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Effect of Teichoic Acid on Resistance to the Membrane-lytic Agent of *Streptococcus zymogenes*

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ABSTRACT

DAVIE, JOSEPH M. (Indiana University, Bloomington), AND THOMAS D. BROCK. Effect of teichoic acid on resistance to the membrane-lytic agent of *Streptococcus zymogenes*. J. Bacteriol. **92**:1623–1631. 1966.—The resistance of *Streptococcus zymogenes* to its own lytic agent has been shown to be due to the production of a specific, inhibitory teichoic acid. A survey of streptococcal strains showed that only strains resistant to the lytic agent produced the specific inhibitor. In addition, the inhibitor can be removed from spheroplasts of resistant strains, thereby making them sensitive to the lysin. Throughout the early part of the growth cycle, the inhibitor is associated with the cell and cannot be found in the medium. During late logarithmic phase, however, the inhibitor is released into the medium by the cells, and therefore is a contributing factor to the apparent lability of the lytic agent. The purified, inhibitory teichoic acid contains ribitol, phosphate, glucose, and D-alanine. The alkaline lability of the biological activity of the teichoic acid was correlated with the hydrolysis of the D-alanine. A streptococcal strain which is sensitive to the membrane-lytic agent produced an inactive ribitol teichoic acid which lacks the ester-linked D-alanine, whereas a lysin-resistant mutant of this strain produces a teichoic acid which contains D-alanine and which has inhibitory activity.

Streptococcus faecalis var. *zymogenes* produces a lytic substance which is active against membranes of gram-positive bacteria and erythrocytes, and which has been termed, accordingly, both a hemolysin and a bacteriocin (2, 3). Most gram-positive bacteria are sensitive to the lytic agent, but *S. zymogenes*, the producing species, is completely resistant. It was, therefore, of interest to determine the mechanism by which *S. zymogenes* resists the action of its own lysin. The data in the present paper indicate that *S. zymogenes* produces a specific inhibitory material which neutralizes the lytic substance. The specific inhibitor, elaborated by all resistant strains of *S. zymogenes*, was not detected in any sensitive strain. It is probable that the inhibitor accumulates in the culture medium during late logarithmic phase of growth, and that it inactivates the soluble lytic agent. The inhibitor was purified and identified as a teichoic acid containing ribitol, phosphate, glucose, and ester-linked D-alanine. These data suggest a biological property of certain teichoic acids which may be important in the protection of the cell against the destructive action of membrane lytic agents.

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MATERIALS AND METHODS

Organisms. The strains of streptococci used in this study have been described previously (2, 3, 6). Briefly, *S. zymogenes* X14 produces the lytic agent (hemolysin-bacteriocine), and *S. faecium* X13 and *S. faecalis* X46 are both sensitive to the lytic agent. An S following the strain number indicates that the strain is resistant to 1 mg of streptomycin per ml.

Media and buffers. Todd-Hewitt (Difco) broth and 1.5% agar were used throughout. Malonate buffer consisted of 0.05 M disodium malonate, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.0001 M ethylenediaminetetraacetate (EDTA), and 1 g of streptomycin per liter (*p*H 7.0). Acetate buffer consisted of 0.2 M sodium acetate (*p*H 6.0). Tris buffer consisted of 0.01 M tris(hydroxymethyl)aminomethane (*p*H 7.0). Three phosphate buffers were used: 8.70 g of KH_2PO_4 and 4.90 g of K_2HPO_4 per liter (*p*H 5.5); 3.60 g of KH_2PO_4 and 7.13 g of K_2HPO_4 per liter (*p*H 7.0); and 0.29 g of KH_2PO_4 and 11.2 g of K_2HPO_4 per liter (*p*H 8.2).

Chemical assays. Protein was measured by the Lowry method (10) with bovine serum albumin as a standard. Total amino acid was measured by the method of Moore and Stein (13) with DL-alanine as a

standard. Total phosphorus was measured by the method of Chen et al. (5) with inorganic phosphate as a standard. Total hexose was measured by the anthrone reaction (20) with glucose as a standard. Glucose was measured by use of the Glucostat reagent (Worthington Biochemical Co., Freehold, N.J.). Pyruvic acid was measured by the 2,4-dinitrophenylhydrazine reaction (9).

Assays for the lytic agent. Production of the lytic agent and assays for both its hemolytic and bactericidal activities have been described previously (2, 6).

Inhibitor assay. The test solution was diluted 1:2 serially in 0.05 ml of malonate buffer by use of the 0.05-ml Takatsy microtitrator loop (International Scientific Corp., New York, N.Y.). A 0.2-ml amount of hemolysin (1 hemolytic unit per ml) and 0.25 ml of 0.8% washed horse erythrocyte (Colorado Serum Co., Denver, Colo.) suspension were added. After mixing, the suspensions were incubated at 37 C until the control tube (hemolysin without inhibitor) lysed. This usually required about 120 min. The last tube which showed no hemolysis was considered the end point, and its dilution served as the titer of the inhibitor. The reciprocal of the dilution represents the number of inhibitor units in that preparation.

Preparation of cell fractions. The following method of preparation of cell fractions was used in all experiments except for the purification of the inhibitor. Cells from a 1-liter culture of actively growing streptococci (optical density, 0.600 at 660 mμ) were harvested, washed twice with phosphate buffer (*p*H 7.0), and resuspended in 10 ml of the same buffer. The suspension was then treated for 90 sec in a Nossal disintegrator (McDonald Engineering Co.,) with an equal volume of Ballotini beads which had been washed previously with 0.1 N HCl and distilled water. This method gave disruption of at least 90% of the bacteria as determined by phase microscopy. Cell walls were sedimented by centrifugation at 4,000 $\times$ *g* for 1 hr. The membrane fraction was then sedimented by centrifugation at 20,000 $\times$ *g* for 30 min. The supernatant fluid was operationally defined as cytoplasm (8).

Thin-layer chromatography. Silica gel G (Research Specialties Co., Richmond, Calif.) was prepared in water, and the plates were dried at 110 to 115 C for 30 min. The following solvent systems were used: solvent A, 1-propanol-ammonia (d, 0.88)–water, 6:3:1 (1); solvent B, chloroform-methanol–17% NH_4OH, 2:2:1; solvent C, phenol–water, 3:1 (w/w). Sugars were made visible by spraying with ammoniacal $AgNO_3$ (21); polyols, by ammoniacal $AgNO_3$, followed by heating at 110 C for 1 hr; and amino acids, by a modified ninhydrin solution (12).

RESULTS

Preliminary studies on the nature of the inhibitor. Preliminary evidence indicated that certain cultures of *S. zymogenes* produced an inhibitor of the lytic agent. There is a rapid loss in activity of the lytic agent during late logarithmic growth phase of the culture. Furthermore, of all gram-positive organisms tested, only those strains of *S. zymogenes* which produce the lytic agent are resistant to its action.

Inhibitory activity was identified in a bacterial fraction by the following procedure. *S. zymogenes* X14, the resistant strain, was disrupted and fractionated by differential centrifugation into cell walls, membranes, and cytoplasm. Each fraction was tested for its inhibitory effect on hemolysin activity of the lytic agent. The fractions containing cell walls and membranes had little or no inhibitory effect on hemolysin activity. However, the cytoplasmic fraction, a potent inhibitor, neutralized approximately 1,000 units of hemolysin. Similar fractions prepared from *S. faecium* X13, a sensitive strain, had little effect on hemolysin activity. As will be shown later, the small amount of inhibitory activity from the sensitive organism was nonspecific inhibition.

Crude cytoplasmic fractions were employed to determine the properties of the inhibitor. The inhibitor was not recovered in the 20,000 x *g* pellet of the cytoplasmic fraction, but was sedimented at 76,000 $\times$ *g*. The inhibitor was stable for 1 hr at 60 C, but lost 50% of its activity after heating at 98 C for 30 min. Although the inhibitor was not inactivated by exposure to 0.1 N HCl for 30 min at 0 C, it exhibited a half-life of 5 min in 1 N HCl at 90 C. The inhibitor was completely inactivated by 0.1 N NaOH at 0 C in 30 min, and was labile to boiling at *p*H 9 for 5 min. Treatment of the inhibitor with the following enzymes under the appropriate conditions caused no loss in activity: trypsin, ribonuclease, deoxyribonuclease, alkaline phosphatase, and phospholipase C and D. The lack of effect of the last two enzymes suggested that the activity of the inhibitor was not dependent upon a phospholipid moiety.

Periodate oxidizes compounds containing vicinal hydroxyl groups. The inhibitor was exposed to 0.5 M sodium metaperiodate at 0 C in the dark, and samples, removed at timed intervals, were diluted into 10% glucose to stop oxidation. Complete loss of inhibitory activity occurred within 1 min. These findings, together with the fact that the inhibitor binds strongly to diethylaminoethyl cellulose, but not to carboxymethyl cellulose (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), suggest that the inhibitor is an acidic carbohydrate.

Purification and identification of the inhibitor. The heat stability, alkaline lability, periodate sensitivity, and acidic nature of the inhibitor suggested that it might be a teichoic acid. The following procedures were employed to isolate the inhibitor from disrupted bacteria and to identify its carbohydrate nature.

Purification was achieved by a method similar to that used by Burger and Glaser for the isolation of polyglycerol teichoic acid from *Bacillus* (4). A 14-liter amount of Todd-Hewitt broth was inoculated with 1 liter of an overnight culture of strain X14, and was incubated at 37 C without shaking or aeration until the optical density reached 0.600 when measured at 660 mμ in a colorimeter (Spectronic-20, Bausch & Lomb, Inc., Rochester, N.Y.). The culture was then cooled in ice water, and the cells were collected by continuous-flow centrifugation at 12,000 × *g*. The total yield consisted of 3.3 × 10^{13} cocci. The cells were washed, resuspended in 100 ml of Tris buffer (*p*H 7), and disrupted by sonic oscillation (model DF101, Raytheon Manufacturing Co., Waltham, Mass.) for 3 hr at 1.20 amp at 2 C. The suspension of disrupted bacteria was centrifuged at 12,000 × *g* for 10 min to remove the larger particulate matter and whole cells. The supernatant fluid was recovered, and the pellet was washed with 20 ml of Tris buffer. After centrifugation, the wash was combined with the initial supernatant fluid. An analysis of this preparation, as well as those achieved with additional purification, for inhibitor activity and chemical content is presented in Table 1. The inhibitory activity was recovered from the supernatant fluid by centrifugation at 100,000 × *g* for 1 hr. After washing, the active pellet was resuspended in 15 ml of Tris buffer (*p*H 7). The bulk of the inhibitory activity was recovered in the 100,000 × *g* pellet, and only minimal activity was detectable in the supernatant fluid.

Further purification of the inhibitor preparation was achieved by extraction with phenol. The 100,000 × *g* pellet was mixed with an equal volume of 88% phenol and was stored at 4 C for 14 hr. After centrifugation at 12,000 × *g* for 10 min, the aqueous phase containing inhibitor was removed, the phenol layer was decanted, and the precipitate was discarded. The phenol layer was washed with an equal volume of water, and the aqueous phase, after centrifugation, was pooled with the initial aqueous extract. This material was shaken at room temperature for 1 hr with an equal volume of chloroform. The aqueous phase was dialyzed against three changes of 300 volumes of distilled water at 2 C, allowing at least 6 hr per change for equilibration. The dialyzed product of inhibitor contained 27.5 mg of protein, material absorbing at 260 mμ, and a considerable amount of inhibitor activity.

The final preparation of purified inhibitor, devoid of protein and nucleic acid, was achieved by starch block electrophoresis of the inhibitor dialysate. A 1-ml amount of the dialyzed material was added to a starch block in phosphate buffer (*p*H 5.5) and was subjected to 100 v (4 v/cm) and 0.5-ma current for 48 hr at 2 C. Sections (1 cm) were eluted with 2 ml of tris(hydroxymethyl)aminomethane (Tris) buffer at 2 C, and the eluate was assayed for 260 mμ absorption and inhibitor activity. The inhibitor activity was found in three peaks (Fig.1). The inhibitor in the peak near the origin had probably complexed with basic or insoluble materials. A second peak was found in the fractions which contained 260 mμ-absorbing material, and probably represented ribonucleic acid (RNA)-bound inhibitor, similar to that reported by Wicken, Elliott, and Baddiley for glycerol teichoic acid (22). The third and largest peak, which moved most rapidly toward the anode, was free of 260-mμ absorbing material. The fractions of this peak were centrifuged at 27,000 × *g* for 10 min to remove particulate starch, and were dialyzed to remove contaminating glucose. The final product was employed for chemical and biological studies.

As shown in Table 1, the hydrolysate of the purified inhibitor contained 0.60 μmoles of glucose per ml and 0.57 μmole of phosphate per ml. The teichoic acid nature of the inhibitor is sup-

TABLE 1. *Summary of purification of inhibitor from Streptococcus zymogenes strain X14*[a]

Prepn	Total inhibitor units	Total protein	Total hexose	Total Pi	Units of inhibitor/ mg of Pi	Purification
		mg	*mg*	*mg*		
Disrupted bacteria	105,000	1,030.	90.	16.4	6,400	—
Precipitate (100,000 × *g*)	76,000	92.5	16.5	6.0	12,800	2
Phenol-extracted material	195,000	27.5	17.	5.5	37,000	5.8
Starch eluate[b]	97,500	<0.4	1.37[c]	0.690	143,000	22.4

[a] The samples were assayed without hydrolysis for protein and hexose; inorganic phosphorus was determined after hydrolysis in 1 N $HClO_4$ for 3 hr at 100 C.

[b] Data from peak III of the electrophoresis run. Values shown were obtained by multiplying data from sample no. 23 times 38.1, to correct for the fact that only 1 ml of the dialyzed material was subjected to electrophoresis.

[c] Measured as glucose.

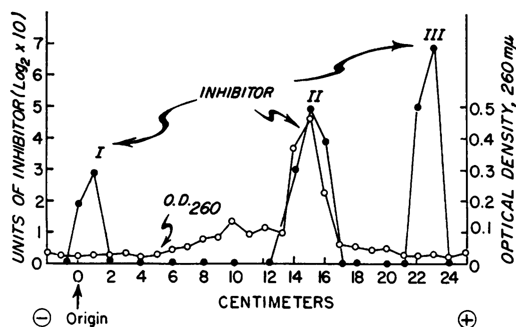

FIG. 1. *Starch block electrophoresis of inhibitor. Starch sections were eluted with 0.01 M Tris buffer (pH 7). Eluate was assayed for 260-mμ absorption and inhibitor concentration.*

ported by its ribitol content, as shown by thin-layer chromatography. Although glucose and ribitol have similar R_F values in most solvent systems, complete separation of two compounds was achieved on Silica gel G with use of solvent A. The spots corresponding to glucose and ribitol were of equal size and intensity, indicating that the inhibitor contains similar amounts of glucose and ribitol. Another difference between ribitol and glucose is the lack of staining of ribitol with anisidine-phthalate (17). The spot in the unknown corresponding to ribitol did not stain with anisidine-phthalate. In addition, the hydrolysate of the inhibitor did not react with glycerol dehydrogenase.

In some solvent systems, the hydrolysate of the inhibitor had a prominent spot which did not correspond to either ribitol or glucose. Ribitol reacts when treated with 1 N $HClO_4$ for 3 hr at 100 C to give 1,4-anhydroribitol (1), and anhydroribitol has the same R_F as the spot in the unknown. This is an additional indication that the inhibitor contains ribitol.

It will be recalled that inhibitor activity was lost when extracts were heated 5 min in 0.01 N NaOH. It is conceivable that loss of activity is dependent upon release of amino acid, because teichoic acids frequently possess ester-linked amino acids which are labile to alkali treatment. Alanine liberated from the inhibitor by alkali was detected by thin-layer chromatography. The chromatogram was developed in two dimensions; first, with solvent B, and, second, with solvent C. The plate was dried at 100 C for 10 min and was sprayed with a modified ninhydrin solution (12). One spot appeared which corresponded to alanine.

Another preparation of inhibitor, which contained 0.21 μmole of phosphate per ml and 0.22 μmole of glucose per ml, was analyzed quantitatively for amino acid content. Equal volumes of sample and 0.02 N NaOH were heated at 100 C for 5 min, and were neutralized. Both the alkali-treated sample and an unhydrolyzed control were assayed for amino acid by use of the quantitative ninhydrin method (13). The unhydrolyzed control had no detectable ninhydrin-positive material, whereas the alkali-treated sample contained 11.2 μg of amino acid per ml (based on alanine).

Proof of the identity of the amino acid was obtained by the use of radioactive compounds. The alkali-treated sample was desalted by passing the sample through a column of Dowex 2-X8 (200-400 mesh, Dow Chemical Co., Midland, Mich.) which had been prepared by the method of Dreze et al. (7). The column was washed with water, and the amino acid was eluted from the column with 1 N acetic acid. The acid eluate was dried under vacuum at 48 C, and the residue was dissolved in distilled water. Two-dimensional thin-layer chromatography of the desalted amino acid solution was done with use of solvents B and C. A 5-μliter amount of C^{14}-D-alanine (1 μc/ml; Calbiochem) was placed with the test spot before chromatographic development. Following chromatography, X-ray film was placed over the dried plate, and both were allowed to remain in the dark. At the end of 5 days, the chromatogram was sprayed with ninhydrin, and the X-ray film was developed. The ninhydrin spot and the spot on the X-ray were exactly superimposable, indicating that the unknown amino acid was alanine.

The optical configuration of alanine was determined as follows: D-amino acid oxidase (Sigma Chemical Co., St. Louis, Mo.), which converts D-alanine to pyruvic acid, was added to desalted alanine from the alkaline hydrolysate of the inhibitor in 0.5 ml of phosphate buffer (pH 8.2). The mixture was incubated at 37 C for 2 hr. An equal volume of 20% trichloroacetic acid was added to remove the protein, and the supernatant fluid was assayed for pyruvic acid (9). Almost quantitative conversion of the alanine to pyruvic acid was found, indicating that the teichoic acid contained only D-alanine.

These chemical data indicate that the purified inhibitor of *S. zymogenes* is a ribitol teichoic acid which consists of Pi, glucose, and D-alanine in a molar ratio of 1.00:1.05:0.62.

Production of inhibitor by other streptococcal strains. The data thus far suggest that the lytic agent produced by *S. zymogenes* is neutralized by a teichoic acid isolated from the same organism. The following experiments indicate that this property of the teichoic acid may be important for the survival of *S. zymogenes*, which elaborates the destructive lytic agent.

Earlier studies identified a low titer inhibitory material in extracts of strains of *S. faecium* which are sensitive to lytic activity; however, this inhibitory activity was not affected by alkali treatment. In addition, the inhibitor from *S. zymogenes* had a remarkable degree of sensitivity to dextran. A concentration as low as 0.05 μg/ml inactivates 50% of the inhibitory activity. The small quantity of inhibitory material from *S. faecium* was not affected by dextran, and it was concluded that the sensitive strain contained a nonspecific inhibitor such as lecithin or a lecithin-like material. With use of the criteria of neutralization by dextran and inactivation by 0.01 N NaOH, a survey of Group D streptococci was made for the presence of the inhibitor like that of *S. zymogenes*. Only *S. zymogenes* strains were resistant to the lytic agent, and only such resistant strains produced the specific inhibitor.

It was of special interest that spheroplasts of sensitive strains of streptococci are rapidly lysed by the lytic agent (6). However, resistant spheroplasts from strain X14 can be rendered sensitive to the lytic agent by suspension in acetate buffer at *p*H 6. In view of the fact that polyglycerol phosphate is removed from streptococci by acetate buffer (11), it is probable that similar treatment removed the inhibitory teichoic acid from the spheroplasts.

Production of inhibitor by resistant mutants of S. faecalis. Studies with resistant mutants derived from sensitive strains confirmed the correlation between resistance and production of inhibitor. *S. faecalis* strain X46S whole cells and spheroplasts, which are extremely sensitive to the lytic agent (6), lack the specific inhibitor. Six mutants of strain X46S which were resistant to the lytic agent were isolated (6), and the inhibitory properties of their cellular extracts were compared to those of the wild-type sensitive strain X46S and those of *S. zymogenes* strain X14. In each case, the resistant mutant produced an inhibitor similar to that of resistant strain X14.

The following studies were designed to compare the chemical composition of the inhibitory teichoic acid isolated from the resistant mutant to that of the noninhibitory teichoic acid isolated from the wild-type strain, with the end in view of identifying the chemical features responsible for the inhibitory property. The inhibitory material from resistant mutant strain X46S-R1 was purified and shown to be a teichoic acid. The method of purification was that used for resistant strain X14, except that the phenol extraction was performed on the 100,000 × *g* supernantant fluid rather than on the pellet, because preliminary tests indicated that the inhibitor was not sedimented at this force. Starch block electrophoresis of the dialysate again revealed three peaks of inhibitor activity. Samples of various stages in the purification of the inhibitor from strain X46S-R1 were assayed (Table 2). In addition, a noninhibitory teichoic acid from the sensitive strain X46S was purified and characterized chemically (Table 3). Because purification of the noninhibitory teichoic acid could not be monitored by assaying for inhibitory activity, the steps employed to achieve its purification were identical to those employed for the inhibitory material from strain X46S-R1. The starch block eluates of X46S and X46S-R1 were centrifuged, dialyzed, and hydrolyzed as described for the inhibitor from strain X14. By use of the same methods as before, the fractions were subjected to thin-layer chromatography. Ribitol and glucose were found in both X46S and X46S-R1 fractions.

The inhibitory activity of the teichoic acid from resistant strain X14 is destroyed by alkali under conditions which cleave off the ester-linked D-alanine. Treatment of the teichoic acid from strain X46S-R1 with alkali destroys inhibitory activity with the release of ninhydrin-positive material. Unlike the teichoic acids of the resistant strains, alkali treatment of teichoic acid from sensitive strain X46S did not liberate detectable ninhydrin-positive material. Thus, inhibitory activity of the teichoic acids may be dependent upon the alanine content. This conclusion is substan-

TABLE 2. *Summary of purification of inhibitor from Streptococcus faecalis strain X46S-R1*[a]

Prepn	Total inhibitor units	Total Pi	Total glucose	Units of inhibitor/ mg of Pi	Purification
		mg	*mg*		
Disrupted bacteria	125,000	20.3	23.4	6,170	—
Supernatant fluid (100,000 × *g*)	62,500	17.3	16.1	3,610	0.58
Phenol-extracted material	12,800	0.860	1.91	14,900	2.42
Starch eluate[b]	3,200	1.64	2.66	1,960	0.32

[a] The samples were hydrolyzed in 1 N $HClO_4$ for 3 hr at 100 C before assaying for inorganic phosphorus and glucose.

[b] Fast-moving peak of inhibitor.

tiated by the data in Table 4 which affords a comparison of the chemical composition of the teichoic acids isolated from the two resistant strains and from the sensitive strain. It is apparent that the degree of resistance to the lytic agent exhibited by the two strains (X14 and X46S-R1) bears a direct relationship to the relative amount of ester-linked D-alanine in the teichoic acid.

Relationship between lytic agent and inhibitor during growth. Decrease in lytic activity of a culture of *S. zymogenes* as growth progresses into late log phase may be dependent upon the loss of inhibitor from the cell and its accumulation in the medium. This view was tested in the following experiment.

Samples of a culture of resistant strain X14 were removed throughout lag and log phase of growth and were assayed for hemolysin concentration in the broth and also for cell-bound inhibitor. After centrifugation of the sample, the hemolysin was detected in the supernatant fluid and the inhibitor was extracted from the sedimented organisms. The pellets were washed once and resuspended to 10^{10} cocci per milliliter in 0.2 M acetate buffer at *p*H 6 and were incubated at 37 C for 1 hr, conditions which cause the release of inhibitor from whole cells (6). After final centrifugation, the supernatant fluid was assayed for inhibitor and protein. The results (Fig. 2) indicate that cell-bound inhibitor was minimal during lag phase and increased markedly at mid-log phase. There was, however, a striking loss of cell-bound inhibitor at a later period in the logarithmic phase. As will be shown in the next experiment, this is accompanied by the occurrence of detectable inhibitor in the culture medium. Hemolysin production reached a peak during log phase of growth, at a time when cell-bound inhibitor was maximal; as growth continued, there was a rapid decline in detectable hemolysin. This decrease in hemolysin may be dependent upon the released inhibitor. This hypothesis was tested by an experiment in which the continued production of hemolysin was measured when means were employed to inactivate the inhibitor.

Previous work had shown that the inhibitor but not the lytic agent was adsorbed to diethylaminoethyl (DEAE) cellulose. Therefore, DEAE cellulose was added directly to the culture medium to bind inhibitor as soon as it was released from the cell. Figure 3 shows the effect of DEAE cellulose on the apparent activity of the lytic agent. A culture of strain X14 in early log phase was divided into two parts; to one part was added 3% DEAE cellulose, and the other part served as control. Periodically, samples of both cultures were removed, and the supernatant fluids were assayed for hemolysin activity. Only the later incubation times of this experiment are depicted in Fig. 3. As in the previous experiment, the lytic agent activity dropped from a peak to less than a detectable amount in 40 min in the control culture. However, the culture containing DEAE cellulose continued to show an increase in concentration of the lytic agent during this period, after which activity declined. The ultimate drop in activity may be dependent upon changing conditions brought about by aging of the culture. To determine whether this result was due to removal of the inhibitor from the medium before it acted on the lytic agent, portions of the samples were heated at 65 C for 30 min to inactivate the lytic agent and then were assayed for inhibitor. Inhibitor became detectable in the control culture at the time when lysin activity disappeared (Fig. 3). The

TABLE 3. *Summary of purification of teichoic acid from Streptococcus faecalis strain X46S*[a]

Prepn	Total Pi	Total glucose
	mg	*mg*
Disrupted bacteria	26.2	26.6
Supernatant fluid (100,000 × *g*)	24.2	19.6
Phenol-extracted material	1.17	3.03
Starch eluate[b]	0.925	2.40

[a] The samples were treated as described in Table 2.

[b] Fraction corresponding to that of strain X46S-R1.

TABLE 4. *Comparison of purified teichoic acids*

Bacterial strain	Sensitivity to lytic agent	Inhibitor units of purified teichoic acid	Relative molar composition			
			Phosphate	Glucose	Ribitol	D-Alanine
X14	Resistant	1,280	1.00	1.05	Present	0.62
X46S-R1	Partially resistant	160	1.00	0.85	Present	0.15[a]
X46S	Sensitive	0	1.00	1.36	Present	0

[a] Identified only as ninhydrin-positive material.

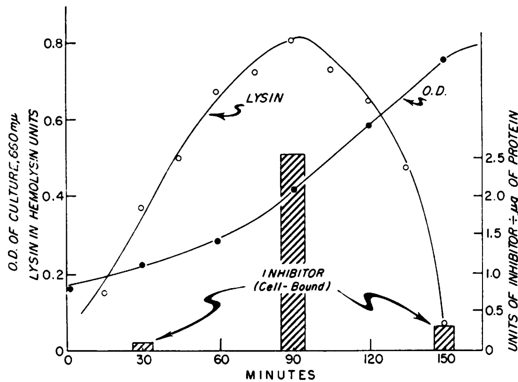

FIG. 2. *Time course of production of the inhibitor, Streptococcus zymogenes strain X14 was diluted 1:50 in Todd-Hewitt broth. Periodically, samples were taken, cells were counted in a Petroff-Hausser counter, and optical density was read. The cells were washed and resuspended to* 10^{10} *cocci per milliliter in 0.2* M *acetate buffer at pH 6. After incubating at 37 C for 1 hr, the cells were removed by centrifugation and the supernatant fluid was assayed for inhibitor and protein. Inhibitor concentration is expressed as the reciprocal of the dilution per micrograms of protein.*

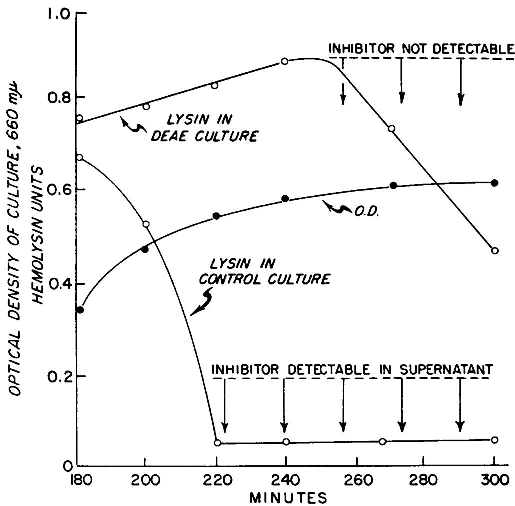

FIG. 3. *Production of lytic agent in presence and absence of DEAE cellulose. Streptococcus zymogenes strain X14 was diluted 1:50 in (a) Todd-Hewitt broth containing 3% DEAE cellulose, and (b) Todd-Hewitt broth alone. Periodically, samples were removed, and the optical density was read in culture b. Both cultures were centrifuged, and the supernatant fluids were assayed for hemolysin activity. The supernatant fluids were heated at 65 C for 30 min to inactivate the lytic agent and were assayed for inhibitor.*

culture containing DEAE cellulose, however, did not have detectable inhibitor activity. These findings suggest that release of inhibitor into the medium may be an important factor in the loss of activity of the lytic agent during growth of the culture.

A consequence of these experiments with DEAE cellulose has been the observation that the hemolysin is not in fact excessively labile to temperature. Rather, the following experiment suggests that the inactivation of the hemolysin by heat is dependent upon the presence of inhibitor. The 180-min samples from both cultures shown in Fig. 3, one containing hemolysin produced in the presence of DEAE cellulose, the other containing hemolysin produced in the absence of DEAE cellulose, were used to determine the heat inactivation of the hemolysin. The lytic agent produced in the absence of DEAE cellulose showed a rapid loss in activity, with a half-life of about 5 min at 46 C (Fig. 4). However, the lytic agent protected from free inhibitor by DEAE cellulose was much more heat-stable, and exhibited a half-life of about 2 hr. Despite this protective effect of DEAE cellulose, it has not been possible to improve the heat stability by increasing the amount of DEAE cellulose. Whether this indicates that the inactivation in the presence of DEAE cellulose represents the true heat lability of the lytic agent, or whether there are small amounts of free inhibitor still present, cannot be answered yet.

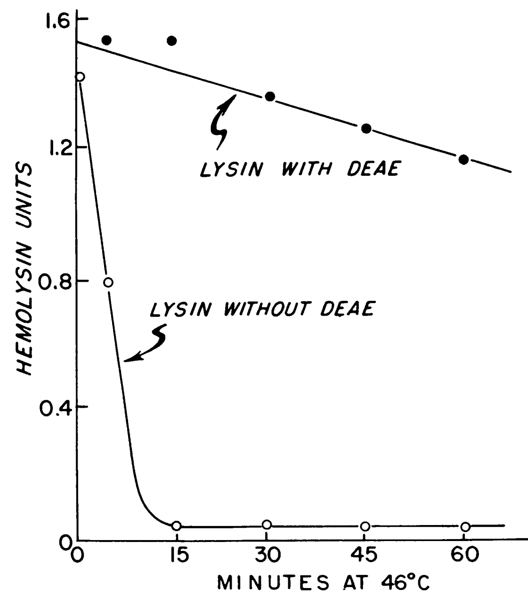

FIG. 4. *Heat inactivation of lytic agent produced in presence and absence of DEAE cellulose. Lytic agent produced in presence and absence of DEAE cellulose as in Fig. 3. Supernatant fluid from mid-logarithmic phase of each culture was heated at 46 C; 1-ml samples were taken periodically and were assayed for hemolysin activity.*

DISCUSSION

The lytic agent of *S. zymogenes* is a positively charged substance which is excreted into the medium during the logarithmic phase of growth. Indirect evidence derived from earlier studies suggested the production of an inhibitor of the lytic agent during the growth of a broth culture. Lytic activity diminished after mid-exponential phase, and the lysin had a half-life of 5 min at 37 C. It is conceivable that this loss in lytic activity is dependent upon either thermal inactivation or the production of an inhibitory substance. In the work reported here, it was shown that a specific lysin inhibitor was associated with the cell until the mid-exponential growth phase, and thereafter was released into the medium. However, if a cationic cellulose is added to the culture during incubation at 37 C, lytic activity is retained, because of the selective neutralization of the inhibitor. Furthermore, lytic agent produced in such a culture was relatively resistant to thermal inactivation.

The finding that an organism resistant to lytic activity produces an inhibitor, whereas a sensitive organism does not do so, suggests that bacterial resistance to the lytic agent may be dependent upon the presence of the inhibitor. Evidence for such a conclusion is as follows:

(i) A survey of streptococci showed that only resistant strains produced the specific inhibitor.

(ii) A mutant derived from a sensitive organism was partially resistant to the lytic agent and produced the inhibitor, whereas the sensitive parent strain did not.

(iii) Resistant spheroplasts, after removal of the inhibitor by acetate buffer, were rendered sensisitive to the lytic agent (6).

Clearly, production of the inhibitor is prerequisite for resistance to the lytic agent. Attempts to isolate sensitive mutants from a resistant *S. zymogenes* strain were uniformly unsuccessful, probably because such a mutation should be lethal.

The inhibitor, purified from strain X14, was identified as a ribitol teichoic acid containing glucose, phosphate, and ester-linked D-alanine. A similar, active teichoic acid could be isolated from the resistant mutant with an alanine content one-fourth that of the teichoic acid from the completely resistant strain. The parent strain produced a ribitol teichoic acid which was devoid of D-alanine and which lacked inhibitory activity. These data indicate that the capacity of the teichoic acid to inactivate the lytic agent may depend on the molar amount of amino acid esterified to the ribitol polymer. The mechanism whereby the alanine content of the teichoic acid confers inhibitory activity is unknown.

There are striking parallels between the results described here and recent work dealing with the transforming systems of pneumococci, streptococci, and *Bacillus*. Competence in pneumococci develops during a narrow range of the logarithmic phase of growth and falls off precipitously as the culture approaches maximal staionary phase (18). This synchronization of a physiological state was correlated with the production of an activator which acts on the surface of a pneumococcal cell, giving it the capacity to take up deoxyribonucleic acid (18). The loss of competence in later log phase was shown by Tomasz and Hotchkiss to be owing to the production of an inhibitor of the competence-conferring factor (19). Competence in Group H streptococci also appears during a brief period of exponential growth (14) and has been shown to be produced in response to an extracellular factor called competase (15). Pakula was unable to demonstrate an extracellular inhibitor of competase and concluded that surface changes were responsible for the loss of competence (14). In addition, Pakula and Hauschild made the observations that growth of competase-treated cells was inhibited, and that the cells appeared to be damaged (16). Of particular interest, Young et al. reported an alteration in the composition of the glycerol teichoic acid in cell walls of *B. subtilis* during the development of competence (23, 24), and therefore suggested that transformation may be influenced by this alteration (F. E. Young and A. P. Jackson, Bacteriol. Proc., p. 35, 1966). In view of the fact that resistance to the lytic agent of S. *zymogenes* is dependent upon teichoic acid production, it is conceivable that other processes such as transformation and autolysis, which depend upon alteration of the cell envelope, may be controlled in part by teichoic acid.

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