# ACTIVE SOLUTE TRANSPORT ACROSS FROG SKIN AND EPITHELIAL CELL SYSTEMS ACCORDING TO THE ASSOCIATION-INDUCTION HYPOTHESIS

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# ACTIVE SOLUTE TRANSPORT ACROSS FROG SKIN AND EPITHELIAL CELL SYSTEMS ACCORDING TO THE ASSOCIATION-INDUCTION HYPOTHESIS

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• *The phenomenon of transport of ions, sugars, amino acids, etc. across frog skin and other epithelial systems has been commonly interpreted on the basis of the membrane-pump theory, according to which asymmetry in solute distribution as well as transport into and out of all living cells results from the permeability properties and "pump" activities of the cell membrane. In the present review, certain findings in the field of transepithelial transport of solutes are given new interpretation on the basis of molecular mechanisms introduced in the association-induction hypothesis, according to which "active transport" of solutes occurs only across bifacial cell systems like frog skin and intestinal epithelium but not in the maintenance of steady levels of solutes in unifacial cell systems such as muscle, nerve, and red blood cells.*

## INTRODUCTION

Since the cell is the fundamental physiologic unit of life, some of the features that define life may be revealed by studying the properties of a cell before and after it has ceased living. One method often used to tell a living from a dead cell is based on the dye-exclusion property. Dyes such as nigrosin, trypan blue, and erythrocin B are excluded by living cells but stain dead cells with vivid colors. In short, the dead cell has lost its exclusion property and hence the ability to maintain itself separate from its environment.

Exclusion is not limited to foreign molecules such as the dyes. It applies to molecules that may be intrinsic to cell functions but require subtle quantitative balance, as in the case of the asymmetrical distribution of $K^+$ and $Na^+$ between cells and their environment. Chemically almost indistinguishable, this pair of ions is sharply segregated by living cells, which can preferentially accumulate $K^+$ to a level many times higher than that in the external medium and, by exclusion, sustain a level of $Na^+$ only a fraction of that found in the same external medium.

The earliest interpretation of this phenomenon of asymmetrical $K^+$ and $Na^+$ distribution was based on the assumption that the cell membrane is absolutely impermeable to both $K^+$ and $Na^+$.[1,2] In following years, revisions were made. First $K^+$, and later $Na^+$, were recognized to be permeant. Then, after the 1940s, membrane impermeability as the basis of asymmetrical solute distribution was abandoned, largely in consequence of direct measurements of solute permeability made possible by the development of radioactive labeling and other accurate techniques. There remained two and only two possible interpretations for the asymmetrical solute distribution typified by $K^+$ and $Na^+$: (*1*) the steady-state model, in which the unequal distribution is due to active transport accomplished by continued operation of energy-consuming membrane pumps; and (2)

the equilibrium model, in which accumulation of $K^+$ and exclusion of $Na^+$ reflect the different physicochemical attributes of the environments inside and outside the cell.

A strong argument often cited in favor of the steady-state model arises from the ion-distribution pattern in giant algal cells such as Valonia macrophysa. Living in sea water, which contains nearly 50 times more $Na^+$ than $K^+$,[3] these cells may retain 3 times more $K^+$ than $Na^+$.[4] The bulk of this $K^+$ and $Na^+$ is found in the cell sap contained in the central vacuole. The sap in fact contains little more than water and salt ions and is therefore not significantly different in this respect from sea water. Clearly, then, the maintained asymmetry of $K^+$ and $Na^+$ distribution in this algal cell can be due only to active transport. Here we have a flawless argument, as far as it goes, but it offers no proof that non-algal cells accumulate $K^+$ and exclude $Na^+$ by the same mechanism. Indeed, giant algal cells with large central vacuoles are not typical of other living cells at all. A large central vacuole is seen solely in old plant cells; young plant cells as well as most procaryotic and eucaryotic cells are "solid bodies."[5]

## CONCEPTS OF MEMBRANE-PUMP THEORY

A majority of cell physiologists adhered to the steady-state model and conventional membrane-pump theory. Most studies of epithelial transport have been built on that theory. Hence epithelial solute transport is widely considered equivalent to selective solute accumulation and exclusion in "simpler" cells, all due to membrane pumps. This approach does not take full account of the difference between unifacial "solid" cells (e.g., human red blood cells, frog muscle cells, squid axon) and bifacial hollow cells (e.g., Valonia). Only the Valonia-type cell contains an enclosed body of simple aqueous solution. Only in Valonia-type cell does a second membrane, the tonoplast, enclose this internal aqueous phase. Fifty years ago, Chambers and Höfler[6] studied the osmotic behavior of the isolated central vacuole and showed that the tonoplast has properties quite different from those of the outer plasma membrane. Indeed, the perfect osmometer-like behavior of plant cells was shown to be due primarily to the tonoplast and not to the plasma membrane.

Of course, giant algal cells are not the only living cell systems that achieve net transport of ions and other small molecules between two similar aqueous phases against concentration gradients. Many epithelial tissues do the same. But instead of the continuous protoplasmic layer characterizing Valonia, epithelial cells joined together in continuous sheets separate the two aqueous phases. Without exception, each of these epithelial cells is bifacial, possessing two different surfaces each facing one of the two aqueous media.

### Proposed Mechanism of the Na-Pump

As pointed out by Ussing and Leaf,[7] "It is the properties of the outward-facing membrane that are unusual, viz., the selectivity for sodium rather than potassium and the absence of a sodium pump. . . . The inward facing cell membrane . . . can be assumed to be of the same nature as the sodium-potassium exchange pump of red cells, muscle and nerve." This opinion clearly shows that in the conventional view, epithelial $Na^+$ transport is the same as in most unifacial non-epithelial cells. But what kind of mechanism, if only in theoretical terms, has been proposed for these Na pumps? The anwer is clearly provided by Glynn and Karlish.[8] Covering the literature over a span of 23 years, they begin their review on the sodium pump with these words: "The recent startling growth of the literature on the sodium pump may make a review timely. . . . If the great

mass of work that has been done had led to the general acceptance, even provisionally and even in outline, of a hypothesis accounting for the working of the pump, we could have described that hypothesis and then considered the evidence for it. Unfortunately no such hypothesis exists. . . ."

I believe that the major reasons behind the difficulty in proposing a mechanism for the Na pump are faulty theoretical assumptions, including that which conceives the lipid layer as the permeability barrier.

#### Theories of Epithelial Transport

A number of theories have been offered relating to different aspects of epithelial transport. With the exception of the sketch of a theory communicated by myself in 1965 (see ASSOCIATION-INDUCTION HYPOTHESIS below) all are based on the membrane-pump theory. They include.

(1) The "two-membrane theory" of Koefoed-Johnson and *Ussing*.[9] This theory was based on the authors' work with isolated frog skin. From electrical potential studies they reached the conclusion that the surface facing the outside has a selective high Na+ permeability and the surface facing the inside has a selective high $K^+$ permeability. According to this well-known "two-membrane" theory the inner membrane has essentially the same properties seen in most living cells including the possession of the Na-K pump in the form of K,Na-activated ATPase. It is the membrane facing the outside solution that is unusual and functions as the seat of transport regulation through the control of Na+ permeability.

(2) The standing osmotic gradient theory of Diamond and *Bossert*.[10] Certain epithelial membranes like the gall bladder, intestinal mucosa, and renal proximal tubule transport salt ions and water in the form of an isotonic solution. Diamond and Bossert suggested that this secretion of an isotonic fluid is due to the pumping of ions into the bottom of the spaces formed between the folds of the baso-lateral membrane of the epithelial cells. The hypertonic solutions thus formed then draw water from the cell, becoming more dilute and eventually isotonic as the fluid moves outward toward the serosal "sink."

(3) Cereijido and *Rotunno's* theory of *pericellular pumping*.[11] In this theory Cereijido and coworkers argue that the transport of Na+ by frog skin involves migration of Na+ along an array of fixed negative sites on the outside surface of the epithelial cells.

(4) The *Na-gradient* hypothesis of sugar and *amino* acid transport. Following the discovery that intestinal transport of D-glucose depends on the presence of Na+ in the mucosal fluid,[12,13] it was suggested by Crane et al.[14] and Crane[15] that glucose, $Na^+$, and a carrier form a ternary complex in the mucosal membrane which then dissociates and delivers Na+ and glucose. The Na+ gradient from the mucosal fluid to the cell interior provides the energy for the inward transport of sugar. That uptake of amino acids by unifacial duck erythrocytes and Ehrlich ascites cells and bifacial kidney cells require Na+ was known even earlier.[16,17] Schultz and Curran[18] further elaborated the Na-gradient hypothesis to include transport of amino acids. Support for the hypothesis came from the study of sugar and amino acid transport into isolated microvilli "vesicles." These isolated microvilli transiently take up sugar or amino acids to a greater concentration than found in the surrounding medium, thereby exhibiting an "overshoot," when the sugar or amino acid is added with a high concentration of Na+ but not in the presence of the sugar transport inhibitor, phloridzin. (For references, see below.)

### THE ASSOCIATION-INDUCTION HYPOTHESIS

The bulk of cell $K^+$ and Na+ in most living cells is not found in a separate aqueous phase as in the central vacuole of *Valo-*

*nia.* These ions, rather, are encountered in the cytoplasm, where proteins make up from 15 to *25%* of the total weight. According to the association-induction (AI) hypothesis,[19,20] it is primarily the cell proteins that provide and maintain a different physicochemical environment in the cell, and it is this different physicochemical environment that gives rise to the accumulation of $K^+$ and exclusion of $Na^+$.[21] Specifioally, the AI hypothesis argues that certain proteins provide a network or matrix of extended polypeptide chains whose alternating positive NH and negative CO groups polarize and orient the bulk of cell water into the state of polarized multilayers.[22,23] Water in this state has decreasing solubility as molecular size and complexity of solutes increases. These same and/or other proteins provide $\beta$- and $\gamma$-carboxyl groups for selective adsorption of $K^+$ over $Na^+$.[24] However, both polarized water (and hence Na+ exclusion) and ion adsorption (and hence $K^+$ accumulation) depend on the existence of the protein-water-ion system in a cooperative high-energy state, called the living state. To maintain this living state, adsorption of ATP (and other key molecules) on certain controlling cardinal sites is essential.[21]

Recently there have been major developments in efforts aimed at choosing between the two diametrically opposed theories of the living cell: *(a)* the steady-state membrane-pump or pump-leak theory and *(b)* the equilibrium-based AI hypothesis. Leaving detailed discussion of this multifaceted, complex problem to a monograph in preparation and several published reviews,[21,25,26] I shall briefly consider here only some criticisms of the AI hypothesis.

(1) *Amount of water of hydration on protein is too small to support the contention that the bulk of cell water differs from normal liquid water.*[27] It is argued that since most native proteins hydrate to the extent of 0.2 to **0.3** g water/g dry protein,[23,27] no more than 7 to 8% of the cell water could be significantly affected by the 15 to *25%* protein content found in most living cells. According to recent surveys as well as experimental studies, however, this conclusion is valid only when proteins are in the globular conformation.[23,28,29] On the other hand, if for one reason or another the protein exists in an extended conformation, long-range polarization of many more water molecules occurs. Water so affected has unusual properties; e.g., reduced solubility for $Na^+$, sugars, and amino acids, in full agreement with the AI hypothesis.[29]

*(2) Strong selective adsorption of $K^+$ over $Na^+$ has not been demonstrated in isolated proteins* in vitro.[30] This objection is no longer valid. Ouabain-sensitive, cooperative, selective $K^+$ binding over Na+ onto one isolated protein (i.e., K,Na-activated ATPase) has been successfully demonstrated *in vitro* by Matsui and coworkers.[31,32] Even more exciting is Edelmann's demonstration of selective accumulation of $K^+$ over Na+ in freeze-dried muscle sections *in vitro*,[33] a finding dealt with in greater detail below.

***(3)*** *If cell K+, which constitutes the bulk of intracellular cation, is in an adsorbed and hence osmotically inactive form, the cell would be unable to maintain normal volume.*[34,35] The linear polymer, polyethylene oxide (PEO)—which carries *no net electric* charge—polarizes water in deep layers. A dialysis bag containing PEO-water shrinks in concentrated Na-citrate solutions and swells in dilute Na-citrate solution in a manner similar to that of living cells placed in hyper- or hypotonic solutions, even though in the case of the PEO-water system there is no semipermeable membrane covering the polymer, the dialysis membrane being fully permeable to Na-citrate.[36] This illustrates the point made by the AI hypothesis to the effect

TABLE I. Data of $K^+$ Concentration ($c_K^i$), $K^+$ Activity ($a_{K^+}^i$), and Activity Coefficient ($a_K^i/c_K^i$) in Various Epithelial Cells Measured with Intracellular K -Sensitive Electrodes

| Authors | Cells | $a_K^i$ (mM) | $c_K^i$ (mM) | $\frac{a_K^i}{c_K^i}$ |
|---|---|---|---|---|
| Khuri et al.[53] | Rat distal renal tubule epithelium | 46.5±1.6 | 136 | 0.34 |
| Khuri et al.[54] | Rat proximal renal tubules epithelium | 54.4±2.5 | 136 | 0.40 |
| Zeuthen and Monge[55] | Rabbit intestinal epithelium | 38 | | |
| White[M] | Conger eel intestinal epithelium | 41.6±1.5 | 146±10 | 0.28 |
| Kimura and Fujimoto" | Bull frog urinary bladder epithelium | 39.3 | 115 | 0.34 |
| Kimura et al.[58] | Toad urinary bladder epithelium | 41±0.5 | 140 | 0.29 |
| De Long and Civan" | Toad urinary bladder epithelium | 4320.6 | 140 | 0.31 |

that reduction of cell water activity is due primarily to its interaction with macro-molecules, not with K+. Thus maintenance of cell volume is not hampered.

*(4) The magnitude of the cellular resting potential demands that the bulk of cell $K^+$ be in a free state.*[37] This contention has no merit because cellular resting potential has been shown to be independent of intracellular $K^+$ and external $Cl^-$ concentration,[19,38,39] thus contradicting the membrane potential theory of Bernstein,[40,41] the ionic theory of Hodgkin and Katz,[42] and the electrogenic potential theory of Mullins and Noda.[43,44] On the other hand, the same data are in full quantitative agreement with the AI model of the cell resting potential as a surface adsorption potential.[19,25,38,39,45,46]

*(5) Mobility of $K^+$ in squid axon and frog muscles is close to that of a solute of similar ionic strength with some slow-down due to mechanical obstruction.*[47,48] $K^+$ mobility in healthy frog sartorius muscle fiber is only ⅛ of that in a solution of 0.1 M KCl.[49] Deterioration of the cells causes increases of mobility approaching that in a dilute salt solution.

*(6) Using ion-selective intracellular electrodes, the $K^+$ activity in nerve and muscle cells is seen to be close to that in a simple* KCl *solution of equal ionic strength.*[50,51] This objection to the AI hypothesis is less readily falsified than those listed above. In the next section, therefore, the question of intracellular $K^+$ activity is discussed in detail.

## THE PHYSICAL STATE OF $K^+$ IN LIVING CELLS

### Evidence for $K^+$ Adsorption

Khuri[52] wrote in a recent review: "Since intracellular activities of the monovalent ions were determined largely in muscle fibers . . . but no activity values were available for epithelia, renal physiologists generally assumed that as in muscle, intracellular $K^+$ is all free. . . . As it turned out, these extrapolations with respect to the physical state of intracellular K . . . were invalidated by the intracellular electrochemical techniques."

Table I presents data on intracellular $K^+$ activities measured in a variety of epithelial cells. Uniformly, the activity coefficient obtained was markedly below that found in a solute solution. Most workers explained the reduced $K^+$ activity as due either to sequestration of the missing free $K^+$ in subcellular compartments or to adsorption of $K^+$ in the epithelial cells. The first explanation can be shown to be highly unlikely. $K^+$ is by far the

major cation in the cells; most $Ca^{2+}$ and $Mg^{+}$ are bound; $Na^{+}$-sensitive microelectrode studies have shown also that there is very little free $Na^{+}$ activity in the epithelial cells (ca. *20* to 25 mM) (see ref. *52*, p. 71). With no significant osmotic activity of other solutes to balance the deficit, sequestration of the missing $K^{+}$ into the limited volume of organelles, which make up less than *50%* of cell volume, would lead to an impermissible osmotic imbalance.* That difficulty leaves the second explanation, $K^{+}$ adsorption, the more reasonable one.

This question must then be raised: is $K^{+}$ all "free" in *muscle cells*, as Khuri maintained? A possible answer, offered some time ago, is that protoplasm is easily damaged or activated by the intrusion of the ion-sensitive electrode which excites and causes liberation of $K^{+}$ from its adsorbing sites.[49,60] The fact that the rest of the cytoplasm, more distant from the microelectrode, may remain intact is irrelevant because it is only the $K^{+}$ activity in the immediate vicinity of the microelectrode tip that is measured. The lower $K^{+}$ activity recorded in epithelial cells suggests that they are less susceptible to activation by the microelectrode than muscle and nerve cells, which are inherently more excitable.

More recently, however, the question of the state of $K^{+}$ in muscle cells has been vastly clarified, mostly through the brilliant work of the young German scientist, Ludwig Edelmann.

*The total intracellular $K^{+}$ concentration is approximately 100 mM. Let us make the generous assumption that the combined volume of mitochondria, nuclei, and other subcellular particles add up to 50% of the cell volume. The measured $K^{+}$ activity is 40 mM. To explain this low $K^{+}$ activity in terms of sequestration the average $K^{+}$ concentration in the subcellular particles would be 100 + (100 − 40) = 160 mM. The sum of measured cytosol $K^{+}$ and $Na^{+}$ would then be 40 + *25* = *65* mM, or less than one-half of the anticipated subcellular particle osmotic activity and thus untenable.

According to the AI hypothesis, the seats of selective $K^{+}$ adsorption are the $\beta$- and $\gamma$-carboxyl groups of certain intracellular proteins.[19,24,60,61] In muscle cells, more than *60%* of the $\beta$- and $\gamma$-carboxyl groups belong to myosin.[61] Since in striated muscle, myosin constitutes the A-bands, one could expect $K^{+}$ to be localized primarily in these bands. Furthermore, since it is generally accepted that in EM sections it is also $\beta$- and $\gamma$-carboxyl groups that bind and are thus stained by cationic uranyl and lead stains, the AI hypothesis could further predict that in normal living striated muscle (as well as in other cells) $K^{+}$ will be distributed at the sites that appear dark in standard fixed EM sections stained with uranium and lead.

Figure 1, assembled from published work of Edelmann,[64] shows that these expectations are indeed accurate. Panel A comprises a small segment of frog muscle fixed and stained with uranium in the usual way. Panel B portrays a similar muscle section that had not been fixed or chemically stained. Instead the muscle had been loaded in the living state with electron-dense $Cs^{+}$. Panels C and D show muscles loaded with thallium ($Tl^{+}$) rather than cesium ($Cs^{+}$). These $Tl^{+}$- or $Cs^{+}$-loaded muscles were then freeze-dried, infiltrated with Spurr's medium, and dry-cut by the method of Edelmann.[64] Panel E shows a cesium-loaded section after it had been washed in distilled water. Panel E shows normal frog muscle with its normal K-content. Figure 1 confirms the expectation that $K^{+}$ in muscle cells is neither free nor evenly distributed but instead adsorbed on $\beta$- and $\gamma$-carboxyl groups belonging to the A-band and Z-lines.

The validity of these conclusions was further confirmed by three different laboratories using three additional techniques: (*1*) radioautography of $^{134}Cs$- and $^{208}Tl$-loaded, air-dried single muscle fibers,[65] (*2*) low temperature (70°K) radioautography of $^{134}Cs$-

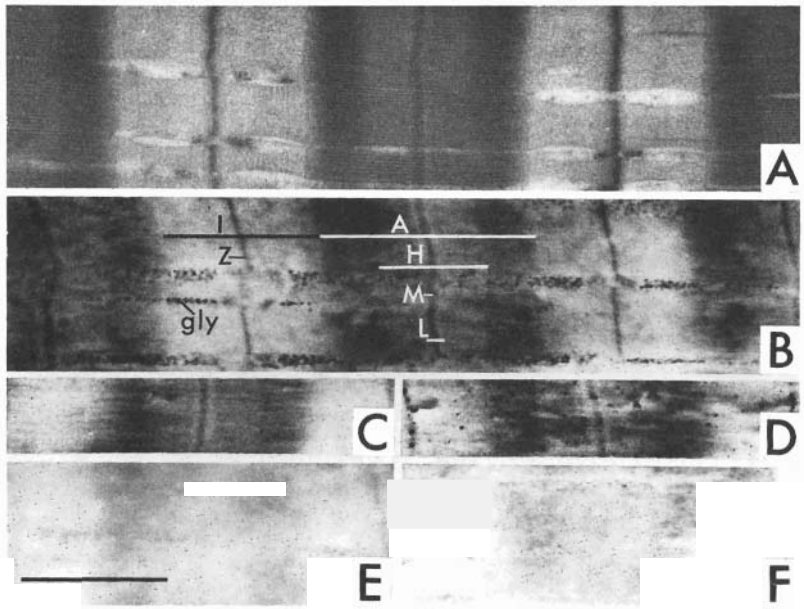


FIGURE 1. Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained with uranium by conventional procedure. (B) EM of section of freeze-dried $Cs^+$-loaded muscle, without chemical fixation or staining. (C) TI+-loaded muscle without chemical fixation or staining. (D) Same as C after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. (E) Section of central portion of B after leaching in distilled water. (F) Normal "K-loaded" muscle. A: from *Edelmann, unpublished. B to F: from Edelmann,*[25] *by permission of Physiol. Chern. Phys.*

loaded, frozen, single muscle fibers,[66] and (3) dispersive X-ray microprobe analysis of $Cs^+$- and $Tl^+$-loaded muscle and of muscle with its normal $K^+$ content.[67] Findings by the latter technique were confirmed by Trombitas and Tigyi-Sebes in isolated air-dried single myofibril of honeybee thorax muscle.[68]

There is no doubt that virtually all of the $K^+$ is specifically adsorbed onto specialized locations in the striated muscle cells. Other experiments with both intact muscle cells[G1] and muscle cells whose postulated membrane pumps had been made totally non-functional (EMOC preparation) showed clearly that the $K^+$ is specifically adsorbed onto the anionic sites and not merely hovering around as free counterions.[69,70] In displacing $K^+$ from adsorption, a pair of univalent cations (e.g., $K^+$ vs. $Cs^+$, $Cs^+$ vs. $Tl^+$) have quantitatively different effects, indicating the major role played by short-range attributes that alone distinguish these ions and that cannot be "felt" without direct contact between the anionic sites and the adsorbed ions.[65]

These findings reinforce the earlier suggestion that ionic activities measured with intracellular ion-sensitive electrodes must be regarded with great caution. That admonition applies not only to excitable cells like muscle and nerve but also to epithelial and other cells. The $K^+$ activity coefficient measured with an intracellular electrode tends to be higher than it should be in totally undisturbed cells. According to the AI hypothesis, virtually all $K^+$ in living cells is in the adsorbed state (Fig. 1).

### Implications of Adsorbed State of $K^+$

OSMOTIC BALANCE. The immobilization of the bulk of intracellular $K^+$ leaves a major

osmotic deficit in muscle cells. One recalls that it is precisely this anticipated "difficulty" that at one time led Hill and many others to conclude that all cell $K^+$ must be free. Since $Ca^{2+}$ and $Mg^{2+}$ as well as a substantial portion of $Na^+$ in frog muscle cells are also in an adsorbed and hence osmotically inactive state, there is no recourse but to accept that the osmotic activity (that is, the lowering of activity of water) must arise from cell proteins. This agrees well with the polarized multilayer theory of cell water in which water activity is reduced by long-range polarization and adsorption. In other words, osmotically speaking, "pure" polarized water behaves like normal liquid water in which substantial amounts of ions or other solutes are dissolved.

CELLULAR ELECTRIC POTENTIAL. The conventional view of the cellular electrical potential, as first suggested by Ostwald[71] and extensively developed by Bernstein,[40] as well as later variants introduced by Boyle and Conway,[41] Hodgkin and Katz,[42] and Mullins and Noda,[43] were all based on the assumption that the bulk of intracellular $K^+$ is in the free state. That in fact the bulk of this $K^+$ is in a localized, adsorbed state makes all these theories inapplicable. Again, the adsorbed state of cell $K^+$ is in harmony with the AI hypothesis' surface adsorption theory of cellular resting potential, which has been experimentally tested and verified.[38,46]

## THE SURFACE BARRIER AND PERMEABILITY

Since Overton introduced his lipoidal membrane theory,[72] the idea that cells and subcellular structures are covered by a membrane whose continuous phase is lipid in nature has taken deep root. Yet there have long been serious questions about the validity of this assumption.[73,74] Seven years ago, for example, I presented evidence that not lipid, but a layer of water polarized in multilayers by surface proteins, constitutes the semipermeable surface barrier.[73]

Work by Stillman et al. on squid axon,[75] by Maloff et al. on inner mitochondria1 membrane,[76] and by Ling and Ochsenfeld on frog muscle and egg[77] produced additional experimental evidence: no change of $K^+$ permeability was observed in any of these systems in response to $K^+$ ionophores. Since there is no question that under similar conditions $K^+$ permeability of phospholipid layers is drastically increased by valinomycin ($10^{-7}$ M) and monactin ($10^{-5}$ M),[78] one concludes that the semipermeable barrier in muscle, nerve, and egg cell as well as in the inner membrane of mitochondria is not a phospholipid layer.

In my view of the cell surface barrier, semipermeability depends on the state of multilayer polarization of cell water near the surface. That state in turn depends on certain proteins assuming the extended conformation. Semipermeability is therefore under close control by hormones or drugs that react with receptor sites on these proteins and so control their conformation. The susceptibility of water to allosteric control offers yet another major advantage to the AI model and suggests new approaches to cell physiology and pharmacology.

### Solute Permeation According to the AI Hypothesis

Before further comparison with the theoretical model of active transport of solute and water across bifacial cells can be given, some additional basic concepts of the AI hypothesis need to be introduced. These concepts include (*1*) the molecular mechanism of selective solute distribution in living cells, (2) the molecular mechanism of selective solute permeability, (*3*) cooperativity in adsorption and desorption of solutes and water, and (4) the control of cooperative adsorption-desorption by a cardinal adsorbent, ATP. Fuller accounts of these concepts and supportive evidence can be encountered else-

where,[19-21,26,79] but we briefly review them here.

SELECTIVE SOLUTE DISTRIBUTION IN LIVING CELLS. There are two basic modes of existence of an intracellular solute: adsorbed on macromolecular sites or dissolved in the cell water. Referring to the particular solute of interest as $p_i$, and its concentration in the cell in moles per kilogram of fresh cells as $[p_i]_{in}$, then

$$[p_i]_{in} = [p_i]_{free} + [p_i]_{ad} \quad (1)$$

where $[p_i]_{free}$ and $[p_i]_{ad}$ are respectively the concentration of free and adsorbed ith solutes in moles per kilogram of fresh cells. The distribution of free ith solute in the cell water follows the Nernst distribution law and thus is described by the linear relation

$$[p_i]_{free} = \alpha q_i [p_i]_{ex} \quad (2)$$

where $\alpha$ is the percentage water content (v/w) of the cell, $[p_i]_{ex}$ is the equilibration concentration of the ith solute in the external medium, and $q_i$ is the average equilibrium distribution coefficient of the ith solute between cell water and the external medium.

Now, if and when the cell water is entirely normal as, say, in a Ringer's solution, $q_i$ would be equal to unity for all permeant solutes. If, however, the cell is in its normal resting state, then according to the AI hypothesis this water is not normal liquid water but in the state of polarized multilayers. As a rule, water in that state has solubilities different from those of normal liquid water, and in polarized water $q_i$ varies with the solute involved. In general, small and simple molecules have q-values close to or equal to 1. The q-value usually decreases with increasing size and complexity of the solute in question. The concentration of hydrated solutes like Na+ in a living cell may be 0.1 or even lower. According to the AI hypothesis, it is the low q-value for Na+ in the cell water, and the unfavorable adsorption energy of $\beta$- and $\gamma$-carboxyl groups in comparison to that for K+, which account for the sustained low Na+ concentration in many kinds of cells.

The adsorbed fraction of the ith solute may be, under the simplest condition, limited to just one type of adsorption site. In this case, the concentration of the ith adsorbed solute may be described by a Langmuir adsorption isotherm:

$$[p_i]_{ad} = ([f]K_i[p_i]_{ex})/(1 + K_i[p_i]_{ex}) \quad (3)$$

where [f] is the concentration of intracellular adsorption sites in moles per kilogram of fresh cells, and $K_i$ is the adsorption constant in units of $(M)^{-1}$. If another solute called the *j*th, and represented as $p_j$, also adsorbs onto the same sites, Eq. 3 becomes

$$[p_i]_{ad} = ([f]K_i[p_i]_{ex})/(1 + K_i[p_i]_{ex} + K_j[p_j]_{ex}). \quad (4)$$

Equation 4 shows that there is a hyperbolic relation between the adsorbed ith solute and the external concentration of the ith solute. Further, this fraction shows competition with other solutes like the *j*th. It also shows *saturability*; i.e., as $[p_i]_{ex}$ increases, $[p_i]_{ad}$ approaches but cannot exceed the value of $[f]$.

Combining Eqs. 1, 2, and 4, we have

$$[p_i]_{in} = \alpha q_i [p_i]_{ex} + ([f]K_i[p_i]_{ex})/(1 + K_i[p_i]_{ex} + K_i[p_j]_{ex}). \quad (5)$$

A somewhat simpler version of this equation was first presented by the Russian physiologist, A. S. Troschin.[80]

### Solute Permeation According to the Membrane-Pump Theory

In the conventional membrane theory, permeation of a solute molecule into a living

cell may be achieved by three mechanisms: (a) free diffusion, (b) facilitated diffusion, and (c) active transport.

Free diffusion, as the name indicates, is envisaged as involving diffusion through the lipid phase of the cell membrane. The rate of solute entry into a cell by free diffusion is usually linearly related to the concentration of the solute in the external medium. As for facilitated diffusion and active transport, fundamentally they are alike. For example, both rest on saturability. The difference between the two mechanisms lies in the relative electrochemical potential of the phase the solute moves into. An active transport mechanism of solute movement is conceived as being against an electrochemical gradient, while facilitated diffusion is conceived as being *not* against an electrochemical gradient. The notion of an uphill movement when applied to unifacial cells is entirely dependent on the basic assumption of the membrane theory that the cell interior is filled with a dilute aqueous solution. Since this assumption has already been disproven (see above), there is no longer a solid foundation for the proposed difference between facilitated diffusion and active transport into single unifacial resting cells such as erythrocytes, muscle cells, or nerve cells. Thus phenomenologically there are two rather than three types of permeation mechanisms: one depends on saturability; the other does not.

Facilitated diffusion is distinguished from free diffusion by at least three outstanding features. First of these is saturability. As already mentioned, the rate of entry of a solute into living cells by this means does not increase linearly with increasing external concentration of the solute. Instead, the rate levels off to approach a fixed value. The second distinguishing feature is competition. That is, the rate of entry of a solute into living cells is inhibited by other solutes of similar nature. In practice, this trait can be easily and quantitatively assessed with the aid of, for example, Linewever and Burk double reciprocal plots of rates vs. external concentration, as in enzyme kinetic studies. The third distinguishing feature is specificity. The rate of entry of a solute into living cells often exhibits a high degree of specificity. Thus the steric orientation of one of the five OH groups in a sugar molecule may have a profound influence on the rate of entry of the sugar. It has been widely considered that facilitated diffusion is mediated through "molecular oarriers" which, like ferry boats, select favored "passengers" and shuttle them back and forth across the lipid membrane barrier. But I have already mentioned convincing evidence against the concept that cells are covered with a continuous sheet of phospholipid. Other such evidence against the lipid barrier concept has been reviewed elsewhere.[75-77] With disproof of the universal existence of a continuous lipid layer the concept of "mobile carriers" also falls, for again like ferry boats, such carriers cannot shuttle without a fluid barrier. Indeed, from the viewpoint of conventional membrane theory, one cannot imagine a better "mobile carrier" for $K^+$ than valinomycin and monactin. The failure of these "ionophores" to cause significant change in $K^+$ permeability in nerve, muscle, and egg cells demands totally different interpretations for the physiological observations of solute permeation. Such alternative interpretations have been available for some time.[19,39,81,82]

### Permeation Mechanisms According to the AI Hypothesis

Since lipid does not constitute a continuous surface barrier and a "mobile carrier" cannot exist without a water-immiscible fluid barrier, clearly a different phospholipid model must be sought to explain the saturable and competitive solute permeation widely observed. Such a model has been suggested.[19,83] It will now be briefly reviewed.

**POLARIZED WATER IN LIEU OF LIPID LAYER AS THE SURFACE BARRIER FOR "NON-SATURABLE" SOLUTE ENTRY.** The near perfect semipermeability of Traube's copper ferrocyanide precipitation membrane led Pfeffer to found the membrane theory.[1] Copper ferrocyanide obviously contains no lipid but is a gel composed of a network of crystalline particles with water-filled interstices on the order of **150 A** in width.[84] These "pores" are many times as wide as the diameter of sucrose molecules (94 **A**); yet this membrane is virtually impermeable to sucrose. More recently I showed that the permeability to water and ten other hydroxylic compounds at three different temperatures through inverted frog skin is in excellent correspondence to that through a sheet of cellulose acetate.[73] Both membranes exhibit highest permeability for water; both are virtually impermeable to sucrose even though the average pore diameter of the "active" layer of the cellulose acetate membrane is more than four times as wide as the sucrose molecule. These data strongly support the concept that water in the state of mulilayers, polarized by a matrix of protein chains carrying polar sites, provides semipermeable surface barriers in living cells as well as their cogent models.

**FIXED POLAR SITES ON SIDE-CHAINS AND "BACKBONE" OF CELL SURFACE PROTEINS IN LIEU OF "MOBILE CARRIERS" AND "PUMPS" AS "GATES" FOR "SATURABLE" SOLUTE ENTRY.** The AI hypothesis offers an interpretation of what is conventionally called facilitated diffusion, as well as of active transport, in terms of an adsorption-desorption mechanism on cell surface adsorption sites. Indeed the mechanism is so simple that it involves few postulations additional to those already described for solute distribution in the bulk phase.

Consider the rate of permeation of labeled $K^+$ into a living cell, typically demonstrating saturability, competition, and a high degree of specificity. Now, the bulk phase distribution of $K^+$ in frog muscle cells is to a first approximation described by Eq. 5. If we consider the cell surface to be primarily a water-protein system like the cell interior, then in principle the cell surface is a two-dimensional rendition of the three-dimensional cell. In other words, like the cell interior, the cell surface will have a continuous layer of polarized water mentioned above, and also anionic $\beta$- and $\gamma$-carboxyl groups distributed at regular distances apart. Furthermore, if an instantaneous photograph could be taken, one would find most of the $K^+$ to be associated with the surface anionic sites, as has been demonstrated for the bulk phase $K^+$ (Fig. **1**). Few $K^+$ molecules would be found between these sites in the interstices filled with polarized water. If then a motion picture could be taken, one would observe two different modes of entry corresponding to each of these instantaneous positions taken by the $K^+$:

(1) Saltatory route. In this mode of entry, $K^+$ enters via the polarized water filling the space away from the charged sites.

(2) Adsorption-desorption route. As the name indicates, for this kind of entry the $K^+$ from the outside must first succeed in occupying one of the anionic sites, followed by a librational motion around that site and then eventual desorption and entry into the cell.

Taking both routes into account, the rate of entry $v_{i,\mathrm{inw}}$ of the ith ion in moles per sec per kilogram of fresh cells can be written as[19,82]

$$v_{i,\mathrm{inw}} = Ak_{i,\mathrm{sal}}[p_i]_{\mathrm{ex}} + \{(k_{i,\mathrm{ad}}[f]K_i[p_i]_{\mathrm{ex}})/(1 + K_i[p_i]_{\mathrm{ex}} + K_j[p_j]_{\mathrm{ex}})\} \quad (6)$$

where A is the total surface area of one kilogram of cells, $k_{i,\mathrm{sal}}$ is the inward rate constant for the entry of the ith solute via the

saltatory route, *[f]* is the molar concentration of surface sites per kilogram of fresh cells, $k_{i,ad}$ is a kinetic rate constant for the desorption of the ith solute from the surface adsorption sites, $K_i$ and K, are respectively the adsorption constants in $M^{-1}$ of the ith and *j*th solute on the surface sites.

Epstein and Hagen[85] in 1952 first successfully analyzed alkali-metal ion entry into barley roots using Michaelis-Menton kinetics, an analysis I soon confirmed for Rb+ entry into frog muscle.[81] Epstein and Hagen like many other investigators who have extended these studies to various types of cells and solutes, adhered to the "mobile carrier" concept. Indeed, with the additional assignment of the non-saturable fraction of solute entry as "leaky," the overall rate equation for solute permeation becomes formally analogous to Eq. 6.( For extensive discussion of this subject from the conventional viewpoint, see Christensen.[86]) However, as mentioned above, the disproof of the lipid layer theory makes untenable the mobile carrier model.

To demonstrate the general validity of Eq. 6 and its basic assumptions, I have shown they can not only describe rate of entry of solutes into living cells but also rate of entry into such inanimate systems as sheets of ion-exchange resin or sheep's wool.[19,39,82] These models have in common with the AI model the attribute of anionic sites fixed in a matrix of polarized water.

While Eq. 6 adequately describes solute entry, especially for neutral solutes, a somewhat more complicated equation is required to describe the entry of ions into frog muscle cells by a mechanism called the triplet adsorption-desorption route. That is to say, in the case where the entrant ion is rather tightly adsorbed by the electrostatic forces, its desorption requires the participation of another free cation. Thus a second $K^+$ from the outside may approach a fixed anion-$K^+$ pair in the right direction, weakening the attraction between the fixed anion and $K^+$, thereby facilitating the latter's entry into the cell. Indeed, we have shown that the rate of entry of labeled Rb+ into frog muscle cells is facilitated by external $K^+$.[82] Clearly the facilitating effect of the second $K^+$ outweighs the competition offered by $K^+$ against Rb+ adsorption.

SPECIFICITY OF SUGAR PERMEABILITY. As noted above, the third characteristic of permeation phenomena is specificity. In the past this has often been attributed to carriers, and is exemplified by sugar entry into cells. Here also, I believe, the present model has distinctive advantages over the oarrier model.

There is little question that the high degree of steric specificity for solute permeation rate can be recognized only by a system that provides a complex of closely spaced sites. This strongly suggests that these sites are provided by proteins. In the AI model, adsorption followed by libration and desorption would be all that is required to achieve a facilitated diffusion when such specific sites are available.

SPECIFICITY IN ALKALI-METAL ION ENTRY; VARIABILITY AND THE C-VALUE CONCEPT. Surfaces of most normal unifacial cells and the basel lateral surfaces of bifacial cells have greater permeability for $K^+$ than for $Na^+$. However, the frog skin surface facing the outside has a higher permeability for Na+ than for $K^+$.[9] What could be the molecular basis for this specificity difference?

In 1952 I suggested a theoretical model of selective adsorption of $K^+$ over Na+ on fixed anionic sites.[24] Stimulated by the later discovery[87] that carboxyl ion exchange resin selects Na+ over $K^+$ while sulfonate ion exchange resin selects $K^+$ over $Na^+$, as well as the work of Eisenman, Rudin, and Casby on glass electrode ion selectivity, I constructed a theoretical model in which the c-value concept was introduced.[19,39] In essence, the c-value measures the electron density of an anionic oxygen atom; high

c-value is equivalent to a high $pK$ value as in acetic acid, low c-value to a low $pK$ value as in trichloroacetic acid. It was then possible to show that a variation of the $c$-value produces changes in the preferential order of selectivity among $Cs^+$, $Rb^+$, $K^+$, $Na^+$, $Li^+$, as well as $NH_4^+$ and $H^+$. Thus $K^+/Na^+$ preference seen in muscle and nerve as well as basolateral membranes of various epithelial cells corresponds to a fairly low c-value; high $Na^+$ over $K^+$ preference seen in a number of apical cell membranes" bespeaks a high c-value. The essence of this work was presented in 1960[39] and in full detail in 1962.[19]

Solitary $\beta$- and $\gamma$-carboxyl groups usually have a pH value of 4 to 5; when carboxyl groups are placed in close proximity to each other as in various carboxy types of ion-exchange resin, the $pK$ value may rise to 9 or even higher![88] Thus the high c-value of apical membranes may be due to $\beta$- and $\gamma$-carboxyl groups in close proximity to each other or paired, whereas the low c-value at the basolateral membrane may be due to isolation of $\beta$- and $\gamma$-carboxyl groups (for evidence, see ref. 89).

In 1962 Eisenman also published a theory of selective ionic adsorption using a much simpler model[90,91] to explain the relation between ion specificity of glass electrodes and the glass composition.[91,92] Ussing and Leaf[7] considered Eisenman's theory to explain the different specificity at the two surfaces of epithelia but rejected it on the ground that the theory does not provide enough specificity. They preferred the "close-fit" hypothesis of Mullins[93,94] in which close-fitting into small holes endows a high degree of specificity in selectivity for $Na^+$ and $Li^+$ over $K^+$ (see ref. 19, p. 548, for tabulated data from literature). Mullins' model required a dehydration of $Na^+$ and $Li^+$ prior to entry into the postulated close-fitting pores—a concept not easily defensible if the cell membrane is a lipid bilayer, and not defensible at all if the membrane is not a lipid bilayer. It is even more difficult to think of rigid pores in a layer of polarized water that would force the dehydration of $Na^+$ and $Li^+$ before their entry.

On the other hand, the low level of ionic specificity attributed to Eisenman's model is not applicable to the association-induction model, in which a high degree of $Na^+$ as well as $K^+$ selectivity has been theoretically calculated.

In brief, I feel that difference in ionic specificity between the two surfaces of the epithelial cells can be adequately explained as a result of the difference in the c-values of the anionic surface sites.

## PROTEIN-WATER-ION: THE COOPERATIVE ASSEMBLY

### Cooperativity in Adsorption and Desorption of Solutes and Water

In the preceding sections I have discussed adsorption in relation to selective solute accumulation as well as selective solute permeability. Thus far the adsorption sites have been considered non-interacting and therefore the adsorption is adequately described by the Langmuir adsorption isotherm. In that isotherm, given as a part of Eq. 5, the concentration of adsorption sites [$f$] and a pair of adsorption constants $K_i$ and $K_j$ (or their ratio, $K_i/K_j$) determine the adsorption at a fixed ratio of $[p_i]_{ex}/[p_j]_{ex}$ in the surrounding medium. In the cooperative adsorption isotherm, these parameters play comparable roles. However, it is the new parameter, the nearest neighbor interaction energy, that opens the door to coherence in adsorption. Thus if the nearest neighbor interaction energy is positive, it means that if one adsorption site adsorbs $K^+$ it would make the two immediately neighboring sites prefer $K^+$ over $Na^+$. Conversely, if the middle site adsorbs $Na^+$, it would make the two immediately

neighboring sites prefer Na+ more than if the middle site were to adsorb K+. The result is *autocooperativity* in ion adsorption. When the nearest neighbor interaction energy is large and positive, the whole system of adsorption sites will adsorb either all $K^+$ or all $Na^+$. "All-or-none" switching can then occur at a "threshold" value of the external $K^+/Na^+$ concentration ratio. Thus autocooperativity among the four heme sites in hemoglobin provides the molecular basis for efficient loading and unloading of oxygen between the lung and the respiring tissues. This type of cooperative adsorption isotherm describes the uptake of $K^+$ or Na+ in various living cells, including frog muscle,[95-97] human lymphocytes,[98,99] and a variety of smooth muscles (ref. 100, p. 22), all showing positive nearest neighbor interaction and a sigrnoid-shaped adsorption curve. Since I have established that initially nearly all intracellular $K^+$ is in an adsorbed state (Fig. 1), this autocooperativity switching from the $K^+$ to the Na+ state clearly bears a fundamental similarity to the oxygenation and deoxygenation of erythrocytes. In both, the seats of interaction are intracellular proteins. As far back as 1908, Moore pointed out the parallelism of oxygen accumulation in erythrocytes and $K^+$ accumulation in cells.[101] In 1965, I sharpened this parallelism by demonstrating that both oxygen taken up by erythrocytes and $K^+$ taken up by frog muscle show autocooperative behavior with similar values of nearest neighbor interaction energy.[96]

### Control of Cooperative Adsorption and Desorption by Cardinal Adsorbents

Autocooperativity provides the basis for the ability of the solute-adsorbing protein to shift its adsorbed solute from one type to another in a stepwise, all-or-none manner. According to the AI hypothesis, such an autocooperative transition is not the property of the protein alone but involves the water molecules, ions, and other substances that interact with the protein molecule at different sites. As a result of the propagated electronic redistribution, all or at least a major portion of the properties of the protein-water-ion assembly are changed. Often, however, it is the physical conformation change that is most noticeable. (For a fuller discussion of the various theories of cooperative interaction, see ref. 25.) Proteins contain specific sites that exert a controlling influence on the all-or-none transitions. These sites are called cardinal sites, and the specific adsorbents interacting with these cardinal sites are called cardinal adsorbents. ATP is a *bona fide* cardinal adsorbent, as are many drugs and hormones, and all share a distinctive feature: a small number of cardinal adsorbent molecules can bring about responses involving a much larger number of non-cardinal sites such as the $\beta$- and $\gamma$-carboxyl groups adsorbing $K^+$ and $Na^+$.

OUABAIN AS A CARDINAL ADSORBENT. Ouabain, like a number of other cardiac glycosides, causes loss of $K^+$ and gain of Na+ in a variety of living cells. In the conventional membrane-pump model, ouabain acts by inhibiting the Na pump. In fact, it was the parallel behavior of ouabain's effect on the K+/Na+ distribution in living cells and its inhibition of isolated K,Na-activated ATPase that gave impetus to the extensive work carried out under the assumption that this K+, $Na^+$-activated ATPase is in fact the Na pump and that when incorporated into phospholipid vesicles this pump can actually translocate both $K^+$ and Na+ against concentration gradients. Careful analysis of the resultant data revealed major inconsistencies of interpretation, so that an alternative interpretation more in accord with the findings was presented in 1980.[102]

That K,Na-activated ATPase can indeed adsorb $K^+$ and Na+ and that this adsorption

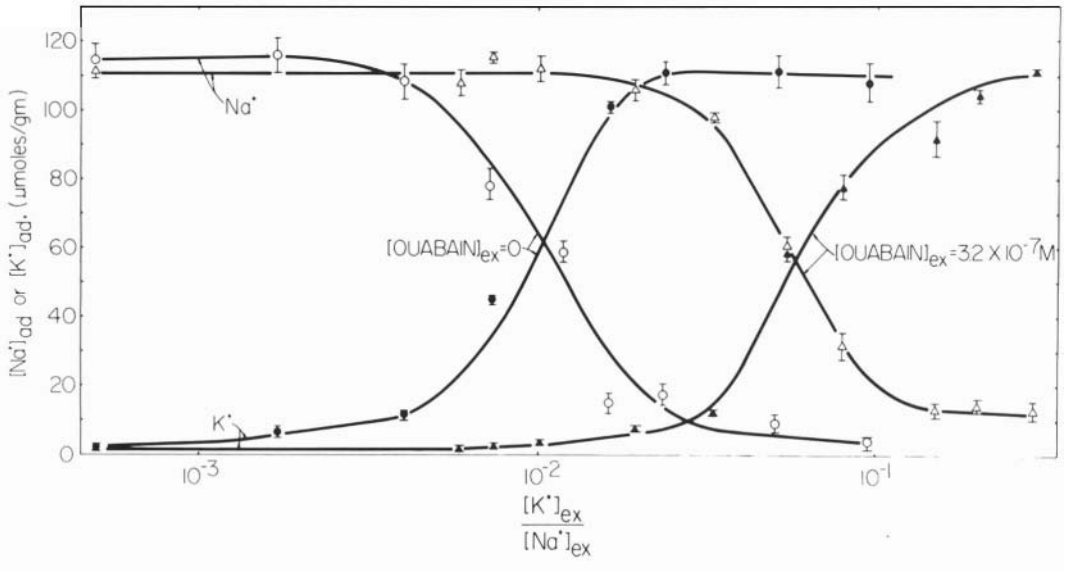


**FIGURE 2. Effect of ouabain ($3.2 \times 10^{-7}$ M) on equilibrium distribution of $K^+$ and $Na^+$. Curves with open ($Na^+$) and filled ($K^+$) circles describe equilibrium distribution data for muscles not treated with ouabain. The point of intersection gives $K^{oo}_{Na \to K}$ of 100. In muscles treated with ouabain ($3.2 \times 10^{-7}$ M), $K^{oo}_{Na \to K}$ has shifted to 21.7.**

is indeed sensitive to ouabain have been demonstrated by Matsui et al.[31,32] as mentioned above under **INTRODUCTION.** The ouabain-sensitive $K^+$ binding on K,Na-activated ATPase also exhibited autocooperativity as had Seen repeatedly demonstrated for $K^+$ binding in intact living cells.

Certainly the data of Matsui et al. indicated that this enzyme may be the seat of some $K^+$,$Na^+$-adsorption in some cells. Nevertheless, the demonstration that the A-band containing another ATPase, myosin, is the seat of adsorption of the bulk of intracellular $K^+$ left little doubt that ouabain acts on $K^+$ and Na+ distribution in frog muscle and other cells by changing the relative preference of many proteins for the adsorption of $K^+$ and $Na^+$. Figure 2, from Ling and Bohr,[97] shows that the Yang-Ling cooperative isotherm can quantitatively describe the $K^+ \rightarrow$ Na+ transition in frog muscle in response to $3.26 \times 10^{-7}$ M ouabain. The figure presents the full range of external $K^+/Na^+$ concentration ratios to demonstrate that the primary effect of ouabain is to shift the intrinsic equilibration constant for the Na+ —, $K^+$ exchange from a value of about 100 to a value of 17; i.e., the constant changes by a factor of 6.

The normal environment of muscle cells contains 2.5 mM $K^+$ and 100 mM $Na^+$, corresponding to a $[K^+]_{ex}/[Na^+]_{ex}$ ratio of $2.5 \times 10^{-2}$. At this ratio of external $K^+/Na^+$ concentration, almost all the intracellular anionic sites are occupied by K+. The effect of exposure to $3.26 \times 10^{-7}$ M ouabain is to reverse the situation completely; the anionic sites become almost entirely occupied by $Na^+$.

It is clear that ouabain, by its adsorption onto the appropriate cardinal sites, alters selectivity in adsorption of the cooperatively linked anionic sites, causing changes in *c*-values in a direction toward reduced preference for $K^+$ over $Na^+$. Therefore it is to be expected that if one increases external $K^+$ concentration or decreases external Na+ concentration by a factor equal to or greater

than 6, the preferential $K^+$ accumulation seen in normal cells will be restored. That this is true is indicated, of course, by the data of Fig. 2.

ATP AS A CARDINAL ADSORBENT. ATP was long considered to carry special chemical bonds—so-called high energy phosphate bonds. But later work[103] established that the enthalpy of hydrolysis of these bonds was not usually high at all. Investigation also established that the favorable free energy of hydrolysis reflects largely the different affinity it produces for $H^+$, $Mg^+$, and $H_2O$.[104,105] These findings, though little celebrated, contributed substantially to better understanding of ATP, one of the most important biological compounds. Certainly the old idea that this package of high energy could do work as a sort of universal fuel became no longer tenable.

The AI hypothesis, on the other hand, by recognizing the role of ATP as a cardinal adsorbent, provides a different mechanism by which ATP energizes biological performance.

#### Maintenance of the Living State: Role of ATP

The conventional concept, still, is that hydrolysis of ATP releases energy stored in its "high energy" phosphate bond to support cellular work. Thus a resting muscle, on receiving its package of energy from the hydrolyzing ATP, enters into the high-energy contracted state. When the ATP is used up, the muscle reverts back to its low-energy relaxed state.

According to the AI hypothesis, quite the opposite is the case. The resting state is seen as a high-energy state; its maintenance does not depend on a steady stream of decomposing ATP but on a steady adsorption of intact ATP onto key cardinal sites. The resting state is a high-energy state much like that of a set mousetrap. It is only when ATP is destroyed, say by hydrolysis, that the muscle seeks its low-energy state, much as a triggered mousetrap seeks its low-energy state.

The simplest and most direct evidence in favor of this concept is the fact that a dead muscle, as a rule, is found in the contracted state. Were the relaxed state the lower energy state, dead muscle all should be fully relaxed and *rigor mortis* would not occur.

Relaxation and shortening are but two of the changes in muscle tissue that occur when its ATP content changes. A parallel phenomenon is the inverse relation observed between muscle shortening and muscle desorption of $K^+$ followed by its release into the medium.[19,24] Perhaps the most convincing and elegant demonstration of this intimate relation was that performed by Wilde and coworkers using perfused turtle hearts.[106] They were able to observe that each heartbeat is accompanied by an exact pulse of labeled $K^+$ release.

ATP-induced changes are not limited to contraction-relaxation and $K^+$ desorption-adsorption, biologically important as they are, but include changes in the physical state of intracellular water.

#### ATP Control of the Physical State of Cell Water

In 1952 I showed that a quantitative relation exists between $K^+$ (and $Na^+$) content and ATP level in frog muscles poisoned by iodacetate.[24] This observation has been repeatedly confirmed and extended.[19,79,107,108] There is also, generally speaking, a reciprocal relation between the $K^+$ and $Na^+$ contents. The latter relation can be explained as due to a mechanism similar to that proposed above for ouabain effects. However, the strict one-for-one exchange seen in ouabain-induced $Na^+$ for $K^+$ exchange was not observed here. Found instead was a parallel between the gain of $Na^+$ with decreasing ATP and the gain of labeled sucrose.[79]

In terms of the AI hypothesis, the low level of (hydrated) $Na^+$ as well as sugars and amino acids in normal living cells is due to the polarized multilayered condition of the cell water. Just as ATP acting as a cardinal adsorbent maintains the protein anionic $\beta$- and $\gamma$-carboxyl groups at a c-value at which $K^+$ is preferred over $Na^+$, so the adsorption of ATP on the same or other proteins maintains the c-value analogs* of the backbone carbonyl groups (and the $c'$-value analogs* of the backbone imino groups) at values that favor long-range water polarization. This single unitary cause for solute exclusion (in contrast to the multiple and separate causes required by membrane-pump theory) is described as the universality *rule*.[74] The rule states that if for one or another reason the solubility of one normally excluded solute is changed, then the solubility of all other normally excluded solutes should change pari *passu*. This rule has been demonstrated in D-arabinose distribution and $Na^+$ distribution in frog ovarian eggs[109] as well as in D-arabinose, sucrose, and $Na^+$ distribution in the IAA-poisoned frog muscles.[110] As the egg cells or muscles were dying their ATP levels gradually fell, and a parallel gain of free $Na^+$, free D-arabinose, and free sucrose occurred.

**ATP Control of Salt Linkage Formation and Dissociation**

As mentioned above, ouabain causes a stoichiometric displacement of $K^+$ by $Na^+$. The additional $Na^+$ taken up has been shown by NMR spectroscopy to be in an adsorbed state.[111] For a while it was asked whether the original assumption used in identifying bound $Na^+$ might not have been erroneous.[112] However, it turned out that although error had been made it was merely quantitative. The "disappearance" of part of the $Na^+$ signal was indeed due to one-site-one-ion specific adsorption of $Na^+$. Thus Monoi's demonstration that the NMR-invisible $Na^+$ in liver homogenate can be made visible by introduction of competing $K^+$ and $Cs^+$, but not of choline, indicates ion-specific adsorption.[113] If $Na^+$ signal disappearance were truly due to a diffuse electric field gradient as suggested by Berendson and Edzes,[112] there would be only valency specificity and not the ion specificity that Monoi found. A theoretical argument against the diffuse electric field gradient concept was presented by Chang and Woessner.[114]

The obedience of $Na^+$ and sugar uptake in poisoned frog muscle to the universality rule, however, shows clearly that the loss of adsorbed $K^+$ is accompanied by a gain of free $Na^+$ (and free sucrose) and is therefore different from ouabain-induced $K^+ + Na^+$ exchange.

But if $K^+$ is lost, what has happened to the $\beta$- and $\gamma$-carboxyl groups (in the muscle A-bands and Z-lines) that normally adsorb $K^+$? According to the AI hypothesis, in the absence of ATP these anionic sites [f], became "masked" by forming salt linkages. Specifically, without ATP those sites [f] prefer as counterions fixed cations [$f^+$] in the form of $\epsilon$-amino groups, guanidyl groups, a-amino groups, and/or histidyl groups, thus forming salt linkages $f^+f^-$.[19,60,115] An expression for the reaction is

$$f^-{\cdot}K^+ + f^+X^- \rightarrow f^-f^+ + K^+ + X^-. \qquad (7)$$

Still uncertain is the nature of the counteranion, $X^-$, adsorbed to the fixed cation before the salt-linkage formation. However, in

*The $c'$-value is a parameter measuring the positive charge density at a cationic group. c-value analogs and $c'$-value analogs refer to the negative and positive charge density at polar groups not bearing net charges."

muscle tissues X– could be creatine phosphate.

If salt linkages are formed among different protein molecules, there may be macroscopic volume shrinkage and loss of water with concomitant change of the state of remaining water. On the other hand, if the salt linkages are formed within a single protein molecule or an aggregate of like protein molecules, the result might be limited to conformation change of the molecule(s).

### Synchronized ATP- and ATPase-Dependent Cyclic Changes

Addition of $Mg^{2+}$ and ATP to a suspension of glycerinated *Paramecia* evoked synchronized beating of the cilia, propelling the dead protozoa in water as if they were alive.[116] An interpretation of this remarkable phenomenon on the basis of the AI hypothesis was offered.[21] In brief, ATP acts as a cardinal adsorbent that causes cilia orientation in one direction, followed by $Mg^{2+}$-induced ATP hydrolysis and resultant protein conformation change that causes cilia orientation in another direction. The beating cycle is then re-initiated by adsorption of fresh ATP. This model establishes the need for a continuous supply of fresh ATP to maintain continuous swimming.

Another fascinating illustration of the inherent ability of protoplasm to act in a synchronized and cyclic manner is the ATPase-dependent ion accumulation and swelling cycle demonstrated in rat liver and pigeon heart mitochondria, initiated in the latter by valinomycin or monactin.[117,118]

The evidence that valinomycin, monactin, etc. do not act as ionophores but rather as cardinal adsorbents has been discussed recently.[25] It only needs to be mentioned here that $Sr^{2+}$, a simple divalent ion with no ionophore property whatsoever, can initiate cycle changes in much the same way observed for valinomycin in other studies.[119]

The oscillatory changes at issue are in perfect synchrony in regard to shrinkage and swelling with concurrent loss and gain of $K^+$, and they involve the operation of an ATPase. It is my belief that this basic protoplasmic trait is essential to a variety of physiological activities including transepithelial transport of water and ions.

### Cooperative Adsorption-Desorption Model of Active Transport Across Epithelia and Other Bifacial Cell Systems

When living cells are incubated in a $K^+$-free or low $K^+$ solution, they gain $Na^+$ and lose $K^+$ until a new equilibrium is reached. In the case of isolated frog muscle, a tenfold reduction in the external $K^+$ concentration (from 25 to 0.25 mM) at constant external $Na^+$ concentration (100 mM) leads to almost total displacement of $K^+$ by $Na^+$ (Fig. 2). If these $K^+$-depleted muscles are returned to a normal Ringer's solution, their $Na^+$ will be stoichiometrically displaced by $K^+$. In this restoration process both $Na^+$ and $K^+$ are "transported" against concentration gradients. This type of phenomenon is referred to as "active transport" by adherents of the membrane-pump theory. However, now that the cellular $K^+$ is known to be virtually all adsorbed (Fig. 1), as is the $Na^+$ that stoichiometrically replaces $K^+$,[120] the phenomenon can no longer be regarded as active transport. Actually the phenomenon represents an exchange adsorption much like that seen in the operation of an ion-exchange resin. The displacement of $K^+$ by $Na^+$ adsorbed on Dowex 50 is not an active transport process but merely the consequence of (*1*) a change in the ratio of $K^+/Na^+$ concentration in the external medium and (2) the fact that electrostatic and other forces, at the ambient concentration ratio, make it more favorable for one ion to congregate in the resin than an alternative ion. Oxygen concentrates in red blood

cells for exactly the same reasons.

If a dialysis bag containing hemoglobin solution is suspended in an oxygen-containing solution, after a suitable length of time an equilibrium will be reached and the amount of oxygen taken up by the sac is quantitatively defined. If now ATP is added to the internal solution, oxygen will begin to move out of the hemoglobin-containing phase into the external solute.[79,121] This outward migration of oxygen is the consequence of ATP interaction with the oxygen-binding protein, hemoglobin. ATP exercises a long-range effect on the affinity of the heme groups for oxygen, a type of effect termed "allosteric" by Monod et al.[122] (A detailed mechanism for such allosteric effect has been offered by the AI hypothesis, which referred to the phenomenon as an indirect *F*-effect.[20,21]) When ATP is removed from the system, oxygen will once more move into the bag, apparently against a concentration gradient. This effect of ATP on the concentration of a solute in the hemoglobin-containing system does not involve the hydrolysis of ATP, since hemoglobin has no ATPase activity.

Movement of the solute oxygen into and out of the above hemoglobin-containing system involves participation of the laboratory worker, who introduces or removes ATP from the system and hence brings about the oxygen transport. But one may imagine another way to achieve the cyclic back-and-forth transport of oxygen. It would occur if the bag contained first, a specific enzyme, an ATPase, which could destroy and thus remove ATP from the hemoglobin-containing phase; second, another enzyme system that could regenerate ATP; and third, a coordinating system that could synchronize the ATP destruction and its subsequent resynthesis and readsorption. Examples of biological systems known to provide just this kind of coordination and synchrony are manifest in the above-mentioned ATPase-dependent swimming of dead paramecia and in the ATPase-dependent oscillatory changes of ion and water uptake and release in isolated mitochondria suspensions.

In summary, through the use of simple models I have shown how a cyclic change of ATP adsorption and hydrolysis can bring about a cyclic change of selective accumulation of a solute from the medium and its subsequent release back into that medium. I shall now try to incorporate this mechanism into a theoretical model able to perform true active transport. The major additional component needed is a one-way valve.

### A Detailed Model of Active Transport of Solutes and Water

As discussed above, the surface permeability barrier of the living cell is not a lipid layer but multilayers of water polarized by cell surface proteins. It is reasonable, then, that synchronized cyclic ion and water release and uptake would involve an alternation between a polarized impermeable state and a depolarized, permeable state of the surface water. If we can imagine that the basolateral or serosal surface has these properties and that the apical or mucosal surface does not undergo cyclic changes but has high c-value anionic sites and hence selective high permeability for $Na^+$, we will then be equipped with the necessary components for a model of active transport I believe to be in best accordance with available data and published observations.

Figure 3 outlines the proposed model in four stages. The ion to be transported from the external solution, $Na^+$, is represented as solid triangles. The mucosal surface is considered to have a higher permeability for $Na^+$ than the resting serosal surface because the serosal surface anionic sites have a high c-value that promotes selective $Na^+$ adsorption. Water in the serosal surface, in the cytoplasm, and in the normal cell surface

exists in the polarized multilayered state at the beginning of the cycle when the cell is at rest. In this active transport model the key role is played by the serosal and cytoplasmic surface proteins, which possess an ATP-binding cardinal site as well as an ATPase activated by the transported ion. It is suggested that the ATP-binding cardinal site is in fact the ATPase site but in a different cooperative state. The model functions in the following sequence:

***Stage* I.** The higher mucosal surface permeability allows both Na+ and water to enter the cell. Once inside, the Na+ and water will proceed to adsorb onto anionic sites on the cytoplasmic proteins under the

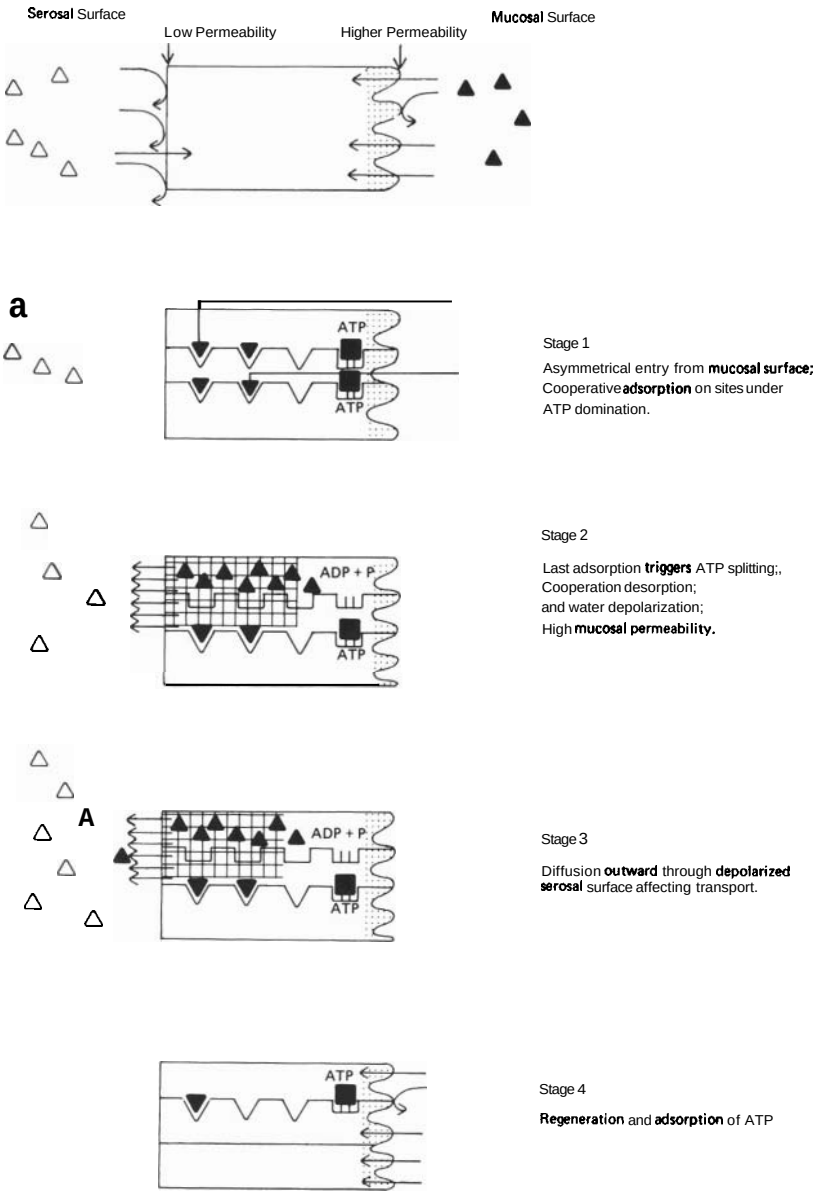


**FIGURE 3.** Cyclic adsorption-desorption model of active transport of Na (△,△) across frog skin, intestinal epithelia, and other bifacial systems.

control of the cardinal adsorbent, ATP, and thus existing in an extended state with their backbones favoring multilayer polarization of water and their anionic side chains preferring $Na^+$.

It may be asked how this stipulation can be reconciled with the view that ATP adsorption favors $K^+$ (rather than $Na^+$) adsorption on cell proteins. The answer is that the ATP control of the c-value ensemble depends on other factors; e.g., secondary cardinal adsorbents. That ATP adsorption favors $K^+$ adsorption is specific only for a specific protein under rigorously defined conditions. It also bears remembering that in active transport across bifacial cells the key cation is not always $Na^+$. Thus in Malpighian tubules of insects, the key ion transported is $K^+$.[123] In any case, the stochastic process of Stage 1 continues until the protein enters into the cooperative $Na^+$ state with a high concentration of adsorbed Na+ locally accumulated.

*Stage 2*. The autocooperative shift to the Na+ state involves the site adjacent to the cardinal site that also adsorbs $Na^+$. This Na+ adsorption then activates the Na,K-activated ATPase activity of the cardinal site, causing the hydrolysis of ATP, thereby entering Stage 2.

With the hydrolysis of ATP, the protein undergoes an autocooperative desorption of Na+ (possibly of $Cl^-$ as well) with concomitant formation of salt linkages, depolarization, and release of water plus liberation of a high concentration of $Na^+$. At the same time or slightly later, the serosal surface protein also undergoes change from the extended to a more helical conformation accompanied by water depolarization.

*Stage 3*. The depolarization of serosal surface water increases serosal surface permeability to ions and osmotic flow of water, permitting rapid exit of both the liberated Na+ and released water through the serosal surface.

*Stage 4*. This last stage is marked by the regeneration of ATP, its adsorption onto the cardinal site, and autocooperative shift back to the Stage 1 condition favoring $Na^+$ adsorption on anionic side chains and multilayer polarization at the serosal membrane. The cycle is now ready to repeat itself.

**Discussion of the Proposed Model**

CYCLIC CHANGES OF ADSORPTION-DESORPTION AS THE BASIS OF ACTIVE TRANSPORT. The cyclic changes of adsorption-desorption proposed in this model may not be easily observable in a multicellular epithelium. However, there is evidence of cyclic changes in the case of single giant algal cells (due largely to the careful work of S. C. Brooks.[124,125]) Thus when *Nitella* cells were exposed to salt solutions containing radioactive K+, $Na^+$, and $Rb^+$, those ions first accumulated in the protoplasmic layer surrounding the central vacuole. Furthermore, the accumulation of these labeled ions was not monotonic but exhibited a distinct periodic increase and decrease, and they reached a concentration in the protoplasm many times higher than that found in the surrounding medium. It was considerably later that the labeled cation reached the cell sap in the central vacuole. Figure 4 (from Brooks[124]) shows the time course of labeled Rb+ accumulation in the sap. The rise phase of sap Rb+ coincided with the fall phase of protoplasmic $Rb^+$, whereas the fall phase coincided with the rise phase of the protoplasmic $Rb^+$. Eventually the sap Rb+ reached concentrations "notably exceeding those present in the immersion fluid." At no time was the concentration in the sap higher than that in the protoplasm.

LOCATION OF THE TRANSPORT MECHANISM RELATIVE TO USSING'S TWO-MEMBRANE MODEL. Our model has incorporated the two-membrane theory of Ussing to the extent of recognizing and utilizing the differ-

ent permeability characteristics of the serosal and mucosal membrane as first discovered by Koefoed-Johnson and Ussing.[9] Otherwise the model is based on the AI hypothesis rather than on conventional membrane-pump theory. The part of the protoplasm involved in the adsorption-desorption cycle has been presented as including the entire cytoplasm as well as the serosal surface, because there is experimental evidence suggesting the involvement of the entire cell content (see below). However, on purely theoretical grounds the protoplasm involved in the cyclic changes may be entirely limited to the serosal surface. Under this condition it would approximate the location of pumping proposed by Ussing's two-membrane theory.

The evidence that the cyclic transport mechanism is not confined to the serosal cell surface includes the following: (*1*) Maddrell showed the rate of transport of $K^+$ and $Na^+$ by the isolated Malpighian tubule of the blood-sucking insect, *Rhodnius*, is strongly correlated with the respective *total* intracellular concentration of each of these ions.[123] (2) Spring and Giebisch showed that the rate of net Na transport in perfused *Necturis* kidney cells is linearly related to the *total* intracellular $Na^+$ concentration.[126] Again, no such linear correlation could be expected if the bulk of cell $Na^+$, a substantial portion of which is adsorbed,[52] is not involved in the transport. (3) Morel showed that after injection of $K^+$ into rabbits the specific activity of urinary $K^+$ quickly attains that found in the renal tissues but does not follow the time course of specific activity in the arterial plasma.[127] The results support the view that it is the $K^+$ which has undergone effective exchange with cell $K^+$ that plays a major role in urinary $K^+$ excretion. Again, this suggests involvement of more than a small amount of adsorbed $K^+$ on the serosal surface in the process (see Table I).

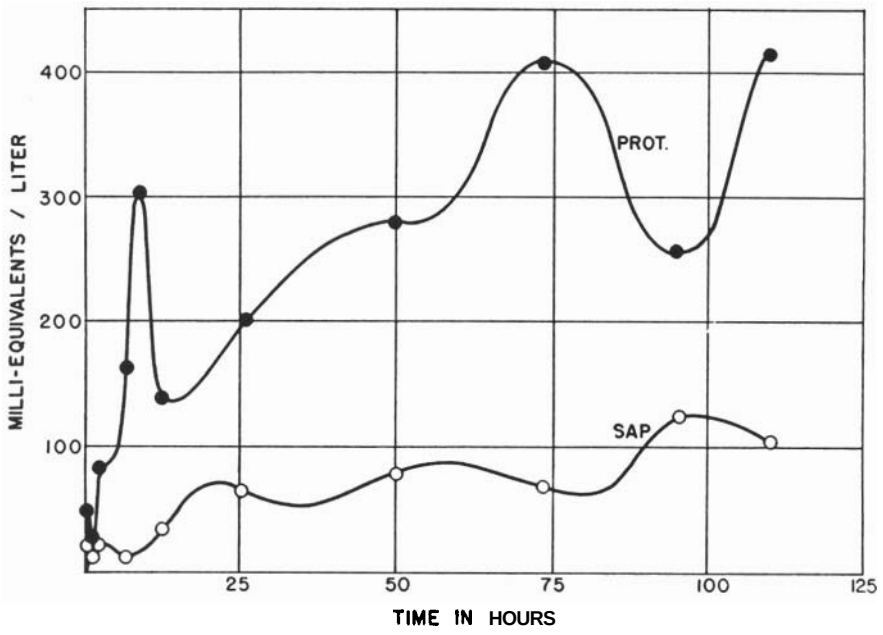


FIGURE 4. Concentration of $Rb^+$ in protoplasm and sap of *Nitella* internodal cells during 120 h of immersion in 0.005 M $Rb^+Cl$ (means of five series). pH = 7.3; continuous illumination contains 0.0001 M $CaCl_2$ (experiment Rb 10). *By permission from J. Cell. Comp. Physiol.*[124]

SOURCE OF ENERGY FOR ACTIVE TRANSPORT. In the present model, the immediate source of energy is stored in the protein-water-ion assembly in the high-energy resting state, and the ultimate source of energy for transport of solutes and water is that used in the synthesis of ATP from ADP and $p_i$. If one assumes that this synthesis is highly effective, with an efficiency approaching 100%, then the energy formed would be proportional to the free energy liberated during ATP hydrolysis. Further, since there is a fixed number of ATP molecules synthesized for each molecule of oxygen utilized, the energy for active transport should be quantitatively related also to the extra oxygen consumed.

Ussing and Leaf have raised the old question of "whether there is a stoichiometric relationship between the number of ions transported and the amount of, say, ATP consumed. . . ." (ref. 7, p. 3). They cited earlier work of their own and others leading to the conclusion that there may be a correlation between Na+ transport and oxygen consumption of, for example, frog skin. On the other hand, the failure to demonstrate such a relation in all tissues may reflect either a predominantly glycolytic source of ATP regeneration or the presence of a high and variable rate of oxygen consumption for cell functions not directly concerned with active transport.

COUPLING OF ION AND WATER TRANSPORT. Gall bladder, proximal kidney tubules, and small intestine are examples of "low resistance" epithelia. They transport water in the form of an isotonic fluid. As mentioned above, the standing gradient osmotic flow theory was proposed by Diamond and Bossert.[10] This theory, a refinement of the earlier double membrane theory of local osmosis to explain the apparent coupling of solute and water flow,[128,129] argued that contrary to earlier assumptions a second membrane is not necessary and that any confined space lined with a membrane possessing both active salt transport and semipermeability could function as a local osmotic coupling space. But Hill[130] pointed out that the feasibility of such functioning depends critically on the osmotic permeability of the membrane involved and that the required permeability range of $10^{-1}$ to $10^{1}$ cm²/sec "lies completely outside that of any living . . . membrane studied to date by at least three orders of magnitude."

In the case of NaCl-coupled water transport, the present adsorption-desorption model may be represented by two sets of alternative equations:

$$(H_2O)_n f^- Na^+ + f^+ \underset{+ATP}{\overset{-ATP}{\rightleftharpoons}} f^- f^+$$

$$\text{to serosal side} \rightarrow \quad \overbrace{+ Na^+ + nH_2O}$$

$$\leftarrow \text{from mucosal side} \qquad (8)$$

or

$$(H_2O)_n f^- Na^+ + (H_2O)_m f^+ Cl^- \underset{+ATP}{\overset{-ATP}{\rightleftharpoons}} f^- f^+$$

$$\text{to serosal side} \rightarrow \quad \overbrace{+ Na^+ + Cl^- + (n+m)H_2O}$$

$$\leftarrow \text{from mucosal side} \qquad (9)$$

As shown in Fig. 3, the synchronized dephosphorylation of ATP at Stage 2 causes simultaneous liberation of Na+ from its adsorption on anionic sites $f^-$ and of water from its polarized multilayered adsorption on the extended polypeptide chain NHCO sites. A normal free aqueous solution of Na+ and $Cl^-$ is momentarily and locally released concomitant with the removal of the barrier to ion permeation as well as to

osmotic water flow at the serosal cell surface. This is so because cooperative multilayer polarization of water drastically reduces osmotic permeability but only moderately reduces diffusive permeability whereas depolarization of water increases both. As a result, an essentially isotonic fluid of $Na^+$ is excreted into the lumen of the "low resistance" epithel a.

It should be mentioned that solute and water transport may be rate-limited by either the frequency of the cycles of adsorption and desorption or the mucosal surface permeability. Antidiuretic hormones when applied to the outside surface of frog skin and toad bladder greatly increase the rate of $Na^+$ transport as well as osmotic water permeability without major effect on diffusional permeability.[131] This suggests that such hormones may depolarize water at the mucosal surface, thereby increasing both $Na^+$ and water permeability at the external or mucosal surface.

While the cyclic adsorption-desorption model given above can explain active coupled salt and water transport with only the serosal surface involved in polarization-depolarization cycling of permeability changes, there is no reason why in different tissues the cycling may not occur at the mucosal rather than the serosal surface, as is known or at least indicated in insect Malpighian tubules, midgut, choroid plexus, and gastric mucosa.

**CONCILIATION OF "HOMOCELLULAR REGULATION OF CELL $K^+$ AND $Na^+$ COMPOSITION" AND "HOMOEPITHELIAL Na TRANSPORT."** Schultz[132] wrote in a recent review on "Transport Across Small Intestine": "The data available at present cannot be readily reconciled with any model that invokes a close relation between transepithelial Na transport and the homocellular regulation of Na and K composition or with the notion that the epithelial cell can be adequately represented by a double membrane model." (p. 769).

These limitations and conflicts are primarily the consequence of the incorrect basic concepts of the membrane-pump theory. The present model does not encounter any internal conflict between a theory of the mechanism of selective $K^+$ accumulation and $Na^+$ exclusion in epithelial cells (and algal cells) and the theory of transepithelial $Na^+$ transport. Indeed, the transepithelial $Na^+$ transport model is built on the foundation of the mechanism for "homocellular" $K^+$ accumulation and $Na^+$ exclusion.

**COUPLING OF $Na^+$ TRANSPORT WITH SUGAR AND AMINO ACID TRANSPORT.** An outstanding event in the study of transepithelial solute transport was the discovery of co-transport of $Na^+$ and sugar and co-transport of $Na^+$ and amino acids across epithelial systems.[12-14] However, the interpretation thus far offered, the Na-gradient concept, is based on the assumption that cell water is free. Since this assumption is now disproven, the subsidiary assumption that an electrochemical gradient of $Na^+$ exists between the mucosal fluid and the cytoplasm is also disproven. I shall now very briefly present a recasting of these important findings in terms of the cyclic adsorption-desorption model of transepithelial transport proposed in this paper.

Surface Protein Adsorption Sites to Replace "Membrane Carriers." In the conventional view, the mutual dependency of $Na^+$ and sugar (or amino acid) in their rate of transepithelial transport is due to the mandatory requirement for formation of a ternary complex of $Na^+$-carrier-sugar to achieve permeation into epithelial cells.[15,18] The carrier coupled either to $Na^+$ or to sugar alone is not able to permeate into the cell or at least permeates very much more slowly. But the fact that the cell membrane barrier is now known not to be due to phospholipid

demands a reconstruction of the conventional model.

As discussed earlier, polarized water offers strong resistance to permeation by sugar or $Na^+$. To enter the cells, these solutes must take the adsorption-desorption route via surface protein sites. Under normal conditions the cell surface proteins offer few or no specific adsorption sites for Na+ or D-glucose. However, in the presence of both Na+ and D-glucose the surface proteins undergo a cooperative transformation to assume a new conformation with a new *c*-value profile. In this new conformation, the surface protein offers both anionic sites of the proper c-value for Na+ adsorption and a combination of sterically suitable backbone and side-chain sites to adsorb D-glucose specifically. The subsequent entry will follow typically the adsorption-desorption route earlier described for simple ion entry. Since D-glucose and Na+ adsorption is cooperative, desorption of D-glucose or Na+ will facilitate the desorption of Na+ and D-glucose respectively, hence the enhanced simultaneous entry of Na+ and D-glucose.

Studies of Isolated Microvilli from Intestinal and Kidney Epithelia. In recent years techniques for isolation of microvilli from intestinal mucosa and from rat or rabbit epithelia have been developed and steadily improved.[133] Typically, the rate of D-glucose (but not L-glucose) or amino acid uptake is greatly accelerated if the Na+ concentration is high in the external medium and low in the microvilli. Pre-incubation of the microvilli in a medium containing high Na+ concentration, inclusion of phoridzin in the incubation medium, or substitution of $K^+$ for Na+ in the external medium all slow down the initial uptake. However, if valinomycin is added to a K-preloaded vesicle, the D-glucose uptake is again accelerated. Similarly, addition of the uncoupler CF-CCP to proton-loaded microvilli also accelerates initial D-glucose uptake rate. The conventional interpretation has been that D-glucose uptake is driven by the Na+ gradient, or in the presence of valinomycin by the $K^+$ gradient, or in the presence of CF-CCP by a proton gradient.

Equilibrium level of D-glucose. The accelerated uptake described above usually appears in the form of an "overshoot." That is to say, the sucrose quickly reaches a peak in the microvilli and then declines until a much lower and steady level is reached. It would seem that the relative height of this level can provide additional insight into the mechanism which maintains or controls the level of D-glucose and other solutes in living cells.

It is generally agreed that in intestinal as well as kidney tubule epithelium the postulated Na pump is located at the serosal surface. It is also widely agreed that there is no outward Na+ pump on the mucosal microvilli surface. The demonstrated osmotic activity of isolated microvilli indicates that most of the microvilli are resealed at the broken end. Thus the isolated microvilli present a unique system that contains no outward *Na* pump of the kind postulated for all living cells. Following the logic of conventional membrane-pump theory, one would expect that the steady level of sugar reached in the microvilli must be equal to that in the bathing solution.

From the data in the literature I have calculated two sets of apparent equilibrium distribution coefficients or p values for D-glucose, L-alanine, and L-lactic acid (Table II). The apparent distribution coefficient at the peak of the "overshoot" is expressed as $\rho_{max}$. The apparent equilibrium distribution coefficient for each of these solutes at the final equilibrium level is represented as $p_e$. The values given in the table were calculated on the assumptions that the external concentration of D-glucose, etc., did not significantly differ from that given as the initial concentration of the medium and that the

microvilli contained 80% water. Table II shows that while $\rho_{max}$ occasionally rose above unity, without exception, $\rho_{eq}$ was below unity and as a rule more or less at the same level as in intact cells. These findings contradict the membrane-pump theory but are in full accord with the AI hypothesis. Water in the cell cytoplasm in the microvilli, as well as elsewhere, exists in the state of polarized multilayers and as such has reduced solubility for all solutes including D-glucose, L-glucose, D-lactate, and L-alanine.

***The overshoot.*** I have already suggested that the synergistic adsorption of $Na^+$ and D-glucose onto surface protein sites facilitates the entry of $Na^+$ and D-glucose into the microvilli. I now suggest that the "overshoot" represents a transient adsorption of D-glucose onto some cytoplasmic proteins. In support of this concept are the following observations: (*1*) As shown in Table II, the apparent maximum $\rho$-value achieved at the peak of the overshoot often exceeds unity. In terms of the AI hypothesis, an above-unity $\rho$-value as a rule is due to selective adsorption or other form of complex formation in the cell. (2) No overshoot is discernible in liposomes incorporating proteins extracted from microvilli.[142,143] In these cases, the vesicles are hollow rather than solid as in the isolated microvilli; the cytoplasmic proteins essential for the transient D-glucose, L-alanine adsorption are of course not present. (3) The strong dependence of the overshoot on anions known for their strong adsorption on anionic sites (e.g., $SCN^-$ vs. $Cl^-$) (see ref. 19, p. 172 for compiled data on relative adsorption energy of anions on proteins in general) suggests that the adsorption of D-glucose involves unmasking of sites when $Na^+$ and $SCN^-$ join in dissociating salt linkages as in Eqs. 8 and 9 in the direction from right to left. (4) It has already been mentioned that the cell surface barrier is not primarily lipid in nature and that valinomycin and monactin could not function as ionophores (for detailed discussion, see ref. 25). Rather, they function as cardinal adsorbents, controlling the electronic as well as steric conformation of certain specific proteins and affecting $K^+$ adsorption. Similarly, CF-CCP acts as a

**TABLE II. Apparent Maximum Equilibrium Distribution Coefficient for D-Glucose, L-Glucose, L-Lactate, and L-Alanine in Isolated Microvilli from Intestinal and Renal Epithelia. $\rho_{max}$ is the apparent distribution at the peak of the overshoot; $\rho_{eq}$ is the final equilibrium value.**

| Authors | Tissue source | Solute studied and concentration | | $\rho_{max}$ | $\rho_{eq}$ |
|---|---|---|---|---|---|
| Hopfer, Nelson, Perrotto, and Isselbacher[134] | rat intestine | D-glucose | 1 mM | 0.15 | 0.15 |
| Murer and Hopfer[135] | rat intestine | D-glucose | 1 mM | 0.2 | 0.1 |
| Aronson and Sacktor[136] | rabbit kidney | D-glucose | 50 $\mu$M | 2.5 | 0.24 |
| Beck and Sacktor[137] | rabbit kidney | D-glucose | 50 $\mu$M | 0.5 | 0.15 |
| Kinne[138] | rat kidney | D-glucose | 1 mM | 1.25 | 0.45 |
| | | L-lactate | 1 mM | 0.61 | 0.31 |
| Kinne, Murer, Kinne-Saffran, Thees, and Sachs[139] | rat kidney | D-glucose | 1 mM | 0.58 | 0.16 |
| Turner and Silverman"" | human kidney (Na) | D-glucose | 1 mM | 1.07 | 0.98 |
| | (K-val) | D-glucose | 1 mM | 0.94 | 0.55 |
| | | L-glucose | 1 mM | | 0.27 |
| Fass, Hammerman, and Saktor[141] | rabbit kidney (Na) | L-alanine | 20 $\mu$M | 0.14 | 0.09 |

cardinal adsorbent to bring anionic sites to a c-value affecting $H^+$ binding (i.e., increases the c-value).

However, the question of why the increased adsorption does not sustain itself but declines is less amenable to explanation. Does it correspond to part of the cyclic change proposed? Or is it only a transient change of a deteriorating system? All these and many other questions can be answered only by future studies. □

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***ACKNOWLEDGMENT AND*** CORRECTION — Regarding my recent contribution Oxidative Phosphorylation and Mitochondria1 Physiology: A Critical Review of Chemiosmotic Theory, and Reinterpretation by the Association-Induction Hypothesis ***(Physiol. Chem. Phys., 13,*** **29, 1981),** I owe thanks to Dr. E. C. Slater of the Universiteit van Amsterdam, Netherlands. In a personal communication he pointed out that the calculation of voltage generated by loss of net charges given on page **34** (line **12,** 1st column to line **38,** 2nd column) apparently rested on a misunderstanding; the Mitchell assessment of ion loss was based on a unit of one liter of mitochondria rather than on a single mitochondrion as I had written. More careful reading has convinced me that Dr. Slater is right. Nevertheless, the correctly calculated voltage would still be much larger than **100 mV.** Hence the correction does not alter the thrust of my published critique or weaken the new interpretation offered.

***— Gilbert N. Ling***