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# INTERACTION BETWEEN TETANOLYSIN AND MYCOPLASMA CELL MEMBRANE

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SUMMARY

1. A partially purified tetanolysin preparation lysed the sterol-requiring *Mycoplasma capricolum* cells but had no effect on *M. capricolum* cells adapted to grow with no or very little cholesterol. The sterol-non-requiring *Acholeplasma laidlawii* cells grown either in a cholesterol-rich or a cholesterol-poor medium were unaffected by the tetanolysin preparation.

2. The lysis of *M. capricolum* cells by the tetanolysin preparation was temperature dependent, inhibited by cholesterol, sublytic concentrations of lucensomycin, and $Mg^{2+}$. The sensitivity to lysis was greatly affected by the age of the culture, being highest in cells from the early logarithmic phase of growth and declining sharply thereafter.

3. Isolated *M. capricolum* membranes were capable of binding large amounts of the tetanolysin activity (up to 30 hemolytic units per $\mu$g membrane protein), 20 times as much as membranes of the adapted strain. The binding of tetanolysin activity to membranes was almost the same at 4, 22, or 37 °C, and was very little affected by the age of the culture. The binding capacity of the membranes was not affected by the removal of 60–70% of membrane proteins by pronase digestion but markedly decreased with the removal of membrane lipids.

4. Of the five polypeptide bands detected in electrophorograms of the partially purified tetanolysin preparation, two bands (mol. wt. 44 000 and 42 000) were found to bind to the cholesterol-containing mycoplasma membrane preparation. EPR spectrometry revealed that the freedom of motion of fatty acid spin labels in the tetanolysin-treated membranes was markedly higher than that in untreated membranes.

5. The concept that tetanolysin interacts specifically with membrane cholesterol resulting in the shielding of cholesterol from its interaction with membrane phospholipids is discussed.

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## INTRODUCTION

Tetanolysin, an oxygen-labile hemolytic protein produced by *Clostridium tetani*, is known to disrupt a variety of cells and organelles [1–3], resulting in the leakage of intracellular constituents into the medium [3]. The mode of action of tetanolysin is not yet fully understood. Studies with another oxygen-labile bacterial lysin, streptolysin *O*, have suggested the participation of cholesterol in the biological effects of this toxin [1, 4] and recently further evidence that cholesterol is the membrane specific binding site for streptolysin *O* has been presented [5, 6]. Our studies were undertaken therefore to examine the possibility that cholesterol is the binding site also for tetanolysin. Mycoplasmas serve as a most convenient tool for such studies because cholesterol is an essential membrane component of these organisms [7]. Furthermore, the recent adaptation of mycoplasma species to grow without added cholesterol [8] provides a good source for control cells. The mechanism of mycoplasma lysis by a partially purified tetanolysin preparation [9] as well as the chemical and physical changes induced in the membranes upon their interaction with the toxin are described.

## MATERIALS AND METHODS

*Preparation of tetanolysin.* Crude culture filtrates of *C. tetani*, Massachusetts $C_2$ strain, were concentrated and applied to Sephadex G100 columns (2.5 × 90 cm) as previously described [9]. Elution was made with 0.025 M sodium phosphate buffer, pH 7.0, by upward flow and the eluate was collected in 5 ml fractions. Chloroform (0.025 ml) was added to each tube as a preservative. Hemolytic activity was eluted mainly in 6–8 fractions (fractions 42–50). For some experiments, a single fraction with the peak hemolytic activity was used. However, for most experiments, 4–6 fractions with hemolytic activity of greater than 100 hemolytic units/ml were pooled. The specific activity of the tetanolysin preparations used was 2000–5000 hemolytic units/mg protein.

*Assessment of tetanolysin activity.* Hemolytic activity in fractions eluted from the column and in samples obtained after binding experiments with mycoplasma membranes was determined as previously described [9]. Serial two-fold dilutions of the toxin were made in 0.2 ml of phosphate-buffered saline (50 parts of 0.15 M NaCl, 10 parts of 0.16 M $NaH_2PO_4$ and 40 parts of 0.13 M $Na_2HPO_4$, pH 7.4) containing 2 % gelatin. To four parts of phosphate buffer, pH 6.6, containing 0.2 % of sodium hydrosulfite and 1 part of toxin dilution, 1 part of 2 % defibrinated sheep red blood cells was added. The tubes were incubated at 37 °C for 2 h. Complete hemolysis was determined by visual examination [9] or calculated by measuring the absorbancy at 570 nm of the supernatant fluid after centrifugation at 1 000 × *g* for 10 min. Hemolytic activity was expressed as hemolytic units based on the reciprocal of the toxin dilution that caused complete hemolysis [9].

*Organisms and growth conditions. Mycoplasma capricolum* (California Kid) and *Acholeplasma laidlawii* (PG8) were grown in 100–500 ml of a modified Edward medium [10] containing 2 % PPLO Serum Fraction, Difco. Each lot of serum fraction required testing for suitability prior to use. In some experiments, the PPLO Serum Fraction was replaced with 0.5 % fatty acid-poor bovine serum albumin

(Sigma, St. Louis, Missouri), and supplemented with 0.5–10 μg/ml of cholesterol. For labeling cells, the medium was supplemented with 50–100 μCi per liter of [*Me*-$^3$H]-thymidine (20 Ci/mmol). For labeling membrane cholesterol, the medium was supplemented with 10–20 μCi per liter of [4-$^{14}$C]cholesterol (56 Ci/mol). Growth was determined by measuring the absorbancy of the culture at 640 nm. The organisms were harvested after 8–24 h of incubation at 37 °C, and were washed once with 0.25 M NaCl. The number of colony-forming units in the washed cell suspensions was determined by the standard dilution-colony count procedure [11]. Protein was determined in the cell suspension according to Lowry et al. [12] using bovine serum albumin as standard. Total cholesterol was determined by the $FeCl_3$ method [13].

*Assessment of osmotic fragility.* Osmotic fragility of the washed mycoplasma cells was determined spectrophotometrically as described previously [14]. Results were expressed as percent lysis in 0.05 M NaCl. In some experiments, osmotic fragility was assessed by measuring the release of radioactivity from [*Me*-$^3$H]thymidine-labeled cells. In these studies, 0.1 ml washed cell suspension (containing 2–3 mg cell protein/ml) was incubated with 2 ml of deionized water, 0.05 M or 0.25 M NaCl solutions. After 15 min at 37 °C the cells were either rapidly filtered through millipore filters (0.22 nm pore size, Millipore Corp., Bedford, Massachusetts) or sedimented by centrifugation at 12 000 × *g* for 15 min. The filters were washed with 20 ml of a cold solution of 0.25 M NaCl containing 0.01 M $MgCl_2$, and dried overnight at 37 °C. Radioactivity of the dried filters or of the supernatant fluids obtained by sedimentation of the cells was counted using a Packard Tricarb Liquid scintillation spectrometer and Aquasol (New England Nuclear, Boston, Mass.) scintillation liquor.

*Assessment of cell lysis by tetanolysin.* For determining mycoplasma lysis by tetanolysin two-fold dilutions of each tetanolysin preparation were made in 1 ml volumes of 0.025 M sodium phosphate, pH 7.0 in 0.25 M NaCl (to be referred to as "phosphate-salt" solution). To each tube 0.05 ml of a washed mycoplasma cell suspension (containing 2–3 mg cell protein/ml) were added and the tubes were incubated at 37 °C for 15 min. The absorbancy of the cell suspension at 500 nm was recorded and the percent lysis of the mycoplasma cells was calculated according to the formula: (absorbance without toxin – absorbance with toxin/absorbance without toxin) × 100. In some experiments, cell lysis by the tetanolysin preparation was calculated by measuring the release of [*Me*-$^3$H]thymidine from mycoplasma cells as described above.

Inhibition of tetanolysin activity by cholesterol was performed by passing tetanolysin (128–256 hemolytic units/ml) at 1 ml per min over a column (20 × 4 mm) containing packed cholesterol crystals (Sigma, St. Louis, Missouri) or by incubating tetanolysin (128–512 hemolytic units/ml) with 2.5–10 mg/ml of cholesterol. The ability of these preparations to lyse *M. capricolum* was then determined. Inhibition of tetanolysin activity by lucensomycin was performed by incubating mycoplasma cells with 6 to 12 μg/ml of the antibiotic for 15 min at 37 °C. The cells were then centrifuged, resuspended in "phosphate-salt" solution, and examined for their sensitivity to tetanolysin.

*Isolation of mycoplasma cell membranes.* Cell membranes were isolated following osmotic lysis of the organisms [15]. The membrane preparations were washed once with deionized water and incubated with 50 μg/ml of deoxyribonuclease (Sigma, St. Louis, Missouri) for 15 min at 37 °C. The membranes were then washed 5 times in

0.025 M sodium phosphate buffer, pH 7.0, resuspended in 2–4 ml of deionized water and stored at −20 °C until used.

*Proteolytic digestion and lipid extraction of cell membrane preparation.* Suspensions of isolated membranes (2.5 mg membrane protein/ml) were treated with 200 μg/ml of pronase (B grade, Calbiochem, Los Angeles, California) for 18 h at 37 °C. To avoid bacterial contamination, sodium azide was added to the membrane suspension to a final concentration of 0.01 %. The digested membranes were collected by centrifugation at 15 000 × *g* for 60 min and washed twice with 5 ml of "phosphate-salt" solution. Lipids were extracted from wet pellets of membranes (containing about 5 mg membrane protein) by two successive extractions with chloroform/methanol (2 : 1, v/v), at 45 °C for 2 h. The extracts were discarded and the extracted membranes were washed twice and resuspended in the "phosphate-salt" solution. The pronase-digested and the lipid-extracted membranes were analyzed for protein [12] and for cholesterol [13].

*Binding of tetanolysin to membranes.* Serial two-fold dilutions of native, digested, or lipid-extracted membrane preparations were made in the "phosphate-salt" solution. To 0.1 ml volumes of each of the dilutions (0.8–200 μg protein/ml), 0.2 ml of a tetanolysin preparation (containing 100–200 hemolytic units/ml) was added and the mixture was incubated for 2 h at room temperature. The resulting membrane-tetanolysin complex was sedimented by centrifugation at 37 000 × *g* for 15 min. The amount of tetanolysin bound to the membranes was obtained indirectly by determining the residual free tetanolysin activity in the supernatant fluid. In some experiments tetanolysin activity in the reaction mixture was determined without a prior sedimentation of the membrane-tetanolysin complex.

*Polyacrylamide-gel electrophoresis.* The sodium dodecyl sulfate gel electrophoresis procedure of Fairbanks et al. [16] was employed with some modifications. Tetanolysin preparations containing 50–200 μg protein were freeze-dried and then solubilized by boiling for 1 min in 0.1–0.2 ml of a solution containing 1 % sodium dodecyl sulfate, 1 mM EDTA, 2 % 2-mercaptoethanol, 10 mM Tris · HCl, pH 8.0, 3.6 M urea, 10 % glycerol and 30 μg/ml Bromophenol blue. Samples (40 μl) of the solubilized material (25–50 μg protein) were placed in wells of 7 % polyacrylamide gel slabs (7.5 cm × 7.5 cm × 0.3 cm) prepared according to Fairbanks et al. [16]. Electrophoresis was carried out in a Pharmacia gel electrophoresis apparatus (Pharmacia, Uppsala, Sweden) for 4–5 h at room temperature using the sodium dodecyl sulfate-buffer system [16], and a current of 30 mA per slab. The gels were fixed and stained with Coomassie blue R-250 [16]. Destaining was obtained by incubating the gels in 10 % acetic acid for 6–8 h at 40 °C with constant stirring. The destaining solution was changed frequently during this period. Molecular weight of polypeptide bands was estimated by comparison to a calibration curve prepared with bovine serum albumin (mol. wt. 67 000), ovalbumin (mol. wt. 45 000), chymotrypsinogen (mol. wt. 25 000) and ribonuclease A (mol. wt. 13 700). All protein standards were products of Worthington Biochemical Corporation (Freehold, N.J.).

*Paramagnetic resonance spectrometry.* For paramagnetic resonance spectrometry studies, a pellet of isolated membranes (1–2 mg membrane protein) was resuspended in 1 ml of the "phosphate-salt" solution containing 100–200 hemolytic units/ml of tetanolysin. After 30 min of incubation at 37 °C the membranes were sedimented by centrifugation at 37 000 × *g* for 30 min. The procedure was then repeated 5 ad-

ditional times with fresh tetanolysin preparations. The final tetanolysin-treated membranes were spin-labeled with 5-nitroxystearate or 12-nitroxystearate (Syvo, Palo Alto, Calif.) by exchange from bovine serum albumin [17]. EPR spectra of the spin-labeled membranes were obtained by a Varian E-3 spectrometer. Results are expressed as the hyperfine splitting ($2T_{||}$) or as $\tau_0$, an empirical motion parameter calculated according to Henry and Keith [18] from the expression: $\tau_0 = 6.5 \cdot 10^{-10} \times W_0[h_0/h_{-1})^{\frac{1}{2}} - 1]$ where $W_0$ is the line width of the mid field and $h_0$ and $h_{-1}$ are the heights of the mid and high field line on a first derivative absorption spectra. Greater freedom of motion is associated with smaller values of $2T_{||}$ or $\tau_0$.

## RESULTS

### *Adaptation of M. capricolum to growth in a cholesterol-poor medium*

*M. capricolum*, a sterol-requiring *Mycoplasma*, was adapted to grow in a modified Edward medium [10] containing 0.5 % fatty acid-poor bovine serum albumin, and as little as 0.5 μg/ml cholesterol. The adaptation was achieved after 10 serial transfers of the organisms in the media containing decreasing concentrations of cholesterol (20–0.5 μg/ml). A single transfer of the adapted strain in a media containing high concentration of cholesterol caused a loss of its ability to grow with low cholesterol concentrations and serial passages were again needed for adaptation. A marked decrease in the cholesterol content of the cells occurred upon the adaptation. The native strain grown with 2 % PPLO Serum Fraction contained 90–110 μg cholesterol per mg cell protein as against 10–15 μg cholesterol per mg cell protein of the adapted strain.

### *Effect of tetanolysin on mycoplasma cells*

Fig. 1 shows that, whereas the native *M. capricolum* cells were lysed by the

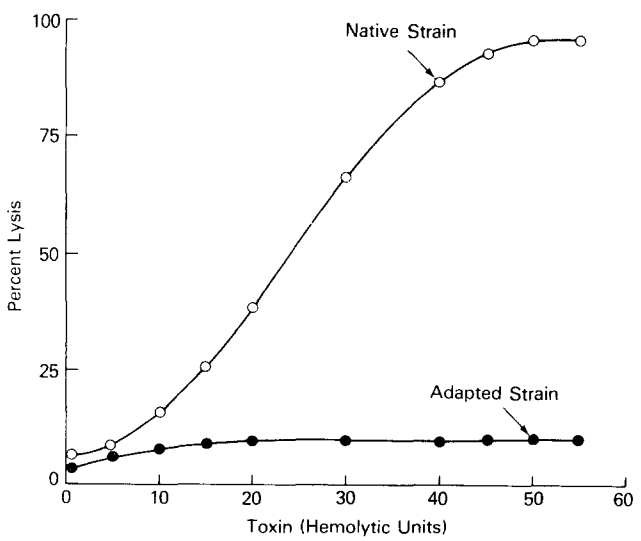


Fig. 1. The susceptibility of the native and adapted *M. capricolum* cells to lysis by tetanolysin preparation.

TABLE I

THE EFFECT OF TETANOLYSIN PREPARATION ON *M. CAPRICOLUM* AND *A. LAIDLAWII* CELLS

Cells were grown in modified Edward medium containing 2% PPLO serum fraction and [*Me*-$^3$H]-thymidine. The cells were harvested, washed in 0.25 M NaCl, resuspended in the different media and incubated at 37 °C for 30 min. The absorbancy of the cells was then measured and after centrifugation radioactivity was determined in the supernatant fluid. HU, hemolytic units.

| Medium | *M. capricolum* | | *A. laidlawii* | |
|---|---|---|---|---|
| | Absorbancy at 500 nm | Radioactivity released (% of total) | Absorbancy at 500 nm | Radioactivity released (% of total) |
| 0.25 M NaCl | 0.65 | 6 | 0.58 | 7 |
| 0.25 M NaCl+10 HU/ml tetanolysin | 0.41 | 16 | 0.57 | 7 |
| 0.25 M NaCl+50 HU/ml tetanolysin | 0.13 | 30 | 0.54 | 8 |
| Deionized water | 0.18 | 34 | 0.08 | 85 |

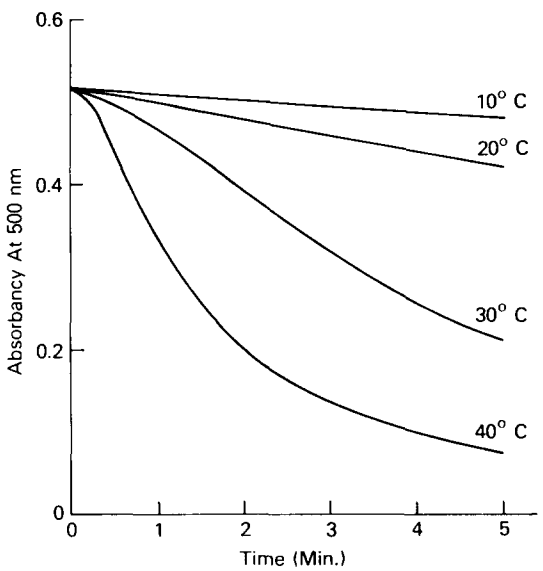


Fig. 2. The effect of temperature on the lysis of *M. capricolum* cells by tetanolysin (40 hemolytic units/ml).

tetanolysin preparation, the adapted cells containing small amounts of cholesterol were not. The sterol-nonrequiring *A. laidlawii* cells grown either in a cholesterol-rich or a cholesterol-poor medium were unaffected by the tetanolysin preparation. The cell lysis was determined either by the decrease in absorbancy of the cell suspension at 500 nm, or by the release of thymidine-labeled components from cells grown with [*Me*-$^3$H]thymidine (Table I). Unlike *A. laidlawii* cells, the radioactivity released from *M. capricolum* cells, even after a complete lysis by an osmotic shock, did not exceed 30–35% of the total radioactivity of the cells, indicating that a great part of *M.*

*capricolum* cellular DNA is tightly associated with cell membrane fragments. Lysis of *M. capricolum* cells by the tetanolysin preparation was temperature-dependent (Fig. 2), and was completely inhibited by either passing the tetanolysin through a column of packed crystals of cholesterol or by incubating the tetanolysin preparation with cholesterol. No lysis of the native *M. capricolum* cells occurred with heated (5 min at 100 °C) tetanolysin preparation. $Mg^{2+}$ partially protected *M. capricolum* cells against lysis by the tetanolysin preparation, but the protection was less effective than that against osmotic lysis. The sensitivity of *M. capricolum* cells to lysis by the tetanolysin preparation was decreased by preincubating the cells (15 min at 37 °C) with sublytic concentrations of the polyene antibiotic lucensomycin (Fig. 3), and was markedly affected by the age of the culture (Table II). Early logarithmic phase cells were more sensitive, decreasing sharply toward the end of the logarithmic phase of growth. Table II shows also that the effect of the age of the culture on the sensitivity of *M. capricolum* cells to lysis by the tetanolysin preparation was more pronounced than cell lysis by osmotic shock. Thus, cells from the late logarithmic phase of growth, though still sensitive to osmotic shock, were unaffected by the tetanolysin preparation. In addition, the susceptibility of the cells to lysis was influenced by the lot of serum fraction used for growth. Lots of serum fraction did vary in their cho-

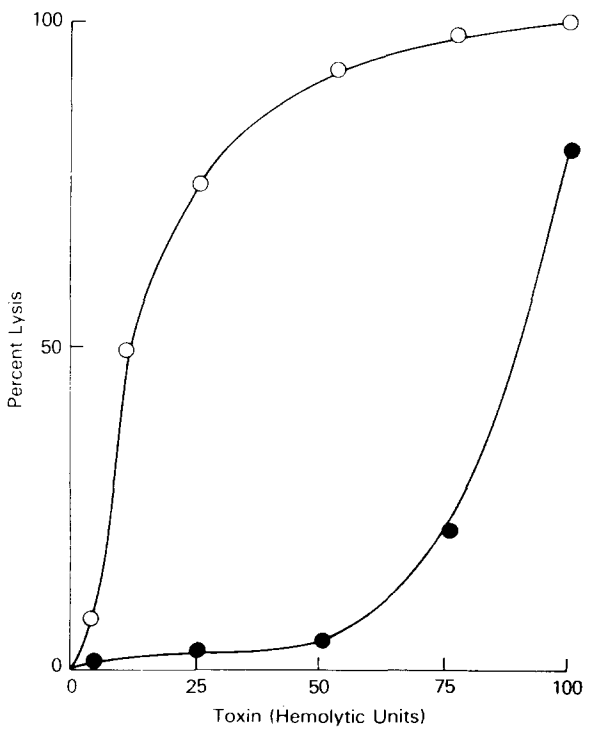


Fig. 3. The inhibition of the tetanolysin-induced cell lysis by lucensomycin. *M. capricolum* cells were incubated with lucensomycin (60 $\mu$g per mg cell protein) for 15 min at 37 °C. The cells were then sedimented by centrifugation at 12 000 $\times g$ for 20 min, resuspended and their susceptibility to tetanolysin was determined. ○, untreated cells; ●, cells treated with lucensomycin.

TABLE II

THE EFFECT OF TETANOLYSIN PREPARATION ON *M. CAPRICOLUM* CELLS HARVESTED AT DIFFERENT AGES OF CULTURE

The cells were grown in a modified Edward medium. Tetanolysin activity and osmotic fragility are presented as % lysis in 40 hemolytic units/ml or 0.05 M NaCl, respectively.

| Age of culture (h) | Absorbancy of culture (640 nm) | Percent lysis | |
|---|---|---|---|
| | | Tetanolysin activity | Osmotic fragility |
| 12 | 0.06 | 92 | 83 |
| 18 | 0.26 | 36 | 85 |
| 24 | 0.31 | 14 | 5 |

lesterol, $Mg^{2+}$ and $Ca^{2+}$ content. Cells grown in some batches of serum fraction appeared more fragile than others and care in washing was required. Since the susceptibility of the cells to lysis was greatest immediately after harvesting and resuspension, the cells required use as quickly as possible. When *M. capricolum* cells grown with [$^{14}$C]cholesterol were subjected to lysis with the tetanolysin preparation, the radioactivity was found exclusively in the membrane fraction rather than in the soluble fraction of the cell, suggesting that cholesterol is not removed from the membranes as a result of its interaction with the tetanolysin preparation.

*Binding of tetanolysin activity to M. capricolum membrane preparations*

*M. capricolum* membranes were capable of binding the tetanolysin activity, resulting in a decrease in the hemolytic activity of the preparation. Fig. 4 shows the

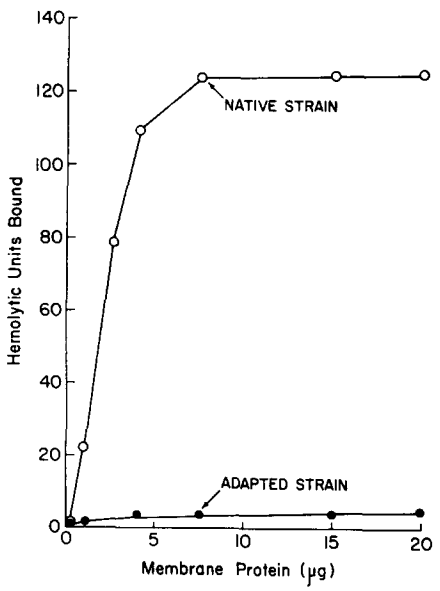


Fig. 4. Binding of tetanolysin activity to isolated membranes of *M. capricolum* native and adapted strains.

high binding capacity of membranes of the native strain and the low binding capacity of membranes of the adapted *M. capricolum* strain. Due to the low activity of the tetanolysin preparations, saturation of binding sites on the membranes was determined using a constant solution ($\approx$ 150 hemolytic units) of tetanolysin and varying concentrations of membranes. Under these conditions maximal binding levels of 25–30 hemolytic units per $\mu$g membrane protein were obtained. Although aged mycoplasma cells were not lysed by the tetanolysin preparation (see Table II), binding of the tetanolysin preparation to membranes from stationary phase cells was only 20–25% lower than to membranes from early logarithmic phase cells. Due to the low activity of the tetanolysin preparation (100–200 hemolytic units/ml) and our inability to obtain a rapid separation of the membrane fragments from the residual free tetanolysin, kinetic and physical studies could not be accurately performed. Throughout our study, membranes were separated from free tetanolysin by centrifugation at 37 000 $\times$ *g* for 30–45 min. Attempts to obtain a rapid separation by filtration through Millipore (0.22 $\mu$m) filters failed, due to the inactivation of the tetanolysin upon filtration. Semi-quantitative experiments showed that binding of tetanolysin to *M. capricolum* membrane preparation at 4, 22, or 37 °C occurred at similar rates. Due to the sensitivity of the tetanolysin preparation to prolonged incubation at 37 °C, most binding experiments were performed at room temperature.

Table III shows that the binding of the tetanolysin activity to *M. capricolum* membrane preparation was not affected by removing 70% of the Lowry reactive material from the membranes by digestion with pronase, but was completely inhibited by removing 93% of membrane cholesterol by extracting the membranes with chloroform/methanol (2 : 1).

The electrophoretic patterns of the partially purified tetanolysin preparation before and after binding to *M. capricolum* membranes are shown in Fig. 5. The native tetanolysin preparation (Fig. 5, gel 1) contained five polypeptide bands (A, B, C, D and $D_2$). After incubation of the tetanolysin preparation with pronase-digested membranes (Fig. 5, gel 2), Bands A (mol. wt. 44 000) and B (mol. wt. 42 000) completely disappeared. The pattern obtained with a native membrane preparation (Fig. 5, gel 3) was different from that obtained with pronase-digested membranes, mainly in the appearance of two polypeptide bands, a diffuse band in the region of Band B and

TABLE III

BINDING OF TETANOLYSIN ACTIVITY TO NATIVE, PRONASE-DIGESTED AND LIPID-EXTRACTED *M. CAPRICOLUM* MEMBRANES

| Preparation | Protein ($\mu$g) | Cholesterol ($\mu$g) | Tetanolysin activity bound (hemolytic units) |
|---|---|---|---|
| Native membranes | 50.0 | 15.0 | 125 |
| | 6.2 | 1.8 | 110 |
| Pronase-digested membranes | 15.0 | 14.0 | 120 |
| | 1.8 | 1.8 | 100 |
| Lipid-extracted membranes | 42.0 | 1.0 | 5 |
| | 5.2 | 0.1 | 3 |

a band in the region of $B_1$ (mol. wt. 36 000). Polypeptide bands similar in mobility and intensity to those released by the tetanolysin preparation could be shown in gels of *M. capricolum* membranes. Neither the binding of the tetanolysin polypeptides nor the release of polypeptides from the native membranes occurred in controls containing a heat-inactivated (100 °C, 5 min) tetanolysin preparation, indicating that an active tetanolysin is required for both binding and release.

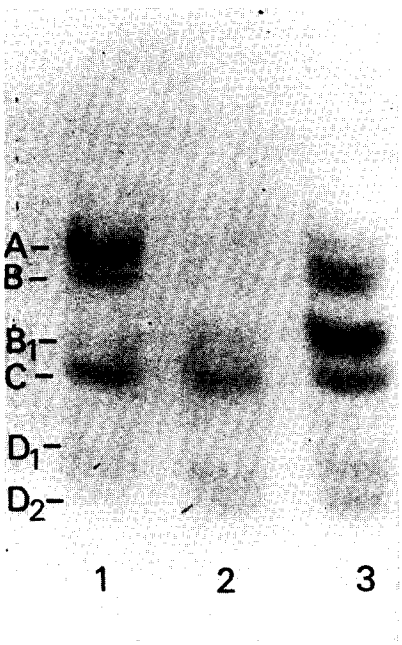


Fig. 5. Electrophoretic patterns in polyacrylamide gels containing sodium dodecyl sulfate of the partially purified tetanolysin preparation (1) and the residue recovered from the supernatant fluid after treating the tetanolysin with pronase-digested (2) or native (3) *M. capricolum* membranes.

*Paramagnetic resonance spectroscopy*

Fig. 6 shows the pronounced temperature dependence of the hyperfine splitting $2T_{||}$ of 5-nitroxystearate in *M. capricolum* membranes treated and untreated with the tetanolysin preparation. At temperatures of 20-50 °C the hyperfine splitting was smaller with the tetanolysin-treated membrane preparation, indicating a greater freedom of motion of the spin-labeled fatty acid in the treated membranes. The greater freedom of motion in the tetanolysin-treated membranes was further demonstrated by calculating $\tau_0$, the empirical motion parameter, from spectra of membrane preparations labeled with 12-nitroxystearate (Table IV). When $\tau_0$ values were plotted versus $°K^{-1}$, a straight line with no transition points was obtained for both treated and untreated membrane preparations.

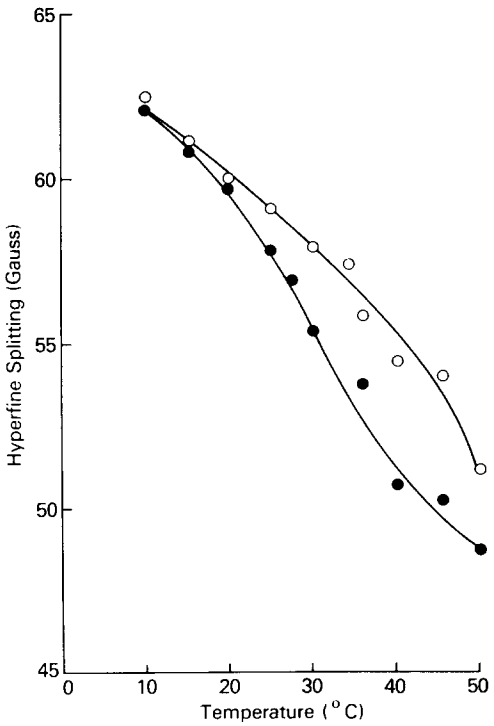


Fig. 6. Temperature dependence of the hyperfine splitting ($2T_{||}$) of 5-nitroxystearate in untreated (○) and in tetanolysin-treated *M. capricolum* membranes (●).

TABLE IV

THE EFFECT OF TETANOLYSIN PREPARATION ON THE MOTION PARAMETERS OF *M. CAPRICOLUM* NATIVE AND PRONASE-DIGESTED MEMBRANES LABELED WITH 12-NITROXYSTEARATE

Values were calculated from EPR spectra obtained at 37 °C.

| Preparation | Motion parameter ($\tau_0 \cdot 10^{-10}$ s) | |
|---|---|---|
| | With tetanolysin | Without tetanolysin |
| Native membranes | 65.5 | 82.1 |
| Digested membranes | 61.7 | 73.4 |

## DISCUSSION

The data presented indicates that the binding site of tetanolysin is cholesterol. We have shown that the native strain of *M. capricolum* containing appreciable quantities of cholesterol (100 μg per mg cell protein) was readily lysed by partially purified tetanolysin preparations whereas the adapted mycoplasma strain containing very low amounts of cholesterol was not lysed. Moreover, the binding of tetanolysin to membranes of *M. capricolum* was not affected by removing 70 % of the membrane pro-

teins by pronase but was greatly reduced by removing lipids from membranes by extraction with chloroform/methanol, supporting the concept that the binding site of tetanolysin resides in the lipid domain. We have also shown that the lysis of *M. capricolum* can be inhibited by prior exposure to cholesterol and to lucensomycin, a polyene antibiotic known to interact specifically with membrane cholesterol [19]. Our findings are in agreement with other studies using streptolysin *O* [4, 6], cereolysin [6], and $\theta$ toxin of *Clostridium perfringens* [20] and support the concept that cholesterol is a common binding site for the oxygen-labile hemolysins of bacteria [1].

Although the lysis of intact mycoplasma cells was dependent on temperature and was reduced with aged cells, the binding of tetanolysin to isolated membrane preparations was not. The primary event, therefore, appears to be the formation of a tetanolysin-cholesterol complex in mycoplasma membranes, an event which is unaffected by temperature and age of cells, whereas the subsequent events which lead to the lysis of cells are affected. It would appear that the tetanolysin-cholesterol primary complexes tend to form aggregates which lead to cell lysis, as was suggested for the filipin-cholesterol interactions in membranes [21]. The aggregation of complexes would proceed via the lateral diffusion of primary complexes in the lipidous matrix of the membrane, a diffusion which is controlled by membrane fluidity [22]. Therefore, it may follow that the formation of aggregates would be restricted in aged cells or at low temperatures when hydrocarbon chains are at low thermal motion [23].

The increase in lipid chain mobility of tetanolysin-treated mycoplasma membranes, observed by EPR analyses, indicates that tetanolysin may shield cholesterol from its interactions with the polar lipids in membranes, interactions which are known to restrict chain mobility [24]. However, membrane proteins have also been shown to restrict lipid chain mobility [25] and, therefore, one must consider the possibility that increased chain mobility may be due, in part, to the release of membrane polypeptides as a result of tetanolysin injury (see Fig. 6).

The mechanism of mycoplasma cell lysis by the tetanolysin preparation may be the result of decreased resistance of the membrane to brittle fracturing because of the reduced molecular cohesion of membrane constituents in the absence of free cholesterol, rather than to the formation of pits and holes in the membrane [26]. Therefore, since cholesterol is not required to maintain the integrity of acholeplasma membrane lipids, the interaction of the tetanolysin or streptolysin *O* [4] with *A. laidlawii* cells will not result in cell lysis. It is possible, however, that the resistance of *A. laidlawii* to tetanolysin may be due to the fact that the cholesterol in acholeplasma cells is not accessible to the tetanolysin preparation because of either the masking effects by other membrane components or because the cholesterol is localized mainly in the inner half of the lipid bilayer of the membrane. The concept that cholesterol present in *A. laidlawii* is inaccessible to tetanolysin gains support from the observations of Pendleton et al. [27] who used antibody ferritin labeling technique to demonstrate that streptolysin *O* and cereolysin can bind to *M. gallisepticum* but not to *A. laidlawii*. The tetanolysin preparation used throughout our study was a partially purified preparation consisting of as many as five polypeptide bands as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis and had a low hemolytic activity ($\approx$ 200 hemolytic units/ml). Only the two major bands (Band A, mol. wt. 45 000, and Band B, mol. wt. 42 000) were adsorbed to *M. capricolum* membranes,

but it is not known whether one or both of the bands are required for the lytic activity of the tetanolysin. In addition to the hemolytic activity, the tetanolysin preparations also contained low neurotoxic, lipolytic and proteolytic activities (Hardegree, M. C., unpublished data) and it is possible that these or other unknown components were responsible for the release of the two membrane polypeptides from membranes treated with the tetanolysin preparation.

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