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Molecular reorganization of lipid bilayers by complement: A possible mechanism for membranolysis

(immune cytolysis/membrane structure/spin labeling)

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ABSTRACT **The interaction between the membrane attack complex (MAC) of complement and flat lipid bilayers was investigated. Using spin-labeled derivatives of phospholipids and cholesterol and electron paramagnetic resonance spectroscopy, we measured the penetration of the MAC into bilayers and its influence on the order of bilayers. The MAC precursor components C5b–6, C7, C8, and C9 did not exert any measurable influence on lipid membranes. Functional C5b–7 was shown to interact strongly with the bilayer surface without deep penetration into the bilayer. Formation of C5b–8 and especially C5b–9 caused a marked change in the anisotropy of spectra from probes located within the hydrocarbon phase. The spectral changes are not caused by changes in probe rotation and, in the case of the cholesterol probes, are not due to direct probe–protein interactions. For these reasons we interpret the spectral changes to be the result of reorientation of ordered bilayer lipids effected by strong binding of phospholipids to MAC proteins.**

We wish to report that the membrane attack complex (MAC) of complement is capable of physically reorganizing lipid bilayers, and that the forming MAC affects polar and nonpolar regions of these bilayers at different stages of its assembly. The study was designed to explore the extent to which interaction between the MAC and the constituents of lipid bilayers occurs.

Previous studies have defined the MAC as a complex consisting of C5b–9 (1–3) and indicated that its membranolytic function is not due to enzymatic activity but to physicochemical forces (4, 5). Mayer (6, 7) has postulated that the MAC forms a doughnut-like structure that, inserted into a membrane, creates a sizable hydrophilic protein channel through the hydrophobic interior of the membrane. In contrast, several other authors favored a model according to which only a subunit of the forming MAC extends itself through the membrane to form a small transmembrane channel (2, 4, 5, 8). A subunit that has been considered as a possible channel-former is the α-γ-chain subunit of C8 (9). The "doughnut" model resembles the mode of action of low molecular weight ionophores such as gramicidin (10), while the "insertional peptide" hypothesis is reminiscent of the ionophoric function of the F_0 subunit in ATPase (11).

Several advances in molecular probe spectroscopy, particularly in the electron paramagnetic resonance (EPR) spin-labeling technique (for review see ref. 12), have made it possible to gain insight into the interactions between these two types of channel-formers and lipids in membranes (13, 14). For this reason we decided to use EPR spectroscopy employing spin labels to investigate the interaction between the MAC and bilayer lipids. In contrast to others (15–17), however, we decided to study these interactions in defined artificial bilayer lipid membranes and not in natural membranes, because the heterogeneous composition of natural membranes together with drastic form changes during membranolysis make it difficult to draw unambiguous conclusions.

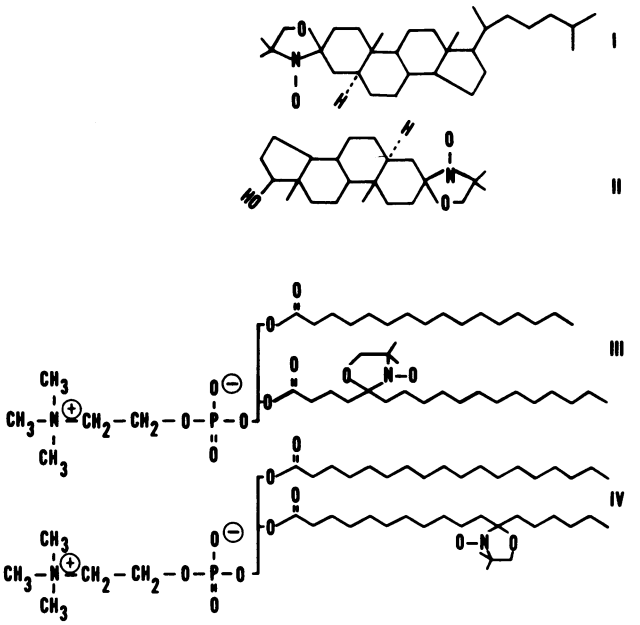

FIG. 1. Chemical structure of spin labels used in the present study.

MATERIALS AND METHODS

Lipids and Spin Labels. Dimyristoyl, dipalmitoyl, distearoyl, and egg lecithin, palmitoyl lysolecithin, and cholesterol were obtained commercially (Avanti Biochemicals, Birmingham, AL, and Calbiochem). [^{14}C]Cholesterol was a product of New England Nuclear. The spin-labeled cholesterol (label **I**) (see Fig. 1) and androstan (label **II**) derivatives and 5-doxylstearate were purchased from Syva (Palo Alto, CA); 1-palmitoyl-2-(5-doxyl)stearoyl lecithin (label **III**) was synthesized from palmitoyl lysolecithin and 5-doxylstearate according to published procedures (18); and 1-stearoyl-2-(12-doxyl)stearoyl lecithin (label **IV**) was from Serdary Research Laboratories (London, ON).

Complement Proteins. Complement proteins C5b–6, C7, C8, and C9 were purified as published (9, 19–21).

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Abbreviations: EPR, electron paramagnetic resonance; MAC, membrane attack complex; doxyl, 4,4-dimethyloxazolidine-*N*-oxyl.

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Cholesterol-Binding Studies. Binding of [^{14}C]cholesterol to C5b–8 and C5b–9 was measured by using a protocol identical to the one used in phospholipid-binding experiments (22).

Lipid Multilayers. Oriented flat lipid multilayers containing 1 mol % spin label were prepared by methods similar to those published by Smith and Butler (23). Because our studies required multiple addition of proteins to one multilayer preparation, we chose small rectangular glass cells (0.4 mm high, 4 mm wide, 50 mm long; Microslides, Vitro Dynamics, Rockaway, NY) as bilayer carriers. The bilayers were hydrated with either phosphate- or barbital-buffered saline. Complement proteins were added sequentially in 15-μl amounts and incubated with the bilayers for 15 min at room temperature. The slides were slowly drained before the next protein was added. To decrease the number of additions, sometimes several proteins were combined in the required stoichiometric concentration and added together; however, C5b–6 and C7 were always added sequentially to prevent formation of fluid phase C5b–7 aggregates, which are inactive. The final measurements were then performed on incompletely drained slides that were closed with putty (Crito-Seal, Scientific Products) to create a humid environment and prevent drying out.

EPR Measurements. The glass slides were mounted into a slotted Teflon rod and centered into the standard quartz dewar flask of the Varian temperature controller (model E-4557) with the aid of a goniometer (Varian model E-229). A more detailed description of the use of such microslides for spin-label studies on lipid bilayers and tissue culture cells will appear elsewhere. EPR spectra were recorded on a Varian E-104 Century line X-band spectrometer interfaced with a Nicolet model 535 signal averager, at 10-mW microwave energy and 2-G (1 G = 10^{-4} tesla) modulation amplitude settings and 25°C.

RESULTS

Structure of lipid multilayers

Phospholipid molecules in an aqueous environment self-assemble to form bilayers. When the assembly process is carried out on a flat glass surface, the lipids form stacked bilayers separated by water layers (24). The hydrocarbon chains are oriented more or less perpendicular to the plane of the bilayer and therefore the whole assembly is anisotropic. When spin labels such as those shown in Fig. 1 are incorporated into lipid multilayers, characteristic EPR spectra are obtained. The main differences between the steroid and the phospholipid spin labels are the orientation of the N—O bond relative to the long axis of the molecule and the fact that the steroid labels, because of their rigid structure, can only rotate around their long axes, while the lipid labels can undergo a flexing motion in addition. Both types of labels yield different spectra depending on whether they are recorded with the plane of the bilayer parallel or perpendicular to the magnetic field. This difference in spectra indicates that the long axes of the probes are not randomly oriented (Fig. 2). The degree of organization within the lipid bilayer was evaluated by using an empirical parameter, the ratio of the peak heights B and C (Fig. 2). For perfect order this ratio approaches 1; for complete disorder it approaches 0. In addition, this ratio is sensitive to the rate of rotation of the probe about its long axis, as is the maximum hyperfine splitting $2A_{max}$ (23).

Multilayers stable enough for multiple applications of protein solutions were most easily obtained with samples containing cholesterol. For this reason, lipid mixtures containing 30–50 mol % cholesterol were used. The spectra shown in Fig. 2 *A* and *B* indicate that lipid multilayers prepared in microslides are well ordered. The observed lineshapes and the B/C ratios of approximately 0.7 are similar to the ones reported in the literature (25). When the magnetic field is perpendicular to the long axis of the probe (parallel to the bilayer surface), the spectra show asymmetric lineshapes with $2A_{max}$ values of 61 G for label **I** in the 50% cholesterol-containing sample and 53 G for label **II** in the 33% cholesterol-containing sample. On the EPR time scale, these hyperfine splitting values are indicative of a relatively slow rotation about the long probe axis. Fast rotation would result in averaging of the x and z components of the hyperfine splitting tensors [6 and 32 G, respectively (23)] to give an effective hyperfine component of $2A_{max}$ = 38 G. When the magnetic field is perpendicular to the bilayer surface, the spectra show a set of three narrow and closely spaced lines with additional components in the low- and high-field wings. The latter maxima and minima are probably caused by sample imperfections because the multilayers could not be trimmed.

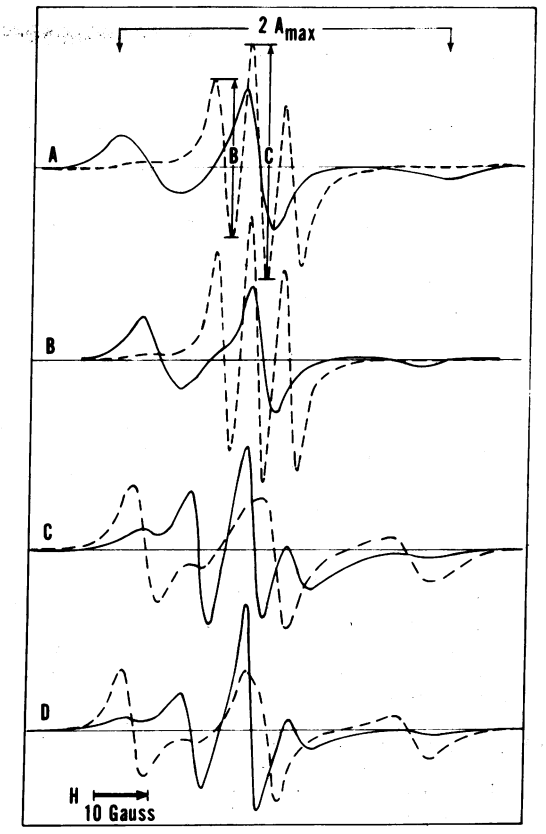

FIG. 2. EPR spectra of lipid multilayers containing different spin labels (% values are molar). (*A*) Label **I** in 50% dimyristoyl lecithin/50% cholesterol; (*B*) label **II** in 67% dimyristoyl lecithin/33% cholesterol; (*C*) label **III** in egg lecithin; (*D*) label **IV** in 50% distearoyl lecithin/50% cholesterol. Solid lines represent the surface of the multilayers parallel to the applied magnetic field H; broken lines represent the surface perpendicular to the magnetic field. EPR spectrometer settings: 10 mW power, 1 G modulation amplitude, 25°C.

Similar information can be obtained from phospholipid spin-label spectra (Fig. 2 *C* and *D*). In this case, however, the largest hyperfine splitting ($2A_{max}$) is obtained when the magnetic field is parallel to the long axis of the probe molecule because of the different orientation of the N—O bond (see Fig. 1). Thus, in this case, one can determine the angle of tilt of the N—O bond with respect to the bilayer normal by plotting $2A_{max}$ as a parameter of the angle between the bilayer surface and the magnetic field, as shown in Fig. 3 (26). The angle of tilt (δ_{app}) is equal to half of $2\delta_{app}$ as estimated from the plot. For egg lecithin containing label **III**, this angle was determined to be between 25° and 30° (Fig. 3), which is in good agreement

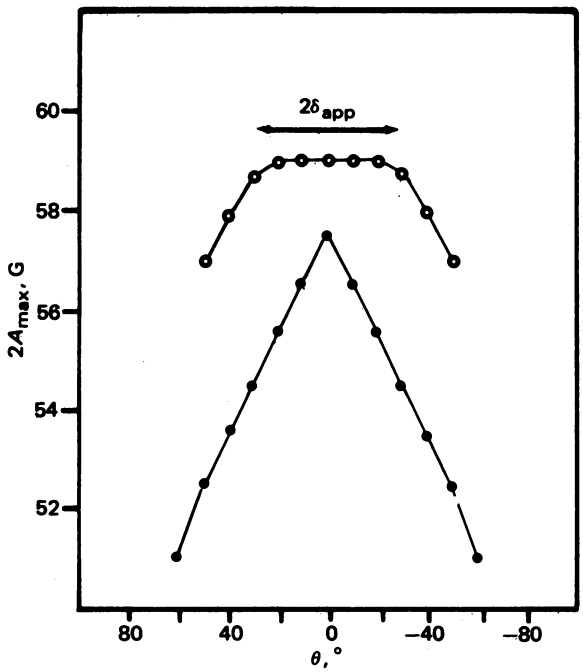

FIG. 3. Experimental plots of $2A_{max}$ vs. θ for label **III** in egg lecithin (○) or label **IV** in a mixture of 50% distearoyl lecithin/50% cholesterol (●). $\theta = 0°$ corresponds to the magnetic field perpendicular to the bilayer surface and δ_{app} is the approximate angle of tilt.

with the published data (26, 27). Addition of 50% cholesterol to lecithin removes this angle and orients the fatty acid chains perpendicular to the bilayer surface.

Effect of complement on lipid multilayers

Single addition of C5b–6, C7, C8, or C9 to spin-labeled multilayers produced no spectral changes, but sequential addition of these proteins had a marked effect on the shape of the various EPR spectra. When multilayers prepared from dimyristoyl lecithin, cholesterol, and label **I** were incubated with C5b–6 and C7 was added to allow formation on the multilayers of C5b–7, the B/C ratio was reduced from 0.7 to 0.43 (Table 1). The same combination of proteins had only a slight effect on spectra recorded with label **II** (Table 1 and Fig. 4). When multilayers doped with label **II** and containing C5b–7 were incubated with C8, the B/C ratio was reduced from 0.68 to 0.52. Addition of C9 to this reaction mixture, which allows formation of the membranolytic C5b–9 complex, resulted in a further reduction of the B/C ratio to 0.36. It is interesting to note that the assembly of the complex has only a moderate effect on the $2A_{max}$ values. In the case of label **II**, a slight increase after addition of C5b–7

Table 1. Effect of complement on lipid multilayers

Dimyristoyl lecithin/ cholesterol, mol ratio	Spin label	Addition	B/C	$2A_{max}$, G
1:1	I	Buffer	0.68	61.0
1:1	I	C5b,6	0.67	60.5
1:1	I	C5b–7	0.43	60.5
2:1	II	Buffer	0.70	53.0
2:1	II	C5b,6	0.68	53.0
2:1	II	C5b–7	0.68	56.0
2:1	II	C5b–8	0.52	56.0
2:1	II	C5b–9	0.36	55.0

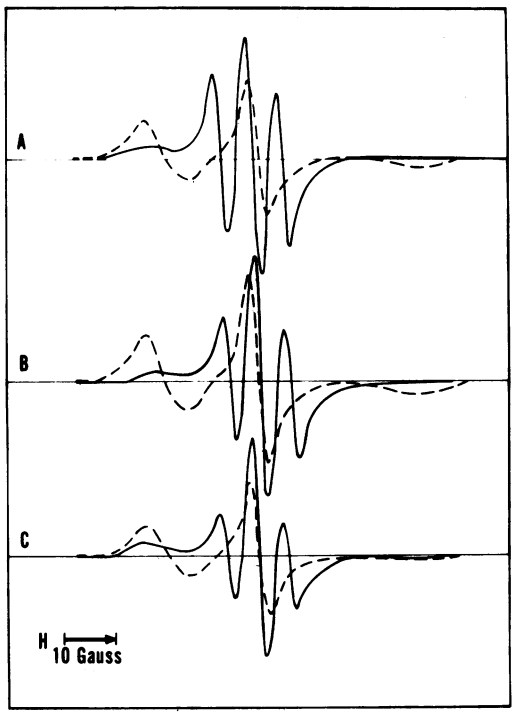

FIG. 4. Effect of MAC assembly on EPR spectra of label **II** in 67% dimyristoyl lecithin/33% cholesterol. EPR spectra after assembly of C5b–7 (*A*), C5b–8 (*B*), and C5b–9 (*C*). Broken lines represent the surface of the multilayers parallel to the applied magnetic field; solid lines represent the surface perpendicular to the magnetic field.

can be observed, while for label **I** these values remain more or less constant (Table 1).

A similar sequence of spectral changes can be observed in lecithin/cholesterol multilayers containing labels **III** or **IV**, although the changes are less pronounced compared to steroid label spectra (Fig. 5). Generally, the spectral changes became more apparent when moderate (30%) amounts of cholesterol and short-chain lipids such as dimyristoyl lecithin were used to prepare the bilayers. However, progressive assembly of the MAC resulted in distinct loss of spectral anisotropy, independent of the type and composition of lipid mixtures. The changes seen with all four labels were only produced when the MAC was successfully assembled on the lipid membrane. When C5b–6

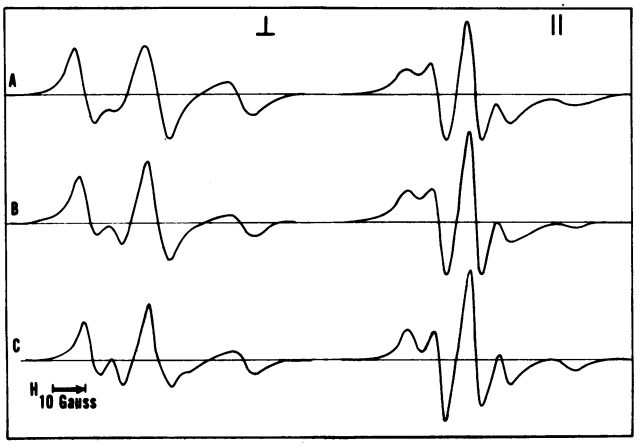

FIG. 5. Effect of MAC assembly on EPR spectra of label **IV** in 50% distearoyl lecithin/50% cholesterol. EPR spectra after assembly of C5b–7 (*A*), C5b–8 (*B*), and C5b–9 (*C*). ⊥ indicates that the bilayer surface is perpendicular to the magnetic field and ∥ that the bilayer surface is parallel to the magnetic field.

and C7 were combined prior to application to the bilayers, subsequent addition of C8 and C9 produced no spectral changes. It is known from earlier work (28) that under such conditions C5b–6 and C7 combine to form large aggregates that are lytically inactive.

DISCUSSION

The experiments presented here are focused on two problems, the depth of penetration of the MAC into lipid bilayers and the interaction between the MAC proteins and bilayer lipids. The answer to the first problem is apparent from the data presented in Table 1 and Fig. 4. It is clear that the C5b–7 complex has a strong influence on the spectrum of a probe molecule (label **I**) located on the surface of a bilayer, whereas another probe (label **II**) located deeper in the hydrocarbon phase was unperturbed. Some perturbation by C5b–7 was also sensed by a probe (label **III**) positioned in the hydrophobic phase but close to the polar/nonpolar interface. Thus, it appears that C5b–7 interacts strongly with the ionic part of the bilayer and may penetrate slightly into the hydrophobic region. In contrast, C5b–8 and C5b–9 produced significant spectral changes suggestive of deeper penetration of these complexes into the bilayer. It is nevertheless possible that such spectral differences are caused solely by structural alterations of the bilayer without intercalation of the MAC. Measurements of *isotropic* nitrogen hyperfine splitting constants (a'_N), which are sensitive to the environmental polarity, should provide more definitive answers.

Solutions to the second problem require a more quantitative interpretation of the different spectra. Such evaluations are based on the work of Smith (23, 25, 29), Griffith (26, 30), and McConnell (27, 31) and their coworkers, who have discussed the types of spectra obtainable in flat lipid multilayers and have developed methods to interpret such spectra. Their results show that both motion and orientation of the probe molecules must be considered in the explanation for the observed line-shape changes. In this respect it is easier to interpret the steroid spin-label spectra, because the rigid steroids can only rotate about their long axes and do not undergo a flexing motion. It is evident from Table 1 that addition of C8 and C9 to C5b–7-containing bilayers to assemble the C5b–8 and C5b–9 complexes has only a minor effect on the maximal hyperfine splitting ($2A_{max}$) values. Thus, the motion of these probes about their long axes is only moderately affected, if at all. In the absence of motional changes it is clear that the decrease in order as measured by the B/C ratio must be caused by variations in the organization of the bilayers. Increase in disorder means that the fatty acyl chains adopt a wider distribution of angles with respect to each other than they would in a normal bilayer structure. This interpretation is corroborated by the results obtained with lipid spin labels (labels **III** and **IV**). Although interpretation of spectra from these labels is very difficult without the aid of computer simulations, it is obvious from Fig. 5 that the progressive assembly of the MAC results in distinct reduction of the spectral anisotropy. The narrowing of the lines, especially in the parallel direction, indicates that also the amplitude or the rate of chain motion or both have increased. By comparison with published spectra on such labels in different systems (12), we can estimate that the average angle between the long axis of these labels and the bilayer normal can be as wide as 65° after complete assembly of the MAC.

Obviously, there are several ways to disorder multilayers, as Schreier-Mucillo *et al.* (25) have discussed, and which they have categorized as (*i*) intralamellar, (*ii*) interlamellar, or (*iii*) nonlamellar disorder. The first possibility may arise from loose packing of molecules, thereby allowing a high degree of mobility with a consequent decrease in spectral anisotropy. It is very unlikely that such a mechanism could explain our results, because the presence of 50% cholesterol suppresses mobility and the spectra of the steroid labels show no indication of rapid motion. The second possibility can occur as a result of lack of coplanarity between the bilayers. It could be argued that the large MAC assembles between the bilayers, thereby creating unequal distances or even "bubbles" between individual bilayers. If this were true, then the inactive, aggregated fluid phase complex should have a similar effect. However, the latter complex did not produce any spectral changes. The last possibility appears to be the most likely one. It is known that certain lipids under certain environmental conditions form nonlamellar structures such as cylinders and micelles, and it is conceivable that complement proteins are capable of inducing such structures.

However, one could also argue that the proteins interact preferentially with the probes and thus the spectral charges are not representative for changes in the organization of the bilayers. This possibility seems very remote in the case of the steroid labels. Experiments designed to measure the binding of cholesterol to the MAC were negative. The C5b–8 complex had no affinity for cholesterol, and the C5b–9 complex bound fewer than 10 molecules. This lack of binding of cholesterol has to be compared with the extremely high binding capacity of the MAC for phospholipids. C5b–8 was found to bind 800 and C5b–9 approximately 1000 molecules of phospholipids (22).

The most likely mechanism that could explain the observed spectral changes is as follows. During the assembly of the MAC the terminal complement proteins undergo profound conformational changes that create specific binding sites for phospholipids. The bound phospholipids are removed from the ordered lipids in the bilayer and form a shell around the protein. Although steroids do not interact *directly* with the proteins, they intercalate between the phospholipids and, in the case of spin-labeled steroids, can thus report on the orientation of the bound phospholipids. At the present time we cannot decide in which way the phospholipids bind to the proteins, whether ionically through the headgroups or hydrophobically through the end methyl groups. If the binding is hydrophobic, then only a small portion (and most likely the end methyl groups) of the hydrocarbon chains is involved in binding. Otherwise, we should have observed spectra of immobilized spin label groups. Such a mode of binding has been shown to occur between the membrane-bound portion of ATPase and bilayer lipids (14).

ATPases are capable of forming transmembrane channels to conduct ions across membranes in a very specific way, and it appears that in this case lipid–protein interactions should be tight and nondisruptive. In contrast, complement causes nonselective leaks that lead to membranolysis and cell death. For this reason we feel that reorganization of the lipid bilayer structure, which, after all, constitutes the basic barrier in membranes, would be an effective mechanism to impair all biological membranes. In fact, it has been shown (32–34) that the boundary areas between different lipid phases are highly permeable to small molecules. Nevertheless, we wish to emphasize that our results do not exclude the possibility that the MAC constitutes a doughnut-like assembly (6) with a hydrophilic center as a passageway for small molecules. Further studies will be necessary to demonstrate whether ion flow occurs through or around the MAC–phospholipid complex.

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