THE PHYSICAL STATE OF SOLUTES AND WATER IN LIVING CELLS ACCORDING TO THE ASSOCIATION-INDUCTION HYPOTHESIS *

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Cells shrink in concentrated aqueous solutions of sodium chloride or sucrose; they swell in more dilute solutions. Similarly, a sac made of parchment paper, dead animal bladder, or copper ferrocyanide gel, filled with an aqueous solution, may gain or lose water in such environments. On the basis of the resemblance among these osmotic behaviors, Pfeffer formulated the membrane theory a century ago.[1] Membranes of the above sort—with high permeability to water and much lower permeability to alcohols, sugars, and salts—were later referred to as semipermeable membranes. Pfeffer suggested that a semipermeable membrane surrounds all living cells and is responsible for the discontinuity of the cell from its aqueous environment.

At the turn of the century, Overton postulated that the plasma membrane consists of a continuous lipid layer.[2, 3] In support of this idea, Overton and, later, Collander [4, 5] demonstrated a linear correlation between the solubilities in oil of nonelectrolytes ranging from monohydric alcohols to glycerol and their relative rates of penetration into living cells, both parameters covering ranges of 5 logarithmic scales.

Later measurement of the surface tensions of living cells showed inconsistency with this lipid membrane theory. The measured surface tension was found to be more than one-hundredfold lower than predicted if the cell surface is lipid.[6–8] A remedial suggestion was then introduced; i.e., the lipid layer is sandwiched between two hydrophilic protein layers, thus diminishing the cell surface tension.[9] The implicit assumption was that these protein layers, themselves, would not alter the cell's permeability.

A serious deficiency persists in this model even after the remedial postulation of protein covering the cell surfaces; i.e., a lipid membrane is not a bona fide semipermeable membrane. Thus, ethyl alcohol is 40 times more soluble in oil than water is.[10] From the relation given by Collander between the oil/water distribution coefficient and permeability, ethyl alcohol should be 100 times more permeant to the cell membrane than water, rather than less permeant. This is hardly acceptable, because it was the much greater permeability of pig's bladder to water than to ethyl alcohol that led Abbé Nollet to the first recorded observation of osmotic phenomena in 1748.[11] To remedy this and other defects, Collander and Bärlund [12] then suggested that the lipid layer had small pores through which water molecules, but not the larger alcohols and sugars, could pass.

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In 1867 Traube,[13] who invented the copper ferrocyanide gel membrane, offered a similar "atomic sieve" idea to explain semipermeability—an explanation abandoned shortly after, since the holes of the semipermeable membrane appeared to be much larger than the sizes of molecules to which the membranes are impermeable (see Reference 11, p. 95; Reference 14, p. 656).

Davson and Danielli[9] then put together the now well-known bimolecular leaflet lipid model of the cell membrane: a double lipid layer sandwiched between protein layers and pierced with small pores. This model, in particular, and the membrane theory, in general, received support when electron microscopy of $KMnO_4$-stained cells, revealed the presence, in a large variety of living cells, of unit membranes consisting of two dark stained lines separated by a lighter line, the total thickness being in the neighborhood of 100 Å.[15] Since electron microscopy has revealed that unsaturated lipid can be stained by heavy metals, the Davson-Danielli model of the cell membrane was widely regarded as having been verified. Thus far, we have followed the development of the membrane theory on the basis of the pattern of interaction with noncharged, water-soluble materials: water and nonelectrolytes. However, it was not water and nonelectrolyte behaviors alone that guided the development of the membrane theory. Other scientists working with ions had also decided to resurrect the sieve idea.[16]

An outstanding phenomenon in the realm of ionic permeability of the living cell is the general tendency of cells to swell in concentrated KCl solution and to shrink in concentrated NaCl solutions. Following the basic tenets of the membrane theory, this could only mean that the cell membrane is permeable to K^+ ions (and Cl^- ion) but impermeable to Na^+ ion. In a historically important paper, Boyle and Conway[17] then postulated that the pores of the cell membrane are of such a critical size that they allow the passage of water, the smaller hydrated K^+ ion, and H^+, Cl^- ion, but not the passage of the larger hydrated Na^+, Ca^{++}, and Mg^{++} ions. This sieve idea also brought into line the phenomenon of electrical potentials of certain living cells, which had long been known to respond to changes in the external K^+ ion concentration but not to external Na^+ ion concentration. Boyle and Conway's sieve idea provided a mechanistic basis for interpreting this difference in terms of Bernstein's theory of cell potentials.[18]

Scientists working with nonelectrolytes have dealt largely with kinetic phenomena: i.e., rates of passage in, out of, or through cells. In their studies even the most impermeable nonelectrolytes have not been shown to be absolutely impermeable.[10, 19] Thus, the ether-water distribution coefficient of the highly impermeable glucose is only some ten times less than that of glycerol,[19] long known to be quite permeable. Boyle and Conway's sieve model, on the other hand, demanded absoluteness in the impermeability of the cell membrane to Na^+ ion. Ironically, before Boyle and Conway's theory was published the experiments of Heppel[20] and Steinbach[21] had clearly shown that Na^+ ion can pass into and out of the cells with relative ease. Boyle and Conway's theory of selective Na^+ ion exclusion was thus disproved.

The Postulation of the Na Pump

According to the membrane theory, the cytoplasm is essentially an aqueous solution of salts and proteins that is no different from a test tube full of buffers,

enzymes, and other proteins; only the cell membrane is considered capable of such vital functions as maintaining the separateness of the cell from its environment. Since it is a well-established fact that Na^+ ion can be pumped across kidney tubules and intestinal mucosa against concentration gradients, the fundamental tenets of the membrane theory demand that these pumps be located in the cell membranes. Thus, the discovery of the permeability of the cell membrane to Na^+ ion left to those who did not question the fundamental validity of the membrane theory no alternative than to postulate Na pumps in the cell membranes to explain the low level of this ion in the cell. Indeed, this was by no means the first time that membrane pumps had been postulated. Long ago, Overton had dealt with the same problem and proposed a similar mechanism using a somewhat different name, i.e., "adenoid" (or secretory) activities, for the transport of nonlipid soluble materials across the cell membrane (see Reference 10, p. 8).

As was mentioned earlier, the idea of Boyle and Conway was theoretically self-consistent. It argued that solutes fall into two categories: permeant and absolutely impermeant. A permeant solute distributes itself between the intra- and extracellular phases according to its electrical charge. If the solute contains no net charge, it will be distributed equally between the cell water and the external water. If the solute is charged, its distribution would follow the rule of a Donnan equilibrium: [22]

$$\frac{[p_i^+]_{in}^{1/n}}{[p_i^+]_{ex}^{1/n}} = \frac{[p_j^-]_{ex}^{1/m}}{[p_j^-]_{in}^{1/m}} = r, \qquad (1)$$

where r is the Donnan ratio, $[p_i^+]_{ex}$ is the concentration of the ith cation of valence n in the external solution, and $[p_j^-]_{ex}$ is the concentration of the jth anion of valence m in the internal solution.

Insofar as impermeant solutes are concerned, the Boyle and Conway theory considered the cell membrane an insurmountable barrier. Thus, for these solutes, the intracellular and extracellular phases are thermodynamically isolated systems and, accordingly, their concentrations in these phases should be independent of one another. Heppel's and Steinbach's experiments disproved Boyle and Conway's sieve idea and forced the postulation of the Na pump because they showed that Na^+ ion is actually permeant and that its distribution does not follow the Donnan distribution ratio found, for example, for K^+ ion. It was thought at that time that Na^+ ion was an exception to the rule that still holds for the other solutes.

As years went by, it became increasingly clear that, in certain types of cells at least, molecules as big as porteins [23] and DNA with molecular weights up into the millions can enter the cells.[24] The permeability of other solutes such as Ca^{++},[25] Mg^{++},[26] sucrose [27] and ATP [28, 29]—long considered impermeant—have also been proven permeant one by one. Yet as a rule, their equilibrium distribution ratios do not follow the rules mentioned above (TABLE 1). It thus became necessary to postulate a pump for each one of these solutes and many such pumps were postulated (TABLE 2); still other pumps, as yet unpostulated, would have to be if the membrane theory is to remain self-consistent.

The Energy Need of the Na Pump

Conway was the first to ask the crucial question: [30] How much energy is needed to operate the Na pump? Two years later, Levi and Ussing [31] were

TABLE 1

A COMPARISON OF THE OBSERVED INTRACELLULAR CONCENTRATIONS OF IONS IN FROG MUSCLES AND THE VALUES PREDICTED THEORETICALLY ON THE BASIS OF THE DONNAN MEMBRANE EQUILIBRIUM *

	Extracellular Concentration	Intracellular Concentration	
		Predicted	Observed
	(mM)	(mM)	(mM)
K^+	2.5	128.0	128.0
Na^+	103.8	5250.0	16.9
Ca^+	2.0	5100.0	5.7
Mg^+	1.2	3070.0	15.8
Cl^-	74.3	1.47	1.04
HCO_3^-	25.4	0.50	9.2
lactate$^-$	3.3	0.064	3.5
orthophosphate	3.1	0.013 †	7.7

* For sources of data and the statistical criteria for their selection, see ref. 46, p. 217.

† Predicted on the basis of Donnan equilibrium, the Donnan ratio r is set at 50.59 from K^+ ion distribution observed. Intracellular pH assumed to be 7.0; valency, 1.43.

TABLE 2

POSTULATED MEMBRANE PUMPS *

Solute	Direction	System	Reference
Na, K	coupled	many cells	169
Ca^{++}	outward	RBC, striated muscle	170, 171
Mg^{++}	outward	frog sartorius	172
Choline$^+$	inward	RBC	173
Amino acids	inward	RBC, muscle, tumor	174–176
D-xylose	inward	rat diaphragm	177
D-xylose	outward	rat diaphragm	178
Na^+	inward	frog sartorius	179, 180
Noradrenaline	inward	vascular smooth muscle	181
Prostaglandins	inward	mammalian liver	182
Curarine	inward	mouse diaphragm	183
Br^-, I^-, ReO_4^-, WO_4^-	outward	Ascites	184
Cu^{+2}	inward	Ascites	185
Aminopterin	inward	Yoshida sarcoma	186
Cl^-	inward	squid axon, motor neurons	187, 188
Mn^{++}	inward	*E. coli*	189
Cl^-	outward	*E. coli*	189
Sugars	inward	*E. coli*	189
Amino acids	inward	*E. coli*	189
Tetracycline	inward	*E. coli*	190

* Data collection was more or less arbitrary and not intended to be comprehensive.

able to answer this question with the aid of an adequate tracer technique. Their answer was that more than 30% of the maximally available energy is needed for the Na pump, assuming that all processes involved are 100% efficient. These processes include conversion of the energy in glucose to a form suitable for operating the pump and the pumping process itself. Later, Harris [32] and Keynes and Maisel,[33] offered evidence to show that a figure of 20% would be more accurate.[34] Apparently, Levi and Ussing were fully aware that these assumptions were overly optimistic. They suggested, therefore,[31, 35] a mechanism whereby the energy need could be reduced. Their postulation is that part of the labeled Na^+ ion efflux observed might have been due to a one-to-one Na^+ ion exchange between the intracellular and extracellular phase. Such an exchange, called Ussing's Exchange Diffusion, would entail no energy expenditure, thereby reducing the calculated rate of Na^+ ion pumping and hence the energy demand.

The postulated mechanism also provides a ready means for testing the question of its own existence if Na^+ ion were removed from the external medium, the fraction of Na^+-ion exchange due to such a mechanism would be reduced to zero. Based on this type of experiment, Hodgkin and Keynes concluded that exchange diffusion does not exist in squid axon,[36] Hoffman and Kregenow found it does not exist in red blood cells,[37] Buck and Goodford found that it does not exist in guinea pig *Taenia coli*.[38] Only Keynes and Swan [39] contended that half of the Na^+ ion efflux from frog muscle is due to exchange diffusion. This conclusion was in conflict with an earlier study by Ling [34] as well as a later study by Ling and Ferguson.[40] The reason for this conflict will be discussed in a later section. In general one may say that this mechanism, postulated to bail out the Na pump, has not been experimentally substantiated.

In later years, Essig made another attempt in the same direction.[41] Using irreversible thermodynamic arguments, he showed that it is possible to overestimate the active transport rate from the unidirectional efflux rate. However, in order for this overestimation to be nonnegligible, a large part of the Na influx must come about through the pump mechanism. In fact, to overestimate the pump rate by 100% for frog muscle Na^+ efflux, the passive permeability channels must offer about six times the resistance to Na flow as the inward active pump. There is no evidence to date, however, that the majority of inward Na flux is active in a normal Ringer's solution.

The Efficiency of the Metabolic Degradation of Glucose and of the Na Pump

Energy in the glucose molecule cannot be directly utilized to operate the postulated pump; it is generally agreed that this energy must first be converted to ATP. The degradation of glucose into CO_2 and H_2O is associated with a standard free energy change of -2850 kJ/mole. The standard free energy of hydrolysis of ATP is accepted as about -29 kJ/mole. Theoretically, therefore, the degradation of one mole of glucose should be able to generate about 100 moles of ATP. Actually, only 36 molecules are generated. The efficiency for the conversion of glucose energy into ATP energy is, therefore, only 36%. Thus, even if the pumping process is 100% efficient, the minimal energy needed according to the figure cited by the proponent of the membrane theory should not be 20% but $0.2/0.36 = 55\%$. However, as will be pointed out

further on, even this figure represents a gross underestimation, due to a serious error in an assumption made in estimating the rate of Na^+ ion efflux.

More Decisive Experiments on the Theoretical Feasibility of the Membrane Pump Theory

Widely cited as an example of the behavior of living cells in general, is the finding in Sepia and Loligo nerves that interference with metabolism slows down the rate of Na^+ ion efflux.[42, 43] Yet, since 1952 it has been pointed out that the Na^+-ion efflux from frog muscles at 0° C is not materially affected by the combined action of iodoacetate and cyanide (44, 45, p. 766). This finding has been confirmed repeatedly.[33, 46] The poisoned muscles can maintain normal K^+ and Na^+-ion concentrations for hours. Since both respiration and glycolysis are blocked, the only remaining energy sources are ATP and creatinephosphate originally present in the tissue. By measuring the fall in concentrations of these compounds and by comparing the maximal available energy with the minimal energy needed for the pump, the senior author of the present paper concluded that the Na pump under this defined condition would consume at least 15–30 times as much energy as was available, again assuming that the pump operates at 100% efficiency.[46]

Additional Unknown Sources of Energy

The question may be raised whether in these IAA-CN-N_2-poisoned muscles, there might not have been some other significant, as yet undiscovered, energy source that might have provided for the energy need of the Na pump. Such possible sources might be enthalpic or entropic or both. An entropic source of free energy, by definition, signifies that part of the system is undergoing disintegration. The functional integrity of these poisoned muscles, as evidenced by their normal contractility and electrical activity, as well as the normal K^+ and Na^+-ion distribution in IAA-CN-N_2 experiment poisoned muscles showed clearly that no such entropic source of energy could have been present. A more likely energy source might be some enthalpy source as yet unidentified.

Hill and Parkinson [47] found that IAA- and N_2-poisoned muscles, when stimulated to exhaustion, yielded 367 cal/g fresh tissue. Hukuda [48] found a somewhat higher amount (420 cal/g). The heat of hydrolysis of one mole of creatinephosphate (CrP) is 10,700 cal † and the heat of one mole of ATP $12{,}500 \times 2 = 25{,}000$ cal.† Normal frog muscles contain about 25 mmoles/kg of CrP and 5 mmoles/kg of ATP. The total heat from the hydrolysis of these components in the presence of muscle proteins could amount to $10{,}700 \times 0.025 + 25{,}000 \times 0.005 = 393$ cal/g, which exceeds the value obtained by Hill and Parkinson, but is somewhat lower than Hukuda's figure. In reality some CrP and ATP hydrolysis must have occurred during the initial 45 minutes of incubation in IAA and O_2 before thermal measurements began. The data given in Table 3 were gathered from unpublished work of Ling. They show that, following precisely the treatments described by Hill and

† These values were measured in the presence of muscle proteins and in part could reflect the heat of neutralization of the proteins (for discussion, see Ref. 46, p. 129).

TABLE 3

CREATIVE PHOSPHATE, ATP, AND LACTATE CONTENTS OF IAA-N_2-POISONED FROG SARTORIUS MUSCLE *

		Expt. No.	Mean ± S.E. (μmoles/g)	ΔH (cal/g)
Creatine phosphate	control	5	26.0 ±0.79	
	experimental	5	23.68 ± 1.83	0.249
ATP	control	5	3.86 ± 0.69	
	experimental	5	2.99 ± 0.68	0.102
Residual lactate production	control	6	3.86 ± 0.46	
	experimental	6	12.38 ± 2.17	0.135
				0.486

* One muscle from each frog was analyzed for its creatine phosphate (CrP) and ATP following isolation (controls). The pairs were treated to the higher concentration of Na-iodoacetate (1/12,500) for 45 min with oxygen bubbling and then analyzed for CrP and ATP. The data so obtained are labeled "experimental." The conditions of the experiments were made to conform as closely as possible to those used by Hill and Parkinson; these included the composition of Ringer's solution, temperature (20.3°C). Lactate contents of muscle treated in a similar manner were referred to as control. Lactate contents after these IAA-poisoned muscle had been stimulated in N_2 until complete exhaustion were called experimental.

Parkinson, sartorius muscles contained enough CrP and ATP to yield a total heat of 351 cal/g. Residual lactate formation added another 135 cal/mole. The total, 486 cal/mole, is ample enough to account for the higher figure obtained by Hukuda.‡ One must emphasize that both Hill and Parkinson and Hukuda have proven that the total heat output is constant and independent both of the temperature and of the frequency of stimulation (0° vs. 20° C). Had there been some additional source of energy not inhibited by the combined action of IAA and pure nitrogen, this could hardly have been the case.

In conclusion, we must state that there is no evidence for the existence of hidden enthalpic or entropic sources of energy large enough to make any difference in balancing the enormous deficit of energy balance. It is now ten years since the original work on this subject was published. The conclusion was mentioned repeatedly in many subsequently published articles;[49–54] nevertheless, it has not aroused much concern among the proponents of the membrane theory. It is as though there were two separate worlds that we inhabit, and that what is primary and what is secondary are totally different in each of them.

‡ Hill & Parkinson compared these values with heat from their estimates, (but not actual analyses) of CrP & ATP changes and reached the conclusion that only half of the observed heat was accounted for. They suggested a number of possible sources for the extra heat. The most significant one was the possibility of a small residual amount of glycolysis. This conjecture was confirmed by Ling (see Table 2).

Evidence for a Major Error in Assessing the Intra-Extracellular Exchange Rate of Na^+ Ion and in the Membrane Permeability of Na^+ Ion

In their first study of Na^+-ion efflux, Levi and Ussing found that their efflux curve could be resolved into two fractions.[31] They naturally assigned the fast fraction to Na^+ ion to the extracellular space and the slower fraction (with a half-time of exchange of about 30 minutes) to the intracellular-extracellular exchange, i.e., as an expression of the pumping rate. Their basic assumption was immediately accepted by most workers, and it has been the foundation for the majority of experimental studies of the Na pump in frog muscle for the last 30 years.

In 1959 Hodgkin and Horowicz[55] showed that the Na-ion efflux from single frog muscle fibers, plotted semilogarithmically, appears as perfectly straight lines; without any extracellular space, the fast fraction seemed to vanish. Undoubtedly, this piece of work reassured most scientists working on this subject of the correctness of Levi and Ussing's original interpretation. Ling, on the other hand, has shown cause to doubt these assumptions. Ling and Ochsenfeld now have evidence which makes it all but certain that this interpretation was erroneous.

Figure 1 shows the K^+-ion efflux curve of a sartorius muscle that had been equilibrated with ^{42}K until its intracellular K^+ ions had reached equilibrium with the labeled K^+ ion. The washing solution contained 45 mM K^+, which so hastened the K^+-ion exchange that in less than 10 hours more than 90% of the labeled K^+ ion had exchanged along a single exponential time course. These data clearly showed that in this case, there was a clearly surface- (or membrane-) limited exchange of K^+ ion, the use of whole sartorius muscle having left its exponential nature unchanged. Figure 2 shows the Na^+-ion efflux curve of a similar sartorius muscle. Even after corrections had been made for the Na^+ ion associated with connective tissue elements, the efflux curve does not follow a single exponential course. The question is: Could the faster fraction or fractions be entirely due to the extracellular space?

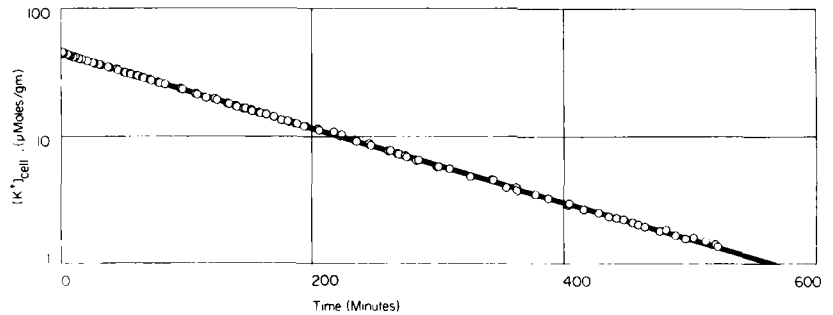

Figure 1. The efflux of ^{42}K-labeled K^+ ion from a sartorius muscle washed in Ringer's solution containing 46.7 mM K^+ ion. A sartorius muscle weighing 75.2 mg was incubated initially in Ringer phosphate solution containing 2.5 mM ^{42}K-labeled K^+ ion at 0°C for 21.5 hr. It was then washed at 25°C in a 3:1 mixture of normal Ringer phosphate with KH_2PO_4-KOH buffer containing 0.135 M K^+, at a pH of 6.9.

Johnson,[56] using the fast fractions to measure the extracellular space, concluded that the extracellular space in the sartorius muscles of *Rana temporaria* and *Rana esculenta* was 37.2% ± 2.9% (S.E.), while Mullins and Frumento [57] arrived at a figure of 22% ± 2% (S.D.) in *Rana pipiens*. TABLE 4 represents a random collection of our own data showing a fast fraction of 19.1% ± 1.21% (S.E.). We submit that these values are too large to represent the true extracellular space. The most compelling reason for this contention derives from the chloride and bromide ion concentration found in frog sartorius muscles.

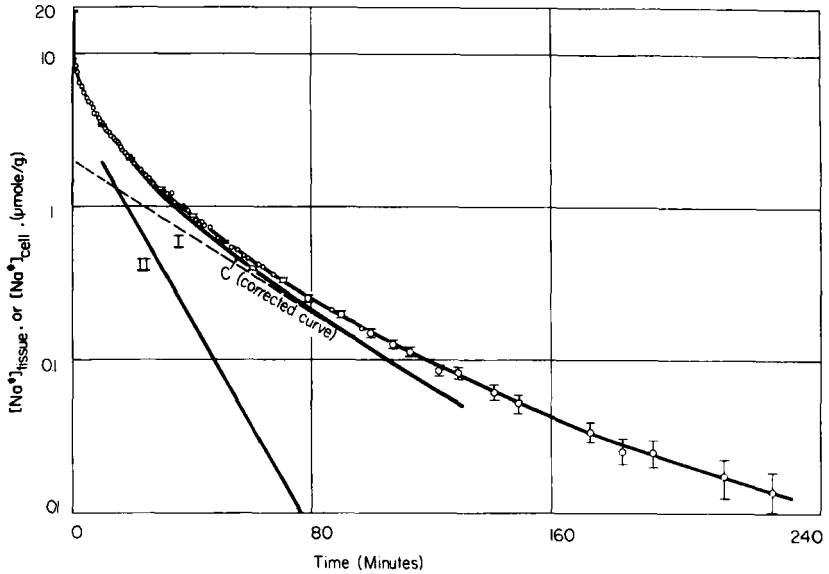

FIGURE 2. Time course of labeled Na^+-ion efflux from a sartorius muscle washed in normal Ringer Phosphate Solution at 25°C. Muscle incubated in Ringer phosphate solution containing ^{22}Na for 16 hr at 4°C. Correction for the connective tissue contribution was made on the basis of a similar efflux curve from a piece of connective tissue isolated from the same leg of the same frog and incubated in the same labeled solution for roughly the same length of time. C is the corrected curve, which can be resolved into Fractions I and II. (From Ling. Courtesy of Physiological Chemistry & Physics.)

In TABLE 5 we have collected all published data on the chloride ion content of frog sartorius muscle known to us up to 1941. Applying the criteria of selection cited in the legend, we find that the acceptable data from Chao,[63] Fenn and Cobb,[64] and Boyle and Conway [17] agree within 5% of each other, giving an average of 9.8 mmoles per kg of frog muscle. The chloride-ion concentration in frog plasma is, according to Fenn, 74.3 mM/kg.[64] Thus, the total chloride space is no more than 15%. Similarly, the equilibrium bromide-ion space is 16.3% ± .6% (from 13 determinations).[65] If the extracellular space were truly 19% or higher, the chloride and bromide-ion concentration in the cell would be negative. This is obviously impossible.

TABLE 4

RANDOM COLLECTION OF OUR DATA ON THE EXTRACELLULAR SPACE—DEFINED AS THE TOTAL FAST-EXCHANGING FRACTION FROM AN EFFLUX CURVE AFTER SUBTRACTING A SLOWER FRACTION *

Date		Incubation Temp.	Incubation Duration	Temp. Washout	$[Na]_{tis}$ (μmoles/g)	$t_{1/2}$	$[Na^+]_{in}$ (μmoles/g)	$[Na^+]^*_{e.c.s.}$ (μmoles/g)	$_{e.c.s.}$* (%)
9–16, 1960	I	2°C	165 hr	00°C	25.3	220 min	4.0	21.3	20.5
	II	2°C	165 hr	25°C	22.6	27 min	5.5	17.1	16.4
9–22, 1960	I	2°C	7 days	0°C	32.9	330 min	7.4	25.5	24.5
9–23, 1960	I	2°C	7 days	0°C	21.2	36 min	6.2	15.0	14.4
	II	2°C	7 days	25°C	32.9	360 min	7.4	25.3	24.3
10–14, 1960	Ia	2°C	2 days	25°C	22.3	30 min	8.1	14.2	13.7
10–21, 1960	IX	2°C	3 days	25°C	23.0	30 min	7.2	15.8	15.2
11– 3, 1960	I	2°C	22 hr	25°C	29.2	43 min	6.8	22.4	21.5
12– 2, 1968	A1	25°C	2 days	25°C	24.9	28 min	10.0	14.9	14.3
	A2	25°C	2 days	25°C	30.5	46 min	9.4	21.1	20.3
7–20, 1971	Ib	25°C	18 hr	25°C	27.5	34 min	9.4	18.1	17.4
	IIb	25°C	18 hr	25°C	40.5	52 min	7.0	33.5	32.2
7–21, 1971	Ia	25°C	18 hr	25°C	30.0	43 min	9.0	21.0	20.2
	IIb	25°C	18 hr	25°C	24.0	32 min	8.1	15.9	15.3
	Ib	25°C	18 hr	25°C	30	60 min	6.0	24.0	23.1
7–23, 1971	Ia	25°C	18 hr	25°C	23.5	37 min	5.0	18.5	17.8
7–29, 1971	Ia	25°C	46 hr	25°C	22.4	26 min	8.0	14.4	13.8
Mean ± S.E.						37.4±2.7			19.1±1.21

* It should be pointed out that, in our opinion, this "extracellular space" is composed of true extracellular space and a fast-exchanging fraction Na^+ ion from the muscle cells.

TABLE 5

CHLORIDE ION CONTENT OF FROG MUSCLE *

	Katz[58]	Urano[61]	Meigs and Ryan[61]	Maurer[62]	Cho[63]	Fenn[64]	Boyle and Conway[17]
Number of detns.	2	2	2	23	94	79	13
Cl content, μmole/g (±S.E.)	11.2 not included	12.5 not included	18.6 not included	15.8±0.8 not included	10.4± included	8.4±6 included	10.5±1.4 included
Average						9.8	

* Criteria for choosing data: number of determinations must exceed 10, and the S.E. must be smaller than 5% of the mean. The first four sets of data were excluded for one or the other of these reasons.

In view of the crucial role played by the extracellular space in this and other problems, we attempted to find new ways to determine the extracellular space, with the following results:

(1). Using a new extracellular space probe, poly-L-glutamate, Ling and Kromash found a ceiling value of 8.9%.[66]

(2). By comparing the sorbitol space and sucrose space of whole sartorius muscle with the sorbitol and sucrose contents of similarly treated, isolated single muscle fibers, Ling and associates reached the conclusion that the extracellular space could not exceed 10%.[67]

(3). As was mentioned earlier, the total Br^--ion concentration in frog sartorius muscle is 16.3% of the extracellular Br^--ion concentrations. After correction for Br^- ion in the connective tissue elements, the Br^- ion efflux from sartorius muscle falls neatly into two fractions. The faster fraction, representing the extracellular space, averages 8.2% ± 0.13% from 13 measurements.

Thus, these independent measurements all point to an extracellular space of less than 10%.§ Since the fast fraction of Na^+-ion efflux is too large to be attributed to the extracellular space, there must be an additional source or sources of rapidly exchanging labeled Na^+ ion.

FIGURE 3 shows that a fast exchanging fraction of Na^+ ion persists in the efflux curves of small bundles of muscle fibers containing 92, 13, 3, and 1 single fiber.[68] Since single muscle has no extracellular (or interfibular) space (the adhering layer of fluid is washed away in a fraction of a second), we concluded that the Na^+-ion efflux from muscle cells is not a simple exponential. This conclusion was in direct conflict with that of Hodgkin and Horowicz, cited earlier. How can this conflict be reconciled?

§ One word of caution. The extracellular space depends upon the procedure used to remove adhering fluids on the tissue surface. In our studies a standardized procedure was adopted and used throughout.[67]

In presenting their findings, Hodgkin and Horowicz cautiously pointed out that their technique did not permit a reading until 10 minutes after washing began, and that readings were taken at 5-minute intervals. The U-tube technique we introduced [69] represented a distinct technical advantage, since we were able to make the first reading a few seconds after washing began and to make readings thereafter at 20-second intervals. In the inset of FIGURE 4 we have redrawn the data of Hodgkin and Horowicz as they were published, showing clear-cut straight lines in semilogarithmic plots. In the main part of the Figure are replots of the data shown in FIGURES 2 and 3, as well as other data on whole sartorius muscles. With the elimination of all points before 10 minutes and the retention of points only at 5-minute intervals, as in Hodgkin and Horowicz, all the unambiguously complex two-component curves have straightened out to assume the appearance of single straight lines.[68] These analyses led us to conclude that the Na^+-efflux curve from single frog muscle fibers is different from that of K^+-ion efflux. At least two fractions emerge from the same cell.

Keynes and Steinhardt [70] had earlier suggested that the faster-exchanging

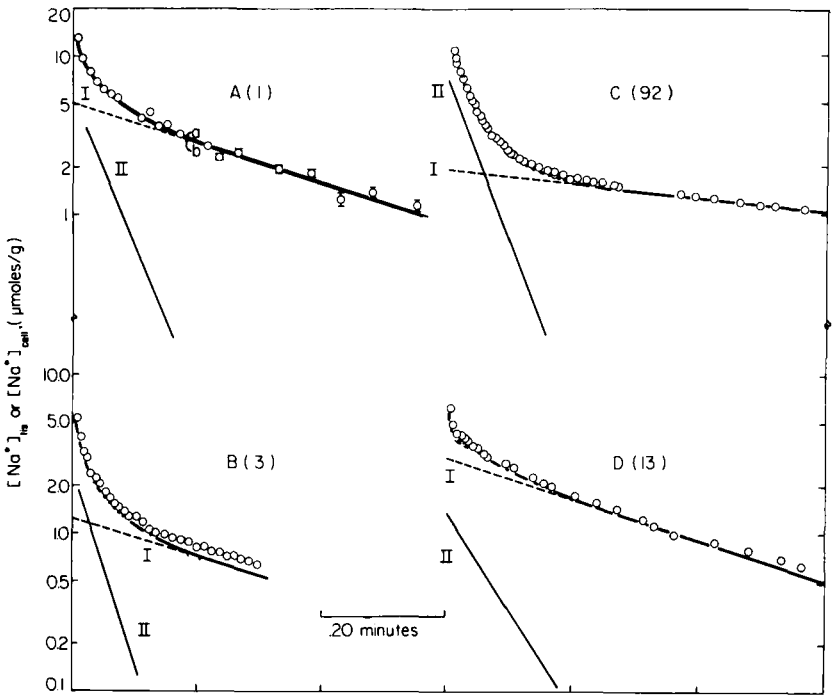

FIGURE 3. Time course of labeled Na^+ ion efflux from a single fiber and small multiple fiber bundles. Number of fibers is indicated on Figure. All fibers were washed in normal Ringer phosphate at 25°C except B, which was washed at 0°C. Correction for connective tissue was made on the basis of a composite curve of Na^{22} ion efflux from similarly incubated connective tissues from four frogs. Curve C is not shown in FIGURE A, since it nearly coincides with the uncorrected curve. (From Ling. Courtesy of Physiological Chemistry & Physics.)

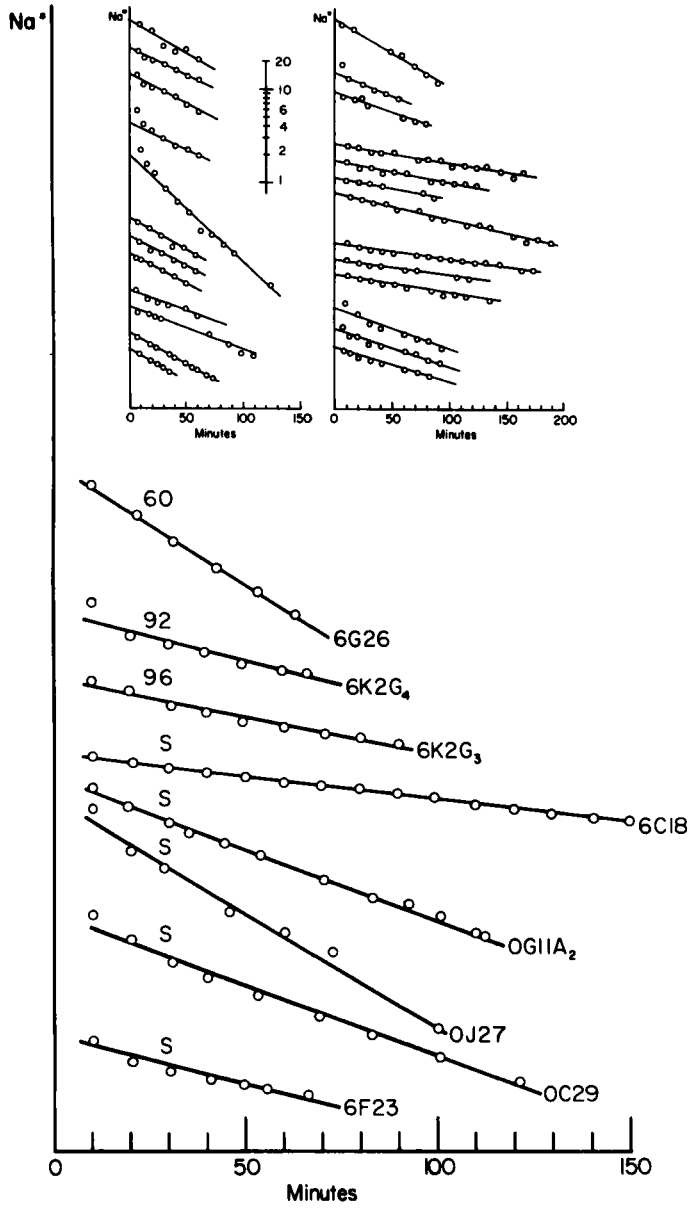

FIGURE 4. Replot of experimental data. The large graphs include some of the data from similar experiments shown in FIGURES 2 and 3, replotted to correspond to time intervals used by Hodgkin and Horowicz.[55] The numbers above the curves refer to the number of fibers in our preparations; the curves marked S were derived from whole sartorius muscles. The inset shows data of Hodgkin and Horowicz redrawn from their figure.

fraction of Na^+ ion came from the sarcoplasmic reticulum. However, this postulation is contradicted by the anatomical demonstration that only the T-tubules are open to the outside,[71] that the T-tubule space, at some 0.2–0.4% in volume,[72-74] can be reached by extracellular space probes, and, therefore, is already included in the below-10% figure cited. However, even if we assume this membrane separating the sarcoplasmic reticulum to have specific high permeability toward small molecules and ions, there are still ample reasons to reject Keynes and Steinhardt's suggestion: (1) No such fast fraction is observable in the Br^--ion exchange.[65] (2) When sartorius muscles are exposed briefly to Ringer solution containing 100 mM K^+ or Rb^+ ion, the efflux curves

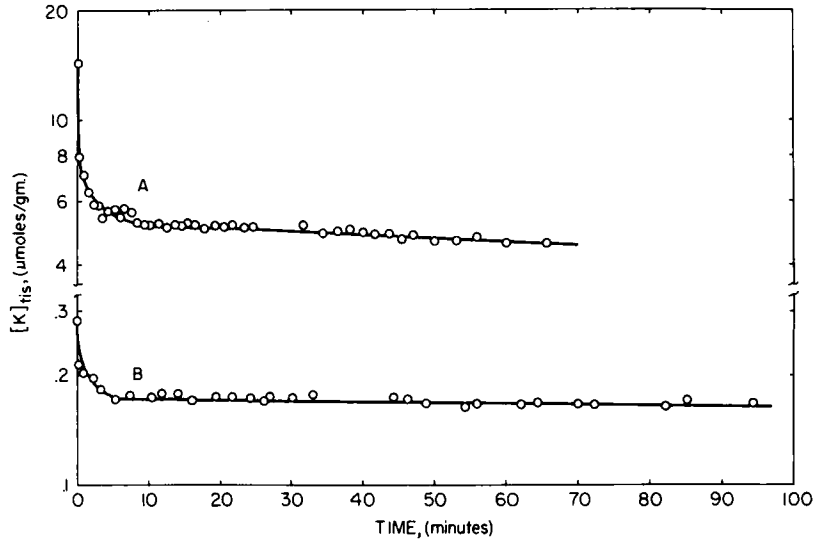

FIGURE 5. K^+-ion-efflux curves of frog sartorius muscles briefly exposed to ^{42}K-labeled Ringer's solution. **A:** 7-minute exposure in 0.118 M KCl with enough glucose to prevent swelling. **B:** 7.9-minute exposure to normal Ringer containing 2.5 mM labeled K^+ ion. All incubation and washout carried out at 0°C. Fast fraction was $\frac{100}{14.3-5.4} = 8.9\%$ in A and $\frac{0.282-0.174}{2.5} = 4.3\%$.

can be resolved into two single fractions—a very fast fraction corresponding to an extracellular space of below 10% and another slow fraction from the cell. No second fast fraction exists (FIGURE 5).

None of these data supports the Keynes and Steinhardt postulation. Facts to be cited below strongly indicate another interpretation.

THE Na^+-ION EFFLUX IN IAA-POISONED MUSCLES FROM NORMAL STATE TO DEATH

We have already mentioned the finding that arrest of respiration and of glycolysis do not materially alter the rate of Na^+-ion efflux at 0° C. In work

soon to be published, Ling and Ochsenfeld have further pursued this study. At 0° C, the Na^+-ion efflux curve can be resolved into three fractions called α, β, and γ, in addition to a very rapidly exchanging fraction similar to the fast fraction seen in data such as those shown in FIGURE 5.[46] When muscles have been treated with a low concentration of iodoacetate (0.1 mM), K^+ ion is gradually lost and Na^+ ion gained; the level of these ions in the cells at any time is determined by the amount of ATP remaining in the cells (Reference 46, p. 252).[75, 76] When ATP reaches the near-zero level, K^+ ion in the cells approaches that in the external medium, and the total Na^+-ion concentration in the cells reaches a concentration about 10 mM higher than that in the external medium. By following the Na^+-ion efflux in IAA-treated muscles after their ATP contents have fallen to different levels, we can make the following observations:

1. The γ-fraction alone or in conjunction with the β-fraction (see below) has been regarded for the last thirty years by the proponents of the membrane theory as representing intra-extracellular Na^+ ion exchange, its rate determined by the membrane's permeability. The half-time of exchange ($t_{1/2}$) of this fraction undergoes some fluctuation, but these fluctuations do not show any definite trend. The total amount of Na^+ ion belonging to this fraction is indicated by the intercept of the fraction at 0 time, which was found to remain unchanged at about 10 μmoles/g of muscle cell from the time the muscle cells were perfectly normal until they were dead.

2. The β-fraction also remains more or less unchanging both in $t_{1/2}$ (ca. 20 min) and in intercept on the ordinate (ca. 10 μmoles/g).

3. The α-fraction also remains unchanged in its $t_{1/2}$ (at about 7 min). In contrast to the other two fractions, however, its intercept on the ordinate, which represents the amount of Na^+ ion belonging to this fraction, rises steadily from a value of about 7 μmoles/g in normal muscle to 100 μmoles/g in dead muscle. From these data one concludes: (1) that the γ-fraction does not represent the bulk of intracellular Na^+ ion and its $t_{1/2}$ does not reflect the rate of intra-extracellular exchange, limited by the cell membrane or its pumping activity. If it did, the total amount of Na^+ ion in this fraction, shown as the intercept on the ordinate, should have increased steadily with the deterioration of the muscle, reaching the same concentration as that in the external solution when the muscle is dead; (2) that the α-fraction, which has long been regarded as representing Na^+ ion in the extracellular space, or according to Keynes and Steinhardt, Na^+ ion in the sarcoplasmic reticulum space, in fact represents neither of these. It is this fraction that increases progressively with deterioration until it reaches the same concentration as in the external medium; the α-fraction must represent intra-extracellular exchange.

We may then ask: Could the exchange rate of the α-fraction be regarded as representing the rate of Na pumping? The answer is that this is also highly improbable; throughout the dying process, the $t_{1/2}$ of the α-fraction remains unchanging. Now the rate of Na^+ ion pumping (J) in terms of the membrane theory is approximately equal to the total measured efflux rate (Reference 52, p. 105):

$$J = \frac{V}{A} k_{Na} [Na^+]_{in}, \tag{2}$$

where

$$k_{Na} = \frac{0.693}{t_{1/2}} \tag{3}$$

A/V is the surface volume ratio of the muscle cells, and k_{Na} is the specific permeability constant in Sec-1. An unchanging $t_{1/2}$ means as unchanging k_{Na}. Meanwhile, $[Na^+]_{in}$ has been steadily increasing with the cell deterioration. Taken together, the data show that in terms of the membrane-pump model, the pumping rate, J, is steadily increasing as the energy source is steadily reduced. A completely dead muscle with no energy source whatsoever pumps faster than ever. This is clearly illogical and it argues against the membrane pump model.

The mistaken significance of the γ-fraction has led to other serious errors that need to be corrected. Thus, the true intracellular-extracellular exchange rate represented by the $t_{1/2}$ of the α-fraction has been shown to be approximately eight times faster than the exchange rate of the γ-fraction (both at 25° C).[68] The minimal energy need of the Na pump in a resting frog sartorius muscle is, therefore, not 54% but $8 \times 54\% = 432\%$, even as we continue to assume that the pumping operation is 100% efficient.

A New Model of the Living Cell According to the Association-Induction Hypothesis

The new model of the living cell presented by the association-induction hypothesis is based on the well-known fact that as a rule living cells are composed of some 20% proteins and 80% water, and on the postulation that in the living cell, proteins, water and solutes exist in a physical state different from that of an aqueous protein solution. This living state of the protoplasm is maintained partly by its structure, which is due largely to the specific interaction of proteins among themselves and with certain key controlling agents called "cardinal adsorbents." A prominent example of a cardinal adsorbent is ATP. The mechanism of control exercised by cardinal adsorbents will be discussed in a subsequent paper in this monograph on the control of cooperative phenomena.[77]

Proteins in the resting cells may not exist in the stable state they assume (e.g., the α-helical state) when isolated and dissolved in water. In this resting state the backbone NHCO groups may be more available to react with water and other molecules, and the side chain functional groups may exhibit properties that can be expressed in terms of a specific set of c-values and c′-values (a measure of the electron density) (Reference 46, p. 58). These basic concepts were derived from theoretical calculations, showing that the same β and γ carboxyl groups may prefer K^+ over Na^+ ion or prefer Na^+ over K^+ ion, depending on the c-value. The c-value, in turn, is controlled and modulated by the cardinal adsorbents.

Juxtaposed surfaces with a checkerboard array of positive and negative charges can polarize multilayers of water hundreds of molecules thick. This is well illustrated by the effect of polished glass surfaces 0.1μ apart in depressing the freezing point of water held between them to below −90° C and in reducing the vapor pressure of the water to near zero, even at 300° C.[78] Evidence that the alternatively positive and negative NHCO sites of proteins in the proper conformation may also polarize water in multilayers has also been discussed recently.[79]

It is important to emphasize that the association-induction hypothesis does not consider all cells at all times to be in the same physical state defined by

one unique assembly of c-values, nor does it hold that all cell water can exist only in one uniquely defined state of polarized multilayers. Indeed, it is the ability to change from one metastable state to another that has made these physical states part of the reversible mechanism that distinguish the living from the dead.

Ion Distribution in Living Cells

The unusual physical state of the protoplasm is reflected in the distribution patterns of ions and other solutes in the living cell. A general equation for solute distribution in living cells has been described [54] and is referred to in another paper in this monograph.[77] For the sake of simplicity we choose to present here a much simpler expression, an equivalent form of which was first used by Troschin to describe sugar distribution in erythocytes.[80]

$$\underset{\text{(total)}}{[p_i]_{in}} = \underset{\text{(free fraction)}}{\alpha q_i\ [p_i]_{ex}} + \underset{\text{(adsorbed fraction)}}{\frac{[f]K_i[p_i]_{ex}}{1 + K_i[p_i]_{ex} + K_j[p_j]_{ex}}}, \qquad (4)$$

where $[p_i]_{in}$ is the intracellular concentration of the ith solute in the cell in μmoles per gram of fresh tissue, $[p_i]_{ex}$ and $[p_j]_{ex}$ are the external concentrations of the ith and jth solutes, respectively, at equilibrium, α is the water content of the cell in ml per gram, and q_i is the all-important equilibrium distribution coefficient of the ith solute in the cell water. Thus, the first term on the right-hand side of Equation 4 refers to the ith solute in the cell water and the second term to the ith solute adsorbed. Equation 4 per se is not uniquely biological, since it is equally applicable to solute distribution in killed cells and in protein solutions. The truly distinguishing part of Equation 4 lies in the high degree of specificity assigned to the values of q_i, K_i, and K_j, which are not usually observed in dead cells or in a simple aqueous solution of proteins.

The Mechanism of Solute Exclusion

A q_i value lower than unity means that the ith solute is excluded from the cell water, as in the case of Na^+ ion. We have already discussed at some length why the absolute ion impermeability concept in the sieve theory and the steady-state concept of the membrane-pump theory are no longer tenable. The association-induction hypothesis has suggested two mechanisms [50] to account for a low q value. These mechanisms may act singly or jointly. Both are based on the fundamental concept that solute distribution represents equilibrium states.

The first of these mechanisms is what we may call a "microcell exclusion effect"; [81] that is, for electrostatic reasons, the volume occupied by a fixed anion and its counter ion K^+ does not permit the accommodation of additional ions. This effect is limited to charged solutes, i.e., ions. The second mechanism applies to both charged and noncharged particles and reflects the unusual physical state of the bulk of the cell water.[50] A quantitative expression for the degree of exclusion for a specific solute, referred to as the ith, is

$$q_i = \frac{(p.f.)_i^{in}}{(p.f.)_i^{ex}} \cdot \exp\ (-\Delta H^\circ/RT), \qquad (5)$$

where $(p.f.)_i^{in}$ and $(p.f.)_i^{ex}$ are the "partition functions" (in the statistical mechanical sense) of the ith solute in the cell water and in the external medium, respectively. These partition functions measure the quantum mechanically permissible energy states that the ith solute may assume (Reference 46, Chap. 1).

When q_i is lower than unity, it may be because $(p.f.)_i^{in}$ is less than $(p.f.)_i^{ex}$ (e.g., exclusion due to water structure) or it may be because $\Delta H°$, the enthalpy change in moving an ith solute from the external medium into the fixed-charge matrix is unfavorable (e.g., the microcell exclusion effect), or it may be both.

These solutes may be excluded from cell water existing in the state of polarized multilayers for two possible reasons: (1) The structure of cell water in multilayers makes it impossible to form as many and/or as strong H-bonds as are found in free solution. Such an enthalpy effect will be seen as a positive $\Delta H°$. (2) The structure of water in multilayers may reduce the rotational freedom of the solute molecules. This reduces the entropy, i.e., the partition function ratios of Equation 5.

In either case, the lowering of q_i value from unity is highly dependent on the nature of the solute molecule. Simple gaseous molecules will have q_i of unity, as neither enthalpy nor entropy mechanism affects their distribution. Similarly, simple molecules such as methanol would have a q_i value higher than ethylene glycol.

According to this theory, $(p.f.)_i^{in}$ lower than $(p.f.)_i^{ex}$ may obtain only if the ith solute is a multiatomic solute, as in the case of various sugars, or de facto multiatomic, as in the case of Na^+ ion due to the layer of water molecules adhering to the monoatomic Na^+ ion. In this aspect, the theory is profoundly different from the theory of nonsolvent water, which is supposed to have no more tendency to dissolve gases than sugars and Na^+ ion.

The Mechanism of Selective Ionic Accumulation

According to the association-induction hypothesis, a typical living cell is seen as a proteinaceous fixed-charge system in which the β and γ carboxyl groups adsorb alkali metal ions in rank orders according to the c-value of these anionic sites. A very important part of the theory is its contention that there is cooperative interaction among these adsorption sites and that cooperative adsorption and desorption under the control of cardinal sites constitutes one of the basic elements of physiological activities. This concept will be dealt with in detail in a later paper in this monograph.[77]

Distribution Patterns of K^+ and Na^+ Ion in Living Muscle Cells

The concentration of K^+ ion in frog plasma and in Ringer's Solution is 2.5 mM; that of Na^+ ion is 104 mM. The intracellular concentration of K^+ ion in frog muscle is about 80 μmoles per gram of fresh tissue and that of Na^+ ion about 20 μmoles/g.

Let us assume that the q value for Na^+ and K^+ ion are similar and equal to, say, 0.1. This will then allow the prediction that $104 \times 0.1 \sim 10$ μmoles/g of intracellular Na^+ ion are in the free state. The remaining 10 μmoles/g

must be adsorbed. On the other hand, the free K^+ ion in the cell is only $2.5 \times 0.1 = 0.25$ μmoles/g. The remaining 79.7 μmoles/g, or better than 99%, must be adsorbed. If Equation 4 has validity, this relatively low adsorption for Na^+ ion and high adsorption for K^+ ion, means that the adsorption constant for K^+ ion, (K_{K^+}) must be about two orders of magnitude greater than that of Na^+, (K_{Na^+}).

Distribution of Na^+ Ion

Given the conditions described, Equation 4 will predict the following. The equilibrium intracellular Na^+ ion concentration, when plotted against the external Na^+ ion concentrations, should have the shape of a hyperbola superimposed on a straight line when the external K^+ ion is normal (2.5 mM). Increase of K^+ ion to 15 mM should abolish the hyperbolic component, since most of the adsorbed Na^+ ion will be chased away, and the distribution curve should now be a straight line representing primarily the free Na^+ ion in the cell. Further increase of K^+ would not make any further change in the Na^+ ion distribution curve. All these anticipated results have been observed. From the slope of the straight line one obtains a q-value of about 0.15.[54]

K^+, Rb^+, Cs^+, and Li^+ Ion Distribution

The K^+ ion, virtually all in an adsorbed state, should follow a Langmuir adsorption isotherm, which can be written in the reciprocal form:

$$\frac{1}{[K^+]_{in}} = \frac{1}{[f]K_{K^+}} (1 + [Cs^+]_{ex} K_{Cs^+}) \frac{1}{[K^+]_{ex}} + \frac{1}{[f]} \qquad (6)$$

This equation predicts that the reciprocal of the equilibrium concentration of labeled K^+ ion in the cell, $[K^+]_{in}$ when plotted against the reciprocal of the external labeled K^+ ion concentration, should yield straight lines, the intercept on the ordinate giving the reciprocal of the adsorption sites [f]. The slope of the straight line should depend on the concentration and adsorption constant of the competing species, which is shown as $[Cs^+]_{ex}$ in Equation 6. These predictions have also been verified.[53] The adsorption constant so obtained showed that the adsorption sites prefer the alkali-metal ion in the order $Rb^+ > K^+ > Cs^+$. Other studies of the equilibrium distribution in muscle cells revealed that they accumulate the five alkali-metal ions in the sequential order of preference: $Rb^+ > K^+ > Cs^+ > Li^+ > Na^+$.[82] This is one of the theoretically predicted orders given in 1962 (Reference 46, p. 77). It must be pointed out, however, that adherence to the Langmuir adsorption isotherm is limited to external K^+-ion concentration equal to or higher than that found in its normal environment. For lower K^+-ion concentration, a more general isotherm is needed.[51]

Nonelectrolyte Distribution

In frog muscle cells, phosphorylation and metabolism of D-glucose comes to a halt at 0° C.[83] Under these conditions, it becomes possible to study the

equilibrium distribution patterns of alcohols and sugars without significant interference from continued degradation of these compounds. FIGURE 6 shows how methanol, ethylene glycol, glycerol, xylose glucose, and sucrose all reach an equilibrium in less than 10 hours and how, from there on, each maintains a level of distribution different from the others. A plot of the intracellular concentration of methanol and D-glucose against the external concentration show a linear relation,[84] suggesting that under these conditions (e.g., the absence of insulin): (1) the bulk of these solutes is not adsorbed but distributed in the cell water; (2) their q-value varies with the number of H-

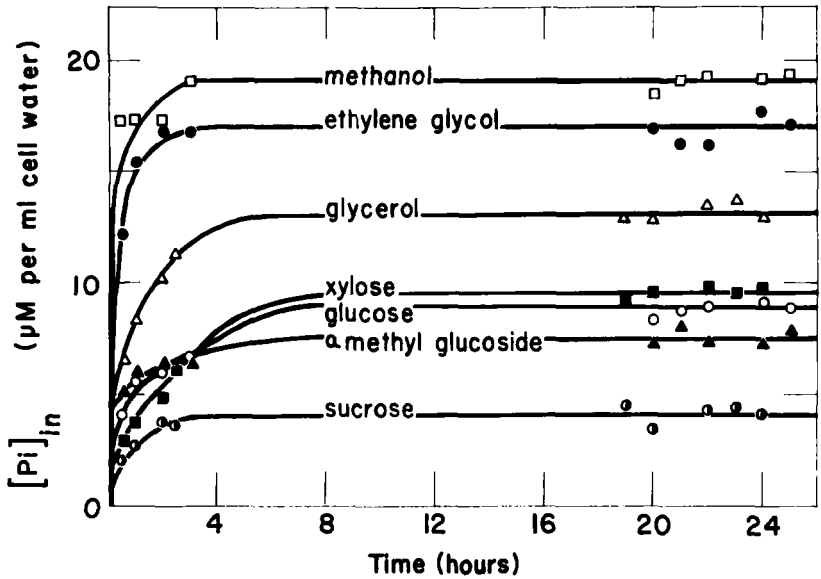

FIGURE 6. The time course of uptake of methanol, ethylene glycol, glycerol, xylose, glucose, and sucrose by frog muscles at 10°C. Muscles isolated the day before and kept overnight at 4°C. The initial concentration of all solutes was 25 mM/l. The final external concentrations were: methanol 17.0, ethylene glycol 17.1, glycerol 18.5 mM/l., xylose 19.7 mM/l., glucose 21.4 mM/l., α-methyl glucsoside 20.8, and sucrose 22.04 mM/l. The q-values are 1.1 for methanol, 0.99 for ethylene glycol, 0.71 for glycerol, 0.48 for xylose, 0.44 for glucose, .36 for α-methyl-glucoside, and 0.18 for sucrose.

bonding groups it possesses; and (3) there is no significant amount of "non-solvent" water that does not dissolve any solutes. All of these indications are in accord with the prediction of the theory stated above.

SELECTIVE ACCUMULATION AND EXCLUSION OF IONS AND NONELECTROLYTES IN ION EXCHANGE RESIN AS A MODEL OF THE LIVING CELL

Wet ion exchange resins have been used as a model of the living cell ever since the introduction of the association-induction hypothesis.[45] According

to the hypothesis, ion exchange resins share important features with living cells because both represent water-filled macromolecular matrix-bearing ionic groups.

Adsorption of Accumulated Ions

Like living cells, certain ion exchange resins can selectively accumulate K^+ over Na^+ ion. Indeed, the early version of the association-induction hypothesis offered a molecular mechanism for the selective K^+ ion accumulation over Na^+ ion in living cells as well as in ion exchange resin [4, 45] in terms of a preferential adsorption of K^+ over Na^+ on fixed anionic sites.¶ At that time, Gregor suggested another theory which involved no specific association of the accumulated K^+ or Na^+ ion with ionic sites.[86] Instead, he suggested that the selection of K^+ ion is the result of a high hydrostatic pressure in the resin. Since hydrated K^+ ion is smaller than hydrated Na^+ ion, the introduction of a mole of K^+ ion into the resin entails less energy expenditure than the introduction of a mole of Na^+ ion; K^+ ion is, therefore, selected over Na^+ ion. The following facts support the association-induction hypothesis, but not Gregor's hydrostatic pressure theory.

The Reversal of Selectivity in Carboxyl Resin

Gregor's theory contends that the selectivity of K^+ over Na^+ reflects an inherent property of this pair of alkali metal ions, and thus predicts the same K^+ over Na^+ selectivity in all negatively charged resin systems. This theory is not in accord with the subsequent finding that in carboxylic ion exchange resins Na^+ ion is selectively accumulated over K^+ ion.[87] || The reversal of selectivity with a change in the nature of the charged groups strongly suggests a more profound role for the specific nature of the anionic groups than that determined by their electrostatic charge. A most straightforward answer to the variability of preference for K^+ or Na^+ ion is that there is adsorption, i.e., the bulk of K^+ or Na^+ ion in either exchange resin exists in an adsorbed state. This intimate association between the counter ions and the fixed anionic sites then offers the chance for resins bearing sulfonate $S(=O)_2-O^-$ and those bearing carboxyl groups, $C(=O)-O^-$ to have profoundly different selectivities for the alkali metal ions.[46]

¶Years later Harris & Rice [85] suggested more-or-less the same mechanism for selective ionic accumulation in ion exchange resin. These authors were apparently unaware of our earlier work on the basis of adsorption on ionic sites.

|| The same observation also showed the inadequacy of the original mechanism that Ling suggested for K^+ ion accumulation which was also based on the hydrated ionic diameter differences [45] and this discrepancy [88] then led to the more general theory of ionic selectivity presented in 1962 as part of the association-induction hypothesis (see Fig. 5).

Competition of Counter Ion Entry into the Resin

Ion exchange resin is a three-dimensional matrix carrying fixed ionic sites. The surface of a resin bead or sheet, on the other hand, is a two-dimensional representation of the three-dimensional bulk phase. If virtually all counter ions are adsorbed on sites in the bulk of the resin, as the association-induction hypothesis contends, this should apply to sites on the surface of the resin as well. As a result, one would anticipate that entry (or exit) of isotope-labeled ion into the resin sheet must be via the process of adsorption into the surface sites followed by desorption. The rate of entry of the ith ion into a resin sheet or bead should be readily derived from Equation 4. In the simplest case,

$$V_i = A_i[p_i]_{ex} + \frac{V_i^{max}\tilde{K}_i[p_i]_{ex}}{1 + K_i[p_i]_{ex} + K_j[p_j]_{ex}}, \tag{7}$$

where V_i and V_i^{max} are, respectively, the initial rate of entry of the ith ion into the resin and the maximum rate of the ith ion entry, when $[p_i]_{ex}$ approaches infinity. The first term on the right-hand side of Equation 7 represents the rate of entry through the water-filled interstices between fixed ionic sites (saltatory route; see Reference 46, Chap. 11), and the second term represents the adsorption-desorption route via the fixed ionic sites on the resin surface. On the assumption that A_i is considerably smaller than $V_i^{max}K_i$, Equation 7 can also be simplified by ignoring the first term on the right-hand side. Written in a reciprocal form, the simplified equation has the form:

$$\frac{1}{V_i} = \frac{1}{V_i^{max}\tilde{K}_i}(1 + \tilde{K}_j[p_j]_{ex})\frac{1}{[p_i]_{ex}} + \frac{1}{V_i^{max}}, \tag{8}$$

which predicts that a reciprocal plot of the initial rate of entry of the ith ion into the resin, V_i, against the reciprocal of the external ith ion concentration should yield straight lines. The slope of these lines varies with the nature and concentration of the competing jth ion. This predicted relationship was actually observed, thereby verifying the assumption that A is indeed much smaller than $V_i^{max}\tilde{K}_i$. This finding can be stated in a somewhat different way: the bulk of counter ions is adsorbed. Few ions are found in the interstitial space in this two-dimensional replica of the three-dimensional resin bulk phase. By inference, we arrive at the conclusion that the bulk of counter ions in the three-dimensional bulk phase is also adsorbed.

Exclusion of Na^+ and Other Ions from the Water in Ion-Exchange Resins

FIGURE 7 shows the equilibrium distribution of labeled Na^+ ion in a sulfonate ion exchange resin. When the external Na^+-ion concentration has reached 1M, the Na^+-ion content in the resin has reached a nearly maximum value. Further, increase of $[Na^+]_{ex}$ is accompanied by a relatively small gain in the intracellular Na^+ ion concentration. We have already presented evidence showing that the bulk of the counter ions in the resin are adsorbed. It is readily understandable why the adsorption sites, once saturated, cannot take up any more Na^+ ion. But the resin contains water. If this aqueous environment is entirely normal, there is no reason why, as $[Na^+]_{ex}$ increases, the resin water should not accommodate more Na^+ ion at the same concentration as that in the external medium.

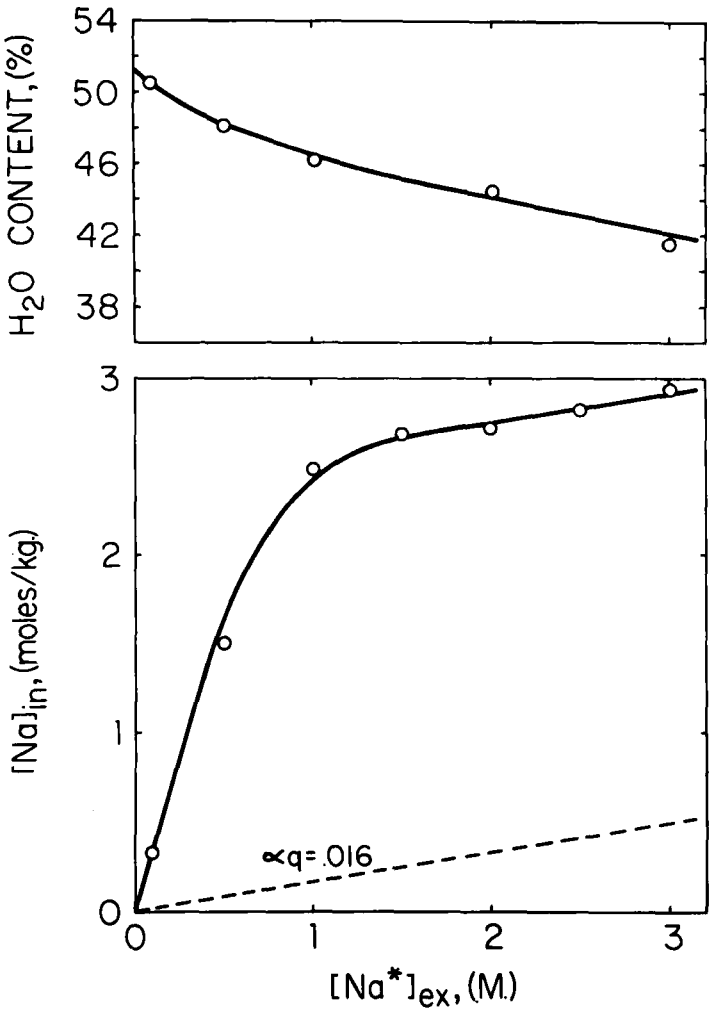

FIGURE 7. The water content and the equilibrium distribution of Na^+ ion in a sulfonate ion exchange resin. Top graph shows variation of water content with Na^+ ion concentration. Sulfonate resin. Bio-rad AG50W-XI.

In reality, this is not so; much less Na^+ ion is gained than in a normal aqueous environment. This phenomenon leaves little doubt that the aqueous environment in the resin excludes Na^+ ion to such a high degree that the q-value is far from unity. Since hydrated Na^+ ion is charged and de facto multiatomic, exclusion of Na^+ ions is most likely to result from both the microcell exclusion effect and from the effect of the reduced solubility of the water existing in a different physical state than normal liquid water. To distinguish between these two effects we need to study the q-value of some other multiatomic but noncharged solutes.

Exclusion of Nonelectrolytes from the Water in Ion Exchange Resin

FIGURE 8 shows the equilibrium distribution of several nonelectrolytes in the water of sulfonate ion exchange resin (cross-linking, 8%; water content, 50%). It is quite clear that the degree of exclusion varies with the complexity of the molecules; it is thus in accord both with the theory of solute exclusion, discussed earlier, on the basis of water existing in the form of polarized multilayers, and with the sequential order of selective exclusion, experimentally demonstrated in living frog muscle cells (FIGURE 6).

EVIDENCE OF WATER EXISTING IN THE FORM OF POLARIZED MULTILAYERS

We have shown that the pattern of Na^+ ion and nonelectrolytes exclusion in living cells bears resemblance to the patterns seen in ion exchange resin. We have interpreted both phenomena as reflecting an unusual physical state of water in both systems (as well as the microcell exclusion effect). Our next question is: Is there other independent evidence that, in such cells as frog muscle, water does exist in the form of polarized multilayers?

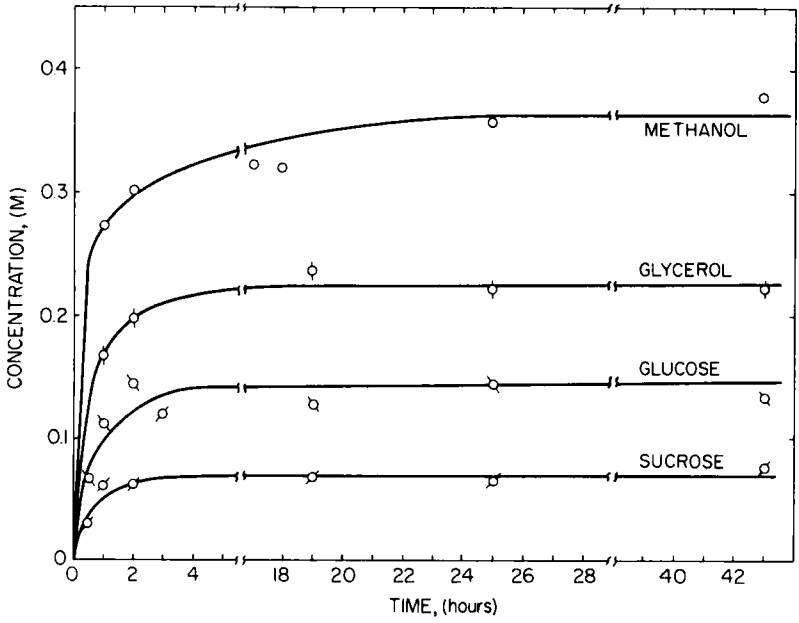

FIGURE 8. Time course of uptake of methanol, glycerol, glucose, and sucrose by sulfonate ion exchange resin. Rexyn RG50 (H^+form) at 0°C. Approximately 5 g of wet resin in 5 ml of solution containing 50 mM acetate buffer at pH 3.75. Final concentration was 0.383M for methanol; 0.382 M for glycerol; 0.485 M for glucose; and 0.458 M for sucrose. The equilibrium distribution coefficients (q-value) were 0.97 for methanol, 0.464 for glycerol, 0.309 for glucose, and 0.153 for sucrose.

Intracellular Freezing Pattern

When supercooled water is seeded with an ice crystal, a typical ice crystal is formed, with featherlike branches going in different directions.[54, 89, 90] This pattern is somewhat distorted, but not basically altered, when ice grows in an aqueous solution of 13% actomyosin.[91] However, the intracellular water in frog muscle supercooled to 3° C will not freeze even if the entire extracellular fluid is frozen. To initiate ice formation in such a supercooled cell, a seeding ice crystal must be brought into direct contact with the cytoplasm. When ice does begin to form, it develops a long spike without branches, in the direction of the long axis of the muscle fiber.[54, 92, 93] If the muscle fiber is twisted, the usually straight ice spikes now grow in a curved manner following the twist. One can readily deduce that it is the longitudinally oriented myofilaments that provide the guidelines.

What seems to be, at first glance, the most puzzling aspect of this type of ice formation is the complete absence of ice crystals growing sideways, either secondarily from the long spikes as branches, or initially at the point of seeding. Since longitudinal spikes can form in any part of the muscle fiber, this means that, throughout the fiber, all directions of ice propagation are forbidden except one. Again, since the bulk of the material at the initiating point is water (which constitutes 80% of the cytoplasm, while protein constitutes only 20%), this means not only that this water is not normal liquid water (which cannot remain supercooled when in contact with seeding crystal) but also that these water molecules must be in the form of a cooperative structure that resists changing to another cooperative state—the tridymite ice-I structure. If the water is not in a crystalline solid state and involves many layers of molecules, the most reasonable interpretation is that these water molecules exist as polarized multilayers.

Bradley Multilayer Adsorption Isotherm

De Boer and Zwikker,[94] and later Bradley,[95] derived equations describing the sorption of gases on solid surfaces in the form of polarized multilayers. It was shown by Mellon and Hoover [96] and by Ling [50] that the Bradley adsorption isotherm best describes the adsorption of water vapor on collagen and on sheep's wool. In 1965 Ling postulated that water in living cells might also exist in the form of polarized multilayers.[50] At that time, there was no experimental evidence to support this hypothesis. In 1969, Ling and Negendank [97] published the results of their investigation of this problem and showed that 95% of the water in frog muscle does follow the behavior predictable on the basis of the Bradley adsorption isotherm:

$$\log\left(\frac{p^\circ}{p}\right) = K_1{}^a K_3 + K_4, \tag{9}$$

when p/p° is the relative vapor pressure; K_1, K_3, and K_4 are constants under a specialized condition; and a is the amount of water sorbed. A residual 5% of the cell water is so tightly bound that it remains adsorbed even at very low vapor pressure. We have also shown that the adherence of cell water to the van't Hoff equation, often cited in support of the membrane theory, is only a partial expression of the Bradley isotherm.[98] In regions of vapor pressure

lower than the limit of hypertonicity achievable in the conventional immersion method, the linear relation between the reciprocal of osmotic pressure and cell water seen in higher water contents no longer holds.

In a forthcoming work, Ling and Negendank further show that the water sorption is due to cellular proteins and not to the ions and other solutes in the cells (which, according to the association-induction hypothesis, are in an adsorbed state). In muscle cells leached virtually free of all K^+ and other solutes, the equilibrium water content at a vapor pressure corresponding to that of a Ringer's solution is at least of the same order of magnitude as that in the living muscle cells (at pH 7.2, 320 g H^2O/100 g dry weight vs. 400 g/100 g in normal muscle). At higher or lower pH, the water content far exceeds that in normal muscles. The predictable patterns of water uptake rule out any incidental capillary condensation interpretation (for evidence against this once-popular view, see recent review in Reference 79). The quantity of water taken up is so large that only the multilayer concept can cope with it.

Some Simple Experiments

According to the membrane theory, it is the cell membrane and pumps located in the cell membrane that maintain the high intracellular K^+-ion concentration and low intracellular Na^+-ion concentration. Recent years have seen the development of techniques whereby the cytoplasm of the giant squid axon can be removed without damaging the cell membrane.[99-101] This squid axon membrane tube filled with various solutions, is able to maintain normal electrical activities which, if the membrane-pump theory is correct, reflect the continued preservation of the intactness of the membrane and the K-Na pump. Thus, if before the open ends are closed, the membrane tube is filled with Ringer's solution, the bulk of the Na^+ ion in the sac should sooner or later be replaced by K^+ ion as a result of the continued activities of the pump. In 1963, Dr. Richard D. Keynes made it known at the Johnson Foundation of the University of Pennsylvania that attempts had been made to check these predictions but that these attempts had failed (Reference 51, p. 95).

More Evidence Supporting the Association-Induction Hypothesis

An equally simple experiment was designed to test the validity of the association-induction hypothesis: to destroy the cell membrane to such an extent that whatever remains of the membrane can be shown to be unable to sustain its postulated function, and then to demonstrate selective K^+ and and Rb^+ ion accumulation over Na^+ ion in this effectively membraneless open-ended cell (EMOC) preparation.[102]

A frog sartorius muscle was mounted in a tube filled with vaseline with only the tibial end protruding beyond the narrow slit of a silicon rubber seal at the end of the tube. The tibial end was next amputated, thereby exposing naked cytoplasm directly to the Ringer's solution containing labeled Rb^+, K^+, Na^+ singly or in pairs. After a few hours the muscles were removed and frozen in liquid air at their natural length, then cut into equal segments 1–2 mm in length. Radioactivity in each segment was then assayed. Typically,

there was selective accumulation of Rb^+ and K^+ ion in the cytoplasm near the cut end after a few hours at 25° C. The peak of accumulation was a little distance away from the open end. For Rb^+ ion this peak, as a rule, reached a height several times greater than the external Rb^+ ion concentration (2.5 mM). On the other hand, at the same location as the Rb^+ ion peak, the Na^+ ion concentration was less than that in the external medium. Longer exposure did not materially alter the picture. The selectivity rank order in the cytoplasm was $Rb^+ > K^+ > Na^+$, in agreement with that in intact frog muscles mentioned above.

However, before we could accept these findings as supporting the association-induction hypothesis, we had to determine: (1) whether there was a regeneration of the cell membrane either at the cut end or inside the muscle cells away from the cut end; and (2) whether Rb^+ and K^+ ion might be accumulated in subcellular compartments with independent uninjured membranes. These questions were answered as follows. Membrane sequestion at the cut end was ruled out by briefly exposing the muscle fiber to labeled sucrose immediately after amputation and several hours after amputation. It was found that the uptake of sucrose through the cut end did not diminish with incubation (if anything, it increased). Regeneration of the cell membrane at the cut end was also ruled out by studying the outward diffusion profile of K^+ and Rb^+ ion. The muscle was first equilibrated with labeled K^+ and Rb^+ ion. Mounted in the same tube as that described above, the tibial end was then cut and exposed to a large body of stirred nonradioactive Ringer's solution. After a certain length of time, the muscle was again cut into small segments and the radioactivity of the segments assayed. The profile of radioactivity along the length of the muscle was then compared with well-known theoretical profiles.

The results show that in general the experimental profile agrees with the theory of diffusion in homogeneous medium. There is no indication that internal membranes developed that would prevent further diffusion of labeled K^+ or Rb^+ outward. Nor was there any indication that a significant quantity of K^+ or Rb^+ ion was sequestered in subcellular compartments. If there were sequestration of K^+ ion in compartments, the profile would be quite different from the theoretical diffusion profile calculated on the basis of a single diffusion coefficient in a homogeneous medium and would have a higher concentration of labeled ion than theoretically predicted in regions close to the cut edge. Actually, the experimental points near the cut edge were close to the theoretical curve.

Taken together, the data are most easily explained by the association-induction hypothesis: that the bulk of intracellular K^+ ion is adsorbed because the adsorbed sites have a much more favorable adsorption energy for K^+ (even more so for Rb^+) than for Na^+ ion. Removal of the cell membrane causes injury to the system, but not such severe injury that we cannot observe a selective adsorption of labeled Rb^+ and K^+ ion over Na^+ ion.

A Critical Evaluation of the Two Opposing Theories on the Nature of Living Cells

Since its introduction a century ago, the membrance theory has been constantly challenged. Its virtually universal acceptance as a proven theory in many textbooks is due, at least in part, to the success which its proponents

have had in convincing a large number of people that this theory has more experimental evidence in its favor than all opposing theories, which have correspondingly less evidence for and more evidence against them. However, the situation has changed now. Indeed, we cannot find any experimental evidence that can be claimed as unequivocally in favor of the membrane theory when compared with alternative theories. In the following section we will discuss in brief a number of factors that have played major roles in advancing the membrane theory and also their current status.

The Osmotic Balance

It was the osmotic behavior of living cells that prompted Pfeffer to formulate the membrane theory, and osmotic arguments later played a major role in advancing the theory to its dominant state. Thus, Hill and Kupalov [101] showed that living cells are in equilibrium with a roughly 0.1 N (or 0.2 osmolar) NaCl solution. They concluded that the intracellular salt, also found in a total concentration roughly 0.2 osmolar (in muscles largely K^+ and creatine phosphate and ATP), must also be free, as Na^+ and Cl^- are free in a 0.1 N solution.

The proteins are omitted from this osmotic balance sheet since, because of their high molecular weights, they add little to the total osmolarity.

However, the assumption that each protein molecule acts on water only as a single osmotically active particle, like a single Na^+ ion, has been shown to be wrong. Low temperature NMR and other studies have produced unequivocal evidence that proteins, in general, react specifically with water [106] (for recent review see Reference 79). Earlier, we showed water uptake by muscle "ghosts" freed of intracellular solutes. These data strongly suggest that cellular proteins play an important part in the so-called osmotic activities that have historically been attributed to free K^+ and other intracellular ions.

High Cytoplasmic Conductance and K^+-Ion Mobility

It has long been claimed that cytoplasmic conductance is high [107] and that the diffusion coefficient of K^+ ion is not too far away from that of free K^+-ion diffusion in an aqueous solution.[108] Both phenomena have long been regarded as strong evidence in support of the membrane theory, which maintains that intracellular K^+ ion is free.

According to the association-induction hypothesis, the bulk of intracellular K^+ ion is not bound in an irreversible way but is reversibly adsorbed. Such adsorbed K^+ ions follow the pattern of behavior of what physical chemists call mobile adsorption (in contrast to localized adsorption).[109] Ions thus adsorbed can move from one adsorption site to another with ease. Supportive of this idea and the theory for this interpretation [46, 49] is the long-known fact that K^+-ion has a higher mobility on glass surfaces than in free solution.[110-112]

Kushmerick and Podolsky [113] recently measured the diffusion coefficients of various substances in frog muscle cytoplasm and found that (with the exception of Ca^{++} ion, which showed a much lower diffusion coefficient) all the other substances showed a diffusion coefficient roughly one-half of that in free solution. Therefore, they concluded that this impartial reduction is due to a

mechanical barrier effect found in cellular structures which effectively lengthen the diffusion path to twice the shortest distance between two points in the longitudinal direction. They did not produce evidence that muscle cells cut into short segments might not have undergone deteriorative changes.

Apparently Kushmerick and Podolsky were unaware of the important work of Tamasige,[114] who showed that the cytoplasmic conductance of single frog muscle fibers at 25° C is also approximately one-half of that of a Ringer's solution. However, Tamasige found that as the temperature lowers the discrepancy does not remain constant, as could be expected to be the case if it were merely a matter of mechanical barriers. Indeed, the conduction reaches a value one-fifth of that of a Ringer's solution at 0° C.

In work soon to be published Ling and Ochsenfeld will present new experimental data on K^+ ion diffusion in frog muscle cells that yield different conclusions than those derived from earlier work.

The Existence of a Continuous Lipid Membrane

Overton's postulation of a lipid membrane is the heart of the membrane theory. Without such a lipid layer, the many postulated pumps would be as meaningful to transport as ferry boats in a dried up river. When Robertson [15] demonstrated the presence of what he called the unit membrane, with a trilayered structure on the surfaces of many cells and subcellular particles, his work stimulated a great surge of studies of artificial lipid membranes, the most prominent among them being those of Mueller and Rudin.[115] The highly popular notion then was that the dark lines represent visual confirmation of the lipid component of the cell membrane postulated by Davson and Danielli,[9] and called the "pauci-molecular bileaflet lipid membrane."

Subsequent investigations, however, strongly suggested that these earlier ideas were not correct. First, the best stain for the unit membrane, $KMnO_4$, was shown not to stain lipids in test tubes.[116] Secondly, after virtually total extraction of lipids from mitochondria [117] and from myelin,[118] the unit membrane structure remained little changed. Thirdly, "microspheres," a pure protein (water) system synthesized from pure amino acids by Fox, exhibit similar unit membrane structure when properly stained.[119] Taken together, these findings strongly suggest that what is revealed at the cell surface is not a lipid membrane but primarily a protein structure.

A Lipid Membrane Is Not a Bona Fide Semipermeable Membrane

The most persuasive evidence for the lipid membrane theory is the demonstration that the permeability of nonelectrolytes through living protoplasm is related to their relative solubility in oil. A good correlation was shown between the permeability of nonelectrolytes and their oil/water distribution coefficient, each spanning about five decades.[10] A major flaw in this theory is the lack of a bona fide semipermeable property in a lipid layer. Our next question is: If the surface layer is not lipid, what other component is present in a large enough quantity in the cell surface that it can form a continuous barrier showing strongly selective permeability toward nonelectrolytes?

We have recently provided experimental evidence to show that this com-

ponent may very well be nothing other than water itself—not normal water, but water existing in the physical state of polarized multilayers.[84] To support this point, we have evaluated the permeabilities of 11 hydrophilic compounds at different temperatures through a living frog skin (from inside to outside), and through an artificial lipid-free, water-containing cellulose acetate membrane. The correlation coefficient between these two sets of permeability data is +0.88. In both cases the permeability to water is higher than to ethyl alcohol and to other solutes studied, as a semipermeable membrane should be. One recalls that the surface tension of living cells must be two orders of magnitude higher if the cell surface is truly covered by a layer of lipid. This requirement led to the postulation of a layer of proteins covering the lipid, thereby reducing the surface tension. The underlying assumption is that this protein layer itself does not offer any resistance to diffusion. There has been no evidence that this assumption is valid.

Experimental evidence has accumulated rapidly to show that proteins as a rule interact with water.[79] The exact correspondence between the semipermeable properties of a living frog skin and a cellulose acetate membrane strongly suggests that the surface proteins interact with water in a manner similar to the interaction of cellulose acetate and water, and that it is this polarized water that serves as the continuous surface barrier which, for three-quarters of a century, has been characterized as a lipid.

The type of membrane used in the experiments reported above has been extensively studied by Schultz and Asunmaa,[120] who were particularly interested in the thin (~2500 Å) "skin" that forms one surface of the membrane. Electron micrographic studies show that this surface is made up of roughly spherical subunits of cellulose acetate in a random array interspersed with pores of 44 Å effective diameter. Using two completely independent methods, these authors determined that the pores are filled entirely with highly structured water having physical properties markedly different from those of bulk water. This "skin" of ordered water sits on top of the bulk membrane matrix, about 100 μ thick, which offers negligible resistance to water or solute diffusion.[121] It is important to realize that the pores are much larger than any solute tested and, therefore, that the selectivity of the membrane must reside in the interaction of the solute with the structured water of the skin. It may be worthy of notice that whereas lipid membrane is not a bona fide semipermeable membrane in regard to alcohol, other membrane models, including pig bladder, parchment paper, and Traube's famous ferrocyanide gel membrane are all bona fide semipermeable membranes. None of these models contains lipid as an essential ingredient; all contain water in a three-dimensional charge-bearing matrix.

In Vitro *Demonstration of K^+ Ion Binding on Proteins*

In 1929, Hoeber [122] analyzed the possibility that selective K^+ ion accumulation might be due to binding onto cellular proteins. He rejected the idea on the grounds that the quantity of ions bound by proteins was very small and that there was no evidence of preferring K^+-ion binding over Na^+ ion. Later work has consistently shown that proteins such as actomyosin that make up the bulk of muscle cell proteins do not selectively adsorb K^+ ion over Na^+ ion, even though some adsorption of a low specificity has been demonstrated.[123] There is no question that these findings ruled out the original idea of K^+ ion

irreversibly bound to cell proteins that was propounded in the earlier part of the century. However, according to the association-induction hypothesis, selective K^+-ion accumulation is not due to an irreversible binding but rather to a reversible selective adsorption. Such adsorbed K^+ ion represents a delicately balanced metastable equilibrium state, much as a bent bow or a set snare represents metastable equilibrium states.

According to this view, drastic steps involved in isolating a protein from a living cell upset this delicate metastable state and are, therefore, no more likely to leave the cell proteins in their K^+ ion-adsorbing states than bits of a smashed radio are likely to be found in a singing state. The metastable K^+-ion adsorption states reflect the unique physical state and molecular environment of the proteins involved: Their fixity in space (Reference 46, Chap. 2); the presence of certain key adsorbents (e.g., ATP, Ca) on cardinal sites; the presence of water in a state different from normal water—all of these are necessary ingredients operating cooperatively to maintain a certain specific c-value of the anionic sites, which in turn determines the ionic specificity for K^+ ion.

It should be mentioned that although it has not been theoretically anticipated that one would find isolated proteins selectively accumulating a large amount of K^+ ion over Na^+ ion, it is well known that individual sites on proteins have been shown repeatedly to exhibit a high degree of selectivity with regard to different alkali-metal ions [124] (Reference 46, p. 397). These observations demonstrate the inherent ability of proteins to perform the elementary process of selecting one alkali-metal ion over another. Even more direct evidence has come from the competitive inhibition study of alkali-metal ions on the rate of entry of K^+ and other ions into frog muscle cells. The data have shown that at 25° C the surface sites prefer K^+, Rb^+, and Cs^+ over Na^+ ion by a factor of about two orders of magnitude. As discussed earlier, the demonstration of pK value of 4.6 has identified these sites as the β- and γ-carboxyl groups of the proteins.[105] Clearly, this finding shows that the same functional groups of proteins in solutions, and as part of the living protoplasm, may act quite differently in their interaction with the alkali-metal ions, thereby reaffirming a major theme of the association-induction hypothesis.

High Intracellular K^+ Ion Activity Measured with an Ion-Sensitive Intracellular Microelectrode

Hinke,[125, 126] Lev,[127] and others [128] have repeatedly observed an activity of K^+ ion in nerve and muscle cytoplasm close to that of free K^+ ion in an aqueous solution, when an ion-sensitive microglass electrode is inserted into a single muscle fiber or a nerve axon. This observation appears to be in agreement with the postulation of the membrane theory that the bulk of intracellular K^+ in a normal resting cell exists in a free state. However, unlike a microelectrode of the Gerard-Graham-Ling type, which measures the electrical potential over the entire cell surface, this ion-sensitive glass electrode, like any glass pH electrode, measures only the activity of K^+ ion in a microscopic film of fluid immediately surrounding the electrode tip.[129] Moreover, this film of fluid is precisely the layer of cytoplasm which the impaling electrode has to displace, in order to reach the inside of a cell. Such cytoplasm, torn from its normal structural framework and pushed against adjacent layers of cytoplasm cannot, in our evaluation, be considered as existing in its normal physiological state;

what is far more likely is that it resembles cytoplasm of a well ground-up muscle. As such, the K^+ ion would be liberated from its normal adsorption sites, thereby giving high K^+ ion activity reading on the ion-specific microelectrode.

More recently, Dick and MacLaughlin,[130] further defending the validity of the recorded K^+-ion activity with an intracellular microelectrode, first agreed with Ling that trauma could conceivably bring about K^+-ion liberation and that the ion-sensitive electrode might indeed register only K^+-ion activity in a microscopic film surrounding the tip of the electrode. They then pointed out, however, that such liberated K^+ ion would rapidly diffuse away, and a lower K^+-ion activity would be subsequently recorded if the K^+ ion in the resting muscle cells is indeed adsorbed. In contrast to this expectation they found that, in a period of observation lasting 30 minutes, there was not much change in the measured (high) K^+-ion activity. From these findings they concluded that the bulk of intracellular K^+ ion in a normal resting cell is in the free state, as the membrane theory predicts.

It would seem that any liberated K^+ ion would diffuse away far more slowly than anticipated. Hodgkin and Keynes provided the needed information,[108] when they observed that labeled K^+ ion taken up at one locus in a squid axon dropped to two-thirds of its initial concentration only after a period of 7 hours (445 minutes). Thus, a half-hour experiment is insufficient to reveal true K^+-ion activity unmasked by the injury-liberated K^+ ion. One may also add that even if an impaling electrode were kept in site for 7 hours, the spreading deterioration near the injury, described earlier in connection with the EMOC preparation, would most likely provide a continued additional source of liberated K^+ ion, thereby invalidating any conclusion one might draw from electrode recordings.

The Temperature Dependence of K^+-Ion Accumulation and Na^+ Ion Exclusion

Often cited in favor of the membrane-pump theory is the well-known reversible loss of K^+ and gain of Na^+ with a lowering of temperature to, say, 0° C. This loss has been taken as indicative of the dependence of normal K^+ and Na^+-ion contents on a continual pumping process that slows down with cooling. However, this interrelation is not in harmony with the fact that frog muscles can be cooled to 0° C without losing K^+ or gaining Na^+.[46]

According to the association-induction hypothesis, the delicately balanced physical state of ions and water in a resting cell provides the cell with the ability to shift reversibly from this resting, inactive state to a thermodynamically more stable state when the cell goes into functional activities. However, if individual sites act independently, it would be virtually impossible to call into prompt action a whole cell containing a vast number of such individual sites. According to the association-induction hypothesis, the need for individual sites to act together is taken care of by the "cooperativity" of the system. This cooperativity in turn arises from the interaction between individual adsorption sites along the partially resonating polypeptide chain of the cell proteins (Reference 46, p. 102).

Since this subject will be discussed in greater detail in a subsequent paper,[77, 131, 132] suffice it to say here that cooperative adsorption of K^+ and Na^+ ion exhibits a critical transition temperature. Above this transition temperature,

K^+ ion is adsorbed on virtually all the adsorption sites. Below this temperature, virtually all the sites are occupied by Na^+ ion. The transition temperature varies with the species of animal and with the types of tissue in question. This is why lowering the temperature to 0° C causes loss of K^+ and gain of Na^+ in many types of mammalian tissues but not in frog muscle. Thus, there is reason to believe that evolution has provided genetic mutation to give animals such as the North American leopard frog protein structures that can be maintained in the K^+ state at a low temperature.

The Dependency of Cellular K^+ and Na^+-Ion Concentration on Metabolism

Poisoning of cells with a variety of metabolic inhibitors brings about a loss of K^+ and a gain of Na^+ ion. Again, this fact has long been considered as evidence that ionic distribution depends on pumping and that the pumping process needs metabolic energy. When a continual supply of metabolic energy is cut off by poisons, the asymmetry in the K^+ and Na^+ ion distribution vanishes. However, we found that in frog muscle cells, arrest of both respiration and of glycolysis did not materially alter the rate of Na^+ ion efflux [45] (Reference 46, p. 198) for a long period of time; this finding was later confirmed by Keynes and Maisel.[33] These and other findings then suggested that it might have been the store of creatine phosphate (CrP) and ATP that provided the energy for the Na pump. However, as described earlier, the energy provided by the hydrolysis of CrP and ATP is far from sufficient to cope with the need of the Na pump.

According to the association-induction hypothesis, the essential function of metabolism in regard to K^+ and Na^+-ion distribution is to provide an adequate supply of ATP, which functions as one of the most important cardinal adsorbents in living cells. Adsorption of ATP on cardinal sites maintains gangs of protein sites at a c-value, so that K^+ ion is preferentially adsorbed over Na^+ ion and water exists in the state of polarized multilayers capable of excluding Na^+ ion.[77] Years after the presentation of this theoretical model, the following experimental findings were reported, giving substantial support to the theory.

(1) It was demonstrated [133, 134] that adsorption of ATP (and 2, 3-diphosphoglycerate) on hemoglobin shifts the affinity for oxygen of a distant heme site, thereby providing a clear-cut *in vitro* demonstration of the indirect F effect (Reference 46, p. 97) exercised by a cardinal adsorbent. Since hemoglobin is not an ATPase, this action involves no hydrolysis of ATP and hence no liberation of the so-called high energy.

(2) Bowen and Mandelkern [135] have clearly shown that the contraction of glycerinated muscle fibers brought about by ATP has no dependence on the hydrolysis of ATP.

(3) Gulati and associates [76] have confirmed the earlier reports of Ling [44, 46, 54] that there is a quantitative relation between the concentration of K^+ ion and that of ATP in the cell; i.e., each mole of ATP adsorbed controls about 20 K^+ (or Na^+) ion adsorption sites. It is, of course, true that this quantitative relation can also be attributed to a dependence of this ATP hydrolysis rate (hence Na^+ ion pumping rate) on the ATP present. This is ruled out, however, from the experiment discussed earlier, which showed that a fall of concentration from normal to zero did not decrease the rate of the intracellular-extracellular Na^+ ion exchange.

Control of K^+ and Na^+-Ion Distribution by Ouabain

Schatzman demonstrated the inhibition of K^+ uptake and Na^+-ion extrusion in human red blood cells by cardiac glycosides.[136] This finding and many others have led proponents of the membrane theory to believe that ouabain is a specific inhibitor of the Na pump. Chief among their reasons are: (1) that the Na^+-ion efflux rate is slowed down by ouabain in normal RBCs and in red cell ghosts, which the proponents of the membrane theory believe to be primarily the cell membrane; and (2) that at a similar concentration ouabain also inhibits an ATPase which is found in the same red cell ghost preparations. The interpretation of ouabain effects in terms of the membrane theory is based on the assumption that it is the rate of hydrolysis of ATP that determines the rate of Na^+ ion pumping. As we have just discussed, this postulation is contradicted by our failure to find any slowing down of the Na^+ ion efflux with partial or complete loss of ATP in IAA-poisoned frog muscle. In addition, if ouabain truly acts by slowing down ATP hydrolysis, one would anticipate that in the range of concentrations (1×10^{-9}– 3.27×10^{-7} M) effective in changing the equilibrium distribution of K^+ and Na^+ ion in frog muscle, ouabain should cause the ATP concentration to rise. Actual measurements showed that ATP remained entirely constant after treatment with ouabain (8×10^{-9}–3.27×10^{-7} M) for 3 days at 25° C.[137]

According to the association-induction hypothesis, ouabain is another cardinal adsorbent, controlling the K^+ vs. Na^+ ion distribution in a variety of living tissues. The quantitative confirmation of this theory will be presented in a later paper in this monograph.[77] Summarizing briefly, we may say that the interaction of ouabain with the specific cardinal sites of the cell brings about a shift in the intrinsic equilibrium constant $K^{\circ\circ}{}_{Na \to K}$, decreasing it from about 100 to about 10.[137] In the presence of the much higher concentration of Na^+ ion than K^+ ion normally found in the surrounding media, a virtually complete replacement of cell K^+ by Na^+ ion follows.[138]

The association-induction hypothesis also accounts for the following facts: (1) A moderate increase of K^+-ion concentration can completely reverse the action of ouabain (2) Ouabain induces a slowdown of Na^+-ion efflux from cells and red cell ghosts. This effect on Na^+-ion efflux is seen as the result of the shift of the K^+-ion binding site to a c-value more strongly adsorbing Na^+. The more strongly adsorbed Na^+ ion will have a higher activation energy for desorption and hence there will be a slowdown of the exchange rate of this fraction (the β and γ fraction). (3) There is a parallel effect of ouabain on the Na^+-ion efflux rate and ATPase activity. In this model one can visualize that the ATPase site is in the same gang containing the ouabain-binding cardinal site and the regular K-Na adsorption sites. The synchronous cooperative action of the whole gang in response to ouabain will create a correspondence in the ATPase activity change and the change in Na^+ ion efflux rate, as was reported.

Michaelis-Menton Kinetics in Ions, Sugars, and Amino Acids

In a wide variety of living cells, the entry of ions, sugars and amino acids into the cells has been shown to follow Michaelis-Menton kinetics.[103-105, 139-144] Proponents of the membrane theory regard this phenomenon as evidence

that carriers or pumps are involved in the entry of these solutes. According to the association-induction hypothesis, the entry of solutes into living cells following Michaelis-Menton kinetics merely indicates the presence of specific adsorption sites for the entrant solute near the cell surface. In support of the hypothesis, it has been shown that ion entry into ion exchange resin sheets, sheep's wool, and even an actomyosin gel, also follows Michaelis-Menton kinetics.[104, 105, 145]

For K^+-ion entry into frog muscle cells, we have specific evidence that the surface adsorption sites are the β and γ carboxyl groups of proteins because the K^+-ion entry is pH-dependent, and from a plot of the rate of entry of K^+ against pH, a pK value of 4.6 was found for the surface adsorption sites.[105] This pK is characteristic of the β and γ carboxyl groups. We believe this finding to be of prime importance, since it rules out a variety of postulated membrane carriers such as valinomycin and polyclic ethers, which are neutral and thus have no meaningful pK value.

Ionic Transport Carriers

We have already pointed out that the existence of a continuous lipid layer is the prerequisite of the carrier-pump model and that the existence of such a layer is seriously in doubt. However, for the sake of argument, let us assume that such a continuous lipid layer does exist. One of the popular views among proponents of the membrane theory is that the carriers are small molecules, such as certain antibiotics,[146-153] and cyclic polyethers,[149] because these molecules have been shown to form specific complexes with K^+ ion, and that when these carriers are added to the aqueous phase in contact with living membranes, or artificial lipid membranes, they actually promote the traffic of K^+ ion.

A fundamental question one must ask in relation to this concept is: How would such carriers distribute themselves between the microscopic cell membrane and the infinitely larger aqueous environment surrounding the cell? In order to ferry its passenger ion, the carrier must be freely mobile—i.e., weakly held—in the membrane. Let us assume that the lipid solubility of these carriers is one million times higher than their water solubility. In this case an amoeba 10 μ in diameter would have a total membrane volume of approximately $\frac{4}{3}\pi\ (5 \times 10^{-4})\ 2 \times 10^{-6} = 1.05 \times 10^{-12}$ cc or 1.05×10^{-5} liter. Let us assume that the ameba lives in a very small fresh-water pond containing a total of one liter of water. In this case, it would have to produce no less than $\frac{1.05 \times 10^{-15} \times 10^{-3}}{10^{-6}} = 1.05 \times 10^{-3} \frac{1 \times 10^{-6}}{1.05 \times 10^{-15}} \sim 10^9$ times more carriers molecules before the carrier concentration in the membrane would be steady enough to function. Each time it rained, the amoeba would have to produce more carrier molecules. This does not consider the confusion that would be created if one organism picked up another organism's carrier from the common water pool, thereby wiping out all the unique transport characteristics between individuals created by eons of painstaking evolution. Similarly, one might anticipate different cells in a multicellular organism to exchange carriers to the point where, eventually, all their permeability properties became the same. All this is less than reasonable.

The Identification and Isolation of Sugars and Other Permeases

Workers in the sugar transport field, accepting the basic tenet of the membrane theory, long ago reached the conclusion that a substance that can differentiate between closely similar sugars must possess a high degree of stereospecificity; it has been generally agreed that this substance must be a protein.[150, 151] Quite in line with this kind of thinking is the clear demonstration that the ability to accumulate a specific sugar is determined by a specific gene.[143] Since all genes are known to specify protein structures, it was concluded that the carrier or permease must also be a protein.

To serve as a permease, a protein must possess the ability to recognize the specific sugar (or other solute) it transports and to bind this sugar. A great deal of effort has been spent in trying to find a protein from a disrupted cell that shows a high affinity for the substance it transports. Fox and Kennedy [152] demonstrated a protein called M-protein, from the membrane-containing particulate fraction of *Escherichia coli*, which can accumulate β-galactoside. This M-protein was shown to have a high affinity for certain β-galactoside. Auraku [153] found that osmotic-shocked *E. coli* loses the ability to accumulate galactose and that this lost ability can be restored by exposure to a protein material collected in the shock fluid. Pardee [154] went one step further and claimed to have isolated and crystallized a sulfate permease from *Salmonella typhimurium*. Kolber and Stein [155] were also able to isolate a specific β-galactoside binding protein from *E. coli*, but in this case only from the cytoplasmic fraction and not from the membrane fraction of the cell extract.

In none of these cases has it been shown that the protein demonstrated can actually perform the postulated task: namely, to transport sugars and other solutes across a lipid membrane model against a concentration gradient. On the other hand, the demonstration of specific binding proteins for ions, sugars, and amino acids at the cell surface or in the cell interior directly supports the fundamental concepts of the association-induction hypothesis, which argues that it is this specific binding that determines the accumulation of many of these solutes in living cells, as well as the Michaelis-Menton kinetics in solute entry.

The Cellular Electrical Potential

If virtually all the K^+ ion in the cell is adsorbed, how can one explain the electric potentials that have been shown to respond to temperature and to external K^+-ion and Na^+-ion concentration, in agreement with the Hodgkin-Katz-Goldman equation according to which all intracellular K^+ ion is free?

The resting and action potentials (ψ) of a variety of living cells have been shown to conform, partially at least, to the Hodgkin-Katz-Goldman equation.[156]

$$\psi = \frac{RT\ln}{F}\left(\frac{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{ex}}{P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex} + P_{Cl}[Cl^-]_{in}}\right), \qquad (10)$$

where P_K, P_{Na}, and P_{Cl} are the membrane permeability constants, to K^+, Na^+, and Cl^- ion respectively. $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{in}$ are the intracellular concentrations of K^+, Na^+, and Cl^- ion, while $[K^+]_{ex}$, $[Na^+]_{ex}$, and $[Cl^-]_{ex}$ are the corresponding concentrations in the extracellular solution. R, T, and F are the gas constant, absolute temperature, and Faraday constant respectively. The

relations of ψ to T and to $[K^+]_{ex}$ and $[Na^+]_{ex}$ have all been verified for the resting potential.[46] However, the potential does not vary permanently with $[Cl^-]_{ex}$, as predicted by the membrane theory.[157] It has also been repeatedly observed that ψ does not vary with changes of $[K^+]_{in}$ or $[Na^+]_{in}$. Thus, Grundfest and collaborators observed no change of potential followed the injection of a high concentration of K^+ or Na^+ ion into squid axon.[158] Falk and Gerard observed no change following a similar injection into frog muscle cells.[159] Koketsu and Kimura [160] observed little change in the resting potential in frog muscle leached free of the bulk of intracellular K^+ ion (an observation confirmed by Dr. M. Neville). Similarly, the rather extensive work of Tasaki and his coworkers led them also to the conclusion that the potential does not change with extensive variation in the perfusion solution.[100, 161-164]

Baker and associates [164] explained the lack of sensitivity to changes in internal K^+ and Na^+-ion concentrations by postulating compensatory changes in P_K. Since no such compensatory changes were ever needed to describe the relation between ψ and $[K^+]_{ex}$, the need for this postulation creates an inconsistency. Since K^+ ion moves rapidly between the intracellular and extracellular phase, if P_K changes with $[K^+]_{in}$, it should also change with $[K^+]_{ex}$.

The major departure of the association-induction hypothesis from the membrane theory consists in its view of the physical state of ions and water in the cytoplasm (Reference 46, Chap. 10). In this theory, the resting and active potentials have no direct relation to ionic permeability.[165-167] Instead, the potentials are seen as phase-boundary potentials between the cell phase and the extracellular phase. The resting potential is created when some of the counter ions (K^+) diffuse away, leaving vacant negatively charged sites near the cell surface. Therefore, in this model, the equation describing the potential is as follows:

$$\psi = \text{constant} - \frac{RT}{F} \ln \{K_k[K^+]_{ex} + K_{Na}[Na^+]_{ex}\}. \qquad (11)$$

A comparison of Equation 11 with the Hodgkin-Katz-Goldman equation (Equation 10) shows that Equation 11 contains neither the intracellular and extracellular chloride-ion concentrations nor the intracellular K^+ and Na^+ ion concentrations. In other words, the parameters in the Hodgkin-Katz-Goldman equation, whose relation to ψ has not been verified, are not found in Equation 11. The remaining parameters T, $[K^+]_{ex}$, and $[Na^+]_{ex}$, whose relation to ψ has already been experimentally verified, are the only parameters constituting Equation 11.

Comparing Equation 11 with Equation 10, one also notices a fundamental difference in the assigned significance of the quotients of the external ionic concentration. These quotients represent membrane permeabilities in one case (membrane theory) and adsorption constants on surface sites in the other (association-induction hypothesis). In an article in this monograph, Edelman has given unequivocal evidence that supports the association-induction hypothesis.[167, 168] In brief, the relative effectiveness of K^+, Rb^+, and Cs^+ ions in lowering the resting potential bears no relation to the membrane permeability of these ions in guinea pig heart muscle, but follows quantitatively their measured adsorption constants on the cell surface.

Active Transport in Intestinal Epithelium Kidney Tubules, Frog Skin, Toad Bladder, and Certain Plant Cells

In each of these systems, the transport of various solutes from one aqueous phase to a similar aqueous phase against a concentration gradient is well established. In each of these systems, a basic anatomical structure is seen: a thin layer of cell protoplasm with two morphologically different surfaces facing two aqueous solutions across which solutes are transported often against a concentration gradient. We do not doubt that in this case active transport occurs. Indeed, on the basis of the general concepts of the association-induction hypothesis, Ling offered a molecular theory for such an operation in 1965.[51] In summary, we conclude that at this moment we know of no experimental findings that contradict the association-induction hypothesis. On the other hand, there is a sizable collection of facts that contradict the membrane theory.

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REFERENCES

1. PFEFFER, W. 1921. Osmotische Untersuchungen. 2nd edit. Engelmann. Leipzig.
2. OVERTON, E. 1899. Vjschr. Naturforsch. Ges. (Zurich) **44:** 88.
3. OVERTON, E. 1907. *In* Handbuch der Physiologie des Menschen. W. Nagel, Ed. Vieweg Braunschweig **2:** 744.
4. COLLANDER, R. 1937. Trans. Farad. Soc. **33:** 985.
5. COLLANDER, R. 1954. Physiol. Plantarum **7:** 420.
6. COLE, K. S. 1932. J. Cellular Comp. Physiol. **1:** 1.
7. HARVEY, E. N. & R. COLLANDER. 1932. J. Franklin Inst. **1:** 214.
8. DANIELLI, J. F. & E. N. HARVEY. 1935. J. Cellular Comp. Physiol. **5:** 483.
9. DAVSON, H. & J. E. DANIELLI. 1952. *In* The Permeability of Natural Membranes. 2d edit. Cambridge University Press. London and N.Y.
10. COLLANDER, R. 1959. *In* Plant Physiology. A Treatise. F. C. Steward, Ed. **2:** 3. Academic Press. New York, N.Y.
11. FINDLAY, A. 1919. Osmotic Pressure. Longmans, Green & Co. New York.
12. COLLANDER, R. & H. BÄRLUND. 1933. Acta. Botan. Fenn. **11:** 1.
13. TRAUBE, M. 1867. Arch. Anat. Physiol. Wiss. Medizin. **1867:** 87.
14. GLASSTONE, S. 1961. Textbook of Physical Chemistry. 2nd edit.: 656. D. Van Nostrand Co. Princeton, N.J.
15. ROBERTSON, J. D. 1960. Progr. Biophys. Chem. **10:** 344.
16. RUHLAND, W. & C. HOFFMAN. 1925. Planta. **1:** 1.
17. BOYLE, P. J. & E. J. CONWAY. 1941. J. Physiol. (London) **100:** 1.
18. BERNSTEIN, J. 1902. Arch. Ges. Physiol. **92:** 521.
19. ROSS, E. J. 1951. J. Physiol. **112:** 229.
20. HEPPEL, L. A. 1940. Am. J. Physiol. **128:** 449.
21. STEINBACH, H. B. 1940. J. Biol. Chem. **133:** 695.
22. BOLAN, T. R. 1932. The Donnan Equilibrium. G. Bell & Sons. London.
23. RYSER, H. J. P. 1968. Science **159:** 390.
24. AVERY, O. T., C. M. MCLEOD & M. MCCARTY. 1944. J. Exp. Med. **79:** 137.
25. TASAKI, I., T. TEORELL & C. S. SPYROPOULOS. 1961. Amer. J. Physiol. **200:** 11.

26. CONWAY, E. J. & G. CUESS-CALLAGHAN. 1937. Biochem. J. **31:** 828.
27. GILBERT, D. 1960. J. Gen. Physiol. **43:** 1103.
28. BOYD, I. A. & T. FORRESTER. 1968. J. Physiol. **199:** 115.
29. CHAUDRY, I. H. & M. K. GOULD. 1970. Biochem. Biophys. Acta. **196:** 320.
30. CONWAY, E. J. 1946. Nature **157:** 715.
31. LEVI, H. & H. H. USSING. 1948 . Acta. Physiol. Scan. **16:** 232.
32. HARRIS, E. J. & G. P. BURN. 1949. Trans. Faraday Soc. **45:** 508.
33. KEYNES, R. D. & G. W. MAISEL. 1945. Proc. Roy. Soc. (Biol.) **142:** 383.
34. LING, G. N. 1955. J. Phys. Med. **34:** 89.
35. USSING, H. H. 1947. Nature **160:** 262.
36. HODGKIN, A. L. & R. D. KEYNES. 1955. J. Physiol. **128:** 28.
37. HOFFMAN, J. F. & F. M. KREGENOW. 1966. Ann. N.Y. Acad. Sci. **137:** 566.
38. BUCK, B. & P. J. GOODFORD. 1966. J. Physiol. **183:** 551.
39. KEYNES, R. D. & R. C. SWAN. 1959. J. Physiol. **147:** 591.
40. LING, G. N. & E. FERGUSON. 1970. Physiol. Chem. Phys. **2:** 216.
41. ESSIG, A. 1968. Biophys. J. **8:** 53.
42. HODGKIN, A. L. & R. D. KEYNES. 1955. J. Physiol. **128:** 28.
43. CALDWELL, P. C. 1960. J. Physiol. **152:** 545.
44. LING, G. N. 1951. Amer. J. Physiol. **167:** 806.
45. LING, G. N. 1952. *In* Phosphorus Metabolism. W. D. McElroy & B. Glass, Eds. **2:** 748. Johns Hopkins Press. Baltimore, Md.
46. LING, G. N. 1962. A Physical Theory of the Living State. Ginn (Blaisdell). Waltham, Mass.
47. HILL, A. V. & J. L. Parkinson. 1931. Proc. Roy. Soc. (London) **B108:** 148.
48. HUKUDA, K. 1931. J. Physiol. (London) **72:** 438.
49. LING, G. N. 1965. Fed. Proc. Symp. **24:** S103.
50. LING, G. N. 1965. Ann. N.Y. Acad. Sci. **125:** 401.
51. LING, G. N. 1965. Persp. Biol. Med. **9:** 87 (1965).
52. LING, G. N. 1966. Fed. Proc. Symp. **25:** 958.
53. LING, G. N. & M. M. OCHSENFELD. 1966. J. Gen. Physiol. **49:** 819.
54. LING, G. N. 1969. Intern. Rev. Cytol. **26:** 1.
55. HODGKIN, A. L. & P. HOROWICZ. 1959. J. Physiol. **148:** 127.
56. JOHNSON, J. A. 1955. Amer. J. Physiol. **181:** 263.
57. MULLINS, L. J. & O. A. S. FRUMENTO. 1963. J. Gen. Physiol. **46:** 629.
58. KATZ, J. A. 1896. Pflügers. Arch. Ges. Physiol. **63:** 1.
59. URANO, F. 1908. Z. Biol. **50:** 212.
60. URANO, F. 1908. Z. Biol. **51:** 483.
61. MEIGS, E. B. & L. A. RYAN. 1912. J. Biol. Chem. **11:** 401.
62. MAURER, F. W. 1938. Am. J. Physiol. 124: 546.
63. CHAO, I. P. 1939. Chinese J. Physiol. **14:** 457.
64. FENN, W. O. & D. M. COBB. 1935. Am. J. Physiol. **112:** 41.
65. LING, G. N. 1972. Physiol. Chem. Phys. **4:** 199.
66. LING, G. N. & M. H. KROMASH. 1967. J. Gen. Physiol. **50:** 677.
67. LING, G. N., M. C. NEVILLE, S. WILL & P. SHANNON. 1969. Physiol. Chem. Phys. **1:** 85.
68. LING, G. N. 1970. Physiol. Chem. Phys. **2:** 242.
69. LING, G. N. & A. SCHMOLINSKE. 1956. Fed. Proc. **15:** 120.
70. KEYNES, K. D. & R. A. STEINHARDT. 1968. J. Physiol. (London) **198:** 581.
71. PORTER, K. R. & M. A. BONNEVILLE. 1964. An Introduction to the Fine Structures of Cells and Tissues. Lea & Febiger. Philadelphia, Pa.
72. PEACHY, L. D. 1965. J. Cell. Biol. **25:** 209.
73. HILL, D. K. 1964. J. Physiol. **175:** 275.
74. HUXLEY, H. E. 1964. Nature **202:** 1067.
75. LING, G. N. 1964. Texas Rep. Biol. Med. **22:** 244.
76. GULATI, J., M. M. OCHSENFELD & G. N. LING. 1971. Biophys. J. **11:** 973.
77. LING, G. N. & M. M. OCHSENFELD. This monograph.

78. HORI, T. 1956. Low Temp. Sci. **A15:** 34. (English Translation No. 62. U.S. Army Snow & Ice Permafrost Establishment, Wilmette, Ill.).
79. LING, G. N. 1972. *In* Water and Aqueous Solutions, R. A. Horne, Ed.: 663. Wiley-Interscience. New York.
80. TROSCHIN, J. S. 1958. Das Problem der Zell Permeabilitat. Fischer. Jena, East Germany.
81. LING, G. N. 1955. Am. J. Phys. Med. **34:** 89.
82. LING, G. N. & G. BOHR. 1971. Physiol. Chem. Phys. **3:** 573.
83. LING, G. N., M. C. NEVILLE, P. SHANNON & S. WILL. 1969. Physiol. Chem. Phys. **1:** 42.
84. LING, G. N. 1970. Intern. J. Neuroscience. **1:** 129.
85. HARRIS, F. E. & S. A. RICE. 1956. J. Chem. Phys. **24:** 1258.
86. GREGOR, H. P. 1951. J. Amer. Chem. Soc. **73:** 642.
87. BREGMAN, J. I. 1953. Ann. N.Y. Acad. Sci. **57:** 125.
88. CONWAY, E. J. 1957. Physiol. Rev. **37:** 85.
89. LING, G. N. 1967. *In* Thermobiology. A. Rose, Ed.:5. Academic Press. New York.
90. HALLET, J. 1965. Fed. Proc. Symp. **24:** 34.
91. MENZ, L. J. & B. J. LUYET. 1961. Biodynamica **8:** 261.
92. CHAMBERS, R. & H. P. HALE. 1932. Proc. Roy. Soc. **B110:** 336.
93. MILLER, C. & G. N. LING. 1970. Physiol. Chem. Phys. **2:** 495.
94. DE BOER, J. H. & C. ZWIKKER. 1929. Z. Phys. Chem. **60:** 1182.
95. BRADLEY, S. 1936. J. Chem. Soc.: 1467.
96. MELLON, S. R. & E. F. HOOVER. 1950. J. Amer. Chem. Soc. **72:** 2562.
97. LING, G. N. & W. NEGENDANK. 1970. Physiol. Chem. Phys. **2:** 15.
98. COPE, F. W. 1967. Bull. Math. Biophys. **29:** 583.
99. BAKER, P. F., A. L. HODGKIN & T. I. SHAW. 1962. J. Physiol. **164:** 330.
100. TASAKI, I., A. WATANABE & T. TAKENAKA. 1962. Proc. Nat. Acad. Sci. **48:** 1171.
101. HILL, A. V. & P. S. KUPALOV. 1930. Proc. Roy. Soc. **B106:** 445.
102. LING, G. N. To be published.
103. LING, G. N. 1953. Proc. 19th Intern. Physiol. Congr. : 566. Montreal.
104. LING, G. N. 1960. J. Gen. Physiol. **43:** Suppl. 149.
105. LING, G. N. & M. M. OCHSENFELD. 1965. Biophys. J. **5:** 777.
106. KUNTZ, I. D., T. S. BRASSFIELD, G. D. LAW & G. V. PURCELL. 1969. Science **163:** 1329.
107. HERMANN, L. 1879. Handbuch der Physiologie. F. C. W. Vogel, Ed. **2:** 3. L. Hermann. Leipzig.
108. HODGKIN, A. L. & R. D. KEYNES. 1953. J. Physiol. **119:** 513.
109. FOWLER, R. & E. A. GUGGENHEIM. 1960. Statistical Thermodynamics. Cambridge Univ. Press. New York.
110. MCBAIN, J. W. & C. R. PEAKER. 1930. J. Phys. Chem. **34:** 1033.
111. MYSELS, K. J. & J. W. MCBAIN. 1948. J. Coll. Sci. **3:** 45.
112. NIELSEN, J. M., A. W. ADAMSON & J. W. COBBLE. 1952. J. Amer. Chem. Soc. **74:** 446.
113. KUSHMERICK, M. J. & R. J. PODOLSKY. 1969. Science **166:** 1297.
114. TAMASIGE, M. 1950. Ann. Zool. Jap. **23:** 125.
115. MUELLER, P., D. O. RUDIN, H. T. TIEN & W. C. WESCOTT. 1963. J. Phys. Chem. **67:** 534.
116. KORN, E. D. 1966. Science **153:** 1491.
117. NAPOLITANO, L., F. LEBARON & J. SCALETTI. 1967. J. Cell. Biol. **34:** 817.
118. FLEISCHER, S., B. FLEISCHER & W. STOECKENIUS. 1967. J. Cell. Biol. **32:** 193.
119. FOX, S. 1971. Presentation at the Second Symp. of Physique Theoretique a la Biologie. Versaille.
120. SCHULTZ, R. D. & S. K. ASUNMAA. 1969. Rec. Prog. Surf. Sci. **3:** 291.
121. RILEY, R. L., J. O. GARDNER & V. MERTEN. 1964. Science **143:** 801.
122. HOEBER, R. 1929. Pflügers Arch. Ges. Physiol. **216:** 540.

123. LEWIS, M. S. & H. A. SAROFF. 1957. J. Amer. Chem. Soc. **79:** 2112.
124. NEVILLE, M. & G. N. LING. 1967. Arch. Bioch. & Biophys. 1967. **118:** 596.
125. HINKE, J. A. M. 1961. J. Physiol. **156:** 314.
126. HINKE, J. A. M. 1959. Nature **184:** 1257.
127. LEV, A. A. 1964. Nature **201:** 1132.
128. VOROBIEV, L. N. 1967. Nature **216:** 1325.
129. LING, G. N. 1969. Nature **221:** 386.
130. DICK, D. A. T. & S. G. A. MCLAUGHLIN. 1969. J. Physiol. **205:** 61.
131. REISIN, I. L. & J. GULATI. 1972. Science **176:** 1137.
132. REISIN, I. L. & J. GULATI. This monograph.
133. CHANUTIN, A. & R. R. CURNISH. 1967. Arch. Biochem. Biophys. **121:** 96.
134. BENESCH, R. & R. E. BENESCH. 1969. Nature **221:** 618.
135. BOWEN, W. J. & L. MANDELKERN. 1971. Science **173:** 239.
136. SCHATZMANN, H. J. 1953. Helv. Physiol. Pharmakol. Acta **11:** 396.
137. LING, G. N. & G. BOHR. 1971. Physiol. Chem. Phys. **3:** 43.
138. GULATI, J. & A. W. JONES. 1971. Science **172:** 1348.
139. EPSTEIN, E. & C. E. HAGEN. 1952. Plant Physiol. **27:** 457.
140. NEVILLE, M. 1962. Ph.D. dissertation. University of Pennsylvania.
141. FISCHER, R. B. & D. S. PARSON. 1953. J. Physiol. **119:** 210.
142. CONWAY, E. J. & F. DUGGAN. 1958. Biochem. J. **69:** 265.
143. COHEN, G. N. & J. MONOD. 1957. Bact. Rev. **21:** 169.
144. CRANE, R. K. & T. H. WILSON. 1958. J. Appl. Physiol. **12:** 145.
145. LING, G. N. & M. M. OCHSENFELD. 1970. Physiol. Chem. Phys. **2:** 138.
146. ANDREOLI, T. E., M. TIEFENBERG & D. C. TOSTESON. 1967. J. Gen. Physiol. **50:** 2527.
147. EISENMAN, G. 1968. Fed. Proc. **27:** 1249.
148. MUELLER, P. & D. O. RUDIN 1968. Nature **47:** 713.
149. PEDERSEN, C. J. 1967. J. Amer. Chem. **89:** 701-7.
150. BOWYER, F. & W. F. WIDDAS. 1953. Membrane phenomena. Discussions Faraday Soc. **21:** 251.
151. STEIN, W. D. & J. F. DANIELLI. 1953. Membrane phenomena. Discussions Faraday Soc. **21:** 238.
152. FOX, C. F. & E. P. KENNEDY. 1965. Proc. Nat. Acad. Sci. **54:** 891.
153. AURAKU, Y. 1968. J. Biol. Chem. **243:** 3116.
154. PARDEE, G. 1967. Science **156:** 1627.
155. KOLBER, A. R. & W. D. STEIN. 1966. Nature **209:** 691.
156. HODGKIN, A. L. & B. KATZ. 1949. **108:** 37.
157. HODGKIN, A. L. & P. HOROWICZ. 1959. J. Physiol. **148:** 127.
158. GRUNDFEST, H., C. Y. KAO & M. ALTAMIRANO. 1945. J. Gen. Physiol. **38:** 245.
159. FALK, G. & R. W. GERARD. 1954. J. Cell. Comp. Physiol. **43:** 393.
160. KOKETSU, K. & Y. KIMURA. 1960. J. Cell. Comp. Physiol. 55: 239.
161. TASAKI, I. & T. TAKENAKA. 1963. Proc. Nat. Acad. Sci. U.S.A. **50:** 619.
162. TASAKI, I. & M. LUXORO. 1964. Science **145:** 1313.
163. CALDWELL, P. C. 1968. Physiol. Rev. **48:** 1.
164. BAKER, P. F., A. L. HODGKIN & T. I. SHAW. 1962. J. Physiol. **163:** 355.
165. LING, G. N. 1967. Naturw. Rundschau **20:** 415.
166. LING, G. N. 1971. *In* Die Zell. H. Metzner, Ed. 2nd edit. : 314. Wissenschaftliche Verlagsgesellschaft. Stuttgart, W. Germany.
167. EDELMANN, L. This monograph.
168. EDELMANN, L. & J. H. BALDAUF. 1971. Proc. First Europ. Biophys. Congr. **3:** 243.
169. SJODIN, R. A. 1971. *In* Biophysics & Physiology of Excitable Membranes. W. J. Adelman, Ed.
170. HASSEBACK, W. 1965. Progr. Biophys. Med. Biol. **14:** 167.
171. SCHATZMANN, H. T. 1966. Experientia **22:** 364.
172. GILBERT, D. L. 1960. J. Gen. Physiol. **43:** 1103.
173. MARTIN, K. 1967. J. Physiol. **191:** 105.

174. SCHOFFENIELS, E. 1967. Cellular Aspects of Membrane Permeability. Pergamon Press. New York.
175. JOHNSTONE, R. M. & P. G. SCHOLEFIELD. 1965. Advan. Cancer Res. **9:** 143.
176. WINTER, C. G. & H. N. CHRISTENSEN. 1965. J. Biol. Chem. **240:** 3594.
177. EICHBORN, J. & O. HECHTER. 1961. J. Gen. Physiol. **45:** 15.
178. RYBOVA, R. 1965. Physiol. Bohesoslov. **14:** 412.
179. ESSIG, A. 1968. Biophys. J. **8:** 53.
180. SJODIN, R. A. 1972. Biophys. Soc. Abstr **12:** 72a.
181. GILLESPIE, J. S. & D. N. H. HAMILTON. 1967. J. Physiol. **192:** 30P.
182. BITO, L. Z. 1971. Pharmacoloq. **13:** 293.
183. EHRENPREIS, S. 1967. Ann. N.Y. Acad. Sci. Disc. (Discussion) **144:** 754.
184. SALVATORE, G., M. SALVATORE & J. WOLFF. 1966. Biochim. Biophys. Acta **120:** 383.
185. NEUMANN, P. Z. & M. SILVERBERG. 1966. Nature **216:** 414.
186. DIVEKAR, A. Y., N. R. VAIDYA & B. M. BRAGANCA. 1967. Biochim. Biophys. Acta **135:** 927.
187. KEYNES, R. D. 1962. J. Physiol. **163:** 19P.
188. ECCLES, J. C., R. M. ECCLES & M. ITO. 1964. Proc. Roy. Soc. **B160:** 197.
189. MINKOFF, L. & R. DAMADIAN. This monograph.
190. HUTCHINGS, B. L. 1969. Biochim. Biophys. Acta **174:** 734.

DISCUSSION

DR. M. U. PALMA: In a simple model system, that is, the agar-water gel, we recently found results that are very similar to your results on selectivity and accumulation. We have found selectivities of a factor of 30 or so, under appropriate conditions.

QUESTION: What is the effect on osmotic activity of the association of the potassium ion to the protein?

DR. LING: This is a very interesting question. If one measures the total number of ionic particles inside the cell and compares this with the number in the system that is in osmotic equilibrium with the cell, one often finds that they match each other. Thus this was found to be the case for frog muscle in equilibrium with Ringer solution. From these observations, the conclusion had been drawn that intracellular ions like K^+ must be free. However, when these questions were being pondered, little was known about proteins and their intense interaction with water, and it was assumed that the osmotic contribution of proteins was no more than that due to the total number of protein molecules in the cell, which was trivial.

In muscle cells leached in distilled water, the muscle "ghosts" I described, there are practically no ions, and yet at the same vapor pressure they take up almost exactly the same amount of water as normal living muscles with their full ionic contents.

I therefore conclude that ions in the cells are not the major factor responsible for the lowering of water activity in these living cells.

DR. H. L. NEWBOLD (*Department of Psychiatry, Lenox Hill Hospital, New York, N.Y.*): I have recently been studying sodium levels in the tissues of schizophrenics. Finding them very low in most cases, I would like to know how this sodium interchange that you describe is affected by various chemicals:

male and female sex hormones, megavitamin doses of niacin, vitamin E, tranquilizers, and so forth. Do you have any comments about this?

DR. LING: According to the association-induction hypothesis, the adsorption of water and ions on the many protein sites is cooperative. That is, groups of these regular sites are functionally linked together and act in unison under the control of what we call a cardinal site. When the cardinal site is vacant, all the regular sites may be in one cooperative state, say the K^+ ion adsorbing state. When the cardinal site is occupied by a hormone, a vitamin, or other agents (which we collectively call cardinal adsorbents), the regular sites may be in an alternative cooperative state, say, the Na^+ ion adsorbing state. The result is seen in changes in the levels of cell Na^+ or K^+ ions as you suggested.

DR. F. W. COPE: The question of osmotic pressure deserves a little more comment. If the ions in the cell do not exert osmotic pressure, then what controls the amount of water in the cell? Dr. Ling has presented evidence that the water content of the cell is governed by the Bradley isotherm for water absorption by the cell proteins. This means that if the vapor pressure of water around the cell is changed, the amount of water in the cell must also be changed. That would seem to be the obvious means of controlling the amount of water in the cell. And this has been expanded upon a little, to see how one can quantitatively relate the concentration of the solutes outside the cell to the swelling of the cell. There are many experiments on the swelling and shrinking of cells as a function of the solute concentration outside the cell. These conform to the van't Hoff equation, which was originally derived on the basis of the osmotic pressure idea. However, one can derive a close approximation to the van't Hoff equation by ignoring all osmotic phenomena, and simply using the Bradley isotherm as the factor controlling the absorption of water by the cell. The basic idea is that the solute concentration outside the cell changes the vapor pressure of the water outside the cell, which causes the Bradley isotherm to move up or down. Therefore the cell-shrinking experiments that have been used to justify the membrane hypothesis and the theory of the osmotic control of cell swelling equally well fit the idea that cell hydration is controlled by the Bradley isotherm.[98]

DR. LING: Ling and Negendank have recently shown that the linear relationship between water content and the reciprocal of osmotic pressure is restricted to only one region of water activity, or partial vapor pressure. Over virtually the entire range of vapor pressure, however, the data are correctly described by the association-induction hypothesis, according to which the bulk of cell water exists in polarized multilayers.

In work to be published, we have shown that muscle "ghosts," muscle leached in distilled water until virtually all intracellular solutes are removed, retain practically the same amount of water as normal muscle (when at the same vapor pressure as a Ringer solution), and that at a high or low pH, more water is taken up, as is well known to be the case in unleached muscles. The question is, why is additional water taken up in such enormous quantities when the pH is low or high? The classical interpretation is that this is due to electrostatic effect. We submit an alternative, namely, that the tendency of cell proteins to take up more water is normally held in check by the salt linkages formed between β- and γ-carboxyl groups and ϵ-amino or guanidyl groups. High or low pH values deionize either the fixed cations or the fixed anions. As a result, the salt linkages are dissociated and more water is taken up.

The evidence for this aspect of the association-induction hypothesis is

twofold. Firstly, actomyosin gel takes up much more water *in vitro* than it does as part of a normal, living muscle.

Secondly, we have evidence against the electrostatic theory. If the increased water uptake at pH 3, for example, is due to an increase in the electrostatic repulsion among fixed anionic charges, this effect should be enhanced if we substitute, say, methanol, which has a dielectric constant of 30, or acetone, which has a dielectric constant of 20, for water, which has a dielectric constant of 81. In fact, the equilibrium volumes of the muscles were greatly reduced in methanol or acetone at pH 3, instead of being further augmented. This shows that the long-range electrostatic effect plays at best a minor role here. It is primarily the tendency of cell proteins to orient water in *deep* polarized multilayers that determines the cell volume in an aqueous environment.

DR. ABRAMSON (*Albert Einstein College of Medicine, Bronx, New York*): I would like to ask two points. One, have you studied temperature effects; have you observed any temperature change that would indicate some conformational changes in the protein, or possibly structure changes in the water? And have you monitored the anions present and determined whether the proteins provide some of the anionic groups that change with structure, or are those free ions that come in or leave as the cations change?

DR. LING: The answer to the first question, whether ion accumulation changes with temperature, is yes. Dr. Reisin, in cooperation with Dr. Gulati, has demonstrated the existence of phase transitions with sharp transition temperatures, which vary with the nature of the cell.

In answer to the question about anions, remember that in muscle tissue they are mostly phosphorylated metabolic intermediates; indeed, they are largely creatine phosphate and ATP. The key role these compounds, and ATP in particular, play in the control of K^+ ion accumulation will be fully dealt with when cooperative phenomena are discussed.

DR. R. DAMADIAN: At least in bacteria, there is a quantitative electrostatic correspondence of anions with cations, both in the unenriched cation state and in the enriched cation state.

Specifically, though, I want to comment on Dr. Newbold's question on the relation between neuroexcitability in tissues and their ion composition, I want to call attention to the dramatic effect of alkali cations on neuroexcitability, namely the effect of lithium carbonate in quieting neuroexcitability in patients with manic depression, and the effect of rubidium in increasing the neuroexcitability of patients in excited states. This is a very important point, because if one tries to explain these data in terms of conventional membrane pumps, one is at a complete loss to explain why lithium quiets the neuroexcitable state and rubidium enhances excitability.

If one specifically deals with the effect of these ions on solvent, however, and takes into consideration the continual increase in the solubility of these ions as we proceed down the periodic table, starting with hydrogen and ending with cesium, one discovers that these ions have a rather profound effect on the total solvent structure, and that the easiest way to interpret the effects of lithium and rubidium on neuroexcitability is simply in terms of the tendencies of these two ions to order water on the inside of the cell.

DR. ALAN MACKENZIE (*Madison, Wisconsin*): I would like to comment on Dr. Ling's reference to Luyet's "freezing patterns." Dr. Ling has shown us several of the many widely different freezing patterns Luyet obtained when he froze selected aqueous systems at various rates. Dr. Ling, you have chosen

to reproduce patterns obtained at rather high subzero temperatures. Would you agree that in these circumstances the growth of ice in the single muscle fibers is very likely restricted to the columnar spaces between the fibrils? With increasing interfacial curvature, the freezing point of water is, I believe, sufficiently depressed to support this contention. Is it not unnecessary to postulate that ice grows within the fibrils at −2.5° C? Interfibrillar propagation could account for the spiralling growth of ice in the twisted muscle fiber.

DR. LING: If spikes form only in the interfibrillar space, it is very difficult to understand why the contracture brought about by caffeine should virtually abolish this rapidly propagated formation of ice spikes. I am less inclined to accept the radius of curvature idea. Chambers and Hale showed in 1932 that at the temperature used in the muscle experiment, extremely fine ice crystals regularly form in amoebas. Thus it is possible to form ice crystals of much smaller radius.

DR. ROGER COOKE (*University of California, Berkeley, California*): Several studies have appeared recently that have measured the self-diffusion coefficient of water inside a muscle, using magnetic field gradients and pulse nuclear magnetic resonance methods. These studies have come to the conclusion that the self-diffusion coefficient of muscle water is similar to that of normal water. Can you reconcile this with your idea of the polarized multilayers?

DR. LING: The idea that water exists in polarized multilayers refers to an equilibrium adsorption phenomenon, whereas a diffusion coefficient is a kinetic property. There is not necessarily a simple, predictable correlation between the two.

Some adsorption involves a severe restriction in the translational motion; this type of adsorption is often referred to as localized adsorption. Other adsorption involves no such restriction; this is often called mobile adsorption. An example of the latter type of adsorption is the adsorption of K^+ ion on glass surfaces. In this case, the diffusion coefficient of the K^+ ion has been clearly shown to be not lower, but actually higher than in free solutions.

I also want to add that studies of radial water diffusion coefficients in frog ovarian eggs and in giant barnacle muscle fibers indicate that they are not the same as in normal water; they are one-half to one-third the value of tritiated water diffusion coefficients in free solution.