BBA 75580

LIPID MONOLAYERS. INTERACTIONS WITH STAPHYLOCOCCAL α-TOXIN*

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(Received August 17th, 1970)

SUMMARY

Staphylococcal α-toxin spreads readily as a film on aqueous media; the film contains 3 and 30 times as much protein as in films of mitochondrial structural protein and in a protein monolayer, respectively. The toxin forms a film also by adsorption from the hypophase. The ability of α-toxin both to spread and adsorb at the air–water interface is increased markedly by 6 M urea.

Penetration of α-toxin into lipid monolayers is influenced greatly by the chemical structure of the lipid; the $\Delta\Pi$ values are in the order cholesterol > phosphatidyl choline > phosphatidyl inositol > sphingomyelin > ganglioside. In line with the known binding of gangliosides by histones, penetration of the basic toxin into the air–water interface is inhibited by ganglioside as well as by sulfatide monolayers.

Inasmuch as film penetration ($\Delta\Pi$) is consistent with anchoring of the protein at the interface, binding of the cationic toxin under the negatively charged monolayers of phosphatidyl inositol, sulfatide, or ganglioside explains the low $\Delta\Pi$ values observed with these lipids and the known ability of this protein to coat the host membrane.

Penetration of α-toxin into lecithin monolayers increases as the protein concentration in the hypophase becomes greater, as the initial pressure of the lipid film becomes smaller, as pH or temperature of hypophase increases.

INTRODUCTION

Staphylococcal α-toxin is a cytolytic protein for which no enzymatic function has been demonstrated[1]. This apparent lack of specific activity and the fact that this protein interacts with and disrupts artificial and natural membrane systems[2] prompt interest in the search for a more general kind of mechanism of action of α-toxin such as penetration of lipid–water interfaces. The behavior of α-toxin at the air–water

* This work is taken from the dissertation presented by A. R. Buckelew, Jr., in partial fulfillment of the requirements for the Ph. D. degree in the Department of Microbiology at the University of New Hampshire, Durham, N.H., in July 1968. The monolayer experiments were done at the Albert Einstein College of Medicine.

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interface and its interaction with lipid monolayers were therefore investigated with the view to find out if the surface activity of this protein could account for properties that relate to its cytolytic action.

MATERIALS AND METHODS

Organism and growth conditions

A human strain of *Staphylococcus aureus* was obtained from Major M. Smith U.S. Army, Japan. This strain was selected because it produced large amounts of α-toxin and relatively little β-hemolysin, thus simplifying the purification of α-toxin.

The cultural conditions for the production of α-toxin were adapted from the methods of CHESBRO *et al.*[3]. Improved yields of α-toxin were obtained by adding 5 units of penicillin G (Eli Lilly and Co.) to the culture medium before inoculation. This procedure, described by RITZERFELD *et al.*[4], gave twice the yield of toxin over controls without penicillin.

Purification of α-toxin

The culture supernatant was dialyzed in 3/4-inch tubing (Fisher Chemical Co., Fair Lawn, N.J.) against three changes of distilled water for 2 days at 4°. The toxin was then recovered according to the method of COULTER[5]. The retentate was adjusted to pH 4.0 with glacial acetic acid. Methanol, precooled at −20°, was added slowly with constant stirring until a concentration of 25% methanol was attained. The mixture was left to stand at 4° for 3 h and then was centrifuged at 12000 × *g* for 30 min at 4°. The pellet was extracted 3 times with 10 ml of 0.15 M sodium acetate followed by centrifugation at 12000 × *g* for 30 min at 4°. The supernatants were collected: the pH was adjusted to 7.5, and this product of crude toxin was stored at −20° if necessary.

Otherwise, 10-ml fractions of the crude α-toxin were applied onto a 300-ml Sephadex G-75 column (LKB Instruments, Rockville, Md.), which was equipped with plunger heads; chromatography was performed at 4°. The column was eluted with 0.15 M sodium acetate at the rate of 7.5 ml/h with the aid of an LKB peristaltic pump Model 4912A, while 1–2-ml fractions were received in an LKB fraction collector. The absorption of the column fractions at 280 mμ was monitored with an LKB instrument, 8300 A Uvicord II.

The hemolytic activity present in the column fractions was determined on rabbit erythrocytes. The reciprocal of the highest dilution causing 100% hemolysis was the hemolytic titer[6]. The combined most active fractions from the Sephadex G-75 column were made 50% saturated with solid $(NH_4)_2SO_4$ and left to stand for 2 h at 4°. The solution was then centrifuged at 24000 × *g* for 30 min, the sediment was discarded, and the supernatant was made 80% saturated with the addition of solid $(NH_4)_2SO_4$, and stored at 4°; the activity of the toxin is preserved this way[6]. When needed for study, the purified α-toxin was recovered in a pellet by centrifugation at 24000 × *g* for 30 min and dissolved in the desired buffer. The activity of these preparations was about 8 hemolytic units/μg of toxin protein.

The purified α-toxin was homogeneous on Sephadex G-75 gel filtration, acetate strip, agarose gel electrophoresis and immunodiffusion. After each one of the first three tests, the toxin retained most of its activity. In immunodiffusion our toxin

preparations formed only one precipitin line against Lederle antisera[6]; heterologous antistaphylococcus sera, lots No. 83 and No. 73 were obtained from Lederle Laboratories. A molecular weight of 43000, which was calculated from gel filtration, agrees with the value obtained by others[1]. The purified α-toxin was devoid of phospholipase A, phospholipase C, and phosphatase activities[6].

Proteins

Several preparations of α-toxin were made by the above method. The lipid-free apoprotein of rat plasma high density lipoprotein was kindly provided by G. Camejo[7]. Streptolysin S, 15000 hemolytic units/mg, was a gift from the late R. Rowen and was prepared by methods already described[8]. Bovine ribonuclease, twice crystallized, was a product of Worthington Biochemical Corp., Freehold, N.J. Protein solutions of 5 mg/ml in 0.04 M phosphate buffer containing 0.1 M NaCl (pH 7.0), were stored for not longer than 3 days at 2°.

Water and buffers

Protein and salt solutions were prepared with water which was redistilled over alkaline permanganate in an all glass system (Corning, Model AG-2, New York); the conductivity of the resulting water was 1 $\mu\Omega^{-1}$ cm^{-1}. Tris buffer, 0.05 M, of the desired pH was used in the study of the influence of pH on film penetration. Unless otherwise stated, all the other experiments were run with 0.04 M phosphate buffer–0.1 M NaCl (pH 7.0). Water and aqueous solutions were stored in polyethylene bottles for not longer than 3 days at room temperature.

Lipids

Cholesterol and phosphatidyl choline from egg were products of Silvana Chemical Co., Millburn, N.J.[9]. Sphingomyelin from beef heart was supplied by Dr. M. M. Rapport[10]. Phosphatidyl inositol was prepared as the ammonium salt by the method of COLACICCO AND RAPPORT[11]. All these lipids were homogeneous when 100 μg were applied to thin-layer plates coated with silica gel H (Merck, Darmstadt, Germany); chromatography was carried out in chloroform–methanol–water–conc. NH_4OH (70:30:4:1, by vol.) and the lipids were detected by staining with I_2 vapors. A preparation II-R70-1, of "highly purified mixed gangliosides from beef brain gray matter" was a gift from Dr. M. M. Rapport; upon thin-layer chromatography, this lipid produced several spots typical of mixed gangliosides which stained both with I_2 vapors for lipid and with resorcinol for sialic acid[12]. The major components were disialo gangliosides. Highly purified and chromatographically homogeneous mono-, di- and trisialogangliosides were kindly supplied by Dr. K. Suzuki: These gangliosides were prepared by injection of [1-^{14}C]glucosamine into young rats and purified according to the conventional methods of extraction and solvent partition and finally by preparative thin-layer chromatography. Chemical and radioactive purity of each ganglioside was at least 96% (ref. 13). The sodium salt of sulfatide[14] was a gift of Dr. M. B. Abramson. The lipid solutions, which contained about 0.5 mg/ml lipid in chloroform–methanol (85:15, by vol.) were stored in glass-stoppered pyrex tubes for not longer than 3 days at 2°.

Apparatus and procedures

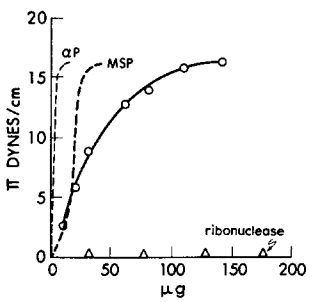
Surface tension was measured by a Wilhelmy plate suspended from a torsion balance[15]. Three methods were used to determine the surface activity of α-toxin:

namely the ability of the protein (a) to spread as a film, (b) to form a film by adsorption from the aqueous hypophase, and (c) to penetrate lipid monolayers. The surface activity was expressed as film pressure; details of the monolayer techniques and a micro-Lowry method for the determination of protein in the subphase have been described (16). The trough consisted of a crystallizing dish coated with paraffin and partitioned into a large film area (18 cm^2) and a small service area. The hypophase, 50 ml, was mixed with a small teflon-coated (1 cm long) magnetic bar.

RESULTS

The toxin formed a film by spreading from its aqueous solutions, whereas ribonuclease did not. Toxin, however, was less active than the apoprotein of high density lipoprotein. And, although the toxin spread as a film as readily as mitochondrial structural protein during the first additions, it became sluggish thereafter. The typical saturation pressure of protein film, 16–18 dynes/cm, was finally reached (Fig. 1). Determination of the quantity of protein in the subphase showed that the toxin film on a area of 33 cm^2, contained 33 μg protein when the surface pressure was 7 dynes/cm and 116 μg at 10 dynes/cm. The latter corresponds to a surface concentration of 3.5 $\mu g/cm^2$, which is nearly 30 times the quantities of protein required to make a "protein monolayer"[17] and 3 times the quantities found in films of mitochondrial structural protein under the same conditions[16,18].

An overall view of the influence of the chemical structure of the lipid on the penetration of α-toxin is presented in Fig. 2. Rate and extent of pressure rise were in the order cholesterol > lecithin > phosphatidyl inositol = sulfatide > sphingomyelin > ganglioside. Only small differences in $\Delta\Pi$ value were observed between the various gangliosides. A trend, however, was clear and consistent with the data in Fig. 3. There, the $\Delta\Pi$ values are in the order lecithin $\gg$ monosialo- > disialo- = trisialoganglioside. Mixed films of lecithin and ganglioside showed also a marked decrease in pressure rise ($\Delta\Pi$) as compared to that of pure lecithin films, and the ($\Delta\Pi$) decrease was proportional to the concentration of ganglioside in the film.



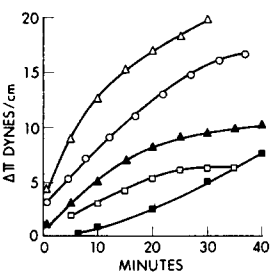


Fig. 1. Formation of protein film after spreading of different quantities of α-toxin, ○—○, at the air–water interface. For comparison: △—△, ribonuclease; MSP, mitochondrial structural protein; αP, apoprotein of rat high density plasma lipoprotein. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl (pH 7.0), 25°; film's area 18 cm^2.

Fig. 2. Influence of the chemical structure of the lipid on the film penetration by α-toxin. △—△, cholesterol; ○—○, egg lecithin; ▲—▲, phosphatidyl inositol; □—□, sphingomyelin; ■—■, ganglioside. Initial film pressure 2 dynes/cm; protein 2 μg/ml. Hypophase as in Fig. 1.

The influence of protein concentration on film penetration by α-toxin was studied with lecithin monolayers (Fig. 4). The curves relating $\Delta\Pi$ to α-toxin concentration were clearly biphasic; the slope for protein concentrations greater than 2 μg/ml was about half that for concentrations below 2 μg/ml.

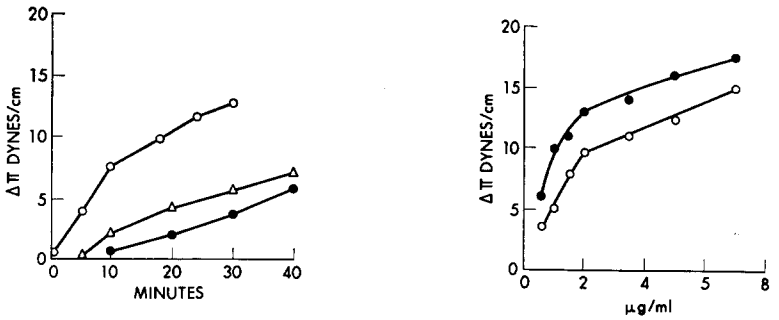


Fig. 3. Interaction of α-toxin with monolayers of monosialoganglioside (△—△) and di- or trisialoganglioside (●—●) as opposed to egg lecithin (○—○). The sodium salt of sulfatide had a $\Delta\Pi$ value slightly greater than that of the monosialoganglioside and similar to that of sulfatide. Initial film pressure 2 dynes/cm; protein 2 μg/ml. Hypophase as in Fig. 1.

Fig. 4. Effect of concentration of α-toxin in subphase on its penetration ($\Delta\Pi$) of lecithin monolayers. ○—○, at 10 min; ●—●, at 20 min. Initial film pressure 2 dynes/cm. Hypophase as in Fig. 1.

As with rat plasma albumin[19] penetration of α-toxin in lecithin monolayers increased as the pH of the subphase was raised between 6 and 10. The plateaus at the pH values 7, 8 and 9, 10 were reproducible (Fig. 5). Experiments below pH 6 were not performed because under such conditions α-toxin precipitates out of solution.

Penetration of α-toxin into lecithin films increased as the temperature of the subphase was raised between 10 and 35° (Fig. 6). Biphasic curves show a markedly greater slope between 20 and 35° than between 10 and 20°.

The influence of the initial pressure of the lecithin film on the penetration of

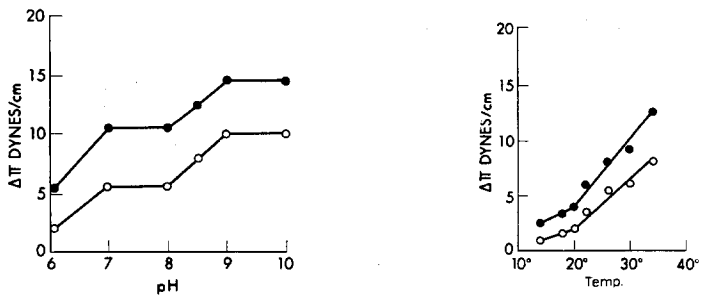


Fig. 5. Effect of pH of hypophase on the penetration ($\Delta\Pi$) of monolayers of egg lecithin by α-toxin. ○—○, at 10 min; ●—●, at 20 min. Phosphate buffer, pH 6.0–8.0; Tris buffer, pH 8.5–9.0. Initial film pressure 2 dynes/cm; protein 2 μg/ml.

Fig. 6. Effect of temperature in hypophase on the penetration ($\Delta\Pi$) of α-toxin into monolayers of egg lecithin. ○—○, at 10 min; ●—●, at 20 min. Initial film pressure 2 dynes/cm, protein 2 μg/ml. Hypophase as in Fig. 1.

α-toxin is shown in Fig. 7. As with many other proteins, the increase in film pressure decreased as the initial pressure of the lipid film was raised. However, the curves relating $\Delta\Pi$ to Π_i are also biphasic, as they show a steep slope for values of Π_i between 2 and 10 dynes/cm and a very small slope for values of Π_i between 10 and 30 dynes/cm.

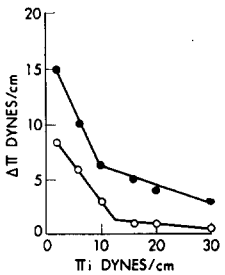


Fig. 7. Effect of initial film pressure (Π_i) on the penetration ($\Delta\Pi$) of egg lecithin by α-toxin ○—○, at 10 min; ●—● at 20 min. Protein 2 μg/ml. Hypophase as in Fig. 1.

DISCUSSION

Significance of data

Formulation of mechanisms and interpretation of data in the interaction of proteins with the air–water interface are still qualitative. Although the increase in film pressure ($\Delta\Pi$) observed after injection of protein under lipid films is referred to as film penetration and is a measure of interaction, it is neither a measure of protein present in the film nor an expression of formation of a lipid–protein complex in the hydrophobic as well as in the hydrophilic regions of the film. Both the protein incorporated in the film and any lipid–protein complexes must be determined independently[16,20]. The physical meaning of Π or $\Delta\Pi$ follows from the definition of surface tension. Since the latter is a property of the liquid (water) and since film pressure is a decrease of surface tension, the Π or $\Delta\Pi$ values are proportional to the number of asymmetric protein structures which become anchored at the interface and to the amount of surface free water displaced during the anchoring. In view of the available data[16,20], the Π or $\Delta\Pi$ values do not encompass fully the biophysical processes which take place at the interface. Beside a $\Delta\Pi$-mechanism which puts minimal monolayer quantities of anchored protein structures in the Π-interface at the expense of the surface free energy of water, two non $\Delta\Pi$-mechanisms put large quantities of protein either in the air–lipid medium or in the aqueous medium below the Π-interface. These mechanisms were described and are responsible for the W, Z, and Ω protein forms respectively[16,20]. Consequently, although in many cases the $\Delta\Pi$ values obey Langmuir's adsorption equation and the relationship of $1/\Delta\Pi$ *vs.* $1/c$ is a straight line[19], they do not reflect in any way the quantities of protein in the film except for the minimal amounts required to make the profile of the Π-interface. Moreover, attainment of equilibrium takes between 2 and 8 h. Apart from the fact that its definition is arbitrary and meaningless and the operations would be impractical and void of biological significance, a meaningful evaluation of the data can be made in terms of kinetic curves, Π or $\Delta\Pi$ *vs.* time, or in terms of Π values at a given time,

provided that the curves do not intersect each other[9]. Conclusively, therefore, $\Delta\Pi$ values reflect the ability of the protein to anchor at the interface irrespective of the quantity and mode of organization. The average thickness of the protein film can be arrived at by dividing the quantity of protein found in the film as $\mu g/cm^2$ by the monolayer quantity, 0.125 ($\mu g/cm^2$ layer), at a pressure of 10–15 dynes/cm. The choice of pressure will depend on the compressibility of the film[16,17].

Ability of α-toxin to spread as a film in the absence of lipid

The staphylococcal α-toxin has a marked tendency to form a film at the air–water interface of dilute salt solutions both by spreading and adsorption, a property that this protein shares with structural proteins[16] and with peptides known to interact with membranous structures[21]. Interestingly, the films which are formed by spreading of α-toxin are very thick, 3 times as thick as the films of mitochondrial structural protein and 30 times as thick as some apolipoprotein films and the hypothetical protein monolayer[17]. This indicates that α-toxin has a remarkable tendency to aggregate at and coat lipid or lipoprotein surfaces, a property which is borne out by the studies of FREER (ref. 22). The ability of the toxin to adsorb at surfaces may be an important factor which relates to the interaction of this protein with biological membranes.

Influence of the chemical structure of the lipid on $\Delta\Pi$

The influence of the chemical structure of the lipid on the penetration of α-toxin (Fig. 2) seems to follow the observations made with other proteins[9,19,20]. Marked is the failure of toxin to penetrate monolayers of gangliosides, a property which is exhibited also by serum albumin[20] and streptolysin S. As with several other proteins, sphingomyelin was penetrated less than phosphatidyl choline[9,19,20]; in the same category was found the acidic lipid phosphatidyl inositol, whereas sulfatide approached the behavior of ganglioside. With mixed lecithin–ganglioside monolayers, the inhibition of penetration of toxin was proportional to the concentration of ganglioside and was felt already with 10 mole% ganglioside. With 50 mole% cholesterol in a lecithin film, the penetration curve approached that of lecithin alone; this confirms a previous observation, whereby equimolar mixtures of cholesterol and egg lecithin preserved a lecithin character in the non-specific penetration by γ-globulin whereas, above 50 mole% cholesterol, the $\Delta\Pi$ values increased linearly with the cholesterol concentration[23].

Although no decision has been made as yet concerning the mechanism of interaction of proteins with sphingomyelin monolayers, cholesterol, phosphatidyl choline (lecithin), and the acidic lipids and gangliosides fall in the three different categories, binding-mediated, free, and binding-inhibited penetrations which have been described[20]. Obviously, when protein is adsorbed by hydrophilic interactions with the ionic or polar groups of the lipid which extend into the aqueous phase out of the Π-interface[24], the routes of access to the Π-interface are obstructed, and the degree of obstruction will depend on the geometry of the hydrophilic groups and on the aggregating effects that the binding can exert on the protein. Gangliosides have a particular biological significance in this context, since preincubation of toxin with ganglioside reduces the lethal effects of toxin[25].

The marked effectiveness of mono- and the slightly greater effectiveness of

disialo- and trisialogangliosides in inhibiting penetration of α-toxin into the air–water interface (Fig. 3), means simply that one acidic group is sufficient to prevent penetration of toxin; the second and third sialic acid groups have little additional effect. Inhibition of penetration suggests binding of the cationic α-toxin on the sialic acid group. This mechanism bears a resemblance to the binding of basic proteins to gangliosides in brain[26,27]. The presence of acidic groups which extend into the aqueous phase from the molecules of ganglioside and sulfatide suggests that a mechanism of binding-inhibited penetration similar to one seen with the albumin–ganglioside and the lipid hapten–antibody systems[16] may operate between α-toxin and these glycosphingolipids. Similarities with the binding of cytochrome *c* to monolayers of acidic lipids are in order[28].

Incubation of toxin with an equal weight of ganglioside prior to injection of the protein under the lipid film caused a marked decrease in the rate and extent of penetration ($\Delta\Pi$) of the toxin into the lecithin monolayer. Only sulfatide had a similar effect. Incubation of α-toxin with cerebroside, sphingomyelin, and lecithin itself did not cause a diminished interaction of the incubation mixture with phosphatidyl choline. One could conclude from these observations that the positively charged α-toxin molecules are prevented from entering the film of negatively charged lipids, probably because they become bound to the lipid's negative charges. The more avid lytic activity of α-toxin when incubated with erythrocytes of certain species, especially the rabbit, and the apparent resistance of the erythrocytes of other species[29] could be related to the relative abundance of negatively charged groups such as sialic acid on the surface of the resistant cells.

Influence of protein concentration in subphase

The biphasic character of the curves relating $\Delta\Pi$ to protein concentration (Fig. 4) is consistent with previous observations[9,30] and could be due to the formation of multiple protein layers[16,20,30,31]. Neither kinetic nor thermodynamic interpretations are presented. This was done intentionally in order not to add another example to the one already available in the literature[19]. A useful interpretation of the physcical meaning of the data cannot be had unless the molecular mechanisms and processes of the films are better understood.

Influence of pH of subphase

The increase of $\Delta\Pi$ values with increasing pH values of the hypophase between 6 and 10 (Fig. 5) was observed also with rat plasma albumin[19], rabbit serum albumin, γ-globulin, ribonuclease, and lysozyme (G. COLACICCO, unpublished results); details of the $\Delta\Pi$ *vs.* pH curve seem to vary from protein to protein. Striking is the possibility that the increase of $\Delta\Pi$ on the alkaline side of the pH scale be related to the expansion that several proteins undergo in that pH range[32].

Influence of temperature

The increase of $\Delta\Pi$ values with the temperature in the lecithin–toxin system (Fig. 6) is consistent with the increase in surface denaturation of the protein at higher temperatures. This could be expected since phosphatidyl choline functions by a mechanism of free penetration[16,20]. This behavior of the $\Delta\Pi$–temperature curve is in marked contrast with the curves obtained in the interaction of γ-globulin with

monolayers of lactosides; the latter showed a maximum in the $\Delta\Pi$–temperature curve. Except for pointing out the differences, we are unable to provide a detailed explanation at present. Preliminarily, however, since lactosides operate by a binding-mediated mechanism[20], it is conceivable that the critical step is the transient binding, which, as such, is expected to have an optimal temperature. Since reorganization of the protein is the consequence of the binding, in the latter's absence, less protein could enter the incompressible lactoside film[9] past the optimal temperature.

Influence of initial pressure of lipid film

The decrease of the $\Delta\Pi$ values with increasing values of the initial film pressures has been observed with other systems[9,19,30]. Noticeable is a greater decrease of $\Delta\Pi$ in the lecithin–toxin system (Fig. 7) as compared to that of the lecithin–globulin[9] and lecithin–albumin[19] systems. Obviously, molecular weights of the protein do not play a role, but molecular geometry could. The unusual tendency of α-toxin to aggregate at the interface could restrict the ways of access of the protein in the Π-interface. The sharp change in slope of the curve relating $\Delta\Pi$ to initial film pressure (Fig. 7) at Π_i = 10 or 12 dynes/cm, could be associated with a marked decrease in the rate of anchoring of protein structures when the area available for penetration reaches a sufficiently small value.

Conclusions and perspectives

The trends reported in the present study were reproduced consistently with several preparations of α-toxin. A cogent relation between behavior of the toxin at surfaces and its biological activity was offered by the observation that the values of Π or $\Delta\Pi$ decreased as the toxin's age increased and the cytolytic activity decreased. Although an enzymatic function for α-toxin has yet to be discovered and cannot be ruled out, it seems reasonable to conclude that the toxin's surface activity may play an important role in its interaction with biological membranes. The foregoing monolayer experiments reveal a remarkable surface activity of this toxin, which is unique in its inhibited penetration into lipid membranes containing gangliosides. Since, the concentration of 2 μg/ml at which these studies have been carried out are smaller than those (5–7 μg/ml) at which the toxin reveals its optimal disruptive action on membranes[6], one can very well anticipate the effects of similar protein concentrations on the lipid monolayers. The absence of disulfide bridges in α-toxin, its ability to form thick films, and to penetrate lipid films though not as violently as melittin[21], indicate that, from the standpoint of surface chemistry, α-toxin is a respectable candidate for processes at the membrane level. Future monolayer experiments may have to consider the fact that in nature membrane surfaces sensitive to toxin have a complement of protein and lipid which could elicit a toxin action greater than the one observed here with monolayers consisting only of lipids.

ACKNOWLEDGMENTS

Supported in part by American Cancer Society Grant P-410, and National Institutes of Health Research Grant HE 09659. A.R. B. was supported by a University of New Hampshire Fellowship and a Terminal Predoctoral Fellowship from Public Health Service 1-F1 Gm-36-513-01.

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