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# pH-Dependent Bilayer Destabilization and Fusion of Phospholipidic Large Unilamellar Vesicles Induced by Diphtheria Toxin and Its Fragments A and B†

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ABSTRACT: The passage by the low endosomal pH is believed to be an essential step of the diphtheria toxin (DT) intoxication process in vivo. Several studies have suggested that this low pH triggers the insertion of DT into the membrane. We demonstrate here that its insertion into large unilamellar vesicles (LUV) is accompanied by a strong destabilization of the vesicles at low pH. The destabilization has been studied by following the release of a fluorescent dye (calcein) encapsulated in the liposomes. The influence of the lipid composition upon this process has been examined. At a given pH, the calcein release is always faster for a negatively charged (asolectin) than for a zwitterionic (egg PC) system. Moreover, the transition pH, which is the pH at which the toxin-induced release becomes significant, is shifted upward for the asolectin LUV as compared to the egg PC LUV. No calcein release is observed for rigid phospholipid vesicles (DPPC and DPPC/DPPA 9/1 mol/mol) below their transition temperature whereas DT induces an important release of the dye in the temperature range corresponding to the phase transition. The transition pH associated to the calcein release from egg PC vesicles is identical with that corresponding to the exposure of the DT hydrophobic domains, as revealed here by the binding of a hydrophobic probe (ANS) to the toxin. This suggests the involvement of these domains in the destabilization process. Both A and B fragments destabilize asolectin and PC vesicles in a pH-dependent manner but to a lesser extent than the entire toxin. Phospholipid vesicle fusion mediated by DT or its fragments was monitored in order to evaluate the possible contribution of this process to the release of the liposome internal content. DT and DTB induced fusion of negatively charged vesicles below pH 5 whereas DTA did not fuse any vesicles whatever the composition tested. The role of DTB peptide domains in the fusion and aggregation processes is discussed.

Diphtheria toxin (DT),[1] produced by *Corynebacterium diphtheriae*, is a 60-kDa polypeptide chain constituted by two fragments A and B linked to each other by a disulfide bridge. These fragments can be separated by site-specific cleavage of the protein and thiol reduction (Collier, 1975). After binding to yet unknown specific cell surface receptors (Middlebrook et al., 1978), the B fragment ($M_r$ 37 240) mediates, via acidic endosomes (Sandvig & Olsnes, 1980; Marnell et al., 1984), the internalization of DT fragment A ($M_r$ 21150) into the cytoplasm where it inhibits the protein synthesis and causes cell death by ADP ribosylation of elongation factor 2 (Pappenheimer, 1977). The importance of acidic pH for penetration of toxin into cells has been experimentally demonstrated

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PE, phosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; egg PC, phosphatidylcholine from egg yolk; DPPA, DL-α-dipalmitoylphosphatidic acid; DPPC, DL-α-dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); SUV, small unilamellar vesicle(s); DT, diphtheria toxin; DTA, diphtheria toxin A fragment; DTB, diphtheria toxin B fragment; TX-100, Triton X-100; $C_{12}E_8$, octa(ethylene glycol) dodecyl ether; PTR, phase transition release; RET, resonance energy transfer; ANS, 1-anilino-8-naphthalenesulfonate; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *p*-xylenebis(pyridinium bromide).

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by the ability of weak bases such as $NH_4Cl$ and chloroquine, which increase pH in cell acidic compartments, to inhibit toxicity (Sandvig & Olsnes, 1980). This protection is removed by briefly exposing cells to acidic pH, suggesting that, in these conditions, DT can directly cross the cytoplasmic membrane (Sandvig & Olsnes, 1980). Little information is available on the molecular mechanism by which the DTB fragment mediates the DTA fragment translocation through the endosomal membrane even though the mode of insertion of DT or DTB into a lipid bilayer has been investigated. Insertion of DT into a lipid layer is not observed at neutral pH (Montecucco et al., 1985). However, at low pH, DT changes its conformation as suggested by the modification of its tryptophan environment, exposes on its surface hydrophobic regions, cryptic at neutral pH (Blewitt et al., 1985), and inserts into the lipid bilayer. Conductance measurements on planar lipid membranes have suggested that, at low pH, DT induces pore formation (Donovan et al., 1981). The B fragment exhibits the same property whereas the A fragment does not modify the conductance of the bilayers (Kagan et al., 1981). On this basis, a translocation model has been proposed in which the B fragment inserts into a bilayer at low pH and forms channels large enough to allow the passage of DTA, in its extended form, through the membrane (Kagan et al., 1981). This model implies, however, that DTA is protected from contact with the hydrophobic lipid region of the membrane. Recently, using membrane-restricted photoactivatable probes, several authors have demonstrated that both A and B fragments were labeled after incubation of DT at low pH either with liposomes (Hu & Holmes, 1984; Montecucco et al., 1985) or with Sendai virus (Zalman & Wisnieski, 1984). Labeling of DTA fragment is not compatible with the pore model, and Bisson and Montecucco (1987) proposed instead a model in which both A and B fragments insert into the lipid bilayer at low pH and the translocation is mediated via the interaction between the two fragments. Although very attractive, this model remains to be demonstrated at a molecular level. During the past few years, valuable information about the interactions between proteins [viral proteins (Tsao & Huang, 1985; Blumenthal et al., 1986), clathrin (Hong et al., 1985), lysin (Hong & Vacquier, 1986)] and membranes has been obtained by studying the destabilization of lipid bilayers promoted by these proteins. Indeed, numerous studies have established that the interaction of a protein with lipid bilayers can alter the structure and change the lipid bilayer's permeability (Allen, 1984). Therefore, in order to provide information about the molecular mechanism of the association of DT to lipid bilayer, we have examined the effect of the pH upon the lipid destabilization induced by DT and its fragments A and B. Destabilization was studied by following the release of an encapsulated dye [calcein (Allen, 1984)] from asolectin or egg phosphatidylcholine vesicles. In this report, in contrast to most studies dealing with DT–model membrane interactions that have been carried out on small unilamellar vesicles (SUV), we used large unilamellar vesicles (LUV) whose curvature and stability better mimic the lipid membrane structure. The regulation of the release by the lipid environment (composition, charge, and fluidity of the vesicles) and the aggregation state of the toxin has been examined. Toxin-induced aggregation and/or fusion of the liposomes was monitored in order to evaluate their contribution to the release of the internal liposome content.

## Materials and Methods

*Proteins.* The monomeric form of the toxin has been purified according to Collier and Kandel (1971) and is chromatographically pure. Complete nicking was obtained as described by Falmagne et al. (1978); ApUp-free and -bound monomers were not separated. DTA and DTB were prepared as described previously (Falmagne et al., 1978) and solubilized in 50 mM Hepes buffer, pH 7.3.

*Lipids.* Egg PC (type V), asolectin (mixed soybean phospholipids), DPPA (sodium salt), and DPPC were purchased from Sigma (St. Louis, MO). Fluorescent phospholipids NBD-PE and Rh-PE were obtained from Avanti Polar Lipids (Birmingham, AL).

*Chemicals.* Calcein (Sigma) was purified by chromatography on Sephadex LH20 (Pharmacia). Calcein was loaded on the column as a sodium salt solution and eluted with water at neutral pH. The concentration of calcein was determined spectrophotometrically by using $7.0 \times 10^4$ as the molar extinction coefficient at 492 nm (Hong et al., 1985). Detergent Triton X-100 was obtained from Sigma and $C_{12}E_8$ from Calbiochem. ANS is a gift from Dr. R. Jeener (Laboratoire de Physiologie Animale, Université Libre de Bruxelles, Brussels, Belgium). All other chemicals were pro analysi.

*ANS Fluorescence.* ANS fluorescence in the absence or presence of DT has been recorded by using a Perkin-Elmer 204 fluorometer at 20 °C. Final ANS concentration is $5 \times 10^{-5}$ M. The $\lambda_{ex}$ is 380 nm, and we measured the fluorescence of ANS in the presence of DT at 470 nm, which is the maximum emission wavelength of ANS in the presence of the toxin at pH 4.5. Buffers used were as follows: for pH 4.2–5.0, acetate (0.1 M) and NaCl (0.15 M); for pH 5–6, phosphate (0.1 M) and NaCl (0.15 M); for pH 7.2, Tris-HCl (0.1 M) and NaCl (0.15 M).

*Preparation of Liposomes.* LUV were prepared according to the extrusion procedure described by Hope et al. (1985) using an extruder (Lipex Biomembranes Inc., Vancouver, Canada). Typically, dry lipid films were hydrated with the appropriate buffer or calcein solutions to produce large multilamellar vesicles at a concentration of 50 mg/mL (60–70 mM). These suspensions were subsequently submitted to five cycles of freezing and thawing. The resulting preparation was extruded 10 times through two stacked polycarbonate filters (Nuclepore Corp., Pleasanton, CA) with a pore size of 0.1 μm by using the extruder and moderate nitrogen pressure (100–200 lb/in.$^2$). During the extrusion of DPPC or DPPC/DPPA suspensions, the temperature of the extruder was maintained at 60 °C by immersing it in warm water. An average diameter of 90 nm can be expected for such LUV (Mayer et al., 1985).

Calcein vesicles were prepared in either 65 mM calcein or 30 mM calcein at pH 7.4 (10 mM Hepes). The nonencapsulated calcein was removed from the liposome suspension according to the minicolumn centrifugation technique (Lelkes, 1984). The minicolumns (2 mL) were loaded with Sephadex G-50 (Pharmacia) and were preequilibrated with Hepes (10 mM), NaCl (0.15 M), EDTA (0.1 mM), and $NaN_3$ (0.02%), pH 7.4. Usually, 100 μL of liposome suspension was processed by this technique, which allows a quick recovery of the liposomes with minimal dilution. The liposome concentration was determined by measuring lipid phosphorus content according to the procedure of Mrsny et al. (1986). The osmolarity of the solutions (calcein, buffers) was fixed to 295 ± 5 mosmol/kg of $H_2O$ (Fiske osmometer).

*Release of Encapsulated Calcein.* Calcein, entrapped at a self-quenching concentration in LUV, increases in fluorescence when it leaked out of the liposomes. Fluorescence was monitored by using a Jobin-Yvon spectrofluorometer (JY3D) with excitation and emission slits of 4 nm. Unless otherwise specified, experiments were done at a lipid concentration of

50 μM and a temperature of 20 °C, and complete dye release was obtained by lysing the LUV with TX-100 (0.25% final concentration). The percentage of total fluorescence is defined as

$$\% F(t) = \left( \frac{I(t) - I_0}{I_f - I_0} \right) \times 100$$

where $I_0$ = the initial fluorescence, $I_f$ = the total fluorescence observed after addition of Triton X-100, and $I(t)$ = the fluorescence at time $t$ corrected for the dilution. In our experimental conditions, in the absence of protein, the leakage rate was less than 1%/10 min. Buffers used were as follows: for pH 4.25–5.50, acetate (10 mM), NaCl (150 mM), and EDTA (0.1 mM); for pH 5.50–6.50, Mes/NaOH (10 mM), NaCl (150 mM), and EDTA (0.1 mM); for pH 7.4, Hepes/NaOH (10 mM), NaCl (150 mM), and EDTA (0.1 mM).

*(A) LUV Containing Calcein (30 mM).* Measurements were done in a semimicrocuvette (800 μL) with an excitation path length of 4 mm and an emission path length of 10 mm and with a front face illumination (illuminated surface oriented 30° from the incident beam). Excitation and emission wavelengths were 490 and 520 nm, respectively. A 10-μL aliquot of LUV containing the dye (pH 7.4) was diluted with 725 μL of buffer of the indicated pH. After pH equilibration, 15 μL of a solution of protein was added and the mixture was thoroughly stirred.

*(B) LUV Containing Calcein (65 mM).* Measurements were done in a 1 × 1 cm cuvette under constant magnetic stirring by using right angle illumination. Excitation and emission wavelengths were 430 and 520 nm, respectively. For the PTR experiments, the suspension (vesicles or protein + vesicles) is placed in a small thermostatable cuvette (1 mL) connected to a water bath circulator. Dilution of the vesicles and mixing with the proteins are made at room temperature, under magnetic stirring. The suspension is then heated rapidly through $T_m$ (60 °C/min) up to 65 °C, and fluorescence is monitored continuously on a Servogor. To determine the fluorescence intensity achieved by release of 100% of the dye, 10 μL of a $C_{12}E_8$ solution was added to dissolve the vesicles (final concentration 0.13%) (TX-100 was not used at high $T$ because of its cloud point). The fluorescence intensities were corrected for the temperature dependence of calcein quantum yield.

*Turbidity Measurements.* Aggregation of LUV was followed by recording the change in absorbance over time at 550 nm in a Shimatzu double-beam UV 190 spectrophotometer (Kyoto, Japan). The total volume of the sample was 750 μL, containing 37.5 nmol of phospholipid. All turbidity measurements were carried out at 20 °C according to the procedure described for leakage in section A above.

*Lipid Mixing–Fusion Assays.* Lipid mixing was determined by changes in fluorescence intensity resulting from fluorescence energy transfer between the head group labeled probes NBD-PE and Rh-PE as described by Struck et al. (1981). Labeled LUV containing both probes at 1% (molar ratio) each were mixed in a 1/9 mole ratio with probe-free liposomes at a final concentration of 50 μM. In the labeled liposomes, the NBD fluorescence is quenched by Rh; upon dilution of the probe during mixing between labeled and unlabeled membranes, the efficiency of transfer decreases and NBD fluorescence increases. The initial fluorescence of the 1/9 (labeled/unlabeled) suspension was taken as 0% fluorescence, and the 100% fluorescence was calibrated by using an equivalent concentration of LUV prepared with 0.1% of each fluorescent phospholipid. The suspensions were irradiated at 450 nm, and the NBD fluorescence was recorded at 530 nm. Other experimental conditions were identical with those described for leakage in section A above.

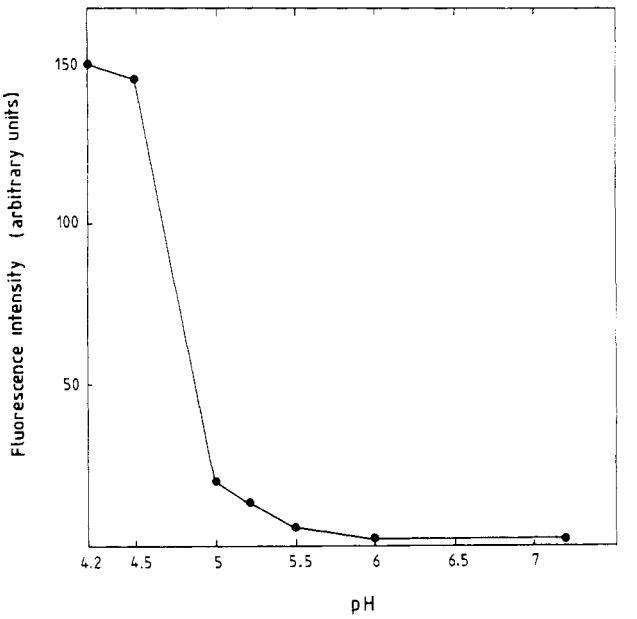


FIGURE 1: Effect of pH upon the fluorescence intensity at 470 nm of ANS ($5 \times 10^{-5}$ M) incubated with diphtheria toxin ($1.5 \times 10^{-6}$ M). The excitation wavelength was 380 nm, and the temperature was set to 20 °C.

## RESULTS

*Effect of pH upon 1-Anilino-8-naphthalenesulfonate Binding to Diphtheria Toxin.* Both emission wavelength maximum ($\lambda_{max}$) and quantum yield of 1-anilino-8-naphthalenesulfonate (ANS) probe are dependent upon the polarity of the surrounding medium: an increase in quantum yield and a blue shift indicate a binding of the probe to hydrophobic regions of proteins (Stryer, 1968). When ANS is incubated in the presence of DT at pH 4.2, the ANS $\lambda_{max}$ is shifted from 510 to 470 nm whereas no modification is observed in the presence of DT at pH 7.2 (data not shown). The fluorescence intensity at 470 nm of ANS incubated with DT at different pHs reveals a transition occurring close to pH 5.0 (Figure 1). An identical pH dependence has been described for the binding of DT to detergent micelles (Blewitt et al., 1985).

*pH-Dependent Calcein Release Mediated by Diphtheria Toxin. (A) Effect of the Liposome Lipid Composition and Physicochemical State.* Figure 2 illustrates the effect of pH and liposome composition upon the leakage induced by diphtheria toxin. At neutral pH, addition of the protein to the vesicles does not cause any leakage of calcein, whatever the bilayer composition. On the contrary, at pH 4.7 (Figure 2B) an immediate release of calcein is observed upon the addition of the protein to asolectin or egg PC vesicles. The progressive enhancement in DT-induced release of calcein, observable when the pH is lowered from 7.4 to 4.5, is strongly dependent on the composition of the vesicles (Figure 2A): at a given pH, the release is always faster with the fluid negatively charged asolectin vesicles as compared to the neutral fluid egg PC vesicles. Moreover, the pH dependence of the initial release rates for egg PC (Figure 2A) shows a transition close to pH 5.0; the transition pH observed in identical experimental conditions with asolectin vesicles is shifted upward (pH 5.25), suggesting the involvement of an electrostatic contribution in

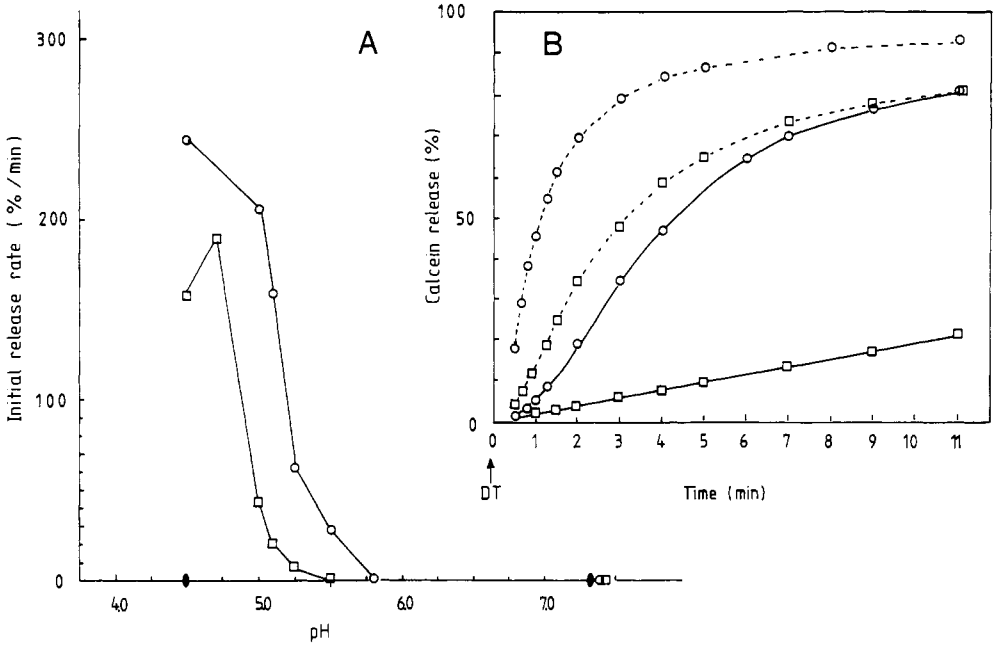


FIGURE 2: Effect of pH upon diphtheria toxin induced leakage of calcein entrapped in asolectin (○), egg PC (□), and DPPC or DPPC/DPPA (9/1) (●) LUV (50 μM) at 20 °C. (A) Initial leakage rates observed at a protein concentration of $10^{-7}$ M. (B) Time course of the release observed when DT ($2.5 \times 10^{-8}$ M) is added at pH 4.7 (---) or 5.0 (—).

Table I: Phase Transition Release Experiments (PTR)[a]

| | pH 4.5 (%) | pH 5.25 (%) | pH 7.4 (%) |
|---|---|---|---|
| DPPC/DPPA | 39 | 21 | 14 |
| DPPC/DPPA + DT ($2.5 \times 10^{-7}$ M) | 76 | 55 | 15 |
| DPPC | 40 | 20 | 13 |
| DPPC + DT ($2.5 \times 10^{-7}$ M) | 73 | 51 | 15 |

[a] LUV (50 μM) containing calcein (65 mM) were taken through the phase transition (20 to 65 °C in 45 s). The 100% leakage point is defined by the post-$C_{12}E_8$ (0.13% final concentration) fluorescence intensity.

the interaction of the toxin with the lipid bilayer. The ability of DT to induce calcein leakage is dependent on the rigidity of the PC-containing vesicles: at 20 °C, rigid vesicles composed of DPPC or DPPC/DPPA (9/1 mol/mol) stayed completely impermeable to calcein upon toxin addition at low pH (Figure 2A) in agreement with observations made by Papini et al. (1987a) with SUV (DPPC/DPPA 9/1 w/w). We have submitted those large vesicles to a phase transition release experiment (PTR: for details, see Materials and Methods) as described by Weinstein et al. (1984). During those experiments, the temperature of the suspension (transferred into a thermostated cuvette) is raised through the phase transition, and the subsequent percentage of dye release observed at 60 °C is reported in Table I for three pHs and two lipidic compositions. In the absence of toxin, the lower the pH, the higher the leakage associated to the phase transition. Lowering the pH from 5.25 to 4.50 corresponds to a 2-fold increase in the leakage. As identical results are obtained with the two liposome compositions regardless of their charge, this enhanced leakage at acidic pH can only be explained by the progressive neutralization of negative charges carried by calcein at low pH (Allen, 1984), which facilitates the permeation process.

At pH 7.4, the addition of DT to the LUV does not modify the leakage during the PTR experiment; this suggests that, during the short time required to bring the system through the phase transition, DT does not experience a thermal denaturation which would expose hydrophobic domains; at pH 4.5, on the contrary, DT triggers an immediate release (≈75%) of calcein. If we assume that the leakage rate reflects the extent of protein penetration into the lipid bilayer, those results show the difficulty for DT to penetrate into tightly packed rigid bilayers. Similar results have been reported when clathrin is added to PE-containing bilayers (Hong et al., 1985). However, during the passage through the lipid phase transition, the coexisting fluid and rigid lipid domains offer the opportunity to the protein to insert into the liposomes if the hydrophobic domain has been unmasked by pH lowering.

*(B) Effect of the DT Concentration and Preaggregation.* Several studies have been performed to determine whether the toxin is associated to the membrane as a monomer or as a dimer or in a more aggregated state (Donovan et al., 1981; Shiver & Donovan, 1987). Study of the calcein release induced on neutral vesicles at pH 5.0 shows that the rate of DT-induced leakage is directly proportional to the amount of protein (in the concentration range studied, 1.5–15 μg/mL), with a log slope of 1.1 (data not shown). However, below pH 5.0 for PC vesicles and below pH 5.25 for asolectin LUV, no linearity is observed between the initial rate of calcein release and either the DT concentration or the square of this concentration. This suggests that the aggregation state of DT within the lipid membrane is strongly dependent upon the pH and the lipid composition of the vesicles. It should be mentioned that DT after different times of preincubation at pH 4.5–5.0 was less effective in inducing leakage of the dye from egg PC vesicles at pH 4.5 (Figure 3). This indicates that, in a highly aggregated state (Blewitt et al., 1985), the toxin partly loses its ability to insert into a lipid bilayer.

*pH-Dependent Calcein Release Mediated by Fragments A and B.* In order to determine which fragment of DT was responsible for the calcein release, DTA and DTB were added to egg PC or asolectin vesicles at different pHs and calcein release was followed as a function of time (Figure 4). Both fragments induce a release of calcein entrapped in egg PC or asolectin vesicles when the pH is lowered, but the comparison of Figure 4 (DTA and DTB concentration: $2.5 \times 10^{-7}$ M) with Figure 2B (DT concentration: $2.5 \times 10^{-8}$ M) shows that the fragments have a much lower potency than the native toxin to induce the release of the dye.

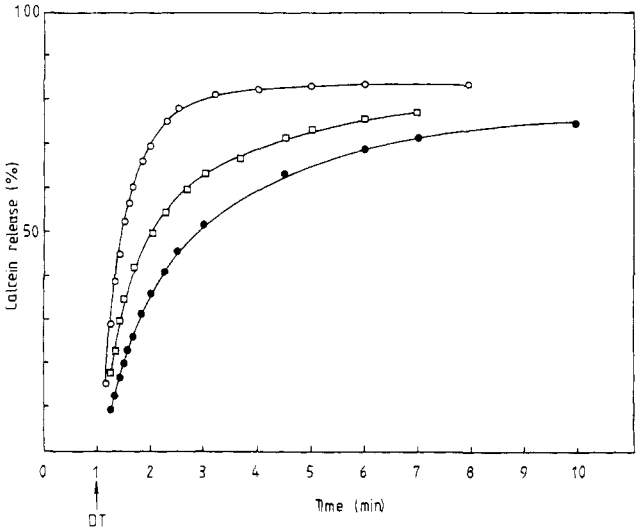


FIGURE 3: Effect of preincubation of DT at pH 4.5 on the time course of the calcein release at 20 °C. DT ($10^{-7}$ M) was added at pH 4.5 on egg PC LUV (50 μM) containing calcein (65 mM). (○) Without prior incubation of the toxin at acidic pH; (□) after an incubation of 30 min at pH 4.5; (●) after an incubation of 3 days at pH 4.5.

Comparison of panels A–D of Figure 4 occasions two further remarks: (1) The effect of DTA can only be observed at a pH lower than 5.0, and the small efflux of the dye (≈25%) from neutral vesicles and that from charged vesicles are quite similar (Figure 4A,B). (2) With DTB there is a large increase of leakage (80%) when the protein fragment is added to the asolectin vesicles (Figure 4C), and the kinetics of release is reminiscent of that observed for DT (Figure 2B). On the other hand, the calcein release induced by DTB on PC vesicles never exceeds 30%, but a pH dependency up to pH 5.50 is obvious (Figure 4D). This indicates that the B fragment is responsible for the sensitivity of DT to the presence of negative charges in the vesicles.

*Protein-Induced Aggregation and Fusion of LUV.* The fusogenic properties at low pH of DT and some of its mutants were established so far for SUV liposomes (Cabiaux et al., 1985; Papini et al., 1987a). It was thus worthwhile to determine whether the calcein releases observed with our LUV populations were correlated (or not) to liposome fusion. For this purpose, protein-induced aggregation and (or) fusion of the vesicles were (was) followed by optical density measurements, and lipid mixing of the liposomes was monitored by the resonance energy transfer method (Struck et al., 1981).

Addition of DT and its fragments to neutral egg PC LUV as well as addition of DTA to asolectin LUV does not cause any significant change in turbidity (data not shown). On the contrary, DT triggers an immediate increase of asolectin LUV turbidity as shown in Figure 5. The kinetics of turbidity increase is markedly dependent on the pH; the turbidity changes are maximum and faster below pH 5.0, which suggests that the protonation of the carboxylic acids of the toxin is a prerequisite for this process. To evaluate the role of the lipid mixing in the turbidity increase, the proteins were added to a suspension of asolectin–Rh-PE–NBD-PE LUV and asolectin LUV (molar ratio 1/9) and the NBD fluorescence was followed at 530 nm (Figure 6). DT induces a rapid lipid mixing of asolectin vesicles when incubated below pH 5.0 (Figure 6A). The identical increase of fluorescence intensity observed after addition of DTB to the asolectin vesicles (Figure 6B) suggests that the domains of DT involved in the fusion process are mostly located in the DTB fragment. No fusion occurs with egg PC vesicles (data not shown), indicating that the calcein release induced by the toxin and its A and B fragments is only due to the destabilization of these vesicles and not to a leaky fusion. DT-induced leakage without significant fusion takes place for asolectin too at pH 5.0 and above. Indeed, at this pH, the fusion remains at a very low level whereas a 90% calcein release is obtained. This immediate leakage of the vesicle content in the presence of toxin explains why we failed to demonstrate at pH 4.5 the mixing of asolectin liposome

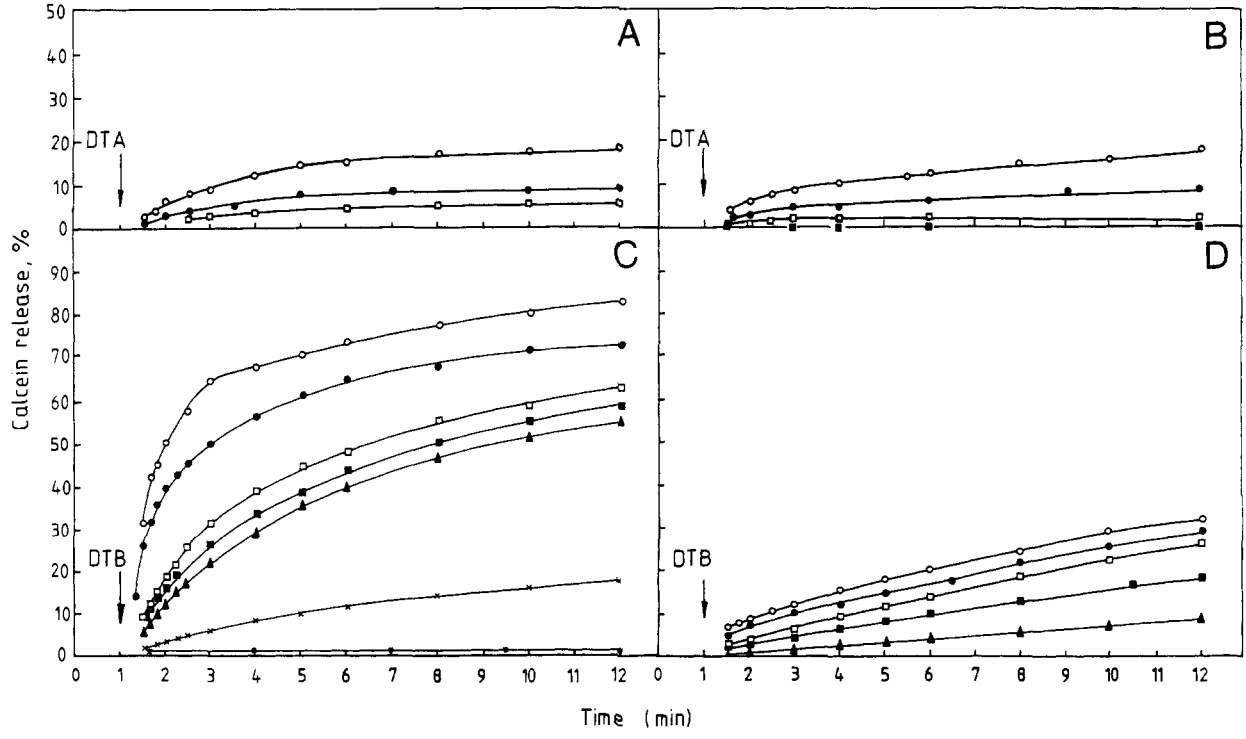


FIGURE 4: Effect of pH on the time course of DTA- (A, B) and DTB- (C, D) induced release of calcein (30 mM) encapsulated in asolectin (A, C) or egg PC (B, D) LUV (50 μM). Proteins $2.5 \times 10^{-7}$ M. $T = 20$ °C. (○) pH 4.25; (●) pH 4.5; (□) pH 4.75; (■) pH 5.0; (▲) pH 5.25; (×) pH 5.45; (*) pH 5.55.

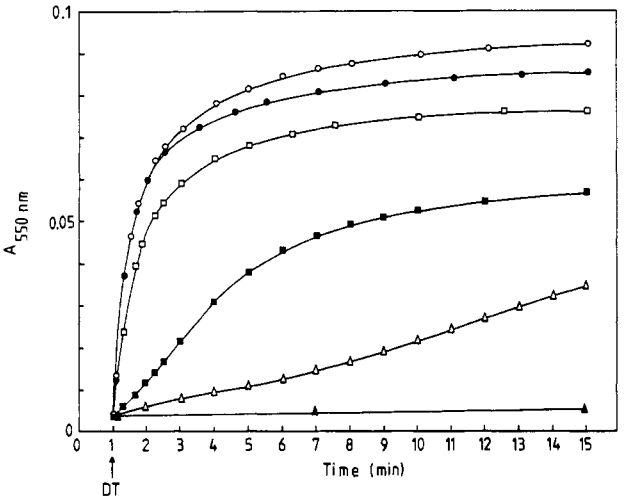


FIGURE 5: Effect of pH on the time course of the DT-induced absorbance change of asolectin LUV (50 μM) at 550 nm. Proteins: 2.5 × $10^{-7}$ M. $T$ = 20 °C. Buffer was used to set the 0 absorbance. (○) pH 4.25; (●) pH 4.5; (□) pH 4.75; (■) pH 5.0; (△) pH 5.1; (▲) pH 5.25. Addition of DTA does not modify the absorbance of asolectin LUV, and the absorbance of egg PC LUV was never modified whatever the protein added.

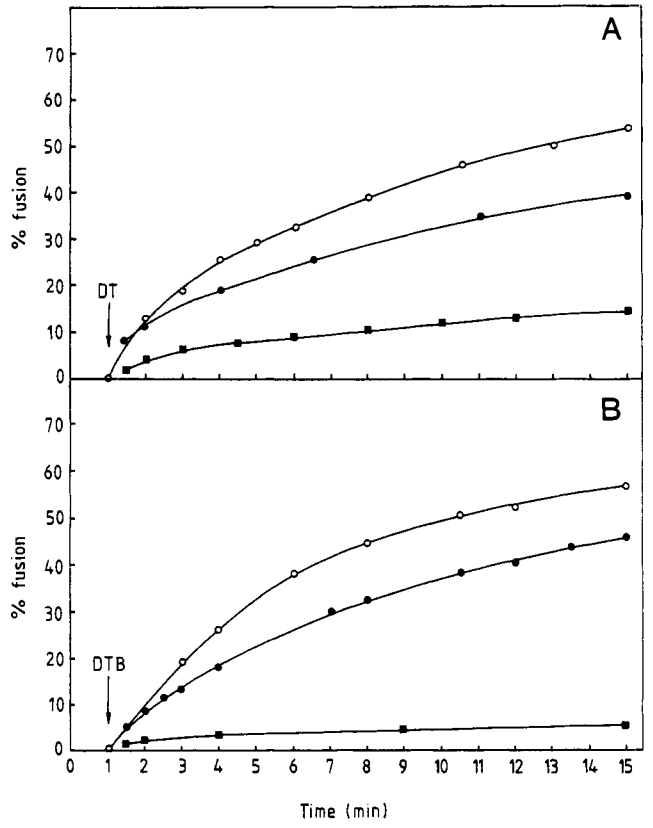


FIGURE 6: pH dependence of DT- (A) and DTB- (B) induced fusion of asolectin LUV (50 μM). The lipid mixing was measured by the resonance energy transfer (RET) assay between NBD-PE and Rh-PE. Each sample contained 50 μM LUV composed of 1 part labeled asolectin and 9 parts unlabeled liposomes. Proteins: 2.5 × $10^{-7}$ M. $T$ = 20 °C. (○) pH 4.25; (●) pH 4.5; (■) pH 5.0.

aqueous phases using the ANTS/DPX assay (Ellens et al., 1984, 1985).

## Discussion

The increase of ANS quantum yield associated with a $\lambda_{max}$ blue shift indicates that exposure of DT hydrophobic domains occurs between pH 5.0 and 4.5. This exposure is accompanied by a toxin conformational change as evidenced from tryptophan fluorescence measurements (Blewitt et al., 1985). The correspondence between the transition pHs observed for the ANS fluorescence (Figure 1) and calcein-release experiments (Figure 2A) suggests that the hydrophobic domains of DT, exposed at low pH, are involved in the destabilization of egg PC LUV.[2] The calcein release would be due to the insertion of these domains into the lipid bilayer.

In agreement with the photolabeling experiments of Montecucco et al. (1985) and Papini et al. (1987b), we observed a stronger interaction of DT with asolectin vesicles than with egg PC vesicles. For identical pH values, the calcein release is always faster for asolectin LUV than for egg PC LUV. Moreover, the transition pH of the calcein initial rate of release from asolectin liposomes is shifted upward to pH 5.25. This shift could originate from the negatively charged surface of the asolectin vesicles, giving rise to high cation concentrations in the diffuse double layer existing at the membrane–solution interface. According to the Gouy equation and considering a mean surface charge density of 3 × $10^{-3}$ charged group/Å$^2$ for asolectin (Letters, 1964; Papini et al., 1987a) (corresponding to around 20% of negative lipids), the calculated surface pH is at least 0.5 pH unit lower than that of the bulk solution [for a detailed discussion see Chung and London (1988)].

In these conditions, at low pH, it is also conceivable that the cationic toxin (isoelectric pH = 5.4 for free monomer; Chung & London, 1988) will concentrate at the membrane–solution interface, causing a faster release of calcein from asolectin vesicles. Moreover, a low surface pH as compared to the bulk pH should favor the acidic pH conformation and therefore trigger the insertion of the toxin into asolectin vesicles even when the bulk pH is maintained above the transition pH of the toxin conformational change. Chung and London (1988) observed a similar effect on the association of DT with neutral or anionic vesicles.

The physical state of the liposomal membrane is another important factor governing the release of dye induced by DT at acidic pH. Indeed, our results suggest that the coexistence of fluid and rigid phases at the transition allows a deeper penetration of the toxin into the hydrocarbon core of the vesicles as evidenced by the increase of calcein release at the transition temperature of the vesicles in the presence of DT (Table I). PTR experiments have previously shown such a preferential interaction with lipids at the transition temperature for, e.g., tubulin, actin (Klausner et al., 1981), and phospholipase A2 (Okimasu et al., 1982).

Both DTA and DTB are capable of releasing entrapped calcein from egg PC and asolectin vesicles, but their efficiency at a given pH is lower than that of the entire toxin. This suggests that the association of the two fragments is required for a maximum destabilization of the lipid layer. This is consistent with a translocation model proposed by Bisson and Montecucco (1987) in which both toxin fragments insert into the lipid bilayer and then fragment A—in association with the B fragment—crosses the membrane, leaving behind fragment B. Since the calcein release mediated by the B fragment occurs at a higher pH than the release mediated by the A fragment, it is reasonable to assume that, during pH lowering in the endosome, the B fragment inserts into the lipid bilayer before

[2] A further support of this hypothesis is provided by the lower release of calcein entrapped in PC vesicles observed at pH 5 in the presence of preaggregated toxin. Indeed, DT aggregates extensively in solution at acidic pH probably as a result of interactions between its hydrophobic sites (Blewitt et al., 1985).

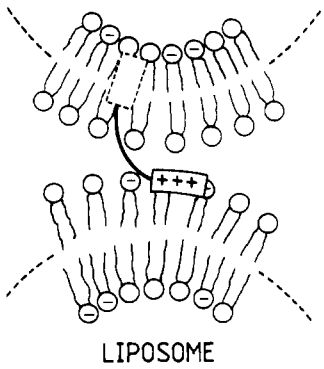


FIGURE 7: Model proposed for the fusion of negatively charged vesicles by DTB. (Box with dashed outline) CB1; (box with crosses) CB4.

the A fragment. In this process, DTB would favor the insertion of DTA into the membrane, thereby forming a complex that would lead to the DTA translocation through the membrane.

Several reports have shown that incubation at acidic pH of DT with liposomes caused intense vesicles aggregation and fusion (Cabiaux et al., 1984, 1985; Papini et al., 1987a). But those studies have been carried out with SUV which are naturally less stable than the LUV used in the present paper (Wilschut & Hoekstra, 1986). For instance, polylysine (Gad et al., 1982, 1985) and GALA (Parente et al., 1988) are "fusogenic" toward SUV but not toward LUV of the same composition.

Our study demonstrates that lipid mixing can be induced by DT or DTB on asolectin LUV; the destabilization of the lipid bilayer resulting from the insertion of DT hydrophobic domains is necessary but not sufficient to ensure the fusion: indeed, a significant calcein release occurred with DT and DTB incubated with egg PC vesicles up to pH 5.25 or asolectin vesicles up to pH 5.50 whereas the lipid mixing requires pH <5.0 and negatively charged asolectin vesicles.

Therefore, the interaction of DT with lipid membrane should proceed in—at least—two steps: (1) One step leads to the destabilization of the vesicles without mixing of their lipidic phases. This phenomenon occurs on PC vesicles below pH 5.25 and on asolectin vesicles between pH 5 and 5.5. (2) The second step leads to a more complex situation in which lipid mixing is observed and occurs only with asolectin vesicles below pH 5. From available data about fusion [for a review see Wilschut and Hoekstra (1986)] it appears that the capacity of a peptide for promoting fusion is to be related to two main characteristics: First, when negatively charged phospholipid vesicles are used, there appears to be a general requirement for the peptide to be positively charged, allowing an initial electrostatic interaction with the lipid bilayer. Second, with only a few exceptions, the peptide reveals an amphipathic character, exposing one or more hydrophobic segments capable of inserting and destabilizing—or modifying the structure of—the lipid bilayer. Recent polarized infrared spectroscopy measurements have demonstrated that domains located in CB1 (residues 147–265) adopt an $\alpha$-helical structure, oriented parallel to the lipid acyl chains of DPPC/DPPA (9/1 w/w) vesicles, and that the peptide CB4 (residues 1–37) binds to the surface of a negatively charged bilayer made of DPPC/DPPA (9/1 w/w) (Cabiaux et al., 1988). As DTB is as fusogenic as DT, we proposed a hypothetical model consistent with the experimental data: the fusion is promoted both by the penetration of the CB1 hydrophobic domain into the bilayer and by the association of a positively charged domain—such as CB4—with the surface of the bilayer (Figure 7). Such interactions should lead to the aggregation of the vesicles, immediately followed by the lipid mixing mediated by the insertion of the hydrophobic domain into the lipid layer. In this model, two distinct domains of DTB would be involved in the fusion process as in the model proposed for the fusion of PS/PE (1/1) SUV by lactalbumin at low pH where hydrophobic and positively charged domains have been involved in the process (Kim & Kim, 1986).

With vesicles made of neutral lipid as egg PC, the aggregation of the vesicles is not allowed and only the insertion of the hydrophobic domains is observed, leading to a destabilization without any fusion. Destabilization without fusion is also observed on the asolectin system above pH 5.0. This could be explained by the inability of the B fragment to bind to the negatively charged surface of liposomes—probably because of its partial protonation above pH 5.0—and to promote aggregation leading to fusion. Again, in these conditions, the insertion of the hydrophobic domains would be responsible for the calcein release. However, the DT-induced destabilization of LUV is not fully explained in terms of the model proposed for DTB as it does not involve the A fragment which seems to give to DT a higher potency to destabilize bilayers but not to fuse them.

## ACKNOWLEDGMENTS

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**Registry No.** DPPC, 2797-68-4; DPPA, 5129-68-0.

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# ATP Does Not Regulate the Reconstituted Glucose Transporter†

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ABSTRACT: ATP has been reported to affect glucose transport in human erythrocytes and resealed erythrocyte ghosts [Jacquez, J. A. (1983) *Biochim. Biophys. Acta 727*, 367-378; Jensen, M. R., & Brahm, J. (1987) *Biochim. Biophys. Acta 900*, 282-290]. In more detailed studies, effects of micromolar levels of ATP on transport in ghosts and inside-out vesicles, and on the fluorescence of ghosts and the purified glucose transporter [Carruthers, A. (1986) *Biochemistry 25*, 3592-3602; Hebert, D. N., & Carruthers, A. (1986) *J. Biol. Chem. 261*, 10093-10099; Carruthers, A. (1986) *J. Biol. Chem. 261*, 11028-11037], have been interpreted as supporting a model in which ATP regulates the catalytic properties of the transporter. Both allosteric and covalent effects of ATP were proposed; among the allosteric effects was a 60% reduction in the $K_m$ for zero-trans uptake. In order to test whether allosteric ATP regulation of the transporter occurs, we reconstituted glucose transport activity into liposomes using erythrocyte membranes without detergent treatment. The effects of ATP, present either outside, inside, or both inside and outside the liposomes, on the transport activity were examined. Effects of ATP on trypsin-treated liposomes, which have only a single orientation of active transporters, were also tested. While the model predicts activation by ATP, only inhibition was observed. This was significant only at millimolar concentrations of ATP, in contrast to the previously reported effects at micromolar levels, and was primarily on the extracellular surface of the transporter. In addition, the ATP effects on reconstituted transport were nonspecific, with similar effects produced by tripolyphosphate. The $K_m$ for zero-trans uptake in trypsin-treated liposomes was unaffected by the presence of 4 mM ATP, also in disagreement with the proposed allosteric effects. While these results do not address possible regulatory effects of ATP due to covalent modifications, they argue against allosteric effects of ATP acting directly on the glucose transporter.

A number of studies of glucose transport in human erythrocytes have dealt with the effects of ATP on the transport rate. Jacquez (1983) reported that depletion of erythrocytes of their ATP lowered the $V_{max}$ for zero-trans uptake of glucose at 5 °C by 70-80%, with no effect on the $K_m$. Hemolysis and resealing in the presence of ATP or ADP reversed the change in $V_{max}$, with half-maximal effects at about 0.6 mM nucleotide

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