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Transfer of Phospholipid and Protein into the Envelope of Gram-Negative Bacteria by Liposome Fusion†

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ABSTRACT: A liposome–bacterial fusion system was developed in order to introduce preformed terminal complement complexes, C5b–9, into the outer membrane of Gram-negative bacteria. Liposomes were prepared from a total phospholipid extract of *Salmonella minnesota* Re595. Fusion between liposomes and *Salmonella* sp. or *Escherichia coli* 17 was dependent on time, temperature, pH, and Ca^{2+} and PO_4^- concentration. Only *Salmonella* sp. with attenuated LPS core regions were able to fuse efficiently with liposomes. It was demonstrated that fusion of liposomes with *S. minnesota* Re595 or *E. coli* 17 under optimum conditions resulted in (i) quantitative transfer of the self-quenching fluorescent membrane probe octadecyl rhodamine B chloride from the liposomal bilayer to the bacterial envelope, (ii) transfer of radiolabeled liposomal phospholipid to the bacterial outer membrane and its subsequent translocation to the cytoplasmic membrane, demonstrated by isolation of the bacterial membranes following fusion, and (iii) delivery of liposome–entrapped horseradish peroxidase (HRP) to the periplasmic space, confirmed by a chemiluminescent assay. Following fusion of liposomes incorporating C5b–9 complexes with *S. minnesota* Re595 or *E. coli* 17, immunological analysis of the isolated membranes revealed C5b–9 complexes located exclusively in the outer membrane.

Liposomes have been used extensively as model systems for the study of membrane interactions and membrane fusion. Fusion is an important event in many processes associated with eukaryotic cell activity and has been extensively investigated in relation to intracellular membrane traffic including endocytosis, exocytosis, and viral genomic delivery to host cells (White et al., 1983; Pastan & Willingham, 1985; De Lisle & Williams, 1986; Morre et al., 1988). In contrast, there is no intracellular vesicle transport system in prokaryotes, and it is at present unclear whether protein-controlled fusion events have any role to play in secretion, conjugation, and cell division in bacteria (Bamford et al., 1987).

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Liposomes have also been used as delivery systems for the introduction of entrapped or incorporated molecules into cells. While the majority of studies have been performed in eukaryotic systems (Doyle & Baumann, 1983; Hallett & Campbell, 1980; Miller & Racker, 1976), liposome-encapsulated ferritin molecules have been successfully transferred into flagellated *Escherichia coli* cell envelope preparations, suggesting that liposome carriers might be used to introduce exogenous macromolecules into the cell envelope of intact Gram-negative bacteria (Lelkes et al., 1984). Jones and Osborn (1977a) developed a procedure for the bulk incorporation of the components of phospholipid bilayer vesicles into the cell envelope of viable *Salmonella typhimurium* cells and were able to demonstrate that whereas phospholipid and cholesteryl ester distributed rapidly and extensively between the outer and cytoplasmic membranes, vesicle-derived lipopolysaccharide or an incomplete precursor of lipid A remained exclusively in the outer membrane. In addition, liposome–bacteria fusion systems have been used to transfect *E. coli* (Fraley et al., 1979) and *Mycoplasma capricolum* (Nicolau & Rottem, 1982) with liposome-entrapped pBR322 plasmid DNA, although the extent of fusion and the rate of transformation were found to be very low.

An efficient and reliable procedure for the fusion of phospholipid vesicles with viable Gram-negative bacteria would facilitate the study of many membrane-associated phenomena. We have been interested in the formation and action of the complement membrane attack complex (Morgan et al., 1986; Stanley et al., 1986; Stanley & Luzio, 1988) and the mechanism of complement-mediated killing of Gram-negative bacteria (Taylor & Kroll, 1985). Following activation of the complement cascade by susceptible cells, the components of the terminal membrane attack pathway (C5–C9) assemble to form stable, discrete macromolecular transmembrane channels (C5b–9 complexes)[1] that, when correctly inserted into the bacterial envelope, are responsible for rapid loss of viability of target bacteria (Taylor & Kroll, 1985). In order to study this interaction, we wished to introduce preformed C5b–9 complexes into the envelope of viable bacteria; to this end, we have developed a fusion system for the bulk transfer of liposome phospholipid and incorporated C5b–9 complexes into the cell envelope of viable *E. coli* and *Salmonella sp.*

Materials and Methods

Chemicals and Reagents. Polyclonal antisera were obtained from Miles Laboratories Inc., Elkhart, IN. Octadecyl rhodamine B chloride (R18) was purchased from Molecular Probes Inc., Eugene, OR, and [2-^{3}H]glycerol was from Amersham Radiochemicals, Amersham, U.K. Luciferin *O*-phosphate was purchased from Novabiochem Ltd., Nottingham, U.K. All other chemicals were obtained from the usual sources and were of the highest purity commercially available.

Bacterial Strains and Medium. The bacteria used in this study are listed in Table I. *E. coli* 17 is a double phospholipase A negative mutant of K12 obtained from Dr. Shochichi Nojima, Teikyo University, Kanagawa, Japan. *Salmonella minnesota* Re595 is a mutant derived from the wild-type *S. minnesota* S218 by Dr. Jacik Hawiger, Vanderbilt University, Nashville, TN.

All strains except *S. typhimurium* LT2 and SH777 are complement sensitive as determined by the method of Taylor and Kroll (1983). Bacteria were grown at 37 °C with aeration in Mueller–Hinton broth.

Phospholipids and Their Analysis. S. minnesota Re595 was grown overnight in Mueller–Hinton broth containing [2-^{3}H]glycerol (0.3 mCi/mL), and total ^{3}H-labeled bacterial phospholipids were extracted according to Osborn et al. (1972). Phosphatidylethanolamine accounted for 77%, phosphatidylglycerol for 13.6%, and cardiolipin for 9.4% of the radiolabel as determined by thin-layer chromatography. Total phospholipid determinations were performed according to the method of Stein and Smith (1982). Phospholipid extracts were stored in chloroform/methanol (2:1) under nitrogen at −20 °C.

Preparation of Liposomes. ^{3}H-Labeled phospholipids in chloroform/methanol (2:1) were dried under nitrogen and dispersed in 2% (w/v) NaDOC at a concentration of 5 mg/mL. Liposomes were prepared by removal of detergent by dialysis against the desired detergent-free buffer. Dialysis was carried out at 4 °C with stirring for 48 h. A constant buffer flow through the dialysis chamber of 150 mL h^{-1} (mL of phospholipid suspension)$^{-1}$ was maintained. Liposomes were labeled with the fluorescent probe R18; a stock solution was prepared by dissolving the probe in DMSO to a concentration of 20 mM (stored at −20 °C). The fluorescent probe was mixed with the phospholipid suspension prior to dialysis to give a self-quenching concentration of 7.5 mol %. Determination of self-quenching of R18 fluorescence was made according to Hoekstra et al. (1984). Liposomes entrapping HRP and luminol were prepared by mixing equal volumes of phospholipid suspension with HRP at 1 mg/mL in NaDOC and luminol at 10 μg/mL in NaDOC prior to dialysis. Free HRP and luminol were removed by passing the liposomes over a Sepharose CL-4B column (0.7 cm × 25 cm) equilibrated with 130 mM NaCl/10 mM sodium acetate/10 mM Na_2HPO_4/5 mM Tris-HCl, pH 7.0 (CAPT buffer).

C5b–9 from human serum was isolated on rabbit erythrocyte membranes following alternative pathway complement activation by the method of Bhakdi and Tranum-Jensen (1982), and purified by column chromatography using the method of Biesecker et al. (1979). The complexed terminal proteins of the complement pathway, C5b–9, were incorporated into liposomes by mixing purified C5b–9 complexes in 2% (w/v) NaDOC with phospholipid before dialysis. A lipid:protein ratio of 5:1 (w/w) was routinely used. Incorporation of C5b–9 was confirmed by rocket immunoelectrophoresis of fractions following 10–45% (w/w) sucrose density gradient centrifugation (Bhakdi & Tranum-Jensen, 1980) and by electron microscopy. PCS revealed the liposomes and C5b–9-incorporated liposomes to have a mean diameter of 210 and 230 nm, respectively. Preparations were stable for 5 days at 4 °C after which time aggregation and altered fluorescent properties became apparent. Electron microscopy revealed the liposomes to be unilamellar structures with apparent diameters of 150–200 nm.

Fusion of Bacteria and Liposomes. Bacteria were grown to $E^{1\,cm}_{600\,nm} = 0.6$ which corresponded to approximately 4×10^8 CFU/mL. The cells were harvested by centrifugation, washed, and resuspended to their original volume in liposome dialysis buffer. Normally, the incubation mixture consisted of 50 μL of cell suspension, 5 μL of R18–liposome preparation (ap-

[1] Abbreviations: C5b–9, terminal membrane attack complex of complement; CAPT buffer, 130 mM NaCl, 10 mM sodium acetate, 10 mM Na_2HPO_4, and 5 mM Tris-HCl, pH 7.0; CFU, colony-forming unit; CM, cytoplasmic membrane; DMSO, dimethyl sulfoxide; GVB^{2+}, veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$; HRP, horseradish peroxidase; LPS, lipopolysaccharide; NaDOC, sodium deoxycholate; OM, outer membrane; PBS, phosphate-buffered saline; PCS, photocorrelation spectroscopy; R18, octadecyl rhodamine B chloride.

proximately 25 μg of phospholipid), and 400 μL of liposome dialysis buffer. Fusion was initiated by adding $CaCl_2$ to a concentration of 20 mM and the mixture incubated at 37 °C in a shaking water bath. After the desired incubation time (usually 45 min), EDTA was added to a final concentration of 25 mM and the mixture maintained at room temperature for 5 min in order to terminate further phospholipid transfer and reverse visible Ca^{2+}-mediated nonspecific aggregation (Jones & Osborn, 1977a). The extent of fusion was assessed by measuring fluorescence (Hoekstra et al., 1984) in a Perkin-Elmer LS-5 luminescence spectrometer with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. All determinations were made at room temperature. A scale of fluorescence was produced by measuring R18-labeled liposome fluorescence before and after addition of Triton X-100 to 0.5% (v/v), the readings being taken as 0% and 100% fluorescence, respectively.

Delivery of HRP to the Periplasmic Space. Liposomes containing entrapped HRP and luminol were fused with *E. coli* 17 as described for R18 liposomes. Following EDTA treatment, cells were washed twice and resuspended to 100 μL in CAPT buffer. Following a recovery period of 45 min at room temperature, light production was measured after adding luciferin *O*-phosphate (0.6 mM), luminol (0.1 μM), and H_2O_2 (1 mM) (final concentrations). The final reaction volume was 500 μL, and light was measured every 10 s over a 30-min period in a LKB Luminometer 1250.

Fluorescence Microscopy. Samples were diluted in liposome dialysis buffer and applied to a microscope slide. A coverslip was pressed firmly over the sample and excess liquid removed from the edge of the coverslip with a tissue. Samples were then examined with a Leitz orthoplan microscope equipped for fluorescence microscopy using a 100× oil immersion phase objective and a 10× ocular lens. Photographs were recorded on Ilford XP-400 film that was push-processed to ASA 1200.

Isolation and Characterization of Bacterial Membranes. Following bacteria–liposome fusion and addition of EDTA, cells were separated from free liposomes by centrifugation through 18% (w/v) sucrose in 50 mM Hepes, pH 7.2. The cell pellet was washed once with PBS, and the membranes were isolated by osmotic lysis of the cells followed by sucrose density gradient centrifugation of membrane preparations (Kroll et al., 1983). Gradients of 4.5 mL were fractionated by inserting a glass capillary tube through the gradient and removing liquid from the bottom of the tube with a peristaltic pump at a flow rate of 12 mL/h. Fractions of 150 μL were collected, and the absorbance at 278 nm of each fraction was determined. The density of the fractions was determined by refractometry using a sucrose standard curve as reference. Radioactivity was measured using LKB Optiphase II scintillant in a Packard 1500 Tri-Carb liquid scintillation counter. NADH oxidase activity was assayed according to the method of Osborn et al. (1972).

Detection of C5b–9. A dot blot procedure was used to assay for C5b–9 complexes in membrane gradient fractions. A nitrocellulose membrane (0.2-μm pore size) was presoaked in PBS for 30 min and then clamped in a standard 96-well dot blot apparatus. Twenty-five-microliter samples were pipetted into the wells and allowed to stand for 15 min; excess fluid was then removed by vacuum. Each well was washed 3 times with PBS; the membrane was removed from the apparatus and blocked by incubation in 0.05% Tween-20 in PBS (PBST) containing 1% BSA for 90 min at room temperature. The membrane was then incubated overnight at 4 °C in PBST + 1% BSA containing a 1:2000 dilution of goat anti-human C9 antibody. Following four 10-min PBST washes, the membrane was incubated for 60 min at room temperature in PBST + 1% BSA containing peroxidase-conjugated rabbit anti-goat IgG antibody. After a further four 10-min washes, peroxidase activity was detected by using 100 mM Tris-HCl, pH 7.4, containing 0.05% (w/v) 3,3-diaminobenzidine and hydrogen peroxide at a dilution of 1:5000. Following color development, the spots were scanned by reflection absorbance at 530 nm using a Joyce-Loebl Chromoscan 3.

Unless otherwise stated, all figures show representative experiments.

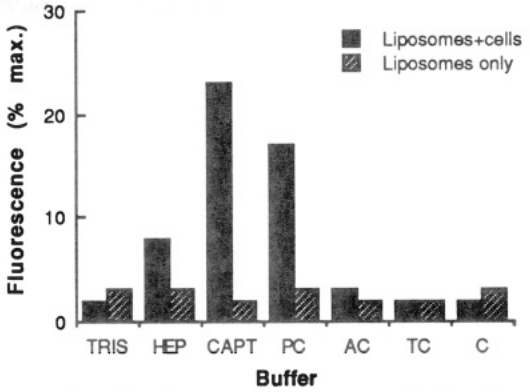

FIGURE 1: Effect of buffer composition on extent of fusion. Liposomes were incubated with and without *S. minnesota* Re595 for 45 min at 37 °C in the indicated buffer. All buffers were at pH 7.0 and contained 20 mM Ca^{2+}. Abbreviations: TRIS, 10 mM Tris-HCl; HEP, 10 mM Hepes; CAPT, 130 mM NaCl, 10 mM sodium acetate, 10 mM Na_2HPO_4, and 5 mM Tris-HCl; PC, 10 mM Na_2HPO_4/145 mM NaCl; AC, 10 mM sodium acetate/145 mM NaCl; TC, 5 mM Tris-HCl/150 mM NaCl; C, 155 mM NaCl. Liposomes were prepared by dialysis of phospholipid and R18 in NaDOC against the buffer under test.

RESULTS

Cell Fusion. Fusion of a lipid bilayer containing self-quenching concentrations of the R18 probe with an unlabeled membrane results in dilution of the probe in the target membrane and a relief of self-quenching (Hoekstra et al., 1984). Figure 1 shows the effect of buffer composition on fusion between *S. minnesota* Re595 and liposomes labeled with self-quenching concentrations of R18. The increase in levels of fluorescence following fusion was found to be similar for *E. coli* 17 under all conditions tested. Fusion required Ca^{2+} ions at a minimum concentration of 10 mM and was temperature dependent. Incubation at 37 °C in the presence of Ca^{2+} resulted in fusion between cells and liposomes whereas incubation at 0 °C failed to produce fusion. Figure 1 shows that, in addition to Ca^{2+}, phosphate appears to play an important role in the fusion process. CAPT buffer provided the best conditions for fusion; Ca^{2+} is an absolute requirement for fusion, and of the other CAPT buffer components, only phosphate will individually provide suitable conditions for fusion. There appears to be a synergistic effect when all are used together, and a phosphate concentration of 10 mM was found to be optimal (data not shown). The 10 mM Tris-HCl and 10 mM Hepes buffers correspond to those used by Jones and Osborn (1977a,b) for fusion between liposomes and *S. typhimurium*. We were unable to achieve fusion in 10 mM Tris-HCl. Some fusion was achieved in 10 mM Hepes although the fluorescence obtained was only one-third of that seen using CAPT buffer for 45 min at 37 °C. Longer incubation times of up to 90 min produced no significant increase in fluorescence with either buffer which suggests that the extent of fusion is diminished in Hepes buffer.

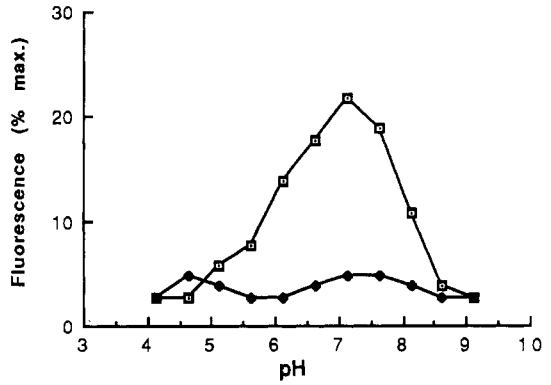

FIGURE 2: Effect of pH on extent of fusion. Liposomes were incubated with *S. minnesota* Re595 for 45 min at 37 °C (□) or 0 °C (♦) in CAPT buffer adjusted to the indicated pH with NaOH or HCl as appropriate. Liposomes were preincubated for 10 min at a new pH before addition of cells resuspended in buffer at the same pH.

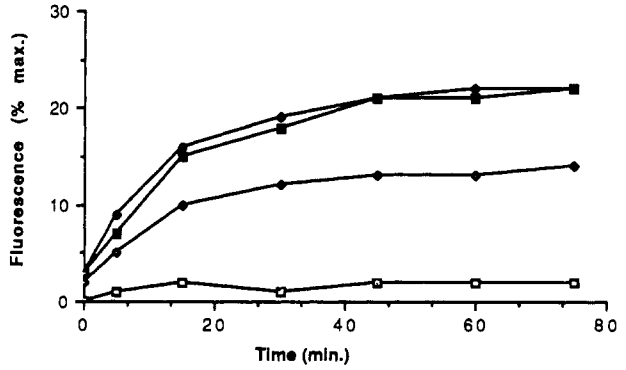

FIGURE 3: Effect of cell concentration on rate and extent of fusion. Liposomes (25 μg of phospholipid) were incubated for the indicated times with *S. minnesota* Re595 at 3×10^7 CFU (♦), 1.5×10^7 CFU (■), and 7.5×10^6 CFU (◊). Liposomes were also incubated in the absence of bacteria (□). All incubations were at 37 °C in CAPT buffer, pH 7, containing 20 mM Ca^{2+}.

The maximum extent of fusion between liposomes and cells in CAPT buffer was found to occur at pH 7.0. No fusion was recorded below pH 5 or above pH 8.5 (Figure 2). A high pH was found to result in extensive aggregation of liposomes and cells which could not be reversed by addition of EDTA.

When the effect of different buffers on liposome–cell fusion was determined, the dialysis buffer used for liposome preparation was the same as that to be used in the test system.

Relief of R18 Self-Quenching. Figure 3 shows that the amount of target membrane available, i.e., number of cells, affects the rate of fusion and the level of fluorescence attained (extent of fusion). At 37 °C, there was a rapid initial rate of fusion with very little fusion activity occurring after about 45 min. Below a threshold level, halving the amount of target membrane presented to the same number of liposomes resulted in a proportional decrease in fluorescence. The concentration of cells used for fusion assays throughout this study was the minimum number producing maximum relief of R18 self-quenching. This corresponds to about 1.5×10^7 cells per 25 μg of liposome phospholipid containing 7.5 mol % R18. Since a further increase in cell number produced no increase in the levels of fluorescence following fusion, it would appear that an experimental infinite dilution of the R18 probe in the bacterial membranes was achieved. However, as addition of Triton X-100 to the same concentration of R18–liposomes produced approximately 4-fold higher levels of fluorescence, it appears that there was still considerable quenching of the probe within the bacterial membrane following fusion. It is unlikely that the lower than expected levels of fluorescence obtained following fusion of R18–liposomes with bacteria are due to bound intact liposomes because fluorescence microscopy of samples following fusion revealed only fluorescently labeled bacteria.

Table I: Bacterial Strains

species	strain	chemotype[a]
Salmonella minnesota	Re595	Re
Salmonella typhimurium	SL1102	Re
	SL1032	Rd1
	SL848	Rc
	HIS642	Ra
	SH777	SR
	LT2	S
Escherichia coli	17	*b*

[a] LPS structures for the various chemotypes of *Salmonella* are as defined in Lugtenberg and van Alphen (1983). [b] *E. coli* K12 core.

Effect of Divalent Cations on Fusion. Replacement of 20 mM $CaCl_2$ with 20 mM $MgCl_2$ or 20 mM $BaCl_2$ in incubating mixtures of cells and R18–liposomes caused visible aggregation similar to that observed with $CaCl_2$, but did not result in any fusion as determined by fluorescence. The fluorescence micrograph in Figure 4A shows the fluorescently labeled bacteria following fusion with R18–liposomes in 20 mM Ca^{2+} at 37 °C. More than 90% of the bacterial cells were fluorescently labeled. Figure 4B shows that the R18–liposomes bound to *S. minnesota* Re595 in the presence of Mg^{2+} at 37 °C, but did not fuse. Since the quenching of R18 in liposomes is not complete, the outline of the bacteria can be traced by the bound fluorescent liposomes. Identical results were obtained when cells were incubated at 0 °C with R18–liposomes in the presence of 20 mM Ca^{2+}. As previously indicated, before taking any fluorescence readings or preparing slides for fluorescence microscopy, it was necessary to add EDTA to the cells and liposomes to reverse nonspecific cation-induced aggregation. Following addition of EDTA, liposomes which were bound, but not fused (as with Mg^{2+}), remained only weakly associated with the bacterial surface since incubation for 20 min at room temperature or vortexing was sufficient to remove bound liposomes.

Liposome Fusion with Other Bacterial Strains. Due to mutations in the gene clusters encoding enzymes involved in LPS synthesis or assembly, the series of *S. typhimurium* mutants listed in Table I possess a progressive attenuation of their LPS (Lugtenberg & van Alphen, 1983). The results shown in Figure 5 indicate that the ability of R18-labeled liposomes to fuse with *Salmonella* is dependent on the degree of substitution of the LPS core. Both Re mutants (*S. minnesota* Re595 and *S. typhimurium* SL1102), which contain only 2-keto-3-deoxyoctonic acid (KDO) in their core region, showed rapid and extensive fusion with the R18–liposomes. As the degree of substitution of the core region increases, so the extent of fusion with R18–liposomes decreases. Wild-type *S. typhimurium* LT2 and *S. typhimurium* SH777 showed no fusion. *S. typhimurium* SH777 lacks the enzyme to polymerize sugar units of its O-side chain but possesses a complete core region.

Isolation and Characterization of Bacterial Membranes following Fusion. In order to confirm that fusion between cells and liposomes had taken place, bacterial membranes were isolated following incubation of *S. minnesota* Re595 and *E. coli* 17 with R18–liposomes. Liposomes were prepared by using ^3H-labeled phospholipids in order to determine whether phospholipid transfer from liposome to cell membrane had occurred. Following incubation of cells and liposomes and addition of EDTA, bacteria were pelleted by centrifugation

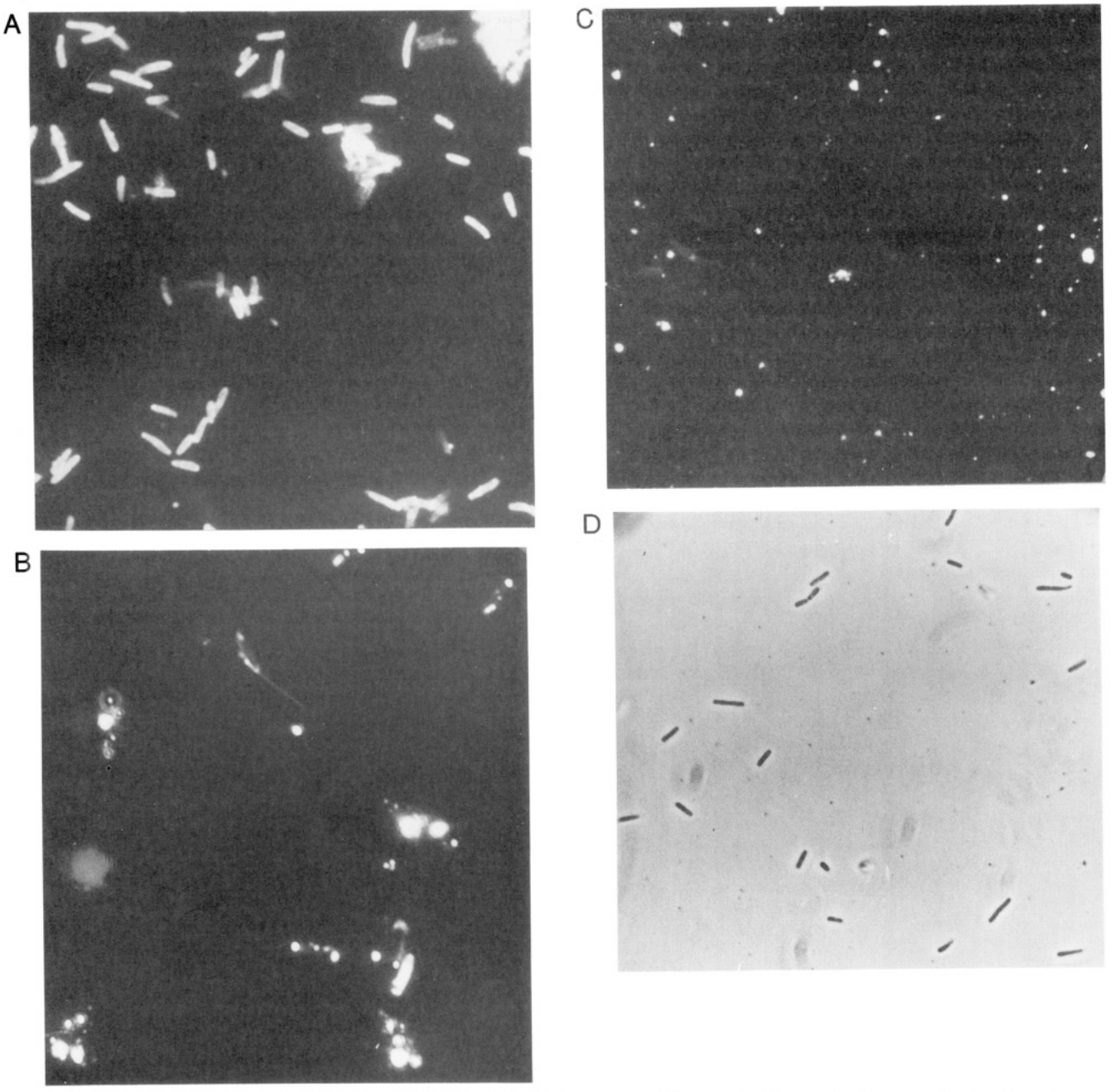

FIGURE 4: Micrographs of incubating *S. minnesota* Re595 and R18–liposomes. Cells were incubated with liposomes in CAPT buffer, pH 7, for 45 min at 37 °C. (A) +20 mM Ca^{2+}; (B) +20 mM Mg^{2+}; (C and D) no divalent cations. (A), (B), and (C) are fluorescent images, and (D) is a phase-contrast image of the same field as (C). Before preparation of all slides, EDTA was added to incubating mixtures.

through 18% sucrose; free liposomes were unable to penetrate the sucrose. When cells and liposomes were incubated under nonfusogenic conditions, e.g., no Ca^{2+} or at 0 °C, 95–100% of radioactivity failed to penetrate the sucrose. Incubation of cells and liposomes in CAPT buffer with Ca^{2+} at 37 °C, however, resulted in greater than 95% of added ^3H counts sedimenting with bacteria.

Membranes were subsequently isolated by cell lysis and separated by 20–60% (w/v) sucrose density gradient centrifugation. The heavier outer membrane (OM) and the cytoplasmic membrane (CM) were identified by their apparent buoyant densities and NADH oxidase content (Osborn et al., 1972). The lighter CM fractions contained approximately 4 times higher NADH oxidase activity than OM (data not shown). After fusion, between 80 and 90% of radioactivity applied to sucrose gradients was always recovered in the membrane fractions, and of this, about 75% was always associated with the CM. These results suggest that fusion of liposomes with *S. minnesota* Re595 or *E. coli* 17 results in bulk transfer of phospholipid to the OM followed by translocation of the phospholipid to the CM. These findings are in broad agreement with those of Jones and Osborn (1977a,b), who have presented a more detailed study of phospholipid translocation in *S. typhimurium*. As previously mentioned, they achieved fusion by incubation of cells and liposomes in 10 mM Hepes, pH 7.2. In our system, when incubations were carried out in 10 mM Hepes, the extent of fusion was considerably lower with only about 15% of liposomal ^3H-labeled phospholipid being transferred to the bacterial membrane (Figure 6b). In agreement with the fluorescence data on fusion (see earlier), longer incubation times of 90 min did not result in further phospholipid transfer (data not shown). Incubation of cells and liposomes under similar conditions in CAPT buffer, pH 7, resulted in the transfer of 80–90% of added liposomal ^3H-labeled phospholipid to bacterial membranes (Figure 6a).

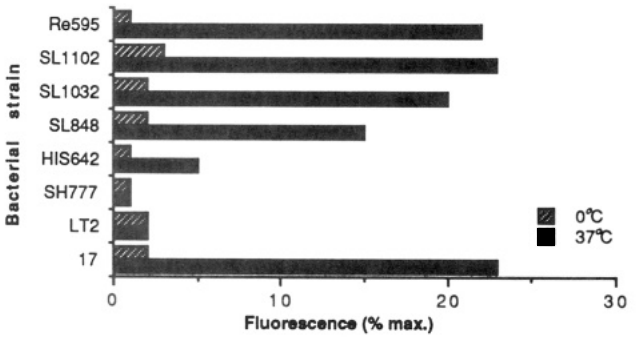

FIGURE 5: Fusion of liposomes with different bacterial strains. Liposomes (25 μg of phospholipid) were incubated with 1.5×10^7 CFU of the indicated bacterial strain at 37 °C in CAPT buffer, pH 7, containing 20 mM Ca^{2+}. Re595 represents a *S. minnesota* strain, and 17 represents an *E. coli* strain. The remainder are strains of *S. typhimurium*. Refer to Table I for further details.

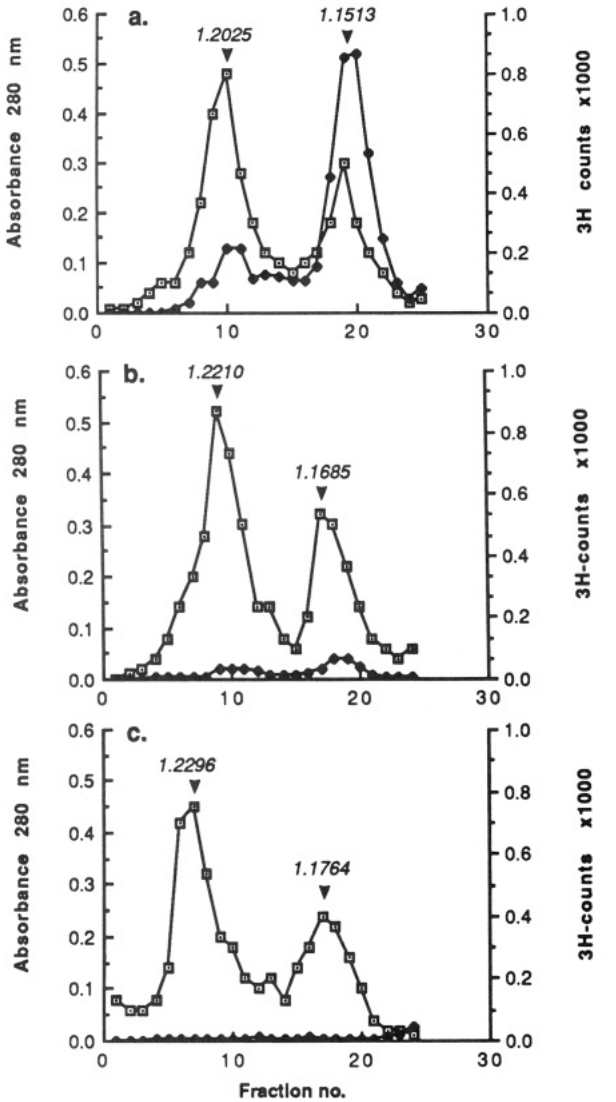

FIGURE 6: Sucrose density gradient centrifugation of *S. minnesota* Re595 membranes following incubation with liposomes. Cells and ^3H-labeled liposomes were incubated for 45 min at 37 °C in (a) CAPT buffer, pH 7, containing 20 mM Ca^{2+}, (b) 10 mM Hepes, pH 7, containing 20 mM Ca^{2+}, and (c) CAPT buffer, pH 7, with no Ca^{2+}. A total cell membrane fraction was prepared as described and applied to the top of the sucrose gradient. Following centrifugation, the gradient was fractionated, and fractions were analyzed by the absorbance at 280 nm (⊡) and ^3H radioactivity (♦). The figures alongside each peak represent density in grams per centimeter cubed.

Both the CM and OM had decreased buoyant densities following fusion. This appears to be a function of the amount

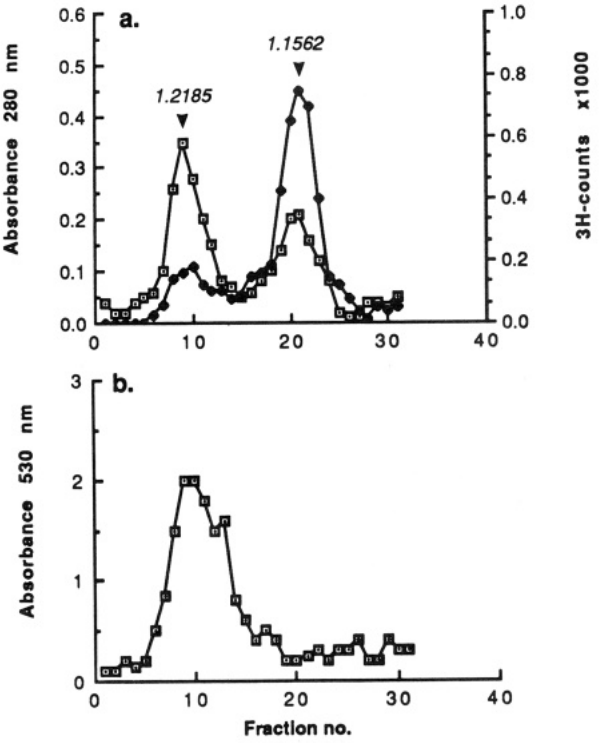

FIGURE 7: Sucrose density gradient centrifugation of *S. minnesota* Re595 membranes following incubation with C5b–9-incorporated liposomes. Cells were incubated for 45 min at 37 °C in CAPT buffer, pH 7, containing 20 mM Ca^{2+}. (a) Analysis of membrane fractions by absorption at 280 nm (⊡) and ^3H radioactivity (♦). (b) Assay for C5b–9. Membrane fractions were assayed for C5b–9 by a dot blot procedure (see Materials and Methods). Following color development, dots were scanned by reflection absorbance at 530 nm.

of transferred phospholipid since the more extensive the fusion, the lighter the membranes (cf. Figure 6a,b).

Transfer of C5b–9 to Bacterial Membranes. Liposomes incorporating C5b–9 complexes were incubated with *S. minnesota* Re595 or *E. coli* 17 under the optimum conditions for fusion. Similar results were obtained with both bacteria. Figure 7a shows that resulting phospholipid transfer and subsequent translocation across the membranes were similar to those found with liposomes containing no C5b–9 complexes (Figure 6a).

After fusion of cells with C5b–9-incorporated liposomes, isolated membrane gradient fractions were assayed for C9 (the major component of C5b–9) by a dot blot procedure. Figure 7b shows that the protein was located exclusively in the OM. The lesser decrease in the buoyant density of the OM can be explained by the effect of C5b–9 incorporation, the transferred, heavier protein molecules negating to some extent the effect of the transferred, lighter phospholipid. A decrease in the buoyant density of the CM was still observed, although again a slightly lesser decrease, presumably because there was no translocation of C5b–9 from the OM. The reason for this lesser decrease in the buoyant density of the CM is not known, but it may be possible that the transferred C5b–9 complexes in the OM are interfering with macromolecular traffic between the two membranes.

Viability of Bacteria following Fusion. Fusion of liposomes incorporating C5b–9 with both *S. minnesota* Re595 and *E. coli* 17 under conditions optimal for fusion and at the concentrations described resulted in an approximate 50% reduction in cell viability. This effect was not attributable to incorporation of C5b–9 into the OM since liposomes without C5b–9 produced a similar reduction in viability. Fusion of liposomes and cells in 10 mM Hepes resulted in a 10% reduction in

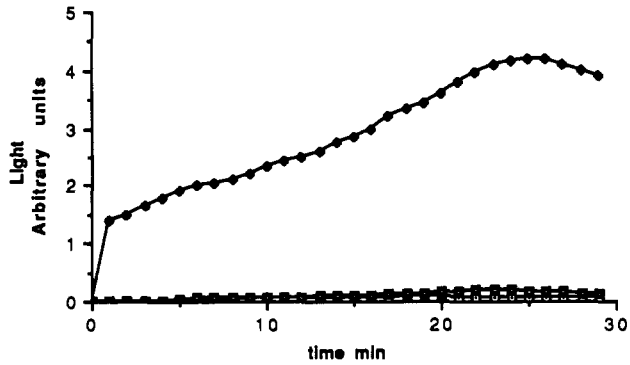

FIGURE 8: Light produced with time following fusion of *E. coli* 17 with liposomes. *E. coli* 17 were incubated with HRP + luminol-entrapped liposomes under fusogenic conditions (♦) and nonfusogenic conditions (□). Following washing of cells and a recovery period, luciferin *O*-phosphate (0.6 mM), luminol (0.1 μM), and H_2O_2 (1 mM) (final concentrations) were added externally to cells, and light production was measured. Luminol and H_2O_2 only were also added externally to washed fused cells (■).

viability. Cell viability was not a prerequisite for fusion with liposomes since *S. minnesota* Re595 exposed to lethal concentrations of lysozyme-free serum would also fuse with R18–liposomes; the extent of fusion, however, was reduced by approximately 75%. A reduction of approximately 25% in fusion efficiency was also found for *S. minnesota* Re595 when in the stationary phase.

Delivery of Liposome-Entrapped Enzyme to the Periplasmic Space. We have demonstrated the delivery of a liposome-entrapped enzyme, HRP, to *E. coli* 17 by linking the reaction of the entrapped enzyme to the reaction of an enzyme located in the periplasmic space. Luciferin *O*-phosphate (M_r 360) added externally to *E. coli* 17 will diffuse across the outer membrane where it is cleaved to produce luciferin and phosphate by the periplasmic enzyme alkaline phosphatase. The luciferin thus liberated can then act as an enhancer (Whitehead et al., 1983) for the reaction:

$$\text{HRP} + H_2O_2 + \text{luminol} \rightarrow \text{light}$$

Luciferin *O*-phosphate will not enhance this reaction unless hydrolyzed to luciferin. Figure 8 shows delivery of liposome-entrapped HRP to the periplasmic space of *E. coli* 17 by measurement of light production following fusion. HRP delivered to the periplasmic space is too large to diffuse across the OM [diffusion limit approximately 600 daltons (Nikaido & Vaara, 1985)]. The presence of luciferin *O*-phosphate outside the fused cells resulted in a 100-fold enhancement of light production, demonstrating that the site of its hydrolysis (the periplasmic space) is continuous with the intraliposomal space containing the HRP and luminol necessary for the light-producing reaction.

Addition of luciferin *O*-phosphate, luminol, and H_2O_2 to cells following fusion with liposomes containing no entrapped HRP did not result in any light production (data not shown).

Discussion

The self-quenching fluorescent probe octadecyl rhodamine B chloride (R18) has been used previously for continuous monitoring of membrane fusion in artificial, viral, and eukayotic systems (Duzgunes et al., 1987; Klappe et al., 1986; Nir et al., 1986). This paper reports on the use of this probe to monitor fusion between liposomes and bacteria. However, due to the conditions needed for fusion of liposomes and bacteria, it was not possible to continuously monitor fusion as in the previously reported assays. The aggregation of liposomes and bacteria caused by the high Ca^{2+} concentrations used results in a considerable decrease in fluorescence, presumably due to fewer particles crossing the light beam. It was therefore necessary to add EDTA to reverse cation-induced aggregation before any fluorescence determination.

Hoekstra et al. (1984) demonstrated that the R18 probe, once inserted into a membrane, will not dissociate from the membrane either by spontaneous diffusion of the free monomers through the aqueous phase or by a collision-mediated transfer process. That the same is true for R18 incorporated into liposomes under the conditions used for fusion in this study is indicated by the following: (i) Free probe added to a suspension of *S. typhimurium* LT2 fluorescently labeled the bacteria due to insertion of the probe into the membrane. No such labeling was achieved by addition of R18–liposomes, which would be expected if there was spontaneous diffusion of the probe through the aqueous medium. (ii) The rate of increase in fluorescence was dependent on the concentration of target membrane (i.e., number of cells) (Frank et al., 1983). (iii) Mg^{2+}, Ba^{2+}, and Ca^{2+} ions all induced massive aggregation of R18–liposomes and cells, but only with Ca^{2+} was any increase in fluorescence detected. It appears unlikely, therefore, that transfer of the probe is occurring between aggregated membranes.

Other evidence for liposome–bacterial fusion rather than association was provided by the isolation of bacterial membranes following fusion assays. ^{3}H-Labeled liposome phospholipids were quantitatively recovered in the CM and OM fractions on sucrose gradients following incubation of *S. minnesota* Re595 or *E. coli* 17 with liposomes under suitable conditions. Protein transfer from liposomes to the bacterial envelope was indicated by the presence of C5b–9 in OM fractions following incubation of cells with liposomes containing incorporated C5b–9.

The final criterion of true liposome–bacterial fusion would be to demonstrate the mixing of liposome and periplasmic space contents. In the present experiments, continuity between the intraliposomal space and the periplasmic space following fusion was shown by delivery of a liposome-entrapped enzyme, HRP, to the bacterial cell. HRP is too large to diffuse across the OM once delivered to the periplasmic space. The assay demonstrating its delivery was based on access of luciferin *O*-phosphate to periplasmic alkaline phosphatase to produce luciferin and its subsequent light-enhancing reaction with HRP, H_2O_2, and luminol. While the measurement of light production provided a simple means of assessing delivery of liposome content to the periplasmic space, the assay presented in this paper is complex. Unfortunately, a simpler assay in which liposome-entrapped firefly luciferase is delivered to the periplasmic space to react directly with luciferin (again produced by periplasmic alkaline phosphatase cleavage of luciferin *O*-phosphate) and ATP to generate light was not possible since the liposome preparation methods inactivated the luciferase. Nevertheless, in the present experiments, the periplasmic production of luciferin from luciferin *O*-phosphate resulted in a 100-fold enhancement of light production from liposome-supplied HRP in the presence of H_2O_2 and luminol. This clearly demonstrated the delivery of liposome-entrapped HRP to the periplasmic space.

Fusion between liposomes and bacteria was dependent on Ca^{2+} concentration, temperature, time, buffer composition, pH, LPS chemotype, and cell and liposome concentration. Jones and Osborn (1977a) reported on the interaction of *S. typhimurium* with phospholipid vesicles, and while much of our data corroborate their findings, there are some discrepancies. Jones and Osborn used either 10 mM Tris, pH 7.5

(1977a), or 10 mM Hepes, pH 7.5 (1977b), as their fusion buffers. We were unable to achieve any fusion with 10 mM Tris in our system and achieved a 5-fold increase in phospholipid transfer from liposomes to bacterial membranes when using CAPT buffer, pH 7, in place of 10 mM Hepes. It would appear from our results that this increased potential for fusion is due to the presence of phosphate in addition to Ca^{2+} in the fusion buffer. A synergistic effect of Ca^{2+} and phosphate on membrane fusion has been previously reported (Fraley et al., 1980). This effect is probably due to the formation of calcium phosphate complexes on the OM which would cause dehydration of the membrane and may also induce phase separation of OM components (Brass, 1986), either of which might facilitate fusion. Under optimum conditions, about 90% of liposomal phospholipid was transferred to bacterial membranes of the Re chemotype. Of the phospholipid transferred to the OM, more than 75% was found to be translocated to the CM. This is considerably more than that reported by Jones and Osborn (1977b), who demonstrated that phospholipids were translocated between the membranes in a relatively nonspecific manner to equilibrium. This difference may be a function of an increased volume of phospholipids transferred and a greater immediate capacity of the CM to accommodate them.

Much emphasis has been placed on the role of Ca^{2+} and its interaction with lipid bilayers during fusion in both artificial and biological systems (Duzunges, 1985; Lucy, 1984; Ohki & Duax, 1986). In many of the systems described, there was found to be an absolute Ca^{2+} requirement for fusion (Papahadjopoulos et al., 1979), although not at the high concentrations found to be necessary for fusion between liposomes and bacteria. It is thought that the effects of Ca^{2+} are probably due to neutralization of negative surface charges and dehydration of cell surfaces (Borovjagin et al., 1987). It has been suggested that hexagonal II phase lipids are important for membrane fusion (Varkleij et al., 1984) and Ca^{2+} is able to trigger a bilayer–hexagonal II phase transition of some phospholipids. It is interesting that cardiolipin, a phospholipid found in Gram-negative membranes and the liposomes used in this study, will undergo such a transition upon addition of Ca^{2+} ions, but not Mg^{2+} (Verkleij et al., 1984). Phosphatidylethanolamine, the major phospholipid of Gram-negative bacteria, can also adopt both bilayer and hexagonal II arrangements, the latter arrangement being favored at temperatures above about 10 °C (Cullis & Hope, 1985). It should be noted, however, that the lipid component of normal *E. coli* and *S. typhimurium* is almost certainly structured into a lipid bilayer (Cronan et al., 1987).

In considering the importance of LPS in fusion between liposomes and Gram-negative bacteria described in this paper, an obvious explanation for the extent of fusion being inversely related to the polysaccharide chain length would be that of steric hindrance. Steric hindrance by the polysaccharide chains may inhibit interaction of liposomes and the cell surface or interaction of Ca^{2+} ions and negatively charged groups on the cell surface, neutralization of which may be necessary for fusion. However, the progressive loss of LPS components from mutants of *S. minnesota* is known to be accompanied by changes in the envelope protein composition (Parton, 1975), and it is therefore not inconceivable that the ability of some Gram-negative bacteria to fuse with liposomes is a consequence of the OM protein composition or concentration associated with LPS type rather than a direct consequence of polysaccharide chain length. Indeed, high concentrations of divalent cations have been shown to cause considerable loss of OM proteins from rough strains of Gram-negative bacteria, but not smooth wild type (Stan-Lotter & Sanderson, 1981). It is interesting that *E. coli* 17 which possesses a complete core region will efficiently fuse with liposomes, unlike *S. typhimurium* strains possessing complete or nearly complete core regions. It is clear that membrane proteins play an important role in membrane fusion between some eukaryotic cells and viruses (Choppin & Scheid, 1980; Doms et al., 1985; White et al., 1983), and Bamford et al. (1987) recently reported a targeted, protein-dependent fusion between the bacterial virus 06 and *Pseudomonas syringae* which was independent of divalent cations and pH. Changes in protein composition of the OM are probably reflected by changes in phospholipid, and it is likely that proteins lost from the OM are replaced by phospholipids (Lugtenberg & van Alphen, 1983). Borovjagin et al. (1987) have shown that high Ca^{2+} concentrations promote the enrichment of phospholipid in the outer leaflet of the OM of wild-type Gram-negative bacteria, and they suggest that membrane phospholipids are of primary importance in the processes leading to membrane fusion. Fusion between liposomes and rough mutant bacteria reported in this paper suggests the presence of phospholipids in the outer leaflet of the OM although it is thought that the OM outer leaflet of many wild-type enterobacteria is completely deficient in phospholipids (Lugtenberg & van Alphen, 1983). Another possibility for the effect of Ca^{2+} may be the loss of OM proteins (Stan-Lotter & Sanderson, 1981) leading to increased levels of phospholipid in the OM, these being responsible for enhancement of liposome–cell membrane contact formation and fusion. The LPS chain length could have a role to play in modulating the effect of Ca^{2+}, if not simply physically distancing liposomes from the cell surface.

Fusion of liposomes with *S. minnesota* Re595 and *E. coli* 17 under optimal conditions leads to a loss of viability of around 50%. Such a reduction was not found by Jones and Osborn (1977a), but the difference may be attributable to more extensive phospholipid transfer. From the preliminary results presented on C5b–9 transfer to the OM and cell viability, it appears that under the conditions and concentrations used, preformed C5b–9 complexes cannot alone cause the death of serum-sensitive bacteria. It is clear, however, that further quantitative work on incorporation of C5b–9 into liposomes and of C5b–9 transfer to bacteria must be undertaken. Studies are under way to demonstrate that the transferred complexes incorporated into the liposomes and transferred to the bacterial OM are still functional as water-filled pores.

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