interactions, tend to interact with each other and precipitate. This is apparently not the case with apo-BHDL which exists in solution in an extended configuration and forms aggregates of limited size. More detailed studies of the structure and properties of apo-BHDL or other apo-HDL's in solution may provide valuable information on the factors which determine their capacity to bind lipids.

Finally, the present work, with the conclusion that most of the protein is located on the surface of BHDL, suggests that protein–lipid interactions are not simply hydrophobic interactions of massive, nonpolar regions of the protein with nonpolar lipid domains, rather, it implies the existence of specific forces between lipids and probably a limited number of amino acid residues.

References


Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Camejo, G. (1969), *Biochim. Biophys. Acta 175*, 290.

Cross, D., and Fisher, H. (1966), *Biochemistry 5*, 880.

Forte, T., and Nichols, A. (1972), *Advan. Lipid Res. 10*, 1.

Goldsack, D. E. (1970), *Biopolymers 9*, 247.

Gotto, A. M., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1119.

Herskovits, T. T. (1965), *J. Biol. Chem. 240*, 628.

Herskovits, T. T. (1967), *Methods Enzymol. 11*, 748.

Herskovits, T. T., and Laskowski, M., Jr. (1961), *J. Biol. Chem. 235*, PC 56.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.

Herskovits, T. T., and Sorensen, M. (1968a), *Biochemistry 7*, 2523.

Herskovits, T. T., and Sorensen, M. (1968b), *Biochemistry 7*, 2533.

Jonas, A. (1972a), *J. Biol. Chem. 247*, 7767.

Jonas, A. (1972b), *J. Biol. Chem. 247*, 7773.

Lindskog, S., and Nilsson, A. (1973), *Biochim. Biophys. Acta 295*, 117.

Mihalyi, E. (1968), *Biochemistry 7*, 208.

Nichols, A. V. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1128.

Nozaki, Y., and Tanford, C. (1971), *J. Biol. Chem. 246*, 2211.

Riddiford, L. M. (1966), *J. Biol. Chem. 241*, 2792.

Scanu, A. (1967), *J. Biol. Chem. 242*, 711.

Scanu, A., and Hirz, R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 890.

Scanu, A. M., Reader, W., and Edelstein, C. (1968), *Biochim. Biophys. Acta 160*, 32.

Scanu, A. M., and Wisdom, C. (1972), *Annu. Rev. Biochem. 41*, 703.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 95.

Teale, F. W. J. (1960), *Biochem. J. 76*, 18.

Teale, F. W. J., and Weber, G. (1959), *Biochem. J. 72*, 15p.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239*, 1415.

Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.


# Mechanism of Ion Escape from Phosphatidylcholine and Phosphatidylserine Single Bilayer Vesicles†

H. Hauser, D. Oldani, and M. C. Phillips*

ABSTRACT: The escape of $^{22}Na^+$ and $^{36}Cl^-$ from egg lecithin and ox-brain phosphatidylserine single-shelled vesicles has been measured at 4°. The mechanism of $Cl^-$ escape involves diffusion across the phospholipid bilayer; this follows first-order kinetics. At pH 5.5 the first-order rate constant ($k_1$) is about three orders of magnitude higher than that for $Na^+$ diffusion and the enhanced $Cl^-$ diffusion probably involves the covalent association of $Cl^-$ ions and protons at the lipid–water interface. This is consistent with the finding that log $k_1$ is inversely proportional to pH. From a kinetic analysis of the $Na^+$ escape and from energetic considerations of the transport of ions across bilayers it is concluded that there are two contributions to the measured cation flux. These are diffusion across the bilayer and collision-induced rupture of the vesicles with concomitant release of the ions encapsulated in the internal cavities of the vesicles. The latter mechanism probably also involves aggregation and/or coalescence of the single-shelled vesicles to larger phospholipid structures; these effects occur more readily at higher temperatures. Since the relative contributions of the two mechanisms are unknown, the experimental data for $Na^+$ cannot be expressed as intrinsic bilayer permeabilities. In contrast, the $Cl^-$ permeabilities seem to represent intrinsic values since at acid pH the diffusion is so fast that the contribution of the collision mechanism is negligible.

Phospholipid bilayers are now considered to play an important part in the structure and function of biological membranes (Stoeckenius and Engelman, 1969; Hendler, 1971; Phillips, 1972) and hence the properties of these bilayers in various model systems have been studied intensively during the past decade. In particular, aqueous dispersions of egg-yolk lecithin, both sonicated and unsonicated, have found wide application as a lipid bilayer model in biochemical and biophysical studies. Bangham *et al.* (1965) and Papahadjopoulous and Watkins (1967) used aqueous dispersions of egg lecithin to study ion flux rates across lipid bilayers. Radioactive ions were incorporated in egg lecithin particles which

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were assumed to consist of completely closed bilayers. The ion fluxes were calculated from the appearance of radioactivity in the external medium surrounding the particles. These early measurements were carried out on lipid dispersions either unsonicated or sonicated under various experimental conditions, and were troubled by several difficulties some of which have been pointed out by Johnson and Bangham (1969) and Hauser *et al.* (1972).

Papahadjopolous and Miller (1967) showed first by electron microscopy that sonicated phospholipid dispersions contain single-shelled vesicles. Huang (1969) isolated by gel permeation chromatography of sonicated egg lecithin dispersions a fraction II which consisted of single-shelled vesicles, and a fraction I containing multilamellar lipid particles. Fraction II vesicles are bounded by a single bilayer and it has been shown (Bystrov *et al.*, 1971; Finer *et al.*, 1972) that the internal cavity is isolated from the external solvent. Obviously, determination of the rate of ion flux from the interior of such vesicles could ideally give the intrinsic ion permeability of a phospholipid bilayer, if the vesicles remain intact for the duration of the experiment and ions only escape by diffusion across the phospholipid bilayers. This treatment ignores the possibility that collisions caused by the thermal motions of the vesicles could give rise to vesicle rupture and release of the contents.

In view of these uncertainties, the present work was undertaken in order to assess the validity of employing single-shelled lipid vesicles in measuring ion fluxes across the lipid bilayer. We report here the measurement of sodium and chloride ion fluxes out of egg lecithin and ox-brain phosphatidylserine single-shelled vesicles. The likely mechanisms of ion escape from the vesicles are deduced by analysis of the kinetics of the ion release and from thermodynamic considerations.

## Experimental Section

*Materials.* Hen egg lecithin, hen egg lysolecithin, and the monosodium salt of ox-brain phosphatidylserine were purchased from Lipid Products, South Nutfield, U. K. If necessary, egg lecithin and phosphatidylserine were purified according to Dawson (1963) and Long *et al.* (1962), respectively. $^{22}$NaCl and Na$^{36}$Cl (specific radioactivities 31 and 4 $\mu$Ci/mg, respectively) were obtained from The Radiochemical Centre, Amersham, U. K. Sephadex G-75 and Sepharose 4B were purchased from Pharmacia, Uppsala, Sweden. All other chemicals used were research grade. Water was deionized, distilled from $KMnO_4$ under nitrogen, and redistilled in a glass apparatus.

*Methods.* PREPARATION OF THE RADIOACTIVELY LABELED PHOSPHOLIPID DISPERSIONS. Egg lecithin and phosphatidylserine dispersions (1% (w/v)) in 0.145 M NaCl solution (pH 5.5) were prepared as described before (Hauser, 1971). The NaCl solution contained 4–20 $\mu$Ci/ml of $^{22}Na^+$ or 8–10 $\mu$Ci/ml of $^{36}Cl^-$. One experiment was carried out with 0.145 M NaCl containing both $^{22}Na^+$ and $^{36}Cl^-$ at specific activities of 5.9 and 5.4 $\mu$Ci/ml, respectively. The phospholipid dispersions (2.5–3 ml) were sonicated for 15–20 min as described before (Hauser, 1971). The yield of single-shelled vesicles was always higher than 93%. No significant chemical degradation of the phospholipids was observed under these experimental conditions: the production of lysophospholipid was less than 3–4 wt %. Immediately after sonication the radioactively labeled ions present in the solvent were separated from the phospholipid vesicles by column chromatography on Sephadex G-75 (2.5 × 33 cm) or Sepharose 4B (2.5 × 33 cm) equilibrated with 0.145 M NaCl which, unless otherwise stated, was carried out at 4°. The durations of the column chromatography experiments were 60–70 min and 6–8 hr, respectively. Fractions (2.0–2.5 ml) were collected by automatic drop counting and both the optical density measured at 280 nm and the radioactivity of each fraction counted with the appropriate counting device (see below). The phospholipid concentration of each fraction of the effluent was determined by phosphorus analysis (Chen *et al.*, 1956).

MEASUREMENT OF THE ESCAPE OF RADIOACTIVELY LABELED IONS FROM LIPID VESICLES. The peak containing the phospholipid vesicles was eluted at the void volume of the Sephadex G-75 column and at an approximate elution volume $V_e$ = 100 ml with the Sepharose 4B column. All fractions comprising the single-shelled phospholipid vesicles were pooled prior to measuring the escape of ions out of lipid vesicles. The diffusion was measured at 4° to minimize lipid decomposition and coalescence of vesicles. Either of the three following methods was used. (1) An aliquot (4–5 ml) of the phospholipid dispersion was pipeted under $N_2$ into a rinsed Visking dialysis tubing. To assist mixing, the bag was knotted so that an $N_2$ bubble was included and dialysis was carried out at 4° against 6–8 ml of isotonic (0.145 M) tracer-free NaCl solution. Before sampling each dialysate for counting, the dispersion and the dialysate were mixed thoroughly by shaking. Aliquots of the dialysates containing $^{22}Na^+$ were counted in a "Tricarb" liquid scintillation spectrometer, Model 3000 (Packard Instrument Co., Reading, U. K.), and combined with the total dialysate after counting. Small aliquots of the $^{36}Cl^-$-containing dialysate were removed and either counted in planchets with a gas flow counter (Nuclear-Chicago Actigraph 3 detector chamber, obtained from Searle & Co. Ltd., High Wycombe, U. K.) or with the "Tricarb" liquid scintillation spectrometer. In the calculation of the total amount of tracer appearing in the dialysate corrections were made for the prior removal of aliquots for counting purposes. (2) The remainder of the pooled phospholipid dispersion was stored under $N_2$ at 4° and at appropriate intervals aliquots of the solvent were filtered using a Sartorius 4.7-mm pressure filter holder with a Sartorius Hygro LSG filter of mean pore size $5 \times 10^{-9}$ $\mu$ (from Sartorius, Göttingen, Germany). The filtrate was counted as before. (3) The phospholipid dispersion was put into a thoroughly rinsed Sartorius collodion bag (pore size about $10^{-8}$ $\mu$) supported by a Sartorius collodion bag holder and the solvent sucked off by means of a water pump. The solvent was counted as before.

All three methods gave results consistent with each other within experimental error. At the end of the diffusion experiment the particle size distribution of the vesicle dispersion was examined by column chromatography on Sepharose 4B and only a little aggregation of the vesicles was detected.

Aliquots of the sonicated dispersions before and after the diffusion experiments were freeze-dried. The residue was dissolved in $CHCl_3$–MeOH (2:1, v/v) and the oxidation and hydrolysis of the phospholipids were monitored by measuring the oxidation index (Klein, 1970) and thin layer chromatographic (tlc) analysis (Hauser, 1971), respectively. With egg lecithin the oxidation index $A_{233}/A_{210}$ (ratio of the absorption at 233 nm to the absorption at 210 nm) increased slightly during sonication in the presence of $N_2$ from 0.135 to 0.160 and returned during the diffusion experiment (~30 days) to its original value. Similarly the oxidation index of phosphatidylserine increased on sonication from 0.120 to 0.185 and a value of 0.140 was measured after ~30 days at the end of the diffu-

sion experiment. The degree of chemical degradation due to hydrolysis is shown in Figure 1 which gives the tlc chromatograms and densitometer tracings of them. After the diffusion experiment several minor bands were detected with both phospholipids: however, the total amount of degradation products in the phospholipid dispersions used for the diffusion measurements in this work did not exceed ~8%. Typical samples are shown in Figure 1b. In some preparations (which were discarded) the hydrolysis products accumulated during the diffusion experiment exceeded 10–15%; in these cases there was concomitant increase in oxidation index ($A_{233}/A_{210} > 0.5$) and in ion permeability to values above those presented here. We believe that the degree of chemical degradation (~8%) incurred during the diffusion experiment did not seriously affect our measurements (*cf.* diffusion measurements carried out with a mixture of lecithin–lysolecithin of 9:1, Table I). The fact that all experiments were carried out at 4° in the presence of 0.02% sodium azide eliminated the possibility of bacterial contamination; no bacteria were visible in electron micrographs of dispersions stored at 4° for 40 days.

Analytical gel permeation chromatography on Sepharose 4B was carried out as described before (Hauser *et al.*, 1970).

## Results

*Characterization of Phospholipid Vesicles.* Ultrasonic irradiation under the experimental conditions used produced a fraction II containing a range of roughly spherical "single-bilayer" vesicles. Figure 2 gives the total surface area and the total internal volume per micromole of egg lecithin as a function of the vesicle diameter over the particle size range obtained. Due to the inhomogeneity in the particle size distribution an average particle diameter of about 250 ± 8 Å had to be used to calculate the total lipid area across which diffusion of the ions took place; Table II summarizes the average vesicle parameters used in the calculation of ion fluxes.

Phosphatidylserine dispersions in 0.145 M NaCl sonicated under identical conditions consist of single-shelled vesicles whose average diameter and internal volume are close to those of egg lecithin vesicles. Some differences in the spread of the particle size distribution were observed which, however, were of little consequence to the ion permeability measurements. For the calculation of ion flux rates and permeability coefficients the same average vesicle parameters were used as for egg lecithin.

*Column Chromatography.* Figures 3a and b are column chromatograms of $^{22}Na^+$-labeled lecithin vesicles obtained with Sephadex G-75 and Sepharose 4B, respectively. In both cases the $^{22}Na^+$ in the external medium was well separated from the vesicle fraction indicating that there was no leakage of $^{22}Na^+$ from the vesicles during chromatography. Figure 3b shows that 6.5% of the total radioactivity was eluted as fraction I (multilamellar) lecithin particles at the void volume of the column; fraction I would not have been separated from the single-shelled vesicle fraction on Sephadex G-75. Therefore, if Sephadex G-75 is used to separate the external $^{22}Na^+$, it is important to sonicate so that fraction I is kept at a minimum (Hauser and Irons, 1972). Figure 3c is the chromatogram of $^{36}Cl^-$-labeled lecithin vesicles obtained on Sephadex G-75. By comparison with Figure 3a it is obvious that there is a significant proportion of the total activity leaking out of the vesicles during the column chromatography. This was allowed for in the analysis of the diffusion experiments summarized in Table III. Chromatograms similar to those in Figures 3a

TABLE I: $Na^+$ Escape from Lecithin and Phosphatidylserine Single-Shelled Vesicles.

| Phospholipid | Duration of Expt (hr) | Self-Diffusion Rate[a] | $k_1$ ($sec^{-1}$) | $k_2$[b] ($sec^{-1}$ (counts/sec)$^{-1}$ ml) | $k_2$[c] ($sec^{-1}$ (counts/sec)$^{-1}$ ml) | $k_3$ | Ion Flux $M$[d] (mol $cm^{-2}$ $sec^{-1}$) | Permeability[e] Coeff (cm $sec^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Egg lecithin | <300 | 0.011–0.046 | 0.30–1.3 × $10^{-7}$ | 8.5 × $10^{-10}$ | | | 0.70–3.2 × $10^{-18}$ | 0.53–2.2 × $10^{-14}$ |
| | | av 0.025 | av 7.0 × $10^{-8}$ | | | | av 1.7 × $10^{-18}$ | av 1.2 × $10^{-14}$ |
| Egg lecithin | 750 | 0.054–0.075 | 1.5–2.1 × $10^{-7}$ | | 1.7 × $10^{-11}$ | 0.056 | 3.0–4.2 × $10^{-18}$ | 2.1–2.9 × $10^{-14}$ |
| Egg lecithin + 10% egg lysolecithin | <300 | 0.0168 | 4.7 × $10^{-8}$ | 4.5 × $10^{-10}$ | | | 1.2 × $10^{-18}$ | 0.83 × $10^{-14}$ |
| | 500 | 0.055 | 1.5 × $10^{-7}$ | | 1.1 × $10^{-11}$ | 0.150 | 3.0 × $10^{-18}$ | 2.1 × $10^{-14}$ |
| Phosphatidylserine | <300 | 0.008–0.018 | 2.2–5.0 × $10^{-8}$ | 9.7 × $10^{-10}$ | | | 0.56–1.2 × $10^{-18}$ | 3.9–8.3 × $10^{-15}$ |
| | | av 0.0095 | av 2.6 × $10^{-8}$ | | | | av 0.65 × $10^{-18}$ | av 4.5 × $10^{-15}$ |
| | 450 | 0.14 | 4.0 × $10^{-7}$ | | 1.1 × $10^{-11}$ | 0.046 | 8.0 × $10^{-18}$ | 5.5 × $10^{-14}$ |

[a] The self-diffusion rate is the percentage of trapped radioactivity appearing in the dialysate per hour. It was obtained from the linear portion of Figure 4c. [b] $k_2$ obtained from least-squares analysis of the experimental data according to eq 2. [c] $k_2$ obtained from least-squares analysis of the experimental data according to eq 18 of the Appendix. [d] The ion flux $M$ was calculated either from the first-order rate constant $k_1$ using the equation $k_1 = MA_0/V$[NaCl], where [NaCl] is the NaCl concentration in moles per centimeter$^3$ and $A_0$ and $V$ are explained in Table II, or from the self-diffusion rate $M$ = (self-diffusion rate)$V$[NaCl]/(3.6$A_0$10$^5$). If the ion flux measurements are carried out with all the precautions discussed by Hauser *et al.* (1972), we consider that these measurements using single-shelled phospholipid vesicles are accurate to within one-half an order of magnitude. [e] The permeability coefficient $P$ was obtained from $P = M$/[NaCl].

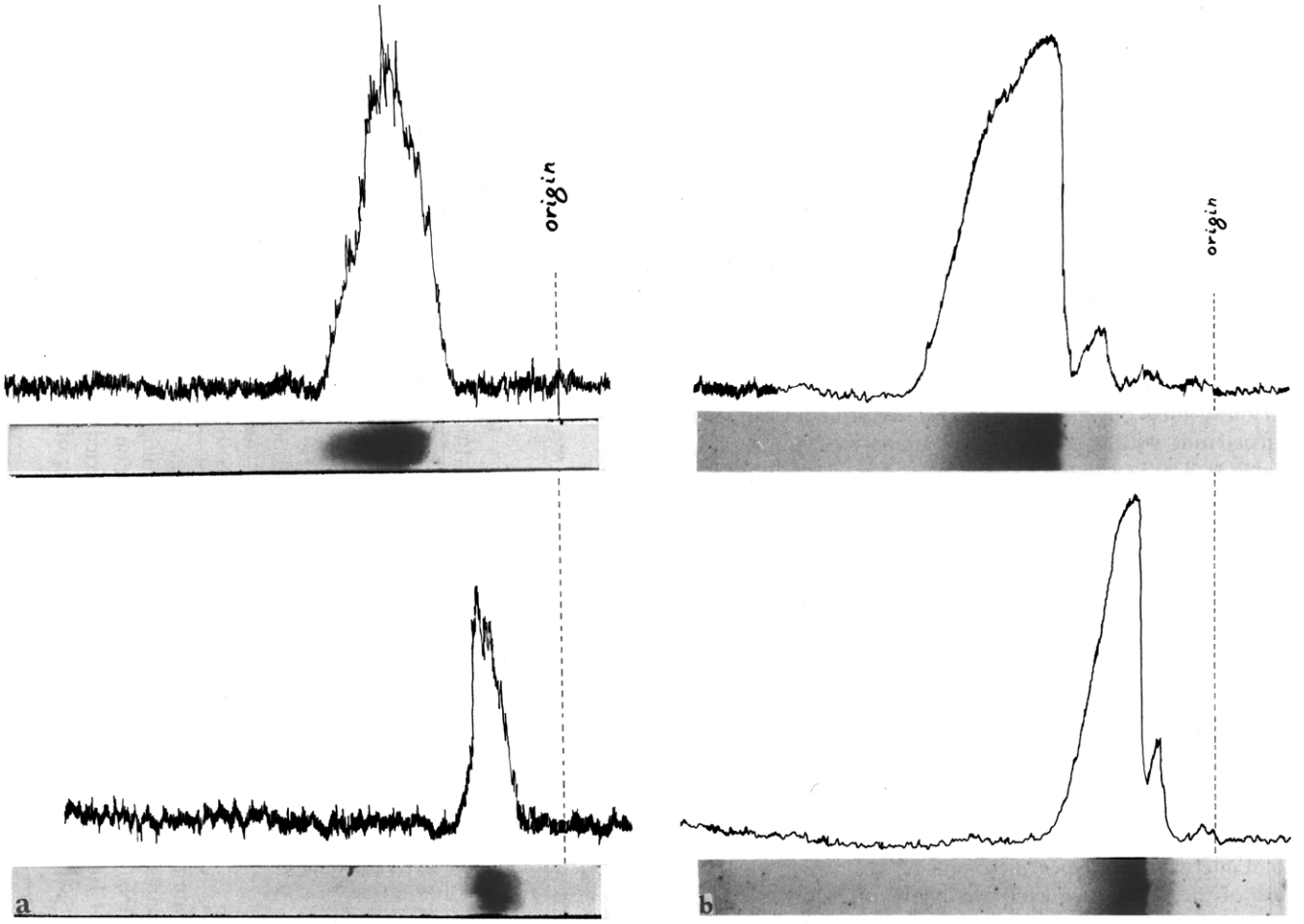


FIGURE 1: Thin-layer chromatograms and densitometer tracings of egg lecithin (a, top) and phosphatidylserine (a, bottom) taken immediately after sonication; (b, top and bottom) the corresponding samples after about 30 days at the end of the diffusion experiments. Silica gel H was used with chloroform–methanol–7 M ammonia (46:18:3, by vol) as the solvent. Application: 1 and 0.7 mg of egg lecithin and phosphatidylserine, respectively. Detection: either with iodine vapor or concentrated sulfuric acid saturated with $K_2Cr_2O_7$. Egg lecithin (b, top) gave three minor bands with $R_F$ 0.21, 0.11, and 0.02 besides the main lecithin band ($R_F$ 0.37). Phosphatidylserine (b, bottom) had $R_F$ 0.17 and showed two minor bands with $R_F$ values of 0.095 and 0.02. For assignments of the lecithin bands see Hauser, 1971. The main degradation product of phosphatidylserine is the lyso compound ($R_F$ 0.095).

and c were obtained with $^{22}Na^+$ and $^{36}Cl^-$-labeled phosphatidylserine vesicles except that with $^{22}Na^+$ some activity was recorded between fractions II and III due to the replacement of $^{22}Na^+$ by $^{23}Na^+$ in the external double layer of the vesicles.

*Ion Flux out of Phospholipid Vesicles.* Since the ionic composition was the same on both sides of the bilayer, passive diffusion rather than a net flux was measured. Table I gives all the values which characterize the movement of $Na^+$ out of lecithin and phosphatidylserine vesicles. Statistical analysis of the data for $Na^+$ escape shows that for a period of up to about 300 hr the experimental values obtained with both lecithin and phosphatidylserine could be fitted reasonably well both by first- (eq 1) and second-order (eq 2) equations,

$$\log [(X_0 - X_\infty)/(X - X_\infty)] = k_1 t/2.3 \quad (1)$$

$$(X_0 - X)/[(X_0 - X_\infty)(X - X_\infty)] = k_2 t \quad (2)$$

though the former usually gave the better fit (Figure 4). $X_0$, $X$, and $X_\infty$ are the concentrations of radioactive ions incorporated in the vesicles at $t = 0$, $t$, and at equilibrium, respectively, and $k_1$ and $k_2$ are the first- and second-order rate constants, respectively. This fit of either equation is probably due to the limited accuracy of the measurements (see Table I). However, when the "diffusion" measurements were extended over longer periods of time, the experimental data deviated both from simple first- and second-order kinetics. This is shown in Figures 4a and b for the escape of $Na^+$ from lecithin vesicles. The slopes of the least-squares lines in Figures 4a and b are the first- and second-order rate constants $k_1 = 8.0 \times 10^{-8}$ sec$^{-1}$ and $k_2 = 8.5 \times 10^{-10}$ (counts/sec)$^{-1}$ ml sec$^{-1}$, respectively (Table I). A reasonably good fit over the whole range of experimental points (Figure 4c) was obtained by assuming that besides the first-order diffusion of ions across the bilayer, ions can also be released as a result of certain energetic collisions between vesicles (see Appendix). A similar analysis was carried out with the experimental data of $Na^+$ escape from phosphatidylserine vesicles. The first- and second-order rate constants obtained from this analysis are given in Table I.

Figure 5 and Table III summarize the $Cl^-$ permeability measurements. The diffusion of $Cl^-$ across lecithin (Figure 5a) and phosphatidylserine bilayers followed first-order kinetics and the first-order rate constants $k_1$ of Table III were obtained from the slope of the least-squares lines in Figure 5a. Figures 5a and b show that with lecithin the $Cl^-$ diffusion is inversely proportional to the pH of the medium and a linear relationship was obtained when log $k_1$ was plotted as a func-

TABLE II: Average Vesicle Parameters Used for Calculating Ion Fluxes.

| | Exptl | Calcd |
|---|---|---|
| Area/egg lecithin molecule | 70 Å$^2$ [a] | |
| Outer vesicle diameter | 250 ± 8 Å [b] | |
| Bilayer thickness | 46 Å [c] | |
| Inner vesicle diameter | | 158 Å |
| No. of egg lecithin molecules/vesicle | | 4000 |
| $A_0$ = outer area/vesicle | | $2.0 \times 10^{-11}$ cm$^2$ |
| No. of molecules on the outer surface of the bilayer | 70.5% [d] | 2800 (70%) |
| No. of molecules on the inner surface of the bilayer | 29.5% | 1130 (30%) |
| Total outer vesicle area/μmol of lecithin | 2950 ± 30 cm$^2$ [e] | ~3000 cm$^2$ |
| Internal vol ($V$)/vesicle | $2.2 \times 10^{-18}$ cm$^3$ [c] | $2.1 \times 10^{-18}$ cm$^3$ |
| Total vol/μmol of lecithin | $0.33 \pm 0.01 \times 10^{-3}$ [c] | $0.32 \times 10^{-3}$ cm$^3$ |

[a] Small, 1967. [b] The vesicle diameter is larger than that previously published by Hauser (1971) because of a slight decrease in the efficiency of the soniprobe. [c] Hauser and Irons, 1972. [d] Finer *et al.*, 1972. [e] The total outer vesicle area per milliliter of dispersion was obtained from the phospholipid concentration, and the ratio of phospholipid molecules on the outside and inside surface of the lipid bilayer (Finer *et al.*, 1972) using a value of 70 Å$^2$ for the area per phospholipid molecule.

tion of the pH (Figure 5b). In order to check whether $Cl^-$ transport only occurs by exchange diffusion, the chloride (0.145 M) of the external medium of $^{36}Cl^-$ containing vesicles was replaced with 0.145 M $NaNO_3$ by column chromatography on Sephadex G-75. The $Cl^-$ diffusion was somewhat increased as compared to that of vesicles dispersed in 0.145 M NaCl indicating that exchange diffusion is not important (*cf.* Pagano and Thompson, 1968).

The large difference in the $Cl^-$ and $Na^+$ permeabilities (*cf.* Tables I and III) is best expressed as the permeability ratio which is 350 for egg lecithin and 270 for phosphatidylserine. Usually $Cl^-$ and $Na^+$ fluxes were measured independently with different preparations; however, with one preparation of vesicles which contained both $^{22}Na^+$ and $^{36}Cl^-$, simultaneous $Na^+$ and $Cl^-$ flux measurements were carried out and the values obtained were consistent with those listed in Tables I and III.

TABLE III: $Cl^-$ Diffusion out of Lecithin and Phosphatidylserine Single-Shelled Vesicles.

| Phospholipid | pH | $k_1$ (sec$^{-1}$) | Ion Flux $M$ (mol cm$^{-2}$ sec$^{-1}$) | Permeability Coeff $P$ (cm sec$^{-1}$) |
|---|---|---|---|---|
| Egg lecithin | 3.1 | $2.7 \times 10^{-4}$ | $6.6 \times 10^{-15}$ | $4.5 \times 10^{-11}$ |
| | 4.3 | $1.3 \times 10^{-4}$ | $3.2 \times 10^{-15}$ | $2.2 \times 10^{-11}$ |
| | 5.5[a] | $3.2 \times 10^{-4}$ | $8.0 \times 10^{-15}$ | $5.5 \times 10^{-11}$ |
| | 6.4 | $3.3 \times 10^{-5}$ | $8.1 \times 10^{-16}$ | $5.6 \times 10^{-12}$ |
| | 6.4[b] | $8.0 \times 10^{-5}$ | $2.0 \times 10^{-15}$ | $1.3 \times 10^{-11}$ |
| | 10.0 | $3.4 \times 10^{-6}$ | $8.3 \times 10^{-17}$ | $5.7 \times 10^{-13}$ |
| Phosphatidylserine | 5.5[a] | $9.0 \times 10^{-5}$ | $2.2 \times 10^{-15}$ | $1.5 \times 10^{-11}$ |

[a] In these experiments the Sephadex G-75 chromatography was carried out at room temperature which may account for the higher fluxes observed. In all other experiments the chromatography was carried out at 4°. [b] The internal cavities contained 0.145 M $Na^{36}Cl$ and the external medium was 0.145 M $NaNO_3$. In all the other diffusion experiments the external medium was 0.145 M NaCl.

## Discussion

*Comparison with Earlier Work.* The ion permeabilities given in Table I are lower than most of the previously published data obtained with various bilayer models. Petkau and Chelack (1970) used the planar "black lipid membrane" model to measure the passive diffusion of $Na^+$ and obtained an $Na^+$ flux at 37° of $9 \times 10^{-14}$ mol cm$^{-2}$ sec$^{-1}$ across brain phospholipid bilayers which probably contained *n*-tetradecane. Pagano and Thompson (1968) measured $Na^+$ and $Cl^-$ fluxes at 30° using large spherical egg lecithin bilayers which probably also contained organic solvent and obtained values of $3.9 \pm 0.2 \times 10^{-13}$ and $9.02 \pm 0.08 \times 10^{-11}$ mol cm$^{-2}$ sec$^{-1}$, respectively, corresponding to a flux ratio $M_{Cl^-}/M_{Na^+}$ of 230. The difficulties with the black lipid membrane model are the relatively small areas across which ion diffusion takes place, the instability of the system (besides serious limitations in the lifetime of bilayers, transient leaks in the region of the torus of the bilayer can occur during manipulations (D. E. Graham, H. Hauser, and M. C. Phillips, unpublished results)), and the inclusion of unknown and possibly variable amounts of organic solvents. In view of these uncertainties the dis-

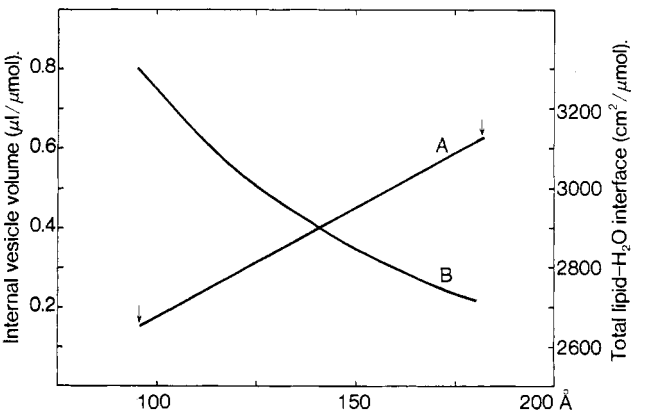


FIGURE 2: The internal vesicle volume (curve A) and the total lipid–water interfacial area (curve B) plotted as a function of the Stokes radius. The two arrows indicate the spread of particle sizes as derived from column chromatography on Sepharose 4B. The calculation of lipid–water interfacial area assumes that the packing of the molecules is similar in the outside and inside layers of the bilayer and that the bilayer thickness is 46 Å.

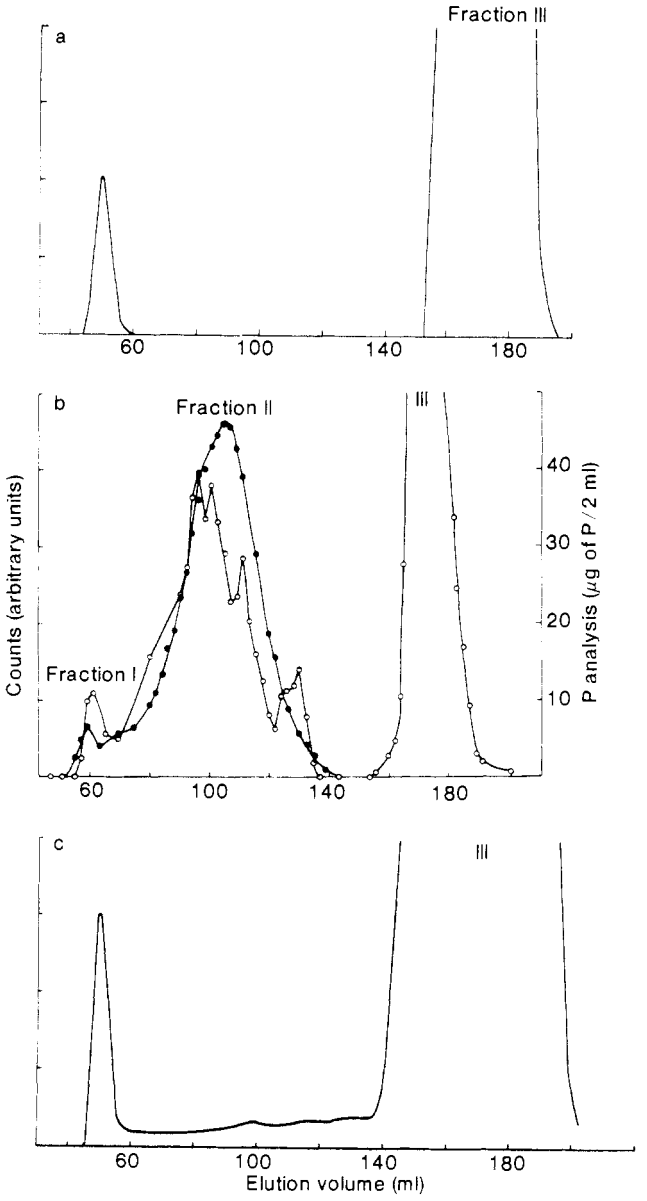


FIGURE 3: (a) and (c) are Sephadex G-75 chromatograms of $^{22}Na^+$- and $^{36}Cl^-$-labeled egg lecithin vesicles, respectively; (b) the chromatogram of $^{22}Na^+$-labeled egg lecithin vesicles fractionated on Sepharose 4B: (○) counts in arbitrary units; (●) $P$ analysis of the eluate. Fraction III = $^{22}Na^+$ in external medium.

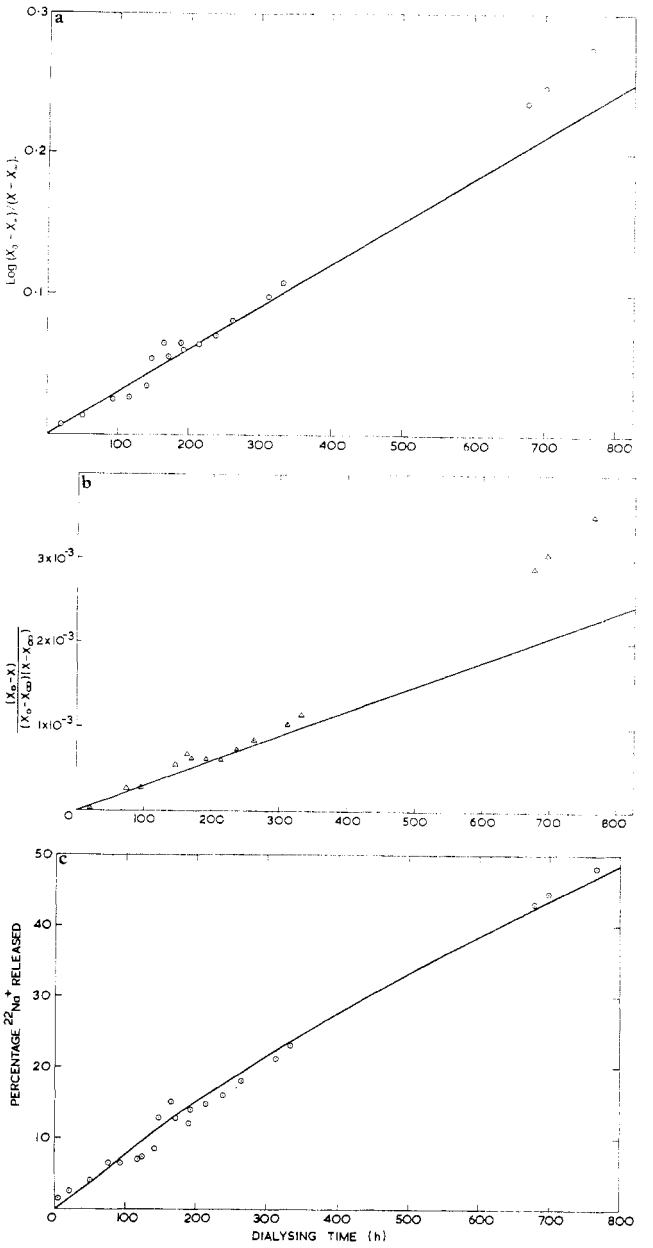


FIGURE 4: Escape of $Na^+$ from egg lecithin vesicles. (a) The experimental data (○) are treated in terms of eq 1 for first-order kinetics; the solid line was obtained by least-squares analysis. (b) The experimental data (△) are treated in terms of eq 2 for second-order kinetics; the solid line was obtained by least-squares analysis. (c) Circles represent the observed proportion $p$ (expressed as percentage) of $Na^+$ released from the vesicles as a function of time. The solid line was computed using $p = 1 - (X/X_0)$ where $X/X_0$ is given by eq 18 of the Appendix. The symbols are defined in the text.

crepancy between these measurements and our results is not surprising.

Some of the earlier ion permeability studies using both unsonicated and sonicated liposomes were not evaluated in terms of flux rates or ion permeability coefficients because the total lipid–$H_2O$ interfacial area was unknown. When interfacial areas were assumed for the flux calculation, the numbers employed were all significantly lower than our value of 2950 $cm^2/\mu mol$ of lecithin. For this and other reasons, which have been pointed out before (Hauser *et al.*, 1972), the early cation and anion permeabilities reported were significantly higher than those in Tables I and III. When the more recent values for cation fluxes at 36° obtained by Papahadjopoulos and coworkers (Papahadjopoulos, 1971; Papahadjopoulos *et al.*, 1972) are adjusted to 4° using appropriate activation energies the results are in agreement with those given in Table I. Our value for the $Cl^-$ flux is somewhat higher than that of Papahadjopoulos so that whereas his flux ratio $M_{Cl^-}/M_{Na^+}$ ranges from 40 to 60 (Papahadjopoulos and Watkins, 1967; Papahadjopoulos and Bangham, 1966; Papahadjopoulos *et al.*, 1972), our value at 4° is of the order of 200–400. The fact that the activation energies for the $Na^+$ flux through lecithin and phosphatidylserine bilayers are greater than those for the $Cl^-$ flux (Papahadjopoulos *et al.*, 1972) partly explains this discrepancy. Another contributing factor is the fact that $M_{Cl^-}/M_{Na^+}$ is pH sensitive and we have operated at different pH values from those of Papahadjopoulos and coworkers.

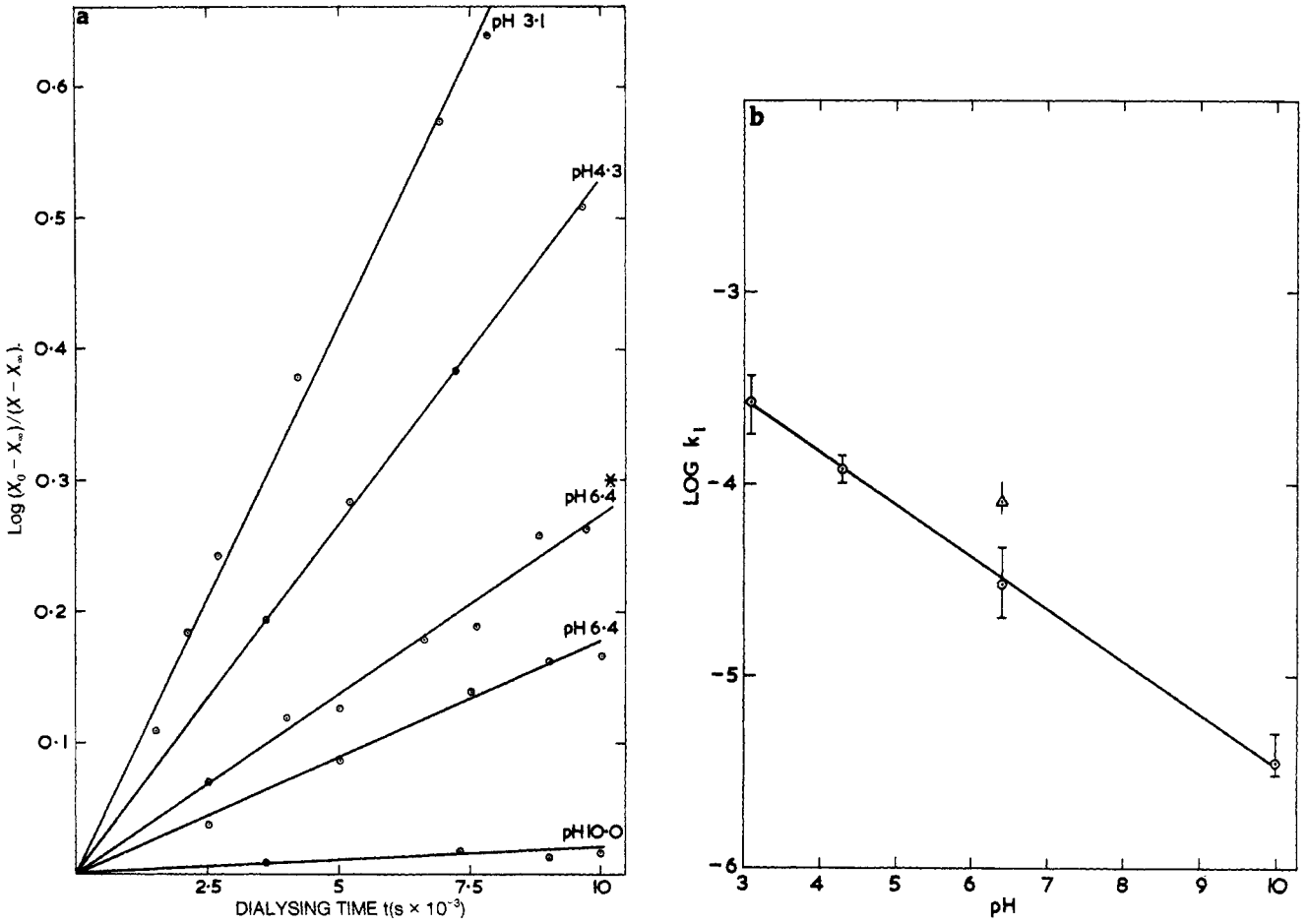


FIGURE 5: Diffusion of $Cl^-$ out of egg lecithin vesicles as a function of pH. (a) Experimental data were analyzed according to first-order kinetics and the solid lines are least-squares lines which have standard deviations of less than 0.06. The pH of the dispersion ranged from 3 to 10. The external medium at the asterisked point was 0.145 M $NaNO_3$. In all other experiments both the internal and external media were 0.145 M NaCl. The pH was adjusted by adding small amounts of either HCl or NaOH. (b) The logarithms of the rate constants $k_1$ obtained from the slopes of the straight lines in Figure 4a are plotted as a function of pH. The bars represent the experimental spread of two–four experiments. The triangle is the experiment with $^{36}Cl^-$-labeled vesicles suspended in 0.145 M $NaNO_3$.

The measurements in Tables I and III for sodium and chloride ions and the recent results of Papahadjopoulos and coworkers are for the most part in agreement, suggesting that both sets of data are reliable. However, whether or not a cation flux of the order of $10^{-17}$ mol $cm^{-2}$ $sec^{-1}$ at 37° is the intrinsic value characteristic of an unmodified phospholipid bilayer depends on whether or not all the ions have escaped from the vesicles by diffusion across the bilayer. The mechanism of ion escape needs therefore to be elucidated and this problem is considered below.

*Kinetic Considerations.* CHLORIDE ION. From studies of the rate of ion escape it follows that $Cl^-$ shows "ideal" behavior and the mechanism of ion escape from both lecithin and phosphatidylserine vesicles is a diffusion process as indicated by the first-order kinetics for $Cl^-$ diffusion at 4°. Our kinetic analysis suggests that the $Cl^-$ permeabilities (Table III) are probably intrinsic values for the diffusion of $Cl^-$ across unmodified phospholipid bilayers, at least at acid and neutral pH values, because the diffusion is so rapid that its measurement is not affected by the collision mechanism (see below). The large difference in $Na^+$ and $Cl^-$ flux rates at acid and neutral pH, together with the experimental finding that the $Cl^-$ permeability is inversely proportional to the pH of the medium, suggests that there is a nonionic contribution to the $Cl^-$ flux. The latter probably involves a covalent association of $Cl^-$ ions and protons at the lipid–$H_2O$ interface. This facilitates the chloride transport into the bilayer by significantly reducing the electric field around the ion pair (Parsegian, 1969). The involvement of a "flip-flop" mechanism as suggested by Kornberg and McConnell (1971) is unlikely because surface chemical measurements (H. Hauser and M. C. Phillips, unpublished results) do not confirm the formation of a lecithin–$Cl^-$ complex, which is a prerequisite of such a mechanism. Furthermore, because of charge repulsion such a complex would be extremely unlikely with phosphatidylserine. Any disturbance or disruption of the bilayer as a consequence of the flip-flop mechanism can also be ruled out as the cause of the enhanced $Cl^-$ permeability because this mechanism does not allow for any cation–anion specificity.

SODIUM ION. The kinetics of escape of $Na^+$ ions are not so simple because the equation for first-order kinetics does not explain the experimental results (Figure 4a) if the duration of the experiment exceeds 300 hr. The time course of the $Na^+$ escape (Figure 4c) from lecithin and phosphatidylserine vesicles can be described in terms of three regions. (1) Up to about 10 hr the percentage of $Na^+$ released exceeds the figures calculated from either first- or second-order kinetics and this is probably due to the presence of some faulty vesicles and/or some leaky large multilamellar particles (Hauser *et al.*, 1972), or, in the case of phosphatidylserine, to the exchange of $Na^+$ in the external double layer. (2) Between 10 and about 300 hr the experimental data can be described by either first- or

second-order kinetics. (3) If the ion release is followed over longer periods of time (Figure 4c), the quantity of ions escaping from the vesicles after diffusion times >300 hr is in excess of that expected from either simple first- or second-order kinetics. It is unlikely that this increase in permeability is due to chemical degradation of the phospholipid because no major chemical degradation was detected by thin-layer chromatography at the end of the diffusion experiment. Furthermore, the incorporation of 10% lysolecithin has little effect on the $Na^+$ permeability (see Table I).

If some of the collisions which occur during the Brownian motion of the vesicles can cause rupture of the bilayers, then the possible kinetics of ion escape become complex. If the vesicles simply collide and occasionally rupture before leaking all their contents and resealing unchanged, then the collision frequency will be constant throughout the experiment and the ion release will follow first-order kinetics. In this case ion release by diffusion across bilayers and by collision-induced rupture would result in the same kinetics. The only difference would be that in the latter case the release of ions would occur in a quantized rather than a continuous fashion. As shown by Hauser and Irons (1972), collision can also result in flocculation (aggregation) and coalescence of the vesicles. If flocculation is rapid and coalescence is slow, then first-order kinetics would again result. However, if coalescence occurs rapidly and large particles which no longer participate in rupture-inducing collisions are formed, then the collision frequency would be rate determining for ion release and the kinetics would be second order.

One possible way of accounting for the complex kinetics of $Na^+$ escape is to assume that the diffusion (first order) and collision–coalescence (second order) mechanisms operate simultaneously and independently (see Figure 4c and Appendix). The theoretical line (solid line in Figure 4c computed from eq 18 and 19 of the Appendix) fits the experimental points satisfactorily. On this basis, the measured fluxes are the result of the two independent mechanisms of ion diffusion across the bilayer and collision-induced rupture and coalescence of vesicles.

If it is assumed that the second-order rate constant reflects the number of collisions which gives rise to ion release then it is possible to estimate the proportion of collisions which are effective and to derive an energy barrier to bilayer rupture. The number of effective collisions, $Z_{eff}$, is related to $k_2$ and the concentration of vesicles. Using eq 9 of the Appendix and the $k_2$ values obtained from least-squares analysis of the experimental data according to eq 18 of the Appendix (see Table I), the number of effective collisions can be estimated as $5 \times 10^6$ collisions $sec^{-1}$ $ml^{-1}$. This figure may be compared with the total number of collisions $Z$ at 4° calculated from the Smoluchowski equation (3) where $r$ is the vesicle radius (125 Å), $D$ is the diffusion coefficient of the vesicles at 4°, $D_4 = 1.1 \times 10^{-7}$ $cm^2$ $sec^{-1}$ (Hauser and Irons, 1972), and $n \cong 2 \times 10^{15}$ vesicles/ml. This gives a total collision rate $Z = 2.5 \times 10^{19}$ collisions $sec^{-1}$ $ml^{-1}$ indicating that at 4°, 1 in $5 \times 10^{12}$ collisions induces rupture of the bilayer and possibly loss of $Na^+$. The energy barrier $E_A$ to effective collision can be estimated from eq 4, which gives a value for the energy barrier to bilayer rupture/coalescence of $E_A = 32RT$ or 5 cal/mol of lipid.

$$Z = 16\pi r D n^2 \quad (3)$$

$$Z_{eff} = Ze^{-E_A/RT} \quad (4)$$

The above analysis suggests that at 4° the vesicles are relatively stable and that diffusion across bilayers is the predominant mechanism of ion release. The situation could be quite different at higher temperatures. The observation of Hauser and Irons (1972) that egg lecithin vesicles can aggregate and coalesce at room temperature to form clusters and/or spherical particles consisting of up to about a dozen bilayers indicates that at room temperature the rate of coalescence is appreciable. The rate of coalescence at 4° seems greatly reduced as evident from the $k_3$ values of Table I. The negative surface charge on phosphatidylserine vesicles (Hauser and Phillips, 1973) does not seem to greatly affect the rate of coalescence. Träuble (unpublished results) has shown by using spin-labeling techniques that the rate of vesicle coalescence is enhanced by increasing temperature. A clear manifestation of the collision mechanism was obtained when $^{22}Na^+$-labeled vesicles prepared from synthetic dilauroyllecithin were fractioned on Sepharose 4B. Because of the reduced chain cohesion with this lecithin the bilayer is relatively unstable and at both 4° and room temperature extensive coalescence was observed by gel filtration and electron microscopy. As a result of this and possibly due to an increase in the intrinsic ion permeability with short-chain lecithin bilayers, all the radioactivity escaped from the vesicles in the course of the column chromatography.

We can conclude that because of the ambiguity in $k_1$ and $k_2$ (see Appendix) the relative contributions of the diffusion- and collision-induced rupture mechanisms are unknown. Since it is difficult to eliminate the collision process, it would seem that the intrinsic sodium ion permeability of lipid bilayers is not readily amenable to experimental determination using vesicles.

Recently, work has shown that the increased curvature of phospholipid vesicles compared to multilamellar liposomes affects the molecular packing in the bilayers of vesicles. The cooperativity of the hydrocarbon chain motions is altered and the cooperative unit is reduced in the small vesicles (Melchior and Morowitz, 1972). Sheetz and Chan (1972) concluded that the molecular packing in lecithin vesicles is less regular than in liposomes; however, the difference must be slight because the partial molar volume of lecithin is the same in both situations and the correlation time for motion of a hydrophobic spin probe incorporated in lecithin bilayers is not significantly affected by sonication of the dispersion (Hauser and Irons, 1972). We can conclude that the permeability coefficients for ion diffusion across vesicle bilayers will be upper limits for the permeabilities of more nearly planar bilayers but any differences are likely to be small.

*Energetic Considerations.* There have been many investigations of the mechanism of ion transport across cell membranes (Davson and Danielli, 1952; Stein, 1967) and lipid bilayers (Bangham, 1968; Mueller and Rudin, 1969) but as yet there is no satisfactory quantitative theory to account for the data given above. Nevertheless, it is possible to consider the general energetics of ion transport across a lipid bilayer to check whether the conclusions drawn from the kinetic analysis are consistent with thermodynamic requirements.

For the permeation of a molecule or ion across a homogeneous lipid bilayer of thickness $\rho$, the permeability coefficient $P$ can be expressed as

$$P = KD/\rho \quad (5)$$

where $K$ is the equilibrium distribution or partition coefficient between the interior of the bilayer and the external aqueous phase and $D$ is the diffusion coefficient for diffusion within the bilayer. Clearly for a given bilayer, any differences in $P$ for permeating species of similar size and shape will be controlled by differences in $K$. This is reflected in the fact that highly water-soluble materials diffuse less readily than oil-soluble materials through phospholipid bilayers (Bangham, 1968; Mueller and Rudin, 1969) and cell membranes (Davson and Danielli, 1952; Stein, 1967). It is to be expected that the essential barrier to ion movement across a lipid bilayer will be the hydrocarbon chain region because $K$ for simple inorganic ions must be extremely small. Experimental values of $K$ for $Na^+$ have not been determined, but theoretical estimates are possible by consideration of the self-energy of an ion when it is situated in the interior of a bilayer.

The self-energy $E$ (Gurney, 1962) of a spherical univalent ion of radius $r$ in a given medium is

$$E = e^2/2\epsilon r \tag{6}$$

where $e$ is the electronic charge and $\epsilon$ is the dielectric constant of the medium. Clearly, the calculation is sensitive to changes in the dielectric constant and the ionic radius. As the values of $r$ and $\epsilon_m$, the dielectric constant of the hydrocarbon chain region of the bilayer, are not known for our situation, we shall only attempt to derive a maximum value for $K$. The free-energy increase for the ion transfer from the aqueous to the hydrocarbon phase is minimized by postulating that the fully hydrated ion is transferred into the hydrocarbon interior. For a fully hydrated sodium ion of corrected Stokes radius = 3.3 Å (Robinson and Stokes, 1959) in water ($\epsilon \simeq 80$), $E \simeq 0.6$ kcal/mol ($\sim 1RT$ at 25°). For the same ion in a hydrocarbon medium ($\epsilon \simeq 2$) $E \simeq 25$ kcal/mol ($\sim 41RT$ at 25°). On this basis, the free-energy increase (eq 6a) for transposing 1 mol

$$\Delta E = \frac{e^2}{2r}\left(\frac{1}{\epsilon_m} - \frac{1}{\epsilon}\right) \tag{6a}$$

of sodium ions from aqueous solution to the interior of a phospholipid bilayer will be about $40RT$. With this information it is possible to estimate the concentration of sodium ions in the interior of the bilayer ($C_b$) from the Boltzmann distribution, if the sodium ion concentration in the aqueous phase ($C_a$) is known, because

$$C_b = C_a \exp(-\Delta E/RT) \tag{7}$$

Substitution into eq 7 indicates that when $C_a = 10^{-1}$ M, $C_b \simeq 10^{-19}$ M. The distribution of sodium ions between the interior of the bilayer and the exterior aqueous phase is given by the coefficient $K = C_b/C_a = 10^{-18}$.

Further information about the mode of permeation can be obtained by comparing the above figures for sodium with those for the better understood water transport. Mueller and Rudin (1969) have estimated that the free-energy increase associated with transporting 1 mol of water into a lipid bilayer is 6 kcal/mol ($\sim 10RT$ at 25°). Substitution into eq 7 indicates that when $C_a \simeq 50$ M, $C_b \simeq 10^{-3}$ M so that $K \simeq 10^{-4}$. This figure is close to experimental values (Mueller and Rudin, 1969; Träuble, 1971) and is associated with a water permeability coefficient $P = 2 \times 10^{-3}$ cm/sec. Träuble (1971) has discussed the movement of water molecules across a phospholipid bilayer in terms of thermal fluctuations (kinks) in the hydrocarbon chains. If it is assumed that water molecules enter into the free volumes of these kinks and migrate across the bilayer together with the kinks, the calculated value for the water permeability is compatible with the experimental value quoted above. Now for a given bilayer, it is clear from eq 5 that

$$\frac{P_{H_2O}}{P_{Na}} = \frac{K_{H_2O}D_{H_2O}}{K_{Na}D_{Na}} \tag{8}$$

and if the intrabilayer diffusion processes are the same with both water molecules and sodium ions, then $D$ will be a property of the chains only and $D$ can be cancelled from eq 8. Substitution of our derived values of $K_{H_2O}$ and $K_{Na}$ leads to $P_{Na} = 10^{-17}$ cm sec$^{-1}$, whereas we measured $P$ as $\sim 10^{-14}$ cm sec$^{-1}$ (Table I).

The difference between experimental and calculated permeability coefficients of at least three orders of magnitude has to be accounted for by postulating an additional mechanism of $Na^+$ diffusion across lipid bilayers, because a difference of greater than $10^3$ between $D_{Na}$ and $D_{H_2O}$ seems unlikely. It is therefore clear that the above energetic considerations are consistent with the conclusion, derived from the analysis of the kinetics of ion flux, that there are two mechanisms of sodium ion release from phospholipid vesicles.

Acknowledgment

We are indebted to Dr. F. Franks for helpful discussions and to Mr. J. Taylor for valuable aid with the mathematical analysis of the experimental data.

Appendix

The following model was used for fitting the experimental data for $Na^+$ release from single-shelled phospholipid vesicles over periods of time exceeding 300 hr (see Figure 4c). It is assumed that $Na^+$ can be released from the internal vesicle cavities by two mechanisms. The first is diffusion across the lipid bilayer. The second is exchange of the ion contents with the external medium during transient collision-induced rupture of the bilayers. In the latter case, the ruptured bilayers of the two vesicles involved in a collision may either reseal with the formation of the two original cavities or they may coalesce forming a single enlarged cavity. In both cases the ion content of cavities involved in energetic collisions will be in equilibrium with the bulk phase, *i.e.* their content of radioactive ions is $<X_\infty$, and is negligible compared with the radioactivity $X$ trapped within the vesicles at time $t$. $X_0$ is the number of counts at time = 0 enclosed in $N_0 = V_T/V$ vesicles per milliliter of dispersion, where $V$ is the average vesicle volume and the total volume $V_T = \Sigma_1^i V$. Between time $t$ and $t + \delta t$ the number of collisions $\delta N$ is proportional to the square of the number of cavities present per milliliter at time $t$, the proportionality constant being the rate constant $k_2'$ of the process. The prime is used to indicate that the units of the constant in eq 9 are different from those of eq 2. $\psi$ is defined as

$$\delta N = (k_2'/2)(\psi N_0)^2 \delta t \tag{9}$$

the number of cavities present at time $t$, per $N_0$. A proportion $k_3$ of these collisions leads to coalescence of the two cavities involved; each of the collisions reduces the total number of

$$-(\delta N/N_0) = -d\psi = (k_2'k_3/2)N_0\psi^2\delta t \tag{10}$$

cavities by one. Integration of eq 10 gives

$$\psi = \frac{1}{1 + (k_2'k_3N_0t/2)} \quad (11)$$

Similarly, the number of collisions $\delta N'$ between $t$ and $t + \delta t$ involving single-shelled vesicles which up to this point have not been involved in energetic collisions will be given by the product of $\theta N_0$, the number of single-shelled vesicles per milliliter [$\theta$ = the number of single-shelled vesicles which have not been involved in collision per $N_0$] and $k_2'\psi N_0\delta t$, the number of cavities which are involved in active collisions. Hence $\delta N'$ is given by

$$\delta N' = k_2'\theta\psi N_0^2\delta t \quad (12)$$

or

$$(\delta N'/N_0) = -\mathrm{d}\theta = k_2'\theta\psi N_0\delta t \quad (13)$$

Integration of eq 13 gives

$$\theta = \psi^{2/k_3} \quad (14)$$

The radioactivity released between $t$ and $t + \delta t$ consists of two contributions, $\delta X_1$ and $\delta X_2$, due to the two mechanisms described above. If it is assumed that every collision energetic enough to induce a transient rupture of the bilayer results in the release of all $^{22}Na^+$ trapped in the cavity, then $\delta X$ is given by

$$\delta X = \delta X_1 + \delta X_2 \quad (15)$$

where

$$\delta X_1 = -k_1X\delta t \quad (16)$$

is the loss due to ion diffusion across the bilayer and

$$\delta X_2 = -\delta N'(X/\theta N_0) = -k_2'\psi N_0X\delta t \quad (17)$$

is the loss due to single-shelled, $^{22}Na^+$-containing vesicles being involved in energetic collisions which is given by the product of the number of collisions $\delta N'$ and the radioactivity trapped in one single-shelled vesicle at time $t$. Substitution of eq 16 and 17 into eq 15 and integration gives

$$X/X_0 = \theta e^{-k_1t} = \left[\frac{1}{1 + (k_2'k_3N_0t/2)}\right]^{2/k_3} e^{-k_1t} \quad (18)$$

In Figure 4c the proportion $p$ of $Na^+$ released from the internal cavities was computed using eq 19 where $X/X_0$ was

$$p = 1 - (X/X_0) \quad (19)$$

taken from eq 18. In a computer program, $\Sigma(p - \hat{p})^2$, where $p$ is the experimental value and $\hat{p}$ is the value computed from eq 18 and 19, was minimized by iterations involving the three parameters $k_1$, $k_2$, and $k_3$. The values for $k_1$, $k_2$, and $k_3$ obtained from this analysis are summarized in Table I. The table also contains the $k_2$ (eq 2) values for the situation where all the $Na^+$ is released by the collision mechanism. The ratio of $k_2$(eq 2)/$k_2$(eq 18) is of the order 50–100 indicating that the diffusion mechanism is dominant and that, at an experimental temperature of 4°, the collision mechanism will only contribute if the diffusion experiment is extended over longer periods of time. With $k_2$ being very small, eq 18 approximates to a first-order equation (20).

$$X/X_0 = e^{-(k_1 + k_2'N_0)t} \quad (20)$$

This explains why in Figure 4 the first-order equation gives a much better fit than the second-order equation. It should be noted that the treatment shown above gives a good estimate of $(k_1 + k_2'N_0)$, but it gives only rough estimates of $k_1$ and $k_2$ because, provided $(k_1 + k_2'N_0)$ remains constant, $k_1$ and $k_2$ can vary greatly without significantly affecting $\Sigma(p - \hat{p})^2$.

## References


Bangham, A. D. (1968), *Progr. Biophys. Mol. Biol. 18*, 29.

Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965), *J. Mol. Biol. 13*, 238.

Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., and Bergelson, L. D. (1971), *Chem. Phys. Lipids 6*, 343.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Davson, H., and Danielli, J. F. (1952), The Permeability of Natural Membranes, New York, N. Y., Cambridge, University Press.

Dawson, R. M. C. (1963), *Biochem. J. 88*, 414.

Finer, E. G., Flook, A. G., and Hauser, H. (1972), *Biochim. Biophys. Acta 260*, 49.

Gurney, R. W. (1962), Ions in Solution, New York, N. Y., Dover Press, p 3.

Hauser, H. (1971), *Biochem. Biophys. Res. Commun. 45*, 1049.

Hauser, H., Finer, E. G., and Chapman, D. (1970), *J. Mol. Biol. 53*, 419.

Hauser, H., and Irons, L. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1579.

Hauser, H., and Phillips, M. C. (1973), *Proc. Int. Congr. Surface Active Substances, VIth, 1972* (in press).

Hauser, H., Phillips, M. C., and Stubbs, M. (1972), *Nature (London) 239*, 342.

Hendler, R. W. (1971), *Physiol. Rev. 51*, 66.

Huang, C. (1969), *Biochemistry 8*, 344.

Johnson, S. M., and Bangham, A. D. (1969), *Biochim. Biophys. Acta 193*, 82.

Klein, R. A. (1970), *Biochim. Biophys. Acta 210*, 486.

Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10*, 1111.

Long, C., Shapiro, B., and Staples, D. A. (1962), *Biochem. J. 85*, 251.

Melchior, D. L., and Morowitz, H. J. (1972), *Biochemistry 11*, 4558.

Mueller, P., and Rudin, D. O. (1969), *Curr. Top. Bioenerg. 3*, 157.

Pagano, R., and Thompson, T. E. (1968), *J. Mol. Biol. 38*, 41.

Papahadjopoulos, D. (1971), *Biochim. Biophys. Acta 241*, 254.

Papahadjopoulos, D., and Bangham, A. D. (1966), *Biochim. Biophys. Acta 126*, 185.

Papahadjopoulos, D., and Miller, N. (1967), *Biochim. Biophys. Acta 135*, 624.

Papahadjopoulos, D., Nir, S., and Ohki, S. (1972), *Biochim. Biophys. Acta 266*, 561.

Papahadjopoulos, D., and Watkins, J. C. (1967), *Biochim. Biophys. Acta 135*, 639.

Parsegian, A. (1969), *Nature (London) 221*, 844.

Petkau, A., and Chelack, W. S. (1970), *Biochim. Biophys. Acta 203*, 34.
Phillips, M. C. (1972), *Progr. Surface Membrane Sci. 5*, 139.
Robinson, R. A., and Stokes, R. H. (1959), Electrolyte Solutions, London, Butterworths, p 126.
Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11*, 4573.
Small, D. M. (1967), *J. Lipid Res. 8*, 551.
Stein, W. D. (1967), The Movement of Molecules across Cell Membranes, New York, N. Y., Academic Press.
Stoeckenius, W., and Engelman, D. M. (1969), *J. Cell Biol. 42*, 613.
Träuble, H. (1971), *J. Membrane Biol. 4*, 193.

# Lipid Requirements for Rhodopsin Regenerability†

Keelung Hong and Wayne L. Hubbell*

ABSTRACT: The regenerability of purified rhodopsin has been determined in phospholipid bilayers, in digalactosyl diglyceride bilayers, and in phospholipid-free solutions of digitonin. A high regenerability in the latter system indicates the lack of an absolute requirement for phospholipid. In lipid bilayers, there is no specific requirement for a particular polar head group or fatty acid species. It is suggested that structural rather than specific chemical requirements are important in maintaining a regenerable configuration of the molecule.

Rhodopsin is an integral component of the disk membranes in vertebrate photoreceptors and plays a central role in generation of the receptor potential. Recent data derived from X-ray diffraction (Blasie, 1972; Blaurock and Wilkins, 1972) and freeze-fracture electron microscopy (Hong and Hubbell, 1972; Chen and Hubbell, 1973) have been interpreted to suggest that an appreciable fraction of the rhodopsin molecule is located within the hydrophobic interior of the membrane and in direct contact with the fatty acid chains of the phospholipids. A study of the interactions between rhodopsin and bilayer phospholipids, and their modulation by light, is a step toward elucidating molecular mechanisms of the early events in phototransduction.

It has previously been shown that purified, delipidated rhodopsin can be incorporated into bilayers of egg phosphatidylcholine (Hong and Hubbell, 1972). Such recombined membranes represent an ideal system for investigating the details of phospholipid–rhodopsin interactions, since the compositional variables may be completely specified. More recently, Chabre *et al.* (1972) have disrupted native rod outer segment (ROS)[1] membranes with Triton X-100 and re-formed bilayers containing rhodopsin by extraction of the detergent with toluene. In the development of such model systems, it is necessary to establish an assay to determine whether or not the protein has maintained its native integrity during the various manipulations involved in the protein purification and recombination with phospholipids. Clearly, functional activity is the ultimate criterion. At present, the *in vivo* function of rhodopsin is not known and until established other criteria must be selected. The first necessary (but not sufficient) criterion that must be met for rhodopsin to be considered native is that the protein have the characteristic visible absorption spectrum. This criterion is met for the protein in many detergent solutions and the recombinant membranes previously described (Hong and Hubbell, 1972). It is possible that rhodopsin may exist in various states of partial denaturation even though the characteristic absorption spectrum is maintained. In this paper the term "rhodopsin" will be used to describe any state of the protein characterized by the native absorption spectrum. A more demanding criterion is that of chemical regenerability. Regeneration yields are dependent on the molecular environment of the protein, and so far have been found to be high only in the native membranes, recombined membranes, and digitonin solution. Rhodopsin solubilized in most detergents is either not regenerable following bleaching or the yields are very low.

Two recent reports in the literature have dealt with the subject of phospholipid requirements for rhodopsin regenerability. Zorn and Futterman (1971) have reported that partially delipidated cattle rhodopsin is not regenerable, and of a variety of phospholipids examined, only phosphatidylethanolamine appeared to be capable of restoring the regenerability of the bleached pigment. Shichi (1971) has reported that the photochemical regenerability of rhodopsin is lost when 20–30% of the phospholipid is extracted from cattle rod outer segment membranes, but is restored upon the addition of phospholipid. Shichi's experiments suggest no specific requirement for the type of phospholipid recombined with the partially delipidated membranes.

High regenerability is a characteristic of native rhodopsin, and the degree of regeneration is, in part, a reflection of the ability of the local environment to maintain a particular, regenerable configuration of the protein. From this point of view, molecular requirements for high regenerability are of interest in defining the nature of rhodopsin–lipid interactions.

The present work is concerned with elucidating such requirements, using the approach of recombining purified, phospholipid-free rhodopsin with chemically defined synthetic and natural lipids.

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[1] Abbreviations used are: ROS, rod outer segments; DTAB, dodecyltrimethylammonium bromide; TrTAB, tridecyltrimethylammonium bromide; TeTAB, tetradecyltrimethylammonium bromide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.