# Dehydration of Model Membranes Induced by Lectins from *Ricinus communis* and *Viscum album*

Peter Pohl, Sapar M. Saparov, Elena E. Pohl, Veronika Y. Evtodienko, Igor I. Agapov, and Alexander G. Tonevitsky

Medizinische Fakultät, Institut für Medizinische Physik und Biophysik, Martin Luther Universität, 06097 Halle, Germany

ABSTRACT The effects of ribosome-inactivating proteins (RIPs) from *Ricinus communis* and from *Viscum album* on the water permeability,  $P_{\rm f}$ , and the surface dielectric constant,  $\varepsilon$ , of model membranes were studied.  $P_{\rm f}$  was calculated from microelectrode measurements of the ion concentration distribution in the immediate vicinity of a planar membrane, and  $\varepsilon$  was obtained from the fluorescence of dansyl phosphatidylethanolamine incorporated into unilamellar vesicles.  $P_{\rm f}$  and  $\varepsilon$  of fully saturated phosphatidylcholine membranes were affected only in the presence of a lectin receptor (monosialoganglioside, GM1) in the bilayer. It is suggested that the membrane area occupied by clustered lectin-receptor complexes is markedly less permeable to water. Protein binding to the receptor was not a prelude for hydrophobic lipid-protein interactions when the membranes were formed from a mixture of natural phospholipids with a high content of unsaturated fatty acids. These membranes, characterized by a high initial water permeability, were found to interact with the RIPs unspecifically. From a decrease of both  $P_{\rm f}$  and  $\varepsilon$  it was concluded that not only water partitioning but also protein adsorption correlates with looser packing of polyunsaturated lipids at the lipid-water interface.

#### INTRODUCTION

The potent plant toxins (see Table 1) ricin (RCA60) and the mistletoe lectins I (MLI) and III (MLIII) are heterodimeric proteins consisting of an A-chain, which has 28S ribosomal RNA N-glycosidase activity, joined to a B-chain, which is a galactose and/or N-acetyl-D-galactosamine-specific lectin (Lee et al., 1994). The binding of the B-chain to cell surface galactose-containing proteins is followed by endocytosis (Sandvig and van Deurs, 1994). The subsequent translocation across the membrane of an intracellular compartment to enter the cytosol is the transport step least understood in the entire cell intoxication process (Raso, 1994; Wellner et al., 1995). Much evidence currently suggests that toxin entry and routing inside cells are not toxin-specific and mimic pathways of physiological molecules (Barbieri et al., 1993). It is believed that the lectin is delivered into the cytosol by the protein transport machinery of the endoplasmic reticulum (ER) after having been transported across the trans-Golgi network in retrograde direction (Sandvig and van Deurs, 1996). Alternatively, membrane destabilization as a result of direct lipid-protein interactions is hypothesized to

Received for publication 18 July 1997 and in final form 7 August 1998. Address reprint requests to Dr. Peter Pohl, Medizinische Fakultät, Institut für Medizinische Physik und Biophysik, Martin Luther Universität, 06097 Halle, Germany. Tel.: +49-345-5571243; Fax: +49-345-5571632; E-mail: peter.pohl@medizin.uni-halle.de.

Veronika Y. Evtodienko's permanent address is A. N. Belozersky Institute of Physicochemical Biology, Moscow State University, 119899 Moscow, Russia.

Igor I. Agapov's and Alexander G. Tonevitsky's permanent address is Institute for Genetics and Selection of Industrial Microorganisms, 1st Dorozhny Proezd, 113545 Moscow, Russia.

Abbreviations used: RCA120, agglutinin from Ricinus communis; RTA, A-chain of ricin; RTB, B-chain of ricin.

© 1998 by the Biophysical Society 0006-3495/98/12/2868/09 \$2.00

be involved in the translocation mechanism (Utsumi et al., 1989; Agapov et al., 1997; Pohl et al., 1998).

From a comparison of the interaction of various lectins with the lipid bilayer, a better insight in the mechanism of translocation across an intracellular membrane barrier into the cytosol is expected. The knowledge of this transport step is crucial for a therapeutic utilization of ribosome-inactivating proteins (RIPs) in the treatment of cancer (Brinkmann and Pastan, 1994), autoimmune (Raso, 1994), and graft-versus-host diseases. The study of the interaction of lectins with lipid membranes is also important because generally, the membrane insertion of water-soluble proteins is of basic interest in membrane biosynthesis and secretion (Montich et al., 1995; Ladokhin et al., 1997).

Protein partitioning into the membrane, which leads to defects in membrane structure, is assumed to introduce some additional bilayer compressibility. Therefore, an enhanced water membrane permeability  $(P_{\rm f})$  is expected (Needham et al., 1988). An increasing amount of water penetrating the hydrocarbon, as reported for small peptides after hydrophobic binding at the bilayer interface, is also expected to translate into higher water permeation rates (Jacobs and White, 1989). Nevertheless, the protection of isolated thylakoids against freeze-thaw damage by some galactose-specific lectins was afforded by a reduction of the hydraulic membrane conductivity (Hincha et al., 1993). The mechanism responsible for this contradictory finding is unknown.

In molecular dynamics simulations it is anticipated that the phospholipid headgroups are engulfed in water, which then intermittently partitions into the region of the hydrophobic aliphatic chains of the fatty acids (Haines and Liebovitch, 1995). As a result of protein adsorption to the membrane surface some of the interfacial water may be expelled (Hoekstra and Wilschut, 1989). In this case, the portion of

**TABLE 1 Proteins** 

Protein	Function	Molecular Mass (kDa)	Subunits	References
Ricin (RCA60)	Type 2 ribosome-inactivating	63	A-chain	(Lord et al., 1994)
	protein		B-chain	
Ricinus agglutinin (RCA120)	Type 2 ribosome-inactivating protein	120	Two A-chains Two B-chains	(Saltvedt, 1976)
Viscumin (MLI)	Type 2 ribosome inactivating protein	120 (in concentrated solutions)	Two A-chains Two B-chains	(Olsnes et al., 1982)
Mistletoe lectin III (MLIII)	Type 2 ribosome inactivating protein	60	A-chain B-chain	(Eifler et al., 1994)
Ricin A-chain (RTA)	Cleaves the <i>N</i> -glycosidic bond at adenine <sub>4324</sub> in 28S rRNA	30		(Endo and Tsurugi, 1987)
Ricin B-chain (RTB)	Binds to galactose or <i>N</i> -acetylgalactosamine residues	33		(Sandvig and van Deurs, 1996)

the membrane surface covered by the protein should be markedly less permeable to water than normal. To test this hypothesis, we have measured membrane water permeability and surface hydrophobicity under the same conditions. For the surface dielectric constant (ε) measurements, a fluorescence spectroscopic method (Ohki and Arnold, 1990; Kimura and Ikegami, 1985) was used, which detects the environmental effect on the membrane surface upon the addition of different RIPs.

The investigations were carried out with bilayers of different composition because it is expected that partitioning of adsorbing molecules into lipid monolayers depends on membrane mechanical properties (Needham, 1995). For water, an increase of membrane compressibility (i.e., a decrease in tension) was reported to be accompanied by a deeper penetration into these bilayers and a progressive increase of bilayer water permeability (Bloom et al., 1991). In the present study, nonspecific nonelectrostatic interactions between the RIPs and lipid bilayers were also found to be lipid-dependent. It was concluded that not only water (Huster et al., 1997), but also protein partitioning correlates with looser packing of polyunsaturated lipids at the lipid-water interface.

#### **MATERIALS AND METHODS**

#### Liposomes

Unilamellar vesicles were made from diphytanoyl phosphatidylcholine (DPhPC), egg phosphatidylcholine (EPC; both from Avanti Polar Lipids, Alabaster, AL), phosphatidylethanolamine (PE), phosphatidylserine (PS), and ergosterol (all from Sigma, Dreisenhofen, Germany). In some of the experiments 10 mol % monosialoganglioside (GM1, Sigma) were added. The lipids were dissolved in a chloroform/methanol mixture. For labeling, dansyl phosphatidylethanolamine (DPE; Avanti Polar Lipids) was given to the lipid at a molar ratio lipid/DPE of 1:100–1:200. Large unilamellar vesicles were prepared by an extrusion technique (MacDonald et al., 1991) using the small-volume apparatus LiposoFast (Avestin Inc., Ottawa, Canada) with filters of 100 nm pore diameter. The final lipid concentration was 25  $\mu$ M in a buffer solution consisting of 100 mM NaCl, 10 mM HEPES, and 10 mM MES.

### Planar bilayers

Planar bilayer lipid membranes (black lipid membranes, BLMs), 0.8 mm in diameter, were spread by a conventional method (Mueller et al., 1963)

across a circular hole, in a diaphragm separating two aqueous phases of a polytetrafluorethylene (PTFE) chamber. By using this technique a considerable amount of solvent remains dissolved in the bilayer. Nevertheless, the water permeability properties of lipid bilayer membranes are intrinsic to the bilayer structure and do not depend on the presence of hydrocarbon solvent in the membranes (Finkelstein, 1987).

The membrane-forming solution consisted of 20 mg DPhPC or 30 mg EPC (50 mol %) and PE (50 mol %) or EPC (50 mol %) and ergosterol (50 mol %) per ml of an *n*-decane/chloroform/methanol mixture (all Merck, Darmstadt, Germany) (volume ratio = 7:2:1). Ten mol % monosialoganglioside GM1 were added to the membrane-forming solution in some experiments. The bathing solution contained 20 mM Tris (Fluka, Buchs, Switzerland), 20 mM MES (Boehringer, Mannheim, Germany) and 100 mM NaCl (Merck, Darmstadt, Germany). It was agitated by magnetic bars.

For monitoring bilayer capacitance a sine wave input voltage (source: Model 33120A, Hewlett-Packard, Loveland, CO) was applied to the membrane. The output signal was first amplified by a current amplifier (Model 428, Keithley Instruments Inc., Cleveland, OH) and then visualized with an oscilloscope. Conductance measurements were carried out with the same amplifier (Model 428, Keithley Instruments Inc.) using the built-in voltage source for voltage clamping.

## Measurements of the hydraulic membrane permeability

It is well known that even in vigorously stirred systems there is usually a stagnant layer adjacent to a membrane that leads to concentration differences, i.e., water that passes through the membrane dilutes the solution it enters and concentrates the solution it leaves (Fettiplace, 1978). From the ion concentration distribution within the unstirred layer (USL) the osmotic permeability of a planar bilayer may be calculated (Pohl et al., 1997). It is assumed that the ion concentration C depends only on the distance x from the membrane and that there is a gradual change of the stirring velocity in the immediate membrane vicinity which can be described by the model of stagnant point flow. Within a USL of the size  $\delta$  ( $-\delta \le x \le \delta$ ) the concentration course is found as (Pohl et al., 1997)

$$C(x) = C_s e^{(-vx/D) + (ax^3/3D)}$$
 (1)

where a, D, and v are the stirring parameter, the diffusion coefficient of the impermeable solute (here Na<sup>+</sup>), and the velocity of the transmembrane water flow, respectively. Fitting Eq. 1 to experimental concentration profiles allows us to find the unknown parameters v and a. With the knowledge of v the transmembrane water permeability  $P_{\rm f}$  can be calculated (Finkelstein, 1987):

$$P_{\rm f} = \frac{v}{C_{\rm osm} V_{\rm W}} \tag{2}$$

where  $V_{\rm w}$  is the partial molar volume of water and  $C_{\rm osm}$  is the near-membrane concentration of the solute used to establish the transmembrane osmotic pressure difference.  $C_{\rm osm}$  has to be corrected for dilution of the urea bulk concentration,  $C_{\rm urea}$ , at the hypertonic side and the transmembrane difference of NaCl concentration  $\Delta C$  that is induced by the volume flow:

$$C_{\text{osm}} = \frac{C_{\text{s}}C_{\text{urea}}}{C_{\text{b}}} - 4\Delta C \tag{3}$$

Concentration changes of sodium ions in the immediate membrane vicinity due to the water flow across the membrane were monitored with the help of microelectrodes. An osmotic gradient was induced by urea (Laborchemie Apolda, Apolda, Germany) added to the trans side of the membrane only. The sodium-sensitive electrodes were made of glass capillaries containing cocktail A of sodium ionophore II (Fluka, Buchs, Switzerland) (Amman, 1986). Their tips had a diameter of  $\sim 1-2 \mu m$ . Electrodes with a 90% rise time below 0.5~s were selected. The experimental arrangement was similar to the one described previously (Pohl et al., 1993). Voltage sampling was performed routinely every second by an electrometer (Model 617, Keithley Instruments Inc.) connected via an IEEE-interface to a personal computer. The microelectrode was moved perpendicular to the surface of the BLM by a hydraulic microdrive manipulator (Narishige, Tokyo, Japan). The touching of the membrane was indicated by a steep potential change (Antonenko and Bulychev, 1991). Since the velocity of the electrode motion was known (2  $\mu$ m s<sup>-1</sup>) the position of the microsensor relative to the membrane could be determined at any instant of the experiment. The accuracy of the distance measurements was estimated to be  $\pm 8 \mu m$ .

The effect of the lectins on the hydraulic conductivity was assessed after adding them to both sides of the BLM.  $P_{\rm f}$  was determined with a total error of  $\sim \pm 2~\mu \text{m/s}$ , and the standard deviation was kept even smaller as a result of averaging 5–10 concentration profiles.

### Lectins

RCA60, RCA120, RTA, and RTB were purified from *Ricinus communis* seeds as described earlier (Tonevitsky et al., 1990). To completely remove RTB, preparations of RTA were additionally purified on Sepharose 4B (Pharmacia, Sweden) with fixed asialofetuin (Sigma). MLI and MLIII were isolated from *Viscum album* (Eifler et al., 1994). Different lectin isoforms were separated on an FPLC chromatograph (Pharmacia) using a Mono S HR column ( $5 \times 5$ ) with a linear NaCl gradient (0-500 mM) in 15 mM citric buffer, pH 4.2.

# Evaluation of local dielectric constant by fluorescence spectroscopy

Lectin-induced changes in the dielectric constant around the polar region of lipid bilayer membranes were obtained from the emission spectrum of a fluorescence probe incorporated in unilamellar vesicle membranes (Kimura and Ikegami, 1985; Ohki and Arnold, 1990). The  $\epsilon$  of the DPE environment in the lipid membrane was calculated from an empirical law that relates the wavelength ( $\lambda$ ) at the maximum of the emission spectrum to its dielectric properties. This experimental relationship has been obtained from DPE fluorescence spectra in organic solvents with known  $\epsilon$  (Kimura and Ikegami, 1985; Ohki and Arnold, 1990). The measurements were performed at a constant temperature of  $20^{\circ}\mathrm{C}$ .

#### **RESULTS**

Neither conductance nor capacitance of planar membranes was affected by the addition of the RIPs;  $1.2 \pm 0.3$  nS cm<sup>-2</sup>,  $1.7 \pm 0.3$  nS cm<sup>-2</sup>, and  $28 \pm 1$  nS cm<sup>-2</sup>, respectively, were measured for DPhPC/GM1, EPC/PE, and EPC/

ergosterol bilayers. The respective membrane capacitances were equal to  $0.39 \pm 0.7~\mu F~cm^{-2}$ ,  $0.42 \pm 0.5~\mu F~cm^{-2}$ , and  $0.47 \pm 0.6~\mu F~cm^{-2}$ . Consequently, it is ruled out that the solubility of the solvent in the planar membrane is changed due to the addition of the RIPs. Furthermore, an eventual augmentation of the water permeability cannot be attributed to channel activities. This result conflicts with an earlier report where RCA60 was shown to increase the conductance of planar bilayers from glycerolmonooleate (Kayser et al., 1981). The lack of carboxifluoresceine leakage from RCA60-treated liposomes (Utsumi et al., 1984), however, supports our result about the invariability of the membrane conductance.

The  $P_{\rm f}$  of pure DPhPC membranes was not affected by RCA60, RCA120, and MLI. The sodium concentration profiles obtained in the presence of the RIPs were not distinguishable from those measured in their absence. Only after the membrane was enriched with monosialoganglioside GM1 (10 mol %), that is known to act as a lectin receptor (Utsumi et al., 1987; Tonevitsky et al., 1990), a measurable drop of  $P_{\rm f}$  from 25 to 23  $\mu$ m/s was induced by RCA60 (Fig. 1). The difference is rather small. It does not exceed the deviation usually measured from one membrane to another (the ratio of the initial to the final membrane permeabilities is equal to 0.92  $\pm$  0.05). A further decrease of  $P_{\rm f}$  from 23

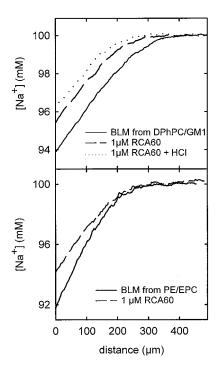


FIGURE 1 Averaged sodium concentration profiles obtained at the *trans* side of a planar membrane made from 90 mol % diphytanoyl phosphatidylcholine and 10 mol % monosialoganglioside (50 mol % phosphatidylethanolamine and 50 mol % egg phosphatidylcholine). The osmotic water flux was induced by 0.8 M urea. The concentration shift near a protein-free membrane is diminished due to the addition of 1  $\mu$ M RCA60. The corresponding hydraulic conductivities ( $P_{\rm f}$ ) are 25  $\mu$ m/s (47  $\mu$ m/s) and 23  $\mu$ m/s (33  $\mu$ m/s). A subsequent pH drop from 7.5 to 4.5 decreased  $P_{\rm f}$  to 21  $\mu$ m/s. Buffer composition: 10 mM Tris, 10 mM MES, 100 mM NaCl.

 $\mu$ m/s at pH 7.5 to 21  $\mu$ m/s at pH 4.5 was calculated from the Na<sup>+</sup> concentration distribution according to Eqs. 1–3. Binding to the galactose residues of GM1 was specific because the initial osmotic permeability of 25  $\mu$ m/s was reestablished after galactose (1 mM) was added to the buffer solution surrounding the BLM. The effect of pH was reversible, too. If in the presence of 1  $\mu$ M RCA60 neutral pH was settled up again or if the membrane was reformed after rupturing in an acidic milieu,  $P_{\rm f}$  was equal to 23  $\mu$ m/s. In control experiments it was established that within the experimental error, the hydraulic permeability of protein-free bilayers made from the DPhPC/GM1 mixture did not vary in the pH interval from 4.5 to 7.5.

Analogous to RCA60, the effect of RTA also appeared to be a function of pH. In Fig. 2 Na $^+$  concentration profiles measured in the vicinity of a GM1-containing membrane are shown.  $P_{\rm f}$  was decreased from 25 to 19  $\mu$ m/s at pH 7.5 and up to 17  $\mu$ m/s at pH 4.5. The ratio of the initial water permeability and the one measured in the presence of RTA was the same for GM1-containing and GM1-free membranes. It was not affected by galactose. These results were expected, because only the B-chain of RCA60 has galactose affinity. RTB was found to have a maximal effect at neutral pH. A permeability of 20  $\mu$ m/s was measured after the addition of 1  $\mu$ M RTB (Fig. 2). A pH reduction did not result in an increased association of RTB to the BLM ( $P_{\rm f} = 21~\mu$ m/s).

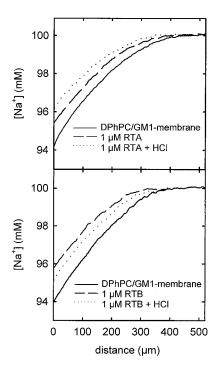


FIGURE 2 Changes of averaged sodium concentration profiles induced by the addition of 1  $\mu$ M of A- and B-chains of RCA60. GM1 content of the DPhPC membrane was 10 mol %. Other conditions as in Fig. 1. The A-chain decreased the initial  $P_{\rm f}$  from 25  $\mu$ m/s to 19  $\mu$ m/s (pH 7.5) and further to 17  $\mu$ m/s (pH 4.5), whereas at pH 7.5 the B-chain diminished  $P_{\rm f}$  from 25  $\mu$ m/s to 20  $\mu$ m/s; it failed to reduce  $P_{\rm f}$  after acidification (21  $\mu$ m/s).

In the case of DPhPC membranes the lectins reduced the parameters  $P_{\rm f}$  and  $\varepsilon$  most effectively at acidic pH provided that a lectin receptor had been incorporated. Most probably, the decreased osmotic permeability at low pH values corresponds to a conformational change of the A-chain because only the isolated A-fragment provoked a decrease of  $P_{\rm f}$  at acidic pH.

At neutral pH both MLI and MLIII reduced the hydraulic conductivity of BLMs containing 10 mol % GM1. Like RCA60 and RTA, these lectins required acidic pH to maximally decrease  $P_{\rm f}$ . The profiles shown for MLIII in Fig. 3 correspond to a drop of the initial hydraulic permeability from 25 to 17  $\mu$ m/s at pH 7.5 and to 14  $\mu$ m/s in an acidic milieu. After addition of galactose competitive to galactose-containing ligands incorporated into model membranes (Lee et al., 1994) an increase of  $P_{\rm f}$  up to the initial value of 25  $\mu$ m/s was observed.

The effect of the RIPs on the water permeability of DPhPC/GM1 membranes was compared with the one of cholesterol that is known to expel water from central regions of the bilayer, thereby decreasing  $P_{\rm f}$  (Subczynski et al., 1994). At acidic pH MLIII is nearly as effective as cholesterol in the highest concentration allowing it to form a bilayer. Forty-five mol % cholesterol in the membrane-forming solution led to a decrease of  $P_{\rm f}$  to 12  $\mu$ m/s under our conditions (see Fig. 5). Half that amount of cholesterol induced an effect close to the one of MLI, MLIII, RTA, or RTB at physiological pH values (see Fig. 5).

In contrast to RCA60, the structurally very similar agglutinin RCA120 did not alter the hydraulic membrane permeability of pure DPhPC or GM1-containing (10 mol %) membranes at physiological or acidic pH, although up to 5  $\mu$ M were added. Membranes made from a mixture of 50 mol % EPC with either 50 mol % natural PE or 50 mol % ergosterol interacted more effectively with the agglutinin.

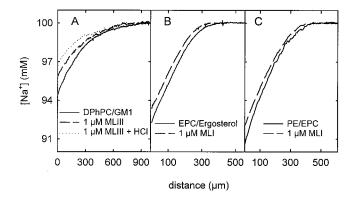


FIGURE 3 Representative sodium concentration profiles measured before and after the addition of (A) 1  $\mu$ M mistletoe lectin III to a DPhPC/GM1 membrane. (B) 1  $\mu$ M mistletoe lectin I to a membrane made from EPC/ergosterol, or (C) 1  $\mu$ M mistletoe lectin I to a PE/EPC membrane. The calculated hydraulic permeabilities are (A) 25 and 17  $\mu$ m/s, (B) 47 and 36  $\mu$ m/s, and (C) 34 and 30  $\mu$ m/s, respectively. In case (A) 14  $\mu$ m/s were measured after pH switching from 7.5 to 4.5. The osmotic gradient was 600 mM urea in (A) and 800 mM in (B) and (C). Except for the lower stirring velocity, all conditions were as in Fig. 1.

Upon the addition of 3  $\mu$ M RCA120,  $P_{\rm f}$  decreased measurably (Fig. 4). Membranes of this composition underwent dramatic changes of their permeability if MLI (Fig. 3), MLIII or RCA60 (Fig. 1), were added. Here, the augmentation of the concentration above 1  $\mu$ M revealed no additional effect.

These dramatic changes of the hydraulic bilayer conductivity are a result of unspecific interactions between the bilayer and hydrophobic domains of the proteins that most probably substitute interfacial water during the process of membrane binding. To test this hypothesis we looked for a lectin-induced increase in membrane surface hydrophobicity. The latter is measurable as a decrease of the apparent dielectric constant in the headgroup region of the phospholipid bilayer (Kimura and Ikegami, 1985). Consequently, a fluorescent probe was used to monitor the local polarity. For vesicular membranes made from DPhPC and doped with DPE a dielectric constant of 32–35 was measured (Fig. 6). This value corresponds well to the one reported for phosphatidylcholine membranes in the literature (Kimura and Ikegami, 1985; Ohki and Arnold, 1990).

For the experiments carried out with liposomes, we were forced to enhance the protein concentration to detect an effect. However, a direct comparison with the concentration used for experiments carried out on planar bilayers is not very useful because here the protein-lipid ratio is difficult to assess. RCA60 exhibited a modest effect on the surface dielectric constant of vesicles made from DPhPC that was not altered by the incorporation of 10 mol % GM1 into the bilayer (Fig. 6). Whereas the fluorophore is located in the glycerol backbone region of the lipid bilayer (Waggoner and Stryer, 1970) the oligosaccharide portion of the GM1 molecule extends beyond the PC headgroup into the fluid space, i.e., the GM1 headgroup is nearly fully extended from the bilayer surface (McIntosh and Simon, 1994). Upon acidification, the polarity of the membrane surface decreased below 12. This dramatic effect was found only in the presence of GM1 (10 mol %) in the vesicular membrane (Fig. 7). Our observation is consistent with a literature

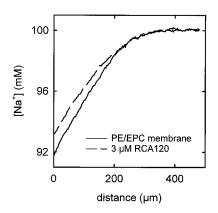


FIGURE 4 Changes of averaged sodium concentration profiles in the vicinity of a PE/EPC membrane (50 mol % each) caused by the addition of 3  $\mu$ M RCA120. The corresponding  $P_{\rm f}$  values are 46  $\mu$ m/s (solid line) and 39  $\mu$ m/s (dashed line). All conditions were as in Fig. 1.

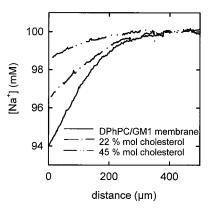


FIGURE 5 Effect of cholesterol on representative sodium concentration profiles in the membrane vicinity. The membranes were composed of 90 mol % DPhPC, 10 mol % GM1 (—); 68 mol % DPhPC, 10 mol % GM1, and 22 mol % cholesterol (— · · —); and 45 mol % DPhPC, 10 mol % GM1, and 45 mol % cholesterol (— · · —). The corresponding  $P_{\rm f}$  values are 26  $\mu$ m/s, 19  $\mu$ m/s, and 12  $\mu$ m/s. All conditions were as in Fig. 1.

report where RCA60 was described to be bound to galactose moieties on the surface of liposomes at neutral pH and to be associated with the bilayer at acidic pH (Utsumi et al., 1987). It was suggested that specific binding to the receptor (GM1) is a prelude for hydrophobic protein-lipid interactions. Under our conditions, this is true only for fully saturated DPhPC membranes. Fig. 6 shows that RCA60 interacts very efficiently with bilayers made from a mixture of lipids (50 mol % PE, 20 mol % PS, 10 mol % EPC, 20 mol % ergosterol). The same holds for MLIII (Fig. 8) and all other RIPs investigated. Addition of 10 mol % GM1 only slightly accelerated protein-induced dehydration at acidic pH (Fig. 7). The surface of totally uncharged membranes (50 mol % EPC and 50 mol % PE) is dehydrated as well (Fig. 9). Electrostatic attraction or repulsion seems to be of minor importance because neither GM1 bearing one negative charge per molecule nor the charged phosphatidylserine (20 mol %) was able to inhibit or promote the changes in

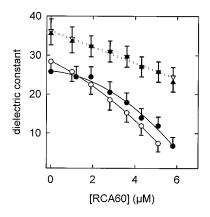


FIGURE 6 Surface dielectric constant of unilamellar vesicles made from DPhPC (*triangles*) or a lipid mixture (20 mol % PS, 20 mol % ergosterol, 10 mol % EPC, 50 mol % PE; *circles*) after addition of RCA60. Filled triangles or circles indicate the additional incorporation of 10 mol % GM1 into the model bilayers.

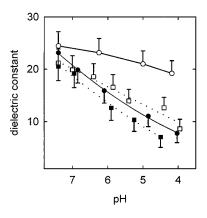


FIGURE 7 Effect of pH on the surface dielectric constant of unilamellar vesicles pretreated with 5.8  $\mu$ M (*circles*) or 3.0  $\mu$ M (*squares*) RCA60. The vesicles were made from DPhPC (*circles*) or a lipid mixture (20 mol % PS, 20 mol % ergosterol, 10 mol % EPC, 50 mol % PE; *squares*). Filled squares or circles indicate the additional incorporation of 10 mol % GM1 into the model bilayers.

surface hydrophobicity (Figs. 6, 7, 9). Furthermore, it is not a special kind of lipid that is required for the interaction with the proteins. For example, substitution of PE by ergosterol did not vary the dehydrating effect of RCA120 (Fig. 9). In agreement with earlier reported results (Hoekstra and Düzgünes, 1986) a modulating action of RCA120 on Ca<sup>2+</sup>-lipid interactions was monitored (Fig. 10). Only small amounts of free Ca<sup>2+</sup> were needed (Hoekstra and Düzgünes, 1986) to shift ε below 12 where fusion is possible (Ohki and Arnold, 1990; Köhler et al., 1997).

#### DISCUSSION

In the present work the interactions of several water-soluble ribosome-inactivating proteins with model bilayers were studied by monitoring the transmembrane water flow and the hydrophobicity of the membrane surface. The four-chain (RCA120 and MLI) and the two-chain (RCA60, MLIII) RIPs (Citores et al., 1993) as well as the subchains

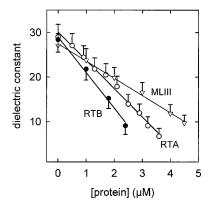


FIGURE 8 Surface dielectric constant of unilamellar vesicles made from 50 mol % EPC and 50 mol % PE after addition of MLIII (*triangles*), RTA (*open circles*), or RTB (*filled circles*), pH was 7.4.

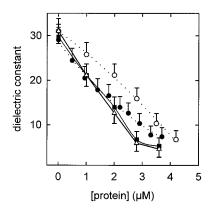


FIGURE 9 Surface dielectric constant of unilamellar vesicles after addition of MLI (dashed line) or RCA120 (solid line). The vesicles were made from 50 mol % EPC and 50 mol % PE (filled squares and circles) or from 50 mol % PC and 50 mol % ergosterol (triangles), or from a lipid mixture (20 mol % PS, 20 mol % ergosterol, 10 mol % EPC, 50 mol % PE; open circles).

RTA and RTB were found to interact with model membranes in a lipid-dependent manner.

By adsorbing to the membrane surface, all lectins decreased the hydraulic conductivity of the bilayer. Assuming that packing defects are introduced by the partitioning of the RIPs into the bilayer, the opposite effect was expected (Needham et al., 1988). At least for small peptides binding to the bilayer, it was found that their distribution is mirrored by water (Jacobs and White, 1989). According to the solubility-diffusion model for water permeation, an enhanced water concentration in the bilayer tends to increase the water permeability (Paula et al., 1996, 1998). The most plausible explanation for the diverging effects of model peptides and lectins is that receptor-mediated lectin adsorption to the membrane surface causes a reduction in diffusion pathways. It is suggested that the RIPs occupy points of water entry into bilayers at the interface. Very recently this case was discussed for ethanol that also decreases  $P_{\rm f}$ , al-

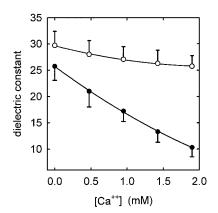


FIGURE 10 Effect of Ca<sup>2+</sup> on the surface dielectric constant of unilamellar vesicles (20 mol % PS, 20 mol % ergosterol, 10 mol % EPC, 50 mol % PE) incubated with 0.7  $\mu$ M RCA120 (filled circles). The dielectric constant of untreated vesicles (open circles) was nearly constant. The buffer solutions (pH 7.4) contained 1 mM EDTA.

though it enhances the water content within bilayers (Huster et al., 1997). Extensive patches of bound lectin coexist with occasional areas that are apparently devoid of glycolipid receptor (Peters et al., 1984b). Because of the high affinity (Grant and Peters, 1984) of RCA60 to GM1 (association constant =  $2.2 \cdot 10^6 \,\mathrm{M}^{-1}$ ), all available receptor molecules may be assumed to be occupied. The clusters formed (Peters et al., 1984a) are, most probable, markedly less permeable to water than the rest of the membrane (Fig. 11). Because the fluorescent dye (DPE) is excluded from the clusters, only a moderate decrease of  $\varepsilon$  was found under these conditions. From the GM1 concentration (10 mol %) the clusters are expected to occupy at least 10% of the membrane area. Accordingly,  $P_{\rm f}$  should be dropped by 10% also. This assumption was confirmed experimentally (Fig. 1). However, the effects induced by MLI and MLIII are larger (compare Fig. 3). Nevertheless, because of its bulkiness, the lectin may act as an additional barrier for water diffusion in an area that is two or three times as large as the area occupied by the receptor.

When the RCA60-lipid complexes are exposed to acidic pH, the protein bound to GM1-liposomes becomes associated with the phosphatidylcholine bilayer (Utsumi et al., 1987); it penetrates deeply into the model membrane (Ramalingam et al., 1994). In this case the protein probably induces a reduction in the mobility of the aliphatic chains (Hincha et al., 1993) that also may tend to decrease the membrane hydraulic conductivity. However, the rate-limiting step of water transport is the permeation through the dense part of the lipid tails, where the resistance is the highest (Marrink and Berendsen, 1994). Therefore, it may be suggested that the lectins induce an increase in lipid packing density in this region, which in turn is expected to reduce water permeation rates (Huster et al., 1997). Indirect support for this hypothesis comes from the observation that

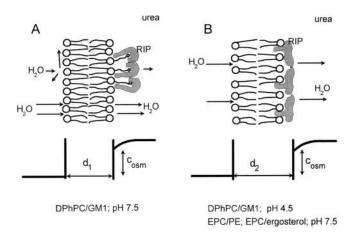


FIGURE 11 Mechanism for the decrease in water permeation. (*A*) Clusters of RIPs bound to the GM1 receptor make a portion of the membrane markedly less permeable to water than normal. Probably, the lectin occupies water diffusion pathways. (*B*) A complete protein layer that adsorbs to the membrane increases the thickness (d) of the osmotic barrier. Although the urea concentration difference remains unchanged, the osmotic gradient ( $C_{\rm osm}/{\rm d}$ ) is decreased.

the dielectric constant of the membrane surface is decreased. The corresponding increase in surface hydrophobicity correlates with an increase in interfacial tension of the membrane (Ohki and Arnold, 1990; Ohki and Zschornig, 1993). The latter then is predicted to be accompanied by a decrease of the water permeability (Evans and Needham, 1986), which was observed in the experiment.

The impact of changes in microviscosity or tension is difficult to assess from our experiments, but another mechanism seems to be more important for the alterations of the hydraulic conductivity. From the sharp decrease of the dielectric constant observed on the membrane surface, it is likely that the surface of the planar membrane is completely covered by the lectin. The size of the osmotic barrier increases (Fig. 11). At the interface, the solubility of the osmolute is changed. Consequently, at a constant urea concentration difference, the osmotic gradient is diminished. As a result, both in the case of DPhPC/GM1 mixtures at acidic pH and mixtures of natural lipids at pH 7.5, the transmembrane water flux is reduced.

The impact of electrostatics on the protein-lipid interactions seems to be rather small because the incorporation of 20 mol % PS did not modify the lectin-induced effects (Fig. 9). Until now it was believed that at physiological pH a receptor is required for RCA60-membrane interactions to occur (Hincha et al., 1993; Utsumi et al., 1987; Ramalingam et al., 1994). This conclusion is based on experiments carried out with PC membranes only. Substituting DPhPC for an EPC/PE mixture ensured that not only RCA60, but the other lectins as well, interacted very efficiently with lipid bilayers not bearing GM1 (Figs. 1, 3, 4, 6–10). Ergosterol was found to be as competent as PE in promoting hydrophobic interactions (Figs. 3 and 9). It is therefore unlikely that protein partitioning requires a distinct species of lipid. Rather, differences in the mechanical membrane properties seem to be involved.

It is the tension that also governs the hydraulic conductivity: the greater the tension, the lower  $P_{\rm f}$  (Bloom et al., 1991; Needham, 1995). For lysolecithin, an increase in bilayer tension was shown to increase its membrane solubility (Zhelev, 1996). The insertion of lysolecithin occurs in two steps. First it accumulates in one of the monolayers that is extended. The resulting increase in tension promotes the formation of monolayer defects. A subsequent collective lipid transport through short-lived monolayer defects then contributes to the apparent lipid transfer rate (Needham and Zhelev, 1995). From these experiments the impact of tension to lysolecithin partitioning into the first monolayer is not evident. It is possible that its intercalation into the first monolayer is hindered by an increase in tension, similar to the partitioning of water. Therefore, this experimental finding (Needham and Zhelev, 1995) does not conflict with our observation that protein adsorption to the membrane surface is promoted by lipids capable of facilitating water partitioning into the bilayer. In fact, the membranes with the lowest permeability for water are poor substrates for protein adsorption. Fully saturated DPhPC bilayers only interact with

the lectins if a specific receptor is present. Membranes from natural lipids, mixtures made from PE/EPC and PE/ergosterol, have a higher initial water permeability and their interaction with the RIPs requires neither GM1 nor acidic pH. Looser packing at the water-lipid interface and a deeper penetration of water into unsaturated bilayers (Huster et al., 1997) is responsible for the differences in water permeation that were found between bilayers made from DPhPC, and EPC/PE or EPC/ergosterol. The fully saturated DPhPC membrane has, as expected, the lowest water permeability. Following the partitioning of water, protein adsorption is governed by membrane tension, too. Our experimental results are in agreement with the prediction (Gawrisch et al., 1995) that a change in lipid-lipid interaction in the hydrocarbon core of the membrane, for example as a result of the introduction of polyunsaturated fatty acids, will alter lipidsolvent and lipid-peptide interactions at the interface.

This project was supported by the Deutsche Forschungsgemeinschaft (Po 533/1-1 and 436 RUS 113/60).

#### REFERENCES

- Agapov, I. I., A. G. Tonevitsky, A. T. Shamshiev, E. Pohl, P. Pohl, R. A. Palmer, and M. P. Kirpichnikov. 1997. The role of structural domains in RIP II toxin model membrane binding. *FEBS Lett.* 402:91–93.
- Amman, D. 1986. Ion-selective microelectrodes. Principles, Design and Application. Springer, Berlin.
- Antonenko, Y. N., and A. A. Bulychev. 1991. Measurements of local pH changes near bilayer lipid membrane by means of a pH microelectrode and a protonophore-dependent membrane potential—comparison of the methods. *Biochim. Biophys. Acta.* 1070:279–282.
- Barbieri, L., M. G. Battelli, and F. Stirpe. 1993. Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta*. 1154:237–282.
- Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes—a perspective. *Q. Rev. Biophys.* 24:293–397.
- Brinkmann, U., and I. Pastan. 1994. Immunotoxins against cancer. Biochim. Biophys. Acta. 1198:27–45.
- Citores, L., J. M. Ferreras, R. Iglesias, M. L. Carbajales, F. J. Arias, P. Jimenez, M. A. Rojo, and T. Girbes. 1993. Molecular mechanism of inhibition of mammalian protein synthesis by some four-chain agglutinins. Proposal of an extended classification of plant ribosome-inactivating proteins (rRNA N-glycosidases). FEBS Lett. 329:59–62.
- Eifler, R., K. Pfueller, W. Gockeritz, and U. Pfueller. 1994. Improved procedures for the isolation of mistletoe lectins and their subunits: lectin patterns of the European mistletoe. *In* Lectins 9: Biology, Biochemistry, Clinical Biochemistry. J. Basu, M. Kundu, and P. Chakrabati, editors. Wiley, New Dehli. 144–151.
- Endo, Y., and K. Tsurugi. 1987. RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J. Biol. Chem. 262:8128–8130.
- Evans, E., and D. Needham. 1986. Giant vesicle bilayers composed of mixtures of lipids, cholesterol and polypeptides. Thermomechanical and (mutual) adherence properties. Faraday. Discuss. Chem. Soc. 267–280.
- Fettiplace, R. 1978. The influence of the lipid on the water permeability of artificial membranes. *Biochim. Biophys. Acta.* 513:1–10.
- Finkelstein, A. 1987. Water Movement through Lipid Bilayers, Pores, and Plasma Membranes. Wiley & Sons, New York.
- Gawrisch, K., J. A. Barry, L. L. Holte, T. Sinnwell, L. D. Bergelson, and J. A. Ferretti. 1995. Role of interactions at the lipid water interface for domain formation. *Mol. Membrane Biol.* 12:83–88.
- Grant, C. W., and M. W. Peters. 1984. Lectin-membrane interactions. Information from model systems. *Biochim. Biophys. Acta*. 779:403–422.

- Haines, T. H., and L. S. Liebovitch. 1995. A molecular mechanism for the transport of water across phospholipid bilayers. *In Permeability and Stability of Lipid Bilayers*. E. A. Disalvo and S. A. Simon, editors. CRC Press, Boca Raton. 123–136.
- Hincha, D. K., I. Bakaltcheva, and J. M. Schmitt. 1993. Galactose-specific lectins protect isolated thylakoids against freeze-thaw damage. *Plant Physiol*. 103:59-65.
- Hoekstra, D., and N. Düzgünes. 1986. *Ricinus communis* agglutininmediated agglutination and fusion of glycolipid-containing phospholipid vesicles: effect of carbohydrate headgroup size, calcium ions, and spermine. *Biochemistry*. 25:1321–1330.
- Hoekstra, D., and J. Wilschut. 1989. Membrane fusion of artificial and biological membranes: role of local membrane dehydration. *In* Water Transport in Biological Membranes. G. Benga, editor. CRC Press, Boca Raton. 143–176.
- Huster, D., A. J. Jin, K. Arnold, and K. Gawrisch. 1997. Water permeability of polyunsaturated lipid membranes measured by O<sup>17</sup> NMR. *Biophys. J.* 73:855–864.
- Jacobs, R. E., and S. H. White. 1989. The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices. *Biochemistry*. 28:3421–3437.
- Kayser, G., E. Goormaghtigh, M. Vandenbranden, and J. M. Ruysschaert. 1981. Ricinus communis toxin interacts specifically with GM 1 ganglioside incorporated into planar lipid bilayers. FEBS Lett. 127:207–210.
- Kimura, Y., and A. Ikegami. 1985. Local dielectric properties around polar region of lipid bilayer membranes. *J. Membr. Biol.* 85:225–231.
- Köhler, G., U. Hering, O. Zschornig, and K. Arnold. 1997. Annexin V interaction with phosphatidylserine-containing vesicles at low and neutral pH. *Biochemistry*. 36:8189–8194.
- Ladokhin, A. S., M. E. Selsted, and S. H. White. 1997. Bilayer interactions of indolicidin, a small antimicrobial peptide rich in tryptophan, proline, and basic amino acids. *Biophys. J.* 72:794–805.
- Lee, R. T., H. J. Gabius, and Y. C. Lee. 1994. The sugar-combining area of the galactose-specific toxic lectin of mistletoe extends beyond the terminal sugar residue—comparison with a homologous toxic lectin, ricin. *Carbohydr. Res.* 254:269–276.
- Lord, J. M., L. M. Roberts, and J. D. Robertus. 1994. Ricin: structure, mode of action, and some current applications. FASEB J. 8:201–208.
- MacDonald, R. C., R. I. MacDonald, B. Ph. M. Menco, K. Takeshita, N. K. Subbarao, and L. R. Hu. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* 1061: 297–303.
- Marrink, S. J., and H. J. C. Berendsen. 1994. Simulation of water transport through a lipid membrane. *J. Phys. Chem.* 98:4155–4168.
- McIntosh, T. J., and S. A. Simon. 1994. Long and shortrange interactions between phospholipid/ganglioside GM1 bilayers. *Biochemistry*. 33: 10477–10486.
- Montich, G. G., C. Montecucco, E. Papini, and D. Marsh. 1995. Insertion of diphtheria toxin in lipid bilayers studied by spin label. *Biochemistry*. 34:11561–11567.
- Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Wescott. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67:534–535.
- Needham, D. 1995. Cohesion and permeability of lipid bilayer vesicles. *In* Permeability and Stability of Lipid Bilayers. E. A. Disalvo and S. A. Simon, editors. CRC Press, Boca Raton. 49–76.
- Needham, D., T. J. McIntosh, and E. Evans. 1988. Thermomechanical and transition properties of dimyristoylphosphatidylcholine/cholesterol bilayers. *Biochemistry*. 27:4668–4673.
- Needham, D., and D. V. Zhelev. 1995. Lysolipid exchange with lipid vesicle membranes. *Ann. Biomed. Eng.* 23:287–298.
- Ohki, S., and K. Arnold. 1990. Surface dielectric constant, surface hydrophobicity and membrane fusion. J. Membr. Biol. 114:195–203.
- Ohki, S., and O. Zschornig. 1993. Ion-induced fusion of phosphatidic acid vesicles and correlation between surface hydrophobicity and membrane fusion. *Chem. Phys. Lipids*. 65:193–204.
- Olsnes, S., F. Stirpe, K. Sandvig, and A. Pihl. 1982. Isolation and characterization of viscumin, a toxic lectin from *Viscum album L.* (mistletoe). J. Biol. Chem. 257:13263–13270.

- Paula, S., A. G. Volkov, and D. W. Deamer. 1998. Permeation of halide anions through phospholipid bilayers occurs by the solubility-diffusion mechanism. *Biophys. J.* 74:319–327.
- Paula, S., A. G. Volkov, A. N. Vanhoek, T. H. Haines, and D. W. Deamer. 1996. Permeation of protons, potassium ions, and small polar molecules through phospholipid bilayers as a function of membrane thickness. *Biophys. J.* 70:339–348.
- Peters, M. W., K. R. Barber, and C. W. Grant. 1984a. Lateral distribution of gangliosides in bilayer membranes: lipid and ionic effects. *J. Neurosci. Res.* 12:343–353.
- Peters, M. W., I. E. Mehlhorn, K. R. Barber, and C. W. Grant. 1984b. Evidence of a distribution difference between two gangliosides in bilayer membranes. *Biochim. Biophys. Acta.* 778:419–428.
- Pohl, P., Y. N. Antonenko, V. Y. Evtodienko, E. E. Pohl, S. M. Saparov, I. I. Agapov, and A. G. Tonevitsky. 1998. Membrane fusion mediated by ricin and viscumin. *Biochim. Biophys. Acta*. 1371:11–16.
- Pohl, P., Y. N. Antonenko, and E. H. Rosenfeld. 1993. Effect of ultrasound on the pH profiles in the unstirred layers near planar bilayer lipid membranes measured by microelectrodes. *Biochim. Biophys. Acta*. 1152:155–160.
- Pohl, P., S. M. Saparov, and Y. N. Antonenko. 1997. The effect of a transmembrane osmotic flux on the ion concentration distribution in the immediate membrane vicinity measured by microelectrodes. *Biophys. J.* 72:1711–1718.
- Ramalingam, T. S., P. K. Das, and S. K. Podder. 1994. Ricin-membrane interaction: membrane penetration depth by fluorescence quenching and resonance energy transfer. *Biochemistry*. 33:12247–12254.
- Raso, V. 1994. Review: immunotargeting intracellular compartments. Anal. Biochem. 222:297–304.

- Saltvedt, E. 1976. Structure and toxicity of pure ricinus agglutinin. Biochim. Biophys. Acta. 451:536.
- Sandvig, K., and B. van Deurs. 1994. Endocytosis and intracellular sorting of ricin and shiga toxin. *FEBS Lett.* 346:99–102.
- Sandvig, K., and B. van Deurs. 1996. Endocytosis, intracellular transport, and cytotoxic action of shiga toxin and ricin. *Physiol. Rev.* 76:949–966.
- Subczynski, W. K., A. Wisniewska, J. J. Yin, J. S. Hyde, and A. Kusumi. 1994. Hydrophobic barriers of lipid bilayer membranes formed by reduction of water penetration by alkyl chain unsaturation and cholesterol. *Biochemistry*. 33:7670–7681.
- Tonevitsky, A. G., O. S. Zhukova, N. V. Mirimanova, V. G. Omylyanenko, N. V. Timofeeva, and L. D. Bergelson. 1990. Effect of gangliosides on binding, internalization and cytotoxic activity of ricin. *FEBS Lett.* 264: 249–252.
- Utsumi, T., Y. Aizono, and G. Funatsu. 1984. Interaction of ricin and its constituent polypeptides with dipalmitoylphosphatidylcholine vesicles. *Biochim. Biophys. Acta.* 772:202–208.
- Utsumi, T., Y. Aizono, and G. Funatsu. 1987. Receptor-mediated interaction of ricin with the lipid bilayer of ganglioside GM1-liposomes. *FEBS Lett.* 216:99–103.
- Utsumi, T., A. Ide, and G. Funatsu. 1989. Ricin A-chain induces fusion of small unilamellar vesicles at neutral pH. *FEBS Lett.* 242:255–258.
- Waggoner, A. S., and L. Stryer. 1970. Fluorescent probes of biological membranes. Proc. Natl. Acad. Sci. U.S.A. 67:579–589.
- Wellner, R. B., J. F. Hewetson, and M. A. Poli. 1995. Ricin: mechanism of action, detection, and intoxication. J. Toxicol. 14:483–522.
- Zhelev, D. V. 1996. Exchange of monooleoylphosphatidylcholine with single egg phosphatidylcholine vesicle membranes. *Biophys. J.* 71: 257–273.