temperatures or increased cholesterol content (Figure 7).

Finally, these experiments illustrate an unusual and potentially very useful application of the vesicle-entrapped pyranine technique (Clement & Gould, 1981a) to study the molecular dynamics of membrane–protein interactions. Additional studies on the effects of membrane surface charge and phospholipid composition, as well as studies utilizing larger ionophoretic proteins, such as the hydrophobic CF_0 portion of the chloroplast ATP synthase complex, are in progress.

References

Benz, R., Stack, G., Janko, K., & Lauger, P. (1973) *J. Membr. Biol. 14*, 339–364.

Benz, R., Frohlich, O., & Lauger, P. (1977) *Biochim. Biophys. Acta 464*, 465–481.

Boheim, G., Hanke, W., & Eibl, H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3403–3407.

Clement, N. R., & Gould, J. M. (1981a) *Biochemistry* (first of three papers in this issue).

Clement, N. R., & Gould, J. M. (1981b) *Biochemistry* (preceding paper in this issue.)

Cogan, U., Shinitzky, M., Weber, G., & Nishida, T. (1973) *Biochemistry 12*, 521–528.

Glickson, J. D., Mayers, D. G., Settine, J. M., & Urry, D. W. (1972) *Biochemistry 11*, 477–486.

Goodall, M. C. (1970) *Biochim. Biophys. Acta 219*, 471–478.

Haydon, D. A. (1975) *Ann. N.Y. Acad. Sci. 264*, 2–16.

Hladky, S. B., & Haydon, D. A. (1970) *Nature (London) 225*, 451–454.

Krasne, S., Eisenman, H., & Szabo, G. (1971) *Science (Washington, D.C.) 174*, 412–415.

Mueller, P., & Rudin, D. O. (1967) *Biochem. Biophys. Res. Commun. 26*, 398–407.

Papahadjopoulos, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330–348.

Racker, E. (1973) *Biochem. Biophys. Res. Commun. 55*, 224–230.

Racker, E., & Hinkle, P. C. (1974) *J. Membr. Biol. 17*, 181–188.

Sarges, R., & Witkop, B. (1964) *J. Am. Chem. Soc. 86*, 1861–1862.

Stark, G., & Benz, R. (1971) *J. Membr. Biol. 5*, 133–153.

Szabo, G., Eisenman, G., & Ciani, S. (1969) *J. Membr. Biol. 1*, 346–382.

Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676.

Urry, D. W., Goodall, M. C., Glickson, J. D., & Mayers, D. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1907–1911.

Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792–1801.

Electric Field Induced Transient Pores in Phospholipid Bilayer Vesicles†

Justin Teissie and Tian Yow Tsong*

ABSTRACT: A study of the voltage induction of transient pores in phospholipid bilayer vesicles is reported. Unilamellar vesicles (dipalmitoylphosphatidylcholine), with a size distribution of 100 ± 30 nm, were prepared by the method of Enoch & Strittmatter [Enoch, H., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145]. The vesicles loaded with [^{14}C]sucrose and suspended in a mixture of 150 mM NaCl and 272 mM sucrose (both are the isotonic solvent for erythrocytes) were exposed to an intense electric field in the range of 20–40 kV/cm, with a field decay time of 5–15 μs. A transient leakage of sucrose label was detected when the field strength exceeded 30 kV/cm. After the field was removed, no slow leakage of the tracer molecules occurred during a 65-h incubation period at the room temperature (23 ± 2 °C). The leakage is attributed to the field-induced transmembrane potential, but not other effects such as the Joule heating or the shock wave associated with the voltage discharge. When this potential exceeded a threshold value of 200 mV, corresponding to an applied field strength of 30 kV/cm, there was a dielectric breakdown of the bilayer structure. Pores which allowed passage of sucrose were formed, transiently. Experiments show that these pores were fully reversible, and no global and permanent damages to the vesicle bilayer were detected. The implication of this membrane potential triggered conducting state of lipid bilayers to biological functions of cells is discussed.

Transmembrane potentials are believed to play a major role in biological processes. Neurotransmission is linked to the transfer of ionic currents across the nerve membrane induced by the action potential (Hodgkin, 1964). Membrane potential changes as a consequence of proton (and other charged species) translocation across the mitochondrial membrane are now considered a "driving force" of the energy transduction (Mitchell, 1977). Release of biogenic amines and other hormones in connection with membrane depolarization is well established in chromaffin granules of the adrenal medulla (Neumann & Rosenheck, 1972; Rosenheck et al., 1975).

Under normal conditions, the phospholipid bilayer in the cell membrane is a poor conducting medium, and it can mimic a capacitor. Species transport when it does occur is supposed to be, and often proved to be, associated with an enzymatic machinery (the so-called "pumps"). Nevertheless, when a transport system is activated, drastic changes in membrane potential are recorded with electrophysiological measurements. Under such conditions, the simplistic model of the phospholipid layer as a nonconducting capacitor may no longer be valid. In fact, electrical fields in the range of 100 kV/cm are induced (i.e., a potential of 50 mV on a membrane with a thickness of 5 nm).

When submitted to a strong external electrical field, the bilayers of closed membranous vesicles are polarized because of the movement of ions along the electric field lines. As a consequence, the electrogenicity of the cell is severely per-

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received August 28, 1980.* This work was supported by National Institutes of Health Grant HL18048.

0006-2960/81/0420-1548$01.25/0

turbed. Such a methodology has been applied in our laboratory for the study of red blood cells (Tsong et al., 1976; Kinosita & Tsong, 1977a,b, 1979). The use of intense electric field of short duration (about 2 kV/cm at 20-μs range) has been shown to induce irreversible pores in the erythrocyte membranes. Thirty-five percent of these channels are localized at the sites of the Na^+/K^+-ATPase (Teissie & Tsong, 1980). But under these conditions, the induced membrane potential is in the 1-V range, and the observed processes may be due to a mechanical dielectric breakdown of the membrane. Other experiments have shown that catecholamines are released from chromaffin granules of bovine adrenomedullary cells submitted to electrical fields (on the order of 20 kV/cm and with a decay time of about 150 μs). Such a transient release has been reported to be reversible (Neumann & Rosenheck, 1972). More recently, by use of charge-pulse experiments on oxidized cholesterol planar lipid membranes (BLM), transient high conductivity states were induced by large membrane potentials (in the 1-V range) (Benz et al., 1979; Benz & Zimmermann, 1980). Those states were fully reversible as compared to similar experiments done on a similar system but with other phospholipids (Tien, 1974). These experiments are conclusive of the electric field induction of transient pores in lipid planar layers. In other words, when the membrane potential exceeds a threshold value, the lipid layer cannot anymore be considered an insulating layer. The field induction of transient pores has yet to be demonstrated in a lipid vesicle system. Especially in view of the fact that questions have often been asked whether the stable pores introduced in our electric perforation of erythrocyte membrane could have occurred at the lipid domain, we have undertaken the present study.

This study is facilitated by recent preparation of closed unilamellar vesicles of pure phospholipids with a diameter of 100 nm (Enoch & Strittmatter, 1979). This vesicle system is more suitable for the electrical pulsation experiments because of the larger diameter and enclosing volume. Transmembranous potentials in the 200-mV range are evoked when the external field strength reaches about 30 kV/cm.

In this communication, we describe electric field induced release of entrapped sucrose in such unilamellar macrovesicles. The leakage is transient, and no permanent damage to the membrane structure has been detected. The transient pores occur only when the membrane potential reaches a value greater than a threshold potential. The biological significance of these results is discussed.

Materials and Methods

Chemicals. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma (PO763). The lipid exhibited a transition width of less than 0.2° in a DSC run, and no further purification was done. ^{3}H-Labeled dipalmitoylphosphatidylcholine ([^{3}H]DPPC) was obtained from Applied Sciences (T 11903). ^{14}C-Labeled sucrose was purchased from New England Nuclear (935.017 NEN). Sodium deoxycholate was prepared from deoxycholic acid (Sigma). All salts were analytical grade. Buffers were 7 mM phosphate buffer (pH 7.2) containing mixtures of isoosmotic NaCl (150 mM) and sucrose (272 mM).

Preparation of Unilamellar Macrovesicles. The general procedure described by Enoch & Strittmatter (1979) was followed with some modifications. Small sonicated vesicles were first prepared at a concentration of about 15 mg/mL. The suspension was then kept at 55 °C (i.e., above the transition temperature), and a small aliquot of a 0.2 M sodium deoxycholate solution was added to give a ratio of detergent to phospholipid of 1:3 to 1:4. The mixture was then incubated

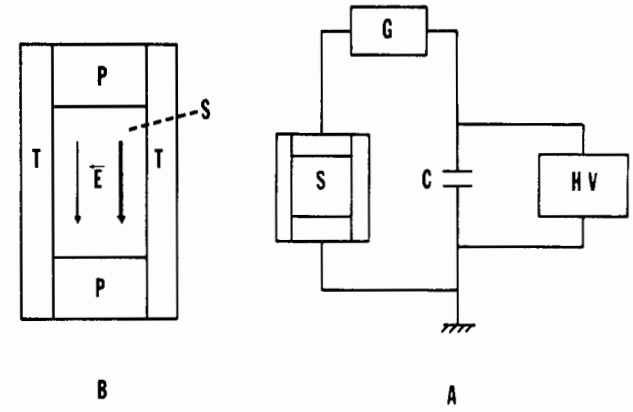

FIGURE 1: Schematic drawing of the voltage pulsation device. (A) General view; (B) enlarged view of the chamber. The sample (S) is placed in a chamber of Teflon (T) between two brass electrodes with platinum coating (P), which are thermostated by circulating water. The high voltage produced by the generator (HV) is stored in the discharge capacitor (C). The voltage is discharged through a spark gap (G). E are the electric field lines.

for 30 min at 55 °C. The detergent was removed by a gel chromatography run at 55 °C (Sephadex G-25, Pharmacia, 1 × 50 cm). The vesicle suspension was concentrated by centrifugation (40 000 rpm, 30 min, Beckman L5 65 centrifuge, Beckman R54 rotor). The pellet was resuspended and the supernatant discarded. The unilamellar macrovesicles so prepared contained less than 0.2 mol % of deoxycholate. [^{14}C]Sucrose-loaded macrovesicles were obtained by performing the sonication and the deoxycholate incubation in a [^{14}C]sucrose-containing solution. Electron microscopy studies and gel chromatography elution (Sepharose 4B and CL-2B, Pharmacia, 1.5 × 50 cm) show that the size of the vesicles is about 100-nm mean external diameter but with a broad distribution (σ = 30 nm).

Electron microscopy was performed by negative staining with 2% ammonium molybdate. The formvar coated grid was first treated by a 1% cytochrome *c* solution (Sigma) in some cases. A drop of macrovesicles solution (1 mM) was first added and dried during 1 min, and a drop of the staining solution was then added and drained off with filter paper after 1 min. This last step was repeated 4 times in order to lower the ionic and sucrose content of the preparation. The grids were examined on a Zeiss 10B electron microscope.

Electric Pulsations. Electrical pulsations were performed with a temperature-jump apparatus (Messanlagen, Germany). In this type of device, a high voltage (up to 40 kV) capacitor (0.05 μF) was discharged into the solution (Eigen & De Maeyer, 1963) (Figure 1). As a consequence, a strong electric field is applied onto the solution during this process. Its amplitude is time dependent and follows an exponential decay. This time course of the field decay can be recorded by the change with time of the optical absorption of dyes (Tsong et al., 1976). This was done by the use of phenol red. Under our experimental conditions the decay time constant ranges from 5 to 25 μs. The observation wavelength was 575 nm, and the signal was recorded by means of a Biomation 805 transient recorder connected to a Tektronix 7704 A oscilloscope and to a Heath SR 204 chart recorder.

The distance between the electrodes was 1 cm, and the magnitude of the initial electric field can be calculated from the intensity of the voltage loading the capacitor.

Due to the current, the temperature of the solution was to increase during the discharge process (about 10 °C for the highest voltage that was used) (Joule heating). For limitation of these thermal effects, the discharge cell was thermostated

at 21 °C by circulating water, and a delay of 3 min was observed between each pulsation when repeated pulsations were done with a given sample. It was checked that this 3-min delay was enough for the solution temperature to return back to its initial value.

Sucrose Determination. Leakage of sucrose was measured by spinning down the macrovesicles as described above and counting the supernatant on a Beckman LS 100 scintillation counter using Liquiscint (National Diagnostic) as scintillation fluid.

Results

Vesicle Response to Electrical Field: A Theoretical Consideration. When a vesicle is exposed to an electric field, the ions in the solution will move along the field lines. But they are held back by the phospholipid bilayer whose conductivity is very low: resistances in the 10^6–10^{10} Ω cm^2 range have been reported (Tien, 1974). As a consequence, the ions accumulate on the surface of the membrane, thus generating a transmembrane potential.

The potential difference across vesicular membranes, induced by an applied electric field, has been shown to possess a maximum value of (Neumann & Rosenheck, 1973)

$$\Delta V = \left\{ \left[1 + \frac{1 + \lambda - 2\lambda^2}{9 + 2(1-\lambda)^2 3d/b} \right] b - \left[\frac{(1 + \lambda - 2\lambda^2)(1 - 2d/b) + 9\lambda}{9 + 2(1-\lambda)^2 3d/b} \right] a \right\} E$$

where a and b are the inner and outer radii, respectively, of the membrane vesicle, d is its thickness, E is the applied electric field, and λ is the ratio between the membrane conductivity and the external buffer conductivity (the conductivity of the internal buffer is supposed to be the same as that of the external buffer).

For phospholipid macrovesicles, at subthreshold external field, the membrane conductivity is null ($\lambda = 0$), and the thickness of the membrane can be neglected when compared to its radius (b = 100 nm, d = 5 nm, $b = 20d$); thus the induced potential is

$$\Delta V = 1.5bE \cos\theta$$

where θ is the angle between the direction of the field and the radius to the point considered on the membrane.

From these relationships, one can easily see that the induced potential is position dependent. In our experimental conditions, it will reach its maximal values at the two loci facing the electrodes ($\theta = 0$ or π).

As described in the case of red blood cells but considering that they were spherical membranes (Kinosita & Tsong, 1977a,b), this potential is not instantaneously induced, but it rises with a first-order kinetic process:

$$\Delta V = 1.5bE \cos\theta\,[1 - \exp(-t/\zeta)]$$

The rise time constant ζ has been calculated to be

$$\zeta = bC(r_i + r_e/2)$$

C is the bilayer capacitance [a value of 0.33 μF cm^2 was reported (Tien, 1974)] and r_i and r_e are the resistivities of the internal and external spaces (100 Ω cm, in our case). This gives a time constant ζ of 0.25×10^{-10} s. Thus, practically, the transmembranous potential is not delayed by this capacitance effect.

But the magnitude of the applied external field decays with time as shown by the experiments with phenol red:

$$E(t) = E(0)\exp(-t/\alpha)$$

where E(0) is the initial value, directly related to the loading voltage of the discharge capacitor. α was determined experimentally and was shown, as expected, to be a function of the ionic content of the buffer: under our experimental conditions, α is in the range of a few to 15 μs.

As a consequence, the induced potential can be expressed as

$$\Delta V = 1.5b \cos\theta\, E(0) \exp(-t/\alpha)$$

It should be noticed that as the charging time of the macrovesicle membrane is very short (less than 1 ns) as compared with other systems like red blood cells (Kinosita & Tsong, 1977), we are working under conditions which are similar to the "charge-pulse" experiments (Benz et al., 1979; Benz & Zimmermann, 1980).

But with so fast a charging process, the dielectric constant of the membrane cannot anymore be considered as constant and equal to its value determined at very low frequency. The relaxation frequency f_0 (i.e., the frequency over which the dielectric constant decreases sharply from its value at low frequency) for a close spherical vesicle was determined to be (Bernhard & Pauly, 1973)

$$1/f_0 = 2\pi C_M d_M (p\rho_i + q\rho_o)$$

where C_M (F cm^{-2}) is the membrane capacity, ρ_i and ρ_o (Ω cm^{-1}) are respectively the specific resistances inside and outside of the cell, p and q are dimensionless form factors ($p = 0.5 \times 10^3$, $q = 10^3$ for a sphere), and d_M is the thickness of the membrane (5 nm). This gives a value f_0 = 0.64 MHz.

Under our experimental conditions (charging time of the membrane in the 10^{-10}-s time range), one should use the time-dependent expression of the induced potential (Bernhard & Pauly, 1973):

$$V = \frac{F_j a_j E}{(1 + (f/f_0)^2)^{1/2}}$$

where F_j is a dimensionless form factor (1.5 for a sphere) and a_j is the radius of the sphere (50 nm).

Any function f(x) can be transformed in a Fourier series (Hildebrand, 1976)

$$f(x) = \sum_{n=1} A_n \sin(nx)$$

if then, as in our case

$$f(x) = \exp(-x/RC)$$

one obtains

$$\exp(-x/RC) = (2/\pi)\sum_{n=1}^{\infty} [n/(n^2+1)][1 - \exp(-\pi)\cos(n\pi)]\sin(nx/RC)$$

if we consider the time of 1 μs, a good fit of the series with the actual value of the function (discrepancy of less than 10%) needs a summation of 24 harmonic sine waves, i.e., frequencies up to more than 0.4 MHz. But for so high frequencies, the induced potential is affected by the dielectric relaxation. Practically, this means that the transmembrane potential is not reached instantaneously but will, in fact, have a rise time in the microsecond range. More precisely, we have

$$V = (3/\pi)aE\sum_{n=1}^{\infty} [n/(n^2+1)][1 - \exp(-\pi)\cos(n\pi)] \sin(nt/RC)/[1 + (n/(2\pi RCf_0))^2]^{1/2}$$

Another point of interest is that when a membrane becomes conductive by means of induced transient pores, the induced

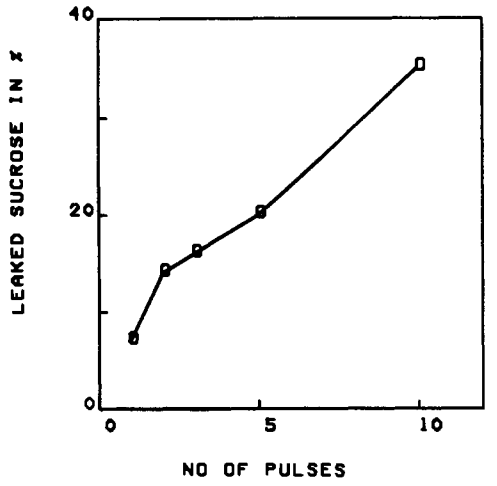

FIGURE 2: Field-induced sucrose release as a function of the number of voltage pulsations. [^{14}C]Sucrose-loaded macrovesicles suspended in a NaCl/sucrose 50:50 (v/v) mixture were pulsed with an initial voltage of 35 kV. After pulsation, the sample was spun down, and the radioactivity of the supernatant was counted. Total radioactivity (100%) was obtained from an unpulsed uncentrifuged sample. Initial temperature was 21 °C. A 3-min time lag was observed between repetitive pulses. The final temperature was lower than 32 °C.

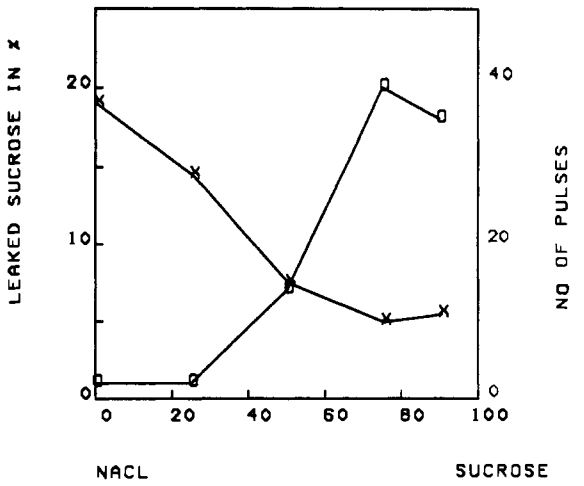

FIGURE 3: Influence of the buffer ionic strength on the leakage of sucrose. [^{14}C]Sucrose-loaded macrovesicles were pulsed with an initial voltage of 35 kV (T = 21 °C), in medium containing different ratios of NaCl to sucrose (both solutions being isoosmotic) (100% NaCl on the left side, 100% sucrose on the right side). Induced leakage of sucrose was determined after one pulse (O) as described in the procedure of Figure 2. The number of pulses (with a 3-min time lag to avoid overheating) needed to observe a 50% sucrose release (×) was obtained from the plot illustrated in Figure 2.

potential is to decrease. As mentioned above, $V = 1.5bE$ is valid only as far as λ is null. If now $\lambda = 1$, that means that the membrane is as conductive as the buffer, one obtains $V = (b - a)E$.

Leakage of Entrapped Tracer Molecules Induced by Electric Field. When a suspension of [^{14}C]sucrose-loaded macrovesicles was submitted to a 35 kV/cm electric field pulsation, a leakage of sucrose was observed. When the pulsation was repeated (up to 10 pulses), the leakage increased. The results are shown in Figure 2. Such a leakage can be referenced by two parameters: the number of pulses needed to obtain 50% of sucrose leakage and the percentage of sucrose which leaks out after one single pulse.

For a given electric field strength, both parameters were found to be a function of the ionic content of the suspension. Pulsations were performed with buffers containing different relative ratios of NaCl and sucrose (both solutions being isotonic). For a 35 kV/cm electric field, it was observed (Figure 3) that (1) the amount of pulses needed to obtain a 50% of sucrose leakage decreased with decreasing ionic content and (2) the amount of sucrose released after one pulsation increased with decreasing ionic content. From Figure 3, it can be seen that both values reach a steady level when the NaCl/sucrose ratio is smaller than 25:75.

Leakage Occurred Only at Field Intensity Exceeding a Threshold Value. Electric field pulsations of increasing magnitude were applied to sucrose-loaded DPPC macrovesicles. Five repetitive pulses were performed following the procedure described above. The results are shown in Figure 4. For intensities smaller than 30 kV/cm, no leakage was observed, and then a strong increase in the leakage was observed for field intensities between 30 and 35 kV/cm. Above 35 kV/cm, a steady level of leakage was detected. From these results it can be concluded that a threshold value of the electric field has to be reached in order to induce the leakage. This threshold value is 30 kV/cm external field, or a transmembrane potential of 225 mV.

Leakage Was Not Due to Effects of the Joule Heating. In an attempt to clarify if the above reported effects might be due to the associated Joule heating, aliquots of sucrose-loaded DPPC macrovesicles were heated at 35 °C for given periods of time. This elevation in temperature (ΔT = 14 °C) was

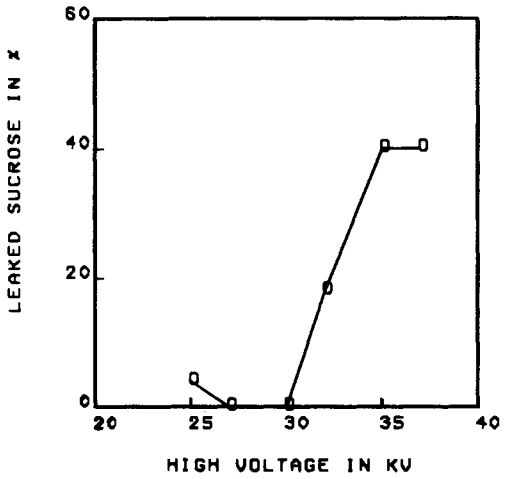

FIGURE 4: Dependence of the sucrose release on the magnitude of the initial voltage. [^{14}C]Sucrose-loaded macrovesicles were suspended in a NaCl/sucrose 25:75 (v/v) mixture at 21 °C. They were pulsed 5 times (with a 3-min time lag) at the indicated initial voltage. Leakage of sucrose was determined as described in Figure 2.

larger than the increase which was associated to the electric field jump (ΔT < 11 °C). No thermally induced sucrose leakage was observed.

Leakage Was a Transient Phenomenon. Sucrose leakage was determined after different given times following the pulsation to obtain evidence that the observed electric field induced sucrose leakage was either a transient process or linked to some irreversible damage in the phospholipid bilayer. No slow leakage, beyond the initial level, was detected for as long as 67 h of incubation at room temperature (23 °C).

Global Damage to the Vesicle Structure Was Not Detected. In an attempt to answer the question of whether the leakage could have resulted from a global damage to the vesicle structure, we have examined the size distribution of vesicle preparation before and after the voltage pulsation by a Sepharose CL-2B chromatography. [^{3}H]DPPC-labeled vesicles were used. In Figure 5 is the size distribution of the untreated sample. No change in the size distribution was observed for a sample 5 times treated with a 35 kV/cm pulse (data not shown). Another experiment using Sepharose 4B chroma-

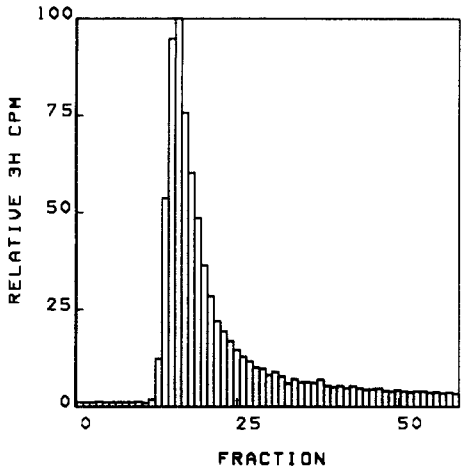

FIGURE 5: Gel filtration of macrovesicles on a Sepharose CL-2B column. [^{14}C]Sucrose-loaded [^{3}H]DPPC-labeled macrovesicles were prepared in a NaCl/sucrose 25:75 (v/v) mixture; 0.7 mL was applied to a column (30 × 1.5 cm) of Sepharose CL-2B. Chromatography was performed at 21 °C with a flow rate of 1 mL/min. The collected fractions were counted for both ^{3}H and ^{14}C radioactivities. The results are plotted for ^{3}H radioactivity taking 100 as the value for the highest peak. No alteration in the size distribution of the vesicle preparation is seen by the voltage treatment (5 pulses at 35 kV).

tography has shown that no detectable level of microvesicles (20-nm diameter) was introduced by the voltage pulsation of the sample (data not given). These results indicate that global damages to the vesicle did not occur by the electric pulsations of the vesicle suspension.

When using the optical detector to monitor transient changes in the turbidity of the vesicle suspension during the voltage discharging process, we have observed a rapid decrease in turbidity arourd 30 μs followed by a slow recovery of signal in a few millisecond time range. These effects have been reported previously (Tsong & Kanehisa, 1977) and were attributed to a transient modification of the vesicle structure, in microseconds, followed by an annealing reaction in millisecond time range. Again, no permanent damage to the vesicles was detected. The fact that the geometry of the vesicles is close to spherical makes it unlikely that the optical signal change was the result of a reorientation effect, as described for linear DNA molecules (Dourlent et al., 1974).

Leakage Occurred Predominately in Large Size Vesicles. As shown in Figure 6 and discussed under Materials and Methods, the size distribution of the vesicle preparation was rather broad. The electron microscopic examination gave a mean size of 100 ± 30 nm diameter. If the sucrose leakage was, in fact, due to the field-induced transmembrane potential, one would expect the leakage to occur predominately in the large size vesicles since the magnitude of the induced transmembrane potential is linearly proportional to the vesicle diameter ($\Delta V_{max} = 1.5\ bE$). To investigate the size dependence of the field-induced leakage, we have done a double-labeled experiment described below.

Unilamellar macrovesicles were prepared with DPPC containing ^{3}H-labeled lipid. The vesicles were loaded with [^{14}C]sucrose. The ratios of ^{3}H and ^{14}C counts in different fractions of the Sepharose CL-2B elution as shown in Figure 5 are plotted in Figure 6A. In the controlled sample, this ratio is uniformly lower than the voltage-treated sample. This is expected since voltage treatment caused leakage of sucrose that reduced the ^{14}C counts. But if we plot the relative ratio of the two samples for different fractions, as given in Figure 6B, there is a linear increase in this value toward a large size vesicle. This indicates that the field induction of sucrose

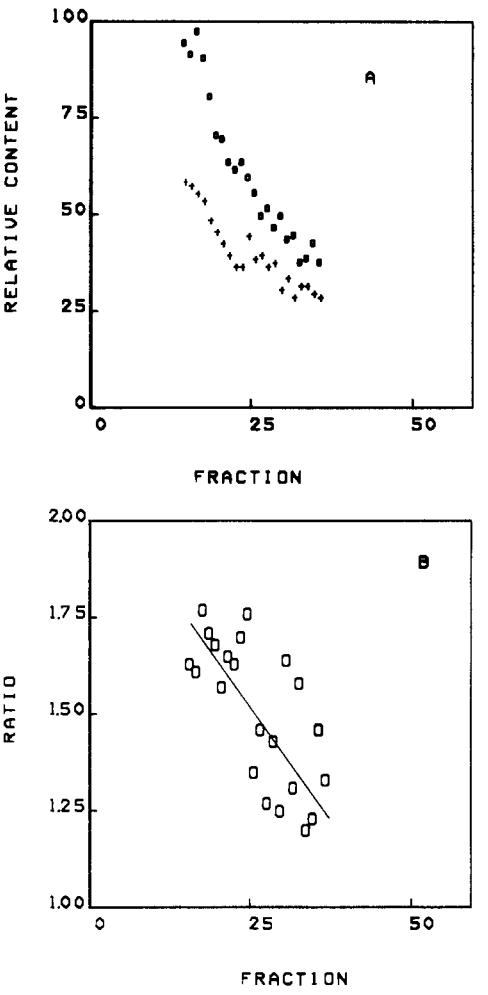

FIGURE 6: Changes in the sucrose content of the pulsed vesicles vs. their sizes. (A) Ratio of ^{3}H to ^{14}C counts plotted for the reference (+) and the pulsed (O) samples for the different fractions of the CL-2B chromatography described on Figure 5. A higher value of this ratio for a given fraction means a higher leakage of sucrose from vesicles. (B) Ratio of the two values (O/×) in (A) plotted for the different fractions of the CL-2B chromatography. The straight line is the least-squares fit of the data points. Its negative slope is indicative that the leakage of sucrose was larger for the higher molecular weight fractions, i.e., the vesicles with the larger diameter. This is expected as the electric field induced membrane potential is proportional to vesicle radius. See text for details.

leakage depended linearly on the vesicle size, as is expected from relations discussed in the theoretical section.

Discussion

The above-described experiments report evidence that transient pores, large enough to allow the leakage of sucrose molecules, are induced across the membranes of phospholipid vesicles by external electric fields. The induced pores are not related to a thermal effect. This conclusion is derived from the following evidence. First, no sucrose leakage has been detected if the heating is applied without the electric field, even if the high temperature state is maintained for a long period (up to 15 min). Second, the sucrose leakage is not a function of the temperature increase. For a given applied initial voltage, i.e., a given heating power (for $W = {}^{1}/_{2}CV^{2}$), or a given temperature increase, the sucrose leakage is a function of the discharge time, i.e., the time during which the field is applied to the suspension. The longer this time is (the lower the ionic content of the buffer), the larger the sucrose leakage is.

Pressure effects are irrelevant to explain the observed effects. Associated to the discharge, a pressure jump is present. But

its magnitude is larger when the discharge time is shorter (Hammes, 1974). If the sucrose leakage was linked to this pressure increase, one would expect a larger leakage for a shorter discharge time. The contrary is experimentally observed.

These induced pores are fully reversible. No change in the macrovesicle structure can be detected after the pulsation except the above-mentioned release of sucrose. Thus, they seem to have a very short lifetime: no slow leakage can be detected by further incubation.

As described in the "theory" section, associated to this electrical pulsation, a strong and almost instantaneous membrane potential evolves across the phospholipid layer. From the relation given previously, one can compute the threshold membrane potential required to induce the pores. Assuming that the opening of channels is instantaneous, one finds a threshold value of 225 mV for a vesicle diameter of 100 nm (an external field strength of 30 kV/cm). Since, as we have shown, the amount of leaked sucrose is a function of the ionic content of the external buffer, we can suggest that these transient pores have a very short lifetime and may just exist as long as the membrane potential is greater than its threshold value.

A simulation of electric field decay with varied discharge time constants (or RC value of the circuit) showed that a simple linear correlation should be observed between the amount of released sucrose and the field decay time. As seen in Figure 3, the release is sigmoidal with respect to sucrose content (which is proportional to the decay time). Two explanations may be given to account for this discrepancy. First, as we discussed earlier, the evolution of field-induced membrane potential is not an instantaneous process. The dielectric relaxation $f_0 = 0.64$ MHz. Thus, the effective transmembrane potential is reduced, and this reduction is much more obvious with a shorter decay time of the applied electric field. Second, the opening of the pores is not an instantaneous process as suggested above. The membrane potential has to be maintained over the threshold value for a given period of time in order to induce the pores. If one assumes that the time required for the opening is the same whatever the ionic strength of the buffer, such a process will drastically decrease the "conductive state" lifetime in a high ionic content buffer. Another consequence of such a kinetic scheme would be that the effective threshold potential is smaller than described above. The time required to induce the pore can be roughly calculated from our experiments. The experimental voltage thresholds are 30 kV in a low ionic strength buffer (NaCl/sucrose = 25:75, discharge time = 15 μs) and 35 kV in a high ionic strength buffer (NaCl = 100, discharge time = 5 μs). If one assumes that the time lag, the threshold voltage, and the size of the vesicles are the same in both buffers, one obtains the time lag of 1 μs. As a consequence, the threshold membrane potential will be 210 mV (assuming that the diameter of the vesicle is 100 nm). The time needed for the opening of the pore may be a mere by-effect of the frequency dependence of the lipid layer dielectric constant. Such a property has been described in the theoretical considerations. The induced potential reaches its steady-state value after a given time which may roughly approximate the inverse of the relaxation frequency f_0: $1/f_0 = 1.5$ μs. The agreement between this theoretical value and our experimental determination of the opening time is very good.

The lifetime of these pores, although short as demonstrated in our experiments, may in fact be larger than the duration of the applied external voltage. As indicated under the theoretical discussions, as soon as the membrane is in a conductive state, one should consider that its conductivity is almost the same as the buffer ($\lambda = 1$). Thus the induced potential is reduced from a maximal value of 225 mV to 15 mV under the assumptions that E is 30 kV, b, 50 nm, and d, 5 nm.

Successive pulsations have been shown to induce an additive release of sucrose. Such a result is to be considered as one more piece of evidence that the lifetime of the transient pore is very short. If the channel stays open, the amount of leaked sucrose would not be proportional to the number of pulsations, but much larger.

Another piece of evidence that the threshold membrane potential is the physical trigger of these transient pores is given by the elution histogram of the Sepharose CL-2B chromatography. The leakage of sucrose is larger for the larger sized macrovesicles.

Molecular mechanisms responsible for the opening of these transient pores are not yet clear. One may consider a gating mechanism. Polar heads of phospholipids are electrical dipoles. In the case of DPPC, NMR studies have shown (Seelig, 1977) that these dipoles parallel the lipid/layer interface. When submitted to the electric field at the level of the membrane ($E = 4 \times 10^5$ V/cm for $V = 200$ mV and $d = 5$ nm), these dipoles may be reoriented. Such an induced conformational change may allow a flux of species like sucrose across the bilayer. This modification would be transient and disappear as soon as the triggering potential disappears. In a description of the dielectric breakdown of BLM (Crowley, 1973; White, 1974), the bilayer was assumed to be elastic but incompressible (i.e., density = constant). The applied field induces a stress on the film (the so-called electromechanical stress) and thins down the membrane. Consequently, the area per phospholipid must increase. Such an expansion of the film may be responsible for the transient pore. In a recently published description of the theory of electric field induced "phase transition" of phospholipid bilayers (Sugar, 1979), a reorganization of the membrane structure is presumed to occur for a given threshold voltage (at a given temperature). For DPPC at 20 °C (i.e., under our experimental conditions), a drastic increase in the phospholipid area must occur (from a threshold point of view) at a voltage of 120–160 mV. This area increase may be considered as the molecular basis of the observed transient pores, e.g., by inducing a loose packing of the lipids. Recent studies on both natural membranes (Zimmermann & Benz, 1980) and BLM (Benz et al., 1979; Benz & Zimmermann, 1980) have described that charge pulses induce high conductivity states in membranes when the membrane potential reaches a threshold value. Such a transient state is fully reversible as in our study and is different from the electrically induced mechanical breakdown already described with BLM (Crowley, 1973) and erythrocytes membranes (Zimmermann et al., 1975; Kinosita & Tsong, 1977a,b). These ionic channels are relevant of both compression of the membrane (as we have suggested for the sucrose transient pores) and ion movement across the membrane induced by the high electric field (Neumcke & Lauger, 1969; Neumcke et al., 1970).

Another interpretation of these pores, radically different from the above-suggested ones, is given by the analysis of electric breakdown of BLM as described by Abidor and colleagues (1979). In that case, spontaneous pores exist in the membrane due to structural defects, whose development is favored by the electric field. An electric field threshold would occur leading to an irreversible damage of BLM. These pores are analogous to the nuclei in the theory of new phase formation (Tsong & Kanehisa, 1977), a process where an increase

in permeation rates was described (Tsong, 1975).

The observation of membrane potential induced transient pores across phospholipid bilayers should have biological significance. We have shown that the threshold voltage is in a range of about 200 mV for pure DPPC bilayers. In the cell membrane, the threshold potential of lipid domain could be much lower. Thus, as soon as the membrane potential exceeds the threshold potential in a biological membrane, one should consider the lipid layer a poor insulating leaflet, allowing a rapid passage of ions or small molecules.

References

Abidor, I. G., Arakelyan, V. B., Chernomordish, L. V., Chizmadzhev Yu, A., Pastushenko, V. F., & Tarasevich, M. R. (1979) *Bioelectrochem. Bioenerg. 6*, 37.

Benz, R., & Zimmermann, U. (1980) *Biochim. Biophys. Acta 597*, 537.

Benz, R., Beckers, F., & Zimmermann, U. (1979) *J. Membr. Biol. 48*, 181.

Bernhard, J., & Pauly, H. (1973) *Biophysik (Berlin) 10*, 89.

Crowley, J. (1973) *Biophys. J. 13*, 711.

Dourlent, M., Hogrel, J. F., & Helene, C. (1974) *J. Am. Chem. Soc. 96*, 3398.

Eigen, M., & De Maeyer, L. (1963) in *Techniques of Organic Chemistry*, p 895, Wiley, New York.

Enoch, H., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145.

Hammes, G. G. (1974) in *Investigation of Rates and Mechanisms of Reactions*, p 147, Wiley, New York.

Hildebrand, F. B. (1976) in *Advanced Calculus for Applications*, p 214, Prentice-Hall, Englewood Cliffs, NJ.

Hodgkin, E. R. (1964) in *Conduction of the Nervous Impulse*, Liverpool University Press, Liverpool.

Kinosita, K., & Tsong, T. Y. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1923.

Kinosita, K., & Tsong, T. Y. (1977b) *Biochim. Biophys. Acta 471*, 227.

Kinosita, K., & Tsong, T. Y. (1979) *Biochim. Biophys. Acta 554*, 479.

Mitchell, P. (1977) *Annu. Rev. Biochem. 46*, 996.

Neumann, E., & Rosenheck, K. (1972) *J. Membr. Biol. 10*, 279.

Neumann, E., & Rosenheck, K. (1973) *J. Membr. Biol. 14*, 194.

Neumcke, B., & Lauger, P. (1969) *Biophys. J. 9*, 1160.

Neumcke, B., Walz, D., & Lauger, P. (1970) *Biophys. J. 10*, 172.

Rosenheck, K., Lindner, P., & Pecht, I. (1975) *J. Membr. Biol. 20*, 1.

Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353.

Sugar, I. P. (1979) *Biochim. Biophys. Acta 556*, 72.

Teissie, J., & Tsong, T. Y. (1980) *J. Membr. Biol. 55*, 133.

Tien, H. (1974) in *Bilayer Lipid Membranes: Theory and Practice*, p 121, Marcel Dekker, New York.

Tsong, T. Y. (1975) *Biochemistry 14*, 5409.

Tsong, T. Y., & Kanehisa, M. (1977) *Biochemistry 16*, 2674.

Tsong, T. Y., Tsong, T. T., Kingsley, E., & Siliciano, R. (1976) *Biophys. J. 16*, 1091.

White, S. H. (1974) *Biophys. J. 14*, 155.

Zimmermann, U., & Benz, R. (1980) *J. Membr. Biol. 53*, 33.

Zimmermann, U., Pilwat, G., & Riemann, F. (1975) *Biochim. Biophys. Acta 375*, 209.

Interaction of Cytochrome *c* with Phospholipid Monolayers. Orientation and Penetration of Protein as Functions of the Packing Density of Film, Nature of the Phospholipids, and Ionic Content of the Aqueous Phase†

J. Teissie*

ABSTRACT: Energy-transfer fluorescence quenching has been used to observe the binding of cytochrome *c* to a lipid assembly. The probe (donor), dansylphosphatidylethanolamine, was dispersed either in dipalmitoylphosphatidylcholine, in phosphatidic acid, or in a mixture of the two lipids. The heme of the protein was the acceptor. When the phospholipids were spread in monolayer at the air–water interface, orientation and penetration parameters of the protein relative to the membrane were obtained. The cytochrome is bound with an orientation such that its heme crevice is fully accessible to the aqueous space. Its penetration in the lipid layer is dependent on the ionic content of the subphase and the initial packing of the film. The perturbation induced in the lipid matrix by the binding appears very localized. The same results were obtained with lipid microvesicles. The type of binding of cytochrome *c* to phospholipids observed here implies that there are specific areas on the protein which appear to be different from those involved in its interaction with cytochrome oxidase and other cytochromes. These conclusions are relevant to the existence of different classes of binding sites for cytochrome *c* in the mitochondrial membrane.

In mitochondria, orientation of heme groups of different cytochromes relative to each other and to the membrane are generally accepted to be critical for electron-transfer efficiency. In the case of cytochrome *c*, ESR measurements using labeled species [3-[[(iodomethyl)carbonyl]amino]-2,2,5,5-tetramethylpyrrolidinyl-1-oxy covalently bound on methionine-65] have shown that a specific area is involved in the binding to charged phospholipid microvesicles (Brown & Wuthrich, 1977; Vanderkooi et al., 1973a,b). Our preliminary paper has shown that this is also the case with native cytochrome *c* (Teissie & Baudras, 1977).

A monolayer of phospholipids spread at the air–water interface provides a useful model system for studying lipid–

† From the Centre de Biochimie et de Genetique Cellulaires du CNRS, 31077 Toulouse, France. *Received February 6, 1980.* This work is a part of the These de doctorat d'etat (Universite de Toulouse III, March 1979, to J.T). Part of this work was presented at the Sixth International Biophysics Meeting, Kyoto, 1978.

0006-2960/81/0420-1554$01.25/0