Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., & Sporn, M. B. (1983) *J. Biol. Chem. 258*, 7155.
Barbarash, G. R., & Quarles, R. H. (1982) *Anal. Biochem. 119*, 177.
Bishop, J. M. (1982) *Adv. Cancer Res. 37*, 1.
Bowen-Pope, D. F., & Ross, R. (1982) *J. Biol. Chem. 257*, 5161.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
De Larco, J. E., & Todaro, G. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4001.
De Larco, J. E., & Todaro, G. J. (1980) *J. Cell. Physiol. 102*, 267.
Deuel, T. F., Huang, J. S., Proffitt, R. T., Baenziger, J. U., Chang, D., & Kennedy, B. (1981) *J. Biol. Chem. 256*, 8896.
Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A., & Antoniades, H. N. (1983) *Science (Washington, D.C.) 221*, 275.
Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., & Sporn, M. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3676.
Haddow, A. (1972) *Adv. Cancer Res. 16*, 181.
Hawke, D., Yuan, P.-M., & Shively, J. E. (1982) *Anal. Biochem. 20*, 302.
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Marquardt, H., & Todaro, G. J. (1982) *J. Cell. Physiol. 104*, 41.
Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De Larco, J. E., Stephenson, J. R., & Todaro, G. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4684.
Raines, E. W., & Ross, R. (1982) *J. Biol. Chem. 25*, 5154.
Roberts, A. B., Lamb, L. C., Newton, D. L., Sporn, M. B., De Larco, J. E., & Todaro, G. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3494.
Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., & Sporn, M. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5339.
Roberts, A. B., Anzano, M. A., Frolik, C. A., & Sporn, M. B. (1982a) *Cold Spring Harbor Conf. Cell Proliferation 9*, 319.
Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., Frolik, C. A., Marquardt, H., Todaro, G. J., & Sporn, M. B. (1982b) *Nature (London) 295*, 417.
Roberts, A. B., Frolik, C. A., Anzano, M. A., Assoian, R. K., & Sporn, M. B. (1983a) in *Methods in Molecular and Cell Biology* (Barnes, D., Sato, G., & Sirbasku, D., Eds.) Alan R. Liss, New York (in press).
Roberts, A. B., Frolik, C. A., Anzano, M. A., & Sporn, M. B. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2621.
Sammons, D. W., Adams, L. D., & Nishizawa, E. E. (1981) *Electrophoresis (Weinheim, Fed. Repub. Ger.) 2*, 135.
Schaeffer, W. I., & Friend, K. (1976) *Cancer Lett. 1*, 259.
Sporn, M. B., Roberts, A. B., Shull, J. H., Smith, J. M., & Ward, J. M. (1983) *Science (Washington, D.C.) 219*, 1329.
Steck, G., Leuthard, P., & Burk, R. R. (1980) *Anal. Biochem. 107*, 21.
Stein, S., Bohlen, P., Stone, J., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 203.
Stiles, C. D., Capone, G. T., Sher, C. D., Antoniades, H. N., Van Wyk, J. J., & Pledger, W. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1279.
Todaro, G. J., Fryling, C., & De Larco, J. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5258.
Tsugita, A., & Scheffler, J. J. (1982) *Eur. J. Biochem. 124*, 585.
Twardzik, D. R., Todaro, G. J., Marquardt, H., Reynolds, F. H., & Stephenson, J. R. (1982) *Science (Washington, D.C.) 216*, 894.
Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J. S., & Deuel, T. F. (1983) *Nature (London) 304*, 35.
Westall, F. C., & Hesser, H. (1974) *Anal. Biochem. 61*, 610.

Interaction of Colicin A with Phospholipid Monolayers and Liposomes†

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ABSTRACT: The insertion of colicin A into monomolecular films and liposomes composed of different phospholipids was studied. Although colicin A was able to penetrate many phospholipid monolayers, it interacted preferentially with negatively charged phospholipids such as phosphatidylglycerol. These interactions are highly dependent on the physical state of the lipid, the ionic strength, and the pH. Amino acid residues with a pK of 5.5 probably govern the lipid–protein interaction. At acidic pH, colicin A was able to insert into phospholipid vesicles and was as strong a penetrating agent as the lytic peptides bee venom mellitin and snake venom cardiotoxins. Below pH 5, colicin A induced aggregation and partial fusion of liposomes. At neutral and basic pH, colicin A penetration ability is limited, and the protein was unable to bind to phospholipid vesicles. However, association of colicin A with lipid vesicles could be achieved at pH 7 by the detergent dialysis technique. The apparent molecular area of colicin A inserted into phosphatidylglycerol films (2000 Å^2/molecule) suggests that a substantial part of the colicin A molecule inserts into the lipid surface.

Colicins A, E1, Ia, Ib, and K have been purified (Konisky, 1973; Schwartz & Helinski, 1971; Goebel, 1973; Jesaitis, 1970; Cavard & Lazdunski, 1979). The primary structure of E1 and A has been deduced from the nucleotide sequence of the gene (Yamada et al., 1982; Morlon et al., 1983a,b). These colicins depolarize the bacterial inner membrane and rapidly cause inhibition of active transport [see Konisky (1978)]. They can form voltage-dependent ion channels in planar bilayers (Schein et al., 1978; Weaver et al., 1981; Pattus et al., 1982, 1983), and they also have been reconstituted with liposomes (Tokuda & Konisky, 1979; Uratini & Cramer, 1981). For

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a review, see Cavard & Lazdunski (1982), Konisky (1982), and Cramer et al. (1983).

These colicin–lipid systems constitute suitable models for answering three fundamental questions: (1) What is the mechanism of insertion of proteins into membranes? (2) What is the mechanism by which an ion channel is gated by membrane potential? (3) What is the three-dimensional structure of the polypeptide within the channel? In this paper, we have addressed the first question. For this purpose, the interaction of colicin A with phospholipid monolayers and liposome has been studied. The advantage of the monolayer technique is that the "quality of the interface" can be varied at will (Verger & Pattus, 1982), enabling a detailed study of protein–lipid interactions.

Materials and Methods

Colicin A was purified as previously described (Cavard & Lazdunski, 1979). For the preparation of ^{35}S-labeled colicin A, colicinogenic bacteria were grown in minimal medium; [^{35}S]methionine (0.3 μCi/mL) was added 10 min after the addition of mitomycin C to the medium. Growth was continued for 2 h, and ^{35}S-labeled colicin A was purified (Cavard et al., 1981).

Dilauroylphosphatidylglycerol (DLPG) was kindly provided by Dr. J. F. Tocanne (Toulouse). Dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylglycerol (DPPG), and dipalmitoylphosphatidylcholine (DPPC) were obtained from Medmark. Egg yolk phosphatidylcholine (egg PC), natural phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylethanolamine (PE) were purchased from Sigma and further purified by preparative thin-layer chromatography (PTLC) (Broekhuyse, 1969). Phosphatidylglycerol and phosphatidylethanolamine were purified from total lipid extract of *Escherichia coli* by bidimensional PTLC. Phospholipids labeled with ^{32}P or ^{3}H were purified from a culture of *E. coli* grown in the presence of [^{32}P]phosphate or [^{3}H]glycerol. All lipids were pure as judged by two-dimensional TLC on HPTLC plates silica gel 60 (Merck) (Broekhuyse, 1969). The phospholipid was dissolved in chloroform or chloroform–methanol (2:1) mixtures at a concentration of 1 mg/mL. It was then deposited gently on the air–water interface with a microsyringe in order to obtain a lipid monolayer.

All precautions required to avoid contamination by tensioactive impurities were described elsewhere (Pattus et al., 1979). Surface pressure (π) was measured with a platinium plate according to the Wilhelmy method (Wilhelmy, 1863). The three-compartment trough described by Lairon et al. (1980) was used for all experiments.

For constant-surface experiments, only the first thermostated circular compartment was used (31-mL) volume, 45.5-cm^2 surface). The three-compartment trough was used to measure surface changes (ΔS) with a surface barostat in constant-pressure experiments (Verger & de Haas, 1973). Constant surface pressure was maintained automatically by the backward movement of a Teflon barrier increasing the surface occupied by the film. When necessary, the film was directly recovered in scintillation vials by suction with a glass capillary (Rietsch et al., 1977), and radioactivity was determined (Beckman Model LS 3800). Surface radioactivity was quantitatively determined with an ionization-chamber counter (Merlin Gêrin Ililog, Probe LMT 13 AP7), with the probe placed 4 mm above the air–water interface.

Lipid vesicles were prepared from a mixture of soybean phospholipids (lecithin type II from Sigma) purified according to Kagawa & Racker (1971). Lipids (10 mg) ^{3}H labeled by 1% of ^{3}H-labeled *E. coli* PE were dissolved in 5 mL of hexane, dried under nitrogen to a thin film in a 1-L flask, and resuspended in 10 mL of tris(hydroxymethyl)aminomethane (Tris)–acetate buffer, 50 mM, and NaCl, 0.1 M, pH 7.0 or 5.0, by a gentle shaking in the presence of glass beads (2-mm i.d.). A total of 2.5 mg of ^{3}H-labeled lipid vesicles was incubated with 90 μg of ^{35}S-labeled colicin A in 350 μL of buffer (pH 7 or 5) for 30 min at 25 °C. The mixture was then layered on a 5-mL linear sucrose gradient (5–35%) and centrifuged for 21 h at 50000 rpm in a SW 60 rotor (Beckman).

Reconstitution of colicin A in lipid vesicles was also performed by the detergent dialysis procedure (Kagawa & Racker, 1971; Dufourcq et al., 1974) with octyl-POE [polydisperse octyloligo(oxyethylene), cmc at 25 °C = 6.6 mM] kindly supplied by Dr. J. P. Rosenbusch (Basel) (Rosenbusch et al., 1982). A total of 2.5 mg of ^{3}H phospholipids dispersed in 1% octyl-POE was mixed with 90 μg of ^{35}S-labeled colicin A in 1% octyl-POE in Tris–acetate buffer, pH 7 (350-μL final volume). The mixture was dialyzed against an excess of detergent-free buffer for 60 h with several buffer changes. The cloudy suspension was layered on the top of a sucrose gradient and centrifuged as described above.

Fractionation of the gradient into 250-μL aliquots was performed with a Buchler Auto Densi Flow II gradient collector. Protein and lipid in each fraction was estimated by counting the ^{35}S and ^{3}H radioactivity.

Negative Staining for Electron Microscopy. Unilamellar vesicles prepared by detergent dialysis with octyl-POE were incubated with colicin A (10^{-8} M). Carbon-coated grids were rendered hydrophilic by glow discharging in air. The grids were touched to the membrane suspension for 30 s, rinsed with distilled water, and then touched to aqueous phosphotungstic acid for 45 s.

Results

Colicin Penetration into Phospholipid Monolayers. Colicin A, unlike detergents, did not solubilize the lipid films. Colicin A at a concentration of 5×10^{-7} M was injected under a film of [^{32}P]phosphatidylglycerol from *E. coli* spread at an initial pressure (π_i) of 30 mN/m. After 25 min, the surface radioactivity was found unchanged whereas mellitin (10^{-5} M) led to an 8% decrease within 5 min.

The penetration of colicin A molecules (4×10^{-8} M) into a lipid film of DMPG was accompanied by a significant increase of surface pressure. The lower the initial pressure of the film, the higher was this increase in film pressure. The observed kinetics reached a plateau within a few minutes. This is in agreement with existing data on the penetration of proteins into lipid monolayers [for a review, see Verger & Pattus (1982)]. The maximum value of surface pressure increase ($\Delta\pi_e$) was plotted as a function of the initial pressure (π_i) at which the toxin was injected in the aqueous phase (Figure 1). A critical pressure for penetration may thus be defined: it corresponds to the extrapolated value of initial surface pressure beyond which there is no increase. This was performed with different phospholipid films, allowing a comparison of the penetration ability of colicin A (Figure 1). Colicin A was found to interact with every phospholipid tested, but the strongest interactions were observed with negatively charged phospholipids. The following affinity sequence was observed: *E. coli* PG $\simeq$ 14-PG > 12-PG, PA, and PI > PE and total phospholipid extract from *E. coli* > PC.

The number of bacteriocin molecules associated with the film was determined by using uniformly ^{35}S-labeled colicin A. As in the case of other toxins (Bougis et al., 1981) and lipolytic

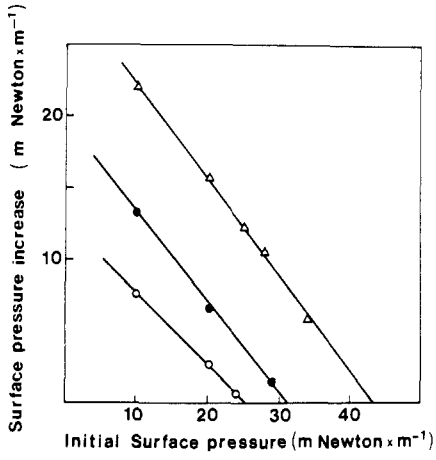

FIGURE 1: Dependence of surface pressure increase caused by colicin A penetration upon initial surface pressures of phospholipid monolayers. Colicin A (4×10^{-2} mg/mL) was injected in the subphase buffer (50 mM Tris–acetate, pH 6, 100 mM NaCl, 1 mM $CaCl_2$) at 30 °C beneath phospholipid monolayers: (△) *E. coli* PG; (●) *E. coli* PE; (○) egg PC.

enzymes (Pattus et al., 1979), only a small proportion of the total amount of protein was associated with the lipid film. We determined that the amount of colicin bound to the film, within the plateau region of surface pressure increase, obeyed the same affinity sequence as the one determined by the measurements of surface pressure increase. There is a direct relationship between the amount of colicin bound to the film and the increase of surface pressure ($\Delta\pi_e$) for a given phospholipid monolayer. Moreover, increasing the chain length of phosphatidylglycerol molecules from 10 to 14 atoms of carbon increased the amount of bound colicin A by a factor of 2.5.

Influence of Temperature and of Physical State of the Phospholipid Monolayer. We compared the penetration of ^{35}S-labeled colicin A in egg lecithin films to the penetration in DPPC films at various temperatures. For the 0–50 °C temperature range, egg lecithin monolayers are in the liquid-expanded state while DPPC monolayers undergo a surface pressure dependent transition between the liquid-expanded state and the condensed state. In Figure 2A, this transition at a surface pressure of 10 mN/m is shown by plotting the area occupied by the DPPC molecules as function of temperature.

As shown in Figure 2B, the fraction of colicin A bound to egg PC monolayers increased monotonically with temperature. A van't Hoff plot of the fraction of colicin A bound (not shown) gave an enthalpy of binding of ΔH = 2.0 kcal/mol. In contrast, colicin A bound weakly to DPPC monolayers in the condensed state. At the transition temperature (28–32 °C), there was a drastic increase of the binding of colicin A to the phospholipid monolayer. As shown in Figure 2A, there is a direct correlation between the abrupt change of the ratio ν (colicin A bound to DPPC per colicin A bound to egg PC) and the phase transiton of the DPPC film occurring when the temperature increases. Similar results were obtained with phosphatidylglycerol monolayers. At 37 °C and pH 6 and at a surface pressure of 20 mN/m DMPG monolayers are in the liquid-expanded state while DPPG monolayers are in the condensed state. After ^{35}S-labeled colicin A injection (4×10^{-8} M), we found 15.5% of colicin A bound to DMPG films and only 2% of colicin A bound to DPPG films.

Influence of pH and Ionic Strength. The pH dependence for the penetration of colicin A injected below DLPG and DLPC films was investigated at an initial surface pressure of

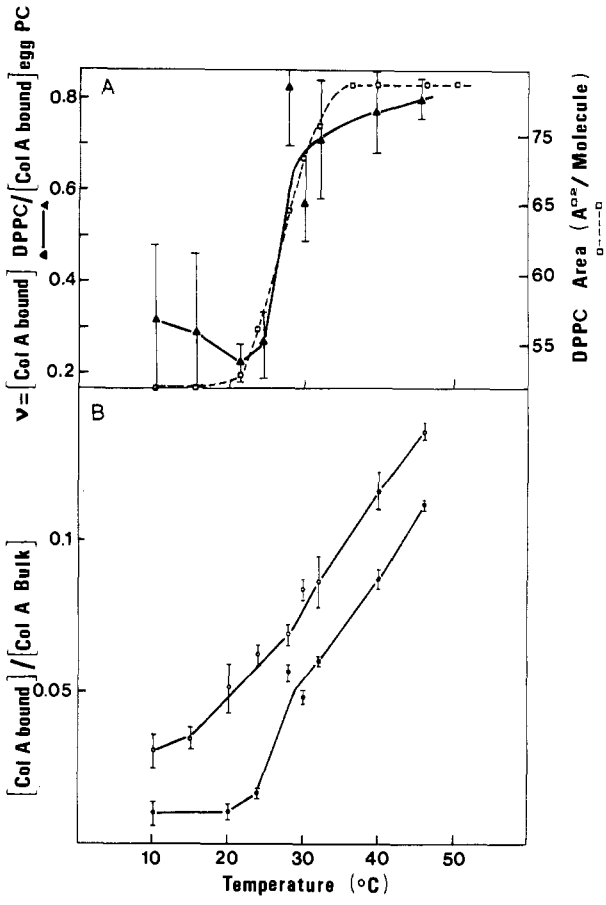

FIGURE 2: Temperature dependence of ^{35}S-labeled colicin A binding to egg PC and DPPC. Conditions are identical with those described in Figure 1 except that colicin A concentration was 2.7×10^{-8} M. (A) The ratio $[\text{colicin A bound}]_{DPPC}/[\text{colicin A bound}]_{eggPC}$ (▲) and the area occupied by DPPC molecules (□) were plotted as a function of temperature. (B) Fraction of colicin A bound to egg PC films (○) and fraction of colicin A bound to DPPC films (●) as a function of temperature.

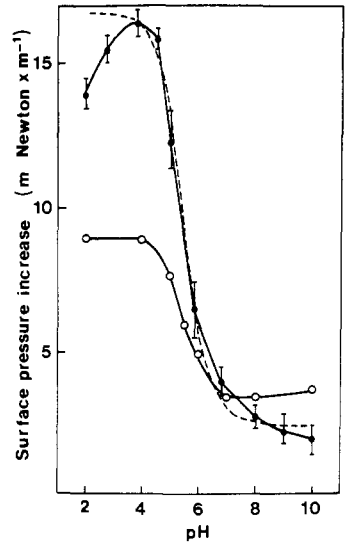

FIGURE 3: pH dependence of surface pressure increase. Colicin A (5×10^{-8} M) was injected in the subphase buffer (20 mM Tris–acetate, 100 mM NaCl, 1 mM EDTA) at 25 °C beneath phospholipid monolayers: (●) DLPG, initial surface pressure 25 mN/m; (○) egg PC, initial surface pressure 15 mN/m. (– –) Theoretical curve assuming that an amino acid residue with a pK of 5.5 "governs" the binding.

25.0 and 15.0 mN/m, respectively (Figure 3). There was an abrupt increase of the penetration ability of colicin A as the

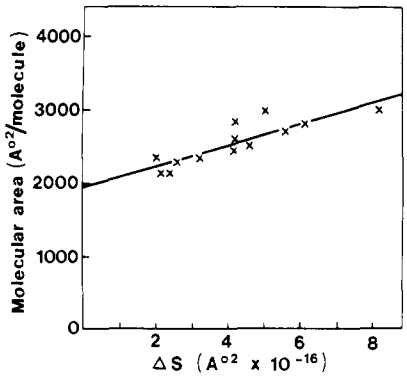

FIGURE 4: Molecular area of colicin A in DLPG monolayers. The apparent molecular area was plotted as a function of the relative surface increase (ΔS) reached with 4.1×10^{-7}, 8.3×10^{-7}, 2.1×10^{-8}, and 4.2×10^{-8} M colicin A. The protein was injected in the subphase buffer (20 mM Tris–acetate, pH 5, 100 mM NaCl, 1 mM EDTA) beneath a DLPG monolayer spread at 20 mN/m (see Materials and Methods).

pH was decreased from pH 6.4 to 4.5. This change was independent from the degree of ionization of the phospholipid head group since identical results were obtained with DLPC, DLPG (Figure 3), and natural PI or PE (data not shown). As shown in Figure 3, the experimental curve fitted quite well with the theoretical titration curve, calculated by assuming that an amino acid residue with a pK of 5.5 governs the penetration of colicin A into the film. The small decrease of $\Delta\pi_e$ between pH 4 and 2 with DLPG films, which is significant, may reflect the loss of negative charge of the phospholipid and correlates well with the condensation of the film occurring between pH 4 and 2 (data now shown). At pH 4, colicin A was a penetrating agent as potent as mellitin or cardiotoxins (Bougis et al., 1981). The penetration was inhibited by high salt concentration with DLPG films. The sodium chloride concentration required to produce half-inhibition of colicin A penetration was 0.8 and 0.2 M at pH 4.5 and 6.0, respectively. At pH 4.5, 10 mM calcium chloride produced the same inhibition. These salt concentrations are similar to those resulting in half-inhibition of cardiotoxin binding to phosphatidylserine monolayers and vesicles (P. Bougis, personal communication; Dufourcq & Faucon, 1978).

Determination of Apparent Molecular Area of Colicin A in a DLPG Film. ^{35}S-Labeled colicin A was used to evaluate the number of bacteriocin molecules associated to the film. By measuring the surface increase at constant pressure, as performed by Bougis et al. (1981) with cardiotoxins, we were able to determine the apparent molecular area of colicin A inserted into a film of DPLG. This value is uncertain for two reasons: first, the phospholipid molecules in direct association with colicin A could have an influence on the surface increases (ΔS), and second, the surface radioactivity measured might represent not only those colicin A molecules directly responsible for the surface increase but also an unknown amount of protein close to the monolayer. In order to reduce experimental errors of measurement of this molecular area generated by the progressive formation of a mixed lipid–colicin film, we measured $\Delta S/S$ values lower than 15%. As well, we determined that, for a fixed ΔS, the molecular area occupied by colicin A was independent from the toxin concentration from 4×10^{-9} up to 5×10^{-8} M. As shown in Figure 4, extrapolation to zero surface increase ($\Delta S/S = 0$) gave the apparent molecular area of the first colicin A molecule penetrating the phosphatidylglycerol monolayer. At pH 5, with DLPG monolayers at a surface pressure of 20 dyn/cm, the extrapolated value was 2000 Å^2/molecule of colicin A.

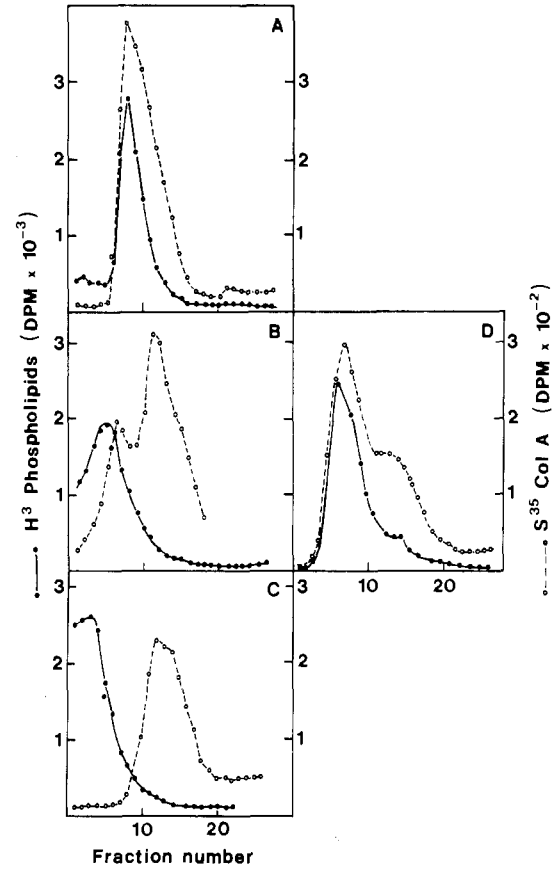

FIGURE 5: pH dependence for binding of colicin A to liposomes. ^{35}S-labeled colicin A was incubated with ^{3}H-labeled preformed liposomes at pH 5 (A) or 7 (B). (C) Colicin A and preformed liposomes were centrifuged separately at pH 7 (the same pattern was obtained at pH 5). (D) Colicin A and soybean phospholipids were reconstituted into liposomes by the detergent dialysis technique at pH 7.

Interaction of Colicin A with Liposomes. In order to confirm and extend some of the monolayer data, the binding of colicin A to liposomes was investigated. Soybean phospholipids were selected as lipid source because these phospholipids were used in previous studies on colicins binding to liposomes and planar bilayers (Tokuda & Konisky, 1979; Schein et al., 1978; Pattus et al., 1982). Colicin A was incubated with preformed soybean phospholipid liposomes at pH 7 and 5 as described under Materials and Methods. The mixtures were spun down in a 5–35% sucrose gradient. Colicin A was found at the middle of the gradient (Figure 5C) whereas soybean phospholipids remained at the top of the gradient so that any interaction of colicin A could easily be detected by this technique. Results, obtained under comparable conditions at pH 5 and 7, are presented in Figure 5. At pH 5 (Figure 5A), nearly all colicin molecules migrated together with lipids to an intermediate density, indicating binding of colicin A to the liposomes. In contrast, at pH 7 (Figure 5B), only a small fraction of colicin A was retarded on the gradient. No clear lipoprotein peak was formed.

However, when colicin A was reassociated with liposomes by the detergent dialysis procedure (see Materials and Methods), a stable lipid–protein complex could be obtained even at pH 7.0 (Figure 5D). No desorption could be detected by centrifugation of the lipid–protein complex and measurement of ^{35}S radioactivity in the supernatant.

As shown in Figure 6, colicin A incubated with unilamellar soybean phospholipid vesicles at pH 4.5 produced aggregation and partial fusion of the lipid vesicles. The kinetics of vesicle aggregation was measured by following the turbidity change

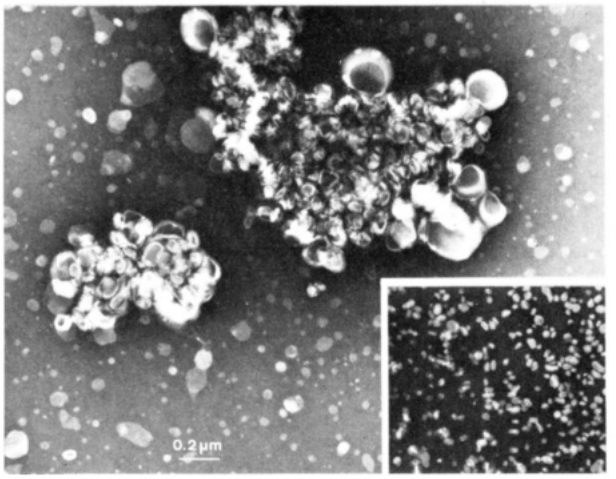

FIGURE 6: Electron micrographs of unilamellar liposomes incubated with colicin A (1.6×10^{-8} M) at pH 4.5 in Tris–acetate, 50 mM, and KCl, 0.1 M. (Inset) Untreated liposomes, same magnification.

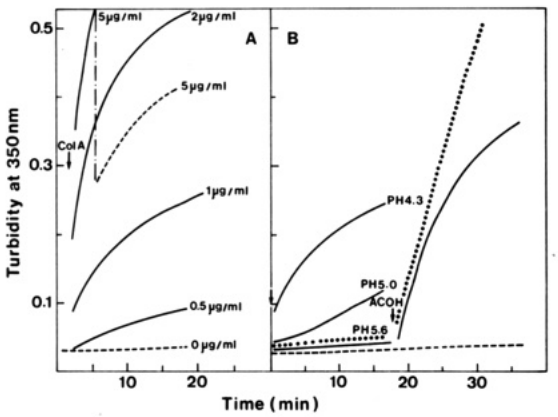

FIGURE 7: Influence of colicin A on turbidity of a unilamellar liposome solution at 350 nm. (A) Soybean liposomes (1 mg in 1 mL, mean diameter 500 Å) prepared by detergent dialysis with octyl-POE were incubated with increasing amounts of colicin A in Tris–acetate, 50 mM, and KCl, 0.1 M, pH 4.2. (B) Effect of pH on turbidity change produced by (—) colicin A (1.6×10^{-8} M) and (···) colicin A (1.6×10^{-7} M), pH 5.6. At the time indicated by the arrow, acetic acid was added to decrease the pH to 4.2. (– –) Control without colicin A, pH 5.6 or 4.2.

at 350 nm (Figure 7A) and occurred only below pH 5.6 (Figure 7B). At pH 5.6, increasing the colicin A concentration by a factor of 10 did not produce significant turbidity change (Figure 7B, dotted line). Although colicin A was known to bind to the vesicles. Subsequent addition of acetic acid to the colicin A–phospholipid suspension sufficient to reach pH 4.2 produced an immediate rise of the turbidity. Control experiments without colicin A showed that this aggregation was specifically induced by the bacteriocin molecules.

Discussion

The results presented in this study indicate that colicin A is a tensioactive protein able to penetrate phospholipid monolayers. This protein interacts preferentially with negatively charged phospholipids such as phosphatidylglycerol, one of the major phospholipids from *E. coli* inner membrane (Kanfer & Kennedy, 1963). This penetration was inhibited by high salt concentrations or by Ca^{2+} ions. The latter condensed the phospholipid film and competed for negative charges at the lipid interface. This indicates the predominance of electrostatic-type interactions during the penetration process. Hydrophobic-type interactions might be also involved since the lipid affinity was reinforced by increasing the length of the hydrocarbon chain from 10 to 14 atoms of carbon.

At basic and neutral pH, however, the penetration ability of colicin A into the phospholipid film is not extensive. From the critical pressure of penetration into different types of phospholipid films, we predict that colicin A will not bind phospholipid liposomes at neutral and basic pH, of which equivalent surface pressure is in the order of 30 mN/m (Blume, 1979). Furthermore, colicin A binds weakly to phospholipid monolayers in the condensed state. In contrast, with pancreatic phospholipase A_2 (Op den Kamp et al., 1974), maximal penetration of colicin A was not observed at the transition temperature where defects in the lipid packing could aid the protein penetration.

There is a drastic influence of pH on the penetration ability of colicin A. At acidic pH, colicin was as strong a penetrating agent as the lytic peptides cardiotoxin III or mellitin. We propose that an amino acid residue on colicin A with a pK of 5.5 appears to regulate this penetration (Figure 3). This indicates that the lipid binding site of colicin A is not influenced by the net charge of the protein (pK = 7.5) and must be a restricted domain in the protein molecule. Measurement of the area occupied by colicin A in DLPG films (2000 Å^2/molecule at a surface pressure of 20 mN/m) indicates that a substantial part of the colicin A molecule inserted into the lipid layer. We cannot, however, estimate how much of the protein interacts with the lipid. A 2000 Å^2/molecule may correspond to the maximal cross-section of a globular protein of M_r 60 000. On the other hand, according to studies on the hydrodynamic properties of colicin E1 and A (Schwartz & Helinski, 1971; F. Pattus, unpublished results), colicin A could be a prolate ellipsoid with major and minor axes of 19 and 370 Å, respectively. We might then conclude that only one-third of colicin A was in direct contact with the lipid layer. A similar conclusion could be reached by assuming that an amino acid residue covers a surface of 10–15 Å^2.

Although such estimates should be taken with caution, they are in agreement with the proposed domains of colicins (Konisky, 1982). The C-terminal part of the bacteriocins would constitute the domain responsible for the killing activity while the N-terminal domain would be responsible for binding to the specific receptor on the outer membrane of the *E. coli* cell. We recently isolated a C-terminal peptide from colicin A (M_r 20 000, 207 residues) that is able to form pores in planar bilayers, similar to those formed by the intact colicin (Martinez et al., 1983).

The predictions of the ability of colicin A to interact with liposomes at various pHs, derived from monolayer results, are confirmed by the direct measurements of the binding of colicin A to soybean phospholipid vesicles. At pH 7, the binding of colicin A to the lipid vesicles was not significant while at pH 5, complete binding was obtained under identical experimental conditions (Figure 5). Furthermore, below pH 5, colicin A induced rapid aggregation and partial fusion of soybean liposomes (Figures 6 and 7). These sensitivities and these fusogenic properties are very similar to those observed with Semliki Forest virus proteins, the targets of which are the acidic lysosomes (Helenius et al., 1980). The above studies indicate that the colicin A target in *E.* coli cells is probably the phospholipids of the inner membrane and, more specifically, the negatively charged phosphatidylglycerol molecules.

The energy barrier that prevents the penetration of colicin into the lipid bilayer at neutral and basic pH may be due to the packing of the polar head groups as found with pancreatic phospholipase A_2 (Pattus et al., 1978). Such an energy barrier can be overcome by reconstitution with a detergent to disrupt

the tight association of the phospholipid molecules. At neutral and basic pH, colicin A was unable to interact directly with liposomes, but as soon as the colicin A was associated with the lipids, a stable complex was formed. No desorption could be detected. This behavior resembles those of membrane proteins that require specific mechanisms for insertion into membranes.

An amino acid residue with a pK_a around 5.5 regulates the colicin A–membrane interactions. The nature of this residue is not yet known. In a previous study on the pore properties of colicin A in planar bilayers (Pattus et al., 1983), we found that the single-channel conductance and the voltage dependence of the pore are regulated by an amino acid residue with a similar pK_a. It is not clear whether the same amino acid residue is involved in the regulation of the binding of colicin A to the phospholipid interface and in electrical properties of the pore. Such drastic changes of these two properties of colicin A could be a consequence of protein aggregation by protonation on an amino acid residue(s). Preliminary experiments carried out by analytical centrifugation of colicin A showed that colicin A aggregated at acidic pHs (unpublished experiments), and further studies are now in progress to determine the molecular structure of colicin A pores.

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Registry No. Colicin A, 12737-31-4; DLPG, 63644-55-3; DLPC, 18285-71-7; DMPC, 13699-48-4; DMPG, 61361-72-6; DPPG, 4537-77-3; DPPC, 2644-64-6.

References

Blume, A. (1979) *Biochim. Biophys. Acta 557*, 32.
Bougis, P., Rochat, H., Piéroni, G., & Verger, R. (1981) *Biochemistry 20*, 4915.
Broekhuyse, R. M. (1969) *Clin. Chim. Acta 23*, 457.
Cavard, D., & Lazdunski, C. (1979) *Eur. J. Biochem. 96*, 519.
Cavard, D., & Lazdunski, C. (1982) *Toxicon 20*, 223.
Cavard, D., Bernadac, A., & Lazdunski, C. (1981) *Eur. J. Biochem. 119*, 125.
Cramer, W. A., Dankert, J. R., & Uratani, Y. (1983) *Biochim. Biophys. Acta 737*, 173.
Dufourcq, J., & Faucon, J. F. (1978) *Biochemistry 17*, 1170.
Dufourcq, J., Bernon, R., & Lussan, C. (1974) *C. R. Hebd. Seances Acad. Sci., Ser. D 278*, 2565.
Goebel, W. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 854.
Helenius, A., Kartenbeck, J., Simmons, K., & Fries, E. (1980) *J. Cell Biol. 84*, 404.
Jesaitis, M. A. (1970) *J. Exp. Med. 131*, 1016.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477.
Kanfer, J., & Kennedy, E. P. (1973) *J. Biol. Chem. 238*, 2919.
Konisky, J. (1978) in *Bacteria* (Ornston, L. N., & Sokatch, J. R., Eds.) Vol. 6, pp 319–359, Academic Press, New York.
Konisky, J. (1982) *Annu. Rev. Microbiol. 36*, 125.
Konisky, J., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 2972.
Lairon, D., Charbonnier-Augeire, M., Nalbone, G., Leonardi, J. C., Hauton, J. C., Piéroni, G., Ferrato, F., & Verger, R. (1980) *Biochim. Biophys. Acta 618*, 106.
Martinez, M. C., Lazdunski, C., & Pattus, F. (1983) *EMBO J. 2*, 1501.
Morlon, J., Lloubès, R., Chartier, M., Bonicel, J., & Lazdunski, C. (1983a) *EMBO J. 2*, 787.
Morlon, J., Lloubès, R., Varenne, S., Chartier, M., & Lazdunski, C. (1983b) *J. Mol. Biol.* (in press).
Op den Kamp, J. A. F., de Gier, J., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 17*, 1170.
Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry 18*, 2691.
Pattus, F., Cavard, D., Verger, R., Lazdunski, D., Rosenbusch, J., & Schindler, H. (1982) *Toxicon 20*, 205–206.
Pattus, F., Cavard, D., Verger, R., Lazdunski, C., Rosenbusch, J., & Schindler, H. (1983) in *Physical Chemistry of Transmembrane Ion Motions* (Spach, G., Ed.) pp 407–413, Elsevier, Amsterdam.
Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313.
Rosenbusch, J. P., Garavito, R. M., Dorset, D. L., & Engel, A. (1982) in *Protides in Biological Fluids* (Peters, H., Ed.) p 171, Pergamon Press, Oxford.
Schein, S. J., Kagan, B. L., & Finkelstein, A. (1978) *Nature (London) 276*, 159.
Schwartz, A. S., & Helinski, D. (1971) *J. Biol. Chem. 245*, 6318.
Tokuda, H., & Konisky, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6117.
Uratani, Y., & Cramer, W. A. (1981) *J. Biol. Chem. 256*, 4017.
Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127.
Verger, R., & Pattus, F. (1982) *Chem. Phys. Lipids 30*, 189.
Weaver, C. A., Kagan, B. L., Finkelstein, A., & Konisky, J. (1981) *Biochim. Biophys. Acta 645*, 137.
Wilhelmy, L. (1863) *Ann. Phys. (Leipzig) 119*, 177.
Yamada, M., Ebina, Y., Miyata, T., Nakazawa, T., & Nakazawa, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2827.