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POTENTIAL DIFFERENCE AND NET FLUX OF WATER IN THE ISOLATED AMPHIBIAN SKIN

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Abstract—The potential difference existing spontaneously across the isolated skin of *Rana temporaria temporaria* L. has been measured simultaneously with the net flux of water arising under the influence of an osmotic gradient. The effect of various compounds on these parameters has been studied. The results obtained suggest that in the isolated frog skin the passive movement of Na and the flux of water take place through spatially separated structures.

IT IS well known that many substances can affect the permeability characteristics of living membranes. It has been shown that the neurohypophyseal peptides increase not only the permeability to water but also the net flux of Na across the frog skin.[1] *d*-Tubocurarine chloride (curare), atropine sulphate, pilocarpine chlorhydrate, pyridine-2-aldoxime methiodide (2-PAM), pyridine-2-aldoxime dodeciodide (2-PAD),[2–6] local anaesthetics,[7] etc. affect also specifically the ion's movement across the frog skin.

An interesting question raised by these observations is to know not only the localization of the site of action of the compound studied but also the spatial arrangement of the chemical architecture responsible for the permeability characteristics affected. It has, for instance, been proposed that quaternary ammonium derivatives would enhance the net flux of Na across the frog skin by increasing the passive permeability to Na of the outer layers of the skin.[5–7] On the contrary, lipid-soluble 4 N derivatives and tertiary amines, because of their lipid solubility, could reach the site of the active transport mechanism of Na located at the inner border of the epithelial cells of the skin.

On the basis of experiments performed with curare, 2-PAM and 2-PAD we have suggested that water and the passive component of the Na flux move through channels spatially separated in the frog skin.[2, 8–9] This paper is an attempt to bring more evidence in favour of this view. It is based on the results of net flux of water and electrical potential difference measurements obtained on the isolated frog skin as affected by the antidiuretic hormone (ADH), curare, atropine and 2-PAM.

MATERIAL AND METHODS

Isolated abdominal skin from *Rana temporaria temporaria* L. are mounted in an apparatus identical in principle to the one described by Koefoed-Johnsen *et al.*[10] Net flux of water arising under the influence of an osmotic gradient is measured simultaneously with the electrical potential difference existing between the solutions bathing the outside and inside of the skin. The exposed area of the skin is 7 cm² and

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the volume of the solution in the outside chamber can be measured with an accuracy of $\pm 5\ \mu l$. The inside solution has the following composition: 113 mM NaCl, 1·9 mM KCl, 1·8 mM $CaCl_2$ and is buffered with phosphate at pH 7·8 (1·5 mM). The outside solution has the same composition but is diluted ten times. The ADH (Pitone Organon) is brought to pH 7·8 and added at the concentration indicated for the inside solution. Concentrated stock solutions of the other compounds studied are prepared and adequate amounts are added to the outside solution. The experiments were performed during the autumn.

RESULTS

1. *Net flux of water across the isolated skin*

While the net flux of water varies considerably (sometimes up to 400 per cent) from one individual to another, it is usually constant for each skin over an experimental period of more than 6 hr.[11–12] We have confirmed this finding and Table 1 shows some results obtained.

TABLE 1. NET FLUX OF WATER ACROSS THE ISOLATED SKIN OF *Rana temporaria temporaria* L.
(Outside solution 1/10 Ringer. Inside solution Ringer.)

Experiment	Period (hr)	Net flux ($\mu l\ hr^{-1}\ cm^{-2}$)
1	0–4	4·0
	4–7	4·1
2	0–1	3·0
	1–3	3·0
3	0–1·5	4·0
	1·5–3	3·8

TABLE 2. ACTION OF CURARE ON THE NET FLUX OF WATER ACROSS THE ISOLATED SKIN OF *Rana temporaria temporaria* L.
(Outside solution 1/10 Ringer. Inside solution Ringer. C = control.)

Experiment	Period (hr)	Conditions	Net flux ($\mu l\ hr^{-1}\ cm^{-2}$)
1	0–1	C	4·0
	1–2	C	4·2
	2–3	curare 200 $\mu g/ml$	19·4
	3–4		19·8
	4–5		20·0
	5–6		21·0
2	0–4	C	3·2
	4–5·5	curare 250 $\mu g/ml$	8·6
	5·5–6·5		8·5

2. *Action of curare at various concentrations*

A few years ago, Kirschner[13] showed that curare increases the net flux of Na across the isolated frog skin. More recently we have shown that, while the effect of curare on the potential difference of the skin of *Rana temporaria* is rather capricious, there is a

consistent increase in the net flux of water.[2, 5] This increase remains unaltered for several hours as shown by Table 2.

We have repeated these experiments using increasing amounts of curare. Fig. 1 shows the results obtained when curare is added to the outside solution in the concentration range 50–1450 μg/ml. It can be seen that the increase in net flux of water is already

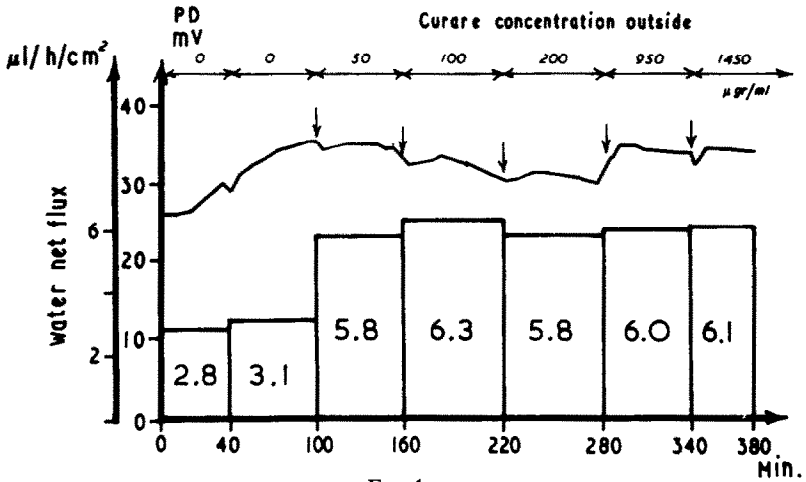

FIG. 1

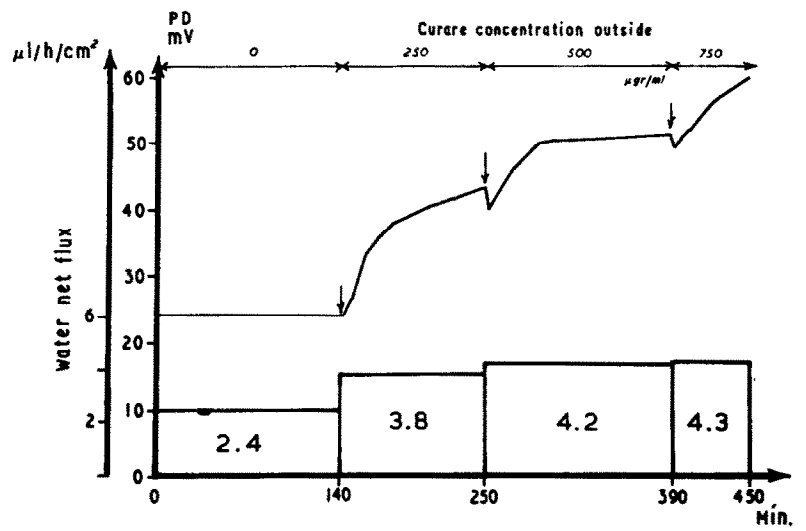

FIG. 2

FIGS. 1 and 2. Action of curare at various concentrations (50–1450 μg/ml) when added outside, on the potential difference and the net flux of water.

maximal at 50 μg/ml. As far as the potential difference is concerned we confirmed previous results, i.e. the response is capricious, but when an effect is observed, the potential difference increased with each adjunction of curare (Fig. 2). At higher concentration (2250 μg/ml) the net flux of water still remains at the value reached for 50 μg/ml.

3. *Action of ADH at various concentrations*

Table 3 shows that after one single addition of ADH (0·1 IU/ml) in the solution bathing the inside of the skin, the net flux of water remains constant over a period of 5 hr.

Fig. 3 shows that the increase in net flux of water is maximum when 0·1 IU/ml of ADH is added to the inside solution. The potential difference still increases at a concentration of 0·2 IU/ml while there is no further change at higher concentrations.

TABLE 3. ACTION OF ADH ON THE NET FLUX OF WATER ACROSS THE ISOLATED FROG SKIN

(Outside solution 1/10 Ringer. Inside solution Ringer. The hormone is added in the solution bathing the inside of the skin at a concentration of 0·1 IU/ml. C = control.)

Experiment	Period (hr)	Conditions	Net flux (μl hr^{-1} cm^{-2})
1	0–1	C	1·6
	1–2	ADH 0·1 IU/ml	3·6
	2–3		3·6
	3–4		3·7
	4–5		3·3
	5–6		3·6
2	0–1	C	2·1
	1–2	C	2·1
	2–3	ADH 0·1 IU/ml	7·1
	3–4		7·1
	4–5		6·8
	5–6		7·0

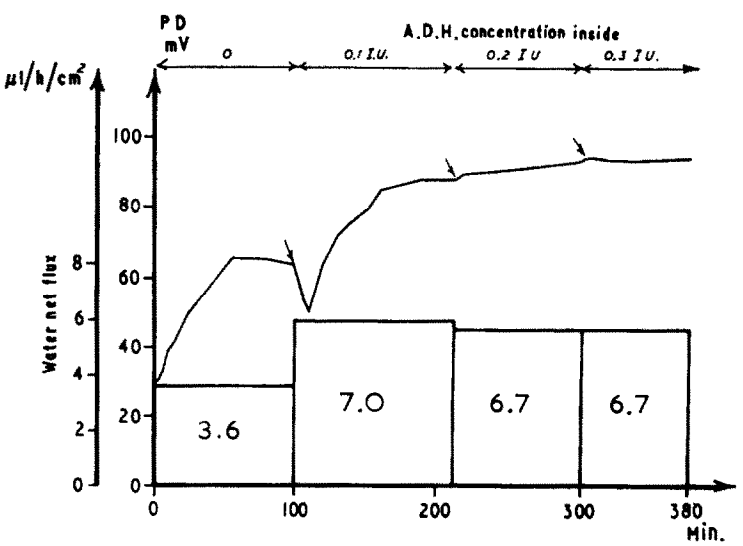

FIG. 3. Action of ADH (0·1 to 0·3 IU/ml) on the potential difference and the net flux of water. The hormone is added inside.

4. *Action of ADH and 2-PAM*

2-PAM increases the potential difference but is without effect on the net flux of water.[5, 9] It is therefore of interest to study the effect of ADH in the presence of 2-PAM. Fig. 4 shows that after addition of 2-PAM (200 μg/ml) there is an increase in potential difference. ADH is then added after 2 hr, at a concentration of 0·1 IU/ml. While there is a further increase in potential difference, the net flux of water remains unaffected.

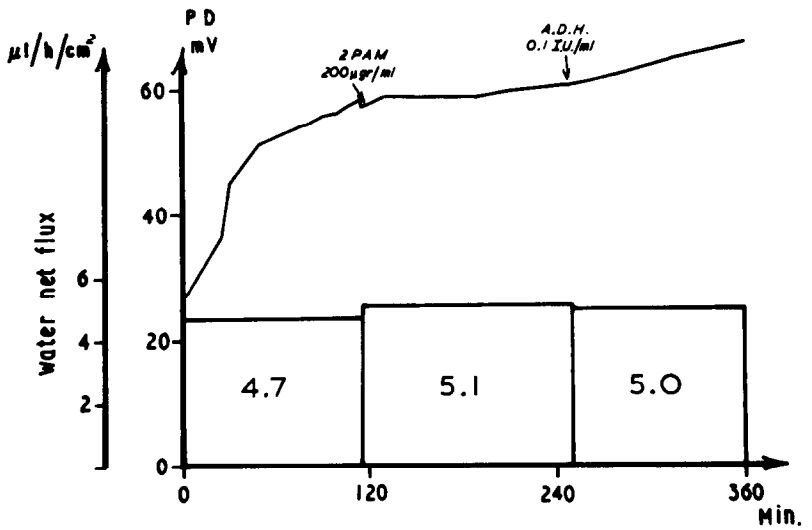

FIG. 4. Action of 2-PAM (200 μg/ml) and (0·1 IU/ml) on the potential difference and the net flux of water. The hormone is added inside, 2-PAM outside.

It is therefore of interest to see how 2-PAM can modify the response of the skin when ADH is added first. Fig. 7 shows the results obtained. ADH is added at a concentration of 0·1 IU/ml to the outside solution. 2-PAM is then added outside at increasing concentrations (from 200 to 800 μg/ml). It can be seen that the effect of 2-PAM on the potential difference is still present, i.e. a slight increase in electrical potential, thus suggesting that the mechanism responsible for this change is not yet fully saturated. On the contrary the net flux of water decreases as if 2-PAM would displace ADH from its active sites.

The decrease in net flux of water within the first hour after addition of 2-PAM is significant and cannot be ascribed to a decrease if the flux of water sometimes observed after the initial effect of a dose of ADH. This spontaneous decrease indeed takes place more than 3 hr after a single application of ADH as already observed by Fuhrman and Ussing[11] and as shown by our results (Table 3).

5. *Action of 2-PAM and curare*

Results obtained by adding curare (200 μg/ml) after application of 2-PAM show that the net flux of water remains at the control value while an effect on the potential difference can be demonstrated (Fig. 5).

6. *Action of 2-PAM and atropine*

When added outside, atropine increases both net flux of water and potential difference. If, however, 2-PAM is added first, the net flux of water remains unchanged while an effect on the potential difference can be demonstrated (Fig. 6).

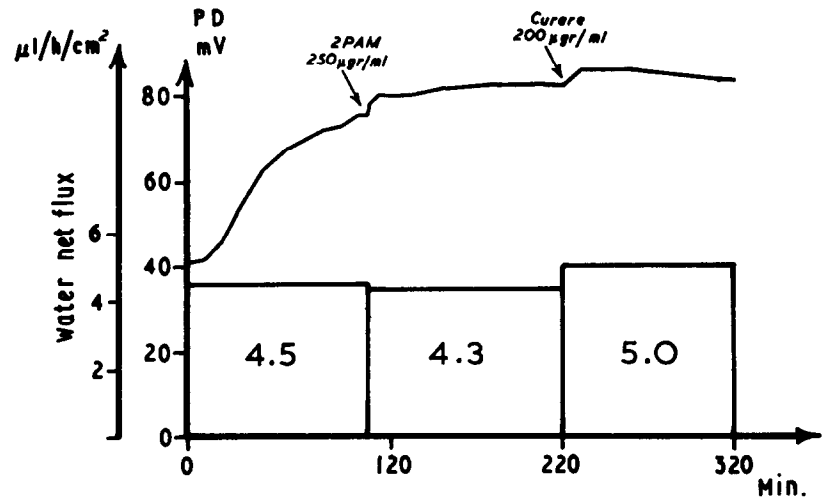

FIG. 5. Action of 2-PAM (250 µg/ml) and curare (200 µg/ml) on the potential difference and the net flux of water. Curare and 2-PAM are added outside.

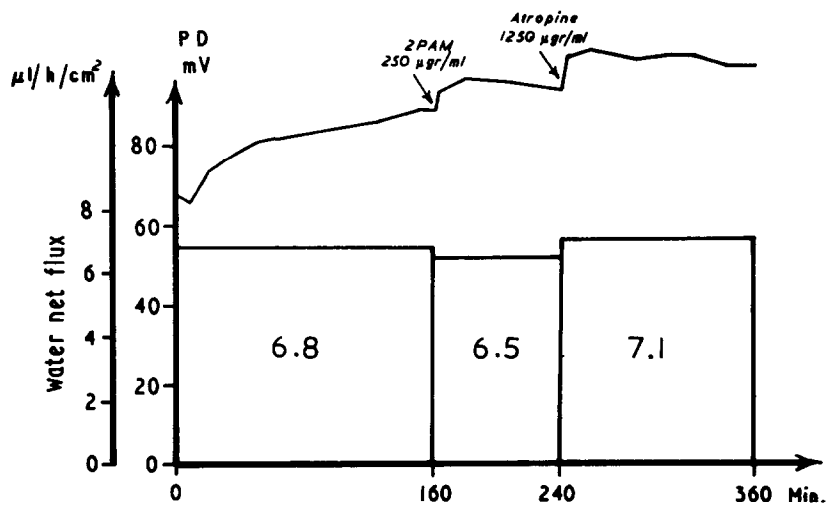

FIG. 6. Action of 2-PAM (250 µg/ml) and atropine sulphate (1250 µg/ml) on the potential difference and the net flux of water. 2-PAM and atropine sulphate are added outside.

7. *Action of curare and ADH*

As shown in Fig. 8, the increase in net flux of water produced by curare remains practically unaltered after application of ADH. On the contrary, the potential difference is modified (Fig. 8).

If curare is added after ADH, all the results obtained show that the net flux of water is slightly increased. Fig. 9 exemplifies the results of a typical experiment. After addition of ADH, the net flux of water goes from 1·5 to 8·1 μl/hr per cm^2. A subsequent adjunction of curare brings the value to 10·7 μl/hr per cm^2. It can also be seen, by comparing the results presented, that while the potential difference always increases under the influence of the compounds used, ADH is nevertheless more effective in this respect.

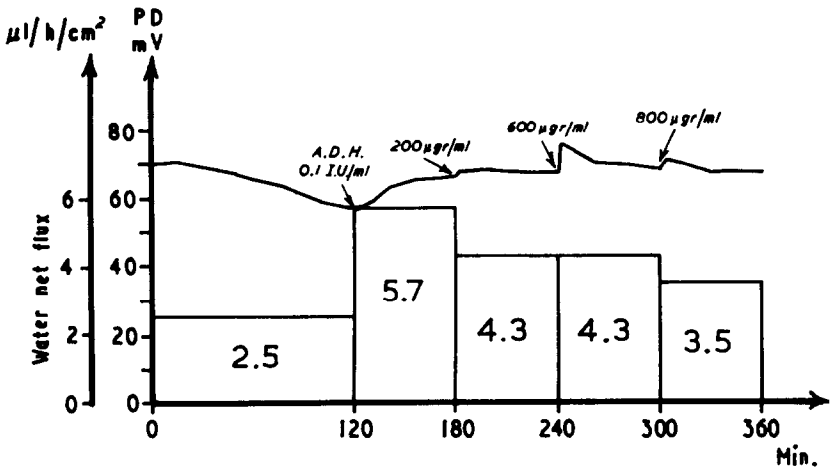

FIG. 7. Action of ADH (0·1 IU/ml) and 2-PAM (200–800 μg/ml) on the potential difference and the net flux of water. ADH is added inside, 2-PAM outside.

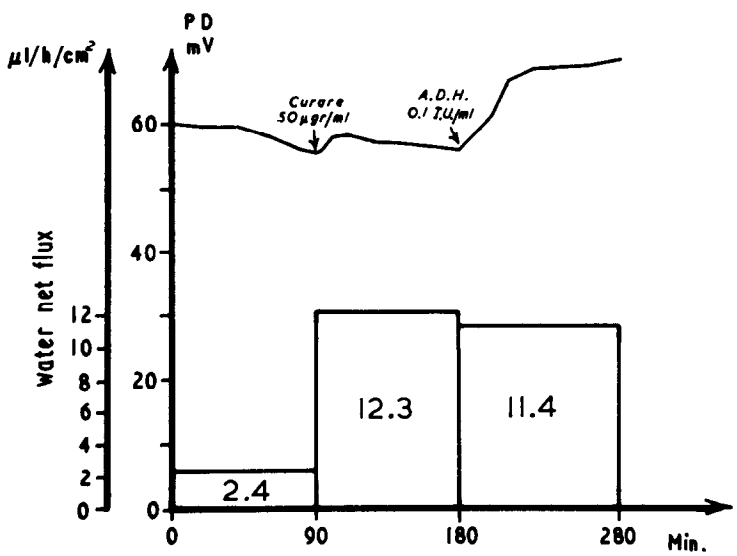

FIG. 8. Action of curare (50 μg/ml) and ADH (0·1 IU/ml) on the potential difference and the net flux of water. Curare is added outside and ADH inside.

DISCUSSION

To explain the effect of ADH, curare and other quaternary nitrogen derivatives, it has been proposed that they act by increasing the passive permeability to Na of the cellular membranes facing outside.[5-7, 14] If one considers how an electrical potential difference is developed across the frog skin, it is easy to understand how an increase in the passive permeability to Na of the cellular membranes facing outward can

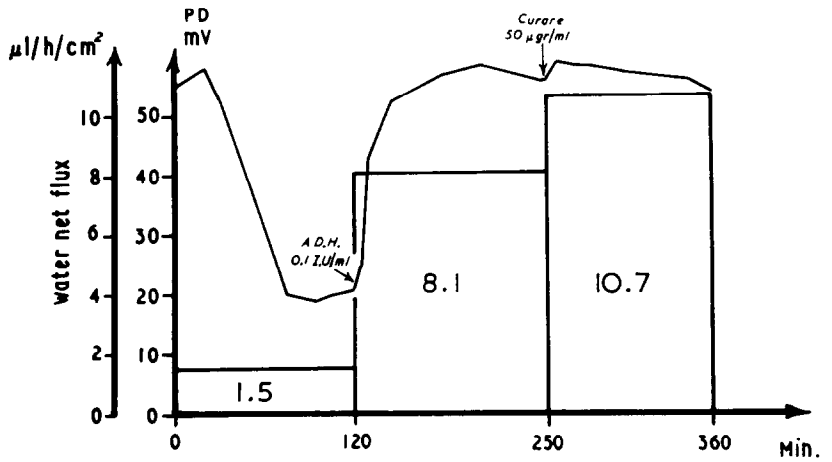

FIG. 9. Action of ADH (0·1 IU/ml) and curare (50 µg/ml) on the potential difference and the net flux of water. ADH is added inside and curare outside.

increase the potential difference. It has indeed been proposed that the potential difference existing across these membranes is related to the Na concentration in the following way[15]:

$$E_o - E_c = \frac{RT}{zF} \ln \left(\frac{P_{Na}\,(Na)_o + P_{Cl}\,(Cl)_c}{P_{Na}\,(Na)_c + P_{Cl}\,(Cl)_o} \right) \qquad (1)$$

where $E_o - E_c$ is the potential difference existing between the outside solution and the intracellular fluid, R the gas constant, T the absolute temperature, z the valence of the ion, F Faraday's number, P_{Na} and P_{Cl} the coefficients of relative permeability of Na and Cl respectively, (Na) and (Cl) the concentrations in Na and Cl, the subscripts o and c refer to the outside and intracellular solutions.

It is thus apparent that an increase in P_{Na} relative to P_{Cl} will induce an increase in potential difference. We have indeed shown[5] that if Cl is replaced by a non-penetrating anion (SO_4 for instance) there is no increase in potential difference after application of a quarternary ammonium derivative in the outside solution. This result, however, does not exclude the possibility that this class of compounds decreases the permeability to Cl.

An increase in P_{Na} should bring about an increase in intracellular Na concentration which in turn, stimulates the active transport mechanism localized, as proposed by Koefoed-Johnsen and Ussing[15], in the cellular membranes facing inwards. Using the toad bladder (personal communication from Dr. A. Leaf), Leaf *et al.* have demonstrated that the intracellular Na concentration increases after application of ADH.

Thus in this interpretation the effect observed on the active transport of Na is related to the increase in intracellular Na concentration the compounds used being without direct action on the mechanism of active transport. This interpretation is at variance with the one advanced by Bourguet and Maetz[16] who proposed that ADH could also stimulate directly the active transport mechanism for Na, since the neurohypophyseal peptides are known to increase not only the net flux of water arising under the influence of an osmotic gradient but also the net flux of Na.

As shown by our results, curare systematically increases the net flux of water, while the effect on the potential difference is less predictable, at least with *Rana temporaria*.[5] On the contrary 2-PAM is without any effect on the net flux of water but systematically increases the potential difference. These results strongly suggest that at the outer boundary of the cell Na and water move through loci affected differently by the compounds used, and that the increase in passive Na permeability is not directly related to a similar increase in water permeability. If for instance, we consider the results obtained by using curare at various concentrations it is apparent that the maximum increase in net flux of water is rapidly obtained while the potential difference still increases as the concentration of curare increases (Figs. 1 and 2). This is also evident if one considers the results obtained using ADH (Fig. 3).

This interpretation finds an experimental support in the results obtained when using two compounds. Figs. 4, 5 and 6 show that, in the case of ADH, curare and atropine, 2-PAM inhibits the effect of these compounds on the net flux of water but not on the potential difference. It would seem therefore that 2-PAM occupies the sites responsible for the increase in water permeability without being able to activate them, while still preventing another activator of these sites, e.g. curare, ADH, atropine, from acting. If this interpretation is correct, by varying the concentration of 2-PAM it should be possible to reverse the effect of an activator of the site responsible for the flux of water. Fig. 7 shows that this is indeed the case. 2-PAM added after ADH still produces an increase in potential difference while the net flux of water is reduced almost to the control value. If compounds having the same effect on both net flux of water and potential difference are used together (curare and ADH for instance) the effect observed can be interpreted as being a matter of compound concentration and relative affinity of the sites in the membrane for the molecule used (Fig. 8). We need, however, more data before being able to express quantitatively, i.e. in terms of dissociation or association constants, the relative affinity of various compounds used for the cellular structures involved.

From the results discussed here it may be proposed that in the frog skin, the molecular architectures responsible for the permeability characteristics to Na and water at the outside membranes, are spatially separated and affected differently by a large number of pharmacodynamically active agents. Some compounds (2-PAM, 2-PAD), while able to occupy the two sites, are, however, unable to activate these sites equally. Other compounds (i.e. atropine, curare, pilocarpine) are good activators of both sites. Compounds of the first series seem to compete for the same sites with the other class of compounds since 2-PAM, a poor activator of the site responsible for water movement, is, however, able to prevent the action of ADH, a good activator of this site.

The hypothesis proposed here explains satisfactorily the results presented some years ago by Barker-Jørgensen[17]. This author has shown that during moulting the

permeability of the frog skin to water increases by a factor of 3–4 while that to Na increases by a factor of 20, thus suggesting that the movement of water and Na are not necessarily linked.

Considering now the results obtained with a great variety of cells and tissues, it would seem that different ionic species move across a membrane through separate channels: in conducting cells for instance, all the results obtained so far suggest that during activity Na and K move through different sites and more recently, it has been shown[18] that ADH affects differently the movement of ^{32}P and ^{86}Rb through the mesentery of the rabbit. It would thus seem that it is a general characteristic of living membranes that each ionic species moves through a separate channel.

As far as the mechanism of action is concerned, little can be said. In our interpretation we assume that the compound involved is bound to the structural elements directly responsible for the specific permeability characteristics of the membrane. Other interpretations are, however, possible. In a recent short communication, Orloff and Handler[19] propose that the effect of ADH on both water and Na movements could be due to the production by the cell of an intermediary substance, 3′:5′-AMP, which in turn would be responsible for the changes in permeability observed. Thus in this interpretation the same molecule, 3′:5′-AMP, induces the change observed. If this view is in agreement with some of our results, it is, however, difficult to reconcile it with other of our findings, as well as with those of Bourguet and Maetz[16]. 2-PAM increases only the potential difference and not the net flux of water. If its action is interpreted in terms of the formation of an intermediary substance, we should then have to postulate that this intermediary is not the same as the one produced under the influence of ADH, or that 2-PAM has a double effect: production of an intermediary, and competition with this intermediary for one common site (the structure responsible for the water movement).

Until more information becomes available, we prefer to think in terms of complex formation between the compound used and some structural elements of the membrane directly related to the permeability characteristics.

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