ION TRANSPORT ACROSS MEMBRANES

Hans T. Clarke, *Editor*

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HANS T. CLARKE, *Editor*
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Preface

The collection of papers in this volume is based upon a Symposium on The Role of Proteins in Ion Transport across Membranes, sponsored by the National Science Foundation and held at the College of Physicians & Surgeons, Columbia University, on October 2 and 3, 1953.

The problem of ion transport across cell membranes has long attracted the attention of biologists in various fields. It is of prime importance for an understanding of the mechanism of bioelectric currents and the conduction of the nerve impulses; it has also played an important role in other studies such as the functions of the kidney and of red blood cells. The recent advances of biochemistry, especially in protein and enzyme chemistry, and the availability of isotopic tracers have made possible rapid progress in the area here under consideration. The problem is reaching a stage where the molecular forces involved have become a subject of more than speculative interest.

Now that experimental biology is approaching the molecular level, active cooperation between biologists, physical chemists, and protein chemists should lead to significant developments in basic theory. History records many such cases of cross-fertilization of ideas. For example, the membrane theory which is still the basis of all modern concepts of nerve impulse conduction was built upon the fundamental work of Ostwald, Nernst, Planck, and other physical chemists and physicists. The plan of this Symposium was not, as is the usual procedure, to bring together investigators working in similar areas for the discussion of limited, specific problems. It was planned, rather, to bring together a small group of physicochemically minded biologists working on ion transport and the role of proteins in this process, with a small group of distinguished physical chemists and protein chemists who are or may become interested in this borderline problem. The study of ion transport across membranes and the interaction between ions and proteins transcends the field of biology and has always been a domain in which physical chemists were interested.

At the suggestion of Dean Willard C. Rappleye a committee was organized, consisting of Robert F. Loeb, H. Houston Merritt, and David Rittenberg, with David Nachmansohn and Hans T. Clarke as organizing secretary and chairman, respectively. This committee decided to limit the number of participants to about fifty in order to ensure an

atmosphere favorable to the exchange of ideas, by informal discussion and personal contacts, between the two groups. As the Symposium was held during only two days, the program was limited to eight papers, given by Drs. Ussing, Wilson, Eyring, Kirkwood, Scatchard, Debye, Sollner and Edsall. Drs. Rittenberg, Louis P. Hammett, I. I. Rabi, and Raymond M. Fuoss served as the chairman at each of the four sessions.

The Symposium was held on the eve of the 25th anniversary of the Columbia-Presbyterian Medical Center. As stated by Dean Rappleye in his opening address, Columbia University is proud to have been host to so many distinguished scientists. The Symposium was an appropriate expression of the spirit of research prevailing at the Center.

The present volume contains the substance of the various addresses, together with six invited articles on allied topics, contributed by various investigators who could not be represented on the program. The organizing committee expresses its gratitude to all the authors of the chapters here assembled. The book is dedicated to the memory of Jacques Loeb, pioneer in the application of the principles of physical chemistry to biological problems, whose intensive studies of ion transport across cell membranes are still bearing fruit, as pointed out by Dr. Osterhout, his associate through many years.

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Note on the Work of Jacques Loeb

W. J. V. OSTERHOUT

It is a pleasure to respond to the suggestion of the Editorial Committee to say something about the pioneer work of Jacques Loeb[a] in applying physical chemistry to biology.

Loeb was one of the first to see the importance to biology of the dissociation theory of Arrhenius, and he applied it with conspicuous success. A few examples will suffice to show the nature of his work.

Loeb's point of view may be illustrated by his experiments with eggs of the fish *Fundulus*, which develop in distilled water as well as in sea water. In a solution of sodium chloride isotonic with sea water, the eggs soon die. This also happens in an isotonic solution of zinc sulfate, but when these two solutions are mixed in suitable proportions normal embryos are produced. From this he concluded that sodium and zinc ions antagonize each other. He found that, in general, the toxic effects of univalent cations could be counteracted by the addition of small amounts of bivalent cations and by still smaller amounts of tervalent cations. It was a novel and important idea that the physiological effects of ions could be predicted by knowing their electrical charges, and it gave a great stimulus to the application of physical chemistry to biology.

Loeb suggested that these effects were due to the action of ions on the permeability of the membrane surrounding the egg. The antagonistic action is quite different as soon as the embryo escapes from the covering. He thus directed attention to the importance of the study of permeability.

A deep interest in the changes which occur in the egg after union with the sperm led him to attempt to analyze and control these by using the techniques of physical chemistry. His first task was to induce development in the absence of sperm (artificial parthenogenesis). This he soon accomplished by varying osmotic pressure, pH, surface tension, and other factors. These experiments aroused great interest and stimulated extensive research which still continues. Loeb concluded

[a] For a fuller account, see the Memorial Volume, *J. Gen. Physiol.* **8**, (1925–1928).

that the first step is a change at the surface of the egg whereby the normal structure begins to break down (cytolysis). If this continues too long, death occurs, but, if stopped at the right time, it is followed by cell division and development.

In the course of Loeb's experiments it became clear that in many cases a knowledge of the properties of colloids is important. The whole subject was at that time in a state of confusion and he sought to clarify it by using the methods of physical chemistry. He therefore began experiments on the colloidal properties of gelatin. An important aspect of this work was his study of the effects of pH. McBain[b] speaks of the invaluable services of Loeb in pointing out the predominant role of pH on amphoteric basic and acidic colloids. In this field his scientific imagination and insight had a clarifying effect, as is evident in his well-known book on proteins and the theory of colloidal behavior.

These examples may suffice to illustrate the character of his work, in all of which he sought, with notable success, to apply the methods of the exact sciences.

In many cases Loeb was able to replace obscure biological ideas by clear-cut, mechanistic conceptions and thereby opened up new fields of research of fundamental importance. His example was inspiring and his influence was great. For this we owe him a debt of gratitude.

[b] J. W. McBain, "Colloid Science," p. 215. D. C. Heath & Co., Boston, 1950.

Ion Transport Across Biological Membranes

HANS H. USSING

Diffusion Through Biological Membranes

At the outset it might be worth while to define briefly what we experimental biologists mean by a biological membrane. I think most of us can agree upon a formulation like this: Whenever we meet, in a living organism or part thereof, a boundary that presents a diffusion resistance to solutes higher than that of the phases separated by the boundary, it is called a membrane. The membrane is often, but not always, anatomically discernible.

The objects we study under the name of biological membranes are extremely diverse. Thus we have membranes on the multicellular level like the gastric mucosa or the frog skin epithelium. Then there are the cell membranes like, for instance, the membranes of the nerve fiber.

Finally, the work of the last few years tends to show that even membranes on the subcellular level are highly important. Notably, the surface of the mitochondria shows membrane-like properties, such as the ability to maintain, and under certain circumstances to create, within the mitochondrium concentrations of a number of substances which differ from those of the surroundings. As an example we may take a table from a recent paper by Bartley and Davies (1952) (Table I). It is seen that the Na ion undergoes a conspicuous concentration in the mitochondria as compared to the surrounding medium.

At first sight there seems very little in common between the nerve fiber membrane and the skin of a frog, or between the tip of a plant root and the gill of a crab. Nevertheless, these different structures show many similarities in the way they handle inorganic ions. Formerly it was generally assumed that the similarities stemmed from the fact that the element determining the behavior of ions was in all cases a cell membrane, or possibly a number of cell membranes placed in series. This may still be true. With the increasing knowledge concerning the ability of mitochondria to concentrate and exclude certain ion species, one may, however, speculate as to whether some of the phenomena involving the transfer of ions across cell boundaries are actually the result of the activity of mitochondria. Discussing this problem at the

TABLE I

INTERNAL-TO-EXTERNAL CONCENTRATION RATIOS FOUND FOR METABOLIZING SHEEP-KIDNEY CORTEX IN MITOCHONDRIA AT 20°C

Substance	*Ratio*	*Concentration in medium after separation (M)*
H^+	2.5	1.6×10^{-7}
Na^+	26	5.9×10^{-4}
K^+	2.0	9.0×10^{-2}
Mg^{++}	4.5	2.0×10^{-4}
Orthophosphate	6.0	1.9×10^{-4}
Adenosine polyphosphates	0.7	4.3×10^{-4}
Pyruvate	0.8	3.5×10^{-3}
Fumarate	8.0	2.8×10^{-4}
Oxaloacetate	0.1	1.8×10^{-3}
α-Ketoglutarate	1.0	6.3×10^{-2}
Citrate	0.8	1.4×10^{-2}

Water content of metabolizing mitochondria = 80%.
Water content of nonmetabolizing mitochondria = 91%.
(After Bartley and Davies, 1952)

conference on active transport at Bangor this summer, Davies pointed out that in secreting kidney cells the mitochondria are arranged longitudinally from the cell wall bordering the lumen of the tubules. During the discussion following Dr. Davies' paper, Professor Wigglesworth then mentioned that, in the salt-reabsorbing part of the Malpighian tubule in insects, the giant mitochondria actually pierce the luminal cell boundary, waving with one end in the pre-urine while having the base well within the cell body.

Alternatively one may imagine that the surface of the mitochondrium and that of the cell have in common certain properties which enable them to handle ions in a characteristic manner, and resist their free diffusion.

This brings us to the point of asking: What is the nature of the cell membrane? Or, to be more cautious, how do the biologists picture the cell membrane? Without having made use of Mr. Gallup's methods, it is probably fair to assume that the concept most widely accepted is that of the lipoid-pore membrane, as proposed by Collander (1937). It implies that the less polar substances penetrate by dissolving in the membrane phase, whereas the polar substances, notably the inorganic ions, penetrate only in so far as the ionic diameter is smaller than the

pore diameter. Although the lipoid-pore theory explains a multitude of experimental facts, it has not remained unchallenged, however. Thus Davson and Danielli (1943) have proved theoretically that the experimental facts obtained by Collander and Bärlund (1933) could equally well be obtained with a nonporous membrane in which the diffusing molecules and ions dissolved. The criticism by Davson and Danielli is an important one since it poses the question of whether polar molecules, like inorganic ions, pass the membrane dissolved in the water that fills the pores or whether they dissolve in a nonwatery medium.

Recently, in the Laboratory of Zoophysiology, Copenhagen, we have approached the question of the existence of pores in cell membranes from an angle which promises some measure of success (*cf.* Ussing, 1952). The approach is based on the following well known facts: If water, or any substance which is insoluble in the membrane phase, is to pass a pore membrane by simple diffusion, the rate is to a first approximation proportional to the total area of the pores. If, however, water is pressed through the pores by hydrostatic pressure or osmotic pressure, the rate is determined by the shape and number of the individual pores and no longer simply by the sum of their areas. We can thus define two independent measures of water permeability, namely, 1) diffusion permeability as determined by the rate of diffusion of isotopic water, and 2) filtration or osmotic permeability determined by the rate of flow induced by a difference in osmotic or hydrostatic pressure. Obviously the difference between the two water permeabilities vanishes if pores are absent so that all water molecules pass by dissolving in the membrane phase.

Thus, by determining both water permeabilities on the same membrane, one can decide whether it has pores or not. Furthermore, the relative magnitudes of the two permeabilities allows an estimate of the pore sizes.

As early as 1935, Hevesy, Hofer, and Krogh (1935) found the osmotic permeability of the frog skin to be 3–5 times greater than the diffusion permeability as determined with heavy water. This has been confirmed by Capraro and Bernini (1952), and the phenomenon has been found to be even more pronounced in the isolated skin of the toad (Koefoed-Johnsen and Ussing, 1953). Prescott and Zeuthen (1953) have determined diffusion and osmotic permeability to water in a number of eggs of fishes and amphibians (Table II).

It is seen that if both permeabilities are expressed in the same units, say μ/sec, the osmotic permeability is always higher than the dif-

TABLE II

PERMEABILITY TO WATER OF DIFFERENT EGGS

Cell type	P_d (μ/sec)	P_f (μ/sec)	P_f/P_d
Frog ovarian egg	1.28	89.1	69
Zebra fish ovarian egg	0.68	29.3	43
Xenopus body cavity egg	0.90	1.59	1.8
Frog body cavity egg	0.75	1.30	1.7
Zebra fish shed, nondeveloping	0.36	0.45	1.3

Diffusion permeability coefficient: P_d.
Filtration permeability coefficient: P_f.
(After Prescott and Zeuthen, 1953)

fusion permeability. In the ovarian eggs the difference is very striking indeed, indicating the presence of large pores. Krogh and Ussing (1937), on the other hand, demonstrated that during the first days of development the trout egg is absolutely impermeable to heavy water. This may suggest that the membrane substance, even between the pores of the membranes of other eggs, is also tight, so that all water penetration takes place through pores.

Egg membranes, however, are probably very specialized, and it would be desirable to get information concerning the two water permeabilities, even in other types of living membranes.

We shall also have to face another criticism of the simple membrane concept, a criticism which was voiced by Krogh (1937a) during a Faraday Society Discussion. Commenting on the proper use of the word membrane he said: "Active transport of a substance is brought about by some kind of dynamic machinery working within the cells, which may in turn be bounded by membranes allowing certain substances to pass and holding others back. I maintain that such membranes are, in the present state of our knowledge, definitely unsuitable for the study of membrane properties, because it is too difficult to distinguish the membrane from the cell of which it is an integral and perhaps variable part."

At that time the importance of active transport as distinct from simple diffusion was not so generally accepted as it is now, and I have heard the comment that Krogh tried to revive the old vitalistic views

concerning the processes of life. But the difficulties of studying simple permeation in the presence of simultaneous active transport processes have proved real enough. If we want to study the passage of ions through cell membranes, then what we need first of all is means to decide which ions are actively transported and which are not.

Apparently the easiest case to deal with is that of a cell in equilibrium or steady state with its surroundings. Under these conditions any ion which is not subject to active transport must obey the simple condition $\bar{\mu}_o = \bar{\mu}_i$ where $\bar{\mu}_o$ is its electrochemical potential in the cell interior and $\bar{\mu}_i$ that of the ion in the bathing solution.

If we had at our disposal reversible electrodes for all ions concerned which could be inserted in the cells, the problem would be easily answered. Unfortunately this is not the case. We therefore have no better choice than to calculate the electrochemical potentials from the concentrations and the electric potential difference across the membrane, as determined with internal microelectrodes, usually making the more or less well justified assumption that the activity coefficients are the same in both phases. Crude as this method may be, it has led to rather consistent results, indicating that K and Cl are in equilibrium with the surroundings in muscle and possibly in nerve. The technique of measuring potentials by aid of microelectrodes, inserted in the cell interior, has recently been developed to a high degree of refinement, and technically it may soon be possible to measure potentials in most cell types of the body; but we are still left with a vague doubt as to whether or not the values found are always thermodynamically significant.

Even more serious is the question of the activity coefficient to be applied. Is it justifiable to treat the probably highly organized cell interior as a dilute solution? Certainly, if the ions of the cell interior are forming complexes with organic cell constituents, our estimate of the equilibrium conditions may be very far off.

It has repeatedly been claimed that myosin and hemoglobin bind potassium in preference to sodium (for references, see Stone and Shapiro, 1948, and Steinbach, 1950). However, work with pure hemoglobin and preparations of muscle particulate matter (Steinbach, 1950) showed no evidence for selective K binding. Rather, in the case of the muscle preparation, there was a distinct preference for sodium.

Thus, unless the spatial arrangement of the protein molecules within the cells changes their ion binding properties profoundly, there is reason to believe that the K ions are free in the popular sense of the word. This is also borne out by the fact that intracellular K has the same

mobility in the direction of the fiber axis as has K in free watery solution (Hodgkin and Keynes, 1950).

The analysis, along the lines outlined here, of the equilibrium conditions for the more important inorganic ions has so far been performed for muscle and nerve only. It would have been tempting to extrapolate the result that K is in electrochemical equilibrium with the surroundings even in other types of animal cells with high K content, if it had not been for the fact that the erythrocytes of most animals also have a high concentration of K relative to the blood plasma. Even though the internal potential of red cells cannot be measured directly, a fair estimate of it can be obtained from the distribution of the freely diffusible ions Cl^- and H^+ between cells and surroundings. This estimate indicates strongly that the electrochemical potential of intracellular K is much higher than that of extracellular K. Thus even in the case of the K ion—which is one of those most carefully studied—it is still largely unknown whether or not it is subject to active transport in most animal cell types.

The equilibrium method for distinguishing between passive diffusion and actively transported ions clearly has its limitations. In the first place, one must know whether the ion species under study is at all able to penetrate the membrane. The introduction of isotopic tracers in biological work has, however, largely overcome this difficulty, since it is possible, without altering the chemical composition of the system, to study the rate of ion exchange across the membranes. A more serious objection to the equilibrium method is that our experimental objects are often far from the equilibrium state. Some membranes, like the frog skin and the gastric mucosa, are continuously transporting certain ions from one boundary to the other. Even single cells in the isolated state usually show continuous changes in ionic composition. It would therefore be desirable if, from the kinetics of ion permeation, one could draw conclusions as to whether a given ion species diffuses passively or whether it is subject to active transport.

The problem of passive diffusion through living membranes can be approached along two lines, different in principle.

In the first place one may integrate the general differential equation for the diffusion of charged particles, making certain *a priori* assumptions as to conditions within the membrane phase. The method was first applied by Goldman (1943) and has recently been developed further by Teorell (1951).

Teorell also tested his equation on collodion membranes and found good agreement between experiment and theory.

The integration depends, however, on the assumption that the membrane is uniform throughout—an assumption which is not even likely in the case of living membranes. If an ion does not behave according to the integrated equation, it can always be blamed upon structural peculiarities of the membrane. It can easily be shown, however, that there is one characteristic function of the permeability to a free passive ion which depends solely on the electrochemical potential difference, being independent of any assumptions as to the membrane structure. This characteristic is in the flux ratio, i.e., the ratio between the total diffusion stream of the ion passing the membrane in one direction and that passing in the opposite direction. If we denote by M_{in} the flux of the ion going inward and by M_{out} that going outward, we have

$$RT \ln (M_{in}/M_{out}) = \bar{\mu}_o - \bar{\mu}_i \qquad (1)$$

where $\bar{\mu}_o$ and $\bar{\mu}_i$ are the electrochemical potentials of the ion in the outside and inside phases, respectively. This relationship has been derived by Teorell (1949) and myself (1949) independently and seems also to be implicit in Eyring's theory of rate processes as applied to diffusion. (Zwolinski, Eyring, and Reese, 1949) In many cases the flux values can be obtained easily from experiments with isotopic tracers.

As an example we may consider the passage of chloride ions through the isolated frog skin. As is well known, the isolated frog skin, when in contact with suitable salt solutions, maintains for many hours an electric potential difference across itself, the inside solution being positive relative to the outside. Some twenty years ago another surprising property of the frog skin attracted the interest of physiologists. Huf (1935) found that the isolated surviving frog skin, when in contact with Ringer's solution on both sides, performs an active transport of NaCl from the outside to the inside solution. Shortly afterwards, Krogh (1937b) observed that frogs in need of salt will take up chloride ions through the skin even from solutions as dilute as 10^{-5} M with respect to NaCl.

Some years ago (Ussing, 1948) the present author advanced the hypothesis that only the Na ions are transported actively the the frog skin whereas the Cl ions follow passively owing to the electric potential difference created by the active Na transport. In order to test this

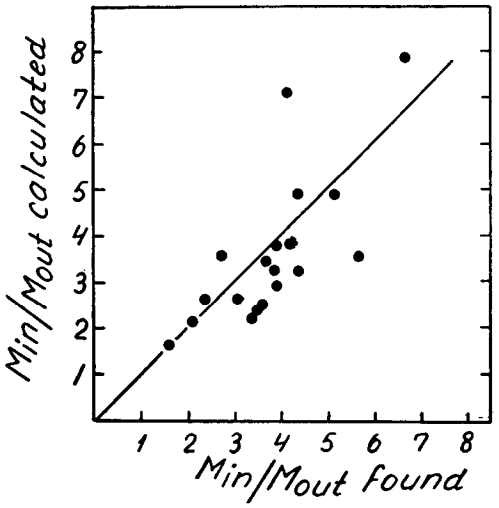

FIG. 1

hypothesis, a series of experiments were performed by my collaborators, Drs. Valborg Koefoed-Johnsen and Hilde Levi, and myself (1952) in which the outflux of chloride was determined by Cl^{36} added to the inside solution, whereas the difference between influx and outflux, i.e., the net Cl flux, was determined by chemical analysis. The spontaneous potential difference across the skin was recorded and the mean calculated for each experimental period. Fig. 1 shows the relatively good agreement between the flux ratios found and those calculated according to equation 1. More recent experiments, in which influx and outflux were determined with the two radioactive tracers Cl^{36} and Cl^{38}, are also in agreement with the assumption of a purely passive behaviour of Cl in the nonstimulated skin.

Linderholm (1952) in Teorell's laboratory has been able completely to verify this finding.

Similar studies of the behavior of K ions in Cephalopod and crustacean nerve have been performed by Keynes (1951) and Keynes and Lewis (1951). Some of their results are shown in Table III. It is seen that the agreement between the flux ratios calculated and those found is excellent.

And that would have been a good time to stop doing experiments, at least as far as our peace of mind is concerned. Quite recently, however, Hodgkin and Keynes (1953) have repeated the experiment on