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Production, Purification, and Preliminary Characterization of a Gonococcal Growth Inhibitor Produced by a Coagulase-Negative Staphylococcus Isolated from the Urogenital Flora

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Coagulase-negative staphylococcus no. 7, isolated from the urogenital flora, was grown on semisolid brain heart infusion medium. Supernatants were obtained by centrifuging frozen and thawed media which had supported the growth of the staphylococci at 37°C. The kinetic of production revealed that the antigonococcal activity was detected at the end of the logarithmic phase of growth and that the maximum activity was obtained after 24 h of incubation. Production of inhibitory activity was detected in cultures grown between 35 and 39°C and in a pH range of 6.9 to 9.4. The inhibitory substance was purified by methanol extraction, acetone fractionation, dialysis, and chromatography on Ultrogel AcA 54. The characterization of the inhibitor showed that it was a lipoprotein or a lipid-associated-protein and that the protein component could be separated from the lipids when chromatographed on Ultrogel AcA 54 in the presence of urea. The inhibitory activity was associated with the protein component which had a molecular weight of approximately 15,900. In polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea, the protein component was dissociated in a subunit estimated to be approximately 1,400 daltons.

The staphylococci are known to produce a variety of antibacterial substances (24), but only few studies have dealt with antigonococcal products produced by these bacteria. Kaye and Levison (14), Saigh et al. (22), and Bisaillon et al. (2) have shown that coagulase-negative staphylococci are among the aerobic organisms present in the vaginal or cervical secretions that most commonly interfere with the in vitro growth of *Neisseria gonorrhoeae*. The study of the inhibitory effects of antigonococcal activity produced by staphylococci has been complicated by the difficulties in obtaining it in a soluble form. Ingraham et al. (12) have detected soluble inhibitory activity in the expressed liquor obtained by centrifuging frozen and thawed agar medium which has supported growth of the staphylococci, and they suggest that it is a staphylococcin similar to that studied by Jetten et al. (13). Lafond et al. (18) have also obtained some active preparations in the liquid phase prepared from semisolid agar cultures of urogenital staphylococci.

The present work was carried out to further study the antigonococcal activity produced by one of the active urogenital staphylococci (no. 7). We describe the production, purification, and preliminary characterization of this gonococcal inhibitor, which appears to be different from the previously described inhibitors of bacterial origin, including staphylococcin 1580.

MATERIALS AND METHODS

Bacterial strains. The antigonococcal inhibitor-producing strain was a coagulase-negative staphylococcus no. 7 of our collection and was selected from the urogenital flora (2) for its broad antigonococcal spectrum and narrow antibacterial spectrum. It was chosen mainly because it has been previously shown to produce an antigonococcal activity on semisolid agar cultures that can be recovered in the supernatant after centrifugation (18). The target strain, *N. gonorrhoeae* G-10, was previously described (2, 18).

Culture media and conditions. The producing strain was propagated on Columbia agar containing 7.5% sheep blood. The solid medium used for cultivation of the gonococcal target strain was GC agar base (GIBCO Diagnostics, Madison, Wis.) enriched with 1% (vol/vol) CVA (GIBCO) and 1.5% (vol/vol) lysed horse blood. *N. gonorrhoeae* strain G-10 was grown in a 5% CO_2 atmosphere at 70% relative humidity and 37°C, and the staphylococcus was grown under aerobic conditions at 37°C.

Production of soluble antigonococcal activities. The crude supernatant was obtained by centrifuging frozen and thawed semisolid brain heart infusion (BHI) medium (GIBCO) which had supported a 24-h growth of staphylococcus no. 7 strain at 37°C as previously described (18). Supernatants from uninoculated semisolid media were used as controls. The supernatants

were adjusted to pH 7.0 and filtered through a 0.2-μm membrane (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). The crude preparations were then kept at −20°C. The optimal temperature of incubation and pH of the medium before inoculation for antigonococcal activity production were also determined. The temperature and pH assayed ranged from 33 to 41°C and 5.9 to 9.9, respectively.

Detection of soluble antigonococcal activities. Quantitative determination of the soluble antigonococcal activity was accomplished by a method similar to the one used by Reeves (21) and modified by Jetten et al. (13) for staphylococcin activity. The test was performed with a series of tubes, each receiving 5×10^7 colony-forming units (CFU) of *N. gonorrhoeae* G-10 in 1.0 ml of fresh BHI medium (GIBCO lot no. 870093) and a decreasing volume of the culture filtrate to be tested, starting with 1.0 ml. The final volume of the tube cultures was made up to 2.0 ml with control supernatant from uninoculated semisolid medium in every case. The tubes were incubated at 37°C for 6 h in 5% CO_2 and under an agitation of 200 rpm in a Psycrotherm incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Bacterial growth was measured by determining the absorbance at 540 nm in a Coleman Junior II A spectrophotometer. The reciprocal of the dilution yielding a 50% increase in absorbance with respect to the control (containing no inhibitor) was taken to be the activity in arbitrary units per milliliter.

Kinetics of production. The kinetics of production of the antigonococcal activity by staphylococcus no. 7 were determined with semisolid agar cultures. The growth curve of the isolate on the semisolid medium was evaluated by a method similar to the one used by Casciato et al. (4) for anaerobic bacteria. At different time intervals, inoculated semisolid cultures were removed from the incubator, CFU counts were carried out, and crude preparations containing soluble antigonococcal activity were prepared as already described. The staphylococci were removed from the surface of the medium with 10 ml of saline, and 10-fold dilutions of the resulting suspension were made. Volumes of 0.1 ml of the proper dilutions were plated on Columbia blood agar, and counts were made 24 h after incubation at 37°C. As a function of time, a growth curve expressed in terms of CFU per plate as well as a curve of the antigonococcal activity (arbitrary units per milliliter) detected could be drawn. We also determined the pH of each culture by measuring it in the crude supernatant.

Chemical analysis. The protein concentration was determined by the method of Lowry et al. (19), with bovine serum albumin as a reference. Polysaccharides were estimated by the anthrone reaction (15), using glucose as a standard. The lipid content was determined by the sulfophosphovanilline method as described by Zollner and Kirsch (30) after extraction of lipids with chloroform methanol by the method of Bligh and Dyer (3). The triolein was used as a standard.

Polyacrylamide and agarose gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out at 10% polyacrylamide by the methods of Laemmli (17) and Weber and Osborn (29) and in presence of 8 M urea by the method of Swank and Munkres (26) in vertical slab gel cells (Bio-Rad Laboratories, Mississauga, Ontario, model 220). The samples were diluted in the sample buffer (17) and heated in a boiling water bath for 2 min. Bromophenol blue was used as tracking dye. A pre-electrophoresis of 2 to 3 h was performed before the addition of the samples. The protein standards (low-molecular-weight SDS-polyacrylamide gel electrophoresis standard) were obtained from Bio-Rad Laboratories. Bacitracine (1,400 daltons) (Sigma Chemical Co., St. Louis, Mo.) and ribonuclease (13,700 daltons) (Pharmacia Fine Chemicals, Dorval, Quebec) were used as standards in the SDS-polyacrylamide gel electrophoresis in the presence of urea. Gels were stained for protein with 0.25% Coomassie blue R 250 in 50% methanol–5% acetic acid and destained in 7.5% acetic acid–5% methanol. Polyacrylamide (7.5%) gel electrophoresis without SDS was carried out in 0.05 M phosphate buffer, pH 7.0, or at alkaline pH by the method of Davis (7). Agarose (1%) electrophoresis was carried out in 0.01 M phosphate buffer, pH 7.0, on glass slides (9 by 11 cm). After electrophoresis, the agarose was dried with an air dryer and stained for protein with 0.5% Coomassie blue R 250 in 45% ethanol–10% acetic acid and destained in this last mixture. The coloration for lipids was done with Sudan black B (28). The antigonococcal activity could be located by embedding in BHI agar medium (Difco Laboratories, Detroit, Mich.) a slice of the agarose gel after electrophoresis, and the gonococcal indicator strain (10^6 to 10^7 CFU) was inoculated over it. After incubation at 37°C for 18 h under 5% CO_2 and 70% relative humidity, the inhibition zone was localized.

Protease treatment. Pronase enzyme was added to a final concentration of 0.1 mg/ml to the purified antigonococcal substance dissolved in 0.01 M phosphate buffer (pH 7.0). The solutions were incubated at 37°C for 1 h. The pronase activity was destroyed by heating at 100°C for 10 min, and the antigonococcal activity was tested. The antigonococcal activity was only lightly affected by this last treatment. Appropriate controls were included. Trypsin treatment was carried out in the same way, except the incubation was 3 h at 37°C.

Chemicals. LKB Ultrogel AcA 54 was purchased from Fisher Scientific Co., Montreal, Quebec, Canada. Agarose, acrylamide, *N,N'*-methylenebisacrylamide, and urea were electrophoresis purity reagent grade purchased from Bio-Rad Laboratories, Mississauga, Ontario, Canada. *N,N,N',N'*-tetramethylenediamine was purchased from Eastman Kodak Co., Rochester, N.Y. SDS was purchased from Matheson Coleman Bell, Norwood, Ohio. Pronase, free of nucleases, B grade was from Calbiochem, San Diego, Calif. Trypsin was from bovine pancreas, type III from Sigma Chemical Co., St. Louis, Mo. The blue dextran 2000 and the globular protein standards for gel filtration (ovalbumin, chymotrypsinogen, and ribonuclease) were in a calibration kit purchased from Pharmacia Fine Chemicals, Dorval, Québec, Canada.

RESULTS

Production of antigonococcal activities. Upon growth of staphylococcus no. 7 on a semisolid BHI medium, a crude preparation was obtained which inhibited *N. gonorrhoeae* G-10 growth. The kinetic of production revealed that the ac-

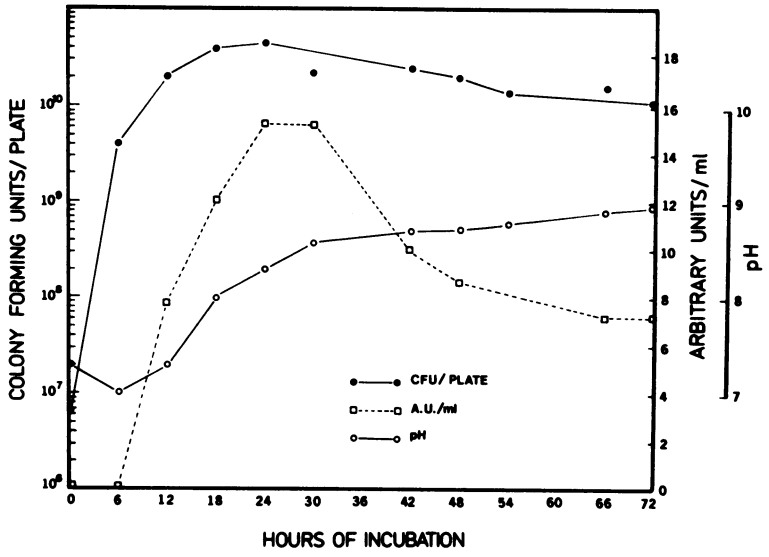

FIG. 1. Kinetic production of the gonococcal inhibitor on BHI semisolid medium by the coagulase-negative staphylococcus no. 7. Samples withdrawn at the time indicated were assayed for antigonococcal activity, number of CFU per plate, and pH of the culture in the crude preparation.

tivity was detected toward the end of the logarithmic phase of growth and that the maximum activity was reached after 24 h of incubation (Fig. 1). There was a marked drop in the activity after 48 h of incubation while the CFU count was still high. At the beginning of the culture, the medium was slightly acidic; this was followed by a pH increase to a value close to 9.0 after 72 h of incubation.

Different BHI media such as Difco lots 668294 and 669679 and GIBCO lots 870092 and 870093 were tried for the production of the inhibitory activity, and the yields obtained were comparable. The SN1 medium (8) was compared with the BHI medium, but no activity was detected on the semisynthetic medium. It was also observed that production occurred on solid (1.5% agar) as well as on semisolid (0.4% agar) BHI medium. Removing the staphylococcal cells present on solid or semisolid medium before freezing and thawing of the culture did not modify the yield of antigonococcal activity found in the crude preparation. In agitated (250 rpm) liquid BHI medium incubated for 24 h, the production of the gonococcal inhibitor was very low, and the yield obtained was about 10 times smaller than the one observed on semisolid culture. No activity was detected in liquid culture when the incubation period was prolonged to 30 h or when it was nonagitated or agitated at 120 rpm.

The optimal incubation temperature for the production of the antigonococcal activity ranged from 37 to 39°C, and no production occurred at temperatures of 33 and 41°C. The pH of the semisolid culture medium before inoculation which enabled some production of the antigonococcal activity varied from 6.4 to 9.4, and the optimum was 7.4.

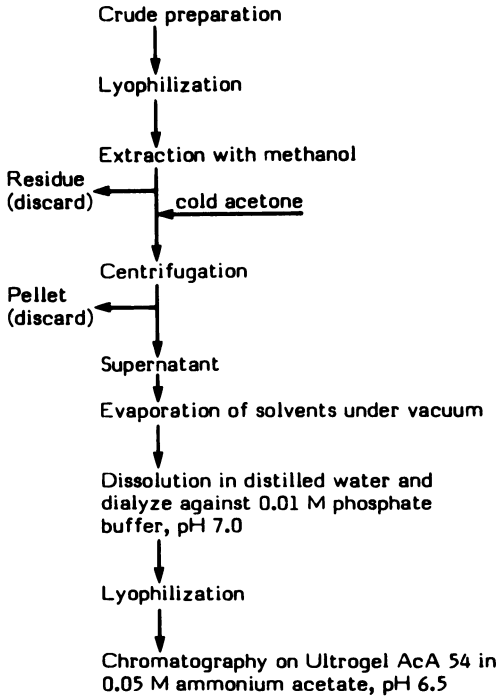

FIG. 2. Purification of the gonococcal growth inhibitor produced by the coagulase-negative staphylococcus no. 7.

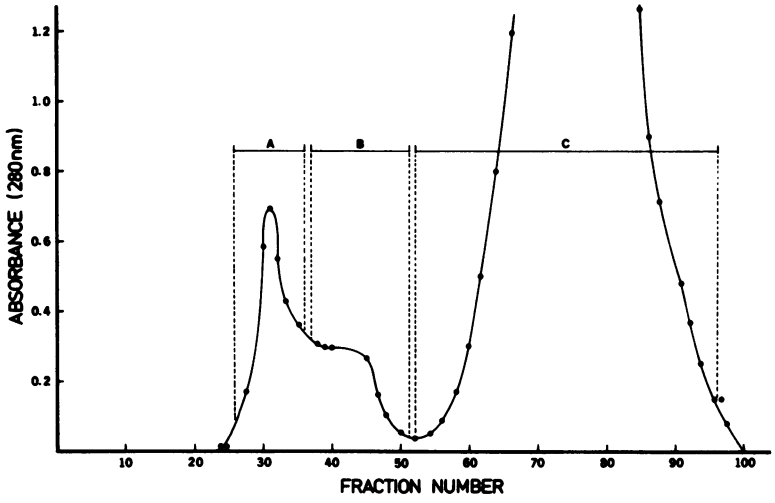

FIG. 3. Ultrogel AcA 54 gel filtration of the gonococcal inhibitor. The elution occurred in 0.05 M ammonium acetate buffer (pH 6.5). The tubes were pooled in three fractions, A, B, and C, and then were lyophilized.

Purification of the gonococcal growth inhibitor. A scheme followed for the purification of the growth inhibitor produced by staphylococcus no. 7 is given in Fig. 2. The crude preparation obtained from the semisolid culture medium was lyophilized and kept at 4°C in a desiccator under vacuum. Dry powder from a volume of 250 ml of the crude preparation was extracted twice at room temperature with 100 ml of methanol. The insoluble substances in methanol were removed by centrifugation at 10,000 × *g* for 20 min, and an equal volume of cold acetone was added slowly to the supernatant at 4°C. A large precipitate appeared which was discarded by centrifugation. The supernatant was evaporated in vacuo, the residue was dissolved in distilled water, and the solution was dialyzed for 24 h against 0.01 M phosphate buffer (pH 7.0). The dialyzed material was lyophilized and dissolved in 0.05 M ammonium acetate buffer (pH 6.5). The solution was applied to an LKB Ultrogel AcA 54 column (2.5 by 80 cm) (LKB Instruments Inc., Rockville, Md.) prepared in 0.05 M ammonium acetate buffer (pH 6.5) (Fig. 3). The flow rate was 50 ml/h, and fractions of 5.0 ml were collected. The peaks were pooled in three fractions named A, B, and C and were lyophilized. The fractions were dissolved in 0.01 M phosphate buffer (pH 7.0), and the activity was mainly found in the peaks A and B (Table 1). The intensity of peak A and its antigonococcal activity were variable and were sometimes missing from one column to another, even when the starting material came from the same batch. This result suggested that the peaks A and B contained the same active substance and that different complexes or aggre-

TABLE 1. Purification of gonococcal inhibitor from coagulase-negative staphylococcus no. 7

Fraction	Vol (ml)	Protein (mg)	Total activity[a] (AU)[b]	Sp act (AU/mg)	Recovery (%)	Purification
Crude prepn	250	5,837.5	4,000	0.7	100	1
Methanol-acetone extract						
Before dialysis	15	—[c]	2,880	—	72	—
After dialysis	23	88.3	2,944	33.3	74	48
Chromatography on Ultrogel AcA 54						
Peak A	5	3.6	765	212.5	19	304
Peak B	5	3.5	380	108.6	10	155
Peak C	5	73.7	135	1.8	3	3

[a] Evaluated against strain G-10.
[b] AU, Arbitrary unit.
[c] The protein content of this fraction was not determined since unusual coloration was present in the chemical assay, suggesting the presence of some interfering substances.

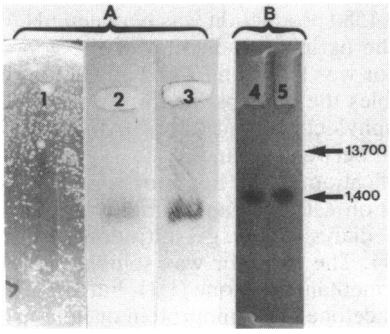

FIG. 4. (A) Agarose (1%) electrophoresis of the gonococcal growth inhibitor (peak A+B). The gels were tested for antigonococcal activity (gel 1) and were stained for protein (gel 2) and lipid (gel 3). (B) SDS-polyacrylamide gel electrophoresis on 10% gels in the presence of 8 M urea in the Swank and Munkres system. The gels were stained for protein. Gonococcal inhibitor peak A+B (gel 4), peak 2 (gel 5) (see Fig. 5). Bacitracine (1,400 daltons) and ribonuclease (13,700 daltons) were used as standards. Samples of the inhibitor were used at a concentration of around 40 μg of protein.

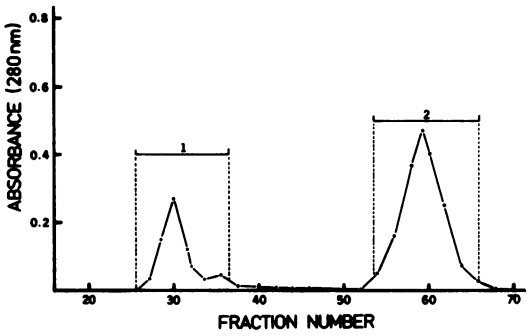

FIG. 5. Ultrogel AcA 54 gel filtration of the gonococcal growth inhibitor. The elution occurred in 0.05 M ammonium acetate buffer (pH 6.5) in the presence of 4 M urea. The tubes corresponding to the two peaks 1 and 2 were pooled and lyophilized.

gations were produced in the purification process.

Agarose gel electrophoresis in 0.01 M phosphate buffer (pH 7.0) was carried out with a sample of the pool of the peaks A and B, and the dried plate was stained for proteins with Coomassie blue and for lipids with Sudan black B. Only one band was found which migrated toward the anode and stained for proteins and lipids (Fig. 4A). The antigonococcal activity was also related to this band. No polysaccharides were detected in the peaks A and B by chemical analysis. The antigonococcal activity was attributed to a lipoprotein or a lipid-associated protein. The lipid component was estimated by chemical analysis to be 8.1% of the dry weight. The lipids were extracted with methanol-chloroform, and the residue obtained in the chloroform extract did not show antigonococcal activity, suggesting that the lipids were not the active component.

Separation of the protein component. To obtain the minimal chemical substance required for the antigonococcal activity, the peaks A and B were pooled and chromatographed on an LKB Ultrogel AcA 54 column (2.5 by 80 cm) in 0.05 M ammonium acetate buffer (pH 6.5) in the presence of 4 M urea. Two peaks were obtained (Fig. 5), and the fractions were pooled and dialyzed for 48 h against 0.01 M phosphate buffer (pH 7.0) to remove the urea. The activity of peaks 1 and 2 was determined; peak 1 contained 8.9% of the original activity with a specific activity of 178, and peak 2 had 78.6% of the original activity with a specific activity of 192. The peak 1 was eluted with the void volume, and this corresponded to a molecular weight equal to or larger than 70,000. The molecular weight of the peak 2 was estimated to be 15,900, as compared with the elution profile of globular protein standards chromatographed under the same experimental conditions.

The peak 2 contained only a protein component; no lipid was found in this fraction as determined by the absence of coloration with Sudan black B and by the chemical analysis for lipids. The urea completely dissociated the lipid of the protein. When the protein inhibitor was rechromatographed on the same column without urea, four peaks were found, corresponding to 13,600, 23,400, 29,900, and 36,900 daltons. Since each peak showed some antigonococcal activity, the inhibitor appeared to form complexes of different molecular size. A pronase treatment confirmed the role of the protein in the antigonococcal activity: 62 and 91% of the activity was lost for the lipoprotein (peak A+B) and the protein (peak 2), respectively, after 1 h of incubation at 37°C. The activity was completely lost with the trypsin enzyme after 3 h of incubation at 37°C. The lipoprotein was relatively stable to heating; a 50% loss of activity was observed after 40 min of incubation at 100°C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence or absence of SDS was used as a criterion of homogeneity of the purified substance. In the presence of SDS, the aggregates or complexes were dissociated, and only one band was observed for the peaks A and B when used separately or in mixture. This band migrated just behind the tracking dye, suggesting that it was a low-molecular-weight protein. Polyacrylamide gel electrophoresis in the presence of SDS and urea (Swank and Munkres system) is known to

achieve the separation and estimation of molecular weight of peptides and proteins between 1,000 and 10,000 daltons. Only one band was found in this gel system for the peaks A and B, and this band corresponded to the same electrophoretic migration as Bacitracine (1,400 daltons) (Fig. 4B). The same electrophoretic migration was also observed with the protein (peak 2) obtained after the chromatography with urea. After electrophoresis without SDS in 0.05 M phosphate buffer (pH 7.0), only one large band was obtained with the peak 2. In alkaline pH, this band was larger and not well defined. This particular profile in gel without SDS could be attributed to the presence of different complexes of the inhibitor and to their breakdown into smaller units during the electrophoresis.

DISCUSSION

The gonococcal growth inhibitor is produced extracellularly by staphylococcal cells in semisolid medium. The inhibitor is produced at the end of the logarithmic growth, a situation different from the one observed for staphylococcin C55 by Dajani and Wannamaker (6) and for staphylococcin A-1262a by Lachowicz (16). Unlike perfringocin 11105 (5), the passage of the inhibitor from the staphylococcal cells to the medium does not seem to require partial lysis of the cells. Other studies have also reported substantial losses of inhibitory activity after prolonged incubation of cultures (6, 16). This effect may be related to the appearance of specific inactivators, like the low-molecular-weight inhibitor of the bacteriocin 1580 found by Jetten et al. (13) in the culture supernatant or after enzymatic digestion of proteases, since staphylococci are known to produce these enzymes (1).

The production of gonococcal growth inhibitor was much higher on solid or semisolid media than in liquid media. Similarly, Jetten et al. (13) showed that upon growth of the cells on a semisolid medium, a crude staphylococcin 1580 preparation was obtained with an activity 20 times higher than that in liquid cultures.

It is well known that the growth conditions of a producer organism strongly influence the yield of the inhibitor (27). As shown by Lafond et al. (18), the production of the gonococcal inhibitor is influenced by the composition of the culture medium. Some components of the BHI medium seem to be required, since in the semisynthetic SN1 medium, no production occurred. In an investigation of conditions for optimal colicin K production, it was shown that the control of the pH of the medium was a critical factor (10). We have found that the gonococcal inhibitor production was increased by adjusting the initial pH of the medium to 7.4, whereas Jetten et al. (13) showed that the optimal pH range for staphylococcin 1580 production was between pH 6.5 and 8.0. The optimal production of the gonococcal inhibitor was between 37 and 39°C. This finding resembles the one obtained by Jetten et al. (13) for staphylococcin 1580 production, which was optimal between 35 and 38°C.

Purification of the inhibitor was achieved by extraction with methanol, fractionation with acetone, dialysis, and gel filtration on Ultrogel AcA 54. The inhibitor was soluble in methanol and in methanol-acetone (1:1), but it was insoluble in acetone. The lipoprotein or lipid-associated protein found in peaks A and B after Ultrogel filtration was present as complexes of different molecular weights varying from 50,000 to 70,000 and more. The largest complexes present in peak A were absent in some purifications, suggesting that these complexes were labile or not done in particular purification. The smallest unit found after Ultrogel chromatography in the presence of urea was estimated to be 15,900 daltons and consisted of proteins. However, in SDS-polyacrylamide gel electrophoresis, the inhibitor was dissociated on a subunit estimated to be approximately 1,400 daltons. The protein component appeared to be quite stable since its activity was not destroyed by urea and the organic solvents used in the purification procedure. However, the activity was completely lost when the preparation was dialyzed against distilled water. The dialysis step in the purification procedure carried out against 0.01 M phosphate buffer (pH 7.0) achieved a good purification of the inhibitor without loss of activity. The gonococcal inhibitor appeared as a protein or a lipid-associated protein which is generally present as complexes of different molecular size. Although our results suggest that some lipids are associated with the inhibitor, we cannot exclude the possibility that this association was done in the culture media or in the purification process. The role of the protein component in the antigonococcal activity is suggested by the loss of activity after pronase and trypsin treatment and by the activity found in peak 2 (protein inhibitor), no lipid being detected in this fraction as revealed by colorimetric assay and coloration in gel with Sudan black B. The possibility that fatty acids carried along during the separation process could be responsible for the inhibitory activity seems unlikely. Indeed, no activity was detected in the residue obtained in the chloroform phase after extraction of peak A+B (lipoprotein or lipid-associated-protein inhibitor) or peak 2 (protein inhibitor) with chloroform-methanol. If fatty acids had been present in our fractions, they would have been easily extracted with this system and their antigonococcal activity, if any, would have been detected in the chloroform extract.

This gonococcal growth inhibitor was different from the staphylococcin 1580 (13) isolated from *S. epidermidis* culture on the basis of its narrow antibacterial spectrum (submitted for publication) and its chemical properties. This bacteriocin is a lipopolysaccharide-protein complex which in the presence of urea was separated into a smaller unit consisting of protein, lipid, and carbohydrate. Our gonococcal growth inhibitor did not contain carbohydrate, and the lipid and protein components were separated in the presence of urea. Our gonococcal inhibitor was also found to be different from the staphylococcins Bac R_1 (20), C55 (6), 414 (9), and A (16), from the lysostaphin (23), and from the epidermidins (11). Recently, Simpson and Davis (25) reported the production of a gonococcal growth inhibitor produced by *Escherichia coli* which was bacteriostatic, had a molecular weight of 1,200 to 2,000, and was not susceptible to proteolytic enzymes.

The gonococcal growth inhibitor reported in this paper appears to be different from all other gonococcal inhibitors of bacterial origin already described in the literature. A further study of the chemical nature and of some properties of this inhibitor is presently being carried out in our laboratory.

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