MEMBRANE LIPID LAYERS VS. POLARIZED WATER DOMINATED BY FIXED IONS: A COMPARATIVE STUDY OF THE EFFECTS OF THREE MACROCYCLIC IONOPHORES ON THE K^+ PERMEABILITY OF FROG SKELETAL MUSCLE, FROG OVARIAN EGGS, AND HUMAN ERYTHROCYTES

G. N. LING AND M. M. OCHSENFELD
Department of Molecular Biology, Pennsylvania Hospital, Eighth and Spruce Streets, Philadelphia, PA 19107.

Moritz Traube's copper ferrocyanide gel membrane was highly permeable to water but not to many ions and other solutes (Traube, 1867). To explain this "semi-permeability", he suggested that this membrane acted as an "atomic sieve", with pores large enough to allow passage of the small water molecules but not that of larger solutes. Further studies of copper-ferrocyanide membranes by Pfeffer ultimately led to his founding of the membrane theory (Pfeffer, 1877). The atomic sieve idea was not supported by later electron and x-ray diffraction studies of the copper ferrocyanide gel which was shown to have "pores" with diameters of the order of magnitude of 100 to 200 Å, far too large to explain the impermeability of this membrane to molecules the size of sucrose (diameter 8.8 Å) (for review, see Glasstone, 1946, pps. 656-657; see also Findlay, 1919, p. 95 for additional evidence).

An alternative concept of the nature of the cell membrane was proposed by Overton and given the name, lipoidal membrane theory (Overton, 1899). Adopting the "solution theory" of osmosis of L'Hermite (1855) and others, Overton hypothesized that all cells are covered by a continuous lipid layer and that the relative permeability of different nonelectrolytes are determined by their solubility in this lipid membrane. Overton's theory received support from Collander who showed that the rates of permeation of a large number of nonelectrolytes into the central vacuoles of Nitella cells were, with some notable exceptions (see below), correlated with their oil/water distribution coefficients (for review, see Collander, 1959); and from Gorter and Grendell (1925) who showed that different types of red blood cells studied possessed just enough lipids to form a continuous bilayer covering of the entire cells.

Among the evidence that seriously contradicted the lipoidal membrane theory was that the lipoidal membrane theory predicts that the cell membrane should be more permeable to ethanol than to water. This prediction, however, contradicts the conclusion from the first recorded demonstration of "semipermeability" by Abbé Nollet, who observed higher permeability of pig bladder membrane to water than to ethanol (see Glasstone, 1946, p. 651). To correct this type of shortcoming, the concept of a mosaic membrane was introduced (Collander and Bärlund, 1933). A mosaic membrane is one in which a continuous lipid membrane is punctured by pores just wide enough to allow the passage of water and other small molecules but to bar passage of other larger solute molecules. As such, the mosaic membrane theory incorporates both the lipoid membrane concept and the atomic sieve idea. Collander and Bärlund, however, made no reference to the evidence then available against the atomic sieve idea.

In the early 40's, Boyle and Conway (1941)

once more invoked and elaborated on Traube's atomic sieve idea. They too made no reference to the evidence against the atomic sieve concept. In their newer version of the atomic sieve theory, a semipermeable, ion-selective cell membrane carries pores of uniform sizes, just wide enough to allow the passage of smaller (hydrated) K', H', and Cl^- with relative diameters (r.d.) of 1.00, 0.20, and 0.98 respectively but not that of the larger (hydrated) Na^+ (r.d. = 1.49), Li^+ (r.d. = 1.95); Ca^{++} (r.d. = 2.51), and Mg^{++} (r.d. = 2.84). This theory provides a mechanism for both selective accumulation of K^+ over Na^+ in living cells and selective K^+ permeability of cell membranes. Though a landmark in cell physiology, this theory was disproven soon after its publication; radioactive tracer studies showed that Na^+ (Cohn and Cohn, 1939; Heppel, 1939), Cd' (Shanes and Bianchi, 1959), and Mg^+ (Conway and Cruess-Callaghan, 1937; Ling et al., 1979) are in fact all permeant.

In 1962 Ling had compiled from the literature 22 sets of data on the radii of hydrated K', Na^+, and Li' averaging respectively 2.15 $\pm$ 0.22 (SE.), 2.69 $\pm$ 0.33, and 3.21 $\pm$ 0.45 Å (Ling, 1962, p. 548). According to Boyle and Conway's figure the radius of hydrated Mg^{++} is 2.84 times larger than that of K^+ and is thus of the order of 6.10 Å which is considerably larger than that of sucrose (radius, 4.4 Å, see Renkin, 1954). Yet Mg" traverses the muscle cell membrane very rapidly ($t_{1/2}$ = 4.7 $\pm$ 0.25 min. 25°C) (see Ling et al., 1983).

The permeability of the cell membrane to large hydrated ions posed a very serious problem for the membrane theory. It shows quite clearly that the cell membrane must contain large pores big enough to allow passage of large hydrated ions like Mg^{++}. Since even the most lipid-soluble solute that Collander presented in his well-known publication, paraldehyde, (see Collander, 1959) still had an oil-water distribution coefficient much lower than unity (i.e., 0.019), there is no reason that any of the 69 solutes Collander studied will choose the unfavorable lipid part of the cell membrane to affect their entry, and refrain from the much easier routes of large pores. When all solutes enter and leave the cells via large pores filled with normal water, the only difference in the permeability rates of water and sucrose through the cell membrane will be that due to the differences in their diffusion coefficients in water (2.44 X 10^{-5} cm^2/sec for H_2O (Wang et al, 1953) and 5.226 X 10^{-6} cm^2/sec for sucrose (Hodgman et al. 1961)), a mere difference of 4.7 fold, which is a far cry from reality. Thus for the inverted frog skin, water permeability at 25°C is more than 9000 times greater than that of sucrose (Ling, 1973).

In 1951 and years following Ling (1951, 1952, 1960) presented the rudimentary form of a new theory of the living cell, which was for a while known as (Ling's) fixed charge hypothesis. In this hypothesis, the proteins of the living cell and its subcellular organelles (e.g., the cell membrane) are joined into a three-dimensional latticework, forming what is called a "fixed charge system". A key concept of this hypothesis was to provide a molecular mechanism, whereby discrimination between K^+ and Na^+ can be achieved. This concept is described in terms of the "principle of full counterion association as a result of charge fixation." Thus in living cells, cations like K^+ and Na^+ are completely or nearly completely adsorbed on (or associated with) fixed anions as part of the proteinaceous fixed charge system of the cells*. It is to be noted that this "basic principle" was diametrically opposed to the prevailing belief at that time of full ionic dissociation in aqueous solutions, at ionic strength of biological systems (ca. 0.1 M).

Association with anionic β- and γ-carboxyl groups carried by aspartic and glutamic acid residues of cytoplasmic proteins allows the only differences between the short-range attributes of K^+ and Na^+ (e.g., size, polarizability)

to be "felt", thereby providing a mechanism for the selective adsorption of K' and hence accumulation of this cation in resting cells. This basic mechanism was also invoked to explain selective and preferential K^+ permeability over Na^+ (Ling, 1952, 1953, 1955). Later these concepts became incorporated into a general theory of the living cells under the title of "association-induction (Al) hypothesis" replacing the older name of (Ling's) fixed charge hypothesis.

In 1965 the AI hypothesis was further extended to include the polarized multilayer theory of cell water (Ling, 1965, 1972). In this subsidiary hypothesis, certain intracellular proteins found throughout the living cell exist in the fully extended conformation forming a three-dimensional matrix.** In this conformation the sequence of positively charged polypeptide NH groups (P sites) and negatively charged CO groups (N sites) polarize in multilayers the bulk of cell water. For both entropic and enthalpic reasons, water in the state of polarized multilayers has decreasing solubility for solutes with increasing size (the "size rule'). The theory of polarized multilayer of cell water provides, on one hand, a nonenergy-consuming mechanism for the exclusion of Na^+ and other large molecules and hydrated ions from water in living cells (in lieu of the Na^+ pump and other pumps); and on the other hand, a possible candidate for the semipermeable barrier of the cell membrane (in lieu of the mosaic membrane concept) (see below).

The original mosaic membrane concept is faulty because its major component, the atomic sieve idea has been counterindicated again and again in the past. Polarized water as the basis for size-dependent permeability is, on the other hand, compatible with semipermeable properties of pores too wide to act as molecular sieves as the copper ferrocyanide gel membrane had demonstrated. Evidence suggesting that polarized water may indeed act as the seat of the semipermeable properties of living cell membrane were presented in 1973 (Ling, 1973).

* It should be pointed out that the existence of fixed charges on the cell membranes and model systems have been known for a long time (Bethe and Toporoff, 1914, 1915; Michaelis, 1925; Toerell, 1935-36; Meyer and Sievers, 1936: Sollner et al., 1941). However, following conventional concept of properties of dilute solutions, counterions of these fixed charges were considered fully dissociated. As a result, no selectivity for the counterions could be achieved since the differences between K^+ and Na^+, for example, are in short-range attributes which are not "sensed" unless they are closely associated with the fixed anions. Counterion selectivity on the basis of close-contact association was a new concept introduced first in 1951 and 1952 (Ling, 1951, 1952, 1960, 1962). Theoretical basis for the effect of charge fixation on enhanced counterion association was discussed and experimental evidence cited (Kern, 1948: Ling, 1962; Ling and Zhang, 1983, 1984).

** The fully extended conformation is a conformation where all inter- and intra-chain H-bonds are broken, and the polypeptides NHCO groups directly exposed to the bulk-phase water. This conformation is different from the "extended" conformation, denoting β-pleated sheet conformation.

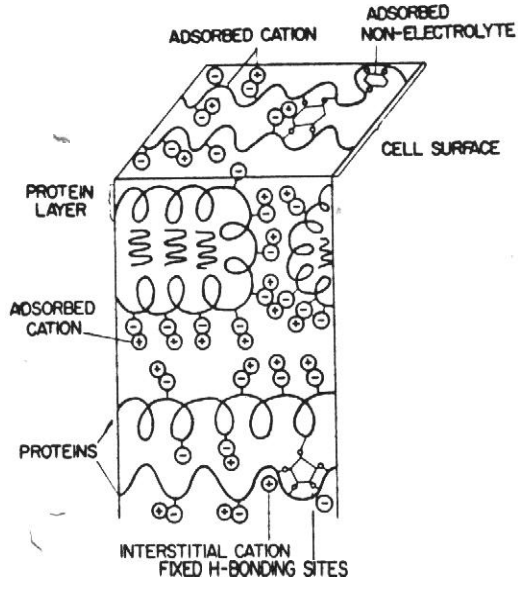

FIGURE 1. Diagram of the surface of a typical cell, including the cell membrane and the underlying cytoplasm. Water molecules, existing throughout in polarized multilayers, have been eliminated here (Ling, 1965).

Figure 1, taken from an earlier review (Ling, 1965) presents a diagrammatic illustration of a part of a cell membrane as primarily a proteinaceous fixed charge system where an aqueous pore is filled with water polarized by the protein in the form of multilayers and lined with an array of fixed anions whose association with entrant cations provides an "adsorption-desorption route" for the selective exchange of ions across the cell membrane (Figure 2). Ions of the opposite charge as the fixed ion (e.g., Na^+) but unable to compete successfully for the fixed anions against preferred counterions (e.g., K^+), ions of the same polarity as the fixed ions (e.g.. Cl^-), and neutral nonelectrolytes may enter the cell via the polarized water by what was designated as the "saltatory route".

Thus far little effort has been made to assess how much of the surface of one specific cell type is covered by a lipid layer and how much is covered by the fixed charge polarized water system and if significant differences exist among different cell types in the relative abundance of the lipid component vs. the fixed charge-polarized water component of the cell membrane.

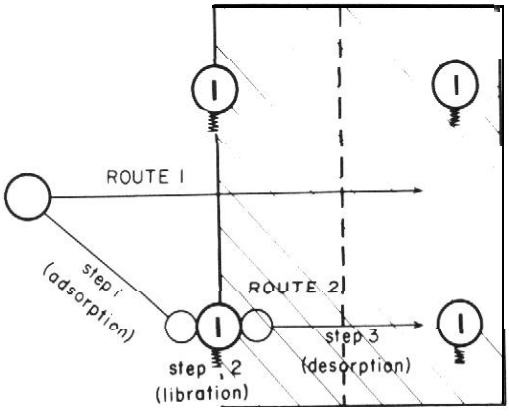

FIGURE 2. Diagrammatic illustration of the two routes of ion entry into a fixed-charge system. Shaded area represents a microscopic portion of the surface of a fixed-charge system in which four fixed anions are represented. Route I is the saltatory route. Route 2, the adsorption-desorption route, involves a sequence of three steps: adsorption, libration around the fixed anion, and desorption. (Ling and Ochsenfeld, 1965.)

Andreoli, Tiffenberg, and Tosteson (1967) showed that black membranes prepared from lipids extracted from sheep erythrocytes have very high electrical resistance ($1 - 3 \times 10^8$ ohm-cm*) which is indifferent to the concentration of KC1 solution bathing the membrane. The introduction of 10^{-7} M valinomycin or mixtures of monactin and dinactin produced drastic decreases of the membrane resistance by as much as 3 or 4 orders of magnitudes if the bathing solution also contained a high enough concentration of KCl (10^{-1}M for valinomycin, 10^{-2}M for monactin-dinactin). It occurred to us that these properties of the macrocyclic antibiotics might offer insight in the relative proportion of lipid phase and fixed-charge polarized water phase of different types of cell membranes.

Figure 3 diagrammatically illustrates the three hypothetical types of cell membranes and their anticipated response to the presence of external K' and K^+ ionophores. Type I, pure lipid membranes; Type 2, mixed lipid-fixed charge-polarized water membranes; and Type 3, pure or effectively pure fixed charge-polarized water membranes. In the presence of external K^+ and K^+ ionophores. Type I, pure lipid membranes; Type II, mixed lipid-fixed charge-polarized water membranes; and Type III, pure or effectively pure fixed charge-polarized water membranes. In the presence of external K', K^+ ionophores drastically increase K^+ permeability of pure lipid membranes, moderately increase K^+ permeability of mixed membranes, but have no effect on the K^+ permeability of pure fixed charge-polarized water membranes.

cells, a highly specialized cell to serve a very specific function, O_2 and CO_2 transport; frog sartorius muscle, which represents the excitable cells whose ionic permeability changes underlie its diverse physiological functions; and the prototypic frog eggs, from which all cell types are derived.

MATERIALS AND METHODS

Sartorius muscle and ovarian eggs were obtained from the North American leopard frogs (Rana pipiens pipiens Schreber). The frogs were force-fed canned dog food twice a week; they were kept in a stainless steel sink with running water at room temperature. Human red blood cells were obtained from freshly drawn heparinized whole blood of healthy adults.

In all cases, we studied the K^+ permeability by measuring the rate of influx of labelled K^+ into the cells during a 14 to 15 minute exposure followed by removal of radioactivity in the adhering fluids or in the extracellular space. Influx studies are superior to efflux studies due to the time-saving efficiency and the freedom from choosing between fractions when efflux curves are complex. More detailed procedures are given below for each tissue studied.

Frog *Sartorius* Muscle *Studies.* After isolation, the sartorius muscles were kept overnight at 4° C in a Ringer's phosphate solution (Nd, 103.7 mM; K^+, 2.5 mM; Ca^{++}, 0.72 mM; Mg^{++}, 1.2 mM; Cl-, 96.6 mM; HCO_3, 6.64 mM; PO_4, 3.2 mM; SO_4, 1.2 mM; dextrose, 24.0 mM).

Loose connective tissue isolated from the legs and thighs of the animals that provided the muscles were treated in the same manner as the sartorius.

Muscle pairs were divided into two groups. One group was incubated in a Ringer's phosphate solution containing varying K^+ concentrations and either 10^{-7}M valinomycin (Sigma Chemical Co. Lot 118C-4025; Boehringer Mannheim Lots 1139205, 1379106); 10^{-7}M nonactin (Boehringer Mannheim. Lot 1468304); or 10^{-7}M monactin (obtained as a gift from Drs. Muller and Scheibli, Ciba-Geigy), Ionophores were initially dissolved in absolute ethyl alcohol which in the final solutions was 0.1%. Incubation of the control group was in a Ringer's phosphate solution of the same composition but without the ionophores. To avoid possible adherence of ionophores on to glass, plastic ware was used throughout the experiment.

The tissues were preincubated in experimental solutions at 27° C for fifteen minutes by one of two methods: (1) immersion in a

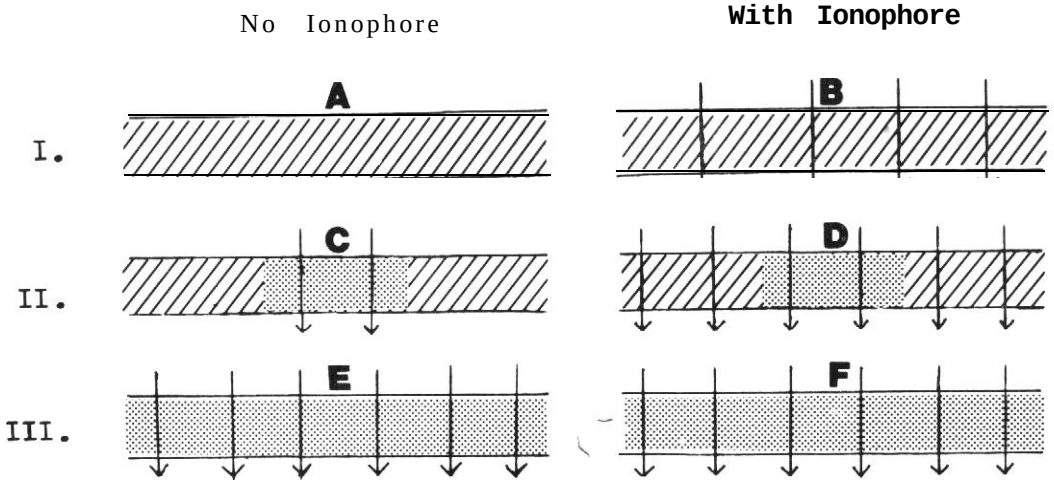

FIGURE 3. Diagrammatic illustration of three types of cell membranes and the effect of ionophores on the rate of K^+ permeation. Number of arrows roughly correspond to extent of K^+ permeation. Type I, pure lipid membrane; Type II, mixed lipid-fixed ion-polarized water membrane; Type 111, pure or effectively pure fixed ion-polarized water membrane.

large volume of solution (200-400 cc) while either bubbling with air or vigorously shaking on a rotating shaker or (2) in a continuous flowing solution at the rate of 30 cc/min through a vessel holding the tissues.

The tissues were removed at the end of pre-incubation, quickly blotted on filter paper wetted with the experimental solution and transferred to a" incubating solution of the same compositions as the preincubation solutions but with ^{42}K label (New England Nuclear Co., Lots 262, 271, 277, 325, 331). The average duration of incubation was 14 minutes, a time short enough to measure the true initial rate of entry (Ling and Ochsenfeld, 1965).

Extracellular space fluids were removed from the muscles and the connective tissues with the aid of the centrifugation procedure described by Ling and Walton (1975a).

Following centrifugation, the muscle tissue was frozen in liquid nitrogen and broken into two pieces: one piece was weighed and dried in 100°C oven to determine the water content of tissue; the remaining piece was extracted in 3 ml. of 5% trichloroacetic acid and the ^{42}K activity measured on a Nuclear-Chicago y-scintillation system. The extract was also analyzed for total K^+ and Na^+ on a Perkin Elmer Model 103 atomic adsorption spectrophotometer.

Final data were corrected for connective tissue elements present in the isolated muscle. Experimental data obtained for the treated connective tissue and a" estimated 5.9% for the weight of connective tissue in centrifuged muscle provided the basics for correction (Ling and Walton, 1975b).

Oocyte Studies. Clusters of 15 eggs from several frogs were dissected free from the ovarian lobe in a cold Ringer's phosphate solution just prior to the experiment. The bulk of the connective tissue attached to the egg was removed leaving a bare minimum for handling with fine forceps. The diameters of eggs used ranged from 1.1 to 1.9 mm.

Incubation solutions were of the exact composition as those used in sartorius studies. The experimental procedure was similar to muscle studies except adhering incubation fluid was removed by a 3-5 second rinse in a large body of nontagged incubation solution. The cluster was the" drained by placing it on filter paper moistened with the nontagged incubation solution and gently blotted.

To measure the contribution of the connective tissue elements adhering to eggs, connective tissues were obtained from the "stalk" of the ovary and from the ovarian tissue lobe after removal of all eggs. These tissues were then incubated and washed in exactly the same manner as the eggs and assayed for radioactivity to provide data for making corrections.

The percentage of extracellular tissues surrounding the egg cells were determined by a method described earlier (Ling and Ochsenfeld, 1977). For the present experiments this percentage varied between 1.1 to 2.7% depending upon the sire of the eggs.

The water content of the egg cluster was determined by treating duplicate egg clusters in the same manner as the experimental egg clusters and drying in vacuo over phosphorous pentoxide.

Red Blood Cell Studies. The basic incubation solutions for human red blood cells were slightly modified Kreb's solutions (composition of basic medium: Na', 143.2 mM; K^+, 5.0 mM; Ca^{++}, 2.9 mM; Mg", 1.2 mM; Cl^-, 130.9 mM; HCO;, 22.5 mM; PO,, 1.2 mM; dextrose, 5.6 mM) containing 1 "nit/ml heparin. Ionophore addition as described under muscle studies.

Red blood cells were mixed at a 10% hematocrit and preincubated with gentle shaking at 26°C under a mixture of 50% O_2, 45% N_2, 5% CO_2 for ten minutes. The suspension was then centrifuged at 27,000 X g.

Cells were resuspended at a IO-30% hema-

tocrit in a solution of the same composition as the preincubation solution but with ^{42}K label (New England Nuclear, Lots 262, 277, 325, 344, 359, 363, 365). The duration of incubation was 15 minutes.

Cells were then separated from the tagged incubation solution by centrifugation in 0.5 ml microcentrifuge tubes for either 10 minutes at 27,000 X g or 4 minutes at 15,600 X g. Part of the red blood cells spun down were weighed fresh and again after drying at 100°C to obtain their water content. Other samples of the spun-down cells were introduced into 5% trichloroacetic acid and analyzed for K', Na^+ and ^{42}K as in muscle studies.

The volumes of extracellular fluids were determined with the aid of ^{14}C-carboxyl inulin (New England Nuclear, Lot 1141-229; ICN, Lot 591163) as follows: Just prior to the final centrifugation of experimental samples, a

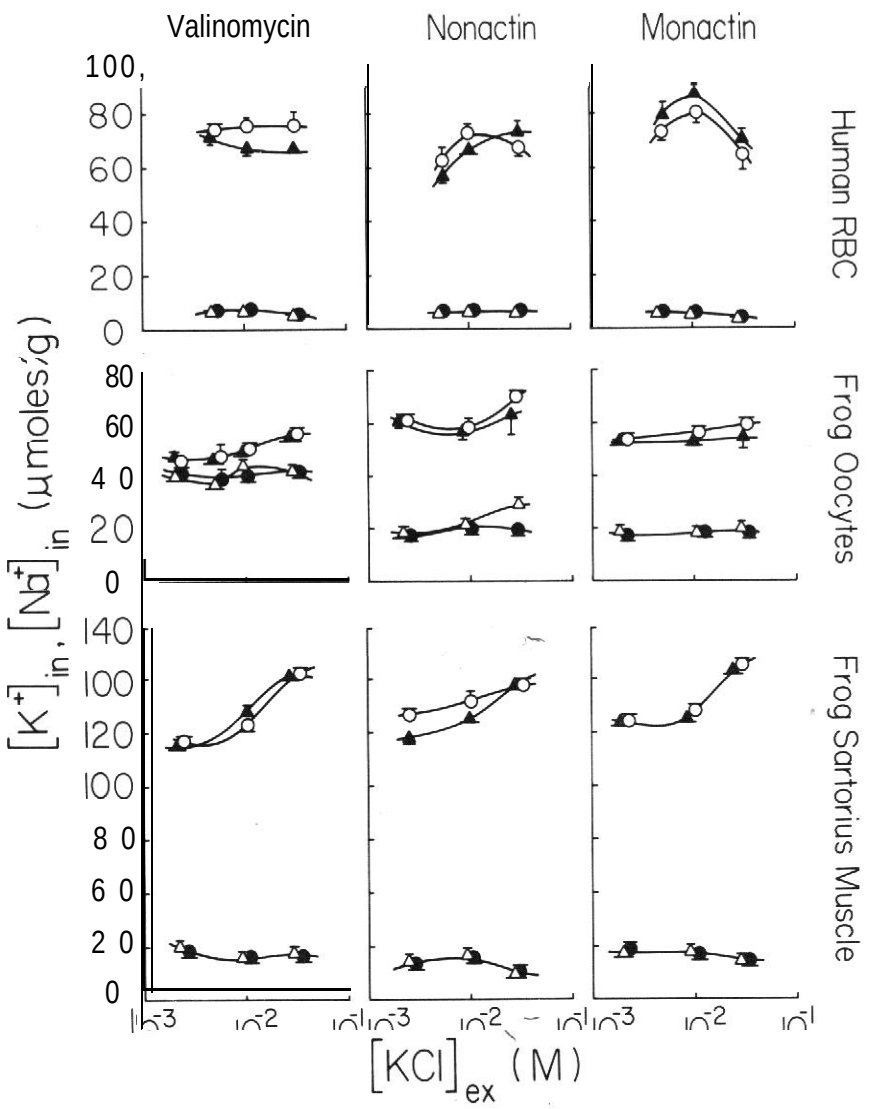

FIGURE 4. Effects of valinomycin (10^{-7}M), nonactin (10^{-7}M), and monactin (10^{-7}M) on the K^+ and Na^+ contents of human red blood cells, frog oocytes, and frog sartorius muscles at different external K^+ concentrations. Antibiotic treated cells (K^+, ▲; Na^+, Δ); their controls (K^+, O; Na^+, ●).

volume of a solution containing ^{14}C carboxyl inulin equal to 1% of the incubation solution was added and mixture gently shaken. In addition to ^{42}K and total analysis, the TCA extract of the pellet was also analyzed for ^{14}C on a Model 3300 p-scintillation spectrophotometer to determine its labelled inulin content used for correcting the radioactive and nonradioactive ionic contaminations in the extracellular space fluid.

In some cases the red cell pellet obtained from the above centrifugation was added to a finite volume of untagged Kreb's solution. After gentle mixing to resuspend cells, the sample was centrifuged as above and the supernatant analyzed for ^{14}C inulin released from cells.

The Calculation of the K Permeability Constant. Assuming first order kinetics, the

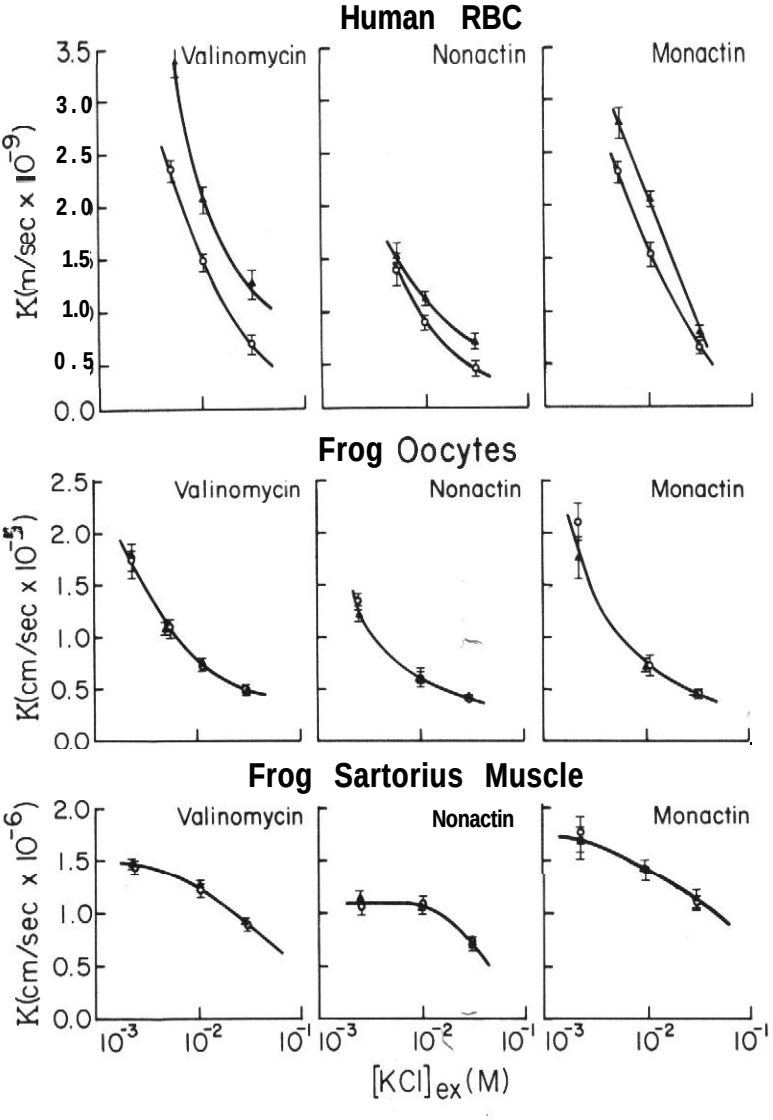

FIGURE 5. Effects of valinomycin (10^{-7}M), nonactin (10^{-7}M), and monactin (IO-'M) on the inward permeability of human red blood cells, frog oocytes, and frog sartorius muscles at different external K^+ concentrations. K^+ permeability given in units of cm/sec. ▲, antibiotic treated. 0, control.

TABLE I. Detailed data on the effects of valinomycin, nonactin, and monactin, all at 10^{-7}M on the (inward) permeability constants (κ) (in units of cm/sec.) to labeled K^+ of frog sartorius muscles.

		Control		Experimental		
	No. of Experiments	$(K^+]_{ex}$ (mM)	$\kappa \times 10^6$ (cm/sec)	$(K^+]_{ex}$ (mM)	$\kappa \times 10^6$ (cm/sec)	$\kappa_{exp}/\kappa_{control}$
Valinomycin	10	0.33	1.28 ± 0.060	0.33	1.21 ± 0.068	0.95
	27	2.45	1.43 ± 0.047	2.41	1.48 ± 0.045	1.03
	17	10.5	1.21 ± 0.051	10.2	1.25 ± 0.054	1.03
	17	31.1	0.890 ± 0.031	30.3	0.903 ± 0.031	1.01
	mean ± S.E.					(I.01 ± 0.02)
Nonactin	6	2.55	1.04 ± 0.066	2.57	1.13 ± 0.062	1.09
	6	10.2	1.06 ± 0.065	10.0	1.03 ± 0.049	0.97
	6	30.9	0.710 ± 0.038	29.8	0.669 ± 0.037	0.94
	mean ± SE.					(I.00 ± 0.05)
Monactin	6	2.32	1.74 ± 0.169	2.32	1.64 ± 0.169	0.94
	6	10.0	1.38 ± 0.100	10.0	1.38 ± 0.107	1.00
	7	30.0	1.07 ± 0.068	30.0	1.11 ± 0.080	1.04
	mean ± S.E.					(0.99 ± 0.03)
	Overall mean ± S.E.					(1.00 ± 0.016)

TABLE II. Detailed data on the effects of valinomycin, nonactin, and monactin, all at 10^{-7}M on the (inward) permeability constants (κ) (in units of cm/sec.) to labeled K^+ of frog ovarian eggs.

			Control		Experimental		
	Expt. No.	Temp.	$(K^+]_{ex}$ (mM)	x x 10^4 (cm/sec)	$(K^+]_{ex}$ (mM)	$\kappa \times 10^4$ (cm/sec)	$\kappa_{exp}/\kappa_{control}$
Valinomycin	V10	27-28°	2.34	1.74 io.157	2.37	1.77 ± 0.129	1.02
	"14		11.65	0.735 1.08 ± 0.033 0.082	11.5	0.774 1.10 ± 0.057 0.024	1.05
	V10, V14		31.8	0.494 ± 0.029	32.3	0.481 ± 0.009	0.97
	mean ± S.E.						(1.02 ± 0.02)
Nonactin	V21	29°	2.45	1.32 ± 0.050	2.54	1.19 ± 0.068	0.90
			10.0	0.606 ± 0.089	10.1	0.604 ± 0.065	1.00
			29.7	0.408 ± 0.013	30.3	0.442 ± 0.028	1.08
	mean ± S.E.						(0.99 ± 0.05)
Monactin	V24	30°	2.28	2.23 ± 0.17	2.28	1.76 ± 0.203	0.79
			11.0	0.718f0.08	10.2	0.713 ± 0.068	0.99
			33.0	0.459 ± 0.028	30.4	0.445 ± 0.012	0.97
	mean ± S.E.						(0.93 ± 0.05)
	Overall mean ± S.E.						(0.98 ± 0.02)

rate of inward permeation of labelled K^+ into the cells is described by the relation

$$\frac{d[K^*]_{in}}{dt} = \frac{A}{V} \kappa_{K^+}^{inward} [K^*]_{ex} , \qquad (1)$$

where $[K^*]_{in}$ and $[K^*]_{ex}$ are the concentration of labelled K^+ in the cells and in the incubation solution. A and V are the surface volume ratios of the cells, and $\kappa_{K^+}^{inward}$ is the inward permeability constant in units of cm/ sec. For the frog sartorius muscle cells the A/V ratios determined are 550 cm^2/gram X 1.05 = 578 $(cm)^{-1}$ (Ling, 1962, p. 208). Due to considerable variation among frog ovarian eggs studied, the A/V ratio equal to 3/r cm^{-1} was individually calculated from the radius (r) of the eggs. Since the eggs used were usually 1.5 to 2.0 mm in diameter, the A/V ratios were in the neighborhood of 30 to 40 cm^{-1}. The surface-volume ratio of human erythrocytes used, 1.57 X 10^4 $(cm)^{-1}$, was based on our own estimates and was somewhat lower than a value of 1.88 X 10^4 if one used for calculation the surface area of an individual erythrocyte (163 μ^2) and its volume (87 μ^3) given by Ponder (1948).

RESULTS

Valinomycin is a powerful uncoupler Of oxidative phosphorylation (McMurray and

TABLE 111. Detailed data on the effects of valinomycin, nonactin, and monactin, all at 10^{-7} M on the (inward) permeability constants (κ) (in units of cm/sec.) to labeled K^+ of human red blood cells. For explanation of the significance of figures in square brackets see text.

			Control		Experimental		
	No. of sets of Expts.	No. of Individual Expts.	$(K^+)_{ex}$ (mM)	$\kappa \times 10^9$ (cm/sec)	$(K^+)_{ex}$ (mM)	$\kappa \times 10^9$ (cm/sec)	$\kappa_{exp}/\kappa_{control}$
Valinomycin	3(13,27,30)	14	5.26	2.24 ± 0.093	5.72	3.67 ± 0.169	1.64
	[2(27,30)]	10	5.22	2.36 ± 0.100	5.69	3.40 ± 0.147	1.44
	4(9,13,27,30)	18	10.1	2.46 ± 0.515	10.2	4.33 ± 0.895	1.76
		14	10.4	1.50 ± 0.055	10.5	2.82 ± 0.342	1.88
	[2(27,30)])	10	10.4	1.49 ± 0.075	10.6	2.09 ± 0.12	1.40
	1(9)	4	14.3	2.14 ± 0.172	14.5	5.97 ± 0.021	2.79
	5(9,13,27,28,30)	33	31.9	1.49 ± 0.166	31.9	2.47 ± 0.33,	1.66
	[2(27,30)]	9	31.0	0.701 ± 0.075	31.0	1.28 ± 0.140	1.83
	mean ± S.E.						(1.80 ± 0.154)
Nonactin	3(17,20,26)	12	5.46	2.68 ± 0.417	5.48	3.94 ± 0.746	1.47
	[1(26)]	4	5.50	1.40 ± 0.148	5.50	1.53 ± 0.121	1.09
	4(17,20,26,31)	24	10.4	1.31 ± 0.161	10.4	1.86 ± 0.322	1.42
	[2(26,31)]	16	10.3	0.908 ± 0.06	10.3	1.12 ± 0.074	1.23
	5(17,20,26,28,31)	34	31.3	1.24 i-0.157	31.3	1.19 ± 0.198	0.96
	[2(26,31)]	10	31.4	0.476 ± 0.065	31.4	0.718 ± 0.082	1.51
	mean ± S.E.						(1.28 ± 0.09)
Monactin	2(25,29)	9	5.02	3.21 ± 0.482	5.19	4.15 ± 0.643	1.29
	[1(29)]	6	5.14	2.30 ± 0.103	5.34	2.78 ± 0.146	1.21
	2(25,29)	9	10.6	3.20 ± 0.964	10.2	3.06 f0.449	0.95
	[1(29)]	6	10.8	1.53 ± 0.104	I0.5	2.05 ± 0.075	1.34
	3(25,28,29)	26	31.0	1.63 ± 0.21	31.0	1.66 ± 0.456	1.02
	[1(29)]	6	31.8	0.656 ± 0.056	31.8	0.795 ± 0.054	1.21
	mean ± S.E.						(1.17 ± 0.63)

Begg, 1959). Exposure to this drug often led to cell deterioration with loss of cell K^+ and gain of cell Na^+ (Tosteson, et al., 1975; Carmeliet and Lieberman, 1975). This propensity to deteriorative loss of K^+ provides one important reason for our choice of influx studies over efflux studies to determine K^+ permeability. To test the predicted effect of the three K^+-specific ionophores on K^+ permeability, the cells used for the test must not be seriously damaged by the drugs. Figure 4 showed that exposure of the three types of cells to valinomycin (10^{-7}M), nonactin (10^{-7} M), and monactin (10^{-7}M) for the duration similar to those used to determine the K^+ permeability produced no or only slight changes in the K^+ and Na^+ concentrations over a range of external K^+ concentration from 2.5 to 30 mM.

The main data collected from all the experiments conducted are summarized in Figure 5 and Tables I, II, and III. Table I shows that the permeability constant, κ (in "nits of centimeter per second) of normal frog sartorius muscles varied considerably. Thus at a" external K^+ concentration "ear 2.5 mM, κ ranged between 1.04 X 10^{-6} to 1.74 X 10^{-6} cm/sec. Nevertheless the control values agree with the range of variations reported in the literature from 0.97 X 10^{-6} cm/sec (Mullins, 1959) to a high as 2.11 X 10^{-6} cm/sec (Katz, 1966) with most of the values earlier reported from this laboratory falling somewhere in between (1.54 X 10^{-6} cm/sec, Ling, 1962; 1.56 X 10^{-6}, Ling and Ochsenfeld, 1965). The agreement with literature values also shows that the inclusion of 0.1% (v/v) of ethanol in the controls did not materially alter κ, a conclusion we also verified experimentally (data not given).

A comparison of the values of κ_{K^+} of control frog muscle with their paired muscles exposed to valinomycin, nonactin, and monactin, all at 10^{-7}M, revealed that "one of these ionophores had any significant effect on the K^+ permeability of frog muscles.

Table II shows that the K^+ permeability of frog ovarian eggs is a" order of magnitude higher than that of frog sartorius muscles. Like frog muscles, the K^+ permeability is also unaffected by exposure to 10^{-7}M valinomycin, nonacti", and monactin.

Table III shows that the K^+ permeability constants of human erythrocytes are five orders of magnitude lower than that of frog ovarian eggs and three orders of magnitude lower than that of frog muscle. I" contrast to both frog tissues, but in general agreement with the report of Tosteson et al. on sheep erythrocytes, 10^{-7}M valinomycin, nonactin, and monactin all significantly increased the K^+ permeability.*** However, the magnitude of the effects was on the average below a factor of 2 — in contrast to the report of 10 to 20-fold increase reported by Tosteson and his coworkers on sheep erythrocytes. Even more modest increases of κ were produced by nonactin and monactin — in the range of 25 to 30%.

The data given in Table III, under the number of sets of experiments, 3 (13, 27, 30) indicates three sets of experiments each of which bear the expt. no. of 13, 27, and 30. On the next line in square brackets [2 (27, 30)] represents the average of only two of the three sets of data. The reason for this selectivity is as follows: In Expt. 13, the values of the extracellular spaces were determined by labelled inulin and corrections were based on

*** The fact that enhanced K^+ permeability of human erythrocytes was not paralleled by a loss of the abilities of these cells to maintain their normal high K^+ content and low Na^+ content (see Figure 1) contradicts the conclusion of Freedman and Hoffman (1979) who cited the total loss of these abilities in erythrocytes exposed to nystatin as evidence in favor of the membrane (and postulated) pumps rather than cytoplasm as the seat of K^+ accumulation and Na^+ exclusion. It would seem more likely that nystatin, a strong poison, produced its effect by injury inflicted on the cells. (For other evidence against the membrane and for the cytoplasm as the scat of selective solute distribution, see Ling and Negendank, 1980; Ling, 1984).

the average values from similarly treated erythrocytes. Later we discovered rather large variation of the extracellular spaces among different samples of erythrocytes. In experiments performed later, correction was based on inulin space determined individually on each sample; as a result, much less variations were seen. These data obtained with individual inulin-space determinations include Expt. 27 and 30. These and other similar experiments performed with individual inulin assay, are presented between the square brackets in Table III and are also the (only) data given in Figure 2.

However, even in human erythrocytes, there are also some unexplained traits of the observed increase of K^+ permeability in response to the three macrocyclic compounds. The data of Andreoli et al. (1967) mentioned above show that in the presence of 10^{-7}M valinomycin, the resistance of artificial lipid membranes demonstrated a 400-fold decrease from 10^7 to 2.5 X $10^4 \Omega/cm^2$, as the external KC1 concentration was increased from 10^{-3} to 10^{-2}M. Thus if the observed increase of K^+ permeation into human red blood cell in response to valinomycin was by a mechanism essentially similar to what Andreoli et al. demonstrated in lipid membrane from sheep erythrocytes, one would expect a 40-fold ***increase*** in K^+ permeability (κ) (in units of cm/sec.) when the external K^+ concentration was raised IO-fold from 10^{-3}M to 10^{-2}M (see equation I). The experimental data (Table I, Figure 3) did not show this trend; κ remained more or less constant. Similarly, if monactin alone and mixture of nonactin and dinactin exercise similar quantitative effects on lipid membrane permeability to K^+ one would anticipate a substantial increase of κ with increase of external K^+ concentration for 10^{-3} to 10^{-2}M. Again no increase of this kind was seen. Thus while consistency of an observed increase of K^+ permeability in response to all three macrocyclic compounds gave us some confidence for the role of these compounds as specific K^+ carriers across the lipid part of the erythrocyte membrane; further work is needed to clarify these apparent discrepancies.

DISCUSSION

I. Most Living Cells Studied Have Type III Membranes. The main conclusion from this study is that none of the three types of cells possess pure lipid membranes which would have demonstrated dramatic increase of K^+ permeability in response to the macrocyclic compounds and that only the human erythrocytes exhibit a mild sensitivity to K^+ ionophores to be expected from mixed membranes. The other two types of cells showed no response to the K^+-ionophores and apparently fall in the category of pure fixed charge-polarized water type, or effectively pure fixed charge-polarized water type.

The failure of frog muscle and frog ovarian egg to respond to each of the three macrocyclic K^+ ionophores reported here confirm earlier failure to detect any increase of K^+ permeation through the liver mitochondrial membrane on exposure to valinomycin (Maloff, et al., 1978) and similar failure to detect any increase in the rate of K^+ permeation Mto squid axon in response to monactin (Stillman, et al., 1970). Taken as a whale, one is led to believe that in fact the majority of cell membranes of living cells that have occupied the greatest share of interest among cell physiologists, resemble one another as well as the liver mitochondrial membrane. They all have membranes with properties similar to that of pure polarized water-fixed charge systems. It is the red blood cell that represents the "odd man out". From the very low K^+ permeability constant, one can safely conclude that the permeability of K^+ through the aqueous channels in erythrocyte membranes are quite low. Of course, otherwise, the erythrocytes could not have exhibited sensitivity to the macrocyclic compound as

they in fact do.

II. The Effects of Polarity of Fixed Charges on Permeability to Cations or Anions. Obviously, the lipid component of the frog muscle and egg membranes offers no significant pathway for K^+ permeation either. In other words, the much higher overall K^+ permeability in these tissues as well as in squid axons and liver mitochondrial membranes, must be due to the much faster rate of permeation through their aqueous channels. What then are the differences between aqueous channels in red cells on one hand and those in frog egg, frog muscle, squid axon, and liver mitochondria? To answer this question, we must go back to the AI hypothesis.

In 1953 Ling suggested that such aqueous pathways can be made to function as selective *ion permeability barrier,* favoring K^+ over Na^+ if the aqueous channels are endowed with fixed anions in the form of β- and γ-carboxyl groups.

The reason that fixed β- and y-carboxyl groups can function as ion selective filters is that most entrant ions must first adsorb on the fixed anion before eventual entry into the cytoplasmic water and only the ion most preferred by the fixed anion and also *readily desorbed* can rapidly enter the cell. This adsorption-desorption route can be blocked by high concentration of a competing ion. At an external concentration of 2.5 mM, the rate of entry of K^+ into frog muscles can be reduced by 90% by the presence of 75 mM Rb' in the external medium (Ling, unpublished), Thus at least 90% of the entrant K^+ takes this adsorption-desorption route showing both saturability and competition characteristics.

The AI hypothesis offered two reasons for the predominance of this adsorption-desorption route of K^+ entry over the "saltatory route", i.e., direct entry through the aqueous phase of the pore without adsorbing onto the fixed anions. The first reason is electrostatic. Like the electrically charged cotton fibers in cotton wads once widely used to stopper sterile flasks, the fixed ions either repel or attract and capture the oppositely charged ions (or bacteria). The second reason is the low solubility as well as low diffusion coefficients in the polarized water in the membrane pore of large and complex molecules and hydrated ions like K^+ or Na^+.

From its low K^+ permeability of the red blood cell membranes one may anticipate that few aqueous pores containing fixed β- and y-carboxyl groups are found. With this in mind, one might anticipate very high electrical resistance of red cell membranes. In fact, the opposite is the case. At 7 to 10 Ω/cm^2, the red cell membrane is one of the least resistive of all cell membranes (Lassen and Sten-Knudsen, 1968; Johnson and Woodbury, 1964; frog muscle: 4000 Ω/cm^2 Shanes, 1958; squid axons: 700 to 1000 Ω/cm^2; Cole and Hodgkin, 1938-39). The low resistance of the red cell membrane is paralleled by a high Cl^- permeability of the red cell membrane, i.e., 1.5 X 10^{-4} cm/sec. (Dirkin and Mook, 1931; Tosteson, 1959). Passow, Schnell and coworkers had extended the concept introduced by Ling for fixed anion-mediated cation permeation (Ling, 1953, 1960), to fixed cation-mediation of chloride permeation (Passow. 1965; Passow and Schnell, 1969).

In summary, our comparative study of the permeability to K^+ shows that selective permeability of the cell membrane to K^+ primarily depends on the density as well as properties of fixed β- and y-carboxyl groups-lined aqueous channels; selective Cl^- permeability depends on the density of fixed cations (e.g., e-amino group and guanidyl group)-lined aqueous channels. We shall examine next another mode of entry of solutes into cells, which does not involve adsorption on and desorption from fixed ions, i.e., the saltatory route (Route I of Figure 2).

III. The Ionic Selectivity Via Saltatory Route. As mentioned above, a high concentration (75 mM) of strongly adsorbed counterion of the same polarity (e.g., Rb^+) inhibits the entry of K^+ (2.5 mM) by 90%. Thus some 10% of the entrant K^+ enters frog muscle by means of a nonsaturable, and noncompetitive saltatory route. In theory, such a route may be via the lipid phase of the cell membrane, but this possibility is ruled out by the extremely low permeability of lipids to ions (see above). In frog muscle, this nonsaturable route of entry exhibits a permeability rate corresponding to a κ of about 10^{-6} cm/sec. and hence still orders of magnitude faster than the total permeation rate of erythrocyte membranes (i.e., 10^{-9} cm/sec.). The question arises, "Why can't K^+ enter via the fixed cation-dominated aqueous pores in the red cell membrane as easily as it enters via the fixed anion-dominated aqueous pores of the frog muscle and eggs?"

The most direct answer is again electrostatic: due to repelling electrostatic barriers the permeability of an entrant ion via the saltatory route is greatly less when the entrant ion is of the same electric charge (Ling, 1984, p. 102). Next we asked the question, "What relative proportion of the cell membrane in frog egg and muscle is occupied by water?"

IV. The Relative Area of the Cell Membranes of Frog Ovarian Eggs and Frog Muscle that are Spanned by Polarized Wafer. Removal of 95% of the lipid components of liver mitochondria membrane did not materially alter the trilaminar structure of EM picture of liver mitochondria (Fleisher, et al., 1967), a striking finding repeatedly confirmed in other tissues and not seriously challenged (see Ling, 1984, p. 382). Whereas the red blood cells contain the highest lipid content in its dry substance (47%) and according to Gorter and Grendell this lipid content is just enough to form a continuous bilayer, the lipid contents of many types of cell membranes are considerably less than that in the red cell membranes (see Dewey and Barr, 1970). Thus the inner membrane of liver mitochondria contains only 20% lipids in its membrane dry matter. The conclusion derived from electron microscopic studies of liver mitochondrial membranes by Sjöstrand and his coworkers: "in this membrane, lipids do not form continuous layers but only 'islands'." (Sjöstrand, 1978) The general similarity of the behavior toward K^+ ionophores of the inner membrane of liver mitochondria and those of squid axons, frog muscle, and frog ovarian eggs suggest similar distribution patterns of lipids, as isolated islands and as such cannot function as an effective diffusional barrier.

V. Evidence of Extensive Coverage of Cell Membranes of Frog Ovarian Eggs and Frog Muscle with (Polarized) Water. By careful analysis of the profiles of influx of labeled water in frog eggs (Ling, et al., 1967) and into isolated single giant barnacle muscle fibers (Reisin and Ling, 1973), the conclusion was reached that in both cases the rate of labeled water exchange is "bulk phase limited", much as diffusion of water and solutes into a filament of agar gel is bulk phase limited. What this means is that the relative area of the cell membrane covered by water is similar to that in any imaginary concentric spherical shell of egg cytoplasm (like that of an onion) or concentric cylindrical shell of muscle cytoplasm. In other words, as a diffusion media for labeled water, the water in the cell membrane is similar to water in the cytoplasm and the relative areas occupied by proteins, lipids, and other barriers to water diffusion are-approximately the same as the inward diffusing labeled water approaches the center of the cell. How can this conclusion be "reconciled" with current opinions of extreme sparsity of membrane pores is the main concern of a following paper (Ling, 1986).

It should be carefully pointed out that the

demonstration of bulk phase limited diffusion does not imply an absence of a permeability barrier at the surfaces of frog egg and frog muscle. Quite the contrary, the cell membrane is decidedly rate-limiting for the exchange of ions, sugars, etc., because the water in the cell membrane being more intensely polarized than the bulk phase water affects the diffusion of water very insignificantly, while it does slow down the diffusion of large and complex molecules intensely (see Ling, 1973).

SUMMARY

The effects of 10^{-7}M valinomycin, nonactin, and monactin on human erythrocytes, frog sartorius muscle, and frog ovarian oocytes in the presence of varying external K^+ concentration were studied. The results showed essentially a consistent but relatively modest increase of the K^+ permeability constant in cm/sec with all three antibiotics on human erythrocytes. No change in response to any one of the antibiotics was observed in frog muscles or in frog ovarian eggs.

These results and reports of similar failure to demonstrate ionophore-mediated increase of K^+ permeability in squid axon and inner membrane of the liver mitochondria led to the conclusion that lipid membrane barrier to ionic traffic may be significant in the human erythrocytes but even here one must regard the evidence as tentative. In contrast, for the majority of other cell types studied, the data indicate the primary, if not exclusive route of ion traffic, is via the nonlipid component of the cell membrane. The evidence that these nonlipid paths are the fixed charge-polarized water layer complex and that they cover much of the cell surface of many types of living cells was discussed.

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