JOURNAL OF BACTERIOLOGY, Aug. 1973, p. 655-661
Vol. 115, No. 2

Properties of a *Streptococcus sanguis* (Group H) Bacteriocin and Its Separation from the Competence Factor of Transformation

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Received for publication 30 April 1973

STH_1, a streptocin elaborated by group H streptococcus strain Challis, is lethal for group H streptococcus strain Wicky and is produced maximally during the exponential growth phase of liquid medium cultures. Crude streptocin preparations are resistant to oxidation and display a biphasic pH stability (stability being maximal at pH 5.0 and 10.0). Survivor studies indicate that streptocin-mediated killing is a "one-hit" phenomenon and proceeds rapidly. The streptocin has been purified 50-fold with $(NH_4)_2SO_4$ fractionation and Sephadex G100 chromatography and appears to exist in equilibrium between two molecular weight forms. Low ionic strength and neutral pH buffers favor the isolation of the 110,000 molecular weight form, whereas high ionic strength and alkaline pH conditions facilitate isolation of the 28,000 to 30,000 molecular weight form. These findings suggest an association-dissociation relationship between macromolecules of 28,000 to 30,000 molecular weight. Purified STH_1 has no "competence factor" (CF) activity. In addition, CF has no STH_1 activity and displays no inhibitory effect on exponential-phase Wicky cultures as determined by absorbancy measurements. It appears, therefore, that initiation of the competent state for transformation in strain Wicky is not necessarily accompanied by gross alterations in cell growth.

Insight into the physiology and molecular biology of bacteriocins has been derived mainly from research on gram-negative bacteria. Such investigations have not only led to significant findings regarding the mode of action of bacteriocins, but have also initiated new thinking on the structure of membranes and the basic control mechanisms of the microbial cell. Gram-positive bacteria, although not extensively studied for bacteriocinogeny, appear to have a less complex cell surface architecture than the gram-negative organisms and may offer a simplified system for exploring the interactions between bacteriocins and cell surfaces.

Several laboratories have described the elaboration of bacteriocins by streptococci (2, 4, 5, 6, 8). Unfortunately, characterization of these factors was impeded by the inability to isolate bacteriocin activity from culture filtrates (6) and the instability of such factors in solution (4).

Group H Challis streptococci synthesize a streptocin (STH_1) which is effective against another group H streptococcus, strain Wicky (12). The bacteriocin is detectable in culture filtrates and is relatively stable at low temperatures. These favorable characteristics made possible the following study on the properties and purification of the Challis bacteriocin.

MATERIALS AND METHODS

Bacterial strains. Group H streptococcal strains Challis and Wicky were used for bacteriocin and transformation studies. These strains were obtained from the Central Public Health Laboratory (London) via R. Pakula. Strain Challis is spontaneously transformable, whereas strain Wicky must be "induced" for transformation by exposure to competence factor (CF) (9).

DNA preparation. Transforming deoxyribonucleic acid (DNA) was purified from strain Wicky Str-r, a mutant resistant to more than 1 mg of streptomycin per ml, by methods described previously (11).

Preparation of Challis filtrates. One-half milliliter of an overnight Brain Heart Infusion (BHI) broth Challis culture was directly inoculated into 90 ml of transformation medium (TM) (11) and incubated at 37 C for 2.0 h. The TM medium culture was then

centrifuged for 24 min at 13,000 × *g* and filter-sterilized with a membrane filter (0.45-μm pore size; Millipore Corp.).

Determination of CF titer. An overnight (18 h) BHI culture of Wicky was diluted 10-fold into TM containing 5% unheated sterile horse serum. One-tenth-milliliter fractions of this dilution were then inoculated into test tubes containing 1.8 ml of TM medium and were incubated at 37 C. After 1.5 h, rate-limiting amounts (0.2 ml) of experimental Challis supernatant fluid (or fractions from a chromatographic column) were added to the Wicky cultures and allowed to incubate for 25 min. Wicky Str-r DNA was then added to a final concentration of 5 μg/ml, incubated with the cells for 15 min, and then hydrolyzed with 0.1 ml of a deoxyribonuclease (DNase) solution (2 mg of DNase per ml, 10 mM $MgSO_4$). Cultures were maintained at 37 C for an additional 2 h for expression of streptomycin resistance. Transformation was quantitatively estimated by a liquid medium technique (11).

Determination of bacteriocin (STH_1) titer. An 18-h BHI broth culture of Wicky was diluted 1:5 into TM medium. One-tenth-milliliter samples of this dilution were mixed with 1.0 ml of solution being evaluated for STH_1 titer. After 30 min at 37 C, 0.5-ml samples were withdrawn and added to 5 ml of TM medium. After 5 to 6 h, absorbancy measurements were recorded at 0.5-h intervals and plotted semilogarithmically. Parallel lines were drawn through control and experimental growth curves. The horizontal distance (D_t) between the control and experimental growth lines was used to calculate the number of susceptible Wicky cells killed by the Challis bacteriocin (12).

The following expression relates the number of surviving cells (after exposure to Challis streptocin) to the experimentally determined value of D_t.

$$N = N_0 \times 2^{-D_t/g} \quad (1)$$

where N = number of surviving cells, N_0 = initial number of cells, and g = generation time of strain Wicky cells in liquid medium. Rearranged, equation (1) becomes:

$$N/N_0 = 2^{-D_t/g} \quad (2)$$

From the Poisson distribution, the multiplicity (m) of streptocin molecules per cell can be related to N/N_0 by equation:

$$N/N_0 = e^{-m} \quad (3)$$

solving equations (2) and (3), one obtains:

$$e^{-m} = 2^{-D_t/g} \quad (4)$$

This last relationship was used to calculate m (an index of streptocin titer). Since g is a constant in equation (4), STH_1 titers were expressed simply as units of D_t (min).

Column chromatography. A Sephadex (G100) chromatograph (Pharmacia) was used for purifying the streptocin activity of Challis filtrates. Crude fractionation was first performed by $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was slowly added (at 4 C) to Challis filtrate with continuous agitation until the solution was 50% saturated. After 2.0 h at 4 C, the precipitate was centrifuged at 12,000 × *g* for 25 min and the supernatant fluid was discarded. The pellet was redissolved in 5 to 6 ml of (i) 0.01 M sodium phosphate buffer, pH 7.7 or (ii) 0.15 M NaCl adjusted to pH 10.8 with 1.0 N NaOH. The 5-ml bacteriocin solution was then applied to a Sephadex G100 column (2.5 by 60 cm) equilibrated with appropriate buffer. The Sephadex bed volume was 320 ml and the flow rate was 55 to 60 ml per h. Fractions of 5.0 ml each were collected at 4 C and then assayed for streptocin titer in the manner described above.

RESULTS

Appearance of STH_1 and CF activities in Challis culture supernatant fluids. One-half milliliter of an overnight BHI broth Challis culture was inoculated into 90 ml of TM medium and incubated at 37 C. Samples (5.0 ml) were removed from the culture every 15 min and centrifuged at 2,000 × *g* for 30 min. The supernatant fluids were filtered through a membrane filter (0.45-μm pore size; Millipore Corp.), readjusted to pH 7.5, (the initial pH of TM medium) and then assayed for CF and STH_1 titers as described in Materials and Methods. CF and STH_1 first appeared in Challis supernatant fluids after 90 min of incubation (Fig. 1). STH_1 activity peaked at 120 min and declined sharply thereafter. CF titers, on the other hand, peaked at approximately 135 to 150 min, and remained at maximal levels for 4 to 6 h.

Effects of culture conditions upon STH_1 production. To determine the optimal culture conditions for streptocin production, Challis cells were grown as stationary or rotating cul-

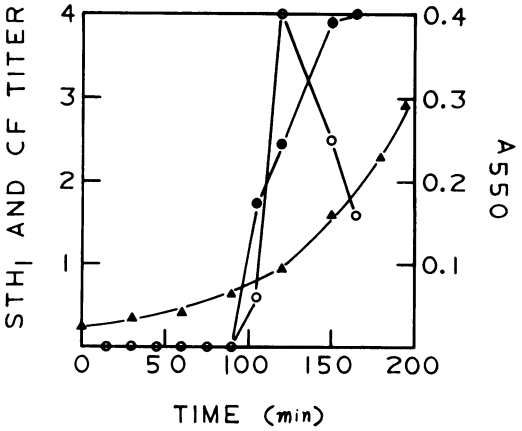

FIG. 1. *Appearance of STH_1 and CF activities in Challis culture supernatant fluid. Challis cells were incubated in TM medium at 37 C for various time periods and then assayed for A_{550} (▲), STH_1 titer (○), and CF titer (●).*

tures. For each of the cultural conditions examined, six test tubes (containing 7.2 ml of TM medium per tube) were used. All TM medium was equilibrated for 1 to 2 h at each environment prior to inoculation with Challis cells.

Stationary cultures were maintained at 37 C in a water bath. Other cultures were inserted into a 30-degree angle test tube rack of a New Brunswick gyratory shaker and rotated at 150 rpm. All test tubes were inoculated with 0.1 ml of a 1:5 dilution of an overnight Challis culture. At 0, 1, 2, 3, 4, 5, and 6 h of incubation, one test tube from each experimental group was measured for A_{550} and assayed for STH_1 titer as above. Figure 2a illustrates the growth of stationary and rotating cultures of strain Challis. Although both cultures eventually attain the same final cell density, they have different generation times: stationary, 50 min; rotating, 75 min.

The streptocin titers from each of these cultures is indicated in Fig. 2b. There is a 1-h temporal separation between the two streptocin maxima. However, comparison of Fig. 2a and b reveals that maximal STH_1 titers appear at similar absorbancy readings (see arrows, Fig. 2a). Thus, aeration (and/or growth rate) seems to have little or no effect on the appearance and maximal amounts of streptocin in Challis supernatant fluid relative to the culture's growth curve.

Several basic characteristics of the crude streptocin were investigated to facilitate purification of the bactericidal factor and furnish a reference point for future studies with purified material.

pH stability of STH_1. Samples (5.0 ml) of Challis filtrate were distributed into eight test tubes. Each test tube was adjusted to a different pH value (from pH 3.0 to 12.0) by using 1 N NaOH or 1 N HCl and then was incubated at 37 C for 35 min. All tubes were then readjusted to pH 7.5 and assayed for residual STH_1 activity. STH_1 is most stable at either pH 5.0 or pH 10.0 (Fig. 3). At pH 7.0, STH_1 is either unstable or rapidly destroyed by extracellular enzymes in the supernatant fluid. Whether the complexity of this pH stability curve derives solely from the nature of the bacteriocin cannot be resolved until studies are performed with purified streptocin.

O_2 stability of STH_1. Two 20-ml portions of Challis supernatant fluid were placed in an ice bath. One portion was aerated by bubbling air into the solution through a Pasteur pipette for 15 min. Both fractions were then assayed for STH_1 titer. Relative to the non-aerated fraction of STH_1, the aerated fraction showed a 17% decrease in activity.

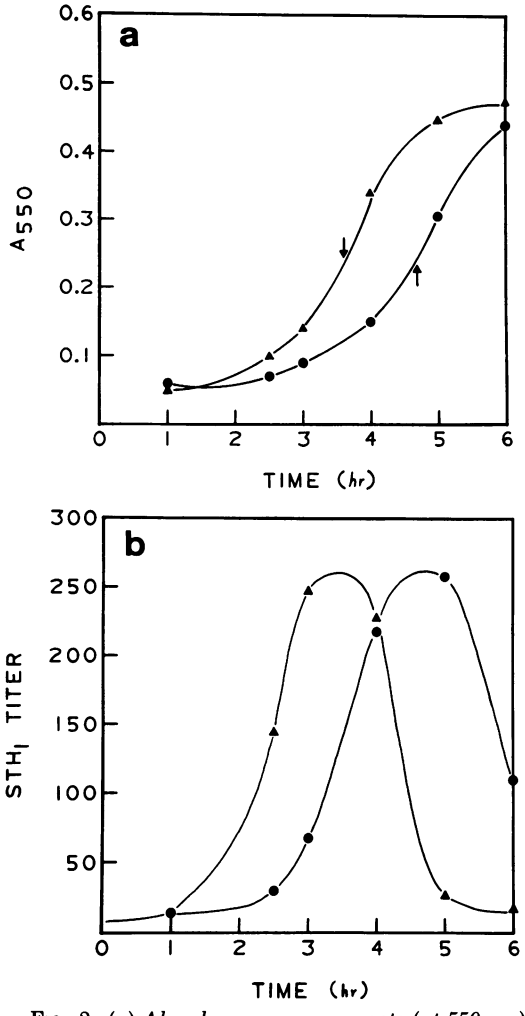

FIG. 2. *(a) Absorbancy measurements (at 550 nm) for stationary (▲) and rotating (●) Challis cultures. Arrows indicate time at which STH_1 titers were maximal. (b) STH_1 titers of supernatant fluid from the stationary (▲) and rotating (●) Challis cultures depicted in Fig. 2a.*

Dose-response relationship for streptocin-induced cell death. Challis filtrate was divided into portions of 0, 1, 2, 3, 4, and 6 ml. To each fraction, sufficient amounts of TM medium were added to adjust total volume to 6 ml. An overnight BHI broth Wicky culture was then diluted 1:5 with TM medium, and 0.1-ml samples were used to inoculate six test tubes containing 3.6 ml of TM medium. After incubation of the Wicky cultures at 37 C for 1 h, the 6-ml dilutions of STH_1 were added to culture tubes.

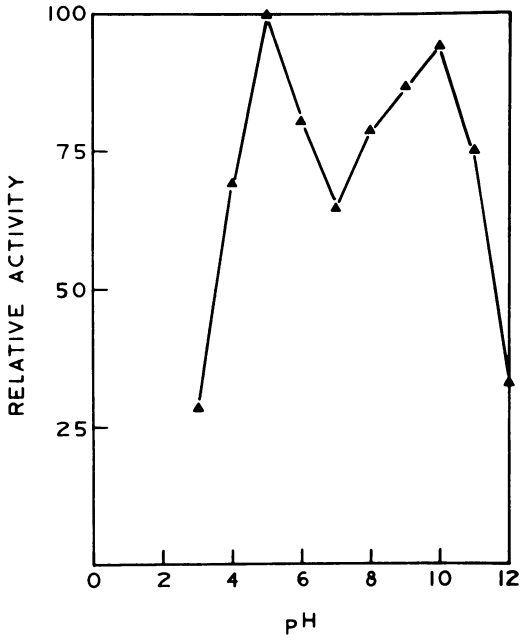

FIG. 3. *pH stability of STH_1. Challis supernatant fluids were adjusted to various pH values, incubated at 37 C for 35 min, neutralized, corrected for volume changes, and assayed for STH_1.*

Killing was quantitated by D_t determination and is plotted in Fig. 4. Extrapolation from the experimental data shown in this figure reveals 1.3 "hit" killing kinetics. The slight deviation from the characteristic one-hit kinetics may possibly result from the crude quality of the streptocin preparation.

Kinetics of streptocin-induced cell death. Previous work from this laboratory has shown that trypsin inactivates 82 to 85% of the Challis streptocin in 5 min at 37 C (12). It was, therefore, possible to utilize trypsin as a tool for investigating the rapidity of interaction between STH_1 and the sensitive Wicky cells. An 18-h BHI broth culture of Wicky was diluted 1:5 with TM medium, and 0.1-ml samples were used to inoculate 3.6 ml of TM medium. The Wicky cultures were incubated at 37 C for 1 h. Two milliliters of STH_1 (from three different preparations) were added to the 3.6-ml Wicky cultures. At various times after addition of STH_1 to the Wicky cells, trypsin was added to 77 μg per ml. Maximal STH_1-induced killing was determined by eliminating the addition of trypsin and thereby allowing full expression of STH_1 activity. In Fig. 5, values for Wicky cell death are expressed as the percentage of total activity present in that particular streptocin preparation. It is evident from the data in this figure that 50% of the total activity in each of the three preparations of STH_1 is expressed in 15 min. This implies that either 50% of the cells are killed in 15 min or that 50% of the streptocin binds to the sensitive cells and thus becomes resistant to trypsin inactivation. Also, it should be noted that when trypsin and STH_1 are added simultaneously to Wicky cells (time 0), approximately 10 to 12% of the total activity of that preparation remains. This observation probably relates to the previous finding (12) that 5 min are required for trypsin to inactivate most of the STH_1 killing potential.

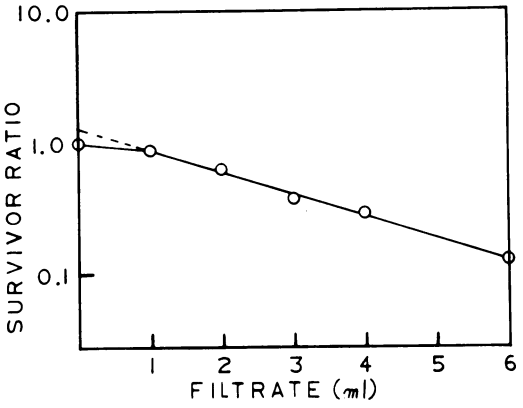

FIG. 4. *Dose-response curve for streptocin-induced cell death. Different amounts of Challis filtrate were diluted to a total volume of 6.0 ml and titered for STH_1. Survivor ratios were calculated from D_t measurements.*

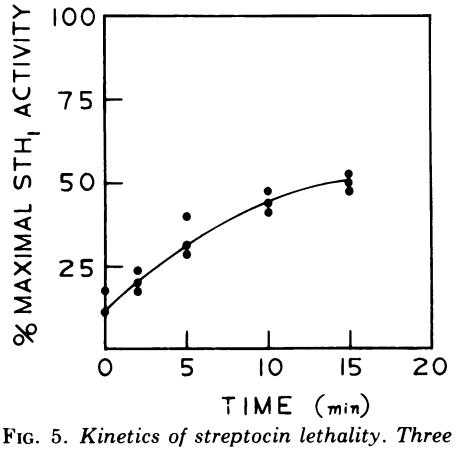

FIG. 5. *Kinetics of streptocin lethality. Three different preparations of streptocin were added to sensitive Wicky cells at 0 time. At times indicated on the abscissa, STH_1 was inactivated with trypsin (77 μg/ml) and cell death was quantitated by D_t measurements. Maximal titers for each STH_1 preparation were determined by eliminating the trypsin treatment.*

In an effort to refine the studies on the nature of STH_1 and its action on sensitive Wicky cells, purification of the factor was attempted by using standard techniques of protein purification.

$(NH_4)_2SO_4$ fractionation of STH_1. Crude Challis filtrate was precipitated by adding solid $(NH_4)_2SO_4$ to 60% saturation, (see Materials and Methods) and the supernatant fraction was discarded. After resuspending the precipitate in 5 to 6 ml of phosphate buffer (0.01 M, pH 7.6), its activity was titered and compared to that of crude Challis filtrate. Eighty to 90% of the total streptocin activity in Challis filtrates was harvested by such procedures.

In another experiment, different levels of $(NH_4)_2SO_4$ saturations were evaluated for their ability to precipitate STH_1. The results are given in Table 1. The majority of STH_1 activity is precipitated by 45% $(NH_4)_2SO_4$. However, a significant portion (20%) of STH_1 does not precipitate until saturation levels of 55% are attained. A possible explanation for the inexact fractionation of STH_1 by this "salting-out" technique may lie in the fact that TM medium contains nucleic acids (present in yeast extract) which can interfere with precipitation.

Sephadex G100 chromatography of STH_1. Challis filtrates (90 ml) were partially purified by precipitation with 50% $(NH_4)_2SO_4$. This precipitate was then dissolved in 5 ml of phosphate buffer (0.01 M, pH 7.6) and applied to a column of Sephadex G100 as described in Materials and Methods. The chromatographic pattern is displayed in Fig. 6. STH_1 activity was eluted in two fractions. The first fraction has an approximate molecular weight (MW) of 110,000 as determined by the method of Andrews (1). The pattern of the smaller fraction is too broad to give an adequate estimation of molecular weight.

It was found that a change of buffer conditions could affect the basic pattern of STH_1 elution from the same G100 Sephadex column. Thus, when precipitated STH_1 was resuspended in saline solution (0.15 M NaCl, pH 10.8) and applied to the column, the elution profile shown in Fig. 7 was obtained. This figure again illustrates that two components of activity are present. However, under these chromatographic conditions, the major component of STH_1 elutes as a substance of MW = 28,000 to 30,000. The shift from a low ionic strength, pH 7.6 buffer to a high ionic strength, pH 10.8 solution has somehow altered the apparent molecular size of the STH_1 substance.

TABLE 1.

$(NH_4)_2SO_4$ saturation (%)	Total precipitated STH_1 activity (%)
0–40	61
40–45	13
45–50	5
50–55	21
55–60	0

Sephadex G100 chromatography of CF. The growth-inhibitory activity of Challis culture supernatant fluid has often been attributed to the action of competence factor (9). In an attempt to demonstrate that CF and STH_1 are different substances, G100 Sephadex chroma-

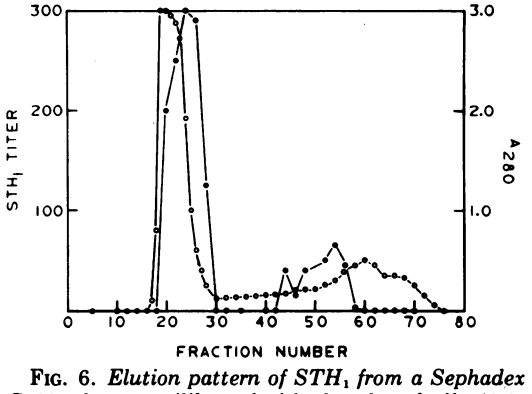

FIG. 6. *Elution pattern of STH_1 from a Sephadex G100 column equilibrated with phosphate buffer (0.01 M, pH 7.6). Challis filtrates were precipitated with ammonium sulfate, dissolved in phosphate buffer, and applied to the column. V_0 = fraction 19. Flow rate = 55 ml/h. Percent recovery = 50%. Symbols: A_{280}, O; STH_1 titer,* ●.

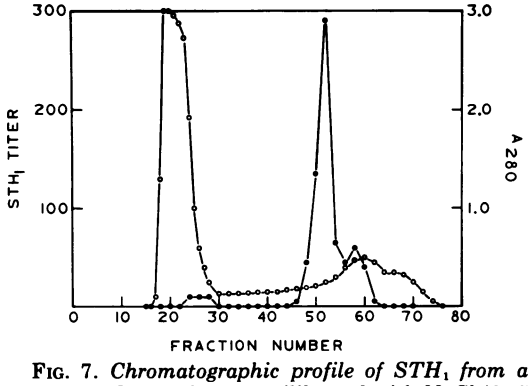

FIG. 7. *Chromatographic profile of STH_1 from a Sephadex G100 column equilibrated with NaCl (0.15 M, pH 10.5). Streptocin precipitate was dissolved in the appropriate NaCl solution, applied to the column, and chromatographed at 57–58 ml/h. V_0 = fraction 19. Percent recovery = 55%. Symbols: A_{280}, O; STH_1 titer,* ●.

tography was employed. Challis filtrates were fractionated with 50% saturating $(NH_4)_2SO_4$, and the precipitate was resuspended in either of two buffers (1) 0.01 M phosphate buffer (pH 7.6) or (2) 0.15 M NaCl, pH 10.8. Regardless of elution system utilized, the chromatographic profiles of CF from the Sephadex columns were identical (Fig. 8).

To evaluate the possibility that STH_1 was an aggregate of CF, the 110,000-MW component of Challis streptocin (Fig. 6) was readjusted to conditions of 0.15 M NaCl, pH 10.8. These conditions appear to result in the "disaggregation" of streptocin into a smaller substance (Fig. 7) which is eluted similarly to CF (Fig. 8). However, this disaggregated STH_1 component exhibited no CF activity. In a similar fashion, preparations of partially purified CF (fractions 59–61 of Fig. 8) were assayed and found to contain no STH_1 activity. Hence, partially purified STH_1 has no CF activity and vice versa.

DISCUSSION

In 1949 Sherwood et al. (13) reported on the production of "antibiotic substances" by beta-hemolytic streptococci. Later, several laboratories began to survey the numerous groups of streptococci in search of correlative evidence for a pathological role of bacteriocins in disease. Although Kuttner (6) found a close relationship between "bacteriocin" elaboration and nephrogenicity of streptococcal group A strains, later work (8) tended to contradict these findings. The word "bacteriocin" has been placed in quotation marks to designate the questionable nature of the substances studied by the aforementioned research groups. The agar-plate assay technique which was used in these investigations can also detect the release of toxic substances other than bacteriocins (10).

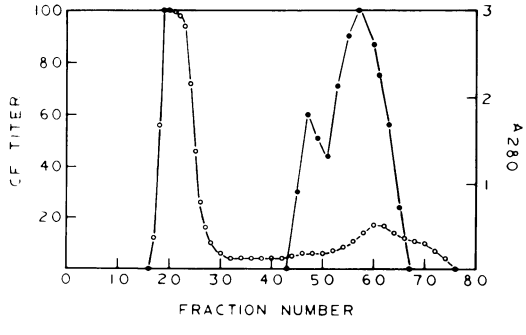

FIG. 8. *Elution of CF activity from a Sephadex G100 column under two different buffer conditions. Streptocin precipitates were dissolved in either (i) phosphate buffer (0.01 M, pH 7.6) or (ii) NaCl (0.15 M, pH 10.5) and applied to a column equilibrated with corresponding buffer. CF activity (●) eluted with a similar pattern regardless of ionic and pH environments. Flow rate = 55 ml/h. Percent recovery = 65%. A_{280}, (○).*

Kuttner's attempt to characterize streptococcal bactericidal factors was thwarted since no activity could be obtained from the supernatant fluids of liquid medium cultures. Kelstrup and Gibbons (4), on the other hand, found that "bacteriocins" were produced by liquid medium cultures of oral streptococci only if the medium's viscosity were properly adjusted with agar, dextran, glycerol, or starch. Recently, Krämer and Brandis (5) have purified a bacteriocin from *Streptococcus agalactiae* strain 73. Appreciable quantities of uninduced bacteriocin could be detected in supernatant fluids from stationary-phase liquid medium cultures. The bactericidal factor, designated B73, appeared to have a molecular weight of 10,000 and was sensitive to proteolytic enzymes.

The description of bactericidal substances in group H streptococci derived from transformation studies with two strains, Challis and Wicky. In order to investigate the initial phases of competence development, researchers have used strain Challis culture supernatant fluids as a source of "competence factor" for inducing the transformable state in Wicky. It was assumed that the growth-inhibitory effect of the Challis supernatant fluids was a result of the action of CF on Wicky cells and that competence, in some manner, was accompanied by a slowed cellular metabolism. Precedence for a correlation between decreased metabolism and competence had been established previously with the *Bacillus subtilis* transformation system (3, 7). However, a recent report by our laboratory (12) suggests that the growth-inhibitory effect of Challis filtrates results from the presence of a bacteriocin that can be destroyed without affecting the competence-inducing ability of the supernatant fluids. It is interesting that the distinct activities of CF and STH_1 appear concomitantly in liquid medium cultures of Challis. This finding implies either that CF and STH_1 are co-regulated by Challis cells, or that membrane permeability changes are responsible for their similar kinetics of release. We have reported here that it is possible to separate physically the bacteriocin and CF activities of Challis filtrates by using Sephadex chromatography. Initiation of the competent state in strain Wicky with partially purified CF is not accompanied by any detectable changes in growth. Although earlier contrasting data have been presented by Pakula and Spencer (9),

these investigators were using unpurified filtrates which may have contained both CF and STH_1 activity.

The strain Challis bacteriocin (STH_1) is a heat-labile, trypsin-sensitive, and oxygen-stable substance. In contrast to the bacteriocin characterized by Krämer and Brandis (5), STH_1 appears in maximal amounts during the exponential growth phase of liquid medium cultures. It displays rapid, single-hit killing kinetics, and estimates from Sephadex G100 chromatography suggest that it may exist as two distinct molecular forms. It is possible that the 110,000-MW form represents a tetramer of the smaller 28,000 to 30,000-MW form and that both varieties may coexist in equilibrium. High ionic strength and high pH favor the smaller molecular weight size. By eluting STH_1 from Sephadex columns with 0.01 M phosphate buffer, we have obtained streptocin which is free of CF activity. Re-chromatography with 0.15 M NaCl (pH 10.8) separates STH_1 from most of the supernatant fluid protein and results in a 50- to 70-fold purification relative to A_{280} material. After rechromatography, approximately 30 to 40% of the original streptocin activity is recovered.

The biological role of bacteriocins is unknown. Whether these substances serve as agents to selectively inhibit organisms competing for the same micro-ecological niche or whether they participate in the normal events of cellular growth remains to be answered. Brock and Davie (2) believe that the bacteriocin of a group D streptococcus is also an hemolysin. Mutation studies indicate that the bactericidal and hemolytic activities of this organism are lost and regained coincidently. Also, both activities show similar heat-inactivation kinetics.

It is difficult to ascribe a cellular function to STH_1. Present efforts to demonstrate an effect of this factor on the producer strain (Challis) have been negative. Regardless of the bacteriocin:cell ratio, Challis cells display no detectable alterations in growth. There could be several explanations for this observation. If the site of action of STH_1 were intracellular, then a membrane permeability barrier might exist which prevents access to this location. Also, there is no reason to assume that the addition of superfluous STH_1 should result in an easily observable change in cell growth. Mutation studies with other bacteriocinogenic strains suggest that the production of bacteriocins can be eliminated without affecting the growth of the organism. It would be important to determine if this relationship prevails for streptococci.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grants HE-03709-15 from the National Heart and Lung Institute, and DE-03615-01 from the National Institute of Dental Research, and by grants from the Office of Naval Research (NR-136-608), the Grainger Fund, and the Hemac Fund.

R.S. is a predoctoral trainee supported by Public Health Service General Research Support Grant (1 S01 Fr5370) and by Microbiology Training Grant (5-T1-GM00724-12) from the National Institute of General Medical Sciences. H.D.S. is the recipient of Public Health Service Research Career Award K6-GM 16284 from the National Institute of General Medical Sciences.

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