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OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIAL PHYSIOLOGY: A CRITICAL REVIEW OF CHEMIOSMOTIC THEORY, AND REINTERPRETATION BY THE ASSOCIATION-INDUCTION HYPOTHESIS

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• *Fundamental assumptions of the chemiosmotic hypothesis of Mitchell are examined. Comparison of these assumptions with experimental data accumulated over the past fifty years leads to the conclusion that the hypothesis has not been supported. A review of important findings concerning the physical state of the major intracellular cation potassium shows clearly that this ion does not exist in a free state but is adsorbed on specific anionic sites. These findings refute the membrane-pump theory but add powerful support for the association-induction hypothesis, on the basis of which a new mechanism of oxidative phosphorylation as well as a wide variety of mitochondrial behaviors are proposed and compared with experimental data.*

SUBJECT OUTLINE

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(1) Inner membrane permeability to K^+ and Na^+. (2) Inner membrane permeability to divalent cations.
c. Anion impermeability

E. Functions of uncoupling agents and ionophores

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5. Synchronous oscillatory changes in mitochondrial swelling, ion uptake, and other properties
6. Mitochondria1 electrical potential

I. INTRODUCTION

Living cells, the basic units of all life, are at once extremely complex and exquisitely coherent. Acquiring understanding of the living cell, small though it is, resembles solving a gigantic multidimensional crossword puzzle into which, instead of words, appropriate physical mechanisms must be inserted. But in this effort, two main obstacles continue to face the cell physiologist. First, the correct physical mechanisms must be known—yet many remain unknown. Second, the task is so vast that it requires division of labor among many scientists over long periods, with each specialized investigation adopting a conceptual foundation dictated by what at the time appears to be the most trustworthy evidence—recent as well as "classic."

A dominant theory of the physico-chemical nature of the living cell is the membrane or membrane-pump theory, proposed more than a century ago by W. Pfeffer.[1] For many good reasons this theory has been broadly accepted. However, the development of modern physics as well as various findings made possible by powerful new tools have led numbers of scientists to conclude that some of those once convincing reasons are in fact wrong or equivocal. New basic concepts concerning the structure and function of the living cell are being forged, including the association-induction hypothesis (AI hypothesis) first elucidated in 1962.[2] Meanwhile, much progress was made in all disciplines concerned with cell physiology. Of the many pertinent areas of specialization, mitochondrial physiology is now one of the most significant because of both the considerable volume of outstanding work in that field and the central importance of oxidative phosphorylation to cell viability. But such advances have so far been viewed predominantly in accordance with the long-held membrane-pump theory. The present paper interprets some of the major recent developments in the physiology of the cell in terms of the association-induction hypothesis. In particular, the work on mitochondria is given attention. (For additional background, see review, ref. 114.)

II. THE CHEMIOSMOTIC HYPOTHESIS

Since Mitchell first proposed the chemiosmotic hypothesis in 1961, it has been reviewed many times[3-13] and therefore will be described only briefly here. According to this hypothesis, the inner membrane of a mitochondrion is impermeable to H^+ as well as to other ions. The respiratory chain is arranged in three loops corresponding to the three coupling sites. By a special vectorial arrangement of the electron-carrying molecules, an H+-adsorbing reaction occurs on the inside surface of the inner mitochondrial membrane (i.e., the surface facing the matrix). A concomitant H+-releasing reaction occurs on the outside surface of that membrane. As a result, an H^+ gradient develops, with the higher concentration of H^+ on the outside of the inner membrane. A reversible ATPase complex on the mitochondrial inner membrane is located in a region impermeable to water but accessible to OH^- from one side of the inner membrane and accessible to H^+ from the other side. Thus ATP hydrolysis would be reversibly coupled to the translocation of OH^- ions across the system with a stoichiometry of one OH^- translocated per ATP molecule hydrolyzed. The proton gradient provides energy for synthesis of ATP.

Mitchell noted, however, that the H^+ gradient alone is not sufficient to supply the energy required for ATP synthesis. Hence the mechanism was further elaborated by hypothesizing the creation of an electrical potential across the inner membrane as a result of asymmetrical electron transport. Mitchell argued that "the sum of the electrical potential difference and the osmotic pressure difference" provides a "protomotive force" (PMF)

for ATP synthesis and other energy-consuming processes.

The major share of this PMF is attributed specifically to a postulated membrane potential of 210 to 270 mV, with the inside phase negative; and a small contribution to the PMF is attributed to a postulated pH difference.[5]

A. Generation of an Electrical Potential Difference by Postulated Electrogenic Pump vs. Law of Macroscopic Electroneutrality

According to the chemiosmotic hypothesis, the mitochondrial inner membrane is a metabolically driven charge-separating machine—or, to use a term often employed by electrophysiologists, an electrogenic pump. Mitchell[8] calculated that the translocation of as much as one milli-equivalent (or 10–▾▾ram ions) of net ionic charges from the inside phase to the outside phase across the mitochondrial membrane would create a macroscopic potential difference of only 100 mV—a startling conclusion!

Consider that in his noted treatise on thermodynamics, Guggenheim[14] posed this question: How big a potential difference would be created across the surface of a spherical body with a radius equal to 1 cm (i.e., 10^{-2} m) and loaded with a quantity of electrical charge of only 10^{-10} gram ions? This quantity is so minute that it is not measurable by ordinary chemical methods, but it is demonstrably equal to $10^{-10} \times 0.965 \times 10^5$ coulombs, or 0.96×10^{-5} coulombs.

Now, in a vacuum the permissivity (ϵ_0) is 1.11×10^{-10} coulombs/volt m. The voltage of the charged sphere, then, is

$$\psi = Q/\epsilon_0 r = (0.96 \times 10^{-5})/(1.11 \times 10^{-10} \times 10^{-2})\ \mathrm{V} = 0.87 \times 10^7\ \mathrm{V}.$$

There we have a quantitative illustration of the law of the conservation of macroscopic electroneutrality. Under that precept it is not possible to achieve a net separation of a chemically detectable quantity of charges between two macroscopic phases (i.e., inside phase and outside phase of a cell or mitochondrion) without creating enormous potential differences.

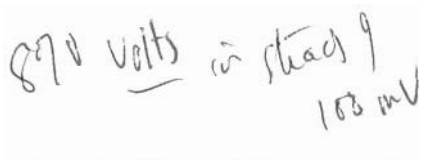
For example, a single isolated rat liver mitochondrion suspended in a lightly buffered 0.25 M aqueous sucrose solution with a dielectric constant of about 100 and maintained in the State 4 "orthodox" conformation is roughly spherical and about 1 μm (10^{-6}) in diameter. If there were a net charge separation of only 10^{-10} gram ions as in Guggenheim's calculation, the electrical potential difference would be

$$\psi = (0.96 \times 10^{-5})/(1.11 \times 10^{-8} \times 10^{-6}) = 1.9 \times 10^{11}\ \mathrm{V}.$$

If there were a charge separation of 10^{-3} gram ions, as assumed by Mitchell,[8] the potential would be 10' ▾ ▾—tremendous compared to Mitchell's finding of 100 mV!

We see that there are at least two errors in Mitchell's calculation. The first is improper use of the thermodynamic equation for the chemical potential of a ***single*** ion in a macroscopic phase, implying that electrical charges can move from one macroscopic phase to another without regard to the law of electroneutrality. The second error arises from equating potential difference across each mitochondrion with potential difference across an aggregate of mitochondria in one liter of solution, thereby in effect artificially creating a mitochondrion of gigantic dimensions. This huge figment, of course, can have no real significance.

Steady electrical potential differences do in fact occur at the interface between two unlike phases; e.g., between solid and liquid, liquid and liquid, liquid and gas. The magnitude of such a difference is usually determined by (*1*) the difference in entropy (or more



exactly, in statistical mechanical terms, the "partition functions") of the charge-carrying particles (free electrons or ions) in the two phases, and (2) the kinetic energy of the particles at the given temperature. The phase that offers greater entropy acquires an excess of one of the charge-carrying particles, and as a result an electrical potential develops. This is the basic mechanism for surface adsorption potentials, diffusion potentials, and the like.

The "electrogenic potential," on the other hand, has a different genesis. It was postulated not according to a consistent logical system based on knowledge of the inanimate world but as a sort of a catch-all for the discrepancies between the potential predicted by conventional membrane-pump theory and the potential experimentally observed. Thus Kernan[15] wrote in his review on the electrogenic pump: "The electrogenic pumping of ions may be recognized by a change of the membrane potential which cannot be accounted for in terms of the passive ion movement and which has some characteristics of metabolic processes." In short, one cannot refrain from viewing the hypothesis of an electrogenic pump as yet another ad *hoc* postulation, with a heavy vitalistic accent.

B. Proton Gradient

Data derived from in ***vitro*** studies with the aid of 5,5-dimethyl-2,3-oxazolidinedione (DMO) led Addanki et al.[16] to the conclusion that the change of pH gradient across the inner membrane of mitochondria at State 4 respiration and uncoupled by 2,4-dinitrophenol is only 0.005 pH units. That finding was confirmed in essence by Rothenberg.[17] The latter author concluded that in Mitchell's model the protomotive force must be due primarily to the membrane potential.

Of course, Mitchell's hypothesis was directed primarily at isolated mitochondria and chloroplasts. Yet if the chemiosmotic approach is to have physiological significance, it must take into consideration the vast differences between the highly artificial 0.25 M sucrose solution and the natural environment of the mitochondrion; i.e., the cytoplasm of the parent cell. Cytoplasm contains a high concentration of pH buffers including solutes such as the various phosphorylated intermediates and, more importantly, proteins and other macromolecules. A rise in proton concentration demonstrable in a suspension of mitochondria in a lightly buffered 0.25 M sucrose solution does not automatically predict a similar proton concentration change when the mitochondria are in their natural environment with its inherently high buffer capacity.

Isolated mitochondria utilizing such substrates as succinate and β-hydroxybutyrate are also different from mitochondria in their natural environment. One major natural energy source for mitochondria is glucose, and the tricarboxylic acid cycle enzymes essential for degradation of glucose are situated inside the mitochondrial matrix, within the inner membrane (ref. 18, p. 512). Like the electron transport system, these enzymes (i.e., isocitrate, α-ketoglutarate, malate dehydrogenase) produce H^+, which therefore would increase H^+ activity at the inside of the inner membrane and thus reduce the gradient of protons created by unidirectional spilling to the outside of the mitochondrial inner membrane, as stipulated in the chemiosmotic hypothesis.

C. Electrical Potential Gradient

Since a sizeable pH gradient is unlikely to contribute to the protomotive force, an electrical potential difference must exist across the inner membrane that can by itself provide the energy needed for ATP formation, ion transport, and transhydrogenation reactions. According to Mitchell, a potential difference of about 210 to 270 mV (inside

negative) would serve this purpose.[3-5,7]

Electrical potential differences across the inner membranes of isolated mitochondria have been measured by a variety of methods. Unfortunately, many of these measurements were indirect, and their validity relied upon the assumptions of either Mitchell's own hypothesis or some other hypotheses.

1. *In Vivo* Measurements

Obviously, the least disputable way of assessing the magnitude and polarity of this potential difference is to measure it directly. To do so, the inside of the mitochondrion must be electrically tapped, as done by Tupper and Tedeschi[19] in the late 1960s using essentially the Ling-Gerard glass capillary microelectrode technique[2"] and the giant chromosomes of fruit flies. Tupper and Tedeschi showed that the measured electrical potential difference was only 10 to 20 mV in magnitude and that the polarity was opposite to that hypothesized by Mitchell. Further, the potential was not affected by metabolic inhibitors or oxidative phosphorylation uncouplers. Also, they reported that resistance across the inner membrane was only 1 to 4 Ω cm^2. They concluded that electrical potential cannot play a major role in oxidative phosphorylation.

In 1977, Maloff, Scordilis, Reynolds, and Tedeschi[21,22] reported another series of electric potential studies. This time the giant mitochondria from mice fed with cuprazone were used. Although grossly oversized, these mitochondria carry out normal oxidative phosphorylation (P:O ratio for succinate was about 1.6). Again an electrical potential difference of the mitochondria of 15 mV was observed, again with a polarity opposite to that demanded by the chemiosmotic hypothesis. And again the resistance was about 2 Ω cm^2, and the potential was indifferent to the presence of succinate, antimycin A, or FCCP.

The iconoclastic import of these findings, in effect torpedoing Mitchell's hypothesis, demanded close scrutiny of their foundation. Indeed, strong criticisms, directed primarily at Tupper and Tedeschi[19] were voiced, as follows.

a. MEMBRANE DISRUPTION. Lieberman and Skulachev,[23] also Skulachev,[12] suggested that the microelectrode technique is unsuitable for measuring the electrical potential in so small and fragile structure as the isolated mitochondrion. In support they pointed out that the inner mitochondria1 membrane has in fact a very high resistance, citing Mitchell's as well as their own calculated value of 10^7 to 10^9 Ω cm^2. They considered that the much lower resistance measured by Tupper and Tedeschi (1 to 4 Ω cm^2) confirmed their view that the mitochondria when impaled by the microelectrode became grossly damaged. Another kind of evidence cited in favor of Lieberman and Skulachev's interpretation came from Lassen et al., who found that impalement of Ehrlich ascites cells[24] and giant red cells of the Conger eel[25] often leads to rapid depolarization ($t_{1/2} = 1$ msec) and consequent loss of sensitivity to external K^+ concentration.

Rebuttal: Maloff, Scordilis, and Tedeschi[26] presented the following rebuttal: (*1*) In the studies by Lassen et al.,[24,25] the rapid decline in electrical potential must have been due to a technical defect because other investigators were able to demonstrate much more stable potentials in both Ehrlich ascites cells ($t_{1/2} =$ 10 sec)[27] and Conger eel red cells (seconds to 1 min).[28] Moreover, in the giant mitochondria of cuprazone-fed mice the electrical potential does not decay at all but remains constant, as has been observed in many other types of small cells.[22] (2) Equally convincing evidence that no serious damage was produced by the impaling microelectrode was the finding by Maloff et al.[22] that exposure to valinomycin changed the polarity of the electrical potential and conferred a K^+ sensitivity

on the electrical potential of the cuprazone-treated mitochondria. Still other data supporting Tedeschi, Tupper, Maloff, and their coworkers' view will be discussed below (p. 37) in relation to measuring the electrical potential of mitochondria in *situ*.

b. ELECTRODE MISPLACEMENT IN THE INTERMEMBRANE SPACE. Skulachev[12] suggested that the microelectrode tip might not have penetrated the inner membrane but only the outer membrane.

Rebuttal: Maloff et al.[22] refuted that suggestion in several ways, only two of which need be mentioned: (1) Advancement of a microelectrode will register an unchanging potential as long as the microelectrode is within the mitochondrion. (2) Impalement of mitochondria freed from their outer membranes (i.e., mitoplasts) gives the same potential as that from intact mitochondria.

c. INPOCKETING OF MITOCHONDRIAL MEMBRANE AT MICROELECTRODE TIP. Rothenberg[m] suggested that the valinomycin-sensitive electrical potential measured by Maloff et al. might have been artificially created when the microelectrode tip still outside the inner membrane was pushed against it, creating a selectively permeable membrane surrounding a KCl-filled microelectrode tip that would then function as a K^+ electrode.

Rebuttal: Maloff et al.[26] pointed out that if the microelectrode tip, enclosed by a valinomycin-treated inner membrane, did indeed function as a K^+ electrode, the potential should not develop when the microelectrode is filled with 2 M NaCl instead of 2 M KCl, since valinomycin is not an ionophore for Na^+. The opposite, however, was observed to be the case. NaCl- or KCl-filled microelectrodes measured the same potential difference.[26] (For a comprehensive recent review, see Tedeschi.[30])

2. In *Situ* Measurements

The conclusions of Tedeschi, Tupper, and Maloff are further strengthened by the elegant studies of Giulian and Diacumakus.[31] These authors were able to measure the electrical potential of cultured HeLa cells with the electrode tip just inside the cell surface and also following the tip's penetration into the Golgi apparatus, nucleus, and mitochondrion. Using thorium dioxide as a marker, they clearly established the placement of the microelectrode tip in the mitochondrial matrix. Yet the potential measured between the inside of the mitochondrion and the cytoplasm was some 15 mV, with the inside negative.

The low negative potential thus measured offers general confirmation of the in *vitro* measurements of Tupper, Maloff, Tedeschi, and their coworkers. It follows that the electrical potential cannot be the major source of energy for phosphorylation, ion transport, and transhydrogenation as postulated by the chemiosmotic hypothesis.

3. Effect of Valinomycin on Resistance

Another explosive observation by Maloff and coworkers[2%]as that even though valinomycin created a K+-sensitive electrical potential difference having characteristics similar to those seen across the surface of many living cells, the introduction of valinomycin in the presence of varying external K^+ concentration ranging from 1 mM to 160 mM did not create changes in the resistance of the mitochondria. Indeed, the electrical resistance of the mitochondrial inner membrane remained at a constant value of 2 MΩ, equivalent to a specific conductance of 2 Ω cm^2. On this ground alone, the chemiosmotic approach can be considered fatally flawed.

D. Is the Mitochondria1 Inner Membrane Impermeable?

We see that taken as a whole, the studies (cited above) by Tedeschi, Maloff, and their coworkers render the chemiosmotic hypothesis untenable. But what of still another basic

assumption of the chemiosmotic hypothesis—the idea that the inner membrane of mitochondria is an ion-impermeable barrier? It is argued that under normal conditions, the postulated pH gradient and electrical potential difference are maintained to promote the continual production of ATP. When an electrochemical ionophore such as valinomycin is introduced into the external K+-containing medium, it will ferry K^+ into the cell, thereby discharging the pH and potential gradients. Other ionophores may discharge the H^+ gradient. With the gradient discharged, production of ATP stops. Thus accounted for is the uncoupling action of K^+ plus valinomycin and other similar ionophore uncouplers. This tenet of the chemiosmotic hypothesis may be separated into the following specific components: (*1*) The inner membrane is primarily a continuous phospholipid bilayer. (2) That membrane is virtually impermeable to ions in general and H^+ in particular. (*3*) Ionophores such as valinomycin, monactin, and the like affect oxidative phosphorylation by changing the permeability of the inner membrane to the ions that it transports. Let us examine these propositions in turn.

1. Is There Enough Phospholipid in the Inner Membrane to Form a Continuous Bilayer Barrier?

The concept of the inner membrane as primarily a continuous sheet of phospholipid came originally from a postulation of Overton[32] made at the turn of the century. However, there is strong evidence that this basic postulation is not true.

A direct answer to the question of the existence of a continuous lipid bilayer is most readily obtained by analyzing the chemical composition of the inner (and outer) mitochondrial membranes. Actually, these analyses have long been on record and the results are widely known[33,34] (see also ref. 18, p. 512). It is the outer membrane, long recognized as offering no barrier to solute movements, that is rich in phospholipids (50%). The inner membrane, on the other hand, contains only 20% phospholipids, the rest of the solids being proteins. Moreover, these percentages were derived from dry-weight analysis. Since all proteins and phosopholipids hydrate and some hydrate extensively[35,36] the total percentage of lipid in fresh living mitochondrial inner membrane must be considerably less than 20%. Still, a possibility remains. Could it be that even this limited amount of lipid is sufficient to spread out into a continuous bilayer?

Gorter and Grendell[37] showed that human red blood cells contain just enough lipid to form a continuous bilayer covering the surface of the cell. But the human red blood cell membrane is also one of the richest (47%) in lipid content (ref. 38, p. 343). It would appear, then, that the 20% total lipid content of the mitochondria1 membrane would not be enough to form a continuous bilayer.

Fleischer and coworkers[39] fixed beef-heart mitochondria in osmium tetroxide, then extracted 95% or more of the lipid with organic solvents. If lipids really existed as a continuous layer in the inner membrane, after the lipid extraction the inner membrane would certainly have become much thinner. In fact, however, the electron micrograph of the defatted membrane showed an unaltered "railroad" structure with spacing similar to that in the unextracted control. This finding, supported by similar results from other laboratories,[40,41] again indicates that a continuous lipid bilayer cannot be a part of the inner membrane structure.

Sjostrand and coworkers,[42,43] using an improved method that prevents denaturation of proteins, concluded that the inner membrane of liver mitochondria does not contain continuous lipid layers.

Maloff, Tedeschi, and their coworkers demonstrated a constant and low membrane resistance indifferent to the pres-

ence or absence of valinomycin and K^+. This is in full agreement with the equally important findings of Stillman, Gilbert, and Robbins.[44] Those authors used voltage-clamp methods to show that monactin, another K+-specific ionophore, has no effect whatsoever on the K^+ conductance of giant squid axon membrane. As yet unpublished work of Ling and Ochsenfeld showed similar lack of effect of valinomycin (10^{-7} M) on K^+ permeability in frog muscles and ovarian eggs. Since it has been unequivocally established that valinomycin[45,46] and monactin[47-49] instantly increase K^+ conductance across artificial phospholipid membranes by several orders of magnitude (see also ref. 38), the findings of Maloff, Tedeschi, Stillman, and their coworkers, as well as Giulian and Diacumakos, leave little doubt that at the inner membrane of the mitochondria (or the outer surface of squid axon, frog muscle, frog egg, etc.) the diffusion barriers are not continuous lipid layers. These data argue against not only the chemiosmotic hypothesis but also the basic lipid membrane concept on which the chemiosmotic hypothesis is founded.

2. Is the Mitochondria1 Inner Membrane Virtually Impermeable to Ions in General and H^+ in Particular?

I have already mentioned that the inner membrane conductance given by Lieberman and Skulachev[23] was 10^7 to 10^9 Ω cm² based on Mitchell's data, whereas the experimentally measured conductance[19,21,22] was only 1 to 10 n cm², a discrepancy of 10^6 to 10^8. Which of the two estimates is closer to the truth?

For answer, let us compare the values with those derived from the study of natural membranes. One of the biomembranes that exists primarily to serve the purpose of electrical insulation is the frog nerve myelin sheath; its resistance is 1 to 1.6 $\times$ 10^5 Ω cm².[50] This is from two to four orders of magnitude lower than that cited by Lieberman and Skulachev[23] for the mitochondrial inner membrane even though myelin contains about 75% of its dry weight as phospholipid while only 20% of the dry weight of the mitochondrial inner membrane is phospholipid.

The living cell membrane of squid axon has a resistance of 500 to 1000 Ω cm².[50] However, the nodal membrane of frog myelinated nerve exhibits only 10 to 20 Ω cm² (ref. 50, p. 53). Glial cells in tissue culture have a membrane resistance of 3 to 10 Ω cm².[51] The membrane resistance of the excitable face of electric eel plates[52,53] is from 1 to 13 n cm² and of the inexcitable face is only 0.1 to 0.4 Ω cm².[53] From these data it is clear that the measured mitochondrial inner membrane resistance of 1 to 10 Ω is entirely reasonable. On the other hand, there is no known record of measured membrane resistance as high as that derived by Lieberman and Skulachev on the basis of Mitchell's data.

As for the question of how the estimate of such an enormously high resistance came to be made, the answer has already been partly given by Maloff et al;[22] the estimate at issue was based on an estimate of H^+ conductance alone. Therefore let us examine the evidence purporting to show that the mitochondrial inner membrane is all but impermeable to H^+—and even less permeable to other ions.

a. H^+ PERMEABILITY. Mitchell and Moyle[54] studied the H^+ permeability of the inner membrane of rat liver mitochondria. They found that the introduction of acid into a mitochondrial suspension is followed by a two-step pH change in the external medium. Initially a fast rise of pH is seen, which the authors attribute to the buffering capacity of the space between the inner and outer membranes. Then a slow rise of pH occurs, which they attribute to entry of H^+ into the space enclosed by the inner mitochondrial membrane. They considered that the rate of the slow change reflect; H^+ permeability of the

inner membrane. Since the half-time of pH change of the slow titration was 1 min at 25°C, Mitchell and Moyle concluded that this indicated "the lowest natural membrane ion conductance" known to them (ref. 54, p. 599).

However, they were mistaken in their calculation. In a surface-limited unidirectional flux of a solute labeled i, the half-time of exchange ($t_{1/2}$) is related to the permeability constant of the solute (κ_i) by the simple relation[2]:

$$\kappa_i = V/A \cdot (\ln 2)/t_{1/2} \qquad (1)$$

where V/A is the volume/surface ratio of the cell. In the case of H^+ permeability of an isolated mitochondrion with a radius (r) 0.5 μm, the V/A ratio is equal to

$$(4/3)\pi r^3/\pi r^2 = (4/3)r = 6.6 \times 10^{-5}\ \text{cm}.$$

Thus $\kappa_{H^+} = 6.6 \times 10^{-5} \times 0.69 \times 60^{-1} = 7.5 \times 10^{-7}$ cm/sec.

For comparison I have collected in Table I the permeability constants for K^+ and Na^+ in three types of cells: frog muscle, squid axon, and human red blood cells. It is obvious from the table that the inner mitochondrial membrane cannot be considered to be unusually impermeable to H^+. This conclusion, derived from Mitchell and Moyle's own data, supports in fact the 1966 conclusion of Chance and Mela,[57] as well as that of Pressman et al.,[58] that the inner membrane is quite permeable to H^+.

b. CATION IMPERMEABILITY. A search of the literature reveals an astonishing abundance of experimental evidence indicating that the inner mitochondrial membrane is quite permeable to other ions as well. True, some related literature asserts that certain ions, such as Cl^-, are impermeant. However, much of the evidence for the latter interpretation was derived not from direct permeability measurements but indirectly from swelling and shrinkage measurements.

TABLE I. K^+ and Na^+ Permeability Constants of Three Types of Cells

	κ_{K^+} (cm/sec)	κ_{Na^+} (cm/sec)
Human red blood cell	[a] 2.4×10^{-10}	[a] 10^{-10}
Squid axon (passive flux)	[a] 5.6×10^{-7}	[a] 1.5×10^{-8}
Frog muscle	[b] 21.1×10^{-7}	[c] 1.2×10^{-7}

[a] From compilation by Jain,[38] Tables 5-9. [b] From Katz.[55] [c] From Harris.[56]

In the early days of cell physiology, when more direct methods were not yet available, cell permeability was defined on the basis of osmotic theory. That is, if a living cell remained shrunken when immersed in a hypertonic solution of a particular dissolved substance X, the cell membrane was considered to be impermeable to substance X. On the other hand, swelling of the cells was taken to indicate permeability of the cell membrane to the substance in question. Based on these criteria, a vast amount of permeability data, including the bulk of the work published by Overton,[1] was collected. Indeed, it was on this basis that the plasma membrane was initially believed to be impermeable to sodium. It was not until the early 1940s that the decisive experiments of Heppel[59] and Steinbach[60] disproved the notion of Na^+ impermeability of cell membranes. The more general implication of their findings was that osmotic swelling or shrinkage could no longer be depended on to determine solute permeability. In 1965 Pressman[G1] drove home the point by discovering a discrepancy between swelling and transport in mitochondria.

1. Inner membrane permeability *to* K^+ *and* Na^+. The bulk of the K^+ in isolated mitochondria readily exchanges with ^{42}K-labeled K^+ in the external medium, as shown by

Stanbury and Mudge[62] and repeatedly confirmed.[63,64] The standard procedure of isolating mitochondria in lightly buffered sucrose (250 mM) involves continued exposure to solutions containing very little K+; the bulk of K^+ remaining in the mitochondria must be sequestered within the inner membrane. The ready exchange of ^{42}K with mitochondria K^+ therefore shows that the inner membrane is permeable to K+.

Under proper conditions K^+ can be taken up from the external medium in a matter of minutes.[65-67] In agreement, Lehninger, Ray, and Schneider wrote that "K^+ is freely and rapidly permeable into mitochondria. . . ."[68]

Gear and Lehninger[69] further demonstrated that the permeability of the inner membrane to K^+ is high and not dependent on respiration or added ATP. In the absence of substrates or in the presence of rotenone or antimycin, addition of NaCl causes displacement of mitochondrial K^+ in 30 sec or less. Gear and Lehninger's studies clearly showed also that the inner membrane is as permeable to Na+ as to K+. Studies of Na+ accumulation in heart mitochondria reported by Settlemire, Hunter,' and Brierly,[70] and Brierly, Settlemire, and Knight[71] support the conclusion that mitochondria are intrinsically permeable to Na^+.

2. Inner membrane permeability to *divalent* cations. Johnson and Pressman[72] showed that in the presence of substrate the mitochondrial inner membrane is permeable to Mg^{2+}. The speed with which Ca^{2+} can enter mitochondria, replacing an equivalent amount of H+, is remarkable. In 15 sec, more than 80% of the Ca^{2+} is accumulated and H^+ rejected.[73,74] Rossi, Azzi, and Azzone[75] demonstrated that in the absence of metabolism, Ca^{2+} can also release K+. They concluded that "the penetration of Ca^{2+} in the intramitochondrial space in the absence of metabolism classifies Ca^{2+} as a permeant cation. . . ."

The conclusion is inescapable, in agreement with Blondin and Green,[76] that the mitochondrial inner membrane is permeable both to H^+ and to other major cations normally found in the environment.

c. ANION IMPERMEABILITY. Although the failure of a solute to cause cell swelling does not necessarily indicate that the solute is impermeant, under most conditions the ability of a solute to induce swelling does indicate permeability. Amoore[77] in 1959 showed that succinate, citrate, and phosphate can accumulate within mitochondria, reaching concentrations many times higher than those in the bathing solution. Spector[65] found much more K^+ accumulation in mitochondria when the anion is glutamate, fumarate, citrate, lactate, or oxalate than when it is chloride. Brierly et al.[78] noted that without a metabolic energy source, a permeant anion and a permeant cation will enter the mitochondria as a pair. Among the anions tested and found permeant were NO_3^-, succinate, fumarate, malate, and trichloroacetate. The Brierly group also concluded that chloride ion is permeant but only at a high nonphysiologic pH, implying that a normal mitochondrion in its normal environment is impermeable to chloride.

In 1975, however, Weiner[79] reassessed the widely held belief that the mitochondrial inner membrane is absolutely impermeable to chloride in the light of the established high chloride permeability of various living cell plasma membranes. Weiner studied the swelling of mitochondria in a wide variety of chloride salts in the presence of uncoupling agents and concluded that "the mitochondrial inner membrane shares with other biological membranes a definite permeability to this ubiquitous anion."

E. Functions of Uncoupling Agents and Ionophores

So far we have dealt with the basic assumptions of the chemiosmotic hypothesis; i.e., the

existence of a pH gradient with lower pH on the outside, a large membrane potential with the outside positive, and the impermeability of the mitochondrial inner membrane to ions in general and H^+ in particular. The evidence, examined above, does not support any of these assumptions.

Why, then, does wide acceptance of the chemiosmotic hypothesis persist? The explanation is that at one time it seemed to offer the most persuasive explanation for certain key observations concerning (*1*) uncoupling agents and ionophores and (*2*) ion gradient-driven ATP synthesis. Next, therefore, let us discuss those observations.

Compounds like 2,4-dinitrophenol (DNP), dicumarol, and carbonylcyanid p-trifluoromethylphenylhydrazine (FCCP) inhibit mitochondrial phosphorylation of ADP to form ATP without inhibiting the rate of respiration. These "uncoupling" compounds also evoke ATPase activity in mitochondria, which are normally devoid of such activity.

According to the chemiosmotic hypothesis, such uncouplers act by serving as specific carriers for H^+ across the phospholipid barrier of the mitochondrial inner membrane[54] which, as mentioned above, is postulated to be impermeable to H^+ and other ions. By mediating H^+ movement across the inner membrane, the uncoupler discharges the protomotive force. Since in this theory the protomotive force provides the energy for ATP synthesis from ADP and P_i, the uncoupler causes oxidative phosphorylation to cease. In support of that view, uncouplers were indeed shown to be capable of promoting proton conductance across artificial phospholipid membranes.[12,38]

Another group of compounds, valinomycin being a typical example, also exercises a strong effect on mitochondrial phosphorylation.[80] However, discovery of the ability of valinomycin to transport K^+ (in preference to Na^+) across phospholipid membranes[45] shifted the emphasis to its role as an "ionophore."[81]

Andreoli et al.,[45] in their study of the ionophoric property of valinomycin across lipid bilayer models, gave the rank order of preference as $H^+ > Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$. Mitchell and coworkers, however, as well as a number of other supporters of the chemiosmotic hypothesis[82,83] (see also ref. 11, p. 58) believed that valinomycin is a specific ionophore for K^+ but not an ionophore for H^+. In support, it was pointed out that Andreoli et al.[45] had studied H^+ transport at a pH much lower than that of the mitochondrial environment.

Moore and Pressman[84] studied the effect of low concentrations of valinomycin on respiring mitochondria suspended in sucrose solution containing various concentrations of KCl up to 10 mM. They noted that valinomycin caused transport of K^+ into the mitochondria with a concomitant release of H^+. The following is a reviewer's account (ref. 11, p. 59) of this basic phenomenon from the viewpoint of the chemiosmotic hypothesis: "In terms of Mitchell's hypothesis, valinomycin, by transporting K^+ across the membrane, allows K^+ to be taken up in exchange for H^+ ejected by the respiratory chain. . . ." Thus even though under the hypothesis valinomycin is not considered to have H^+-transporting ability, and the membrane *per se* has no H^+ permeability, H^+ for K^+ exchange could nevertheless occur across the inner membrane by a mechanism called "exchange diffusion."*

Since each molecule of valinomycin has been shown to form a cavity in which exactly one K^+ ion fits,[39] and it is as part of this valinomycin-K^+ complex that K^+ is trans-

*Ussing's exchange diffusion[85] is limited to exchange of the *same* species of ion across the cell membrane, therefore is quite distinct from what Mitchell conceived as "exchange diffusion." It is of interest even though Ussing's exchange diffusion could not be experimentally verified in recent studies?"

ported across a phospholipid membrane, it is not clear how valinomycin-mediated K^+ transport could be linked to a counter H^+ transport in a membrane supposedly impermeable to H^+ and containing no H+-transporting ionophore.

Another contradiction is the requirement of inorganic phosphate (P_i). As a rule, uncouplers elicit increased oxygen consumption in the absence of ADP. Moore and Pressman[84] and Azzone and Azzi[81] clearly showed that valinomycin in the presence of K^+ elicits an increase in respiration. However, this is true only if P_i is added, even though valinomycin by itself induces K^+ and H^+ exchange. This relevant point apparently is ignored by the chemiosmotic hypothesis interpretation of the uncoupler action of valinomycin plus K^+ mentioned above (p. 42).

When a large oxygen pulse is given to mitochondria suspended in an anaerobic environment that contains substrate ATP, low K^+ (0.35 mM), and valinomycin, protons are ejected and an almost equivalent amount of K^+ is taken up and concentrated inside the mitochondria to a calculated level of 48 mM (ref. 11, p. 29). Under the chemiosmotic hypothesis, a one-to-one exchange of K^+ ions for H^+ ions is seen as the means of maintaining osmotic equilibrium; i.e., the same concentration of osmotically active particles in the form of free H^+ originally present in the mitochondria is traded for an isosmotic concentration of free K+. But is that idea realistic?

A concentration of 48 mM is not unusually high for an intracellular cation such as K+. However, an equivalent amount of H^+ is equal to a pH of 1.32! It is not likely that this amount of free H^+ would exist in the surviving mitochondria, since (*1*) such a low pH would denature most of the enzymes in the mitochondria and (2) the mitochondrial matrix is highly concentrated in proteins. Protons entering in their free form would rapidly titrate acidic groups bearing pK_a values much higher than 1.32 (e.g., β- and γ-carboxyl groups, $pK_a = 4.6$). Thus the bulk of the quantity of H^+ displaced by the K^+ must have been in the adsorbed form. This would in turn produce an osmotic imbalance—a difficulty that again cannot be resolved without violating the basic tenet of the membrane-pump theory; i.e., that the bulk of intracellular K^+ and cell water exist in the free state. This subject will be dealt with below (p. 74).

Equally difficult to reconcile with Mitchell's chemiosmotic hypothesis are the following observations:

(*1*) Rossi, Azzi, and Azzone[75] observed that the level of Ca^{2+} accumulated in mitochondria in the presence of metabolic inhibitors (rotenone, antimycin, or KCN) is also increased by valinomycin. High external K^+ concentrations decreased the steady levels of accumulated Ca^{2+}. These findings in conjunction with the later work of Massari, Balboni, and Azzone[88] suggest that the steady Ca^{2+} level accumulated in the mitochondria also varies quantitatively with the valinomycin concentration. *But valinomycin is not an ionophore for Ca^{2+} or any other divalent ion.* It follows that the effect of valinomycin on mitochondrial ion distribution requires interpretations not easily derived from the chemiosmotic hypothesis or any ion-pump hypothesis.

(2) Massari, Balboni, and Azzone[88] observed that different concentrations of valinomycin do not permit the K^+ concentration in rat liver mitochondria to reach the level determined by the postulated electrical potential difference, as would be expected under the chemiosmotic hypothesis. *Instead, the steady levels of accumulated K^+ are determined by the concentration* of *valinomycin!* This important finding also invalidates the method used by Mitchell and coworkers to determine the electrical potential across the inner membrane by taking the K^+ gradient observed in the presence of valinomycin.

F. ATP Synthesis

Perhaps the most convincing support for Mitchell's hypothesis was provided by evidcnce that H^+ and other ionic gradients across membranes can provide energy for the synthesis of the high-energy phosphate bonds of ATP. Consideration began in 1966 with Jagendorf and Uribe,[89] who reported that isolated spinach chloroplasts would synthesize ATP from ADP plus P_i, without illumination or metabolism, if the chloroplasts simply were exposed first to an acid medium and then to an alkali medium. It seemed that the consequent proton gradient forced an H^+-ATPase to work backward to synthesize ATP. This exciting finding was soon confirmed in other preparations. Reid, Moyle, and Mitchell[90] showed that in liver mitochondria a reverse change of base to acid causes ATP formation from ADP plus P,. Cockrell, Harris and Pressman[91] showed that in rotenone-treated rat liver mitochondria loaded with K^+ and suspended in a K^+-free sucrose medium the addition of valinomycin brought about a rapid loss of K^+ and concomitant ATP synthesis. Rossi and Azzone[92] showed that if K^+ accumulation was brought about by aerobic metabolism, treatment with rotenone also brought about a slow leak of K^+ and concomitant ATP synthesis. These and similar findings (see below) convinced many former skeptics that the chemiosmotic approach had validity.

But soon unexpected observations began to appear. In 1970, Kanazawa, Yamada, and Tonomura[93] observed formation of ATP from ADP and a phosphorylated Caw-dependent ATPase from *fragmented* sarcoplasmic reticulum of muscle tissues (see also refs. 94, 95).

These latter findings, to be taken up again in Section IV below (p. 48), heralded a revolution in our understanding of oxidative phosphorylation. In anticipation, one notes that this central process of cell metabolism, ATP synthesis, does not rely on the existence of a "chemiosmotic" gradient maintained by an intact, membrane-enclosed structure.

G. Conclusions Regarding the Chemiosmotic Hypothesis

From the above review it is clear that many new and old experimental results render the chemiosmotic hypothesis untenable. A new and different interpretation of the findings must be attempted in order to construct an alternative hypothesis compatible with data both in agreement with and at odds with the chemiosmotic hypothesis. The failure of that hypothesis is tightly interwoven with the failure of the more general membrane-pump theory, which since 1877 has been the prevailing theory of cell physiology and the foundation for most ongoing biomedical investigation. Accordingly, the remainder of this paper shall first review an alternative theoretical model of ion transport in the cell and then show how this model can be used to interpret much of the data collected with regard to mitochondria1 function.

III. THEORY OF THE LIVING CELL

Since the publications of Claude[m] and later of Hogeboom, Schneider, and Palade,[97] a brilliant chapter has been added to the history of biochemical research concerning two key issues in cell physiology: oxidative metabolism and photosynthesis. The isolated mitochondria and chloroplasts that perform these specialized functions, however, are part and parcel of the cell. As such, they exhibit the properties of swelling, differentiated permeability, solute accumulation and exclusion, electrical potential, dependence on energy metabolism, and responsiveness to metabolic poisons and biologically active compounds. These properties, to all appearances, are similar to those of whole living cells.

It was natural that most investigators of

mitochondria and chloroplasts took as a starting point the fundamental theory of the living cell at the time: the membrane or membrane-pump theory, a theory originally introduced by Pfeffer.[1]

A. Basic Features of the Membrane-Pump Theory

Essentially, the postulates of the membrane-pump theory may be summarized as follows:

1. Water. The bulk of cell water is free.

2. *Ions.* The major intracellular cation K^+ is free in a dilute aqueous solution.

3. Cell surface barrier. The cell is surrounded by a plasma membrane, the major components of which are phospholipids. These form a continuous layer broken occasionally by islets of proteins that may or may not connect the two aqueous phases separated by the membrane. The phospholipid layer is the seat of selective permeability for solutes and water.

4. Membrane pumps. In the plasma membrane are found a large variety of so-called pumps that regulate, usually with a high degree of specificity, the steady-state levels of permeant solutes in the cell. Energy needed for the pumping may be supplied by hydrolysis and by liberation of the energy conserved in the high-energy phosphate bonds of ATP.

5. Cell volume. The cell behaves like an osmometer. Its swelling and shrinking depend on the presence of an intact cell membrane. Free K^+ and other ions and solutes provide just enough osmotic pressure to counterbalance that created by free Na^+ and other ions and solutes in the external medium.

6. Resting potential. The electrical potential difference measured between the inside and outside of a living cell is regarded as a "membrane potential" and is determined primarily by K^+ (and Na^+) ionic concentration gradients according to Donnan's theory of membrane equilibrium or its variant, the Hodgkin-Katz-Goldman model. Electrical potentials that cannot be explained by the Donnan theory are often attributed to special "electrogenic" pumps (as mentioned above, p. 35).

B. Basic Features of the Association-Induction Hypothesis

The membrane-pump theory, however, is by no means the only theory of the living cell. One alternative, the sorption theory, was proposed by the Russian investigator A. S. Troschin[99] (see also Nosonov, ref. 99). Other concepts of the living cell were advanced by Ernst.[100] Another and more complete theoretical structure, known as the association-induction hypothesis (AI hypothesis), was first presented in an elementary form by Ling in 1951[101] and 1952[102] and in a more complete version in 1962' (for later reviews, see refs. 103-106). Over the years, as experimental data and interpretive concepts accumulated and were systemized, the AI hypothesis has taken on the force of a formalized theory. But although more formal and more general, the current version of the AI hypothesis still departs radically from the membrane-pump theory in describing the living cell. The basic AI tenets are as follows:

1. *Water.* The bulk of cell water exists in a physical state different from that of normal free liquid water. This state is characterized by dynamic polarized multilayers formed by interaction with a matrix of extended and more or less parallel polypeptide chains in which the repeating sequences of CONH groups are directly exposed to the bulk water.

2. *Ions.* The bulk of intracellular K^+ is adsorbed on β- and γ-carboxyl groups of intracellular proteins.

3. Cell surface barrier. The surface barrier

is semipermeable. It consists primarily of polarized water, but includes surface proteins that offer fixed ionic and other sites essential for selectivity in solute permeability.

4. Pumps and solute exclusion. Pumps in epithelial cell systems (e.g., frog skin, toad bladder, intestinal epithelium) would involve, as a rule, the entire cell--endowed with asymmetrical surfaces. Maintenance of the steady level of solutes in resting cells in general is not due to continually operating membrane pumps but reflects the combination of two basic mechanisms. The first mechanism is selective adsorption on macromolecular sites that tend to increase intracellular concentrations to above those in the external medium (e.g., K^+). The second mechanism is reduced solubility in the polarized-multilayered cell water that tends to decrease intracellular concentrations of solutes to below those in the external medium (e.g., Na^+). The larger and more complex the solute, the lower its equilibrium level in the cell water.

5. *Cell volume.* Maintenance of cell volume as a rule is not directly dependent on an intact cell membrane, nor does it depend much on the small amount of free ions in the cell. Instead, it primarily reflects the reduced water activity in the state of polarized multilayers and the reduced solubility of the major external solute, Na^+.

6. *Resting potential.* The resting potential is not a Donnan or membrane potential but a surface adsorption potential. Its magnitude is determined by the nature and density of fixed anionic sites on the cell surface and the nature and concentration of the external ions adsorbed at these sites.

C. Discriminatory Experimental Evidence

It is obvious from the above that in many ways the membrane-pump theory and the AI hypothesis are irreconcilably different.

A great deal of experimental work aimed at providing decisive proof for one or the other approach has been and continues to be reported. Since many of the earlier findings have been reviewed,[101-106] and since an updated and more comprehensive treatment in the form of a monograph is forthcoming, I shall limit myself here to brief discussion of several key pieces of evidence in support of the AI hypothesis.

1. Energy Requirements of Pumps

Under controlled conditions, the Na^+ pump in muscle, though but one of many energy-requiring pumps postulated by membrane-pump theory, would consume 15 to 30 times the total energy available in the cell.[2] The essence of this finding has been confirmed by studies of cells other than muscle cells.[107,108] Accordingly, three remedial hypotheses (exchange diffusion, sarcoplasmic reticulum sequestration, and a nonenergy-consuming pump) were advanced to save the Na pump; but all were subsequently disproved.[86]

The energy impasse is eliminated by the AI hypothesis. Since the resting cell exists in a metastable equilibrium state, no continual energy expenditure is required for maintenance of the asymmetrical solute distribution. Furthermore, the maintenance of cell ion levels does not depend on the rate of a "pumped" flux, as shown in muscle depleted of ATP[109] and in lymphocytes at varying temperatures.[110]

2. Membrane vs. Cytoplasm as the Seat of Discrimination in Solute Distribution

Sheaths of intact squid axon membrane without cytoplasm, capable of .normal electrical activities[111] and ATP-dependent Na^+ efflux[112] can be prepared. Sacs of these sheaths with the ends tied should offer an unusually favorable preparation for a decisive test of the Na^+-pump concept. However, repeated attempts to demonstrate actual pump-

ing of K^+ or Na^+ against an electrochemical gradient in the presence of ATP produced no positive results.[106]

On the other hand, frog muscle cells devoid of a functional cell membrane pump are capable of accumulating K^+ and excluding Na^+ in ways similar to cells with intact membranes.[113] Similarly, "white" erythrocyte ghosts without cytoplasm, but with intact membranes and K,Na-activated ATPase, do not transport K^+ or Na^+ against concentration gradients.[114,115] Only "red" ghosts that retain considerable amounts of cytoplasmic proteins[116,117] are able to transport limited amounts of K^+ and Na^+ against concentration gradients.[115,118]

These findings again show that it is the cytoplasmic protein-water system and not the membrane that is responsible for K^+ accumulation and Na^+ exclusion.

3. The Adsorbed State of K^+

Since its inception in 1951, the AI hypothesis has suggested that the β- and γ-carboxyl groups represent the seat of selective K^+ adsorption in living cells.[2,101] In voluntary muscle cells, more than 60% of these groups are found in the myosin.[101,119]

Now, myosin in muscle cells, rather than being evenly distributed, is localized exclusively in the A bands.[120,121] The work of Hodge and Schmidt[122] shows that uranyl ion, the cationic electron microscope stain, binds to the β- and γ-carboxyl groups of the aspartic and glutamic acid residues of protein respectively. The observation that uranium stains primarily the A bands strongly supports the AI hypothesis, which predicts that the bulk of intracellular cations must be localized in the A bands and in other cytological structures that appear dark after uranium staining. In other words, if one could somehow visualize K^+ in the living, resting muscle cell, the picture would most likely resemble the electron micrograph of a fixed muscle cell preparation stained with uranium.

This theoretical expectation has recently been confirmed by a series of observations by Edelmann. He first replaced the K^+ in living frog muscle with the electron-dense cesium ion—a stoichiometric exchange leaving the muscle cells functionally intact.[123] He then applied a simple but highly effective new freeze-drying technique[123,124] to the muscle cells. The specimen was infiltrated with Spurr medium at low temperature, and the sections were dry-cut. Figure 1A is an electron micrograph of muscle fixed in glutaraldehyde and stained with uranium only in the conventional manner (Edelmann, unpublished). Figure 1B, on the other hand, is Edelmann's ***unfixed*** and ***unstained*** frog muscle cell loaded in the living state with Cs^+.[124-126] Figures 1A and 1B match each other in almost all details. Figures 1C and 1D show muscles loaded with thallium (Tl^+) rather than Cs^+. Figures 1E and 1F are respectively a Cs^+-loaded section that has been washed and a normal K^+-loaded section. The dark areas in Figs. 1B, 1C, and 1D mark the cytological structures that selectively adsorb Cs^+ (and hence K^+) in the resting state.

Compelling as they are, before these findings can be accepted as confirming the predictive value of the AI hypothesis, two questions must be resolved.

(I) Could the Cs^+ seen in the region of the A band merely be free counterions hovering in the vicinity of fixed negative charges rather than adsorbed? A negative answer is provided by demonstration that the alkali-metal ion uptake in frog muscle is ion-specific, indicating expression of short-range attributes detectable only by direct contact, and is not just valence-specific, as would have been the case if Cs^+ or K^+ existed as free counterions.[119] Thus Cs^+ is only one-third as effective as K^+ in displacing adsorbed ^{42}K-labeled K^+ from muscle cells, and such ion specificity persists in muscle cell preparations

devoid of an intact cell membrane or of postulated membrane pumps.[127]

(2) Could artifacts of the special technique used be responsible for the observed results? This possibility is ruled out by studies in which four different techniques were used, including (a) autoradiography of dried single muscle fibers at 25°C carried out by Ling;[127,128] (b) autoradiography of frozen fresh single muscle fibers at −190°C carried out by Edelmann;[129] (*c*) dispersive X-ray microprobe analysis on single frog muscle cells by Edelmann[126] (confirmed using isolated single *myofibrils* from honeybee thorax muscle[130]), and (d) laser microprobe mass spectrometry (LAMA) and X-ray microanalysis demonstration of selective K^+ and Cs^+ uptake over Na^+ in the A-bands of freeze-dried embedded muscle section by Edelmann.[131]

Two of these methods—dispersive X-ray microprobe analysis and LAMA—also identified K^+ localized in the A bands without the use of Cs^+ or Tl^+ surrogates. Thus selective K^+ adsorption in living muscle cells, central to the AI hypothesis, has been substantiated thoroughly and unequivocally in recent years.

4. Consequences of K^+ Binding in Living Cells

To repeat, the membrane-pump theory and the AI hypothesis, internally consistent as they are, cannot be reconciled with each other. Free K^+ in living cells is an indispensable and integral part of the membrane-pump theory. Confirmation that the bulk of cell K^+ is in an adsorbed state has disallowed the conventionally accepted state of osmotic equilibrium, since K^+ makes up the bulk of the solutes found in living cells. By the same token, the membrane and Donnan theories of cellular potential are also invalidated, since they require that all muscle-cell K^+ must be free to account for a resting potential of some 90 mV.[55,132]

Common sense—though admittedly not rigorous proof—at one time led Monod and Jacob[133] to suggest that "anything found to be true of E. coli must also be true of elephants." Is what is true of cells also true of cell organelles? I have shown that whole striated muscle cells selectively accumulate K^+ over Na^+, and that this phenomenon is due to selective adsorption of K^+ (and exclusion of both K^+ and Na^+ from cell water). The mitochondria are intrinsic to these and other living cells. So it is not surprising that mitochondria have been shown to also selectively accumulate K^+.[134,135] Indeed, even fragments of mitochondria accumulate K^+ over Na^+, as demonstrated by Gamble[136] in 1957. Thus common sense would dictate a great unlikelihood that mitochondria would achieve a similar selective K^+ accumulation by resorting to postulated ion pumps of the conventional or chemiosmotic type.

IV. THE SOURCE OF ENERGY FOR ATP SYNTHESIS

Mitchell's chemiosmotic hypothesis is a modern extension of the membrane-pump theory. Beginning in microbiology, its generalization to mitochondria and chloroplasts and to other intact cells has been vigorously pursued (for examples, see refs. 13, 137; for critique, see ref. 114). The fundamental difficulties described above and elsewhere that argue against the membrane-pump theory[114] also argue against the chemiosmotic hypothesis. It thus becomes timely to attempt an interpretation of mitochondrial behavior in general and of oxidative phosphorylation in particular in terms of the association-induction hypothesis.

In the chemiosmotic model, ATP formation is due to a "protomotive" force arising in part from a H^+ gradient across the inner membrane of mitochondria and chloroplasts. One influential set of observations that persuaded many scientists to accept the chemiosmotic approach began with Jangendorf and Uribe's[89] report, mentioned earlier, to the ef-

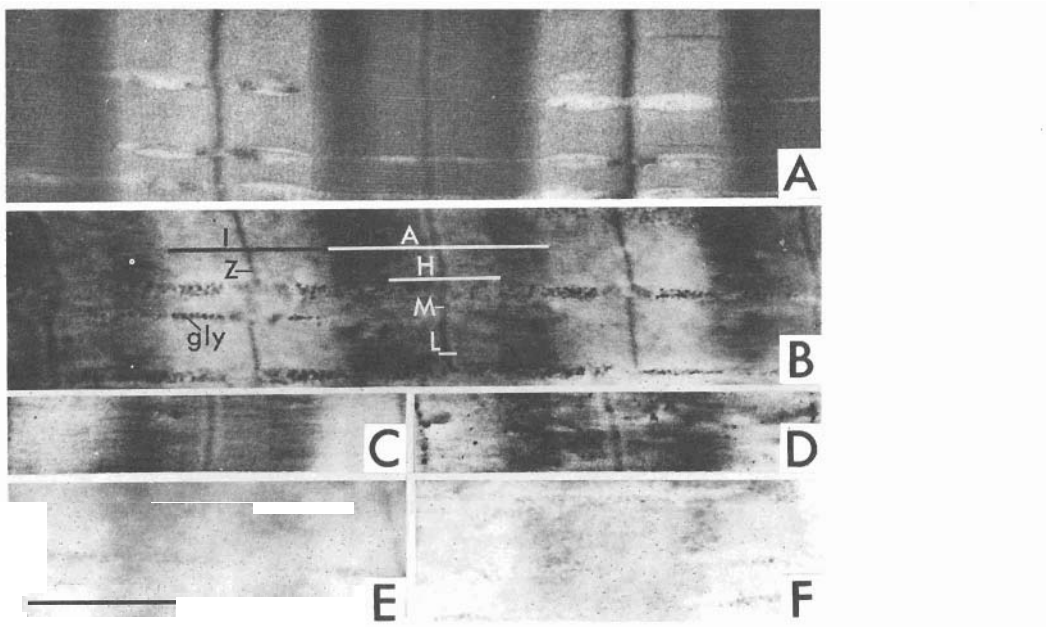


FIGURE 1. Electron micrographs of frog sartorius muscle. **(A)** Muscle fixed in glutaraldehyde only and stained with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+-loaded muscle, without chemical fixation or staining. **(C)** TI+-loaded muscle without chemical fixation or 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,[125] by permission of Physiol. Chem. Phys.*

fect that spinach chloroplasts synthesize ATP if exposed first to an acid and then to an alkaline solution. This was soon followed by reports from Reid et al.[90] and Cockrell et al.,[91] who demonstrated ATP synthesis associated with ionic gradients in liver mitochondria. Similar observations were made with red blood cells.[138,139] Then rabbit muscle sarcoplasmic reticulum vesicles preloaded with Ca^{2+} and incubated in the presence of EGTA were found to show rapid release of Ca^{2+} accompanied by ATP synthesis.[140-142] These findings also appeared to demonstrate that ATP was formed using energy derived from dissipation of the ionic gradient across the membrane. But against that view, Kanazawa et al.[93] in 1970 showed that sarcoplasmic reticulum (SR) fragments "phosphorylated"* with ATP could later transfer their phosphate groups to ADP, thus synthesizing ATP. Boyer et al.[143,144] showed that a small amount of the "phosphorylated" enzyme could be formed when the SR was not loaded with Ca^{2+}. Kanazawa[145] and Masuda and de Meis[146,147] showed that vesicles were "phosphorylated" in the absence of a Ca^{2+} concentration gradient. Clearly Kanazawa and coworkers[145] by 1972 had successfully demonstrated the formation of ATP from ADP and P_i in the absence of ion gradients either in the step generating the phosphoenzyme or in the subsequent step generating ATP.

In 1975 Knowles and Racker[148] confirmed this finding by demonstrating the synthesis of ATP from ADP and P, in purified Ca^{2+}-ATPase from the SR without a Ca^{2+} gradient. Knowles and Racker proposed that "the energy for ATP formation is derived from the interaction of Ca^{2+} with the protein."

A parallel observation was made in the

* Quotation marks around "phosphorylated," etc., signify a lack of knowledge whether a covalent bond is formed between P_i and protein. P_i adsorption, for example, may be an alternative.

same year by Taniguchi and Post[149,150] using purified Na^+,K^+-activated ATPase from guinea pig kidney. They demonstrated the synthesis of ATP without an ionic gradient and concluded that "binding of sodium ion to a low-affinity site on phosphoenzyme formed from inorganic phosphate is sufficient to induce a conformational change in the active Enter which permits transfer of the phosphate group to adenosine diphosphate." For later reference it is to be noted that "phosphorylation" by P_i of both Ca^{2+}-activated ATPase and K^+,Na^+-activated ATPase requires Mg^{2+} and K^+.[148,150] On the other hand, Mg^{2+} inhibits ATP synthesis from ADP and the "phosphorylated" enzyme. Instead, a different ion is needed for the task — Ca^{2+}-activated ATPase and Na^+ for Na^+, K^+-activated ATPase.

These revolutionary findings cast a new and different light on the subject of ATP synthesis. *The emphasis is shifted from ionic or "osmotic" gradients, central to the membrane-pump theory, to ion adsorption on proteins, central to the AI hypothesis.* Indeed, the findings lead to revision of a paramount

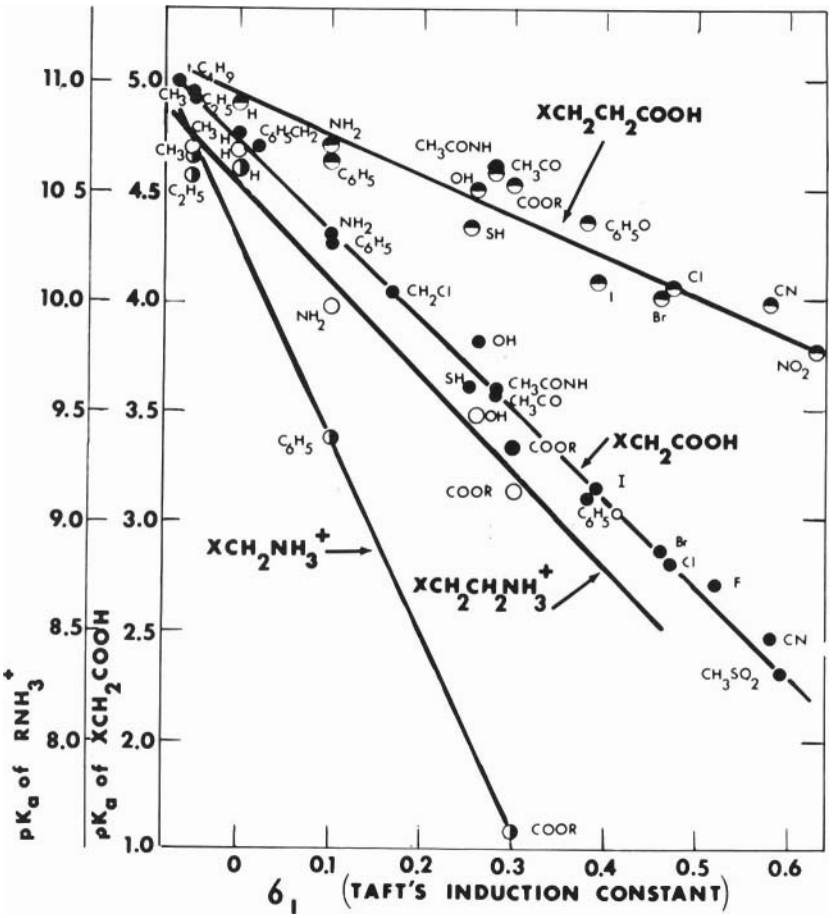


FIGURE 2. Relation between Taft's induction constant σ_I and acid dissociation constant pK_a of α-substituted acetic acid (XCH_2COOH), 6-substituted propionic acid (XCH_2CH_2COOH), a-substituted methyl-ammonium ion ($XCH_2NH_3^+$) and 6-substituted ethyl-ammonium ion ($XCH_2CH_2NH_3^+$). In these formulae, X represents the substituent, which varies. Abscissa represents the σ_I of each substituent indicated in graph. Ordinate gives the acid dissociation constant of that particular substituted compound as it is indicated on the graph. *From Ling,'" by permission of Tex. Rep. Biol. Med.*

question regarding mitochondria1 oxidative phosphorlylation : How does the mitochondrion, without the aid of a biochemist, carry out ,two-step manipulations equivalent to laboratory procedures? The first step involves exposing the ATPase to Mg^{2+} (and sometimes K^+) and P_i, thus producing the "phosphoenzyme." The second step involves removing the Mg^{2+} or K^+ already present and replacing it with a different cation, either Ca^{2+} or Na^+ as the case may be. Additionally, today we must ask how adsorption of Ca^{2+} or Na^+ creates the phosphate transfer from the ATPase to ADP to synthesize ATP?

V. PROTEINS ACCORDING TO THE AI HYPOTHESIS: HIGH- AND LOW-ENERGY STATES OF PROTEIN-ION-WATER SYSTEMS. PRIMARY INDUCTIVE EFFECT

In recent years, the terms "high-energy state" and "low-energy state" have become widely applied to biological systems although the precise meanings of those terms are often not explicitly stated. The following is a brief review of the concept of the high-energy living state according to the AI hypothesis.

As an analogy, consider a chain of soft-iron nails loosely joined end to end with pieces of string. If a strong magnet is brought close to one of the terminal nails, magnetization of this nail will cause the next nail in the sequence to be attracted to the opposite end of the first nail; the second nail will, in turn, attract and magnetize the third nail, and so on. If iron filings are placed in the area surrounding the chain, they will be picked up by the magnetized nails in a more or less ordered manner. Thus approximation of the strong magnet will propagate magnetic polarization of a number of the nail elements accompanied by an increase in the magnetic interaction not only between the magnet and each individual nail but also between the nails and the iron filings around them. Along with a gain of magnetic *energy* in this system, there is also a loss of *entropy;* i.e., a more ordered state is created from a more random state.

Another model would be a chain of electrical insulators placed end to end. If an electrically charged rod is brought close to one of the terminal insulators, electrical polarization or induction will propagate through the chain of insulators much as the magnetization propagated through the soft nails in the example above. In this case, it is electrical polarization and energy from an electrostatic interaction that are enhanced. The magnetically polarized nails or the electrically polarized insulators represent a higher energy state, much like the high tide brought to a still higher energy state by the synergistic action of sun and moon alignment. When the large magnet or the electrifying rod is removed, or when the sun and moon are no longer aligned, the magnetic poles, the electrons, or the water, as the case may be, revert to their normal low-energy, high-entropy state and in so doing can perform work with the released energy. It is also worth noting that in the polarized high-energy state the electron density is higher at some sites than during the unperturbed state but lower at other sites. *The* key *issue is the perturbation of the entire linked system from its low-energy equilibrium position.*

According to the AI hypothesis, the unique partial resonance of the polypeptide chain in proteins enhances propagation of an electrical polarization or *inductive effect* through this chain. The energy elevation, as in the model system of nails in a sea of iron filings, is not limited to elements of the protein itself but critically involves all the other polarizable elements of which the proteins are a part. *Thus propagated high- and low-energy states refer not to a single component but to all the closely associated components of living cells: water, proteins, and ions existing as a cooperative unit.*

But induction is not the only basic mecha-

TABLE II. Relation Between Inductive Index and Ionization Constants of Oxygen Acids

	Compound H—OX	10^3I for–OX	pK_a 25° (1)		Compound H—OX	10^3I for–OX	pK_a 25° (1)		Compound H—OX	10^3I for–OX	pK_a 25° (1)
Halogen group	**H–OClO$_3$**	455.4	**~ −8(2)**	Phosphorus group	H–OPO(OH)–OPO(OH)$_2$	305.7	**1.0(18°)**	Carbon group	H–OCOCOOH	269.8	1.2
	H–OClO$_2$	389.9	**~ −3(2)**		**H–OPO(OH)–OPO$_2^-$(OH)**	298.4	**1.5(18°)**		H–OCOOH	257.6	3.9; **~3.7(2)**
	H–OClO	319.5	2.3; **2.0(23°)**		H–OPO(OH)–OPO$_2^-$–OPO$_2^-$(OH)	297.5	2.3		H–OCHO	245.7	3.8
	H–OIO$_2$	311.2	0.8		H–OPO(OH)$_2$	288.1	2.1		**H–OCOCOO$^-$**	245.3	4.3
	H–OIO(OH)$_4$	257.6	1.6		H–OPH(O)(OH)	280.0	2.0		H–OCOCH$_2$CH$_2$SiMe$_3$	242.9	4.9
	H–OCl	243.2	**7.4(27°);7.0(2)**		**H–OAsO(OH)$_2$**	275.6	2.2		H–OCOMe	242.5	4.7
	H–OBr	237.6	8.5; **8.7(2)**		H–OPO(Me)(OH)	275.6	**2.3(3)**		H–OCOCH$_2$SiMe$_3$	242.0	5.2
	H–OI	225.7	9.7; **11.0(2)**		H–OPH$_2$(O)	270.9	**1.0; 2.0(2)**		H–OSi(OH)$_3$	228.3	9.**7(30°); 10(2)**
	H–OIO$_2^-$(OH)$_3$	192.6	8.3		H–OPO(Me)$_2$	263.2	**3.1(3)**		H–OGe(OH)$_3$	228.3	**8.5(2)**
	H–OIO$_3^=$(OH)$_2$	127.7	12.4		H-ONO	257.8	**3.2(20°);3.2(30°)**		H–OCOO–	191.3	10.3
	H–OIO$_4^=$(OH)	62.7	**>15**		H–OAsO(Me)$_2$	252.4	6.1		H–OSiO–(OH),	162.8	**11.7(30°)**
	H–OIO$_5^{-4}$	−2.3	**>15**		H–OPO$_2^-$–OPO$_2^-$–OPO$_2^-$(OH)	232.0	6.3				
					H–OPO$_2^-$–OPO$_2^-$(OH)	230.9	6.6				
					H–OPO$_2^-$–OPO$_2^-$–OPO$_3^=$	230.8.	8.9				
					H–OPO$_2^-$(OH)	**222.6**	**7.2**				
Sulphur group	**H–OSO$_3$H**	367.8	**~ −3(2)**		H–OPO$_2^-$–OPO$_3^=$	221.7	9.6; **8.4(18°,** 3)	Boron group	H-OBO	251.9	9.1
	H–OSeO$_3$H	332.8	**~ −0.5**		**H–OAs(OH)$_2$**	220.0	9.4		**H–OB(OH)$_2$**	217.0	9.2
	H–OS$_2$O$_2^-$	317.9	1.6—1.7		H–OPO$_2^-$(H)	214.5	6.6		**H–OAl(OH)$_2$**	214.0	11.2
	H–OSO$_3^-$	302.2	1.9; 2.0		H–OAsO$_2^-$(OH)	210.4	7.0		**H–OB(OH)–C$_6$H$_5$**	210.7	9.9
	H–OSO(OH)	297.4	1.9; **1.9(2)**		**H–OAsO$_2^-$(Me)**	210.2	**7.1(3)**				
	H–OSeO(OH)	280.3	**2.5(18°)**		H-ONH,	191.1	8.0		**H–OB(OH)–CH$_2$C$_6$H$_5$**	205.2	9.1
	H–OSeO$_3^-$	**267.5**	**2.0(30°)**		**H–OPO$_3^=$**	157.1	**12.0(2)**		**H–OB(OH)–CH$_2$CH$_2$C$_6$H$_5$**	203.2	10.0
	H–OTeO(OH)	260.8	2.5		**H–OAsO$^-$(OH)**	154.9	13.5		**H–OB(OH)–Bu-*n***	202.0	10.7
	H–OTe(OH)$_5$	233.1	7.8		**H–OAsO$_3^=$**	145.2	**11.5(18°)**		H-OBO-(OH)	151.0	**12.7(~20°)**
	H–OSO$_2^-$	228.8	**7.0(18°)**		**H–OAsO$_2^=$**	89.8	**>15(32°)**		**H–OGaO$^-$(OH)**	150.5	**10.3(18°)**
	H–OSeO$_2^-$	231.9	**8.0(18°)**						**H–OBO$_2^=$**	**85.1**	**13.8(~20°)**
	H–OTeO$_2^-$	195.9	7.8						**H–OGaO$_2^=$**	83.0	**11.7(18°)**
	H–OTeO$^-$(OH)$_4$	168.3	10.3								

(1) **Landolt-Börnstein,** 1%0 *Zahlenwerte und Funktionen,* **Sechste Auflage,** Band II, Teil 7, S. **842—45.**
(2) Pauling. 1949 ***General Chemistry,*** p. 3%.
(3) Crofp and Korolnpoff, 1953 *J.* Am. *Chem.* **Soc., 75,** *3379,*

From Chiang and Tai,[168] by permission of Scientia Sinica.

nism involved in propagated high- and low-energy biological states. Equally basic is electron density-dependent preferential ion adsorption at charged sites, tracing to the differences in electron density between polarized and non-polarized states. Both these mechanisms will now be discussed.

A. Inductive Effect as the Basis of Energy and Information Transfer Over Distance

After the major discovery of ATP and its pivotal role in cell metabolism and function, ATP was believed to contain a high-energy bond in which an unusually large amount of potential energy was stored. Since the 1950s it has become clear that this concept is not correct. The enthalpy of hydrolysis of the ATP phosphate group is not unusually high;[151] the free energy of hydrolysis of ATP is largely due to the liberation of H^+ in an environment maintained at physiological pH as well as to other extraneous factors.[152-154]

That ATP may serve a physiological function without undergoing hydrolysis has been repeatedly suggested. Riseman and Kirkwood,[155] Botts and Morales,[156] and Ling in 1952[102] believed that ATP adsorbed on muscle protein affected the protein conformation by a direct electrostatic repulsive effect mediated through space. The view that I finally adopted, however, is different; stress is placed on the inductive effect of adsorbed ATP mediated through the protein molecule.[2] Indeed, the inductive effect permits (*1*) determination of the secondary and higher protein structure and (2) the properties of protein molecules due to their primary structure as well as their environment and past history.[2,104,106,157,158] The mode of action of the inductive effect falls into two categories: inductive effect over a short range, referred to as the ***direct*** F-effect (describing the combined inductive or I effect and the direct electrostatic D-effect); and an ***indirect*** F-effect involving a propagated mechanism, or domino effect, reaching over longer distances.

1. Universal Applicability of the Inductive Effect

The inductive effect, formally presented by Lewis,[159,160] is the basis of a major aspect of the structure-activity relations in organic chemistry, often termed "linear free-energy" relations.[161-167] The work of Hammett[163] and Taft[165,166] was closely associated with this effect and as a rule their equations and constants describe relations between substituents and alterations in the equilibrium and kinetic properties of close and distant functional groups. In 1963 Chiang and Tai[168] succeeded in transforming these empirical rules into equations that yield precise quantitative predictions based on independent physical constants of atomic electronegativity, bond length, and molecular structure.

The Chiang-Tai theory made it feasible to calculate the entire constellation of properties of even very large and complex molecules from their molecular structure and thus represents a major step beyond the limited conventional empirical approach. The Chiang-Tai theory also made it possible to advance the AI hypothesis beyond the mere deduction that primary structure determines all properties of a protein molecule.[2,104] At last structure and properties of all side chain, backbone, and other functional groups actually could be calculated according to the aggregate inductive effects. The precise molecular structures of numerous pure proteins have now been determined, creating an opportunity for major advances in protein chemistry.

2. Target Groups of the Inductive Effect

The inductive effect is universal in that it can be applied to all properties and behaviors of complex molecules. To illustrate its role under the AI hypothesis in determining the properties of proteins, three examples are now discussed: the acid dissociation constant

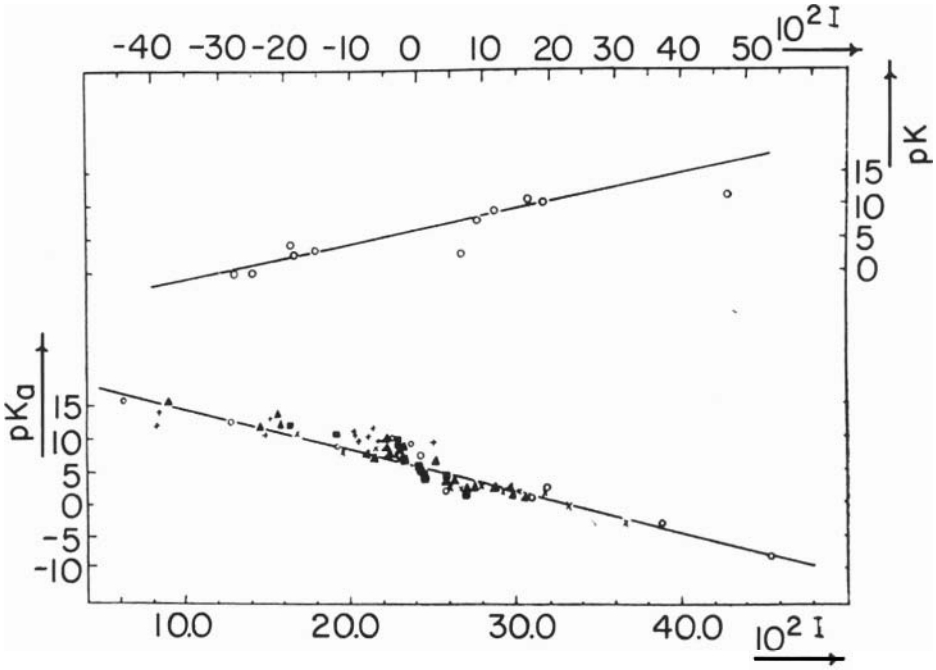


FIGURE 3. Relations between inductive index and ionization constants of oxygen acids and metallic hydroxides. Oxygen acids and the theoretically calculated inductive indices are those listed in Table II. Acid dissociation constants were collected by Chiang and Tai from the literature. ***From Chiang and Tai,[168] by permission of Sci. Sin.***

(or pK_n), H-bonding strength, and oxidation-reduction potential.

a. THE ACID DISSOCIATION CONSTANT (pK_a). It is well known that although acetic acid and trichloroacetic acid possess the same carboxyl group, substitution of the more electronegative chloride atoms for the three H-atoms causes a drastic change in the pK value of the carboxyl group from **4.7** to less than **1.** This is the classic example of the inductive effect.

Figure 2 plots the pK_a values of various substituted fatty acids against the Taft induction constants of each of the substitutions and exhibits the standard linear relation. The value and scope of the Chiang-Tai theory can be seen from the four graphed cases, chosen from the much more extensive and equally accurate data the authors presented in their **1963** paper.[168] Table II presents (*1*) the acid dissociation constants of **73** oxygen acids, organic and inorganic, and (2) their theoretically calculated "induction indices" derived from the literature. Figure **3** from Chiang and Tai,[168] shows the linear relation between these two parameters for all **73** acids.

b. H-BONDING STRENGTH. That the proton-donating power and proton-accepting power of H-bond groups are determined by the induction effect is well known.[2,157,169,171] Figure 4 illustrates how the H-bond strength, like the acid dissociation constant, is determined by Hammett's[163] inductive constants for para-substitutes of aromatic molecules in a predictive manner.

c. OXIDATION-REDUCTION POTENTIAL. Figure 5, also from Chiang and Tai,[168] shows the linear relation between thiol and sulfide reactivity obtained from the literature and those authors' theoretically calculated induction indices. Thiol reactivity reflects the oxidation-reduction potential of the sulfhydryl group, which is also inductively controlled.

3. The Inducing "Groups"

The linear energy relation most extensively studied is that between covalently linked groups. However, because of its basically electrostatic nature, the inductive effect should extend to other closely associated groups; e.g., groups held together by ionic bonds or H-bonds. However, for an adsorbed entity to have a significant effect, the adsorption must involve more than a trivial amount of energy. To illustrate this point, Table III (reproduced from an earlier publication[2]) shows the much greater enthalpy of ATP adsorption compared to the enthalpy of ATP hydrolysis. The table also shows the very strong coulombic energy between K^+ and negatively charged carboxyl groups as part of the "absolute" enthalpy of adsorption of the K^+ ion.

One example of a strong inductive effect exercised by a dissociable ion is the proton of a carboxyl group. The inductive constant of a dissociated COO^- group is totally different from that of an undissociated COOH group. Other examples showing that H-bonded groups can exert a highly significant inductive effect have been reviewed elsewhere.[157]

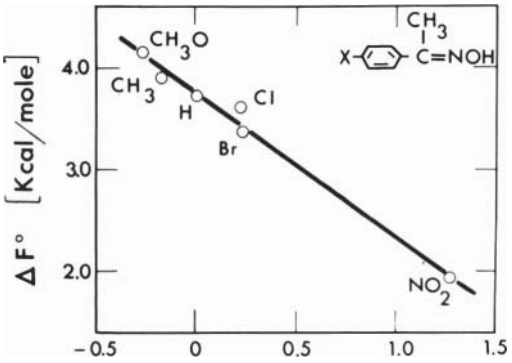


FIGURE 4. Relation between Hammett's o-constant and the free energy of dimerization of *para*-substituted acetophenoneoximes. Structural formula of para-substituted acetophenoneoximes appears in right upper corner. Abscissa represents Hammett's u-constant for each substituent indicated as X in the structural formula and more explicitly indicated in the graph. The free energy of dimerization of each substituted compound was calculated from Reiser's data and on the assumption that only dimers but no higher polymer existed. Frorn Ling,[1] *by* permission of Tex. *Rep.* Biol. *Med.*

4. Additivity and Reversibility of the Inductive Effect

The effects produced by two or more inducing groups on the same target group are additive. Also, such effects are completely reversible; that is, as A affects B, B affects A in a reciprocal manner. Although each inducing group tends to have a quantitatively different effect, in general the inducing group can either draw electrons toward itself or donate electrons. These basic traits are fundamental to assessment of complex multicomponent interactions, as discussed later below (p. 81).

B. c-Value Concept, Linear Model, and Theoretically Calculated Change in Preference for K^+, Na^+, H^+, and NH_4^+

The preceding discussion of the inductive effect prompts this question: What is the significance of the pK_a values of, for example, the β- and γ-carboxyl groups of a protein other than as indicators of their affinity for protons? The answer, according to the AI hypothesis, is that variations in pK_a values are a partial expression of the electron density of these acidic groups. This is significant indeed, since it is the electron density that determines protein specificity in ionic adsorption as well as in maintenance and conformational change. Before this concept can be fully presented, it is necessary to introduce a new parameter, the c-value.

The familiar pK_a value is not an independent variable. It expresses a specific interaction between an acidic group and a proton. The c-value, on the other hand, is the underlying parameter that gives rise to the pK_a value. The c-value, given in Angstrom units,

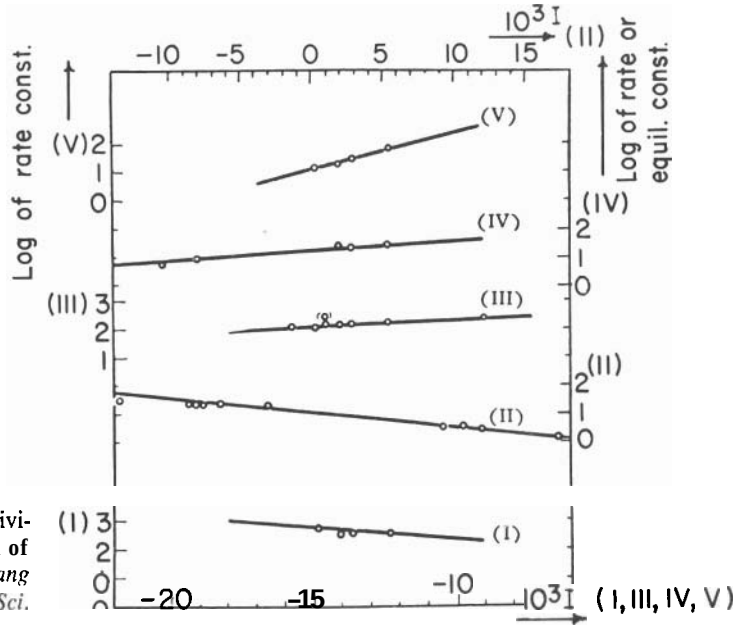


FIGURE 5. Chemical reactivities of acetals and ethers and of thiols and sulfides. *From Chiang and Tai,*[168] *by permission of Sci.*

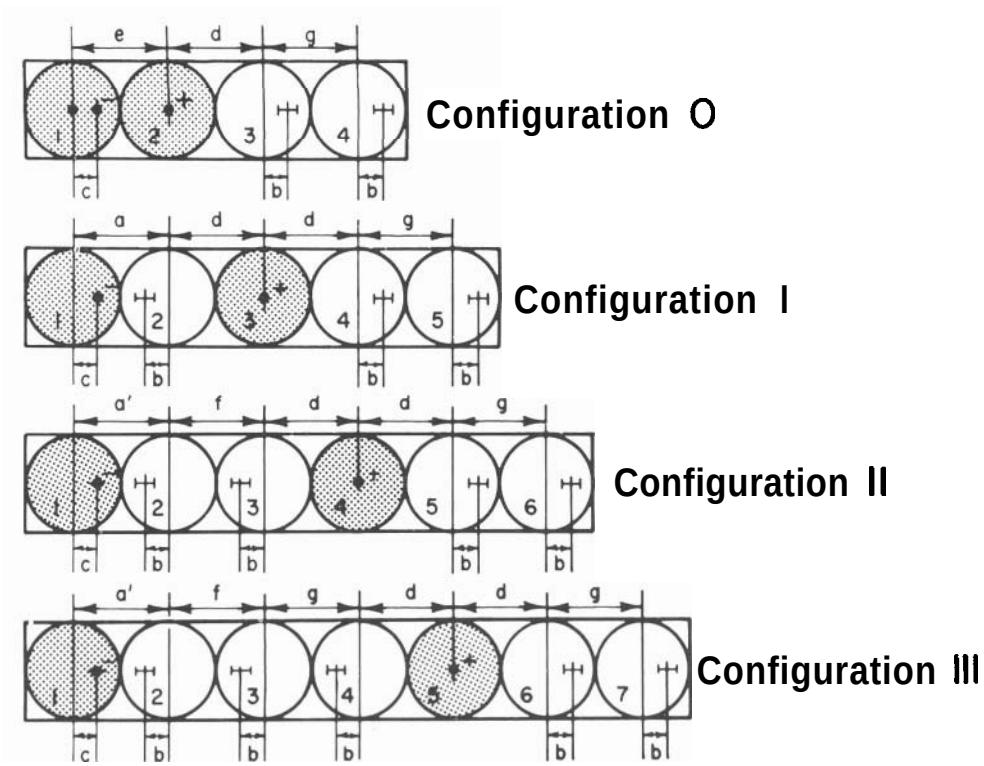


FIGURE 6. The linear model. The interaction energies were calculated for each of the monovalent cations in each of the four configurations of fixed anions and water. *From Ling.*[1]

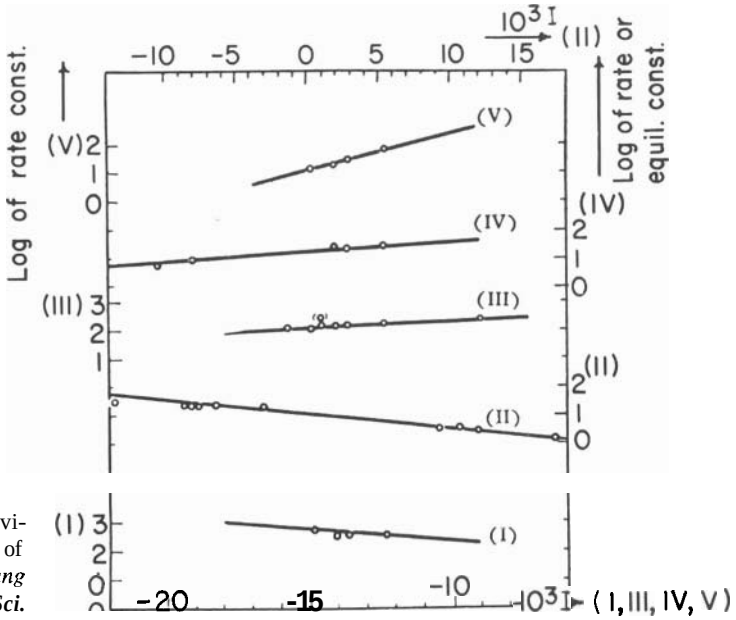


FIGURE 5. Chemical reactivities of acetals and ethers and of thiols and sulfides. *From Chiang and Tai,*[168] *by permission of Sci.*

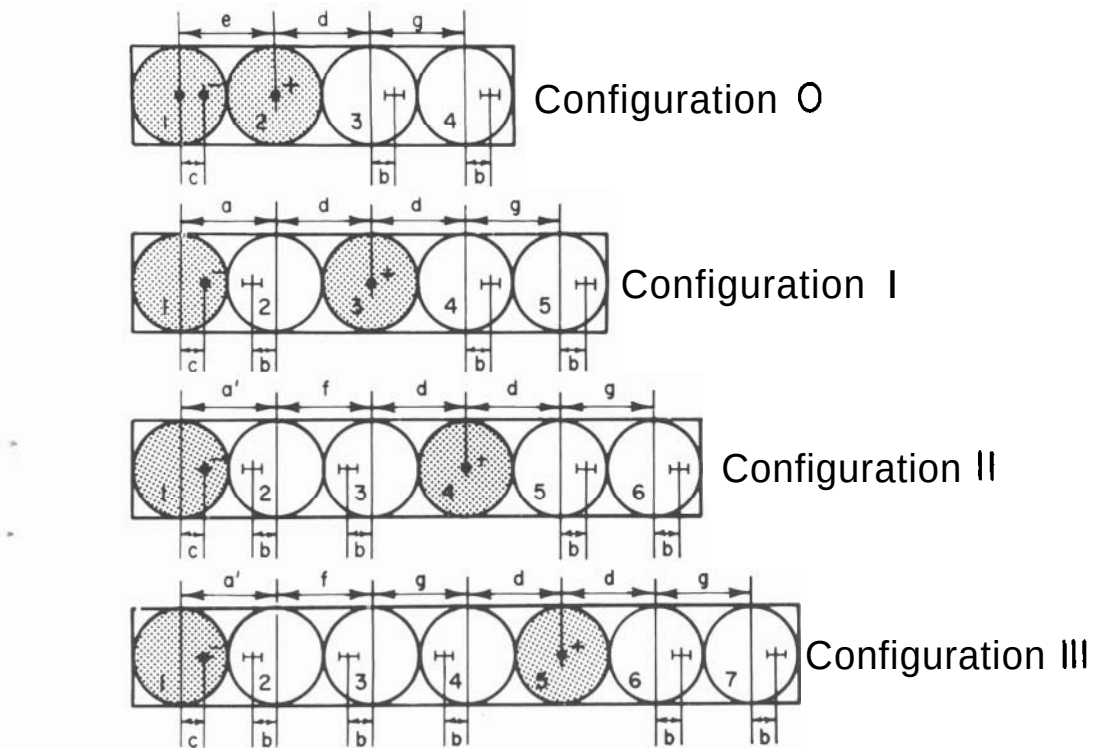


FIGURE 6. The linear model. The interaction energies were calculated for each of the monovalent cations in each of the four configurations of fixed anions and water. *From Ling.*[1]

from these data, the average energy of association. This enables calculation of theoretical curves that show either the adsorption energy (Fig. 7) or the selectivity coefficient (Fig. 8), measured against K^+, of the five alkali-metal ions in addition to H^+ and NH_4^+ as a function of the c-value. Note how the H^+ preference becomes greatly increased with the increase in anion site polarizability.

We now know that alkali-metal ions are

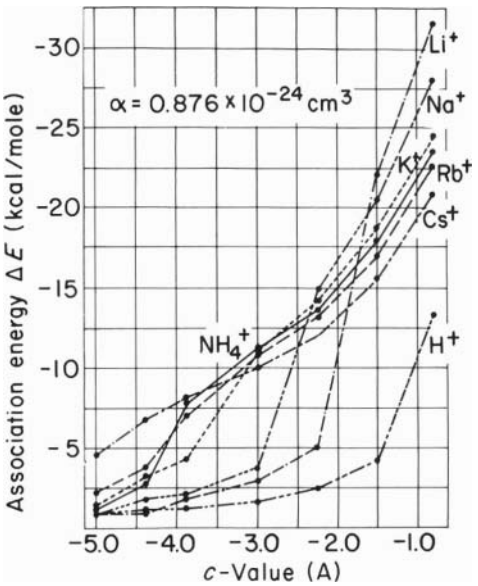


FIGURE 7. Relation between calculated association energy ΔE of various cations and c-value of the anionic group. Polarizability of anionic site, α, is 0.876×10^{-24} cm³. *From Ling.*[2]

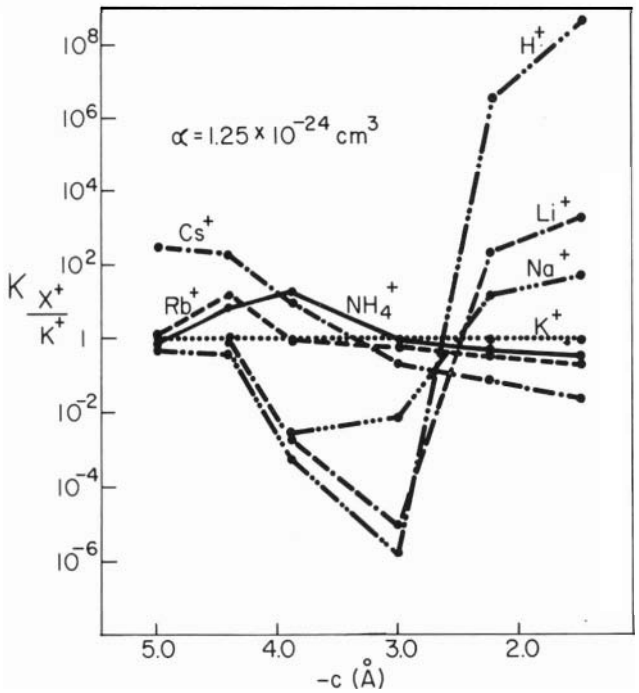


FIGURE 8. Relation between selectivity ratios of various cations and c-value. The K^+ ion is taken as unity and selectivity ratios are calculated from association energies. *From Ling.*[1]

adsorbed in the pattern of one ion to one site in the sulfonate and carboxyl types of ion-exchange resins (Ling and Brady, unpublished). Figure 9 (adapted from Bregman[178]) bears a resemblance to Fig. 8, but in Fig. 9 the abscissa represents the percentage of the cross-linking agent, divinyl benzene (DVB). An increase of pK_a value with increasing DVB has indeed been observed by Gregor and coworkers.[179] Figure 9 is essentially a plot of cation selectivity coefficients against the pK_a or c-value, as in Fig. 8. The general similarity between the theoretical data of Fig. 8 and the experimental data of Fig. 9 is obvious. Still, for emphasis, two features of the theoretical curves (Figs. 7, 8) are worth mentioning:

(1) Small changes in c-value could involve large changes in, even a reversal of, selectivity between a pair of ions (e.g., between K^+ and Na^+, between NH_4^+ and Na^+, etc.). The reason for the low preference for Na^+ at low c-values is that at equilibrium Na^+ exists in a higher conformation (i.e., it is more hydrated—see Fig. 6). The reason for the high preference for Na^+ at high c-values is that Na^+ then exists at a lower conformation (i.e., dehydrated). This relation is important to remember because, according to the AI hypothesis, it explains the different effects of Na^+ and K^+ on enzyme activity in general and on ATP synthesis in particular (see p. 67 below).

(2) The NH_4^+ group may represent a prototype of the positively charged ϵ-amino group of lysine side chain or the positively charged guanidyl group of an arginine side chain.

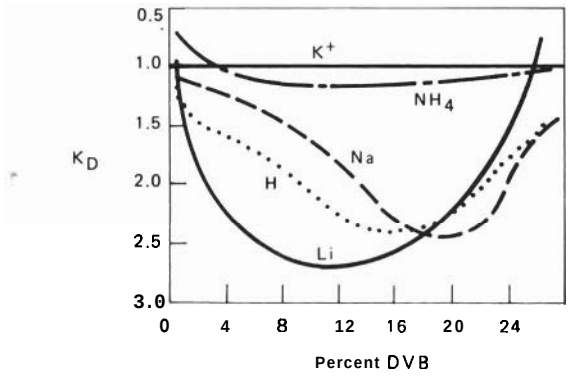


FIGURE 9. Relative selectivity coefficients of H^+, NH_4^+, and alkali-metal ions compared to K^+ in sulfonate ion exchange resin with varying percentage of the cross-linking agent divinyl benzene (DVB). ***Redrawn from Bregman'" by permission*** of ***Ann. NY Acad. Sci.***

C. Complex Interaction Between Biologically Active Agents

The additive nature of the inductive effect presents theoretical models that yield complex multicomponent interaction patterns of biological activity, of which enzyme activity is a well-known example. Figure 10 shows theoretical curves compared to actual experimental observations reported in the literature.[103] From these examples one sees that an agent promoting a particular effect in one direction may inhibit the same effect at a different concentration or upon variation in the concentration of a second agent. Simple nomenclature such as "uncouplers" or "inhibitors," useful and adequate under one set of conditions, may not apply under other conditions—in fact, may describe an effect totally opposite to that seen under the first conditions. [Note that complex patterns of interaction have been reviewed and theoretically derived elsewhere (see ref. 2, chapts. 7, 14)].

D. Cooperative Behavior of Living Cells: Interpretation Based on the AI Hypothesis

Many physiological manifestations of living cells exhibit all-or-none characteristics, as exemplified by muscle contraction. nerve excitation, and egg activation. "Denaturation" is a similar well-known all-or-none property of proteins.

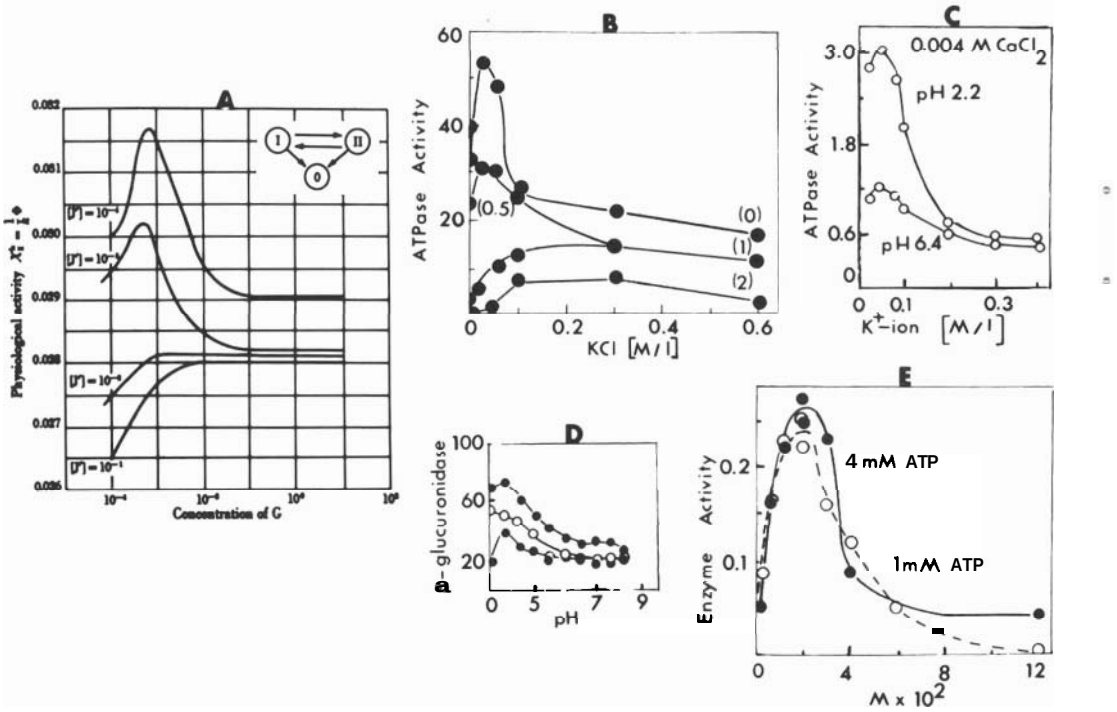


FIGURE 10. Dose-activity relations experimentally observed in graphs B, C, D, E demonstrate resemblance in pattern of physiological response to the theoretical curves shown in A. (A) Theoretical curves from a system in which two receptor sites and one effector site are arranged in linear order shown in inset (i.e., receptor-receptor-effector type). The physiologically active site is represented as 0 and the two flanking receptor sites as I and II. J′ and G are the biologically active entities. (B) Myosin B ATPase activity in the presence of varying concentrations of KCl in M/l and UO_2Cl in mM/l. ***From Bowen and Kerwin.'" (C)*** Effect of pH and of KCl on ATPase activity of myosin. ***From Mommaerts and Green.***[81] (D) Glucuronidase activity. Each curve represents an enzyme from a different source organism. ***From Wakabayashi and Fishman."'*** (E) Enzymatic synthesis of deoxycytidinephosphate. Abscissa refers to concentration of Mg^{2+} ion. ***After Richard et al.,***[183] ***from Ling,***[102] ***by permission of Tex. Rep. Biol. Med.***

All-or-none phenomena occur widely in the inanimate world, too. For instance, one of the earliest studied materials, β-brass, in statistical mechanical terms exhibits cooperative behavior distinguished by the presence of non-zero near-neighbor interaction energy. This is chosen to illustrate that the word "cooperativity," although extensively used in recent years with regard to protein behavior, denotes a range of phenomena not all of which are consistent with the statistical mechanical definition. But in 1962, I suggested that all-or-none changes in protein (such as denaturation) or in ***vivo*** (such as nerve excitation and muscle contraction) can be truly described as cooperative in the statistical mechanical sense, with the inductive effect providing the primary component of the near-neighbor interaction energy.[2] I now want to show that this description may apply to mitochondrial behavior just as it applies to that of intact whole cells.

1. Cooperative Adsorption Isotherm of Yang and Ling

In 1964 Yang and Ling[1''] presented a general cooperative adsorption isotherm, us-

ing the one-dimensional Ising method* for a circular or long linear chain of adsorption sites. All the sites on this chain are assumed to be similar. Each site can adsorb either of two alternative adsorbents described as i and *j*, including the case where i or *j* may represent a vacant site. As a rule, there is nearest-neighbor interaction, that is to say, the relative affinity for i or *j* at a particular site depends not only on the site itself and the nature of i or *j* but also on what occupies the two flanking sites. The adsorption isotherm derived describes the concentration of the ith adsorbed solute $[p_i]_{ad}$ as follows:

$$[p_i]_{ad} = [f]/2 \{1 + \frac{\xi - 1}{[(\xi - 1)^2 + 4\xi \exp(\gamma/RT)]^{1/2}}\} \quad (2)$$

where [f] is the total concentration of the adsorption sites, and R and T are the gas constant and absolute temperature, respectively. ξ is defined as follows:

$$\xi = ([p_i]_{ex}/[p_j]_{ex}) \cdot K^{\circ\circ}_{j\to i} \quad (3)$$

where $[p_i]_{ex}$ and $[p_j]_{ex}$ are the concentrations of the i and *j* solutes in the surrounding medium in equilibrium with the polymer. $K^{\circ\circ}_{j\to i}$ is the intrinsic equilibrium constant for the *j* to i exchange. The intrinsic free energy of the $j \to i$ exchange is $\Delta F^{\circ\circ}_{j\to i} = -RT \ln K^{\circ\circ}_{j\to i}$. The nearest-neighbor interaction energy, $-\gamma/2$, represents the additional energy during a *j* to i exchange without a change of the number of nearest neighboring *i-j* pairs. For example, consider a segment with 3 sites. The first site adsorbs an i, the middle site a *j*, and the third site a *j*. If the middle site *j* exchanges for an i ($ijj \to iij$) the free energy change is equal to $\Delta F^{\circ\circ}_{j\to i}$, because no new i, *j* neighboring pairs are created. On the other hand, for an exchange of the type $jjj \to jij$, two new *ij* pairs are created. Since each new pair adds an additional energy of $-\gamma/2$, the total free energy charge is $\Delta F^{\circ\circ}_{j\to i} + 2(-\gamma/2) = \Delta F^{\circ\circ}_{j\to i} + \gamma$. Figure 11 shows three different types of adsorption curves with different nearest-neighbor interactions. The middle simple hyperbolic curve corresponds to $-\gamma/2 = 0$ and is in fact a Langmuir adsorption isotherm without nearest-neighbor interaction. The flattened "hyperbolic" curve corresponding to a negative $-\gamma/2$ value is described as hetero-cooperative; in this case it is energetically more favorable to have neighbors of different rather than similar species. More important is the case where the value of $-\gamma/2$ is positive, the curve being sigmoid. In this type of isotherm, referred to as "autocooperative," it is energetically more favorable to have neighbors of the same species.

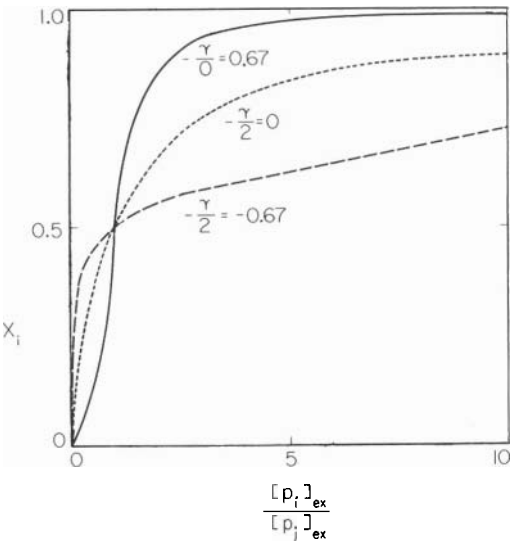


FIGURE 11. Linear plot of the Yang-Ling cooperative adsorption isotherm of the ith solute at varying rate of concentration of the ith and *j*th solutes in the bathing medium. x_i is the mole fraction of adsorption sites occupied by the ith solute. $-\gamma/2$ is the nearest-neighbor interaction energy. *From Ling,[7] by permission of Pergamon Press.*

*In 1958 Zimm and Bragg[184] used the same one-dimensional Ising method to derive an equation for the specific phenomenon of helix-random coil transition in proteins. Their equation, though dealing with a different phenomenon, bears a formal resemblance to the equation presented here.

2. Oxygen Dissociation of Hemoglobin and Related Phenomena

One of the most familiar autocooperative adsorption isotherms is the oxygen-dissociation curve of hemoglobin. Since the discovery that the binding of oxygen by hemoglobin follows a sigmoid-shaped curve, three types of quantitative or semiquantitative analyses have been made. Let us examine these in turn.

a. EMPIRICAL ANALYSIS (HILL'S EQUATION). In 1910, A. V. Hill[186] presented the following empirical equation that, because of its simplicity, has been the most extensively used to describe sigmoid adsorption isotherms and related phenomena:

$$\bar{x} = Kx^n/(1 + Kx^n) \qquad (4)$$

where $\bar{x}$ is the mole fraction of heme sites binding oxygen, x is the concentration of oxygen in the surrounding medium, and K is the equilibrium constant for oxygen binding. Usually log $\bar{x}/(1 - \bar{x})$ is plotted against log x:

$$\log[\bar{x}/(1 - \bar{x})] = n\log x + n\log K. \quad (5)$$

It has long been recognized that n determines the degree to which the oxygen binding curves take on a sigmoid configuration. Since Eq. 5 is empirical, n has no specific physical meaning. Wyman[187] believed that "n is closely related to the average free energy of interaction of the sites." Wyman did not, however, provide an explicit quantitative formalization of this relation.

b. THE AAKM THEORIES. More recent analytical theories include those of Adair,[188,189] Atkinson et al.,[190] and Koshland et al.[191] In each of these theories an assumption is made that the successive binding constants for oxygen on the four heme sites in a hemoglobin molecule are not the same but increase by some unspecified mechanism. On the other hand, in the symmetrical model of Monod et al.[192] the four subunits of the hemoglobin molecules are assumed to exist in one of two states. The affinity of all four units in one state is uniformly different from that of the four units in the other state.

c. THE YANG-LING MODEL: COMPARISON WITH OTHER MODELS. The cooperative isotherm described by Eq. 2 has been applied to the binding of oxygen to the hemoglobin molecule,[193] yielding the Yang-Ling analytical model. While a detailed comparison of this theory with those mentioned above is beyond the scope of the present paper, a few key differences are pointed out to facilitate development of its main theme.

(*1*) The AAKM theories were all proposed specifically to deal with hemoglobin as a tetramer containing four subunits, each with one binding site. Equation 2 suffers no such restriction. It deals with all types of proteins that contain a single chain or any number of subunits, and each chain or subunit may contain one or more binding sites (see p. 63 below).

(2) The AAKM theories neither consider a nearest-neighbor interaction nor provide a mechanism for it. Equation 2, as part of the AI hypothesis, offers a specific mechanism.

(*3*) The AAKM theories offer no explicit interpretation of the Hill coefficient n, whereas Eq. 2 does provide an explicit explanation of n as follows:

From Eq. 2, the mole fractions of sites adsorbing the ith solute ($\bar{x}_i$) and jth solute ($\bar{x}_j$) are

$$\bar{x}_i = \frac{[p_i]_{ad}}{[F]} = \tfrac{1}{2}\left\{1 + \frac{\xi - 1}{[(\xi - 1)^2 + 4\xi\exp(\gamma/RT)]^{1/2}}\right\} \qquad (6)$$

and

$$\bar{x}_j = 1 - \bar{x}_i.$$

Thus

$$\ln \frac{\bar{x}_i}{1-\bar{x}_i} = \ln \frac{[p_i]_{ad}}{[p_j]_{ad}}$$

$$= \ln \frac{[(\xi-1)^2 + 4\xi \exp(\gamma/RT)]^{1/2} + \xi - 1}{[(\xi-1)^2 + 4\xi \exp(\gamma/RT)]^{1/2} - \xi + 1} \quad (7)$$

Figure 12 shows that this equation accurately describes the oxygen binding of horse hemoglobin studied by Lyster (Ling, refs. 104, 194). In a plot of $\ln[\bar{x}_i/1 - \bar{x}_i]$ against $\ln[p_i]_{ex}/[p_j]_{ex})$ at the locus at which half the sites are occupied by i, the slope of Eq. 7 is $\exp(-\gamma/2RT)$. In fact, a straight line drawn through the log-log plot at the half-saturation point can be derived from Eq. 1 as

$$\ln \frac{\bar{x}_i}{\bar{x}_j} = n \ln \frac{[p_i]_{ex}}{[p_j]_{ex}} + n \ln K^{\circ\circ}_{j \to i}. \quad (8)$$

In the case where i represents oxygen molecules and j is unspecified, Eq. 8 becomes

$$\ln \frac{\bar{x}_{O_2}}{1-\bar{x}_{O_2}} = n \ln pO_2 + n \ln K_{O_2}. \quad (9)$$

This is formally identical to the Hill equation in a log-log plot. Here, however, the Hill coefficient n is an explicit function of $-\gamma/2$:

$$n = \exp(-\gamma/2RT). \quad (10)$$

3. Three Types of Autocooperative Adsorption on Proteins

Figures 13 and 14 show two additional types of in *vitro* adsorption isotherms: the binding of the cation dodecyltrimethylammonium bromide (DTAB) on bovine serum albumin (Fig. 13) from Few et al.[195] and the binding of phenol on collagen (Fig. 14) in the form of dried cowhide powder from Kiintzel and Schwank.[196] In each case, as in Fig. 11, the points represent experimental data and the solid curves are theoretical according to Eq.2.

In the case of oxygen binding on hemoglobin, the binding sites are the heme prosthetic groups, one oxygen to each heme in each of the four subunits of the hemoglobin molecules. In the case of bovine serum albumin, the binding sites are the β- and γ-carboxyl groups; the maximum number of DTAB ions bound roughly equals the total number of β- and γ-carboxyl groups of the protein known from chemical analyses.[157] In the case of phenol binding, the maximum number of phenols bound approaches the total number of backbone NHCO groups of the protein.[193,196]

The oxygen binding on hemoglobin represents ligand binding on prosthetic groups; DTAB binding to carboxyl side chains of bovine serum albumin unravels the tertiary structure of the protein; phenol binding on collagen peptide groups unravels the secon-

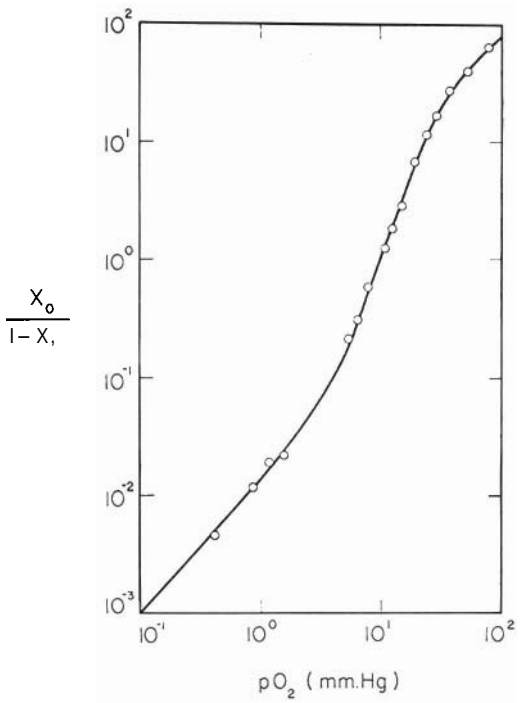


FIGURE 12. Log-log plot of data of Lyster on oxygen uptake by horse hemoglobin at pH 7.0, 19°C. *From Ling,[104] by permission of Int. Rev. Cytol.*

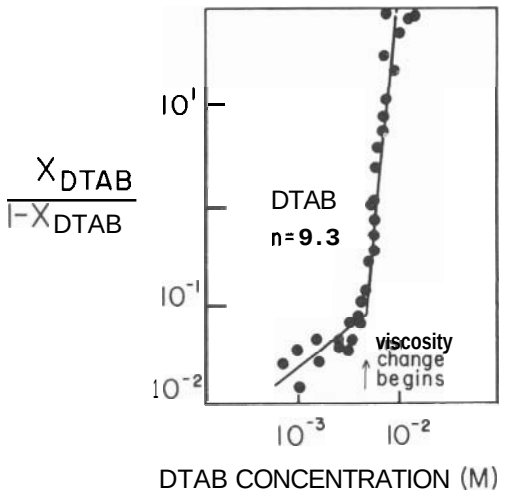


FIGURE 13. Binding of dodecyltrimethylammonium bromide by bovine serum albumin. Data from Few et al.[195] X_{DTAB} refers to the mole fraction of sites occupied by DTAB. *From Ling.'"*

dary structure. In spite of the great diversity of the particular sites involved they all exhibit autocooperative site-to-site interaction, describable by a positive value for $-\gamma/2$. As such, they show the most significant and important traits of all autocooperative interactions: the tendency to exist in an all-or-none" manner in either one of two alternative discrete states. Another highly significant featore of autocooperative adsorption is the phenomenon of a "threshold" or critical ligand concentration. Thus the twofold increase in the concentration of DTAB from 5×10^{-3} M to 10×10^{-3} M changes the protein conformation from one in which the β- and γ-carboxyl groups are locked in their native configuration (probably mostly in the form of salt linkages) to one in which these anionic groups bind cationic DTAB.[195] Similarly, a small change of the phenol concentration switches cowhide collagen backbone NHCO groups from H-bonding within the chain to H-bonding to phenol molecules.[196] Clearly, cooperativity is not limited to four or any other number of identical or similar subunits but occurs between neighboring sites wtihin the same protein chain, as in the case of DTAB and phenol binding.

E. Autocooperativity in Selective Solute Accumulation in Living Cells

According to the AI hypothesis, autocooperative adsorption-desorption behavior is a general and fundamental property of protoplasmic proteins and may be expected to play a major role in other biological phenomena including the selective accumulation of solutes in the living cell as well as in its organelles (mitochondria, nuclei, etc.).[2] With that in mind, let us return to the familiar oxygen-dissociation curve of red blood cells.

Oxygen is a solute present in the blood. In arterial blood, the oxygen in the plasma amounts to about 0.44 ml per 100 ml, while

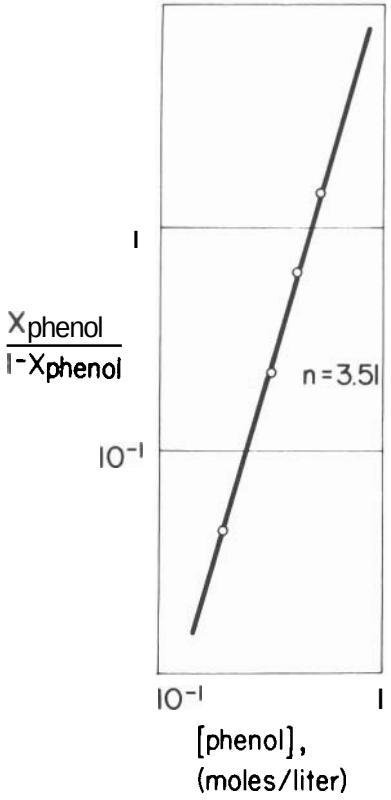


FIGURE 14. Binding of phenol by cowhide collagen· Data of Küntzel and Schwank.[196] *From Ling,[193] by permission of Fed. Proc.*

in the red blood cell the oxygen amounts to about 42 ml per 100 ml of cell water. Therefore, there is nearly a 100-fold selective accumulation of oxygen in red blood cells.

The inset of Fig. 15 shows a plot of the *in vivo* oxygen-binding curve of human red blood cells. Despite minor differences, this curve and the *in vitro* binding curve of hemoglobin shown in the large graph of Fig. 15 are quite similar. Indeed, this is accepted knowledge; oxygen binding to an intracellular protein, hemoglobin, is the basis of selective oxygen accumulation in red blood cells.

Now, another solute found in the blood cells is K^+. Its concentration in the plasma is about 5 mM while that in erythrocytes, as well as in other cells, is many times higher (about 150 mM). As far back as 1910, Benjamin Moore of Oxford University[1,2] pointed out this parallelism and suggested selective K^+ binding onto intracellular protein, rather than absolute membrane impermeability and permanent entrapment — as was then widely thought to be the cause of selective accumulation of K^+ in living cells. The powerful array of evidence cited earlier (e.g., Fig. 1) leaves little doubt that similar to oxygen accumulation in red blood cells, K^+ accumulation is also a matter of selective adsorption on (the β- and γ-carboxyl groups of) intracellular proteins. Figure 15 shows that similar to oxygen uptake in intact red blood cells, equilibrium levels of K^+ in intact frog muscle cells also showed a sigmoid-shaped curve and a positive nearest-neighbor interaction energy $(-\gamma/2)$ quite close to that of oxygen binding in hemoglobin.[193,199] Indeed the sigmoid K^+ uptake curve as well as the concomitant mirror image Na^+ uptake curve has been observed in a variety of living cells including frog voluntary muscle[193,199] (see Fig. 15), guinea pig smooth muscle of *Taeniae coli*,[200] canine carotid arteries,[201] rabbit myometrium,[202] Ehrlich ascites cells (Reisin and Gulati, unpublished), and human lymphocytes.[203] Just as relatively small changes in the external oxygen concentration can shift the oxygen uptake via its adsorption on hemoglobin, small concentration changes of K^+ (or, more precisely, the K^+/Na^+ ratio) shift the alkali-metal ion uptake between alternate adsorption states.

A frequent question is, "If indeed myosin or other intracellular proteins adsorb K^+ or

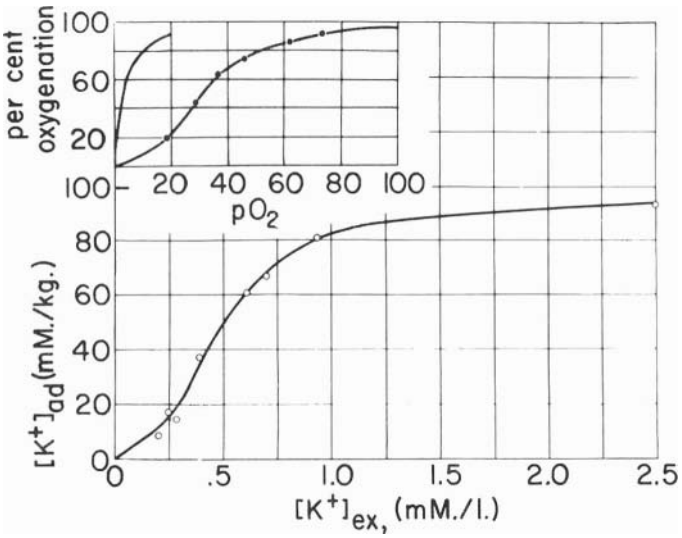
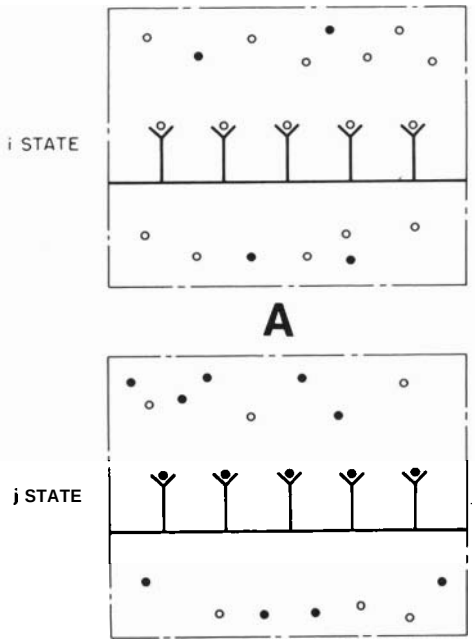


FIGURE 15. Equilibrium K^+-ion concentration in frog sartorius muscle in solutions with low K^+-ion concentrations but high Na^+-ion concentrations. Inset shows oxygen uptake by human erythrocytes (broken line with filled circles) and by myoglobin (solid line). *From Ling,[193] by permission of Fed. Proc. Data of inset from Eastman et al.[197]*



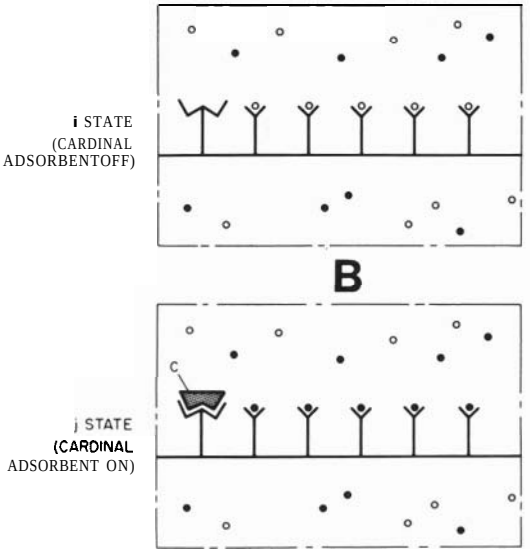


FIGURE 16. (A) Cooperative shifts between *i* and *j* states due to a change in the relative concentration of the i and *j* solutes in the environment. (B) Cooperative shifts between *i* and *j* states due to adsorption/desorption of cardinal adsorbent C in an environment with unchanging *i* and *j* concentrations. ***From Ling and Ochsenfeld,[158] by permission of Ann. NY Acad. Sci.***

Na+ autocooperatively, why has similar selective adsorption of K^+ not been demonstrated in ***vitro?"*** The answer, in terms of the AI hypothesis, is related to the specific ways in which different cells carry out their physiological functions. Shuttling between two environments with different concentrations of the ligand (oxygen) is the way red blood cells perform their function; other types of cells carry out their functions involving cooperative shifts of $K^+ \rightleftharpoons Na^+$ states within the same environment with more or less constant K^+ and Na^+ concentrations. In this case the simple cooperative shift between two states as a result of variations in ligand concentration is of little use. Other factors must be brought into the system to achieve the refined autocooperative shifts. This third category of delicate and consequently unstable interaction offers an explanation for the difficulty in demonstrating selective K^+ accumulation in ***vitro*** and will provide a key mechanism for mitochondria1 function.

F. Control by Cardinal Adsorbents of Shifts Between Two Cooperative States

The shifts between two alternative states, as seen in oxygen-binding on hemoglobin, DTAB binding on bovine serum albumin, or phenol binding on collagen, are brought about by varying the external concentration of the adsorbing species and hence a change in $[p_i]_{ex}/[p_j]_{ex}$ of the term ξ shown in Eq. 3. This type of cooperative shift is illustrated in Fig. 16A. Shown in Fig. 16B is a different mechanism that brings about a shift in the cooperative state in an environment containing an unchanging concentration of the adsorbent~p_i and p_j and of the ratio $[p_i]_{ex}/[p_j]_{ex}$. The change in this case is brought about by a change of $K^{\circ\circ}_{j \rightarrow i}$. The latter change, in terms of the AI hypothesis, is the result of a propagated inductive effect, or indirect F-effect, initiated by the electronic perturbation created by the interaction of a key site, called

the cardinal site, with a cardinal adsorbent. This phenomenon is analogous to the effect of the magnet or of the electrically charged rod upon the chain of nails or the insulators, respectively, as previously discussed.

Figure 16 illustrates a step-by-step propagated indirect F-effect initiated by the cardinal adsorbent (C). Whereas the diagram illustrates the stepwise mechanism of the all-or-none transition from one to the other state, the result also brings on a change in the reactivity of all the functional groups of the side chains due to the difference in the net electron-donating and electron-withdrawing effects of an a−a+ entity compared to those of the b−b+ entity where a+b+ are proton-donating groups or molecules and a−b− are proton-accepting groups or molecules. These affected side chains could include SH groups, with altered chemical reactivity, or β- and γ-carboxyl groups, with altered c-values, as examples of targets of the inductive effect. Although the normal physiological function of hemoglobin in red cells require no cardinal site-mediated cooperative transition, the oxygenation-deoxygenation curve has been known to be influenced by 2,3-diphosphoglycerate and inositol hexaphosphate[204] and has been quantitatively described by a set of equations derived from the basic Eq. 3.[205] Similarly, the adsorption of K^+ and Na^+ by frog muscle cells can be controlled by the cardiac glycoside ouabain, again quantitatively described by a similar set of equations derivable from Eq. 3.[206] One difference between hemoglobin and myosin in this parallel behavior is that the myosin system is much less stable. The process of isolating myosin from its complicated "environment" has led to an even lower energy state and a loss of its specificity in K^+ and Na^+ adsorption. Thus the direct demonstration of this specificity in ion adsorption has been possible only by using the freeze-drying techniques developed by Edelmann (see p. 47 above) and applied to the intact muscle cell.

VI. TENTATIVE MODEL OF ASSOCIATIVE-INDUCTIVE COUPLING MECHANISM FOR ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

Thanks to the work of Kanazawa, Kanazawa and Boyer, Taniguchi and Post, and Knowles and Racker (see Section IV above) ATP formation from P_i and ADP has been shown in model enzyme systems to involve two steps. First, "phosphorylation" of the ATPase by P_i is achieved by interaction with Mg^{2+} and/or K^+. Second, phosphorylation of ADP by the "phosphoenzyme" is achieved by interaction with Ca^{2+} and/or Na^+. To initiate ATP production these two steps are manipulated by equilibration of the enzyme and P_i in a Mg^{2+}- and/or K^+-containing medium, followed by dialyzing or otherwise removing the Mg^{2+} and/or K^+ and transferring the "phosphoenzyme" to the second ionic environment containing Ca^{2+} and/or Na^+. Thus production of ATP in ***vitro*** involves a sequential exposure of the protein enzyme to two different ionic environments.

A. The Coupling Mechanism Hypothesis

To account for these phenomena, I introduce here a detailed hypothesis resting on the assumption that in the first environment the ATPase ***adsorbs*** Mg^{2+} and/or K^+, and in the second environment the enzymes shift to ***adsorb*** Ca^{2+} and/or Na^+. Since there are no living beings in living cells to manipulate the ionic environment of the mitochondria, and since mitochondria do not migrate from one ionic environment to another like circulating erythrocytes, clearly a mechanism different from manipulation or migration must exist if the change from adsorption of Mg^{2+} and/or K^+ to that of Ca^{2+} and/or Na^+ is necessary to bring about the phosphorylation of ADP and the production of ATP. I now suggest such a mechanism, which may be formally hypothesized as follows:

Postulate 1. The generation of ATP is the consequence (as noted above) of two discrete steps: "phosphorylation" of ATPase by P_i promoted by adsorption of Mg^{2+} and/or K^+ on the enzyme—and phosphorylation of ADP by the "phosphoenzyme" when the enzyme adsorbs Ca^{2+} and/or Na^+.*

Postulate 2. Since free Mg^{2+}, Ca^{2+}, K^+, and Na^+ are available and more or less constant in the natural environment of the mitochondria, the shift in adsorption from Mg^{2+} and/or K^+ to Ca^{2+} and/or Na^+ is brought about by an autocooperative all-or-none shift of c-value ensemble and a consequent change of the relative preference of the adsorption for Mg^{2+} over Ca^{2+} or for K^+ over Na^+. With reference to Eqs. 2 and 3, such a change in these relative preferences is achieved by a change in ξ, not through altering the ratio of the concentration of the two alternative ionic species (K^+ vs. Na^+, Ca^{2+} vs. Mg^{2+}) but through changing $K^{\circ\circ}_{j\to i}$, the intrinsic equilibrium constant for the *j*th (Mg^{2+} or K^+) to the ith (Ca^{2+} or Na^+) ionic adsorbent (see p. 80 below for further discussion.)

Postulate 3. The respiratory chain units are cooperatively linked, through operation of the indirect F-effect, to the ATPase at each coupling site. As discussed earlier (p. 63), autocooperative linkage can occur among sites on the same protein chain and among closely associated sites on different protein chains (e.g., cooperative interaction among the four subunits of a hemoglobin molecule). This postulate offers the propagated inductive effect as a bridge between the respiratory chain centers and the ATPase site.

Postulate 4. The autocooperative linkage between the respiratory chain center and the ATPase allows the c-value ensemble of the ATPase to be controlled by the electron state of the respiratory chain center: when the respiratory chain center is in the reduced state, the c-value ensemble of the ATPase has a discretely different set of values. At one set of c-values, Mg^{2+} and/or K^+ would be preferentially adsorbed; at the other set, Ca^{2+} and/or Na^+ would be adsorbed. In other words, the associated pyridine nucleotide, ubiquinone, and the iron in the iron sulfur proteins and in the cytochromes act as "cardinal adsorbents" on the cooperatively linked ATPase much as oxygenation and deoxygenation of the heme iron in a hemoglobin molecule can allosterically affect the pK_a value of distant anionic groups [the alkaline Bohr effect (see p. 70 below)]. Thus each cycle of oxidation and reduction of the respiratory chain center is potentially capable of generating one ATP molecule.

In summary, the coupling mechanism hypothesis holds that the coupling between "electron transport" and "phosphorylation" is through cooperative shifts, mediated by the induction effect, between two different states of the ATPase system, permitting sequential formation of enzyme phosphate complex (EP) and then of ATP from ADP and EP. Selective ionic adsorption plays a key role in the mechanism. This hypothesis has its roots in (*1*) the universality of the inductive effect in chemistry, (2) the strong evidence that cellular cations exist in an adsorbed state, and (*3*) the observed indications that selective cation adsorption on proteins in living cells is controlled by cardinal adsorbents. Additional support can be found in the behavior of several other model systems (see below).

The proposed coupling model represents a special aspect of the basic concept of the living cell presented in the AI hypothesis. As such, this coupling mechanism has little in common with chemiosmotic coupling mechanisms, which are based entirely on the lipid membrane concept. However, the present model, at least in formal terms, bears some

***The specific ions mentioned in these postulates are used to illustrate the principles involved. In other systems, different ions may be operative. Thus in spinach chloroplasts, H^+ rather than K^+ would more likely play the equivalent role.**

resemblance to Slater's chemical intermediate hypothesis[mi] and even the conformation hypotheses of Boyer[208] and Green.[76] Kell's review[209] demonstrates an open-mindedness regarding new and different ideas and holds the promise of more unity and coherence in the field of cell physiology.

B. Comparison with Other Models

While the usefulness and validity of the present model remain to be decided by future experimental studies, our knowledge to date should allow us to determine whether oxidation and reduction of prosthetic groups can indeed influence selective ionic adsorption, since it has already been established that such adsorption leads to ATP systhesis. This oxidation-reduction mechanism applies espe–cially to the two heme proteins, hemoglobin and cytochrome c. Unlike cytochrome c, hemoglobin does not undergo the transition from Fe^{3+} to Fe^{2+}. However, it is also well known that the oxidation-reduction equilibrium of hemoglobin (Fe^{2+}) and methemoglobin (Fe^{3+}) bears a close resemblance to oxygenation-deoxygenation equilibrium (ref. 194, p. 188).

1. Heme-Heme Interaction and the Bohr Effect

In his 1979 review on hemoglobin, Perutz[210] stated that the regulation of heme's oxygen affinity by the structure of globin could in principle be accomplished by inductive effects (ref. 210, p. 383). Nevertheless, he reiterated his mechanical interpretation of heme-heme interaction. Much of Perutz's argument in favor of the mechanical model was based on studies of hemoglobin crystals and evidence that structures similar to those seen in the crystalline state are maintained in solution. Space does not permit an exhaustive analysis of alternative theories; however, contrary to Perutz's analysis, the long-range effect could be modulated primarily through an inductive mechanism, with mechanical steric factors playing an auxiliary role. Supporting that view are the following observations:

(*1*) In solution, hemoglobin does not maintain the kind of mechanical rigidity needed for a purely mechanical interpretation of heme-heme and other interactions. Ease of oxygen uptake and release of hemoglobin both in solution and as part of the living erythrocyte are absolutely essential and well known by observation. The behavior of hemoglobin crystals does not allow such ready uptake and release. When deoxyhemoglobin crystals combine with oxygen, the protein crystals break up;[211] crystals of oxyhemoglobin firmly retain oxygen when in a low-oxygen environment (ref. 187, p. 280).

(2) The mechanical feature of a rigid T-structure in the deoxygenated state and a relaxed R-structure in the oxygenated state is not compatible with the lower entropy associated with the oxygenated state rather than the deoxygenated state.[212]

(3) The rigid, impervious deoxygenated state of hemoglobin is not readily reconcilable with the binding of as many as 10 molecules of bromthymol blue to each chain of the deoxygenated state but not to chains of the oxygenated state.[213]

(4) Reactivity of the sulfhydryl groups is strongly influenced by whether the hemoglobin is in the oxygenated or deoxygenated state, much more reactivity being exhibited in the oxygenated state. Yet a simple mechanical steric hindrance cannot easily explain why only oxygenated hemoglobin reacts with mercuric benzoate and neither oxygenated nor deoxygenated hemoglobin react with iodoacetate.[214] A combination of steric hindrance and a difference in the induction-dependent oxidation-reduction potentials of these reagents seems to provide a more complete explanation (see Fig. 5).

2. Oxidation-Reduction Controlled Autocooperative Ion Adsorption Shifts

The observations cited above, in short, make it seem that cooperative transitions between oxygenated and deoxygenated states mediated by the inductive effect are more ca-

pable of reconciling findings that cannot be readily explained by the mechanical steric interpretation alone. Oxygenation. and deoxygenation, like oxidation and reduction, can indeed control the transitions between the two cooperative states, and in each state, as observed, many if not all of the functional groups exhibit different properties. It should next be asked, then, whether these transitions also involve shifts in the states of ion binding? In answer, one can cite observations of cytochrome c, hemoglobin, and pyridine nucleotides.

a. CYTOCHROME C. In this, the most extensively studied cytochrome of the respiratory chain, Margoliash et al.[215,216] have demonstrated that ferrocytochrome binds only cations, including Mg^{2+}, whereas ferricytochrome binds only anions, including Cl^-, P_i, and ADP. Thus oxidation-reduction changes clearly can alter ion binding in an all-or-none manner.

b. HEMOGLOBIN. Although hemoglobin is not a part of the electron transport system, its similarity of behavior makes it another useful model of the cytochromes. It has long been known that the effects of KC1 and NaCl on the in *vitro* oxygen-dissociation constant of hemoglobin are different.[217,218] Tosteson and coworkers,[219] for example, have shown that in the red cells of patients with sickle-cell anemia, deoxygenation produces not only sickling but a profound change in the intracellular levels of K^+ and Na^+. Since it is well established that sickle-cell anemia involves only one gene and one faulty amino acid residue in the p-chain of hemoglobin, the Tosteson findings suggest two conclusions. First, hemoglobin carries sites which, under physiological conditions, adsorb K^+ selectively. Second, the faulty amino acid in the p-chain permits deoxygenation of the heme group, altering the c-value of the sites that adsorb K^+ such that a value is reached at which the preference for K^+ over Na^+ is reduced.

Although the oxygenation-deoxygenation process differs from oxidation-reduction in degree, it is clear that oxygenation also involves a strong electrostatic effect and so is likely to produce similar short- and long-range induction effects. With this parallel in mind, the familiar Bohr effect and reverse Bohr effect are seen to be outstanding confirmation that prosthetic group reaction may lead to changes in the pK_a value (and hence the c-value) of acidic groups. In the case of lamprey hemoglobin,[220] the large and steep Bohr effect is compatible with autocooperative interaction even among the anionic groups, most likely β- and γ-carboxyl as already demonstrated in the case of bovine serum albumin interacting with DTAB (Fig. 13).

c. PYRIDINE NUCLEOTIDES. The most direct evidence that the oxidation-reduction state of the mitochondria1 electron transport system can determine cooperative adsorption of cations essential for ATP synthesis has been provided by Lehninger et al.[221] in both rat liver and heart mitochondria as well as in Ehrlich ascites cells. Those workers showed that the mitochondria take up Ca^{2+} and hold it as long as the pyridine nucleotide is in the reduced state; when the nucleotide is oxidized, Ca^{2+} is promptly released.

VII. INTERPRETATION OF OTHER MITOCHONDRIAL PROPERTIES IN TERMS OF THE AI HYPOTHESIS

A. Additional Basic AI Concepts

The preceding discussions have demonstrated how the series of experimental findings initiated by Jangendorf and Uribe's[89] studies of chloroplasts in spinach provided what was considered definitive support for the chemiosmotic hypothesis. Such interpretation was then called into question by the revolutionary findings of Kanazawa and others. A coupling mechanism based, under the AI hypothesis, on ionic association and

the inductive effect as well as oxidation and reduction of the respiratory chain, was proposed above to better account for the observations.

The AI hypothesis is sufficiently powerful, however, to allow cogent reinterpretation of other significant findings that until now have been interpreted almost entirely on the basis of modified versions of the membranepump theory. These findings have to do with the mechanism of action of uncouplers and ionophores, with steady oscillatory swelling and shrinkage, and with mitochondria1 electrical potentials. Hence certain additional fundamental concepts of the AI hypothesis must be discussed.

1. Physical State of the Bulk of Cell Water in Living Cells

According to the AI hypothesis, the bulk of cell water exists as polarized multilayers in a matrix of extended protein chains containing an alternating array of negatively charged CO groups and positively charged NH groups. Water in the state of polarized multilayers differs from normal liquid water—as found, for example, in isotonic Ringer's solution—but also differs profoundly from ice. In an ice crystal (i.e., Ice I), individual water molecules are fixed in positions that can be revealed by "instantaneous" photography. Neither normal liquid water nor water in the state of polarized multilayers can reveal much long-range structure in an instantaneous photograph. However, normal liquid water and water in the state of polarized multilayers are also profoundly different from each other in that if many serial photographs are taken from a fixed position, the photograph of liquid water will remain free of structure while water in the state of polarized multilayers will give sharper and sharper images as the number of exposures is increased. Thus water in the state of polarized multilayers can be said to possess structure—not the usual kind familiar to crystallographers, to be sure, but dynamic structure, perhaps more like a sea-scape as seen by impressionist painters.[35,41,222,223]

This theory of cell water has received experimental confirmation.[224,225] It has been shown that when the CO and NH groups of a protein molecule are locked in internal H-bonds (e.g., a-helix, β-structure), the water surrounding the molecule is minimally affected by the protein. On the other hand, if because of structural changes or the presence of denaturants that disrupt secondary structure, the CO and NH groups in these proteins are directly exposed to bulk-phase water, the properties of the water will be altered. In this altered state, water partially excludes Na^+, sucrose, and glycine, much as living cells do. Furthermore, the apparent equilibrium distribution coefficient, p, between polymer-oriented water and external normal water decreases with increasing size and complexity of the solute molecules, showing a ρ-value close to unity for small and simple molecules like methanol but lower for larger molecules like sucrose or inulin,[225] much as has been demonstrated for living cells.[105] These findings support the AI hypothesis concerning the mechanism for the partial exclusion of Na^+ and other solutes in whole living cells or isolated parts of living cells. They also offer indirect evidence in support of another tenet of the AI hypothesis, according to which hormones, drugs, and other biologically active agents can manipulate cell properties by controlling shifts of protein conformation from α-helical or other close structures, which accommodate water largely in the free state, to the extended open structure, which orients deep layers of water with reduced solubility as well as permeability to Na^+, sucrose, and other solutes.[102,104]

2. Swelling and Shrinking

According to the AI hypothesis, the existence of the bulk of cell K^+ in an adsorbed and thus largely osmotically inactive form re-

moves about half the osmotic activity of the living cell compared with that of the essentially isotonic solution of dissociated NaCl in the tissue fluid. This consideration alone renders untenable the idea that living cells behave like osmometers, as assumed by the chemiosmotic hypothesis.

Indeed, it has been shown that the linear relation between cell volume and external ("impermeant") solute concentration is largely fortuitous and due to the limited range of solubility of sucrose, salts, etc. in water used to prepare the hypertonic solution. When the much larger range of water activity provided by exposing the cell *indirectly* (via an air phase) to different concentrations of sulfuric acid and sodium chloride are applied in cell shrinkage studies, the swelling-shrinkage curve is no longer linear as required by the osmometer concept. Rather, that curve assumes the familiar S-shape typical of polarized multilayer adsorption.[226] Osmotic pressure, after all, is not an actual pressure but an expression of the reduced water activity in the cell; it is a "pressure" not exerted solely by free intracellular ions but also to a large extent by the long-range effect of the extended protein chains.

In support of this view, it has been found that (*1*) muscle cells swell in hypotonic solution to the same degree whether or not the membrane is intact;[227] (2) as shown by Troschin[m] and other Soviet scientists,[99,228,229] cells shrink in a concentrated solution of permeable solutes (e.g., urea, alanine); and (3) cells swell in hypotonic salt solutions and shrink in hypertonic solutions, greatly resembling the behavior of dialysis bags filled with solutions of polymers that contain oxygen atoms spaced two water molecules apart as in an extended polypeptide chain.[224,225] These polymers are like urea-denatured proteins—able to polarize water in multilayers. When the polymer solution is enclosed in standard dialysis tubing and exposed to dilute or concentrated salt solutions, the sacs will swell or shrink even though the wall of the dialysis tubing is obviously freely permeant to the salt ions.[230] On the other hand, if the dialysis bag is filled with a solution of a polymer that does not polarize water in multilayers, the sac will only swell and will not shrink permanently.

But polarization and orientation of water represents only one aspect of the swelling-shrinkage problem. To maintain cell volume, often there are also restraining forces against expansion. The AI hypothesis proposes that the restraining forces may reside among the salt-linkages between protein chains.[2,231] It is interesting to note that in recent years the importance of salt linkage has gained more and more recognition, resulting to some degree from the development of precise X-ray crystallography.[232]

According to the AI hypothesis, a high concentration of KCl causes swelling of muscle cells with or without an intact membrane[227] owing to dissociation of the restraining salt-linkages.[2,102,231] Thus

$$f^+f^- + K^+ + Cl^- \rightleftharpoons f^+Cl^- + f^-K^+$$

where f^+ represents fixed cations (e.g., ϵ-amino, guanidyl groups) and f^- represents fixed anions (e.g., β- and γ-carboxyl groups). The preference of the fixed anion f– at its specific c-value for K^+ over Na+ and the preference of the fixed cation f^+ for Cl^- rather than SO_4^{2-} account for the effectiveness of KCl in causing swelling and the ineffectiveness of NaCl or K_2SO_4 at the same concentrations.

3. Polarized Water Rather than Membrane Lipid as Seat of Selective Permeability

In 1973, I presented evidence that a membrane containing polarized water in multilayers (i.e., hydrated cellulose acetate membrane) much more accurately reproduces the selectivt: permeability properties of the living cell surface than does a pure lipid layer as

envisaged by Overton[32] and demonstrated in a model containing an olive oil or ether layer.[41,233] Lipid layers suffer the serious shortcoming of not being truly semipermeable; i.e., more permeable to water than to ethanol. Rather, a bona *fide* lipid layer is anti-semipermeable; i.e., more permeable to ethanol than to water. Since ethanol is about 50 times more soluble in lipid than in water, if we assume a linear relation between oil/water distribution coefficient and permeability, even a 10% lipid coverage of the cell surface could make the cell 5.5 times more permeable to ethanol than to water. In fact, reversed frog skin, for example, is twice as permeable to water as to ethanol.[233]

The important findings of Stillman et al.[44] and Maloff et al.,[22] mentioned earlier, showed that monactin and valinomycin do not increase K^+ permeability of the axon plasma membrane and the mitochondria1 inner membrane, respectively. This constiuted additional and compelling evidence negating the basic assumption of the lipid membrane approach.

The AI hypothesis holds that polarized water serves the barrier role too long mistakenly assigned to a continuous lipid layer, and specific fixed anionic and H-bonding sites of surface proteins serve the roles mistakenly attributed to mobile carriers or pumps.[234,235] It has been shown repeatedly that specificity, competition, and saturability frequently taken as evidence for pumps or carriers can often be easily demonstrated with simpler model systems carrying fixed sites — such as cation exchange resin beads, sheep's wool,[2,236,237] or even a layer of membraneless protein gel.[238]

4. Resting Potential as Surface Adsorption Potential

The cellular resting potential is conventionally felt to be a membrane potential, as first suggested by Ostwald,[239] further developed by Bernstein[240] and Donnan,[241] and revised by Hodgkin and Katz[242] and others.[7,15] According to the AI hypothesis, however, the resting potential is a surface adsorption potential; its magnitude is dependent on the density and nature of fixed anionic sites (i.e., β- and γ-carboxyl groups) of the cell surface proteins and on the concentration and nature of counterions in the external solution.[2,236,243-245] Furthermore, there is site-to-site interaction among β- and γ-carboxyl groups in the cytoplasm (e.g., as demonstrated with myosin).[193,195,199-203] The equation for the resting potential, ψ, derived from the cooperative adsorption isotherm given by Eq. 3, is as follows:

$$\psi = \text{constant} + \frac{RT}{F} \ln \frac{1}{[K^+]_{ex}} \left\{1 + \frac{\xi - 1}{[(\xi - 1)^2 + 4\xi \exp(\gamma/RT)]^{1/2}}\right\}. \quad (12)$$

Since its presentation in 1979,[246] this equation has proved useful in quantitatively explaining many features of the cellular resting potential previously published. Its usefulness in the realm of mitochondrial behavior will be discussed in a later section (p. 89).

B. Important Findings in Mitochondria1 Physiology and Some New Interpretations

1. Swelling and Its Reversal by ATP

Ever since the technique of isolating mitochondria in lightly buffered 0.25 M sucrose was introduced, the phenomenon of mitochondrial swelling has attracted investigators. Recall, however, that in order to preserve the normal elongated shape and staining properties of isolated mitochondria, sucrose at more than three times isotonic strength (i.e., 0.88 M) had to be used.["] Isolation of mitochondria in what is now the conventional medium

of 0.25 M sucrose is clearly a compromise between convenience and best preservation. Brenner-Holzach and Raaflaub[247] noted in 1954 that spontaneous swelling of mitochondria occurs when their ATP content falls below a certain level. Seven years later Nakao et al.[248] noted that as the ATP concentration in human erythrocytes declines, the biconcave cells first develop crenation and then, when the ATP content reaches a still lower and critical concentration, the red cells swell into a spherical shape. In isolated mitochondria as in erythrocytes, then, it is the concentration of ATP and not its rate of hydrolysis that determines shrinkage or swelling. In other words, it is not hydrolysis of ATP releasing a packet of energy believed to reside in a "high-energy phosphate bond,"[151-154] but rather the presence of ATP per se, that determines the normal mitochondrial or cellular shape and size. The view that ATP itself maintains the shape and structure of cells and its constituent parts by its adsorption on cell proteins was first expressed by Ling in 1952.[102]

However, to account for the mitochondrial findings under the AI hypothesis, a further revision of accepted membrane-pump and chemiosmotic concepts needs to be considered. For example, there is ooly one type of swelling and shrinkage according to the membrane-pump theory. It posits that the activity of water is higher in a hypotonic solution than in an isotonic solution but lower in a hypertonic solution. As a result, water moves from a hypotonic solution into the cell or from the cell into a hypertonic solution to reach thermodynamic equilibrium. Does this thesis hold? Let us compare it with that of the AI hypothesis, which proposes three types of swelling/shrinkage mechanisms active in living cells and subcellular organelles, with consequent changes in cell and/or organelle volume, as follows: (1) Simple changes due to equilibration with water that has altered activity. (2) Changes due to dissociation or association of macromolecular salt-linkages. (3) Changes due to polarization or depolarization of water. Let us examine these in turn.

a. TYPE I. SIMPLE SWELLING AND SHRINKAGE. For nearly a century the majority of biologists have believed that maintenance of osmotic equilibrium is governed by a semipermeable membrane and free intracellular K^+ and other solutes that balance the osmotic activity of free Na^+, Cl^-, and other solutes in the external medium. However, compelling evidence indicates that K^+ is in fact adsorbed within the cell (see p. 47 above). Cells without an intact membrane continue to swell normally in hypotonic solution. Addition of permeant solutes such as urea nevertheless causes cell shrinkage. Clearly, then, the osmometer concept is no longer tenable. Far more convincing is the AI view that maintenance of cell volume in an isotonic NaCl or sucrose solution represents a condition of balance among several opposing trends of water movement. The circumstances are:

First, certain intracellular proteins exist in an extended conformation, forming a matrix throughout the cells. In this conformation, these proteins maintain the bulk of cell water in a state of polarized multilayers. Water in this state has reduced activity. If proteins and polarized water were the only constituents of the cell, and if the cell were immersed in distilled water, the cell would swell because the activity of the distilled water is greater than that of the polarized water; water would naturally move toward the phase with less water activity.

Second, if the external medium were a sodium chloride solution (0.1 M), the activity of the extracellular water would be lower than that of distilled water, and consequently, water would begin to move from the hypothetical water-filled cell toward the external solution.

Third, as water moved out of the cell, Na^+

and Cl^- would move into it. If the cell water were simply normal liquid water, NaCl would reach the same concentration within the cell as in the external solution. When this condition would be reached, intracellular water activity would again be lower than extracellular activity owing to the presence of water-polarizing proteins, and again the cell would swell. However, if the cell water were not normal liquid water but existed in the state of polarized multilayers, the extent of water movement would be different. In that state, water has reduced solubility for Na^+ and Cl^-; i.e., the equilibrium distribution coefficient* for Na^+ as chloride, of $q_{Na(Cl)}$ would be below unity, say 0.1. Under this condition, water would reach a different equilibrium level between the cell and its environment. The total reduction of water activity in the cell, then, would be due to the combined effects of first, the extended protein chains; second, the reduced levels of Na^+ and Cl^- in the cell water; and third, the tension or mechanical pressure due to cohesive salt-linkages and H-bonds among the protein chains.

Under the AI hypothesis, therefore, simple swelling and shrinking imply that neither the mechanical tension nor the q-value for NaCl or other solutes present in the external solution has changed during the process.

b. TYPE II. DISSOCIATIVE SWELLING. In a previous section, mention was made of salt-linkage participation in the maintenance of cell volume by restraining the cell from gaining more water. Salt-linkages can be dissociated by salt ions, which would cause swelling. However, this dissociative action is limited to salt ions that can compete successfully against the affinity of the fixed anions for the fixed cations and vice versa.[227,231] Swelling of normal frog muscle in isotonic KCl involves no depolarization of cell water exemplifying Type IIA swelling.[231]

Furthermore, under the AI hypothesis the relative affinity of dissociating cations is not constant but varies with the c-value of the anion groups in a manner shown in Figs. 7 and 8. Since the c-value is not a constant but can be controlled by very low concentrations of cardinal adsorbent, which modulates the affinity of fixed ion pairs relative to that for free competing ions. Swelling of ATP-depleted frog muscle in isotonic NaCl (or Ringer solution) involves depolarization of cell water exemplifying Type IIB swelling.

c. TYPE III. DESORPTIVE SWELLING. In both simple swelling and dissociative swelling, no fundamental disturbance occurs in the physical state of the intracellular adsorbed ion (e.g., K^+).

An entirely different kind of swelling occurs if the water remains polarized while K^+ becomes desorbed. Under this condition the total osmotic activity of the cell will exceed that of an isotonic Ringer solution. As a result the cell will swell.

Type III swelling may occur during the early stage of cell damage and ATP depletion and is usually a transient phenomenon.

2. Reappraisal of Previous Reports According to the AI Hypothesis

a. ATP AND SWELLING/SHRINKAGE OF FETAL AND ADULT RAT LIVER MITOCHONDRIA. Pollak[249] isolated mitochondria from the livers of fetal and adult rats, placed the material in concentrations of sucrose ranging from 50 to 500 mM, and recorded the optical extinction at the wavelength of 545 nm until constant readings were obtained. Liver mitochondria from 5-day prenatal rats showed no swelling or shrinkage throughout the range of

*Note that the q-value, which refers only to solutes in the cell water or gel phase in comparison to the external concentration, is different from the ρ-value, the *apparent* equilibrium distribution coefficient, which may include adsorbed or other non-dissolved components.

concentrations. Mitochondria from adult rats showed pronounced shrinkage, whereas those from the 3-day and I-day prenatal rats showed moderate shrinkage. The virtually total insensitivity of the 5-day prenatal rat liver mitochondria to a 10-fold increase in external sucrose concentration could mean only that the mitochondrion is totally permeable to sucrose and—equally important—that it did not exclude sucrose. Otherwise, the mitochondria would have shrunk as mentioned earlier.

Prior to Pollak's study, Nakazawa et al.[250] had shown that the ATP and ADP contents of total rat liver mitochondria are very low during gestation but steadily increase from five days before birth to adulthood. Addition of ATP to the mitochondrial suspension greatly improves the respiratory control. Furthermore, the addition of ATP and Mg^{2+} enabled respiring fetal mitochondria to release H^+ in response to the addition of Ca^{2+}, as occurs in adult mitochondria.

Pollak[251] further demonstrated that both ATP-induced shrinkage of fetal liver mitochondria and enhancement of respiratory control depend not on hydrolysis of ATP but on the presence per se of ATP, which interacts with the mitochondrial inner membrane. In 1973 Stoner and Sirak[252] demonstrated that contraction induced in bovine heart mitochondria by ATP, ADP, and pyrophosphate has nothing to do with their hydrolysis, since they are equally or more active in the presence of oligomycin plus cyanide. These authors concluded that "inner membrane contraction occurs as a result of adenine nucleotide binding. . . ."

To explain the data, a suggestion made several years ago[2,226] is reiterated here. In adult resting cells, ATP is a major cardinal adsorbent that maintains certain matrix as well as membrane proteins in an extended water-polarizing conformation. Loss of ATP from the cell causes Type III and/or Type IIB swelling; gain of ATP reverses it.

b. Mg^{2+} AND Ca^{2+} vs. SWELLING/SHRINKAGE. It is possible that ATP is not the only cardinal adsorbent controlling water polarization? Mg^{2+} may be a co-cardinal adsorbent working synergistically with ATP. In the event that Mg^{2+} is competitively displaced from its site by Ca^{2+}, the effect of Mg + ATP may then be reversed. This possibility is suggested by findings reported by Hunter et al.[253] Those authors found that mitochondrial swelling induced by exposure to hypotonic solutions does not involve uncoupling of oxidative respiration. From this they concluded that "our results on hypotonically swollen mitochondria dismiss the long-held view that swollen mitochondria must be damaged and uncoupled. . . ." This type of swelling may be considered Type I.

One also recalls that in 1955 Hunter and Ford[2''] had noted likewise that simple osmotic swelling did not interrupt normal oxidative phosphorylation. Apparently no cooperative changes of the c-value ensemble or of the state of water occur. Hunter et al. had found that the addition of Ca^{2+} produce an increase of sucrose-available space from about 85% to nearly 100%. Furthermore, this increase is not specific to sucrose but applies to choline and glucose as well. All these responses can be promptly reversed by the addition of ATP and Mg^{2+}. Again, I suggest that addition of Ca^{2+}, like depletion or lack of ATP, causes Type IIB swelling; ATP and Mg^{2+} reverse this swelling by causing Type IIB shrinkage, producing long-range water polarization, and restoring low q-values for sucrose as well as other normally excluded solutes. Again, this interpretation is generally consistent with that of Hunter et al.,[253] who concluded that the effects of ATP and Mg^{2+} require neither electron flow nor energy but rather the mere accessibility to Ca^{2+} of some internal sites.

c. PASSIVE OSMOTIC SWELLING. While substrate oxidation or external ATP are essential for swelling, such as that induced by Ca^{2+} and a variety of other agents studied by Lehninger and many others.[18] Brierly et al.[78] found that isolated heart mitochondria will swell even when substrate or ATP is absent; however, they will also swell in the presence of a permeant anion and a permeant cation. In terms of the AI hypothesis, permeant anions, defined primarily on the basis of their capacity to induce swelling, are anions preferentially adsorbed by fixed cationic sites on cell proteins. Their presence therefore greatly enhances the probability of Type II swelling because they displace salt-linkages (see Eq. 11).

In contrast, salts of the same cation and the more weakly adsorbed anion Cl^- may not be able to effect any swelling. As an example, the fact that mitochondria can undergo swelling in KNO_3 or $NaNO_3$ but not in KCl or NaCl suggests that NO_3^- is more strongly adsorbed than Cl^-. However, Brierly and Jarkowitz[255] have also shown that mitochondria will not swell in 100 mM K or $NaNO_3$ if the pH is neutral but will swell in a pH of 8. This also agrees with the present interpretation. Salt-linkages are formed between β- and γ-carboxyl groups on the one hand and α-amino, histidine, ϵ-amino, and guanidyl groups on the other. A change of even one pH unit causes de-ionization of α-amino and histidine, the H^+ competing with Na^+ and K^+ and thus weakening the ability of $NaNO_3$ and KNO_3 to disrupt the salt-linkages.

Type II swelling as illustrated here is of the simple kind. A more complicated type, which depends on the action of cardinal adsorbents, is discussed in a later section.

3. Ion and Substrate Transport

Extensive investigation has been accorded the transport of ions, substrates, and other solutes in and out of living cells. Recent evidence (reviewed above, p. 38) indicates that Overton's postulation of the continuing lipid layer to serve as a selective permeability barrier is erroneous. The AI hypothesis, however, offers a feasible alternative: water polarized by macromolecules has been shown to reproduce more accurately the permeability properties of living cells.[233] Such a model is entirely in harmony with the findings that monactin has no effect on K^+ permeability of squid axon plasma membrane,[11] and valinomycin has no effect on K^+ permeability of the mitochondria1 inner membrane[22] or the cell surface of frog muscles and ovarian egg cells (Ling and Ochsenfeld, unpublished data). The cell membrane contains primarily protein and water. Phospholipid serves a secondary though important role, that of stabilizing the structure;[41] and except in special cases, as in the myelin of myelinated nerves, the phospholipid does not form a continuous barrier. Polarized water has the distinguishing feature of possessing nearly normal solubility and permeability for small molecules, such as water itself or methanol, but progressively lower solubility and permeability for larger and more complex molecules and hydrated ions.[225,256,257]

While surface proteins offer extended polypeptide chains to polarize deep layers of water, they also offer side chains that can adsorb ions and other solutes. Counterions enter exchange-resin beads and cells not by going between the fixed anionic sites but by a route involving, first, adsorption onto these sites and, second, libration around the sites and eventual desorption and entry.[2,236,237,243,258] Solutes too large and complex to enter through the polarized water may enter or leave by an adsorption-desorption mechanism on the inner membrane protein sites. The kinetics of this system are similar to those described for enzyme activity by Michaelis and Menton;[259] i.e., the adsorption-desorption mechanism exhibits the competition and saturability conventionally regarded as demonstrating a carrier mechanism.

Other fascinating findings from Pollak's laboratory illustrate the usefulness of the association-induction model. For instance, Sandor and Pollak[260] showed that the initial rate of entry as well as the levels of ^{14}C-labeled ATP in adult liver mitochondria are inhibited by atractyloside. In contrast, the initial entry rate of fetal mitochondria is considerably faster than that in the adult and is totally indifferent to atractyloside. After a peak uptake of ^{14}C-labeled ATP was reached at about 1 min, the level of labeled ATP began a decline, falling to nearly half peak level at the end of 5 min. While the accumulated level of [^{14}C]ATP was declining it also was developing a responsiveness to atractyloside. Sandor and Pollak interpreted their data to indicate the rapid, atractyloside-insensitive entry of ATP into the permeable fetal mitochondria. But once inside, "ATP interacts with the inner mitochondria1 membrane to transform its configuration and permeability characteristics . . . resulting in a diminished matrix space so that labeled ATP may be regarded as literally squeezed out together with water as the matrix becomes very condensed. . . ."

This view of course is in full harmony with my interpretation, given above, of Pollak's studies on reversal of Type IIB swelling. Here a new and intriguing feature is presented—atractyloside further reduced the level of ^{14}C-labeled ATP 2 min after exposure to non-labeled ATP. It is not likely that this could have been due to simple interference from an adenine nucleotide "translocase" operating at the inner membrane. Were that the case, atractyloside would have had no effect on the level of ^{14}C-labeled ATP since the latter was already in the mitochondria and could not leave. A useful future experiment would be to expose fetal mitochondria to ^{14}C-labeled ATP in both the presence and absence of atractyloside to determine with greater certainty whether initial exposure to and accumulation of ATP indeed render atractyloside sensitive to further ATP influx.

Sandor and Pollak's experimentation[260] clearly showed that atractyloside sensitivity is dependent on prior interaction of the fetal mitochondria with ATP, suggesting that maintenance of the polarized water surface barrier, as well as the presence of specific ATP binding sites, require that certain cardinal sites be occupied by ATP.

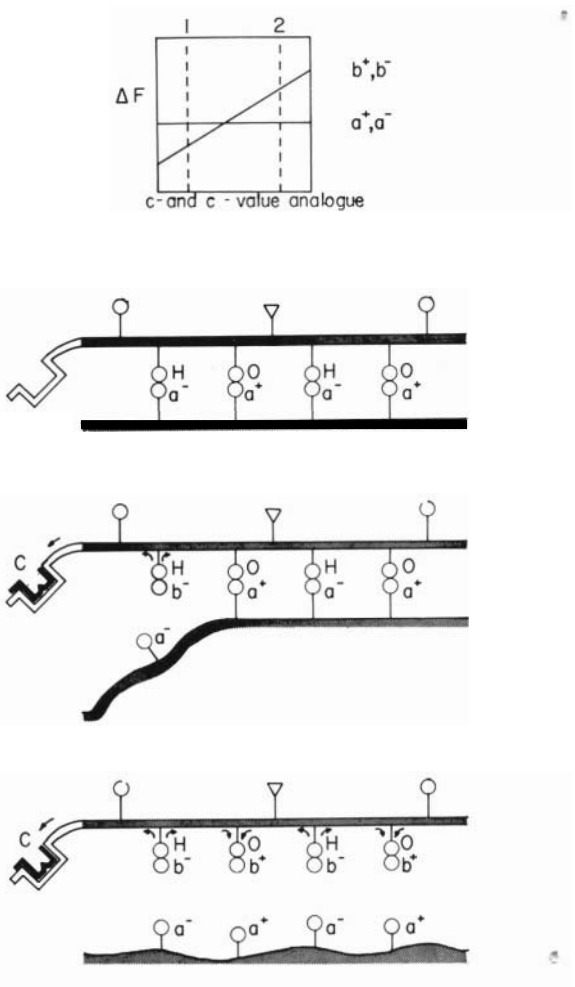


FIGURE 17. Diagram of cooperative transition induced by a cardinal adsorbent. Top figure shows the variation of the free energies of adsorption with changes of c-value analog of the C=O groups and *c'*-value analog of the NH group. Figures below demonstrate stepwise displacement of b^+ and b– (NH and C=O groups on the lower polypeptide) by a+ and a^- in consequence of interaction with cardinal adsorbent, C, at extreme left. Overall result of cooperative transition is dissociation of the two peptides (or uncoiling of a helix). *From Ling,"' by permission of Int. Rev. Cytol.*

It is also interesting to recall some much earlier observations. Lehninger et al.[68] in 1957 reported that ATP-induced contraction of mitochondria swollen in thyroxine is most effective in the presence of KCl. McFarlane and Spencer[66] and Gamble'[67] also observed that both ATP and K^+ are essential for the prevention of swelling. These various findings agree with the theoretical model presented in Fig. 17 and introduced more than a decade ago[104] in which adsorption of K^+ on β- and γ-carboxyl groups parallels alteration of the protein backbone from extended conformation and long-range polarization of water. Further, in a study of intact frog muscle cells, Ling and Ochsenfeld[158] demonstrated a qualitative correlation between selective K^+ adsorption and the degree of sucrose and Na^+ exclusion by cell water.

4. Uncouplers, Ionophores, Ca^{2+}, Mg^{2+}, ATP, and Other Agents as Cardinal Adsorbents

Why do many chemical agents, although different in type and reactive with different components of the mitochondria, produce essentially the same physiological effect? This question was first raised by Hunter and Ford[254] in 1955 and again 20 years later by Siliprande et al.,[261] who noted the almost identical effects produced by inorganic phosphate, the sulfhydryl-oxidizing agent diamide, and the divalent-ion "ionophore," A23187. On the other hand, certain agents that could be expected to produce a specific effect not only fail to do so at times but may even produce an opposite effect! Thus oligomycin, an inhibitor of oxidative phosphorylation, can at certain concentrations actually stimulate oxidative phosophorylation in both the forward and reverse direction.[262] Valinomycin, a specific K^+ ionophore that uncouples oxidative phosphorylation in the presence of K^+, may stimulate oxidative phosphorylation.[263] Yet valinomycin in the total absence of K^+ or any other ion inhibits electron transport in isolated chloroplasts.[264,265] Moreover, valinomycin, which does not combine with or transport Ca^{2+} or any other divalent ion across experimental phospholipid membranes, nevertheless promotes accumulation in mitochondria of Ca^{S+} in competition with K^+.[75] Monactin, a specific K^+ ionophore, does not increase K^+ conductance in squid axon membranes[44] nor does valinomycin in the presence of K^+ increase K^+ conductance in isolated liver mitochondria.[22]

To date, attempts to account for these puzzling phenomena have stemmed chiefly from the prevailing view in the field of mitochondrial physiology, a view based on Overton's lipoidal theory of the cell membrane. Specifically, the inner membrane is supposed to be a continuous sheet of phospholipid serving as a selective permeability barrier. Let us, however, review the data in the light of the totally different assumptions of the AI hypothesis.

a. Mechanism of Action of Valinomycin on Mitochondrial K^+ Accumulation. According to conventional membrane-pump theory, the major intracellular cations, K^+ and Na^+, are in the free state. According to the AI hypothesis, some solutes in the cell are in the free state—but others are not. The equilibrium level of the solutes in the free state, as a rule, is less than or equal to the concentration of the solute in the external medium according to the value of q. The remaining fraction exists in one or more adsorbed states, the equilibrium level of each being described primarily by the parameter ξ and to a lesser extent by $-\gamma/2$ (see Eq. 2). Now, $\xi = ([p_i]_{ex}/[p_j]_{ex})K^{\circ\circ}_{j\to i}$. Thus the level of adsorbed ith solute $[p_i]_{ad}$ depends on (1) the ratio of its concentration to that of the major competing ion, p_j, in the external medium; and (2) $K^{\circ\circ}_{j\to i}$, the intrinsic equilibrium constant for the i-to-j exchange adsorption.

With this dependency in mind, we can extend the AI hypothesis by suggesting that vir-

tually all K^+ in isolated mitochondria in an external medium containing a low concentration of K^+ is adsorbed on the β- and γ-carboxyl groups of mitochondrial proteins. Potentially these sites can also adsorb H^+, Rb^+, Cs^+, Li^+, Ca^{2+}, and Mg^{2+} as well as fixed cations that form salt-linkages. Whether these sites adsorb and thus accumulate K^+ or some other ion depends on the c-value and on the proximity of the sites to one another.[86] The "ionophore" valinomycin may function as a cardinal adsorbent and affect the c-value of cation-adsorbing sites.

In retrospect, the theory that adsorption is the basis of selective K^+ accumulation, presented in 1951[266] and 1952,[102] was considered favorably by Berger,[135] who was then studying mitochondrial physiology under Lardy, who had expressed a similar view.[134] Others, like Gamble,[136] Judah et al.,[267] Rossi et al.,[75] and Cereijo-Santalo,[268] at various times also referred to ion binding. Nevertheless, the majority of investigators continued to believe that K+, once inside the mitochondrial inner membrane, must be in the free state or else the cell would suffer osmotic imbalance and shrink. That recurrent theme, although contrary to observation, worked against the concept of K^+ adsorption from the first, just as it does now. Indeed, many years ago this argument against the notion of "bound K^+" in whole cells was effectively supported by Hill,[269] who convinced many that cell K^+ must be free.[100] However, as mentioned above, in terms of the AI hypothesis the loss of osmotic activity due to K^+ adsorption is compensated for by the long-range polarization of water and thus presents no problem.

One crucial difference between the chemiosmotic and AI hypotheses with regard to valinomycin is that in the chemiosmotic view valinomycin can manipulate only the rate of K^+ entry but not the final steady-state level of K+. The reason given is that the inner membrane by itself is K+-impermeable. In the AI model, on the other hand, valinomycin modulates the steady-state level in a quantitative manner. In 1972, Massari et al.[88] showed that steady-state levels of K^+ not only accumulate in the presence of various concentrations of valinomycin but indeed do vary with changes in that concentration. These findings show that valinomycin does not just open a "closed door" to K^+ but is related quantitatively to accumulations of K^+ and Ca^{2+}.

Another possible interpretation is that valinomycin might affect the steady-state level of K^+ by manipulating the rate of operation of a K^+ pump. However, membrane pumps as the basis of maintaining the steady-state level of K^+ in whole resting cells have been disproven.[2,86,106] Of all the pumps postulated for cells,[105] the Na-K pump in muscle cells, under controlled conditions, alone would consume at least 15 to 30 times more energy than the total energy available to the whole cell.[2] The idea of additional pumps to control ion flux across the mitochondrial membranes, whose total surface area is 20 times greater than that of the plasma membrane surface, (ref. 270, p. 30), thus consuming many times more energy than the pump originally postulated to exist in the plasma membrane, is untenable.

Does valinomycin in truth interact with mitochondria by combining with cardinal sites? An interesting finding by Griffith[271] offers some clues. He found that the mitochondria of certain mutant yeasts lacked sensitivity to valinomycin with respect to K^+ transport. Since a mutation of this kind is usually the result of an error in the DNA replication, and since DNA specifies protein sequence in the mutant, a part of the mitochondrial protein must be faulty. I suggest that this faulty or missing part contains the cardinal site that reacts with valinomycin. When Moore and Pressman[54] first reported on the mechanism of action of valinomycin on mitochondria, they concluded that "the triggering of K^+ transport by valinomycin implies the existence of a mitochondrial recep-

tor site. . . ." In terms of the AI hypothesis, such a receptor site would be called a cardinal site. However, contrary to Pressman, in the AI model the cardinal sites control K^+ distribution not via a K-pump but by altering the affinity for adsorption of K^+ on the other (non-cardinal) protein sites.[158]

b. IONOPHORES, UNCOUPLERS, AND OTHER CARDINAL ADSORBENTS: INDUCTION OF *c*-VALUE CHANGE AS BASIS OF THEIR ACTION ON MITOCHONDRIAL ION DISTRIBUTION. The variety of biologically active agents seems endless. In terms of the AI hypothesis, this enormous variety resembles that found in the shapes of the "keys" described in Paul Ehrlich's lock-and-key analogy regarding drugs. Though greatly varied in shape, however, the key can do only two things: open a door or close a door. Cardinal adsorbents also have but two options: donate electrons or withdraw electrons. Thus these agents can be classified into electron-donating cardinal adsorbents (EDC) or electron-withdrawing cardinal adsorbents (EWC). But complexity is introduced by steric factors and the multiplicity of attachment sites, which influence the specificity of agents in achieving the EDC or EWC effect. Another variable, of course, is the degree of intensity of the EDC and EWC actions. All these indicate that whereas the basic action may be simple, the actual observed effect may be extremely complex.

Earlier (p. 67) it was pointed out that a strong EDC in a cooperatively linked site tends to increase electron density (or the c-values of all the negatively charged sites) and also the positive charge density (or the *c'*-values of all the positively charged sites). Hence as a first simple assumption we may expect that strong EDCs increase while strong EWCs decrease the *c*- and *c'*-values of many of the sites in the proteins of the mitochondria. But there are pitfalls in the application of this simple concept. For example:

(*1*) One EDC displacing another EDC may create an electron-withdrawing effect if the first EDC is a weaker electron donor than the second EDC. Conversely, one EWC displacing another EWC may produce an electron-donating effect. Thus lack of awareness of a preexisting agent occupying the same cardinal site—which is often the case-could lead to conclusions opposite to the truth.

(2) A stronger EDC reacting with a cardinal site adjacent to an anionic site that is the first member of an anionic-cationic sequence may increase the c-value of all the anionic sites and the *c'*-value of all the cationic sites (see Fig. 18). On the other hand, if the cardinal site reacting with the stronger EDC is flanked by a cationic site which in turn is followed by a sequence of anionic and cationic sites, the result will be just the opposite: a decrease of the c-value of the anionic sites as well as of the *c'*-value of the cationic sites.

(3) The multiplicity of and interactions among cardinal sites (Fig. 10) constitute another source of confusion. An agent may produce opposite effects at different concentrations.

(4) Assessment of rates of fluxes vs. steady-state levels is tricky. Strictly speaking, changes in c-value can be assessed only on the basis of changes brought about in steady levels of ions accumulated in the mitochondria. Unfortunately, such data are relatively rare and often the c-values must be approximated. Because of this ambiguity, c-value changes of bulk-phase sites may be confused with properties of the surface sites.

Because of such pitfalls, the following analyses, based on data collected by investigators with entirely different theoretical backgrounds, may need considerable future revision.

Indeed, the difficulties are so formidable, that one may well ask whether there is any point in making even a preliminary assessment of the properties of EDC or EWC? The answer is a strong affirmative, because the behavior of mitochondria in particular, and of living cells in general, exhibits a high de-

gree of internal coherence. The best demonstration of this is the oscillating response of isolated mitochondria, to be discussed later. For the moment, in order to determine if an agent is electron-withdrawing or electron-donating, we must rely on the following:

(i) Theoretical data (Figs. 7, 8) describing change in relative preference between a pair of adsorbed cations in response to the agent applied.

(ii) The reasonable assumptions that oxidation or respiration generally involves loss of electrons from the system and hence a c-value decrease, and that reduction of anaerobiosis generally involves a gain of electrons and hence a c-value increase.

1. Respiration and anaerobiosis. A cation-exchange resin can be made to replace all its counterions; it cannot be made to lose them altogether. As long as there are n moles of fixed anionic sites in the resin, n moles of countercation must also be present. A living cell shares a number of important attributes with a simple ion-exchange resin bead, but in death — whether brought about by heat or by metabolic inhibition — all of the cell's accumulated ions may be lost. The AI hypothesis has long argued that this is because living cells are neither cation-exchange resins nor anion-exchange resins but a mixed amphoteric ion-exchange system on flexible chains.[2,102] In cell death, adsorbed ions are displaced by the formation of salt-linkages between the fixed anions and fixed cations that in the normal resting cell adsorb free cations and free anions, respectively. There are two reasons for the propensity of living cells to assume a final salt-linkage formation. The first is that the loss of entropy when a salt-linkage is formed is less than that when two free ions are adsorbed; however, if this were the only reason, no free ions would ever be adsorbed.

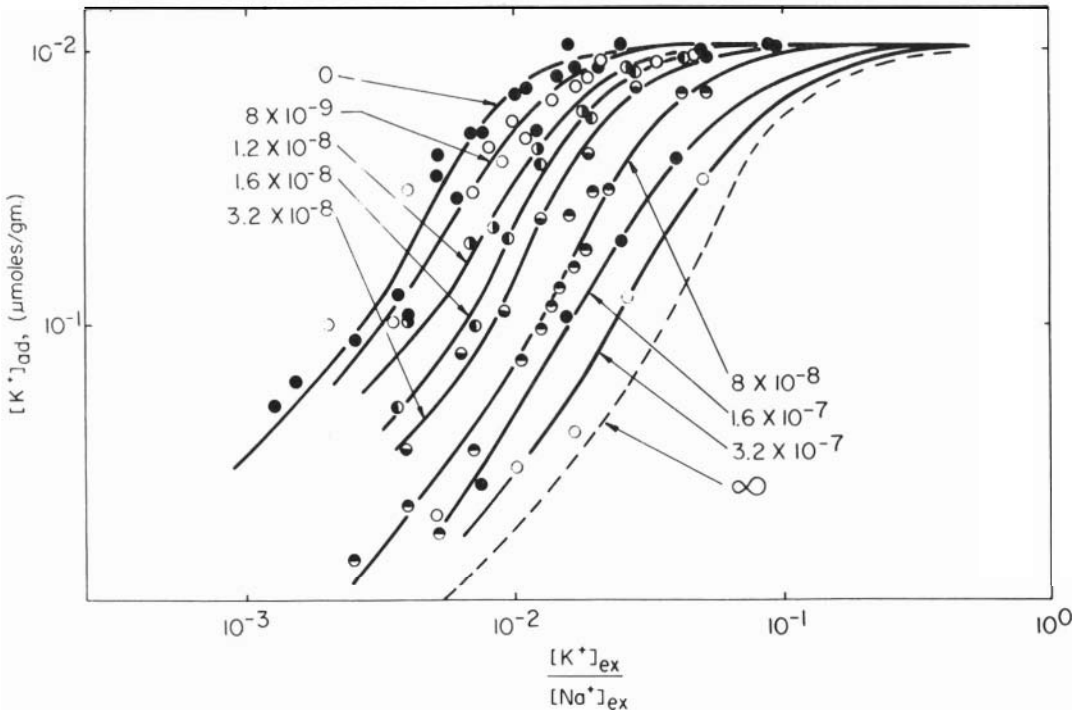


FIGURE 18. Effect of different concentrations of ouabain on equilibrium distributions of K^+ ion in frog muscles. Each point is the average of 4 determinations. Standard errors not shown to avoid confusion. Numbers refer to equilibrium molar concentrations of ouabain in the external media. ***From Ling and Bohr,[206] by permission of Physiol. Chem. Phys.***

The second reason is that in the dying state, electrons flow from fixed cationic sites and other sources to fixed anionic sites as well as to backbone carbonyl groups (raising their c-value or. c^r-value analogs), and the high c-value anionic sites then combine with high c^r-value fixed cationic sites, forming tight salt-linkages. A similar high electron density of the carbonyl oxygen of the backbone promotes the formation of strong H-bonds with peptide NH groups of high positive charge.[104] The internally neutralized amphoteric system is also seen in "dead" proteins, such as sheep's wool and hair.[2]

Mitochondria are not dead in any of the six metabolic states defined by Chance and Williams[272] and Chance.[273] However, it is possible that in State 4 the c-value ensemble is fairly high, in agreement with the highly reduced state of DPNH measured.[272] Referring to Figs. 7 and 8, one can then predict substantial retention of H^+ in State 4 even in a medium of very low H^+ concentration (i.e., pH 7.4). In State 1, the respiratory carriers are in a somewhat more oxidized state than in State 4.[272] Yet Gear and Lehninger,[69] showed that the relative effectiveness of alkali-metal ions in displacing K^+ and H^+ follows the rank order $Li^+ > Na^+ > K^+ > Rb^+$—a preference corresponding to that of an anionic site of relatively high c-value (Figs. 7, 8).

Under the AI hypothesis, I suggest that the mitochondrion may be brought to the highest c-value when the entire respiratory chain is in the reduced state (high substrate concentration but no oxygen). The c-value increases are accompanied by c^r-value increases at the fixed cationic sites and extensive salt-linkage formation occurs, "squeezing out" both adsorbed cations (e.g., K^+) from anionic sites and adsorbed anions from cationic sites (e.g., ATP), consistent with the data of Gamble and Hess.[274] The contraction concomitant with salt-linkage formation (Type II shrinkage) is then seen as a strong increase of light-scattering. On the other hand, respiration, which shifts the chain to a more oxidized state, by definition would lower the c-value. In the theoretically calculated curves (see ref. 2, Fig. 4.10) one notices that H^+ is greatly preferred over K^+ at high c-value and that this preference reverses itself at low c-value. In agreement, it has been known for some time that respiration favors K^+ retention in isolated mitochondria.[65,135,136] That is to say, respiration reduces the c-value to a level at which K^+ preference is increased in relation to H^+.

2. "Ionophores." Valinomycin causes additional uptake of K^+ and release of H^+ in respiring rat liver mitochondria.[84] As just discussed, respiration tends to maintain the electron chain in an oxidized state, corresponding to a low c-value state. The introduction of valinomycin reduces the c-value still further to a level at which the preference of K^+ over H^+ is further increased, and a K+-for-H+ exchange then follows. So it would seem that valinomycin is an EWC. Since A23187 has a similar effect on K+-for-H+ exchange,[275] it may also be an EWC.

Pressman showed that gramicidin brings about a gain of both K^+ and Na^+ exchange for H^+ from a medium containing 1.5 mM K^+ and 1.9 mM Na^+.[276] Consulting Figs. 7 and 8, one sees that a more moderate decrease of the c-value than that brought about by valinomycin would bring about the uptake of both K^+ and Na^+. Gramicidin is thus a weaker EWC than valinomycin.

Pressman also described the response of the mitochondria to gramicidin when the oxygen was becoming exhausted: ". . . Upon approach of anaerobiosis (i) K^+ ejection occurred first. . . (ii) followed by Na^+ ejection, (iii) H^+ uptake and (iv) mitochondria1 contraction," concomitant with H^+ ejection. Again referring to Figs. 7 and 8, one sees that the better part of this sequence of events can be explained by a steady increase in the c-value of the anionic sites. Why then was

the H^+ accumulated at a high c-value also released with an increase of light-scattering after total anaerobiosis? I suggest that at such high c-values, salt-linkage formation and the concomitant Type II shrinkage become energetically more favorable than continued H^+ adsorption.

3. Uncouplers. It has long been known that 2,4-dinitrophenol (DNP) causes a loss of K^+ and a gain of H^+[267] in respiring mitochondria.[62,67,135,136] That this K^+ loss *cum* H^+ gain involves an H+-for-K+ exchange caused by a DNP-induced increase in the preference for H^+ over K^+ is further supported by the following observations. First, DNP inhibits H^+ liberation in response to the addition of external K^+.[67] Second, increasing K^+ and other alkali metal ions can prevent DNP-induced uptake of H^+ in the rank order of effectiveness $K^+ > NH_4^+ > Na^+$.[267] Third, while valinomycin causes K^+ gain and H^+ loss, DNP and valinomycin together cause K^+ release—a release that can be prevented by raising the external K^+ concentration.[274] From these findings it would seem that DNP has an effect opposite to that of respiration and valinomycin and must therefore primarily be an EDC.

In the same way, one can understand why DNP also prevents KCl-induced (Type II) swelling.[273] But how does DNP increase oxygen consumption, uncouple oxidative phosphorylation, and activate ATPase activity? At this stage, only general statements can be made in answer.

It has long been known that K^+ is necessary for the respiratory activity of mitochondria. This requirement as first reported by Pressman and Lardy[277] was disputed.[278] However, later work including that of Krahl et al.,[279] Kimmich and Rasmussen,[280] and Gomez-Puyou et al.[281,282] has removed any doubt that the original conclusion of Pressman and Lardy was essentially correct. The AI hypothesis suggests that rapidly oscillating cooperative adsorption and desorption of K^+ accompany the propagated oxidation-reduction cycles along the respiratory chain. Such rapidly oscillating cooperative adsorption-desorption depends on a delicate balance between the c-value and the appropriate pair of counterions. DNP, like ADP acting as an EDC, perturbs this state of balance and as a result the oxidation rate is increased. In general agreement with this suggestion, it will be recalled that DNP stimulates respiration only at low concentrations. At higher concentrations DNP actually depresses respiration.[283,284] Similarly K+, which is necessary to increase DNP-induced oxygen consumption at low concentrations, inhibits respiration at high concentrations. These seemingly contradictory behavior patterns are to be expected from the kind of complex electronic interactions illustrated in Fig. 10.

That continued respiratory activity depends on a delicately poised state of the respiratory chain proteins adsorbing K^+ is also in harmony with the following findings: (*1*) K+-depletion causes inhibition of respiration.[280,285] (2) Valinomycin and gramicidin, which tend to increase K^+ in isolated mitochondria, increase respiration.[84,263,286,287] (3) Nigericin, which causes K^+ loss, inhibits respiration.[288,289] (4) ADP-induced respiratory control index increase depends on K^+.[281] Loss of respiratory control parallels loss of K^+ content.[282] (5) DNP added as a supplement to valinomycin causes loss of K+, after which respiration stops; addition of KC1 restores respiration.[280]

Other evidence supports the notion that in normal mitochondria the delicately balanced adsorption and desorption of K^+ depends on both Mg^{2+} and ATP acting as cardinal adsorbent~:(1) ATP, Mg, and diphosphopyridine nucleotide all restore mitochondria1 respiration and oxidative phosphorylation.[254] (2) Mg^{2+} is required for DNP-activated ATPase activity and oxidative phosphorylation.[290] *(3)* Respiration of fetal rat liver mitochondria is not stimulated by ADP. The

respiratory control index is increased by exposure to both ATP and to an ATP analog that cannot be hydrolyzed.[250]

The even more prominent effect of DNP and other uncouplers on mitochondria is inhibition of oxidative phosphorylation. Thus, as pointed out earlier, there is a delicate shift of the ATPase from a Mg and/or K state, which favors the promotion of the phosphorylated ATPase, to a Ca and/or Na state, in which ATP is synthesized. Another externally applied agent able to disturb the c-value would undermine the delicate balance and oscillation between the Mg and/or K state and the Ca and/or Na state and thus hinder or arrest oxidative phosphorylation. The key role of the electronic or c-value balance of the ATPase sites can be illustrated by a graph (Fig. 19) reproduced from Estrada-O et al.,[291] showing intramitochondrial ATPase activity in relation to H^+ activity and an EDC, nigericin. If one bears in mind that increasing pH means decreasing H^+ concentration, the complex pattern of interaction is seen to broadly resemble the theoretical curve calculated on the basis of the AI hypothesis with a pair of interacting agents (Fig. 10).

4. Thiol ***reagents.*** Crompton et al.[292] showed that Mg^{2+} is retained in anoxic rat heart mitochondria, which produces Ca^{2+} efflux; Mg^{2+} is lost during succinate oxidation, which retains Ca^{2+}. If oxidation causes a decrease of c-value and respiration inhibition causes an increase in c-value, as argued above, then Mg^{2+} would be preferred over Ca^{2+} at a higher c-value. That respiration favors Ca^{2+} accumulation in the presence of both high and low external Ca^{2+} concentration has been known for a long time (for review, see Lehninger, ref. 293). It is known further that the three energy-conserving sites contribute equally to the support of Ca^{2+} uptake,[294] and that respiratory inhibitors including antimycin A and uncoupling agents, if added after Ca^{2+} has been accumulated, produce rapid release of Ca^{2+} back into the medium.[295,296] The simple AI interpretation, of course, cannot apply to the opposite conclusion of Harris et al.[297] who found that the inhibition of respiration favors retention of mitochondria1 Ca^{2+}, or to the later findings from Lehninger's laboratory that Ca^{2+} uptake parallels the reduced state of pyridine nucleotide.[221] Clarification of these apparent conflicts can be achieved only by future experiment.

As of now, precise theories on alkali earth ion selectivity variation with c-value changes are unavailable. However, my experience with the alkali-metal ions indicates that as a rule

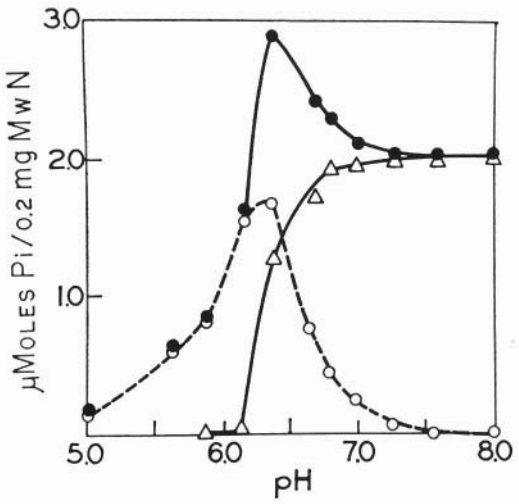


FIGURE 19. Effect of pH on K+-dependent ATPase from mitochondria stimulated by nigericin. Reaction system contained 6 mM tris-ATP, 10 mM triethanolamine-HCl, 8 mM L-histidine, 20 mM acetate, and 50 mM KCI. Vessel contents were adjusted with HCI to the indicated pH values, and 10 μg of nigericin per ml were added to' the corresponding media. Mitochondria containing 0.2 to 0.4 mg of nitrogen were added in 0.3 ml of 0.25 M sucrose. Final volume was 1.0 ml. Open circles = spontaneous ATPase induced by the pH change in K^+ media and hypotonic conditions. Filled circles = activity of ATPase induced by nigericin at different pH values. Triangles = corrected curve of the ATPase activity stimulated by nigericin, obtained after subtracting the spontaneous ATPase from the ATPase in the presence of nigericin. Numbers refer to equilibrium molar concentrations of ouabain in the external media. ***From Estrada-O et al.,[291] by permission of J. Biol. Chem.***

the ions of lesser atomic weight tend to be preferred at higher c-values. Hence an assumption that Mg^{2+} over Ca^{2+} selectivity should increase with increased c-value seems reasonable and may be adopted for the present. Another line of evidence that Ca^{2+} is preferred at low c-values has been reported by Rossi et al.,[75] who showed that valinomycin increases Ca^{2+} uptake, and already presented above (p. 83) is the argument that valinomycin may decrease the c-value. Some general support for the conclusion that Ca^{2+} is preferred at low c-value can also be derived from the more recent work of Siliprandi and coworkers.[261,298] Those authors showed that diamide causes Mg^{2+} loss and Ca^{2+} uptake in rat liver mitochondria.[298] This effect of diamide and other thiol oxidizing agents (e.g., tellurite and selenite) in enhancing the deleterious action of Ca^{2+} on mitochondria is antagonized by a reduction of thiol groups (e.g., by dithioerythritol), which enhances the protective action of Mg^{2+} as well.[298] Since thiol oxidation, like respiration, removes electrons from the mitochondria, it may reasonably be expected to lower the c-value also. The effect of diamide suggests that Mg^{2+} is preferred at a high c-value. A23187, like diamide, causes release of Mg^{2+}[149] and would appear to act as an EWC, as already suggested (p. 83). This agrees with Siliprandi's conclusion that diamide and A23187 exhibit similar effects.[298]

Siliprandi et al.[299] showed additionally that diamide is antagonized by carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). If so, the primary action of this uncoupler on mitochondria would appear to be that of an agent increasing the c-value. This agrees with the suggestion (see p. 84 above) that DNP is an EDC, with the observation that both DNP and FCCP favor H^+ uptake at the expense of K^+,[300] and with the earlier observation that DNP and FCCP stop Ca^{2+} uptake.[295]

5. ATP and ADP. Wehrle and Pedersen[301] found that ATP greatly stimulates Ca^{2+} uptake by rat liver mitochondria. This makes ATP a c-value-reducing agent, or EWC, as would be expected from its ability to support K^+ accumulation in muscle.[101,102,158] That ATP can act as a c-value-reducing cardinal adsorbent is a matter of considerable significance. First, it would explain why extraneous ATP and respiration often exert similar effects. That these effects of ATP do not involve the hydrolysis of ATP can be deduced from the important work of Nakazawa et al.,[250] who showed that fetal liver mitochondria have no ATP and do not release H^+ on exposure to pulses of Ca^{2+}. But addition of ATP with Mg^{2+}, in the presence of both rotenone and oligomycin, brings about full liberation of H^+ during Ca^{2+} pulses as in mitochondria from the adult rat liver. This observation agrees with the thesis that H^+ is preferred at a high c-value and Ca^{2+} at a low c-value and that ATP lowers the c-value so that Ca^{2+} can more effectively displace H^+. That ATP, without undergoing hydrolysis, can maintain the selective accumulation of K^+ over Na^+ in whole living cells, of course, has been a main theme of the AI hypothesis from the first.[2,102,104,106]

Clearly the various mitochondria1 studies cited in the present paper have expanded our understanding of how ATP acts as a cardinal adsorbent, in accordance with the AI hypothesis. Let us, then, return now to the finding discussed earlier (p. 76): that ATP, in the presence of oligomycin, can alter the osmotic response of mitochondria to sucrose. This behavior, in terms of the AI hypothesis, strongly suggests that ATP causes long-range polarization of water — and with it, a decrease in the q-value of sucrose. Can this suggested ATP action then be connected to the c-value-lowering effect? Again, the answer is affirmative, in agreement with previously published extensions of the AI model. I suggested in 1964 and 1969 that the backbone CONH group tends to assume a-helical, nonwater-

polarizing conformations when the side chains are strongly electron-donating.[103,104] Decreasing c-value from the position of a properly located cardinal site for ATP may indeed be expected to be the condition for long-range polarization of water.

Concerning ADP, several sets of independent evidence suggest that it may be electron-donating and thus exert an effect opposite to that of ATP:

First, Azzone and Azzi[57] showed that swelling of mitochondria under anaerobic conditions was reversed by either ADP or DNP. Earlier (p. 84) the reason was presented for suggesting that DNP is an EDC.

Second, Rossi and Azzone[92] showed that upon the addition of rotenone, K^+ accumulated aerobically in mitochondria in the presence of valinomycin is slowly lost, and ADP accelerates this loss. Earlier (p. 83) valinomycin was shown to be an EWC.

Third, Crompton et al.[292] showed that ADP, like anaerobiosis, inhibits Mg^{2+} release. (These authors offered an interpretation different from the electron-donating effect proposed here, attributing their result indirectly to reduction of internal P_i.)

Fourth, Hoser et al.[302] found that the addition of ADP slowed Mg^{2+} release in a way similar to that brought about by nigericin. (These authors also explained this action as being due to internal P_i removal.)

If ATP and ADP indeed have opposite effects on the c-value, this would offer an interesting mechanism for the reversible biological work performance controlled by ATP.[2,106]

5. Synchronous Oscillatory Changes in Mitochondrial Swelling, Ion Uptake, and Other Properties

Since Lardy and Graven[m3] and Pressman[304] simultaneously reported damped oscillatory changes in mitochondria1 swelling and ion transport, there has been intense interest in the subject (for review, see ref. 304). To produce these oscillatory changes, the following factors, described in conventional terminology, are needed. (*1*) An energy source, which could be substrate plus O_2 or ATP. (2) An alkali-metal ion. **(3)** "Permeant anions," such as P_i or acetate. ***(4)*** An "ionophore" or other agent that can increase the permeability of the alkali-metal ion. (5) Suitable pH.

The oscillatory changes at issue occur in cycles lasting one to several minutes under the proper conditions, as demonstrated for example by Chance and Yoshioka[306] in pigeon heart mitochondria and apparently can go on indefinitely. The following synchronized changes have been demonstrated for each cycle, although not all in phase and not all in the same system:[305-308]

(*1*) Volume increase and decrease.
(2) Uptake of alkali-metal ion and its release.
(3) Release of H^+ and its reuptake.
(4) Uptake of permeant anion and its release.
(5) Oxidation of pyridine nucleotide followed by its reduction.
(6) Oxidation of cytochrome b followed by its reduction.

The most extensively studied systems are rat liver and pigeon heart mitochondria in media containing valinomycin and potassium phosphate.[305,306] Since it is now clear that valinomycin does *not* increase K^+ permeability (p. 39) and that both "permeant" and "impermeant" anions are actually permeant, interpretation of the oscillatory phenomenon according to the AI hypothesis is quite different.

Equation 2 describes the cooperative adsorption isotherm, which dictates that an all-or-none autocooperative transition occurs when the parameter ξ changes. Such change occurs in response either to a change in the ratio of two competing adsorbents or to a change in the intrinsic equilibrium constant $K^{\circ\circ}_{j\to i}$. That constant, in turn, is under the

control of cardinal adsorbents.[158] Ouabain at a concentration of 3.26×10^{-7} M is capable of converting frog muscle from an all K^+-adsorbing state to an all Na^+-adsorbing state, provided the existing ratio of medium K/Na is close to $1/K_{j-i}$.[206] If, on the other hand, either K or Na is too high, 3.26×10^{-7} M ouabain, or indeed any concentration of ouabain, would not be able to convert the cell from one state to the other.

Earlier (p. 83) it was suggested that the combination of oxidation of the respiratory chain plus valinomycin brings the c-value to a level that, if K^+ is present in high enough concentration in the external medium, will bring about a K+-for-H+ exchange. If the external K^+ is at a certain level but not too high, the K^+/H^+ exchange would be in a critical state; i.e., capable of changing from one state when K^+ is adsorbed to another state when H^+ is adsorbed, correlated with a cyclic change in the oxidation-reduction state of the mitochondria.

The observation that repeated oscillation also demands the presence of "permeant" anions suggests that the actual change involves not only K+-for-H+ exchange but also salt-linkage dissociation:

$$f^-H^+ + K^+ \rightleftharpoons f^-K^+ + H^+ \qquad (13)$$

$$f^-f^+ + K^+ + P_i \rightleftharpoons f^-K^+ + f^+P_i. \qquad (14)$$

Salt-linkage dissociation in turn allows swelling, which is, of course, the Type II swelling described earlier (p. 75).

In agreement with this interpretation, Chance and Yoshioka[306] have established that for continued oscillation (i.e., with minimal clamping factor), the external K^+/H^+ concentration ratio must fall within a narrow range (between 0.9×10^4 to 1.5×10^4) for the pigeon heart system in valinomycin-K+-containing solutions. A much higher ratio of alkali-metal ion/H^+—about 3×10^5—is required for rat liver mitochondria in the presence of various nonactin homologs.[307]

Significantly, it has been observed that valinomycin,[306,308] gramicidin,[307] EDTA,[309] monazomycin,[310] and Sr^{2+} [311] all serve the same role in promoting oscillatory responses. These results agree with the basic AI concept that reagents, alone or viaintermediates (e.g., Mg^{2+} vs. EDTA), act by shifting the c-value of the protein-water-ion systems.

The remarkable oscillatory phenomenon demonstrates nature's splendid coordination not only within each complex organelle but within the entire population of a complex mitochondrial suspension. Not only do the widely varied aspects of the protein-water-ion system undergo coordinate changes but these coordinated changes are perfectly synchronized in the time dimension also.

The AI hypothesis has long postulated similar oscillating processes to underlie transepithelial solute transport as well as ciliary beating and other cyclic changes, just as in the present paper it has postulated similar though probably much faster cyclic changes as the basis of oxidative phosphorylation. Carrying that line of reasoning a step further, I now suggest that such a cyclic event may underlie the accumulation of Ca^{2+} phosphate in the form of hydroxyapatite in mitochondria when they are "maximally loaded" with Ca^{2+}; i.e., in the presence of ATP.[270,293,294] The event may be described thus:

(*1*) Selective adsorption of Ca^{2+} on anionic sites displaces H^+ and, along with P_i dissociates salt-linkages (Eqs. 13, 14). The adsorptions are favored by the lowered c-value due to ATP adsorption on cardinal sites.

(2) Ca^{2+} adsorption activates ATPase activity, leading to ATP hydrolysis.

(*3*) With the opposing effect of ATP removed, water is depolarized and increase occurs in q-values of solutes within it.

(4) As a result, more Ca^{2+}, P_i, and ATP enter mitochondrial water.

(5) ATP becomes readsorbed, causing water polarization and decrease of q-value of Ca^{2+} and P_i.

(6) The decrease of q-value causes precipitation of calcium-phosphate.

(7) The decrease in c-value triggered by the readmitted or regenerated ATP causes the cycle to repeat itself.

A similar cyclic event may also be responsible for the precipitation of Ca^{2+} as oxalate in microsomal vesicles reported by Hasselbach and Makinose.[312]

6. Mitochondria1 Electrical Potential

According to a later version of the chemiosmotic hypothesis,[5] the electrical potential difference across the mitochondria1 inner membrane constitutes the bulk of the protomotive force that provides energy for ATP synthesis and other energy-requiring functions of mitochondria. In earlier portions of the present article, the evidence was broadly reviewed and the conclusion reached that the hypothesis is no longer tenable. Among the many reasons given was the crucial fact that the electriacl potential, as measured using the least disputed method (microelectrode recording), is far too small to match that minimally required by the chemiosmotic model. It was also shown that the method chosen by Mitchell and others to demonstrate a larger potential difference (measuring the K^+ gradient in the presence of valinomycin) rests upon the view that the simple Nernst equation describes the electrical potential. This view was shown to be incorrect.[88]

Moreover, it has been demonstrated that the steady-state level of K^+ accumulation in mitochondria in response to valinomycin is not a constant[88]—as would be required if valinomycin alone renders an impermeable membrane permeable to K^+—but varies quantitatively with the concentration of valinomycin.[89] This leaves little doubt that one cannot assess the potential by the K^+ concentration gradient. In contrast, as mentioned previously, dependence of the steady-state level of K^+ in mitochondria on valinomycin concentration agrees with the AI hypothesis, which interprets this effect on the same basis as it interprets 2,3-diphosphoglycerate concentration-dependent steady-state level of oxygen in hemoglobin.

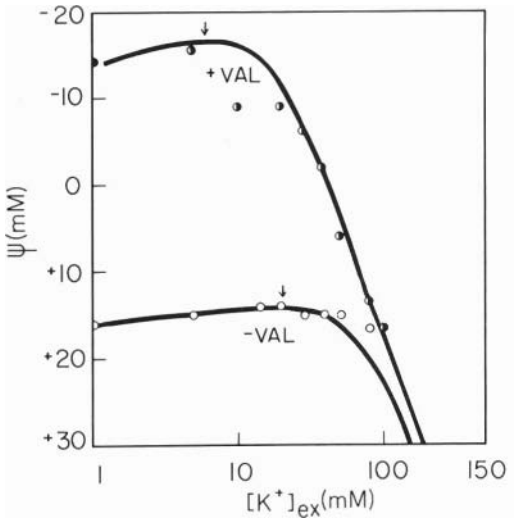


FIGURE 20. Resting electrical potential of isolated giant mitochondria from cuprazone-fed mice in the presence and absence of valinomycin. Solid lines are theoretically calculated according to Eq. 12 (see text). The intrinsic equilibrium constant for exchange of an unidentified cation (possibly H^+) and K^+ increased by a factor of 3.3 in response to valinomycin treatment. The nearest-neighbor interaction energy, $-\gamma/2$, remained at 0.201 Kcal/mole. The polarity of ψ is that used by Maloff, et al. ***Data points from Maloff, Scordilis, Reynolds, and Tedeschi,[42] by permission of J. Cell Biol.***

In intact frog muscle cells, decisive experimental evidence indicates that it is the surface anionic sites, their autocooperative ionic adsorption specificity, the concentration of external K^+ and other monovalent cations that determine the magnitude of the resting potential.[243-246] Bulk-phase selective adsorption of K^+ has only an indirect relation to the resting potential. Figure 20 demonstrates that the AI hypothesis can just as readily and qualitatively explain the electrical potential data from the giant mitochondria of cupra-

zone-treated mouse liver as measured by Maloff et al.[22] Here the experimental points were taken from Maloff et al. (Fig. 6); the solid lines are plots of the theoretical equation (Eq. 12) that describes the mitochondrial potential as a surface adsorption potential. Magnitude as well as polarity of the potential depends on the *c*-value of the anionic sites on a microscopic surface of the inner membrane, and there is autocooperative interaction between these anionic sites. The data are adequately explained by assuming that the outer surface of the inner membrane is covered primarily with anionic sites, which in the absence of valinomycin have relatively less affinity for K^+ when compared with an as yet undetermined cation, X^+. Addition of valinomycin, which acts as a cardinal adsorbent, changes the relative affinity of K^+ vs. X^+ by a factor of only 3.33; i.e., $K^{\circ\circ}_{X\to K}$ is increased by this factor. The nearest-neighbor interaction energy remains unchanged at 0.20 Kcal/mole.

What then is the competing cation, X^+? Without additional data, we cannot be certain. Maloff et al. used 1 mM 2(*N*-morpholino)ethane sulfonic acid (MES) (pK_a 6.15) as a buffer but did not specify what was used to bring the pH to 7.4. A reasonable assumption is that this was potassium hydroxide (KOH) and that the K^+ then introduced was added to K^+ introduced as methane sulfonate. If so, the X^+ could only be H^+. In that case $K^{\circ\circ}_{X^+\to K^+}$ would be $K^{\circ\circ}_{H^+\to K^+}$. The effect of valinomycin is to increase $K^{\circ\circ}_{H\to K}$ from 1.5×10^5 to 5.0×10^5. These are very large numbers but not beyond the range of expected selectivity when the anionic sites have a fairly high polarizability. Chance and Yoshioka[306] found that an external K^+/H^+ concentration ratio of 1.2×10^4 is required to maintain oscillations with minimal damping in valinomycin-treated pigeon heart mitochondria. Graven et al.,[307] on the other hand, found that in nonactin-treated rat liver mitochondria a ratio of alkali-metal cation/H^+ of 3×10^5 was required. It was pointed out earlier that these ratios may be expected to be close to the $K^{\circ\circ}_{H^+\to K^+}$ values.

In frog muscle and other living cells, the resting potential is accurately described also by Eq. 12, with or without externally added cardinal adsorbents. Thus ouabain at 3×10^{-7} M has the effect of decreasing $K^{\circ\circ}_{Na^+\to K^+}$ by a factor of about 10. The most remarkable aspect of the effect of valinomycin on the mitochondria1 electrical potential is that this agent increases $K^{\circ\circ}_{X^+\to K^+}$. Thus in a limited way valinomycin seems to function like ATP.

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