COUNTERARGUMENTS AGAINST ALLEGED PROOF OF THE NA-K PUMP IN STUDIES OF K' AND NA^+ DISTRIBUTIONS IN AMPHIBIAN EGGS

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• ***With the aid of an ingenious technique called the reference phase method, Horowitz and his coworkers tested the alternative theories of solute distribution in living cells. Many interesting and significant findings were reported. However, a careful study of their papers led me to the belief that their claim of a proof for the Na pump theory was unwarranted. By taking into account other relevant information in the literature one can argue that their data in fact support an alternative hypothesis, the association-induction hypothesis.***

INTRODUCTION

There are three and only three types of mechanisms that can produce a sustained difference in the concentration of a substance in two contiguous spaces: Mechanism I — the existence of an insurmountable energy barrier between them; Mechanism 2 — the continual operation of a pump; Mechanism 3 — different physicochemical environments within the two spaces.

As a rule, a normal living cell contains K' at a concentration many times higher than that in the surrounding medium; in contrast, it contains Na^+ at a concentration many times lower' than that in the surrounding medium. The earliest theory offered to explain this asymmetrical K' and Na^+ distribution belongs to Mechanism 1, i.e., the cell membrane is absolutely and permanently impermeable to K' and to Na^+. This theory was disproved by radioactive tracer (and other) experiments. In its place, the membrane-pump theory was suggested. In this theory the asymmetry of ion distribution is due to postulated membrane pumps which continually transport K' in and Na' out of the cell at the expense of metabolic energy. This theory obviously belongs to the category of Mechanism 2. A third type of theory falling into the category of Mechanism 3 is exemplified by Troshin's sorption theory (1966) and by the association-induction (AI) hypothesis. In the AI hypothesis the asymmetrical ion distribution is due to a combination of (i) preferential specific adsorption of K' on β- and γ-carboxyl groups which raise the K' concentration to levels higher than in the surrounding medium and (ii) partial exclusion of Na' (and K') from cell water which exists in the state of polarized multilayers with reduced solubility for large and complex molecules and hydrated ions like Na^+ (Ling, 1984).

Horowitz and his coworkers (1979a, b) injected melted 10-20% gelatin solution into salamander eggs. The cool temperature of the egg caused the injected gelatin solution to gel and to form discrete droplets which were used as a "reference phase" (RP). After equilibration for varying lengths of time, the egg was frozen in liquid nitrogen and by cryodissection, the cytoplasm of the egg and the gelatin droplet were then removed and analyzed separately for K', Na', and H_2O contents. With this technique, Horowitz and Paine created an ingenious model of the

living cell that offers new opportunities to test the alternative theories mentioned above. In this model, part of the cell interior is occupied by its original normal real cytoplasm (and nucleus) while another part is occupied by the RP which has the essential properties of the cell cytoplasm as postulated by the membrane-pump theory. Thus the RP contains none or little proteins that might polarize water; nor does it selectively adsorb substantial amounts of K' over Na'. It is surrounded by a functional cell membrane and in this cell membrane are located the postulated K', Na' pumps. The following are the decisive findings anticipated:

If the membrane-pump theory is correct, the K' and Na^+ concentrations in the reference phase should be the same as in the real cytoplasm. The concentration of K' found in the RP should be much higher than in the external medium and that of Na' much lower than in the external medium.

If the association-induction hypothesis is correct, and if indeed .both K' accumulation and Na' extrusion are due to the cytoplasmic proteins, then clearly the reference phase should neither accumulate K' nor exclude Na'. In other words, the water in the gelatin phase is expected to contain K' and Na^+ at the same concentrations as each is found in the Ringer solution bathing the salamander eggs.

AN EXAMINATION OF THE ALLEGED PROOF OF THE MEMBRANE-PUMP THEORY

Horowitz and coworkers measured the K' and Na' concentrations in the RP and compared these concentrations with those found in the normal cytoplasm and in the Ringer solution. They summarized their main findings in the opening section of the paper to be referred to as Paper I (Horowitz et al., 1979): "We will show that in oocytes free cytoplasmic cation concentrations, measured by the RP, differ from those of the cell's bathing solution. This demonstrates unambiguously that cells maintain Na' and K' activity gradients between cytoplasm and the extracellular medium, and provides proof of active cation transport, which, though widely accepted, has been questioned." (Horowitz et al., 1979, p. 34).

I shall next examine the experimental evidence that was seen as ***proof*** of the postulated Na^+ pump theory.

The prediction of the AI hypothesis, that the RP K' and Na' concentrations should be equal to those in the Ringer solution, cannot be tested without certainty that the 18% gelatin truly does not either interact with the water in the RP or adsorb K' or Na'; and by far more importantly, without certainty that full equilibrium of K', Na^+ and any other electrically charged solute has been attained between the RP and the external Ringer solution.

In regard to the first requirement, there are now extensive data showing that an 18% gelatin gel ***does*** significantly alter the solvency of the bulk phase water (Ling and Ochsenfeld, 1983). Thus the apparent equilibrium distribution coefficient (p-value) for Na-citrate in 18% gelatin gel is about 0.9 and not 1.0. There is also evidence that gelatin at this concentration range binds alkali metal ions (see Veis, 1964, page 109). However, these departures from an ideal RP are minor. Nevertheless, we cannot anticipate on the basis of the AI hypothesis ***equality*** of K' and Na' concentration in the gelatin RP and in the external Ringer solution; we can only anticipate approximation of the two sets of concentrations.

The second requirement of full equilibration being reached for all electrically charged solutes in the RP and external solution is of paramount importance. Indeed, unless true equilibrium has been established for K' and Na^+ as well as all other electrically charged solutes between the RP and the external solu-

tion, no comparison can be made between the K, Na' concentrations in the RP and those predicted by the AI hypothesis. Unfortunately, this crucial point was not appreciated.

The fact that the reference phase K' concentration has ***not*** reached equilibrium with K' in the external Ringer solution is clearly shown by Horowitz and Paine's own data given in the Table III on page 51 of their paper to be referred to as Paper II (Horowitz and Paine, 1979). Here the specific activity of K^{42} had reached only 33% of that in the external Ringer solution after 7.9 hours of incubation (5°C) in the longest experiment reported. Yet the RP K^+ data that formed the foundation of their above-mentioned "proof' were collected after an incubation time of less than 5 hours (5°C) (page 39 of Paper I).

This failure to allow K^+ equilibrium to be reached invalidates the main conclusion mentioned above.

The K' and Na' concentrations in the RP considered as offering proof of the active transport or pump theory were $129 \pm \mu eq/ml$ of RP water for K' and 21 ± 1 $\mu eq/ml$ of RP water for Na'. These values were obtained from oocytes which Horowitz et al. call normal. The definition of a normal or N-oocyte is that the ratio of K' concentration in the RP phase over the sum of K' and Na' concentration in the RP, $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$, exceeds 0.8 (Paper I, page 40). A $C_K^{RP}/(C_K^{RP} + C_{Na}^{RP})$ value exceeding 0.8 is that predicted by the membrane-pump theory.

Thus by defining what are ***normal*** oocytes, the authors have, perhaps unconsciously, already selected the data in favor of one of the two contending theories, the membrane-pump theory. By defining what are ***damaged*** or d-oocytes, they have also'eliminated for further consideration on this issue the data that favor the alternative hypothesis.

In defining the normal and damaged oocytes the authors used the word "damaged" in an unusual context. Thus they did not outright reject the data from the damaged cells. Instead those data, rejected for one specific purpose mentioned above, were retained and used as additional evidence in favor of the membrane-pump theory (isotherm shown in Figures 2 and 3 on page 49 of Paper II). So clearly the data obtained from the damaged oocytes were valid for one purpose but invalid for another. A clear explanation for this decision was not given.

From the three sets of data of K^{42} exchange between RP and external medium provided in Table III of Paper II mentioned above (12% exchange at 1.9 hrs.; 14% exchange at 3.5 hrs.; 33% exchange at 7.8 hrs.) one can roughly estimate how long an incubation at 5°C would it take for the K' in the RP to reach near equilibrium (i.e., 99% exchange) if the oocytes remained in the same condition prevailing in the first 7.8 hours. This was found to be about 90 hours or 3½ days. According to Horowitz et al. (1979) "oocytes incubated in this Ringers at 5°C remain normal, as judged by cellular K^+ and Na^+ levels, for at least 3 d." (page 35 of Paper I). It would seem natural that the authors might have in some experiments allowed their incubation to have lasted considerably longer than 5 hours. In fact it was by increasing the incubation time that they obtained many of the data points with low RP K' and high RP Na^+ shown in the isotherms in Figures 2 and 3 of Paper II (Horowitz, personal communication).

Data given in Figures 2 and 3 of Paper II were used to assess the space available to freely exchangeable K' by the "isothermal" methods. They include many more points than those values given by what were called normal oocytes obtained after less than 5 hours of incubation. At the lower (left-hand side) end of Figure 2 of Paper II, for example, the K' concentration in the RP after long incubation times is no longer 129 $\mu eq/ml$ obtained from normal oocytes but as low as 4 $\mu eq/ml$ and thus approximate to that in the

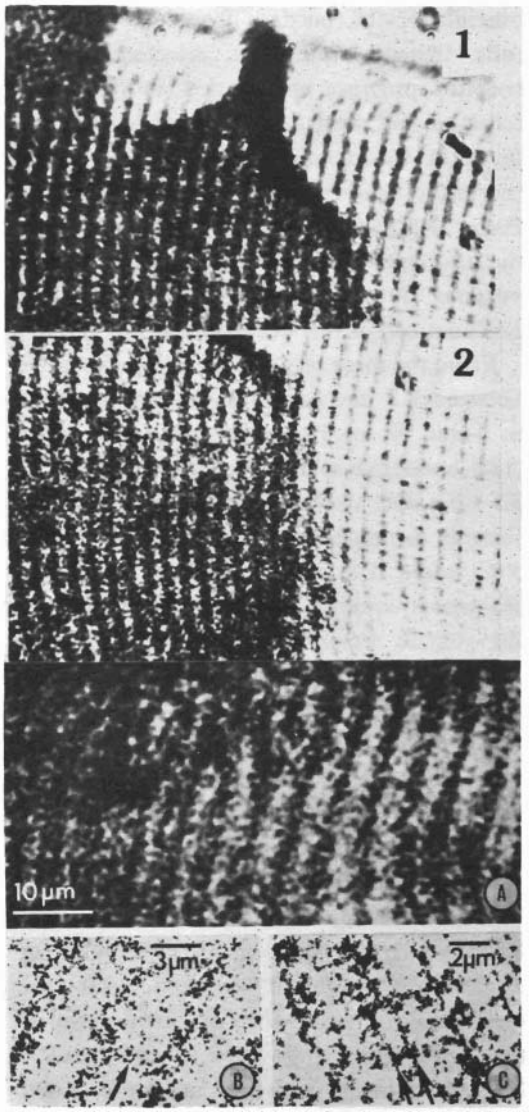

FIGURE 1. **(1 and 2) Autoradiographs of air dried CS^{134}-loaded frog sartorius muscle where the photoemulsion covered only part of the muscle fibers (Ling, 1977a). (A to C) Autoradiographs of frozen-hydrated frog muscle fibers. A. Light microscopic autoradiogram of a stretched Cs-loaded fiber. B. Electron microscopic autoradiogram of a stretched Cs-loaded fiber. The sarcomere length is about 4.4 pm. Between two dark bands (A bands) a line of silver grains indicates the Z-line (arrow). C. Electron microscopic autoradiogram of a stretched Rb-loaded fiber. The sarcomere length is about 3.3 pm. Arrows indicate dark lines at the outer edges of an A band (From Edelmann, 1980a, reprinted by permission of Histochemistry).**

external Ringer solution (2.5 µeq/ml). By comparing the data of RP K^+ concentration given in Figure 2 in Paper II with the RP Na' concentration in Figure 3 of Paper I one finds that the Na' concentration in the reference phase that yielded very low C_K^{RP} (e.g., 4 µeq/ml) is fully as high as that in the Ringer solution (i.e., 105 mM) or even higher.

In concluding this discussion of K' concentration in the RP, I can state that those data that can legitimately test the AI hypothesis (i.e., those from cells in which equilibrium was reached between K' in the RP and the external medium by longer incubation) substantiates the AI hypothesis and refutes the membrane-pump theory. The data points which led Horowitz et al. to the opposite conclusion were obtained before equilibrium was reached and therefore could not be considered as valid evidence. Next I shall discuss the Na^+ concentration in the RP, which did reach isotopic exchange equilibrium with Na^+ in the external Ringer solution.

In contrast to radioactively labelled K', the specific activity of the radioactively labelled Na' in the reference phase reached the same level as that of labelled Na^+ in the Ringer solution in only 4 hours (5° C). Nevertheless, the reference phase Na' concentration was only 23.7 µeq/ml while that in the Ringer solution was 115.5 µeq/ml. Since the concentration of gelatin is too low to provide effective polarization of the cell water, how can this low level of Na^+ in the RP be sustained without the intervention of a membrane Na' pump?

Thus the low level of labelled Na^+ distribution in the RP seems to have offered a proof of the pump theory. In fact, this is not true.

The RP is enclosed by a layer of cytoplasm and beyond that, a semipermeable cell membrane. Within the duration of time in which oocytes remain "N-oocytes" (i.e., $<$ 5 hours) the K' accumulated in the RP remained little changed at close to 125 µeq/ml. That is, to all intents and purposes, K' behaved as an

impermeant cation in what is known as a Donnan membrane equilibrium. As such, the presence of one impermeant cation species on one side (i.e., the RP phase) causes the equilibrium concentration of permeant ions of the same electrical sign to be decreased on the same side. Thus as long as a substantial amount of K' has not yet reached equilibrium with K' in the external solution, and remains trapped in the reference phase, it will act as "impermeant" cation. As a result, permeant cations of the same electrical sign, like Na', will remain at a lower concentration than on the other side of the semi permeable membrane in the external medium.

From these considerations one can draw a second conclusion: the low level of Na^+ would be maintained as long as a high concentration of K' is entrapped in the RP. There is no need of introducing an Na^+ pump. It would consume a lot of energy and serve no useful purpose.

In the next section, I shall examine the question, "Where did the high level of K' in the RP of N-oocytes come from?" To provide the essential background knowledge I shall begin by discussing the physical state of K' in normal resting muscle cells because in these cells we now have clear knowledge that virtually all K' exist in an adsorbed state. I shall then review the highly inconsistent findings of cell K' activity monitored with a K^+-specific intracellular microelectrode. I shall

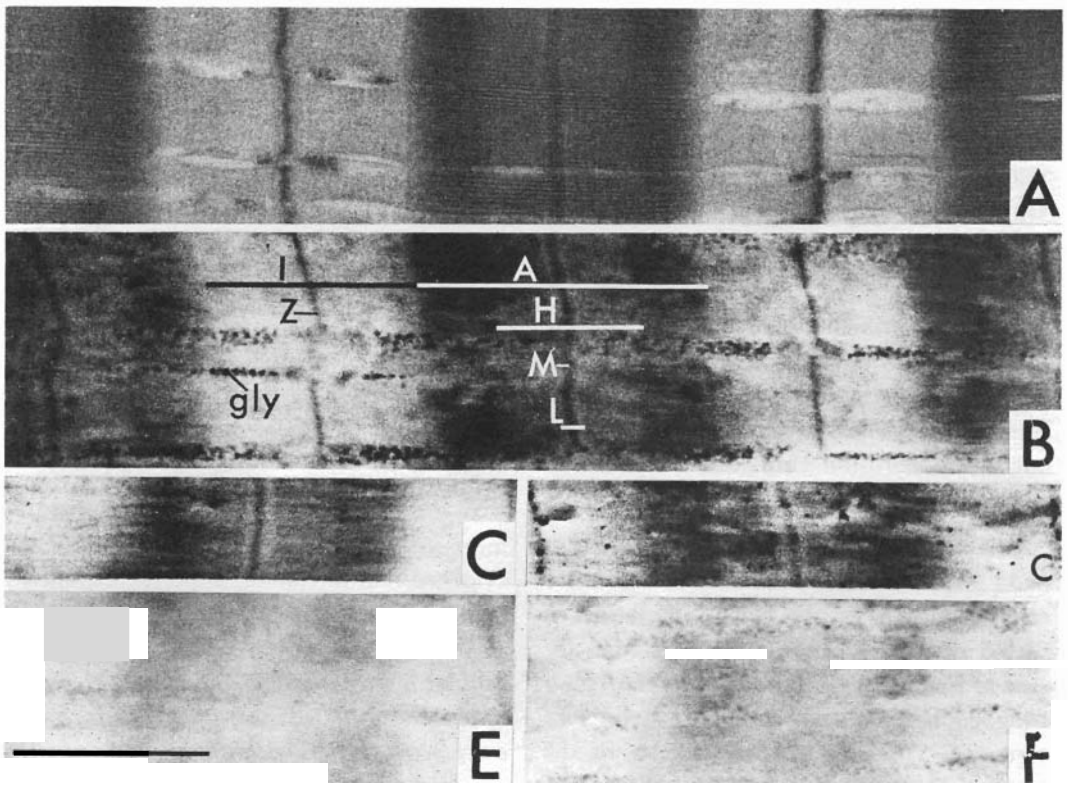

FIGURE 2. Electron micrographs of frog sartorius muscle. A. Muscle fixed in glutaraldehyde only and stained only with uranium by conventional procedure. B. EM of section of freeze-dried Cs^+-loaded muscle, without chemical fixation or staining. C. TI'-loaded muscle without chemical fixation or staining. 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 and Z-line. E. Section of Central portion of B after leaching in distilled water. F. Normal "K^+-loaded" muscle. Scale bar 1 μm. (A from Edelmann, unpublished, B-F from Edelmann (1977)).

then suggest where the high level of RP K' in the N-oocytes came from.

THE PHYSICAL STATE OF K' IN RESTING MUSCLE CELLS

Within the last seven years, three different laboratories in three different countries, using two different kinds of voluntary muscle cells (frog sartorius muscle and isolated honey bee thorax muscle myofibrils) and ***four indepen-det techniques*** have come to the unanimous conclusion that the bulk of intracellular K' in voluntary muscle cells is not evenly distributed but is localized at the edges of the A bands and the Z-line which bisects the I band. The four different techniques are autoradiography of both air-dried (Figure 1, Ling, 1977a) and frozen muscle cells (Figure 1, Edelmann, 1980a); transmission electron microscopy (Figure 2, Edelmann, taken from Ling, 1981); dispersive x-ray microprobe analysis (Edelmann, 1978; Trombitas and Tigyi-Sebes, 1979; Edelmann, 1983); and laser microprobe microanalysis (LAMMA) (Edelmann, 1980b).

Other experimental studies have shown that K' localized at the A bands and Z-line and the competing cations must be in close contact with the adsorption sites in a one ion-one site adsorption (Ling and Ochsenfeld, 1966; Ling, 1977b, 1984).

In summary, the localization of K' at the A band and Z-line, the obedience of the entire cell K' content to the general equation of solute distribution including a major absorbed fraction of K' described by the Yang-Ling cooperative adsorption isotherm (see Equation 5 below), the dependence of the relative effectiveness in displacing K' on the short-range attributes of cations, and the independence of the activity of postulated membrane pumps, when viewed together, led to the conclusion that most of the intracellular K' is adsorbed on specific sites by the one ion-one site close contact mechanism (Ling, 1984).

FREE K' ACTIVITY MEASURED WITH AN INTRACELLULAR K' ELECTRODE THAT IS HIGHER THAN THE TOTAL K^+ CONCENTRATION IN MUSCLE, INTESTINAL MUCOSA, AND FROG EGG

Model studies showed that ions adsorbed on anionic sites are "invisible" to the ion-selective electrode (Ling and Zhang, 1983a, 1984). Since the bulk of muscle K' is adsorbed and very little cell K' is free, one would expect if one can introduce a K' ion-specific electrode into a muscle cell, the measured free K^+ activity would be ***uniformly*** much smaller than the total K'concentration in the muscle cells. What was actually observed could not be more different. As shown in the top row of Table I, the activity measured with the intracellular electrode was lower than, equal to, or higher than the total K' concentration. Further down Table I, one notices that this high degree of variability of the ratio of free K' activity over total K^+ concentration measured occurred in other tissues including amphibian eggs. In contrast, the ratio of measured Na' activity over the total Na' concentration is consistently well below unity.

A statement I made in 1969 has become more appropriate as we now have clear cut evidence that in resting, unperturbed muscle cells, K' is largely adsorbed and therefore should be "invisible" to the K'-sensitive intracellular microelectrode: ***A capillary electrode of the Gerard-(Graham)-Ling type measures an electric potential difference across the entire surface of the muscle cell. An ion-specific electrode, on the other hand, acts quite differently. It measures the activity of ions only in a microscopic layer of the medium immediately surrounding the tip of the electrode. Thus, with a capillary-***

electrode, reasonably accurate recordings of resting and action potentials can be obtained from punctured cells; but with the ion-specific electrode the ionic activity can be measured only in that part of the protoplasm with which the electrode is in close contact, that is, when the structural integrity has been destroyed or at least grossly perturbed.

According to the association-induction hyporhesis the protein-water-ion system of a living cell is labile. In response to external disturbance it may transform in an all-or-none manner from its normal resting state, in which the K^+ ion is adsorbed, to the "excited" state in which the bulk of K^+ ion is released from adsorption. In this state, the adsorption sites may either adsorb other free cations for example, Na^+ ion) or fixed cations for example the ε-amino group).

I have shown recently (Ling, 1984) that all the divergent results illustrated by the data shown in Table I can be explained on the basic principle stated above and the assumption that Na^+ adsorption represents a more stable state following the local perturbation. For the present discussion, I shall begin with the data obtained from barnacle muscle cells because these data appear the most contradictory to what one would have expected from the adsorbed state of K in muscle cells.

To explain the measured K^+ activity *higher* than the average K concentration, one must direct one's attention to several other aspects of the AI hypothesis: (i) the multilayer polarization of virtually all the cell water, (ii) the "size rule" controlling solute exclusion from this water, (iii) multilayer polarization of the cell water, like K adsorption, is metastable and (iv) selective K adsorption and Na^+ exclusion from cell water depend on two

TABLE I. K^+ and Na^+ activities measured with intracellular ion-selective electrodes in comparison with total concentration of these ions in the cells. $(a_K/C_K)_{ms}$ is the experimentally measured ratio of free K^+ activity and total K^+ concentration; $(a_K/C_K)_{th}$ is theoretically anticipated on the assumption that the cell K^+ is free.

Cell Type	C_K (mM)	a_K (mM)	C_{Na} (mM)	a_{Na} (mM)	$\left(\frac{a_K}{C_K}\right)_{ms}$	$\left(\frac{a_K}{C_K}\right)_{th}$	Animal	Source
	169f2.5		51.6±3.3(12)	13.5±0.8			Carcinus maenus	Hinke (1959)
		84±1.5(12)	55.4±4.3(6)	12 to 16	0.55	0.75	Honiaris vulgaris	
	126±2.0(260)	94f4.0(260)	28.4±3		0.75	0.75	Rana temporaria	Lev (1964)
muscle		to 99f.004						
	127f3(150)	97.8 (150)	29f5	5.5				
			28f3	5.3				
	168±2(46)	193±5(10)	81±(46)	14±1(16)	1.14	0.65	Balanus nubilis	McLaughlin & Hinke (1966)
	113±2(11)	82			0.73	0.75	Bufo bufo	Dick & McLaughlin (1969)
oocytes	93f2(8)	120±3(22)	73f3(8)	6 f 1(22)	1.29		Rana pipiens	Palmer, Century, and Civan (1978)
						0.75		
	104±1(8)		77f1(8)		1.15			
	146	38.5			0.27	0.75	Amphioma	White (1976)
intestinal	54/86	65/85			1.0	0.75	Rana pipiens	Lee & Armstrong (1972)
mucosa					1.2			

related but separate mechanisms.

The evidence in support of the polarized multilayer theory of cell water has been reviewed in detail in In ***Search of the Physical Basis of Life*** (Ling, 1984) (see also Ling, 1983; Ling and Murphy, 1983; Ling and Ochsenfeld, 1983). Particularly clear-cut is the evidence provided by recent quasi-elastic neutron scattering (QENS) studies of Trantham et al. (1984). These authors showed that ***all*** the water in the living cell (brine shrimp cysts) suffers motional restrictions: the translational diffusion coefficient is reduced to 1/3 that of normal liquid water; the rotational diffusion coefficient to 1/10, thereby fully confirming the polarized multilayer theory of cell water which predicted both translational and rotational restriction of all the cell water (Ling, 1965a, 1969; Ling et al., 1967). Just as important was the finding of Rorschach (1984) that a 35% PEO solution exhibits QENS entirely similar to that of brine shrimp cysts. Other studies revealed that water in a 35% PEO solution shows reduced solubility for Na' and other solutes normally found at low levels in living cells (Ling and Ochsenfeld, 1983). NMR (Ling and Murphy, 1983), and high frequency dielectric relaxation properties (Kaatze et al., 1978), are in full agreement with conclusions drawn from QENS studies and the predictions from the polarized multilayer theory of cell water.

With this brief background sketch, I now summarize the events that I believe have caused the high K' activity recorded in living cells in excess of the average K' concentration in the cell as reported by Hinke, his coworkers and others:

(i) Initially virtually all intracellular K' is adsorbed single on β- and γ-carboxyl groups and all the cell water is adsorbed in multilayers on the extended chains of certain "matrix proteins."

(ii) The insertion of the K' selective microelectrode disrupts the protoplasm in the microscopic region surrounding the microelectrode tip, causing both the liberation of K' adsorbed on β- and γ-carboxyl groups and the depolarization of multi-layers of polarized water. The thin film of depolarized water surrounding the microelectrode tip, thus functions also as a reference phase (RP) bearing resemblance to the gelatin droplet RP in the salamander eggs. Both contain nearly normal liquid water with near unity q-values for K^+, Na^+, etc.

(iii) This disruption-induced K' liberation and water depolarization spread outward into healthy areas of cytoplasm away from the focal region of initial injury. The absolute and relative rates of propagation of K^+ liberation and water depolarization as well as the extents of these two processes gave rise to the wide divergence of a_K^{free}/C_K^{av} measured (Table I) (Ling, 1984). For other independent evidence of the slow spreading of mechanical injury-induced K' liberation and q-value rise in voluntary muscle cell see Ling (1978); there, the cytoplasm was not torn apart by intracellular injection of gelatin solution or by the insertion of a microelectrode but by the stroke of a sharp razor blade.

The K' liberated from the peripheral regions, where water polarization has either not yet occurred or occurred to a lesser extent than immediately around the electrode tip, will "pour" into the regions of more complete water depolarization and higher q-value in the RP immediately surrounding the microelectrode tip. This "immigrant" K' when added to the locally liberated K' will raise the ratio $(a_{K+}^{free}/C_{K+}^{av})$ to above unity as observed in the barnacle muscles, frog oocytes and other tissues shown in Table I. Let us now temporarily leave events occurring at the RP surrounding the microelectrode tip in a barnacle muscle cell and return to the gelatin RP in the salamander eggs.

Horowitz et al. wrote, ". . . comparison of RP and cytoplasm demonstrates that free and total cation concentrations in cytoplasm differ markedly for both Na' and K'. In the

case of Na^+, only a small portion of the cytoplasmic cation is free, whereas for K' a seeming paradox exists, in that the concentration of free cation is higher than the cytoplasmic concentration of which it is putatively only a component." (Paper I, page 34.) The reader may notice that one can equally well apply this description of K' and Na^+ distribution in the RP of the salamander eggs to the K' and Na' distribution in the RP surrounding the microelectrode tip which Hinke and his coworkers observed in the barnacle muscles, Lee and Armstrong observed in intestinal epithelium and Palmer et al. observed in mature frog eggs. The four sets of phenomena are entirely similar. In frog muscle, there is no longer any doubt that virtually all the K' is adsorbed to begin with. This leaves little room to consider that in frog and 'salamander eggs virtually all the cytoplasmic K' is free as postulated by the membrane theory.

Concluding this section, I can state that it seems highly probable that the high concentration of K^+ found in the RP of salamander eggs within the first 5 hours after the introduction of the RP, originated from adsorbed K' in the cytoplasm which became liberated in response to the local trauma created by the injection of gelatin solution and during subsequent spreading of the deterioration to peripheral areas. In the peripheral areas, the depolarization of cytoplasmic water may have been incomplete, with a q-value for K' lower than that in the RP. As a result, a major portion of the liberated K^+ would "pour" into water in the RP raising the total level therein to higher than the average cytoplasmic K' concentration. Due to an electrostatic (Donnan) effect, the level of RP Na' was kept low as a consequence of the entrapment of K'. After a sufficiently long period of incubation, both the K' and Na' concentrations in the RP approached those in the surrounding Ringer solution, in agreement with the AI hypothesis.

HOROWITZ AND PAINE'S EQUATION FOR ION DISTRIBUTION AND AN ALTERNATIVE

In his sorption theory, A. S. Troshin introduced an equation (the Troshin equation) describing solute distribution in living cells (Troshin, 1951, 1966). This equation includes a non-saturable term corresponding to free solute in the cell water and a saturable term corresponding to adsorbed, complexed solute:

$$C_C = C_S K \left(1 + \frac{A_m}{C_S K + a}\right), \qquad (1)$$

or

$$C_C = C_S K + \frac{A_\infty}{C_S K + a}, \qquad (2)$$

(free) (adsorbed)

where C_S is the concentration of the solute in the surrounding medium. K is a "coefficient of proportionality characterizing the properties of the aqueous phase of the cell water." A_∞ is the limit of adsorption and a, a constant.

Horowitz and Paine also presented a two term equation:

$$C_i^C = q_s C_i^{RP} \left(1 + \frac{{}^{max}C_i^{C2}}{q_s C_i^{RP} + A_i}\right). \qquad (3)$$

This equation represents the intracellular concentration of the ith solute in terms of the ith solute concentration in the RP. ${}^{max}C_i^{C2}$ is the maximum adsorption of the ith species, considered to be exclusively in the yolk platelets. A_i is not Troshin's a. Rather it is equal to q_s/k_i, where k_i is the binding constant of the ith solute. q, is not Troshin's K, which represents solubility. Rather it is the cytoplasmic sucrose space and is a fractional number representing the fraction of cell water avail-

able to sucrose based on the idea that the cell has two kinds of water: one is simply normal liquid water and is that corresponding to all the cytoplasmic water but not including water in the yolk platelets; the other is non-solvent water corresponding to all the water in the yolk platelets.

Equation 3 can also be written as

$$C_i^C = q_s C_i^{RP} + \frac{^{max}C_i^{C2}\, C_i^{RP} k_i}{1 + C_i^{RP} k_i} \qquad (4)$$

(free) (bound)

Equations 3 and 4 tell us nothing about the relation between intracellular and extracellular ion concentration.

I shall raise five questions concerning the basic assumptions of this model:

Question I: Can we propose a pumping mechanism for the maintenance of K' and Na' concentration in an amphibian egg cell, after it has been shown that a progeny of the egg cell, namely, amphibian voluntary muscle does not have enough energy to operate similar pumps?

More and more pumps have been proposed both at the plasma membrane and at the even larger surfaces of subcellular particles (e.g., mitochondria, sarcoplasmic reticulum). There is not even a hint how the muscle cell can manage all these energy needs. One pump alone, the Na pump in frog muscle has been shown to require 15 to 30 ***times*** as much energy as the cell commands (Ling, 1962); three remedial postulations to keep the Na pump afloat had all been experimentally disproven (Ling et al., 1979). The answer to Question 1 is "No." "What is true for E. coli must also be true for elephants" (Monod and Jacob, 1961); clearly what is true for frog muscle must also be true for frog oocytes.

Question 2: Can K' and Na' in the yolk platelets be considered at once nonexchangeable and in thermodynamic equilibrium with K' and Na' in the cytoplasmic water?

q_s in Equations 3 and 4 is defined as the "sucrose space." This space (alone) is equally and completely accessible to all solutes including exchangeable K', Na^+, Cl^- and sucrose while no other space in the cell is accessible to these and other solutes. K^+ and Na' found in the yolk platelets thus must be non-exchangeable with free K', Na^+ in the cytoplasm. Yet it was stated by Horowitz and Paine in Paper II that the K' and Na' in the yolk platelets are in thermodynamic equilibrium with cytoplasmic K' and Na^+. Being in thermodynamic equilibrium can only mean that the yolk platelets are exchangeable with K' and Na'. The answer to Question 2 is therefore also negative.

Question 3: Does non-solvent water exist?

The concept of non-solvent water, in spite of a long history, remains a mystic notion without unequivocal evidence demonstrating its existence. Even solid gold can dissolve lead when they are in contact for a long period of time. Extensive study of model systems, some of which in the past had been considered to possess non-solvent water, told quite a different story. Thus more than 80% of the water in a 25% solution of polyvinylmethyl ether appears not to be available to Na_2SO_4: only 30% of the same water is not available to sucrose; and virtually all this water is available to methanol (Ling et al., 1980a). Similarly only 70% of the water in solutions of 15 urea-denatured proteins is available to sucrose; yet 99.1% of the same water is available to urea (Ling, et al., 1980b). Sucrose distribution therefore cannot be used as a criterion separating normal water and non-solvent water. Indeed there is no reason to believe that liquid water anywhere exhibits all-inclusive non-solvency for solutes implied in the non-solvent water concept. This then is the answer to Question 3.

Question 4: Can (yolk-platelet-free) egg cells maintain their high K' and low Na' concentration by one mechanism (Mechanism 11), while the descendents of the egg cells

maintain their similar high K' and low Na' concentrations by an altogether different mechanism (Mechanism III)?

Stripped of all its platelets, the model of K' and Na^+ in amphibian eggs as given by Horowitz and coworkers would be a simple membrane-pump model. Certainly the K' and Na' distribution properties of the younger ova before they become laden with yolk platelets are very similar to those of other somatic cell types like voluntary muscle, erythrocytes, and nerve, which are after all the descendents of the egg cells. The following are a few selected highlights which have made it virtually certain that muscle, nerve, and erythrocytes maintain their K^+ and Na' levels by a Type III mechanism (for a more comprehensive review see Ling, 1984):

(1) Muscle: (i) In frog muscle cells virtually all the major intracellular cation, K, is not free but adsorbed. (Ling, 1984.) (ii) In frog muscle whose membrane (and postulated) pumps have been made non-functional, selective K' accumulation and Na^+ extrusion persist as usual (Ling, 1978).

(2) Nerves: The cytoplasm (axoplasm) of squid axon can be removed without any damage to the function of the axon membrane (Oikawa et al., 1961, Baker, et al., 1961). When filled with sea water fortified with ATP and its open ends tied, the axon membrane sacs provide an ideal model to test the Na pump theory. Extensive efforts have been made in this direction, but net ion transport against a concentration gradient has not been demonstrated (see Ling, 1965b, page 95).

(3) Erythrocytes: Human red blood cells can be freed of virtually all their cytoplasm. These white ghosts have an intact membrane and active Na-K activated ATPase. Nevertheless such white ghosts in the presence of ATP do not transport K' and Na' against concentration gradients (Ling and Tucker, 1983). Ghosts that do transport K' and Na' against concentration gradients are solid. In fact, the extent of K' accumulation and Na' extrusion is quantitatively related to the concentration of cytoplasmic proteins remaining in the ghosts (Ling, 1984; Ling, Zodda, and Sellers, 1984).

From the above, one finds that the answer to Question 4 must be that it is improbable.

Question 5: Do we need to propose two different mechanisms for similar asymmetrical K' and Na' distribution properties of the whole cell and one of its constituent parts?

Yolk platelets in mature salamander eggs make up 2/3 of the volume of the cytoplasm. These platelets contain K' and Na^+ at concentrations quite different from those in the surrounding cytoplasm. Horowitz and Paine went to considerable efforts to show that this segregation is not due to compartmentation (i.e., absolute membrane impermeability to ions which, of course, is a form of Mechanism 1), but is due to a combination of non-solvent water and K' adsorption, which seemed to fall into the category of Mechanism 3.

Yet there is evidence that there is a great deal of similarity between water and ions in the yolk platelets and elsewhere in a mature amphibian egg. Thus Ling, Ochsenfeld, and Karreman (1967) long ago showed that all the water in mature frog eggs, which must include those in all the yolk platelets, fully exchanges with titrated water in the Ringer solution in about 4 minutes with an overall diffusion coefficient in the cytoplasm differing from that of normal water only by a factor of 2. Ling and Ochsenfeld (1977) have also shown that the total K' (including that in the platelets) and the slowly exchanging Na' of the frog oocyte all become readily exchangeable in the presence of metabolic poisons. Clearly metabolism is involved in the preservation of yolk platelet K' and Na' contents as they are found in the other parts of the frog eggs.

One may then raise the question, are there such profound differences between the larger units we call amphibian eggs and the smaller units we call yolk platelets? Both contain

protein and water. Both contain K' and Na' at concentrations different from those found in the immediate surrounding medium. Both sets of asymmetrical ion distribution depend upon metabolism. How can one then assign one set (i.e., the eggs) to membrane pumps while citing an altogether different mechanism for the other set (i.e., the platelets) to achieve the same purpose? The answer to Question 5 is, therefore, that it is improbable.

AN ALTERNATIVE, SIMPLE EXPLANATION OF K' AND NA' DISTRIBUTION IN ALL LIVING CELLS AND THEIR SUBCELLULAR PARTICLES

I now suggest that distribution patterns of K', Na^+ and other solutes in all kinds of living cells, including frog muscle, squid axon, human red cells, and amphibian eggs, as well as all subcellular particles, including the yolk platelets, can be adequately described by one general equation of solute distribution (Ling, 1965b, 1984). Using K' distribution as an example, we have

$$[K^+]_{cell} = \alpha q_K [K^+]_{ex} + \sum_{L=1}^{N} \left\{ \frac{[f]_L}{2} \left[1 + \frac{\xi_L - 1}{(\xi_L - 1)^2 + 4\xi_L \theta_L} \right] \right\} \quad (5)$$

where $[K^+]_{cell}$ is the K' concentration in the cell in moles/kg fresh cell weight, α is the average water content, and q_K the average equilibrium distribution coefficient of K' in the cell water which may exist in somewhat different states in different parts of the cell. $[f]_L$ is the concentration in moles/kg fresh cell of the Lth type of adsorption sites, among a total of N types:

$$\xi_L = \frac{[K^+]_{ex}}{[Na^+]_{ex}} K^{\infty}_{Na \to K(L)} . \quad (6)$$

$[K^+]_{ex}$ and $[Na^+]_{ex}$ are the K' and Na' concentrations in the external medium. $K^{\infty}_{Na \to K(L)}$ is the intrinsic equilibrium constant $Na^+ \to K'$ exchange on the Lth type of sites. The parameter θ_L is:

$$\theta_L = \exp(\gamma_L / RT) , \quad (7)$$

where $-\gamma_L/2$ is the nearest neighbor interaction energy of the Lth type of sites, R and T are the gas constant and absolute temperature, respectively. Equilibrium solute distribution in living cells that have been carefully examined thus far are adequately described by this equation (for a full account, see Ling, 1984, Chapter 11). Clearly when Equation 5 is applied to amphibian eggs, one or more of the N types of adsorption sites would correspond to those present in the yolk platelets. On this specific point, my view and those of Horowitz and Paine agree.

I conclude this discourse with a quotation of "William of Occam's Razor": "entia non sunt multiplicandor praeter necessitatem" (the simplest of competing theories be preferred to the more complex). Or even more appropriate for the present case, as stated by Sir William Hamilton (1853) in his "Law of Parsimony": "Neither more, nor more onerous causes are to be assigned than are necessary to account for the phenomenon."

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