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Electrolytes Control Flows of Water across the Apical Barrier in Toad Skin: The Hydrosmotic Salt Effect

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Summary. The reversible dependence of skin osmotic water permeability (L_{PD}) upon the ionic concentration of the outer bathing solution – which we have called "hydrosmotic salt effect" (HSE) – was studied in the isolated skin of the toad *Bufo marinus ictericus*. The skin osmotic water flow (J_V) was measured as a function of outer bathing solution osmolality (O_e). L_{PD}, calculated as $(J_v/\Delta\pi)_{\Delta P=0}$ (where $\Delta\pi$ and ΔP are the osmotic and hydrostatic pressure differences across the skin, respectively) was constant when O_e was altered with sucrose, a nonelectrolyte. In contrast, L_{PD} increased continuously in the hypotonic range as O_e was raised from zero (distilled water) with NaCl or KCl. The HSE could also be evoked in the condition of reversed osmotic volume flow. with the outer bathing medium made hypertonic with sucrose.

Diffusional ^{14}C-sucrose permeability, measured in the $J_v=0$ condition to prevent solvent drag of sucrose in the paracellular pathways, indicate that the hydrosmotic salt effect cannot be explained by assuming a paracellular permeability increase, due to tight junction opening, but might be interpreted as due to changes in the osmotic water permeability of the apical membranes of the most superficial cells of the epithelium.

The hydrosmotic salt effect can be elicited in control skins and in vasopressin-stimulated skins, on top of the hormonal response.

The time course of the hydrosmotic salt effect is substantially different from that of the hydrosmotic response to vasopressin. Its half-time is 4 to 5 times faster than that of vasopressin action, with individual values as short as 1.5 min.

The time courses of the hydrosmotic salt-effect onset and reversibility are exponential, clearly contrasting with the typical sigmoidal shape of vasopressin onset and washout time courses.

Based on time course data and on speed of response we postulate that the mechanism underlying the hydrosmotic salt effect is due to modifications of existing water pathways in the apical membrane, rather than to incorporation and removal of water permeability units in this structure.

Key words toad skin · water channels · water permeability · permeability control

Introduction

The antidiuretic hormone-cyclic AMP system is the fundamental process regulating water permeability in epithelial barriers such as the mammalian collecting duct and the urinary bladder and skin of amphibians.

Recent studies indicate that ADH, cyclic AMP and its analogues, and other related substances induce the appearance of intramembranous particle aggregates in the P face of the apical membrane of the epithelial cells (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & Di Scala, 1975; Harmanchi, Kachadorian, Valtin & Di Scala, 1978; Brown, Grosso & De Sousa, 1980) which may represent water-permeable patches through which water flows in aqueous channels.

Not much information is available regarding the post cyclic AMP steps prior to the appearance of aggregates in the apical membrane. However, there are indications suggesting the existence of a post cyclic AMP regulation (Ripoche, Bourguet & Parisi, 1973; Hardy, 1978 and 1979; Taylor, Eich, Pearl & Brem, 1979; Parisi, Chevalier & Bourguet, 1979). Very elegant low temperature results have been presented by Parisi, Chevalier and Bourguet (1980*a*, *b*) showing that cell acidification in frog bladder may switch the water channels from open to closed state, indicating that water permeability can be regulated at two different levels: by altering channel density in the apical membrane or by shifting channel permeability between open and closed states.

The present results indicate that increasing the ionic strength of the outer bathing solution in toad skin, by adding salts to the distilled water bathing the outer skin surface, markedly increases skin osmotic water permeability. This increase is attributed to changes in the apical membrane of the most superficial epithelial cells rather than to an increase of the paracellular water permeability.

Preliminary results have been reported at the XIV Latinamerican Congress of Physiological Sciences, São Paulo, Brazil (Benedictis & Lacaz-Vieira, 1981*a*) and the VII International Biophysics Congress – Satellite Symposium: "State, Distribution and Transport of Ions in Epithelia", Can-Cun, Mexico (Benedictis & Lacaz-Vieira, 1981*b*).

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Materials and Methods

The experiments were carried out in the abdominal skin of the toad *Bufo marinus ictericus* bathed on the inner side by NaCl-Ringer's solution and on the outer side by distilled water or solutions of different composition, as described in Results. The osmotic flow (J_v) was measured by two different methods. Steady-state determinations of J_v were performed by the gravimetric method of Bentley (1958) adapted to the skin, using a bell-shaped chamber of 18 cm^2 of area. A piece of skin was tightly fixed to the chamber border with several turns of string and the lateral area of the chamber covered with Teflon ribbon to isolate the edge-damaged area and to prevent undue amount of solution to be retained in this region during the weighing procedure (Fig. 1). The chamber was connected to the shaft of an electric motor of 100 rpm and kept at an angle of 45° to the vertical. In most cases, skins were mounted with the epithelial side facing outwards. The chamber compartment was half filled with Ringer's solution and the chamber partially immersed in the outer bathing solution contained in a small bowl. Stirring and aeration of the bathing solutions were obtained by rotation of the chamber. Due to rotation, a film of solution, continuously renewed, covered the skin areas above the bathing solutions. Every 5 min the chamber was disconnected from the motor shaft, the outer skin surface carefully dried with filter paper, the chamber weighed to a precision of 10 mg, and immediately coupled to the motor shaft to start a new period of measurement. The osmotic flow (J_v) was calculated by the regression line of chamber weight as a function of time, considering the relative density of the transported solution to be equal to 1.

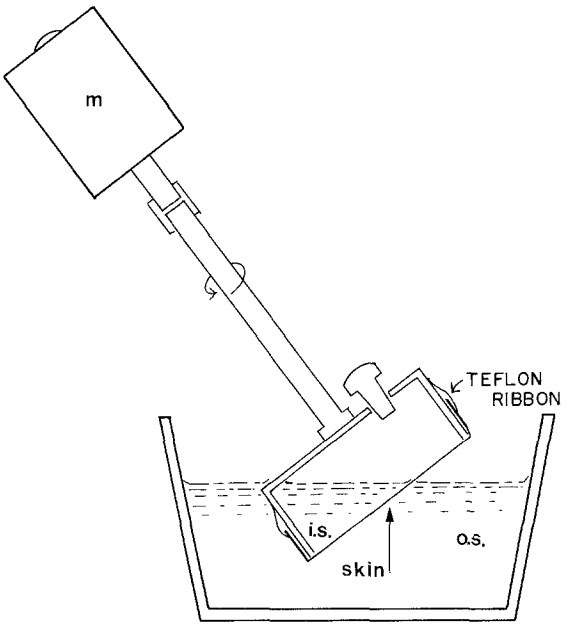

Fig. 1. Diagram of the rotating chamber used for measuring volume flow (J_v) across the skin by the gravimetric method. A Teflon ribbon was strapped around the lateral chamber surface to cover and isolate the area of skin damaged by several turns of string used to fasten it to the chamber. The area of skin exposed to the bathing solutions was 18 $cm^2 \cdot m$=electric motor of 100 rpm. *i.s.* and *o.s.* indicate inner and outer solutions, respectively

The osmotic flow time-course measurements were carried out by the volume-clamp method. This is an adaptation of the method of Bourguet and Jard (1964) except for not being automatic but controlled by the operator which maintained the level of a liquid meniscus at the tip of a hypodermic needle (gauge 8) at a constant position, under microscopic observation. The skins were horizontally mounted with the epithelial surface facing upwards, as shown in Fig. 2. The area of skin in contact with the bathing solutions was 1.7 cm^2. The volume of solution in the lower compartment was kept constant by means of a precision syringe coupled to a micrometer which permitted volume adjustments of the order of 60 nl. During the whole experimental period, the inner and outer bathing solutions were vigorously stirred, the lower compartment by means of a magnetic stirring bar coupled to a magnetic stirrer and the upper compartment by means of a propeller driven by an air turbine. The solution volume in the upper compartment was 2 ml. Solute concentration in this compartment was increased by microliter injections of concentrated salt or sucrose solutions. To return to distilled water, all the upper solution was aspirated and replaced after several rinses by distilled water, this taking no more than 45 sec.

The skin ^{14}C-sucrose permeability was determined in the absence of an osmotic gradient across the skin to prevent solvent drag upon labeled sucrose in the paracellular pathways. A different type of rotating chamber, described by Procopio and La-

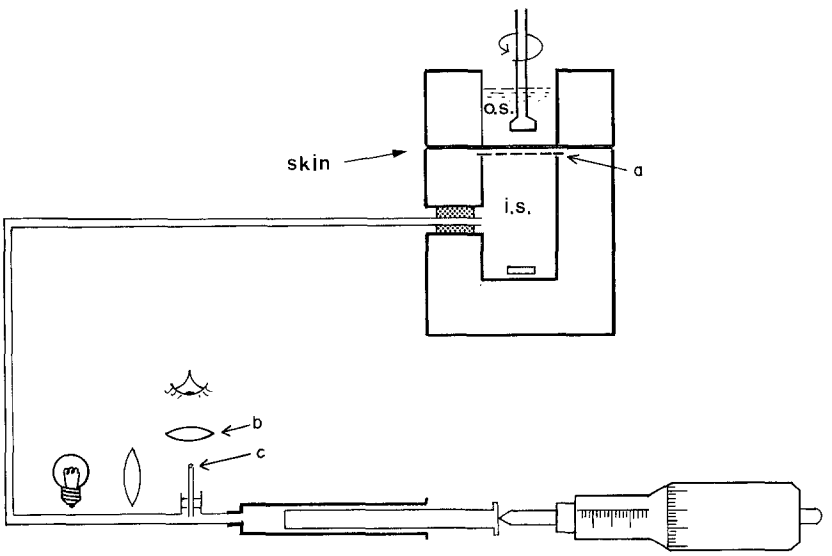

Fig. 2. Diagram of the chamber used for measuring volume flow (J_v) across the skin in the volume-clamp method. The level of a liquid meniscus, at the tip of a hypodermic needle (gauge 8) was kept constant by the movement of a micrometer coupled to a syringe which permitted volume adjustments of the order of 60 nl. (*a*) nylon mesh; (*b*) steroscopic microscope; (*c*) meniscus. The exposed area of skin was 1.7 cm^2. Outer (*o.s.*) and inner (*i.s.*) bathing solutions were stirred by an air-driven propeller and a magnetic stirring bar, respectively

caz-Vieira (1977) was used in these experiments, exposing an area of skin of 4.9 cm^2. In these experiments skins were bathed internally by NaCl-Ringer's solution and externally by different solutions with the same osmolality, adjusted to that of the Ringer's solution by sucrose addition. Approximately 25 μCi of ^{14}C-sucrose (New England Nuclear) were added to the inner compartment. Skins were equilibrated for approximately 30 min, and then after every 5 min all the outer bathing solution was removed by suction into counting vials and immediately substituted by similar fresh solution or by a different solution, according to the experimental protocol. Special precautions were taken to prevent the effects of skin edge damage on the labeled sucrose efflux, by using hemichambers provided with a circular groove (4 mm wide and 0.4 mm deep, filled with high vaccum silicone grease) located at the internal rim of the hemichamber surface in contact with the epithelial skin surface (Varanda & Lacaz-Vieira, 1978, 1979; Bevevino & Lacaz-Vieira, 1981).

NaCl-Ringer's solution had the following composition (in mM): NaCl, 115; $KHCO_3$, 2.5; and $CaCl_2$, 1.0; pH 8.2 after aeration and osmolality of 230 mOsm. The osmolality of the outer bathing solution (NaCl, KCl, or sucrose) was calculated from their molal concentration according to Tables (Handbook of Chemistry and Physics, CRC Press, 59th edition, 1979, p.D-265) and were measured occasionally in a Fiske OM® Osmometer (Fiske Associates). Vasopressin was obtained from Sigma Chemical Company. The results are presented as mean ± standard error.

Results

1. Osmotic Volume Flow (J_v) as a Function of the Osmolality of the Outer Bathing Solution

In these experiments most of the steady-state J_v values were obtained by the gravimetric method and a few by the volume-clamp method, both methods giving similar results. The isolated skin was bathed by NaCl-Ringer's solution on the inner surface. On the outer surface, it was initially bathed by distilled water and subsequently by solutions of increasing osmolality by addition of NaCl, KCl, or sucrose.

Figure 3 shows steady-state J_v values as a function of the osmolality due to NaCl addition to the outer bathing solution. It can be seen that for osmolality values below 30 mOsm[1], despite a reduction in the osmotic pressure difference across the skin due to increasing outer medium NaCl osmolality, J_v increases to reach a peak around 30 mOsm, decreasing subsequently for further increase in osmolality.

Figure 4 shows clearly that the J_v increase obtained by raising NaCl concentration in the outer compartment is not related to fluid transport coupled to the active Na transport, since amiloride (10^{-4} M), which blocks the active Na transport, does not prevent J_v increase by addition of NaCl to the outer bathing medium.

[1] x mOsm = x mOs kg^{-1} refers to a solution exerting the same osmotic pressure as an ideal solution of a nondissociating substance presenting a molality of x moles of solute per kg of solvent.

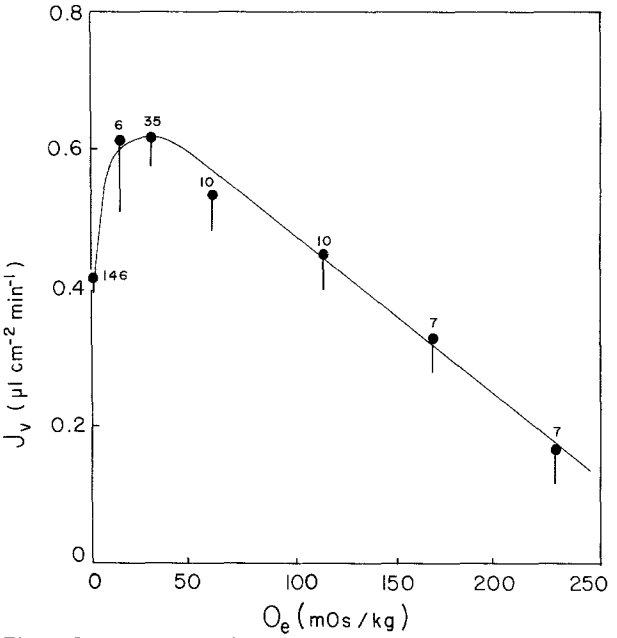

Fig. 3. Osmotic volume flow (J_v) across toad skin as a function of outer bathing solution osmolality (O_e) which was adjusted with NaCl. Inner skin surface was bathed by NaCl-Ringer's solution. The numbers of experiments are given in the figure

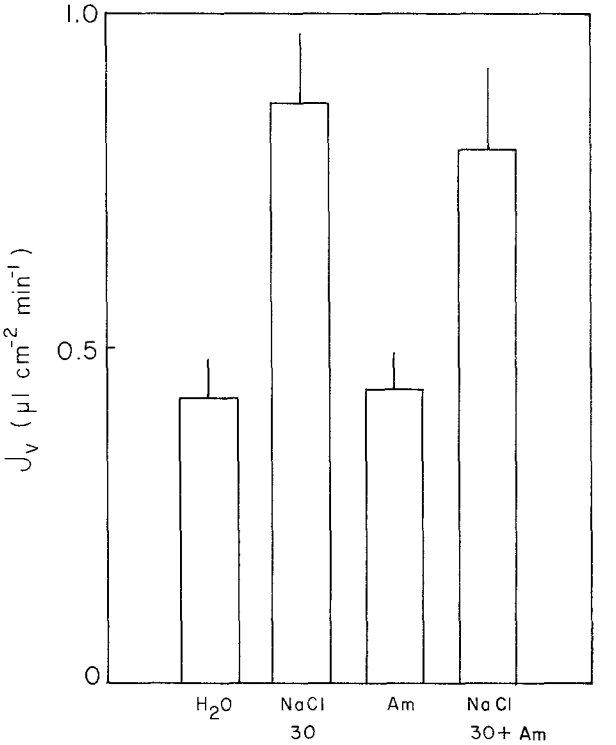

Fig. 4. Effect of the substitution of water by NaCl solution (30 mOsm) in the outer compartment and the action of amiloride 10^{-4} M (Am) in the outer solution. For each skin, the external solution was substituted in the following sequence: H_2O, NaCl 30 mOsm, Am 10^{-4} M, and NaCl 30 mOsm plus Am 10^{-4} M. Differences between J_v values for H_2O and NaCl groups and for Am and NaCl plus Am groups are highly significant ($P<0.01$ paired t test, $n=6$). The difference between J_v values for NaCl and NaCl plus Am groups is also significant ($P<0.01$, paired t test, $n=6$). The inner bathing solution was NaCl Ringer's solution

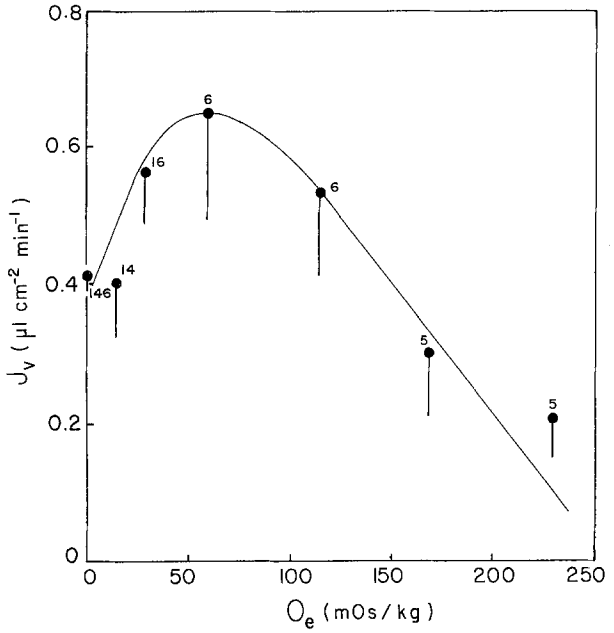

Fig. 5. Osmotic volume flow (J_v) across the skin as a function of outer bathing solution osmolality (O_e) which was adjusted with KCl. Inner skin surface was bathed by NaCl-Ringer's solution. The numbers of experiments are indicated in the figure

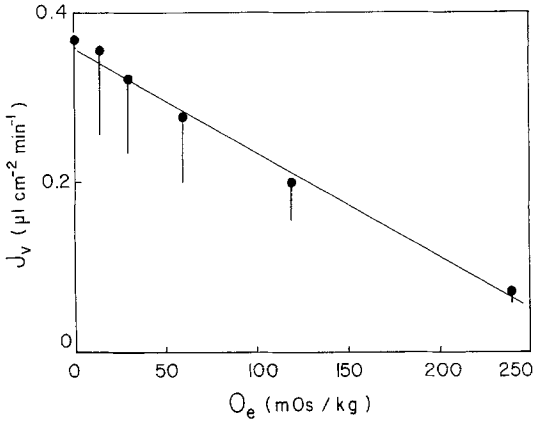

Fig. 6. Osmotic volume flow (J_v) across the skin as a function of outer bathing solution osmolality (O_e) which was adjusted with sucrose. The inner skin surface was bathed by NaCl Ringer's solution. ($n=5$)

Figure 5 shows that results similar to those obtained with NaCl (Fig. 3) can be obtained by increasing KCl concentration in the outer compartment, except for the J_v peak, which in the case of KCl is shifted to higher osmolality, in the region of 60 mOsm.

Contrasting with the results obtained with NaCl or KCl in the outer medium, we see (Fig. 6) that the reduction of the osmotic pressure difference across the skin, by raising the external osmolality with sucrose, a nonelectrolyte, induces only a steady decrease of J_v with increasing osmolality.

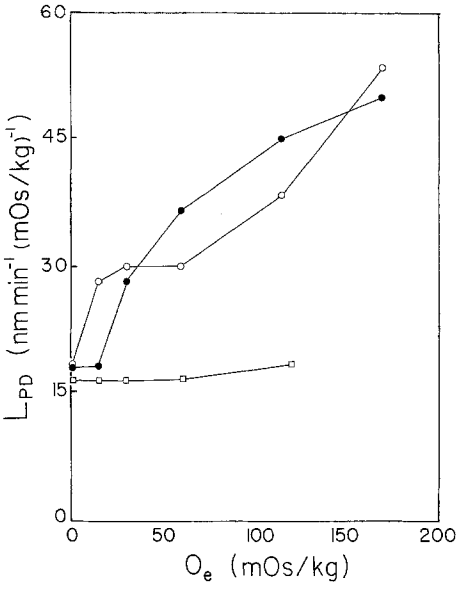

Fig. 7. Osmotic permeability (L_{PD}) of toad skin calculated according to Eq. (2) (*see* text) as a function of the osmolality of the outer bathing solution, which was altered with sucrose (□), KCl (●), or with NaCl (○). The inner bathing solution was NaCl-Ringer's solution

A few experiments were performed with external solutions of other salts ($NaNO_3$, Na_2SO_4, K_2SO_4, $CaCl_2$, LiCl, $BaCl_2$, choline Cl) which were surveyed in groups of two skins for each solution. The results indicate that these salts are also capable of inducing J_v increase.

The results of the experimental groups discussed above indicate that the presence of ions in the outer bathing medium leads to an increase of skin osmotic permeability as compared to control values obtained with distilled water in the outer compartment. Figure 7 shows a plot of skin osmotic permeability coefficient, L_{PD}, as a function of outer bathing medium osmolality, in experiments with NaCl, KCl, or sucrose as osmotic solutes in the outer medium. L_{PD} was calculated from the nonequilibrium thermodynamic relationship (Katchalsky & Curran, 1965):

$$J_v = L_P \Delta P + L_{PD} \Delta \pi \quad (1)$$

where L_P is the hydraulic permeability coefficient, L_{PD} the osmotic permeability coefficient, ΔP the hydrostatic pressure, and $\Delta \pi$ the osmotic pressure difference across the skin. From Eq. (1) we have

$$L_{PD} = (J_v/\Delta \pi)_{\Delta P = 0}. \quad (2)$$

It can be seen from Fig. 7 that L_{PD} remains unchanged when the outer bathing medium osmolality is increased with a nonelectrolyte, such as sucrose. However, with NaCl or KCl as osmotic solute in the

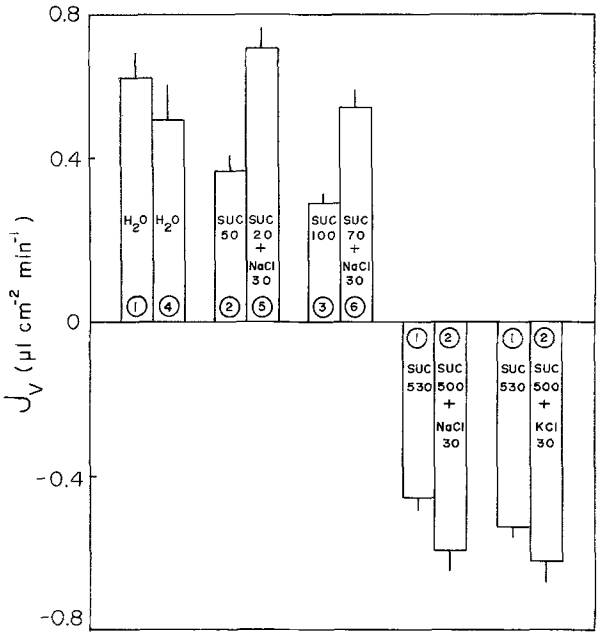

Fig. 8. Comparison of volume flow (J_v) across paired groups of skins subjected to same external osmolality, one with pure sucrose solution and the other with NaCl 30 mOsm plus sucrose to complete osmolality to that of the paired group. Numbers in circles indicate the sequence of external solution substitution in each group. Three distinct groups are presented: one in the hypotonic range and two others with external hypertonic solutions, one with NaCl and the other with KCl. Skins were bathed on the inner surface by NaCl-Ringer's solution. For groups *1* and *4* the differences were not statistically significant ($P=0.18$, paired t test, $n=8$). For groups *2* and *5*, and *3* and *6*, the differences were significant ($P<0.001$, paired t test, $n=8$). For the hyperosmolar experimental groups, the differences were significant: for groups *1* and *2* (NaCl) ($P<0.02$, paired t test, $n=9$); and for groups *1* and *2* (KCl) ($P<0.05$, paired t test, $n=8$)

outer compartment, L_{PD} increases markedly and continuously while raising the osmolality of the outer bathing solution, indicating that the osmotic permeability of the skin increases progressively as the ionic strength of the outer bathing medium is raised.

2. The Ion-Induced Osmotic Permeability Increase Occurs in a Wide Range of Osmolality in the Outer Bathing Medium

These experiments were carried out to test whether the ion-induced L_{PD} increase could be seen for different values of outer bathing medium osmolality, which was increased by addition of sucrose. Results can be seen in Fig. 8, which shows that an increase in outer bathing medium sucrose concentration leads to a reduction of J_v (as already shown in Fig. 6) and reverts it when the skin osmotic pressure difference is reversed. Comparing outer bathing solutions of equal osmolatities, we see that, with distilled water in the outer compartment, J_v values are not statistically different. However, there is a significant difference between the other three pairs of experiments, with external medium osmolalities of 50, 100, and 530 mOsm, the group containing sucrose plus 30 mOsm NaCl showing always higher J_v values than the corresponding group of identical osmolality containing sucrose only. Figure 8 also shows that a similar behavior was observed in another experimental group of 530 mOsm total osmolality, with KCl replacing NaCl.

3. Effect of Increasing NaCl Concentration or Acidification of the External Bathing Solution upon Permeability of Skin to Sucrose

These experiments were carried out in order to discriminate between the possibilities that the osmotic permeability increase, induced by raising the ionic strength of the outer solution, is a consequence of an increase of paracellular water permeability due to opening of tight junctions or due to an increase in water permeability of the apical membranes of the most superficial cells of the *stratum granulosum*. The rationale underlaying these experiments assumes that an increase in paracellular pathway permeability due to opening of tight junctions would be detectable by unidirectional fluxes of extracellular markers, e.g., ^{14}C-sucrose. On the other hand, an increase of water permeability of the apical membranes of the most superficial epithelial cells, where the large resistance to transepithelial water flow is located, (MacRobbie & Ussing, 1961) would not be expected to affect unidirectional fluxes of extracellular markers.

The experiments were carried out at $J_v=0$ by adding sucrose to the outer medium to eliminate the osmotic gradient across the skin, in order to rule out any contribution of solvent drag upon ^{14}C-sucrose movement in the paracellular pathways. Efflux measurements were chosen in order to permit ready changes of the external bathing medium without disturbing ^{14}C-sucrose specific activity in the "hot" compartment.

Skins were bathed on the inner surface by NaCl-Ringer's solution to which ^{14}C-sucrose was added to give a specific activity of 4.67 mCi/mmol. In the control period (12 skins) the outer skin surface was bathed by sucrose solution (230 mOsm) and five determinations of ^{14}C-sucrose efflux were carried out to calculate sucrose permeability (P_D^{suc}) and to evaluate its time stability. The mean value of P_D^{suc} for the five consecutive determinations for all skins was 0.168 ± 0.012 nm sec^{-1} ($n=12$). Then the outer bathing medium was sequentially substituted by a solution containing NaCl 30 mOsm plus 200 mOsm

of sucrose in one group (group A, 8 skins) or by a solution of NaCl 230 mOsm in the other group (group B, 4 skins), five determinations of P_D^{suc} being carried out in each case. For group A, in the control condition (external solution containing 230 mOsm of sucrose), mean P_D^{suc} was 0.171 ± 0.016 nm sec^{-1} and after the substitution, 0.218 ± 0.022 nm sec^{-1}, corresponding to a value 1.27 times greater than the control one ($P < 0.01$, paired t test, $n = 8$). For the group *B*, with sucrose 230 mOsm external solution, P_D^{suc} mean value was 0.160 ± 0.017 nm sec^{-1}, and after the substitution, 0.197 ± 0.017 nm sec^{-1}, corresponding to a value 1.23 times greater than the control one ($P < 0.02$, paired t test, $n = 4$). In sequence, the external medium was again substituted by sucrose solution (230 mOsm) with pH adjusted to 2.1 with H_2SO_4 (6 skins, 4 from group A and 2 from group B) or with HCl (6 skins, 4 from group B and 2 from group A), five consecutive determinations of P_D^{suc} being carried out for each case. The results are presented in Table 1.

Acidification of the external bathing medium, which is a procedure known to increase tight junction permeability (Fischbarg & Whittembury, 1978) was tested upon P_D^{suc}.

Acidification of the external sucrose solution with H_2SO_4 leads to a progressive increase of P_D^{suc}, which after 25 min reaches a value 23 times greater than the control one. Acidification of the external sucrose solution with HCl has a more drastic effect upon P_D^{suc}, which after 25 min reaches a value 141 times greater than the control value (Table 1).

4. Effect of Acidification of the External Bathing Solution upon Skin Osmotic Permeability

These experiments were performed in two groups of skins. In the first, J_v was measured in six skins with external bathing solutions replaced in the following order: distilled water, H_2SO_4 pH 2.1, distilled water, and HCl pH 2.1. In the other group of six skins, the solutions were replaced in the following order: distilled water, HCl pH 2.1, distilled water, and H_2SO_4 pH 2.1. Results are presented in Fig. 9. L_{PD} values were calculated according to Eq. (2). In both experimental groups, the substitution of distilled water by distilled water acidified to pH 2.1 with H_2SO_4 induces no significant increase of J_v and consequently of L_{PD} (for the first group, $P = 0.42$, paired t test, $n = 6$, for the second group, $P = 0.17$, paired t test, $n = 6$). On the other hand, the substitution of the distilled water by distilled water acidified to pH 2.1 with HCl induces a highly significant increase of J_v (consequently of L_{PD}) (for both groups, $P < 0.01$, paired t test, $n = 6$).

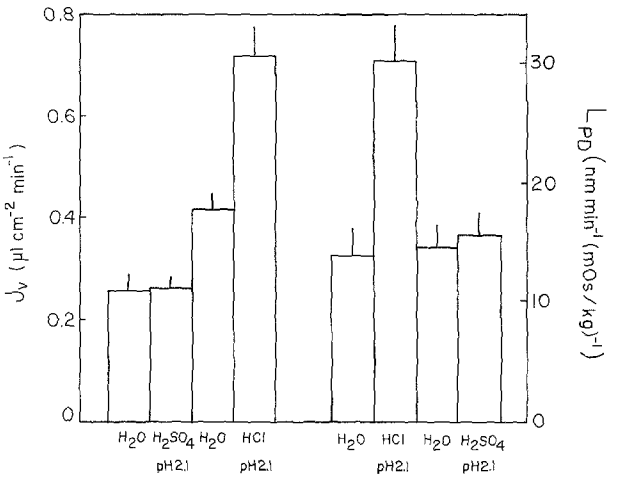

Fig. 9. Osmotic volume flow (J_v) in toad skin and the effect of acidification of the distilled water bathing the outer skin surface with H_2SO_4 or with HCl to pH 2.1. In one group ($n = 6$) each skin was sequentially bathed by H_2O, H_2SO_4, H_2O, and HCl. In the other ($n = 6$), the sequence was: H_2O, HCl, H_2O, and H_2SO_4. The inner bathing solution was NaCl-Ringer's solution. For the first series: difference between H_2O and H_2SO_4 groups was not significant ($P = 0.42$, paired t test, $n = 6$). Between H_2O and HCl groups the difference was significant ($P < 0.005$, paired t test, $n = 6$). For the second series: difference between H_2O and HCl groups was significant ($P < 0.005$, paired t test, $n = 6$). Between H_2O and H_2SO_4 groups the difference was not significant ($P = 0.17$, paired t test, $n = 6$)

5. Time Courses of Hydrosmotic Salt Effect Onset and Reversibility and the Effect of Vasopressin

These experiments were carried out to evaluate the time course of J_v increase upon addition of KCl to the outer bathing solution and its reversibility induced by the substitution of the ion containing outer bathing solution by distilled water. J_v was measured by the volume clamp method. Figure 10 shows the results of a representative experiment in which the outer bathing medium, initially distilled water, had its KCl concentration increased to 60 mOsm by a pulse of 60 µl of 1 M KCl solution. After the new J_v steady-state was attained, the outer compartment was rinsed several times and filled with distilled water. The time course was followed until a stationary condition was reached, showing the reversibility of the phenomenon. Figure 11 presents a similar experiment performed after the osmotic water flow had been stimulated by a supramaximal dose of vasopressin (0.1 U/ml) in the inner compartment.

These experiments permit comparison of the onset time courses of the hydrosmotic salt effect and of vasopressin stimulation. Two important differences characterize these two responses. First, the temporal evolution of the hydrosmotic salt effect is much faster than that of the hydrosmotic response to vasopressin. Second, the time course of vasopressin action

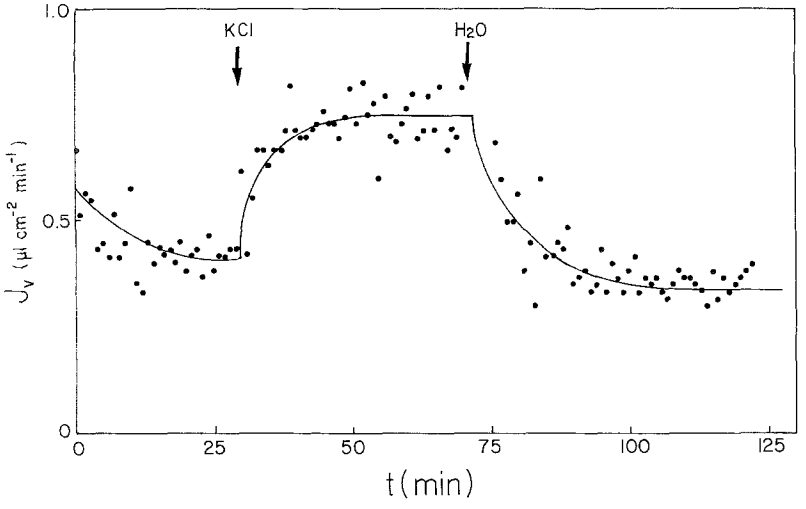

Fig. 10. Onset and reversibility time courses of osmotic volume flow (J_v) in a representative experiment. Inner bathing solution was NaCl-Ringer's solution. The outer bathing solution was initially distilled water. At the arrow indicated KCl, a pulse of 60 µl of 1 M KCl solution was added to the outer compartment (volume 2 ml) to raise the osmolality of the outer bathing solution to 60 mOsm. After a new steady-state was reached, the outer compartment was drained, rinsed several times, and filled with 2 ml of distilled water. For the onset of the response, J_v halftime was 2 min. For the reversibility, the halftime was 7 min. In this particular example, KCl induced a J_v increase of 82%. The points represent measurement at 1-min intervals

follows a sigmoid curve, while that for the hydrosmotic salt effect is exponential. These results also show that the hydrosmotic salt effect can be elicited on top of vasopressin stimulation, as shown in Fig. 11. Mean values for the $t_{\frac{1}{2}}$ of J_v onset induced by a step rise in KCl concentration from 0 to 60 mOsm is 3.9 ± 0.7 min. For the reversibility, induced when distilled water replaces the ionic solution in the external compartment, the halftime is 4.8 ± 0.8 min ($n = 5$).

Discussion

The aim of the present work was to study modulations of skin osmotic water permeability induced by changes in the ionic concentration of the outer bathing medium. Different aspects of this phenomenon, which we have called "hydrosmotic salt effect" were studied: (i) The dependence of the osmotic volume flow (J_v) and of the skin osmotic permeability (L_{PD}) upon the osmolality of the outer bathing solution, which was varied with electrolytes and nonelectrolytes. (ii) The hydrosmotic salt effect onset and reversibility time-courses. (iii) The path of fluid flow across the epithelium.

Different mechanisms could be underlying the hydrosmotic salt effect. One possibility would be action at the tight-junction level. These structures might show permeability changes in connection with changes of the outer medium ionic strength, analogously to permeability changes associated with hypertonicity (Ussing & Windhager, 1964; Erlij & Martinez-Palomo, 1972) or acidification (Fischbarg & Whittembury, 1978) of the outer bathing medium. Another mechanism would be at the apical cell membrane level. The osmotic permeability of the

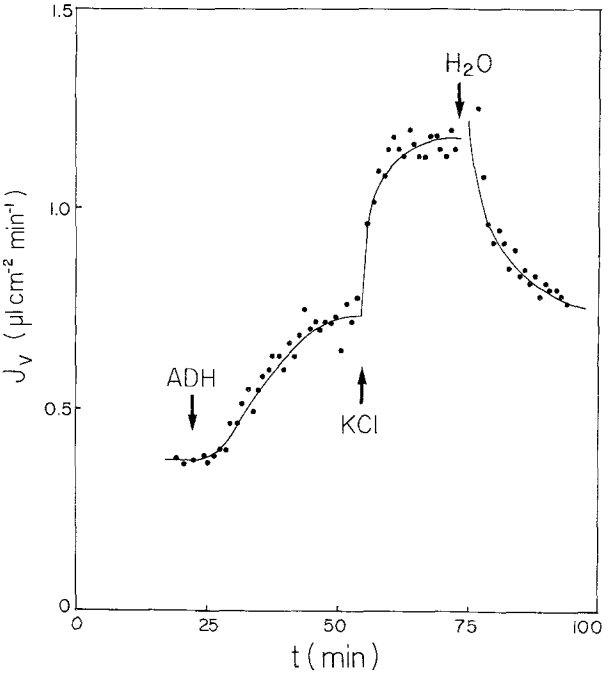

Fig. 11. Hydrosmotic response to vasopressin and superimposed hydrosmotic salt effect time-courses in a representative experiment. Inner bathing solution was NaCl-Ringer's solution. Outer bathing solution was initially distilled water. Vasopressin was added to the inner compartment to give a final concentration of 0.1 U/ml. After the response to vasopressin was completed, 60 µl of 1 M KCl was added to the outer compartment to raise KCl concentration to 60 mOsm. After a new J_v steady state was reached, the outer compartment was drained, rinsed several times, and filled with distilled water. The half-time of vasopressin action was, in this particular case, 13.5 min and that of the hydrosmotic salt effect induced by KCl, 1.5 min

apical membrane of the most superficial cells of the *stratum granulosum*, which is the most important barrier to water movement across the epithelium (MacRobbie & Ussing, 1961) could vary under the influence of changes in ionic strength of the outer

bathing medium. In this case, the hydrosmotic salt effect could be mediated by the activation of the cyclic-AMP system (Orloff & Handler, 1961; 1962) with changes in the density of water channels in the apical membrane or, induced by a direct effect upon the apical membrane, modifying water channel permeability. In this last case, changes in single water channel permeability might reflect changes in the pore molecular structure or be a consequence of modifications of water structure within the pore lumen.

When outer bathing solution osmolality (O_e) is increased with sucrose, a nonelectrolyte, J_V decreases linearly with O_e, as sown in Fig. 6. In this condition J_V can be given by (Katchalsky & Curran, 1965)

$$J_V = L_{PD}(\pi_i - \pi_e) \tag{3}$$

or

$$J_V = L_{PD} RT(O_i - O_e) \tag{4}$$

where π is the osmotic pressure, O the osmolality, R and T have their conventional meanings, and i and e refer to inner and outer compartments, respectively. $L_{PD} = -\sigma L_P$ where σ is the reflection coefficient and L_P the hydraulic conductivity coefficient. The osmolality of the inner bathing medium was kept constant in all experiments (230 mOsm) (NaCl-Ringer's solution). In the experiments with pure sucrose solution bathing the outer skin surface, the linear dependence of J_V on O_e (Fig. 6) indicates independence of L_{PD} upon outer medium osmolality. This is better seen in Fig. 7 for a plot of L_{PD} as a function of O_e.

In a clear contrast with the experiments using sucrose to alter O_e are the experiments with NaCl as osmotic solute in the outer compartment. With NaCl, the relation between J_V and O_e shows a maximum around 30 mOsm (Fig. 3). The increase of J_V with increasing O_e observed for low osmolality values, despite a concomitant reduction of the osmotic driving force ($\pi_i - \pi_e$) reflects an increase of L_{PD} with increasing NaCl concentration in the outer solution. Figure 7 shows that L_{PD}, calculated according to Eq. (2) as $J_V/(\pi_i - \pi_e)$, increases not only for the low NaCl concentration range, but does so continuously as the NaCl concentration of the outer bathing medium is raised.

An alternative explanation to interpret the hydrosmotic salt effect would be the contribution of a volume flow coupled to the active sodium transport (Keynes, 1969; Diamond, 1978). However, this hypothesis can be discarded since the phenomenon is still present when the active Na transport is blocked by amiloride (Fig. 4). The small difference seen in Fig. 4 between J_V in the absence ($J_V = 0.86 \pm 0.08$ µl cm^{-2} min^{-1}) and in the presence of amiloride ($J_V = 0.79 \pm 0.09$ µl cm^{-2} min^{-1}) ($P < 0.01$, paired t test, $n = 6$) reflect some fluid transport coupled to the active transepithelial Na transport that was inhibited by amiloride. The experiments with KCl solution in the outer compartment (Fig. 5) enable us to discard a participation of fluid transport coupled to the active Na transport in the genesis of the hydrosmotic salt effect, since K ions are not actively transported across the skin. The dependence of J_V upon O_e in the experiments with KCl in the outer compartment (Fig. 5) is similar to that of the experiments performed with NaCl (Fig. 3) except for the peak value which is displaced to higher osmolality values, near 60 mOsm. Reasons for this difference are unknown. Figure 7 shows that L_{PD}, as in the NaCl group, increases continuously as O_e is raised with KCl.

The hydrosmotic salt effect occurs over the whole hypotonic concentration range in the outer medium (Figs. 7 and 8), as well as in the condition of reversed osmotic flow induced by hypertonic sucrose solution in the outer compartment (Fig. 8). These results are interesting not only because they show that the hydrosmotic salt effect can be observed in a wide range of outer solution osmolality but, particularly, because they indicate that the hydrosmotic salt effect seems not to be a flux-dependent function and, therefore, it should occur even in the $J_V = 0$ condition.

In order to decide upon the path involved in the hydrosmotic salt effect, diffusional sucrose permeability (P_D^{suc}) was measured with ^{14}C-sucrose (Results, Section 3). The rationale underlying these experiments assumes that an increase of tight junction permeability would be detectable by unidirectional fluxes of extracellular markers, as labeled sucrose, but water permeability increase of the apical membrane would not be expected to affect sucrose unidirectional fluxes, since sucrose is considered to be excluded from the cell compartment (Franz & Van Bruggen, 1967; Whittembury, Martinez, Linares & Paz-Aliga, 1980). The particular condition of $J_V = 0$ was elected in order to eliminate solvent drag of labeled sucrose along the paracellular pathways (Andersen & Ussing, 1957). It could be argued that for $J_V = 0$ the hydrosmotic salt effect might not be present. We do not think, however, that this is a reasonable assumption for the following reasons: (i) L_{PD} increases continuously when NaCl of KCl outer solution concentration is increased, despite a concomitant reduction of J_V towards $J_V = 0$. Besides, there are no reasons to assume a discontinuity or an abrupt change of L_{PD} behavior as J_V approaches zero. (ii) The hydrosmotic salt effect is seen in the presence of normal as well as reversed osmotic flux

Table 1. ^{14}C-sucrose permeability (P_D^{suc}) of toad skin as a function of external bathing solution composition[a]

Period (min)	P_D^{suc} (nm s^{-1}) External bathing solution (mOsm)				
	Sucrose 230	Sucrose 200 + NaCl 30	NaCl 230	Sucrose 230 + H_2SO_4 pH 2.1	Sucrose 230 + HCl pH 2.1
0 – 5	0.145 ± 0.017	0.288 ± 0.033	0.250 ± 0.026	1.54 ± 0.29	4.00 ± 1.49
5 – 10	0.162 ± 0.013	0.201 ± 0.019	0.190 ± 0.018	2.59 ± 0.46	13.12 ± 5.94
10 – 15	0.164 ± 0.011	0.203 ± 0.021	0.186 ± 0.021	3.15 ± 0.56	17.95 ± 7.72
15 – 20	0.177 ± 0.010	0.202 ± 0.023	0.181 ± 0.010	3.51 ± 0.63	21.34 ± 8.50
20 – 25	0.194 ± 0.025	0.194 ± 0.016	0.178 ± 0.010	3.86 ± 0.56	23.75 ± 8.72
Mean for all skins	0.168 ± 0.012 (n = 12)	0.218 ± 0.022 (n = 8)	0.197 ± 0.017 (n = 4)	– (n = 6)	– (n = 6)

[a] P_D^{suc} was measured in efflux experiments in the $J_V = 0$ condition. For each external bathing solution, the P_D^{suc} measurements were carried out in five consecutive periods of 5 min each.

(Fig. 8). Therefore, it is reasonable to assume it to be present in the intermediary condition of $J_V = 0$.

The results of section 3 (Table 1) show that the substitution of the sucrose solution (230 mOsm) on the outer skin surface by a solution containing NaCl (30 mOsm) plus sucrose (200 mOsm) causes an increase of P_D^{suc} of 27%. On the other hand, the substitution of the sucrose solution (230 mOsm) by a NaCl solution (230 mOsm) induces a smaller increases of P_D^{suc} of the order of 23%. This observation, together with the fact that L_{PD} increases continuously when the external NaCl concentration is raised (Fig. 7) is a strong argument in favor of the interpretation that the small effect of external NaCl upon P_D^{suc}, which is not concentration dependent, might not be associated to the strongly NaCl concentration-dependent increase of L_{PD} shown in Fig. 7. The minor NaCl effect upon P_D^{suc} could be considered a secoundary and superimposed effect of the ionic strength upon the permeability of the paracellular pathway. This receives some support from the results of Gonzales, Kirchausen, Linares and Whittembury (1978), indicating that the ionic strength may play a role upon tight junction permeability, at least concerning the ability of hypertonic urea outer bathing solution in opening tight junctions, which is more effective at high than at low ionic strength. This increase could also be due to a small increase of P_D^{suc} with time, as suggested by Table 1 column 2.

Acidification of the external medium, a maneuver that leads to tight junction opening (Fischbarg & Whittembury, 1978) leads in our case to a marked increase of P_D^{suc} as shown in Table 1. With an external sucrose solution (230 mOsm, pH adjusted to 2.1 with H_2SO_4), the observed P_D^{suc} values are well above the control ones obtained with an external sucrose solution of the same concentration but without pH adjustments (pH around 5.5), and they increase progressively with time during five consecutive P_D^{suc} measurements. Considering the last P_D^{suc} value obtained with the external sucrose solution acidified with H_2SO_4 ($P_D^{suc} = 3.86 \pm 0.56$ nm sec^{-1}, $n = 6$, Table 1), the relative increase regarding the control situation (sucrose solution without addition of acid, $P_D^{suc} = 0.168 \pm 0.012$ nm sec^{-1}, $n = 12$) is of the order of 22 times. When the external sucrose solution had its pH adjusted to 2.1 with HCl instead of H_2SO_4, the effect upon P_D^{suc} is much more drastic. The relative increase of the last P_D^{suc} value observed with external sucrose solution acidified with HCl to pH 2.1 ($P_D^{suc} = 23.75 \pm 8.72$ nm sec^{-1}, $n = 6$), regarding the control situation (sucrose solution without addition of acid, $P_D^{suc} = 0.168 \pm 0.012$ nm sec^{-1}, $n = 12$), is of the order of 140 times. These results obtained with low pH external bathing solution show that tight junction opening can be clearly detected by the labeled sucrose efflux experiments and the P_D^{suc} increase is of one to two orders of magnitude.

The fact that at the same pH (2.1) HCl is much more effective upon tight junction opening suggests that the sites of action of protons, leading to tight junction opening, are not readily accessible from the outer bathing solution. They might be deeply located within the tight-junction structure, in a region not as easily accessible to sulfate as to chloride ions.

To get a better insight into the genesis of the hydrosmotic salt effect, particularly into the locus responsible for its origin, it is important to compare the effects of low external pH upon both L_{PD} and P_D^{suc}.

Acidification of the external bathing solution with HCl to pH 2.1 leads to a significant increase of L_{PD}, which is, however, never larger than twice the control value obtained with pure sucrose solution, as can be readily computed from J_V data presented in Fig. 9. On the other hand, acidification of the external solution with HCl to pH 2.1 has a dramatic effect upon P_D^{suc}, which increases more than a hundred times, as already discussed (Table 1). This com-

parison indicates that P_D^{suc} is a much more sensitive parameter of tight junction opening than L_{PD}. This is expected, if we consider that the sucrose efflux occurs mainly through the paracellular pathway while J_V is expected to have a large transcellular component. On the other hand, acidification of the external solution to the same pH (2.1) with H_2SO_4, which does not significantly affect L_{PD} as can be deduced from the absence of effect upon J_V (Fig. 9), has a strong effect increasing P_D^{suc} (Table 1). We may adduce, therefore, that a minor increase of tight junction permeability, as that induced by acidification with H_2SO_4, which is not large enough to reflect upon L_{PD}, is perfectly detectable by P_D^{suc} measurements. These results grant us permission to conclude that the hydrosmotic salt effect, characterized by a L_{PD} increase induced by raising NaCl concentration in the outer bathing solution (or the concentration of other salts, as KCl) is not due, at least predominantly, to an increase in tight junction permeability, since, according to the reasoning presented above, tight junction opening would be expected to be accompanied by a large increase of P_D^{suc}, relatively more important than that of L_{PD}, which is in fact not observed, as shown in Table 1.

Concluding, we put forward the hypothesis that the hydrosmotic salt effect is a consequence of osmotic permeability increase of the apical membranes of the most superficial epithelial cells, where the most significant barrier to water movement across the cells is located.

It is interesting to point out that the hydrosmotic salt effect can be elicited both in vasopressin untreated skins, immediately or several hours after mounting them, or in skins stimulated by supramaximal doses of vasopressin (0.1 U/ml) as shown in Figs. 10 and 11. The observation that the hydrosmotic salt effect can be evoked several hours after mounting the skin rules out the participation of endogenous antidiuretic hormone in the phenomenon. Bentley (1957), working with intact toads (*Bufo marinus*), reported that NaCl solutions of increasing concentrations in the outer compartment potentiated the uptake of water in response to vasopressin over part of the hypotonic range and postulates an electro-osmotic effect to explain his results. More recently Dicker and Elliot (1967), working also with intact toads (*Bufo melanostictus*), presented results consistent with the hydrosmotic salt effect.

The hydrosmotic salt-effect onset and reversibility time-courses are shown for a single experiment in Fig. 10. For the onset of the response, induced by a 60 mOsm KCl solution in the outer compartment, the observed mean half time ($t_{\frac{1}{2}}$) was 3.9 ± 0.7 min ($n = 9$). For the reversibility, when distilled water replaced the KCl solution in the external compartment, $t_{\frac{1}{2}}$ was 4.8 ± 0.8 min ($n = 5$). Halftimes as short as 1.5 min were observed for the onset of the hydrosmotic salt effect induced by 60 mOsm KCl in the outer compartment. Figure 11 shows that two important differences characterize the time course of the hydrosmotic salt effect and of the hydrosmotic response to vasopressin. First, the temporal evolution of the hydrosmotic salt effect is much faster than that of the hydrosmotic effect of vasopressin. Normally, the $t_{\frac{1}{2}}$ of the onset of the hydrosmotic salt effect is about one-fifth of that for the onset of vasopressin action. This difference strongly suggests that the hydrosmotic salt effect is not mediated by stimulation of the cyclic-AMP system, but might be induced by the activation of some step after the generation of cyclic AMP, possibly by a direct action upon the apical membrane itself. Second, the time course of vasopressin action follows a sigmoid curve, while that for the hydrosmotic salt effect is exponential, a possible indication that different mechanisms are involved in the two processes. Vasopressin action upon J_v displays a latency time, while the hydrosmotic salt effect starts almost immediately upon addition of KCl to the outer compartment (Fig. 11). It has been recently proposed that the time course of antidiuretic hormone action in frog bladder results from addition of permeability units in the apical membrane that increase in number during the development of the permeability response (Parisi, Bourguet, Ripoche & Chevalier, 1979). A similar mechanism might be operative in amphibian skins, since the time course of hormonal action is sigmoid as for the toad bladder, and the morphological observations of Brown et al. (1980) show that intramembranous particle aggregates, considered to be low resistance water pathways, are plugged into the apical border by isoproterenol or vasopressin stimulation. Thus, we postulate that the exponential behavior of the hydrosmotic salt-effect onset response (and reversibility response, which is also exponential, as shown in Fig. 10) indicates that the mechanism of increasing water permeability is not due to incorporation of new water permeability units into the apical border of the most superficial epithelial cells but is due to modification of existing water pathways in this structure. In artificial collagen membranes, it has been shown that permeability to solutes and water is strongly dependent upon the salt concentration of bathing solution (Bartolini, Gliozzi & Richardson, 1973), and this behavior is due to membrane phase transitions. In our case, it is also possible that membrane proteins change their configuration as a function of external bathing solution ionic strength. A definite con-

clusion whether or not aggregates are inserted into the apical membrane in the course of the hydrosmotic salt effect is awaiting freeze-fracture studies.

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