 Removal of the α-amino group gives an analog with 10 per cent of original affinity; methylation of α-amino group, 0.8 per cent; substitution of D-cysteine or homocysteine for L-cysteine, 0.4 per cent; additional peptide bond on α-amino group, 0.01 per cent or less; increase size of ring by addition of tyrosine between position 1 and 2, 1 per cent.

Changes in Position 2 Removal of hydroxyl group, 2.2 per cent of original affinity; methylation of hydroxyl group, 1.7 per cent; change from phenylalanine to leucine, 0.56 per cent.

Changes in Position 3 Isoleucine to alloisoleucine, 6.2 per cent of original affinity; to leucine, 5.7 per cent; to valine, 2.2 per cent; to phenylalanine, 2.0 per cent; to norvaline, 1.0 per cent and to norleucine, 0.25 per cent.

Changes in Position 4 Glutamine to asparagine, no change in affinity; glutamine to serine, at least 40 per cent of the original affinity.

Changes in Position 6 Amide for tripeptide side chain, 0.28 per cent of original affinity.

Changes in Position 8 Arginine to lysine, 25 per cent of original affinity; to isoleucine, 8.0 per cent; to citrulline, 6.6 per cent; to leucine, 4 per cent.

Changes in Position 9 Glycine to sarcosine, 62 per cent of original affinity.

Although these findings indicate the profound effect which relatively small structural changes produce upon the affinity of neurohypophyseal hormones for this tissue, it is important to recognize that an extremely high degree of chemical purity of each analog is required if the results are to be meaningful. For example, if the 1-(hemi-*d*-cystine)-oxytocin were contaminated with as little as 0.4 per cent of oxytocin then the results obtained could be due to contamination with this material rather than due to the intrinsic biological activity of the (D) isomer. This degree of contamination lies beyond the resolving power of most of our present day separation techniques, therefore the results obtained with the less active analogs must be considered only as the best approximation to the true activity which is currently possible.

The present data (Tables I–III; Figs. 2 and 3) suggest a number of conclusions concerning the structural requirements for hormonal activity on this amphibian membrane. (*a*) The integrity of the disulfide bond appears to be essential for the intrinsic activity of the hormone molecule. The fact that a dose of 10^{-2} M cysteine is required to prevent hormonal action is of interest because, if the postulated receptor sulfhydryl group actually exists, then it has a reactivity toward the hormonal disulfide at least 10^6 times greater than the sulfhydryl of free cysteine. (*b*) The size of the disulfide closed ring is not critical because both hemi-homocysteine oxytocin and 1*a*-tyrosine lysine vasopressin had full intrinsic activity. (*c*) None of the charged groups on the vasotocin molecule appears essential; the desamino, and 2-phenylalanine analogs were active, as was the ring amide containing neither proline nor arginine. Nor is the character of the non-polar group in position 3 or 4 critical. No analogs with substitutions in position 5 were available for study. Although it is possible to remove the tyrosyl hydroxyl group in position 2 and still preserve intrinsic activity, the substitution of serine for an aromatic amino acid in that position resulted in an inactive analog at least at the dose tested. However, 2-leucine oxytocin had full intrinsic activity despite its extremely low affinity for the receptor site. Thus, the prime requirements for hormonal activity appear to be a 20- to 23-membered ring closed by a disulfide bridge. The aromatic ring and phenolic hydroxyl in position 2 are very important in enhancing the affinity of hormone for its receptor site. Further work is

required to establish whether yet larger or smaller ring structures may possess activity.

It should be noted that the tripeptide side chain, the α-amino nitrogen in position 1, and tyrosyl hydroxyl group in position 2 are not essential for intrinsic activity although they greatly enhance the affinity of hormone for receptor site. In fact, the data suggest that a high degree of molecular complementarity is required between hormone and receptor site in order to retain a high degree of hormone-receptor site affinity. Any change, even the relatively minor one of substituting lysine for arginine in position 8 or alloisoleucine for isoleucine in position 3 results in significant loss of affinity. The latter observations also indicate the important role that steric factors undoubtedly play in the low affinity of a number of the analogs for the receptor site.

The analysis of the effect of hydrogen ion concentration upon hormonal response (Figs. 4–6) indicates that it may be possible to obtain useful information about hormone-receptor interactions by an analysis similar to that employed in the study of enzyme-substrate interactions (21). The data must certainly be interpreted with caution. However, they indicate that there is an ionizable group on the receptor which is important in determining the magnitude of the hormonal response. Clearly they do not unequivocally establish the nature of this group, but are consistent with the previous proposal that the interaction between hormone and bladder involves an SH group on the receptor and the hormonal disulfide bond (18, 23), and would predict that little tritium-labeled hormone would be bound at pH 6.4, a previously established fact (18).

Other peptides (*e.g.*, angiotensin) not possessing disulfide bonds are inactive in this system, but do possess oxytocic activity in other tissues (*e.g.* myometrium) (14). The most likely explanation for this difference is that angiotensin and oxytocin interact with different uterine receptor sites to initiate myometrial contraction.

The S-S bond may be necessary for oxytocin action on the toad bladder but not on the uterus, the nature of the chemical interaction between hormone and receptor sites in these two different tissues being fundamentally different. In this regard, it is noteworthy that the 2,*O*-methyl oxytocin has been shown by Berankova, Rychik, Jost, Rudinger, and Sorm (24) to be a competitive inhibitor of oxytocin on the rat uterus and that 2-leucine oxytocin (25) has a diminished intrinsic activity in this same system. These same two analogs, prepared in the same laboratory, had no such effect upon the amphibian bladder. In fact, both possessed full intrinsic activity at appropriate doses. These data suggest that there is an essential difference in the nature of the chemical interactions responsible for the initiation of hormone action in these two tissues. However, it must be borne in mind that the methyl group on the

oxygen of the tyrosine moiety might interfere with the proper orientation of the disulfide bond and thereby prevent its interaction with a specific group on the receptor site. A difference in receptor site topography might then explain the fact that the same analog can possess full intrinsic activity in one tissue and serve as a competitive inhibitor in another. This striking difference between the response of the amphibian bladder and the uterus to analogs modified in the 2 position certainly deserves further critical study, particularly because the natural hormones for these two tissues (AVT and oxytocin) possess the same ring structure. If hormones and receptor sites possess a high degree of molecular complementarity, it might be expected that the structure of the region of the receptor site complementary to the region of the hormone molecule around the tyrosine in position 2 would be quite similar. If such is the case, then the data suggest that the catalytic step in hormone-receptor interaction is different in the two tissues. There remains, of course, the alternative possibility that the alteration of the hormone molecule in position 8 (outside the ring) sufficiently alters the topography of the molecule around position 2 so that the complementarity at the receptor sites is different in the different tissues. In the latter case, the catalytic step could be the same and the difference in tissue response be due to alterations in orientation of hormone analog to receptor site. The work of Martin and Schild (26) also suggests that the nature of the catalytic event in hormone receptor interaction differs in different tissues for the structurally related neurohypophyseal hormones. They found that thiols prevented the effect of these hormones upon the myometrium but not upon vascular smooth muscle.

It is of interest that insulin, even at high concentration, was ineffective even though it possesses a 20-membered ring similar to that found in the neurohypophyseal hormones. In contrast oxytocin and vasopressin have been found to mimic certain effects of insulin (27, 28). Also some of the actions of insulin can be inhibited and others mimicked by sulfhydryl-blocking reagents (29, 30), all of which points to the possibility that one or more of the disulfide bonds of the insulin molecule may be of functional importance. The lack of insulin effect upon the bladder would not seem compatible with this thesis. However, it is quite possible and even likely that its molecular size and shape are such that its disulfide bond cannot be oriented in a proper manner with reactive groups on the bladder receptor site. Large doses of insulin do not inhibit the response of the bladder to oxytocin suggesting that the affinity of insulin for the bladder receptor sites is of an extremely low order.

In contrast to the high degree of specificity that the neurohypophyseal hormones and insulin possess for their natural receptor sites, they all have a rather similar degree of reactivity with membrane components involved in mitochondrial swelling (31). Furthermore, other non-hormonal agents pos-

sessing either sulfhydryl or disulfide bonds cause a similar response in the mitochondrial system but cannot mimic the natural effects of these hormones (23). In view of these differences and because of the subtle differences in the response of two natural receptors to the same synthetic analog, it seems unlikely that the mitochondrial system will be helpful in defining the sequence of events in the action of these hormones upon their specific target tissues. Although the differences in the behavior of the specific tissue receptor sites and the mitochondrial sites suggest that the general significance and the nature of the chemical interactions are different, the mitochondrial effects are of interest *per se*.

The present data and that previously reported (18, 23) are all consistent with the following hypothesis. The interaction of hormone with bladder receptor site can be considered a two-step process consisting of (*a*) the multipoint attachment of hormone to receptor by ionic, hydrogen, and hydrophobic bonds, and short range forces; and (*b*) the interaction of a receptor sulfhydryl group with the hormonal disulfide bond. Changes in hormonal structure, other than disruption of the disulfide bond, influence primarily the *affinity* of hormone for receptor site (step 1). The disulfide-sulfhydryl interchange (step 2) appears to be responsible for initiating the chain of events leading to the observed physiological effect. There are a number of possible ways in which this latter reaction might induce the tissue response. It could, as previously suggested (18) initiate directly a structural alteration in the mucosal membrane of the bladder by triggering a wave of disulfide-sulfhydryl interchanges. However, if the vasopressin-like actions of cyclic AMP and theophylline (20) on bladder permeability are not adventitious pharmacologic phenomena, this simplest of models is no longer tenable. In any case, it seems likely that there are multiple events between hormone-receptor interaction and the final change in permeability. Only further study can establish the nature of these events, but the role of cyclic AMP certainly deserves critical attention.

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