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The State of Water in the Outer Barrier of the Isolated Frog Skin

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Summary. The flux of water across the outer barrier of the frog skin is generall regarded as the rate-limiting step in the movement of water across the whole membrane This paper presents some evidence that, at room temperature, the flux of water acros the outer barrier occurs through water in a non-liquid state. The organization of water i a non-liquid state lowers the diffusion coefficient of water through water by severa orders of magnitude. The study employs a method recently developed in this laborator which permits measurement of unidirectional fluxes at the outermost part of an epithelia membrane mounted as a flat sheet. Only above 25 °C is the activation energy for the flo of tritiated water (4.3 kcal mole^{-1}) similar to the one observed in free water (4.6 kca mole^{-1}). At temperatures around 15 °C, the energy of activation is 8.5 kcal mole^{-1}. A temperatures near 0 °C, at which the frog lives only part of the year, the energy o activation is 16.7 kcal mole^{-1}.

The balance of water of amphibia depends to a great extent on th ability of the skin to regulate the movement of water under a variety o osmotic and vapor-pressure gradients (Machin, 1969). One of the mai barriers to water movement across epithelial membranes is assumed to b located at the outer anatomical border (MacRobbie & Ussing, 1961; Lea & Hays, 1962). However, it is difficult to evaluate the permeability to wate of this particular barrier on the basis of transepithelial measurements per formed in steady state (Hays, 1968). Rotunno, Vilallonga, Fernández an Cereijido (1970) have recently developed a method to evaluate the uni directional flux of Na from the outer bathing solution into the epithelium (J_{OT}). This method is employed in the present study to obtain informatio on the penetration of water across the outer border of the frog skin.

Studies on the state of water in biological systems indicate that a larg fraction may be not in a free liquid state but in a more structured one (*se* Fogg, 1965; Whipple, 1965). Whereas the diffusion coefficient of water a

25 °C is 2.4×10^{-5} cm^2 sec^{-1} (Wang, Robinson & Edelman, 1953), the diffusion coefficient in the highly structured lattice of ice is 1.0×10^{-10} cm^2 sec^{-1} (Kuhn & Thürkauf, 1958). The fact that a change in water structure may change the diffusion coefficient by several orders of magnitude suggests that the organization of water in a membrane may play a considerable role in the permeation of water. Also, many peculiarities of a membrane's permeability might in fact be a consequence of a variation in the state of the water it contains. Studies carried out by Hays and Leaf (1962), for instance, suggest that the movement of water across toad urinary bladder occurs through channels containing structured water, and that the enhancement of water permeability elicited by neurohypophysial hormones involved the melting of such structures. The present study indicates that the low permeability to water of the outward-facing membrane may be due to the fact that, at room temperature, this membrane contains water in a non-liquid state.

Materials and Methods

The material used was the abdominal skin of the frog *Leptodactylus ocellatus* (L. 1758). All the experiments were carried out over a period of two months (May and June) corresponding to late autumm. Animals of both sexes were kept in moist sinks and had access to water which was previously gassed with compressed air for 2 days. The skin was mounted as a flat sheet between two lucite chambers with an exposed area (rectangular) of 8.9 cm^2. The chambers contained identical Ringer's composed of 115 mM NaCl, 2.4 mM $KHCO_3$, and 1.0 mM $CaCl_2$. The temperature at which each experiment was run was achieved by mounting the whole setup inside a cold or a warm room set at a given temperature. In this way, not only the membrane and the solutions, but the chambers, the dissecting tools, the syringes used to measure J_{OT} (*see* below), etc., were equilibrated at the same temperature. This temperature was recorded in the solution bathing the skin after the incubation period and prior to the measurement of J_{OT}. Once mounted, the skins were left in the chambers for about 20 min. The chambers were then emptied, and the J_{OT} was measured with the technique described by Rotunno *et al.* (1970). The basic principle of this technique is as follows. Once the chambers are thoroughly emptied, a dual simultaneous-infusion pump delivers Ringer's solution containing tritiated water (THO) into the chamber in contact with the outer side, and nonradioactive Ringer's into the chamber in contact with the inside. The solutions are injected into the bottom of the chambers, and the level rises steadily, reaching the upper border in 48 sec. When the solution reaches the upper border, the exposure is quickly interrupted and the skin is plunged deep, first in cold isotonic sucrose solution for 1 sec, and then into a thermos flask containing liquid nitrogen (− 196 °C). In this way, the lower border was exposed for the whole length of the infusion (48 sec). All other parts of the skin were exposed for a fraction of 48 sec. This fraction depends on the distance to the floor of the chamber. The exposed part is cut into 10 transverse sections, each representing an uptake time of THO. While still frozen, each sample is put in a plastic test tube with 2.0 ml of distilled water covered with Parafilm (Marathon) and left to extract in a shaker for more than 12 hr. Two samples of 500 μliters were withdrawn, put in 10 ml of liquid scintillation solution (Bray, 1960), and counted in a Packard Tri-Carb Scintillation Counter. Two

samples of 500 μliters of the loading solution were also counted. This permitted calculatic of the amount of water taken up by each piece of skin. The slope of the curve relati the amount of water taken up per square centimeter and the exposure time gives tl flux of water in μliters per square centimeter and per second.

Results and Discussion

Fig. 1 shows the experimental points obtained in representative exper ments at several temperatures. Points obtained at lower temperatures fa in a straight line during most of the experimental period (50 sec). Th points obtained at higher temperatures after 25 to 30 sec tend to fall belo the initial rate of uptake, indicating that the back diffusion of tracer (cell - outer solution) is no longer negligible. Accordingly, only the initial five c six points are used to compute the flux. As can be seen in Fig. 1, even at th highest temperatures used, an acceptable straight line is obtained.

The permeability coefficient is defined as

$$P \equiv J_{OT}/C_O - C_T .$$

As mentioned above, the fact that in the earlier part of the uptake th experimental points fall in a straight line indicates that during this perio $C_O \gg C_T$. Therefore, P is calculated as the value of the tracer flux divide by the specific activity of THO in the outer Ringer's solution.

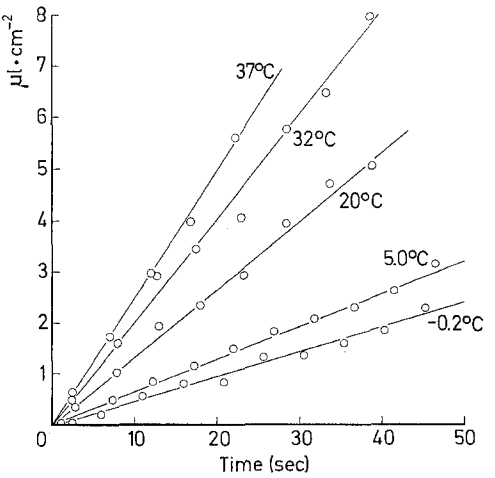

Fig. 1. The uptake of water as a function of time in frog skins equilibrated at tempera tures ranging from −0.2 to 37.0 °C. In the curves at 20, 32 and 37 °C, the latest point are omitted. They would fall below the straight line

The Effect of Unstirred Layers

Solid bodies immersed in fluid become surrounded by a static liquid film: the unstirred layer (Noyes & Whithey, 1897; Nernst, 1904). Although the rate of injection of Ringer's into the chambers insures efficient mixing, the movement of water in the unstirred layer occurs by diffusion. It follows that the diffusional flux J_{OT} might be controlled or influenced by diffusion across the unstirred film. The role of the unstirred layer becomes important when the diffusion coefficient in the membrane is high, when the membrane is very thin, or when the stirring is poor (Helfferich, 1962). The relationship between the measured permeability (P_{meas}) and the "true" permeability of the membrane (P_{true}) is given by the following equation (Dainty & House, 1966):

$$\frac{1}{P_{meas}} = \frac{1}{P_{true}} - \frac{\delta}{D}, \tag{1}$$

where δ is the thickness of the unstirred layer, and D is the diffusion coefficient of THO in water. According to this equation, the difference between P_{meas} and P_{true} will be larger at high P_{meas} or at low D. The highest P_{meas} in the present study was that at 37 °C: 2.31×10^{-4} cm sec^{-1} (mean of four measurements). At this temperature, the diffusion coefficient D is 3.1×10^{-5} cm^2 sec^{-1} (Wang *et al.*, 1953). The value of δ for the outer unstirred layer of the frog skin was estimated as 25 to 50 μ (Kidder, Cereijido & Curran, 1964; Dainty & House, 1966). Therefore, under this condition, P_{true} would be 2.38×10^{-4} cm sec^{-1}, i.e., 3% higher than the measured permeability. The other situation in which the restriction in the unstirred layer becomes important is when D is low. One of the lowest values of D in the present studies was that at 5 °C: 1.39×10^{-5} cm^2 sec^{-1}. Under this condition, P_{meas} is 8.0×10^{-5} cm sec^{-1} (mean of four measurements), and P_{true} would be 8.11×10^{-5} cm sec^{-1}, i.e., 1.3% higher than P_{meas}. The largest number of observations at a given temperature was seven at 20 °C. This gave a value of $1.53 \pm 0.07 \times 10^{-4}$ cm sec^{-1} (i.e., it has a standard error of 4.6%). It means that the correction of 1.3 and 3% in the values of the permeabilities that would be required to correct for the unstirred layer fall within the experimental error, and therefore shall not be taken into account. The conclusion that the unstirred layer *on the outside* is not very influential was also reached by Hays and Franki (1970) who studied the effect of stirring in the diffusion of water in toad urinary bladder.

The Effect of Temperature on the Diffusional Flux of Water

Fig. 2 shows the values of the ln P as a function of the absolute temperature T. Each point represents a single experiment, which in turn re-

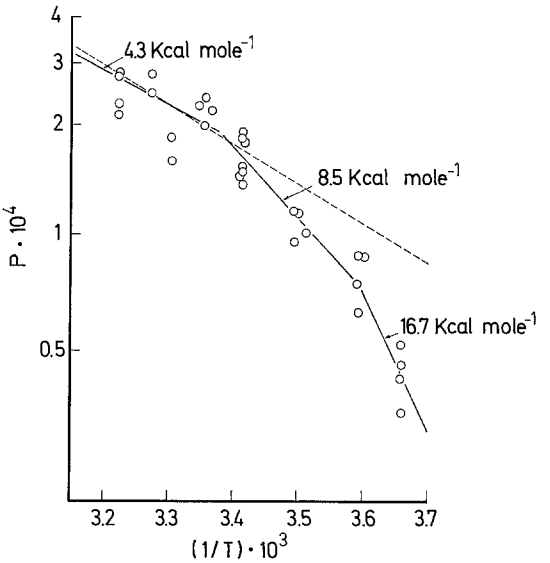

Fig. 2. Logarithmic plot of P against $1/T$ for the skin of *Leptodactylus ocellatus*. Each point represents a single determination. The straight lines for a given set of observations were fitted by the method of least squares. The dashed line corresponds to pure free water

presents 6 to 10 periods of uptake in the same skin. The points depart considerably from a single straight line, suggesting that the state of water or the state of the membrane (or both) is not the same throughout the range of temperature studied. The straight lines of Fig. 2 were fitted by the method of least squares. They represent an apparent activation energy of 4.3, 8.5, and 16.7 kcal mole^{-1}, respectively. The apparent activation energy of THO diffusion in free solution is 4.6 kcal mole^{-1} (Wang *et al.*, 1953). An apparent activation energy closely related to this value is generally taken as an indication that the water flux proceeds through a diffusion pathway containing water in free solution. Conversely, an activation energy larger than this value is assumed to indicate that the state of water is more structured than in free solution, and that energy should be spent to create vacant lattice points where the migrating molecules could jump. The activation energies in experiments carried out at 25 °C or higher are close to those corresponding to the flow of THO in free solution. Activation energies in this range are also observed in human erythrocytes (Jacobs, Glassman & Parpart, 1935), dog erythrocytes (Vieira, Sha'afi & Solomon, 1970), and lobster nerve (Nevis, 1958). The activation energies for water flux occurring below 25 °C are considerably greater than the ones in free solution. It is interesting to notice that the frog *Leptodactylus ocellatus* lives most of the year below this temperature.

Activation energies higher than necessary for the flow in free solution were also found in eggs of *Arbacia punctulata* (Lucke & McCutcheon, 1932), sheep erythrocytes (Widdas, 1951), Ehrlich ascites tumor cells (Hempling, 1960), toad bladder (Hays & Leaf, 1962), and plant cells (House & Jarvis, 1968).

The freezing pattern of cellular water (Ling, 1969), its spectra of nuclear magnetic resonance (Chapman & McLaughlan, 1967; Fritz & Swift, 1967; Cope, 1969) the chemical activity of intracellular Na (Hinke, 1959), and the nuclear magnetic resonance of Na in several tissues (Cope, 1967), in particular the frog skin (Rotunno, Kowalewski & Cereijido, 1967; Reisin, Rotunno, Corchs, Kowalewski & Cereijido, 1970), indicate that the compartements in the cell do not seem to contain an entirely free solution. Since the pioneer work of Schmitt, Bear and Ponder (1936), the cellular membranes which limit those compartments have been found to possess a highly ordered structure (Finean, 1962). Therefore it is not surprising that the water channels in biological barriers should contain non-liquid water.

Fedyakin (1961) has demonstrated that the water contained in capillaries with radii of the order of 200 Å and less has a more compact structure than free water, and that its thermal expansion curve does not show breakups as it does in the bulk. This was taken as an indication that the structure of the water inside small capillaries does not rearrange as the temperature is varied within certain limits. If extrapolations to biological systems are valid, Fedyakin's observation would suggest that the structural changes in the aqueous channels of the outer barrier would not originate in the water of the channels, but would be a consequence of structural changes in the other components of the cell membrane and the cytoplasm. In this connection, it may be of interest that the several delicate crystal-like structures formed by lipidic components of the cells suspended in water can switch from one configuration to another as the temperature is changed (Luzzati, 1968). The views expressed here rest on the assumption that water penetration involves water-filled pores. An alternative explanation would be provided by a change in the solubility of water in the membrane.

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