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^{3}H-Oxytocin Binding Sites in the Isolated Frog Skin Epithelium: Relation to the Physiological Response

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SUMMARY

The uptake of [^{3}H]oxytocin by the isolated frog skin epithelial layer was studied under conditions that permitted simultaneous measurement of the biological response to the labeled hormone. Preliminary experiments showed the incorporation of a small amount of [^{3}H]tyrosine into newly formed proteins since this incorporation can be blocked by first incubating the tissue with unlabeled tyrosine, puromycin, or cycloheximide. All further experiments were performed after the blockade of [^{3}H]tyrosine incorporation. Measurement of [^{3}H]oxytocin uptake as a function of the concentration of the labeled hormone in the medium showed the existence of two sets of binding sites. The sets differed in binding capacity and in affinity for oxytocin and its analogues. The sites with low binding capacity (1–2 pmoles/g) probably correspond to the receptors involved in the biological response (increase in active sodium transport), in accordance with the following criteria: (a) an apparent K value for binding (2.5 nM) identical with that determined from the dose-response relationship obtained for the same preparation; (b) a faster time course for binding than for the biological response; (c) [^{3}H]oxytocin binding in the presence of arginine-8-oxytocin and lysine-vasopressin similar to what might be expected from the relative biological potencies of these two analogues; and (d) parallel inhibition of the binding and biological response by *O*-methyltyrosine-carba-1-oxytocin, a competitive inhibitor of oxytocin on the frog skin epithelium. The second set of sites is characterized by a lower affinity (apparent K value, about 50 nM and a higher binding capacity (about 20 pmoles/g).

After the labeled hormone had been washed from the medium, a significant amount of radioactivity was still present in the tissue, despite the complete reversal of the biological response. This fraction was released when dithiothreitol (10 mM) was added to the incubation medium during the rinsing period; it might correspond to covalent binding of the hormone to the structure through disulfide bonds and be unrelated to the biological effect. This interpretation is supported by the observation that *O*-methyltyrosine-carba-1-oxytocin, an oxytocin competitor lacking the disulfide bond, did not suppress this nonspecific binding of [^{3}H]oxytocin.

INTRODUCTION

The biochemical approach to the mechanism of action of neurohypophysial hormones on frog skin and toad bladder epithelial cells has recently developed on a large scale; however, little is known about the first step of hormonal action on the target tissue, i.e., interaction with specific molecular receptor sites. In a previous paper (1) we reported the results of experiments in which pieces of isolated frog skin epithelium

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were incubated with [^{3}H]oxytocin of high specific activity (2).

Evidence for specific binding of the hormone to the structure was obtained on the basis of the following criteria: saturation as a function of either time or oxytocin concentration, and reduction of binding by oxytocin analogues of high biological activity, but not by analogues devoid of activity. It was suggested that the oxytocin-binding sites observed might be those involved in the onset of the biological response, in spite of the fact that the apparent K value (concentration of hormone giving half the maximal binding) for [^{3}H]oxytocin binding in these experiments (20 nM) was somewhat greater than that previously estimated from dose-response curves (5 nM). Another unexplained observation was the fact that lysine-vasopressin, although poorly active in *Rana esculenta* (3), definitely reduced [^{3}H]-oxytocin binding in some experiments.

These discrepancies led us to re-examine the problem under conditions in which both hormone binding and hormone action could be measured on the same preparation.

MATERIALS AND METHODS

The experiments were performed on isolated skin epithelial layers from frogs (*R. esculenta*) kept in tap water. The epithelium was separated from the underlying corium as follows. The isolated ventral skin (about 40 cm^2) was first fixed over a glass pipe 5 cm in diameter, with the internal surface face downward. The pipe was filled with Ringer's solution (Na^+, 112 mM; K^+, 3.2 mM; Ca^{++}, 1 mM; Cl^-, 119 mM; HCO_3^-, 2.5 mM; pH 8.1) containing 5 mM glucose and 40 units/ml of collagenase, type I. It was immersed in aerated Ringer's solution for 90 min at 35°; a hydrostatic pressure of 15 cm H_2O was applied to the internal surface of the skin. The combined effects of the hydrostatic pressure and collagenase ensured the splitting of the epithelium from the corium.

In most cases about 15 cm^2 of epithelial layer were obtained. Four to six circular pieces (1.3 or 2.5 cm^2) were punched out of the same epithelium. Each piece was spread on nylon mesh, mucosal surface downward, and mounted horizontally between two cylindrical Lucite chambers. The epithelium was bathed on both sides with aerated, buffered Ringer's solution (Tris-HCl, pH 8, 2.5 mM) containing 5 mM glucose. The transepithelial potential difference was continuously recorded through calomel electrodes. The increase in potential difference caused by neurohypophysial hormones was used as a test of the stimulation of active Na^+ transport (natriferic response). After 30 min of incubation in Ringer's solution, the transepithelial potential difference had stabilized and the sensitivity to oxytocin was then checked. For each experiment, we used fragments punched out of the same epithelium and exhibiting similar initial potential differences and responses to a given dose of oxytocin (5 and/or 11 nM). [^{3}H]Oxytocin was then added to the serosal medium, and [^{14}C] inulin was introduced at the same time for the measurement of extracellular spaces. The pieces of epithelium were removed from the chambers either at the peak of the biological response or after washing out of the hormone and the complete reversal of this response. In order to reduce the extracellular radioactivity, the serosal medium was removed and the chamber was quickly rinsed three times with Ringer's solution, each rinse lasting 10 sec. It was established that in the course of this rapid washing procedure most of the [^{14}C]inulin and extracellular [^{3}H]oxytocin disappeared, whereas intracellular or bound ^{3}H radioactivity remained almost unchanged. All tissue samples were blotted on filter paper before counting.

Two alternative experimental procedures were followed. (a) After weighing, the tissue sample was directly solubilized in Soluene (0.5 ml) at 70° for 20 min. (b) The blotted epithelium was frozen in liquid nitrogen, and the resulting solid was pulverized and weighed in tared test tubes. The proteins were then precipitated with trichloracetic acid (10%, 0.3 ml). After centrifugation, the supernatant fluid was separated from the pellet, which then was solubilzed in Soluene (0.5 ml) at 70°.

In both cases the solubilized material was transferred to counting vials containing 8 ml of Bray's scintillation medium (methanol, 100 ml; naphthalene, 60 g; ethylene glycol, 20 ml; 1,4-bis[2-(4-methyl-5-phenyloxazo-

lyl)]benzene, 200 mg; and 2,5-diphenyloxazole, 4 g, in 1000 ml of dioxane). The supernatant solution was counted in the same way after removal of the trichloracetic acid with ether. Aliquots of incubation media and the [^{14}C]insulin and [^{3}H]oxytocin standards were subjected to the same treatment. Control experiments have shown that, when tracer amounts of [^{3}H]oxytocin were added together with trichloracetic acid to frozen, pulverized epithelium, almost all the radioactivity was recovered in the supernatant fraction.

Counting was performed with a Nuclear-Chicago spectrometer under conditions suitable for discriminating between the two isotopes. The total radioactivity counted was corrected for background and incomplete discrimination. The tissue radioactivity content was corrected for extracellular radioactivity and expressed as picomoles per gram, fresh weight.

Tritiated oxytocin. Synthetic oxytocin[1] was tritiated at the tyrosine residue by a modified version (1, 2) of the method described by Agishi and Dingman (4). The labeled product[2] had a specific activity of 24 Ci/mmole. [The peptide concentration was determined according to Lowry *et al.* (5), using pure synthetic oxytocin as the standard.] The biological activity was deduced from avian depressor, uterotonic, and hydrosmotic tests, and amounted to 440 IU/mg of pure peptide. The radiochemical purity was determined by ascending thin-layer chromatography on cellulose plates. Tritiated oxytocin was spotted as a tracer together with 20 nmoles of unlabeled oxytocin as a carrier, and the chromatogram was developed for 15 hr at 4° with butanol–acetic acid–water (4:1:5 by volume). Cellulose powder was scraped off successive strips (width, 1 cm), and the peptide was eluted with acetic acid (0.5%). Radioactivity and avian depressor activity were measured on the successive eluates. Figure 1 reveals the absence of labeled impurities and illustrates the identical behavior of the labeled and unlabeled molecules. The radiochemical purity of the labeled peptide was 100% at the beginning of our experiments (see Fig. 1). It possessed only 80% of its initial biological activity 4 months later, at the end of the experimental period. The tritiated oxytocin was kept at 4° (pH 4.6).

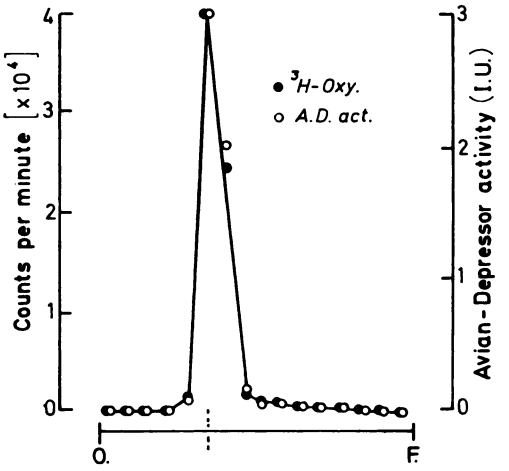

FIG. 1. *Radiochromatogram of* [^{3}H]*oxytocin*
A tracer amount of the tritiated material was spotted together with 20 nmoles of pure, unlabeled oxytocin. ●, ^{3}H radioactivity; ○, avian depressor (A.D.) activity of unlabeled oxytocin (international units).

Other chemicals. The unlabeled peptides were obtained as follows: oxytocin (Syntocinon), lysine-vasopressin, and arginine-8-oxytocin, from Sandoz; and *O*-methyltyrosine-carba-1-oxytocin, as a gift from Drs. S. Jost and T. Barth of the Czechoslovak Academy of Sciences. [*carboxyl*-^{14}C]-Inulin (3.6 mCi/mg) was purchased drom New England Nuclear Corporation; collagenase of *Clostridium histolyticum* (type I), from Sigma Chemical Company; puromycin dihydrochloride (lot 3027), from Nutritional Biochemicals Corporation; cycloheximide (Actidione, U 4527, ST 713, 942 μg/mg), from the Upjohn Company; dithiothreitol (lot 92039), from Calbiochem; and Soluene, from Packard Instrument Company.

RESULTS

Previous experiments (1) showed uptake of radioactivity by tissue incubated in the

[1] Kindly provided by Dr. R.A. Boissonnas, Sandoz, Basel.

[2] We are very much indebted to J. L. Morgat and the Service de Biochimie, C.E.N., Saclay (P. Fromageot), for the tritiation and purification of oxytocin.

presence of [^{3}H]oxytocin. If the tissue radioactivity (in excess of that of the extracellular fluid) measured at the peak of biological oxytocin action corresponds to binding of the labeled hormone molecules to specific receptor sites, this radioactivity uptake should be reversed after removal of the labeled hormone and returning to the biological control level. The reversal of [^{3}H]oxytocin binding to the tissue was studied in preliminary experiments.

Paired epithelium pieces were incubated in the presence of [^{3}H]oxytocin (40 nM). At the peak of the biological response the control fragment was removed for counting as described above. At the same time the labeled hormone was washed from the incubation medium containing the experimental fragment, which was removed after rinsing in fresh Ringer's solution for 1 hr, i.e., after the biological response had been completely reversed. The amount of ^{3}H radioactivity corresponded to 15.1 ± 0.38 pmoles of [^{3}H]oxytocin per gram (n = 3) in the control tissue, and to 9.3 ± 0.6 pmoles/g in the paired, washed tissue.

Under these conditions more than half the radioactivity uptake appeared to be irreversible. This raised the question of oxytocin hydrolysis and [^{3}H]tyrosine incorporation into newly synthetized proteins, and experiments were performed to check this possibility. Paired pieces of epithelial layer were incubated in test media containing either puromycin (200 μg/ml) or unlabeled tyrosine (1 mM); control media contained neither of these compounds. An hour and a half later, both [^{3}H]oxytocin and [^{14}C]tyrosine were added to all the media, up to a final concentration of 40 nM, and after 20 min the radioactivity was washed out and the epithelia were rinsed with normal Ringer's solution for 1 hr. The ^{14}C and ^{3}H radioactivities found in the test pieces were expressed as percentages of those measured in the corresponding control pieces. As shown in Table 1, nearly all ^{14}C uptake by the tissue was inhibited by either puromycin or dilution with nonradioactive tyrosine, indicating that tyrosine incorporation into proteins was efficiently blocked; under the same conditions, a significant amount of ^{3}H radioactivity was still present. Similar results were obtained when cycloheximide (20 μg/ml) was used instead of puromycin to inhibit protein synthesis. These observations suggest that part of the tissue radioactivity uptake which cannot be reversed by washing out the hormone should correspond to covalent binding other than incorporation into proteins.

TABLE 1

Effect of puromycin and unlabeled tyrosine on incorporation of ^{14}C and ^{3}H after incubation with [^{14}C]tyrosine and [^{3}H]oxytocin

Paired fragments of the same epithelial layer were used. Test fragments were treated with puromycin or unlabeled tyrosine for 1.5 hr and then incubated for 20 min in the presence of [^{14}C]tyrosine and [^{3}H]oxytocin (each 40 nM). After rinsing for 1 hr in fresh Ringer's solution, both fragments were removed for radioactivity measurements. Results are the means ± standard deviations of four separate pairs of tissue fragments.

Treatment	Residual radioactivity in test piece	
	[^{14}C]Tyrosine	[^{3}H]Oxytocin
	% control	*% control*
Puromycin (200 μg/ml)	13 ± 4	26 ± 18
Unlabeled tyrosine (1 mM)	2 ± 1	41 ± 13

The existence of irreversible [^{3}H]oxytocin binding to proteins is also shown by the experiments described in Table 2. In this series, tyrosine incorporation into proteins was blocked by previous incubation of the epithelium for 90 min in the presence of cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM). [^{3}H]Oxytocin (11 nM) was then added, and incubation proceeded until the peak of the biological response was reached. One of each pair of epithelium pieces was then quickly rinsed and frozen; the other piece was washed every 5 min with fresh incubation solution, from which only the hormone was omitted, until complete reversal of the biological response. In three experiments, 10 mM dithiothreitol was added to this washing-out solution, and the washed pieces were also frozen. In all these experiments the proteins were precipitated with trichloracetic acid as described under

TABLE 2

Reversibility of [³H]oxytocin binding to proteins after blockade of tyrosine incorporation

Paired fragments were incubated for 90 min in the presence of cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM) [³H]Oxytocin (11 nM) was then added to the incubation medium. Twenty minutes later, the control fragment of each pair was quickly rinsed and frozen. The radioactivity present in the trichloracetic acid pellet was measured as indicated under MATERIALS AND METHODS. The experimental fragment was washed, until reversal of the biological response was complete, in a medium containing either cycloheximide and tyrosine or cycloheximide, tyrosine, and dithiothreitol (10 mM). The radioactivity in the trichloracetic acid pellet was measured as described above.

Treatment	Radioactivity in pellet		Reversibility
	Control	Experimental	
	pmoles/g		%
Cycloheximide + tyrosine	0.59	0.18	68
	0.59	0.16	73
	1.10	0.40	64
	0.64	0.27	58
	1.90	0.36	81
	1.76	0.90	50
	1.26	0.67	47
	1.71	0.95	45
	2.80	1.07	64
	1.58	0.40	75
	1.24	0.63	50
			61 ± 12[a]
Cycloheximide, tyrosine, and dithiothreitol	1.19	0.13	93
	2.20	0.14	94
	1.80	0.10	94
			94 ± 1[a]

[a] Mean ± standard deviation.

MATERIALS AND METHODS, and the radioactivity in the supernatant fractions and pellets was counted separately. For the pieces removed at the peak of the biological response, the radioactivity recovered in the supernatant fluid represented 10–50% of that measured in the pellet. No residual radioactivity was found in the supernatant fraction of the washed pieces, whichever rinsing solution was used. The results obtained for the acid-precipitated protein fraction of both pieces of each pair are listed in Table 2. Despite a large scatter in the data from pair to pair, it may be concluded that when incorporation into proteins is blocked, about 60% of the radioactivity precipitated with the protein fraction at the peak of hormonal action may be removed by washing out the hormone. The greater part of the remaining 40% may be released during the washing-out period if 10 mM dithiothreitol is present in the rinsing solution. This last observation suggests covalent binding of [³H]oxytocin through disulfide bonds.

The time course of cellular uptake of ³H after the addition of labeled oxytocin was studied both with and without inhibition of protein synthesis. In the absence of puromycin (Fig. 2, upper) the tissue uptake of ³H may be divided into two components, one of which increases linearly with time

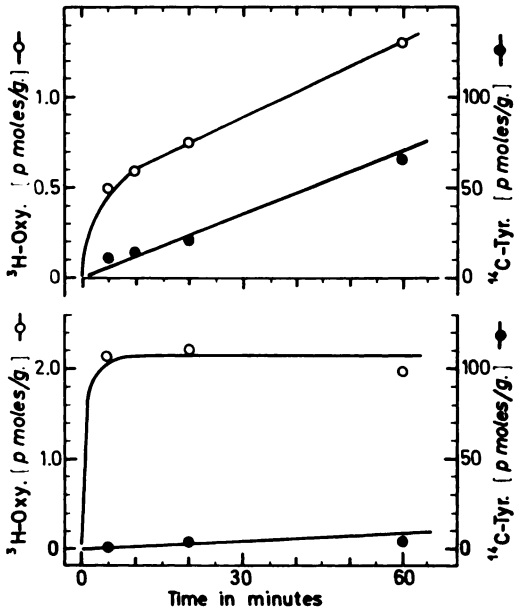

FIG. 2. *Time course of [³H]oxytocin uptake and [¹⁴C]tyrosine incorporation in the presence and absence of puromycin*

For each of the two experiments, several pieces of epithelium from the same animal were used. The preparations were incubated for 90 min in Ringer's solution containing either no addition (upper) or puromycin (200 μg/ml). [³H]Oxytocin (11 nM) and [¹⁴C]tyrosine (11 nM) were added to the internal incubation medium. After 5, 10, 20, and 60 min, fragments were removed and total nonextracellular ³H and ¹⁴C were measured as described under MATERIALS AND METHODS.

and the other of which is rapidly saturated. When puromycin was used the linear component disappeared, as well as the linear incorporation of [^{14}C]tyrosine (Fig. 2, lower). In the latter case, ^{3}H uptake was maximal after 5 min of contact. In all further experiments, the incorporation of radioactivity into proteins was blocked by the addition of inhibitors of protein synthesis and unlabeled tyrosine to the incubation medium. Thus the so-called nonextracellular radioactivity or cellular uptake corresponded to reversibly bound [^{3}H]oxytocin plus labeled oxytocin bound through disulfide bonds. Figure 3 shows the results of an experiment using cycloheximide (20 μg/ml) plus unlabeled tyrosine (1 mM), in which the time courses of radioactivity uptake and the biological response (judged from the change in transepithelial potential difference) were compared after the addition of [^{3}H]oxytocin (11 nM). After 2 min the binding was already 70% of that measured after 20 min. Comparison with the time course of the biological response clearly indicates that binding preceded the onset of the response.

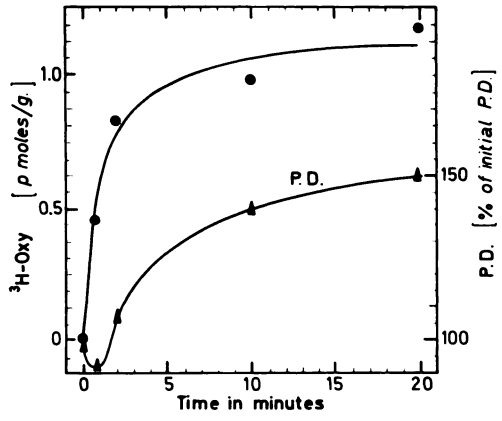

FIG. 3. *Time course of [^{3}H]oxytocin uptake compared with biological response*

Four pieces of the same epithelium were first incubated in the presence of cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM) for 90 min. The transepithelial potential difference (P.D.) was monitored continuously as a function of time. At zero time, [^{3}H]oxytocin (11 nM) was added. The four fragments were removed 0.75, 2, 10, and 20 min later, respectively. Nonextracellular radioactivity was measured as the sum of the activities in the trichloracetic acid pellet and supernatant fraction (see MATERIALS AND METHODS).

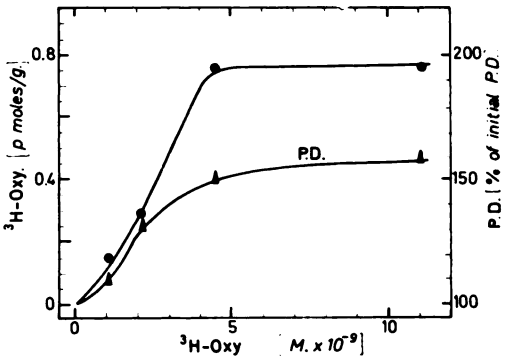

FIG. 4. *Dose dependence of radioactivity uptake and biological response of isolated epithelium incubated with submaximal concentrations of [^{3}H]oxytocin*

Conditions for initial incubation and radioactivity measurement were the same as described for Fig. 3. Different pieces of the same epithelium were incubated in the presence of [^{3}H]oxytocin for 20 min. P.D., potential difference (▲), [^{3}H]-oxytocin uptake (●).

In another series of experiments, binding to the tissue was analyzed as a function of [^{3}H]oxytocin concentration. The doses of labeled hormone were chosen to fall within the range of submaximal biological effects. Incubation was performed in the presence of cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM), and was stopped when the biological response was complete (after about 20 min), assuming, as indicated by Figs. 2 and 3, that the equilibrium state for the biological response corresponded to an equilibrium state for binding. Figure 4 shows that both the radioactivity uptake and biological response were detected from the same threshold concentration, that both curves became saturated above the same concentration, and, finally, that a similar value (about 2.5 nM) was found for the hormonal concentration producing half the maximum biological response and for that producing half the maximum binding of [^{3}H]oxytocin (K value). Comparable observations were made in all these experiments, although somewhat different absolute values were found from frog to frog. In each experiment, K values for binding and biological response fell within the same range (Table 3).

In previous experiments (1) performed

TABLE 3

Comparison of K values determined from dose-response relationships and binding curves

The experiments were similar to those for Fig. 4. The K value was defined as the hormone concentration giving half the maximal biological response (increase in transepithelial potential difference) or half the maximal binding.

Expt.	K values	
	Biological response	Binding of [^{3}H]-oxytocin
	nM	*nM*
1	3.6	3.1
2	2.0	2.6
3	9.7	4.4
4	7.7	5.5

without measuring the biological response to the labeled hormone, S-shaped binding curves were found, with an apparent K value for binding ranging from 20 to 50 nM; these values were higher than those found from dose-response relationships in the frog skin. Since they are also higher than those observed in the isolated epithelium in the present series, further experiments were performed using a wide range of oxytocin concentrations. As exemplified by Fig. 5, the binding curve obtained showed two steps. The first plateau was observed for [^{3}H]oxytocin concentrations ranging from 5 to 20 nM; maximum biological responses were already present with these relatively low concentrations. A second plateau was observed for higher hormone concentration (50–60 nM); both the binding capacity corresponding to this second set of sites (10 pmoles/g or more) and their apparent K value (about 55 nM) lie within the range previously described (1). These experiments show that the frog skin epithelial layer apparently contains two sets of oxytocin-binding sites, which differ both in their binding capacity and in their affinity for the hormone.

In order to test the specificity of oxytocin binding to the high-affinity sites, two series of experiments were conducted. In the first series, [^{3}H]oxytocin uptake was measured in the presence of equimolar amounts of unlabeled peptide; for the test piece of each pair the peptide was either arginine-8-oxytocin or lysine-vasopressin, and for the control piece it was oxytocin. The natriferic activities of these peptides were 1100 units/mg for arginine-8-oxytocin (3), 8 units/mg for lysine-vasopressin (3), and 450 units/mg for oxytocin. After incubation with cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM), each piece was treated with the unlabeled analogue (final concentration, 5.5 nM); 20 min later, when the absence of biological effect (lysine-vasopressin) and the maximum response (oxytocin and arginine-8-oxytocin) had been checked (preparations poorly responsive to oxytocin were discarded), [^{3}H]oxytocin was added to all preparations (final concentration, 5.5 nM). After another 20 min the pieces of epithelium were removed and treated for radioactivity measurement. In order to calculate the amount of oxytocin bound to the tissue, the radioactivity per gram, fresh weight, was divided by the specific activity of oxytocin in the medium (the specific activity of the tritiated hormone for experiments in which arginine-8-oxytocin or lysine-vasopressin was used, and half this specific activity in control

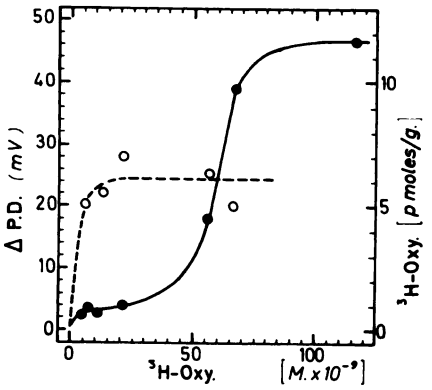

FIG. 5. *Dose dependence of radioactivity uptake and biological response of isolated epithelium incubated with sub- and supramaximal concentrations of [^{3}H]oxytocin*

Pieces of two epithelia exhibiting the same sensitivity to oxytocin were used. Experimental conditions were similar to those of Fig. 4. [^{3}H]Oxytocin uptake (●) was calculated from the sum of radioactivity recovered in the trichloracetic acid pellet and supernatant fraction (see MATERIALS AND METHODS). O, change in potential difference (ΔP.D.).

experiments in order to account for the dilution of [^{3}H]oxytocin with equimolecular amounts of unlabeled oxytocin).

Table 4 gives the results of eight such experiments. Despite some scatter of the data, the amount of oxytocin bound to the controls (0.6–1 6 pmoles/g) fell within the range observed when pure [^{3}H]oxytocin was used in the same final concentration (Table 2); there was no difference in total oxytocin uptake, whether or not the tritiated hormone was diluted with previously added, unlabeled hormone. It may be concluded that free exchange did take place on binding sites between bound, unlabeled molecules and added, free, labeled molecules. When lysine-vasopressin was used, the amount of oxytocin bound to the structure was the same as that of the paired control; in addition, the dose of lysine-vasopressin used did not affect the transepithelial potential difference. When arginine-8-oxytocin was used, the amount of oxytocin bound to the structure was reduced by about half, as compared with the control; moreover, the dose of hormone analogue used produced a maximum increase in the transepithelial potential difference. Thus the biologically active analogue, arginine-8-oxytocin definitely inhibited oxytocin binding, whereas the inactive analogue, lysine-vasopressin, had no effect. These observations suggest that binding of the hormone to high-affinity sites is related to its biological action, although the inhibition observed with arginine-8-oxytocin was less than expected.

TABLE 4

Binding of oxytocin in the presence of lysine-vasopressin or arginine-8-oxytocin

Paired pieces of epithelium were used. Incorporation of [^{3}H]tyrosine was blocked by prior treatment with cycloheximide (20 μg/ml) and prior unlabeled tyrosine (1 mM). Control pieces were incubated for 20 min with oxytocin (5.5 nM), and experimental pieces, with the same concentration of either lysine-vasopressin (experiments 1–4) or arginine-8-oxytocin (experiments 5–8). All preparations were then incubated with [^{3}H]oxytocin for an additional 20 min. Total nonextracellular radioactivity was measured as described under MATERIALS AND METHODS. In the controls that received unlabeled oxytocin, the radioactivity per gram, fresh weight, was divided by the final specific radioactivity of [^{3}H]oxytocin (= half the initial specific activity).

Expt.	Bound radioactivity		Bound oxytocin		Ratio of experimental to control
	Control	Experimental	Control	Experimental	
	cpm/g		*pmoles/g*		
1	2,872	6,485	0.62	0.70	1.10
2	7,133	15,748	1.54	1.70	1.10
3	3,520	4,817	0.76	0.52	0.70
4	2,686	7,133	0.58	0.77	1.35
					1.06 ± 0.27[a]
5	4,076	4,076	0.88	0.44	0.50
6	4,540	4,446	0.98	0.48	0.49
7	7,411	7,596	1.60	0.82	0.51
8	4,446	5,373	0.98	0.58	0.59
					0.52 ± 0.05[a]

[a] Mean ± standard deviation.

In the second series of experiments, *O*-methyltyrosine-carba-1-oxytocin was used. This analogue was shown to have no biological activity per se on the frog skin, and to act as a competitive inhibitor of the oxytocin natriferic effect.[3] The control piece of each experimental pair was treated with [^{3}H]oxytocin at a concentration designed to produce the maximum increase in potential difference; this concentration ranged from 4.4 to 11 nM, depending on each frog's sensitivity to the hormone. The other piece was treated with the same amount of [^{3}H]oxytocin plus a dose of *O*-methyltyrosine-carba-1-oxytocin (1–5 μM) calculated to obtain a concentration ratio ranging from 200 to 900, depending on the experiment. Both pieces of each pair were quickly rinsed 20 min after the addition of the hormone, and the radioactivity was counted. All tissues were incubated in the presence of cycloheximide (20 μg/ml) and unlabeled tyrosine (1 mM).

Both the biological effect of [^{3}H]oxytocin and the tissue radioactivity uptake were reduced in the presence of *O*-methyltyrosine-carba-1-oxytocin as compared to the control; the percentage of inhibition by the analogue was calculated for each effect in every pair, and mean values have been plotted in Fig. 6 against the *O*-methyltyrosine-carba-1-oxytocin to [^{3}H]oxytocin concentration ratios. It

[3] T. Barth, unpublished results.

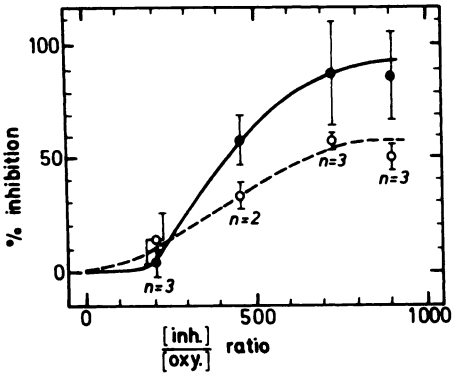

FIG. 6. *Inhibition by O-methyltyrosine-carba-1-oxytocin of radioactivity uptake and biological response to [³H]oxytocin*

Paired pieces of the same epithelium were treated as described in Fig. 4 to inhibit [³H]tyrosine incorporation. The control piece of each pair was treated with [³H]oxytocin at a concentration giving nearly the maximal increase in potential difference. The experimental piece also was treated with various amounts of *O*-methyltyrosine-carba-1-oxytocin simultaneously. Incubation in the presence of the labeled hormone was carried out for 20 min. Total nonextracellular radioactivity was measured as described in the text. For each pair the percentages of inhibition of uptake (○) and biological response (●) were calculated and plotted as a function of the *O*-methyltyrosine-carba-1-oxytocin to oxytocin concentration ratio. Values are means ± standard deviations; *n* = number of experiments.

is clear that both kinds of inhibition increased with the concentration ratio, and that half-maximal inhibition was obtained for a similar inhibitor to oxytocin concentrations ratio (about 400). Despite large variations, inhibition of the biological effect at the highest doses of *O*-methyltyrosine-carba-1-oxytocin used was definitely larger (85–90%) than the corresponding inhibition of radioactivity binding (50–60%). This discrepancy can be quantitatively accounted for if it is assumed that the fraction of tissue radioactivity which could not be released by washing out the hormone corresponds to a disulfide covalent binding process not inhibited by *O*-methyltyrosine-carba-1-oxytocin.

DISCUSSION

The experiments described in this paper were performed at lower [³H]oxytocin concentrations than those previously reported (1). Much of the radioactivity present in the tissue at the peak of the biological response to oxytocin could not be released by washing out the hormone. A large fraction of this irreversible binding corresponds to the incorporation of [³H]tyrosine into newly synthetized proteins, since it may be blocked by adding either inhibitors of protein synthesis or unlabeled tyrosine. Although this incorporation into proteins could be accounted for by less than 0.1% contamination of the [³H]oxytocin with [³H]tyrosine, such contamination appears improbable in view of the procedure used for preparing the labeled hormone. An alternative explanation could be an undetectable degree of hydrolysis of the hormone either by radiolysis or by enzyme degradation during the incubation period. In any case, this cause of error can be almost completely suppressed by using unlabeled tyrosine and/or puromycin or cycloheximide, a procedure which apparently did not modify either hormonal binding to more specific sites or its biological response. However, the radioactivity uptake measured at low [³H]oxytocin concentrations may have represented the sum of at least two different binding processes even when protein synthesis was blocked, since a significant fraction of the radioactivity present in the tissue at the peak of the biological response was not removed by washing with normal Ringer's solution for 1 hr. When 10 mM dithiothreitol was added to the washing solution, the greater part of this bound radioactivity was released, suggesting that some oxytocin was covalently bound to the structure as a result of a disulfide-sulfhydryl exchange reaction between oxytocin molecules and free sulfhydryl groups of proteins. Such an exchange reaction has been suggested by Schwartz *et al.* (6). An important question is whether this reaction is involved in the molecular mechanism of hormonal action. The observation that *O*-methyltyrosine-carba-1-oxytocin (an analogue that lacks the disufide bond) may almost completely suppress the effect of oxytocin by competitive inhibition[3] strongly suggests that rupture of the disulfide-oxytocin bond is not required for its action (7–9). Moreover, only 60% of the bound radioactivity

can be displaced by the analogue, suggesting that the remaining 40% is not related to the mechanism of the hormonal action and could correspond to the disulfide-bound fraction.

The previously described presence in this structure of a set of binding sites for oxytocin with a K value ranging from 10^{-8} to 10^{-7} M has been confirmed. By simultaneously measuring both the biological response to the hormone and its binding to the structure, it has also been possible, to prove that binding of the hormone to these sites cannot be related to its physiological effect, since the full biological response was observed at hormonal concentrations too low to produce any detectable binding to these sites. The possible role of the low-affinity sites is not clear. It is difficult to assume that the large number of these sites could constitute the spare receptors whose existence has been postulated on pharmacological grounds, since spare receptors should not have a different affinity for the hormone from that of other receptors. The existence of spare receptors may simply indicate that incomplete saturation of a single set of receptor sites is sufficient to produce the maximum hormonal responses (10–12).

One could argue that low-affinity sites in the frog skin might be oxytocin receptors whose physiological action is not yet known. However, the low affinity of these sites for both oxytocin and arginine-8-oxytocin (1) makes this assumption improbable, since occupancy of the sites would be nearly zero at arginine-8-oxytocin concentrations measured in the frog's blood under physiological conditions. The over-all saturation curve (Fig. 5) cannot be described simply as the sum of two Langmuir absorption isotherms corresponding to high- and low-affinity receptor sites, as just discussed. It might, however, indicate highly cooperative binding to low-affinity sites.

Interactions of peptidic hormones with two sets of sites—one of high and one of low affinity—were also reported in the action of iodinated adrenocorticotropin on the adrenal gland (13) and in that of [^{3}H]angiotensin on the isolated rabbit aorta (14). Whatever the system studied, it is not yet possible to draw any conclusion about the role of these low-affinity sites.

Binding curves showed two successive saturation plateaus when the hormone concentration in the medium was increased. The bound radioactivity corresponding to the first plateau might partly represent oxytocin molecules bound to specific receptor sites (i.e., sites involved in the initiation of the biological response). Thus these sites proved to be saturable as a function of time, and the time course of binding was faster than that of the biological response. Half-saturation of the sites and half the biological response were obtained at roughly the same oxytocin concentrations. Finally, lysine-vasopressin, which is inactive on the frog skin, did not compete with oxytocin for binding; on the other hand, arginine-8-oxytocin an agonist analogue, definitely reduced [^{3}H]oxytocin binding, although not as much as expected from its high activity.

As far as the high-affinity binding sites are concerned, the very small amount of reversibly bound radioactivity did not make it possible to identify this fraction as unmodified [^{3}H]oxytocin. In any case, the hormone in the medium was not modified by the incubation procedure, as judged from thin-layer chromatography profiles. Taking into account the above limitations, it was not possible to deduce from the binding curves precise values either for the affinity constant of oxytocin for its specific receptor sites (sites binding oxytocin reversibly at low concentrations) or for the binding capacity of these sites. A rough estimate of the K value for the binding of oxytocin to its specific receptor sites is about 2–5 nM. The binding capacity of these sites would be in the range of 0.5–2 pmoles/g. It can be calculated from this last value that if these sites were uniformly distributed over all the epithelial cells, each cell would contain about 1000 sites; if, on the contrary, the location of these sites were restricted to the serosal border in contact with the basement membrane, the density of sites in this membrane would be about $30/\mu^2$.

REFERENCES

1. J. Bockaert, S. Jard, F. Morel and M. Montegut, *Amer. J. Physiol.* **219**, 1514 (1970).
2. J. Morgat, H. Lam Thanh, R. Cardinaud, P. Fromageot, J. Bockaert, M. Imbert and F. Morel, *J. Label. Comp.* **6**, 276 (1970).

3. S. Jard, J. Maetz and F. Morel, *C. R. Hebd. Seances Acad. Sci. Paris* **251**, 788 (1960).
4. Y. Agishi and J. F. Dingman, *Biochem. Biophys. Res. Commun.* **18**, 92 (1965).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
6. I. L. Schwartz, H. Rasmussen, M. A. Schoessler, L. Silver and C. T. O. Fong, *Proc. Nat. Acad. Sci. U. S. A.* **46**, 1288 (1960).
7. S. Hase, T. Morikawa and S. Sakakibara, *Experientia* **25**, 1239 (1969).
8. I. Polacek, I. Krejci, H. Nesvadba and J. Rudinger, *Eur. J. Pharmacol.* **9**, 239 (1970).
9. R. Walter and V. du Vigneaud, *J. Amer. Chem. Soc.* **88**, 1331 (1966).
10. E. J. Ariëns, *Advan. Drug Res.* **3**, 235 (1966).
11. P. Eggena, I. L. Schwartz and R. Walter, *J. Gen. Physiol.* **56**, 250 (1970).
12. I. Krejci, V. Pliska and J. Rudinger, *Brit. J. Pharmacol. Chemother.* **39**, 217 (1970).
13. J. Richard, J. Lefkowitz, J. Roth and I. Pastan, *Science* **170**, 633 (1970).
14. M. Baudouin, P. Mayer and M. Worcel, *Biochem. Biophys. Res. Commun.* **42**, 434 (1971).