

The Effect of Hypertonic Media on Water Permeability of Frog Urinary Bladder

*Inhibition by catecholamines
and prostaglandin E₁*

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ABSTRACT The frog urinary bladder undergoes, in some conditions, a marked increase of its water permeability when incubated in hypertonic media. This increase was observed with various nonpermeant solutes. It seems to result from the shrinkage of an osmo-sensitive compartment of the tissue, probably the epithelial cells. Many similarities were found between this effect and the physiological increase in water permeability (hydrosmotic response) elicited by anti-diuretic hormone (ADH): both were dependent on the physiological state of the animals, and although the response was slower after hyperosmolar than after hormonal challenge, the patterns of response were similar, and in both cases markedly dependent on bathing solution temperature. Norepinephrine and prostaglandin E₁, which in this tissue reduce the hydrosmotic action of ADH, presumably by inhibiting the adenyl cylase also reduced the effect of hyperosmolarity. Conversely this effect was potentiated by incubation in the presence of oxytocin, exogenous cyclic AMP, and theophylline, conditions in which the intracellular concentration of cyclic AMP is increased. These data demonstrate that the response to hyperosmolarity is elicited, at least partly, by mechanisms also involved in the physiological hydrosmotic response to ADH.

INTRODUCTION

It is generally accepted that neurohypophyseal peptides enhance the permeability of a number of epithelial membranes by increasing the intracellular concentration of 3',5'-adenosine-monophosphate (cAMP). This nucleotide would act as a mediator of hormonal action. There is yet no clear knowledge on the hypothetical biochemical steps after cAMP production and on the fundamental molecular changes which must be at the origin of the permeability effects.

Hypertonicity of the incubation medium also alters the permeability prop-

erties of a number of biological membranes (Bentley, 1964; Ussing, 1965, 1966; Brodsky and Schilb, 1965; Earley et al., 1962; Urakabe et al., 1970; Eggena et al., 1969, 1970). The mechanism involved is largely unknown, but the higher efficiency of nonpermeant solutes indicates that a shrinkage of an osmo-sensitive compartment is involved in the epithelial reaction and thus suggests a rather unspecific mechanism of action (Diamond, 1966).

The present experiments were designed to study the relationship that might exist between the increase in the net flux of water observed in the frog urinary bladder under the influence of hypertonic media (Bentley, 1964) and the physiological hydrosmotic (water transfer) action of antidiuretic hormone (ADH) in this tissue. Many similarities were observed in the time-course of the two reactions and, as in the case of the response to the hormone, the response to hypertonic media was found to be highly dependent on the previous physiological state of the animals. Moreover, a strong inhibition of the response to hypertonicity was observed under the action of both catecholamines¹ and prostaglandin E₁, agents known to depress the response to antidiuretic hormone by interfering with the production of cAMP. Conversely it was also demonstrated that the response to hypertonicity is strongly potentiated by ADH, exogenous cAMP and theophylline.

These data strongly support the hypothesis that hypertonic media could act on a biochemical step also involved in the physiological reaction to the hormone, weakening the hypothesis of a nonspecific mechanism.

MATERIAL AND METHODS

Frogs (*Rana esculenta*) originating from central Europe, were purchased from Burgaud, 85-St. Hilaire de Rietz, Vendée, France, and kept at 2°C in running tap water. In some experiments animals were kept in this condition up to the day of the experiment. In other cases, they were transferred in water at 22°C for approximately 1 wk before their use.

The bladders were removed from pithed frogs and mounted between lucite chambers of a two channel device so that an area of 1 cm² was exposed for each hemibladder.

The serosal face was bathed with a Ringer's solution (Na⁺, 114.5 meq; K⁺, 5 meq; Ca⁺⁺, 1 meq; Cl⁻, 119 meq; HCO₃⁻, 2.5 meq; pH 8.1 when bubbled with air). The mucosal side was bathed with the same Ringer's solution except that NaCl concentration was reduced to 5.6 mM. This solution is largely hypotonic so that under all the conditions described in this work, there was a large osmotic gradient across the tissue.

The net flux of water across the bladder was measured by a technique previously described (Bourguet and Jard, 1964) which allows us to record the magnitude of the water movement every minute. Transepithelial potential and short-circuit current were recorded by the technique of Maetz et al. (1959).

The neurohypophysial peptide used was synthetic oxytocin (Syntocinon, Sandoz

¹ A preliminary report on these results was published elsewhere (Ripoche et al., 1969, Parisi et al., 1969).

Pharmaceuticals, Basel, Switzerland). cAMP was purchased from Schwartz Bio Research Inc., Orangeburg, N. Y. and theophylline from Serlaco, Paris.

Commercial preparations of phentolamine (Regitine, CIBA Pharmaceutical Co., Basel, Switzerland), isoproterenol (Isuprel, Winthrop Laboratories, Paris), norepinephrine (Levophed, Winthrop Laboratories), and propranolol (Propanolol, I.C.I., Macclesfield, England) were utilized without further purification. Dichloroisoproterenol was a gift of Expandia, Paris. PGE₁ was a gift of Dr. J. E. Pike, UPJOHN Co., Kalamazoo, Mich.

RESULTS

The incubation in hypertonic media increases the permeability of the isolated bladder of *Rana esculenta* to water and simultaneously promotes a reversible decrease of net sodium transport (Fig. 1), as first reported by Bentley in 1964. These two effects however probably do not share a common mechanism; they are not necessarily linked and their time-course differs widely. In this work, we

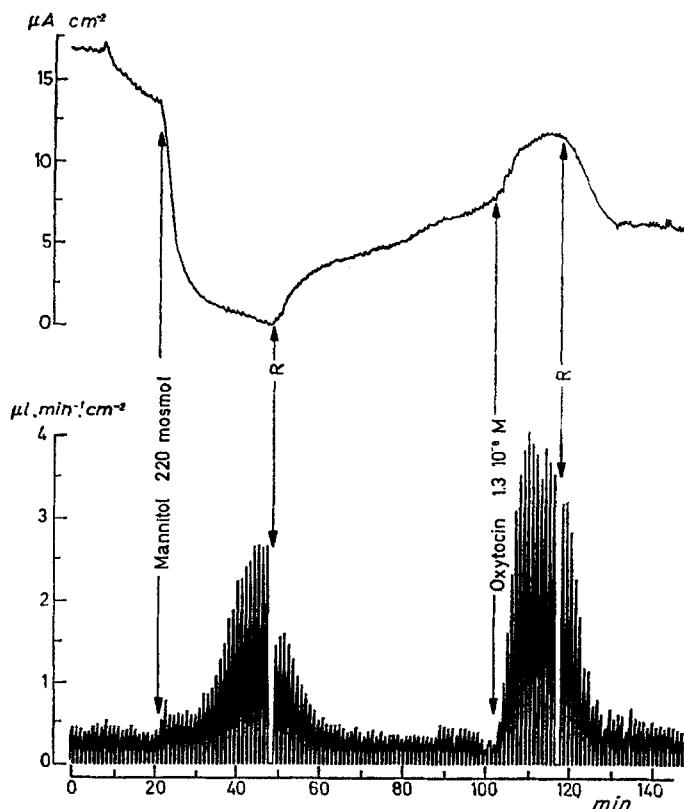


FIGURE 1. Influence of mannitol and oxytocin on net water flux and short-circuit current across *Rana esculenta* urinary bladder. Mannitol was added to the serosal medium. At "R," the serosal solution was replaced by fresh Ringer's solution.

will consider only the increase in water permeability, the so-called hydrosmotic effect of hypertonic media.

Influence of the Physiological State of the Animals

The intensity of the observed hydrosmotic reaction is widely variable from one preparation to the other.

As the hydrosmotic response to neurohypophysial peptides is markedly depressed in bladders from animals maintained at low temperature, the action of hypertonic media was determined in bladders from two groups of animals kept, for at least 8 days, in tap water at low (2°C) or at high temperature (22°C). The stimulus in this series was the addition of mannitol, 220 mosmol, to the serosal solution; the measurements were carried out on isolated bladders incubated at room temperature. Fig. 2 shows that maintaining the animals at low temperature considerably reduces the increase in net water flux induced in the isolated bladders by such a stimulus.

Characteristics of the Response

By many of its characteristics, the hydrosmotic response to hypertonic media compares to the physiological hydrosmotic response of the bladder to anti-diuretic hormone. The effect was fully reversed (see Fig. 3) when the osmotic pressure of the bathing media was reduced to its initial level, and the sub-

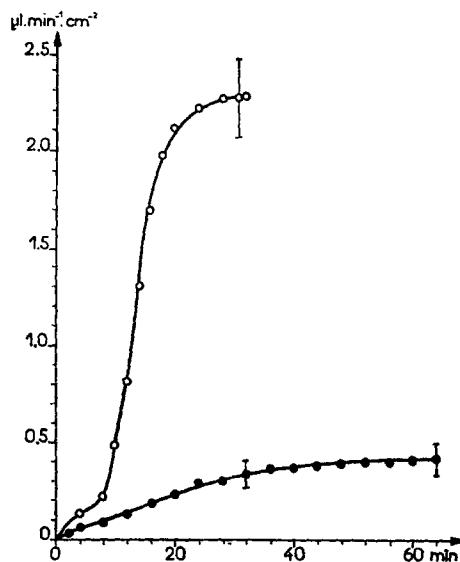


FIGURE 2. Influence of previous adaptation of animals to low temperature on the response to hyperosmolarity. Mannitol (220 mosmol) was added to the serosal medium at $t = 0$. Mean response of bladders from animals kept at 22°C (open circles, $n = 11$) and 2°C (filled circles, $n = 26$).

sequent sensitivity of the bladder was not impaired. When the osmolarity of the serosal solution alone is increased the observed variation in net water flow results in two phenomena: (a) an increase in the osmotic gradient or driving force, and (b) an increase in tissue permeability. However, as previously reported by Bentley (1964), Fig. 4 shows that the increase in net water flux is

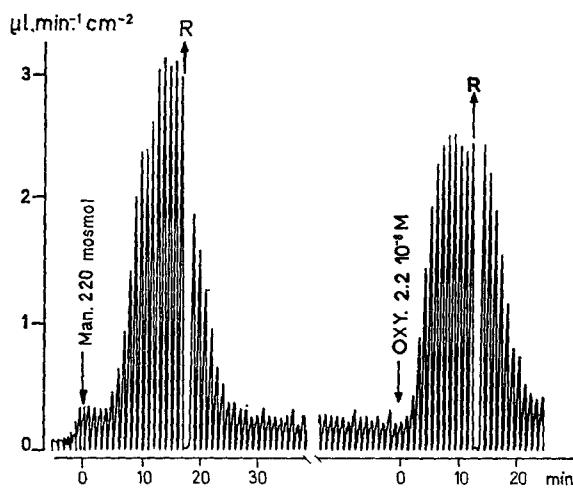


FIGURE 3. Record of typical hydrosmotic responses to mannitol (Man.) and to oxytocin (OXY), obtained sequentially on the same hemibladder. Both compounds were added to the serosal medium. It can be noted that removal of mannitol (R) which reduces the driving force to half of its value, is immediately followed by a drop of net water flux.

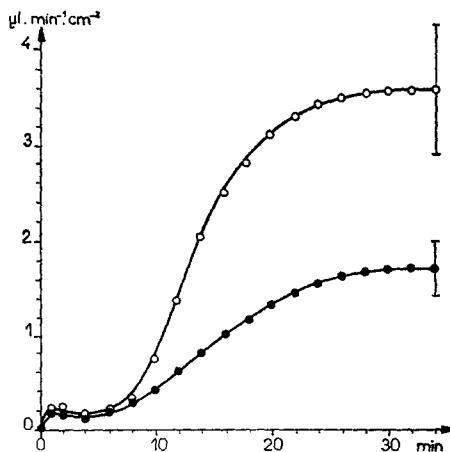


FIGURE 4. Hydrosmotic response to mannitol 220 mosmol added at time zero to serosal solution (open circles, $n = 7$) or to both serosal and mucosal solutions (filled circles, $n = 10$).

also observed when the solute is added simultaneously to the serosal and mucosal solutions and the osmotic gradient is kept constant. No qualitative difference was observed when the solute was added to the serosal and mucosal solutions or when the serosal side alone was made hypertonic, except that the observed net flux was proportionally higher in the latter case. Therefore, in most of the subsequent experiments, mannitol was added to the serosal side only.

The response pattern is also similar to that of oxytocin. The response is however significantly slower, the mean half-time increasing from 7.3 ± 0.35 min ($n = 24$) for oxytocin to 18.9 ± 1.7 min ($n = 24$) for mannitol at room temperature. The time-course of the response is markedly dependent on the temperature of the incubation medium, as is the case for oxytocin. (Rasmussen et al., 1960; Bourguet, 1966). In a series of experiments the mean half-time, i.e. the time required to reach 50% of the maximal increase, was determined on the same bladders at 30° and 20°C , and was found to be 5.9 ± 0.2 min and 13 ± 1.6 min, respectively, giving a mean Q_{10} of 2.38 ± 0.38 ($n = 7$).

Nature of the Solute Used as Stimulus

In a series of experiments, osmolarity was raised with equivalent amounts of different solutes. NaCl was compared to mannitol, as a poorly permeant electrolyte, and urea, as a rather easily permeant nonelectrolyte. Fig. 5 illustrates

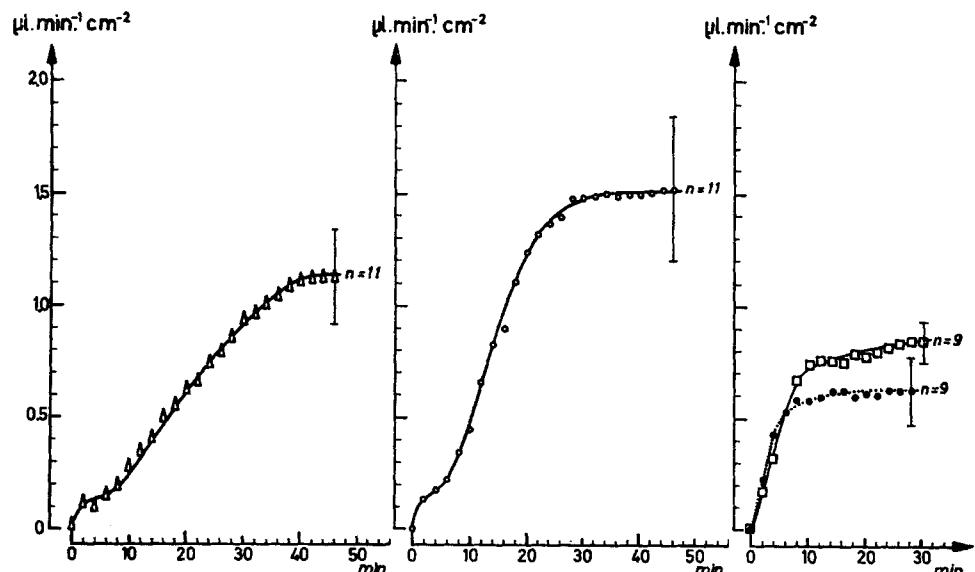


FIGURE 5. Mean hydrosmotic responses to hypertonic media. Ringer's solution was made hypertonic by the addition of mannitol (220 mosmol; open triangles), NaCl (115 mM; open circles), urea (220 mosmol, filled circles and 500 mosmol, open squares). Compounds were added at time zero to the serosal medium alone.

the mean increase in net water flux after the addition, at time zero, of one of these compounds. The effectiveness of NaCl (115 mM) was $1.510 \pm 0.33 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ compared to an increase of $1.12 \pm 0.21 \mu\text{l}$ after the addition of mannitol (220 mosmol) on control hemibladders. The mean half-times of the responses were, respectively, 16.8 ± 1.9 and 17.2 ± 2.8 min (mean of 11 bladders). As previously reported by Bentley (1964), the addition of urea to the serosal medium increased the net water flux to a much lesser extent. The observed increases were $0.62 \pm 0.15 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ with 220 mosmol and $0.84 \pm 0.09 \mu\text{l}$ with 500 mosmol ($n = 9$). A higher concentration of urea (1 M) was tested in another series and was found to increase the net water flux by $4.64 \pm 0.58 \mu\text{l}$ vs. $2.20 \mu\text{l} \pm 0.4$ for NaCl 115 mM in control hemibladders ($n = 9$).

Influence of Adenyl Cyclase Inhibitors

The similarity of the responses to hypertonic media and to oxytocin prompted us to study the effect of mannitol in the presence of agents known to inhibit the response to ADH such as norepinephrine (NE) and prostaglandin E₁. (PGE₁). (Handler et al., 1968; Orloff et al., 1965). Incubation with NE (10 μM) reduced the response to oxytocin ($2.2 \times 10^{-8} \text{ M}$) to $62.9 \pm 5.4\%$ ($n = 5$) of the control without any significant change in its time-course. Responses to mannitol and to NaCl (115 mM) were also reduced by NE, respectively, to 66.4 ± 9.4 ($n = 10$) and $16.53 \pm 3.61\%$ ($n = 8$) of the control, and contrary to the oxytocin response, were considerably slowed. The mean half-time of the response to mannitol was increased to 66.4 ± 0.40 min vs. 12.4 ± 0.75 min ($n = 10$) in this series control. Fig. 6 illustrates the responses to mannitol, NaCl, and oxytocin, both in control and pretreated hemibladders.

The ability of NE to reverse the developed response was also investigated (Fig. 6). The response to oxytocin was rapidly reversed to $37.2 \pm 7.4\%$ ($n = 7$) of its maximal value while the response to mannitol was not modified by subsequent addition of NE. The effect of NE on the increment in net water flux elicited by making the serosal solution hypertonic with sodium chloride was more difficult to assess, the net water flux exhibiting in this series of experiments a spontaneous decrease. A statistically significant although reduced effect was observed with a higher concentration of NE (100 μM); the mean difference between paired values in this series was $-0.75 \pm 0.28 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ ($n = 8$), $P < 0.05$. This effect was slow, the above difference was observed 80 min after the addition of NE.

To make sure that norepinephrine action was not prevented by the prolonged incubation in hypertonic medium, bladders were subsequently challenged with oxytocin. Fig. 7 shows that in these conditions, NE still significantly reduces the response to the neurohypophysial peptide.

Higher concentrations of NE (up to 100 μM) were also found without effect

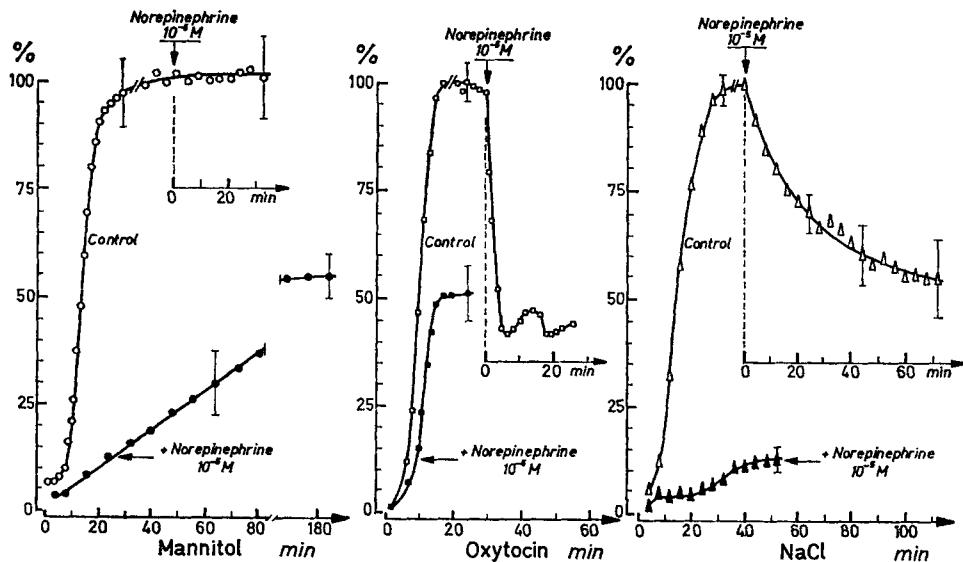


FIGURE 6. Inhibition of hydrosmotic response by norepinephrine. Comparison of pretreatment and reversal of action on the responses to mannitol (220 mosmol, $n = 10$), oxytocin (22 nM, $n = 5$ in the pretreatment series; $n = 7$ in the reversal series), and NaCl (115 mM, $n = 8$ in the pretreatment series; $n = 11$ in the reversal series). Agonists were added to the serosal medium at $t = 0$. NE was added at $t = -15$ min in the pretreatment series. In the reversal series, this time was considered as a new $t = 0$, although, the time elapsed between the addition of the agonist and of NE varied slightly from one preparation to the other. Experiments were done on paired hemibladders. One of them was pretreated with NE (filled symbols), the other was taken as a control (open symbols). When the response was developed the control hemibladder was in its turn treated with NE. The increase in net water flux was expressed for each pair, as a per cent of the maximum increase observed in the control hemibladder, except for the reversal series where the increase in net water flux at the time of addition of NE was taken as 100%.

on the response to mannitol, once developed. Finally a few experiments were also carried out on the effect of NE on the increment in water flow when both bathing solutions were made hypertonic by addition of 220 mosmol mannitol. In this series, the mean increment in net water flux was reduced from $1.34 \pm 0.21 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ in control to $0.23 \pm 0.04 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ after NE ($100 \mu\text{M}$) (mean difference between paired values: $1.11 \pm 0.18 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$, $n = 8$, $P < 0.001$).

The inhibitory action of norepinephrine was completely prevented by phentolamine ($100 \mu\text{M}$) but not by dichloroisoproterenol ($100 \mu\text{M}$) or propranolol (1mM) (Fig. 8). Isoproterenol did not alter the response to hypertonic media. The effect of phentolamine was not fully reversible: after the washout of phentolamine, the response to mannitol was still reduced, but no longer slowed by norepinephrine (Table I).

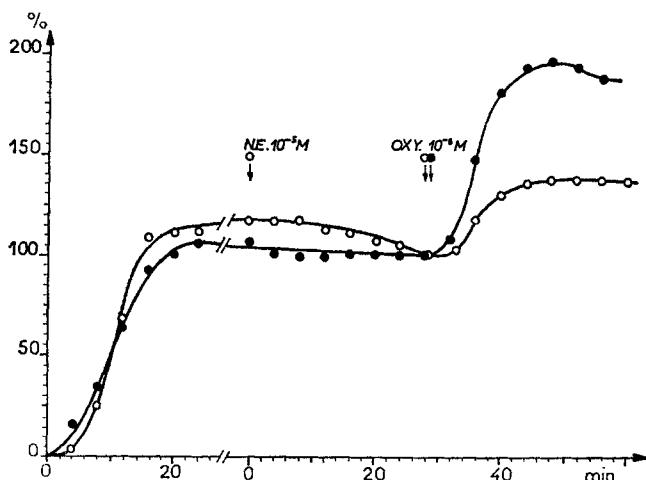


FIGURE 7. Inhibition by norepinephrine (NE) of a subsequent response to oxytocin, in presence of hyperosmolar serosal medium. Both hemibladders were challenged with mannitol 220 mosmol at $t = 0$. Increase in net water flux was expressed in as a per cent of the value reached by the control hemibladder (filled circles) at the plateau. (Mean of six experiments).

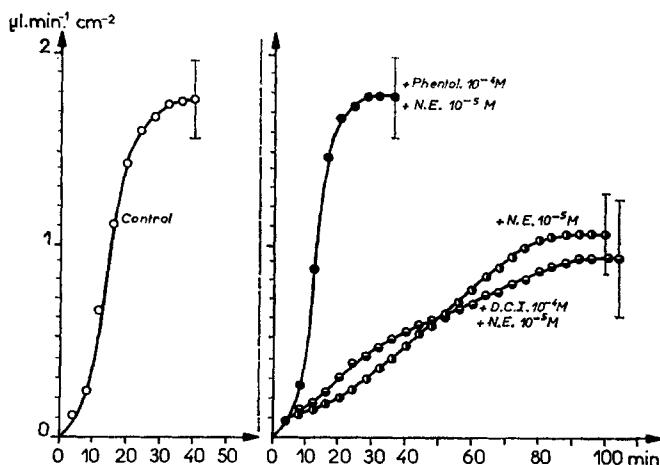


FIGURE 8. Influence of phentolamine (phentol., $n = 9$) and dichloroisoproterenol (D.C.I., $n = 9$) on the inhibition of hydrosmotic response to mannitol (220 mosmol, added to serosal side at $t = 0$) by norepinephrine (NE).

In line with the hypothesis of a reduced enzymatic activity of adenyl cyclase in the presence of NE, addition of cAMP (10^{-8} M) to the incubation medium abolished the inhibitory slowing action of norepinephrine on mannitol response.

According to Orloff et al. (1965), prostaglandin E₁ significantly inhibits the hydrosmotic response of the toad urinary bladders to vasopressin and, in some

TABLE I
EFFECT OF PHENTOLAMINE ON THE INHIBITION OF HYDROSMOTIC
RESPONSE TO MANNITOL BY NOREPINEPHRINE (NE)

Sequence of stimulation by mannitol, 220 mosmol	n	$t_{\frac{1}{2}}^*$	Δmax
		min	$\mu\text{l} \cdot \text{min}^{-1} \text{cm}^{-2}$
Alone	9	14.72±1.03	3.37±0.47
After NE 10^{-5} M‡	7	46.14±3.35	1.70±0.29
After NE 10^{-5} M + phentolamine‡ 10^{-4} M	9	12.78±0.70	3.10±0.40
After NE 10^{-5} M‡	9	14.33±1.13	1.56±0.21

* $t_{\frac{1}{2}}$ is the time required to reach an increase in net water flux equal to 50% of its maximal value (Δmax).

‡ NE and phentolamine were added to the serosal solution 15 min before the treatment by mannitol and maintained in the bath during the treatment.

circumstances to theophylline, but not to cAMP. Fig. 9 (upper trace) shows that the inhibition of oxytocin by PGE₁ (10^{-7} M) is similar to the one induced by NE. The hydrosmotic response to the hormone is inhibited but not slowed by incubation in the presence of PGE₁, and reversed if the product is added after the response had developed. We have also observed that the hydrosmotic response to mannitol is diminished after incubation in the presence of PGE₁ (Fig. 9 lower trace). As in the case of NE, the effect of hypertonicity is no longer reversed by this compound once it has developed.

Influence of Agents Increasing Intracellular cAMP Levels

The drastic influence of adenyl cyclase inhibition on the response to hypertonicity led us to study the effects of various agents which increase tissue cAMP levels, such as oxytocin, incubation in presence of exogenous cAMP or of theophylline. Their effect was studied on bladders obtained from animals adapted to 2°C and exhibiting reduced sensitivity to these agents. A record of an experiment with theophylline is shown in Fig. 10. Mean values of the response to hyperosmolarity (mannitol 220 mosmol) in control experiments (filled symbols) or in presence of threshold concentration of one of these agonists (open symbols) are given in Fig. 11. The resulting response is much greater than the value obtained by summing the responses to agonists acting alone and taking into account the final osmotic pressure difference.

DISCUSSION

Incubation of epithelial tissues in hyperosmolar media considerably alters their biological properties. Some of the observed modifications seem to result from the shrinkage of a cellular osmo-sensitive compartment. Indeed, poorly permeant solutes such as mannitol or NaCl appear to be the most effective agonists to trigger variations of net water flux, while more easily permeant compounds such as urea, used in equivalent concentrations, constitute a much

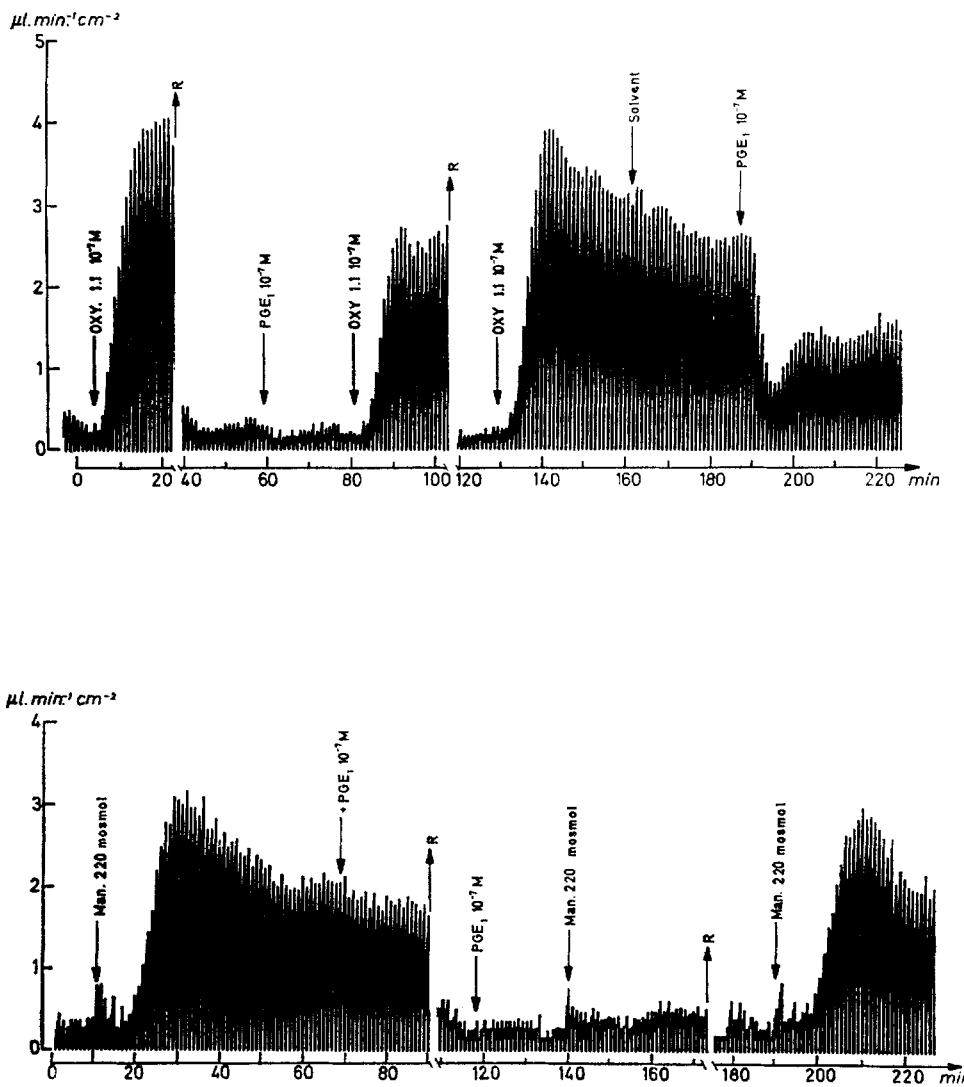


FIGURE 9. Inhibitory action of prostaglandin E₁ (PGE₁) on the hydroosmotic action of oxytocin (upper trace) and mannitol (lower trace). Comparison of pretreatment and action on the developed response. Contrary to what is seen in the case of oxytocin, PGE₁ is without effect on the developed response to mannitol although it completely abolishes the subsequent response of the bladder when given as a pretreatment.

less effective stimulus. The shrinkage of some tissue compartment would naturally lead to variations in the concentrations of a great number of components, as well as to mechanical alterations of all types of membranes and of intercellular junctions. This fact has led to the opinion that the mechanism of action of hyperosmolarity would be unspecific and that biological effects

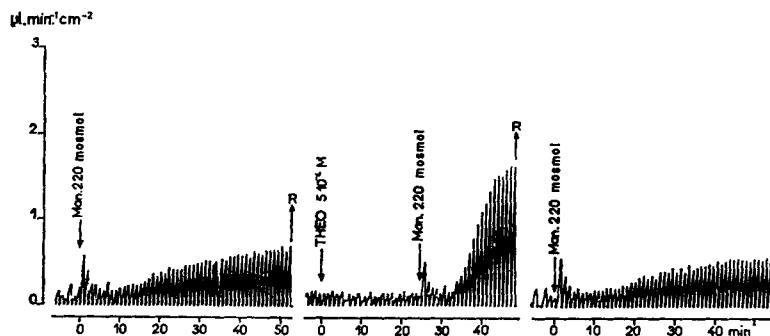


FIGURE 10. Potentiation, by a subthreshold concentration of theophylline (THEO) of the hydroosmotic response to mannitol (Man.). Typical record of responses obtained successively on the same hemibladder. The agonists were added to the serosal side only. The reduced response to mannitol is typical of a bladder from an animal adapted to a low temperature.

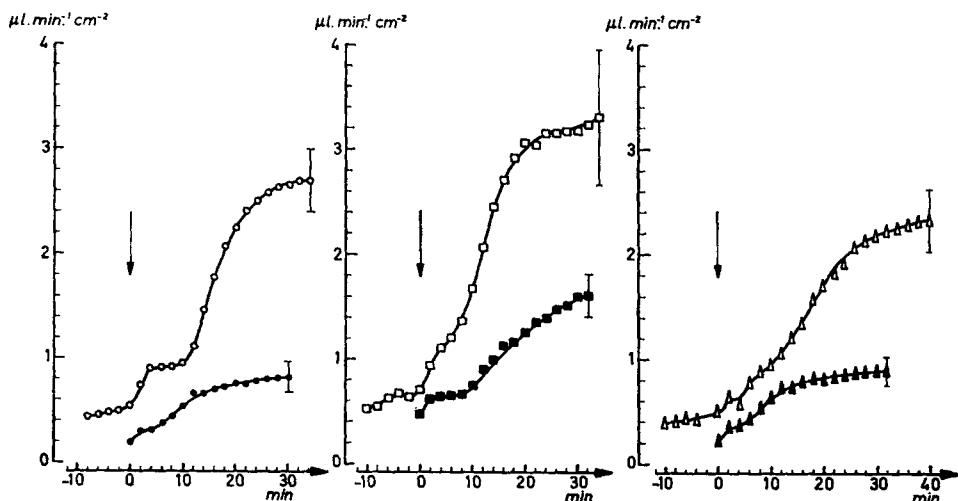


FIGURE 11. Influence of threshold concentrations of agents increasing intracellular cAMP levels on the hydroosmotic response to hypertonic media. Mannitol, 220 mosmol, was added at $t = 0$ to the serosal solution of both control (filled symbols) and test (open symbols) hemibladders. Test hemibladders were pretreated 20 min before the addition of mannitol by oxytocin (2.2 nM, circles), theophylline (1 mM, squares), or cAMP (2.5 mM, triangles). Mean responses ($n = 9$) of bladders from animals adapted at low temperature.

would eventually result from physical alterations of membranes. Our results suggest that in frog urinary bladder, more specific mechanisms are involved and that the different alterations, such as variations of ion and water permeability, do not share the same mechanisms.

It has been proposed, for a long time that natriferic and hydroosmotic re-

sponses to neurohypophysial peptides involve independent hormonal receptors and consequently separate pools of the second messenger cAMP (Bourguet and Maetz, 1961; Petersen and Edelman, 1964). The opposite actions of hyperosmolar media on sodium and water transport across frog bladder, are further evidence of the gross independence of these phenomena. Sodium net transport, as estimated by short-circuit current undergoes a strong inhibition, detectable as early as 1 min after the increase in osmolarity of bathing media, while the net water flux on the contrary increases slowly after a latency period (Fig. 1) and undergoes an evolution in many respects similar to that of the physiological response to ADH.

Different conditions related to the physiological state of the animals as well as various compounds which mimic the neurohypophysial hormones were found to interact with the mannitol response, but the most spectacular interferences were observed with inhibitors of the physiological hydrosmotic reaction.

Norepinephrine, which has been reported to reduce the hydrosmotic response to antidiuretic hormone in toad urinary bladder, was also found to reduce the response to hypertonic media. PGE₁ exhibited the same properties, even when NaCl was used as an agonist, an observation at variance with the situation encountered in *Bufo marinus* bladder (Egguna et al., 1970).

These two agents, moreover, considerably slowed the time-course of the response to mannitol, and there was no apparent correlation between these two effects, an observation in line with the fact that the oxytocin response is reduced without any associated increase in the half-time.

This effect of NE is linked to the stimulation of α -adrenergic receptors. Indeed, isoproterenol did not exhibit any activity on this material and the effect of NE was completely prevented by 0.1 mM of phentolamine, an α -adrenergic receptors blocker, but not by 0.1 mM dichloroisoproterenol, which blocks β -receptors. Prevention of adrenergic inhibition by phentolamine was not entirely reversible. After the washout of phentolamine, NE still reduced the flux observed at the peak of the response to mannitol, but no longer slowed the response, lending support to the idea of a lack of correlation between the two effects.

The action of NE has been attributed to an inhibition of the adenyl cyclase activity resulting in a reduced level in tissue cAMP. In agreement with such a mechanism, we observed that during incubation in presence of a liminal concentration of exogenous cAMP, the influence of NE on mannitol response was no longer observed.

The preceding results thus indicate that a given rate of adenyl cyclase activity and a normal level of tissue cAMP determine both the time-course and the equilibrium value of the response to hypertonicity. In apparent contradiction with this conclusion, we observed that, once developed the response to

hypertonic media is much less sensitive to a treatment with either NE or PGE₁. This lack of effect is not due to a reduction of cyclase inhibition by NE during incubation with hypertonic media. This hypothesis can be ruled out by the observation that NE still inhibits in these conditions a subsequent response to oxytocin.

As shown by Fig. 6, the response to NaCl is significantly more sensitive to NE than that of mannitol. This is observed not only when NE is used as a pretreatment but also in the reduction of the increment once it has been established. This difference was not further investigated, mainly because of the difficulty in obtaining dose-response curves for these stimuli which precluded an exhaustive study of the different concentrations of agonists and inhibitors.

It is clear however from the comparison of equilibrium values in reversal and pretreatment series that the increment in permeability produced by mannitol or sodium chloride is only weakly and slowly reversed by NE once it has been established, a situation qualitatively different from that encountered with oxytocin.

As an agonist of the system, hyperosmolarity seems thus characterized both by its dependence on the presence of cAMP for the development of the response and relative independence for the maintenance of this response. This last point is at variance with the rapid reversal of oxytocin action, which is observed after the addition of either NE or PGE₁. Such a reversal suggests that the persistence of an increased permeability requires a sustained increase in the level of cAMP and presumably a new equilibrium between the production, accelerated by cAMP, of a subsequent intermediary compound and its destruction.

Such a scheme could also account for the action of hyperosmolarity if it is assumed that a high level of one of the intermediary compounds is produced in hypertonic media through an impairment of its breakdown.

This hypothesis finds some support from the observation that hyperosmolarity significantly slows the reversal of the hydrosmotic response to classical stimuli. In a previous report (Ripoche et al., 1971) it was shown that the reversal time of hydrosmotic action after challenges with oxytocin, theophylline, and cAMP was significantly increased for three types of stimuli by the addition of mannitol 110 mosmol to both serosal and mucosal media. Such findings are in line with a reduced inactivation of some intermediary of action.

The poor reversal by NE or PGE₁ of the action of hyperosmolarity suggests that cAMP concentration is not a limiting step as in the case of oxytocin, and makes more likely the accumulation of a subsequent intermediary compound. This would require the addition of a supplementary stage of control to the presently accepted scheme of ADH action. This stage should be subsequent to the accumulation of cAMP and would be partly controlled by some osmo-sensitive compartment of the tissue.

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