Membrane Permeability to Macromolecules Mediated by the Membrane Attack Complex†

Justine A. Malinski and Gary L. Nelsestuen*
Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
Received May 6, 1988; Revised Manuscript Received July 25, 1988

ABSTRACT: A simple and well-defined system of purified phospholipids and human complement proteins was used to study membrane permeability to macromolecules mediated by the membrane attack complex (MAC) of complement. Large unilamellar vesicles (LUVs) of phosphatidylcholine (PC) or phosphatidylserine (PS) containing trapped macromolecules [bovine pancreatic trypsin inhibitor (BPTI), thrombin, glucose-6-phosphate dehydrogenase (G6PD), and larger molecules] were used to monitor permeability. Membrane permeability to macromolecules was measured by thrombin inhibition by an external inhibitor or by separation of released molecules by gel filtration. Membrane-bound intermediates (C5b-8 or C5b-9_3) were stable for hours, and macromolecular permeability occurred without fragmentation, fusion, or aggregation of the vesicles. Quantitative membrane binding by C5b-7 as well as essentially quantitative release of thrombin was obtained for PS vesicles. MAC binding to PS-LUVs approximated the theoretical Poisson distribution curve for full release of vesicle contents by one complex per vesicle. Reactions with PC-LUVs occurred with some fluid-phase MAC assembly. Therefore, results from experiments with these vesicles were interpreted in a relative manner. However, the values obtained closely corroborated those obtained with PS-LUVs. At low C9/C5b-8 ratios, the size of the lesion was proportional to the C9 content of the MAC. Half-maximum release of BPTI, thrombin, and G6PD, by a single MAC per vesicle, required approximately 3, 5, and 7 C9/C5b-8 (mol/mol), respectively. Larger molecules (≥118-Å diameter) were not released from the vesicles. Release of G6PD (95.4-Å diameter) required 45% of saturating C9. Therefore, it appeared that the last half of the bound C9 molecules did not increase pore size and the pore which released G6PD approached the diameter of the closed circular lesion measured (by others) in electron micrographs (~100 Å). The results were consistent with the formation of a stable membrane pore by a single complex per vesicle in which C9 molecules line only one side of the pore at low C9/C5b-8 ratios and maximum pore size is attained by incomplete, noncircular polymers of C9.

Complement proteins C5b, C6, C7, C8, and C9 constitute the membrane attack complex (MAC)[1] of complement and assemble in the following sequence [reviewed in Muller-Eberhard (1986) and Mayer (1984)]:

$$\text{C5b} \xrightarrow{\text{C6}} \text{C5b-6} \xrightarrow{\text{C7}} \text{C5b-7} \xrightarrow{\text{PL}} \text{PL-C5b-7} \xrightarrow{\text{C8}} \text{PL-C5b-8} \xrightarrow{n\ \text{C9}} \text{PL-C5b-9}_n$$

The mechanism of generating membrane permeability by the MAC has been investigated from many standpoints. Electron micrographs of the completed complex show circular "doughnut" shaped lesions with an inner diameter of about 100 Å (Borsos et al., 1964), and membrane permeability was proposed to arise from a protein-lined pore (Mayer, 1972; Bhakdi & Tranum-Jensen, 1978). However, subsequent studies showed that the MAC lesion varied in size (Boyle et al., 1979; Giavedoni et al., 1979; Delmasso & Benson, 1981) depending on the number of C9 molecules per MAC (Ramm et al., 1982, 1985). A mechanism for generating heterogeneous pores has been difficult to understand. Recently, Tschopp (1984) and Amiguet et al. (1985) proposed a structure consisting of noncircular C9 polymers. If one side of a C9 oligomer forms an extremely tight hydrophobic interaction with a membrane bilayer while the other side is hydrophilic, the phospholipid bilayer facing the second side is forced to interact with the aqueous environment. This could generate membrane permeability (Tschopp, 1984). An alternative "leaky patch" model proposes that the MAC produces membrane permeability through local phospholipid disorder (Esser et al., 1979; Esser, 1982).

Despite many studies, certain aspects of MAC function remain controversial. A problem may arise from use of whole serum as the complement source and/or cells as the target membrane. While cells may provide apparent physiological targets for the MAC, they have significant disadvantages which limit quantitative evaluations and can give rise to heterogeneous observations. The observed number of complexes per cell needed for permeability has varied from 8–15 for sheep erythrocytes (Kolb & Muller-Eberhard, 1974) to as many as 300–850 (Sims, 1983; Bauer et al., 1979). The time necessary to reach the end point of release from cells can be 3 h or more (Ramm et al., 1982, 1985; Sims & Lauf, 1978, 1980). Protein-mediated processes may inactivate or eliminate MACs so the number of complexes per cell may not remain constant. In addition, the number of C9 molecules bound per MAC was reported to decrease from 15 to 7 as the number of complexes per rabbit erythrocyte was increased from 2500 to 25 000 (Podack et al., 1982). A different study found that C9 binding was constant (three or four per MAC) over a wide

† This work was supported in part by Grant HL 15728 from the National Institutes of Health.

[1] Abbreviations: MAC, membrane attack complex; LUVs, large unilamellar vesicles (diameter about 100 nm); SUVs, small unilamellar vesicles (diameter about 30 nm); PC, phosphatidylcholine; PS, phosphatidylserine; BPTI, bovine pancreatic trypsin inhibitor; G6PD, glucose-6-phosphate dehydrogenase; AT III–heparin, antithrombin III plus heparin complex; C5b-9_n, complex formed by the addition of *n* C9/C5b-8 (mol/mol); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-2960/89/0428-0061$01.50/0

range of MACs per cell (Stewart et al., 1984). Some of these differences may arise from the variable ability of whole cells to withstand attack by complement. Proteins known as homologous restriction factors have been shown to protect against the MAC (Schonermark et al., 1986; Zalman et al., 1986). Lysis is least efficient with proteins and erythrocytes from the same species (Hansch et al., 1981).

This study examined membrane permeability in a simple system with purified components. The results suggested an ordered assembly that proceeded with stable intermediates and which did not produce vesicle fusion or dissolution. The size of the membrane pore was dependent on the number of C9 molecules per MAC. Maximum pore size was generated when only about 50% of the C9 molecules was bound. These properties support and expand a model for membrane permeability generated by a porelike structure that is only partially lined with protein.

Materials and Methods

Proteins and Heparin. C5b-6 (Podack et al., 1978), C7 (Silversmith & Nelsestuen, 1986a), C8 (Steckel et al., 1980), C9 (Biesecker & Muller-Eberhard, 1980), bovine α-thrombin, and antithrombin III (Pletcher & Nelsestuen, 1982) were purified as described. Hirudin, BPTI, torula yeast G6PD, equine apoferritin, bovine fibrinogen, and bovine thyroglobulin were purchased from Sigma Chemical Co., St. Louis, MO. Unfractionated porcine heparin was donated by Riker Laboratories, 3M Co., Northridge, CA. Extinction coefficients used to calculate protein concentrations were as follows: 1.9 mL mg^{-1} cm^{-1} for C7, 1.6 mL mg^{-1} cm^{-1} for C8, 0.99 mL mg^{-1} cm^{-1} for C9, 0.61 mL mg^{-1} cm^{-1} for antithrombin III, 1.95 mL mg^{-1} cm^{-1} for thrombin, and 0.825 mL mg^{-1} cm^{-1} for BPTI. Molecular weights were as follows: 120 000 for C7 (Podack et al., 1978), 150 000 for C8 (Steckel et al., 1980), 71 000 for C9 (Biesecker & Muller-Eberhand, 1980), 56 000 for antithrombin III (Nordenman et al., 1977), 39 000 for α-thrombin (Mann et al., 1971), and 6500 for BPTI (Kassell et al., 1963). A considerably different extinction coefficient for C7 has been published (DiScipio & Gagnon, 1982). However, the value given above correlated closely with the mass of functional protein present in the C7 preparations (Silversmith & Nelsestuen, 1986a,b) and was used in this study.

Vesicles. Highly purified (>98% purity, manufacturer's estimate) PC (egg yolk) and PS (bovine brain) were purchased from Sigma Chemical Co., St. Louis, MO. Large unilamellar vesicles with trapped proteins were prepared by the extrusion technique of Hope et al. (1985). Phospholipids (120 mg), dried from organic solvent under vacuum at 50 °C in an agitating bath (6 h), were resuspended in buffer [2 mL of 200 mM Tris buffer, pH 9.2, for PS-LUVs and in 50 mM Tris buffer, pH 7.5, containing 100 mM NaCl–0.02% NaN_3 (standard Tris buffer) for PC-LUVs], containing protein (2–3 mg of thrombin, 4000 units of G6PD, or 5 mg of BPTI, apoferritin, fibrinogen, or thyroglobulin). This was freezed/thawed 6 times and then forced through a 0.1-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) 10–12 times (200–400 psi) by using a high-pressure extruder (Sciema Technical Services Ltd., Richmond, BC, Canada). The final suspension was gel filtered on a Sepharose 2B column (1.5 × 25 cm) equilibrated in Tris buffer. Vesicles of pure PS were also passed through a dextran sulfate–Sepharose CL-4B affinity column (0.75 × 10 cm) in order to remove thrombin which adhered to the external surface of the vesicles.

Vesicles containing trapped thrombin could be stored for up to 3 months at 4 °C without significant release of trapped thrombin. The activity released by Triton X-100 as well as the *Z*-averaged diameter of the vesicle preparation remained unchanged. One batch of PS obtained from the manufacturer did not retain thrombin for long periods and was not used.

The *Z*-averaged hydrodynamic diameters of the vesicle preparations were determined by quasi-elastic light scattering with a Langley/Ford LSA2 spectrophotometer coupled to a Langley/Ford 1096 autocorrelator (Silversmith & Nelsestuen, 1986c). The diameters were very consistent. Five preparations of PC-LUVs containing trapped thrombin were 109.7 ± 10 (SD) nm while four preparations of PS-LUVs were 83.0 ± 4 nm. The molecular weight of the vesicles was calculated from the volume of a shell of phospholipid (density = 1.0 g/mL) 4.0 nm thick and with an outer radius equal to the measured vesicle radius. These averaged 7.0×10^7 g/mol for PC-LUVs and 4.5×10^7 g/mol for PS-LUVs.

Phospholipid concentrations were determined by assay of organic phosphate (Chen et al., 1956). A value of 25 was used for the phospholipid/phosphorus weight ratio.

Radiolabeling of Proteins. Reductive methylation of C9 was accomplished (Jentoft & Dearborn, 1983, 1979) by the sequential addition of $NaCNBH_3$ (150 μL of 0.1 M) and [^{14}C]H_2CO (5.4 μCi in 15 μL; New England Nuclear, Boston, MA; 53 mCi/mmol) to 1.0 mg of C9 in 1 mL of 200 mM phosphate buffer (pH 7.7). The reaction was stored for 7 h at 0 °C followed by exhaustive dialysis against standard Tris buffer. The [^{14}C]C9 had a specific activity of 1.4×10^6 cpm/mg and retained 85% of its specific activity for release of thrombin from PS-LUVs.

Radiolabeled BPTI, apoferritin, fibrinogen, and thyroglobulin were obtained in a similar manner. $NaCNBH_3$ (500 μL of 0.1 M) and [^{14}C]H_2CO (250 μCi; 55 mCi/mmol) were added to 5 mg of protein in 250 μL of 50 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. After 8 h at room temperature, unreacted [^{14}C]H_2CO was removed by exhaustive dialysis against Tris buffer. This procedure yielded radiolabeled protein with a specific activity of 4×10^7 cpm/mg.

The following are examples of control studies run in addition to those shown under Results. Polyacrylamide gel electrophoresis (12% running gel; Laemmli, 1970) and autoradiography showed that [^{14}C]BPTI comigrated with underivatized protein as a single radioactive component. Thrombin activity as well as [^{14}C]BPTI elution from gel chromatography (Sephadex G-75) and migration on SDS–PAGE were unaltered by coincubation of these proteins. Gel filtration on Sephadex G-75 (1.5 × 50 cm) showed that the [^{14}C]BPTI and thrombin which remained outside the vesicles eluted at the correct positions. G6PD which had been released from vesicles eluted from a calibrated Sepharose 6B column (1.5 × 90 cm) as a single peak (V_e/V_0 ov 1.86 ± 0.042) corresponding to a molecular weight of about 200 000. This agreed with the molecular weight of the tetramer (205 000; Yue et al., 1969).

When treated with human α-thrombin, human C9 is cleaved at a single site to yield two fragments which remain noncovalently associated and fully active (Biesecker et al., 1982). However, bovine α-thrombin did not cleave human C9 to a detectable extent. Human C9 (0.80 mg/mL) which had been incubated with bovine α-thrombin (0.04 mg/mL) for 1 h at 37 °C in 50 mM Tris buffer (pH 7.8) containing 100 mM NaCl still migrated as a single band on SDS–PAGE [7.5% acrylamide running gel and a 5% stacking gel by the procedure of Laemmli (1970)].

MAC Assembly and Release of Macromolecules. Proteins were diluted immediately before use with buffers containing 50% glycerol (v/v); C5b-6 and C7 were diluted with 25 mM

phosphate buffer (pH 7.5) containing 50 mM NaCl while C8 and C9 were diluted with 25 mM Tris (pH 7.5) containing 50 mM NaCl. MAC assembly involved sequential addition of C7 (slowly added over 0.5–2.0 min) and C8 plus C9 to a mixture of vesicles and C5b-6 in Tris buffer. To minimize fluid-phase MAC assembly, C5b-6 was present in only slight excess over C7. Each protein addition was followed by a 5-min incubation. When a thrombin inhibitor was added at the conclusion of MAC assembly, the mixture was incubated another 2 min to allow complete inhibition. MAC assembly on PS-LUVs used a final reaction volume of 245 μL.

Thrombin activity was then measured by the addition of 5 μL of 6 mM S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride; Helena Laboratories, Beaumont, TX). Release of *p*-nitroaniline (duplicate measurements) at room temperature was followed by monitoring $A_{405\ nm}$ in a Beckman DU-8 spectrophotometer. Absorbance changes from untreated vesicles (less than 2% of the activity released by 1% Triton X-100) were subtracted as a background. Triton X-100 (1% final concentration) caused immediate and maximum release of thrombin. Control experiments showed that none of the individual complement proteins, the fluid phase C5b-9_{20} complex, or the membrane-bound C5b-7 complex were capable of cleaving S-2288 or permitting substrate access to thrombin.

MAC assembly on PC-LUVs which contained entrapped [^{14}C]BPTI plus either thrombin or G6PD was carried out in a total volume of 500 μL. Sepharose 4B and 6B columns (1 × 28 cm each) were used to separate released thrombin and G6PD, respectively. The vesicles gave an optical density peak. Radioactivity and enzyme activity coincident with this peak arose from unreleased proteins. Fractions containing released enzymes were pooled and assayed immediately. Thrombin activity was measured as described above, and G6PD was measured by NADPH production (E_{340} = 6200 M^{-1} cm^{-1}) after addition of Glc-6-P (10 μL of 300 mM) plus $NADP^+$ (25 μL of 20 mM containing 70 mM $MgCl_2$) to 215 μL of the pooled column fractions. The ΔA per minute reported for thrombin and G6PD represent total activity if all the enzyme in the sample was present in one assay. That is, the observed ΔA per minute was much lower but was corrected for sample size and dilution.

C5b-8 released no detectable thrombin or G6PD activity (≤2.5% of the total activity added). Gel filtration of reaction mixtures which consisted of vesicles plus fluid-phase C5b-9_{20}, the MAC proteins alone, or vesicles without MAC proteins did not release detectable levels of trapped thrombin or G6PD. However, some background levels of free [^{14}C]BPTI were present in these controls. Chromatography of vesicles alone, individual MAC proteins plus vesicles, or fluid-phase C5b-9_{20} plus vesicles showed that 8.5% (5300 cpm) of the total added radioactivity (63 000 cpm) was free from the vesicles. Assembly of membrane-bound C5b-8 released an additional 9.5% of the radioactivity. These values were subtracted as background.

Release of trapped proteins was carefully monitored as a function of time. It was found that maximum release occurred within minutes at room temperature (data not shown). The titration curves shown under Results therefore represent end-point values and are not kinetically determined.

Direct Binding of [^{14}C]C9 to PC-LUVs. The MAC was assembled by the procedures outlined above with [^{14}C]C9 and PC-LUVs containing either trapped thrombin or G6PD. The reaction mixtures were applied to columns of Sepharose 2B (1 × 28 cm) which separated vesicle-bound MACs from fluid-phase MACs as well as the released enzymes from the vesicles (see Results). Reaction mixtures which lacked C7 served as controls to determine nonspecific [^{14}C]C9–phospholipid interaction. The latter was always less than 2.7% of the total radioactivity added to the sample.

Probability Calculations. Theoretical curves were generated from the Poisson distribution

$$p(x) = \mu^x e^{-\mu}/x! \quad (1)$$

where $p(x)$ is the population with x complexes bound at a given ratio of MAC/vesicle (μ). The limiting component was C7, and the MAC/vesicle ratio was obtained from the C7 and vesicle concentrations. The calculations assumed that the MAC did not preferentially associate with vesicles containing more or less than the average amount of entrapped protein.

The probability that a vesicle had no MACs [$p(0)$] equals $e^{-[MAC/vesicle]}$. Assuming that 1 MAC/vesicle released the entire contents of that vesicle, the percent of release at each MAC/vesicle ratio becomes equal to the probability that a vesicle has at least one MAC bound: $P(1) = [1 - p(0)] \times 100$ (see below). In general, the probability that a vesicle has at least n complexes bound, $P(n)$, where $n = x + 1$ is

$$P(n) = \{1 - [p(0) + p(1) + p(2) + \ldots + p(x)]\} \times 100 \quad (2)$$

The Poisson distribution was used in an identical manner to calculate the probability that vesicles containing only one C5b-8 complex had x C9 bound at known molar ratios of C9/C5b-8 (μ).

When more than one C5b-8 was present on the vesicle, two models for C9 binding were considered. In one model, the C5b-8 complexes did not collaborate or act together in producing a membrane lesion. In this case, the percent of vesicles bearing one C5b-8 with n or more C9 molecules becomes

$$F(n) = [1 - e^{\{-mP(n)\}}] \times 100 \quad (3)$$

(DeLisi et al., 1980); where m is the average number of C5b-8 complexes per vesicle and $P(n)$ is as described above.

In the second case, the complexes were assumed to form perfect collaborative interactions. That is, the same degree of membrane permeability was produced on a vesicle containing five C9 molecules regardless of whether these were all associated with the same C5b-8 complex or with several different C5b-8 complexes. For this situation the C9 content of each vesicle was obtained assuming random additions of C9 to C5b-8. At an average of three C5b-8 per vesicle, the number of vesicles containing one, two, three, etc. complexes were calculated from eq 1. For each population of vesicles, the total number of C9 per vesicle was estimated assuming a random Poisson distribution of C9 over all complexes. This was solved for the single case where permeability to thrombin occurred when a total of five C9 molecules were present on a vesicle.

Results

Titration of Vesicles with MAC. Vesicles containing trapped thrombin were titrated with limiting C5b-7 and excess C8 and C9. This produced the maximum lesion size for every MAC formed (Figure 1A). Thrombin was fully released from PS-LUVs while only 65% was released from PC-LUVs. The latter property could arise from the low reactivity of C5b-7 with PC-LUVs which enhances competition from, and formation of, fluid-phase C5b-7 (Silversmith & Nelsestuen, 1986c). In fact, Richards et al. (1986) reported that only 25–40% of a fluorescent dye could be released from PC-LUVs. The 65% release shown in Figure 1A was obtained by slow addition of C7 to solutions of C5b-6 and vesicles to minimize

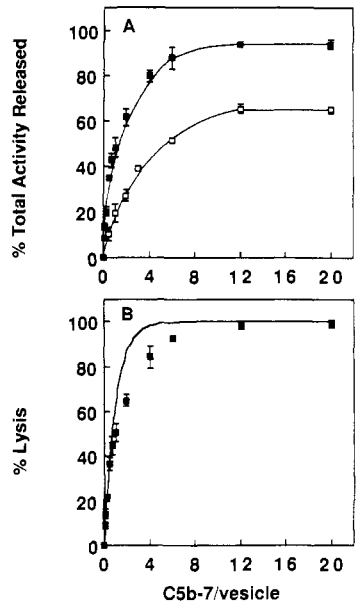


FIGURE 1: Association of the MAC with LUVs detected by exposure of trapped thrombin. Experimental details and probability calculations are described in detail under Materials and Methods. (A) shows thrombin release (mean value ± SD, $n = 2$) from PS-LUVs (■) (4.3×10^{-13} mol; constant final volume of 245 μL) with C5b-7 and excess C8 and C9. A similar titration of PC-LUVs (□) (2.6×10^{-12} mol; in a constant final volume of 245 μL) with C5b-7 and excess C8 and C9 is shown. The rate of S-2288 cleavage is shown as a percentage of the activity exposed by 1% Triton X-100. The maximum ΔA_{405} per minute for PS-LUVs was 0.860, 94% of the Triton released rate. The maximum ΔA_{405} per minute for PC-LUVs was 0.433, 65% of the Triton released rate. (B) shows a comparison of the data obtained for PS-LUVs (■) and the theoretical curve (—) anticipated for random distribution of MACs with equal and total thrombin release from each vesicle as the result of one MAC per vesicle. Maximum release by 20 MAC per vesicle was assigned 100%.

fluid-phase MAC assembly (Silversmith & Nelsestuen, 1986b). However, it appeared that fluid-phase assembly was a factor for all conditions using PC-LUVs so that PS-LUVs were a better choice for quantitative studies. Nevertheless, parallel experiments with PC-LUVs provided corroborative evidence and suggested that the high-charge density of PS-LUVs did not influence the pore generated by the MAC.

Figure 1B shows thrombin released from PS-LUVs compared to the theoretical expectation for full release of thrombin as the result of 1 MAC/vesicle. At low MAC/vesicle ratios, curve shape as well as close correlation of experimental and theoretical curves supported a model of random, noncooperative binding of C5b-7 to the vesicles. A random distribution has also been reported for MAC binding to SUVs (Silversmith & Nelsestuen, 1986b). Above about 0.30 MAC/vesicle there was some deviation from the theoretical curves. This was considered to be within the probable error of the assumptions made in generating the theoretical curve. No attempts were made to improve the curve fit by altering the assumptions (e.g., by assuming a size distribution for the vesicles with selective interaction based on size or by assuming a low level of selectivity for a vesicle subpopulation). Most subsequent experiments were performed at molar ratios less than 0.30 MAC/vesicle for which the probability of more than one complex per vesicle was low, $P(2) = 3.3\%$ (eq 2), or at high levels of MAC where release was nearly quantitative.

Vesicle size before and after the addition of protein showed that MAC binding did not cause vesicle fusion, aggregation, or fragmentation. The Z-averaged diameter of the vesicles increased from 89.0 ± 2.0 to 95.6 ± 2.0 nm and from 85.8 ± 0.7 to 107.0 ± 5.4 nm upon the addition of 3 or 10 MAC/vesicle, respectively. This change was of the magnitude expected for simple protein binding to the lipid surface and was similar to the 12.8 ± 1 nm increase in diameter ($2R_h$) found for PC-SUVs with 3.9 MAC vesicle measured by quasi-elastic light scattering [see Table I of Silversmith & Nelsestuen (1986b)]. This change in diameter was inadequate to suggest significant vesicle fusion, aggregation, or fragmentation. Parallel determinations showed that 82% and 95% of the thrombin were made accessible by 3 and 10 MAC/vesicle, respectively.

Overall bilayer stability agreed with previous studies with PC-SUVs which showed that C5b-9 assembly did not cause fusion, fragmentation, or aggregation of vesicles. Vesicle fusion or aggregation was monitored by fluorescence energy transfer between appropriate probes attached to phospholipids in separate vesicle populations. The addition of C5b-9 to the vesicles caused no change in the fluorescence emission spectrum, indicating that the two probes were not placed in close proximity to each other through aggregation, fusion, or other events (Silversmith & Nelsestuen, 1986b). In addition, the sedimentation characteristics of the MAC–vesicle complex in sucrose density gradients also supported MAC assembly on monomeric vesicles without fusion or fragmentation (Silversmith & Nelsestuen, 1986b).

Characteristics of Thrombin Release and Stability of MAC Intermediates. Vesicles without MACs remained intact during gel filtration, and thrombin was not released by either 300 (Figure 2A) or 100 mM (Figure 2B) NaCl added to the buffer. In fact, these vesicle preparations were very stable and could be stored at 4 °C for up to several months without release of thrombin. At 0.30 MAC/vesicle, 29% of the detergent-released thrombin activity was accessible to substrate but still eluted with the vesicle fractions (Figure 2D, open triangles). Increasing the salt concentration in the column elution buffer to 300 mM NaCl resulted in elution of 35% of the thrombin as free protein (Figure 2C). The amount of released thrombin was very close to that expected for 0.30 MAC/vesicle (Figure 1B). The MAC therefore caused release of thrombin from PS-LUVs, but high salt was needed to dissociate the thrombin–PS interaction.

Incomplete complexes consisting of C5b-8 were ineffective in releasing thrombin; only 4% of the total thrombin activity eluted as free protein (Figure 2E, open triangles). Excess C9 was then added to these vesicles (fractions 5 and 6), and they were gel filtered a second time. In the second pass through the column, 26% of the total thrombin activity eluted as free thrombin (Figure 2E). The C5b-8 formed on the vesicle had been stable for several hours at room temperature and could still add C9 and release the expected amount of thrombin.

When the MAC was initially assembled with three C9/C5b-8, about 8% of the total thrombin was released (Figure 2F, open triangles). Excess C9 was added to the column fractions containing vesicles (Figure 2F, fractions 5 and 6), and as expected, an additional 20% of the thrombin activity was released (Figure 2F, open squares). Therefore, MAC intermediates containing an average of three C9 molecules were stable for several hours at room temperature and remained competent to bind additional C9 molecules. Experiments similar to those in Figure 2 utilized PC-LUVs and gave similar results (data not shown). These experiments were valuable in that thrombin did not associate with the PC vesicle surface; when a MAC-mediated lesion was produced, the thrombin was released from the vesicle and eluted from gel filtration as free thrombin without the use of buffers containing high salt. These observations extended previous studies which

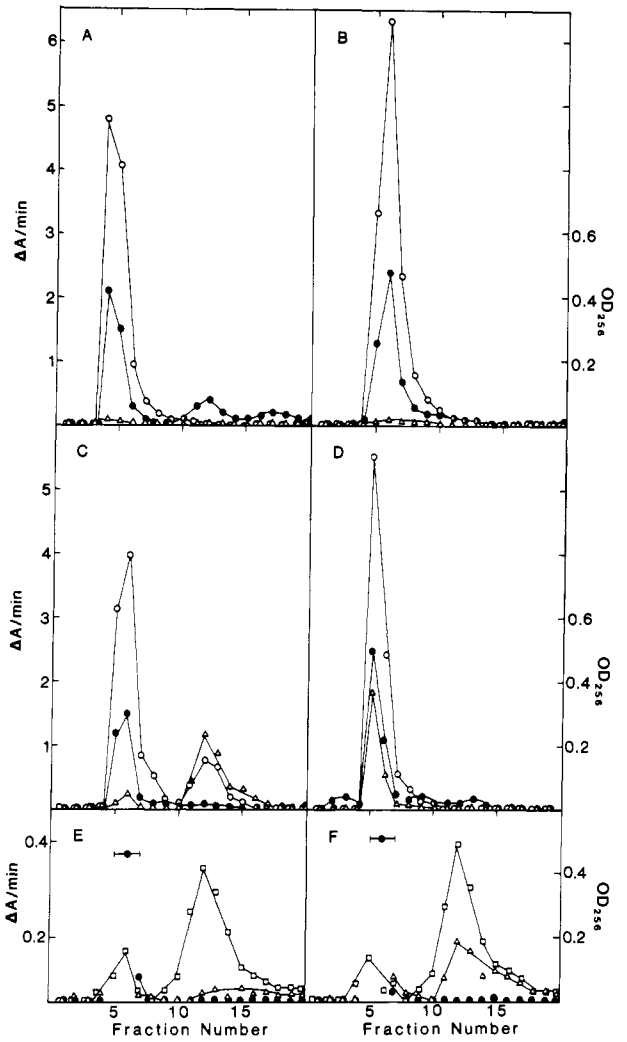


FIGURE 2: Gel filtration of PS-LUVs containing thrombin. Fractions (0.5 mL) from a Sepharose 6B column (0.75 × 23 cm) were assayed for OD_{256} (●) and thrombin activity (ΔA_{405}/min) in the absence (△) and presence of (○) 1% Triton X-100. (A) and (B) show elution profiles of PS-LUVs (9.4×10^{-12} mol) bearing no MACs in buffer containing 300 (A) and 100 mM NaCl (B), respectively. (C) shows the elution profile for the same amount of PS-LUVs which were treated with limiting C5b-7 (2.5×10^{-12} mol) followed by excess C8 and C9. Elution was with buffer containing 300 mM NaCl. (D) shows the same experiment as (C) except that the elution buffer contained 100 mM NaCl. (E) shows an experiment identical with that of (C) except that C9 was omitted. A single average OD_{256} for fractions 5 and 6 is shown. These fractions were pooled, mixed with excess C9, and reapplied to the same column. Fractions from this second chromatography were assayed for thrombin activity (□). In (F), C5b-9_3 was assembled and eluted from the column as in (C). Fractions 5 and 6 were pooled and treated with excess C9. The resultant mixture was rechromatographed, and activity in the absence of Triton (□) was measured.

showed that membrane-bound C5b-7 remained active for hours (Silversmith & Nelsestuen, 1986b).

Thrombin–Substrate Interaction during MAC Assembly. Complexes consisting of C5b-8 (Figure 3A) or C5b-9 (Figure 3B) produced membrane lesions which allowed thrombin access to a low molecular weight substrate (S-2288). About one molecule of C8 per C5b-7 produced maximum C5b-8 associated activity (Figure 3A). Larger ratios of C5b-8/vesicle increased the absolute rate of substrate cleavage but none of this activity was subject to inhibition by hirudin added to the medium (data not shown). Thus, C5b-8 complexes allowed some substrate access, but multiple C5b-8 complexes did not cooperate to produce a structure which allowed permeability

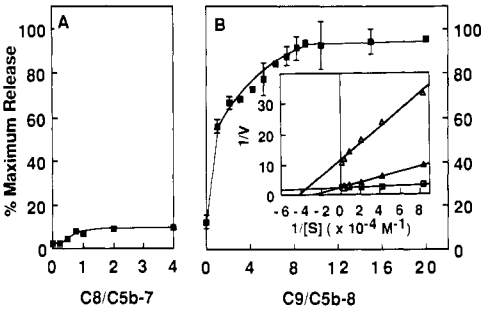


FIGURE 3: Accessibility of thrombin as a result of C5b-8 and C5b-9. In (A), C5b-8 (■) was assembled on PS-LUVs (8.6×10^{-13} mol, constant final volume of 245 μL) by the sequential addition of C5b-7 (6.2×10^{-13} mol) and C8. In (B), C9 was added (■) to C5b-8 (2.5×10^{-13} mol) assembled on PS-LUVs (9.4×10^{-13} mol). Thrombin activity is shown as the percent of maximum released activity (mean value ± SD, $n = 2$). Maximum ΔA_{405} per minute at saturating C9 was 0.530, 34.3% of the Triton released activity. (Inset) Double reciprocal plot for thrombin activity toward S-2288 in PS-LUVs (33 μg, 7.3×10^{-13} mol) made available by C5b-9_1 (△) and C5b-8 (▲); C5b-7 (4.9×10^{-13} mol) was the limiting component. Also shown are data for free thrombin (9.6×10^{-13} mol) in the presence (■) and absence (□) of PS-LUVs (33 μg). The latter contained no trapped thrombin. Velocity units are ΔA_{405} per minute. Rate determinations for free thrombin were made in buffer containing 1% poly(ethylene glycol).

to thrombin or hirudin. The addition of excess C9 to the same vesicles bearing eight C5b-8/vesicle resulted in 84% thrombin release which could then be inhibited by hirudin (data not shown).

The thrombin activity made available by C5b-8 was unchanged in assays where the C5b-6, C7, and C8 were preincubated with excess anti-C9 antibodies (data not shown). In contrast, C9 was unable to increase pore size in the same assays which contained the anti-C9 antibodies. Therefore, the low level of substrate penetration induced by C5b-8 was a function of that complex and did not arise from C9 contamination.

The amount of thrombin made available by MAC assembly could reflect the number, size, and/or stability of the pores that were generated. Kinetic parameters for S-2288 (Figure 3B, inset) showed that V_{max} provided by C5b-9_1 was greater than V_{max} provided by C5b-8 (0.419 and 0.090 ΔA_{405}/min for C5b-9_1 and C5b-8, respectively), while K_m for these complexes remained nearly the same. Since infinite substrate concentration could not produce the same V_{max}, it appeared that not all of the C5b-8 complexes created a membrane lesion adequate for S-2288 permeability. It is possible that some C5b-8 complexes bound without complete transmembrane insertion or that the membrane pore resulted from cooperative action of two or more C5b-8 complexes per vesicle. The accessible thrombin was always equal to or less than the number of vesicles that contained more than one complex. In these experiments (Figure 3, inset) 14% of the vesicles had two or more MACs (eq 2).

Free thrombin displays an extremely low K_m for this substrate (Figure 3, inset, squares; 3 μM for human thrombin, manufacturer's description). Consequently, the high K_m observed for thrombin exposed by C5b-8 and C5b-9_1, 2.5×10^{-5} M and 3.7×10^{-5} M, respectively, probably resulted from restricted diffusion of substrate through the C5b-8 or C5b-9 membrane lesions.

Transmembrane Macromolecular Interaction Due to C9/C5b-8. Vesicles bearing one or zero MACs per vesicle (0.27 C5b-8/vesicle) required 4.9 C9/C5b-8 for half-maximum inhibition of thrombin by hirudin (Figure 4A), and the titration correlated closely with theoretical curves for five or six C9/C5b-8 required for inhibition. This number of C9

Table I: Hydrodynamic Constants of Macromolecules

protein	$D_{20,w}$ ($\times 10^7$ cm^2/s)	R_h[a] (Å)	f/f_o	M_r	reference
BPTI	14.1	15.2		6500	W. Gallagher[b]
inulin		15.2			Pappenheimer (1953)
hirudin	10.8	19.9	1.42	10800	Markwardt (1970)
thrombin	8.76	24.5	1.16	39000	Harmison & Seegers (1962)
G6PD					
dimer	5.7	37.2	1.17	101600	Yue et al. (1969)
tetramer	4.5	47.7	1.19	205000	Yue et al. (1969)
AT III–heparin	4.9	44.0		70000	Pletcher et al. (1986)
apoferritin	3.61	59.4	1.14	466900	Rothen (1944)
thyroglobulin	2.49	86.1	1.49	669000	Salvatore et al. (1965)
fibrinogen	2.02	110.0	2.35	330000	Bloomfield et al. (1967)

[a] Calculated from the diffusion coefficient with the Stokes–Einstein relationship. [b] Unpublished results.

molecules was required for a membrane lesion that was large enough to admit hirudin or release thrombin. To determine which of these explanations was correct, inhibition by antithrombin III–heparin as well as by hirudin was measured with thrombin trapped in PC-LUVs (Figure 4A, inset). Inhibition by either inhibitor occurred at the same ratio of C9/C5b-8. Due to fluid-phase C5b-8, the actual ratio of membrane-bound C9/C5b-8 in this experiment could not be accurately determined. However, these results showed no discrimination due to the different sizes of hirudin and AT III–heparin (Table I). Similar titrations of thrombin (trapped in PS-LUVs) inhibition by AT III–heparin and hirudin were also indistinguishable (data not shown). Therefore, release of thrombin and not entry of inhibitor into the vesicle was measured in the C9 titrations shown in Figure 4.

When the molar ratio of C5b-8/vesicle was increased to 3 (Figure 4B, data points) 50% inhibition occurred at 3.2 C9/C5b-8. Theoretical curves for two extreme cases were considered. In the first case, C5b-9 complexes acted as totally independent units in generating membrane permeability (Figure 4B, right-hand curve). In the second (Figure 4B, left-hand curve), C5b-9 complexes interacted in a collaborative manner on the vesicle surface so that any combination of C5b-9 complexes binding a total of five C9 molecules per vesicle would allow macromolecular permeability. Since thrombin release at three MACs per vesicle deviated somewhat from the theoretical expectation (Figure 1B), interpretation of thrombin–hirudin interaction due to C9 additions was necessarily qualitative. At low C9/C5b-8 ratios the shape of the curve suggested the collaborative model, while at higher C9/C5b-8 ratios the results agreed with independent complexes.

Figure 4C shows a different representation of the data. From this plot it is clear that higher C9/vesicle ratios were needed when a larger number of complexes were present on the vesicle. The midpoint of inhibition at three MAC per vesicle occurred at 9.7 C9/vesicle. This simple comparison appeared to rule out a perfectly collaborative model where any combination of C9 molecules per vesicle generated permeability. If this were the case, the C9/vesicle ratio needed for membrane permeability should not vary greatly with the number of MACs per vesicle.

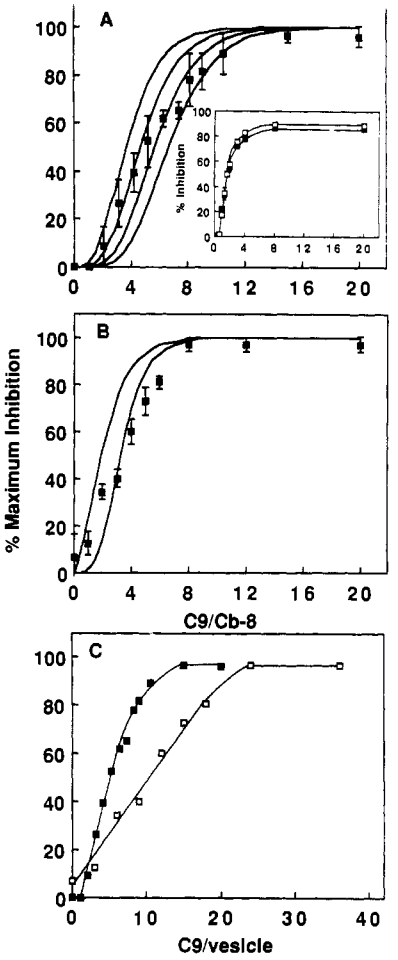


FIGURE 4: Hirudin inhibition of thrombin released from PS-LUVs. (A) shows the C9 titration of vesicles (9.4×10^{-13} mol) bearing 0.27 equiv of C5b-8. The activity in the presence and absence of hirudin (1 unit) was measured, and the percent of maximum inhibition achieved (mean value ± SD, $n = 2$) at saturating C9 is shown (■). Maximum inhibition was 46% of the exposed thrombin. Solid curves are theoretical expectations for thrombin release by n or more C9 molecules (eq 2) per C5b-8 complex. From left to right the curves are for n equal to 4, 5, 6, and 7. (Inset) shows a similar C9 titration of PC-LUVs (2.3×10^{-13} mol) to which C5b-8 (7.0×10^{-13} mol) had been added. The percent thrombin inhibition (mean value ±SD, $n = 2$) by hirudin [1 unit (■)] and heparin plus antithrombin III [4×10^{-8} and 2.8×10^{-7} M, final concentrations, respectively (□)] are shown. (B) shows C9 titration of vesicles (5.6×10^{-13} mol) bearing an average of three C5b-8 complexes per vesicle. Inhibition of thrombin activity by hirudin was expressed as in (A) (■). Theoretical curves were generated for random C9 binding to C5b-8 complexes. The complexes were either assumed to be collaborative or noncollaborative (left and right curves, respectively) in release of thrombin. In each theoretical case five C9 molecules were assumed to allow complete release of thrombin. In (C) The data from (A) (■) and (B) (□) were replotted as a function of C9 per vesicle.

Development of the Pore. Several mechanisms of generating macromolecular permeability are possible. For example, the addition of C9 to C5b-8 could produce pores that vary in size depending on the number of C9 molecules per C5b-8. These pores would exhibit differential release of different sized macromolecules. Alternatively, MAC could add C9 up to a critical level that produced a large disturbance in the membrane and simultaneous release of all macromolecules. To examine these possibilities, titrations were carried out with PC-LUVs containing [^{14}C]BPTI and either thrombin or G6PD (Table I). The released proteins were separated from the vesicles by gel filtration (Figure 5); the appropriate fractions were pooled and assayed for radioactivity or enzyme activity. The results clearly showed differential release corresponding

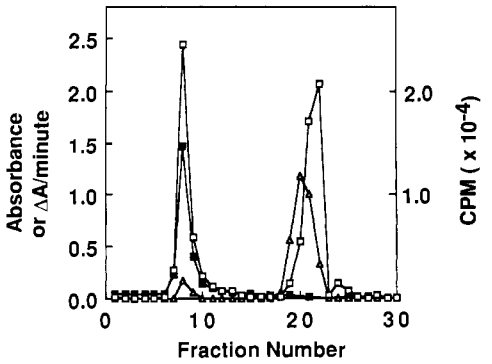


FIGURE 5: Gel filtration of PC-LUVs containing [^{14}C]BPTI and thrombin. The MAC was assembled by the addition of C9 (1.5×10^{-10} mol) to C5b-8 (5.75×10^{-11} mol) and vesicles (5.75×10^{-12} mol). Fractions from a Sepharose 4B column were assayed for OD_{250} (■), thrombin activity (Δ), and radioactivity due to [^{14}C]BPTI (□) as described under Materials and Methods.

to 1.95 versus 3.1 C9/C5b-8 for half-maximum release of [^{14}C]BPTI and thrombin, respectively (Figure 6A), and 2.1 versus 5.8 C9/C5b-8 for [^{14}C]BPTI and G6PD, respectively (Figure 6B). Since maximum release of the enzymes was much less than quantitative, most vesicles had zero MACs and others had one complex.

Figure 6B (inset) shows a comparison of the C9/C5b-8 ratio required for half-maximum release and the hydrodynamic radius of the trapped protein. While the number of C9 molecules per C5b-8 complex was only valid for relative comparisons (fluid-phase assembly would use an unknown portion of the added C9), it is clear that release of larger molecules from PC-LUVs required more C9 and that the size of the lesion increased in an approximately linear manner as C9 was added.

Midpoints of macromolecular release appeared to be a valid point for comparison of different titrations. That is, the C9/C5b-8 ratio at the midpoint corresponded reasonably well with the theoretical number of C9 molecules needed for release; if four C9 molecules were needed for release of a macromolecule, then 50% of the MACs would have approximately four C9 molecules bound [$P(n)$ = 50%] when half-maximum release occurred. This can be seen by comparing the theoretical curves in Figure 4A. The C9/C5b-8 ratio at the titration midpoint was always within 10% of the theoretical value for n. Titration midpoints were therefore used for subsequent comparisons.

Vesicles were prepared which contained [^{14}C]BPTI plus either [^{14}C]apoferritin, [^{14}C]fibrinogen, or [^{14}C]thyroglobulin (Table I). The two radioactive molecules were separated by gel filtration after MAC assembly. In each case MAC binding (20 C9/C5b-8) released [^{14}C]BPTI but not the larger molecule. The effective diameter of the pore created by MAC binding to PC-LUVs must therefore be greater than 95.4 Å ($2R_h$), the diameter of G6PD, but less than 118.8 Å ($2R_h$), the diameter of apoferritin. This measurement compared favorably with the numerous measurements of complete MAC tubules in electron micrographs where the inner diameter is found to be about 100 Å (Tranun-Jensen et al., 1978; Podack et al., 1982; Tschopp, 1984). Retention of apoferritin in PC-LUVs also agreed with the experiments of Dalmasso and Benson (1981), who followed protein release from resealed sheep erythrocyte ghosts. These authors used whole human serum as the complement source and found that all markers smaller than apoferritin could be released. This finding also showed that the maximum diameter of the MAC lesion generated in PC membranes by an individual MAC appeared to be very similar to that generated in sheep erythrocyte membranes by many MACs.

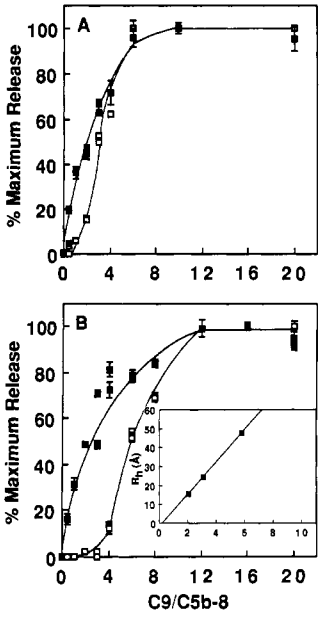


FIGURE 6: Release of different sized macromolecules from PC-LUVs. (A) shows the C9 titration of vesicles (5.75×10^{-12} mol) containing trapped [^{14}C]BPTI and thrombin to which C5b-8 (5.75×10^{-11} mol) had been added. The activity (mean value ± SD, n = 2) of released [^{14}C]BPTI (■) and thrombin (□) was measured following gel filtration similar to that shown in Figure 5 (fractions 18–25). The percent of maximum activity obtained at saturating C9 is shown (mean value ± SD, n = 2). Maximum release of [^{14}C]BPTI was 48% of the total radioactivity (63 000 cpm) and maximum release of thrombin was 35% of the activity released by 1% Triton X-100 (ΔA_{405}/min = 5.11). (B) shows the C9 titration of PC-LUVs (1.02×10^{-13} mol) containing trapped [^{14}C]BPTI and G6PD and which contained C5b-8 (9.3×10^{-13} mol). Released [^{14}C]BPTI (■) and G6PD (□) activity were measured as described above. Maximum release of [^{14}C]BPTI was 36% of the total added radioactivity (29 300 cpm), and maximum release of G6PD was 17.7% of that released by 1% Triton X-100 (ΔA_{340}/min = 10.6). (Inset) shows the hydrodynamic radii of the released proteins (Table I) as a function of the C9/C5b-8 ratio required for half-maximum release.

Direct Binding of [^{14}C]*C9 to Phospholipid-Bound C5b-8.* An alternative approach was needed to allow more quantitative estimation of the number of C9/C5b-8 required for protein release from PC-LUVs. The MAC was assembled on vesicles containing either thrombin or G6PD by the addition of radiolabeled C9 to membrane bound C5b-8. The reaction mixtures were gel filtered on columns of Sepharose 2B which separated fluid-phase MACs from membrane-bound MACs and allowed quantitation of the latter (Figure 7A). Figure 7B shows a typical elution profile for release of the trapped enzyme. Similar chromatography was performed for every [^{14}C]C9/C5b-8 ratio shown. Release of the macromolecules was expressed relative to saturation of the membrane with C9 (Figure 8 insets).

Half-maximum release of thrombin (Figure 8A) occurred at a fractional C9 saturation of 0.32 while half-maximum release of G6PD (Figure 8B) occurred at 0.45 of saturation. If 16 C9/C5b-8 is used as the saturation value for C9, 5.1 C9 molecules were required for half-maximum release of thrombin while 7.2 C9 molecules were required for half-maximum release of G6PD. The value for thrombin agreed extremely well with half-maximum release obtained by direct quantitation with PS-LUVs, 4.9 C9/C5b-8 (Figure 4A). Maximum release of thrombin and G6PD in these experiments was 14.7% and 28.3%, respectively, so that these titrations should represent the action of a single MAC.

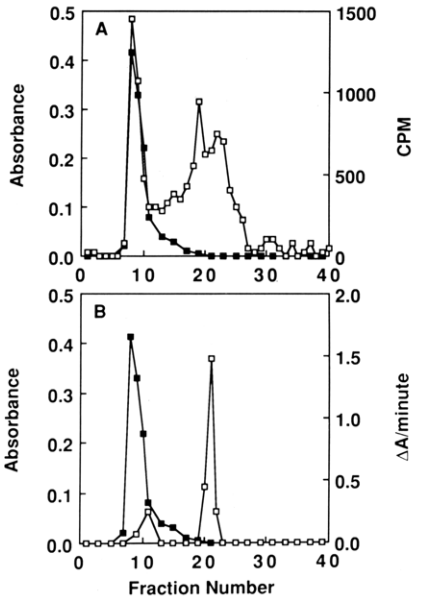


FIGURE 7: Binding of [^{14}C]C9 to PC-LUVs containing G6PD. (A) shows the elution of PC-LUVs (1.1×10^{-11} mol) to which an excess of C5b-8 (2.45×10^{-11} mol) and [^{14}C]C9 (7.3×10^{-11} mol) were added sequentially. Fractions were assayed for OD_{250} (■) and radioactivity (□) as described under Materials and Methods. In (B), fractions were assayed for OD_{250} (■) and G6PD activity (□) as described under Materials and Methods.

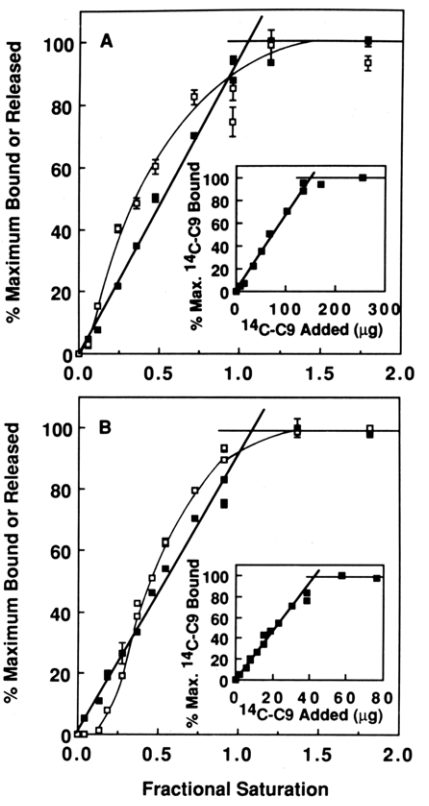


FIGURE 8: Relative membrane saturation with [^{14}C]C9 and release of macromolecules from PC-LUVs. (A) shows [^{14}C]C9 bound (■) to phospholipid C5b-8 (1.2×10^{-10} mol of C5b-8 and 1.2×10^{-11} mol of vesicles) and thrombin activity released (□). These are plotted as the percent of maximum [^{14}C]C9 bound or thrombin activity released (mean value ± SD, $n = 2$) and are compared to the degree of saturation with C9. (B) shows [^{14}C]C9 bound (■) to phospholipid C5b-8 (2.7×10^{-11} mol of C5b-8 and 2.7×10^{-12} mol of vesicles) and G6PD activity released (□). These are plotted as the percent of maximum [^{14}C]C9 bound or G6PD activity released (mean value ± SD, $n = 2$) and are compared to the degree of saturation with C9. (Insets) show membrane-bound [^{14}C]C9 as a function of actual [^{14}C]C9 added. In each case (insets) the point of intersection with the horizontal line was set to one (full saturation with C9). The quantity of [^{14}C]C9 added was expressed in (A) and (B) as a fraction of this value. Maximum release of thrombin ($\Delta A_{405} = 1.59$) was 14.7% of the Triton-released thrombin activity and maximum G6PD activity released ($\Delta A_{340} = 3.10$) was 28.3% of the total activity.

Figure 9 shows the hydrodynamic radii of thrombin and G6PD (open squares) plotted as a function of the fractional C9 saturation necessary to produce half-maximum release. The hydrodynamic radius of apoferritin is also shown, but since it was not released, it is plotted at a fractional saturation greater than 1.00. The binding of [^{14}C]C9 from both titrations in Figure 8 is also shown (closed squares). It is apparent from Figure 9 that release of macromolecules required far less than saturating C9. Furthermore, the data suggested that pore size increased in an approximately linear fashion up to about 50% of saturation with C9 and then ceased to increase for the final half of the C9 molecules.

Discussion

Study of MAC assembly on phospholipid vesicles avoids many of the problems of cells or cell ghosts. For example, vesicles are metabolically inert; they lack proteins which inactivate MAC intermediates and do not have the ability to cause endocytosis or to release complexes from the membrane. In agreement with these predictions, MACs appeared extremely stable at all levels of assembly on vesicles. Like previous studies on MAC assembly (Silversmith & Nelsestuen, 1986a,b), the approaches used here provided two mechanisms for data analysis. One was dependent on absolute determination of protein and phospholipid concentrations (Figure 4) while the other analyzed membrane permeability in a relative sense (Figures 6–9). The latter approach avoids the necessity of accurate determination of concentrations of components which is especially important for the phospholipid where molecular weights are necessarily approximate. However, the two approaches gave similar results for membrane permeability to thrombin (4.9 versus 5.1 C9 per complex for PS- and PC-LUVs, respectively, at half-maximum release by one MAC per vesicle). This corroboration reinforced the conclusions with each approach.

Most previous studies with phospholipid vesicles have utilized SUVs (e.g., Silversmith & Nelsestuen, 1986b,c; Tschopp & Podack, 1981; Hu et al., 1981) or multilammelar vesicles

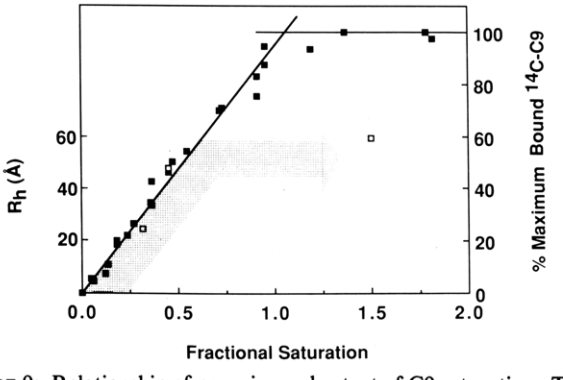
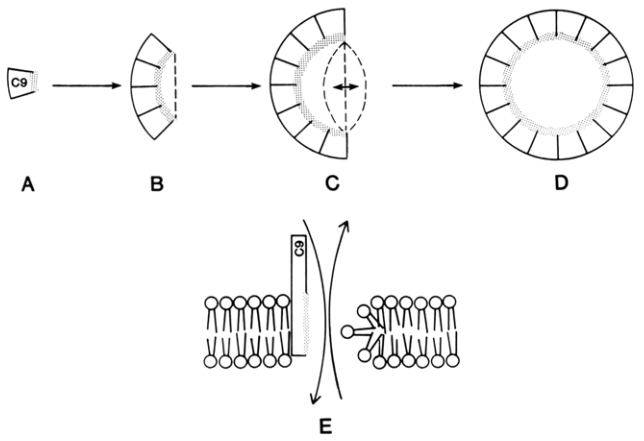


FIGURE 9: Relationship of pore size and extent of C9 saturation. The hydrodynamic radii of the *released* proteins, thrombin, and G6PD (□) and the [^{14}C]C9 that was bound (■) from Figure 8 are plotted as a function of fractional saturation with [^{14}C]C9. Apoferritin, which was *not released* from the vesicles, is plotted at a point beyond full saturation with C9 (the third open square, $R_h = 59.4$ nm). The shaded line represents pore size which reaches an abrupt maximum between 47.7 and 59.4 nm.

(Katoaka et al., 1973). However, the trapping efficiency of SUVs is very low, and high molar concentrations of solute are needed to provide significant trapping. LUVs have a much

Scheme I: Model for Membrane Permeability[a]



[a] Only the C9 molecule is shown. One side of the growing C9 polymer is proposed to be hydrophobic (solid side) and to interact with the hydrocarbon region of the membrane. The other side is hydrophilic (shaded line) and repels the hydrocarbon of the phospholipid. This forces a cross section of the bilayer to form a minimum energy interface with water (the dashed lines in B and C which may consist of structures shown in E). The lipid side of the pore might fluctuate around an energy minimum (C) to produce the maximum pore size at 50% assembly of C9.

higher trapping efficiency and have low surface curvature. Since the MAC does not penetrate LUV structure as well as it does SUV structure (Silversmith & Nelsestuen, 1986c), quantitative binding required the use of acidic phospholipids such as PS. Membranes of PC were useful only for relative determinations (Figure 8).

Release of macromolecules from LUVs followed a one-hit model where a single MAC was responsible for generating a lesion adequate to release all macromolecules in a single vesicle. Evidence of cooperation among MACs was restricted to low C9/C5b-8 ratios. At higher levels of C9 there appeared to be little or no cooperation.

The observations reported here show the following properties of macromolecular permeability generated by MAC: First, release of macromolecules from LUVs occurred through stable lesions with well-defined maximum dimensions. Second, C9 enlarged the lesion in approximate proportion to the ratio of C9/C5b-8. Third, fully functional lesions were formed at low C9/C5b-8 ratios; as few as three C9 allowed release of BPTI (on the basis of the ratio of five C9/C5b-8 for half-maximum release of thrombin from PS-LUVs, three C9/C5b-8 are required for half-maximum release of BPTI from PC-LUVs). Fourth, the maximum lesion size generated (about 95.4 Å, $2R_h$ for G6PD) was very close to the internal diameter of the protein-lined channel of the complete MAC observed in the electron microscope ($\sim$100 Å). Fifth, the maximum lesion was produced when only about half of the maximum number of C9 molecules was bound.

These results are most compatible with membrane-permeability models presented by Tschopp (1984) and Amiguet et al. (1985) for the MAC and by Bhakdi et al. (1985) for streptolysin O. Streptolysin O is a bacterial toxin that forms very large transmembrane channels which are often incomplete. In the models suggested by the above authors, pores formed by incomplete polymers were lined with protein on only one side. The other side of the pore was maintained in an unknown manner by phospholipid in contact with the aqueous environment. For streptolysin O, electron micrographs of noncircular polymers show a semicircular deposit of stain into an area, presumed to be free of phospholipid, on the hydrophilic side of the polymer.

The proposed membrane lesion is shown in Scheme I. One side of the C9 molecule is proposed to be hydrophobic (solid lines) and to interact with the hydrocarbon chains of the phospholipid bilayer in the manner normally described for intrinsic membrane proteins. The other side of the C9 molecule is proposed to be hydrophilic (shaded lines) and to repel the hydrocarbon portion of the membrane bilayer. The phospholipid molecules in contact with the hydrophilic side of the C9 polymer will form the most stable structure possible. Although we do not wish to present a precise description of this structure, a possible structure similar to that suggested by Tschopp (1984) is shown in Scheme IE. This structure will never be as low in energy as a bilayer, but the wall of hydrophilic protein simply prevents formation of a continuous bilayer. This structure could provide an opening with some degree of dynamic behavior (illustrated in Scheme IC by the dotted lines and arrows) centered around an energy minimum. Minimum energy should be achieved when the interaction of the membrane cross section with water is minimized. As the C9 polymer grows, minimum contact should consist of a straight line of lipid–water contact stretching between the two ends of the C9 polymer (Scheme IB,C). This could open up a pore with one dynamic side (the lipid) that varies around the energy minimum and is partially open all the time. Fluctuations (Scheme IC) might produce the maximum pore size at half C9 addition. Completion of the protein channel by the last half of the C9 molecules (Scheme ID) will not increase pore size but will produce membrane structure that is thermodynamically more stable and protein structure that is resistant to degradation [closed circular C9 has been shown to be resistant to dissociation in SDS and to digestion by trypsin (Podack & Tschopp, 1982; Podack, 1984)].

Scheme I suggests that membrane pores do not need to be entirely lined by protein. The ability to produce functional pores at low C9/C5b-8 ratios may be essential. In human serum the C9/C5b-8 ratio is 2 (Bhakdi & Tranum-Jensen, 1984), and the MAC may rarely consist of a full circle of 16 C9 molecules. Since the structure proposed in Scheme I is a single unit that consists of a chain of compact protein units, the terms pore, channel, cut, or incision, but not leaky patch, would appear to be appropriate descriptive terms for this lesion.

This structure shown in Scheme I may also be useful for understanding the mechanism of protein insertion and translocation across membranes in processes such as protein synthesis where the forming peptide chain passes through the membrane of the endoplasmic reticulum (Blobel & Dobberstein, 1975), posttranslational transport of proteins encoded by nuclear DNA across one or two bilayers into the mitochondria (Beattie et al., 1966), or translocation of toxins such as diphtheria toxin into cells (Pappenheimer, 1977). This model might even be considered as an alternative for pores that are currently thought to be completely protein lined. This mechanism of generating permeability is especially attractive for channels that are selectively permeable on the basis of size only. Further studies will hopefully suggest the range of structures that might function by this mechanism.

Registry No. MAC, 82986-89-8; C9, 80295-59-6.

References

Amiguet, P., Brunner, J., & Tschopp, J. (1985) *Biochemistry 24*, 7328–7334.

Bauer, J., Podack, E. R., & Valet, G. (1979) *J. Immunol. 122*, 2032–2036.

Beattie, D. S., Basford, R. E., & Koritz, S. B. (1966) *Biochemistry 5*, 926–930.

Bhakdi, S., & Tranum-Jensen, J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5655–5659.
Bhakdi, S., & Tranum-Jensen, J. (1984) *J. Immunol. 133*, 1453–1463.
Bhakdi, S., Tranum-Jensen, J., & Sziegoleit, A. (1985) *Infect. Immun. 47*, 52–60.
Biesecker, G., & Muller-Eberhard, H. J. (1980) *J. Immunol. 124*, 1291–1296.
Biesecker, G., Gerard, C., & Hugli, T. E. (1982) *J. Biol. Chem. 257*, 2584–2590.
Blobel, S., & Dobberstein, B. (1975) *J. Cell Biol. 67*, 835–851.
Bloomfield, V., van Holde, K. E., & Dalton, W. O. (1967) *Biopolymers 5*, 149–159.
Borsos, T., Dourmashkin, R. R., & Humphrey, J. H. (1964) *Nature (London) 202*, 251–252.
Boyle, M. D. P., Gee, A. P., & Borsos, T. (1979) *J. Immunol. 123*, 77–82.
Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Dalmasso, A. P., & Benson, B. A. (1981) *J. Immunol. 127*, 2214–2218.
DeLisi, C., Boyle, M., & Borsos, T. (1980) *J. Immunol. 125*, 2055–2062.
DiScipio, R. G., & Ganon, J. (1982) *Mol. Immunol. 19*, 1425–1431.
Esser, A. F. (1982) in *Biological Membranes* (Chapman, D., Ed.) pp 277–325, Academic, London.
Esser, A. F., Kolb, W. P., Podack, E. R., & Muller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1410–1414.
Giavedoni, E. A., Chow, Y. M., & Dalmasso, A. P. (1979) *J. Immunol. 122*, 240–245.
Hanch, G. M., Hammer, C. H., Vanguri, P., & Shin, M. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5118–5121.
Harmison, C. R., & Seegers, W. H. (1962) *J. Biol. Chem. 237*, 3074–3076.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.
Hu, V. W., Esser, A. F., Podack, E. R., & Wisnieski, B. J. (1981) *J. Immunol. 127*, 380–386.
Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.
Jentoft, N., & Dearborn, D. G. (1983) *Methods Enzymol. 91*, 570–579.
Kassel, B., Radicevic, M., Berlow, S., Peansky, R. J., & Laskowski, M. (1963) *J. Biol. Chem. 238*, 3274–3279.
Katoaka, T., Williamson, J. R., & Kinsky, S. C. (1973) *Biochim. Biophys. Acta 298*, 158–179.
Kolb, W. P., & Muller-Eberhard, H. J. (1974) *J. Immunol. 113*, 479–488.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Mann, K. G., Heldebrandt, C. M., & Fass, D. N. (1971) *J. Biol. Chem. 246*, 5994–6001.
Markwardt, F. (1970) *Methods Enzymol. 19*, 924–932.
Mayer, M. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2954–2958.
Mayer, M. M. (1984) *Complement 1*, 2–26.
Muller-Eberhard, H. J. (1986) *Annu. Rev. Immunol. 4*, 503–528.
Nordenman, B., Nystrom, C., & Bjork, I. (1977) *Eur. J. Biochem. 78*, 195–203.
Pappenheimer, A. M. (1977) *Annu. Rev. Biochem. 46*, 69–94.
Pappenheimer, J. R. (1953) *Physiol. Rev. 33*, 387–423.
Pletcher, C. H., & Nelsestuen, G. L. (1982) *J. Biol. Chem. 257*, 5342–5345.
Pletcher, C. H., Cunningham, M. T., & Nelsestuen, G. L. (1986) *J. Biol. Chem. 261*, 4143–4147.
Podack, E. R. (1984) *J. Biol. Chem. 259*, 8641–8647.
Podack, E. R., & Tschopp, J. (1982) *J. Biol. Chem. 257*, 15204–15212.
Podack, E. R., Kolb, W. P., & Muller-Eberhard, H. J. (1978) *J. Immunol. 120*, 1841–1848.
Podack, E. R., Tschopp, J., & Muller-Eberhard, H. J. (1982) *J. Exp. Med. 156*, 268–282.
Ramm, L. E., Whitlow, M. B., & Mayer, M. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4751–4755.
Ramm, L. E., Whitlow, M. B., & Mayer, M. M. (1985) *J. Immunol. 134*, 2594–2599.
Richards, R. L., Habbersett, R. C., Scher, I., Janoff, A. S., Schieren, H. P., Mayer, L. D., Cullis, P. R., & Alving, C. R. (1986) *Biochim. Biophys. Acta 855*, 223–230.
Rothen, A. (1944) *J. Biol. Chem. 152*, 679–693.
Salvatore, G., Vecchio, G., Salvatore, M., Cahnmann, H. J., & Robbins, J. (1965) *J. Biol. Chem. 240*, 2935–2943.
Schonermark, S., Rauterberg, E. W., Shin, M. L., Loke, S., Roelcke, D., & Hansch, M. (1986) *J. Immunol. 136*, 1772–1776.
Silversmith, R. E., & Nelsestuen, G. L. (1986a) *Biochemistry 25*, 841–851.
Silversmith, R. E., & Nelsestuen, G. L. (1986b) *Biochemistry 25*, 852–860.
Silversmith, R. E., & Nelsestuen, G. L. (1986c) *Biochemistry 25*, 7717–7725.
Sims, P. J. (1983) *Biochim. Biophys. Acta 732*, 541–552.
Sims, P. J., & Lauf, P. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5669–5673.
Sims, P. J., & Lauf, P. K. (1980) *J. Immunol. 125*, 2617–2625.
Steckel, E. W., York, R. G., Monahan, J. B., & Sodetz, J. M. (1980) *J. Biol. Chem. 255*, 11997–12005.
Stewart, J. L., Monahan, J. B., Brickner, A., & Sodetz, J. M. (1984) *Biochemistry 23*, 4016–4022.
Tranum-Jensen, J., Bhakdi, S., Bhakdi-Lehnen, B., Bjerrum, O. J., & Speth, V. (1978) *Scand. J. Immunol. 7*, 45–56.
Tschopp, J. (1984) *J. Biol. Chem. 259*, 7857–7863.
Tschopp, J., & Podack, E. R. (1981) *Biochem. Biophys. Res. Commun. 100*, 1409–1414.
Yue, R. H., Noltmann, E. A., & Kuby, S. A. (1969) *J. Biol. Chem. 244*, 1353–1364.
Zalman, L. S., Wood, L. M., & Muller-Eberhard, H. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6975–6979.