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STUDIES ON STAPHYLOCOCCAL ALPHA TOXIN-MEMBRANE INTERACTION

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by

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ABSTRACT

STUDIES ON STAPHYLOCOCCAL
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The mechanism of staphylococcal alpha toxin-membrane interaction was investigated in light of the apparent lack of enzymatic funtion of this exoprotein. The influence of various lipids on alpha toxin activity was also studied. Three types of membranc systems were used in this study: living systems, ie., bacterial protoplasts and spheroplasts and mammalian erythrocytes; isolated C^{14}-labeled Streptococcus faecium cell membranes; and simulated membranes, ie., lipid monolayers of different compositions.

Ganglioside and, to a lesser extent, sulfatide inhibited the lysis of rabbit erythrocytes and bacterial protoplasts by alpha toxin. These lipids also inhibited the interaction of alpha toxin with lecithin monolayers.

C^{14}-labeled membranes of Streptococcus faecium were degraded by the action of alpha toxin causing a release of many components.

Alpha toxin had the ability to form a film at an air-water interface. This surface activity was increased in the presence of urea.

It was concluded that the surface activity of staphylococcal alpha toxin is an important property of the molecule for its ability to lyse erythrocytes, and that the presence of negative charges in membranes may inhibit lysis by alpha toxin.

INTRODUCTION

Staphylococcal alpha toxin damages a wide range of vertebrate cell types. Numerous studies point to the cell membrane as the site of action for alpha toxin activity, but the mechanism by which alpha toxin disrupts membrane structure and function is poorly understood.

The problem of the mechanism of alpha toxin activity was approached in this thesis by studying the interaction of alpha toxin with various membrane systems. The attack was made on three fronts: the interaction of alpha toxin with living membrane systems, ie., bacterial protoplasts and spheroplasts and mammalian erythrocytes; isolated cell membranes of Streptococcus faecium, and membrane simulators, ie., lipid monolayers.

The effects of pH, temperature, and urea treatment on the surface activity of alpha toxin was studied in the monolayer system. The influence of ganglioside, known to inhibit the lethal effect of alpha toxin, on the interaction of alpha toxin with erythrocytes, protoplasts, and lipid monolayers was compared with the effects of other lipids.

It was hoped that these basic studies might help explain the activity of alpha toxin in respect to its apparent lack of enzymatic activity. Furthermore, increased knowledge of the mechanism of alpha toxin-membrane interaction might shed some light on the puzzling species specificity of staphylococcal alpha toxin hemolytic activity.

LITERATURE REVIEW

The early literature on staphylococcal alpha toxin has been reviewed by Elek (20) and by van Heyningen (56). Most of the early work does not differentiate between the various staphylococcal hemolysins. In 1900 Kraus and Clairmont (31) noticed that certain Staphylococcus colonies caused lysis of rabbit erythrocytes but not human erythrocytes when grown on blood-smeared agar plates. Decades passed before successful isolation of the substance responsible for this phenomenon, alpha hemolysin. In 1945, Duthie and Wylie (19) greatly improved yields of alpha hemolysin by incubating broth cultures of Staphylococcus aureus in an atmosphere of CO_2 and O_2 on a culture shaker. The first reasonable purification was performed by Wittler and Pillemer (59) using a combination of acid precipitation and methanol precipitation of culture supernatants.

The names lysin, hemolysin, and toxin have all been used in referring to the staphylococcal exoprotein causing hemolysis of rabbit erythrocytes, dermonecrosis in rabbits, and a lethal effect. Most modern workers use the term alpha toxin (2, 15, 56); this is the name which will be used through the remainder of this thesis.

Bernheimer (2, 4) and Coulter (15) have recently reviewed much of the material to be discussed below.

The organism most often used for alpha toxin production

has been the Wood 46 strain of Staphylococcus aureus. Most workers have produced alpha toxin in broth cultures incubated under CO_2 and O_2 on a culture shaker at 37° C for various periods of time.

Kumar, et al., (32) and Lominski and Arbuthnott (34) used a semisolid medium. Bernheimer and Schwartz (6) used a culture medium composed of dialyzed yeast extract, acid hydrolyzed casein, glucose, and vitamins.

Ritzerfeld, et al., (50) noticed that sublethal quantities of penicillin increased alpha toxin yields. Friedman (22) found that D-cycloserine would increase yields of alpha toxin, but found no increase in production with penicillin. Cripps and Work (16) demonstrated the inhibition of alpha toxin production in the presence of high concentrations of NaCl.

Most workers have used some variation of solvent precipitation for the initial steps of alpha toxin purification. Marucci (43) used ethanol precipitation followed by dialysis. Lominski and Arbuthnott (34) used methanol-acetic acid precipitation. Jackson's method (29) involved ethanol precipitation of a zinc-lysin complex followed by chromatography on hydroxy apatite. Coulter (15) obtained a highly pure product utilizing Sephadex column chromatography and continuous flow electrophoresis of methanol-acetic acid precipitate.

Madoff and Weinstein (39) obtained an electrophoretically homogeneous product following a procedure which involved

dialysis of a zinc precipitate, Sephadex chromatography, paper curtain electrophoresis, and DEAE-cellulose chromatography. Bernheimer and Schwartz (6) used $(NH_4)_2SO_4$ fractionation and continuous flow electrophoresis to obtain a product estimated to be about 85% pure.

The molecular weight of purified alpha toxin has been variously determined as 10,000 to 15,000 (32), 8,000 to 10,000 and 40,000 to 50,000 (25), and 44,000 (6). Coulter (15) has hypothesized the existence of several molecular weights, the average being 33,000. Chesbro, Stuart, and Burke (12) have reported that staphylococcal nuclease also occurs as multiple molecular weight forms.

Alpha toxin is usually described as a protein, but there is controversy over the amount and significance of carbohydrate in the preparations of various workers. Kumar, et al., (32) reported the presence of carbohydrate in their preparations. Goshi, Cluff, and Norman (24) demonstrated a 6 - 7% carbohydrate content in their preparations, and reported a loss of activity when they treated their preparations with alpha or beta amylase.

Bernheimer and Schwartz (6) and Coulter (15) tested for carbohydrate by the anthrone reaction. They reported that if present, carbohydrate must make up less than 1% of their preparations.

The amino acid composition of the preparations of Bernheimer and Schwartz (6) and Coulter (15) were basically

similar, and showed large amounts of aspartic acid and no cyst(e)ine. Coulter reported histidine and arginine as N-terminal amino acids, but could not demonstrate the presence of two peptides after treatment with mercaptoethanol and sulfite. Preparations treated with mercaptoethanol yielded only arginine as an N-terminal amino acid.

Aggregates of alpha toxin have been observed under several conditions. Low pH has long been known to precipitate alpha toxin (59). Freer, Arbuthnott, and Bernheimer (21) described the conversion by heating of 3S alpha toxin to a nonhemolytic, non-lethal 12S aggregate, which upon treatment with 8M urea, was reconverted into the hemolytic 3S form. They called alpha toxin treated in this manner heat purified (HP) alpha toxin. Small amounts of a 12S component were also observed by Bernheimer (2) in his preparations of alpha toxin. The 12S aggregate of alpha toxin appeared as a ring shaped structure, approximately 90 A in diameter, when observed under the electron microscope (21). Similar structures were observed on erythrocyte membrane fragments and lipid spherules after treatment with HP alpha toxin. Freer, Arbuthnott, and Bernheimer (21) attributed this second type of ring structure to an alpha toxin-lipid micelle formation.

Most workers agree that the lethal, dermonecrotic, and alpha hemolytic effects of alpha toxin preparations reside in one protein or a closely related group of proteins. This is known as the unitarian theory.

The unitarian theory has been disputed by Haque (26), who was able to demonstrate alpha toxin species with different hemolytic specificities. McClatchy and Rosenblum (44) have isolated staphylococcal mutant strains which were not hemolytic, but retained lethal and dermonecrotic activities and immunologic specificity.

Toxin preparations have been reported to be lethal for a variety of animals such as mice (2), rabbits (4), and frogs (35). The cause of the lethal effect is not known, but Thal and Egner (55) and Bernheimer (4) have suggested that it may be due to a direct effect on smooth muscle. Thal and Egner (55) have speculated that the dermonecrotic effect may also be due to an effect on smooth muscle; namely that the effect is due to a spastic action on vascular smooth muscle.

The hemolytic effect is extremely variable from one species of erythrocyte to another. The rabbit erythrocyte is the most sensitive. Human and guinea pig erythrocytes are only about 0.1% as sensitive as the rabbit erythrocyte (14). The kinetics of alpha toxin hemolysis have been studied by a number of workers resulting in different conclusions as to the enzymatic nature of the phenomenon. Lominski and Arbuthnott (34) concluded that alpha toxin is probably an enzyme, while Cooper, Madoff, and Weinstein (14) came to the opposite conclusion based on their observations that the degree of hemolysis was directly proportional to

the quantity of toxin and that repeated exposure of the hemolysin to erythrocytes resulted in a linear loss of activity. Lominski and Arbuthnott (34) found the absolute titer to remain the same in similar experiments. Marucci (43) reported that despite varying degrees of lysis during the first exposure of alpha toxin to rabbit erythrocytes, the lysis of new cells by the supernatant was identical, indicating that hemolysin was not used up or fixed. Orsi and Poggiolini (46) reported that part of the hemolysin is bound to the erythrocyte after very short contact and that the per cent of bound hemolysin increased with time of contact and concentration of erythrocytes. The hemolysin could not be removed by successive washings or concentrated urea solution.

Although Robinson, Thatcher, and Montford (51) reported that alpha toxin was proteolytic, this observation has not been confirmed. No specific enzymatic activity has been attributed to alpha toxin (4). Coulter (15) could not find any phospholipase activity in his preparation.

In addition to the classic hemolytic, dermonecrotic, and lethal effects, a number of other activities have been attributed to alpha toxin. Bernheimer and Schwartz (8) and Monohar, et al., (41) have observed the lysis of rabbit blood platelets due to the action of purified alpha toxin. Szmigielski, et al., (53) reported that alpha toxin was toxic for rabbit polymorphonuclear leukocytes. Bernheimer and Schwartz (7) observed the lysis of leukocyte lysomes by purified alpha toxin.

Madoff, Artenstein, and Weinstein (38) obtained results suggesting damage to the cell membranes of human amnion and rabbit kidney tissue culture cells upon treatment with purified alpha toxin as judged by the release of herpes simplex virus and intracellular protein labeled with S^{35}-labeled methionine. The same authors (37) described the visible effects of alpha toxin on Ehrlich ascites carcinoma cells. The nucleus became prominent and intranuclear bodies and cytoplasmic granules appeared. Balloon-like extensions of the cell membrane developed and persisted. Ehrlich ascites carcinoma cells treated with alpha toxin "in vitro" would not preliferate in mice. The authors proposed that alpha toxin acted by altering the permeability characteristics of the cell membrane. Pahal, et al., (49) found that alpha toxin inhibited both the short-circuit current and potential difference of isolated toad bladder, suggesting an alteration in epithelial ion movement.

Bernheimer and Schwartz (3, 9) observed the lysis of bacterial protoplasts and spheroplasts in the presence of relatively large amounts of alpha toxin. Under similar conditions, Bernheimer and Davidson (5) demonstrated the lysis of mycoplasma.

The importance of alpha toxin for staphylococcal pathogenicity has been demonstrated by several authors, although other factors such as the growth rate (Cheshro and Wamola, unpublished results) may be more important in determining

the virulence of staphylococcal strains. Taubler, Kapral, and Mudd (54) reported that in contrast to strains producing alpha toxin, alpha toxin negative mutants seeded on sutures implanted in mice were almost devoid of lesion production. Houser and Berry (28) grew staphylococci in diffusion chambers implanted in mice and demonstrated the production of alpha toxin "in vivo".

Alpha toxin has been shown to have an effect on artificial membrane systems. Weissman, Sessa, and Bernheimer (58) demonstrated the lysis of artificial lipid spherules by alpha toxin. Spherules made of lecithin, cholesterol, and dicetyl phosphate or lecithin and dicetyl phosphate alone were lysed as evidenced by the release of trapped chromate ions or glucose from the lipid spherules. Lipid monolayers made up of the same proportions of lipids as were used in making lipid sperules were penetrated by HP alpha toxin contained in the aqueous subphase (21).

Lipids, especially gangliosides, have been observed to influence the activity of alpha toxin. In 1937, Weinstein (57) noted that large amounts of lecithin inhibited the production and activity of staphylococcal "hemolysin". North and Doery (45) reported that mice were spared the lethal effects of a crude staphylococcal toxin preparation when it was incubated with a ganglioside preparation from bovine brain before injection. Dain, Madoff, Tien, and Weinstein (17) confirmed these results. They found complete

elimination of toxicity when purified alpha toxin was incubated at 37° C with any of four fractions of a ganglioside preparation.

METHODS AND MATERIALS

Organism and Cultural Methods

A human strain of Staphylococcus aureus (obtained from Major M. Smith, while he was a graduate student in this laboratory) was used throughout these studies for the production of alpha toxin. The strain, designated # 9, was used because it produced large amounts of alpha toxin and relatively little beta hemolysin, thus simplifying the purification of alpha toxin.

A completely dialyzable medium was prepared in the following manner: 60 g N-Z-Amine Type A, an enzymatic digest of casein (Sheffield Chemical Company, Norwich, New York) and 60 g yeast extract (Baltimore Biological Laboratories, Baltimore, Maryland) were dissolved in 400 ml distilled water and placed in 1 1/8 inch dialysis tubing (Fisher Chemical Company, Fair Lawn, New Jersey). The dialysis tube was placed in 3 liters of distilled water contained in a 4 liter Erlenmeyer flask, in which was dissolved 7.5 g NaCl, 7.5 g KCl, and 4.5 g $(NH_4)_2SO_4$. Several ml of chloroform were added to prevent growth. After about 72 hours at 4° C and occasional agitation, the dialysis tube and its contents were discarded, and 12 g K_2HPO_4 and 6 g sodium succinate were added to the dialyzed medium. The pH was adjusted to 7.4.

All but 200 ml of the dialyzable medium was divided

equally among six heavy wall 4 liter Erlenmeyer flasks provided with one hole rubber stoppers. A glass tube, to which was attached a heavy wall rubber tube equipped with a pinch clamp, was placed in each stopper. In addition, 100 ml of the medium were placed in each of two 500 ml Erlenmeyer flasks and all the flasks were covered with aluminum foil. The flasks were autoclaved at 110° C and 10 pounds pressure for 10 minutes. After autoclaving and cooling, 5 units of Penicillin G (Eli Lilly and Company, Indianapolis, Indiana) were aseptically added to each of the 4 liter flasks.

Colonies of strain #9 which exhibited especially broad zones of alpha hemolysis on 1% sheep blood agar, were inoculated into 100 ml of the dialyzed medium and incubated 14 hours at 37° C on a rotary shaker (G-10 Gyrotory Shaker at setting #7; New Brunswick Scientific Company, New Brunswick, New Jersey). At the end of this time, 100 ml of sterile medium was added to the starter culture which was incubated an additional three hours. The 200 ml of starter culture was then divided equally into each of the six four-liter vacuum flasks, which had been prewarmed at 37° C.

The atmosphere was evacuated from the flasks and replaced with 20% CO_2-80% O_2. This procedure was repeated every 3 hours during a 9-hour incubation at 37° C on a rotary shaker (G-10 Gyrotory Shaker at setting #7; New Brunswick Scientific Company). After 9 hours, several ml chloroform were added to each flask, and the flasks were

stored at 4° C overnight. The cultures were centrifuged at 10,000 x g for 20 minutes at 4° C in a refrigerated centrifuge (Model R-2; Ivan Servall, Inc., Norwalk, Connecticut), and the cells were discarded. The culture supernatant was the starting material for alpha toxin purification.

Purification of Alpha Toxin

The culture supernatant was dialyzed in 3/4 inch dialysis tubing (Fisher Chemical Company, Fair Lawn, New Jersey) against three changes of distilled water for two days at 4° C. After dialysis, alpha toxin was extracted by the method of Coulter. The retentate was adjusted to pH 4.0 with glacial acetic acid. Methanol, precooled to -20° C, was added slowly with constant stirring until the per cent methanol was 25. This mixture was allowed to stand at 4° C for three hours. Then the mixture was centrifuged at 4° C at 12,000 x g for 30 minutes, and the precipitate was extracted three times with 10 ml 0.15 M sodium acetate, followed each time by centrifugation at 12,000 x g for 30 minutes at 4° C. The supernatants were saved, and the final precipitate was discarded. The pH of the resultant partially purified alpha toxin was adjusted to 7.5. The product of this procedure was stored at -20° C.

As soon as possible after the methanol precipitation, 10 ml fractions of the partially purified alpha toxin were applied onto a 350 ml Sephadex G-75 (Pharmacia Fine

Chemicals, Inc., Piscataway, New Jersey) column (LKB Instruments, Inc., Rockville, Maryland) equipped with plunger heads. The column was eluted with 0.15M sodium acetate at a rate of 7.5 ml/hr with an LKB 4912A peristaltic pump while 1 to 2 ml fractions were collected in an LKB fraction collector. The column and fraction collector were contained in a refrigerator at 4° C. The 280 mμ absorption of the fractions from the column were monitored with an absorptiometer (LKB 8300 Λ Uvicord II).

Every fifth fraction was titered for alpha hemolytic activity. Starting with 1:100 dilutions of each sample to be titrated, twofold dilutions were made in 0.85% NaCl (1:200, 1:400, 1:800, etc.). Into 10 x 1 cm test tubes were placed 0.5 ml portions of each dilution, and 0.5 ml 1% citrated rabbit erythrocytes (washed two times with 0.85% NaCl) were added to each tube. After mixing, the tubes were incubated at 37° C for 60 minutes. The reciprocal of the highest dilution causing 100% (sparkling) hemolysis was called the hemolytic titer of a given sample.

The most active fractions from the Sephadex G-75 column were combined and made 50% of saturation with $(NH_4)_2SO_4$ and kept at 4° C for two hours. The solution was then centrifuged at 24,000 x g for 30 minutes, the precipitate discarded, and sufficient $(NH_4)_2SO_4$ was added to the supernatant to make the solution 80% saturated. The purified alpha toxin could be recovered from this solution by centri-

fuging at 24,000 x g for 30 minutes and dissolving the precipitate in the desired buffer.

Electrophoresis

Cellulose acetate electrophoresis was carried out on 25 x 180 mm cellulose acetate strips (Carl Schleicher & Schuell Company, Keene, New Hampshire). The strips were placed in a Shandon electrophoresis apparatus (Consolidated Laboratories, Inc., Chicago, Illinois) with filter paper wicks making contact with 0.03M borate buffer, M/5000 ethylenediaminetetraacetic acid, pH 8.6. To the center of a strip was applied 10-20 µl of purified alpha toxin in 0.075M phosphate buffer, pH 7.6. A constant voltage (250V) was maintained for one hour with a Spinco Duostat regulator D.C. power supply (Beckman Instruments, Inc., Palo Alto, California). The current was about 5 ma. The strips were dried at 70° C and stained 5 minutes in a saturated solution of Amido Black 10 B in methanol-glacial acetic acid, 9:1. The background was cleared by rinsing several times in 10% acetic acid in distilled water.

Agarose electrophoresis was carried out in a LKB type 3276 paper electrophoresis apparatus (LKB Instruments, Inc., Rockville, Maryland). A 1 cm thick block of 0.8% agarose was made on the apparatus with a filter paper wick impregnated in the agarose at each end of the block. The buffer was 0.03M borate buffer, pH 8.6. A channel 3.5 x 0.5 cm was cut in the center of the agarose block, and 0.5 mg of alpha toxin protein (specific activity = 6.5 HU*/µg) was

* hemolytic unit

applied in the channel. A constant voltage of 100 volts was maintained for twenty hours with an LKB power supply, type 3290B. The agarose block was then removed from the apparatus, dried, strained with Amido black stain for 5 minutes, and rinsed several times with 10% acetic acid in distilled water as described above.

In another experiment, 5 mg of alpha toxin were applied to an agarose gel block and run 40 hours at 100 volts. The agarose strip was then cut every 0.5 cm into cross sections, and a cm^2 piece of each cross section was placed on a 1% sheep blood cell plate. Hemolysis of the erythrocytes, resulting in clearing around the pieces of agarose after incubation at 37° C, demonstrated the presence of alpha toxin. The remainder of each cross section was extracted by freezing the strips, centrifuging at 10,000 x g, and collecting the supernatant liquid. The supernatant was then assayed for hemolysin activity and protein.

Protein was determined by the method of Lowry as modified by Litwack (33). Commercially available Folin-Ciocalteau reagent (Fisher Chemical Company, Fair Lawn, New Jersey) was used. The standard curve was prepared using crystalline bovine serum albumin, fraction V (Calbiochem, Los Angeles, California). Optical density was determined with a Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Company, New York, New York) using a filter with a band pass of 620-680 mμ.

Immunodiffusion

Immunodiffusion studies were carried out using a modification of the double-diffusion-in-gel technique of Ouchterlony. Agar was prepared as follows: noble agar (Difco, Detroit, Michigan), NaCl and distilled water in the proportions 1:1:98 plus 1:10,000 merthiolate. Impregnation agar was 0.1% noble agar, 0.05% glycerine in distilled water. 55 x 14 mm Petri dishes were used. A thin layer of molten impregnation agar was poured into the bottom of each dish and allowed to harden, whereupon five milliliters of molten agar were poured into each dish and allowed to harden. Wells were cut with a 22 gauge bullet shell while the plate was positioned on a diagram of the well pattern. The agar was removed from the wells by suction with a Pasteur pipette attached to a vacuum line. Each well had a volume of 1 ml.

Following preparation of the plates, the wells were filled as described in the Results section. The anti-alpha toxin titers of the bovine heterologous anti-staphylococcus sera (kindly supplied by Dr. C. Bartley) were as follows: #1, 190; #4, 64; #11, 96; #12, 96. The plates were then covered and incubated in a moist chamber at 22° C for 48 hours.

Tests for Lipase Activity

An attempt was made to demonstrate phospholipase A activity of alpha toxin by the method of Marinetti. A stock egg yolk suspension was prepared by emulsifying one egg yolk in 0.9% NaCl to make a final volume of 100 ml. The working

suspension used for the detection of phospholipase A was a 10-fold dilution of the stock suspension in 0.9% NaCl. In each test, 1 ml of the working suspension was diluted with 4.9 ml of 0.9% NaCl and mixed in standard 10 ml Klett tubes (Klett Manufacturing Company, New York, New York). To each tube was added 0.1 ml of test solution and the optical density was measured at five and ten minute intervals in a Klett-Summerson Photoelectric Colorimeter using a filter with a bandpass of 660-740 mμ. The absorbancy change between 5 and 10 minute intervals was taken as the relative amount of phospholipase A activity.

Phospholipase C activity of alpha toxin was tested for as follows: 800 hemolytic units of alpha toxin (specific activity = 6.5 HU/μg) in 0.5 ml distilled water were incubated with 1 mg of lecithin (Calbiochem, Los Angeles, California) or sphingomyelin (B grade; Calbiochem) in 0.2 ml distilled water, 0.4 ml sodium acetate, pH 6.8, and 0.1 ml 1% bovine albumin fraction V (Calbiochem) in 10 x 1 cm test tubes at 37° C for 1 hour. The reaction was stopped by boiling for 10 minutes.

After the tubes were cooled, 0.2 ml 5M tris-(hydroxymethyl) aminomethane (Tris) buffer, pH 8.0 and 0.05 ml of a 0.5 mg/ml solution of alkaline phosphatase (Worthington Biochemical Corporation, Freehold, New Jersey) were added to each tube and the tubes were incubated for an additional 20 minutes at 37° C. After this time, 1 ml of 14% trichloracetic acid was added to each tube to stop the reaction.

The tubes were centrifuged at 12,000 x g, and the supernatants were saved for the next step. Simultaneously, standards were run in which 0.2 ml of 0.6 ml of a stock solution of phosphocholine (Calbiochem), 200 µg/ml, were incubated with 0.4 ml sodium acetate buffer, pH 6.8, 0.2 ml 5M Tris buffer, pH 8.0, and 0.05 ml alkaline phosphatase solution. 0.4 ml distilled water was added to the 0.2 ml phosphocholine tube to bring it to volume. The tubes were incubated and centrifuged as above.

To 1 ml of each supernatant was added 4 ml sodium acetate buffer, pH 4.2, 0.5 ml 1% ascorbic acid in distilled water, and 0.5 ml 1% ammonium molybdate (Allied Chemical Corp., New York, New York) in 0.05M sodium acetate, pH 4.2. The samples were held 15 minutes at 4° C in the dark. The optical density of the samples was measured in a Klett-Summerson Photoelectric Colorimeter after 15 minutes and again after 30 minutes using a filter with a band pass of 620-680 mµ.

An attempt to demonstrate lipase activity by thin layer chromatography of incubation extracts was also made. 1 mg amounts of five phospholipids: phosphatidyl inositol (General Biochem, Chagrin Falls, Ohio), phosphatidyl ethanolamine (Pierce Chemical Corp.), phosphatidyl serine (Applied Science Laboratories, Inc., State College, Pennsylvania), lecithin (Mann Research Labs, Inc., New York, New York), sphingomyelin (Calbiochem), and ganglioside IIR70A1 (kindly

supplied by Dr. M.M. Rapport, Albert Einstein College of Medicine, Bronx, New York), were incubated as emulsions in 0.5 ml of 0.03M phosphate buffer, pH 7.2 containing 3200 hemolytic units of alpha toxin (specific activity = 6.5 HU/μg), 0.5 ml 0.03M phosphate buffer containing 3200 hemolytic units boiled alpha toxin, or 0.5 ml 0.03M phosphate buffer containing 5 mg phospolipase C (Clostridium perfringens; Worthington Biochemical Corp.) for 90 minutes at 37° C. The mixtures were then extracted with 0.25 ml $CHCl_3$ at 4° C for 1 hour. After this time, 50 μl amounts of the organic phase were spotted on a thin layer plate. The thin layer plates were coated with Silica gel G (E. Merck AG, Darmstadt, Germany) of 0.25 mm thickness. The plates were activated by heating at 100° C for 30 minutes and were stored in a desiccator until use. The solvent system was chloroform: methanol: water, 75:25:4, and the location reagent was iodine vapor.

Molecular Weight Determination

The molecular weight of alpha toxin was estimated from its elution volume from a Sephadex G-75 column. Five ml of alpha toxin (about 60,000 HU) in 0.15M sodium acetate were applied to a 350 ml (V_t) Sephadex G-75 column and run as described in an earlier section. The elution volume of alpha toxin was 95 ml (V_e), while the void volume was 52 ml (V_o). The partion coefficient, K_{av}, was calculated as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

A K_{av} value of 0.144 was obtained for strain #9 alpha toxin, which corresponds with a molecular weight of about 43,000.

Preparation of C^{14}-Labeled Membranes and Whole Cells of Streptococcus faecium HF8AG

The organism used for the preparation of C^{14}-labeled whole cells and membranes was Streptococcus faecium HF8AG, from the University of New Hampshire Microbiology Department culture collection. This organism was innoculated from a nutrient agar stab culture into the defined broth medium shown in Table 1, to which 450 µc of hydrolyzed yeast cells and 50 µc glucose C^{14} (International Chemical and Nuclear Corporation, City of Industry, California) had been added. The culture was incubated 24 hours at 37° C. Cells were harvested by centrifugation and washed once in phosphate buffer. An amount of cells was frozen at -20° C; these were the C^{14}-labeled whole cells. The remaining cells were suspended in 1/10 the culture volume of 0.5M sucrose, phosphate buffer, to which was added 200 µg lysozyme/ml (B grade; Calbiochem, Los Angeles, California). The suspension was held 90 minutes at 37° C.

Formation of protoplasts was monitored at intervals during this time by three methods: loss of the gram + reaction; phase microscopy; and development of osmotic

TABLE I

DEFINED MEDIUM FOR STREPTOCOCCUS FAECIUM

ADENINE SO4	20 mg	CA PANTOTHENATE	800 ug
GUANINE HCl	20 mg	$MgSO_4$	0.4 g
URACIL	20 mg	NaCl	20 mg
XANTHINE	20 mg	$FeSO_4$	20 mg
THIAMINE HCl	2.0 mg	$MnSO_4$	20 mg
PYRIDOXINE HCl	4.0 mg	Tween 80	10 µl
RIVOFLAVINE	2.0 mg	CASAMINO ACID	0.09%
NICOTINIC ACID	800 µg	L CYSTEINE	0.56 g
FOLIC ACID	20 µg	L TRYPTOPHAN	0.14 g
BIOTIN	20 µg	GLUCOSE	0.6%
		DISTILLED WATER	1000 ml

fragility. In the first method, gram stains were made of samples of the suspension and examined microscopically; a gram-reaction was considered evidence of protoplast formation. Samples placed under the phase microscope were considered converted to protoplasts when the diplococci changed from a grey appearance to a dark, swollen appearance. In the third method, 0.5 ml portions of the cell suspension were mixed with 5 ml of distilled water, and the optical density was checked in a Klett-Summerson Photoelectric Colorimeter with a band pass of 485-550 mμ. When a sample failed to show a lower optical density than the previous sample, protoplast conversion was considered complete.

Protoplasts were washed once in 0.5M sucrose, phosphate buffer and resuspended in 0.05% NaCl. C^{14}-labeled membranes were recovered from the lysed protoplasts by centrifugation at 25,000 x g and were subsequently stored at -20° C.

Effect of Strain #9 Alpha Toxin on C^{14}-Labeled Whole Cells and Membranes

Incubations of alpha toxin with C^{14}-labeled whole cells and membranes of Streptococcus faecium were carried out in 1 x 10 cm heavy wall glass centrifuge tubes. C^{14}-labeled membranes and whole cells were diluted in 0.5M phosphate buffer to give the desired number of counts per minute (cpm). To 0.5 ml amounts of C^{14}-labeled membranes (4 cpm) in 0.5M phosphate buffer, pH 7.2, were added 0.5 ml alpha toxin solution in 0.5M phosphate buffer, pH 7.2,

containing 800 hemolytic units (specific activity = 6.5 HU/μg) or 0.5 ml boiled alpha toxin. In the same manner, 0.5 ml amounts of C^{14}-labeled whole cells (5 cpm) were added to 0.5 ml alpha toxin solution or boiled alpha toxin. Tubes containing 0.5 ml C^{14}-labeled membranes or 0.5 ml C^{14}-labeled whole cells plus 0.5 ml phosphate buffer were included as controls. The suspensions were incubated for 30, 60, or 90 minutes at 37° C with occasional shaking to insure uniform suspension of membrane fragments and whole cells. After these time periods, 0.2 ml of glacial acetic acid was added to each tube.

The tubes were centrifuged at 12,000 x g, and the 1 ml supernatants were plated on 5 cm planchets and dried on a hot plate. THe planchets were placed in a gas flow, thin window Geiger apparatus, and the radioactivity of the samples was measured with a scaler-timer (model 135; Baird Atomic, Inc., Cambridge, Massachusetts). The background radioactivity was 33 cpm.

Descending paper chromatography was performed as follows: the dried contents of each planchet was extracted with 0.1 ml of 10% isopropanol in distilled water. 50μl amounts of each extract were spotted on a 17 x 14 inch piece of filter paper (#2043A; Carl Schleicher & Schuell Company, Keene, New Hampshire). The chromatogram was run in an air-tight chromatography cabinet (Research Specialties Company, Berkeley, California) for 36 hours. The solvent system was

n-butanol: glacial acetic acid: water, 120:30:50. After drying the paper chromatogram was placed against an X-ray film (Cronex; E. L. DuPont de nemours and Company, Wilmington, Delaware) and placed in a dark drawer under a weight so as to insure complete contact. After six months, the film was developed as follows: 5 minutes in D-19 developer (Eastman Kodak Company, Rochester, New York), washed 2 minutes in 3% acetic acid, and fixed 10 minutes in Kodak fixer, followed by washing in running water for 30 minutes.

The paper chromatogram was then cut into strips: one containing the 30 minute samples; one the 60 minutes samples; and one the 90 minute samples. One strip was sprayed with 0.2% ninhydrin in acetone for amino acid location; another with ammoniacal silver nitrate reagent for carbohydrate location; and the third with rhodamine B reagent for phospholipid.

Preparation of Bacterial Protoplasts and Spheroplasts

Bacterial protoplasts and spheroplasts were prepared in Dr. Bernheimer's laboratory (New York University Medical School, New York, New York).

Spheroplasts of Eschericia coli W were prepared as described by Bernheimer and Schwartz (9). One volume of an 18 hour culture of Escherichia coli W was added to 10 volumes of Neopeptone meat infusion broth and incubated on a rotary shaker at 37° C. When the optical density (500 mμ) reached 1.5, 50 ml of the culture were centrifuged, the cells washed in 50 ml 0.01M Tris buffer, pH 8.0, and suspended in 0.6M

sucrose, Tris buffer, pH 8.0. 0.5 mg of lysozyme (Armour Pharmaceutical Co., Kankakee, Illinois) and 1 ml of ethylenediaminetetraacetate (EDTA) at pH 8.0 were added to the suspension, which was incubated 25 minutes at 37° C. At this time most of the cells were transformed to spheroplasts as evidenced by their transformation from rod shaped cells into swollen round bodies when observed by phase microscopy. The spheroplasts were washed in 0.6M sucrose, 0.03M Tris buffer, pH 8.0 and suspended in the same solution containing 0.01M $MgCl_2$, to give an optical density of 0.6.

Protoplasts of Streptococcus pyogenes C203S were prepared by the method of Bernheimer (3) by exposing the cocci to phage associated lysin. One volume of Neopeptone meat-infusion broth, incubated 5 hours at 37° C, 20 ml of the culture were centrifuged, and the cells were washed twice with 0.05M phosphate buffer, pH 7.0 and 0.06M KCl. The cells were suspended in 1 ml of 0.05M phosphate, pH 7.0, 1.2M NaCl, 0.01M $MgCl_2$, 0.01, cysteine and 2 mg of phage-associated lysin. The suspension was incubated 20 minutes at room temperature followed by centrifugation and resuspension of the protoplasts in the same solution, minus the phage enzyme and cysteine, to an optical density of 0.6.

Sarcina lutea (from the culture collection of the Bacteriology Department, New York University School of Medicine) protoplasts were also prepared by the methods of Bernheimer and Schwartz (9). The organisms were grown at

30° C in a shaker incubator in 1% peptone (Difco), 0.1% yeast extract (Difco), and 0.5% NaCl, pH 7.5. 40 ml of the culture were washed in one volume of 0.03M phosphate buffer, pH 7.2 and suspended in 0.125 volume of 1.5M sucrose, 0.03M phosphate buffer, pH 7.2, to which was added 100 µg/ml of lysozyme. After 15 minutes at room temperature, the suspension was diluted in sufficient 1.5M sucrose in 0.03M phosphate buffer, pH 7.2, containing 0.01M $MgCl_2$, to give an optical density of 0.6 to 0.7. Protoplast formation was confirmed by the methods described in the section on formation of C^{14}-labeled membranes.

Lysis of Bacterial Protoplasts and Spheroplasts by Strain #9 and Wood 46 Alpha Toxin

Dr. Bernheimer's strain Wood 46 alpha toxin and strain #9 alpha toxin were directly compared for their ability to lyse bacterial protoplasts and spheroplasts in Dr. Bernheimer's laboratory. Both preparations of alpha toxin were titrated against rabbit erythrocytes by the same method simultaneously. Dr. Bernheimer's titration method differed from that described above in that 50% lysis of the rabbit erythrocytes was taken as an end point of the titration, rather than 100% lysis. To 700 µl of spheroplast or protoplast suspension contained in a pre-warmed cuvette were added 2400 hemolytic units of strain #9 alpha toxin (specific activity = 8 HU/µg) or 870 hemolytic units of Wood 46 alpha toxin contained in 60 µl of 0.03M borate buffer, pH 8.3. The optical density at 500 mµ was continuously recorded for 30

minutes in a double beam spectrophtometer equipped with a curvette holder through which circulating water maintained the temperature of the cuvette at 35° C. A second cuvette containing protoplasts or spheroplasts plus 100 µl 0.03M borate buffer was run simultaneously with the test cuvette. A decrease in optical density of the test cuvette was considered evidence of lysis. Phase microscopy was used to substantiate the apparent lysis.

Influence of Lipids on the Lysis of Sarcina lutea Protoplasts by Strain #9 Alpha Toxin

Protoplasts of Sarcina lutea were prepared as described above. Into 5 x 0.5 cm test tubes was placed 0.5 mg ganglioside IIR70A1, or 1 mg of sulfatide (kindly supplied by Dr. S. Abramson, Albert Einstein College of Medicine), lecithin (Calbiochem), or sphingomyelin (Calbiochem), and the solvents were removed "in vacuo". To each tube was added 8000 hemolytic units of strain #9 alpha toxin (specific activity = 6.5 HU/µg) in 0.1 ml 0.01M phosphate buffer, pH 7.0, and the tubes were well shaken and incubated 30 minutes at room temperature (about 25° C). The contents of each tube was then tested for its ability to lyse protoplasts of Sarcina lutea. To 0.9 ml protoplast suspension contained in a Bausch and Lomb Microcell Cuvette (Bausch & Lomb, Rochester, New York) was added the contents of an incubation tube. The cuvettes were each in turn placed in

a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, California) equipped with a cuvette holder through which circulating water maintained the temperature at 35° C. The optical density at 500 mμ was recorded every five minutes for at least 30 minutes. A control protoplast suspension to which was added 0.1 ml 0.01M phosphate buffer, pH 7.0, and a suspension to which was added 8000 hemolytic units of alpha toxin were included in the experiment.

Influence of Lipids on Rabbit Erythrocyte Lysis By Strain #9 Alpha Toxin

To 10 x 1 cm test tubes were added 0.92 mg ganglioside, 1 mg lecithin, or 1 mg sphingomyelin in duplicate, and the solvent removed "in vacuo". To one of each pair of tubes was added 3 μg of strain #9 alpha toxin (about 20 hemolytic units) in 0.5 ml 0.01M phosphate buffer, pH 7.0, and to the other was added 0.5 ml of the same buffer. The tubes were shaken well and incubated 30 minutes at room temperature. To each tube was added 0.5 ml of 1% rabbit erythrocytes in 0.85% NaCl. The cells were incubated at 37° C for 60 minutes and lysis was recorded every five minutes as follows: ± = partial lysis; + = complete lysis; and - = no apparent lysis.

In another experiment 90, 180, 460, 740, and 920 μg amounts of ganglioside were placed in test tubes in duplicate and the effect of these various amounts of ganglioside on rabbit erythrocytes was determined as described in the previous paragraph.

The possibility of binding of ganglioside to rabbit erythrocytes was tested by incubating 50 µg amounts of C14-labeled ganglioside (kindly supplied by Dr. S. Suzuki, Albert Einstein College of Medicine) at 37° C with 0.5 ml samples of 1% rabbit erythrocytes in 0.85% NaCl for 20 minutes. After incubation, the suspensions were centrifuged at 500 x g for 15 minutes at room temperature. The cell buttons were resuspended in 0.1 ml 0.85% NaCl, and the cells and supernatants were deposited separately on 5 cm planchets and dried on a hot plate. Radioactivity of the samples was measured as described in a previous section.

Monolayer Studies

Monolayer studies were performed in the laboratory of Dr. G. Colacicco, Biochemistry Department, Albert Einstein College of Medicine, Bronx, New York.

Proteins

The lipid-free protein subunit of rat plasma high density lipoprotein (HDL) was prepared by G. Camejo (10). The lipid-free protein has been described as being soluble in aqueous solutions of neutral salts and homogeneous when subjected to gel electrophoresis and boundry sedimentation. Streptolysin S was kindly supplied by R. Rowen, Albert Einstein College of Medicine, Bronx, New York. RNA (CORE; Worthington Biochemical Corporation, Freehold, New Jersey), used in purifying the streptolysin S, was a possible contaminant in streptolysin S and was therefore included in the monolayer experiments as a control. Crystalline beef

ribonuclease was obtained from Worthington Biochemical Corporation. Several preparations of alpha toxin, prepared as described above, were used in this study. Protein solutions were stored at 2° C in pyrex glass tubes at a concentration of 5 mg/ml in 0.04M phosphate buffer, 0.1M NaCl pH 7.0 ± 0.05.

Lipids

Cholesterol (Sylvania Chemical Company, Millburn, New Jersey) was recrystallized several times. Phosphatidylinositol was supplied by Dr. G. Colacicco. Egg lecithin, sphinogomyelin, and ganglioside IIR70A1 were supplied by Dr. M. Rapport (Albert Einstein College of Medicine). Sulfatide was supplied by Dr. S. Abramson (Albert Einstein College of Medicine). All the lipids were homogeneous on thin layer chromatography. Lipids were stored at a concentration of about 0.5 mg/ml in chloroform-methanol, 85:15, for no longer than one week in glass tubes.

Water and Buffers

Protein solutions and buffers were prepared with distilled water, redistilled over alkaline permanganate in an all-glass still (Corning Glass Works, Corning, New York). The conductivity of the water was 1 micromho cm-1. Buffers used in the effect of pH on protein-lipid interaction were 0.05M Tris, pH 6.96, 8.02, 8.42, and 9.06. Unless otherwise stated, all other experiments were run with 0.04M phosphate, 0.1M NaCl, pH 7.0 ± 0.05. Buffer solutions were stored in polyethylene bottles for not longer than one week.

Apparatus and Procedures

Lipids and proteins were brought into contact by a modification of the monolayer technique of Doty and Schulman (18). Protein was injected into the aqueous subphase under a monolayer film of lipid, spread over a circular, fixed area trough. The trough (13) consisted of a pyrex crystallizing dish, 6 x 3.5 cm, with a glass barrier 4 cm long, 2 mm wide, and 1.5 cm high annealed across it. The upper edge of the barrier was 3 mm below the edge of the dish. The upper half of the dish and the barrier were made hydrophobic with a coat of paraffin (m.p. 52° C; Fisher Scientific Company) made by dipping the open end of the dish into molten paraffin.

The dish was completely filled with buffer (60 ml; 25 $\pm$ 1° C), and the liquid surface was cleaning by blowing a gentle stream of nitrogen gas onto it from one side and collecting the impurities from the other side with a disposable pipette attached by a heavy wall tubing to a vacuum line. The final volume was 50 ml. The two surfaces formed were separated by the barrier with a common aqueous chamber below. The larger surface (18 cm^2) was used to spread the lipid monolayer. The smaller surface was used to inject the protein into the common subphase without disturbing the monolayer film.

Films were made by applying lipid solution to the aqueous surface carefully with a 10 µl Hamilton syringe

(Fisher). Mixing of the aqueous subphase with Teflon-coated magnetic stirrer (Tri-R Instruments, Rockville Centre, New York) was started after one minute, allowing time for the lipid solvent to evaporate. The stirring caused no change in surface tension. Protein solution was injected into the aqueous subphase with a Hamilton microsyringe on the other side of the barrier. Unless otherwise stated, the protein concentration in the subphase was 2 µg/ml.

Surface tension was measured by means of a thin sand-blasted platinum plate (2.5 cm long) connected by a thread to the arm of a torsion balance (Federal Pacific Company, Newark, New Jersey). Changes in suface tension of 0.2 dynes/cm could be measured reproducibly.

RESULTS

Preparation and Purification of Staphylococcal Alpha Toxin

The yield and specific activity of alpha toxin from each step in the purification procedure are shown in Table 2. The incorporation of 1 unit of penicillin G per 100 ml of dialyzed culture medium consistently resulted in the production of 3200 hemolytic units of alpha toxin per ml of culture supernatant. Dialysis of the culture supernatant did not cause any loss in alpha hemolytic activity. The average yield from the 25% methanol precipitation at pH 4 was 30%.

Table 3 shows the effect of varying the percent methanol and the yields from subsequent washings, according to Coulter's (15) methods. All of the alpha hemolytic activity placed on the Sephadex G75 column could be recovered; however, only about one half of the alpha toxin from the column was kept in order to prevent contamination from neighboring protein peaks. The elution profile from the column shown in Figure 1 consists of two major peaks absorbing at 280 mμ. The second peak contained the alpha hemolytic activity. The failure of the total column volume to return to 100% 280 mμ was due to slow elution of an unknown brown substance from the column.

Treatment of alpha toxin from the Sephadex column with 50% saturated $(NH_4)_2SO_4$ eliminated an immunodiffusion precipitin line obtained when the untreated alpha toxin preparation was reacted against heterologous staphylococcal bovine antisera (Plate 1). The supernatant from the 50% saturated $(NH_4)_2SO_4$ treatment was stored in 80% saturated $(NH_4)_2SO_4$ in

TABLE II

STRAIN #9 ALPHA TOXIN: YIELDS

FROM PURIFICATION PROCEDURE

Step	Protein	Hemolytic Units	Total Hemolytic Units	Yield	Specific* Activity
	mg/ml	/ml		%	
Culture Supernatant	10	3200	3,040,000	100	0.32
Dialysis	10	3200	3.040,000	100	0.32
Methanol ppt	180	25,600	1,024,000	32.5	1.42
Sephadex Column	0.99	6400	512,800	20	6.5
$(NH_4)_2SO_4$	0.6	3200-6400	160,000	5	8.

* hemolytic units/µg

TABLE III

EFFECT OF METHANOL CONCENTRATION

ON RECOVERY OF STRAIN #9

ALPHA TOXIN

Methanol	Hemolytic Units/ml			
%	1st Extract	2nd Extract	3rd Extract	4th Extract
15	1600	800	200	---
20	3200	800	200	---
25	3200	1600	400	200
30	6400	1600	400	200
40	6400	1600	400	200

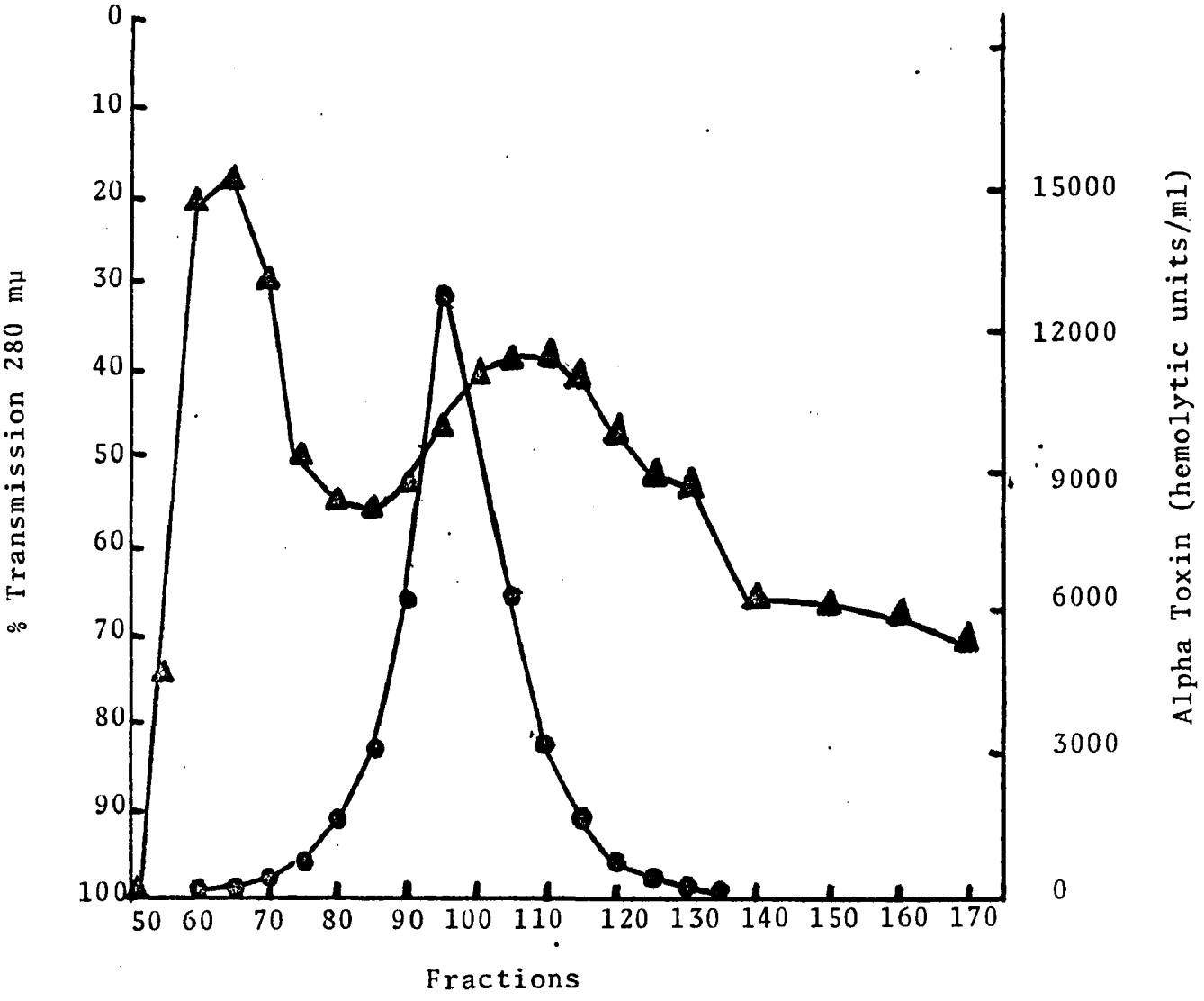


FIGURE 1. Elution of 280 mμ absorbing material and alpha hemolysin from a 350 ml Sephadex G75 column. See Methods and Materials for details.
Symbols: ▲ , % transmission at 280 mμ; ● , hemolytic units/ml alpha toxin.

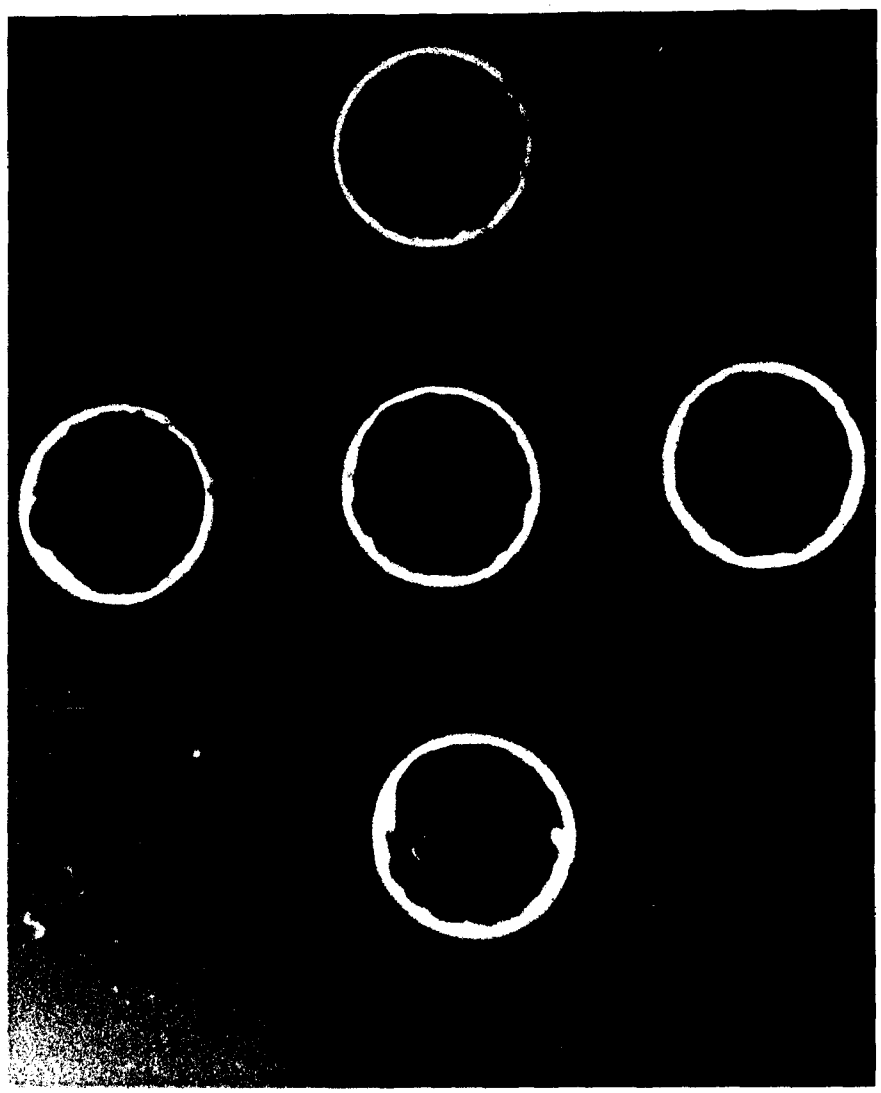


PLATE I. Immunodiffusion of strain #9 alpha toxin from Sephadex G75 column before $(NH_4)_2SO_4$ precipitation in upper and lower wells against bovine heterologous antistaphylococcal serum in center well. Narrow band was eliminated by $(NH_4)_2SO_4$ precipitation.

order to maintain its activity. The storage in $(NH_4)_2SO_4$ resulted in a further loss of activity, but the amount of alpha toxin activity recovered from the 80% saturated $(NH_4)_2SO_4$ remained constant for as long as two years, and had the additional benefit of providing a convenient means of concentrating the alpha toxin preparation. The $(NH_4)_2SO_4$ precipitate could be dissolved in the desired volume of buffer.

The Effect of Penicillin on Alpha Toxin Production

The addition of small amounts of penicillin G to the culture medium resulted in double yields of alpha toxin and beta toxin Smith's strain #9 as shown in Table 4. The effect on nuclease production was ambiguous due to the small amounts of this substance produced by strain 39. Protein content of the culture supernatant was also increased by 30% in the presence of 2.5 units of penillin G. Addition of larger amounts of penicillin to the cultures resulted in decreased culture turbidity and smaller yields of alpha toxin and beta hemolysin. Penicillin was routinely added to culture media in order to increase yields of alpha toxin as a result of these experiments. Table 5 shows the effect of penicillin on alpha toxin production by other strains.

Characterization of Alpha Toxin Preparations

Immunodiffusion of purified staphylococcal alpha toxin against heterologous bovine antisera usually resulted in the formation of one precipitin line. Occasionally immunodiffusion against an individual bovine antiserum resulted in the formation of two or three precipitin lines. Comparison of strain #9 alpha toxin with Dr. Bernheimer's Wood 46 strain alpha toxin

TABLE IV

EFFECT OF PENICILLIN G ON ALPHA TOXIN PRODUCTION

BY STAPHYLOCOCCUS AUREUS

Units of Penicillin G/ 500 ml Culture Medium	Alpha Toxin Titer	Beta Hemolysin Titer	Protein mg/ml
---	1:1600	1:200	7
5	1:3200	1:200	10
10	1:3200	1:400	10
15	1:800	---*	5

*none detected

TABLE V

PENICILLIN G INFLUENCE ON ALPHA TOXIN PRODUCTION BY THREE STRAINS OF STAPHYLOCOCCUS AUREUS

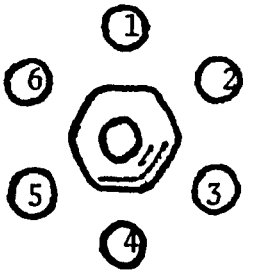
Strain	Units of Penicillin G/ 500 ml Culture Medium	Alpha Toxin Titer
Strain 9	0	1:1600
	2.5	1:3200
Wood 46	0	1:800
	2.5	1:400
UNH 15	0	1:400
	2.5	1.800

using the immunodiffusion technique showed that both preparations produced only one precipitin line against Lederle antisera, while both preparations produced two precipitin lines against certain UNH bovine antisera. These results are illustrated in Figures 2a and 2b. Immunodiffusion against beta hemolysin antisera did not result in the formation of a precipitin line.

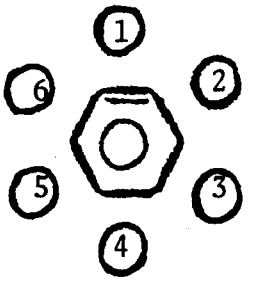
Electrophoresis of purified toxin on cellulose acetate strips in borate buffer, pH 8.6, followed by staining with Amido Schwartz reagent, revealed two bands migrating slowly toward the cathods (Figure 3). Agarose electrophoresis of a purified alpha toxin preparation followed by staining with Amido Schwartz reagent revealed one broad band also migrating slowly toward the cathode at pH 8.6 as in the case of cellulose acetate electrophoresis. Figure 3 shows the results from an agarose electrophoresis experiment. When one cm square pieces were cut from the agarose gel and placed on sheep erythrocyte agar plates, the alpha hemolytic activity was demonstrable as clear zones around the squares containing alpha toxin and corresponded with protein concentration and alpha hemolytic activity eluted from strips cut every cm across the agarose strip. About 10% of the alpha hemolytic activity could be recovered.

The molecular weight of alpha toxin was calculated from its elution volume from a standardized Sephadex G75 column. The molecular weight determined in this manner was about 43,000.

The mouse LD50 was 3.5 µg of alpha toxin injected intraperitoneally. The rabbit minimal dermonecrotic dose was about 0.5 µg. The specific hemolytic activity was between 6.5 and 10 hemolytic units per µg of protein.



A



B

FIGURE 2a: Immunodiffusion of UNH alpha toxin against various heterologous staphyiococcal antisera. Well contents: 1, UNH bovine #1 antiserum; 2, UNH bovine #11 antiserum; 3, UNH bovine #4 antiserum; 4, UNH bovine #13 antiserum; 5, Lederle antiserum lot #83; 6, Lederle antiserum lot # 73. Center well: 5000 hemolytic units UNH alpha toxin.

FIGURE 2b: Immunodiffusion of Dr. Bernheimer's alpha toxin against various heterologous staphlococcal antisera. Center well: 4000 hemolytic units alpha toxin. Wells numbered 1 through 6 are the same as in Figure 2a.

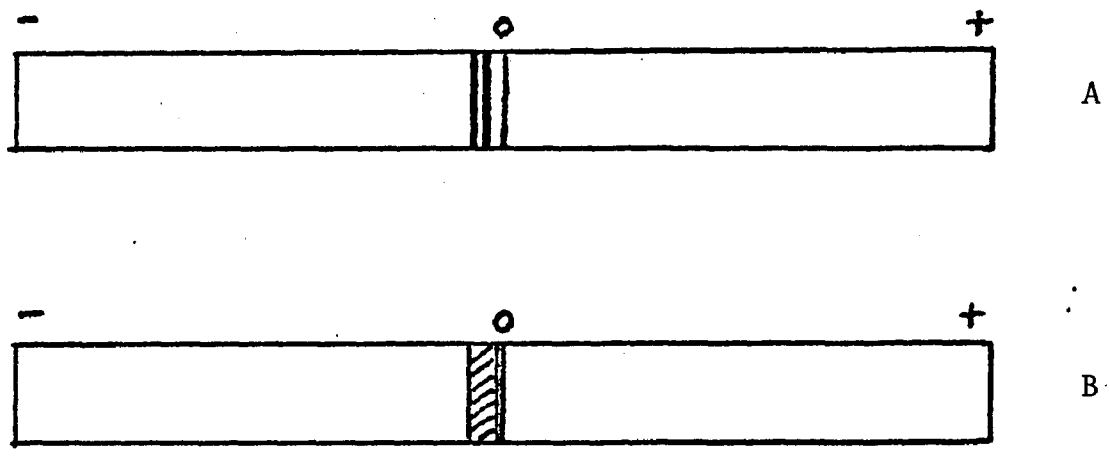


FIGURE 3a: Cellulose acetate electrophoresis of purified alpha toxin. "O" represents the origin.

FIGURE 3b: Agarose gel electrophoresis of purified alpha toxin. "O" represents the origin.

Determination of Lipase Activity in Alpha Toxin Preparations

The apparent activity of alpha toxin on various membrane systems made it important to check the preparations for lipase activity. It was also important to be certain of any lipase activity of alpha toxin preparations before embarking upon monolayer studies. No significant phospholipase A activity was found with the Marinette egg yolk test. Nor could significant phospholipase C or D be demonstrated against lecithin or sphingomyelin. Furthermore, when various phospholipids were allowed to interact with alpha toxin, followed by extraction with C:M, 2:1 and thin layer chromatography of the reaction mixture, no change in the number or size of spots or the Rf values of the lipids could be detected. The lipids included in this experiment were phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine (lecithin), sphingomyelin, and ganglioside.

Polysaccharide Component of Alpha Toxin

Kumar et al. (32) and Goshi et al. (24) reported the presence of a polysaccharide component in alpha toxin, while other workers have failed to attach significance to its presence in alpha toxin preparations. Therefore it was decided to examine alpha toxin by hydrolysis followed by paper chromatography of the hydrolyzate for the presence of polysaccharide.

Three sugars were present in purified alpha toxin. Glucose and galactose were identified by comparison with standards on paper chromatography. The third sugar was not identified. The amount of carbohydrate in purified alpha toxin was estimated by comparing the size of the spots with known amounts of sugars

on paper chromatography. The polysaccharide was about 1% by weight in purified alpha toxin.

Lysis of Bacterial Protoplasts and Spheroplasts

The discovery by Bernheimer and Schwartz (9) that alpha toxin will lyse bacterial protoplasts and spheroplasts prompted studies to ascertain the lytic activity of our preparations on these osmotically sensitive forms. Protoplasts of Sarcina lutea and Streptococcus pyogenes and spheroplasts of Escherichia coli were lysed by preparations of purified alpha toxin from Smith's strain #9. It is evident from examining Figures 4,5, and 6 that protoplasts of Sarcina lutea were the most sensitive to lysis. The peculiar initial rise in optical density of the Sarcina lutea protoplast suspension before lysis by alpha toxin has been explained by Bernheimer, who demonstrated by microscopic observation that protoplast tetrads are separated causing an initial increase in optical density before the drop in optical density due to lysis. Equal numbers of hemolytic units of the UNH preparation of alpha toxin were compared with Bernheimer's preparation of alpha toxin for protoplasts and spheroplast lysis. The UNH preparation was found to have about 2/3 less lytic activity on bacterial protoplasts and spheroplasts. Lysis of Streptococcus faecium protoplasts could not be demonstrated when 0.5 mg of UNH alpha toxin was used.

Effect of Lipids on Bacterial Protoplast Lysis

The effect of ganglioside on the lethal effect of alpha toxin and the effect of ganglioside and sulfatide in the monolayer system (see below) led to experiments designed to determine

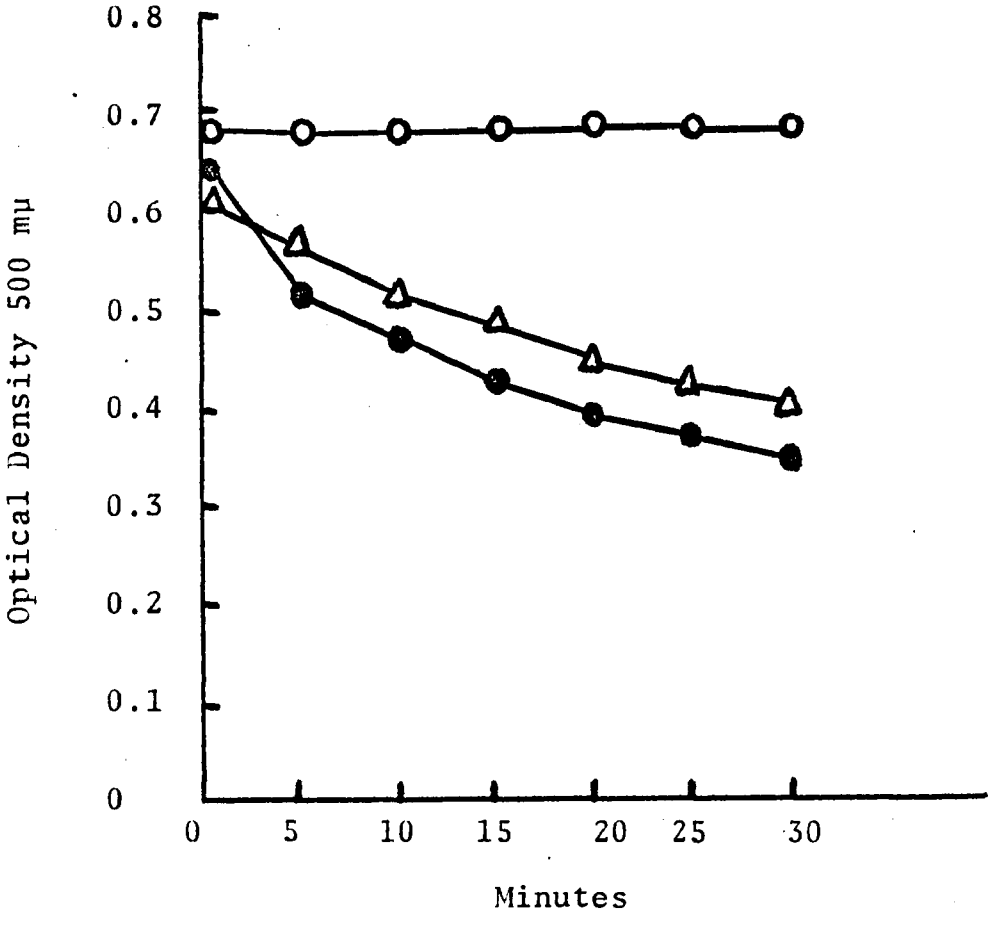


FIGURE 4. Comparison of the lysis of Escherichia coli spheroplasts by UNH alpha toxin and Dr. Bernheimer's alpha toxin. Symbols: ○ , control, spheroplasts plus .03M borate pH 8.3; △ , 2400 hemolytic units of UNH alpha toxin; ● , 870 hemolytic units of Dr. Bernheimer's alpha toxin.

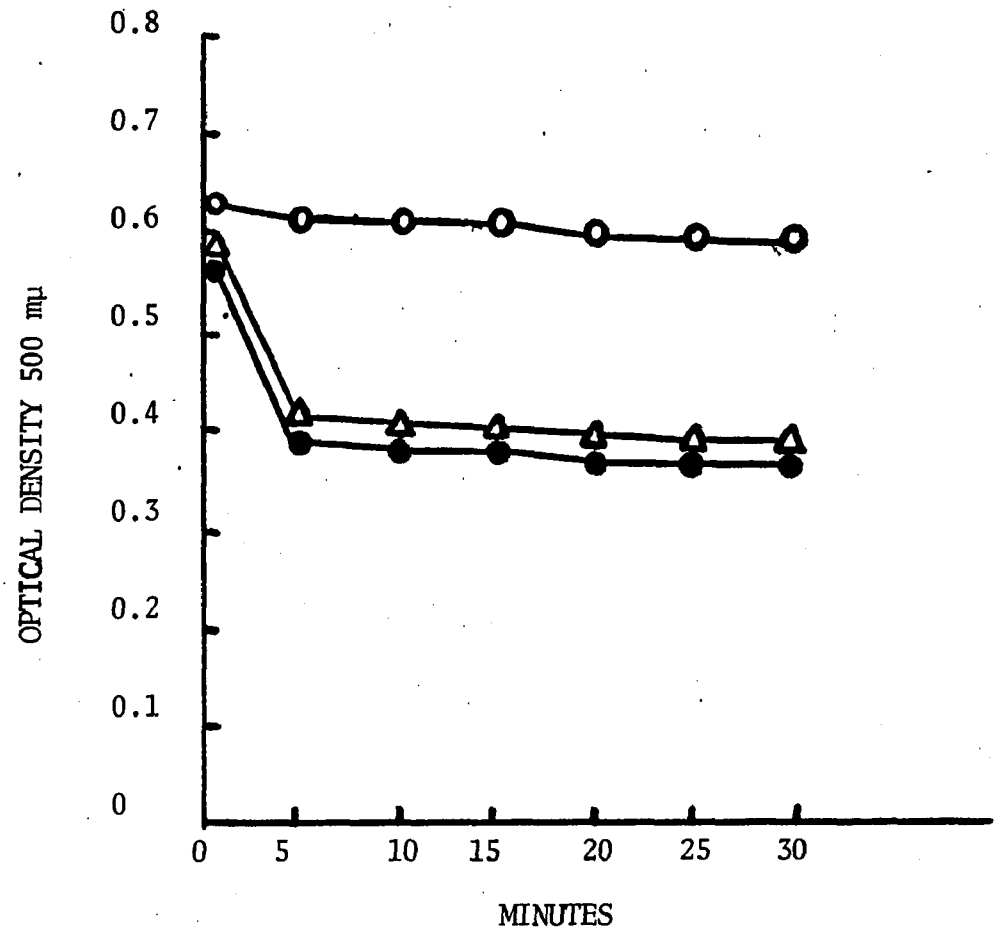


FIGURE 5. Comparison of the lysis of Streptococcus pyogenes protoplasts by UNH alpha toxin and Dr. Bernheimer's alpha toxin. Symbols: O, control, protoplasts plus .03M borate pH 8.3; Δ, 2400 hemolytic units of UNH alpha toxin; ●, 870 hemolytic units of Dr. Bernheimer's alpha toxin.

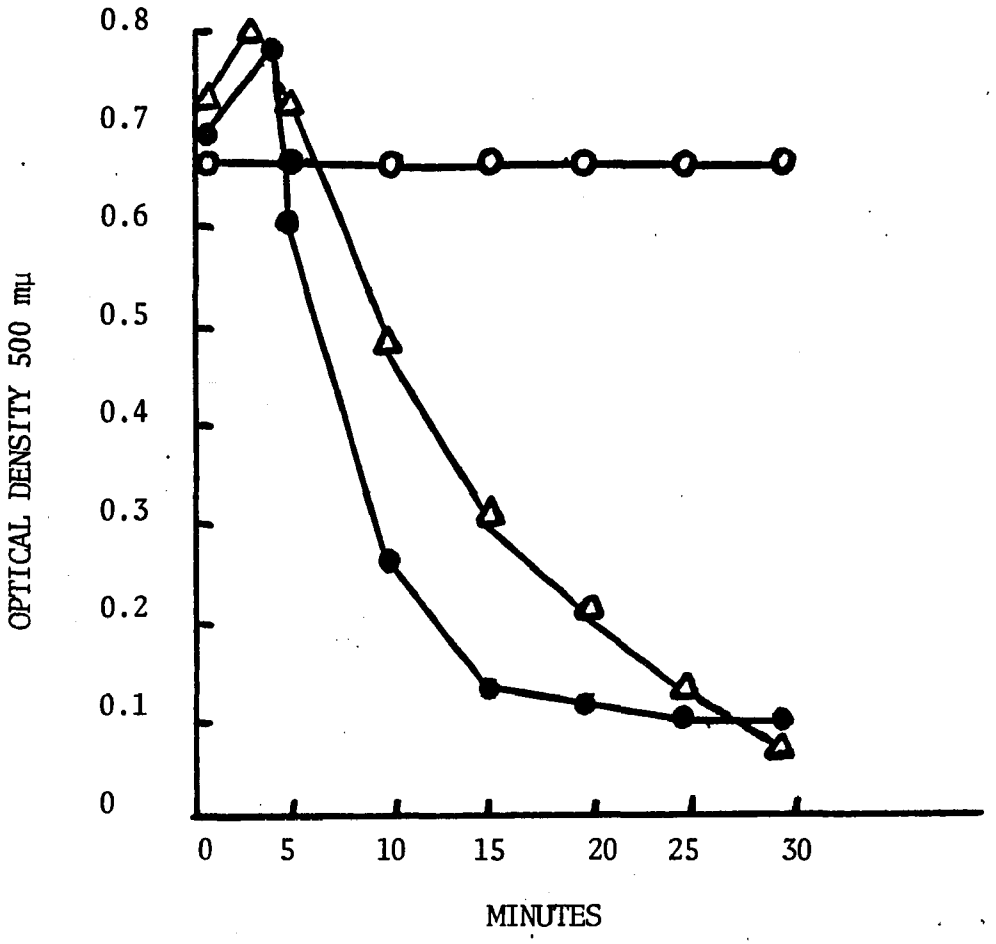


FIGURE 6: Comparison of the lysis of Sarcina lutea protoplasts by UNH alpha toxin and Dr. Bernheimer's alpha toxin. Symbols: ○ , control, protoplasts plus .03M borate pH 8.3; △ , 2400 hemolytic units of UNH alpha toxin; ● , 870 hemolytic units of Dr. Bernheimer's alpha toxin.

the effect of lipids on the protoplast lytic activity of alpha toxin. When alpha toxin was incubated for one hour at 22° C with one half its weight of ganglioside, the lytic effect for Sarcina lutea was completely inhibited as shown in Figure 7. The molar ratio of ganglioside to alpha toxin necessary to achieve inhibition of protoplast lysis was about 16:1. Lecithin, sphinogomyelin, and sulfatide had no effect on the lytic phenonenon when similarly incubated with alpha toxin 1:1 by weight before incubation with the Sarcina lutea protoplasts.

Effect of Lipids on Rabbit Erythrocyte Lysis

Experiments on the effects of lipids on rabbit erythrocyte lysis by alpha toxin were performed to see if ganglioside would inhibit erythrocyte lysis as it inhibited protoplast lysis. One ml suspensions of 1% rabbit erythrocytes were incubated at 37° C with 3 µg of alpha toxin in the presence of increasing amounts of ganglioside. Partial inhibition of erythrocyte lysis occurred in the presence of ganglioside at the 0.5 mg level as shown in Table 6. No lysis occurred after one hour in the presence of ganglioside above the 0.75 mg level. Incubation in the presence of mg amounts of sphingomyelin or lecithin had no effect on rabbit erythrocyte lysis by alpha toxin. This data is presented in Table 7. The molar ratio of ganglioside to alpha toxin necessary to inhibit rabbit erythrocyte lysis was about 5000:1.

In order to determine if ganglioside was exerting its effect by adsorbing to the rabbit erythrocytes, C^{14}-labeled ganglioside was incubated with erythrocytes, followed by

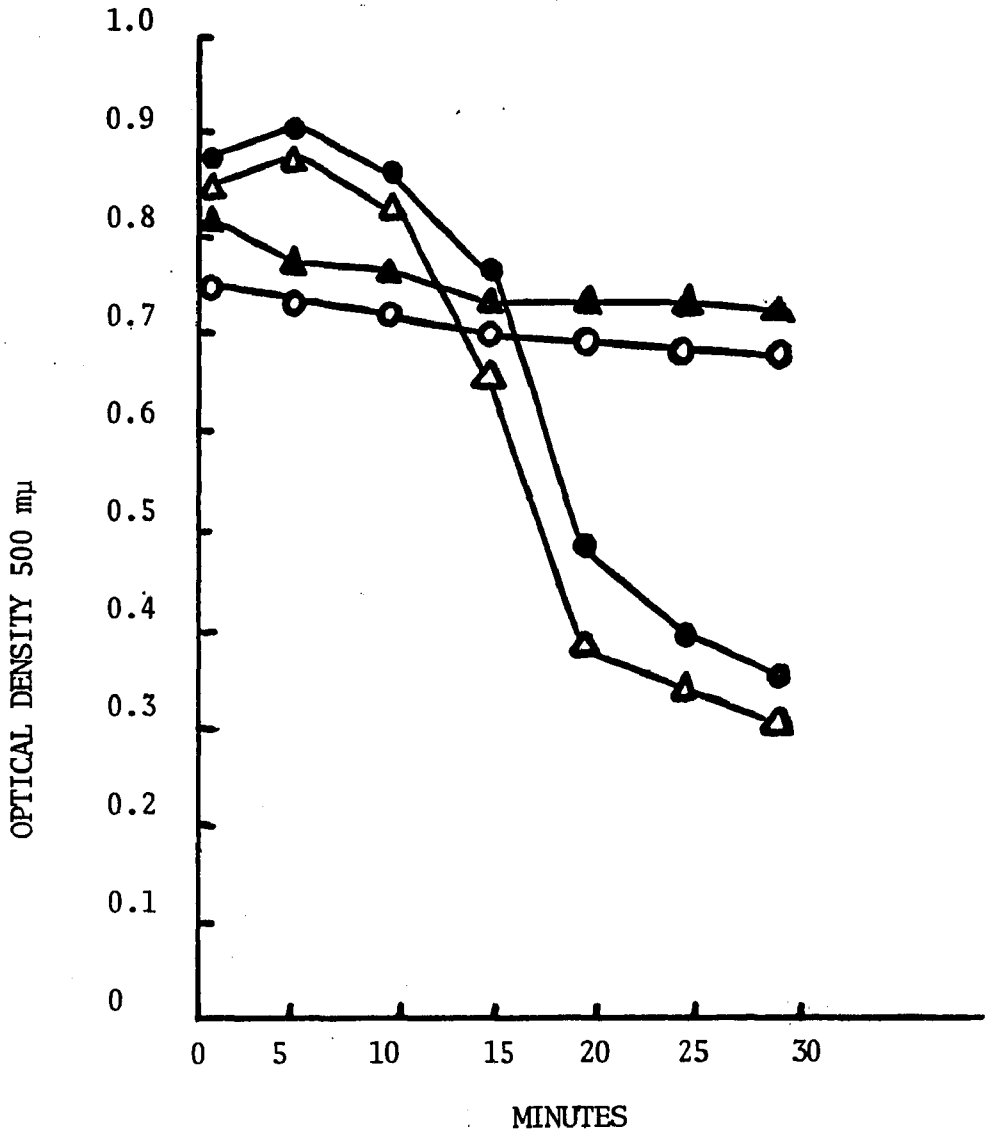


FIGURE 7: Influence of lipids on the lysis of Sarcina lutea protoplasts by alpha toxin. Symbols: ○, control protoplasts plus .01M PO_4 buffer pH 7.0; △, 8000 hemolytic units alpha toxin; ▲, alpha toxin and ganglioside 1:1/2 by weight; ●, alpha toxin and sulfatide 1:1 by weight.

TABLE VI

INHIBITION OF ALPHA TOXIN

RABBIT ERYTHROCYTE LYSIS

BY GANGLIOSIDE

µg Ganglioside	µg Alpha Toxin	ml 1% Rabbit Erythrocytes	Minutes 5	10	15	30	45	60
90	3	0.5	+	+	+	+	+	+ *
	0		-	-	-	-	-	-
180	3	0.5	+	+	+	+	+	+
	0		-	-	-	-	-	-
460	3	0.5	+	-	-	±	±	+
	0		-	-	-	-	-	-
740	3	0.5	-	-	-	-	-	±
	0		-	-	-	-	-	-
920	3	0.5	-	-	-	-	-	-
	0		-	-	-	-	±	+

*
+ = complete lysis
- = no lysis
± = partial lysis

TABLE VII

EFFECT OF LIPIDS ON ALPHA TOXIN

RABBIT ERYTHROCYTE LYSIS

µg Alpha Toxin	ml 1% Rabbit Erythrocyte	mg Ganglioside	mg Lecithin	mg Sphingomyelin	Minutes 5	10	15	35	45	60	
3	0.5	0.92	---	---	-	-	-	-	-	-	*
0					-	-	-	-	-	-	
3	0.5	---	1.0	---	±	+	+	+	+	+	
0					-	-	-	-	-	±	
3	0.5	---	---	1.0	+	+	+	+	+	+	
0					-	-	-	-	-	-	
3	0.5	---	---	---	+	+	+	+	+	+	
0					-	-	-	-	-	-	

* + = complete lysis
- = no lysis
± = partial lysis

centrifugation and measurement of the radioactivity in the cell button and supernatant. All of the radioactivity was recovered from the supernatant. No radioactivity was found associated with the erythrocytes, while 146 and 151 counts per minute were found in the supernatant in two trials.

Effect of Alpha Toxin on Streptococcus faecium C^{14}-Labeled Whole Cells and Membranes

An attempt was made at identifying the numbers of components released from bacterial membranes by interacting alpha toxin with C^{14}-labeled Streptococcus faecium followed by paper chromatography of the reaction mixture and autoradiography of the chromatogram. It can be concluded from Table 8 that no significant radioactivity was released from C^{14}-labeled whole cells of Streptococcus faecium while about 10% of the total membrane activity was released after one hour incubation with 400 hemolytic units of alpha toxin at 37° C (specific activity = 6.5 H.U./µg). Paper chromatography of the supernatant from centrifuged reaction mixtures showed one spot reacting with silver nitrate reagent and two spots reacting with ninhydrin reagent. Autoradiography of the paper chromatogram, Plate 2, showed several spots not all of which corresponded with the spots on the paper chromatogram. Examination of Plate 2 reveals that the same spots released by interaction with alpha toxin are present in trace amounts in the supernatants from unreacted membranes.

Surface Activity of Alpha Toxin

Some definitions must be made in order to present the data in this section with clarity. Surface tension is related to

TABLE VIII

EFFECT OF ALPHA TOXIN ON C^{14}-LABELED STREPTOCOCCUS FAECIUM WHOLE CELLS AND MEMBRANES

Alpha Toxin	Boiled Alpha Toxin	C^{14} Membranes	C^{14} Whole Cells	Counts/min released		
				15	30	60
800 HU*	---	10^4 cpm	---	932	917	1051
800 HU	---	---	10^5 cpm	97	81	101
---	1 mg	10^4 cpm	---	148	77	195
---	1 mg	---	10^5 cpm	75	44	55
---	---	10^4 cpm	---	112	122	---
---	---	---	10^5 cpm	74	92	---

* HU = hemolytic units

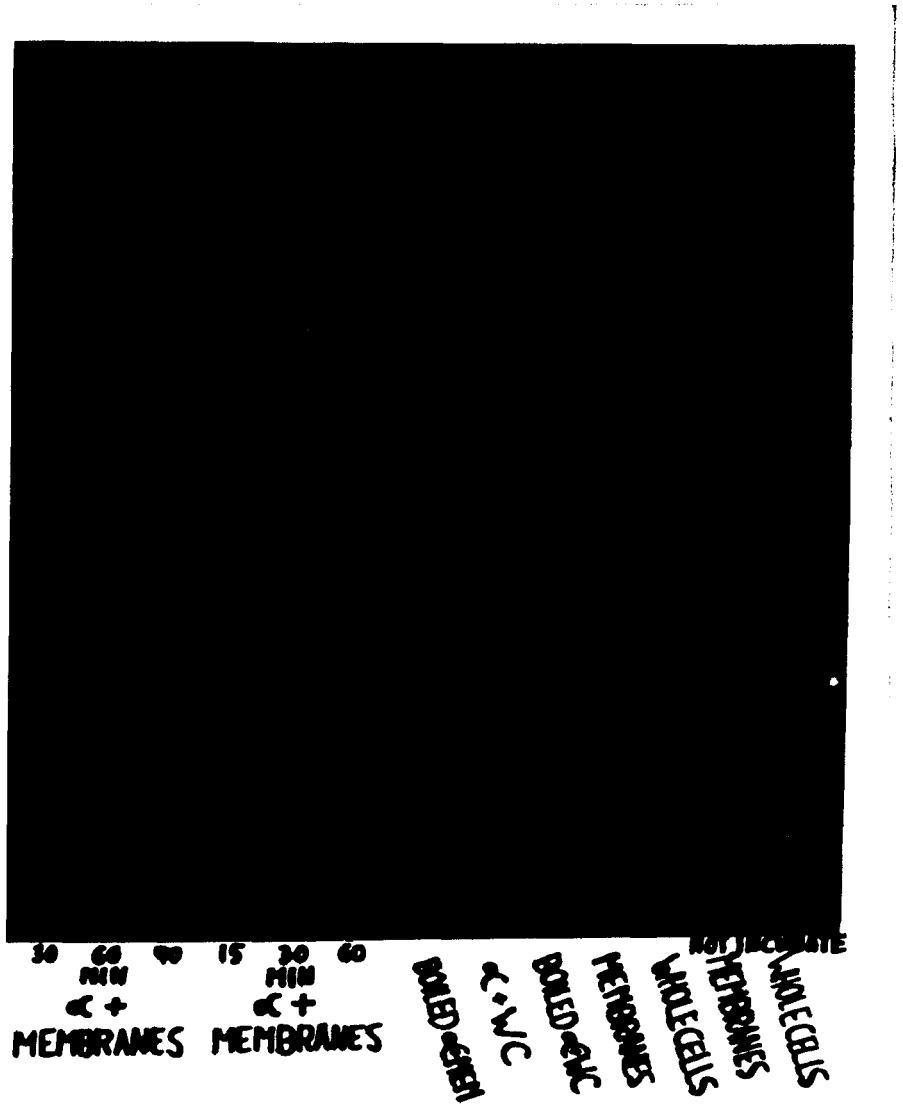


PLATE II. Autoradiography of a paper chromatography upon which was spotted C^{14}-labeled Streptococcus faecium membrane or whole cell-alpha toxin reaction supernatant.

pressure as follows:

$$\Delta\Pi = (\gamma_o - \gamma_t)$$

where $\Delta\Pi$ is the change in surface pressure, γ_o is the surface tension of water, and γ_t is the surface tension at experimental time, t. The initial surface pressure, Π_i, is the loss of surface tension due to the presence of a film. The increase in surface pressure, $\Delta\Pi$, is the further loss of surface tension caused by an interaction of a film with a substance from the solution below the air-water interface or surface (from the subphase).

Alpha toxin had the ability to collect at the air-water interface, forming a film, from the aqueous subphase. Figure 8 presents data showing that ribonuclease, albumin, and streptolysin S produced no film from the subphase when compared with alpha toxin, while the HDL-protein collected at the surface more rapidly. The effect of alpha toxin concentration in the subphase on its ability to form a film from the subphase is presented in Figure 9. The comparison of different experiments is shown in terms of $\Delta\Pi$ at the time interval indicated. The kinetic curves did not intersect each other, and the differences at 10 or 20 minutes were generally the same as at 40 minutes, at which time the film formation was complete or nearly so. Figure 9 shows that a maximum film pressure is approached with an alpha toxin concentration of 7.5 µg/ml in the subphase.

A film of alpha toxin could also be formed by carefully placing alpha toxin solution drop by drop on the

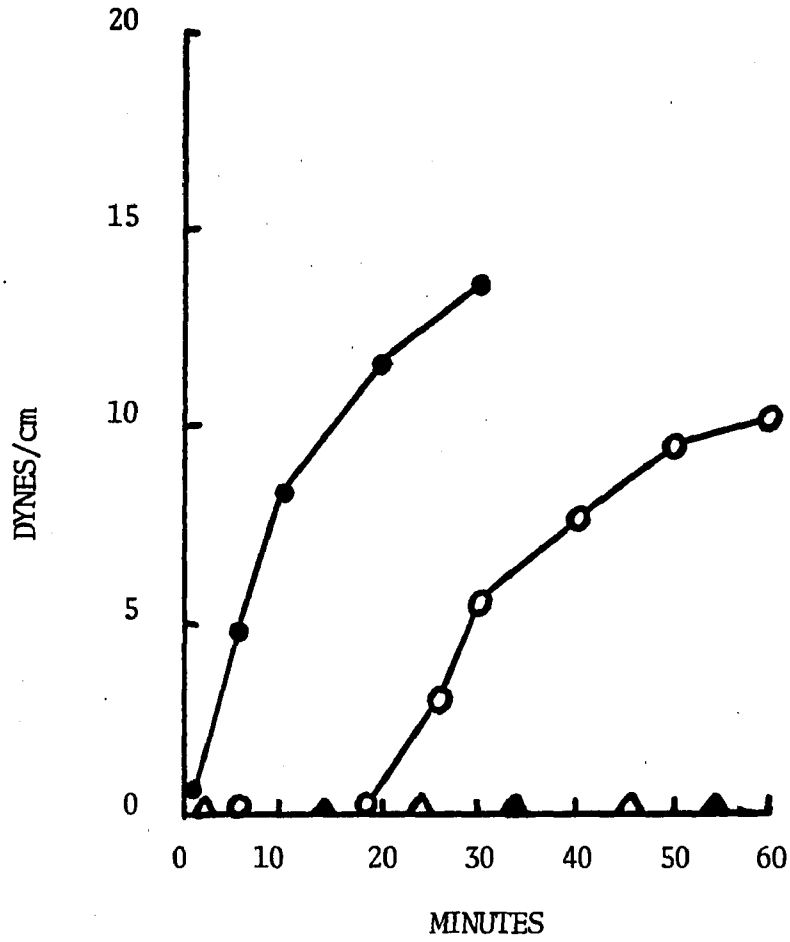


FIGURE 8: Ability of various proteins to form films from the subphase in the monolayer system. Symbols: ●, HDL-protein; ○, alpha toxin; △, ribonuclease; ▲, streptolysin S.

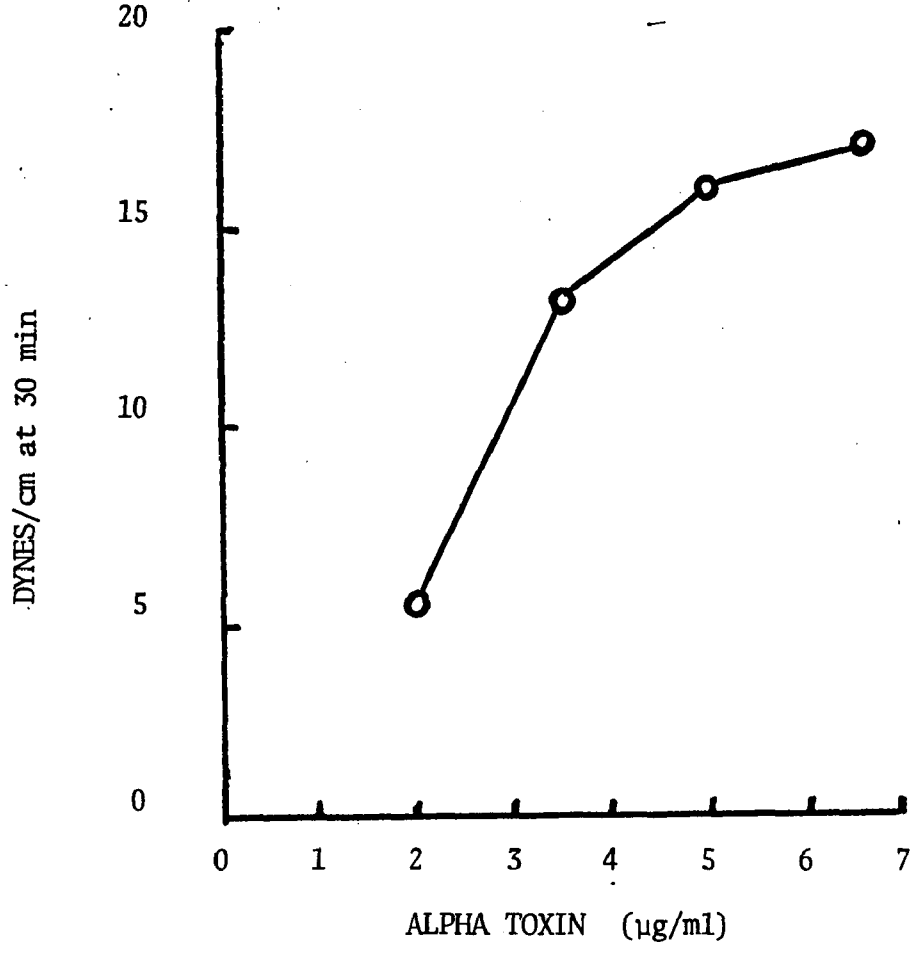


FIGURE 9: Effect of concentration on the ability of alpha toxin to form a film from the subphase in the monolayer system.

surface. Figure 10 presents the pressures attained by increasing amounts of alpha toxin placed on the surface. Protein analysis of the subphase demonstrated that the protein films formed in this manner contained 33 µg of protein at 7 dynes film pressure and 116 µg at 10 dynes.

Interaction of Alpha Toxin with Lipid Monolayers

The interaction, as evidenced by $\Delta\Pi$, of alpha toxin with various phospholipid, glycolipid, and cholesterol monolayers is presented in Figure 11. The interaction of ribonuclease with some of these monolayers is shown in Figure 12. Alpha toxin interacted to a slightly larger extent with these systems than did ribonuclease. In the case of ganglioside, however, alpha toxin interacted considerably less than did ribonuclease. Figure 13 compares the interaction of alpha toxin with mono-, di-, and trisialic acid ganglioside and sulfatide monolayers with the interaction of alpha toxin with lecithin monolayers. Alpha toxin interacted slightly better with mono- and disialic acid gangliosides than with trisialic acid gangliosides. The interaction of alpha toxin with sulfatide monolayers was intermediate between its interaction with lecithin and ganglioside monolayers.

Figure 14 shows the effect of pH on the interaction of alpha toxin with lecithin monolayers. The rate of interaction increased between pH 6 and pH 7, leveled off and increased again between pH 8 and pH 9, again leveling off

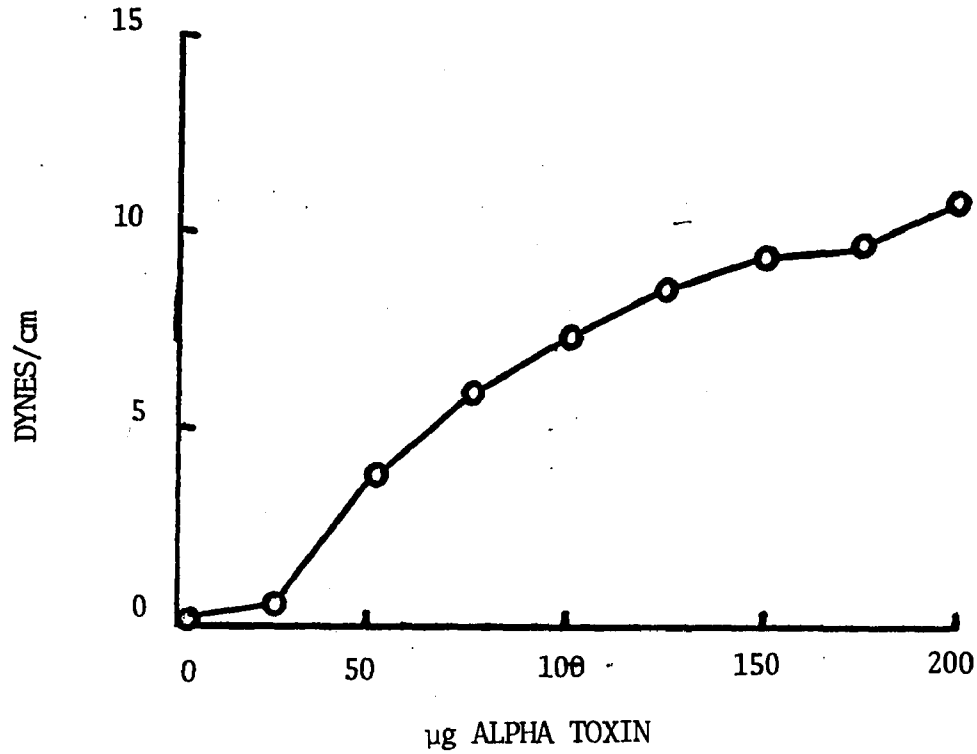


FIGURE 10: Formation of a film by dropping alpha toxin solution at the air-water interphase of the monolayer system.

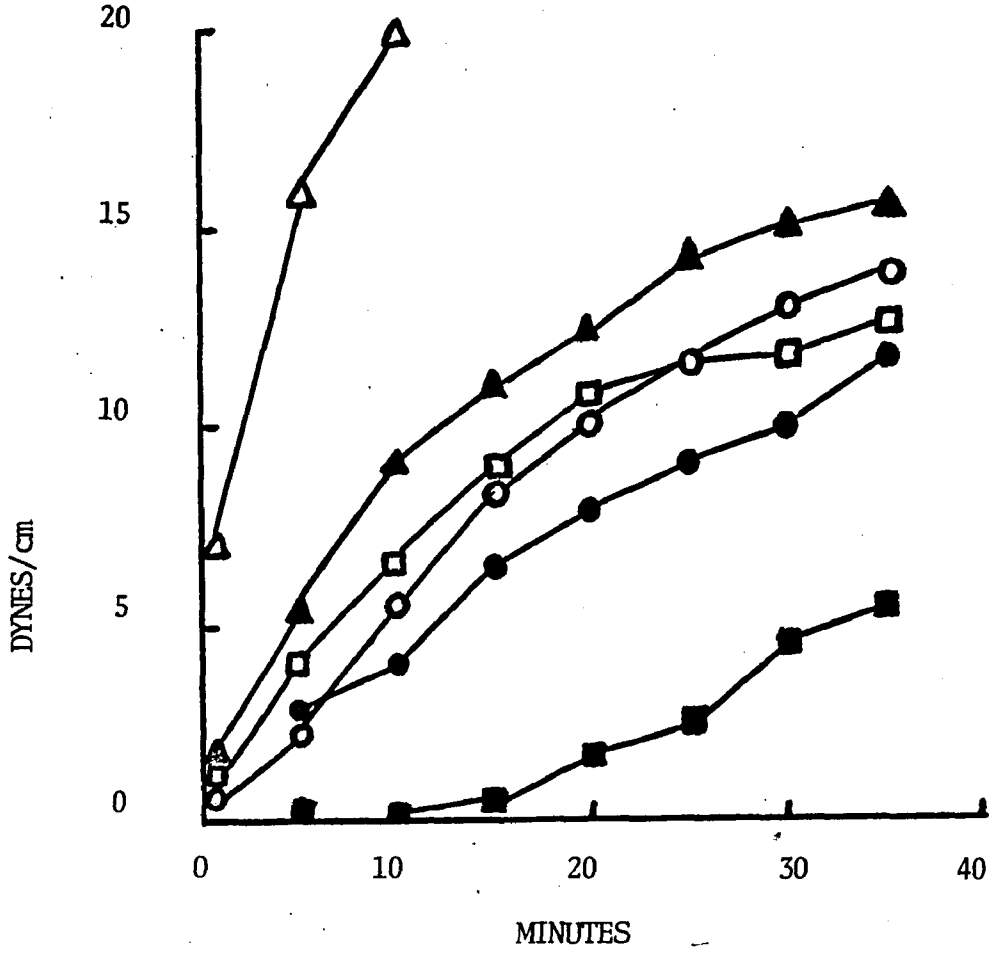


FIGURE 11: Interaction of alpha toxin with various lipid monolayers. Symbols: △, cholesterol; ▲, phosphatidylinositol; □, sphingomyelin; ○, lecithin; ●, cerebroside; ■, ganglioside.

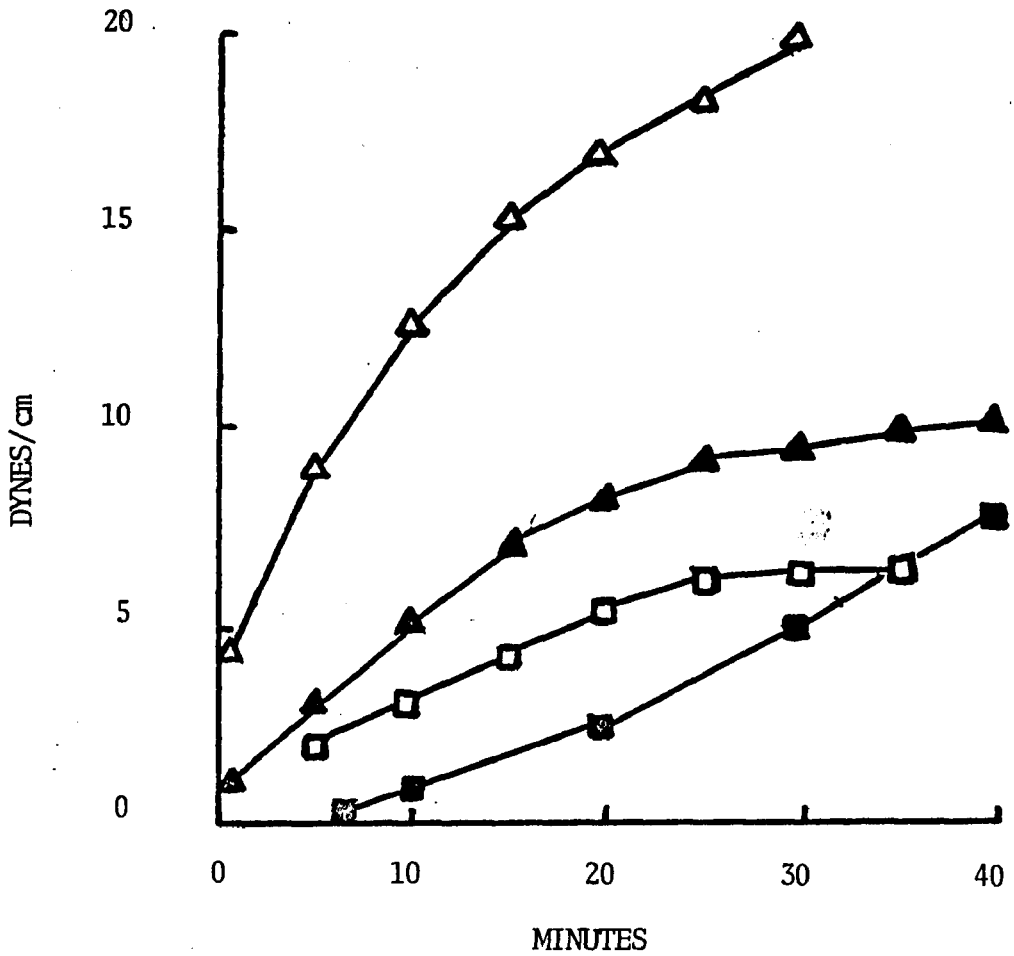


FIGURE 12: Interaction of ribonuclease with various lipid monolayers. Symbols: △, cholesterol; ▲, phosphatidylinositol; □, sphingomyelin; ■, ganglioside.

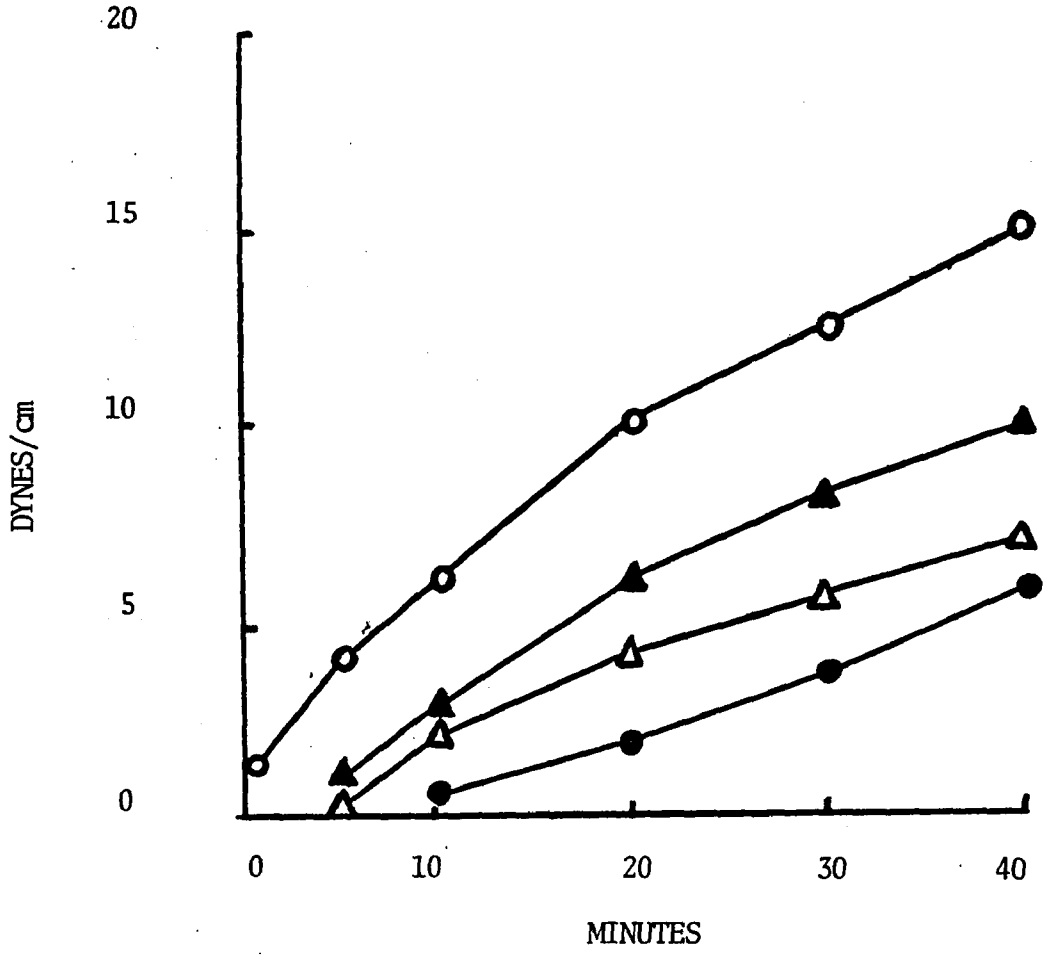


FIGURE 13: Interaction of alpha toxin with negatively charged lipid monolayers compared with interaction with lecithin monolayer. Symbols: ○, lecithin; ▲, sulfatide; △, mono-sialic acid ganglioside; ●, di- and tri-sialic acid ganglioside.

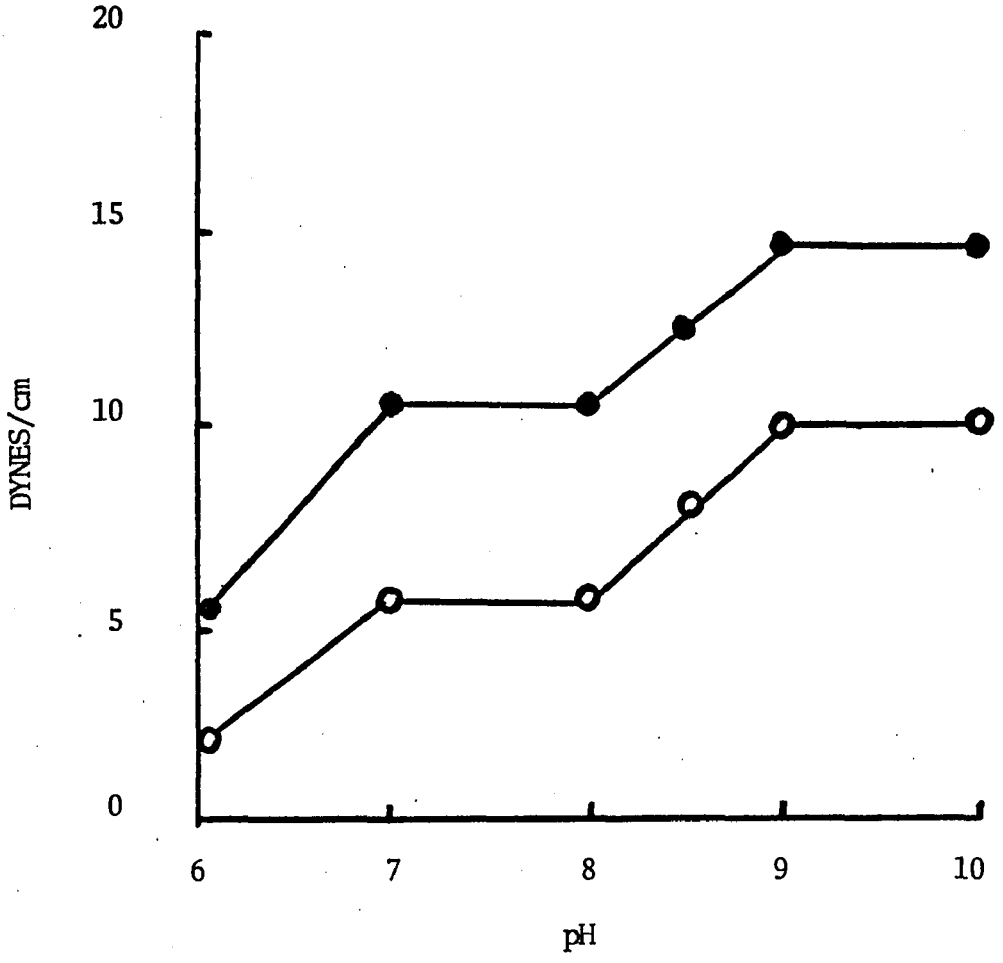


FIGURE 14: Effect of pH on the interaction of alpha toxin with lecithin monolayers. Symbols: ○ , $\Delta\pi$ at 10 minutes; ● , $\Delta\pi$ at 20 minutes.

above pH 9. The effect of temperature on the alpha toxin-lecithin interaction is presented in Figure 15. The rate of interaction was gradually increased with increasing temperature. Figure 16 shows the consequences of increasing the initial pressure, Π_i, of the lecithin monolayer before interaction with alpha toxin. The rate of interaction was much slower at high film pressures than at the 2 dyne pressure used in the above experiments. Increasing the concentration of alpha toxin in the subphase increased the rate of interaction with lecithin monolayers as revealed in Figure 17.

Interaction of Alpha Toxin with C^{14}-Lecithin Monolayers

The decrease in surface tension observed when a protein is interacted with a lipid monolayer had always been assumed to involve a penetration of the monolayer by protein molecules resulting in an increase in film pressure. It is possible, however, that protein molecules might be pulling lipid molecules out of the film which would make the change in pressure during a protein-lipid monolayer interaction extremely difficult to interpret. Therefore, monolayers were made as before with C^{14}-labeled lecithin and were allowed to interact with alpha toxin and streptolysin S. Streptolysin S is reported to have the ability to bind lecithin and so it was thought that streptolysin S might pull lecithin molecules out of the monolayer film, resulting in a low change in pressure. Table 9 reveals that less that 10% of the C^{14}-lecithin applied as a monolayer

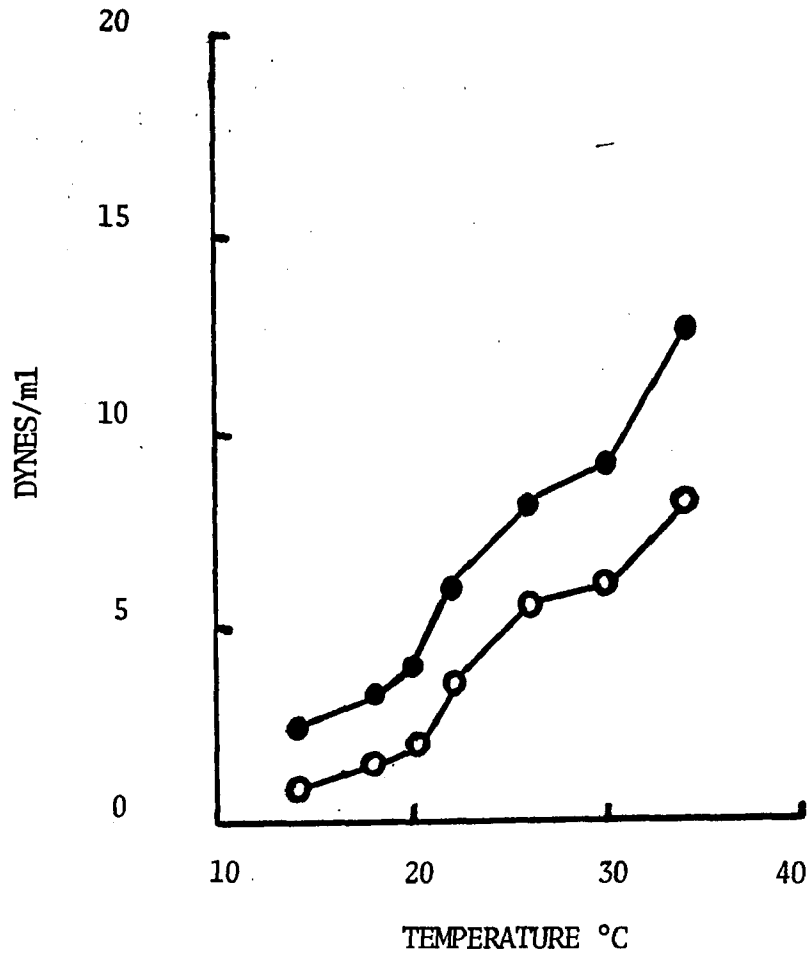


FIGURE 15: Effect of temperature on alpha toxin-lecithin monolayer interaction. Symbols: ○ , $\Delta\pi$ at 10 minutes; ● , $\Delta\pi$ at 20 minutes.

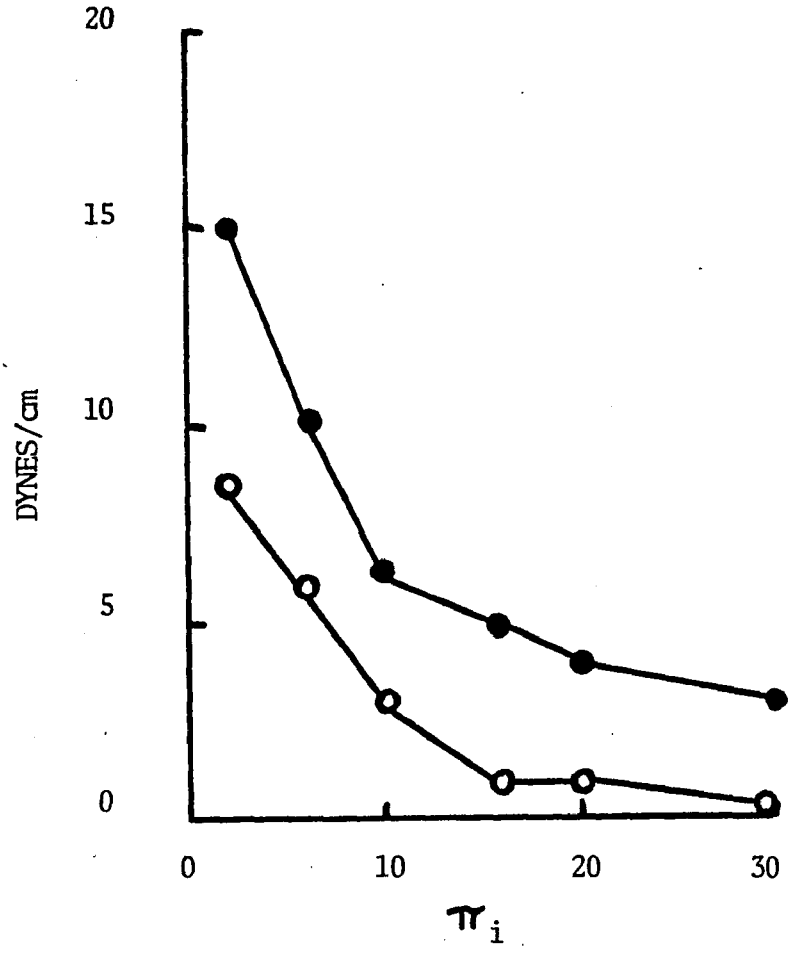


FIGURE 16: Effect of initial film pressure, π_i, on alpha toxin-lecithin monolayer interactions. Symbols: O, $\Delta\pi$ at 10 minutes ●, $\Delta\pi$ at 20 minutes.

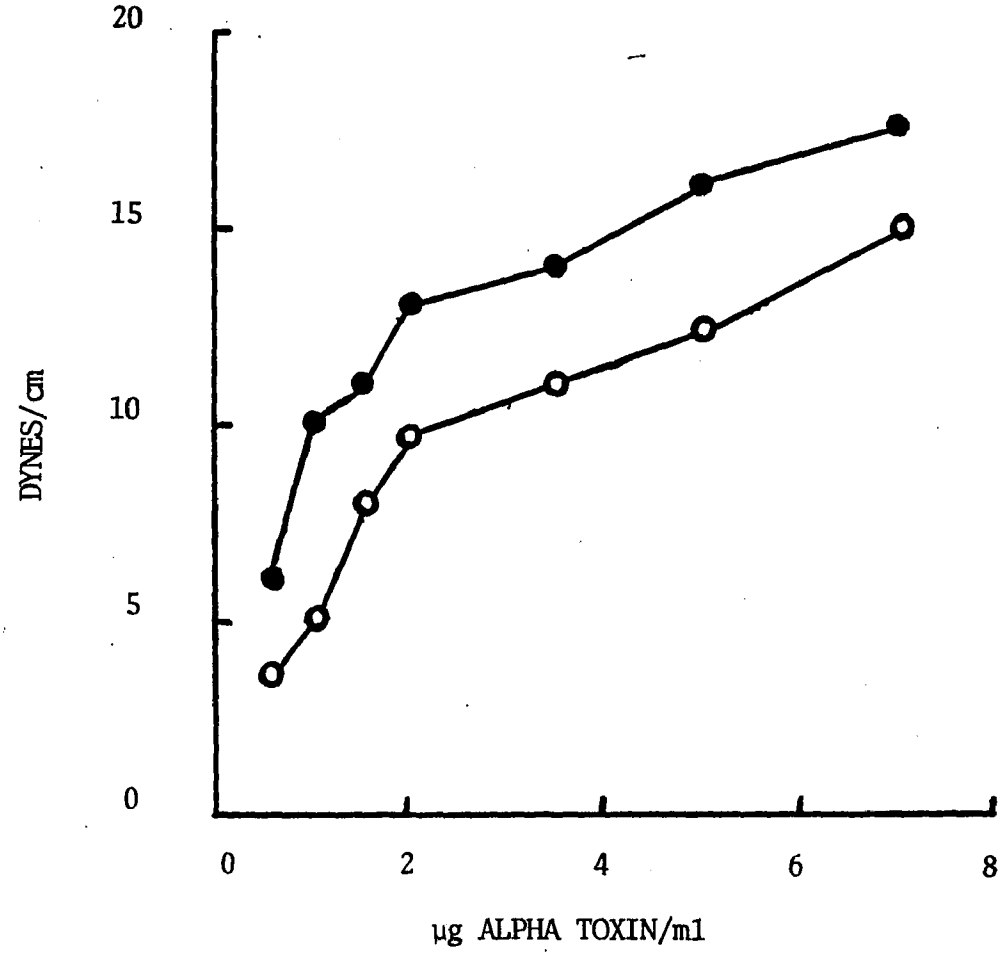


FIGURE 17: Effect of alpha toxin concentration in the subphase on alpha toxin-lecithin monolayer interactions. Symbols: ○, $\Delta\pi$ at 10 minutes; ●, $\Delta\pi$ at 20 minutes.

TABLE IX

% C^{14}-LECITHIN IN SUBPHASE OF THE MONOLAYER SYSTEM AFTER VARIOUS PROTEIN C^{14}-LABELED LECITHIN INTERACTIONS

Substance in Subphase	Counts/min in Subphase	% C^{14}-Lecithin* in Subphase
Alpha Toxin 2 µg/ml	2110	5.2
Streptolysin S 2 µg/ml	1480	3.6
RNA Core of Streptolysin S 2 µg/ml	1850	4.6

* 40,500 cpm C^{14}-lecithin in the monolayer film

can be found in the subphase after interacting with streptolysin S or alpha toxin for 30 minutes. Furthermore, the RNA core used in purifying streptolysin S showed no significant amount of C^{14} in the subphase after interaction with a C^{14}-lecithin monolayer.

Interaction of Alpha Toxin with Mixed Lipid Monolayers

In order to more closely mimic the biological situation, mixed films were made and allowed to interact with alpha toxin. The results of using cholesterol-lecithin monolayers, 50:50, 60:40, and 75:25 by weight, are shown in Figure 18. As might be expected from the changes in pressure observed when alpha toxin was allowed to interact with lecithin or cholesterol alone, the rates of interaction were intermediate between those of pure lecithin or cholesterol monolayers.

The slow interaction of alpha toxin with ganglioside films, together with the literature reports of the effect of ganglioside on the lethal activity of alpha toxin, made it of interest to investigate the effect of alpha toxin on mixed films of ganglioside and lecithin. Figure 19 presents results from such experiments in which 10% ganglioside by weight in a lecithin film was sufficient to reduce the change in pressure upon interaction with alpha toxin.

Preincubation of Alpha Toxin with Lipids before Interaction with Lecithin Monolayers

North and Doery (45) incubated alpha toxin with ganglioside before testing the effect of ganglioside on the lethal

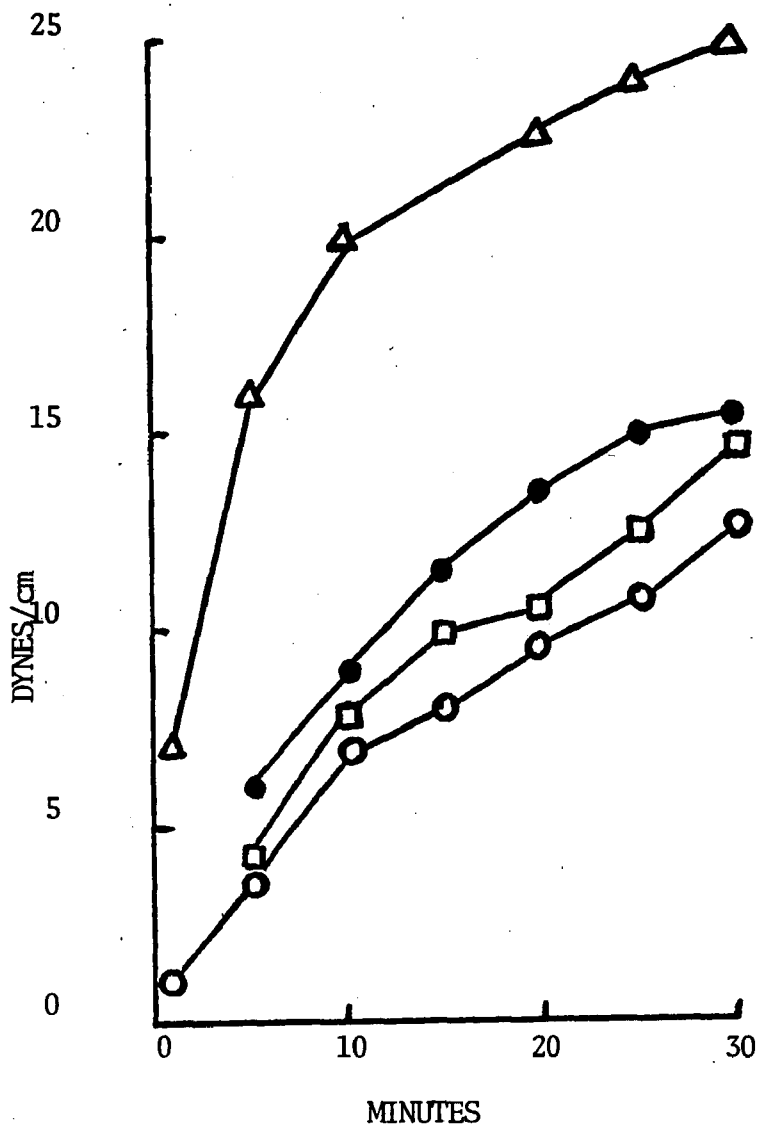


FIGURE 18: Interaction of alpha toxin with mixed cholesterol-lecithin, cholesterol, and lecithin monolayers. Symbols: ○, lecithin; □, 60:40 cholesterol:lecithin by weight; ●, 50:50 cholesterol:lecithin by weight; △, cholesterol.

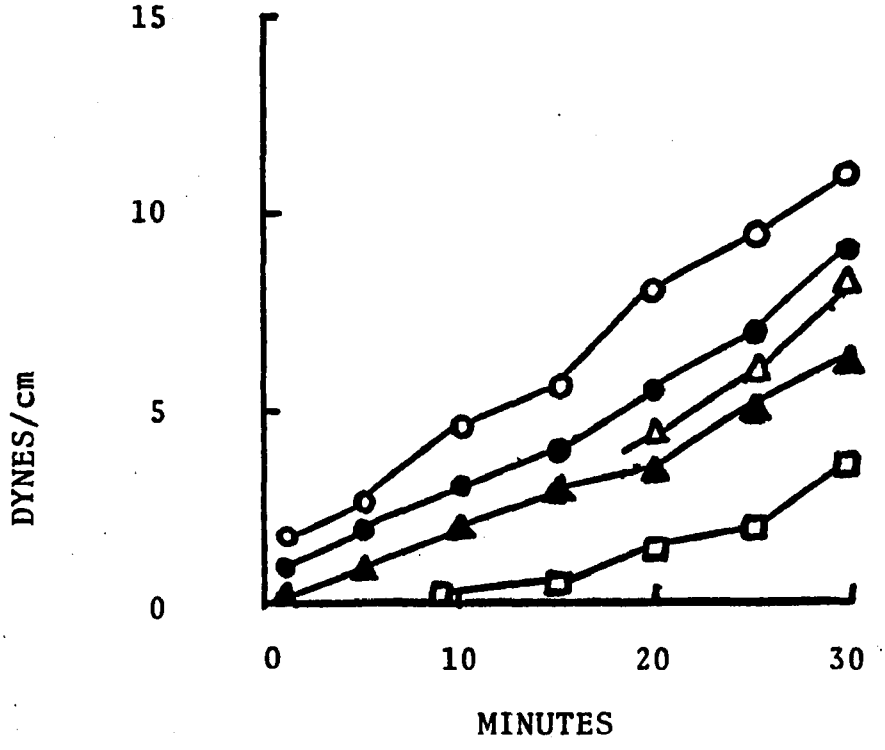


FIGURE 19: Interaction of alpha toxin with mixed ganglioside-lecithin, ganglioside and lecithin monolayers. Symbols: ○, lecithin; ●, 10:90 ganglioside: lecithin by weight; ▲, 20:80 ganglioside:lecithin by weight; □, ganglioside.

effect of alpha toxin. It therefore seemed relevant to try incubating alpha toxin with ganglioside and other lipids before interacting the protein with lecithin monolayers. The effect of ganglioside incubation on the interaction is related in Figure 20. Ganglioside incubation was observed to result in a marked decrease in interaction with lecithin monolayers. To a lesser extent, sulfatide also decreased interaction while lecithin and sphingomyelin had no effect.

Comparisons between Alpha Toxin and Streptolysin S in the Monolayer System

Streptolysin S is another bacterial exoprotein which lyses many different kinds of cells and disrupts lipid spherules. A comparison was made between the behavior of streptolysin S and alpha toxin in the monolayer system. The lack of any ability to collect at the surface from the subphase by streptolysin S was reported in a previous section and Figure 10. Streptolysin S also interacted with lipid monolayers at a slower rate than alpha toxin. Figure 21 presents the interactions of Streptolysin S with lecithin and ganglioside monolayers.

Effect of Urea on Protein Film Formation and Protein-Lipid Monolayer Interactions

Urea is known to have profound effects on the tertiary structure of alpha toxin and many other proteins. The influence of 6M urea in the subphase of the monolayer system on the abilty of alpha toxin to form a film is shown in Figure 22. The formation of protein films from the subphase

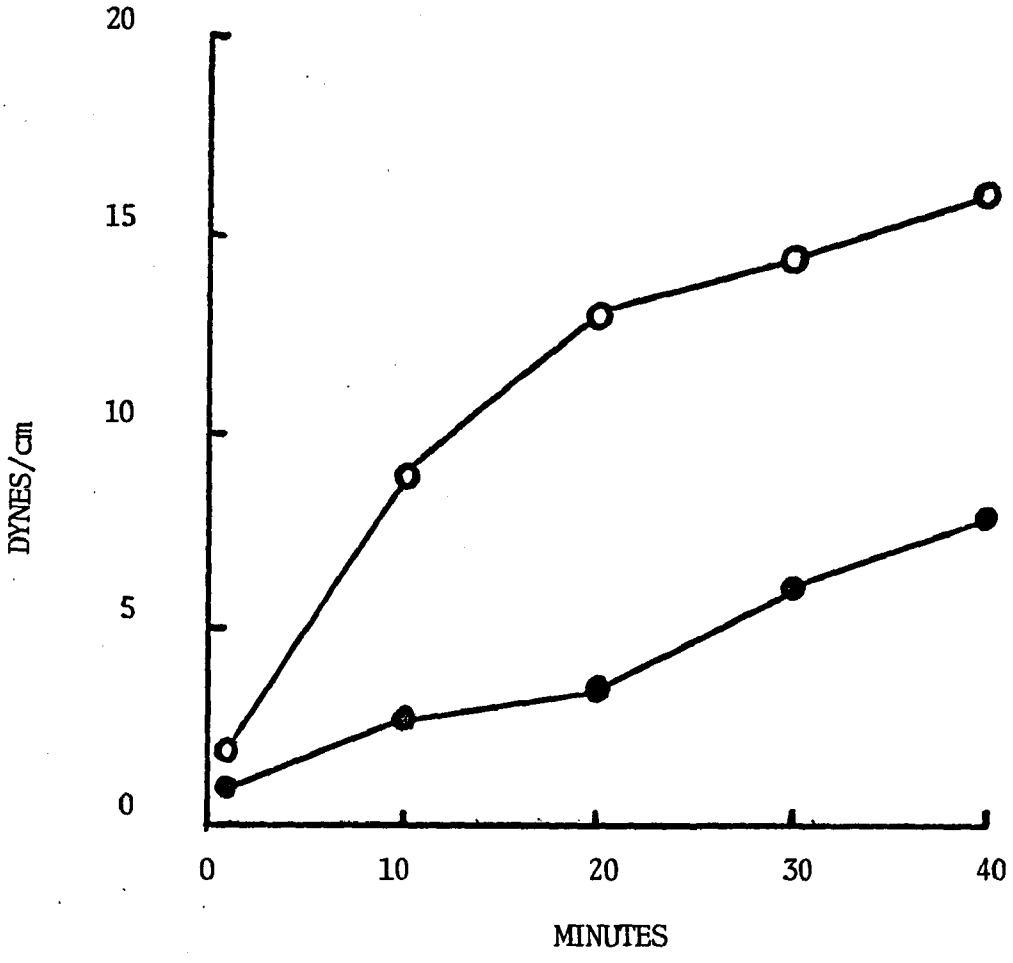


FIGURE 20: Effect of incubation of alpha toxin with ganglioside, 1:1 by weight, before interaction with lecithin monolayers. Symbols: ○ , alpha toxin not incubated; ● , alpha toxin incubated with ganglioside.

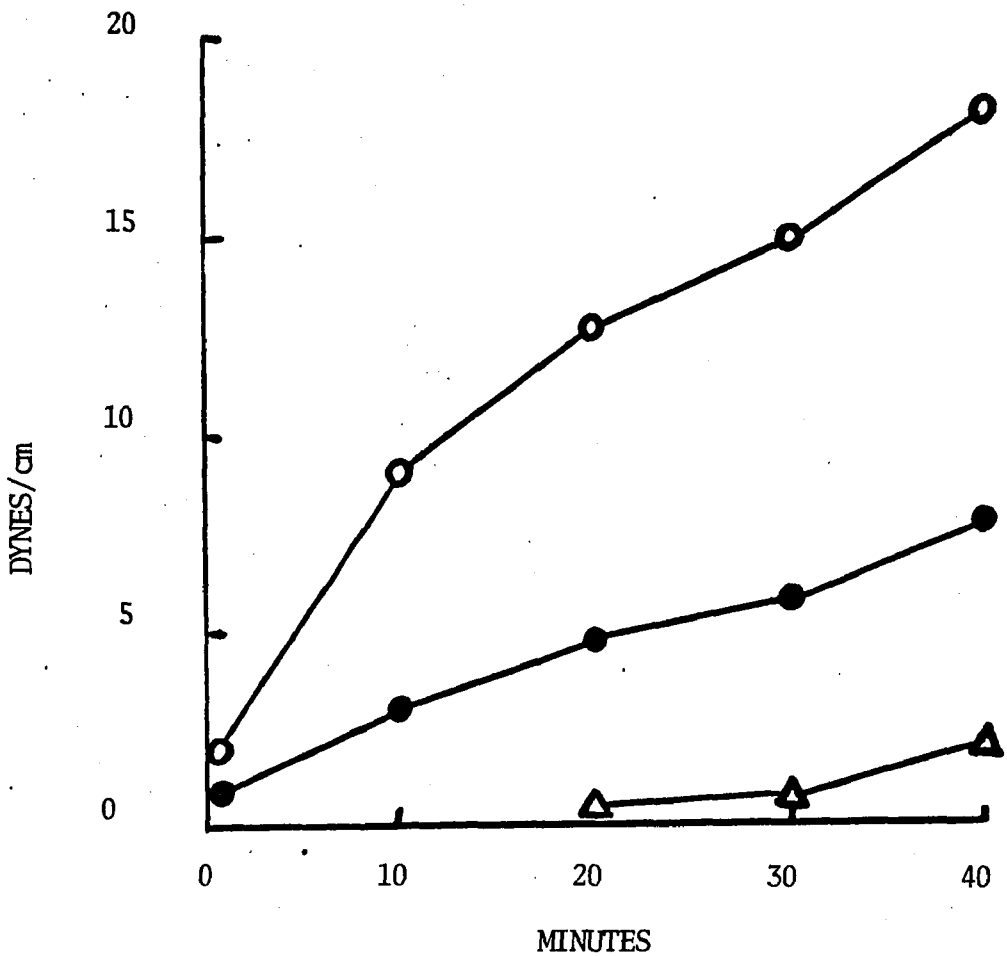


FIGURE 21: Interaction of streptolysin S with lecithin and ganglioside monolayers. Symbols: △, streptolysin S interacted with ganglioside; ●, streptolysin S interacted with lecithin; ○, alpha toxin interacted with lecithin.

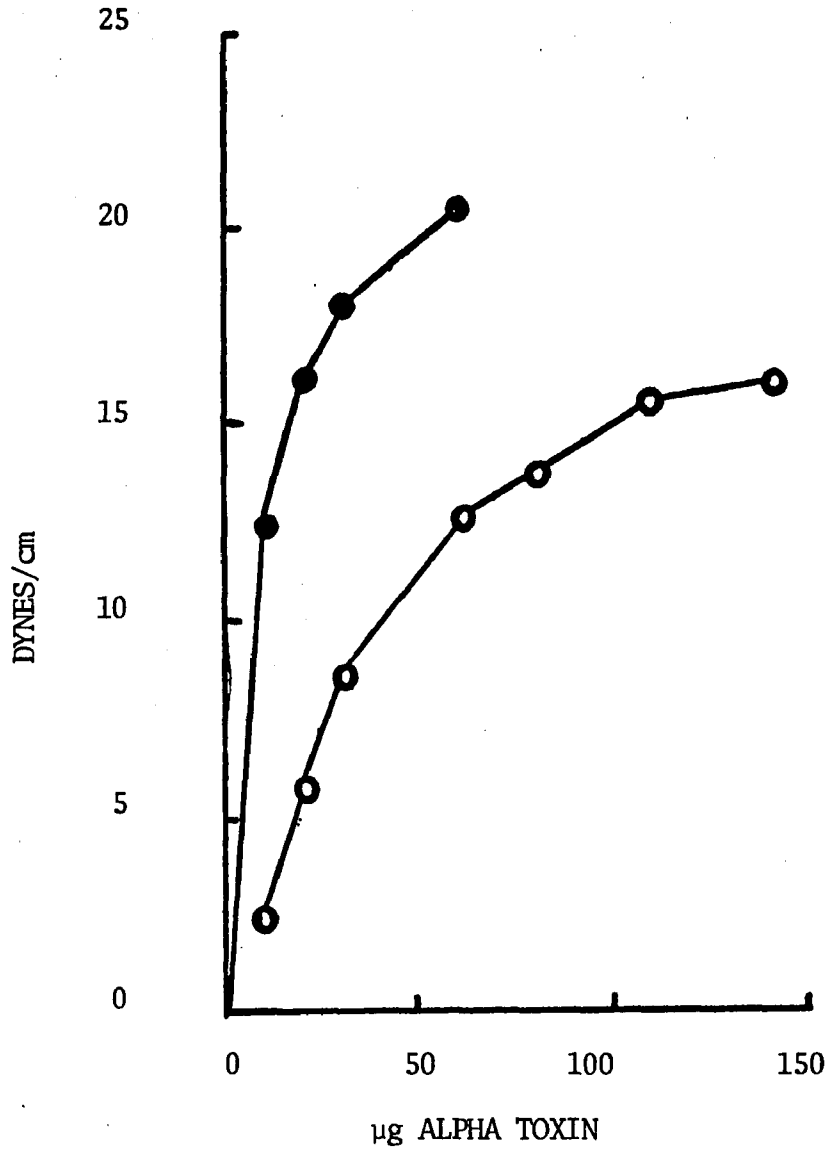


FIGURE 22: Influence of 6M urea on alpha toxin film formation. Symbols: ○, alpha toxin solution dropped on air-water interphase of monolayer system without 6M urea; ●, alpha toxin film formation on 6M urea solution.

and by dropping alpha toxin solutions on the air-water interphase were both increased in rate in the presence of 6M urea. The maximum pressure attained was also increased to 20 dynes. The effect of 6M urea on the ability of streptolysin S to form a film was more extensive. There was a complete lack of ability to form a film by streptolysin S in phosphate buffer, but in the presence of 6M urea, streptolysin S exhibited sufficient film formation to build a pressure of 11 dynes in 40 minutes as related in Figure 23.

6M urea in the subphase also caused an increase in the rate of protein-lipid interaction and maximum increase in pressure when alpha toxin was interacted with lecithin monolayers as seen in Figure 24. The effect of 6M urea on alpha toxin-ganglioside film interactions was large. The data presented in Figure 25 shows that 0.5M urea was sufficient to cause an increase in interaction between alpha toxin and ganglioside as evidenced by increase in film pressure. A $\Delta\Pi$ of 15 dynes in 30 minutes was attained in the presence of 6M urea.

The Effect of Galactose on Alpha Toxin-Lecithin Monolayer Interaction

Due to the presence of galactose residues in the ganglioside molecule, it was decided to test the influence of galactose in the subphase on alpha toxin-lecithin interactions. Figure 26 shows that 0.1M galactose in the subphase had no influence on the interaction.

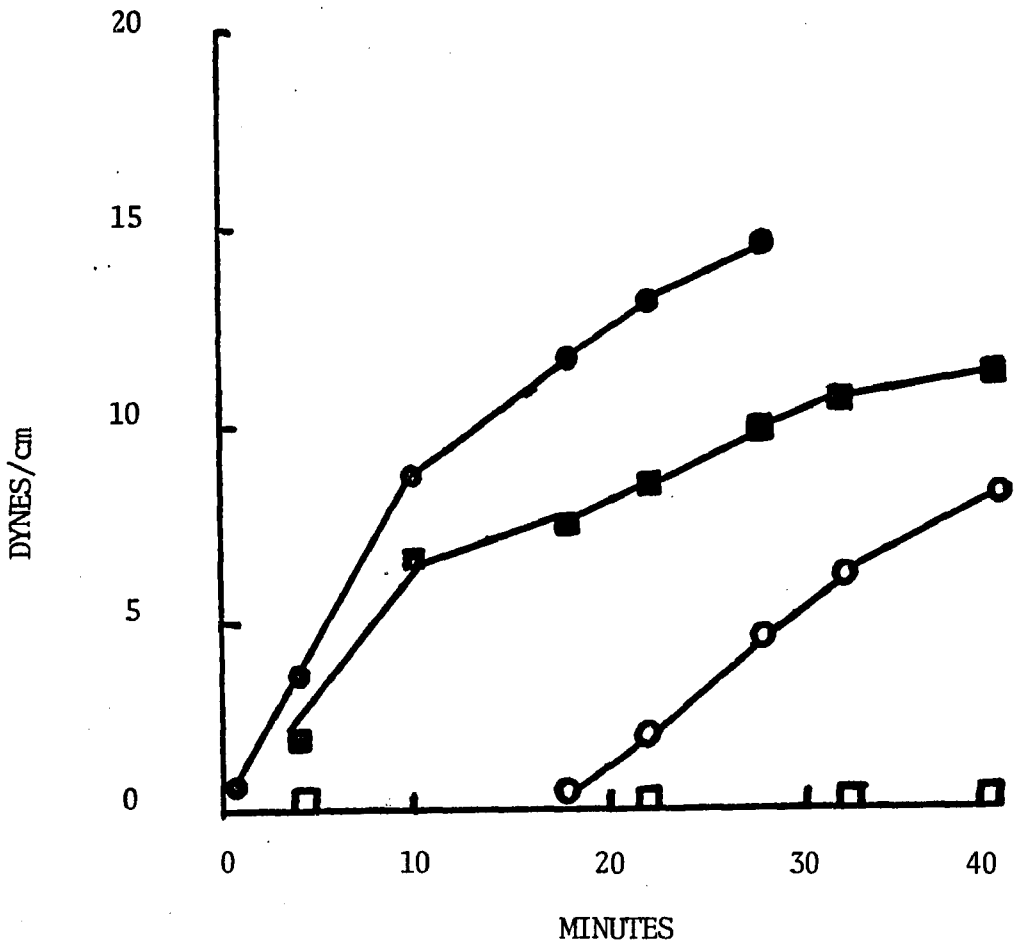


FIGURE 23: Influence of 6M urea on the ability of alpha toxin and streptolysin S to form films from the subphase. Symbols; ● , alpha toxin in 6M urea; ○ , alpha toxin withour urea; ■ , streptolysin S in 6M urea; □ , streptolysin S without urea.

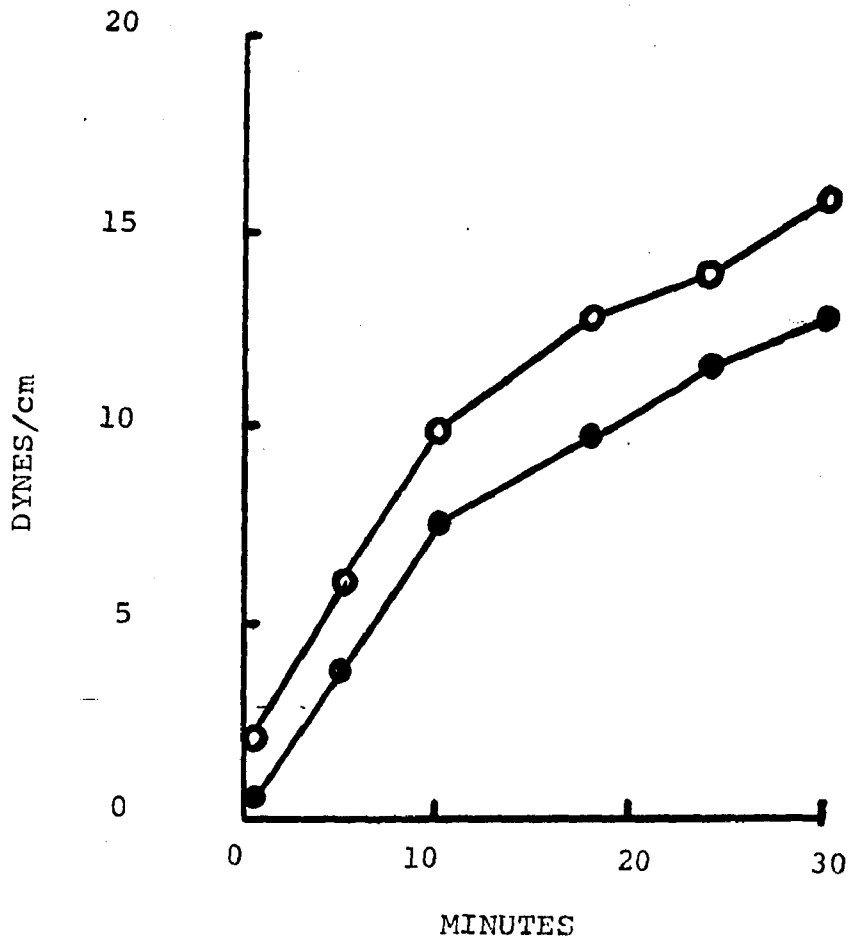


FIGURE 24: Influence of 6M urea on the interaction of alpha toxin with lecithin monolayers. Symbols: ○, interaction in 6M urea; ●, interaction in PO_4 buffer.

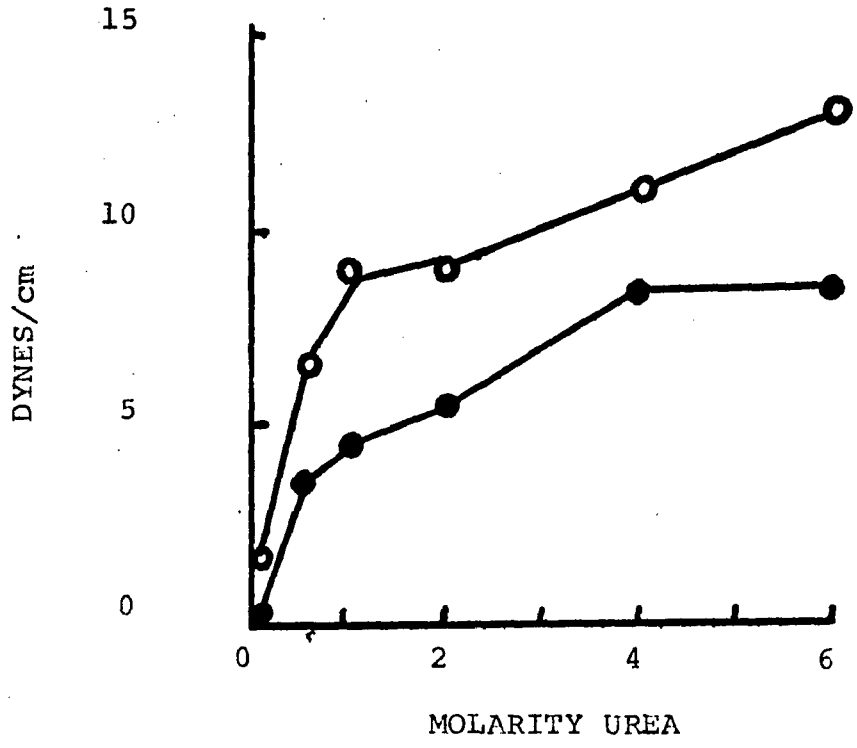


FIGURE 25: Influence of urea on the interaction of alpha toxin with ganglioside monolayers. Symbols: ●, $\Delta\pi$ at 10 minutes; ○, $\Delta\pi$ at 20 minutes.

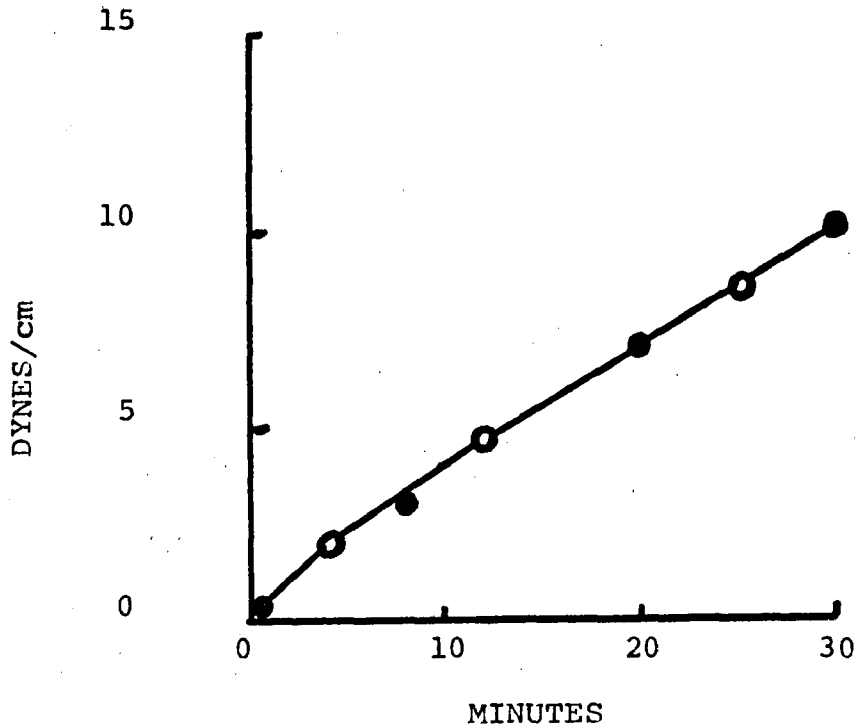


FIGURE 26: Alpha toxin-lecithin monolayer interaction in the presence and absence of 0.1M galactose. Symbols: ● , interaction in 0.1M galactose; ○ , interaction in PO_4 buffer.

DISCUSSION

Cultural Methods

The first attempts at producing alpha toxin resulted in yields from the culture supernatant varying from 400 hemolytic units/ml to 1600 units/ml. It was discovered that several factors were important in achieving a consistently high yeild of 3200 units/ml. Care had to be taken to adjust the gas proportions in the culture flasks every three hours during growth. In addition, the flasks had to be shaken in such a manner as to assure a swirling motion of the broth. It was thought that this action assured more uniform exposure to the gases and reduced foaming to a minimum.

Another important factor in obtaining consistently high yields in the culture supernatant was the incorporation of one unit of penicillin G/100 ml of dialyzed media before inoculation. This amount of one unit was arrived at by trial and error. Too much penicillin caused a decreased yield, probably by inhibiting growth. This conclusion was supported by the observation that culture with decreased yields were less turbid. Too little penicillin had no effect and yields remained low. The amount of penicillin necessary to increase the yield of alpha toxin varied from strain to strain, as reported above, and was arrived at empirically.

Cripps and Work (16) reported that 4-4.5% NaCl in the growth medium of Staphylococcus aureus, inhibited exoprotein

production by this organism. Marquis (42)has demonstrated the contraction of Bacillus megaterium cell walls by non-plasmolyzing NaCl solutions. Perhaps the decrease in exoprotein production observed by Cripps and Work in the presence of NaCl, was due to a tightening of pores in the cell wall of Staphylococcus aureus, preventing the exit of the exoproteins from between the cell membrane and cell wall. Conversely, the effect of penicillin might be to loosen the cell wall lattice, resulting in larger pores in the wall and an easier exit for exoproteins trapped between the cell membrane and wall, thus resulting in greater yields of alpha toxin in culture supernatants. It is of interest to note that Friedman (22) has reported a large increase in staphylococcal alpha toxin production in the presence of sub-lethal amounts of D-cycloserine. Although Friedman did not report an increase of alpha toxin production in the presence of penicillin or bacitracin, he may not have been working with the right concentration. For as Ritzerfeld, Winterhoff, and Kienitz (50) have shown, and as was confirmed in this work, the range of effective concentration for stimulation of exoprotein production is narrow.

Purification of Alpha Toxin

The overall yields from the purification of alpha toxin by methanol precipitation and Sephadex column chromatography are comparable to those obtained by other workers as presented in Table 11. The largest losses were incurred during the acetic acid-methanol precipitation step. This loss was

was probably due to denaturation of alpha toxin protein. Greater losses were observed if the methanol was allowed to become warm or if the precipitation was not carried out in the cold. Special care was also taken to insure that the precipitates and washings were kept at 0-4° C during the centrifugation steps. Approximately 12% of the loss was due to the fact that only the peak fractions of the Sephadex eluent were kept to insure purity of the final product. Some of the alpha hemolytic activity precipitated in 50% saturated $(NH_4)_2SO_4$ and a small amounts stayed in solution in 80% saturated $(NH_4)_2SO_4$, resulting in further losses.

Properties of Purified Alpha Toxin

Different strains of Staphylococcus aureus produce alpha toxins in different molecular weights. Alpha toxin from Smith's strain #9 had a molecular weight of about 43,000. This value is in agreement with Bernheimer's Wood 46 strain alpha toxin for which he has reported a molecular weight of 44,000. Coulter (15) argues that alpha toxin exists in several polymeric forms in rapid equilibrium and arrives at an average molecular weight of 30,000 for alpha toxin.

Although Butler reported an isoelectric pH of 6.4 for alpha toxin, there is now general agreement that the toxin is a basic protein which migrates slowly towards the cathode at pH 8.6. Bernheimer reported the existence of at least four peaks on density gradient electrophoresis with alpha toxin activity. On starch gel electrophoresis,

Bernheimer (6) reported one band moving slowly toward the cathode at pH 8.6. This thesis revealed the appearance of two bands moving slowly towards the cathode on cellulose acetate electrophoresis at pH 8.6. This result also agrees with that of Madoff and Weinstein, who also report the appearance of two bands under similar experimental conditions. Coulter's discovery of two N-terminal amino acids in purified alpha toxin, histidine and arginine, is consistent with the existence of more than one molecular form. Since there was a time lag between Sephadex chromatography and electrophoresis, there is also the possibility that polymers of different molecular weights form during the time lag. The precipitation of the Sephadex fractions with $(NH_4)2SO_4$ could also cause the formation of different molecular weight forms, explaining the appearance of more than one band on cellulose acetate electrophoresis.

Alpha toxin is known to aggregate into high molecular weight forms upon mild heating, and it is suspected by Coulter that there is a gradual formation of high molecular polymers upon standing in PO_4 buffer at 0° C, resulting in precipitation and inactivation. It has been observed in this study, that precipitation occurred after storage in PO_4 buffer for one week resulting in a halving of hemolytic activity. Coulter reported a half life of four days for purified alpha toxin stored in PO_4 buffer.

Alpha toxin precipitation was accelerated by adjusting the pH to 5.5, but there was no inactiviation and the pre-

cipitate was dissolved if the solution was neutralized immediately. This phenomenon is interesting, because proteins are generally least soluble near their isoelectric pH. Alpha toxin is less soluble at an acid pH and more soluble as the pH approaches its isoelectric pH.

No enzymatic activity for a specific substrate was demonstrated in this study. Other workers have made extensive investigations and have also failed to demonstrate a specific substrate for alpha toxin enzymatic activity. However, kinetic studies of alpha toxin hemolytic action, as well as the effects of inhibitors, temperature, salts and pH, lead one to hesitate before ignoring the possibility of an enzymatic function for alpha toxin.

The presence of a polysaccharide in our preparations of alpha toxin is in agreement with the results of Kuman, et al., (32) and Goshi, Cluff and Norman (24). The importance of the sugars have been demonstrated by the loss of activity after treatment of alpha toxin with glycosidase.

Alpha toxin prepared in this study behaved in accordance with the "unitarian theory". Table X shows that the preparations were lethal for mice, dermonecrotic for rabbits, and hemolytic. The specific activities of alpha toxin from Smith's strain #9 in general agree with those of Bernheimer and Schwartz and Jackson, while the specific hemolytic activity appears to be higher than those reported by Goshi, Cluff, Norman and Lominski, Arbuthnott, and Spence (2).

TABLE X

SPECIFIC ACTIVITIES OF PURIFIED ALPHA TOXIN PREPARATIONS*

Mouse LD_{50} μg	Rabbit Dermonecrotic Dose μg	Hemolytic Unit μg	Authority
ca. 1.0 (iv)	ca. 1.0	0.05	Bernheimer and Schwartz
ca. 1.0 (iv)	0.5-1.0	0.0084	Lominski, Arbuthnott, and Spence
3.9 (ip)	0.6	0.2	Jackson
12	2.4	0.012	Goshi, Cluff, and Norman
3.5 (ip)	ca. 0.5	0.15	this author

* adapted from Bernheimer (2)

Protoplast and Spheroplast Lysis

Alpha toxin prepared by the above methods lysed bacterial protoplasts and spheroplasts of several species. It is of interest, however, that while only about 0.15 µg of alpha toxin was necessary to lyse one ml of 1% rabbit red blood cells, 500 µg were necessary to lyse one ml suspension of bacterial protoplasts or spheroplasts made up to give an optical density of between 0.5 and 0.6 at 500 mµ. Furthermore, the same number of hemolytic units of our preparation was only one third as active against protoplasts and spheroplasts as an equal number of hemolytic units of Dr. Bernheimer's preparation. There are at least two possible explanations for these differences.

It is possible that bacterial protoplasts and spheroplasts are more resistant to alpha toxin than rabbit erythrocytes. Indeed the horse erythrocyte requires about 1000 times more toxin for lysis than does the rabbit erythrocyte. However, the differences in specific activity per hemolytic unit for protoplast and spheroplast lysis from one preparation to another argues that there may be more than one kind of activity being expressed by the alpha toxin molecule. Bernheimer has reported that aggregated alpha toxin (12S) is much more lytic for bacterial protoplasts and spheroplasts than 3S alpha toxin. It is possible that protoplast and spheroplast lysis and perhaps the lysis of the more resistant species of erythrocytes may be due to the action of the more aggregated alpha toxin molecules. Bernheimer has also

reported that delta lysin, a heat stable staphylococcal exoprotein, is much more lytic for protoplasts and spheroplasts than alpha toxin. A small amount of contaminating delta lysin in our preparations might explain the low specific activity for protoplast and spheroplast lysis and the differences in specific activity per hemolytic unit.

The effect of lipids on bacterial protoplast and spheroplast lysis will be discussed in a later section.

Effect of Alpha Toxin on C^{14}-Labeled Streptococcus faecium Membranes

The degradation of C^{14}-labeled membranes by staphlococcal alpha toxin gives further evidence for its possible involvement as a membrane active agent. The demonstrated release of many components from C^{14}-labeled Streptococcus faecium membranes indicates that alpha toxin may act by some nonspecific mechanism in the manner of a detergent in this situation. The relatively large amounts of toxin necessary to demonstrate a release of C^{14} from the membranes might point to some mechanism other than the enzymatic. On the other hand, a small amount of 12S alpha toxin or delta lysin may be responsible, thus necessitating a large amount of toxin preparation for activity on C^{14}-labeled Streptococcus faecium membranes.

Although isolated membranes of Streptococcus faecium were degraded by alpha toxin, the same preparations of alpha toxin failed to lyse lysozyme induced protoplasts of Streptococcus faecium, even when subjected to treatment with mg

amounts. A possible explanation may be that the C^{14}-labeled membranes were weakened by the fractionation procedure and subsequent storage at -20° C.

Ability of Alpha Toxin to Form Films at Air-Water Interfaces

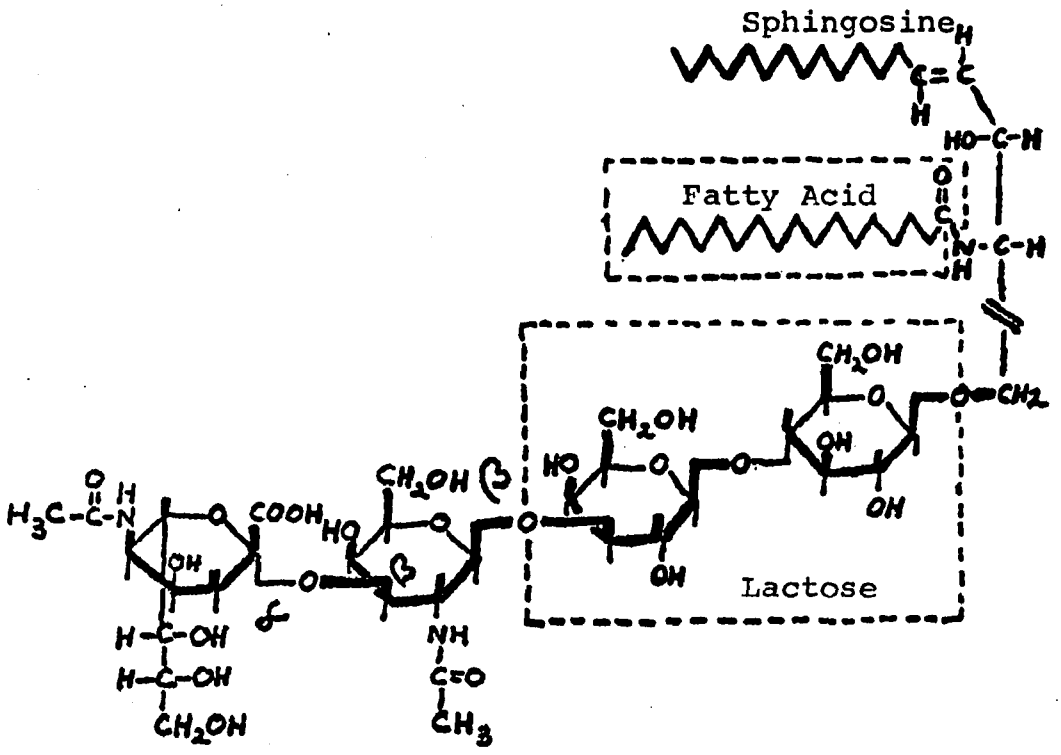
It is apparent from the results of experiments conducted with alpha toxin preparations in a circular trough, that the toxin has a definite tendency to form a film at the air-water interface of dilute salt solutions, as demonstrated by the increase in surface pressure when alpha toxin solution was dropped on the surface. Even more interesting is the ability of alpha toxin to collect at the air-water interphase when the toxin preparations are injected under the surface. The ability to collect at the surface may be important in the interaction of alpha toxin with biological membranes.

The ability to go to the air-water interface could not be demonstrated with ribonuclease, albumin, or streptolysin S. Streptolysin S had less surface activity, although it is known to interact with various types of biological membranes. These results indicate that alpha toxin may act by a different mechanism than streptolysin S. The absence of disulfide bridges in alpha toxin may be an important structural feature in determining its surface activity. The absence of disulfide bridges is a property of membrane proteins and lipoproteins and may be a characteristic of all-lipid associated proteins. The surface activity of the HDL-protein has been attributed to the absence of disulfide bridges in that mole-

cule by Camejo, Colacicco, and Rapport (10). When disulfide bridges are present, high salt concentrations or denaturing agents are necessary for protein film formation.

Interaction of Alpha Toxin with Lipid Monolayers

A comparison of the magnitude and rate of interaction of alpha toxin preparations with various kinds of phospholipids and glycolipids, indicates that alpha toxin interacted to a lesser degree with other kinds of lipids. The differences in interaction between cerebroside, sphingomyelin, and ganglioside are especially significant. Alpha toxin penetrated sphingomyelin and cerebroside films at about the same rate as with lecithin, while alpha toxin penetrated ganglioside and sulfatide films at a lower rate. A comparison of the structures shows that ganglioside shares the sphinogosine structure with sphingomyelin and cerebroside as illustrated in Figure 27. Further examination shows that ganglioside differs from cerebroside in that the former contains the negatively charged sialic acid group. Sulfatide is also similar in structure to cerebroside, differing only in the presence of the negatively charged sulfate molecule in the structure of sulfatides. One could conclude from these results, that the positively charged alpha toxin molecules are prevented from entering the films of negatively charged lipids. The molecules of alpha toxin might be bound by these negative charges under the film, thus preventing entry into the film.



Ganglioside

Sphingomyelin
(partial formula)

Cerebroside
(partial formula)

Sulfatide (partial formula)

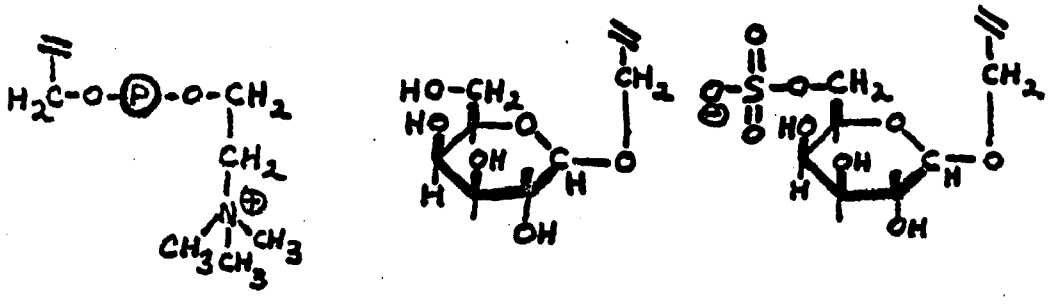


FIGURE 27: Chemical formulas of ganglioside, sphinomyelin, cerebroside, and sulfatide. After Karlson (30).

Effect of Preincubation of Alpha Toxin with Lipids before Interactions with Lecithin Films, Protoplasts, or Rabbit Erythrocytes

Results from studies involving the preincubation of alpha toxin with various lipids before interaction with lecithin films are consistent with the idea that the positive charge on the alpha toxin molecule is important in determining the extent of interaction with lecithin films.

A similar effect is seen when alpha toxin is preincubated with ganglioside before interaction with protoplasts of Sarcina lutea or rabbit erythrocytes. Since results of experiments with C^{14}-labeled ganglioside indicate that ganglioside does not appear to coat the erythrocytes, one could conclude that the lipid is covering the positive charges on the alpha toxin molecule and preventing the protein from interacting with the erythrocytes and protoplast membranes.

Interaction of Alpha Toxin with Mixed Lipid Films

An alternative explanation to the idea that the differences in activity of alpha toxin on procaryotic and eucaryotic cells is due to contamination with delta lysin or 12S alpha toxin can be presented if one assumes that the surface activity of alpha toxin is its most important propety in determining its ability to lyse cells. Studies on mixed films show a significant difference in the ability of alpha toxin to penetrate films containing cholesterol and those containing no cholesterol. Since procaryotic membranes

contain no sterol, one might expect alpha toxin to enter these membranes to a lesser extent if the mechanism of alpha activity is a surface phenomenon; however, it should be noted that cholesterol films are less compressible, leading to a higher change in pressure with ribonuclease as well as alpha toxin.

Mixed films of ganglioside and lecithin again demonstrate the inhibition of alpha toxin interaction with lecithin by the presence of ganglioside in the system. It is thought that the negative charges of the ganglioside block the positively charged alpha toxin from entering the film, perhaps causing a layer of alpha toxin protein to accumulate under the film.

Results indicating that alpha toxin is more able to penetrate lecithin films above the isoelectric point of alpha toxin would indicate that the protein penetrates films more readily when negatively charged. In this case, the negative charges of ganglioside might repell negative charges on the alpha toxin molecule. However, since most experiments were run at pH 7, one would expect alpha toxin to have a net positive charge in these experiments.

The Effect of Urea on Surface Activity

The increases in surface activity exhibited by alpha toxin when in the presence of increasing amounts of urea implies that unwound protein chains are more surface active than proteins with a more globular tertiary structure. As discussed above, the absence of disulfide bridges in the

alpha toxin, leading presumably to a more linear tertiary structure, may be an important property of the alpha toxin molecule, contributing to its surface activity, and possibly, to its biological activity. The fact that other proteins such as streptolysin S, normally less surface active than alpha toxin, become very surface active in the presence of 6M urea, is consistent with the theory that linear molecules are more surface active than globular molecules.

A comparison of the effects of urea on the surface activity of alpha toxin with the effects of urea on alpha toxin aggregates as reported by Freer, Arbuthnott, and Bernheimer (1, 21) lead to some interesting conclusions. Bernheimer (2) has reported the existence of a 12S contaminant in his stage 5 (3S) alpha toxin, which roughly corresponds to purified strain #9 alpha toxin. Heating of stage 5 alpha toxin at 60° C for 1 minute resulted in conversion of 3S alpha toxin to a 12S aggregate. Although the 12S aggregate is much less hemolytic and lethal than the 3S form (1), it has a much higher specific activity for protoplast and spheroplast activity (Bernheimer, personal communication) and is more disruptive for lipid spherules (21). The 12S aggregate appeared as a ring-shaped structure about 90 A in diameter, composed of six subunits, when examined with the electron microscope.

Treatment of the 12S aggregate with 8M urea resulted in a recovery of hemolytic activity. A removal of the urea by

dialysis caused the formation of a precipitate and a loss of hemolytic activity. The precipitate was the inactive 12S aggregate of alpha toxin. This 12S aggregate could then be dissolved in 8M urea and some hemolytic activity was recovered. Arbuthnott, Freer, and Bernheimer (1) call the supernatant remaining after the removal of urea heat purified (HP) alpha toxin.

Structures similar to the 12S rings with 5 to 7 subunits were found adhering to membrane fragments of lysed rabbit erythrocytes and lipid sperules after treatment with 3S (HP) alpha toxin. Furthermore, human erythrocyte ghosts showed ring structures on membranes after treatment with heat purified alpha toxin, even though these cells are relatively insensitive to alpha toxin hemolysis. Collected monolayers also showed some ring structures after interaction with alpha toxin. Freer, Arbuthnott, and Bernheimer (21) attributed this second type of ring shaped structure to alpha toxin-lipid micelle formation. However, the close resemblance of this type of structure with the 12S ring structure is striking.

The increase in surface activity exhibited by alpha toxin in the presence of urea is probably due to a disaggregation of 12S alpha toxin molecules, resulting in an easier access to the surface, because of the smaller radius and more linear structure of disaggregated alpha toxin.

One conclusion which can be drawn from these results is

that the surface activity of alpha toxin must be important in the lysis of rabbit erythrocytes, but not necessarily for the lysis of bacterial protoplasts and spheroplasts or lipid spherules since these bodies are lysed readily by 12S alpha toxin.

Figure 28 is a hypothetical diagram of the molecular transformations of alpha toxin based on consideration of the results of Freer, Arbuthnott, and Bernheimer (21), and the effects of urea on the surface activity of alpha toxin.

The charge of the surface is also apparently important and may offer an explanation for the great differences in specific activity of alpha toxin for various species of erythrocytes. The resistant species may possess more negative surface charge, resulting in a failure of alpha toxin to penetrate the cell membrane.

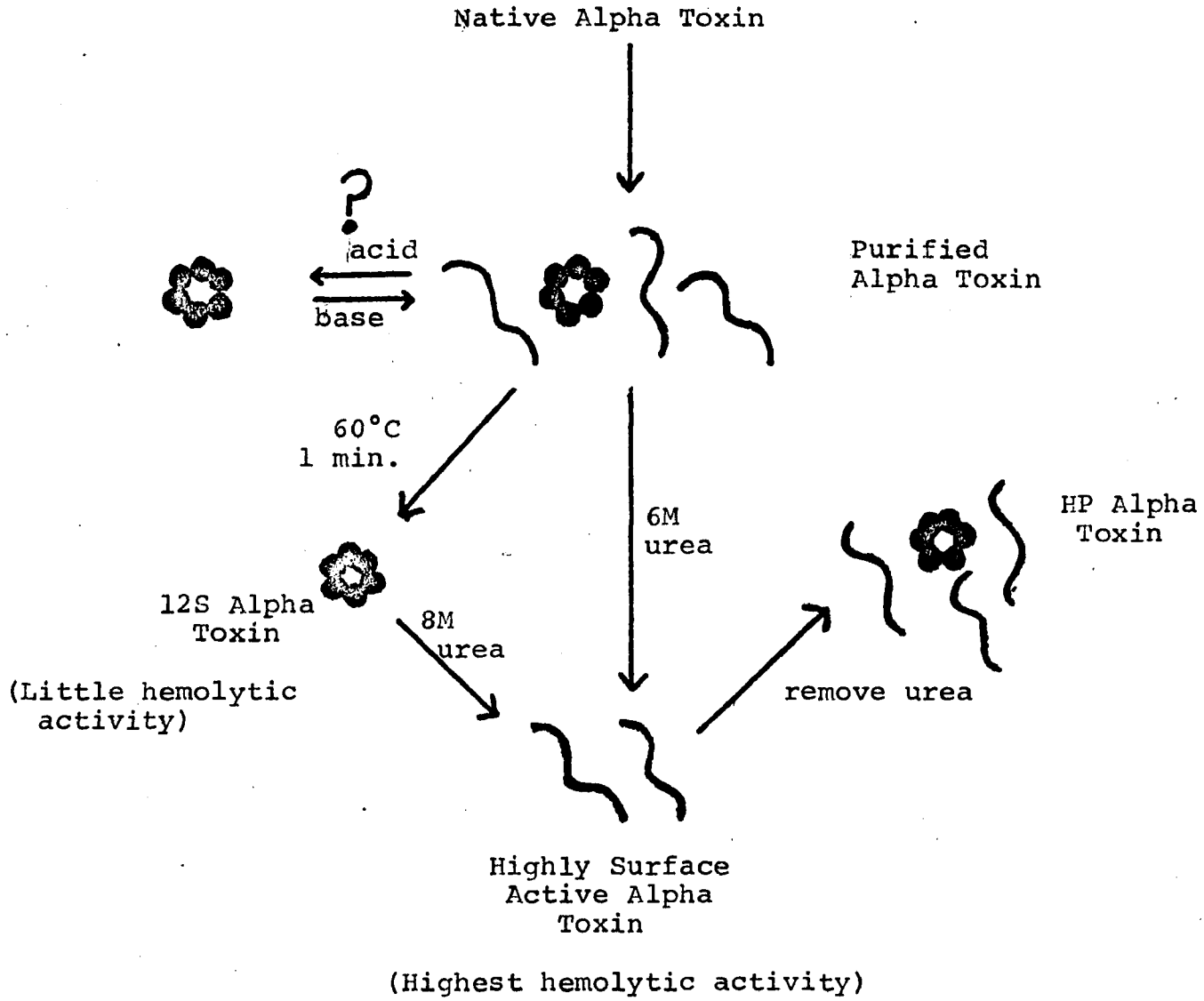


FIGURE 28: Hypothetical diagram of the molecular transformations of staphylococcal alpha toxin.

BIBLIOGRAPHY

1. Arbuthnott, J. R., Freer, J. H., and Bernheimer, A. W. 1967. Physical states of staphylococcal alpha toxin. J. Bacteriol. 94:1170-1177.

2. Bernheimer, A. W. 1965. Staphylococcal alpha toxin. In Whipple, H. E. (ed). The Staphylococci: Erologic Perspectives. Univ. N. Y. Acad. Sci. 128:112-121.

3. Bernheimer, A. W. 1966. Disruption of wall-less bacteria by streptococcal and staphylococcal toxins. J. Bacteriol. 91:1677-1680.

4. Bernheimer, A. W. 1968. Cytolytic toxins of bacterial origin. Science 159:847-851.

5. Bernheimer, A. W. and Davidson, M. 1965. Lysis of pleuroprnemonia-like organisms by staphylococcal and streptococcal toxins. Science 148:1229-1231.

6. Bernheimer, A. W. and Schwartz, L. L. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. 30:455-468.

7. Bernheimer, A. W. and Schwartz, L. L. 1964. Lysosomal disruption by bacterial toxins. J. Bacteriol. 87:1100-1104.

8. Bernheimer, A. W. and Schwartz, L. L. 1965. Effects of staphylococcal and other bacterial toxins on platelets "in vitro". J. Pathol. Bacteriol. 89:209-223.

9. Bernheimer, A. W. and Schwartz, L. L. 1965. Lysis of bacterial protoplasts and spheroplasts by staphyloccal alpha toxin and streptolysin S. J. Bacteriol. 89:1387-1392.

10. Camejo, G., Colacicco, G., and Rapport, M. M. 1968. Lipid monolayers: interactions with a lipid-free protein subunit of plasma lipoprotein. J. Lipid Res. in publication.

11. Chesbro, W. R. 1961. Lysozyme and the production of osmotic fragility in enterococci. Univ. J. Microbiol. 7:952-955.

12. Chesbro, W. R., Stuart, D., and Burke, J. J., II. 1966. Multiple molecular weight forms of staphylococcal nuclease. Biochem. Biophys. Res. Corn. 23:783-792.

13. Colacicco, G., Rapport, M. M., and Shapiro, D. 1967. Lipid monolayers: interaction of synthetic dihydroceramide lactosides and proteins. J. Col. Interface Sci. 25:5-15.

14. Cooper, L. Z., Madoff, M. A., and Weinstein, L. 1964. Hemolysis of rabbit erythrocytes by purified staphylococcal alpha toxin. I. Kinetics of the lytic reaction. J. Bacteriol. 87:127-135.

15. Coulter, J. R. 1966. Production, purification, and composition of Staphylococcal alpha toxin. J. Bacteriol. 92:1655-1662.

16. Cripps, R. E., and Work, E. 1967. The accumulation of intracellular macromolecules by Staphylococcus aureus grown in the presence of sodium chloride and glucose. J. Gen. Microbiol. 49:127-137.

17. Dain, J., Madoff, M., Tien, E., and Weinstein, L. 1965. Inhibition of the lethal effects of purified alpha toxin by brain ganglioside. Bacteriol. Proc. p. 60.

18. Doty, P. and Schulman, J. H. 1949. Formation of lipo-protein monolayers. I. Preliminary investigation on the adsorption of proteins onto lipid monolayers. Dis. Faraday Soc. 6:21-27.

19. Duthie, E. S., and Wylie, J. A. H. 1945. The influence of shaking on the "in vitro" production of soluble bacterial toxins. Brit. J. Exp. Pathol. 26:130-136.

20. Elek, S. D. Staphylococcus pyogenes and Its Relationship to Disease. pp. 230-236. E. E. S. Livingstone Ltd., 1959.

21. Freer, J. H., Arbuthnott, J. P., and Bernheimer, A. W. 1968. Interaction of staphylococcal alpha toxin with artificial and natural membranes. J. Bacteriol. 95:1153-1168.

22. Friedman, M. E. 1968. Inhibition of staphylococcal enterotoxin B. formation by cell wall blocking agents and other compounds. J. Bacteriol. 95:1051-1055.

23. Garvin, J. E. and Karnovsky, M. L. 1956. The titration of some phosphatides and related compounds in a non-aqueous medium. J. Biol. Chem. 221:211-222.

24. Goshi, K., Cluff, L. E., and Norman, P. S. 1963. Studies on the pathogenesis of staphylococcal infection. V. Purification and characterization of staphylococcal alpha hemolysin. Bull. Johns Hopkins Hosp. 112:15-30.

25. Hallander, H. O. 1963. Fractionation of staphylococcal toxins by gel fractionation. Acta Pathol. Microbiol. Scand. 59:543-552.

26. Haque, R. 1967. Identification of staphylococcal hemolysin by an electrophoretic localization technique. J. Bacteriol. 93:525-530.

27. Hough, L. 1950. Application of paper partition chromatography to the separation of the polyhydric alcohols. Nature. 165:100.

28. Hanser, E. D., and Berry, L. J. 1962. Studies of Staphylococcus aureus toxins with the aid of peritoneal diffusion chambers of graded porosities. VIII Inter. Cong. for Microbiol. Abs., E33. A. 11. p. 113.

29. Jackson, A. W. 1963. Purification of staphylococcal-alpha lysin. Can. J. Biochem. Physiol. 41:219-229.

30. Karlson, P. Introduction to Modern Biochemistry. pp. 230-232. Academic Press, 1963.

31. Kraus, R., and Clairmont, P. 1900. Uber Hamolysin und Antihamolysine. Wien. Klin. Wachr. 13:49-56. Cited by Elek, S. D. 1959.

32. Kumar, S., Loken, K. I., Kenyon, A. J., and Lindorfer, R. K. 1962. The characterization of staphylococcal toxins. II. The isolation and characterization of a homogeneous staphylococcal protein possessing alpha hemolytic dermonecrotic, lethal, and leucocidal activities. J. Exp. Med. 115:1107-1115.

33. Litwack, G. Experimental Biochemistry. pp. 145-150. John Wiley & Sons, Inc., 1960.

34. Lominski, I. and Arbuthnott, J. P. 1962. Some characteristics of staphylococcal alpha toxin. J. Pathol. Bacteriol. 83:515-520.

35. Lominski, I., and Arbuthnott, J. P. 1965. Effect of Staphylococcus alpha toxin on some cold-blooded animals. J. Pathol. Bacteriol. 89:387-390.

36. Lominski, I., Arbuthnott, J. P., and Spence, J. B. 1963. Purification of Staphylococcus alpha toxin. J. Pathol. Bacteriol. 86:258-262.

37. Madoff, M. A., Artenstein, M. S., and Weinstein, L. 1963. Biologic activity of purified staphylococcal alpha toxin. II. Effect of alpha toxin on Ehrlich ascites carcinoma cell. Yale J. Biol. Med. 35:382-389.

38. Madoff, M. A., Artenstein, M. S., and Weinstein, L. 1963. Biologic activity of purified staphylococcal alpha toxin. I. Effect of alpha toxin on cells in tissue culture. Yale J. Biol. Med. 35:373-381.

39. Madoff, M. A., Weinstein, L. 1962. Purification of staphylococcal alpha hemolysin. J. Bacteriol. 83:914-919.

40. Marinetti, G. V. 1965. The action of phospholipase A on lipoproteins. Biochem. Biophys. Acta 98:554-565.

41. Manohar, M., Muheswaran, S. K., Frommes, S. P., and Lindorfer, R. K. 1967. Platelet damaging factor, a fifth activity of staphylococcal alpha toxin. J. Bacteriol. 94:224-231.

42. Marquis, R. E. 1968. Salt-induced contraction of bacterial cell walls. J. Bacteriol. 95:775-781.

43. Marucci, A. A. 1963. Mechanism of action of staph-glucoccal alpha hemolysin. I. Some factors influencing the measurement of alpha hemolysin. J. Bacteriol. 86:1182-1188.

44. McClatchy, J. K. and Rosenblum, E. D. 1966. Biological properties of alpha toxin mutants of Staphylococcus aureus. J. Bacteriol. 92:575-579.

45. North, E. A. and Doery, N. M. 1961. Inactivation of staphylococcal, tetanus, and diptheria toxins by ganglioside. Brit. J. Exp. Pathol. 42:23-29.

46. Orsi, N. and Poggiolini, D. Ricerche sull'ativita emolitica dell' alpha emolisma stafilococca. Riv. Biol. 55:361-373.

47. Ouchterlony, O. 1962. Diffusion-in-gel methods for immunological analysis, II. Progr. Allergy. 6:30-154.

48. Pollock, M. R. and Richmond, M. E. 1962. Low cyst(e)ine content of bacterial extracellular proteins: its possible physiological significance. Nature. 194:446.

49. Rahal, J. J., Plant, M. E., Rosen, H., and Weinstein, L. 1966. Purified staphylococcal alpha toxin: effect on epithelial ion transport. Science. 155:1118-1120.

50. Ritzerfeld, W., Winterhoff, D., and Kienitz, M. 1965. Steigerung der Hamolysin-Produktion von Staphylokokken unter Penicillineinfluss. Zentr. Bakteriol. Parasitenk., Abt. I. 199:478-483.

51. Robinson, J., Thatcher, F. S., and Montford. 1960. Studies with staphylococcal toxins. V. Possible identification of alpha hemolysin with a proteolytic enzyme. Can. J. Microbiol. 6:183-194.

52. Smith, I. Chromatographic and Electrophoretic Techniques. p. 95. Interscience, 1960.

53. Szmigielski, S., Jeljaszewitz, J., and Zak, C. 1967. Reaction of rabbit leukocytes to staphylococcal alpha hemolysin "in vivo". J. Enf. Dis. 117:209-214.

54. Taubler, J. H., Kapral, F. A., and Mudel, S. 1963. Role of alpha toxin in lesion formation by Staphylococcus aureus on sutures subcutaneously implanted in mice. J. Bacteriol. 86:51-57.

55. Thal, A. P., and Egner, W. 1961. The site of action of the Staphylococcus alpha toxin. J. Exp. Med. 113:67-81.

56. van Heynigingin, W. E. Bacterial Toxins. pp. 64-66. Blackwell, 1950.

57. Weinstein, L. 1937. The effect of lecithin on streptococcal and staphylococcal hemolysin. J. Inf. Dis. 60:209-212.

58. Weissman, G., Sessa, G., and Bernheimer, A. W. 1966. Staphylococcal alpha-toxin: effects on artificial lipid spherules. Science. 154:772-774.

59. Wittler, R. G., and Pillemer, L. 1948. The immunochemistry of toxins and toxoids. V. The solubility of staphylococcal toxin in methanol-water mixtures under controlled conditions of pH, ionic strength, and temperature. J. Biol. Chem. 174:23-29.