The Formation and Properties of Thin Lipid Membranes from HK and LK Sheep Red Cell Lipids

THOMAS E. ANDREOLI, J. ANDREW BANGHAM, and DANIEL C. TOSTESON

From the Departments of Physiology and Pharmacology and Medicine, Duke University, Durham, North Carolina

ABSTRACT Lipids were obtained from high potassium (HK) and low potassium (LK) sheep red cells by sequential extraction of the erythrocytes with isopropanol-chloroform, chloroform-methanol-0.1 M KCl, and chloroform. The extract contained cholesterol and phospholipid in a molar ratio of 0.8:1.0, and less than 1% protein contaminant. Stable thin lipid membranes separating two aqueous compartments were formed from an erythrocyte lipid-hydrocarbon solution, and had an electrical resistance of $\sim 10^8$ ohm-cm^2 and a capacitance of 0.38–0.4 μf/cm^2. From the capacitance values, membrane thickness was estimated to be 46–132 A, depending on the assumed value for the dielectric constant (2.0–4.5). Membrane voltage was recorded in the presence of ionic (NaCl and/or KCl) concentration gradients in the solutions bathing the membrane. The permeability of the membrane to Na^+, K^+, and Cl^- (expressed as the transference number, T_{ion}) was computed from the steady-state membrane voltage and the activity ratio of the ions in the compartments bathing the membrane. T_{Na} and T_K were approximately equal (~ 0.8) and considerably greater than T_{Cl} (~ 0.2). The ionic transference numbers were independent of temperature, the hydrocarbon solvent, the osmolarity of the solutions bathing the membranes, and the cholesterol content of the membranes, over the range 21–38°C. The high degree of membrane cation selectivity was tentatively attributed to the negatively charged phospholipids (phosphatidylethanolamine and phosphatidylserine) present in the lipid extract.

INTRODUCTION

The relative permeabilities of high-potassium (HK) and low-potassium (LK) sheep red cell membranes to sodium and potassium ions are quite different (1, 2). The difference in cation selectivity may be a consequence of the physicochemical properties of the membrane lipids in the two genetically determined cell types. In order to explore this possibility, the ionic permeability properties of artificial membranes prepared from HK and LK sheep erythrocyte lipids were characterized.

In 1938, Langmuir and Waugh (3) described the formation under water of "black" lecithin-benzene films of short duration. More recently, Mueller, Rudin, Ti Tien, and Wescott (4–7) described a technique for the formation of stable membranes 60–90 A thick from a complex lipid extract of cattle brain and tocopherol dissolved in chloroform-methanol. Since these membranes are analogous to bimolecular black soap films formed in air (8), they provide an interesting synthetic model of the bimolecular lipid leaflet originally proposed by Davson and Danielli (9) as the fundamental unit of biological membranes. Subsequently, Huang et al. (10) formed similar membranes from purified lecithin and an aliphatic hydrocarbon in chloroform methanol. Using optical methods (10, 11), they computed membrane thickness to be 61 A and, more recently, have described the water permeability properties of these membranes (12). Schwan et al. (13) have calculated the capacitance of these membranes to be 0.6–0.7 $\mu f/cm^2$. Hanai et al. (14, 15) have described the formation of lecithin-decane membranes in the absence of chloroform-methanol with a resistance of $\sim 10^8$ ohm-cm^2 and capacitance of 0.38 $\mu f/cm^2$. From their capacitance measurements, the thickness of the hydrocarbon core of the membranes was calculated to be 48 A. These authors have also studied the effects of cholesterol incorporation, protein adsorption (16), and geometric distortion (17) on the conductance and capacitance of the lecithin-decane membranes. The formation of similar membranes has been described by others (18–21), and van den Berg (22) has described a new technique for the formation of membranes of similar composition with large areas (50 mm^2) from lipid-water interphases.

In the present study, thin lipid membranes were formed from HK or LK sheep red cell lipids in hydrocarbon solvents. This paper describes the electrical resistance and capacitance of such thin membranes, a method for intermittent or constant flow of fluid in one of the chambers bathing the membrane, and the relative conductances of the membrane to Na^+, K^+, and Cl^- in the presence of ionic concentration gradients.

METHODS

Preparation of Lipid Extracts

The extraction was begun 1–2 hr after collection of approximately 150 ml of blood, drawn from the jugular vein of HK and LK sheep into heparin. The plasma and buffy coat were removed by centrifugation, and the red cells were washed three times in cold (0–4°C) 0.17 M NaCl. The packed red cells were hemolyzed at room temperature with an equal volume of deionized water, and the lipids were extracted with isopropanol-chloroform, according to Rose and Oklander (23). During the extraction, the samples were bubbled with N_2 and capped to minimize lipid peroxidation. The isopropanol-chloroform extract was taken to dryness under N_2 and partial vacuum in a flash evaporator at 30°C. The dried residue was reextracted by Ways

and Hanahan's modification (24) (Procedure III) of the Folch method (25), and the chloroform phase was dried in the flash evaporator. The dried residue was again extracted with *circa* 200 ml of chloroform (26), filtered through solvent-washed Whatman 2 filter paper, and taken to dryness in the flash evaporator. Inclusion of the final rectification with chloroform was necessary, since a small amount of chloroform-insoluble, methanol-soluble material was invariably collected on the filter paper. The dried residue was dissolved in chloroform, and stored as aliquots of about 400 μl under N_2 at -15°C. Under these conditions, the lipids could be used for membrane formation for at least two months without change in properties.

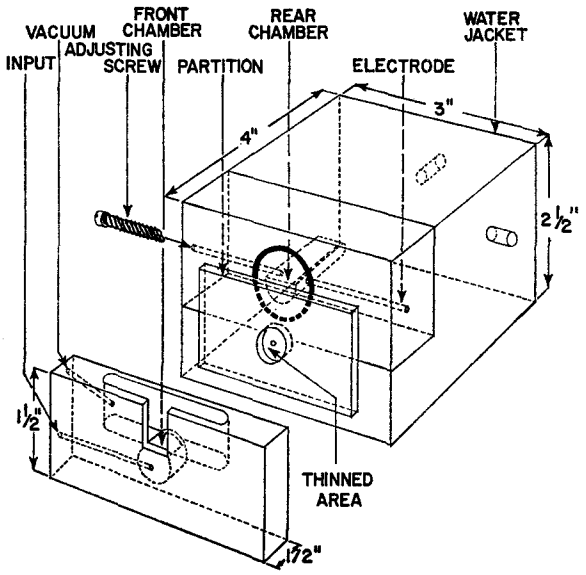

FIGURE 1. Apparatus for the formation of lipid membranes. The bolts used to fasten the front and rear chambers are not shown.

Cholesterol was removed from the lipids by acetone extraction. Approximately 0.8 ml of the extract was dried under a stream of N_2. The residue was taken up in 2.0 ml of acetone and allowed to stand for 15 min. The clarified supernatant was decanted and the entire procedure repeated five times. The residue was dried, dissolved in chloroform, and stored under N_2 at -15°C. Purified ovolecithin (27) was kindly given to us by Dr. A. D. Bangham.

Formation of Thin Lipid Membranes

The membranes were formed on a polyethylene partition separating two Lucite chambers (Fig. 1). The central portion (9.5 mm diameter) of a polyethylene plate 3.2 mm thick was thinned to 0.3 mm and the center of the thinned area was perforated with a needle 1.0–2.5 mm in diameter. The thinned area was slightly abraded with very fine sandpaper, and the entire partition was initially washed in detergent and 50% ethanol and rinsed exhaustively with deionized water. Subsequently, the parti-

tions were stored under decane. Prior to use, the partition was allowed to dry for at least 1 hr; otherwise, "leakage" resistances developed. An aliquot of lipid was dried under N_2, and dissolved in the appropriate solvent, ordinarily decane (14), at approximately 40 mg lipid/ml solvent. The thinned area of the partition was prepainted with the lipid-hydrocarbon solution, and bolted securely between the front and rear chambers. The rear chamber was entirely filled with fluid, and the lipids were applied to the partition by the brush technique of Mueller et al. (4, 5). Following an experiment, the partitions were rinsed with water, dried, and transferred to decane. If lipid solvents other than decane were used, the partition was rinsed in petroleum ether and equilibrated with the appropriate solvent. In our experience, difficulty in membrane formation or electrical "leakage" could usually be traced to contamination of the partition; consequently, the above procedure was rigorously followed.

The rear chamber (Fig. 1), the inflow solutions for the front chamber, and the inflow tubing from the pump to the front chamber were surrounded by water jackets through which termostatted water could be circulated. If a constant flow through the front chamber was maintained, the temperature in the chambers could be controlled to ± 1°C over the range 20–40°C. Unless otherwise specified, experiments were carried out at room temperature (22–24°C) (14).

The membranes were observed through a low-power (25 ×) microscope. Interference colors, when present, were easily visible, and areas with a grey sheen were ordinarily detectable on the blackened membranes. Invariably, some bowing of the membranes occurred, always in a backward direction. The bowing was probably due to seepage of fluid between the partition and the rubber "o" ring of the rear chamber, and varied considerably both in rate and degree of incidence. In early experiments, the membranes were restored to a planar microscopic appearance by tightening a nylon screw threaded into the rear chamber (Fig. 1). Subsequently, a 0.2 ml calibrated glass and Teflon microsyringe (RGI Instruments, RGI Instruments, Inc., Vineland, N. J.) filled with the rear chamber solutions was substituted (15) for the adjusting screw. Although the volume of fluid added to the rear chamber in each adjustment varied considerably (0.2–5 μl), and was at least partially dependent on the interval between adjustments, the microsyringe technique provided a reasonably precise method for maintaining a planar membrane (see below).

Flow System

Initial attempts to flow simultaneously into front and rear chambers were uniformly without success. Consequently, the rear chamber was entirely filled with fluid and sealed, and solution changes were carried out in the front chamber only (Fig. 1). Liquid was pumped into the bottom of the front chamber at a rate of 4–8 ml/min with a peristaltic [Harvard Instrument Co., Boston, Mass. (600–1200)] or piston (Beckman 746) pump, and aspirated from the top of the chamber through a 19 gauge needle. The front chamber's fluid level was constantly maintained higher than that in the rear. The composition of the solution in the front chamber at any time (C_t) could be computed from the expression:

$$C_t = C_R - (C_R - C_F)e^{\frac{-Qt}{V}} \qquad (1)$$

where C_R and C_F are the initial molar concentrations in the reservoir and front chamber respectively, Q is the flow rate (ml/min), and V is the front chamber volume (ml). Alternatively, the chloride content of aliquots of the fluid in the front chamber was measured directly in a Buchler-Cotlove chloridometer (Buchler Instrument Co., Fort Lee, N. J.). No significant effect of flow per se on the membranes was observed, except perhaps to increase the degree of bowing to a slight extent.

Measurement of Electrical Properties

Fig. 2 indicates the equivalent electrical circuit for measurement of membrane resistance (R_M), capacitance (C_M), and voltage (V_M). It is similar to the one utilized by

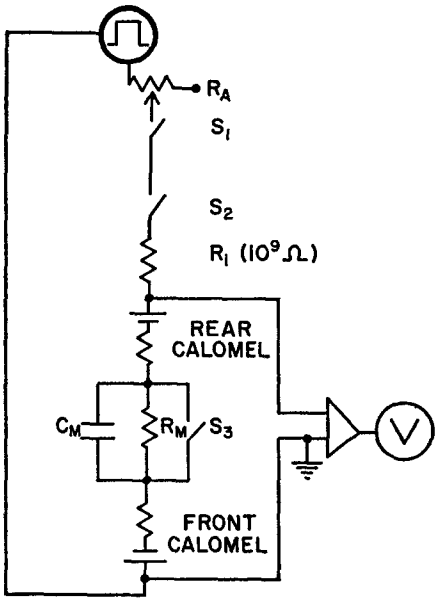

FIGURE 2. The equivalent electrical circuit for membrane studies. For abbreviations, see Methods.

Hanai et al. (15, 16). Calibrated 10–60 mv pulses were supplied by a 1 v DC reference unit (Instrulab, Inc., Dayton, Ohio) modified by an attenuator (R_A) and switch (S_1). Calomel-KCl electrodes (Beckman) were connected by 2% agar-saturated KCl bridges to the chamber. Potentials were increased fivefold with a Biotronik MS-1A preamplifier (Biotronik, Chapel Hill, N. C.), and recorded directly on a Varian recorder (Varian Associates, Palo Alto, Calif.) (V). Prior to formation of the membrane, a shunt (S_3) was placed across the electrodes to permit balancing of the preamplifier and recorder. DC resistances were calculated from Ohm's law, and capacitance, by a DC transient method (15, 16).

Others (13, 14, 16) have shown that the capacitance of thin membranes is frequency-independent (28). Fig. 3 shows recordings obtained when a pulse was applied to a membrane. Stable plateau voltages were always obtained, and the time constant could be measured directly, even when the film was bowed.

To insure the absence of an inherent electrical shunt circuit in the apparatus, an

unperforated partition was placed between the two chambers. The DC resistance between front and rear solutions was greater than 10^{11} ohms.

For the measurement of membrane potentials (V_M), the membrane circuit was separated from the potential generator by opening a mechanical switch (S_2). Transference numbers were calculated from the expressions:

$$V_M = \sum_{i=1}^{n} T_i E_i \tag{2}$$

and

$$1 = \sum_{i=1}^{n} T_i \tag{3}$$

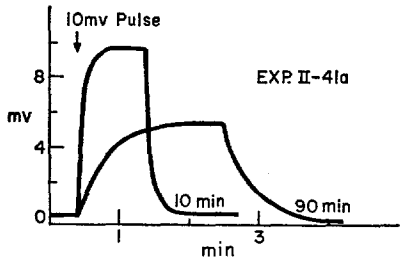

FIGURE 3. Recordings obtained when a DC pulse was applied to the membrane early and late in its life-span. At 90 min, the film was visibly bowed. Lipid preparation = LK 412, 43 mg/ml in decane. Aqueous phase = 0.1 M NaCl, 0.01 M Tris Cl, pH 6.8.

where T_i is the ionic transference number, defined as $\frac{Gi}{Gm}$, where Gi is the conductance of the ith ion, Gm the total membrane conductance, and E_i the equilibrium potential for the ith ion. Equilibrium potentials were calculated from the expression:

$$E_i = \frac{RT}{Z_i F} 2.3 \log \frac{C_i^F f_i^F}{C_i^B f_i^B} \tag{4}$$

where R = gas constant, T = °K, Z_i = valence, F = Faraday's number, C_i^F and C_i^B = concentration of ion in the front and rear chamber respectively, and f = activity coefficient.

Analytical Methods

Red cells were counted on a Coulter counter. Cholesterol was determined by Rosenthal's modification (29) of the method of Zlatkis (30). Lipid phosphorus was determined by a modification (31) of the method of Bartlett (32). Protein was solubilized with sodium deoxycholate (33), and determined by the method of Lowry et al. (34). All regents were of the highest commercial grade available, and chloroform was redistilled.

RESULTS

Composition of Lipid Extracts

Table I shows the composition of the lipid extracts obtained from HK and LK sheep red cells. The yield of lipid/cell is lower than reported values (35, 36) and varied over a threefold range (0.6–2.0 $\times$ 10^{-10} mg/cell). Although Table I indicates a greater yield of lipid/cell with the HK preparation, the difference was not consistent and was sometimes reversed. The low yields probably reflect the relative inefficiency of the extraction procedure with large sample volumes. However, the cholesterol/phospholipid ratio by weight (assuming an average molecular weight of 750 for phospholipid) was 0.4-0.5, in accord with the results of de Gier and van Deenan (37), and implies uniformity of lipid composition in the preparation. There was less than 1% protein contamination in the extract.

TABLE I

COMPOSITION OF LIPID EXTRACTS

Analytical determinations were carried out as described in Methods. The molecular weight of phospholipid was assumed to be 750.

Preparation	Extract/cell	Cholesterol	Phospholipid	Cholesterol/ phospholipid	Protein
	mg	*% by weight*	*% by weight*	*M*	*% by weight*
LK 520	0.745×10^{-10}	27	59	0.87	0.35
HK 527	1.84×10^{-10}	26	51	1.02	0.70

Properties of the Membrane

Following application of the lipid, a small central area of membrane was formed with a heavy surrounding torus. During the first 2 min, there was rapid radial drainage and the torus became nearly imperceptible, making estimation of the latter's contribution to membrane area impractical. Simultaneously, interference colors were noted. Discrete areas of "black" abruptly appeared and coalesced at 3–4 min. The "black" areas continually increased, and by 6–10 min, nearly the entire membrane appeared blackened. However, small discrete areas of interference colors, crudely estimated as approximately 10% of the membrane area, frequently persisted for the duration of the membrane. Once formed, the membranes were stable for 60–90 min, and occasionally for as long as 3 hr.

Fig. 4 illustrates the time course of membrane resistance and the effect of bowing for two separate experiments. When adjusted to a planar appearance, membrane resistance remained constant for the duration of the film and was ordinarily in the range 1.0–2.0 $\times$ 10^8 ohm-cm^2 (13, 14). Resistances as

high as 3.5×10^8 ohm-cm^2 were occasionally observed. However, resistances below 10^8 ohm-cm^2 could invariably be attributed to a bowed membrane and restored to control values, as shown in Fig. 4. An exception to the latter occurred within 5–10 min prior to rupture, when decreases in resistance ($<5 \times 10^7$ ohm-cm^2) were sometimes noted. In contrast to the observations of others (14, 22), abrupt discontinuities in membrane resistance were not observed. It is interesting to note the high degree of reproducibility in the resistance values recorded shortly after the membrane was visually adjusted to a planar appearance. They also were essentially the same as the resistance

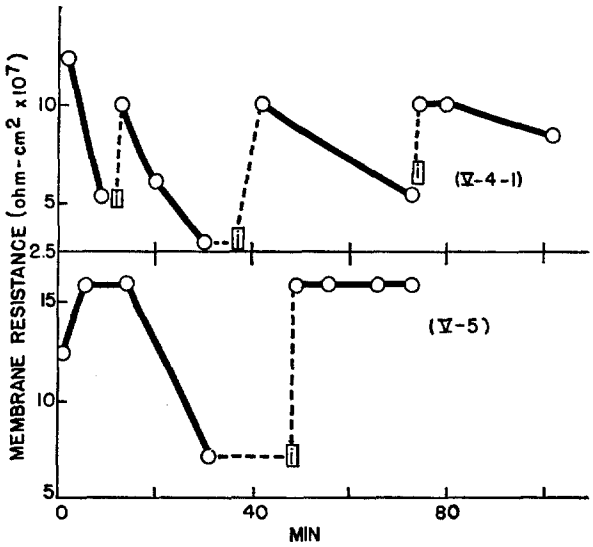

FIGURE 4. Membrane resistance for two different experiments. At the indicated times (i), the membrane was adjusted to a planar microscopic appearance with a microsyringe as described in Methods. The numbers in parentheses indicate the experiment. Lipid preparation = HK 615, 46 mg/ml in decane. Aqueous phase = 0.17 M KCl, 0.01 M Tris·Cl, pH 7.0.

values for an unbowed, unadjusted membrane. A similar correlation was noted with membrane capacitance and voltage (see below).

Fig. 5 shows the time course of membrane capacitance for the same experiments illustrated in Fig. 4. Membrane capacitance for a black membrane was in the range 0.3–0.4 μf/cm^2 and was stable for the duration of the membrane. These values are approximately the same as those reported by Hanai et al. (14) for a lecithin-decane membrane, but lower than those reported by Schwan et al. (13). The initial rise in capacitance during the first 10 min was usually observed, although at varying rates, and probably is due both to thinning of the membrane, as well as to variable degrees of bowing. In experiment V-5, for example, adjustment of the membrane to a planar

appearance during the first 10 min reduced the capacitance only slightly when compared to its subsequent effect.

Membrane resistance and capacitance were the same for membranes formed from HK or LK sheep erythrocyte lipids dissolved in decane. Membrane stability, DC resistance, and capacitance (14) were independent of temperature over the range 21–38°C. These properties were also independent (within the range of precision permitted by our methods) of pH (5.1–7.2) and the kind (NaCl, KCl, Tris Cl) and concentration (0.005–0.25 M) of electrolyte in the aqueous phase (10, 14). Most experiments were performed

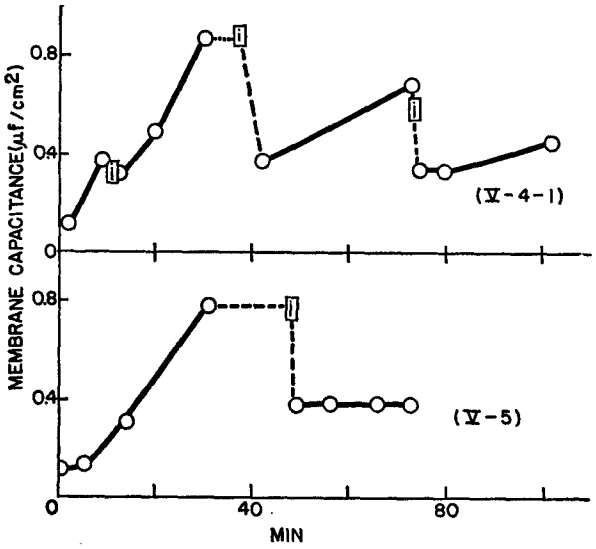

FIGURE 5. The time course of membrane capacitance. The experiments are the same as those illustrated in Fig. 4. Capacitance measurements were made simultaneously with the resistance measurements in Fig. 4.

in unbuffered 0.01 or 0.1 M NaCl or KCl (pH ~5.6) to simplify calculation of ionic transference numbers.

Membrane Potential

Fig. 6 illustrates the development of membrane voltage as solution changes were carried out in the front chamber. The potential was identical in magnitude but opposite in sign when the direction of the NaCl concentration gradient was reversed by changing the initial concentration of electrolyte in the front and rear chambers. In either case, the slope of the tracing was steeper when the solution change was in the direction of increasing concentration, and an initial lag period occurred prior to the development of membrane voltage. The latter could be attributed to washout of the initial medium from the pump and tubing leading to the front chamber.

The calculation of ionic transference numbers from the steady-state membrane voltage, as described in Methods and utilized in Tables II–VII, depends on the applicability of equations 2 and 4 to the system. These equations are based on the assumptions that electric charge traverses the membrane only in ionic form, and that ionic concentration and electrical potential differences are the only significant driving forces for ion transport. In the case where NaCl is the only solute in the solutions bathing the membrane, rearrangement of equation 2 yields:

$$V_M = \left(\frac{RT}{F}\right)(2T_{Na} - 1)(2.3)\left(\log \frac{a^F_{NaCl}}{a^B_{NaCl}}\right) \qquad (5)$$

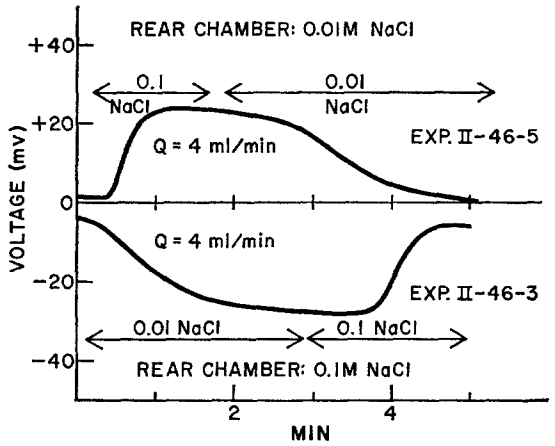

FIGURE 6. Membrane voltage with a tenfold concentration gradient between bathing solutions. In Experiment II-46-5, 0.01 M NaCl was the initial solution in both chambers. In Experiment II-46-3, 0.10 M NaCl was the initial medium. The indicated solution changes were carried out in the front chamber as described in Methods. Lipid preparation = LK 412, 43 mg/ml in decane.

where a_{NaCl}, the ionic activity, is defined as

$$a_{NaCl} = C_{NaCl} f_{NaCl}. \qquad (6)$$

Hence, if the initial assumptions are valid, membrane potential should be a linear function of the logarithm of the actual ionic activity ratio in the chambers bathing the membrane (equation 5). The results of an experiment designed to evaluate this hypothesis are shown in Fig. 7. The membrane was formed in 0.001 M NaCl; during a change in NaCl concentration in the front chamber from 0.001 M to 0.1 M, flow was interrupted at varying intervals during the transient and the membrane potential was recorded. Simultaneously, the salt concentration in the front chamber was measured directly. From the slope of the line in Fig. 7 (32 mv) and equation 5, T_{Na} was com-

puted to be 0.77, in agreement with the values recorded in Table II (see below).

The apparent asymmetry in the time course of V_M, seen in Fig. 6, can be rationalized in terms of equation 5. From the latter, it can be seen that the rate of development of electrical potential differences across the membrane is greater when the initial concentration of electrolyte in the front chamber is lower than the final concentration, at which V_M is measured. Additionally,

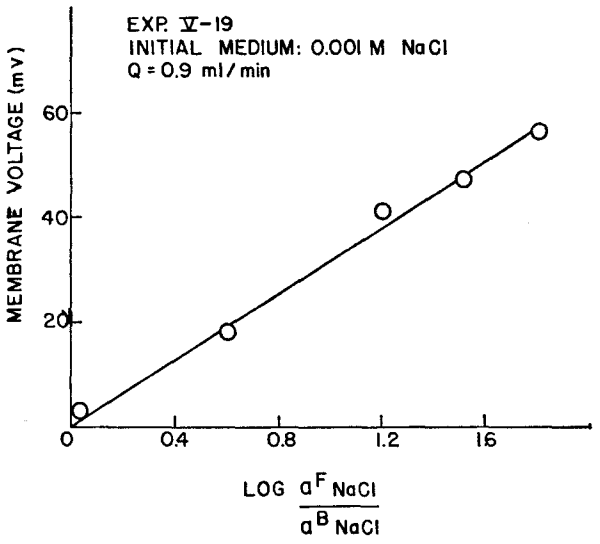

FIGURE 7. V_M as a function of log. $\frac{a^F_{NaCl}}{a^B_{NaCl}}$. The initial medium in both chambers was 0.001 M NaCl; 0.1 M NaCl was allowed to flow into the front chamber. At intervals during the transient, flow was stopped, membrane voltage was recorded and simultaneously an aliquot of fluid was aspirated from the front chamber for chloride analysis, as described in Methods. Lipid preparation = HK 727, 40 mg/ml in decane.

if this interpretation is valid, calculation of a^F_{NaCl} from equation 5 (assuming the values of T_{Na} and T_{Cl} computed from the steady-state value of V_M) should yield a symmetrical curve with respect to time, as illustrated in Fig. 8. Hence, in addition to explaining the apparent asymmetry in the time course of V_M in Fig. 6, this result provides added confirmation that T_{Na} and T_{Cl} are independent of E_{Na} and E_{Cl}.

The values for membrane voltage produced by a given concentration ratio of electrolyte were reproducible within a 10% range during the duration of a membrane, if the latter was planar (Fig. 9). Experiment III-13 in Fig. 9 represents an example of a membrane that bowed at an extremely rapid rate, but which could still be restored to uniform voltage, resistance, and capacitance. At 48 min, its resistance was 10^8 ohm-cm^2 and its capacitance

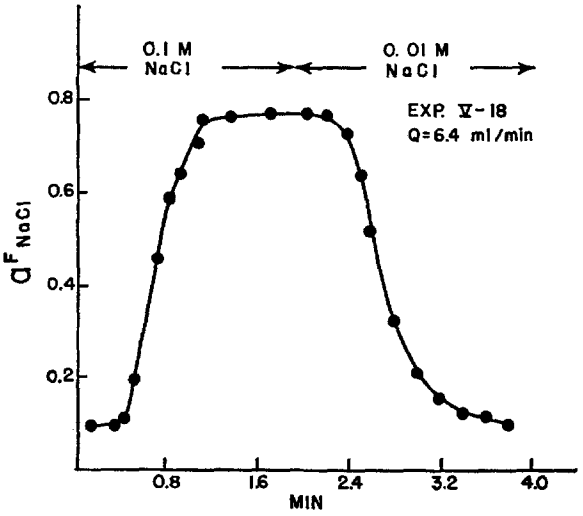

FIGURE 8. The time course of NaCl activity (a^F_{NaCl}) in the front chamber, computed from equation 5 (●—●). The initial medium in both chambers was 0.01 M NaCl. Lipid preparation = HK 615 A, *circa* 40 mg/ml in decane.

0.375 μf/cm^2. Since membrane potential in the presence of a given activity ratio of salt did not change over the temperatures 21–38°C, the transference numbers for Na, K, and Cl appear to be independent of this variable over this range.

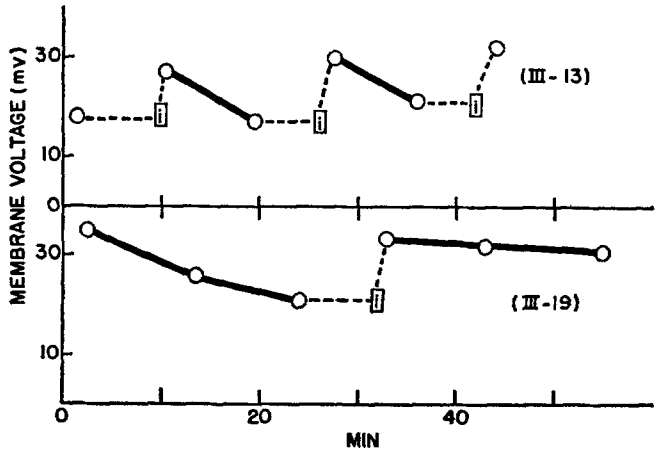

FIGURE 9. The time course of membrane voltage for two separate experiments. At the indicated time (i), the membrane was restored to a planar appearance with an adjusting screw. The numbers in parentheses indicate the experiment. In Experiment III-13, lipid preparation = LK 520, 45 mg/ml in decane. Aqueous phase = 0.1 M NaCl, rear chamber; 0.01 M NaCl, front chamber. In Experiment III-19, lipid preparation = HK 527, 45 mg/ml in decane. Aqueous phase = 0.01 M NaCl, rear chamber: 0.1 M NaCl, front chamber.

Table II summarizes a large experience with synthetic membranes prepared from different HK or LK lipid preparations in decane in which membrane voltage was measured as a function of the NaCl or KCl concentration ratio in the solutions bathing the membrane. In all instances, the cation

TABLE II

IONIC TRANSFERENCE NUMBERS IN THIN LIPID MEMBRANES

Membrane voltages were determined for a 0.1–0.01 M ratio of NaCl or KCl in the solutions bathing the membrane for the four different lipid preparations indicated, each dissolved in decane at approximately 45 mg/ml. The maximum voltage observed for a number of different membranes (indicated by the number in parentheses) made from the same lipid preparation was used to calculate mean membrane voltage. For each lipid preparation, the direction of the concentration ratio was reversed in two of the experiments, as illustrated in Fig. 6. Ionic transference numbers (T_{ion}) were compute as described in Methods.

Lipid preparation	Electrolyte	Concentration ratio	Mean membrane voltage	T_{Na}	T_K	T_{Cl}
			mv			
HK 420	NaCl	10	39 (4)	0.85	—	0.15
HK 420	KCl	10	35.2 (5)	—	0.82	0.18
HK 527	NaCl	10	33 (4)	0.80	—	0.20
HK 527	KCl	10	38 (4)	—	0.85	0.15
Mean HK				0.82	0.84	0.17
LK 412	NaCl	10	35 (4)	0.82	—	0.18
LK 412	KCl	10	37.8 (5)	—	0.84	0.16
LK 520	NaCl	10	28 (4)	0.75	—	0.25
LK 520	KCl	10	30 (4)	—	0.77	0.23
Mean LK				0.78	0.80	0.20

transference number was four to five times greater than T_{Cl}, and the values were reproducible between different lipid preparations. Slightly greater cation transference numbers were obtained when the membranes were formed from HK red cell lipids, but the differences were small and within the range of experimental error in the system. Similarly, T_K was slightly greater than T_{Na}, but, under the experimental conditions, the differences were too slight to be significant. To evaluate the relative permeabilities of Na^+ and

K^+ more adequately, bi-ionic potentials were measured at a constant ionic strength, as illustrated in Table III. A small but consistent membrane voltage was observed, indicating a slightly greater permeability of both HK and LK lipid-decane membranes to K^+ in comparison with Na^+.

TABLE III

BI-IONIC POTENTIALS IN THIN LIPID MEMBRANES

Membrane voltages at constant ionic strength were measured for the two lipid preparations indicated, each dissolved in decane at 45 mg/ml. Mean voltages were calculated as in Table II. Neglecting small differences in activity coefficients, the chloride ratio in the solutions bathing the membrane was unity, and E_{Cl} was zero. T_{Cl} was assumed to be 0.185 (the mean of the observations in Table II), and cation transference numbers were computed as described in Methods. The numbers in parentheses indicate the number of membranes made from the same lipid preparation.

Experiment	Lipid preparation	Chamber medium		V_M	T_K	T_{Na}
		Rear	Front			
III-3	LK 412	0.1 M KCl 0.001 M NaCl	0.001 M KCl 0.1 M NaCl	−2.6 (2)	0.42	0.39
III-10	HK 420	0.1 M KCl 0.001 M NaCl	0.001 M KCl 0.1 M NaCl	−2.1 (2)	0.42	0.39

Since the rear chamber was sealed, bulk osmotic flow was theoretically nil. However, it is conceivable that the ionic selectivity of the lipid membranes is affected by the difference in osmolarity of the solutions bathing its surfaces. In the experiments listed in Table IV, the osmolarity of solutions bathing

TABLE IV

IONIC TRANSFERENCE NUMBERS UNDER VARIED OSMOTIC CONDITIONS

Membrane voltages were determined under isosmotic conditions for lipid preparation HK 527, 45 mg/ml in decane. The control values for this lipid preparation are in Table II.

Experiment	Lipid preparation	Chamber medium		T_{Na}	T_K	T_{Cl}
		Rear	Front			
III-34	HK 527	0.1 M NaCl	0.01 M NaCl 0.16 M sucrose	0.82	—	0.18
III-35	HK 527	0.1 M KCl	0.01 M KCl 0.16 M sucrose	—	0.82	0.18

the membrane was varied by the addition of sucrose, and membranes were formed from a lipid preparation whose control values are listed in Table II. As shown by Tables II and IV, the transference numbers for Na^+, K^+, and Cl^- were essentially identical under both sets of conditions, and independent of the total osmolarity of the electrolyte solutions.

To exclude the possibility that the ionic permeability of the lipid membranes was dependent on a polar contaminant in the decane, the effect of using other hydrocarbons as the solvents for the red cell lipid extract was tested. As Table V illustrates, the ionic transference numbers were in the same range when the solvent was heptane, octane, or decane purified by chromatography on alumina (14). Although the data are not shown here, membrane resistance and capacitance were the same as with the lipid-decane system. No differences in membrane stability were noted when alumina-chromatographed decane was the lipid solvent. However, the membranes were unstable and ruptured within 10–20 min when heptane or octane was the solvent.

TABLE V

IONIC TRANSFERENCE NUMBERS WITH DIFFERENT LIPID SOLVENTS

Membrane voltages were measured as previously described except that the lipids were dissolved in the indicated solvent at approximately 45 mg/ml. Decane was chromatographed on a 2 × 15 cm acid alumina column. The polyethylene partition was equilibrated with the appropriate solvent as indicated in Methods.

Experiment	Lipid preparation	Chamber medium		T_{Na}	T_K	T_{Cl}
		Rear	Front			
III-30-1	HK420-heptane	0.1 M KCl	0.01 M KCl	—	0.88	0.12
III-30-2	HK 420-octane	0.1 M NaCl	0.01 M NaCl	0.90	—	0.10
III-30-3	HK 420-octane	0.1 M KCl	0.01 M KCl	—	0.85	0.15
III-33-1	HK 328–alumina-treated decane	0.1 M NaCl	0.01 M NaCl	0.87	—	0.13
III-33-2	HK 328–alumina-treated decane	0.1 M KCl	0.01 M KCl	—	0.75	0.25

The composition of the membrane was not directly determined and was, of course, probably quantitatively different from that of the lipid extract. Nevertheless, an attempt was made to evaluate the effect of the various components of the lipid extract (Table I) on ionic permeability. As shown in Table VI, acetone extraction removed more than 95% of the cholesterol in the lipid extract. However, the ionic transference numbers were essentially unchanged, implying that cation selectivity is dependent on the phospholipid moiety of the lipid extract. The reverse situation could not be evaluated directly, since we were unable to form membranes from a cholesterol-decane solution in the absence of phospholipid. However, Ti Tien has described the formation of membranes from cholesterol oxidation products (19).

Since sheep erythrocyte lipids contain a relatively high percentage of negatively charged phosphatidylethanolamine and phosphatidylserine (37),[1]

[1] C. F. Reed. Personal communication.

it was of interest to compare the ionic permeability of these membranes with that of membranes prepared from the uncharged phospholipid lecithin. As shown in Table VII, lecithin-decane membranes were considerably less cation selective than the erythrocyte lipid-decane membrane, and T_K and T_{Na} were only slightly higher than T_{Cl}. In contrast, Bangham et al. (38) have shown that liquid crystals prepared from the same lecithin and having a

TABLE VI

IONIC TRANSFERENCE NUMBERS FOR ACETONE-EXTRACTED LIPIDS

Lipid preparation HK 420 was extracted with acetone as described in Methods. The control and acetone-extracted preparation were each dissolved in decane at approximately 45 mg/ml.

Experiment	Lipid preparation	Cholesterol	T_{Na}	T_K
		%		
III-5-8	HK 420	26.3	0.85	0.82
III-26-28	HK 420, extracted with acetone	0.16	0.84	0.83

high permeability to water are considerably more permeable to Cl^- than to Na^+ or K^+.

DISCUSSION

The membranes described in this study most closely resemble those prepared initially by Hanai et al. (14), differing primarily in the type of phospholipid

TABLE VII

IONIC TRANSFERENCE NUMBERS FOR LECITHIN-DECANE MEMBRANES

The membranes were formed from purified ovolecithin (27, 28) dissolved in alumina-chromatographed decane (Table V) at 42 mg/ml.

Experiment	Chamber medium		T_{Na}	T_K	T_{Cl}
	Rear	Front			
III-36	0.1 M KCl	0.01 M KCl	—	0.59	0.41
III-37	0.1 M NaCl	0.01 M NaCl	0.55	—	0.45

used in the preparations, and in the cholesterol content. The erythrocyte lipid-decane membranes have a high resistance ($\sim 10^8$ ohm-cm^2), and a capacitance of 0.3–0.4 μf/cm^2. These values are in agreement with those reported by Hanai et al. (14), and were essentially the same for cholesterol-depleted membranes (Tables VI and VII). From Table I, it can be seen that the cholesterol/phospholipid molar ratio is 0.8-1.0 (assuming a molecular weight of 750 for the phospholipid). Hence, one would expect a comparatively small contribution from the cholesterol to the capacitance of the phos-

pholipid-hydrocarbon membrane (16). However, differences in the cholesterol content between different preparations could partially account for the range of capacitances observed in our experiments.

If the membrane is treated as a parallel-plate capacitor (14, 15), its thickness can be determined if the dielectric constant is known. Fricke (42) originally assumed a value of 3 for lipid membranes. Hanai et al. (14, 15) showed that no dielectric dispersion was produced by the polar groups of lecithin, and, from the bulk dielectric constant of model apolar compounds, computed a dielectric constant of 2.07. However, this value may be doubtful, since the dielectric constant for a material in bulk solution and in a thin membrane may not be the same because of specific orientation of molecules in the latter case. From optical thickness determinations and electrical capacitance measurements, Schwan et al. (13) have calculated a dielectric constant of 4.5 for lecithin-hydrocarbon membranes. With the capacitance range of the membranes reported here, one can compute a membrane thickness of 46–61 A using 2.07 as the dielectric constant, and 100–132 A with a dielectric constant of 4.5. Although these values clearly represent an approximation, the thickness values estimated with the lower dielectric constant are in accord with the thicknesses of the lipid portion of biological membranes as measured by X-ray diffraction (40) and electron microscopy (39, 41).

Some inferences are possible concerning the effect of bowing on the membranes. Within the limits of experimental error, membrane voltage, resistance, and capacitance remained constant for a visually planar film, implying that the planar membrane has a uniform composition, even after bowing. Although both resistance and capacitance are area dependent (17) there is no a priori reason to suppose ion selectivity should be influenced by surface area, if membrane composition is uniform. In practice, membrane voltage was independent of the diameter of the hole on which the membranes were made over a twofold range.[2] However, when the films were bowed, membrane voltage declined. Consequently, it is possible that the bowed membrane undergoes a reversible change either in molecular architecture or in composi- which alters its ionic permeability. The fall in membrane voltage with bowing cannot be attributed to the development of irreversible low resistance "leakage" pathways at the edge of the membrane (17), since membrane resistance returned to initial values when the film was restored to a planar state. In contrast, the leakage pathways described by Hanai et al. increased during the lifetime of the membrane (17). In addition, "leakage" pathways ordinarily had very low capacitances, regardless of surface area.[2]

Of primary interest in the study was the development of a simple method for measuring the relative ionic permeabilities of artificial lipid membranes whose thickness is of the same order of magnitude as cellular membranes,

[2] T. E. Andreoli and D. C. Tosteson. Unpublished observation.

with particular reference to a comparison of these properties with those of the natural membranes from which the lipids were derived. The evaluation of ionic permeability in this study depends on the valid application of equations 2 and 4 to the system. As indicated previously, it has been assumed that ionic concentration and electrical potential differences are the significant driving forces for ion transport. Other possible driving forces for ion transport include temperature, pressure, and the activity of other components, both water and solutes, in the system. However, for any given experiment, the system is both isothermal and isobaric. Activity differences for water cannot have contributed significantly toward movement of one ion with respect to another, e.g. Na with respect to Cl, since V_M was unaffected by variations in osmolality of the bathing solutions if the ionic concentration was constant (Table IV). A more direct demonstration of the validity of equations 2 and 4 in describing the zero current membrane voltage in the system is provided in Fig. 7, where the concentration of salt was measured directly. The constant slope of the line relating V_M to $\log \frac{a^F_{NaCl}}{a^B_{NaCl}}$ indicates that T_{Na} and T_{Cl} are independent of E_{Na} and E_{Cl} over the concentration range 0.001 to 0.1 M NaCl. Additionally, there is good agreement between T_{Na} as calculated from equation 2 (Table II) and measured directly (Fig. 7). A symmetrical curve of ionic activity as a function of time (Fig. 8) was obtained from equation 5 and the asymmetrical voltage-time curve obtained during solution changes (Fig. 6), further substantiating the conclusion that V_M is dependent on the logarithm of the ionic activity ratio in the solutions bathing the membrane. However, the experiments provide no information about the physical mechanism by which the membrane maintains T_K and T_{Na} four to five times higher than T_{Cl}.

The fact that the membranes described in this report prefer cations to anions may be rationalized in terms of their chemical composition. Although there are no gross differences between the lipid composition of the two types of cells,[1] they both contain substantial amounts of negatively charged phospholipids [phosphatidylethanolamine and phosphatidylserine (37)[1]]. If the negative charges were fixed on the membrane surfaces, they would be expected to permit easier access to cations than anions. The fact that removal of cholesterol from the membranes produces no change in the electrical properties of the membranes (Table VI) suggests that the phospholipids are the determinants of the membrane's cation selectivity. Furthermore, the observation that membranes prepared from the uncharged phospholipid lecithin display less selectivity for cations (Table VII) supports the conclusion that the charge of the phospholipids is the decisive factor.

The experiments show clearly that the ionic permeabilities of the membranes of intact sheep red cells and artificial membranes formed from sheep

red cell lipids are radically different. Mammalian red cells are about 10^6 times more permeable to Cl than to Na or K (43). Furthermore, the ratio of Na to K permeability is about 0.6 for HK but 0.2 for LK sheep red cells (2). By contrast, artificial membranes prepared from the lipids extracted from these cells are about equally permeable to Na and K but four to five times more permeable to these ions than to Cl (Tables II, III). The differences in Na and K permeability characteristic of HK and LK cells could have been detected easily if they were present in the lipid membranes. Several possible explanations for this difference between the biological and artificial membranes are tenable. The protein components of the natural membranes may be important determinants of the ionic permeability properties of these structures either directly or through interactions with lipids. This explanation is consistent with the fact that the electrical resistance of the intact red cell membrane is 10^{-6}–10^{-8} of that observed in the lipid films (43). It is also possible that lipids alone regulate the ionic permeability of the intact membrane, but that some critical trace components were lost in the extraction. Finally, the orientation of the lipids in the artificial membrane may be different from that in the biological membrane due to the absence of essential ionic components such as calcium. A decision between these and other conceivable alternatives must await further experiments.

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