per band is the upper limit for resolving phage T7 DNA from T7, C5 LG3 DNA.

The only biological macromolecules known to induce the enhanced fluorescence of ethidium bromide are nucleic acids (Le Pecq & Paoletti, 1966). The successful use of ethidium bromide fluorescence enhancement to detect DNA and DNA bacteriophages during zone sedimentation therefore suggests the use of this technique for the isolation from cell lysates of viruses and DNA-containing viruses precursors. For instance, we have used zone sedimentation in ethidium bromide–sucrose gradients combined with buoyant density sedimentation in ethidium bromide–sodium iothalamate gradients (Serwer, 1975) to purify the DNA–protein complexes and fast sedimenting DNA isolated from lysates of bacteriophage T7-infected *E. coli* by Serwer (1974a,b). A description of this work will be the subject of a future communication.

The fluorescence technique is more sensitive than visible light scattering for detecting T7 phage and possibly for detecting other viruses. Some cellular structures that scatter visible light do not induce ethidium bromide fluorescence enhancement (Le Pecq & Paoletti, 1966), suggesting that virus bands in sucrose gradients may be less readily obscured by other material if the fluorescence enhancement procedure is used rather than light scattering. Ethidium bromide–sucrose gradients may be of use in isolating viruses for which a biological assay has not yet been developed.

Acknowledgments

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References

Appleyard, G. (1967) *J. Gen. Virol. 1,* 143.
Bancroft, F. C., & Freifelder, D. (1970) *J. Mol. Biol. 54,* 539.
Burgi, E., & Hershey, A. D. (1963) *Biophys. J. 3,* 309.
Freifelder, D. (1970) *J. Mol. Biol. 54,* 567.
Kellenberger, E., & Sechaud, J. (1957) *Virology 3,* 256.
Leighton, S. B., & Rubenstein, I. (1969) *J. Mol. Biol. 46,* 313.
Le Pecq, J.-B., & Paoletti, C. (1966) *Anal. Biochem. 17,* 100.
McLaren, A. D., & Shugar, D. (1964) *Photochemistry of Proteins and Nucleic Acids,* MacMillan Co., New York, N.Y.
Saigo, K. (1976) *J. Mol. Biol. 107,* 369.
Serwer, P. (1974a) *Virology 59,* 70.
Serwer, P. (1974b) *Virology 59,* 89.
Serwer, P. (1975) *J. Mol. Biol. 92,* 433.
Serwer, P. (1976) *J. Mol. Biol. 107,* 271.
Sharp, P. A., Sugden, B., & Sambrook, J. (1973) *Biochemistry 12,* 3055.
Simon, M. N., & Studier, F. W. (1973) *J. Mol. Biol. 79,* 249.
Studier, F. W. (1969) *Virology 39,* 562.
Welsh, J. N., & Adams, M. H. (1954) *J. Bacteriol. 68,* 122.
Yamamoto, N. (1958) *J. Bacteriol. 75,* 443.

Specific Binding of a Cardiotoxin from *Naja mossambica mossambica* to Charged Phospholipids Detected by Intrinsic Fluorescence†

Jean Dufourcq* and Jean-Francois Faucon

ABSTRACT: The fluorescence of intrinsic tryptophan in cardiotoxin II from *Naja mossambica mossambica* is a sensitive probe of interactions between the toxin and phospholipid vesicles. The formation of the lipid–protein complexes leads to more than a threefold increase in the fluorescence intensity and a blue shift of 10–15 nm. Cardiotoxin II does not bind to neutral or zwitterionic phospholipids, but interacts specifically with negatively charged phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid. The association constant of the lipid–protein complex is $>10^6\ M^{-1}$, and its stoichiometry is 7 ± 1 lipid molecules per protein molecule, when only one negative charge is borne by the lipid molecule. Results obtained with lipid mixtures can be explained only by assuming that cardiotoxin induces the formation of clusters or lateral phase separations between negative and neutral phospholipids. The binding is reversible and is mainly due to electrostatic interactions between the basic residues of cardiotoxin and the phosphate and/or carboxylic groups of the phospholipids. The complexes can be dissociated either by an increase in ionic strength or by pH effects. Cations compete with cardiotoxin, with an efficiency that increases in the order $Na^+ = K^+ \ll Mg^{2+} < Ca^{2+} < Mn^{2+}$. These results can account for the known actions of cardiotoxin on cells or biological membranes, and we propose that negatively charged phospholipids are the binding site of cardiotoxin on natural membranes.

Snake venom toxins can be separated according to their pharmacological effects into neuro- and cardiotoxins. Both types of toxins have potent effects on membranes, but the neurotoxins are highly specific for only a few cell targets, whereas cardiotoxins display a general action on many kinds of membranes (Yang, 1974). Cardiotoxins are described as direct lytic factors, due to their ability to break down the plasmic membrane. They are generally nonenzymatic proteins, although several toxins have recently been characterized as phospholipases, the major example being β-bungarotoxin (Strong et al., 1976; Howard & Truog, 1977).

Lipids have been implicated in the binding of cardiotoxin

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to cell membranes since purified phospholipids compete efficiently with whole cells (Patel et al., 1969; Zaheer et al., 1975). More recently, studies of the binding of cardiotoxin to axonal membranes have led to the conclusion that membrane lipids probably are the binding site of the toxin because the number of sites per membranes area unit is higher than the total amount of protein in the membrane (Vincent et al., 1976).

Direct interactions between cardiotoxins and pure lipid systems seemed to be essential to clarify whether or not lipids alone can account for the biological effects of the venom toxins. Furthermore, this approach should give information on the interaction mechanism. This is particularly interesting, since lipid–protein interactions play an important part in many functions of biological membranes.

From the known sequences (Louw, 1974), cardiotoxin II (V_2^{II}) from *Naja mossambica mossambica* venom has a single Trp. In this paper, we have measured changes in the intrinsic fluorescence of the Trp to detect the lipid–protein interactions. This technique has previously proved useful in studies of lipid–protein complexes involving membrane proteins (Dufourcq et al., 1975), hormones (Schneider & Edelhoch, 1972), apolipoproteins (Morrissett et al., 1973), phospholipases (Van Dam-Mieras et al., 1975), and toxins like melittin (Mollay & Kreil, 1973; Dufourcq & Faucon 1977) or the cholera toxin (Mullin et al., 1976).

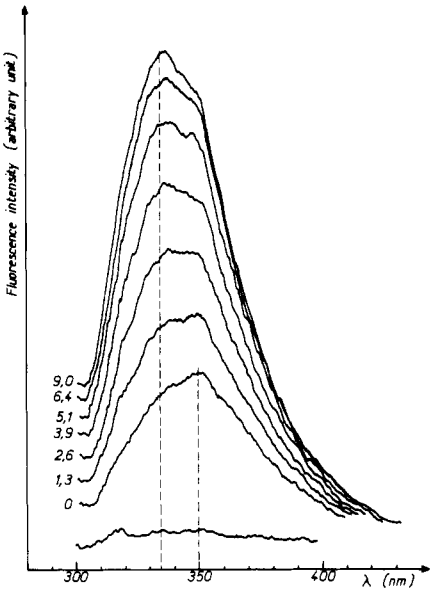

FIGURE 1: Emission spectra of cardiotoxin in the presence of increasing amounts of PS. CTX concentration, 1 μM, in 20 mM Tris–acetate buffer, 1 mM EDTA, pH 7.5. The lipid to protein molar ratio is indicated for each spectrum in the figure.

Materials and Methods

Cardiotoxin II (CTX)[1] from *Naja mossambica mossambica* was purified according to Jover (1976) and was a gift from the Laboratoire de Biochimie, Faculté de Médecine, Secteur Nord, Bd P. Dramard, 13015 Marseille, France.

Some phospholipase A_2 activity was detected by thin-layer chromatography (eluent: $CHCl_3$, CH_3OH, H_2O, 65:25:4 v/v), and it was inhibited in all cases by addition of 1 mM EDTA. In such conditions no lyso compound could be detected after incubation with phospholipids even during several hours.

Phospholipids were purified in the laboratory, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from hens egg yolk (Singleton et al., 1965), phosphatidylserine (PS) from bovine brain (Rouser et al., 1963), and phosphatidylinositol (PI) from baker's yeast (Trevelyan, 1967). Experiments have also been carried out with commercially available phospholipids from Lipid Products (U.K.), in particular phosphatidic acid (PA), sphingomyeline (Sph), and PS. All these lipids showed single spots by TLC.

The selected phospholipids were suspended in 20 mM Tris–acetate buffer, pH 7.5, and sonicated for about 15 min at 4 °C under nitrogen atmosphere, with an Annemasse F 50 sonicator. As was shown by Huang (1969) and by Hauser & Phillips (1973), it mainly resulted in single shelled vesicles, which were used directly. When lipid mixtures were used, appropriate aliquots of benzene solution of each component were mixed and then colyophilized; afterward the homogeneous lipid mixture was dispersed in buffer and sonicated as usual. Measurements of lipid–protein interactions were performed "in situ" in the fluorescence cuvette. The protein was generally dissolved in 20 mM Tris–acetate buffer, pH 7.5, and its concentration was varied in the micromolar range.

Fluorescence experiments were performed on a FICA 55 MK II differential spectrofluorometer, which automatically recorded corrected excitation and emission spectra. Unless otherwise mentioned, all the spectra were obtained in standard conditions, i.e., excitation wavelength 280 nm, excitation and emission slits 7.5 nm, temperature 25 °C. When wavelength shifts are plotted, they are measured as the wavelengths of the midpoint at half-height of the spectrum. Despite the increase of turbidity during experiments, its effect on the emission spectrum has been shown not to be significant (Dufourcq & Faucon, 1977).

Results

1. *Binding of Cardiotoxin II to Negatively Charged Phospholipids.* Cardiotoxin II contains one Trp and two Tyr residues, and so is a class B protein (Teale, 1960). As can be seen in Figure 1, its fluorescence spectrum is mainly due to the Trp residue. The emission maximum is centered around 350 nm, the wavelength characteristic of Trp residues in a polar environment, i.e., exposed to the solvent. A small contribution of Tyr residues can, however, be detected, by comparing the spectra obtained by exciting the protein either at 280 nm or 295 nm, where only tryptophan absorbs.

When single shelled vesicles of negatively charged phospholipids are added to the protein solution, a significant blue shift and a large increase of the emission intensity are observed. This indicates that the Trp environment changes to a less polar one, a result which can only be interpreted by the formation of a lipid–protein complex. Simultaneously, one can observe a large increase of the turbidity of the sample which is probably due to the breakdown of the vesicular structure.

The fluorescence spectra obtained upon addition of phosphatidylserine are shown in Figure 1. A 15-nm blue shift is observed, from 350 to 335 nm. The concomitant increase in intensity at 335 nm is plotted in Figure 2 vs. the lipid to protein molar ratio R_i. The Trp emission increases until a plateau is reached at 3.2 times the original intensity. This corresponds to a R_i value of 7 ± 1. Moreover, the observed effect is unchanged when the protein concentration is varied from 1 to 10 μM.

Similar experiments have also been performed with two other negatively charged phospholipids, namely, phosphati-

[1] Abbreviations used: CTX, cardiotoxin II; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; Sph, sphingomyelin; R_i, lipid to cardiotoxin II molar ratio.

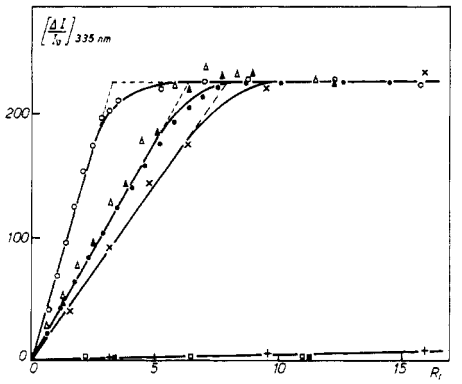

FIGURE 2: Relative increase in the fluorescence intensity ($\Delta I/I_0$) of CTX at 335 nm upon addition of negatively charged and zwitterionic phospholipids in 20 mM Tris-acetate buffer. Unless otherwise mentioned, [CTX] = 10 μM, pH 7.5, and [EDTA] = 1 mM. R_i = lipid to protein molar ratio; (△) PS; (●) PI; (■) PC; (+) PC-PE (60/40); (□) Sph; (○) PA (pH 9.2); (▲) PS ([CTX] = 1 μM); (X) PC after 30-min incubation in the presence of 1 mM Ca^{2+} (without EDTA).

dylinositol and phosphatidic acid. PI gives results identical with those described above for PS as shown in Figure 2.

Upon addition of PA vesicles, the fluorescence intensity of CTX still increases linearly by more than 200%. In this case, when the experiment is carried out at pH 9.5, PA bears two negative charges per molecule. The plateau is then reached for $R_i = 3.5 \pm 0.5$ instead of 7 (Figure 2).

2. *Binding to Zwitterionic Phospholipids.* In contrast to negatively charged lipids, zwitterionic phospholipids such as phosphatidylcholine, phosphatidylethanolamine, or sphingomyelin, have no effect at all on the fluorescence spectrum of CTX for R_i values up to 100 at CTX concentrations of 10 μM and with a buffer solution containing 1 mM EDTA (Figure 2). This does not really prove that no binding occurs between these lipids and the toxin. So, in order to ensure this conclusion, we have performed the following control experiment: the toxin is incubated with a large excess of a selected phospholipid dispersion ($R_i \simeq 200$), and centrifuged (50.2 TI Beckman rotor 200 000g during 1 h) in such a way that practically all the lipid is precipitated. The presence of CTX in the pellet or in the supernatant is then detected by its absorbance and fluorescence spectra. When a negatively charged phospholipid is used, all the toxin is complexed with the lipid, and so it is recovered in the pellet. On the contrary, with a zwitterionic lipid, one observes that CTX remains in solution in the supernatant, which confirms the lack of lipid-protein interaction in this last case.

However, when binding experiments with PC are performed *in an EDTA free buffer,* a slow time increase of the fluorescence intensity is observed. Its rate is greatly enhanced by small amounts of Ca^{2+} (1 mM). Then, as shown in Figure 3, a plateau is reached after 10 to 20 min, depending on the R_i value. The intensity values at the plateaus, plotted vs. R_i in Figure 2, are quite similar to those previously obtained with negatively charged phospholipids.

Similar kinetics and binding curves are obtained with vesicles constituted by mixing PC and PE (molar ratio 60/40). On the contrary, no effect at all is observed on the fluorescence spectrum of CTX when Sph vesicles are added, whatever the buffer composition.

Moreover, it must be pointed out that lipid analysis by TLC shows a total and rapid hydrolysis of all the lipids, excepted Sph, on incubation with CTX in the presence of mM amounts of Ca^{2+},, whereas 1 mM EDTA totally prevents formation of lyso derivatives. This clearly proves that the toxin used contains

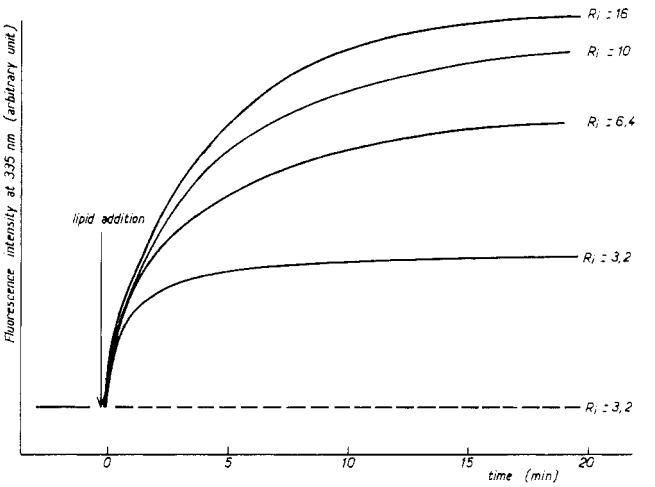

FIGURE 3: Kinetics of the changes in the fluorescence intensity of CTX at 335 nm following the addition of various amounts of PC. R_i = lipid to protein molar ratio (20 mM Tris-acetate buffer, pH 7.5). (Dashed Line) In the presence of 1 mM EDTA; (solid line) in the presence of 1 mM Ca^{2+}.

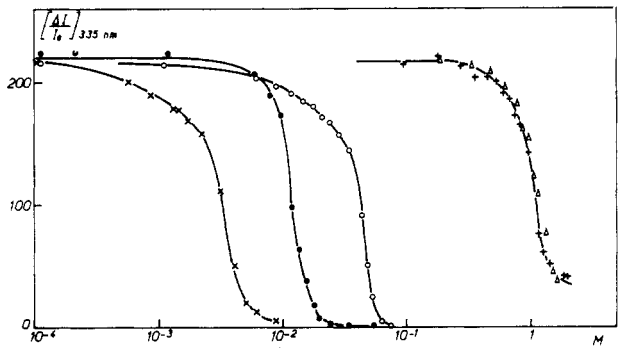

FIGURE 4: Concentration effect of various cations on the fluorescence intensity of the CTX-PS complex. R_i = 4.5, pH 7.5. (X) Mn^{2+}; (●) Ca^{2+}; (○) Mg^{2+}; (+) Na^+; (△) K^+.

some phospholipase A activity. Similar lipase contamination has also recently been emphasized (Visser & Louw, 1977). In our experiments, the amount of phospholipase impurity has been noticeably reduced by antiphospholipase A_2 serum precipitation. Unfortunately, in the conditions used in binding experiments with very high protein to lipid ratios, phospholipase activity still remained noticeable.

Taking in account these last results, the observed effects on the emission spectrum of CTX in the presence of Ca^{2+} can be easily explained by the binding of CTX to negatively charged fatty acids produced by hydrolysis of the zwitterionic lipids, the lyso components remaining unbound. This has been confirmed by direct interaction of lysolecithin and fatty acids with CTX.

3. *Effect of Ionic Strength on the Cardiotoxin Phospholipid Complexes.* In order to characterize the hydrophobic and/or electrostatic nature of the interactions involved in the stabilization of the toxin-lipid complexes, it was useful to look at the effect of an increase of the ionic strength.

The results obtained with the CTX-PS complex are summarized in Figure 4. At low cation concentrations, the fluorescence intensity is unaffected. At higher concentrations, it sharply decreases, ultimately reaching the value which corresponds to the protein free in solution. In the same way, the wavelength of the emission maximum is also reversed to a value close to that of the free toxin. The presence of free toxin has been ensured by a simple centrifugation experiment: when the

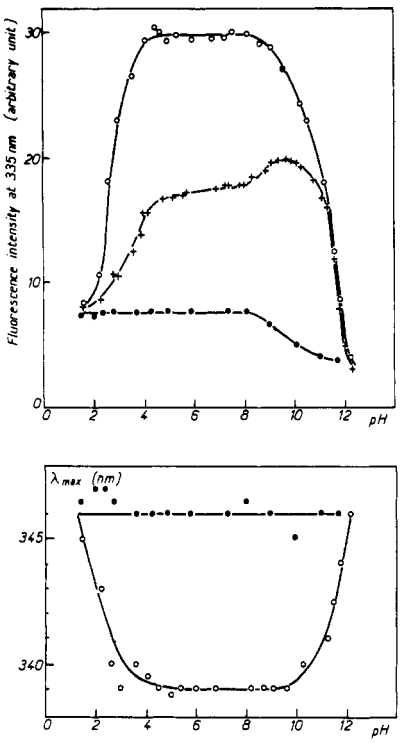

FIGURE 5: Changes in the fluorescence parameters (intensity at 335 nm and wavelength of the emission maximum) of cardiotoxin induced by pH. (●) CTX alone; (+) CTX partially bound to PS (R_i = 3.2); (○) CTX totally bound to PS (R_i = 12.8). pH values were adjusted by addition of NaOH or HCl solutions to a 20 mM Tris-acetate buffer, 1 mM EDTA.

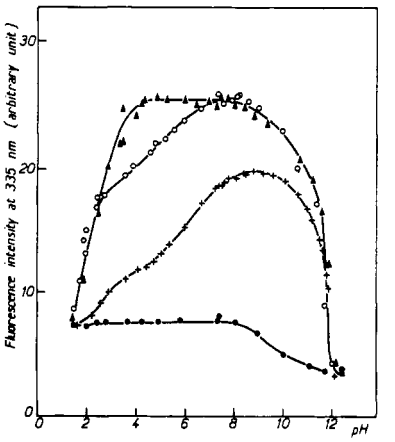

FIGURE 6: Changes in the fluorescence intensity of cardiotoxin bound to PA induced by pH. (●) CTX alone. CTX-PA: (+) R_i = 2.1; (○) R_i = 8; (▲) R_i = 35. pH values were adjusted by addition of NaOH or HCl solutions to a 20 mM Tris-acetate buffer, 1 mM EDTA.

TABLE I: Cation Concentrations Needed to Reach 50% Dissociation of the CTX-Phospholipid Complexes.

Cation concn (mM)	Phospholipid involved		
	PA	PS	PI
Mn^{2+}	0.04	3.2	24
Ca^{2+}	0.4	11.5	35
Mg^{2+}	17	42	80
Na^+-K^+		1000	

lipid–protein complex is centrifuged during 1 h at 200 000g in the presence of an appropriate cation concentration, the recovered pellet contains only lipids, all the protein remaining in the supernatant. So, an increase in the ionic strength of the medium results in the dissociation of the PS–CTX complex.

All the cations used are not equally efficient in dissociating the lipid–protein complex. In the case of phosphatidylserine, the half-effect concentrations range from 3 mM for Mn^{2+} to 1 M for K^+ and Na^+. It must, however, be mentioned that the magnitude of these values should be accepted with caution, especially in the case of Ca^{2+}, because of the presence of the phospholipase impurity.

The two other negative phospholipids studied, PI and PA, lead to similar results, but the half-effect concentrations noticeably differ. By looking at these concentrations, summarized in Table I, it is evident that there are large differences in their values, depending on the phospholipid head group for a given cation. Whatever the phospholipid used, cations compete with cardiotoxin and their efficiency always increases in the same order: $Na^+ = K^+ \ll Mg^{2+} < Ca^{2+} < Mn^{2+}$.

4. *Effect of pH on the Lipid–Protein Complexes.* The fluorescence parameters of pure CTX are plotted vs. pH in Figure 5. No change in the wavelength of the emission maximum can be detected in the whole pH range studied, i.e., from 2 to 12. While the fluorescence intensity is constant from pH 2 to 8, a twofold decrease is observed between pH 8 and 12. This decrease is paralleled by the appearance of a new absorption band at 295 nm in the protein spectrum; this results from the ionization of the Tyr residues as previously shown on neurotoxin (Chicheportiche et al., 1972). Thus, the observed quenching of the toxin fluorescence can be attributed to nonradiative energy transfer between the Trp and tyrosinate residues (Conti & Forster, 1975; Shinitzky & Fridkin, 1976).

In Figures 5 and 6 the fluorescence intensities of CTX totally or partially bound to PS and PA are plotted vs. pH. In both cases the intensities drop above pH 10 and reach the intensity of free protein above pH 12, the half-effect occurring around pH 11.5. Moreover, the wavelength of the emission maximum shifts toward the value of free protein (Figure 5). Since no change in the charge of the phospholipids, especially PA, is expected in this pH range, these effects have to be related to some changes occurring in the protein. The quenching of the Trp fluorescence by tyronisates can induce a decrease in intensity, but it cannot explain any wavelength shift. Another possibility is that in this pH range Lys and Arg residues can be titrated, leading to a change in the net charge of the protein. The observed effects would then be interpreted as a dissociation of the complexes due to the weakening of electrostatic attractions. This explanation is consistent with the results obtained by increasing the ionic strength of the media (see above).

Another decrease in fluorescence intensity occurs at low pH. The pH at which the change occurs depends upon the phospholipid used (Figures 5 and 6). This change is followed by a wavelength shift toward 350 nm, the observed spectra being identical with that of free protein at pH 2. We conclude that the lipid–protein complexes are dissociated. In this pH range, no change is expected for the protein charge. The breakdown of the complexes must be related to the protonation of acidic polar head groups, leading to neutral or positively charged phospholipid species. The pHs at half-dissociation of the complexes have to be compared with the pKs of the lipids involved. For PS, it occurs at pH 3 to 3.5; for PA the half-effect is observed at pH 2.5. These values are in good agreement with those previously determined on the lipid systems, respectively pK 3.5–4.0 for PS (Seimiya & Ohki, 1973; Hauser et al., 1976)

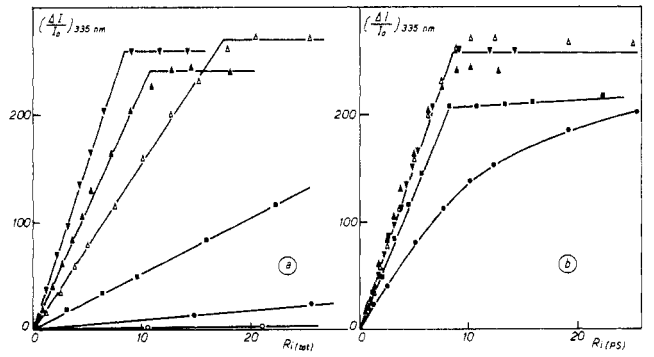

FIGURE 7: Relative changes in the fluorescence intensity of CTX at 335 nm upon binding to binary mixtures of PC and PS at pH 7.5, 1 mM EDTA; [CTX] = 10 μM. PC-PS mixtures are quoted by their PS molar fraction: (○) PS 0%; (●) PS 5%; (■) PS 20%; (△) PS 50%; (▲) PS 70%; (▼) PS 100%. (a) R_i (total) is the molar ratio of total lipids to CTX. (b) R_i (PS) is the molar ratio of PS only to CTX.

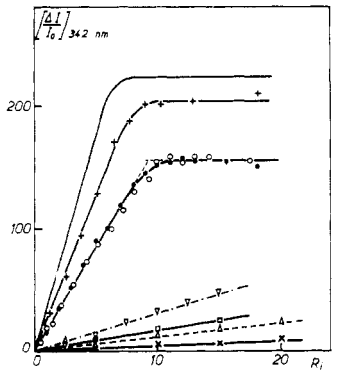

FIGURE 8: Relative changes in the fluorescence intensity of CTX upon binding to various lipid mixtures; R_i is the reduced molar ratio of negative lipids to protein. With 20 mM Tris-acetate buffer, pH 7.5, 1 mM EDTA. (+) PS-cholesterol (1:1). Lipid mixture with the composition of axonal membranes: 22.2% PE; 17.7% PC; 10.4% Sph; 7.7% PS; 0.6% PI; 0.4% PA; 41% cholesterol). (●) [CTX] = 1 μM; (○) [CTX] = 10 μM. Competition with divalent cations, [CTX] = 10 μM: (▽) [Ca^{2+}] = 20 mM; (X) [Ca^{2+}] = 50 mM. (□) [Mn^{2+}] = 20 mM; (△) [Mg^{2+}] = 50 mM. (Solid Line) Results obtained with pure PS.

and pK 3.0 for PA (Traüble & Eibl, 1974; Galla & Sackmann, 1975).

Furthermore, in the case of PS, after a plateau from pH 5 to pH 8.5, a new increase in fluorescence intensity is observed centered around pH 9. This value is close to the ammonium pK = 7.5 (Seimiya & Ohki, 1973; Hauser et al., 1976). Moreover, this intensity increase is only observed when part of the protein is still free in solution. No change is detected when there is a large excess of lipids, and all the protein is already bound. We interpret this effect in terms of an increased affinity of the protein for PS when its ammonium group charge is removed.

In a similar way, with PA, for values higher than pH 4.5, the new increase in intensity centered around pH 6.5 probably arises from an increase in protein binding due to the ionization of the second acidic function, although its pK is equal to 9 (Traüble & Eibl, 1974) or 8.0 (Galla & Sackmann, 1975). This discrepancy could be due to the sensitivity of this pK to ionic strength and so to the displacement of protons by protein on formation of the complex.

Finally, one has to mention that at neutral pH no binding has been detected with PE. When pH increases in the range 7.5 < pH < 11, a detectable binding occurs resulting from the negative global charge of PE above the ammonium p$K \simeq 7.5$ (Seimiya & Ohki, 1973).

5. *Binding of CTX to Lipid Mixtures.* Natural membranes are composed of a wide variety of lipids. Since CTX is known to interact with various kinds of plasmic membranes, erythrocyte, cell cultures (Zaheer et al., 1975), or axonal membranes (Vincent et al., 1976), the results obtained for the interactions of CTX with pure phospholipids must be augmented by the study of CTX behavior in the presence of lipid mixtures.

CTX binds only to pure phospholipids bearing a negative charge. Consequently, we examined first what happens when CTX is allowed to interact with mixtures of PC and PS. The relative increases in intensity at 335 nm vs. the total phospholipid to protein molar ratio are plotted in Figure 7a. It is obvious that, as the mole fraction of PC increases in the lipid system, the plateau is reached for higher R_i values. But, as is seen in Figure 7b, when the same experimental results are plotted vs. the reduced molar ratio of PS to CTX, all the experimental curves lie close together. Only the mixtures containing very small amounts of PS give smooth curves instead of straight lines before the plateau. We take this as an indication that, even in a mixture, CTX only binds to PS; the PC molecules seem not to be involved in the binding process. The same conclusion is reached from results obtained when binding experiments are performed with PS-cholesterol mixtures (Figure 8).

Finally, since direct binding experiments have been reported (Vincent et al., 1976), between ^{3}H-labeled CTX and axonal membranes, it was checked whether or not in similar conditions, binding could be detected by direct interactions of CTX with a mixture having the same lipid composition as axonal membrane (fraction II) (Balerna et al., 1975). The mixture of phospholipids in vesicular form is added to CTX in the same way as described above. The wavelength of the emission maximum is shifted only until 342 nm, as in the case of PS-cholesterol mixtures. The effect on the fluorescence intensity is plotted in Figure 8 vs. the molar ratio of negatively charged phospholipids to CTX. A plateau is reached for $R_i \simeq 8$–9, a value again very close to that obtained with pure PS, which is the major negative lipid component of the mixture. Even in this case, it seems that CTX interacts only with the negative lipids of the mixture. Furthermore, binding is not concentration dependent from 1 to 10 μM. However, it depends on the ionic strength (Figure 8), and it is interesting to notice again that a few tenths millimolar concentration of divalent cations prevents the formation of the complex. The sequence of the cation efficiency for the competition with CTX is the same as that found with pure phospholipids, i.e., $Mn^{2+} > Ca^{2+} > Mg^{2+} \gg Na^+ = K^+$.

Discussion

The above results clearly show that cardiotoxin II does not form complexes with neutral or zwitterionic lipids, but strongly interacts with negatively charged lipids such as phosphatidylserine, phosphatidylinositol, or phosphatidic acid. The observed effects on the fluorescence spectra are consistent with a decrease of the polarity of the Trp environment which can be explained either by a direct interaction of this residue with the phospholipid hydrophobic core, and/or by a conformational change of the protein resulting in a lesser accessibility of Trp to water. Conformational changes induced either by pH (Chicheportiche et al., 1972) or solvent effects (Menez et al., 1976) have already been reported in the case of neurotoxins, leading to different exposure of the aromatic residues and to changes on the helical content.

The high specificity of CTX for negative lipids remains valid

when binding experiments are carried out using lipid mixtures vesicles. If we assume that the bilayer structure is preserved after CTX binding, this implies that CTX can promote an heterogeneous distribution of neutral and negative phospholipids within the plane of the bilayer, i.e., the existence of clusters or of lateral phase separations. It has already been shown that such phenomena often occur in lipid mixtures, either spontaneously (Shimshick & McConnell 1973) or induced by cations (Ito et al., 1975) and also, by proteins (Galla & Sackmann, 1975; Birrell & Griffith, 1976; Boggs et al., 1977). This conclusion is obviously also in agreement with the existence of a "lipid halo" around membrane proteins, as it has been proposed by several authors (Jost et al., 1973; Lee et al., 1974; Faucon et al., 1976).

The prominent part played by negatively charged lipids in the binding of CTX seems to indicate that the stabilization of the lipid–protein complex involves mainly electrostatic forces. This is confirmed by the effect of mono- and divalent cations which are able to dissociate the complex, and also by the pH effects. These clearly show that the amount of bound CTX is closely related to the number of negative charges borne by the lipid bilayer, and also to the charge of the toxin.

In these conditions, it is obvious that, if hydrophobic interactions occur, they play only a minor part in the stabilization of the CTX–phospholipid complexes. The basic residues of the protein, mainly Lys, probably interact with the negative groups either phosphate or carboxylic of the phospholipids. By looking at the toxin sequence, one can notice that up to five ammonium groups are located close to Trp. So, the formation of ionic bonding would promote a contact between Trp and the bilayer which could explain all the observed effects on the fluorescence spectrum.

Two interesting parameters are in principle accessible from these kinds of binding experiments, namely, the association constant K_a and the stoichiometry of the lipid–protein complexes. In fact, in the concentration range used, from 1 to 10 μM, all the studied lipid systems lead to typical stoichiometric binding curves, i.e., one observes a linear increase of the relative change in fluorescence intensity (which is directly proportional to the amount of bound protein). This means that K_a is, in all cases examined here, large ($>10^6$ M^{-1}) and cannot be determined directly by fluorometric titration experiments. An estimate of K_a can be inferred from the Ca^{2+} concentration necessary to reach the midpoint of the complex dissociation. According to Hauser et al. (1976), the association constant of Ca^{2+} for PS is equal to 10^4 M^{-1}. This leads to a K_a value around 2×10^7 M^{-1} for the CTX–PS complex. Nevertheless, this value could be affected by the presence of the phospholipase impurity.

The R_i value for which the plateau is reached is a direct estimation of the stoichiometry of the complex: 7 or 8 when only one net negative charge is borne by the phospholipid, and 3.5 with PA when it bears two charges. If we assume for CTX molecule the same geometry as that recently determined for short neurotoxins (Low et al., 1976; Tsernoglou & Petsko, 1976, 1977), and that the bilayer structure is preserved, it is possible to calculate the maximum amount of bound protein per surface unit at maximum packing. This leads to calculated values of 4 to 6 phospholipids per protein which compare well with the experimental ones, provided that the two faces of the phospholipid bilayer are covered by a sheet of proteins. So the stoichiometry of the complexes is not inconsistent with the existence of a bilayer, but there is as yet no experimental evidence to support this point of view.

Discussion has been limited, until now, to the description of the interaction mechanism between CTX and phospholipids. It remains to be seen whether or not CTX–lipid interactions can account for the results obtained with biological membranes by other authors. First, it seems that the very high affinity of CTX for negative phospholipids is consistent with the dose–response curves of cardiotoxins interacting with membranes or cells. Indeed, CTX concentrations needed to induce contracture of the chick biventers cervicis muscle (Lin et al., 1976), to lyse different strains of cells, or to inhibit ATPase activities (Zaheer et al., 1975; Vincent et al., 1976) always lie in the micromolar range. Moreover, from the binding of labeled [^{3}H]CTX to axonal membranes, Vincent et al. (1976) conclude that the toxin added to the membrane preparation binds *quantitatively* to its receptor site until the membrane is saturated, i.e., when 42 nmol of CTX is added per mg of membrane protein.

Since the composition of axonal membranes is known (Balerna et al., 1975), the CTX concentration needed for saturation of axonal membranes can be easily related to the amount of negatively charged phospholipids contained in these membranes. This simple calculation leads to a molar ratio $7.2 < R_i < 8.5$ of negatively charged lipids per toxin molecule. This value is in very good agreement with those we obtain, with a lipid mixture having the same composition as axonal membranes ($R_i = 9 \pm 1$) (Figure 8) and with pure negatively charged lipids $R_i = 7 \pm 1$ (Figure 2).

Another point which can be readily explained by the binding of CTX to negative phospholipids is the effect of cations. Vincent et al. (1976) have shown that 50% inhibition of CTX binding to axonal membranes is obtained at 12.5 mM Ca^{2+}. This value is practically the same as that (11.5 mM) necessary to dissociate 50% of the CTX–PS complex (Figure 4), which is quite in agreement with the fact that PS is the major negative lipid component in axonal membrane. In the same way, Lin et al. (1976) have shown that a few tenths of millimolar divalent cation concentration inhibited the muscle contracture induced by CTX, the efficiency increasing in the order $Mg^{2+} < Ca^{2+} < Mn^{2+}$, which is again consistent with the results obtained with phospholipids (see Table I).

Finally, our results strongly support the mechanism first proposed by Zaheer et al. (1975) to explain the inhibition by CTX of the (Na^+, K^+)ATPase activity of a variety of cell systems. It is indeed well known that (Na^+, K^+)ATPase activity is generally lipid dependent, PS being the most effective lipid in reversing the activity inhibition (Kimelberg, 1976). Since CTX strongly interacts with negative lipids, one can imagine that it competes efficiently with the lipid moiety of the (Na^+, K^+)ATPase complex. This enzyme would then be depleted of its normal lipid environment, resulting in a decrease of its activity.

In conclusion, even though protein–protein interactions, as those detected between CTX and acetylcholinesterases (Lin et al., 1977) cannot be totally ruled out, it seems that most of the actually known physiological actions of CTX can be explained by a specific binding of the toxin to the negatively charged phospholipids of biological membranes. Further studies are now in progress to determine: (i) the localization of the toxin site, i.e., the residues involved in the binding; and (ii) the effect of CTX on the structure and dynamics of the lipid bilayer.

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References

Balerna, M., Fosset, M., Chicheportiche, R., Romey, G., & Lazdunski, M. (1975) *Biochemistry 14,* 5500.
Birrel, G. B., & Griffith, O. H. (1976) *Biochemistry 15,* 2925.
Boggs, J. M., Wood, D. D., Moscarello, M. A., & Papahadjopoulos, D. (1977) *Biochemistry 16,* 2325.
Chicheportiche, R., Rochat, C., Sampieri, F., & Lazdunski, M. (1972) *Biochemistry 11,* 1681.
Chipperfield, A. R., & Whittam, R. (1973) *J. Physiol. 230,* 467.
Conti, C., & Forster, L. S. (1975) *Biochem. Biophys. Res. Commun. 65,* 1257.
Dufourcq, J., & Faucon, J. F. (1977) *Biochim. Biophys. Acta 467,* 1.
Dufourcq, J., Faucon, J. F., Bernon, R., & Lussan, C. (1975) *FEBS Lett. 57,* 112.
Faucon, J. F., Dufourcq, J., Lussan, C., & Bernon, C. (1976) *Biochim. Biophys. Acta 436,* 283.
Galla, H. J., & Sackmann, E. (1975) *Biochim. Biophys. Acta 401,* 509.
Hauser, H., & Phillips, M. C. (1973) *J. Biol. Chem. 248,* 8585.
Hauser, H., Darke, A., & Phillips, M. C. (1976) *Eur. J. Biochem. 62,* 335.
Howard, B. D., & Truog, R. (1977) *Biochemistry 16,* 122.
Huang, C. (1969) *Biochemistry 8,* 344.
Ito, T., Onishi, S., Ishinaga, M., & Kito, M. (1975) *Biochemistry 14,* 3064.
Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 480.
Jover, E. (1976) Diplôme d'Etudes approfondies, Marseille.
Kimelberg, H. K. (1976) *Mol. Cell. Biochem. 10,* 171.
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Toon, P. A., & Warren, G. B. (1974) *Biochemistry 13,* 3699.
Lin, S. Y. S., Huang, M. C., & Lee, C. Y. (1976) *J. Pharm. Exp. Ther. 196,* 758.
Lin, S. Y. S., Liao, C., & Lee, C. Y. (1977) *Biochem. J. 161,* 229.
Louw, A. I. (1974) *Biochim. Biophys. Acta 336,* 470.
Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., & Richardson (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2991.
Menez, A., Bouet, F., Tamiya, N., & Fromageot, P. (1976) *Biochim. Biophys. Acta 453,* 121.
Mollay, C., & Kreil, G. (1973) *Biochim. Biophys. Acta 316,* 196.
Morrisett, J. D., David, J. S. K., Pownall, H. J., & Gotto, A. M., Jr. (1973) *Biochemistry 12,* 1290.
Mullin, B. R., Aloj, S. M., Fishman, P. H., Lee, G., Kohn, L. D., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1639.
Nicholls, P. (1974) *Biochim. Biophys. Acta 346,* 261.
Patel, T. N., Braganca, B. M., & Bellare, R. A. (1969) *Exp. Cell. Res. 57,* 289.
Rouser, G., Kritchevsky, G., Heller, D., & Lieber, E. (1963) *J. Am. Oil. Chem. Soc. 40,* 425.
Schneider, A. B., & Edelhoch, H. (1972) *J. Biol. Chem. 247,* 4992.
Seimiya, T., & Ohki, S. (1973) *Biochim. Biophys. Acta 298,* 456.
Seto, A., Sato, S., & Tamiya, N. (1970) *Biochim. Biophys. Acta 214,* 483.
Shimshick, E. J., and McConnell, H. M. (1973) *Biochemistry 12,* 2351.
Shinitzky, M., & Fridkin, M. (1976) *Biochim. Biophys. Acta 434,* 137.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42,* 53.
Strong, P. W., Goerke, J., Oberg, S., & Kelly, R. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 178.
Teale, F. W. J. (1960) *Biochem. J. 76,* 381.
Traüble, H., & Eibl, H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 11,* 214.
Trevelyan, W. E. (1967) *J. Lipid Res. 8,* 281.
Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68,* 1.
Tsernoglou, D., & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 971.
Van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14,* 5387.
Vincent, J. P., Schweitz, H., Chicheportiche, R., Fosset, M., Balerna, M., Lenoir, M. C., & Lazdunski, M. (1976) *Biochemistry 15,* 3171.
Visser, L., & Louw, A. I. (1977) *Biochim. Biophys. Acta 491,* 349.
Yang, C. C. (1974) *Toxicon 12,* 1.
Zaheer, A., Noronha, S. H., Hospattanka, A. V., & Braganca, B. M. (1975) *Biochim. Biophys. Acta 394,* 293.