Fluxes and Distribution of Sodium in Frog Skin

A new model

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The exchange of substances between higher organisms and the environment takes place at the level of epithelial membranes constituted by one or more layers of cells. Experimental models (17, 31, 50) and theoretical analysis (13, 45) generally assume that any molecule going across an epithelial membrane must traverse at least two plasma membranes: one to get into the epithelial cells and another to get out at the opposite side of the cell. Models constructed on this basis have been helpful in understanding how the intestine absorbs amino acids, how urine is formed in the kidney, or how the gall bladder concentrates the bile. However, it is increasingly difficult to reconcile the predictions of those models with the information obtained in the last few years. At the center of the problem is the remarkable ability of the epithelial membranes to transport Na^+ (Huf, reference 25) and the coupling of this phenomenon to the movement of almost any substance capable of undergoing net transfer in absence of a difference of electrochemical potential gradient (7, 10, 48). Studies carried out in our laboratory on the movement and distribution of Na in the frog skin led us to develop a working model, and it is our purpose to present here some of its features. Our discussion will be divided into two parts. In the first one we will analyze some of the properties of the transcellular models and show that they are at variance with certain experimental observations and even teleological expectancies. In the second we will present our model, discuss part of the experimental justification and try to explain from our point of view some very well-known phenomena that are hardly understood on the basis of the transcellular models.

TRANSCELLULAR MODELS

According to these models (17, 50) (Fig. 1), Na movement and distribution have three main characteristics: (*a*) Na is supposed to be homogeneously distributed in a unique cellular compartment owing to essentially free diffusion from cell to cell through zonulae and maculae occludentes; (*b*) Na is assumed to enter, by a passive mechanism from the outside bathing solution, into the cell compartment through an outward facing membrane located at the outer anatomical border of the epithelium; (*c*) the active step in Na net movement is assumed to be located at the bound-

ary between the cells and the interspaces which are in turn directly connected with the inner bathing solution. This model explains satisfactorily the Na-transporting properties of a frog skin mounted between two identical Ringer's solutions under short circuit conditions (51). Yet one wonders whether an explanation so useful to the biophysicist could also be of some help to the frog.

In 1937 Krogh (32) observed that frogs are able to take up sodium chloride from

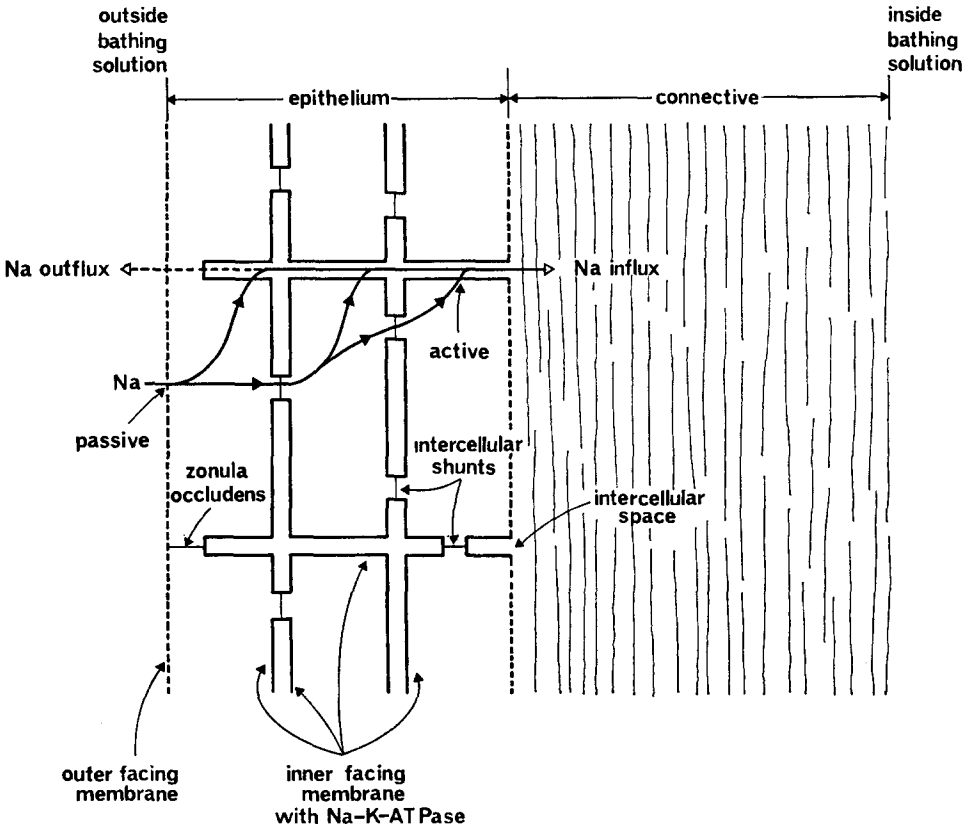

FIGURE 1. Transcellular model of distribution and movement of sodium across frog skin. See text.

the surrounding medium even if the Na concentration in the latter is as diluted as 10^{-5} M. The transcellular models would predict that, since Na enters the cell compartment by a passive mechanism, the concentration in the cells should be lower than 10^{-5} M.[1] Since this Na concentration seemed unreasonably low, Rotunno, Pouchan, and Cereijido (41) carried out a series of studies of the Na concentration in the epithelial cells of the frog skin bathed on the outside by a solution containing 5–10 mM Na. The conclusions were (*a*) that under those circumstances, the concen-

[1] Krogh did not use a short circuit technique that would have made the electrical potential of the cell compartment more negative with respect to the outside bathing solution.

tration of Na in the cells was 50–100 mM and (*b*) that if all the Na is contained in a unique cellular compartment as required by the transcellular models, then the large difference in concentration was not explained on the basis of a passive distribution across the outward facing membrane and required an active step. However they also studied the distribution of Na-K–dependent adenosine triphosphatase (Na-K–ATPase) and concluded that, if the stoichiometrical relationship between molecules of ATP split per Na^+ ion transported is one-third, there is not enough Na-K–ATPase at the outer border to account for the active transport at that level. It is interesting to note that electron microscope studies carried out by Farquhar and Palade (17) indicated that at the outer border there is no ATPase at all, Na-K sensitive or not.

To solve this somewhat puzzling situation Rotunno et al. suggested that Na in the epithelium was contained in at least two different compartments, one of them being directly involved in Na^+ transport. This suggestion has since been proved by studies of Cereijido and Rotunno (5) in which in 90 min only 37% of the Na in the cells exchanged with ^{22}Na in the Ringer's solution (^{22}Na flux across the frog skin equilibrated in less than 30 min). Rotunno, Kowalewski, and Cereijido (40) carried out an analysis of the nuclear magnetic resonance of the Na contained in the frog skin and demonstrated that only 39% of the total Na is free as Na^+ ion and that the rest is bound. If only 37–39% of the Na in the epithelium is free as Na^+ ion there is no problem in explaining the high Na concentration in the epithelium when it is bathed by 10 mM Na on the outside. However, this conclusion was based on some assumptions. To replace them by experimental evidence we developed a technique that makes it possible to calculate the concentration of Na from the amounts of Na and H_2O measured directly in the epithelium.[2] In tissues bathed by solutions with 1 mM Na on the outside the concentration of Na in the epithelium was 52.5 ± 0.3 mM (26 experiments). The ^{22}Na-distribution method used indicated that 70.5 ± 2.7% (28 experiments) of this Na was exchangeable. It means that the Na concentration in the transporting compartment should be as high as 37 mM.[3] The concentration predicted on the basis of a passive distribution is 2 mM, i.e., one order of magnitude lower. We have also shown that only 8–13% is directly involved in ion transport and that the transporting compartment as well as the nontransporting compartments are distributed throughout the whole epithelium (5). We need then a model with an Na-transporting compartment distributed over the whole epithelium but with an active step at the outward facing membrane. The need of a new model arises also from the following considerations.

(*a*) Ussing (48) and Franz and Van Bruggen (18) have demonstrated that by making the outside bathing solution hypertonic it is possible to produce net influx of several substances that otherwise behave passively. Where does the driving force arise? Solvent drag may be discarded because, as proved by Franz and Van Bruggen (18), the hyperosmolarity of the outside bathing solution produces a net water flux oriented in the opposite direction from the flux of the solute undergoing net transfer. Franz and Van Bruggen suggested the possibility that the same high concentration

[2] Cereijido, M., C. A. Rotunno, and I. Reisin. *J. Physiol. (London)*. In press.

[3] This is a minimun estimate which assumes that the trasporting compartment is a chemical compartment and Na is diluted in the water of the whole cell. If, on the contrary, it were a physical compartment the amount of water would be lower and the concentration of Na higher.

of solute that makes the outer bathing solution hyperosmolar might elicit first an increase in the intercellular gap by shrinking the epithelial cells and then by diffusing its concentration gradient. Under these conditions almost any other molecule added in tracer amount to the outside bathing solution may show a net transfer. This was attributed to a drag of the molecules added in tracer amounts by the hyperosmolarity-making molecules.

Two important characteristics of this phenomenon are that both the hyperosmolarity-making molecule and the molecule undergoing net transfer remain extracellular (18) and that, as Ussing (48) clearly demonstrated, the presence of Na is of absolute necessity to produce the hyperosmolar-induced net transfer of small solutes. It stops not only if Na is removed from the outside solution but also if the active transport of Na is inhibited with metabolic poisons. Let us summarize the situation: by making the outside bathing solution hyperosmolar one may produce a net movement of almost any molecule small enough to penetrate the zonula occludens; although this net flux remains extracellular, it is coupled to the Na-active transport that is assumed to occur across the cytoplasm of the cells. We suggest that this coupling would be better understood if the route of the Na transport were close to the extracellular space.

(*b*) There are many circumstances in which an epithelial membrane may lose its ability to transport Na and yet maintain its electrochemical balance (11, 26, 34). According to the transcellular models it is difficult to explain how the pumps that should take care of both functions would tell one function from the other.

(*c*) Table I shows the value of fluxes of Na in different biological systems. One point is immediately evident: Na seems to cross epithelial membranes more easily than single plasma membranes. This might be just fortuitous but, if transcellular models are correct, how is it that Na^+, or any other ion. whose ability to cross a lipid bilayer is so poor (Vreeman, reference 52) goes so easily across *two* plasma membranes?

A MODEL OF Na TRANSPORT

In trying to give an interpretation to our data and to those phenomena mentioned above, we have developed a new model (Fig. 2). In this part we will enumerate its properties and, at the same time, give some justifications for the choice and show the advantages they bring.

(*a*) The polar groups of the outer leaflets of lipids form a system of fixed charges (Eisenman, Rudin, and Casby reference 14; Ling, reference 37) with high selectivity for Na and Li and with low selectivity for K, Cs, and Rb.

(*b*) When an ion from the outside bathing solution reaches the outward facing membrane, the tendency to penetrate the bilipid membrane and to get into the cell is very small compared with the tendency to travel tangentially by jumping from fixed polar group to fixed polar group in a triplet saltatory route, i.e., the ion will go around rather than penetrate the cell. We favor this route because it presents lower energy barriers and because the rate of triplet formation, being proportional to the absorption energies of the ions, would exert discrimination among different cations (Ling, reference 36).

(*c*) The ions may travel until they reach the zonula occludens area. This area constitutes a narrow path in which the low selectivity of the cell surface for certain ions becomes a serious restriction to their diffusion. The order of selectivity (permeability) of this path is Li > Na ≫ K (29, 35).

(*d*) Ions on the outer leaflets of the inner-facing membrane of a cell may reach the outer leaflet of a neighbor cell by crossing at the level of the desmosomes. Kelly (27), working with newt skin, has presented some evidence that the extracellular material of the desmosomes is arranged as pillars or partitions which are continuous with or layered upon the outer unit cell membrane. Some preliminary observations carried out at our laboratory have indicated that in the frog skin the outer leaflet

TABLE I
UNIDIRECTIONAL FLUXES OF SODIUM*

	pmole sec^{-1} cm^{-2}	References
Red blood cells (human)	0.11	Glynn (20)
Abdominal muscle (frog)	5	Keynes (28)
Sartorius muscle (frog)	10	Keynes (28)
Nerve (Sepia and Loligo)	33	Hodgkin and Keynes (23)
Urinary bladder (toad)	140	Frazier and Leaf (19)
Skin (frog)	834	Zadunaisky, Candia, and Chiarandini (55)
Skin	415	Curran and Gill (12)
Skin	390	Ussing (46)
Ileum (rabbit)	2760	Schultz and Zalusky (42)
Ileum (rat)	1330	Curran (9)
Cecum (guinea pig)	2160	Ussing and Andersen (49)

* Taken in the active direction. *Outfluxes:* red blood cells, muscles and nerve. *Influxes (or mucosal to serosal side)*: urinary bladder, skin, ileum, and cecum.

may constitute a continuum throughout which an ion may diffuse without leaving the cell membranes. In this way Li and Na may travel all around the surface of the epithelial cells. K, although present in far lesser amounts, may ramble around the outer or inner membranes; it cannot go from one to the other because that would require its passing through the tight junctions. This view is supported by studies of the space constants carried out by Courtney (6); these indicated that the Na current travels though a syncytial region.

(*e*) The outer nonlipid leaflet covering the innerfacing membrane, forms a layer of fixed charge which is highly selective for K and prevents Na from passing. Na can only cross this barrier in the inward direction by an active mechanism. This membrane would thus behave as a Na-impermeable, K-sensitive barrier (39).

(*f*) The pumps are located on all the cell membranes facing the labyrinth of intercellular spaces of the epithelium (17). If the ion traveling on the outer surface of the cell happens to have affinity for the pumps, then it may be helped to leave the polar groups, to cross the Na impermeable barrier, and to reach the interspace. Na fulfills this requirement and therefore will be pumped; Li will not. Even when Li can enter the epithelium (21), it might not be pumped and will accumulate in the

surface. It would be worth noticing that, although Li can, to a certain extent, activate the Na-K-ATPase implicated in active transport, the addition of other cations (K for instance) leads to an inhibition of this activity (43). From this point Li

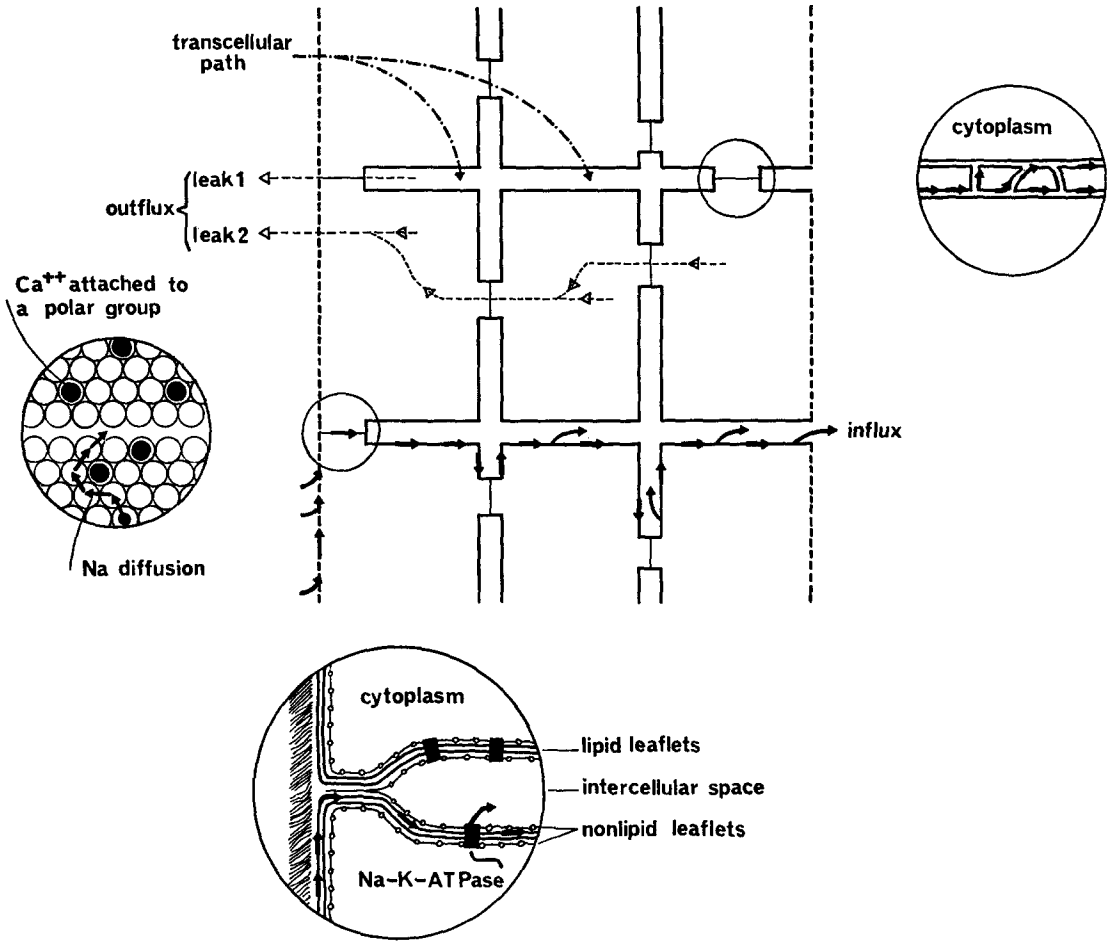

FIGURE 2. Model of distribution and movement of sodium across frog skin. Sodium attaches to fixed charges on the outward facing membrane and diffuses by jumping from one fixed charge to the other (circle on the left). The path might be blocked by ions that are not necessarily permeable. Sodium crosses the zonula occludens and reaches the inner facing membrane of the outermost cell layer. Sodium can also reach the membranes of other cells by diffusing on the extracellular side of the intercellular bridges (circle on the right). The polar groups where Na migrates are separated from the intercellular space by a Na-impermeable barrier (not shown in the figure). Pumps on the inner facing membrane translocate sodium from the membrane to the intercellular space across the Na-impermeable barrier (circle at the bottom). Under certain conditions, (sodium concentration outside relatively high, intracellular electrical potential negative with respect to the outer solution, presence of ADH, etc.) the transcellular path might play a role.

could diffuse or enter the cell and accumulate. Since the Na-selective polar groups are contained between two barriers of low Na-permeability, the site left by a pumped Na can only be refilled by sidewise migration. The over-all effect is to move the empty site towards the outerfacing membrane where another Na can be adsorbed. Mechanical water pumps have the *tip* of the pipe at the bottom of the well, not the motor. Likewise we envision the mechanism for pumping ions *across* the skin as one whose power units are all over the epithelium but whose action is manifested at the outward facing membrane. Li can enter the pipe but will not be pumped. For a very short while, though, it may contribute to the short circuit current (in this connection see Kidder, Cereijido, and Curran, reference 29). This pumping mechanism would offer an explanation of the previously mentioned, puzzling finding of Rotunno et al. (41) that, while the electrochemical potential gradient between the sodium in the outer solution and that in the cells is not explained on the basis of a passive distribution and requires an active step, the pumping mechanism is distributed over the whole epithelium.

(*g*) When current is passed in the inward direction, it will be easily carried by the Na ions. Current in the outward direction will run until the pore gets stuck with K ions that are continuously going across the inner facing membrane. The resistance will then increase. Candia's observations that the frog skin behaves like a semiconductor (2) lends some support to this point of view.

(*h*) Although the zonula occludens might be reached directly, most ions will absorb first at any place in the surface to be in turn funneled into the pore.[4] Owing to high preference of Na over K, this ion will only affect the mechanism at high K and low Na concentrations on the outside bathing solution. This view may explain Ussing's observation that, if a solution with 100 mM K and 0 Na is used on the outside, the inward resistance of the skin increases slowly, while replacing the high K solution for one with high Na decreases the resistance rapidly (48). According to our model, in the first step it will take some time for K to wash away Na from the fixed charges. In the second step Na will easily retake the polar groups where it is preferred.

(*i*) Swelling decreases the size of the pore until Na passage at this level acquires the characteristics of a single file diffusion. Movement of any molecule (even Na) going in the outward direction will be severely restricted. Shrinking increases the pore size and permits more molecules to travel in the outward direction. The influx of Na will not suffer modifications although the increased outflux of Na decrease the short circuit current. This mechanism offers a different explanation of Ussing's observation (47) that shrinking decreases and swelling increases the value of the short circuit current but the Na influx does not change significantly. It also offers an explanation of the findings of Ussing (48) and Franz and Van Bruggen (18) that, provided the Na transporting mechanism is working, a hyperosomlar solution on the outside will produce a net transport of almost any small molecule present in the outside bathing solution. We suggest that a molecule small enough to enter the enlarged cell gap will be dragged along *by sodium*. This coupling between the solute flux and the

[4] These pores are not to be confused with those described by Whittembury (54).

conjugate force of the Na flux may also explain all the other relationships reported between the solute flux and Na (see Ussing, reference 48).

(*i*) The epithelial cells have mechanisms for regulating their K- and Na-concentration levels as any other cell in the body. In this respect they are relatively independent of the hazards of the environment. Their participation in the process of transport arises as a specialization that requires a further degree of organization and is, therefore, more vulnerable. Thus, in any situation the conservation of the steady state of the cell would prevail. If the supply of energy is made scant by dinitrophenol, fluoracetate, iodoacetate, azide, or diethyl malonate, the cells will lose their ability to transport Na *across* the epithelium but will maintain their ion composition until the metabolic inhibition becomes more intense (11, 26). It is also pertinent to note that certain xanthines may stop the net transport without disturbing the electrolyte balance of the cells (34).

(*k*) The fact that the route of Na transport of the model goes across the skin without entering the cytoplasm should not be taken to imply that the outward facing membrane of the epithelial cells is impermeable to Na. When the concentration of Na in the outer solution is high enough or the electrical potential inside the cell is made negative with respect to the bathing solutions by means of a short circuit current (3, 24, 54), the Na entry into the cell and subsequent extrusion toward the interspace may play a significant role in the over-all Na transport. It is conceivable that a substance capable of increasing the permeability of the outward facing membrane of the cells will put the "cell circuit" to work and thus enhance the net transport. This might be the role of the ADH. Note that, if this is so, the sites for ADH action and those for Ca^{++} action will be different but will be placed in parallel on the same layer as required by the evidence obtained by Herrera and Curran (22). The increased permeability of the outer membrane will permit water to penetrate into the cells where the concentration of Na is relatively high.

(*l*) The Na leak toward the outside bathing solution will have two components: an intercellular one that will follow the tight junction route (50) and a cellular one constituted by Na from the interspace that penetrates the cell, reaches the outermost cell layer via the intercellular shunts (38,50), and crosses its membrane toward the outer solution. The evidence, obtained by Cereijido and Curran (3), indicating that the electrical potential inside the cell remains unaffected despite 40-fold change in the Na concentration of the bathing solution and 5-fold change in the short circuit current suggests that, if the leak toward the outside really comes from the cell, it should be quite independent of the electrochemical potential gradient across the whole skin. This view is in keeping with Candia's observation that the Na outflux behaves as if an important fraction of it were independent of the electrochemical potential gradient (2).

As a corollary of the Na-transporting mechanism's making itself evident at the outer facing membrane while being distributed all over the epithelium, most substances capable of modifying the Na transport or the fraction of the electrical potential which depends on Na will appear as acting at the outward facing membrane (in this connection see Cereijido and Curran, reference 3, and Essig and Leaf, reference 15). It should also be noted that an array of electrogenic pumps (1, 11) mounted

on a conducting membrane as proposed here will give rise to an electrical potential profile that will increase continuously as the membrane is farther and farther from the outside bathing solution. This view might offer some basis for the interpretation of the challenging electrical potential profiles obtained by Snell and Chowdhury (44). This mechanism will also saturate at high concentrations of Na in the outside bathing solutions (4, 30).

In summary, we had to abandon the recent versions of the transcellular models mainly because they failed to explain our experimental data. We developed a new one whose essential feature is the location of the Na-pumping route in the plasma membrane of the epithelial cells rather than through their cytoplasm. It offers a possible explanation of how the membrane works not only when it is mounted between two chambers with Ringer's solutions but also when the frog is swimming in a pond with fresh water.

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