AN ELECTRONIC MECHANISM IN THE ACTION OF DRUGS, ATP, TRANSMITTERS AND OTHER CARDINAL ADSORBENTS. II. EFFECT OF OUABAIN ON THE RELATIVE AFFINITIES FOR Li^+, Na^+, K^+, AND Rb^+ OF SURFACE ANIONIC SITES THAT MEDIATE THE ENTRY OF Cs^+ INTO FROG OVARIAN EGGS

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• *Ouabain enhanced the inhibitory effects of Li^+, Na^+, and K^+ on the rate of Cs'permeation into frog ovarian eggs while it reduced the inhibiting effect of Rb^+. The data agree with earlier demonstrated effects of ouabain on the rank order of selective accumulation of the Jive alkali-metals in frog muscles and on the relative effectiveness of glycine, Li^+, Na^+, K^+, Rb^+, and Cs^+ in inhibiting the rate of entry of Cs^+ into frog sartorius muscle. In all three cases, the ouabain behaved as an electron-donating cardinal adsorbent (EDC) causing a rise of the electron density (c-value) of the β- and y-carboxyl groups in the cell cytoplasm (for selective accumulation) and on the cell surface (for selective ion permeation). Explanations based on the association-induction hypothesis were offered why an EDC like ouabain does not initiate cell activation (like veratridine does) and why Ca^{++} and tetradotoxin delays or inhibits physiological and artificial cell activation.*

In 1977 Neumann and Bernhardt pointed out "the rapid and transient permeability changes in excitable membrane appear to reflect special cases of a more general dissipative principle that is based on activator-receptor interaction" and *"the major unsolved problem of bioelectricity is the molecular mechanism underlying the permeability changes in excitable biomembranes"* (Neumann and Bernhardt, 1977, p. 118). Four years later, Hucho in his review on acetylcholine receptor studies pointed out that there was no answer yet to the question, "How does the binding of an agonist bring about an increase of K^+ and Na^+ permeability?" (Hucho, 1981). In 1984 Walker, Richardson and McNamee voiced a similar opinion, emphasizing the still unresolved problem mentioned by Neumann, Bernhardt, and Hucho years earlier. This seems strange since a hypothesis to explain the agonist-binding induced membrane permeability change has been already in existence for a quarter of a century (Ling, 1960, 1962) and its key postulates confirmed in years following (see below, also Ling, 1982, 1984). A question arises: "Why are these reviewers apparently unaware of these developments which are directly relevant to their questions?" It appears that there may have been a breakdown in communication between the scientists who are proponents of the popular view and those offering a self-consistent alternative (see Hazlewood, 1979; Catchpole, 1981).

Another perplexing consideration is that in 1962 Ling presented detailed evidence that the prevailing popular theory of the living cell (the membrane-pump theory) taught as fact in most textbooks, is in fact untenable. Out of the endless number of membrane pumps required by the theory (at the plasma membrane and membranes of subcellular particles), one pump alone would consume at least 15 to 30 times as much as the total

energy the cell commands (Ling, 1962, 1965, 1984). Even this set of figures is a gross underestimation of the disparity between maximally available energy and minimally required energy for the Na pump, because in the computation, it was assumed that ATP and creatine phosphate (CrP) contain large qualitatives of utilizable free energy in their phosphate bonds. Yet, we now know that no such utilizable free energy exists in these phosphate bonds (see George and Rutman, 1960, for the definitive review on the subject). In the 25 years following Ling's 1962 publication, no one has published serious challenges of the experimental findings nor his conclusions. However, three remedial postulations have been made (and described below) in order to keep the Na pump concept afloat. Each, in turn, has been shown to be contradicted by experimental facts.

a. *Ussing's* exchange diffusion: mandatory Na^+ for Na^+ exchange to minimize energy need for pump disproved by demonstration of rapid Na^+ efflux in a Na^+-free environment. (Hodgkin and Keynes, 1955; Buck and Goodford, 1966; Ling and Ferguson, 1970; Hoffman and Kregenow, 1966).

b. Sequestration of cell Na^+ in SR to reduce concentration of Na^+ in cells: disproven by demonstration that most Na^+ is evenly distributed in cells (Ling and Walton, 1975; Somlyo et al., 1977).

c. Nonenergy-consuming Na Pump (Glynn, 1977): In this remedial hypothesis, intended to reduce the energy need of the Na pump, it was postulated that the inward diffusing Na^+ into the cells also goes through the postulated Na^+ pump and in that process turns the "engine" backward, generating ATP which is then used to pump the Na^+ out, maintaining its steady low level. It was argued that only net extrusion of Na^+ requires metabolic energy. Since the resting cell does not gain or lose Na^+, there is no net transport of Na^+. The pumping of Na^+ in resting cells therefore requires no energy expenditure. The foundation of this speculation (see Glynn and Lew, 1970) has long been shown to be questionable. Thus, after much effort intended to demonstrate the conversion of energy obtained by dissipating ion concentration gradients into ATP, within a short period of three years the decisive work of Kanazawa (1972), Boyer et al. (1972), Masuda and de Meis (1973), Taniguchi and Post (1975), and Knowles and Racker (1975) unanimously established that it was not the presence of an ion gradient that generates the ATP (for details, see Ling, 1984, p. 514). These findings removed the experimental foundation on which Glynn's nonenergy-consuming pump could be based. However, to make certain that this remedial hypothesis is not slighted unfairly, I consider some other relevant experimental facts.

If a large share of inward Na^+ movement is via the Na pump, then exposure of the cell to the specific Na pump inhibitor, ouabain (a concept widely believed among proponents of the Na pump concept) should have arrested or at least slowed down the inward Na^+ flux. In fact, ouabain has no influence on inward Na^+ flux rate (Horowicz and Gerber, 1965; Ling and Ochsenfeld, unpublished).

If outward Na^+ flux depends on inward Na^+ flux, then removal of the outside high-inside low Na^+ gradient by replacing the external NaCl with isotonic sucrose or other non-Na chloride salts should have reduced the rate of Na^+ efflux. In fact, as shown under a. the reduction of external Na^+ concentration, has no retarding effect on the rate of Na^+ efflux (Hodgkin and Keynes, 1955; Buck and Goodford, 1966; Ling and Ferguson, 1970).

In 1962 Ling formally introduced a new theory of the living cell, the association-induction (AI) hypothesis. World-wide testing and confirmation of the basic concepts and predicions of the AI hypothesis have been completed in the 25 years following.

This work in aggregate, perhaps overlooked, forms the essence of a revolution in cell physiology. A text presenting the details will appear bearing the title, "A Revolution in Physiology of the Living Cell; Its Completion and Beyond" (Ling, to be published.)

In general one may say that it is the testing and confirmation of ***association*** aspects of the AI hypothesis that have been completed. The induction or control aspect of the AI hypothesis is still in its early stage of testing and development. Indeed, the present communication presents results of efforts in this direction. As background, we may briefly mention that in the AI hypothesis, drugs, transmitters, and many other "cardinal adsorbent~" by their adsorption or desorption trigger the protein-water-ion system from its high (negative) energy resting state to a different and discrete autocooperative low (negative) energy state often seen as the active state of that physiological function. Cardinal adsorbent~(CA) fall into three categories: electron-withdrawing cardinal adsorbents (EWC); electron donating cardinal adsorbents (EDC); and electron-indifferent cardinal adsorbents (EIC). The specificity of a drug is, as pointed out by Paul Ehrlich, primarily in the specific structure and electronic characteristics of the receptor sites. Among living matter with similar receptor sites, diverse physiological manifestations may be created in response to a specific drug or CA due to the location of the protein-water-ion involved.

With the brief sketch in the background, we shall discuss the specific physiological function to be investigated: membrane permeability to ions according to the AI hypothesis.

It is now established that the rates of entry of K', Rb^+, and Cs' into frog voluntary muscle and various other living plant and animal cells follow "Michaelis-Menton" kinetics, showing both saturability and competition (Epstein and Hagen, 1952; Ling, 1953, 1955, 1962, 1984). These traits indicate that during their entry into the cell, these ions do not diffuse freely in an isotropic and homogeneous medium but must first combine with a limited number of surface sites or carriers.

There are serious theoretical reasons against the carrier concept (see Ling et al., 1974, p. 40). Hodgkin and Huxley (1952) rejected the carrier concept on the basis of failure to observe an anticipated electric response if the Na' movement during an action potential is mediated by carriers. Discovery of ion-specific antibiotics "ionophores" (e.g., valinomycin) led to extensive search for their natural counterparts but with little success. Miiller, who with Rudin introduced the black lipid membrane technique, dolefully remarked, "At lot of us have spent a wasted ten years or so trying to get these various materials into bilayers ..." (Miiller, 1975).

The most constant solid component in the cell membrane is protein and not lipids (Dewey and Baar, 1970). Very much simpler explanations were long ago offered for the Michaelis-Menton kinetics observed in ion permeability into living cells. Indeed studies of various inanimate models including ion exchange resin sheets, sheep's wool, isolated actomyosin gel, etc., soon led to the recognition that the mere possession of fixed anions at the surface of a solid or gel is enough to give rise to similar Michaelis-Menton type of kinetics in the entry of cations into the systems as observed in living cells (Ling, 1960, 1962).

In the case of frog muscle, pH titration of the inward K^+ flux rate into resting cells established the anionic groups at the surface as β- and γ-carboxyl groups with a pK_a value of 4.75 (Ling and Ochsenfeld, 1965) providing strong supportive evidence for one of the earliest predictions of the AI hypothesis (Ling, 1952, 1953).

From 1955 to 1960, Ling made two other suggestions which are also inconsistent with conventional beliefs, but are the logical

followups of the earlier concepts that selective accumulation of K' over Na' is the result of preferential adsorption of K' over Na^+ on the β- and y-carboxyl groups of cytoplasmic proteins (Ling, 1984): (1) the resting potential is not a membrane potential but a surface adsorption potential created by the presence on the cell surface of K' preferring fixed β- and y-carboxyl groups (Ling, 1955, 1959, 1960); (2) changes in the electron density or c-value* of the β- and y-carboxyl groups during an action potential converts these fixed anionic groups from preferring K^+ over Na^+ to preferring Na^+ over K^+ (Ling, 1957, 1960) giving rise to the "overshoot" of an action potential.

The basis for this contention is the result of a set of theoretical computations, the results of which were published first in 1960 and then in detail in 1962 (Ling, 1962) and illustrated in Figure 1. Note that in comparison with K', the relative affinities of Cs', Rb^+, NH_4^+, and H' all change with changes of the c-value. The most notable is the change of the relative affinity of Na^+ (K_{Na^+}/K_{K^+}) which swings from very low value at low c-value to very high value at high c-value. Thus with c-value increase of the surface β- and y-carboxyl groups, the cell will shift from one behaving like a K^+ electrode to that behaving like a Na^+ electrode. In the course of the next 15 years these predictions have been experimentally confirmed as follows:

As mentioned above Ling and Ochsenfeld in 1965 first demonstrated that the membrane carboxyl groups of resting frog muscle mediating K^+ traffic has a pK_a of 4.6 to 4.7. The alkali-metal ion adsorption strength follows the rank order $Rb^+ > Cs^+ >$ K' $> Na^+$, which corresponds roughly to a c-value of –4.5 Å (Figure 1). The anionic sites mediating the inward Na^+ current during an action potential have a higher pK_a. In squid axon it was given as 5.2 by Hille (1968, 1975) and 6.5 by Stillman et al. (1971). The rank order of relative permeability is $Li^+ > Na^+ > K^+ > Rb^+ >$ Cs' (Chandler and Meves, 1965), suggesting that the anionic sites during activation have risen to a much higher c-value (ca. –2.3 Å).

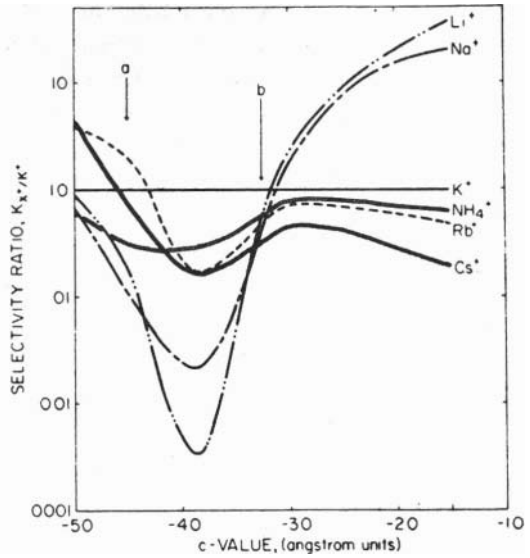

FIGURE 1. The relation between the selectivity ratios of various cations and the c-value. The K^+ ion is taken as unity and selectivity ratios are calculated from the association energies (Polarizability of anionic group: 2.0 × 10^{-24} cm^3) divided by 2. (Ling and Bohr, 1971).

*The definition of c-value: It is well known that acetic acid, a weak acid (pK_a = 4.65), becomes a very strong acid (pK_a < 1.0) when the H atoms on its methyl group are replaced by the more electronegative (with greater power to draw electrons toward itself) chlorine atoms. This is a classical example of the inductive effect. The pK_a's measure a specific interaction (i.e., with H^+). An independent parameter, called c-value was introduced. Beginning with a singly charged oxygen atom with the unit charge located at the center of the atom, the total inductive effect of whatever complex molecule on which the singly charged oxygen atom is borne, can be simulated by a displacement of the unit negative charge either toward or away from the interacting cation (e.g., H^+, K^+, Na^+). The displacement in Angstrom units is called the c-value. Thus a high c-value signifies a higher electron density and would correspond to a high pK_a such as in the case of acetic acid. A low c-value signifies a lower electron density and corresponding to a low pK_a such as in the case of trichloroacetic acid. (A more rigorous and complete definition is given in Ling, 1962, p. 59; see also Ling, 1984, p. 156.)

These experimental confirmations of a key prediction of the AI hypothesis presented long before the experimental studies were undertaken (Ling, 1960, 1962) was a major event especially when it concerns an issue of central importance to cell physiology. Failure to recognize this prediction of the AI hypothesis and its experimental confirmation has led to some misunderstanding. Thus 16 years after Ling first introduced cell surface carboxyl groups as serving the role of selective mechanism in K', Na' permeability and eight years after Ling's introduction of the quantitative theory of c-value (pK_a value) vs. alkali-metal ion selectivity, Hille installed "selectivity filters" in the form of high pK_a-value (i.e., high c-value) carboxyl group in the "Na channel" which is discrete and physically separate from "K channel" equipped with a low pK_a-value (i.e., low c-value) carboxyl groups*. (Hille, 1968, 1975).

The failure to recognize Ling's earlier findings notwithstanding, a key prediction of the AI hypothesis has been confirmed: the β- and γ-carboxyl groups mediating ion permeation at rest and during activation do indeed have different c-values. Our next objective is to look for evidence to decide whether carboxyl groups with high c-values that mediate the Na^+ current and the carboxyl groups of low c-value that mediate K' traffic are in fact different electronic states of the same carboxyl groups according to the AI hypothesis or are permanently and unchangingly different ones?

In fact, experimental evidence also exist that give support to the concept of changing c-value of the surface carboxyl groups. Thus Khodorov found that ion permeability of the open Na channel of the Ranvier nodes of frog nerve changes from $Na^+ > Li' > Tl^+ > NH_4'$ > guanidine > K' to $Tl^+ > NH_4^+ > Na'$, Li' > K' > guanidine > Rb' > Cs' in response to the South American frog poison betrachotoxin. A similar report was earlier made by Mozhayeva et al. (1977) on frog Ranview nodes after exposure to the alkaloid, aconitine. That the same "channel" can change its ionic specificity in response to the cardinal adsorbent, betrachotoxin was soon confirmed by Huang et al. (1979) on cultured mouse neuroblastoma cells.

Again, the relation of this observation to the AI model was not recognized. Nevertheless, the finding of Khodorov, Mozhayeva et al., and Huang et al. had clearly established another major concept of the AI hypothesis, i.e., a cardinal adsorbent may alter the specificity of the same opened surface carboxyl groups mediating cation entry. In terms of the AI hypothesis this change could only be due to a further modulation of the c-value which has already risen by the normal activation process to a very high value (e.g., –2.3 Å, see below). Judging from the large increase of the relative permeability of NH_4^+, Rb', Cs^+, and K' the change brought about by betrachotoxin on the active Na channel might very well involve both a decrease of c-value of the β- and γ-carboxyl groups concerned and a concomitant further depolarization of the polarized water (for evidence of water depolarization at the active membrane site, see Ling, 1984, p. 493; 1988, Figure 112).

With two of the major predictions of the AI hypothesis of action potential and underlying molecular mechanism confirmed, one by Hille (1968) and the other by Mozhayeva, Khodorov, Huang, and others, we are further testing the broader generalization of drug induced changes of the c-value of β- and γ-carboxyl groups by extending the study of the effect of ouabain on the binding energies of five alkali-metal ions in another type of living cell — frog ovarian eggs. We are

***No reference was made to our earlier work even though the first theory that protein carboxyl groups in cell membranes provide a mechanism for selective ionic permeability (Ling, 1952, 1953) and the first *quantitative* theory showing that carboxyl groups with high pK_a do prefer Na^+ over K^+ while low pK_a-value carboxyl groups prefer K^+ over Na^+ were both presented many years before (Ling, 1957, 1960, 1962, 1965).**

testing the prediction that the cell surface of the frog oocyte contains also β- and y-carboxyls under the control of cardinal sites. And if so, we would also like to know if in this case ouabain also acts as an electron donating cardinal adsorbent or EDC as we have already found from the earlier studies of the bulk phase β- and y-carboxyl groups on the edge of the A bands and Z-line (Ling and Bohr, 1971) as well as on the cell surface β- and y-carboxyl groups of frog voluntary muscle (Ling and Fu, 1987).

MATERIALS AND METHODS

All chemicals used were of C.P. grade. Ouabain was obtained from Sigma Chemical Co., St. Louis, MO (Cat. # 03125, Lot # L5F-0551). All experiments were performed on clusters of isolated mature ovarian eggs of leopard frogs (Rana pipiens pipiens, Schreber).

1. Preincubation — As a rule a cluster of eggs (and a piece of loose connective tissue from the same ovarian region of the animal) were isolated on the date of the experiment and preincubated in a 250 cc of "731 culture fluid" (see Ling and Bohr, 1969 for composition) with or without 10^{-6} M ouabain for 4½ hours. The flasks were kept in a constant temperature room (25° C) and shaken gently on a rotary shaker at the rate of 100 rpm.

2. Incubation — Eggs or connective tissue were taken out of the preincubation solutions, blotted on filter paper dampened with preincubation solution, and then placed in 1 ml of incubation solution containing 2.0 mM tagged CsCl (for composition see Table I) and 20 mM of competing alkali-metal ions for 13 min. at 25° C shaken at the rate of 100 rpm.

3. Soaking, weighing and counting — The eggs or connective tissues were removed from the incubation solution, blotted dry, and placed in 1.1 cc of washing solution of the same composition as that of the preincubation solution. Consecutively, the egg was transferred to a different vial after washing for an increasing length of time: 3, 10, 20, 30, 50, 80, 120, 300, 450 seconds. After the last washing the cluster of eggs (or connective tissue) was introduced into a counting tube containing 1 cc of 0.1 N HCl, and the radioactivity assayed on a y-counter. 1.0 ml of the tagged "soaking" solution was also counted.

4. Methods of isolating the connective tissue and estimation of the percentage of connective tissues in the egg clusters — Each grape-like egg cluster of 10 to 12 eggs was first weighed before they were crushed between wet filter paper sheets followed by rinsing in normal Ringer phosphate solution. The process was repeated a number of times until the

TABLE I. Composition of Incubation Solution

Stock Solution		NaCl (0.118M)	$CaCl_2$ (0.0845M)	Tris‡ (0.118M)	XCl‡‡‡ (0.118M)	Cs*Cl (0.118M)	Sorbital (0.118M)	NRP‡‡	Ouabain (10^{-4} M) in NRP‡‡
Volume added (ml)	Control	2.5	0.125	I	10	1.0	43.78	0.59	—
	Expt.	2.5	0.125	1	10	1.0	43.78	—	0.59
Final Concentration (mM)	Control	5.0	0.18	2.0	20	2.0	175		none
	Expt.	5.0	0.18	2.0	20	2.0	175		10^{-6}

‡ Adjust the pH of incubation solution to 7.4 using 0.118 M Tris Base and 0.118 M Tris HCl.
‡‡ NRP, normal Ringer phosphate solution (for composition, see text).
‡‡‡ XCl refers to LiCl, NaCl, KCl, or RbCl.

dark egg material was all removed. After a final blotting the connective tissues thus isolated were weighed. Table II shows 12 sets of data thus obtained, yielding an average connective tissue content of 2.18% ± 0.34% in the egg clusters. Similarly isolated connective tissues were also used for efflux studies to provide the data for correcting the contribution of the connective tissues to the efflux curves of the egg clusters which contain both connective tissues and eggs.

5. Correction for the connective tissue contribution to the ion efflux — From the amount of labeled Cs' remaining in the egg cells at the end of washing and the total radioactivity in each of the washing solutions in different tubes, one can reconstruct the time course of labeled Cs' efflux and plot semilogarithmically. A similar curve obtained from similarly treated connective tissues from the same frog ovary is then subtracted point-by-point from the egg curve on the basis that the isolated egg contains 2.18% of its fresh weight in the form of fresh connective tissue and the resultant concentrations then divided by (1 — 0.022) = 0.978 to yield the concentration of labeled Cs' per gram of egg cells.

TABLE II. Weight percentage of "connective tissues" in ovarian egg clusters. After the egg clusters had been weighed, the connective tissue in the cluster was isolated by crushing the egg cluster between wetted filter paper and repeated blotting and rinsing in Ringer's solution.

Egg Cluster No.	Weight of Eggs and Connective Tissue	Weight of Connective Tissue	Percentage of Connective Tissue Weight (%)
1	2.0470	0.0320	1.56
2	1.2070	0.0222	1.84
3	1.2253	0.0266	2.17
4	1.2351	0.0336	2.72
5	1.1714	0.0231	1.97
6	1.2635	0.0303	2.39
7	1.0030	0.0242	2.41
8	0.8766	0.0209	2.38
Mean ± S.D.			2.12 ± 0.34

6. Separation of three fractions of effluxing labeled Cs' — After correcting for the connective tissue contribution the data on semilogarithmic scale are resolved into individual logarithmic fractions by the method of successive peeling (Ling, 1962, p. 327; Ling, 1980).

DATA ANALYSIS

From past studies, permeation of alkali-metal ions like K^+, Rb^+, and Cs' into many cell types is primarily via the "adsorption-desorption route" (Ling, 1962, p. 297). The rate of Cs' permeation in the presence of Rb' is therefore described by the following equation:

$$V_{Cs} = \frac{V_{Cs}^{max} \tilde{K}_{Cs} [Cs^+]_{ex}}{1 + \tilde{K}_{Cs} [Cs^+]_{ex} + \tilde{K}_{Rb} [Rb^+]_{ex}}, \quad (1)$$

where V_{Cs} is the rate of permeation of labeled Cs^+. $\tilde{K}_{Cs}$, $\tilde{K}_{Rb}$ are respectively the adsorption constants of Cs^+ and Rb^+ on the surface anionic sites. $[Cs^+]_{ex}$ and $[Rb^+]_{ex}$ are the external Cs' and Rb' concentration respectively. V_{Cs}^{max} is the maximum rate of Cs' entry when $[Cs^+]_{ex}$ approaches infinity (Ling, 1962; Ling and Ochsenfeld, 1965).

When the concentration of labeled Cs' ion (2 mM) under study is lower than the competing ion, Rb' (20 mM), one may, as an approximation, assume $\tilde{K}_{Rb} [Rb^+]_{ex} \gg 1 + \tilde{K}_{Cs} [Cs^+]_{ex}$. In that case,

$$V_{Cs} \doteq \frac{V_{Cs}^{max} \tilde{K}_{Cs} [Cs^+]_{ex}}{\tilde{K}_{Rb} [Rb^+]_{ex}} \quad (2)$$

Define $\rho_{Rb} = \tilde{K}_{Rb}/\tilde{K}_{Cs}$; $\rho_K = \tilde{K}_K/\tilde{K}_{Cs}$; $\rho_{Na} = \tilde{K}_{Na}/\tilde{K}_{Cs}$; $\rho_{Li} = \tilde{K}_{Li}/\tilde{K}_{Cs}$; for normal eggs and ρ_{Rb}', ρ_K', ρ_{Na}', and ρ_{Li}' for eggs exposed to ouabain, and let V_{Cs}' (and $V_{Cs}^{max\prime}$) be the rate of Cs' permeation into frog eggs exposed to ouabain and V_{Cs} (and V_{Cs}^{max}) be the rate of permeation into control untreated eggs. In the preceding paper of this series, we have

shown that the maximum rate of Cs' entry into frog sartorius muscle remained the same in the presence and absence of ouabain, i.e., $V_{Cs}^{max} = V_{Cs}^{max\prime}$ (Ling and Hu, 1987, Figure 2). Assuming that this equality holds also for frog ouabain eggs as well, one can then derive the following approximate equation:

$$\frac{V_{Cs}' - V_{Cs}}{V_{Cs}} = \frac{\rho_{Rb} - \rho_{Rb}'}{\rho_{Rb}'}. \qquad (3)$$

RESULTS

We studied the efflux of labeled Cs' from mature frog ovarian eggs. The control clusters of 9 to 12 eggs had been incubated for 4% hours at 25°C in a preincubation Ringer solution without ouabain and then exposed for 13 minutes to an incubation solution containing 20 mM LiCl and 2 mM ^{134}Cs labeled Cs'. The results are shown as data points of the upper curve in Figure 2. The curved line labeled A immediately below these data points is the efflux curve after correction has been made for the contribution of connective tissues, the efflux curves of which is shown as Curve B. This and other similar corrections were made on the basis that connective tissue makes up 2.2% of the wet weight of the egg clusters (Table II). Note that labeled Cs^+ concentration in the corrected curve A should be divided by (I — 0.978) to yield the correct value in units of μmoles per gram of egg cells.

Figure 3 represents a similar set of curves before and after corrections for the connective tissue contribution, only the preincubation and the incubation solution of the eggs (as well as the connective tissues) contained beside 2 mM labeled Cs', and 20 mM LiCl,

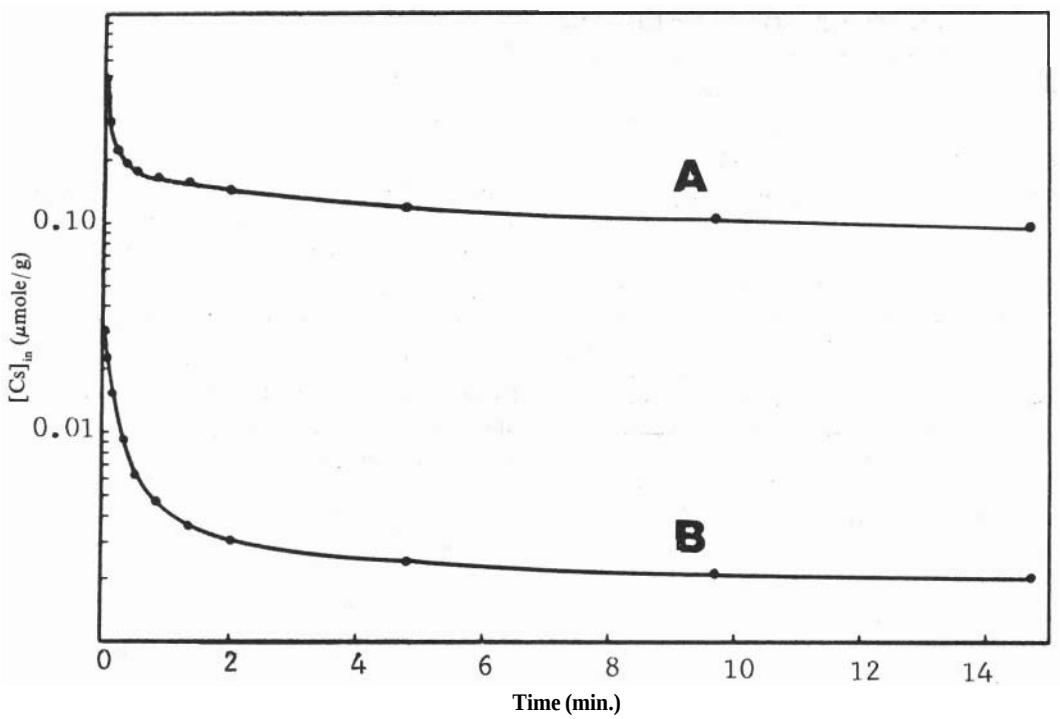

FIGURE 2 **Labeled Cs^+ efflux curves of control frog ovarian eggs and control tissues in the presence of 20 mM LiCl. Curve A represents efflux curve for the ovarian eggs after correction for the contribution from the connective tissue in the egg cluster, estimated at 2.2%. Individual points are original data points. Curve B is the efflux curve of the connective tissue.**

also 10^{-6} M ouabain. In other data to be described below, the connective tissue efflux curves are not explicitly presented. Only the experimental data point and the correlated efflux curves are shown.

Figures 4 to 6 are Cs^+ efflux curves from egg clusters treated in a similar way as that in Figures 2 and 3, except that the egg clusters were incubated for 13 minutes in Ringer solution containing labeled Cs' (2 mM) and 20 mM of NaCl, KCl, or RbCl respectively instead of 20 mM of LiCl as in the experiment described in Figure 2. The curves with solid circles are from ouabain-treated eggs and the curves with empty circles are the controls. Quantitatively, LiCl and KC1 at 20 mM both became more effective in reducing labeled Cs^+ entry into the eggs after ouabain treatment. In contrast, ouabain lowered the effectiveness of Rb' in reducing the rate of Cs^+ entry into the ovarian eggs (Figure 6).

None of the four sets of Cs^+ efflux curves (both control and experimental) represents simple straight lines. Each can be resolved into three different fractions as illustrated in Figure 7. Following past practices, the slowest fraction is named Fraction I, the next slowest Fraction II, and the fastest Fraction III (see Ling, 1962). We then extrapolated each fraction to zero time to obtain the initial concentration of labeled Cs' in each fraction and the data obtained converted to the rate of entry of that fraction in units of moles of labeled Cs^+ per min. per gram of fresh cells. Since most of the eggs used were 1.5 to 2.0 mm in diameter, the surface/volume ratio is usually in the range of 30 to 40 cm^{-1} (Ling and Ochsenfeld, 1986). This value can be used to

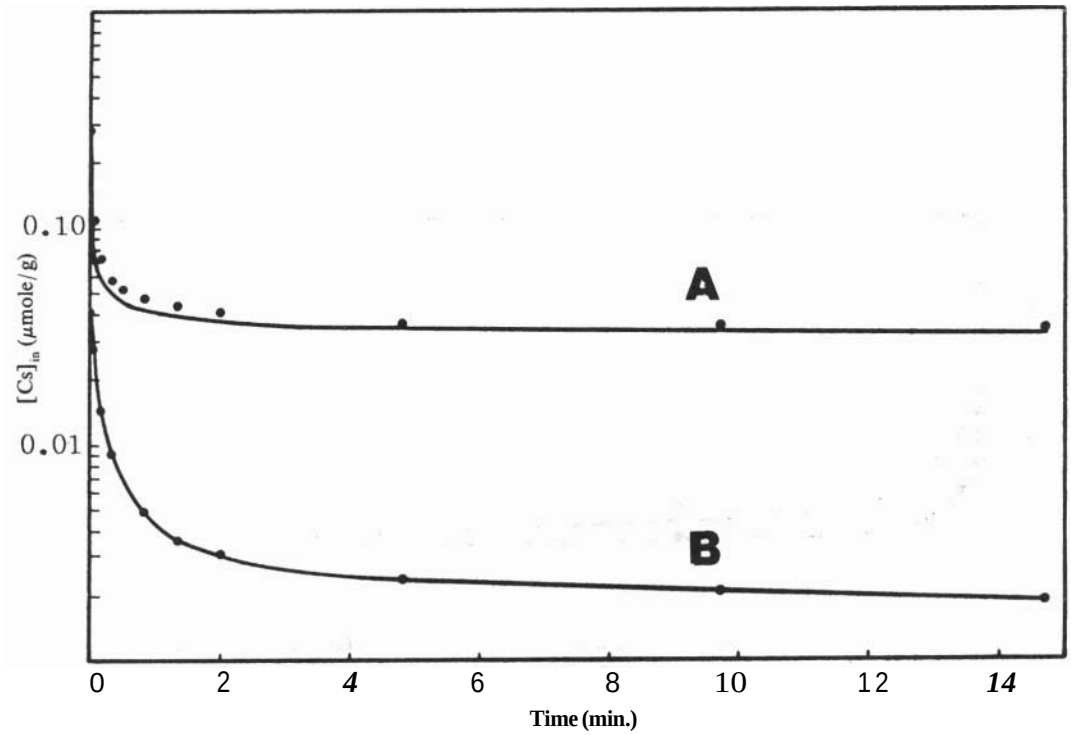

FIGURE 3. Labeled Cs' efflux curves of ouabain-treated frog ovarian eggs and connective tissue similarly treated with ouabain, both in the presence of 20 mM LiCl. Curve A represents efflux curve from the ovarian eggs after correction for the contribution from the connective tissue in the egg cluster. Curve B is the efflux curve of the connective tissue.

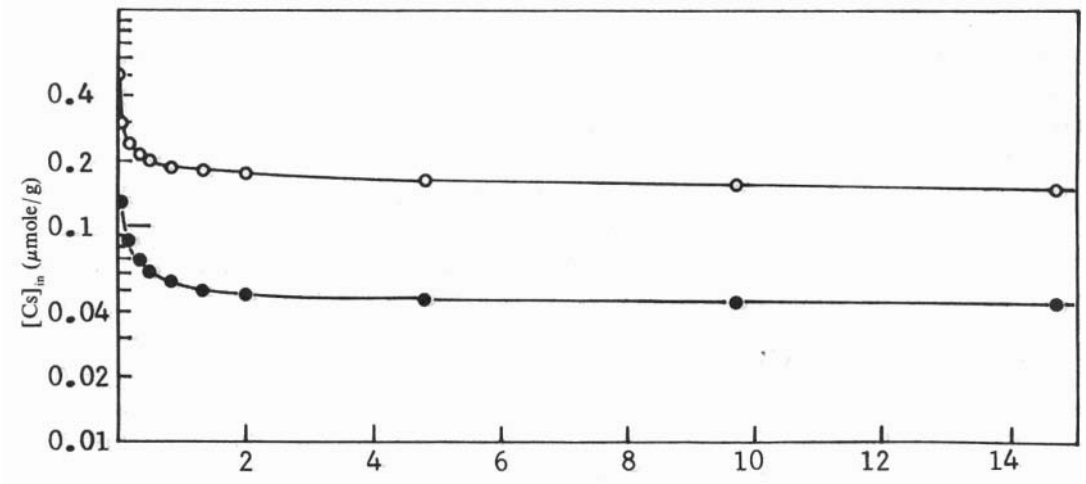

FIGURE 4. Efflux curve of labeled Cs^+ from frog ovarian eggs. Egg clusters were preincubated 4.5 hrs. and incubated 13 mins, in a Ringer's solution containing 2 mM labeled Cs^+ and 20 mM NaCl. Control eggs (—o—o—) were preincubated and incubated in the same manner as the experimental eggs (—●—●—) except that the preincubation and incubation solutions of the experimental eggs contained 10^{-6} M ouabain.

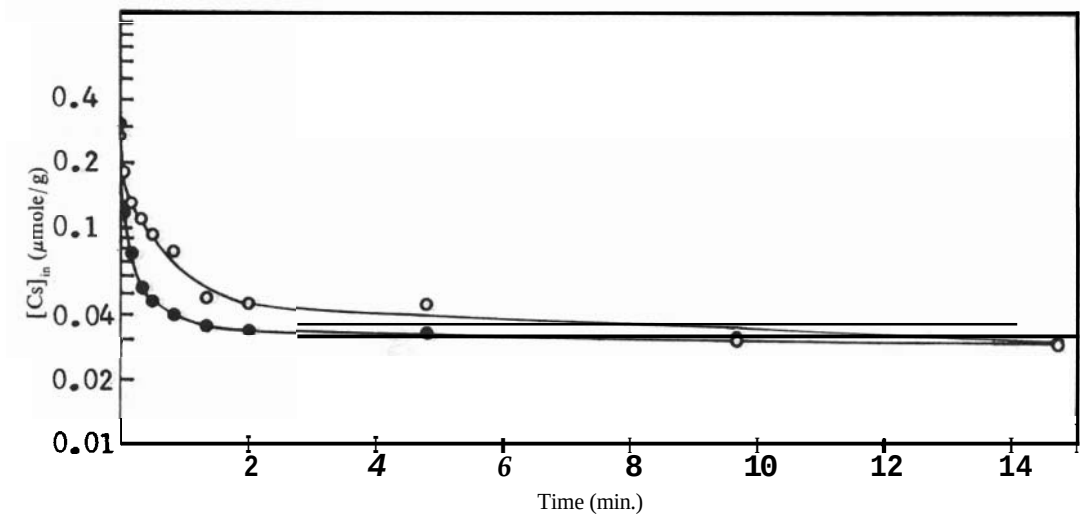

FIGURE 5. Efflux curve of labeled Cs^+ from frog ovarian eggs. Egg clusters were preincubated 4.5 hrs. and incubated 13 mins. in a Ringer's solution containing 2 mM labeled Cs^+ and 20 mM KCl. Control eggs (—o—o—) were preincubated and incubated in the same manner as the experimental eggs (—●—●—) except that the preincubation and incubation solutions of the experimental eggs contained 10^{-6} M ouabain.

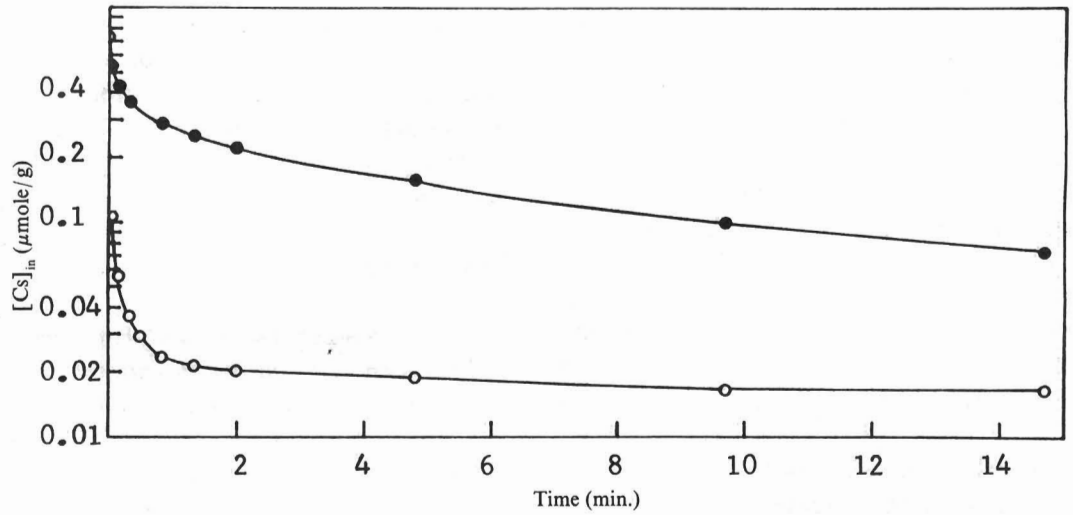

FIGURE 6. Efflux curve of labeled Cs^+ from frog ovarian eggs. Egg clusters were preincubated 4.5 hrs. and incubated 13 mins. in a Ringer's solution containing 2 mM labeled Cs^+ and 20 mM RbCl. Control eggs (—o—o—) were preincubated and incubated in the same manner as the experimental eggs (—●—●—) except that the preincubation and incubation solutions of the experimental eggs contained 10^{-6} M ouabain.

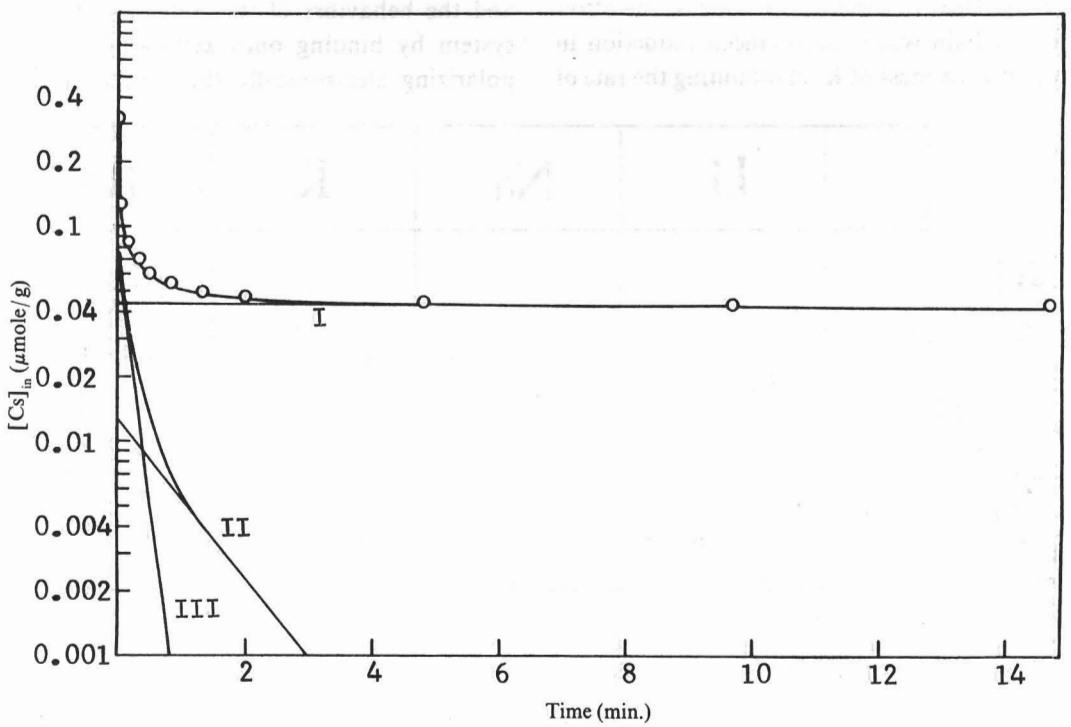

FIGURE 7. Efflux curve of labeled Cs^+ demonstrating the resolution of the efflux curves into three fractions I, II, III. Empty circles are experimental points. Solid curve line just under the experimental points was based on a smoothed line drawn through the points (not shown) from which correction for the connective tissue contribution (2.2%) was subtracted. Extrapolation of each resolved straight line to zero time yields the magnitude of each fraction after dividing by 0.98 to restore the concentration in unit cell weight.

convert the permeability into units of moles per min. per cm^2.

Figure 8 summarizes the results of the four sets of completed experiments. The data are presented as $(V_{Cs}' - V_{Cs})/V_{Cs}$, where V_{Cs} and V_{Cs}' are as defined above in the presence of 20 mM competing Li^+, Na^+, K^+, or Rb^+ respectively.

Ouabain treatment consistently increased the effectiveness of Li^+ in inhibiting Cs^+ entry in all four sets of experiments performed. Ouabain also increased in almost all cases the effectiveness of Na^+ in inhibiting the entry of Cs^+ belonging to Fractions I and II. In only one case was the effectiveness of Na^+ reduced as a result of ouabain treatment (Fraction III).

As far as K^+ is concerned, in three out of the four sets of data, ouabain enhanced its effectiveness in inhibiting the rate of Cs^+ entry. However in one set of eggs, the effect of ouabain was a pronounced reduction in the effectiveness of K^+ in inhibiting the rate of Cs^+ entry belonging to all three fractions.

Finally, all four sets of data obtained unanimously demonstrated a reduction of the effectiveness of Rb^+ in suppressing Cs^+ entry (all three fractions) in response to ouabain treatment.

DISCUSSION

Accord Between Theory and Experiment and Between Experiments on Different Target β- and γ-carboxyl Groups in Different Cell Types. A central theme of the AI hypothesis is that the living matter or protoplasm is primarily an autocooperatively associated system of water-protein-ions, maintained at a high energy living state. Specific part of the protein functions as cardinal (receptor) sites. Drugs, hormones, transmitters, Ca^{++}, ATP modify the energy states and other properties and the behaviors of the water-protein-ion system by binding onto key cardinal sites polarizing electronically the protein-water-

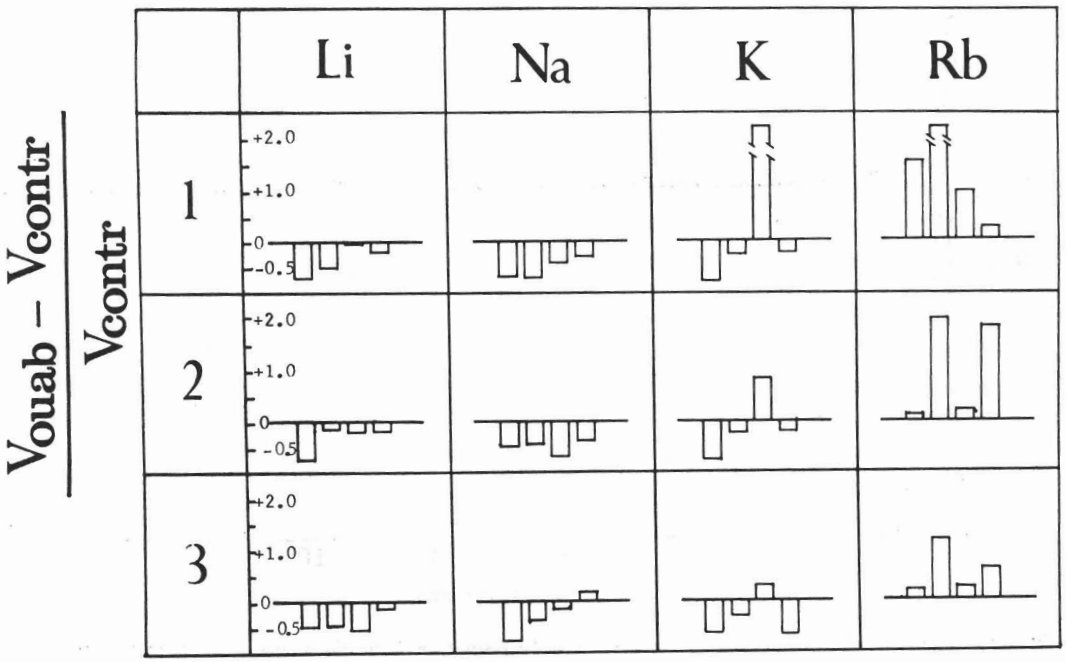

FIGURE 8. The effect of ouabain (10^{-6} M) on the rate of entry of labeled Cs^+ (2 mM) in the presence of 20 mM of competing Li^+, Na^+, K^+, or Rb^+. The *influx* rate during a 13 min. incubation of the egg clusters is determined from the zero time intercepts from the efflux curves and was resolved into three separate fractions, I, II, and III.

ion system. The physiological manifestation of the changes brought about by the cardinal adsorbent reflects the location of the water-protein-ion systems in the cells. Thus preferential adsorption of K^+ on the β- and γ-carboxyl groups in the bulk of cell substance creates the phenomenon of selective equilibrium ionic accumulation. A similar preferential adsorption of K^+ on the β- and γ-carboxyl groups on the cell surface gives rise at once to the creation of the resting potential and selective ionic permeability. The findings on the effect of ouabain on the permeability of frog ovarian eggs permit us to observe generalized response pattern, i.e., between a prototypic cell, the egg and a highly specialized cell, muscle. More specifically, we want to find out if ouabain produces similar effects on the underlying mechanisms in the equilibrium ion accumulation in frog muscle and on the permeability of these ions into frog ovarian eggs.

Figure 1 reproduces the theoretical curves relating the c-value of β- and γ-carboxyl groups to the adsorption energies of the five alkali-metal ions, NH_4^+, and H^+. As mentioned earlier Ling and Bohr compared the patterns of the relative adsorption energies of the five alkali-metal ions experimentally determined in normal frog muscle and that after exposure to ouabain, a tentative suggestion was made that in the resting muscle the cytoplasm β- and γ-carboxyl groups (now known to reside primarily on the edges of the A bands and the Z-line) have a c-value of −4.5 Å. After ouabain treatment, the c-value rose to a value of −3.95 Å. Ling and Bohr thus demonstrated that theoretical pattern changes agree with the observed equilibrium distribution patterns of the five alkali-metal ions in frog muscle in response to ouabain.

Column 2 of Table III displays values of $(\rho_X - \rho_X')/\rho_X'$ based on the *theoretical* data from Figure 1 with the c-values of the β- and γ-carboxyl groups of normal cells assumed to have a value of −4.50 Å and the corresponding c-value of ouabain-treated cells to have a value of −3.95 Å. Column 3 on the other hand, shows $(\rho_X - \rho_X')/\rho_X'$ obtained from the *experimental* equilibrium accumulation data of the absence of ouabain from Ling and Bohr, 1971. The last columns are $(\rho_X - \rho_X')/\rho_X'$ derived from the *experimental* permeability data from this study.

TABLE III. The relative adsorption constants for the alkali-metal ion, X, is given as $\rho_X = K_X/K_{Cs}$ when K_X and K_{Cs} are the adsorption constants in the control sites and $\rho_X' = K_X'/K_{Cs}'$ refer to ouabain-treated sites. $(\rho_X - \rho_X')/\rho_X'$ of muscle cytoplasm were from Ling and Bohr (1971, Figure 5). Theoretical $(\rho_X - \rho_X')/\rho_X'$ were also taken from the same article (Figure 4). $(\rho_X - \rho_X')/\rho_X'$ on frog egg surface sites were obtained from the $(V_X' - V_X)/V_X$ which equals $(\rho_X = \rho_X')/\rho_X'$ (see text). Theoretically predicted values correspond to a c-value of −4.50 Å for control sites and −3.95 Å for ouabain-dominated sites determined earlier (Ling and Bohr, 1971) and were assumed to be the same in the cytoplasmic sites in frog voluntary muscle and the cell surface sites of frog ovarian eggs.

Theoretical		Experimental			
		Muscle Cytoplasmic Sites	Eggs Surface Sites		
	$\frac{\rho_X - \rho_X'}{\rho_X'}$	$\frac{\rho_X - \rho_X'}{\rho_X'}$	$\frac{V_{Cs}' - V_{Cs}}{V_{Cs}} = \frac{\rho_X - \rho_X'}{\rho_X'}$		
			Fraction I	Fraction II	Fraction III
Rb^+	+1.86	+3.34	+2.78 ± 1.8	+1.04 ± 0.51	+0.56 ± 0.23
K^+	−0.32	+0.52	+0.52 ± 0.95	−0.098 ± 0.34	−0.32 ± 0.22
Na^+	−0.83	−0.97	−0.52 ± 0.11	−0.49 ± 0.07	−0.28 ± 0.20
Li^+	−0.79	−0.89	−0.36 ± 0.15	−0.31 ± 0.15	−0.40 ± 0.09

In all three sets of values, one from theory, two from experiments, the lowest $(\rho - \rho')/\rho'$ value is always when Na^+ is the inhibiting ion, and the highest $(\rho - \rho')/\rho'$ value is always when Rb^+ is the inhibiting ion. In all three, the $(\rho - \rho')/\rho'$ follows the order $Rb^+ > K^+ > Li^+ > Na^+$. With so many assumptions and simplifications involved, the general agreement between theory and two sets of experimental observations must be considered very satisfactory.

The accord between theory and the two sets of independently derived experimental data described in Table III offers significant affirmation of the theory derived on the basis of the central role of the inductive effect as the underlying molecular and electronic mechanism in the control of cell functions by drugs and other cardinal adsorbents.

Once more the present data shows that ouabain acts as an EDC. The general similarity in the extent of c-value change suggests that each cardinal adsorbent seems to have a well-defined effect on the c-value of distant groups regardless of where they are in the cell.

How Can So Few Control So Many? It is not yet possible to assess the number of β- and γ-carboxyl groups at the cell surface that mediate Cs^+ entry into frog muscle cells or ovarian eggs and therefore difficult to assess just how many β- and γ-carboxyl groups are under the control of the cardinal sites binding ouabain. However, it is quite easy to estimate how many β- and γ-carboxyl groups at the edges of A bands and the Z-lines of frog muscle cells that have switched their counterions from K^+ to Na^+ in response to ouabain at a low concentration. Thus in the experiments described by Ling and Bohr (1971) roughly 100 μmoles of adsorbed K^+ per gram of fresh muscle cells was displaced by an equal amount of adsorbed Na^+ in consequence of interaction of ouabain. Roughly speaking, each muscle weighing about 100 mg. had been incubated for 3 days at 25° C in 1 liter of "731 solution" containing initially 3.27×10^{-7} M ouabain (Ling and Bohr, 1969). Again as a first approximation, we can assume that all the ouabain initially introduced into the incubation solution became adsorbed in the cell. Then the total number of ouabain adsorbed on the cardinal sites would be equal to 3.27×10^{-7} moles. The total number of β- and γ-carboxyl groups that have switched their countercations are $10^{-4} \times 10^{-1} = 10^{-5}$ moles. Thus at least $10^{-5}/3.27 \times 10^{-7} = 31$ β- and γ-carboxyl groups were under the control of each cardinal site occupied by a ouabain molecule. In truth, only a part of the ouabain in the incubation solution could be taken up by the muscle. Thus the number of β- and γ-carboxyl groups controlled by each ouabain occupied cardinal site might be ten times higher or still more. At first look, this number seems far too large. How can so few control so many? Yet we also know that the few-control-many mechanism is vital to any complex systems that function coherently. So perhaps our initial disbelief is couched in a grossly distorted concept of living matter and how it functions.

Indeed as our knowledge of protein systems expands, the concept of proteins as rigid solids have long passed. Rather they are now known as dynamic system, moving intramolecularly from one configuration state to another through segmental motion, folding-unfolding, "breathing", etc. (Careri et al., 1975; Englander and Rolfe, 1973; Gurd and Rothgeb, 1979). What these findings tell us is that, when examined as a whole, the free energy difference between alternative conformation states, are rather small due to the presence of many compensatory changes. As an illustration, we shall cite the in vitro study of the allosteric control of oxygenation and deoxygenation of hemoglobin by the binding on remote cardinal sites of 2,3-diphosphoglycerol (2,3-DPG), inositol hexaphosphate or ATP (see Chanutin and Curnish, 1967; Benesch and Benesch, 1969; Ling, 1970, 1984,

p. 222).

Even on first look, the phenomenon is remarkable because each hemoglobin molecule binds one molecule of 2,3-DPG to the two β-chains and yet this binding of *one* molecule of 2,3-DPG alters the properties of all *four* heme groups, two of which are on the β-chains and two others on the α-chains. Thus in an environment of suitable oxygen tension, the binding of each mole of 2,3-DPG will cause the liberation of four molecules of oxygen from the four heme sites. However, this 1→4 control is not the only event accompanying the change. With deoxygenation, six pairs of salt linkages between the α- and β-chains are formed (Perutz, 1970), protons are taken up (Bohr effect), hemoglobin becomes less soluble in water (Edsall, 1958), gains in entropy (Manwell, 1958) (probably associated with the release of bound water, see Lauffer, 1975), gains in affinity and binding capacity for bromthynol blue (Antonini et al., 1963), loses reactivity in SH groups on cystein residues (Riggs, 1961), etc., etc. Clearly the binding of one molecule of 2,3-DPG or ATP brings about an extensive alteration of both the steric and electronic conformation of the proteins. By focusing attention on one specific manifestation, one may get a highly distorted picture. Indeed to illustrate this point, we shall consider a thought experiment.

Suppose we can tether end to end a chain of frictionless see-saws and with specially designed multiple telescopes observe not the entire see-saw chains but only say the left side seats of all the individual see-saws. A mouse is then deposited on one of the terminal seats of the see-saw chain. What one sees would be an almost magical and hence superficially improbable lifting of an endless number of see-saw seats by one mouse.

Why Ouabain Binding Does Not Bring About Cell Activation. The question arises, if ouabain is an EDC producing a c-value increase of the cell surface β- and γ-carboxyl groups, and activation of the muscle and nerve involves a c-value increase of the same β- and γ-carboxyl groups, why ouabain does not activate the cell and create an action potential? A possible answer is that ouabain does not increase the c-value far enough.

As mentioned earlier, ouabain seems to bring about a consistent c-value increase in all the phenomenon we examined. That is, ouabain acts as an EDC. However, it is also true that in all cases examined the extent of the c-value increase is uniformly modest corresponding to a rise from −4.50 Å to −3.95 Å.

We have already mentioned that during activation of squid axons, the rank order of ion permeability becomes totally inverted to become $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. Indeed Chandler and Meves reported in 1965 the permeability ratio relative to Na^+ to be 1.1 : 1 : 0.083 : 0.025 and 0.015. If one uses the simple approximate relation between ion permeability rates and relative ion adsorption constant shown in Equation 3 one can say that the observed permeability ratios roughly correspond to a c-value of −2.30 Å from the theoretical curves shown in Figure 1 with a theoretical adsorption constant relative to Na^+ of 1.2 : 1.0 : 0.1 : 0.033 and 0.011.

Drugs That Cause Sustained "Opening" of the So-called "Na Channels" and Drugs That Suppress It. There are evidence which support the notion that it requires a cardinal adsorbent (or its equivalent) that can cause a large enough c-value increase to cause activation. The fact that in different types of cells at different cytological locations, ouabain produces a similar moderate c-value increase suggests that only certain types of agents can cause the c-value increase to reach the activation point. The fact that some toxins (e.g., betrachotoxin) and drugs (e.g., veratridine) do indeed cause sustained opening of the so-called "Na channels" is in harmony with this view.

But perhaps an even more interesting sup-

port comes from the agents that are known to block or inhibit activation. Only two need to be discussed: Ca^{++} and tetradotoxin.

According to the AI hypothesis, the c-value increase and decrease produced by different cardinal adsorbents on the same sites are additive (i.e., the "additivity principle", see Ling, 1962, p. 95). Ca^{++} a doubly charged cation, should be an electron withdrawing cardinal adsorbent or EWC. As such one would anticipate that by increasing the Ca^{++} concentration one should annul the required c-value rise that accompanies the generation of an action potential. In fact this is true. Increasing external Ca^{++} concentration moves the Na^+ activation to a more positive potential (Hille, 1976). Conversely, decreasing Ca^{++} concentration creates a voltage dependent large increase of Na^+ current (Frankenhauser and Hodgkin, 1957; Almers et al., 1984).

The extensive evidence demonstrating the adsorbed state of the bulk of cell K^+ adds further definitive evidence that the resting potential (ψ) cannot depend on the intracellular free K^+ concentration but is quantitatively predicted by the new equation of the resting potential based on the surface adsorption theory. From analysis of the quantitative effects of different cardinal adsorbents on the resting potential measured in the presence of a constant high external Na^+ concentration (i.e., 100 mM) and varying external K^+ concentration, the conclusion was reached that those agents that cause hyperpolarization (i.e., rise of the resting potential) increases the relative affinity of K^+ over Na^+ seen as a rise of the intrinsic adsorption exchange constant $K^{oo}_{Na \to K}$ in agreement with a c-value decrease. On the other hand, cardinal adsorbents that cause depolarization (i.e., fall of the resting potential) cause a decrease of $K^{oo}_{Na \to K}$ in consequence of a c-value increase. On this basis, ouabain acts as an EDC because it causes depolarization. Ca^{++}, on the other hand, causes hyperpolarization and is once more recognized as an EWC.

Now tetradotoxin has long been recognized as a Na channel blocker. Suggestion has been made that its cationic groups interact with the carboxyl group of the Na^+ channel. It is also of interest that tetradotoxin has also been known for its hyperpolarizing action (Freeman, 1971), suggesting that an important electronic mechanism of its blocking activity may be due to its action as an EWC, which cancels the large c-value increase essential for the creation of the action potential.

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