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# Poly(aspartic acid)-Dependent Fusion of Liposomes Bearing the Quaternary Ammonium Detergent [[[(1,1,3,3-Tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium Hydroxide

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ABSTRACT: Addition of the quaternary ammonium detergent [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA[OH]) and the fluorescent probes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-NBD-PE and *N*-Rh-PE, respectively) to liposomes composed of phosphatidylcholine (PC) and cholesterol (chol) resulted in the formation of fluorescently labeled liposomes bearing DEBDA[OH]. Incubation of the anionic polymer poly(aspartic acid) (PASP) with such liposomes resulted in strong agglutination, indicating an association between the negatively charged PASP and the positively charged liposome-associated DEBDA[OH]. Addition of PASP to a mixture of fluorescently labeled and nonlabeled liposomes, both carrying DEBDA[OH], resulted in a significant increase in the extent of fluorescence, namely, fluorescence dequenching. The degree of the fluorescence dequenching was dependent upon the ratio between the nonfluorescent and the fluorescent liposomes, upon the temperature of incubation, and upon the amount of DEBDA[OH] which was associated with the liposomes. Electron microscopic observations revealed that large liposomes were formed upon incubation of liposomes bearing DEBDA[OH] with PASP. The results of the present work strongly indicate that the fluorescence dequenching observed is due to a process of PASP-induced liposome-liposome fusion.

Membrane fusion is an important event in many biological processes such as endocytosis, exocytosis, fertilization, and infection of cells by animal viruses (Poste & Nicolson, 1978). The complexity of biological membranes and the difficulty in studying and analyzing their isolated components have promoted the use of model systems for the elucidation of the molecular mechanism of membrane fusion (Düzgünes, 1985).

The most widely used model system has been fusion between liposomes composed of negatively charged phospholipids such as phosphatidylserine (PS)[1] (Papahadjopoulos et al., 1975; Düzgünes et al., 1981) and cardiolipin (Wilschut et al., 1982). Fusion of such liposomes can be promoted by either divalent metals such as $Ca^{2+}$ (Wilschut et al., 1980) or by polycations such as polylysine (Gad et al., 1985; Uster & Deamer, 1985) and polyhistidine (Wang & Huang, 1984). Increasing the molar content of phosphatidylcholine (PC) is inhibitory, while addition of phosphatidylethanolamine (PE) increases the fusion competence of phospholipid vesicles (Uster & Deamer, 1985).

Fusion between liposomes can be quantitatively followed through the use of fluorescently labeled probes and energy-transfer methods (Struck et al., 1981). With these methods,

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[1] Abbreviations: chol, cholesterol; DEBDA[Cl], [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium chloride; DEBDA[OH], [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide; DQ, fluorescence dequenching; HTC, hepatoma tissue culture cells; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PASP, poly(aspartic acid); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; REV, reverse-phase evaporated vesicles.

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it has recently been demonstrated that also vesicles composed of the cationic surfactant dodecyldimethylammonium bromide are able to fuse following the addition of anionic molecules such as dipicolinic acid (Rupert et al., 1985).

In our laboratory, we have shown that the cationic surfactant [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA[OH]) confers fusogenic activity upon liposomes composed of neutral lipids such as PC and cholesterol (chol) (unpublished experiments). Liposomes bearing DEBDA[OH] were able to induce agglutination and promoted cell–cell fusion when incubated with either human erythrocytes or hepatoma tissue cultured cells (HTC). Neither agglutination nor fusion was promoted by incubation of liposomes bearing DEBDA[OH] and neuraminidase-treated cells. These results indicate that binding of these liposomes to cell plasma membranes is mediated by association between the positively charged DEBDA[OH] and cell surface negatively charged groups (unpublished experiments).

The results of the present work demonstrate that liposomes bearing DEBDA[OH] are also able to undergo fusion among themselves. It is shown that the negatively charged polymer PASP is able to promote agglutination and induce fusion of liposomes composed of PC/chol and bearing DEBDA[OH]. Fusion was monitored by the use of fluorescently labeled liposomes and energy-transfer methods, as well as by electron microscopic techniques.

## Materials and Methods

*Chemicals.* Phosphatidylcholine (PC) from egg yolk, cholesterol (chol), [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA[OH]), poly(aspartic acid) (PASP), and polylysine were purchased from Sigma. [$^{14}$C]PC was purchased from Amersham. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Biochemicals (Birmingham, AL); [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium chloride (DEBDA[Cl]) was from Fluka. SM-2 Bio-Beads were from Bio-Rad. All other chemicals used were of analytical grade.

*Preparation of Fluorescently Labeled Reverse-Phase Evaporated Vesicles (REV).* REV bearing the fluorescent molecules *N*-NBD-PE and *N*-Rh-PE were prepared by a slight modification of the method described by Struck et al. (1981). In short, PC (4 mg) and chol (2 mg), both in chloroform, were mixed with chloroform solutions of 80 μg of *N*-NBD-PE and 100 μg of *N*-Rh-PE. When needed, [$^{14}$C]PC (45 μCi/mg of PC) was added to the chloroformic solutions of the above phospholipids. The mixture was evaporated to dryness by a nitrogen stream, and the thin-layer obtained was dissolved in 1 mL of ether, followed by 0.4 mL of 150 mM sodium acetate, pH 7.4 (acetate buffer). All subsequent steps of REV preparation were essentially as described before (Szoka & Papahadjopoulos, 1978). The liposomes obtained were suspended to give 3 mg/mL PC (PC/chol, 1:0.5, in acetate buffer) and kept at 4 °C until use. Nonfluorescent liposomes were prepared by the same method except that the fluorescent lipids were omitted. During the present work, the amount of liposomes was determined by the PC content; however, in all the experiments described, the liposomes used were composed of PC and chol at a ratio of 1:0.5.

*Preparation of Liposomes Bearing DEBDA[OH].* The preparation of liposomes bearing DEBDA[OH] was performed as follows: DEBDA[OH] was dried from its methanolic solution and incubated in toluene at a concentration of 20–30 mg/mL for 30 min at 37 °C and kept until use at room temperature. Before use, the toluene was evaporated by a nitrogen stream, and the dry layer obtained was resuspended in acetate buffer to give a final concentration of about 30 mg/mL.

Unless otherwise stated, 0.9 mg of the above DEBDA[OH] suspension was added with continuous vortexing to a volume of 0.3 mL of the liposome suspension (0.9 mg of PC). Following incubation for 15 minutes with gentle shaking at 37 °C, SM-2 Bio-Beads were added, at a ratio of Bio-Beads: DEBDA[OH] of 50:1 (w/w), to remove free detergent. At the end of a 20–30-min incubation at room temperature with vigorous shaking, the amount of PC and DEBDA[OH] content was estimated. The amount of DEBDA[OH] that remains associated with the liposome bilayer, to a large extent, depends on the w/w ratio between the Bio-Beads and the detergent added as well as on the time of incubation. To assure the complete removal of free DEBDA[OH] from the liposome suspension, systems containing identical amounts of DEBDA[OH], in the absence of lipids, were incubated under the same conditions with SM-2 Bio-Beads. A quantitative estimation revealed that under these conditions no more than 10% of the liposomes (as was determined by their PC content) were lost during the incubation period with SM-2 Bio-Beads. It should be added that liposomes bearing DEBDA[OH] can also be prepared by adding DEBDA[OH] directly to the suspension of liposomes without the use of SM-2 Bio-Beads to remove free DEBDA[OH]. However, in such cases the amount of added DEBDA[OH] should be titrated carefully in each experiment.

*Fluorescence Measurements.* All fluorescence measurements were performed in a Perkin-Elmer LS-5 spectrofluorometer. Nitrobenzoxadiazole (NBD) fluorescence was measured at 471-nm excitation and 527-nm emission, maintaining the excitation slits narrow to reduce light scattering interference. Absorbance of samples was kept at <0.1 OD to reduce inner-filter effects. For temperature-controlled experiments, a constant flow of water was passed through the cuvette holder. Unless otherwise stated, the temperature was maintained at 23 °C.

*Determination of Fluorescnce Dequenching.* Percentage of fluorescence dequenching (DQ) (Nichols & Pagano, 1981) was determined according to

$$DQ = 100[1 - (F_\infty - F_t)/(F_\infty - F_0)] \quad (1)$$

where $F_\infty$ (total fluorescence) is the fluorescence obtained after solubilization of the liposomes with 0.2% Triton X-100 and correction for the quenching effect of Triton X-100 (×1.5), $F_t$ is the measured fluorescence of the liposomes at different times of incubation, and $F_0$ is the fluorescence obtained before the incubation period.

*Analytical Methods.* PC was estimated by the method of Charles and Stewart (1980). For a quantitative estimation of fluorescently labeled liposomes, [$^{14}$C]PC was used as a marker (100 dpm/μg of PC). The Bradford method (Bradford, 1976), which is used for determination of polypeptides, can also be used for the quantitative determination of DEBDA[OH], with DEBDA[Cl] as a standard. DEBDA[OH] associated with liposomes was determined in 90% methanol by its light absorption at 274 nm, with DEBDA[Cl] as a standard.

## Results

*Effect of DEBDA[OH] on Fluorescently Labeled Liposomes.* Fusion between liposomes can be estimated with fluorescently labeled liposomes and energy-transfer methods

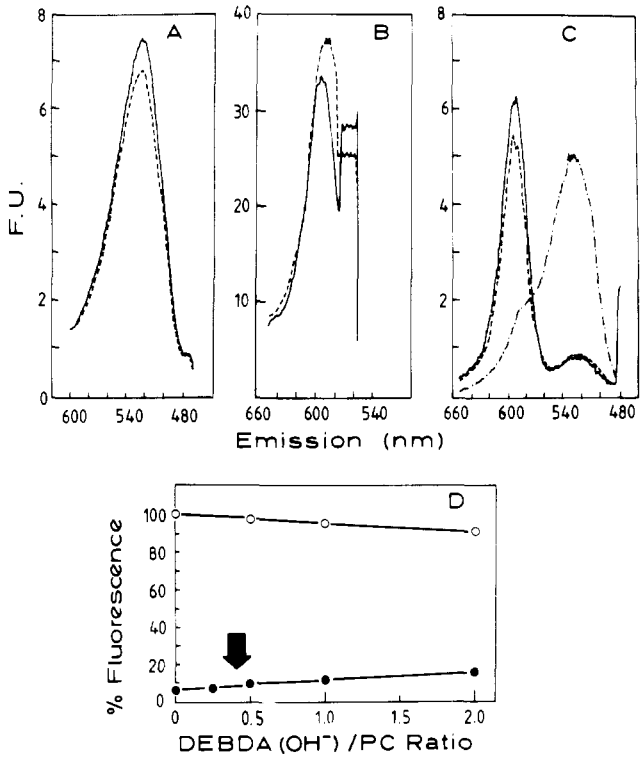


FIGURE 1: Effect of DEBDA[OH] on the emission spectra of fluorescently labeled liposomes. Fluorescence emission of REV (100 μg/mL) carrying either *N*-NBD-PE (A), *N*-Rh-PE (B), or both (C) was monitored in the absence (—) or presence (– –) of DEBDA[OH] (50 μg/mL) and with Triton X-100 (0.2% w/v) (–·–), as described under Materials and Methods. In (D), increasing amounts of DEBDA[OH] were added to REV bearing either *N*-NBD-PE (○) or *N*-NBD-PE and *N*-Rh-PE (●), to give the indicated ratios of DEBDA[OH]:PC (w/w). Following determination of fluorescence, Triton X-100 was added to a final concentration of 0.2% (w/v), and the fluorescence obtained was considered as 100% after correction for the quenching effect of Triton X-100. The DEBDA[OH]:PC (w/w) ratio in the liposomes used in the present work is indicated by the arrow.

(Struck et al., 1981). However, before studying whether DEBDA[OH] confers fusogenic properties upon liposomes, it was important to find out whether this quaternary ammonium detergent has any specific effect on the emission spectra of fluorescently labeled liposomes.

The results in Figure 1, A and B, show that under the conditions used, addition of DEBDA[OH] to REV bearing either *N*-NBD-PE or *N*-Rh-PE did not have any effect on the fluorescence peak positions and only slightly reduced the fluorescence intensity.

Addition of DEBDA[OH] also had very little effect on the quenched fluorescence of REV bearing *N*-NBD-PE and *N*-Rh-PE within the same lipid bilayer and on the fluorescence obtained after solubilization of these liposomes with Triton X-100 (Figure 1, C and D). It should be noted, however (Figure 1D), that addition of increasing concentrations of DEBDA[OH] to REV bearing both fluorescent molecules caused a small and gradual increase in the extent of fluorescence. The net increase in the fluorescence was only about 10% of the total fluorescence, even when the ratio of DEBDA[OH]:PC was as high as 2:1 (w/w). It is noteworthy that most of the experiments described in the present work were performed with liposomes bearing DEBDA[OH] at ratios of DEBDA[OH]:PC (w/w) of 0.6 or below. It appears therefore that the presence of DEBDA[OH] does not interfere with the energy-transfer measurements which were, in the present work, used to study fusion between liposomes. Preliminary experiments (not shown) in our laboratory have shown that addition

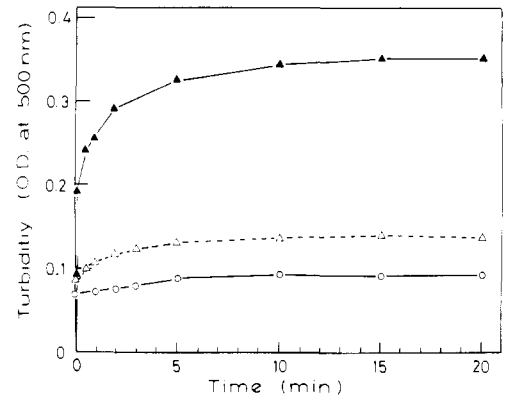


FIGURE 2: PASP-induced aggregation of liposomes bearing DEBDA[OH]. REV (△) and REV bearing DEBDA[OH] (▲) (DEBDA[OH]:PC, w/w, ratio of 0.4) were prepared as described under Materials and Methods. Aggregation of liposomes (60 μg in 0.6 mL of acetate buffer) was monitored in the absence (○) or in the presence (△, ▲) of 20 μg of PASP by the absorbance at 500 nm at room temperature.

of DEBDA[OH] to PC/chol liposomes loaded with carboxyfluorescein (CF) caused instantaneous release of the enclosed CF. These results may indicate that the added detergent is reaching the inner layer of this liposome membrane. However, addition of DEBDA[OH] did not induce release of proteins such as bovine serum albumin (BSA) from loaded liposomes showing—as was also inferred from the fluorescence measurements—that no disruption of the liposome occurred under the conditions used.

*Effect of Poly(aspartic acid) on Liposomes Bearing DEBDA[OH]*. The insertion of the positively charged detergent DEBDA[OH] into the membranes of REV caused their repulsion, as was revealed by fluorescence microscopic observations (not shown). On the other hand, addition of the anionic polymer PASP induced massive agglutination of the positively charged liposomes. This was inferred from results showing a significant increase in turbidity upon addition of PASP to liposomes bearing DEBDA[OH] (Figure 2). Moreover, fluorescence microscopic observations revealed the presence of large agglutinates in such a suspension. No increase in the turbidity was observed following addition of PASP to liposomes lacking DEBDA[OH], indicating that the agglutination was caused by association between the negatively charged PASP and the positively charged DEBDA[OH] (Figure 2).

Addition of PASP to a mixture of fluorescently labeled and nonlabeled REV caused, in addition to heavy agglutination, a fast and high increase in the fluorescence intensity (Figure 3A). As can be seen, up to 60% of the quenched fluorescence was recovered within 3–5 min after the addition of PASP. No increase in fluorescence was observed when these liposomes were incubated in the absence of PASP (Figure 3A). The extent of fluorescence dequenching was highly dependent on the amount of PASP added, reaching a maximum degree upon the addition of 5 μg of PASP to 40 μg of liposomes bearing DEBDA[OH] (Figure 3B). The addition of polylysine prior to PASP strongly inhibited the induction of fluorescence dequenching (Figure 3C). Conversely, no reduction in the fluorescence intensity was noted when polylysine was added after PASP, showing that polylysine, by itself, does not have any effect on the fluorescence intensity. Under such conditions, some decrease in the turbidity was noted (not shown), indicating partial disagglutination of the PASP-induced agglutinates.

The fluorescence dequenching observed might have resulted from intermixing of the REV lipids due either to lipid–lipid exchange processes or to fusion of the liposome bilayers.

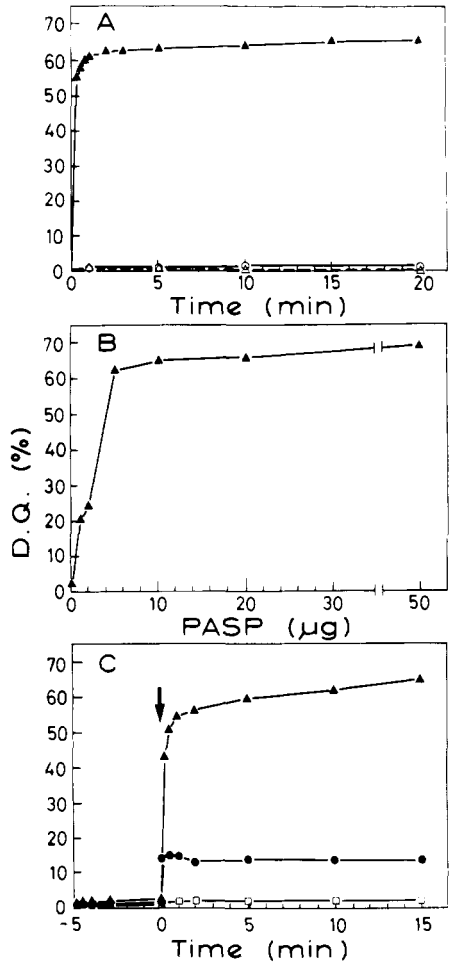


FIGURE 3: Effect of PASP on the fluorescence of liposomes bearing DEBDA[OH]. (A) (1) (▲) PASP (20 μg) was added to a mixture of fluorescently labeled (6 μg) and unlabeled (120 μg) liposomes (0.6 mL in acetate buffer), both of which contained DEBDA[OH] (DEBDA[OH]:PC, 0.4 w/w). (2) (△) As above, except that DEBDA[OH] was absent from the liposomes. (3) (○) The same as above (1) but in the absence of PASP. Following addition of PASP, fluorescence was monitored at room temperature, and the dequenching percentage was calculated as described under Materials and Methods. (B) As in (A-1), except that the indicated amounts of PASP were used. The fluorescence was determined following 30 min of incubation with PASP at room temperature. (C) As in (A-1), except that prior to the addition of PASP (arrow), polylysine at 0 (▲), 100 (●), and 200 μg (□) was added to suspension of liposomes.

Theoretically, solubilization of the phospholipid vesicles by the addition of PASP should also result in fluorescence dequenching. The results in Figure 4 support the view that the fluorescence dequenching observed is due to intermixing of the liposome lipids and not to their solubilization by the added PASP. As can be seen in this figure, no fluorescence dequenching was induced by the addition of PASP to a suspension containing only fluorescently labeled liposomes bearing DEBDA[OH]. On the other hand, a gradual increase in the extent of fluorescence (fluorescence dequenching) was obtained upon incubation of increasing amounts of nonfluorescent with fluorescently labeled liposomes in the presence of PASP (Figure 4). No increase in the extent of fluorescence dequenching was observed upon the addition of PASP to such a mixture of liposomes lacking DEBDA[OH].

The results in Figure 5 show that the rate and the extent of fluorescence dequenching were dependent upon the amount of DEBDA[OH] present in the liposome preparation. High rates and maximum extent of fluorescence were obtained with liposomes containing DEBDA[OH] at a DEBDA[OH]/PC ratio of 0.3–0.4 (w/w) and above (Figure 5).

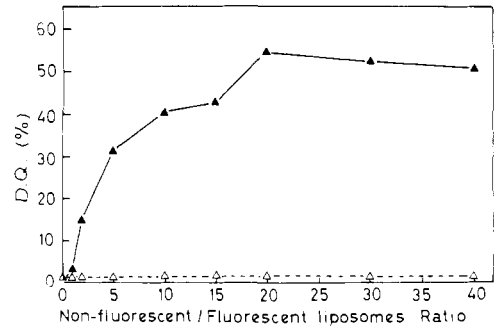


FIGURE 4: PASP-induced fluorescence dequenching: dependency on the ratio between nonfluorescent and fluorescent liposomes. Fluorescently labeled liposomes (6 μg), lacking (△) or bearing DEBDA[OH] (▲) (DEBDA[OH]:PC ratio of 0.4 w/w), were mixed with increasing amounts of nonfluorescently labeled liposomes, lacking or bearing DEBDA[OH], respectively, in 0.6 mL of acetate buffer. The fluorescence was determined following 20 min of incubation with PASP, as described in Figure 3B.

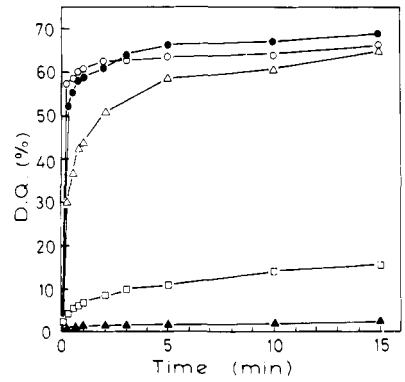


FIGURE 5: PASP-induced fluorescence dequenching: dependency on the amount of DEBDA[OH] in the liposomes. Fluorescently labeled liposomes (6 μg) were mixed in 0.6 mL of acetate buffer with nonfluorescently labeled liposomes (120 μg), both of which contained DEBDA[OH] at the following DEBDA[OH]:PC ratios: 0.1 (▲), 0.15 (□), 0.3 (△), 0.4 (●), and 0.6 (○). After addition of 20 μg of PASP, fluorescence dequenching was determined as described in Figure 3A.

Support for the view that the fluorescence dequenching observed, upon addition of PASP to a suspension of liposomes bearing DEBDA[OH], is due to liposome–liposome fusion was obtained from electron microscopic studies. Incubation of these liposomes with PASP caused a progressive increase in their size (Figure 6). Indeed, extremely large liposomes appeared only in samples incubated with PASP (compare liposomes in Figure 6A and those seen in Figure 6B, C). Only fusion could lead to the formation of such huge liposomes.

*Effect of Temperature and Ionic Strength.* The results in Figure 7 show that induction of fluorescence dequenching by PASP was temperature dependent. A slower rate and lower extent of fluorescence dequenching were observed upon incubation at low temperatures such as 6 °C, as compared with that obtained at 23 °C and above (Figure 7). Furthermore, the fluorescence dequenching observed was dependent upon the ionic strength of the incubation medium. No increase in fluorescence was observed upon the addition of PASP to liposomes bearing DEBDA[OH] and suspended in a buffer of low salt concentration (5 mM sodium acetate, pH 7.4). Under these conditions PASP also failed to promote an increase in the turbidity of the liposome suspension showing that no aggregation of liposomes occurred in a medium of low ionic strength (not shown). Opposed to this, high rates and maximal fluorescence dequenching were obtained by the addition of PASP to liposomes suspended in 150 mM buffer (standard condition used in the present work). As can be seen (Figure

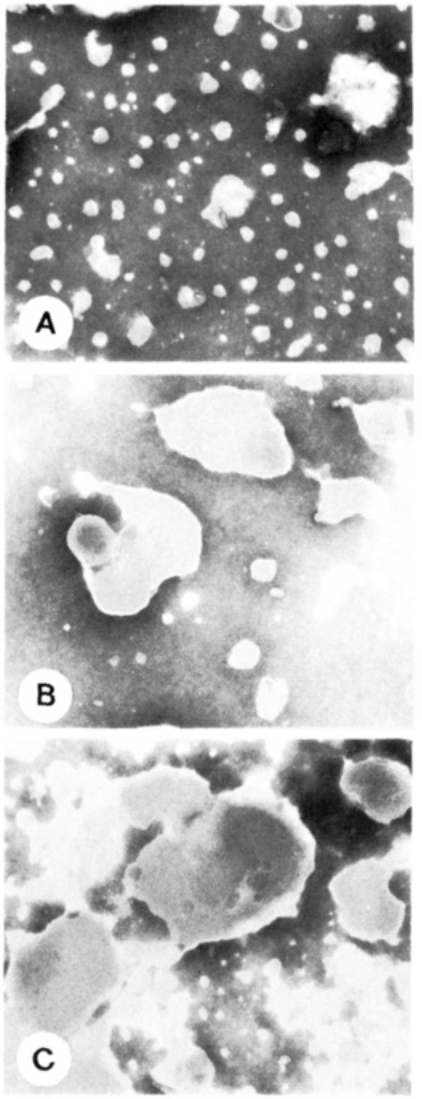


FIGURE 6: Fusion of liposomes bearing DEBDA[OH]: electron microscopic studies. Liposomes composed of PC/chol and bearing DEBDA[OH] (6 μg of PC with a DEBDA[OH]:PC ratio of 0.4 w/w) were prepared as described under Materials and Methods. Following 0 (A), 1 (B), and 5 (C) min of incubation with PASP (20 μg, as described in Figure 2), SM-2 Bio-Beads were added to give a ratio (w/w) of Bio-Beads:DEBDA[OH] of 50:1. At the end of a 30-min incubation at room temperature with vigorous shaking, the liposomes were removed and were negatively stained with phosphotungstic acid, as previously described (Vainstein et al., 1984). Magnification 36700×. In our experiments, we have noted that the addition of phosphotungstic acid (PTA) to liposomes bearing DEBDA[OH] caused an increase in the suspension's turbidity, the induction of fluorescence dequenching, and the formation of huge liposomes (not shown). It is possible that, similar to PASP, also the negatively charged PTA induces fusion of liposomes bearing DEBDA[OH]. Therefore, to study the effects of PASP by electron microscopic observations and, on the other hand, to avoid the effects of PTA, the DEBDA[OH] molecules were removed with the aid of SM-2 Bio-Beads from the liposome suspension. However, it appears that some residual DEBDA[OH] remained associated with the liposomes. This may explain our observation that relatively large liposomes are seen following negative staining in a system incubated in the absence of PASP (A).

8A), intermediate rates and extents of fluorescence dequenching were obtained in liposomes suspended in 50–100 mM buffer. The inhibition of fluorescence dequenching observed in a medium of low ionic strength was completely reversed by the addition of sodium acetate, as is demonstrated by the results shown in Figure 8B. This clearly indicates that the integrity of the liposomes was preserved during their incubation in a medium of low salt concentration. At the high

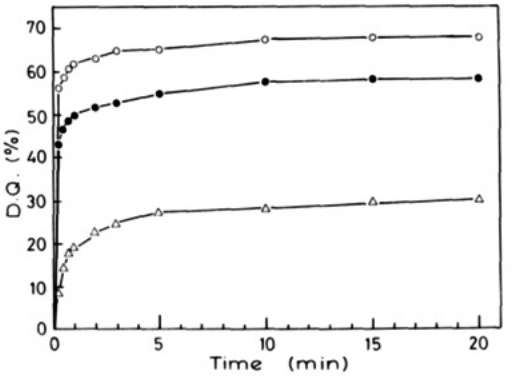


FIGURE 7: Effect of temperature on the PASP-induced fluorescence dequenching. PASP was added to a mixture of fluorescently labeled liposomes and nonlabeled liposomes bearing DEBDA[OH], as described in Figure 3A, and the fluorescence was monitored at 6 (△), 23 (●), or 37 °C (○).

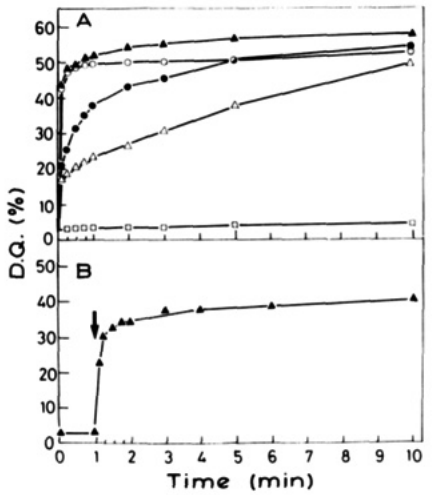


FIGURE 8: Effect of ionic strength on the PASP-induced fluorescence dequenching. (A) PASP was added to a mixture of fluorescently labeled and nonlabeled liposomes bearing DEBDA[OH] (DEBDA-[OH]:PC ratio of 0.4 w/w), as described for Figure 3A, except that the reaction was carried out with liposomes suspended in acetate buffer at the following concentrations: 5 (□), 50 (△), 75 (●), 100 (○), and 150 mM (▲). (B) PASP was added to a mixture of liposomes suspended in 5 mM acetate buffer as in (A). Following 1 min of incubation, concentrated buffer was added (arrow) to reach a final concentration of 150 mM. Fluorescence dequenching was determined as described under Materials and Methods.

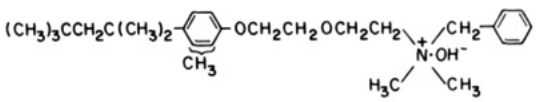

FIGURE 9: Structural formula of the quaternary detergent [[[(1,1,3,3-tetramethylbutyl)cresoxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA[OH]). Molecular weight of 443.7.

concentration used (150 mM), acetate buffer was found to be able to preserve the pH of the medium. The rate and extent of the PASP-induced fluorescence dequenching were pH independent within the range of 5–10 (not shown).

## Discussion

The results in the present work clearly show that DEBDA-[OH] (Figure 9) confers fusogenic properties upon liposomes composed of PC and chol. Microscopic observations and energy-transfer methods have demonstrated that addition of the negatively charged polymer, namely, PASP, to liposomes bearing DEBDA[OH] promotes their strong agglutination and subsequent liposome–liposome fusion. Neither agglutination

nor liposome–liposome fusion was observed when PASP was added to PC/chol liposomes lacking DEBDA[OH]. It is conceivable that the negatively charged PASP interacts electrostatically with the liposome-associated DEBDA cation. Evidently, such interaction will cause neutralization of the liposome's positively charged groups, thus inducing their agglutination and allowing a close proximity between the liposome bilayer. Moreover, due to its polymeric characteristics, PASP probably serves as a bridge between the phospholipid vesicles, causing the formation of huge agglutinates. Tight attachment between two lipid bilayers bearing DEBDA[OH] may induce destabilization and reorganization of the bilayer structure, a process that eventually will probably lead to liposome–liposome fusion. In addition to PASP, certain other negatively charged polymers such as dextran sulfate or DNA were able to promote agglutination and fusion of liposomes bearing DEBDA[OH] (not shown).

The view that DEBDA[OH] can mediate binding of liposomes to a negatively charged group of polymers or biological membranes is also evident from our recent experiments showing that $^{14}C$-labeled liposomes bearing DEBDA[OH] efficiently attached to human erythrocytes. Very little binding, if any, was observed with erythrocytes when negatively charged surface groups (sialic acid residues) have been removed by treatment with neuroaminidase (not shown).

Lipid bilayers containing molecules with detergent-like activity are unstable and amenable to membrane fusion processes (Lucy, 1978). The presence of lysolecithin as well as other derivatives of phospholipids has been demonstrated to confer fusogenic properties upon biological membranes or liposomes (Lucy, 1978; Martin & MacDonald, 1976). Liposomes composed of neutral lipids and lysolecithin have been shown to fuse extensively with erythrocyte membranes. Attachment of such liposomes to the erythrocyte membranes was promoted by the positively charged molecule stearylamine (Martin & MacDonald, 1976).

Vesicle–vesicle fusion was also observed, following the addition of the negatively charged dipicolinic acid to vesicles composed of the positively charged surfactant didodecyldimethylammonium bromide (Rupert et al., 1985). It is evident that partial neutralization of the positively charged detergent promotes heavy agglutination and allows a close proximity between the vesicle membranes. Obviously, close contact between vesicles bearing detergent may cause destabilization, resulting in membrane–membrane fusion (Lucy, 1978; Martin & McDonald, 1976).

The possibility that the reduction in energy transfer observed after the addition of PASP to a mixture of fluorescently labeled and unlabeled liposomes is due to contact-mediated lipid–lipid exchange and not to liposome–liposome fusion cannot be excluded. However, it seems that this is unlikely because of the following reasons: (a) The fluorescent probes *N*-NBD-PE and *N*-Rh-PE are practically unsusceptible to lipid–lipid exchange processes (Arvinte & Hildenbrand, 1984; Pagano et al., 1981), and (b) no reduction in the degree of energy transfer was observed, following agglutination of liposomes bearing DEBDA[OH], by reagents which do not cause neutralization of the positively charged DEBDA[OH]. Indeed, preliminary experiments have shown that the addition of avidin molecules to liposomes carrying covalently attached biotin induces strong liposome–liposome agglutination. Induction of such agglutination in a mixture of fluorescently labeled and unlabeled liposomes bearing DEBDA[OH] did not result in any significant change in the fluorescence intensity (unpublished experiments). Another reason (c) is that electron microscopic studies clearly revealed the formation of larger membrane vesicles after incubation of PASP with liposomes composed of PC/chol and bearing DEBDA[OH] (Figure 6).

The fluorescence dequenching observed could also result from solubilization of the liposomes, as in the case upon addition of Triton X-100 (Figure 1). However, our results clearly show that no fluorescence dequenching occurred when PASP was added to a population of only fluorescently labeled liposomes (Figure 4). Furthermore, even the addition of high amounts of DEBDA[OH] to fluorescently labeled liposomes caused only a very small and insignificant increase in the fluorescence intensity (Figure 1D).

The ability of liposomes to interact with PASP and undergo a process of liposome–liposome fusion was mainly dependent on two parameters: (i) the ratio of DEBDA[OH] to the lipid used (Figure 5) and (ii) the ionic strength of the incubation medium (Figure 8). Heavy agglutination and a high degree of fluorescence dequenching were observed only in liposomes incubated in a medium of relatively high ionic strength and with a ratio (w/w) of DEBDA[OH] to PC of 0.15 and above (Figures 5 and 8). Neither agglutination nor liposome–liposome fusion was observed when liposomes bearing DEBDA[OH] were incubated in a medium of low ionic strength, probably indicating that a certain amount of salt is required to allow electrostatic interaction between PASP and liposome-associated DEBDA[OH]. As opposed to other liposome–liposome fusion systems (Papahadjopoulos, 1978; Wilschut et al., 1985), temperature seems to be a less restrictive factor in this fusion system. Hence, liposome–liposome fusion was also observed upon incubation at relatively low temperatures such as 6 °C (Figure 7).

The results presented in this paper, together with previous work (Rupert et al., 1985; unpublished results), clearly show that liposomes bearing quaternary ammonium detergents possess fusogenic activity and are able to fuse both among themselves and with biological membranes. Liposome–liposome fusion can be induced, as was demonstrated in the present work, by the negatively charged polymer PASP. This raises the possibility that such liposomes will fuse with any membrane vesicle possessing a high density of negative charges. It is conceivable that this indeed occurs upon incubation of DEBDA[OH] containing liposomes with biological membranes. It will be of interest to study whether such liposomes can be used for the microinjection of macromolecules into cells.

**Registry No.** Chol, 57-88-5; PASP, 25608-40-6; PASP, SRU, 26063-13-8; DEBDA[OH], 26248-39-5.

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# Identification of a Receptor Binding Region on the $\beta$ Subunit of Human Follicle-Stimulating Hormone†

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ABSTRACT: Mouse epidermal growth factor (mEGF) and the $\beta$ subunit of human follicle-stimulating hormone (hFSH) (hFSH-$\beta$) have been shown to inhibit binding of intact hFSH to its testes membrane receptor in vitro. Both hFSH-$\beta$ and mEGF contain the tetrapeptide sequence Thr-Arg-Asp-Leu (TRDL). Previous results demonstrated that synthetic TRDL inhibited binding of intact hFSH to receptor. We therefore investigated the possibility that TRDL was located on an exposed region of FSH-$\beta$ using a polyclonal antiserum to hFSH [NHPP anti-hFSH batch 4 (AB4)] which recognized determinants on intact hFSH and its $\beta$ subunit, but not the $\alpha$ subunit. Pituitary FSH preparations from several mammalian species produced parallel inhibition curves in a heterologous [AB4 and $^{125}$I-labeled ovine FSH ($^{125}$I-oFSH)] radioimmunoassay with relative potencies similar to those observed for the same preparations assayed by radioligand receptor assay. This antiserum also competitively inhibited $^{125}$I-FSH binding to receptor. Thus, AB4 appeared to recognize antigenic determinants that are highly conserved and located at or near regions involved with hormone recognition of receptor for FSH. Synthetic TRDL inhibited 50% of $^{125}$I-hFSH binding to antiserum at a concentration of 1.36 mg/tube ($9 \times 10^{-3}$ M). Other tetrapeptides (Thr-Pro-Arg-Lys and Lys-Thr-Cys-Thr) had no inhibitory activity at comparable concentrations. A mixture of the free amino acids T, R, D, and L inhibited radioligand binding only at significantly higher concentrations than TRDL. The presence of TRDL in a receptor contact region of FSH was further suggested by its ability to inhibit the in vitro biological response to oFSH by cultured Sertoli cells at noncytotoxic levels. The reduced potency of TRDL relative to FSH in all three assay systems suggests that it represents only a portion of a larger determinant on the intact hormone and its $\beta$ subunit. These results support a model of hormone–receptor interaction involving multiple, discrete contact points (determinants), some of which influence binding while others may be involved with initiation of a cellular response. Our results further suggest that TRDL is a constituent of one FSH receptor binding region.

Although our knowledge of receptor recognition sites for glycoprotein hormones is still imprecise, their existence is usually inferred from structure–function studies of hormone–receptor interactions (Ward, 1978; Pierce & Parsons, 1981). Receptor recognition of individual hormones in serum occurs through formation of high-affinity hormone–receptor complexes. The interactions between hormone and receptor are thought to be characterized by the same general features as other protein–protein interactions that are well understood from studies such as X-ray crystallography. Electrostatic, van der Waals, and hydrophobic interactions are responsible for noncovalent association at protein–protein interfaces. Such contact surfaces can comprise structural features that include a sequence of adjacent amino acids in the polypeptide as well as residues that come into close proximity in the folded structure. Such sequences of adjacent amino acids have been extremely useful probes of antigenic structure (Geysen et al., 1987) and, more recently, of hormone contact surfaces that are important for interactions with their receptors (Keutman et al., 1987).

While investigating the nature of follicle-stimulating hormone (FSH)[1] binding to gonadal receptors, we observed that

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