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Pivotal Ideas

Does Active Transport Exist?

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Introduction

When my teacher, Professor August Krogh, got the Nobel Prize for his work on the physiology of capillaries, he bought a summerhome in Lynæs, a small village on a fiord north of Copenhagen. Frequently on his holidays, he would walk down to the harbor to have a talk with the fishermen who came in with their catch. He noted then that, instead of bringing the fish to the auction hall right away, the fishermen usually transferred them to a "hyttefad," a floating wooden box with numerous small holes which allowed the harbor water to get in without allowing fish to get out. Asked why they used this routine, the fishermen answered that the harbor water was so nutritious that the fish would gain substantially in weight in the course of a few days. Krogh did not quite believe the explanation, but secretly thought it more likely that the fish which were caught in the salty Kattegat would take up water from the brackish fiord. In any case, the incident inspired him to initiate a series of studies on the handling of electrolytes by waterliving animals. The studies were carried out both by himself and by guest visitors to his laboratory. For references, *see* his Croonian lecture (Krogh, 1946). To summarize some of the important findings: salt-depleted frogs and goldfish (Krogh, 1937, 1938) could take up Na^+ and Cl^- from exceedingly dilute solutions. Both ion species must be subject to active transport since Na^+ can be absorbed from Cl^--free solutions and Cl^- from Na^+-free solutions. K^+, however, was never taken up from dilute media. Active transport obviously could only be observed with classical analysis, if the system had already been perturbed by salt-depleting of the animals. Krogh suspected, however, that active uptake by the species in question was always in operation, making up for inescapable loss due to diffusion. If so, the operation of such a "pump and leak" could only be observed with a suitable isotopic tracer. At that time the cyclotron had just been invented, making it possible to produce tracers for biologically important electrolytes such as Na^+, K^+, Cl^- and phosphorus. Krogh therefore found it imperative that our University should get a cyclotron to supply the biologists with traces. It turned out to be impossible to raise the necessary funds in Denmark, but thanks to a joint application from August Krogh and Niels Bohr to the Rockefeller Foundation, the cyclotron was built and became operational just when the Second World War started. Some of the earliest experiments, showing exchange of K^+ and Na^+ across the membrane of erythrocytes, were actually performed in Copenhagen by the inventor of the tracer method George Hevesy (Hevesy & Hahn, 1941). Professor Hevesy was at that time a visiting scientist in Niels Bohr's Institute and a personal friend of August Krogh.

The German occupation of Denmark in 1940 made experimental work in our laboratory increasingly difficult. In fact, both August Krogh and Niels Bohr had to leave the country to avoid being rounded up by the Nazis. Krogh, who stayed in Sweden, planned to continue his studies of active and passive ion transport with the aid of radioactive isotopes as soon as the war ended. He worked out a detailed application to the Rockefeller Foundation. Aided by a small group of young scientists, he intended to start the project as soon as the war was over.

Key words: Active transport — Flux ratio — Short circuit — Exchange diffusion

The Rockefeller Foundation was willing to finance the project, but the war lasted longer than Krogh had expected, and he retired from his professorship (and position as department head) in 1945. He therefore decided to find a senior scientist who could direct the project in its initial phase and asked me to take over. At that time, I had just obtained tenure at the Laboratory of Zoophysiology and was involved in studies of protein turnover in rats, using deuterium-labeled amino acids (Ussing, 1938). I was reluctant to leave this promising project. However, I realized that Krogh's project was important and must not go astray in the initial phase. I accepted the task and decided to join the project for a year or two. I was mistaken. It was for life.

As mentioned already, Krogh was convinced that active transport of at least Na^+ and Cl^- and very likely other ionic species did exist, but that view was by no means shared by all authorities. The physicochemists, especially, felt that Na^+ and K^+ are so similar that they could not be told apart by chemical bonding. On the other hand, the hydrated Na^+ ion was much larger than the hydrated K^+ ion. Permselective artificial membranes certainly could let K^+ pass much faster than Na^+. Thus, the distribution of the two ion species might be determined by the pore size of living membranes.

A beautiful model, describing the distribution of Na^+, K^+, and Cl^- between striated muscle (frog Sartorius) and the bathing medium, was developed by Boyle and Conway (1941). Their assumptions were that the fiber membrane had small pores, allowing K^+ and Cl^- (which have the same diameter) to pass freely, whereas the larger Na^+ was too big to pass. Furthermore, Boyle and Conway assumed that, initially, the Na^+ content of the fibers was low but constant, and that fibers have a content of nondiffusible anions (proteins, phosphate esters etc.). A double Donnan distribution of the diffusible ions would then define the distribution of the diffusible ions as well as the membrane potential (which was estimated from the injury potential, measured at the cut end of the fibers). The composition of the medium was varied over a wide range, and there was good agreement between experiments and theory.

The weak point of the Boyle-Conway theory was undoubtedly the assumption of an absolutely Na^+-tight cell membrane, and by the time we started our project there was evidence from experiments on muscle with tracer-Na that fiber Na^+ did exchange with Na^+ in the medium; Dean (1941) had proposed a model where the cell Na^+ was kept low by a "sodium pump." The reception this proposal got from the experts was lukewarm, to put it mildly. This may sound surprising today, but one has to remember that, as mentioned above, a chemical distinction between Na^+ and K^+ was considered unlikely. In any case, when we planned our new project in Copenhagen, a renewed study of striated muscle was high on the priority list.

Our Team Is Formed

Our team at the outset consisted of Hilde Levi (physicist), one of Hevesy's former associates, C. Barker Jørgensen (physiologist), and myself. Later we were joined by Valborg Koefoed-Johnsen (one of my former graduate students) and K. Zerahn (inorganic chemist).

We started with two projects: (1) determination of the rate of exchange of sodium in Sartorius muscle, and (2) the possible role of hormones in the regulation of active ion exchange between fresh water animals and the medium.

First, the Sartorius muscle project: we loaded the isolated muscles with ^{24}Na, and followed the washout of the isotope. The washout curves could be resolved into two exponentials, from which we tried to calculate the contributions from the fibers and the interspaces. Since the efflux of Na^+ from the fibers must take place against the chemical as well as the electrical potential, it should be a measure of active extrusion of Na^+.

The Concept of Exchange Diffusion

I then compared the supposedly active sodium transport rates with the metabolic rate, as taken from the literature, and found to my surprise that the energy available did not suffice to bring about the supposedly active sodium transport. Of course, the discrepancy might be due to experimental errors or errors of calculation, but I suddenly realized that the fundamental assumptions might be wrong. In fact, an exchange of an ion species across a membrane *can* proceed without consumption of free energy, even if the ion is present at different electrochemical potentials at the two sides of the membrane (Ussing, 1947, 1948; Levi & Ussing, 1948). However, this is only possible if the passage of the ion in one direction is strictly coupled to the transport of a similar ion in the opposite direction, so that the free energy made available by the downhill transport is used for the uphill transport.

Formally, such an exchange could be brought about by a carrier molecule in the membrane which has a specific binding site for the ion in question, but which can only pass from one side to the other if the site is filled. However, as we already realized at the time, the transporter might be a site in a molecule which can flip-flop between two positions, alternately exposing the site to the two bathing solutions.

The phenomenon of exchange diffusion later was found to be quite widespread. It is of great functional importance in cases where two or more substances share

the same exchange system, for example, chloride and bicarbonate in red cells. However, the mere possibility of exchange diffusion meant that Krogh's original plan for the use of isotopes must be abandoned. Furthermore, it became desirable to develop a terminology for describing and interpreting tracer experiments. We shall return to these problems in a moment.

Vasopressin Stimulates Na^+ Uptake

The Axolotl project had yielded promising results (Jørgensen et al., 1946). Starving axolotls were kept in a container with a known (low) content of Na and Cl^- and known radioactivities of ^{24}Na and ^{38}Cl. From the change of these four quantities with time, it was possible to calculate uptake and loss of Na^+ and Cl^- as functions of time. Under the assumption that uptake and loss followed different paths, it was possible to demonstrate that "uptake" of both ions was stimulated by vasopressin and uninfluenced by oxytocin, whereas the "secretion" was stimulated by oxytocin, but not by vasopressin. We suggested that uptake was localized to the skin, whereas the loss was taking place via the kidneys. So far so good. But were we seeing active transport of Na^+, Cl^- or both? And were "uptake" and "excretion" really via the pathways we assumed? Was it not possible that part of the "excretion" could take place via the skin, and, if so, could not part of the "uptake" as well as the "excretion" be exchange diffusion? All these questions might be answered if we did the experiments on isolated skin, but anybody who has tried to skin an axolotl will agree that it is tricky.

The Isolated Frog Skin

I therefore decided to do a series of experiments on isolated frog skin (1949*a*). The skin was mounted as a diaphragm, separating two, well-mixed and well-aerated solutions. The potential difference between them could be measured in the traditional way via KCl bridges and kalomel cells. Isotopes could be added to one side and samples taken from the other.

Much was already known about the physiology of the frog skin, which has been a favorite object of electrophysiologists since du Bois-Reymond (1848) observed the electrical potential difference between its inside and outside. Thus, Galeotti (1904) had demonstrated that the potential difference (inside positive) across the skin depended on the presence of Na^+ (or Li^+) in the outside bath, and Huf (1935) had shown that the isolated skin could bring about net transport of chloride from the outside to the inside when bathed with Ringer solution on both sides. In our experiments (Ussing, 1949*a*), the inside solution was always frog's Ringer, whereas the outside solution was varied between ordinary Ringer and 1/100 Ringer.

With respect to sodium transport, the result was clear: there was a net transport of Na^+ inward even when the inside concentration was 100 times higher than that of the outside, and the potential difference across the skin remained inside positive, so that the net sodium transport would take place against the electrical as well as the chemical potential. For the description of the individual tracer experiment, a new terminology was introduced. Previously, authors had used terms like permeability, transport or exchange when describing tracer experiments. These terms tended to convey information on the underlying transport mechanism. In fact, the result of the tracer experiment in only one direction could be the result of any combination of diffusion, active transport, exchange diffusion, and solvent drag. Therefore, a neutral term was needed which only told exactly what kind of experiments had been performed. For that purpose, the term "flux," in combination with the direction, was proposed (for example, influx/efflux, forward flux, backward flux, etc.). The physical meaning of the difference between forward flux and backward flux, i.e., the net flux, is immediately clear. Just as in a chemical reaction, the net reaction rate is the difference between the forward and backward reactions. It was tempting, then, to compare the ratio between forward and backward flux with the affinity of a chemical reaction. This consideration led me to study the properties of the flux ratio. Was it possible to use the flux ratio of ions for distinguishing between active and passive transport?

The Flux Ratio Equation

Qualitatively, it was clear from our early experiments (Ussing, 1949*a*) that the sodium flux ratio (in/out) was high, even when the transport went against a concentration as well as a potential gradient. Thus, any passive component must be very small, indeed. Net chloride uptake via the frog skin was only seen when the electrochemical potential was higher in the outside solution than in that of the inside. The combined effects of an electrical and a chemical gradient across a single, homogeneous membrane had at that time been treated by Goldman (1944) and Teorell (1949). To integrate the expression for the net flux across the thickness of the membrane, however, it was necessary to make assumptions. In Goldman's solution, the electric field was assumed to change linearly, whereas Teorell assumed a constant concentration gradient for the ion in question. Of course, neither of these assumptions could be used in the case of a frog skin, which is a very complex

structure. Beginning from the outside there is a thin cornified layer; then follows a multilayered epithelium, followed by a basement membrane and a thick serosa. I noticed, however, that although the Goldman and Teorell equations for the flux in one direction gave different results, they always gave the same result for the flux ratio. Moreover, the expression for the flux ratio did not require any information concerning the membrane, but was determined by the electrochemical potential difference between the two solutions.

I thus suspected that the flux ratio, even for a complex structure like a frog skin, would be the same. Indeed, I succeeded in proving that this is the case (Ussing, 1949*b*). In essence, the reason for this simple solution is that, although there may be any number of "hurdles" which an ion has to pass going from one boundary to the other, the same hurdles have to be passed by an ion going in the opposite direction. Incidentally, I had a hunch that the flux ratio might also be time independent, so that it would be constant from the first appearance of the tracers. Years after, we succeeded in showing this to be true (Ussing, 1978; Sten-Knudsen & Ussing, 1981). In the following years, the flux ratio for chloride fluxes through isolated frog skin was tested carefully, and the flux ratios in nearly all cases fitted the criteria for passive transport. It should not be forgotten, however, that very early in the game, Krogh had demonstrated that salt-depleted frogs will take up chloride from extremely low concentrations, even in the absence of sodium in the medium. Many years later this process was to be localized to special mitochondria-rich cells (Voûte & Meier, 1978; Kristensen, 1981), and the mechanism normally is silent.

In our early experiments (Koefoed-Johnsen, Levi & Ussing, 1952), the flux ratio for chloride could be calculated satisfactorily on the basis of the electric potential difference between the inside and outside, the chemical concentrations, and the activity coefficients calculated from the ionic strength of the solutions.

Solvent Drag

From the very beginning of the study, I was well aware of an additional factor which might influence the flux ratio, *viz* the "solvent drag." If there is a net movement of water through the transporting cells, it might influence the flux ratio even for passively transported species.

Frog skin epithelium belongs to the so-called tight epithelia where the water permeability normally is low. The water permeability, however, can be stimulated dramatically with anti-diuretic hormone (vasopressin, added to the inside solution). Cursory experiments (Koefoed-Johnsen et al., 1952) did not bring about any deviation of the flux ratio from that calculated on the basis of the difference in electrochemical potential. The reason we now know is that water goes through water channels, whereas chloride passes through specific chloride channels which do not admit water.

In principle, it was possible to describe the effect of solvent drag on the flux ratio of a "passive" ion species, i.e., a species which was not simultaneously exposed to active transport (Ussing, 1952; Koefoed-Johnsen & Ussing, 1953). Like in the case of "simple" passive transport, the equation was valid even for multilayered tissues, but, whereas the simple flux ratio equation requires only information concerning the difference in electrochemical potential between the two "bathing solutions," the equation including solvent drag requires in addition the forward and backward flux of one test species.

Quite generally, it has turned out that, on a cellular basis, it is rarely necessary to look for solvent drag, because the ion channels are very specific, so that, for instance, neither sodium, potassium, nor chloride channels are leaky to water, whereas water itself goes through specific water channels. Recently, however, the solvent drag equation has acquired great importance in the analysis of transport of electrolytes and water through the paracellular pathways of leaky epithelia where solvent and solutes use the same pathway (Ussing & Eskesen, 1989; Ussing & Nedergaard, 1993).

The Short-Circuited Frog Skin

During a stay at the Donner laboratory, University of California in Berkeley in 1948–49, I gave a seminar on sodium transport in frog skin. During the discussion, one of the biology students objected strongly to my view that the active transport of Na^+ was the source of the skin potential. He pointed out that his former teacher, Professor E.J. Lund, in his book *Bioelectric Fields and Growth* had shown it to be an oxydation-reduction potential. Actually, the potential was measured through the usual kind of KCl bridges, which, of course, do not pick up redox potentials. However, the book referred to a paper by Lund and Stapp (1947) who had managed to bring about a partial short circuit of the frog skin via lead-lead chloride bridges. He could draw current from the skin for hours. I did a quick calculation and found that the current was of the same magnitude as the net sodium flux through the skin, extrapolated to zero potential. The method could not give full short circuit, and lead is poisonous, but if full short circuit could be obtained under ideal conditions, it would be a way to prove my point: that the active sodium transport, more or less shunted by the passive flux of chloride, is responsible for the skin potential.

At that time, the idea was completely new. The frog skin potential had been studied extensively. Ga-

leotti (1904) noticed that the potential required the presence of Na^+ (or Li) in the outside medium and proposed that the skin was more permeable for Na^+ in the inward than in the outward direction. This explanation was not favorably received since it seemed to violate the second law of thermodynamics. When radioactive Na^+ became available, Katzin (1940) noticed that Na^+ passes faster inward than outward through the skin. He used the term irreciprocal permeability, but he did not discuss the relation of the phenomenon to the frog skin potential.

Over the years the frog skin potential was subjected to numerous studies. For reference, *see,* for instance, Ussing et al. (1960). As late as 1946, Meyer and Bernfeld proposed in a careful study that the potential arose from hydrogen ions diffusing faster from the cells to the inside bath than does bicarbonate, so that the formation of metabolic CO_2 was the real source of the potential. Other explanations were based on the (erroneous) assumption that biopotentials were maintained by different adsorption of ions to the two sides of the membrane.

In short, I was well aware of strong opposition if I proposed that active sodium transport (considered by many physicochemists to be unlikely or impossible) were the sole basis for the skin potential.

I finally realized that, if an appropriate EMF is put in series with the skin potential and adjusted so as to keep the potential drop across the skin equal to zero, it would be possible to bring about a true short circuit of the skin. At the same time, the inward and outward sodium fluxes could be measured (*see* Fig. 1). By the time I had the plan ready, there were only two weeks to the deadline for summaries for the International Physiology Congress in 1950. I discussed the plan with Karl Zerahn, who was then working with phosphate turnover in yeast. I knew him as an excellent experimentalist. He agreed to join me in the frog skin project. After one week, we had set up the apparatus and after two weeks, we knew that the theory was correct: the influx minus the outflux of sodium through the frog skin was exactly equal to the short-circuit current.

In the shorted skin setup, the electrochemical potentials of all components of the bathing solutions are identical on the two sides. Thus, no component has the "right" to pass faster in one direction than in the other. Experiments with radioactive chloride show that, indeed, the flux is the same in both directions.

The contributions of ionic species other than Na^+ to the short-circuit current are quite insignificant. K^+, in particular, crosses the skin only insignificantly. Even when one-third of the Na^+ in the two bathing solutions is replaced by K^+, the short-circuit current is still equal to the net sodium transport.

The experiments with the short-circuited frog skin thus had shown, beyond a reasonable doubt, that active

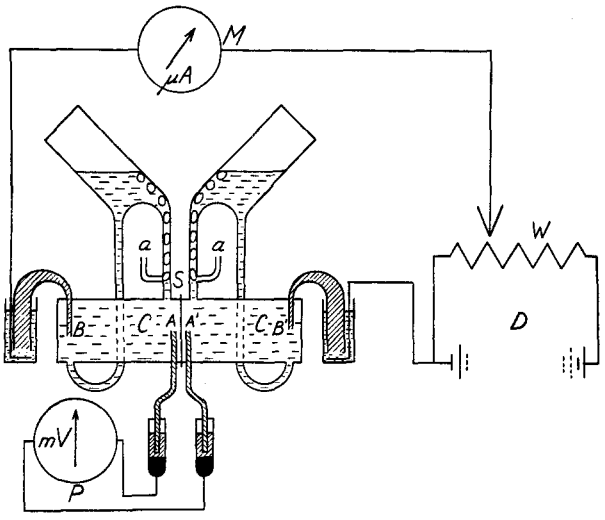

Fig. 1. Diagram of apparatus used for determining Na flux and short-circuit current. (*C*) Celluloid chamber containing 40 ml Ringer on each side of the skin. (*S*) Skin. (*a*) Inlets for air. (*A* and *A'*) Agar-Ringer bridges, connecting outside and inside solutions, respectively, with calomel electrodes. (*B* and *B'*) Agar-Ringer bridges used for applying outside EMF. (*D*) Battery. (*W*) Potential divider. (*M*) Microammeter, and (P) Tube potentiometer. (After Ussing and Zerahn, 1951.)

sodium transport did exist. This made the short-circuited frog skin ideal for the study of the relationship between active transport and metabolism. Independently, Zerahn (1956) and Leaf and Renshaw (1957) were able to demonstrate that there was a stoichiometric relationship between short-circuit current and oxygen consumption (over and above the consumption in the absence of Na^+ transport). In fact, 18 Na^+ were transported across the skin per extra O_2 consumed.

At that time, other scientists had proposed a stoichiometric relationship between ion transport and metabolism. Thus, Conway (1955) claimed for Na^+ transport a one-to-one relationship between ions taken up and electrons passing the respiratory chain. The true efficiency of the Na pump was, however, 4.5 times higher.

The Two-Membrane Model of Epithelia

The short-circuiting method had the great advantage that it demonstrates beyond a doubt that active transport of Na^+ did exist, and allowed the study of the relationship between metabolism and transport. However, it did not predict the skin potential and it did not localize the individual steps in the transport process. It was essentially a "black box" approach. After a while, however, we were tempted to look into the box. From the very beginning of our project, we held the view that the asymmetry of the ion transport must be due to differences in transport properties between the outward- and

inward-facing epithelial membranes. We had noted a peculiar effect on the Na^+ transport exerted by the K^+ concentration of the inside solution. Both skin potential and short-circuit current decreased when the K^+ concentration increased. On the other hand, K^+ had no specific effect if the concentration was changed on the outside. Thus, it was tempting to assume that the outward-facing membrane of the cells was tight to K^+ and permeable for Na^+, whereas the inward-facing membrane was permeable to K^+. The Na pump had then to be placed in the inward-facing membrane. If the pump were placed in the outward facing-membrane, the cells would be flooded with Na^+, and K^+ would be squeezed out via the K^+ conductance of the inward-facing membrane.

This view was not shared by others in the field. Thus, Linderholm (1954) assumed that the Na^+ ions of the outside solution did not enter the epithelial cells, but passed between them until, at the level of the basement membrane, they reached the transport mechanism. He based this assumption mainly on a study by Ottosen et al. (1953) who, with microelectrodes, had found only one potential jump on the way through the skin.

We decided to attack the problem from two different angles. Tom Hoshiko (guest scientist) and Lise Engbæk from our neurophysiological laboratory would make a fresh attempt to find the potential steps in the frog skin epithelium, whereas Valborg Koefoed-Johnsen and I would do a classical study of the skin potential under conditions where the shunting effect of permeating anions was minimized.

The microelectrode study turned out to be more difficult than anticipated, but qualitatively the result was clear: there was more than one potential jump when going from one side of the epithelium to the other (Hoshiko & Engbæk, 1956).

The "old-fashioned study" (Ussing & Koefoed-Johnsen, 1956; Koefoed-Johnsen & Ussing, 1958) of the skin potential under the conditions of minimized anion shunt gave a convincing result. To eliminate the shunting effect of the chloride ions, we replaced chloride in the outside and inside bathing solutions with sulfate, which can neither penetrate the outside nor the inside cell membranes. This treatment is well tolerated by the cells and gives rise to high and stable skin potentials. Equally, high potentials could be produced by addition to the outside solution of Cu^{2+} to a final concentration of 10^{-5} M. The procedure was based on an accidental observation. The Cu^{2+} treatment did not affect the handling of Na^+ and thus did not influence the short-circuit current, but it blocked completely the inward and outward chloride fluxes. Both methods of blocking the anion shunt gave preparations which behaved exactly as predicted: the outside-facing membrane, within a range of Na^+ concentrations in the outside bath from 1 to 100 mM, behaved like a Na^+ electrode, whereas the inward-facing membrane responded to changes of the K^+ concentration of the inside bath as if it were a K^+ electrode.

These observations, in connection with the fact that K^+ does not contribute to net cation movements, led to the model shown in Fig. 2.

The active transport mechanism (the "sodium" pump) is located at the inward-facing membrane of the epithelial cell. It pumps Na^+ from the cell to the inside solution, and the transport is coupled with a K^+ transport in the opposite direction. Since the membrane is impermeable for free Na^+, but permeable for K^+, the result is a high K^+ and a low Na^+ concentration in the cell. The outward-facing cell membrane is supposed to be passively permeable to Na^+, but tight to K^+, and therefore the net result is transport of Na^+ from the outside to the inside bathing solutions. It is obvious that the result would have been the same, if the pump were a pure Na pump. A Na/K pump was proposed, because the sodium transport stopped if the inside bathing solutions were made K^+ free. In the first version of our model, we proposed a one-to-one relationship between Na^+ and K^+, because such a relationship had been proposed for the red cell membrane. Years later, it was shown that the Na^+ ratio for the ion pump of frog skin was 3/2 like in other Na pumps.

A crucial detail in the frog skin model is the existence of specific Na^+ conductance in the outward-facing membrane, but since Hodgkin et al. (1949) had provided evidence for the existence of transient Na^+ and K^+ conductances during the action potential of nerve axons, it was permissible to assume that such specific ion conductance could exist permanently in epithelia.

Conclusion

Since its publication, the two-membrane theory has been generally accepted as a prototype of a transporting epithelium. It should be remembered, however, that whereas it gives a quantitative measure of the active sodium transport, the coupling ratio between Na transport and K recycling has to be estimated by other methods (*see* Skou, 1957). We are reminded of our initial conclusion: that active transport can only be observed with certainty in systems where net transport of the species in question is going on.

Also, the procedures described in the foregoing are powerful only for tight epithelia where paracellular shunt paths can be neglected.

The finding (Ussing, 1978; Sten-Knudsen & Ussing, 1981) that the flux ratio is time independent, i.e., constant from the first appearance of the tracers on the

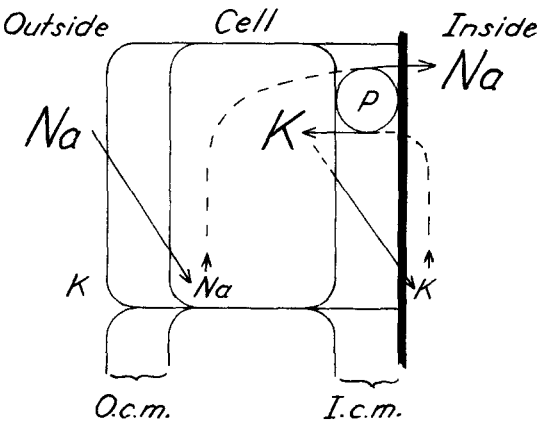

Fig. 2. Simple two-membrane model of epithelium. *Unbroken arrows:* Passive though highly selective diffusion; *dashed arrows,* movements of Na^+ and K^+ through the pump. (After Koefoed-Johnsen and Ussing, 1958.)

"receiving" side, makes it possible, at least theoretically, to analyze multipathway systems like leaky epithelia (*see,* for example, Ussing & Eskesen, 1989 and Ussing & Nedergaard, 1993).

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