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Low pH-induced Fusion of Liposomes with Membrane Vesicles Derived from *Bacillus subtilis**

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We have investigated the pH-dependent interaction between large unilamellar phospholipid vesicles (liposomes) and membrane vesicles derived from *Bacillus subtilis*, utilizing a fluorescent assay based on resonance energy transfer (RET) (Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) *Biochemistry* 20, 4093–4099). Efficient interaction occurs only with negatively charged liposomes, containing cardiolipin or phosphatidylserine, as revealed by the dilution of the RET probes from the liposomal bilayer into the bacterial membrane. The initial rate of fluorophore dilution increases steeply with decreasing pH. The interaction involves a process of membrane fusion, as indicated by (i) the proportional transfer of cholesteryl-[1-^{14}C] oleate, ^{14}C-labeled egg PC, and the RET probes from the liposomes to the bacterial vesicles, (ii) the formation of interaction products with an intermediate buoyant density, and (iii) the appearance of colloidal gold, initially encapsulated in the liposomes, in the internal volume of fused structures as revealed by thin-section electron microscopy. Treatment of *B. subtilis* vesicles with trypsin strongly inhibits the fusion reaction, indicating the protein dependence of the process. Vesicles derived from *Streptococcus cremoris* or from the inner membrane of *Escherichia coli* also show low pH-dependent fusion with liposomes. The fusion process described in this paper may well be of considerable importance to studies on the mechanisms of membrane fusion and to studies on the structure and function of bacterial membranes. In addition, the fusion reaction could be utilized to deliver foreign substances into bacterial protoplasts.

Enrichment of the lipid bilayer portion of biological membranes with exogenous phospholipids provides a valuable tool in studies on the role of specific protein-protein and protein-phospholipid interactions in membrane function. Schneider *et al.* (1, 2) have investigated the effects of membrane lipid enrichment on the rate of electron transfer in the inner mitochondrial membrane. Lipid enrichment has also been reported for thylakoid membranes (3) and for energy-transducing bacterial membranes, such as the chromatophore of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* (4, 5).

In the above studies liposomes (phospholipid vesicles) composed of mixed soybean phospholipids were used as a source of exogenous lipids. Lipid transfer from the liposomes to the biological membranes was achieved by incubation at acidic pH and the mechanism of transfer was suggested to involve a process of membrane fusion (1–5). However, the evidence presented does not rule out alternative mechanisms of lipid transfer, such as a unidirectional flow of individual lipid molecules from the liposome to the biological membrane.

In the present study we have applied a kinetic assay based on fluorescence resonance energy transfer (RET[1]) to study the interaction between liposomes of different compositions and membrane vesicles derived from *Bacillus subtilis*. The assay allows to monitor continuously the dilution of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE) from labeled liposomes into unlabeled membranes, as revealed by an increase of the donor (*N*-NBD-PE) fluorescence. There is strong evidence indicating that *N*-NBD-PE and *N*-Rh-PE do not exchange between membrane vesicles, even when the vesicles are aggregated (6–9). The RET assay has been applied to monitor fusion of pure phospholipid vesicles (6, 7, 10–12). In several such liposome systems the mixing of membrane lipids, as revealed by the RET assay, appeared to correlate well with the mixing of aqueous vesicle contents (10–12). Moreover, the kinetics of lipid mixing during liposome fusion determined with the RET assay are essentially the same as the kinetics of lipid mixing revealed by an alternative assay for lipid mixing, recently developed in our laboratory (13). Therefore, dilution of the *N*-NBD-PE and *N*-Rh-PE from liposomes into unlabeled membranes provides a reliable measure for fusion.

Utilizing the RET assay we here show that membrane vesicles derived from *B. subtilis* fuse efficiently with negatively charged liposomes. The fusion reaction is independent of divalent cations, activated by low pH, and mediated by one or more protein components in the bacterial membrane.

EXPERIMENTAL PROCEDURES

Chemicals—Bovine heart cardiolipin (CL), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), bovine brain phosphatidylserine, *N*-NBD-PE and *N*-Rh-PE were obtained

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[1] The abbreviations used are: RET, resonance energy transfer; CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine.

from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol and egg phosphatidylcholine (PC) were from Sigma. Cholesteryl-[1-^{14}C] oleate was purchased from The Radiochemical Centre (Amersham, United Kingdom). ^{14}C-labeled egg PC was prepared as described (14). Parinaroylphosphatidylcholine was a generous gift from Dr. K. W. A. Wirtz (Department of Biochemistry, University of Utrecht, The Netherlands). Trypsin and soybean trypsin inhibitor were from Sigma. Octyl glucoside was from Boehringer Mannheim. All other reagents were of the highest purity available.

Bacterial Vesicles—B. subtilis W 23 was grown at 37 °C with vigorous aeration in a medium containing 0.8% trypton (Difco Laboratories, Detroit, MI), 0.5% (w/v) NaCl, and 25 mM KCl. Logarithmically grown cells were harvested at an absorbance at 660 nm of 0.8–1.0. Membrane vesicles were prepared as described by Bisschop and Konings (15), except that 10 mM HEPES, 50 mM sodium citrate (pH 7.4) was used instead of potassium phosphate buffer (pH 8.0). It has been shown that the membrane orientation of a large majority of *B. subtilis* vesicles prepared this way is right-side-out (16). *Escherichia coli* ML 308.225 was grown aerobically at 37 °C on minimal medium A (17). Inner membrane vesicles were prepared as described (18). *Streptococcus cremoris* Wg 2 (prt$^-$) was grown anaerobically on MRS broth at a controlled pH of 6.4 and membrane vesicles were prepared as described (19). Concentrated vesicle preparations were stored at −150 °C. Phospholipid content of the vesicle preparations was determined, after extraction of the lipids (20), by phosphate analysis (21). Protein was determined according to the modification of the Lowry procedure described by Peterson (22).

Liposomes—Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation (23, 24) in 100 mM NaCl, 10 mM HEPES (pH 7.4), sized by extrusion (25) through Unipore polycarbonate membranes (Bio-Rad) with a pore size of 0.2 μm and centrifuged in an Eppendorf microfuge during 15 min to remove any residual larger vesicles. The concentration of the liposome preparations was determined by phosphate analysis (20).

Fusion Assays—In the RET fusion assay 0.5 mol % each of *N*-NBD-PE and *N*-Rh-PE were incorporated in the bilayer of the liposomes. Fluorescence measurements were carried out in a final volume of 2.0 ml of 100 mM NaCl, 10 mM sodium acetate, 10 mM sodium phosphate, adjusted to the desired pH. The buffer in the cuvette was maintained at 25 °C (unless indicated otherwise) and stirred continuously. After addition of the liposomes the reaction was initiated by injecting, with a Hamilton syringe, a small volume (50–100 μl) of a concentrated bacterial vesicle suspension, appropriately diluted in 100 mM NaCl, 10 mM HEPES (pH 7.4). The increase of the *N*-NBD-PE fluorescence, due to dilution of the fluorophores into the bacterial membrane, was recorded continuously. Fluorescence was measured in a Perkin-Elmer MPF 43 spectrofluorometer at excitation and emission wavelengths of 465 and 530 nm, respectively. A cut off filter (<515 nm) was placed between sample and emission monochromator. For calibration of the fluorescence scale the initial residual fluorescence of the liposomes was taken as the zero level and the fluorescence at infinite probe dilution as 100%. The latter value was determined by addition of Triton X-100 (0.5%, v/v) to the liposomes and subsequent correction of the fluorescence intensity for sample dilution and for the effect of Triton on the fluorescence quantum yield of *N*-NBD-PE (6). Calibration was done at the pH of the corresponding measurement.

Fig. 1 shows the *N*-NBD-PE fluorescence intensity of liposomes containing different concentrations of *N*-NBD-PE and *N*-Rh-PE, relative to the intensity at infinite probe dilution which was set to 100%. The fluorescence decreases with increasing fluorophore concentrations due to increasing resonance energy transfer efficiency (6). At relatively high probe concentrations, the transfer efficiency does not increase proportionally with the surface density of the fluorescence donor and acceptor (6). However, as can be seen in Fig. 1, starting at probe concentrations of 0.5 mol % each, dilution of the fluorophores results in an essentially linear increase of the *N*-NBD-PE fluorescence intensity. *N*-NBD-PE fluorescence and energy transfer efficiency were unaffected by low pH down to a value of 3.0 (not shown).

Alternatively, fusion was measured by monitoring the relief of self-quenching of parinaroylphosphatidylcholine (25) during its dilution from the liposomal bilayer into the bacterial membrane. Measurements were carried out as described above for the RET assay, with excitation and emission wavelengths of 325 and 420 nm, respectively, without the use of a cut off filter. A narrow excitation slit was used to prevent photodegradation of the probe.

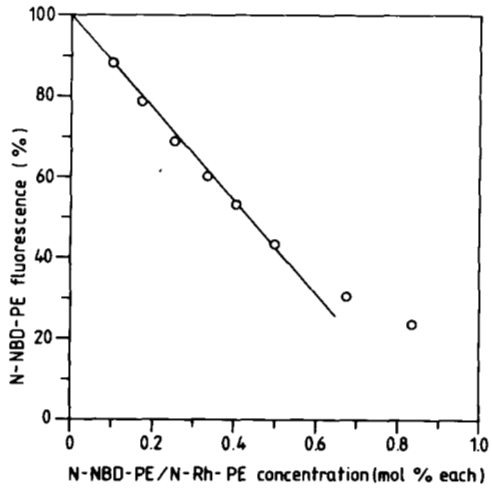

FIG. 1. ***N*-NBD-PE fluorescence intensity of phosphatidylserine LUV, containing different concentrations of *N*-NBD-PE and *N*-Rh-PE.** In all cases the ratio of *N*-NBD-PE and *N*-Rh-PE was 1:1. The fluorescence intensity at infinite fluorophore dilution was set to 100%.

Sucrose Density Gradient Centrifugation—B. subtilis membrane vesicles were incubated for 15 min at 25 °C with liposomes, containing 5 mol % *N*-NBD-PE, at 1.2 mM phospholipid phosphorus concentrations each in 100 mM NaCl, 10 mM sodium acetate, 10 mM sodium phosphate at either pH 4.0 or pH 8.5. The suspension was mixed with 42% (w/v) sucrose in 100 mM NaCl, 10 mM potassium phosphate, 1.0 mM EDTA (pH 8.0) to give a final sucrose concentration of 7%. Subsequently, 1.0 ml of the resulting suspension was layered on a sucrose gradient in the NaCl/phosphate buffer, with sucrose at the following concentrations (w/v): 15% (6 ml), 30% (3 ml), 34% (3 ml), 38% (3 ml), 42% (3 ml), 46% (3 ml), 50% (3 ml), 54% (3 ml), 65% (3 ml). After the addition of an overlay consisting of the NaCl/phosphate buffer, the gradients were centrifuged in a Sorvall SS-90 vertical rotor at 34,000 × *g* during 2 h at 4 °C. The gradients were fractionated and the fractions analyzed for fluorescence intensity and protein content (22). The density of the fractions was determined by refractive index measurements.

Binding Assay—B. subtilis membrane vesicles were incubated for 15 min at 25 °C with liposomes, containing either 0.5 mol % *N*-NBD-PE and 1.0 mol % cholesteryl-[1-^{14}C]oleate (20 Ci/mol) or 0.5 mol % *N*-NBD-PE and 6 mol % ^{14}C-labeled egg PC (3.4 Ci/mol), in 100 mM NaCl, 10 mM sodium acetate, 10 mM sodium phosphate at either pH 4.0 or pH 7.4. Phospholipid phosphorus concentrations of the bacterial vesicles and the liposomes were 0.25 mM each. Subsequently, the mixtures were centrifuged for 5 min in an Eppendorf microfuge. Radioactivity and *N*-NBD-PE fluorescence were determined in the initial reaction mixtures and in the supernatants after centrifugation.

Electron Microscopy—CL/DOPC (molar ratio, 1:1) LUV, containing colloidal gold, were prepared according to Hong *et al.* (27). *B. subtilis* membrane vesicles were incubated for 15 min at 25 °C with the gold-containing liposomes in 100 mM NaCl, 10 mM sodium acetate, 10 mM sodium phosphate at pH 4.0 or pH 8.0. The mixtures were centrifuged in an Eppendorf microfuge for 5 min and the pellets were prepared for thin-section electron microscopy as described (27). Sections were examined in a Philips EM 300 instrument.

RESULTS

Low pH-induced Interaction between Fluorescently Labeled Liposomes and B. subtilis Membrane Vesicles—Fig. 2 shows the fluorescence development observed upon addition of *B. subtilis* vesicles to CL/DOPC LUV, labeled with *N*-NBD-PE and *N*-Rh-PE, at different pH values. At neutral pH a slow increase of fluorescence intensity was seen, reflecting the dilution of the fluorophores into the bacterial membrane. With decreasing pH the rate and extent of fluorescence development increased steeply. Particularly at pH values below 4.5 very fast probe dilution was observed as can be seen in Fig. 3, where the initial rate of fluorescence increase is plotted

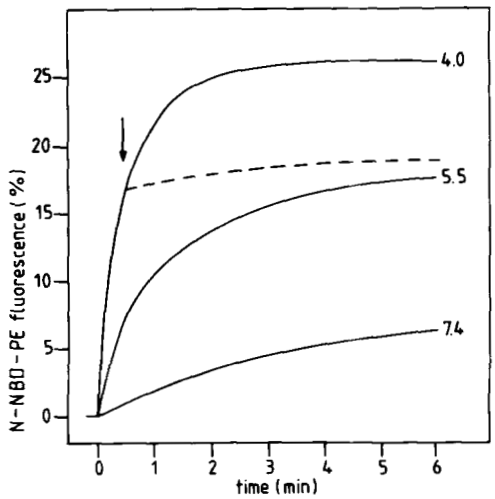

FIG. 2. **Fluorescence development upon interaction between *B. subtilis* membrane vesicles and CL/DOPC (molar ratio, 1:1) LUV, labeled with *N*-NBD-PE and *N*-Rh-PE, at different pH values.** *N*-NBD-PE fluorescence was recorded continuously. The ratio of the liposomal to bacterial phospholipid phosphorus concentration was 1:1 and the total phospholipid phosphorus concentration 50 μM. The *dashed line* represents the fluorescence development upon readjustment of the pH to 7.4, by addition of a small aliquot of NaOH (*arrow*), after initiation of the reaction at pH 4.0.

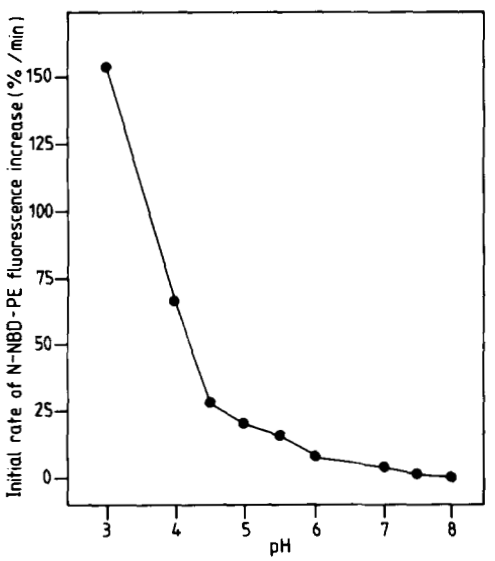

FIG. 3. **Initial rate of *N*-NBD-PE fluorescence increase upon interaction between *B. subtilis* membrane vesicles and fluorescently labeled CL/DOPC (molar ratio, 1:1) LUV as a function of pH.** Values were calculated from tangents drawn at $t = 0$ to fluorescence tracings, as presented in Fig. 2, recorded at high chart speeds.

as a function of pH. The reaction rate at neutral pH was not enhanced by the addition of Ca^{2+} (5 mM) and the presence of EDTA (1.0 mM) did not affect the fluorescence increase at any pH value (not shown). In the entire pH range studied no probe dilution was detected, when labeled CL/DOPC LUV were mixed with unlabeled liposomes of the same composition, indicating the lack of interaction between the liposomes themselves and excluding a low pH facilitated transfer of the fluorophores through the aqueous medium. When, after initiation of the reaction between liposomes and *B. subtilis* vesicles at pH 4.0, the pH was readjusted to neutral, the fast reaction was arrested instantaneously and the fluorescence continued to increase slowly, at a rate similar to the rate observed after initiation of the process at neutral pH (Fig. 2).

Lipid dilution from liposomes into the bacterial membrane vesicles was not restricted to *N*-NBD-PE and *N*-Rh-PE, as indicated by an experiment in which liposomes containing parinaroylphosphatidylcholine were used. Parinaroylphosphatidylcholine, when present in the liposomal bilayer at a sufficiently high concentration, shows fluorescence self-quenching and, therefore, the dilution of the probe into an unlabeled membrane can be monitored continuously (25). Using liposomes composed of an equimolar mixture of CL and parinaroylphosphatidylcholine, we observed dilution of the probe into *B. subtilis* membrane vesicles. Moreover, the pH dependence of fluorescence increase in this system was essentially identical to that observed with the RET assay (not shown).

Dilution of fluorescent lipids from liposomes into the bacterial membranes could either occur through a process of membrane fusion or through transfer of individual molecules. *N*-NBD-PE and *N*-Rh-PE have been shown to be nonexchangeable (6–9), suggesting that the interaction observed involves membrane fusion. Additional evidence against transfer of individual molecules was obtained from the binding experiment shown in Table I. *B. subtilis* vesicles were incubated at either pH 4.0 or pH 7.4 with CL/DOPC LUV, labeled with *N*-NBD-PE and another nonexchangeable marker, cholesteryl-[1-^{14}C]oleate (28). In a parallel experiment liposomes containing *N*-NBD-PE and ^{14}C-labeled egg PC were used. After centrifugation, under conditions such that liposomes alone did not sediment at all, the amounts of fluorescent and radioactive lipids remaining in the supernatant were determined. As shown in part A of Table I, at pH 4.0 association of the liposomes with the bacterial membrane vesicles was extensive. The fraction of the labeled lipids remaining in the supernatant (about 20%) may represent unbound liposomes. It is more likely, however, that a fraction of the interaction products did not sediment, as indicated by the presence of a similar percentage of the bacterial membrane protein in the supernatant. At neutral pH an only limited interaction of the CL/DOPC liposomes with the *B. subtilis* vesicles was observed (Table I, part A). The importance of this binding experiment lies in the observation that all three labels were removed from the supernatant to the same extent, indicating that in either CL/DOPC liposome preparation the *N*-NBD-PE and the radioactive lipid behaved as part of one unit. This result virtually rules out the possibility of transfer of individual molecules through the aqueous phase or during a transient interaction between liposomes and bacterial vesicles. It should be emphasized, however, that the binding experiment *per se* does not discriminate between membrane fusion and irre-

TABLE I

Association of CL/DOPC and DOPC LUV with B. subtilis membrane vesicles

The experiments were carried out as described under "Experimental Procedures."

Label	Part A, binding of CL/DOPC LUV[a]		Part B, binding of DOPC LUV[a]
	pH 4.0	pH 7.4	pH 4.0
	%		%
^{14}C-labeled egg PC	78.3	31.3	2.3
Cholesteryl-[1-^{14}C]oleate	79.0	31.7	3.3
N-NBD-PE	80.9	30.7	2.3

[a] Calculated from the concentrations of labels in the initial reaction mixtures and in the supernatants after centrifugation.

versible binding of the liposomes to the bacterial membrane vesicles. Therefore, we subsequently examined the mixing of internal aqueous compartments of the interacting vesicles.

Mixing of Internal Contents—Mixing of aqueous vesicle contents was investigated by incubating *B. subtilis* vesicles with liposomes containing colloidal gold (27) and subsequent examination of the interaction products by thin-section electron microscopy. *Panels A* and *B* of Fig. 4 show the gold-loaded liposomes and bacterial vesicles, respectively. After co-incubation at pH 4.0, large fused structures were seen with gold particles within the enclosed volume (*panels D* and *E*), whereas unfused liposomes could no longer be detected. *Panel F* shows a fusion intermediate, consisting of a liposome interacting with three bacterial vesicles at pH 4.0. Incubation at pH 8.0 did not result in transfer of gold into the bacterial vesicles: separate gold-containing liposomes and *B. subtilis* vesicles were observed (*panel C*). In control experiments, where *B. subtilis* vesicles were incubated at pH 4.0 with free colloidal gold either in the absence or presence of empty liposomes, no gold particles were observed inside the vesicles (not shown). Therefore, the presence of gold particles in the *B. subtilis* vesicles can only have been the result of fusion with liposomes.

Sucrose Density Gradient Centrifugation—After incubation at pH 4.0 of *B. subtilis* vesicles with CL/DOPC liposomes, labeled with *N*-NBD-PE, sucrose density gradient centrifugation revealed a major band (Fig. 5*B*) with a density (1.11 g/ml) intermediate between the densities of the pure vesicles (1.17 g/ml) and the pure liposomes (1.04 g/ml). This band contained virtually all of the *N*-NBD-PE and most of the bacterial membrane protein. Some protein appeared at very high densities and presumably represented aggregated hydrophobic proteins (29). Incubation of the bacterial vesicles with CL/DOPC liposomes at pH 8.5 and subsequent sucrose density gradient centrifugation resulted in two bands at the respective densities of the vesicles and the liposomes (Fig. 5*A*).

Protein Dependence of the Fusion Reaction—In order to investigate the possible involvement of membrane proteins in the fusion reaction, *B. subtilis* vesicles were treated with trypsin at pH 7.4. After addition of trypsin inhibitor, fusion activity at pH 4.0 was examined utilizing the RET assay. As shown in Fig. 6 (*curve c*), trypsin pretreatment strongly inhibited the fusion reaction. The initial rate of fusion was approximately 6% of that observed with untreated vesicles (*curve a*). *B. subtilis* vesicles pretreated with a mixture of trypsin and an excess of trypsin inhibitor showed the same fusion activity as untreated vesicles (Fig. 6, *curve b*). Pretreatment of the vesicles with other proteolytic enzymes, such as chymotrypsin, pronase, and papain, also produced a virtually complete inhibition of the fusion activity (not shown). These results indicate that the fusion reaction is dependent on one or more protein components in the bacterial membrane.

Effect of Liposomal Lipid Composition—The above experiments were carried out with negatively charged liposomes, containing a high concentration of CL. To investigate the

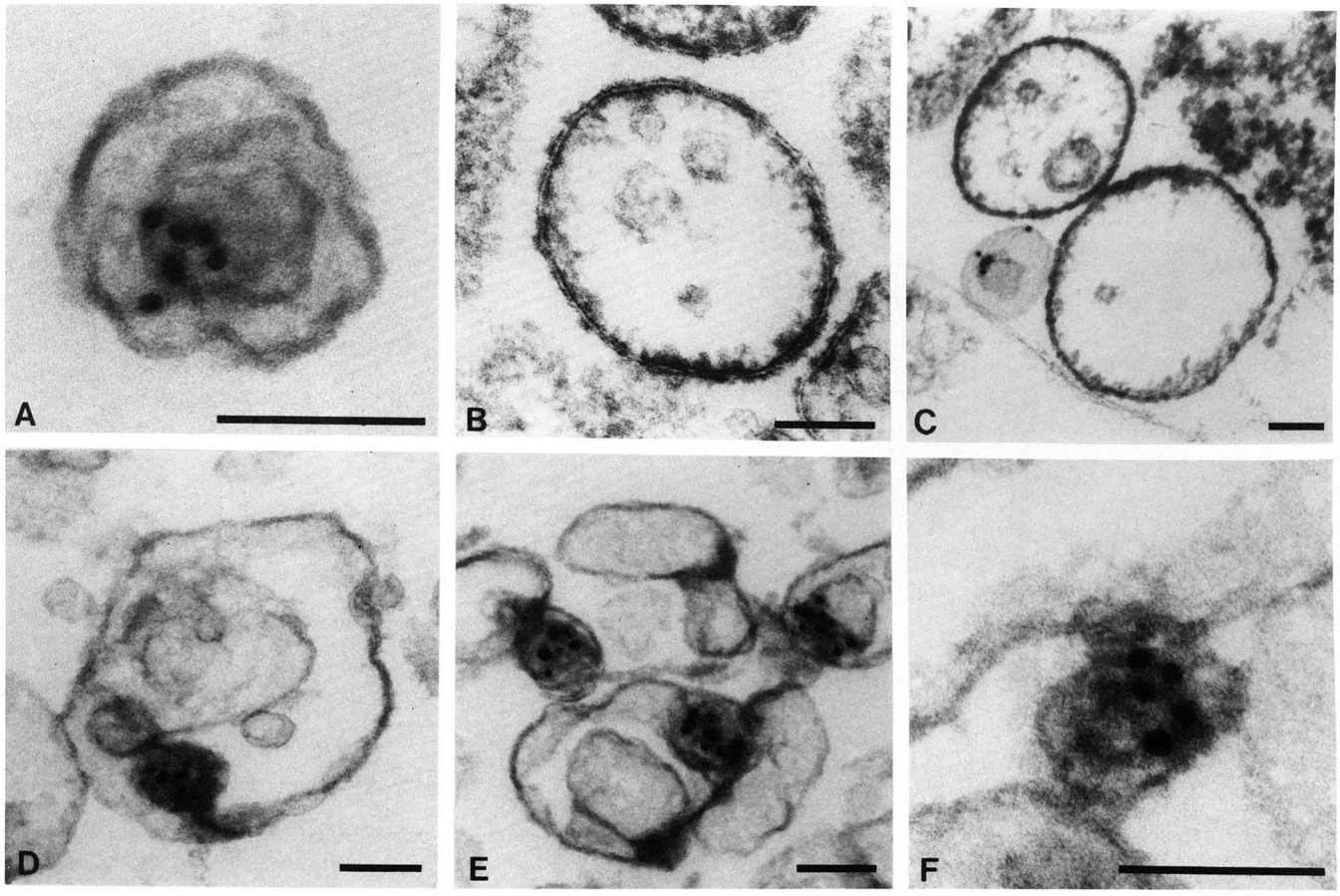

FIG. 4. **Thin-section electron micrographs of CL/DOPC (molar ratio, 1:1) LUV, containing colloidal gold, *B. subtilis* membrane vesicles, and interaction products at pH 4.0 and pH 8.0.** *Panel A*, gold-containing liposome; *Panel B*, *B. subtilis* membrane vesicles; *Panel C*, mixture of liposomes and bacterial vesicles at pH 8.0; *Panels D–F*, mixtures of liposomes and bacterial vesicles at pH 4.0. *Bar* represents 0.1 μm.

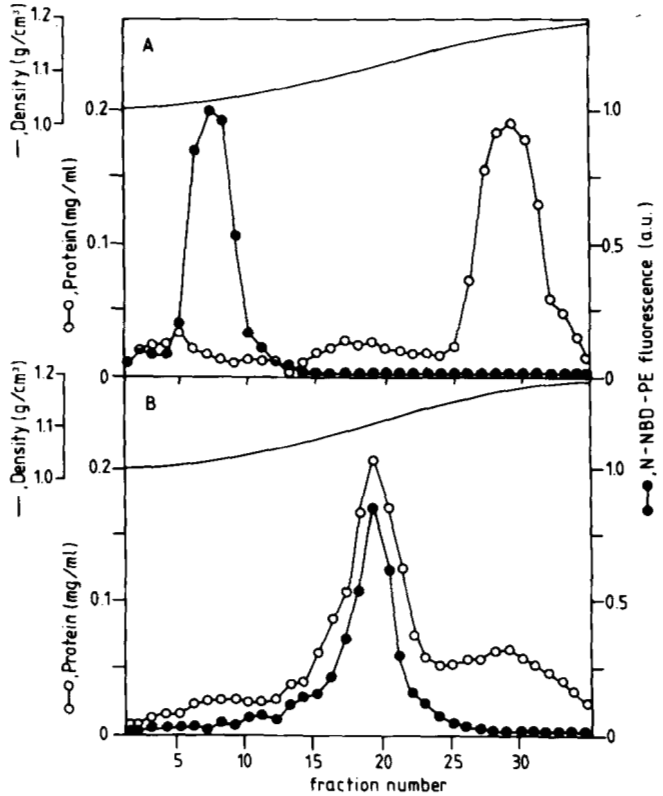

FIG. 5. **Sucrose density gradient analysis of the interaction products of *B. subtilis* membrane vesicles and CL/DOPC (molar ratio, 1:1) LUV, labeled with 5 mol % *N*-NBD-PE.** *Panel A*, a 1:1 mixture of liposomes and bacterial vesicles after preincubation at pH 8.5. *Panel B*, a 1:1 mixture of liposomes and bacterial vesicles after preincubation at pH 4.0.

requirements of the fusion reaction in terms of liposomal charge and composition, fluorescently labeled liposomes of different composition were prepared and examined for their ability to fuse with *B. subtilis* vesicles at low pH. The results are shown in Table II. The fusion reaction showed an absolute requirement for negatively charged phospholipids, such as CL or phosphatidylserine, in the liposomal bilayer. No fusion was observed with liposomes composed of the zwitterionic DOPC either in the absence or presence of DOPE or cholesterol. Incorporation of cholesterol in negatively charged liposomes slightly enhanced the rate of fusion. On the other hand, incorporation of DOPC had an inhibitory effect (Fig. 7 and Table II).

Part B of Table I shows the results of an experiment, in which the extent of binding of DOPC liposomes to *B. subtilis*

TABLE II

Fusion between B. subtilis membrane vesicles and liposomes of different compositions

Fusion was measured at pH 4.0, as described in the legend to Fig. 2, with fluorescently labeled LUV of different compositions.

Liposomal lipid composition[a]	Initial rate of *N*-NBD-PE fluorescence increase[b]	Final level of *N*-NBD-PE fluorescence
	%/min	%
CL	83	27
CL/cholesterol (3:4)	92	28
CL/DOPC (1:1)	51	25
CL/DOPC/cholesterol (1:1:2)	60	29
PS	40	24
PS/DOPC (1:1)	6	12
PS/DOPC/cholesterol (3:3:4)	12	16
DOPC	0	0
DOPC/DOPE (1:1)	0	0
DOPC/cholesterol (3:2)	0	0

[a] Ratios in the lipid mixtures, indicated in parentheses, were molar ratios.

[b] Rates were calculated as described in the legend to Fig. 3.

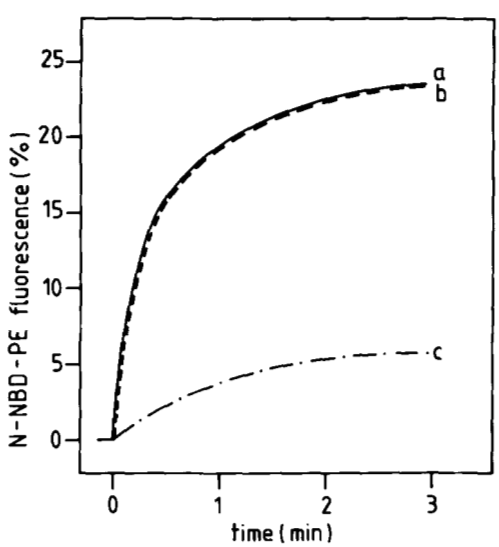

FIG. 6. **Effect of trypsin pretreatment on the fusion activity of *B. subtilis* membrane vesicles.** Bacterial vesicles (0.5 μmol of phospholipid phosphorus) were treated with trypsin (5 μg) in 0.5 ml of 100 mM NaCl, 10 mM HEPES (pH 7.4) for 10 min at 37 °C in the absence or presence of trypsin inhibitor (20 μg). Fusion at pH 4.0 with CL/DOPC (molar ratio, 1:1) LUV was measured as described in the legend to Fig. 2. *Curve a*, control; *curve b*, vesicles simultaneously pretreated with trypsin and trypsin inhibitor; *curve c*, vesicles pretreated with trypsin (after the incubation 20 μg of trypsin inhibitor was added).

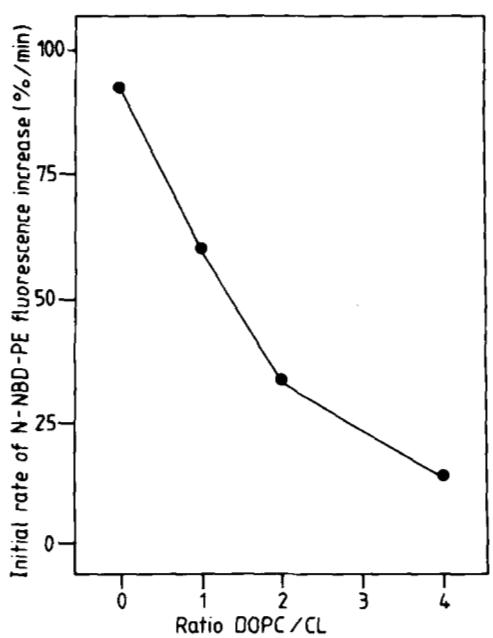

FIG. 7. **Effect of DOPC content of CL/DOPC LUV on fusion with *B. subtilis* membrane vesicles at pH 4.0.** Fusion was measured as described in the legend to Fig. 2 with liposomes composed of mixtures of CL and DOPC (molar ratios are indicated), *N*-NBD-PE and *N*-Rh-PE. The initial rate of *N*-NBD-PE fluorescence increase was determined, as described in the legend to Fig. 3.

vesicles at pH 4.0 was determined. The liposomes contained either ^{14}C-labeled egg PC and *N*-NBD-PE or cholesteryl-[1-^{14}C]oleate and *N*-NBD-PE. Virtually no binding of the liposomes to the bacterial vesicles was detected, explaining the absence of fusion between liposomes of this composition and the bacterial membranes. The lack of binding of DOPC liposomes to the bacterial membrane vesicles at pH 4.0 was confirmed by sucrose density gradient analysis (not shown). Part B of Table I shows once again that the fluorescent and radioactive labels in the liposomal bilayer behaved as part of one unit: none of the labels was preferentially transferred to the bacterial vesicles. This further corroborates the above conclusion that transfer of individual lipid molecules between liposomes and bacterial membranes does not occur.

Quantitation of the Fusion Reaction—In the RET assay, at a 1:1 ratio of labeled to unlabeled membrane vesicles complete mixing of the lipids in the system is expected to result in a 50% increase of *N*-NBD-PE fluorescence relative to the intensity at infinite probe dilution (Fig. 1). The final level of fluorescence intensity observed at a 1:1 ratio of labeled CL/DOPC liposomes and unlabeled *B. subtilis* vesicles was 25% of the *N*-NBD-PE fluorescence at infinite probe dilution (Fig. 2, Table II), which thus represents approximately half the level expected for complete lipid mixing. In search for an explanation for this apparent suboptimal level of fluorescence increase we considered the following possibilities. First, only a fraction of the liposomes and/or the bacterial vesicles fuses. Second, after fusion, bacterial membrane proteins affect the *N*-NBD-PE fluorescence quantum yield and/or the resonance energy transfer efficiency between donor and acceptor lipid.

In order to test the first possibility, we determined the extent of fusion at temperatures ranging from 5 to 30 °C, *i.e.* under conditions where the initial rates of fusion can be expected to be different. The results in Fig. 8 show that at all temperatures studied the same final level of probe dilution was obtained. This strongly suggests that the level of approximately 25% *N*-NBD-PE fluorescence intensity is the maximal level that can be obtained in this system and, thus, argues against the involvement of only part of the liposomes or the bacterial vesicles in the reaction.

With respect to the second possibility: *B. subtilis* vesicles were fused at pH 4.0 with CL/DOPC liposomes, labeled with 0.5 mol % of *N*-NBD-PE only. No change in the fluorescence intensity was observed (not shown), excluding an effect of bacterial membrane proteins on the fluorescence quantum yield of *N*-NBD-PE. To examine a possible effect of bacterial proteins on the energy transfer efficiency between *N*-NBD-PE and *N*-Rh-PE, we prepared "mock" fusion products of *B. subtilis* vesicles and CL/DOPC liposomes, labeled with 0.5 mol % each of *N*-NBD-PE and *N*-Rh-PE, at different ratios. This was done by solubilization of the mixtures with octyl glucoside and subsequent reconstitution of mixed membrane vesicles by slow dialysis of the detergent (30). In Fig. 9 (*curve b*) the *N*-NBD-PE fluorescence intensity of these vesicles is plotted as a function of the ratio of liposomal to total phospholipid in the mixtures. The fluorescence increased linearly with increasing dilution of the fluorophores. Remarkably, in the entire range of dilutions the *N*-NBD-PE fluorescence in the mixed membranes was approximately 30% lower than the theoretically expected intensity (Fig. 9, *curve a*; see also Fig. 1), indicating that the bacterial membrane proteins cause a relative enhancement of the energy transfer efficiency possibly by locally concentrating the fluorophores. *Curve c* in Fig. 9 presents the final extents of *N*-NBD-PE fluorescence after low pH-induced fusion between the liposomes and the bacterial vesicles at different ratios. Again the relationship between fluorescence and probe dilution was linear. Comparison of *curves b* and *c* shows that during fusion a degree of lipid mixing is achieved corresponding to approximately 80% of that in the mock fusion products.

Fusion Capacity of Other Bacterial Membranes—In order to determine whether low pH-dependent fusion activity is specific for vesicles derived from *B. subtilis*, we tested the capacity of membrane vesicles from the Gram-positive *S. cremoris* and of vesicles from the inner membrane of the Gram-negative *E. coli* to fuse with CL/DOPC liposomes, utilizing the RET

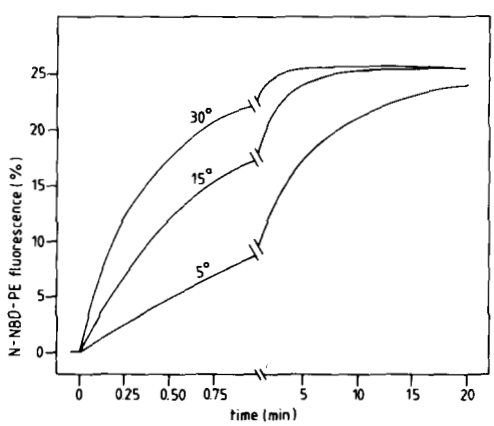

FIG. 8. **Fusion between *B. subtilis* membrane vesicles and CL/DOPC (molar ratio, 1:1) LUV at different temperatures.** Fusion was measured as described in the legend to Fig. 2. The pH was adjusted to 4.0 at the temperatures indicated.

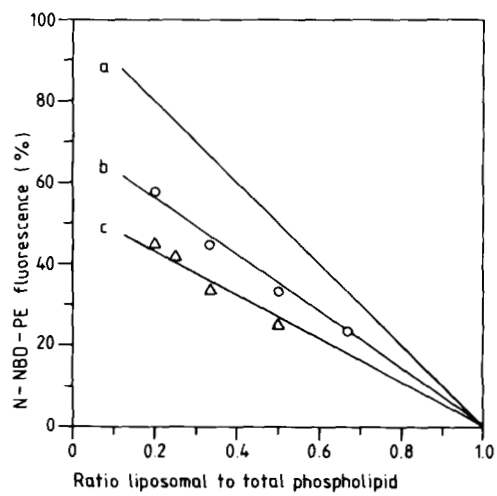

FIG. 9. **Fusion of *B. subtilis* membrane vesicles with CL/DOPC (molar ratio, 1:1) LUV at different ratios of bacterial vesicles to liposomes.** Fusion was measured at pH 4.0, as described in the legend to Fig. 2, except that the bacterial vesicle concentration was varied from 25 to 100 μM phospholipid phosphorus. Data points on *curve c* represent the final levels of *N*-NBD-PE fluorescence. Mock fusion products were prepared by addition of an equal volume of 60 mM octyl glucoside to mixtures of the liposomes and the bacterial vesicles (to give final concentrations of liposomal phospholipid and octyl glucoside of 25 μM and 30 mM, respectively), incubation at 37 °C for 1 h and subsequent slow dialysis against 100 mM NaCl, 10 mM HEPES (pH 7.4) to remove the detergent (Ref. 30). Data points on *curve b* represent the *N*-NBD-PE fluorescence intensities of the mock fusion products at either pH 7.4 or pH 4.0. The intensities at infinite fluorophore dilution were set to 100% and the intensity of the liposomes was taken as the zero level (see "Experimental Procedures"). *Curve c* represents the theoretically expected fluorescence intensity after complete mixing of all the lipids in the system, ignoring a possible effect of membrane proteins on the energy transfer efficiency between *N*-NBD-PE and *N*-Rh-PE.

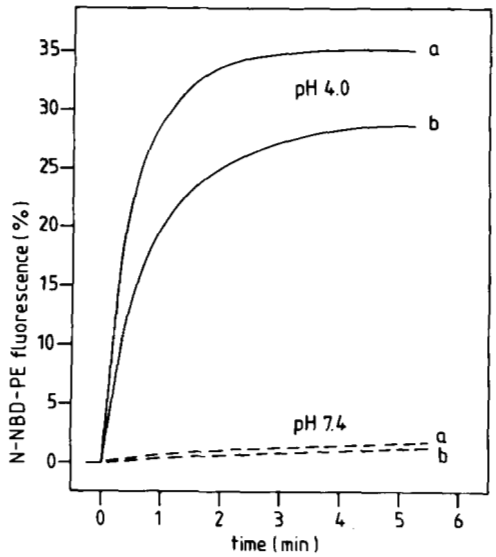

FIG. 10. **Fusion of *S. cremoris* membrane vesicles and *E. coli* inner-membrane vesicles with CL/DOPC (molar ratio, 1:1) LUV at pH 4.0 and pH 7.4.** Fusion was measured as described in the legend to Fig. 2. *Curves a, E. coli; curves b, S. cremoris.*

assay. With either vesicle preparation efficient fusion was observed in a pH-dependent fashion, very similar to that seen with *B. subtilis* vesicles (Fig. 10).

DISCUSSION

In this paper we have demonstrated that at low pH, membrane vesicles derived from *B. subtilis* fuse with negatively charged liposomes. Evidence for fusion is based on the occurrence of both mixing of membrane lipids (Figs. 2 and 3) and mixing of internal contents (Fig. 4) as well as on the formation of interaction products with an intermediate buoyant density (Fig. 5). The observation that not only *B. subtilis* membrane vesicles, but also vesicles derived from *S. cremoris* or *E. coli* have the capacity to fuse with liposomes at low pH (Fig. 10) as well as the occurrence of lipid enrichment of chromatophores of *R. sphaeroides* upon incubation with liposomes at low pH (Refs. 4 and 5) suggest that fusion capacity may be a general property of bacterial membranes.

Recently, Lelkes *et al.* (31) have demonstrated delivery of aqueous contents from liposomes into flagellated cell envelopes from *E. coli.* Although the extent of interaction observed by these authors was small, their results support our conclusion with respect to fusion between liposomes and bacterial membranes. The low extent of interaction observed by Lelkes *et al.* (31) is not surprising, as their experiments were carried out at neutral rather than acidic pH (*cf.* Figs. 2, 3, and 10).

The use of the RET assay allows an accurate quantitation of the initial kinetics as well as the final extent of the fusion reaction. At pH values of 4 or below fusion between *B. subtilis* vesicles and negatively charged liposomes, particularly those containing high contents of CL, occurs within seconds to quite considerable extents (Figs. 2 and 3, Table II). The results in Fig. 8 show that neither part of the liposomes nor part of the bacterial vesicles remains unfused. This is consistent with the observation that virtually all of the liposomes become associated with the bacterial vesicles (Table I, Fig. 5*B*) and that no unfused liposomes are detected by electron microscopic examination of the fusion products. The extent of lipid mixing, based on increase of *N*-NBD-PE fluorescence intensity after fusion at pH 4.0 of CL/DOPC liposomes with the bacterial membranes represents approximately 80% of that in a corresponding, completely randomized, system (Fig. 9). That this extent is 20% lower than the maximal level may be due to incomplete availability of the bacterial lipid for probe dilution during fusion. *B. subtilis* vesicles as used in this study are known to contain a fraction of intravesicular membrane material (32). This fraction is unlikely to contribute to the dilution of the fluorophores after fusion of the liposomes with the outermost membrane of the vesicles.

An important result of the present study is the observation that the fusion reaction is mediated by one or more protein components in the bacterial membrane (Fig. 6). On the basis of our results, one cannot discriminate between a role of these proteins in the initial attachment between the bacterial vesicles and the liposomes, in the fusion process itself or in both. The absolute requirement of the fusion reaction for negatively charged lipids in the liposomal bilayer (Table II) strongly suggests that the initial interaction of the bacterial vesicles with the liposomes is electrostatic in nature, involving positively charged groups on the bacterial membrane. It is quite conceivable that such an interaction is enhanced at low pH due to increased protonation of membrane proteins. The results in Table I, showing that there is little interaction of CL/DOPC liposomes with the bacterial vesicles at neutral pH, support this notion.

With respect to the mechanism of the fusion reaction itself, one may speculate that a mechanism is operating similar to that involved in the low pH-dependent fusion of certain viruses, induced by viral glycoproteins. The best characterized example is influenza virus. The fusion protein of this virus, the hemagglutinin, contains an unusually apolar stretch of amino acid residues, which is exposed at low pH due to a conformational change of the protein. This hydrophobic segment is thought to be directly involved in the fusion reaction possibly by penetrating into the target membrane (33, 34). Obviously, in order to establish whether a similar mechanism is involved in the fusion of bacterial membranes at low pH, the fusion proteins have to be identified and isolated. Characterization of these fusion activities may provide further insight into the mechanisms by which proteins induce and modulate membrane fusion (33–40).

A possible physiological function of the bacterial fusion activity remains unclear. As for Gram-negative bacteria, such as *E. coli*, the fusion activity may serve to establish transient fusion sites between the inner and outer membrane, allowing the transfer of membrane components. Interestingly, evidence has been presented indicating that the transfer of proteins (41, 42) and phosphatidylethanolamine (43) from the inner to the outer membrane of *E. coli* requires a membrane potential and a pH gradient across the membrane.

The presence of a fusion activity in bacterial membranes is of considerable importance to studies on the structure and function of bacterial membranes. Not only can the lipid to protein ratio or the lipid composition of bacterial membranes be varied by fusion with liposomes of different compositions (4, 5), it is also possible to insert membrane proteins, reconstituted in liposomes, into bacterial membrane vesicles. For example, recently we have observed the low pH-induced functional incorporation of bacteriorhodopsin, reconstituted in liposomes, into membrane vesicles derived from *S. cremoris* as evidenced by the generation of a light-induced proton motive force and the occurrence of light-driven transport of Ca^{2+} in the interaction products.[2] Another potentially important application may be the use of liposomes as vehicles to deliver foreign compounds, such as proteins or nucleic acids, into bacterial protoplasts (31, 44). In addition, native or

[2] A. J. M. Driessen, unpublished observations.

reconstituted bacterial membrane vesicles may be used to deliver encapsulated substances into cultured cells, either through fusion with the cellular plasma membrane induced by a transient pH drop in the medium or via the endocytotic pathway.

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