Interaction of Diphtheria Toxin with Model Membranes†

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ABSTRACT: Low pH is believed to trigger membrane penetration by diphtheria toxin in vivo. The effect of pH upon the binding of the toxin to unilamellar model membrane vesicles was determined by using a fluorescence quenching assay. A series of studies were undertaken to determine the effect of lipid composition upon the binding of lipids to the toxin. The binding of toxin to various small unilamellar vesicles of zwitterionic or anionic lipids was similar in extent and was accompanied by deep penetration of the toxin into the fatty acyl chains, in agreement with previous studies. However, the transition pH, which is the pH at and below which toxin binding becomes significant, depended upon the fraction of anionic lipids, being highest with model membranes composed totally of anionic lipids (pH 5.8) and lowest with membranes composed of zwitterionic lipids (pH 5.2). Except for vesicle charge, the transition pH was independent of the nature of the lipid polar groups used. High ionic strength, which had no effect on the transition pH with zwitterionic vesicles, was found to shift the transition pH with totally anionic vesicles to pH 5.2. This suggests that both direct protein–lipid electrostatic interactions and the ionic double layer, which gives rise to a low local pH around anionic vesicles, contribute to the shift in the transition pH. The effect of lipid composition upon the kinetics and strength of binding was also examined. At low pH, binding was rapid and tight. Binding to vesicles containing 20 wt % anionic phosphatidylglycerol was faster and tighter than binding to vesicles of zwitterionic phosphatidylcholine. In addition, binding to phosphatidylcholine vesicles was decreased markedly both when vesicle size was large and when toxin was preincubated in the absence of lipid, whereas these behaviors were not observed with vesicles containing 20% phosphatidylglycerol. These differences probably arise from electrostatic interactions as well. We conclude that the interaction of toxin with lipid vesicles at low pH has both hydrophobic and electrostatic components. This has important implications for both the behavior of the toxin in vivo and the design of experiments for study of toxin behavior in model membranes.

Diphtheria toxin is a protein produced by *Corynebacterium diphtheriae*. The toxin is secreted as a single polypeptide (M_r 58 348) but can be readily cleaved into two fragments, or subunits, A (M_r 21 167) and B (M_r 37 199), which are joined to each other by a disulfide bond (Pappenheimer, 1977; Collier, 1982; Uchida, 1983). Several studies have demonstrated that toxin enters cells via receptor-mediated endocytosis and that it then reaches the cytoplasm by penetration through the membrane of an acidic organelle, which is triggered by a low-pH-induced change in toxin conformation (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981; Marnell et al., 1984). Once the A subunit is released in the cytoplasm, it catalyzes an ADP-ribosylation of elongation factor 2, halting protein synthesis and causing cell death (Pappenheimer, 1977). The molecular mechanism by which toxin penetrates membranes and translocates its A subunit is unknown, although several models have been proposed (Donovan et al., 1981; Hu & Holmes, 1980; Kagan et al., 1981; Misler, 1984; Zalman & Wisnieski, 1984; Montecucco et al., 1985). Elucidation of the mechanism of membrane penetration and translocation by diphtheria toxin is an important goal as it may have important implications for designing immunotoxins, for understanding the viral fusion proteins that mediate viral penetration through acidic organelles, and for understanding the insertion and translocation of newly synthesized membrane and secreted proteins.

Several studies have examined interactions between toxin and lipid. Photolabeling studies have detected penetration of the toxin into the hydrocarbon chains of the bilayer at low pH (Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Montecucco et al., 1985). Consistent with this result, toxin binds to nonionic detergent micelles at low pH (Sandvig & Olsnes, 1981; Blewitt et al., 1984). It has also been shown that toxin induces channel formation in model membranes at low pH (Donovan et al., 1981; Kagan & Finkelstein, 1981), which implies that membrane penetration must occur. However, less is known about the effect of lipid structure on toxin–lipid interaction. Alving et al. (1980) measured binding of various lipids to toxin and concluded specific toxin–phosphate interactions were important in binding. However, these investigators did not distinguish between intrinsic and extrinsic interactions or systematically vary vesicle structure.

In previous studies, we have investigated the nature and cause of the hydrophilic to hydrophobic conformational change triggered by low pH. Using a new fluorescence quenching technique to assay hydrophobicity (Blewitt et al., 1984; London, 1986), we found (Blewitt et al., 1985; Zhao & London, 1986) that the low-pH-induced transition from the hydrophilic state to the hydrophobic state involves a highly cooperative conformational change which is denaturation-like [as first suggested by Pappenheimer (1982)] and have suggested mechanisms whereby protonation of acidic and/or basic residues could trigger such changes (London et al., 1986). Using fluorescence quenching, we have now investigated both the nature of toxin–lipid interaction and the effect of lipid composition in greater detail. The effects of lipid charge, headgroup structure, and vesicle size have been examined. We find the pH of the hydrophilic to hydrophobic conformational

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change is dependent on lipid charge and have been able to show that there is an electrostatic component in the toxin–lipid interaction at low pH. On the basis of these results, some of the earlier conclusions about the nature of toxin–lipid interaction and how it is affected by lipid composition must be revised.

Experimental Procedures

Materials. Partially purified diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada), and the various forms of the toxin were purified as previously described (Collier & Kandel, 1971; McKeever & Sarma, 1982). Free monomers were used in most of the experiments. This preparation contained a mixture of molecules nicked between A and B subunits and intact toxin as judged by sodium dodecyl sulfate (SDS)[1] gel electrophoresis. After long storage, the dimer fractions were found to contain some monomer (20–30%), probably due to slow dissociation. Purified toxin was stored at 4 °C in 5 mM Tris/tartarate, 1 mM EDTA, and 0.02% sodium azide, pH 8. Unlabeled lipids and 12-doxyl-PC were purchased from Avanti Polar Lipids (Birmingham, AL). DPPE-pyrene was purchased from Molecular Probes (Eugene, OR). 6-Pyrene-PC was purchased from KSV-Chemicals (Helsinki, Finland). Asolectin (mixed soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY). Phosphorylcholine chloride (calcium salt) and inositol hexaphosphate (phytic acid) were purchased from Sigma.

The purity of the lipids was checked by TLC on Adsorbosil Plus plates (Alltech Associates, Deerfield, IL) using $CHCl_3$/methanol/H_2O, 65:35:5 (v/v), for uncharged lipids, zwitterionic lipids, and DOPG and using both $CHCl_3$/methanol/concentrated NH_3, 65:25:5 (v/v), and $CHCl_3$/methanol/acetic acid/H_2O, 25:15:4:2 (v/v), for DOPA and DOPS. All lipids showed a single spot when detected with a phosphate spray and by charring (Dittmer & Lester, 1964), except for 6-pyrene-PC, which after a long storage period showed slight breakdown into fatty acid and lyso-PC. The concentrations of the lipid solutions were determined by gravimetric or phosphate analysis, and the agreement between manufacturers' specifications and the experimentally determined concentrations was good. All lipids were used without further purification.

Sample Preparation. Mixtures of the appropriate lipids in chloroform were prepared, dried under a stream of nitrogen, and then dried further under high vacuum for 30–60 min. For sonication, the lipids were resuspended in 150 mM NaCl at a concentration of 5 mg/mL, deoxygenated with nitrogen, and then sonicated in an argon-flushed tube placed in a bath sonicator (Laboratories Supply Co., Hicksville, NY). Samples were sonicated for 20-min periods until nearly optically clear (60–120 min total). The sonicated vesicles were stored under argon at room temperature if they were kept overnight. They were mixed with the appropriate buffer just prior to use. This method was used to prepare vesicles for pyrene quenching measurements unless otherwise noted. For ethanol injection, dried lipids were dissolved in absolute ethanol at a concentration of 5 mg/mL and then diluted 100-fold into the appropriate buffer. This method was used to prepare vesicles for measurement of 12-doxyl-PC quenching. Large unilamellar vesicles (LUV) were prepared by freeze–thawing sonicated vesicles 2–3 times in a dry ice/acetone bath. The size of some vesicle preparations was determined by quasi-elastic light scattering using a Nicomp particle sizer.

Unless otherwise stated, 1-mL samples contained 50 μg/mL phospholipid, 10 mM buffer, 5 mM EDTA, and 150 mM NaCl. Binding was initiated by the addition of a small aliquot (2.5 μL) of free monomer toxin to the solution to give a final concentration of 2.5 μg/mL. Fluorescence was measured after incubation for 0.5–1 h at room temperature. The buffers used were sodium formate for pH 3 and 3.4, sodium acetate for pH 4–6, and Tris-HCl or sodium phosphate for pH 7. No differences were found between toxin behavior in Tris or phosphate buffers. Sample pH was determined after measurement of fluorescence.

Fluorescence. Fluorescence measurements were taken on a Spex 212 Fluorolog spectrofluorometer. Intensity in samples of 1 mL was measured in quartz cuvettes having 1-cm excitation/0.3-cm emission path lengths. The excitation wavelength was 280 nm, and emission was measured at 330 nm. Slit widths with a nominal band-pass of 2.25 nm (excitation) and 4.5 nm (emission) were used. The intensity of background samples without toxin was subtracted from each reading. Inner filter effects due to absorbance in samples containing pyrene were corrected for by multiplying the observed fluorescence by the factor $10^{0.5A_{280}+0.15A_{330}}$, where A_{280} and A_{330} refer to the absorbance of pyrene lipid in the samples when measured in 1-cm cuvettes (London, 1986).

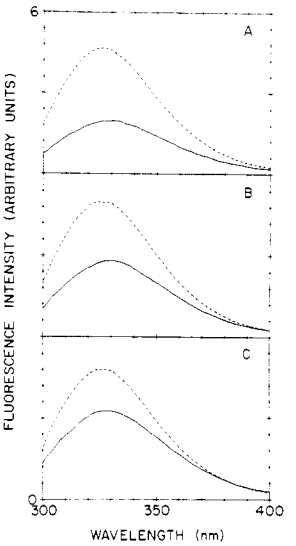

FIGURE 1: Effect of pH upon the intrinsic fluorescence of diphtheria toxin. Samples were prepared as described under Experimental Procedures except that a concentration of 5 μg/mL toxin was used. Dashed line, pH 7; solid line, pH 4. Shown are corrected emission spectra in the (A) absence of model membranes, (B) presence of egg PC SUV, and (C) presence of 20 wt % DOPG in egg PC SUV. The excitation wavelength was 280 nm.

Results

Effect of pH upon Diphtheria Toxin Binding and Penetration of SUV: Detection by Fluorescence Quenching. Figure 1 shows the effect of pH upon the fluorescence of diphtheria toxin both in the presence and in the absence of SUV model

[1] Abbreviations: ApUp, adenylyl(3′,5′)uridine 3′-phosphate; DOPA, dioleoyl-*sn*-glycerol 3-phosphate; DOPG, dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPS, dioleoyl-*sn*-glycero-3-phosphoserine; DOPE, dioleoyl-*sn*-glycero-3-phosphoethanolamine; 12-doxyl-PC, 1-palmitoyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphocholine; egg PC, egg diacyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; IHP, inositol hexaphosphate; LUV, large unilamellar vesicle(s); PI, diacyl-*sn*-glycero-3-phosphoinositol; 6-pyrene-PC, 1-palmitoyl-2-(6-pyrenylhexanoyl)-*sn*-glycero-3-phosphocholine; DPPE-pyrene, *N*-(1-pyrenesulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; SUV, small unilamellar vesicle(s); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

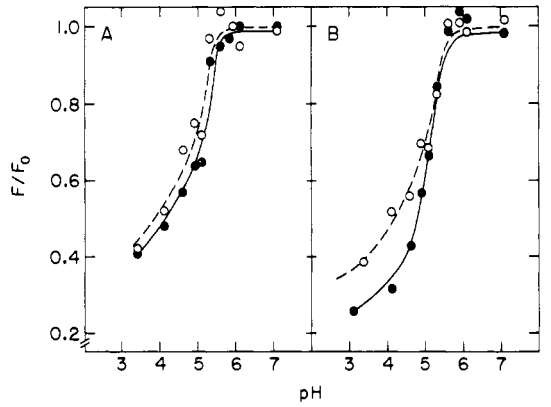

FIGURE 2: Binding and penetration of SUV model membranes by diphtheria toxin. F/F_0 is the ratio of fluorescence in the presence of vesicles containing quenchers [5 wt % DPPE-pyrene (closed symbols) or 20 wt % 12-doxyl-PC (open symbols)] to that in the presence of vesicles without quenchers. The composition of the sample solutions is given under Experimental Procedures. (A) Egg PC SUV prepared by ethanol injection; (B) 20 wt % DOPG in egg PC SUV.

membranes. At neutral pH, both the fluorescence intensity and emission wavelength maximum (λ_{max} is about 326 nm) are unaffected by the presence of SUV. At low pH, there is a decrease in intensity and a red shift in λ_{max} (to about 329 nm) in both the presence and absence of lipid. In the presence of SUV, the decrease in intensity is somewhat less than in their absence. This pH dependence of fluorescence and difference in fluorescence intensity in the presence and absence of lipid at low pH are similar to those previously observed in the presence and absence of detergent (Blewitt et al., 1985).

The effect of SUV upon the pH-induced decrease in fluorescence intensity suggests toxin is interacting with lipids at low pH. To examine the interaction in more detail, a fluorescence quenching assay was used to detect binding of toxin to model membranes. In this assay, binding is detected by the long-range energy-transfer-induced quenching of the fluorescent Trp residues of membrane-bound toxin caused by pyrene-labeled lipid. The degree of quenching is given by F/F_0, which is the ratio of fluorescence in samples containing 5 wt % pyrene-labeled lipid (F samples) to that in samples containing lipid without quencher (F_0 samples). The closed circles in Figure 2 show the quenching-detected binding of diphtheria toxin to SUV as a function of pH. At neutral pH, F/F_0 is approximately 1, indicating there is negligible binding. At low pH, there is a transition to a state in which there is considerable binding to both egg PC and 20 wt % DOPG SUV as shown by the marked decrease in F/F_0. The transition occurs close to pH 5, in good agreement with the pH at which the toxin binds to detergent micelles (Blewitt et al., 1984, 1985). Very similar results are seen for vesicles prepared by ethanol dilution and sonication (cf. Figures 1A and 2A), or when 6-pyrene-PC is substituted for DPPE-pyrene (data not shown).

A lower limit to the extent of binding (60–80%) is given by the percent quenching by pyrene [$(1 - F/F_0) \times 100$]. This gives only a lower limit of the extent because bound protein is not 100% quenched (see Discussion). Gel filtration studies suggest virtually all of the protein actually binds at low pH (data not shown).

An important question is whether binding is accompanied by deep penetration of the toxin into the bilayer. This was assessed by the short-range quenching of Trp residues by 12-doxyl-PC, which has a spin-label attached to the 12-carbon of the 2-position fatty acyl chain (see Discussion). The open circles in Figure 2 shows strong quenching by 12-doxyl-PC,

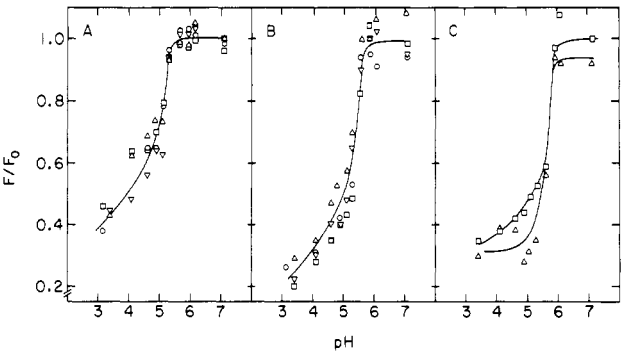

FIGURE 3: pH-dependent binding of diphtheria toxin to sonicated liposomes of various lipid compositions. Samples were prepared as described under Experimental Procedures. F/F_0 is the ratio of toxin fluorescence in quenching vesicles containing 5 wt % DPPE-pyrene, or 6-pyrene-PC in the case of DOPS-containing samples, to that in samples containing lipid without quencher. (A) SUV containing zwitterionic lipids: egg PC (circles); 20 wt % bovine sphingomyelin in egg PC (squares); 20 wt% PE in egg PC (triangles); or 15 wt % cholesterol in egg PC (inverted triangles). (B) SUV containing mixed anionic and zwitterionic lipids: asolectin (circles); 20 wt % DOPG in egg PC (squares); 20 wt % DOPS in egg PC (triangles); 20 wt % DOPA in egg PC (inverted triangles). (C) SUV containing anionic lipids: DOPG (squares); DOPS (triangles).

indicating that penetration is deep for both egg PC and 20% DOPG SUV, in agreement with previous photolabeling studies using other lipid mixtures (Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Montecucco et al., 1985). It should also be noted that binding and penetration parallel each other as a function of pH, in agreement with previous results (Hu & Holmes, 1984). This suggests that they are linked events.

The observation that the pH profiles of binding, of penetration, and of fluorescence in the absence of quencher are all very similar regardless of whether a probe is present, or which probe is used, strongly suggests that binding is not significantly perturbed by use of these probes. This is further supported by the similarity of the pH profile of quenching to the pH profile determined for toxin cross-linking by membrane-associated hydrophobic photolabeling agents in the studies noted above.

It should be noted that the similarity in the level of quenching by pyrene lipid and spin-label lipid shown in Figure 2 is due to the combination of both the different quenching strengths and concentrations of each probe, and these values should not be directly compared. It was found that the levels of quenching could be decreased or increased arbitrarily by decreasing or increasing the fraction of probe-labeled lipids in the membrane (data not shown).

Effect of pH upon Binding of Toxin to SUV of Various Lipid Compositions. Figure 3 shows the effect of lipid composition both on the extent of pyrene quenching and on the pH at which the toxin undergoes a transition to a lipid binding state. The extent of quenching is similar for all of the various lipid compositions, with quenching being slightly stronger for vesicles containing anionic lipids. This may be due to a slightly different extent of binding or a subtle difference between the conformation of toxin bound to zwitterionic vesicles and anionic vesicles. More strikingly, the pH of the transition to the lipid binding state is affected by lipid composition. For zwitterionic vesicles, the transition occurs at pH 5.1–5.2. It is the same in the presence of 20% PE, 20% sphingomyelin, or 15 wt % cholesterol (Figure 3A). When 20% anionic (negative)[2] lipids are used, the transition pH is slightly shifted

[2] The exact charge on these lipids is a function of pH, membrane concentration, and ionic strength (see Discussion).

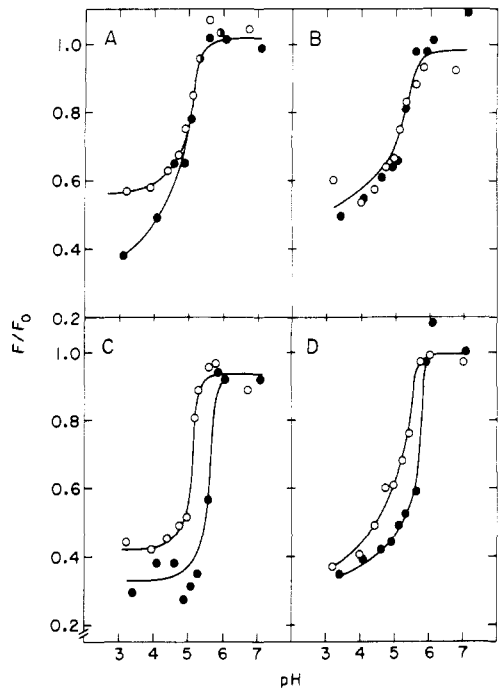

FIGURE 4: Effect of high salt concentrations on binding of diphtheria toxin to sonicated liposomes. Samples for panels A–C were prepared as described under Experimental Procedures except some solutions contained 950 mM NaCl. Samples for panel D were prepared in 0.8 mL of buffer with 150 mM NaCl and incubated for 0.5 h at room temperature, then 0.2 mL of 4 M NaCl was added, and samples were incubated another 0.5 h before measurement of fluorescence. Open symbols: 950 mM NaCl; closed symbols, 150 mM NaCl. F/F_0 was measured as described in Figure 3. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC; (C) DOPS SUV; (D) DOPG SUV.

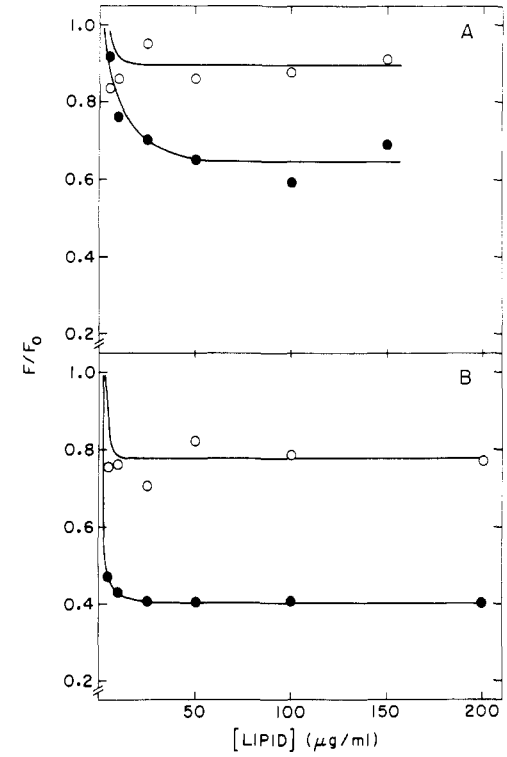

FIGURE 5: Effect of lipid concentration on the binding of toxin to sonicated liposomes. Samples made up as described under Experimental Procedures were modified as described in Figure 10 for pH-neutralized samples, except that the total lipid used in each sample was varied as indicated. F/F_0 was measured as described in Figure 3. Open symbols, samples which had pHs neutralized as described in Figure 10; closed symbols, samples with a final pH of 4.6. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC SUV.

upward to pH 5.2–5.4, and quenching is slightly stronger (Figure 3B). The behavior is the same whether the anionic lipid is DOPA, DOPG, or DOPS. Results are similar with asolectin (mixed soybean phosopholipids), which contains about 30% anionic lipids, primarily PI (London & Feigenson, 1979). It should also be noted that in preliminary experiments under these conditions we also have not been able to detect binding of toxin to SUV containing 20% phosphatidylinositol phosphate in egg PC above pH 5.5 (data not shown). A larger shift in the transition pH to about pH 5.8 was observed in vesicles composed of the anionic lipids DOPS or DOPG (Figure 3C). From these results, it appears that the amount of charge on the vesicle has some influence on the transition pH but lipid head-group structure does not.

Effect of Salt Concentration on the Transition pH. The shift in transition pH observed in vesicles containing anionic lipids suggests that electrostatic interactions could play a role in controlling toxin–lipid interactions (see Discussion). Such an interaction should be antagonized by electrostatic shielding at high ionic strength, resulting in a suppression of the shift in transition pH back to the values observed with zwitterionic lipids. For this reason, the effect of increased salt concentration on the transition pH was measured.[3] Figure 4C shows that in 950 mM NaCl the transition pH observed with DOPS vesicles is pH 5.2, close to the value seen for egg PC vesicles. This shift represents a reversible phenomenon, as shown by the observation that toxin–DOPG samples prepared in 150 mM NaCl have a transition pH of 5.8 but exhibit a decrease of transition pH to 5.3 after addition of NaCl to 950 mM (Figure 4D). To show that the decrease in transition pH in 950 mM NaCl is not due to a direct effect of salt upon toxin, the pH dependence of the toxin conformation of the toxin in the absence of lipid was measured in 950 mM NaCl. The conformational transition pH [which can be detected by changes in solution fluorescence (Blewitt et al., 1985)] was actually seen to be slightly *increased* by 0.1–0.2 pH unit in 950 mM NaCl relative to that in 150 mM salt (data not shown). Further control experiments show there is no significant effect of salt upon the transition pH for toxin in the presence of egg PC vesicles (Figure 4A). It also should be noted that no marked effect is observed with 20% DOPG vesicles (Figure 4B), probably because the expected decrease in transition pH involved is very small (0.1–0.2 pH unit), and may be hidden by the direct effect of high salt upon toxin noted above.

Because toxin does not encounter totally anionic membranes in vivo, we focused on the behavior of toxin with egg PC and 20% DOPG vesicles as representative examples in the remaining experiments.

Binding Isotherms and Binding Kinetics. The strength and kinetics of binding are important parameters for understanding the toxin–model membrane interaction. Figure 5 shows the binding curves for toxin with egg PC and 20% DOPG SUV. In both cases, binding is tight, but it is clearly much tighter with 20% DOPG present, with a half-saturation point of 8 μM and ≤ 2 μM for egg PC and 20% DOPG vesicles, respectively. However, the experimental scatter is too large to allow precise determination of K_d and the number of lipids per toxin "binding site". In addition, interpretation of binding at the lower lipid concentrations is complicated by the fact that under these conditions the model membranes are composed largely

[3] This series of experiments could not be done at very low salt concentration because of the large changes in toxin behavior at low salt concentration (Blewitt et al., 1984).

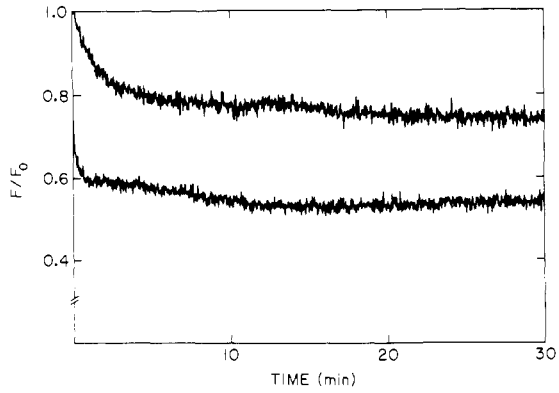

FIGURE 6: Kinetics of diphtheria toxin binding to sonicated liposomes at pH 4.8. Samples were prepared as described under Experimental Procedures. Fluorescence readings were started 15 s after the addition of the toxin, and the time dependence of F/F_0 (defined as in Figure 3) was then measured. Top curve, egg PC SUV; bottom curve, 20 wt % DOPG in egg PC SUV.

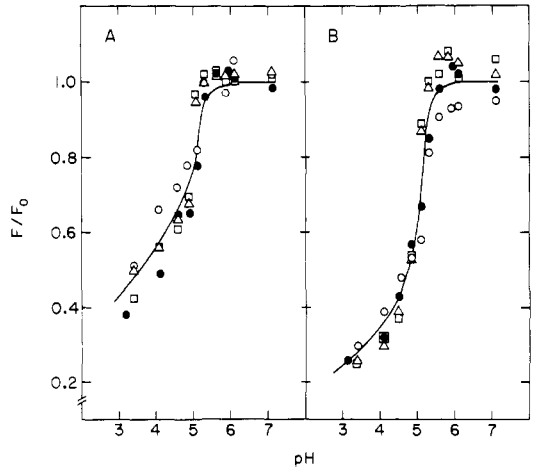

FIGURE 7: Binding of different forms of diphtheria toxin to sonicated liposomes. Samples were prepared as described under Experimental Procedures. F/F_0 was measured as described in Figure 3. Symbols for types of toxin are as follows: open circles, bound monomer; squares, free dimer; triangles, bound dimer; closed circles, free monomer. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC SUV.

of toxin, which will affect both the degree of quenching and the overall membrane structure, and by the fact that the association may not be completely reversible (see below).

Figure 6 shows the kinetics of toxin binding to egg PC and 20% DOPG SUV at pH 4.8. Binding is fast with a half-time of about 1 min for egg PC vesicles and <15 s for 20% DOPG vesicles. Very similar kinetics of binding were found at pH 4.6. It should be noted that previous investigators have also reported rapid insertion of toxin into model membranes (Hu & Holmes, 1984; Donovan et al., 1985).

Effect of Form of Toxin and Presence of Ligands upon Binding to Liposomes. Diphtheria toxin exists in several forms at neutral pH, including bound monomer (containing the dinucleotide ApUp), free monomer, bound dimer, and free dimer. Previous studies have established that the most cytotoxic form of diphtheria toxin is the monomer (Carroll et al., 1986). Therefore, it was of interest to compare the binding of various forms of purified toxin to liposomes. Figure 7 shows the results of binding of toxin to egg PC and 20% DOPG SUV. The transition pH is the same for the various forms of toxin, and at most, only slight differences in the level of quenching can be detected. This result is not surprising, as it has previously been shown that the hydrophilic to hydrophobic transition is very similar for the various forms of toxin (Blewitt et al., 1984, 1985). Furthermore, low pH destroys tight ApUp binding (Collins & Collier, 1987) and causes both aggregation and dissociation of the dimers found at neutral pH (Blewitt et al., 1985).

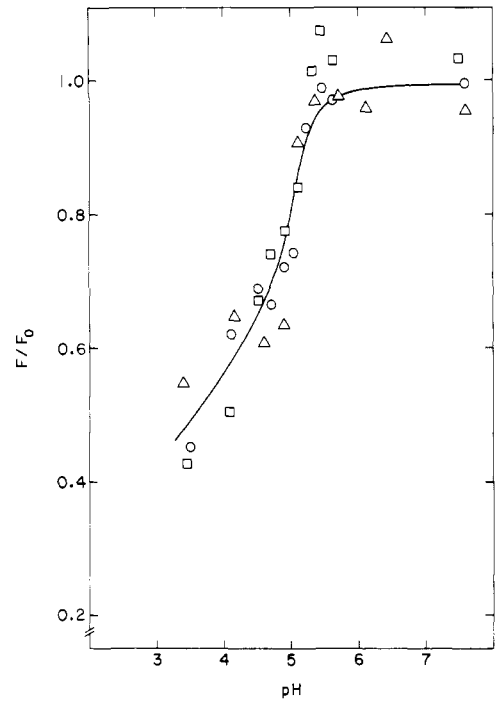

FIGURE 8: Effect of possible inhibitors on binding of diphtheria toxin to sonicated liposomes at various pHs. Possible inhibitors indicated below were included in the buffer. All other conditions were as described under Experimental Procedures. F/F_0 was measured as described in Figure 3. 4 mM phosphocholine and egg PC SUV (circles); 4 mM phosphocholine and 20 wt % DOPG in egg PC SUV (squares); 0.5 mM inositol hexaphosphate with 20 wt % DOPG in egg PC SUV (triangles).

In addition, it has been suggested that phosphate binding sites on the toxin can interact with both ligands and phospholipid head groups in lipids (Alving et al., 1980). We tested if phosphate-containing compounds could affect toxin–lipid association. Figure 8 shows the effect of the presence of inositol hexaphosphate and phosphocholine upon binding to toxin to SUV. Inositol hexaphosphate is a ligand binding at the anion binding site of the toxin (Collins & Collier, 1984), and phosphocholine had been reported to be an inhibitor of toxin binding to liposomes (Alving et al., 1980). As in the case of the ligand ApUp, a significant effect on binding is not observed, suggesting significant competition between these ligands and lipid binding does not occur at low pH under our experimental conditions (see Discussion).

Effect of Preincubation of Toxin in Solution on Binding to Liposomes. Toxin aggregates extensively in solution at low pH, probably due to the interactions via hydrophobic sites on the toxin molecules (Blewitt et al., 1985). We were interested in how the aggregation of the toxin would affect its binding to liposomes. In order to determine the effect of aggregation, toxin first was preincubated in solution at various low pHs to allow it to aggregate, and then vesicles were added followed by a further incubation prior to measuring binding. The results show that both egg PC and 20% DOPG SUV are capable of binding toxin that has been preincubated in solution (Figure 9). However, quenching by egg PC vesicles is greatly reduced. Presumably, this reflects competition between the binding of toxin to itself and its binding to the lipid bilayer. Alternately, quenching may be reduced because large clusters of aggregated toxin are only superficially associated with vesicles such that many are beyond the range of pyrene quenching (see Dis-

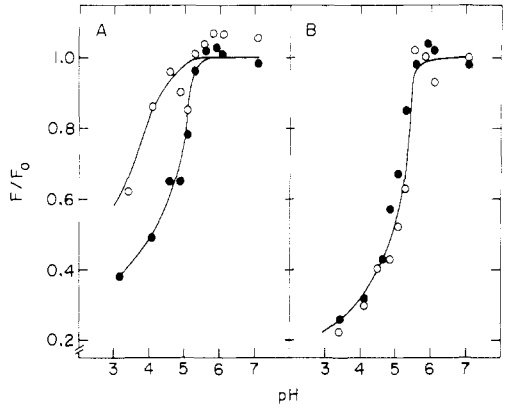

FIGURE 9: Effect of preincubation of toxin at low pH without lipid upon binding of toxin to sonicated liposomes. For preincubation experiments, samples were prepared as described under Experimental Procedures except that toxin was added to buffer and allowed to incubate at room temperature for 15 min before the addition of SUV. The samples were then further incubated for 30 min at room temperature before fluorescence was measured. F/F_0 was measured as described in Figure 3. Open circles, toxin preincubated in the absence of lipid; closed circles, behavior without preincubation. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC SUV.

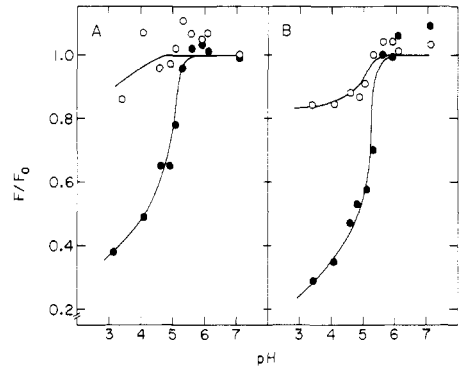

FIGURE 10: Effect of neutralizing pH on the binding of toxin to liposomes. F/F_0 was measured as described in Figure 3, and samples were prepared as described under Experimental Procedures except for the modifications made for neutralizing pH. In these experiments, samples at various pH were prepared in 0.5 mL of buffer. After incubation for 0.5 h at room temperature, a 0.5-mL aliquot of 100 mM Tris or phosphate buffer containing 150 mM NaCl at pH 7.4 was added. These samples were then incubated a further 0.5 h before fluorescence was measured. The final pH of all pH-reversed samples was 7.1–7.4. Open circles, samples where the pH has been reversed; closed circles, samples where the pH has not been reversed. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC SUV.

cussion). In contrast, there is no effect of toxin aggregates upon binding in the presence of 20% DOPG SUV. This may imply that any aggregates formed upon preincubation "dissolve" upon association with 20% DOPG vesicles. In support of this idea, studies of toxin aggregation suggest that some mild detergents do partially prevent aggregation at low pH (L. A. Chung and E. London, unpublished observations).

Reversibility of Toxin Binding upon Increasing pH. Much evidence suggests that after low-pH-induced membrane penetration in vivo, diphtheria toxin becomes exposed to the neutral pH cytoplasm and that the A subunit is then released (see the introduction). Therefore, it was of interest to examine the effect of reversal of low pH upon toxin bound to lipid at low pH. Most quenching by egg PC or 20% DOPG vesicles is lost upon reversal of pH (Figure 10), and the degree of reversibility is independent of lipid concentration (see Figure 5). However, the low-pH-induced change in toxin fluorescence in the presence of vesicles without quencher (i.e., F_0) is not reversed (Figure 9). Therefore, there are at least two changes

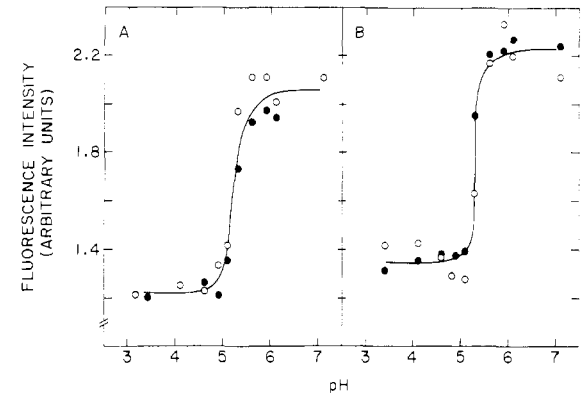

FIGURE 11: Fluorescence curves for diphtheria toxin in the presence of sonicated liposomes before and after pH neutralization. These curves are the protein fluorescence intensities for the samples which do not contain quencher (F_0 values) described in Figure 10. Closed circles, samples where the pH has been neutralized to 7.1–7.4; open circles, samples where the pH has not been neutralized. (A) Egg PC SUV; (B) 20 wt % DOPG in egg PC SUV.

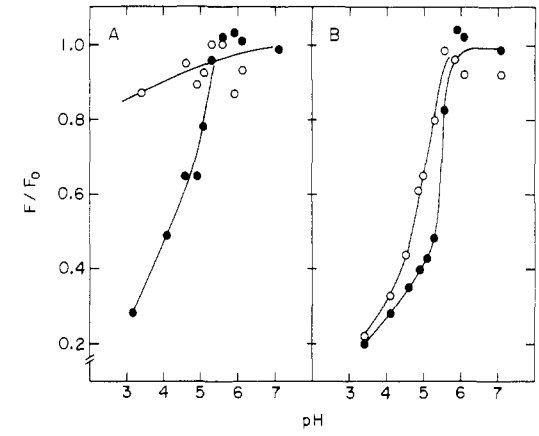

FIGURE 12: Effect of vesicle size on binding of toxin to liposomes. Samples were prepared as described under Experimental Procedures, and F/F_0 was measured as described in Figure 3. Freeze–thawed vesicles (LUV), open circles. Sonicated SUV, closed circles. (A) Egg PC; (B) 20 wt % DOPG in egg PC.

in toxin conformation, one largely reversible and the other irreversible.

It should be noted that similar reversibility behavior was previously observed for toxin associated with detergent micelles at low pH, except that although quenching was largely reversible when pH was neutralized after pH 3 incubation, it was not reversible after a pH 4 incubation (Blewitt et al., 1985). This was shown to be a probable consequence of stronger irreversible toxin aggregation at pH 4 relative to pH 3. Since increased irreversibility after pH 4 incubation is not observed with lipid-bound toxin, it suggests that the lipid-bound toxin is not as highly aggregated at pH 4 as is toxin in the presence of detergent micelles at pH 4.

Comparison of Toxin Binding to SUV and Freeze–Thaw Vesicles. To crudely examine whether vesicle size affects the binding and insertion of toxin, binding to sonicated SUV and much larger freeze–thawed vesicles was compared. Figure 12 shows that the apparent extent of binding to egg PC freeze–thawed vesicles at low pH is very small. Therefore, large vesicle size can greatly affect binding. In contrast, the binding of toxin to 20% DOPG freeze–thawed vesicles is very similar to binding by sonicated vesicles (except near pH 5, which results in a small shift in the apparent transition pH). In both cases, freeze–thawing resulted in a large increase in turbidity and approximate vesicle size determined by quasi-elastic light scattering, which suggests large vesicles are formed both by

egg PC and by 20% DOPG vesicles (data not shown). This implies the difference between behavior with 20% DOPG and egg PC does not involve a difference in the size or amount of large vesicles present. Nevertheless, it is hard to rule out the presence of a small amount of SUV remaining in the 20% DOPG sample to which toxin binds.

Discussion

Fluorescence Quenching Assay of Binding and Penetration. The novel fluorescence quenching assay used to detect binding deserves further comment. It is based on the quenching of intrinsic Trp fluorescence (donor) by energy transfer to pyrene-labeled lipid (acceptor). For this pair, R_0 is 26–27 Å (London, 1986). For most of these studies, we chose a head-group-labeled lipid probe, a pyrene-labeled DPPE. In samples containing 5% DPPE-pyrene, there should be moderate to strong quenching of any Trp residues within the bilayer or within 40 Å of the bilayer surface, according to the amount of energy transfer calculated by the method of Dewey and Hammes (1980). Given the dimensions of a protein, this means that significant quenching should occur upon binding of a protein to the bilayer, whether the binding is penetrating (intrinsic) or surface (extrinsic). It should be noted that the degree of quenching is dependent both upon the difference in Trp and pyrene depths and upon the lateral position of the Trp in the protein (i.e., their distance from the lipid–protein interface), and, therefore, it is not possible to precisely predict the degree of quenching from theory at present.

To measure whether toxin penetrates the bilayer, a similar assay was devised. In this case, 12-doxyl-PC was chosen as a quenching probe. In this case, the quencher is a nitroxide group which is deeply buried within the bilayer. Its quenching is short range, having an effective critical radius of 11–12 Å (Chattopadhyay & London, 1987), and, therefore, strong quenching by this probe should only occur if a protein penetrates deeply into the bilayer.

These binding and penetration assays should be generally applicable to a variety of membrane reconstitution systems. They have the advantages of being rapid and sensitive. Also, they should be intrinsically pH independent, as there are no ionizable groups on the quenching moieties involved. Confirming this, we have previously observed the following: (1) the absorbance spectra of the pyrene probe are not affected by low pH (London, 1986), and thus the R_0 for Trp to pyrene energy transfer will not be pH dependent; (2) both the ESR intensity of the quenching nitroxide group on 12-doxyl-PC and quenching of the uncharged polypeptide gramicidin A in model membranes containing 12-doxyl-PC are virtually identical at pH 4 and 7 (data not shown).

Effect of Electrostatic Interactions on Toxin–Lipid Interaction. This study shows a clear difference between toxin behavior with anionic and zwitterionic vesicles. Electrostatic interactions are very likely to be involved in this behavior since the effect of anionic lipids is independent of head-group structure, and because the toxin is cationic at low pH.[4] One way electrostatic interactions are manifested is as an increase in the pH of the transition between the hydrophilic and membrane binding states of the toxin in the presence of anionic lipids. There are several ways that electrostatic interactions could cause this shift in transition pH. Direct toxin–lipid electrostatic interactions will shift the transition pH upward by shifting the equilibrium between toxin conformations away from the soluble conformation and toward the membrane binding conformation. These interactions could be of two types. First, there will be an affect arising from the (Gouy–Chapman) diffuse double layer. The double layer is a local region of solution around anionic vesicles in which electrostatic interactions give rise to disproportionately high cation concentrations (McLaughlin, 1977). The cationic toxin will be nonspecifically concentrated in the double layer, resulting in a high local toxin concentration around anionic vesicles, and thus causing increased binding due to mass action. Second, once inserted, toxin may bind to anionic lipids more tightly than zwitterionic ones. The differences between egg PC and 20% DOPG binding isotherms and binding kinetics at low pH also may involve such direct electrostatic interactions although differences in SUV size could also play a role (see below). It should be noted that electrostatic effects should weaken a bit at pH 5 relative to lower pH due to decreased charge on the toxin.

In addition to these direct effects, an additional electrostatic effect arises from the local region of high cation concentration in the double layer. The cation-concentrating behavior results in a local pH that is lower than bulk solution pH, and therefore could trigger binding of the toxin upon toxin–anionic vesicle collision even when the bulk pH is above the transition pH. The effect of the double layer upon local pH can be roughly calculated from the equation:

$$pH_{x\ \text{Å from surface}} = pH_{\text{bulk}} + \psi_{\text{at } x\ \text{Å}} F/2.303RT$$

Where ψ is the electrostatic potential, F is the Faraday constant, R is the gas constant, and T is the temperature in degrees kelvin (assumed to be 25 °C for these calculations) (Tsui et al., 1986). The electrostatic potential can be calculated from the Gouy–Chapman equation (McLaughlin, 1977; Winiski et al., 1986). For vesicles composed of 100% anionic lipids, the surface pH will be about 1 pH unit less than the bulk pH in 150 mM NaCl. However, local pH is very dependent on distance from the surface of the vesicle, and for totally anionic lipids in 150 mM NaCl, it will decrease to about 0.3 pH unit at 10 Å from the surface (McLaughlin, 1977). Without knowing the arrangement of pH-sensitive groups on the toxin and their interactions with the vesicle surface upon collision, it is difficult to calculate the precise contribution of local pH, although it is likely to have a significant effect on toxin behavior. Other factors complicating precise calculation include how long toxin remains close to a vesicle after collison and the difference between calculated and experimental surface charge due to lipid asymmetry (Michaelson et al., 1973; Berden et al., 1973) and due to head-group protonation.[5]

Supporting evidence for the role of these electrostatic effects comes from the results of increasing ionic strength. Increasing [NaCl] from 150 to 950 mM completely suppresses the electrostatic potential at 10 Å from the membrane surface (McLaughlin, 1977), and therefore should suppress both the local pH and high local toxin concentration. Consistent with this behavior, high salt concentration suppresses the shift in

[4] The isoelectric pH of diphtheria toxin has been reported to be 6 (Pappenheimer, 1979). We find a value of about 5.4 for our free monomer toxin under nondenaturing conditions.

[5] This should have only a small effect because, except in the case of pure DOPS in 150 mM NaCl, where charge will be greatly reduced at pH 4 and below, anionic lipid protonation should only be a significant factor near pH 3. This is because the apparent carboxylate pK_a of pure DOPS in the liquid-crystalline state [3.5–4.5; see references in Cevc et al. (1981)] is higher than that of phosphate, which is the protonatable group in the other anionic lipids (Träuble & Eibl, 1974; Träuble et al., 1976; MacDonald et al., 1976; Cevc et al., 1981). Furthermore, the apparent pK_a is lower both when anionic lipid is mixed with zwitterionic lipid and in higher salt (Träuble et al., 1976; MacDonald et al., 1976; Tsui et al., 1986), further decreasing lipid protonation.

transition pH seen with fully anionic vesicles. We conclude that electrostatic effects of the above types must be involved, although further studies are needed to evaluate the contribution of each component of this interaction.

It should be noted that our assessment of electrostatic effects differs in some respects from that in the earlier report of Alving et al. (1980). In contrast to our results, they reported a strong variation of the extent of lipid binding upon specific anionic lipid composition which they attributed to specific "exposed" phosphate–toxin interactions. Furthermore, we did not find inhibition of binding by phosphorylated ligands that they reported. We believe the fact that pH was apparently not controlled in the experiments involved, and was reported to be in the range pH 5–6, is most likely to be the major source of this apparent discrepancy. In this pH range, the apparent binding will be extremely sensitive to the exact pH because it is where the sharp conformational transition occurs. In addition, the fact that they used large multilamellar vesicles, which can greatly reduce the degree of binding in some cases (see Figure 11), may be involved. It should be noted that their pH profile of binding, which involved buffered samples, did detect increased binding at low pH, in agreement with our results, photolabeling experiments, and BLM studies. It also should be noted that participation of a combination of hydrophobic and electrostatic effects has been identified in the binding of *Psuedomonas* exotoxin A to model membranes (Farahbakhsh et al., 1986).

Effect of Vesicle Size upon Binding. The results presented in this report suggest that insertion of toxin is affected by vesicle size. There are two possible explanations for the decreased binding of toxin to larger egg PC vesicles. It is possible that the lateral packing of lipids in SUV is sufficiently imperfect, due to increased spacing between head groups in the outer leaflet, that a small portion of the hydrophobic acyl chains are exposed to the aqueous environment, allowing rapid toxin insertion before aggregation of toxin in solution, which competes with binding, can occur. In larger vesicles, where the barrier to entry may be greater due to tighter-head group packing, aggregation would effectively compete with insertion. In addition, insertion of toxin may partly relieve the energetically unfavorable vesicle curvature found in SUV, which would lead to tighter binding to SUV.

In contrast, the binding of toxin to 20% DOPG is apparently not as affected by vesicle size. One might predict the effect of vesicle size on binding should be decreased when 20% anionic vesicles are used, since such vesicles give rise to tighter and faster binding than zwitterionic ones, which in turn should effectively reduce the effects of aggregation.

It should be noted that a similar effect of vesicle size upon protein insertion has been observed for cytochrome b_5 (Greenhut et al., 1986). It should also be noted that diphtheria toxin can fuse model membranes at low pH (Cabiaux et al., 1984), so that the ultimate size of toxin-containing vesicles will be larger than their original size.

Reversibility of Binding of Toxin to Vesicles upon Neutralization of Low pH. It has been reported that a relatively neutral pH cytoplasm is necessary for translocation of toxin across cell membranes (Sandvig et al., 1986). Therefore, the effect of reversing low pH upon toxin may provide important clues to toxin translocation in vivo. Hu and Holmes (1984) showed that toxin preincubated in the absence of vesicles at low pH could subsequently bind to vesicles at neutral pH, indicating some retention of low-pH conformation occurs after pH neutralization. However, it has also been observed that hydrophobic photolabeling of membrane-bound diphtheria toxin is partially reversed by neutralizing the pH before photolysis (Montecucco et al., 1985).

Our study shows that the quenching of toxin is greatly reduced but not eliminated when the pH is reversed.[6] There are several possible explanations for the residual quenching observed after pH neutralization. First, the toxin–lipid K_d may have uniformly increased slightly, allowing some toxin molecules to dissociate. This is ruled out by the binding curves after pH neutralization, shown in Figure 4. These roughly parallel the binding curves at low pH, but the extent of quenching is greatly decreased. An alternate explanation is that a small subpopulation of toxin remains irreversibly associated but that most toxin dissociates totally upon pH neutralization due to a large increase in K_d. It is also possible that all toxin has dissociated but some molecules are trapped within the vesicles, occasionally approaching the membrane surface where some quenching would occur. Finally, it is possible that toxin has not dissociated but has changed conformation such that a large domain no longer interacts with the membrane. This interpretation would be consistent with the results of the previous studies noted above, and our preliminary results obtained by directly assaying toxin–vesicle association favor this explanation (unpublished observations), but further work is necessary to define the changes occurring when the pH is reversed, and to determine their functional role, if any.

Relationship to Toxin–Lipid Interaction in Vivo. An important question is whether lipid composition modulates the transition pH in vivo, as it does in vitro. Since it is very likely that toxin penetrates endosomal membranes in vivo (Morris et al., 1985), it is necessary to consider the lipid composition of endosomes. It has been reported that the endosomal lipid composition is very similar to that of the plasma membrane (Evans & Hardison, 1985). Therefore, the percent of anionic lipids is relatively small, and the expected transition pH is unlikely to be increased more than 0.1–0.2 pH unit above that observed with zwitterionic lipids. In addition, it should be noted that in vivo toxin binding to receptor may influence the nature of toxin–lipid interactions either directly through toxin–receptor interaction or indirectly by effects upon toxin aggregation and surface concentration. However, these possibilities must remain hypothetical until the receptor is characterized and until it is known whether toxin remains receptor-associated at low pH.

It should also be noted that the interaction with lipid observed in this report appears to be both tight and rapid enough to be involved in membrane penetration by toxin in vivo. This is based upon the fact that it takes toxin at least minutes to enter the cytoplasm (Marnell et al., 1984), which is longer than the time for insertion in vitro, and that the effective lipid concentration sensed by a toxin trapped in an organelle in vivo, which can be readily calculated (London et al., 1986), is far above the concentration necessary to obtain binding and insertion in vitro.

The effect of temperature upon lipid association should also be considered. In previous studies, we found the behavior of toxin at 23 and 37 °C to be very similar, although at 37 °C the effects of thermal denaturation begin to influence and complicate toxin behavior (Blewitt et al., 1985; Zhao & London, 1986). One previous study did note thermal effects on toxin interaction with lipid at 0 °C (Hu & Holmes, 1984), but the phospholipid used was dimyristoylphosphatidylcholine,

[6] Because of toxin-induced pore formation (Donovan et al., 1981; Kagan & Finkelstein, 1981), toxin-induced vesicle fusion (Cabiaux et al., 1984, 1985), and acetate permeability, any pH gradient across the vesicles in our samples should be transient at most.

which has a liquid-crystal to gel transition in the temperature range studied, further complicated by the presence of cholesterol. Therefore, it is not clear whether the effects involved were due to thermal effects on toxin or lipid. In the presence of egg PC SUV, we find lipid binding behavior to be similar at 23 and 37 °C except for a slight increase in transition pH (unpublished observations), as is also observed for toxin in solution (Blewitt et al., 1985). Hu and Holmes (1984) also found low-pH behavior similar at 23 and 37 °C. Nevertheless, this will be an important area for further studies.

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Registry No. IHP, 83-86-3; DOPA, 61617-08-1; DOPG, 62700-69-0; DOPS, 70614-14-1; DPPE-pyrene, 76341-59-8; 12-doxyl-PC, 55402-86-3; 6-pyrene-PC, 103625-33-8; cholesterol, 57-88-5; phosphocholine, 107-73-3.

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