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# Disassembly of viral membranes by complement independent of channel formation

(membrane attack complex/nystatin/melittin/hemolysis/bacteriolysis)

ALFRED F. ESSER*, RICHARD M. BARTHOLOMEW*, FRED C. JENSEN†, AND HANS J. MÜLLER-EBERHARD*

Departments of *Molecular Immunology and †Cellular and Developmental Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

*Contributed by Hans J. Müller-Eberhard, August 13, 1979*

**ABSTRACT** **We have compared the effects of the complement membrane attack complex (MAC), nystatin, and melittin on the envelope of murine leukemia viruses to determine if channel formation alone is sufficient to cause membranolysis. Nystatin is a channel former and mellitin is not, although both are hemolytic. Whereas MAC and melittin disintegrated the viral membrane, nystatin had no effect on morphology, integrity, and infectivity of the virus. Incorporation of the antibiotic into the viral membranes was demonstrated by measurements of the characteristic fluorescence of nystatin in membranes and the dose-dependent increase in viral density after uptake of the antibiotic. The density of nystatin was measured to be 1.26–1.27 g/cm$^3$. Proof for the formation of functional nystatin channels was obtained by light scattering measurements. Exposure of untreated virus to hypotonic conditions increased viral light scattering because of osmotic swelling but otherwise had no effect on the integrity of the virus. Nystatin channel formation abolished the light scattering change, showing that the antibiotic had impaired the viral permeability barrier. We interpret these results to indicate that virolysis by MAC is not caused by channel formation and, conversely, in the absence of colloid-osmotic effects, channel formation by itself is not sufficient to disassemble a viral membrane.**

Complement in conjunction with specific antibody is capable of lysing lipid-enveloped viruses (reviewed in ref. 1). Clear-cut demonstration of immune virolysis was provided by Oroszlan and Gilden (2), who measured the release of radiolabeled RNA from mouse leukemia viruses (retroviruses). Later experiments with retroviruses indicated that human complement alone, without specific antibody, could lyse retroviruses (3–5). The mechanism of lysis of enveloped viruses was assumed to be similar or identical to complement-mediated lysis of mammalian cells, a more extensively studied and better understood reaction.

In complement-dependent cytolysis, five complement proteins—C5b, C6, C7, C8, and C9—become physically attached to the outer membrane of a cell. Without further enzymatic modification, these proteins form a multimolecular complex, the membrane attack complex (MAC), that lyses cells by purely physicochemical means (reviewed in ref. 6). Loss of the natural impermeability of the cell membrane to small solutes leads to colloid-osmotic swelling of the cell, rupture, and release of internal proteins. Mayer (7, 8) theorized that the MAC forms hydrophilic protein channels; this action is reminiscent of the channel-forming (9) and hemolytic (10) properties of the antibiotics nystatin and amphotericin B.

In contrast to Mayer's channel theory, we recently proposed (11, 12) that the MAC is membranolytic not because of channel formation, but because of its strong phospholipid binding capacity, through which it is enabled to reorient the lipid bilayer structure of membranes.

Examining our hypothesis, we have compared the effects of nystatin, melittin, and the MAC on the envelope of murine leukemia virus (MuLV) to determine if channel formation alone is sufficient to cause membranolysis. Nystatin is a channel former and melittin is not, although it is membranolytic. Our results show that whereas the MAC and melittin are virolytic, nystatin has no effect on the morphology and infectivity of the virus.

## MATERIALS AND METHODS

**Materials.** Nystatin, amphotericin B, and melittin were purchased from Sigma. Rauscher MuLV, prepared by Electro-Nucleonics (Bethesda, MD), was supplied by the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute. Freshly harvested Moloney, xenotropic (ATS-124) murine, and (BN-p 454-9) rat leukemia viruses were propagated as described (6). Fresh human serum (Community Blood and Plasma, San Diego, CA) frozen at −70°C was used as a source of complement.

Uranyl formate was prepared according to published procedures (13); all other chemicals were of the best grade commercially available. Buffer osmolarities were measured with a freezing-point depression osmometer (Osmette, Precision Systems, Sudbury, MA).

**Virolysis Assays.** Virus samples (100 μg or approximately $10^{10}$ virions in 0.1 ml) treated with complement, nystatin, melittin, or hypotonic buffer were layered on top of linear sucrose density gradients [20–65% sucrose in Veronal-buffered saline (VBS; 4.95 mM sodium barbital/145 mM NaCl, pH 7.3)] and centrifuged at 4°C for 1.5 hr in an SW 50.1 rotor (Beckman) at 280,000 × $g_{max}$. The gradients were divided into 11 fractions by using a Buchler, model Densi-Flow IIC, fractionator. All fractions were made 0.5% in Triton X-100 to disintegrate the virus, and in each fraction the concentrations of internal protein p30 and envelope glycoprotein gp70 were measured by radioimmunoassays (RIAs) as described by Izui *et al.* (14). Reverse transcriptase (RNA-directed DNA polymerase) was measured by determining the incorporation of [$^3$H]dTMP into $(dT)_{12-18}$-$(rA)_n$ as described by Welsh *et al.* (3).

**Infectivity Assays.** Monolayers (24 hr old) of secondary passage NIH Swiss mouse embryo fibroblasts were infected with 1 ml of nystatin-treated or control virus (1 × $10^8$ plaque-forming units/ml) in the presence of Polybrene at 4 μg/ml. Treatment of virus with different amounts of nystatin dissolved in a constant volume of dimethyl sulfoxide ($Me_2SO$) lasted 30 min; control virus samples were treated with an equal volume of $Me_2SO$ or with tissue culture medium. The cultures were

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Abbreviations: MAC, membrane attack complex of complement; VBS, Veronal-buffered saline (4.95 mM sodium barbital/145 mM NaCl, pH 7.3); MuLV, murine leukemia virus; RIA, radioimmunoassay; $Me_2SO$, dimethyl sulfoxide.

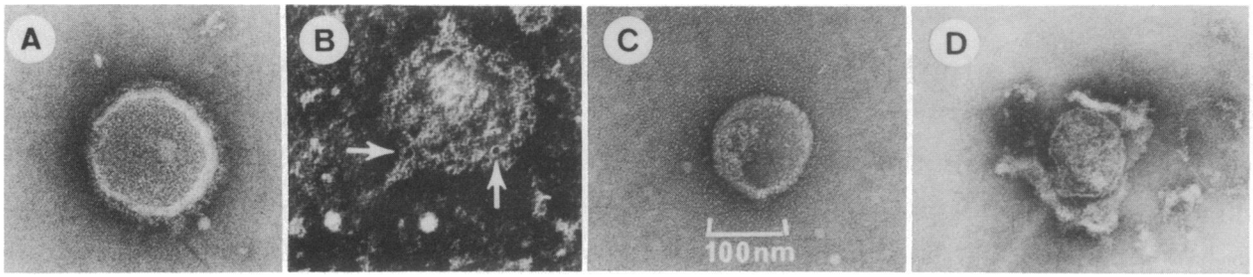


FIG. 1. Electron microscopic appearance of freshly prepared xenotropic (ATS-124) MuLV negatively stained with uranyl formate is shown in *A*. Rat leukemia virus (BN-p454-9) after treatment with human complement is shown in *B* and Rauscher MuLV treated with 22.5 μg of nystatin or 50 μg of melittin in *C* and *D*, respectively. Note the appearance of typical complement lesions in *B* and the "peeling off" of the envelope by melittin in *D*.

refed after 24 and 96 hr and at day 7 the supernatants, after addition of Triton X-100 to 0.5%, were assayed for reverse transcriptase activity.

**Electron Microscopy.** Virus preparations were negatively stained with either 1% uranyl formate dissolved in 0.15 M ammonium formate (pH 3.8) or with 1.3% sodium phosphotungstate dissolved in 10 mM sodium phosphate (pH 7.2) and examined and photographed in a Hitachi model 12 electron microscope at 75 kV. Some virus samples were fixed with 2.0% (vol/vol) glutaraldehyde for 10 min prior to staining. Bacitracin was used as a grid-wetting agent (15).

**Density Measurements.** Nystatin was dissolved in dimethyl formamide (spectrophotometric grade, Mallinckrodt) or $Me_2SO$ (spectrophotometric grade, Aldrich) and the densities of different concentrated solutions were measured at 25.30°C with a Paar DMA 60/601 digital density measuring system (Mettler). According to the equation

$$(1 - \bar{v}\rho_2) = (\rho - \rho)_2/c$$

[in which $c$ is the concentration (g/cm$^3$) of the dissolved material, $\rho$ the density of the solution, and $\rho_2$ the density of the solvent], $\bar{v}$ can be determined from the slope of the straight lines in $\Delta\rho/c$ diagrams. The densities at 25.30°C for the particular solvent lots used here were measured to be 0.9449 g/cm$^3$ for dimethyl formamide and 1.0952 g/cm$^3$ for $Me_2SO$.

**Fluorescence Spectroscopy.** Nystatin fluorescence was recorded with an Aminco–Bowman spectrofluorometer with ratio attachment and 2-mm (11-nm bandpass) excitation slits and 3-mm (17.5-nm bandpass) emission slits.

**Assay of Osmotic Permeability.** Osmotic permeabilities of viruses were measured by light scattering as described by Bittman *et al.* (16) with the modification that steady-state 90° light scattering was monitored in the fluorometer instead of forward light scattering in a photometer as described originally. In brief, 25 μg of virus was added to 1 ml of VBS and light scattering was normalized. Then the solution was diluted 1:3 by addition of either 2 ml of VBS or 2 ml of $H_2O$, and the resulting light scattering was measured again. Effects of nystatin were assessed by adding the antibiotic dissolved in $Me_2SO$ to the viral suspension prior to the dilution step.

## RESULTS

Electron microscopic examination of leukemia virus preparations revealed approximately spherical particles of about 120- to 150-nm diameter (Fig. 1*A*) that resembled those published earlier by Nermut *et al.* (17) and Luftig and Kilham (18). Incubation of such viruses with whole human serum as a source of complement resulted in complete disintegration of the virus. Immediate fixation with glutaraldehyde after addition of serum sometimes allowed observation of the disassembly process (Fig. 1*B*). In contrast, incubation of the virus with nystatin in concentrations as high as 45 μg/10$^{10}$ virions did not produce obvious morphologic changes (Fig. 1*C*). Similarly to complement, however, melittin also caused destruction of the viral membrane (Fig. 1*D*).

That nystatin was indeed incorporated in functional form into the viral membrane was shown in three different ways. First, incubation of the virus with the antibiotic changed the position of the virus after isopycnic centrifugation (Fig. 2 *inset*). The isopycnic position was directly dependent on the nystatin concentration (see below). Second, when the gradients were fractionated and analyzed for nystatin by fluorescence spectroscopy, we observed (Fig. 2) a strong virus-associated fluorescence with emission and excitation spectra characteristic of membrane-embedded nystatin (19, 20). Incorporation of nystatin into the virion produced an approximate 20-fold increase in fluorescence intensity. The fluorescence yield was similarly increased when liposomes prepared from lecithin and cholesterol were added to an aqueous solution of nystatin (data not shown). Third, when freshly harvested leukemia virus was exposed to hypotonic (96 mOsm) buffer a small increase in light

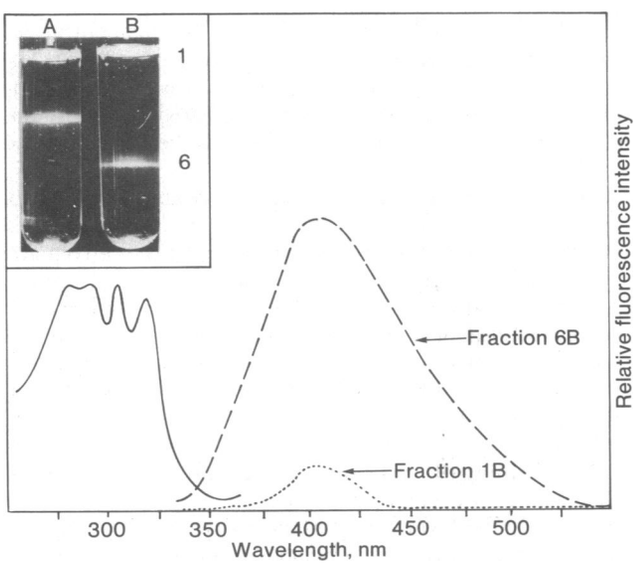


FIG. 2. Fluorescence excitation spectrum (—) (emission λ = 400 nm) and emission spectra (- - -) (excitation λ = 306 nm) of 45 μg of nystatin incorporated into 100 μg of Rauscher MuLV and purified by isopycnic centrifugation. (*Inset*) Photographs of 100 μg of untreated Rauscher MuLV (*A*) and 100 μg of Rauscher MuLV incubated in darkness with 45 μg of nystatin for 30 min (*B*) and centrifuged for 1.5 hr at 280,000 × $g_{max}$ in linear sucrose density gradients (20–65%). Fluorescence of fraction 1 of gradient B was recorded at a 30-fold higher sensitivity compared to fraction 6B.

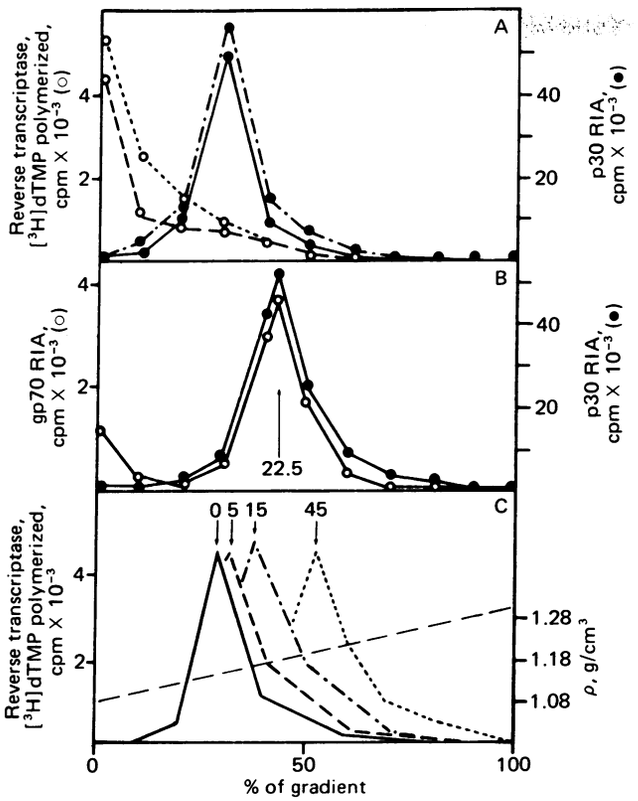


FIG. 3. (*A*) Isopycnic sedimentation behavior of Rauscher MuLV untreated (——), or after 30-min exposure to hypotonic (70 mOsm; VBS diluted 1:4 with water) buffer (-·-), or after incubation (30 min, 20°C) with an equal volume of human serum (- - -) or 50 μg of melittin (– –). The gradients were analyzed for reverse transcriptase activity (○) or by RIA specific for p30 (●). (*B*) Isopycnic sedimentation of 100 μg of Rauscher MuLV incubated in darkness (30 min, 20°C) with 22.5 μg of nystatin. Gradients were analyzed by RIAs specific for gp70 (○) or p30 (●). (*C*) Dose-dependent shift of viral density after incubation of 100 μg of Rauscher MuLV with increasing amounts of nystatin. The numbers above each peak correspond to the amount (in μg) of nystatin.

scattering was observed because of osmotic swelling of the virus. Incubation with nystatin prevented light scattering changes, indicating that this agent had perforated the viral permeability barrier (Table 1). It should be noted that the light scattering changes were observed only on freshly harvested virus; no swelling could be observed after freezing and thawing the virus.

Possible virolysis by hypotonic buffer and by nystatin was then assessed by measuring the release of internal viral components. Intact Rauscher MuLV equilibrates at a density of 1.14 g/cm$^3$. As shown in Fig. 3*A*, incubation of the virus in hypotonic (70 mOsm) buffer for 30 min at 37°C did not affect the integrity of the virion, because no release of p30 was detected, and the particles banded at the same position. Incubation with an equal volume of whole human serum or with 50 μg of melittin resulted in the complete release of reverse transcriptase. However, treatment of the virus with nystatin did not result in release of p30 or of significant amounts of gp70; both proteins still cosedimented at the same position (Fig. 3*B*). Additionally, nystatin did not enlarge the small amount of gp70 that is always released during sucrose density gradient centrifugation. Notably, the nystatin-treated virus samples shifted to higher isopycnic densities in a dose-dependent fashion (Fig. 3*C*). This increased viral density resulted from uptake of the antibiotic. Partial specific volumes ($\bar{v}$) of 0.794 in $Me_2SO$ and of 0.786 in

Table 1. Prevention of osmotic swelling of Moloney MuLV by nystatin

| Virus (25 μg/3 ml) | Tonicity, mOsm | Addition | Light scattering,* recorder deflection, mm |
|---|---|---|---|
| Freshly harvested | 290 | None | 68 ± 8 |
| Freshly harvested | 96 | None | 90 ± 8 |
| Freshly harvested | 290 | 5 μl of $Me_2SO$ | 65 ± 6 |
| Freshly harvested | 96 | 5 μl of $Me_2SO$ | 88 ± 8 |
| Freshly harvested | 290 | 20 μg of nystatin in 5 μl of $Me_2SO$ | 65 ± 6 |
| Freshly harvested | 96 | 20 μg of nystatin in 5 μl of $Me_2SO$ | 68 ± 7 |
| Frozen and thawed once | 96 | None | 70 |

* The 90° light scattering was measured at 500 nm; excitation and emission bandwith was 11 nm; sample temperature, 30°C. Data are given ± SEM.

dimethyl formamide were calculated from density measurements of different nystatin solutions (Fig. 4), indicating a density of 1.26–1.27 g/cm$^3$ for the solvent-free substance. This value is in excellent agreement with the observed change in viral density from 1.14 to 1.18 g/cm$^3$ after addition of 45 μg of nystatin to 100 μg of virus (Fig. 3*C*).

Incorporation of nystatin had no effect on the infectivity of the virus. Treatment of $10^8$ virions with amounts of nystatin as

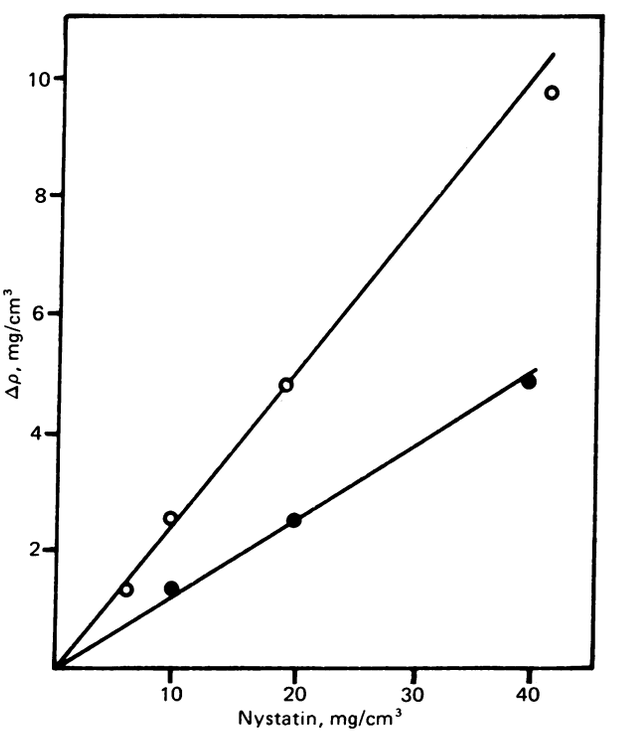


FIG. 4. Δρ/*c* diagram for nystatin dissolved in dimethyl formamide (○) or $Me_2SO$ (●).

Table 2. Effect of nystatin on infectivity of Rauscher MuLV

| Addition to infecting Rauscher MuLV | Time after infection, days | Reverse transcriptase activity, [$^3$H]dTMP polymerized, cpm |
|---|---|---|
| 5 $\mu$l of medium | 0 | 500 |
| 5 $\mu$l of medium | 7 | 49,500 |
| 5 $\mu$l of $Me_2SO$ | 7 | 43,000 |
| 0.04 $\mu$g of nystatin in 5 $\mu$l of $Me_2SO$ | 7 | 60,000 |
| 0.2 $\mu$g of nystatin in 5 $\mu$l of $Me_2SO$ | 7 | 34,500 |
| 5.0 $\mu$g of nystatin in 5 $\mu$l of $Me_2SO$ | 7 | 40,700 |

Infecting Rauscher MuLV was at $1 \times 10^8$ plaque-forming units/ml in all cases.

high as 5 $\mu$g did not prevent subsequent replication (Table 2).

## DISCUSSION

Our results are clear evidence that the morphology, integrity, and infectivity of an enveloped virus such as Rauscher MuLV are unaffected by treatment with a channel-forming agent such as nystatin. The virus appears intact because the envelope (gp 70) and internal (p30, reverse transcriptase) proteins band in a single peak at a density different from that of the free proteins. The Rauscher MuLV preparation we used had already lost most of its loosely attached gp70 during purification, as indicated by the absence of surface knobs otherwise visible by electron microscopy of fresh preparations. That this more tightly bound portion of gp70 was not liberated is taken as evidence that nystatin does not perturb the envelope strongly enough to be disruptive. Channel formation by nystatin requires the presence of cholesterol in the membrane (21, 22) There is ample evidence that viruses budding from the cell membrane take lipids from the host cell, and, in the case of retroviruses, the concentration of cholesterol in the viral membrane appears to be enriched compared to the host membrane (23).

That nystatin has entered the membrane is clearly evident from the results of the fluorescence experiments and the nystatin-dependent shifts in density after isopycnic centrifugation. In addition, the light scattering experiments show that the incorporated agent has also formed a functional channel, thereby destroying the viral permeability barrier. This result is in agreement with numerous studies on the behavior of nystatin in model and natural membranes (reviewed in refs. 21 and 22) and the studies of Bittman and coworkers (16) in particular. These investigators reported that filipin, another polyene antibiotic and channel former, also prevented osmotic swelling of vesicular stomatitis virus. In addition, they found that it did not dissociate membrane components from vesicular stomatitis, influenza, and Rauscher leukemia virions, although it formed typical ultrastructural lesions in the viral membrane (24). The fact that such channel formation did not result in virolysis, in contrast to the hemolysis encountered with erythrocytes, can be explained by the absence of a sufficient osmotic gradient across the viral membrane. Exposure to hypotonic (70 mOsm) conditions did not cause release of internal proteins or significant amounts of envelope proteins, nor did it shift the density of the virus (Fig. 3A). Other investigators (25) have reported that osmotic shock treatment results in the selective release of the envelope protein without destruction of the core. However, in this case the virions were first exposed to a hypertonic sucrose solution (36% or 2100 mOsm) and then diluted with 0.1 M NaCl buffer to 15% sucrose (490 mOsm). Thus, the two experiments are not directly comparable.

Cells such as erythrocytes are very sensitive to hypotonic conditions because of relatively high concentrations of internal solutes and because of the permeability barrier of their limiting membrane. Leukemia viruses need not compartmentalize low molecular weight metabolites, and their predominant internal constituents are two molecules of single-stranded RNA. Thus, there may not be a large osmotic gradient across the viral membrane. Only 1 $\mu$g of nystatin is sufficient to lyse $10^8$ erythrocytes within 30 min at 37°C (10). The ratio between the surface areas of Rauscher MuLV and erythrocytes is about 0.0007 and therefore 0.07 $\mu$g of nystatin should be sufficient to achieve the same membrane concentration in $10^{10}$ virions. The highest nystatin concentration tested here was 64-fold higher than that calculated above.

In contrast to the benign influence of nystatin channels on viral membranes, complement and melittin both caused membranolysis. It has been claimed that the MAC forms channels in membranes (7, 8) but that melittin cannot do so because its hydrophobic segment is apparently too short to span a membrane (26). However, both proteins cause leaks in lipid bilayer membranes (27, 28), and changes in conductivity can be recorded across such membranes (26, 29, 30). Whereas polyene antibiotics such as amphotericin or nystatin produce discrete resistance changes of defined magnitude and duration (31), melittin and MAC cause a nondiscrete decrease in electrical resistance (26, 32). Common to both proteins is their strong lipid binding capacity; the $1.7 \times 10^6$ dalton MAC can bind approximately 1400 lecithin molecules (12) and the $3 \times 10^3$ dalton melittin up to 25 (32, 33). Furthermore, spin label studies on ordered lipid bilayers have shown that MAC and melittin can reorient lipids (11, 34).

Considering these accumulated data, we postulate that virolysis by MAC or melittin is not caused by formation of channels in the viral membrane; conversely, in the absence of a colloid-osmotic effect, channel formation by itself is not sufficient to disassemble viral membranes. Similar arguments can be raised with respect to the bactericidal action of complement. Gram-negative bacteria already have natural channels in their outer membrane allowing the passage of molecules smaller than 700 daltons (35). Assembly of the MAC from purified precursors on the bacterial surface nevertheless leads to cell death (36). Again, mere channel formation cannot account for this result. In contrast, sufficient lipid binding capacity, strong enough to compensate for the cooperative interactions between ordered lipid molecules and reorientation of such ordered bilayer lipids into domains more micellar in nature (11), may cause the observed lytic effects.

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