THE INHIBITION OF NEISSERIA GONORRHOEAE BY A BACTERIOCIN FROM PSEUDOMONAS AERUGINOSA

by

Patrick H. Vaughan

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Stephen A. Morse, Ph.D.,

(Professor in Charge of Thesis

[redacted]

(Chairman, Graduate Council)

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INTRODUCTION

Bacteriocins are antibiotic substances of protein nature produced by certain strains of bacteria which are thought to be active only against strains of the same or closely related species (38). This species specificity and their chemical nature distinguish bacteriocins from classical antibiotics (1). Bacteriocins represent a chemically heterogeneous group of substances ranging from simple proteins such as colicin k (2) to particles resembling bacteriophage components (3). Members of this latter category are thought to be defective bacteriophages (4). Nevertheless these particles have the characteristic properties of bacteriocins. These properties are: (1) bacteriocins kill sensitive cells without multiplying within them, (2) bacteriocinogenic factors are perpetuated by plasmids or integrated genetic material, (3) bacteriocins are released from producing cells like temperate phages, and (4) bacteriocins may be produced spontaneously in low numbers or require induction with chemicals or ultraviolet light (5).

A major problem when working with bacteriocins is expressing a unit of activity. For this reason a Lethal Unit may be defined as the minimum amount of bacteriocin that will kill a sensitive indicator cell. Where analysis has

been possible, a Lethal Unit has been found to vary from 1 to 300 molecules (6,7). These differences may be explained by assuming that not all receptors which specifically bind bacteriocin will lead to cell death. To avoid this problem, a unit of activity is defined as the reciprocal of the highest dilution showing complete inhibition times the quantity applied (8). Thus, no attempt is made to determine the number of bacteriocin molecules in one unit.

S-type pyocins. There are two types of bacteriocins (pyocins) produced by Pseudomonas aeruginosa; the S-type which are low molecular bacteriocins and the R-type which resemble bacteriophage components. Pyocin S2 has been characterized (7) and shown to be a simple protein having a molecular weight of approximately 72000 and containing no carbohydrate or phosphorous. It may be distinguished from the R-type pyocins on the basis of a higher diffusion rate in agar which yields a larger zone of inhibition, by its sensitivity to pronase digestion and by its inability to be inactivated by antiserum against pyocin R. Ito et al (9) observed that S and R-type pyocins could be differentiated both by their size of inhibition zone and by the sensitivity of S-type pyocins to trypsin, chymotrypsin and Nagarase. In addition, all S-type pyocin activity passed through a Diethylaminoethyl (DEAE) cellulose column whereas the R-type activity was retained. S-type have no

discernible structure under the electron microscope. Homma (10) isolated a simple protein from the cell wall of P. aeruginosa. This protein appeared to be associated with the endotoxin. However, its bacteriacidal activity and its inactivation by pronase and Nagarase suggests that it may be an S-type pyocin.

R-type pyocins represent a class of bacteriocins which are similar to the pyocin R first isolated from P. aeruginosa strain R by Jacob (12). He was able to determine that its synthesis was inducible by ultraviolet light. A detailed chemical analysis was not made until 1962 when Kageyama and Egami (13) succeeded in purifying a high-titered pyocin preparation. They were able to determine that this R-type pyocin was a protein which was first produced in the induced cells and later released in high numbers via cell lysis.

Relationship between pyocin and bacteriophage. As stated earlier, the R-type pyocins are now considered to be defective bacteriophages. Early studies began with the finding of a lysogenic phage Ps 3 (14) which was neutralized by anti-pyocin R sera. Antisera directed against Ps3 was effective in neutralizing both pyocin R and R2. Similar observations were made by Homma (15) who isolated three temperate phages which morphologically (without the heads)

resembled pyocin R and which were neutralized by anti-pyocin R antisera. Three variant phages were found which did not resemble pyocin R nor were they neutralized by anti-pyocin R sera. They did however, bear a striking resemblance to pyocin 28, a long rod-shaped particle characterized by Takeya et al. (16). This type of pyocin is not classified with the R-type pyocins owing to its unique morphology. Other similarities have been noted. Yui (17) found distinct amino acid similarities when pyocin R was compared to phage T2 and Higerd et al. (18) noted rosette formation by pyocin R which also occurred with bacteriophages.

Morphology of pyocins. Since purification techniques (13, 18) have become available, the morphology of pyocins has been studied extensively. The R-type pyocins are much more complex than the simple proteins. Ultrastructural studies indicate that these bacteriocins are rod-shaped particles and consist of a contractile sheath surrounding an inner core.

Ishi, Nishi, and Egami (17) were the first to describe the presence of two forms of the R-type pyocins; the contracted and uncontracted particles. No evidence was presented that these pyocins were capable of contraction but they did suggest that the sheath and core were composed of different subunits. Urea (2M) caused complete disap-

pearance of the core structure leaving the sheath intact. The core was extremely labile to acid (pH 2.4) but resistant to alkali (pH=11.0). This was in contrast to the behavior of the sheath which was partially degraded by alkali but left intact by the acid. Other workers (20) showed that 8M urea degraded the cores whereas 10^{-4}M p-chloromercuribenzoate destroyed the sheaths.

Govan (21) demonstrated that these particles were indeed capable of contraction and that this process was required for killing. Using pyocin 21 whose morphology is similar to those of strain R pyocins (100x15nm for the uncontracted particle, 45x17nm for the contracted sheath) several important observations were made. If pyocins were combined with sensitive cells at 0°C, adsorption occurred. However, no contraction was visualized under the electron microscopy and no inhibition was observed. When the incubation temperature was increased to 37°C both contraction and cell death occurred. Moreover this adsorption was firm since the particles were removed with the cells by centrifugation at 2850xg. In addition, when the sheaths were removed with 0.02% SDS no adsorption was found. That the sheaths are required for biological activity was reinforced by Shinomiya (22) who, using mutants without sheaths showed that no biological activity was present.

Higerd et al. (20) observed the spontaneous production of pyocins after 8 hours growth of the producer strain. However, 90% of the pyocins observed by electron microscopy were in the contracted state. In a later investigation (18) these authors found that contraction was promoted by a variety of physical and chemical treatments and that pyocin activity was directly proportional to the percentage of uncontracted particles. Concentrations of magnesium chloride in excess of 0.5M, 1% Formalin, low pH sonic treatment, 1% osmium tetroxide and freezing and thawing caused contraction of the pyocin. The $MgCl_2$ concentration was the only treatment which caused reversible contraction since when dialyzed against lower concentrations, the pyocins resume their relaxed state. While the mechanism causing contraction has not been elucidated, Higerd et al. (18) postulated that the removal of some stabilizing or structural component such as ATP or calcium ions was involved. Evidence for this hypothesis was suggested by the observation that irreversible contraction occurred with prolonged dialysis against 0.02M $MgCl_2$.

Morphogenesis of pyocin R. A detailed series of analysis of the morphogenesis of pyocin R has been presented (17, 23, 24). Yui (17) found that 0.1 N NaOH degraded the pyocin into a contractile sheath and other subunits. The particle weight of the sheath was approximately 7.2×10^6

daltons and sedimented with a value of 89 S. Polyacrylamide gel electrophoresis suggests that there are four subunit species (35000, 32000, 29000, and 26000 daltons) in the sheath which comprise 53% of the total particle weight of pyocin R (23). These results differ from those of Garyaev and Poglzov (24) who, using the same strain, described an $S20_w$ of 103S for pyocin R instead of 90.5S. The estimated particle weight of the sheath was 4.9×10^6 daltons.

The sheath does not appear to be a simple hollow cylinder (23) but rather has a hollow cone attached to the top. This cone does not seem to have a strong quaternary structure since it is readily lost. It is estimated to be approximately 5 to 10 per cent smaller than the rest of the sheath and accounts for the bullet shaped appearance reported by Higerd et al (18). The number of turns of the sheath has been variously described as 33 (23) or 34 (24). Assuming a molecular weight of 7.2×10^6 daltons and that the main subunit occupies 73 per cent of the particle weight, then there are approximately six subunits per turn of the helix.

Biosynthesis and assembly of pyocins. Shinomiya (25) examined the biosynthesis and assembly of pyocins both by measuring the counts per minute incorporated into antigen-antibody preciptates and by the assay of serum blocking

power. Using strain M16 which produces R2 pyocin particles, he was able to obtain 22 subunits for the whole pyocin. No cleavage of structural proteins was observed. Pyocin specific proteins appear at 10-15 minutes post induction with the first active particles appearing 10 minutes later. Pyocin protein synthesis accounted for approximately 2-5 per cent and 30-40 per cent of the protein synthesis in non-induced and induced cells respectively. However, subsequent work (26) revealed that certain proteins were not precipitated with anti-pyocin R sera unless they were first integrated into mature particles. Pulse-labeling experiments demonstrated that pyocin proteins were synthesized in proportion to their final concentrations in the mature particles. These subunits were integrated with first order kinetics and with subunit dependent rate constants.

Abnormalities of pyocin particles. Abnormalities of the pyocin particles have been reported and consist of polysheaths (18) and poly-cores (18). Amako and Yasunaka (27) noted that the formation of polysheaths occurred during the treatment of pyocins with sodium dodecyl sulfate (SDS). Further experiments showed that 0.05 M NaCl was required for polysheath formation and that the optimum concentration of SDS was between 0.05 and 0.2 per cent. Since each polysheath consisted of multiples of the unit length pyocin sheath, it was concluded that these long rods

were formed by the aggregation of two or more (maximum observed was equivalent to 19 sheaths) single sheaths. SDS is an anionic detergent (28) and presumably causes binding at the ends of the sheaths. Therefore the ends would represent regions rich in positively charged proteins. This argument is strengthened by the fact that no aggregation occurs in the side to side configuration. A recent report (22) describes strains of P. aeruginosa which produce defective pyocin particles. 29 out of 42 strains produced polysheath structures but in addition two of these strains produced pyocins having other morphological defects. The first lacked the sheath component while the second had abnormalities of the base plate. However, both these pyocins could adsorb to sensitive bacteria. One of the most interesting findings was one which supported the concept of relatedness among R-type pyocins. The particle defective in the sheath (strain P15-16) could undergo <u>in vitro</u> complementation with another mutant to yield a fully active pyocin. There is evidence that assembly of sheath components around the core results in an active particle. All of the mutants which could complement P15-16 were of the polysheath type. Labeled P15-16 particles (45s) after complimentation regained the original 90.5S sedimentation valve and were precipitated by anti-pyocin R1 sera. These mutants have also led to a better ultrastructural analysis of R-type pyocins. Base plate and tail fibres have been described

previously (18) however resolution was poor thus preventing detailed structural analysis. Through the use of the sheathless mutant these structures can be readily seen. The baseplate is a structure approximately 10 nm in height and 15 nm in diameter. It also appears to have a thin end plate 25 nm in diameter to which are attached tail fibres 40 nm in length and 2 nm wide. Since these mutants adsorb to sensitive cells it was determined that attachment was by these tail fibres as previously postulated (21).

Pyocin receptor substance. The receptor substance for R-type pyocins has been analyzed. Ikeda and Nishi(29) digested sensitive cells with lysozyme and EDTA and found that most of the receptor was solubilized and separated from the cell membrane. They suggested that the receptor activity was localized within the cell wall. An approximation of 4.9×10^5 daltons for size of the receptor was obtained. This differs greatly from that of 4.2×10^7 daltons obtained by Weidel et al. for the T5 phage receptor. Pyocin inactivation by adsorption to the receptor was found to be dependent upon both temperature (37C) and salt concentration (0.1-0.2M NaCl).

Ikeda and Egami originally used trichloroacetic acid extraction (30) and later phenol extraction (31) to study the receptor. The receptor activity was dissociated into

subunits of 12000-16000 molecular weight with sodium deoxycholate but reassociated upon its removal. Chemical analysis of the lipopolysaccharide without the "lipidA" component showed a polysaccharide component of approximately 1300-1500 daltons. The polysaccharide contained glucose, rhamnose, heptose, glucosamine, galactosamine, quinovosamine, fucosamine, and a 2-keto-3-deoxy-sugar acid. No real comparison could be made to the resistant cell chosen for analysis since it was resistant not only to pyocin R but also to R3 and R4. It was thus not clear whether or not these receptor sites were the same or represented defects in more than one site. Relatedness of the receptor sites for the various pyocins was however obtained by Ito and Kageyama in 1970 (14). The mutants they used when resistant to pyocin R, were often resistant to all the rest including phage PS3. The sensitivities to the various agent could be arranged in linear order such as R3-R4-R2-R. Any mutant selected was in general resistant to those to the left of it. If one assumes a branching out of the R receptor site then resistance to a pyocin type can be explained by a loss of part of the LPS moiety.

Genetic analysis of pyocin R2. While other bacteriocins are synthesized from genetic information carried within plasmids (33) the genetic information for R-type pyocins is integrated within the host genome. Data is presently

available for pyocin R2 only (34,35). Kageyama has mapped a bacteriocinogenic factor both by conjugation (34), and transduction (35). Through conjugation the pyocin marker was found to be linked to the tryptophan (trp) marker. Since more than 98 per cent of the trp+ recombinants inherited the female chloroamphenecol (chl) trait (the chl marker is known to be distant to the trp-pyocin (py) markers) it was concluded that there was no possibility of transfer of the py marker via a plasmid. In addition the non-pyocinogenic character could also be transferred from the male to the female strain via recombination. A generalized transducing phage was found which would transfer the pyocinogenic character. Using phage F116 the results indicated that no co-transduction was possible between py R2 or Trp and met-9, pro-3,arg-5, lys-2 or ilv-6. However, there was co-transcution between the trp or py R2 markers and that of str. This yielded the following gene order; str-trp-1-pyR2. Since the pyocins are considered to be defective phage the pyocin locus on the bacterial chromosome may correspond to the prophage site of a temperate phage. Kageyama et al. (36) have tried unsuccessfully to cure the pyocinogenic factor with acridine orange, acriflavin and irradiation with ultraviolet light.

Mode of action of R type pyocins. The mode of action of bacteriocins depends on the bacteriocins studied.

Colicin M (37) exerts its effect on the cell membrane causing lysis. Bacteriocin JF246 from Serratia marcenscens (38) completely inhibits DNA and protein synthesis as does colicin k (39). Colicin E3 (40) seems only to effect protein synthesis.

Two initial observations were made concerning pyocin inhibitory activity. Higerd(20) found that 10^{-3}M Ethylenediaminetetraacetic acid (EDTA) and 0.7M NaCl had no effect on pyocin activity. However, as was observed with colicin k (41), animal sera enhanced the inhibitory activity of R-type pyocin. Aged human and rabbit sera increased the activity of the R type pyocin proportionately with increasing concentrations of serum. Unlike colicin k, there was no enhancement of activity with purified hemoglobin nor with sera from bovine, calf, chicken or horse.

Another report (36) on the biological activity of pyocin R suggests that membrane damage occurs after adsorption of the pyocin to its receptor similar to that observed with a bacteriocin of Bacillus megaterium. Increasing concentrations of pyocin caused an increase in the amount of ultraviolet absorbing material released from cells of P. aeruginosa (36). In addition, a bacteriolytic enzyme was found in the lysates of mitomycin C induced cells of P. aeruginosa, which was active against the pyocin sensitive and pyocin resistant (producer strain) cells. No further characterization of the muramidase-like enzyme was reported.

However, its activity was inhibited by anti-pyocin R sera suggesting that it may be associated with the pyocin particle. The addition of pyocin to sensitive cells produces an immediate cessation of RNA, DNA, and protein synthesis (42). However, there was no degradation of DNA into small TCA-soluble fragments. Measuring the incorporation of radioactive phosphate into the cold trichloracetic acid (TCA) soluble cell fraction demonstrated that synthesis of ATP was reduced but not completely abolished. Therefore, the primary site of pyocin inhibition does not seem to be energy metabolism. Thus, the pyocin R appears to behave like colicin k (63) except that pyocin treated cells cannot be trypsin rescued. Growing cells are much more sensitive to the pyocin (42) than are cells in the stationary phase of growth.

Kaziro and Tanaka (43) presented evidence that protein synthesis was inhibited after the addition of an R type pyocin from P. aeruginosa strain k. Extracts from pyocin treated cells were unable to support poly U-dependent incorporation of ^{14}C - phenylalanine. Analysis of the ribosomes by ultracentrifugation demonstrated the disappearance of the 69S peak which was replaced by two new peaks at 50S and 37S. Kaziro (43) concluded that the rapid inhibition was due to the ribosomal inactivation since the supernatant fraction was fully active when combined with ribosomes from normal cells.

Pyocin typing systems. Pyocin typing has been in use for approximately a decade now. Holloway (45) and Papavassiliou (46) suggested that the considerable variation in pyocin activity could be useful in differentiating strains of P. aeruginosa. Since then, two general procedures have evolved. The first, involves the assay of pyocins spontaneously produced in agar. This method was originally used by Darrell and Wahba (47) and later modified by Gillies and Govan (48). The unknown strain is allowed to incubate on a trypticase-soy agar plate after which the growth is scraped off. Indicator strains are applied perpendicular to the original growth and the inhibition of growth resulting from diffused pyocins recorded. Gillies (48) reported that 88.4 percent of the strains tested were typable by this method. In a later paper, Govan (49) noted that aberrant results were obtained if the temperature and time of incubation were not carefully controlled. He also observed that 5.7 percent of strains examined had altered patterns of inhibition after storage for several months. Pyocin type 1 was the most commonly isolated type (49). Therefore, eight additional indicator strains were added which yielded eight distinct sub types (a-h) of type 1.

There are several problems associated with this first technique. Wahba (50) reported that pyocins are inactivated by undefined cellular substances upon prolonged incubation. Secondly, while some strains produce large num-

bers of pyocins giving clear zones of inhibition, others produce less pyocin showing incomplete inhibition. Thus, no clear positive or negative result may be assigned (51), and reporting of results may vary from day to day. Lastly, some types of pyocin (ie. group 1) are so common that other methods must be used with pyocin typing. Gillies (48) circumvented this problem by adding more indicator strains while Heckman et al. (52) assigned another broad class for those strains which were not typable.

The second method involves production of pyocins in broth. Osman (53) used pyocins which had been spontaneously produced after 24 hours of growth in nutrient broth. He was able to assign 101 isolates of P.aeruginosa into 10 groups using sensitivities to four pyocins. Farmer and Herman (54) modified this basic broth technique. During logarithmic growth unknown strains are treated with mitomycin C which induces both pyocin and phage. These lysates were then tested against twenty-seven indicator strains and the zones of clearing were determined to be either the result of pyocin or phage. After the production pattern had been tested the sensitivity pattern was analyzed using twenty-four standard pyocin-phage lysates. The result was fifty-one characteristics which gave an "epidemiological fingerprint". This method has several advantages. Mitomycin C induction produces larger numbers of pyocins, and strains thought to be non producers by the scrape and streak method

(48) produced pyocin after mitomycin C induction. Finally, by using a relatively short incubation period of eight hours, the decrease in pyocin activity due to proteolytic enzymes (50) was eliminated. Induction with mitomycin C induces bacteriophage which may complicate the reading of results (54). Rampling (55) developed a technique for obtaining phage-free lysates for pyocin typing. The method required a high titer-pyocin preparation and involves irradiating the induced lysates with ultraviolet light at 254nm. Treatment at a dose of 1116 ergs per mm^2 per min. for 30 minutes prevented plaque formation and at the same time preserved sixty-five per cent of the pyocin activity. Jones et al. (56) have developed a simplified typing technique. This method uses trypticase soy broth with added 1% potassium nitrate thereby eliminating the need for aerating the cultures. However, since these cultures are not induced, difficulties in interpreting the results as well as interference by bacteriophage occurs.

Inhibition of Neisseria gonorrhoeae by other bacteria. A paucity of information exists concerning the inhibition of N.gonorrhoeae by bacteriocins. No bacteriocin has been found for the gonococcus (58). Walstad et al. (57) demonstrated growth inhibition by phospholipid and free fatty acids liberated during the growth of N. gonorrhoeae. A slight growth inhibition has been observed by a heat-and

proteinase-labile substance from staphylococci (60). Geizer (61) also observed inhibition by substances produced by other organisms, however, no analysis or identification of the inhibitory substance(s) was reported.

STATEMENT OF PROBLEM

Geizer (61) mentions the inhibition of N. gonorrhoeae by P. aeruginosa. Since it is known that P. aeruginosa produces both S-and R-type pyocins, it was conceivable that this inhibition might be due to such bacteriocins. However, the presence of free fatty acids or phospholipids (57) could not be excluded.

Accordingly the primary questions asked in the research presented in this thesis are as follows:

1. What is the nature of the inhibitor produced by Pseudomonas aeruginosa?

2. What is the spectrum of activity of this inhibitor within the genus Neisseria?

MATERIALS AND METHODS

Organisms. Clinical isolates of N. gonorrhoeae were used in these studies. The specific properties of strains CS-7, JW-31, and 72H870 were previously reported (81). Strains from disseminated gonococcal infections were obtained from K. Holmes (U. S. Public Health Service Hosp., Seattle, Washington). Additional clinical isolates from non-disseminated gonococcal infections were obtained from the Multnomah County Health Dept., Portland, Oregon. Known serological types of N. meningitidis were obtained from H. Schneider (Walter Reed Army Institute of Research, Washington, D. C.). Strains of N. lactamica and other species of Neisseria were obtained from D. Hollis and D. Kellogg, Jr. (CDC, Atlanta, Ga.). The identity of all organisms was confirmed by cell morphology in gram-stained smears, oxidase reaction, and the production of acid from specific carbohydrates (59). T-1 through T-4 colony types (75) from single isolates were subcultured and maintained by selective passage.

A strain of P. aeruginosa (Pa-103) was obtained from P.V. Liu (University of Kentucky, Lexington). This strain was used throughout this investigation as a source of R-type pyocin. The titer of the crude and purified pyocin preparations was determined using P. aeruginosa strain Ps-7 (74) (obtained from E. Fisher, Portland State University,

Portland, Oregon).

Medium. The basal medium contained the following per liter: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. The final pH of the medium was 7.2. When used for the production of R-type pyocins by P. aeruginosa, glycerol (1%vol/vol) and monosodium glutamate (8.46g/liter) were added after autoclaving. When used for the growth of Neisseria spp., a growth factor supplement (1%vol/vol) identical in composition to IsoVitaleX enrichment (BBL) but lacking glucose, $NaHCO_3$ (42 mg/liter), and glucose (5 g/liter), was added after autoclaving. GC agar (Difco) plates containing glucose (5 g/liter) and growth factor supplement (1% vol/vol) were used where indicated.

Induction R-type pyocins. An overnight culture of P. aeruginosa was centrifuged (2,100 x g for 10 min.) and resuspended to one-tenth the original volume in a solution containing 0.85% NaCl and 0.1% cysteine hydrochloride at pH 6.5.A 1% (vol/vol) inoculum was used and the cultures incubated on a gyrotory shaker at 37°C. When the turbidity of the culture reached approximately 150 Klett units, mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 1 ug/ml. Incubation was continued until extensive lysis of the culture occurred (Fig. 1). This lysis normally occurred within 3 hours after the addition of the mitomycin C. The mitomycin C-induced culture

was centrifuged at 2,400 x g for 30 min. to remove cellular debris and the resulting supernatant treated with chloroform (5% vol/vol). This supernatant fraction was designated as crude pyocin.

Purification of the R-type pyocin. Crude pyocin preparations were further purified by a modification (21) of the method of Kageyama and Egami (13). Briefly, this procedure consisted of the slow addition of 1 M $MnCl_2$ (60ml per liter of lysate), while stirring, to the crude pyocin preparation. After adjusting the pH to 7.5 with 1M NaOH, the resulting precipitate was removed by centrifugation (2,400 x g for 15 min.). The supernatant was designated partially purified pyocin. Further purification was accomplished by the addition of solid $(NH4)_2\ SO_4$ to 70% saturation and incubating overnight at 4°C. After centrifugation (2,400 x g for 30 min.) at 4°C, the pellet containing the pyocin activity was dissolved in 50 ml of 0.01 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 7.5) containing 0.01 M $MgCl_2$ and 0.01 M $MgSO_4$ and dialyzed overnight at 4°C against 2 liters of the same buffer. The preparation was then centrifuged at 100,000 x g for 90 min. (type 40 rotor, Spinco model L-265B ultracentrifuge). The gelatinous pellet was gently dissolved in 20 ml of buffer and chromatographed on DEAE-cellulose (De-52, Whatman Biochemicals Ltd. Kent, England) previously

washed and equilibrated with the same buffer. An 8ml sample of pyocin was applied to a 1.5 x 28 cm column and allowed to adsorb for 1 hour. The column was washed with 200 ml of buffer to remove material not adsorbing to the DEAE-cellulose. The pyocins were then eluted with 800 ml of an NaCl gradient (0 to 1.0 M) in 0.01 M buffer. Five-ml fractions were collected and analyzed for absorbances at 280 nm and pyocin activity. The fractions exhibiting pyocin activity were pooled, dialyzed against 0.01 M Tris buffer (pH 7.5) to remove NaCl, and then concentrated by ultracentrifugation (100,000 x g for 90 min.). All chromatographic procedures were carried out at 4°C.

Adenosine diphosphate ribosyl (ADP-R) transferase activity. An enzyme mixture containing aminoacyl transferase activity was prepared from crude extracts of rabbit reticulocytes by a modification (68) of the method of Allen and Schweet (69). ADP-R transferase was measured by the procedure of Collier and Kandel (68). Briefly, the assay mixture in a total volume of 65 ul contained 50 mM tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 8.2), 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA), 40 mM dithiothreitol, 25 ul of the reticulocyte enzyme, 0.367 uM adenine-^{14}C NAD (specific activity 136 mCi/mmol) and various amounts of purified pyocin preparation, P. aeruginosa exotoxin A or diphtheria toxin fragment A as indicated.

The reaction mixture was incubated at 25°C for 5 min. then stopped by the addition of 65 ul of 10% trichloroacetic acid (TCA). The TCA-insoluble precipitates were collected, washed and counted on a Nuclear-Chicago low background counter as previously described (70).

P. aeruginosa exotoxin A was produced and purified by the method of Liu (71) as previously described (72). Diphtheria toxin fragment A was kindly provided by R.J. Collier (Dept. of Bacteriology, University of California at Los Angeles). Protein was determined by the method of Lowry et al. (73) using bovine serum albumin as a standard.

Pyocin typing. Pyocin typing was performed using the broth method as described by Jones et al. (57). The ALA set of eighteen strains of P. aeruginosa (obtained from B.H. Minshew, University of Washington School of Medicine) was used for indicator strains (57). The pyocin type and pattern are reported by the notation described by Farmer and Herman (54).

Inhibition in liquid media. Overnight cultures of N. gonorrhoeae strain Cs7, JW-31, 72H870, and 72H873 were centrifuged (2,100 g for 10 min.) and resuspended to one-tenth their original volume in a solution containing 0.85% NaCl and 0.1% cysteine hydrochloride (pH 6.5) Klett flasks containing 50 mls of basal media were inoculated with 0.5

ml of the cell suspension. Cultures were incubated on a gyrotory water shaker at 37°C. When the turbidity reached approximately 100 Klett units, (Klett meter, No. 54 filter, Klett-Sommerson Co.), 5.0 ml of the cells were removed and replaced by 5.0 ml of the pyocin preparation ($2x10^5$ units/ml). Incubation was then continued and the turbidity monitored as above. To determine the concentration dependence of the inhibitor, N. gonorrhoeae strain 72H870 was prepared as above with the exception that 275 ml of the culture was grown in one flask until the turbidity reached approximately 50 Klett units. Aliquots (50 ml) of culture were pipetted into 5 Klett flasks and the incubation continued. When the turbidity reached approximately 100 Klett units, 5.0ml, 1 ml, 0.1 ml, and 0.0 ml of the pyocin preparation ($2x10^5$units/ml) was added and incubation continued.

Assay of pyocin activity. Organisms being tested for susceptibility to the R-type pyocins were grown overnight on GC agar plates. A suspension of these organisms was prepared in a diluent containing 0.85% NaCl and Cysteine hydrochloride at pH 6.4 and adjusted to a Klett reading of 50-60. GC agar plates were inoculated by means of a swab dipped into the cell suspension. After drying undiluted or serially diluted pyocin preparations (5 ul) were applied to the surface of the agar plate. All plates were incubated overnight at 37°C with increased CO_2 (5%) prior

to being read. Pyocin titres are expressed as 200 times the highest dilution which shows complete inhibition. The cross-streak method of Fredericq (1) was used to assay inhibition of N. gonorrhoeae by spontaneously produced pyocins.

Adsorption of pyocin 611 131 to N. gonorrhoeae JW-31 and P. aeruginosa PS-7. Overnight inocula of the N. gonorrhoeae and P. aeruginosa were prepared as described above. Cultures were inoculated and incubated at 37°C on a gyrotory shaker until the turbidity reached 100 Klett units. Aliquots (6.0 ml) were removed from the culture and 1.0 ml of the purified pyocin preparation (2×10^6 units/ml) was added. Cells were incubated at 37°C for 45 min. Following centrifugation (10,000 RPM for 20 min.) the supernatants were filtered through a 0.45 u filter (Nalgene Co.). The resulting filtrate was then serially diluted and 5 ul amounts applied as above in the spot tests. In this manner the filtrate from N. gonorrhoeae strain JW-31 was applied to the P. aeruginosa strain PS-7. The plates were then incubated overnight at 37°C with increased CO_2 (5%) prior to being read.

Protease treatment of R-type pyocins. Purified pyocins were diluted to a final concentration of 1×10^4 units/ml in 0.01 M Tris-hydrochloride buffer (pH 7.5)

containing 0.01 M $MgCl_2$ and 0.01 M $MgSO_4$. Protease from Streptomyces griseus (Type VI, Sigma Chemical Co., St. Louis, Mo.) or from Bacillus subtilis (Type VIII, Sigma Chemical Co.) were added at a final concentration of 1.0 mg/ml. The sensitivity of the pyocin to trypsin was tested by preparing a similar dilution in 0.05M Tris-hydrochloride buffer (pH 8.1) containing 0.01 M $CaCl_2$; trypsin (Type III, Sigma Chemical Co.) was added at a final concentration of 0.5 mg/ml. Controls consisting of samples diluted in the appropriate buffer were also included. All samples were incubated at 37°C for 3 hours and then titered against P. aeruginosa strain PS-7.

Production of radioactive pyocin particles. An overnight culture of P. aeruginosa strain PA-103 was centrifuged (2,100 x g for 10 min.) and resuspended to one-tenth the original volume in a solution containing 0.85% NaCl and 0.1% cysteine hydrochloride at pH 6.5. A 1% (vol/vol) inoculum was used and the culture incubated on a gyrotory shaker at 37°C. When the turbidity of the culture reached approximately 140 Klett units, 0.1 ml of an H^3 amino acid mixture (1.0 mCi/ml, New England Nuclear, Boston, Mass.) was added. This was followed 1 minute later by the addition of 1.0 ug/ml of mitomycin C. After lysis occurred, the pyocin was purified as described previously.

Studies on the adsorption of labeled pyocin to N. gonorrhoeae. Selected strains of N. gonorrhoeae were grown to 240 Klett units (approximately 2 x 10^9 cfu/ml). Labeled pyocin (0.25 ml/2.25 ml cells) was added and the incubation carried out at room temperature. At 0, 2, 15, and 30 minutes, samples were removed and centrifuged at 10,000 x g for 15 minutes to remove cells. The supernatant (0.15 ml) was counted in a Beckman Liquid Scintillation Counter following the addition of 15.0 ml of Tritosol scintillation fluid (76).

RESULTS

I. Production of the inhibitory factor.

The inhibitory factor was produced in low concentrations during the growth of P. aeruginosa strain PA-103 both in complex medium (Table 1) and by the method of Fredericq (1). The addition of mitomycin C (MMC) (1ug/ml) to logarithmic phase cells caused extensive lysis of the culture within 3 hours which resulted in a 500 fold increase in the concentration of the inhibitory factor (Table 1). The inducible nature of this factor suggested the involvement of a bacteriophage or a bacteriophage product. However, no plaques were observed when dilutions of supernatants from both induced and non-induced cultures were spotted on N. gonorrhoeae strain JW-31. Instead, the area of growth inhibition increased in turbidity with increasing dilution suggesting the presence of a bacteriocin. The activity was eliminated with successive agar-plug transfers of the zone of inhibition on N. gonorrhoeae strain JW-31. Finally there was no difference in the pattern of induction when cells of P. aeruginosa were grown in GC broth or in TSB, both containing glycerol and MSG (Figure 1). Therefore all subsequent work utilized the GC broth.

II. Purification of the inhibitory factor.

The inhibitor was partially purified from the

supernatants of mitomycin C induced cultures of P. aeruginosa strain PA-103 as described in Materials and Methods. Further purification could be obtained by DEAE-cellulose chromatography (Figure 2). The inhibitor was eluted with an NaCl gradient of 0.0 - 1.0 M. Two peaks containing inhibitory activity were observed. The major peak (A) eluted at an NaCl concentration of 0.06 M and contained more than 90 per cent of the inhibitory activity. A minor peak (B) eluted at an NaCl concentration of 0.91 M and contained less than 10 per cent of the activity. The fractions comprising peak A were pooled, dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.5) to remove the NaCl and concentrated by ultracentrifugation (100,000 x g for 90 min.). This preparation containing 2×10^6 units/ml of activity was used for the remainder of these studies.

III. Characterization of the inhibitor.

A. Electron microscopy. Negative-stained preparations of the purified inhibitory factor were examined in the electron microscope. Particles resembling R-type pyocins were observed in both the contracted and uncontracted states (Figure 3). In the uncontracted state, these particles measured 1156 Å in length by 165 Å in width. In the contracted state, the particles consisted of an inner core (1156 Å in length by 82 Å in width) surrounded by a contractile sheath (495 Å in length by 206 Å in width).

Between 20 and 30 per cent of the particles observed in these preparations were in the contracted state. Neither intact bacteriophage or bacteriophage ghosts were seen in any of the negatively-stained preparations. When the pyocins were combined with sensitive cells of N. gonorrhoeae (Figure 4) many contracted particles can be seen to be adsorbed to the bacterial surface as well as in the vicinity of the bacteria. However, when the pyocins were added to the resistant cells of N. ovis (Figure 5) no adsorption was evident. Bullet-shaped particles are also present, but only in the uncontracted state. The contracted particles lose their conical appearance of the top portion of the sheath and the core appears to protrude from the top.

B. Resistance to proteolytic enzymes. R-type pyocins may be differentiated from S-type pyocins by their resistance to proteolytic enzymes (7). Therefore, the effect of various proteolytic enzymes on the inhibitory activity of the purified pyocin preparation was examined. The results (Table 2) indicate that the titer did not decrease with either trypsin or protease treatment, when compared with an untreated control having a titer of 2×10^6 units/ml. These data therefore, suggest that the inhibitory activity was not due to an S-type pyocin.

C. Relationship of the inhibitor to protein toxin. Many strains of P. aeruginosa produce a protein toxin (exotoxin A) which inhibits mammalian protein synthesis (70). The toxin catalyzes the transfer of the ADP-ribose moiety of NAD to elongation factor 2 (Ef-2) resulting in an ADP-ribosyl-Ef-2 complex which is inactive in peptide chain elongation (70, 72). The possibility that the inhibition of N. gonorrhoeae was due to the presence of this toxin in the pyocin preparation was investigated. The results (Table 3) show that neither the crude nor the purified preparation from P. aeruginosa strain PA-103 contains ADP-ribosyl transferase activity. Thus, it seems unlikely that the inhibitory effect of the pyocin preparation is due to exotoxin A.

D. Pyocin typing. The type of the pyocin in both the partially purified and purified preparations was determined as described in Materials and Methods. The pyocin typing pattern did not change during the purification and suggests that one or more pyocin types were not removed during chromatography on DEAE cellulose. The pyocin pattern is 611 131.

IV. Effect of the R-type pyocin on the growth of N. gonorrhoeae.

A. Inhibition in liquid media. In order to determine the relative time sequence of inhibition and to verify the results obtained on solid media, various concentrations of pyocins were added to growing cultures of strains of N. gonorrhoeae. Figure 6 shows that the pyocins exerted their effect rapidly and that the strains tested behaved in a similar manner. A concentration dependent inhibition of N. gonorrhoeae occurred upon addition of type 611 131 (Figure 7). At high concentrations of pyocin (2×10^4 units/ml) a complete inhibition of growth occurred within one hour and was accompanied by extensive lysis of the culture.

B. Inhibitory spectra of pyocin type 611 131. Aliquots of the purified pyocin preparation were spotted on lawns prepared from clinical isolates of N. gonorrhoeae. Typical patterns of inhibition are shown in Figure 8. The zone of inhibition was clearly evident in all strains examined. A negative control of the producer strain P. aeruginosa PA-103 was also included in all assays. The inhibition of various Neisseria species is shown in Table 4. All isolates of N. gonorrhoeae from both the disseminated and non-disseminated infections, were inhibited. However, outside this species inhibition was more restricted; only 3 of 20 strains of N. meningitidis and 5 of 16 strains of N. lactamica were inhibited. There was no correlation observ-

ed between the serological group and inhibition of N. meningitidis.

C. Colony type variation. N. gonorrhoeae exhibits changes in colonial morphology which have been correlated with changes in virulence. Therefore colonial variants were tested as to their sensitivity to inhibition by the pyocin 611 131. No correlation between colony type and sensitivity was observed. All 4 colony types of the strain 1138 were sensitive to the pyocin.

D. Adsorption of pyocins to JW-31 and PS-7. An assay was performed to detect residual pyocin activity after incubation with cells of N. gonorrhoeae strain JW-31 and P. aeruginosa strain PS-7. This would account for the difference in titer observed with pyocin 611 131 was applied to P. aeruginosa strain PS-7. Table 5 indicates that the supernatants from the incubated pyocin-GC suspensions (cells in excess) contained residual pyocin particles would could inhibit P. aeruginosa strain PS-7. When the reverse experiment was performed using JW-31 as the indicator for the pyocin-PS-7 suspension, no residual activity was detected.

E. Isolation of a mutant resistant to the pyocin. When cells of JW-31 were tested for sensitivity to the pyocin 611 131, minute colonies were observed in the center

of the zone of inhibition. These colonies were picked and restreaked onto GC agar plates. When again tested with the pyocin, no zone of inhibition was seen. These mutants were confirmed to be N. gonorrhoeae. When tested with a different pyocin (type 383 122) normal zones of inhibition were seen.

F. Adsorption of ^{3}H-labeled pyocins to bacteria. The objectives of this experiment were twofold. First, to determine the extent of the binding of pyocins to Neisseria and secondly, to see if the pattern of binding was the same as that with P. aeruginosa.

It is known that the pyocins are adsorbed to sensitive P. aeruginosa both at 0°C and at room temperature (36). This binding is essentially complete within 15 minutes at which time approximately 90% of the added pyocins have been bound. From these results, it appears that while cell death is a function of the incubation temperature (21), the maximum amount of binding is not.

The N. gonorrhoeae and pyocins were prepared as listed in Materials and Methods. Cell excess was achieved by combining 4.5ml of cells (2×10^8 cfu/ml) and 0.5ml of the labeled pyocin preparation (approx. 10^6 units/ml). After incubation at room temperature and counting, the results (Table 6) show that when the pyocins are combined with

sensitive N. gonorrhoeae, adsorption is essentially complete within 15 minutes. However, in this case, the maximum amount of binding attained was 69 per cent. When the pyocins were combined with the non-sensitive N. ovis, no binding was observed.

Cultural conditions	Incubation time (hours)	Pyocin activity (units/ml)
Non-induced	18	3.5×10^3
Induced	3	2×10^6

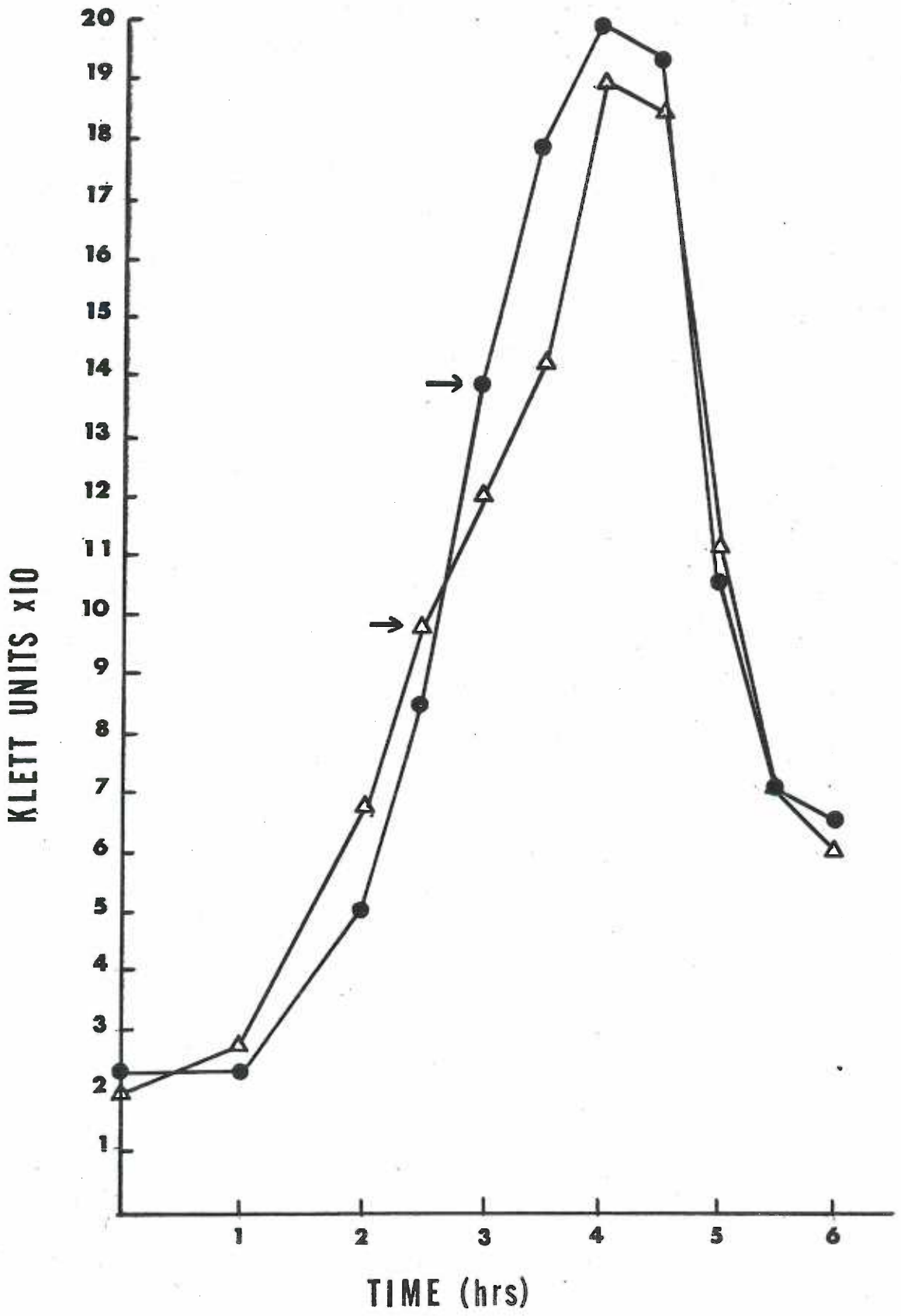
Table 1. Effect of mitomycin C on the production of the gonococcal inhibitory factor.

P. aeruginosa strain PA-103 was grown in GC broth as listed in Materials and Methods. Mitomycin C was added at a final concentration of 1.0 ug/ml. Titering was performed on P. aeruginosa strain PS-7.

Figure 1

A comparison of mitomycin C inductions of P. aeruginosa strain PA-103 using two kinds of media.

An overnight inoculum was resuspended to one-tenth the original volume in a solution containing 0.85% NaCl and 0.1% cysteine hydrochloride. Incubation was continued at 37°C. Symbols: ●, GC broth with added supplements; Δ, Trypticase soy broth with 5% glycerol and 1.0M monosodium glutamate. The arrows indicate the point at which mitomycin C (1 ug/ml) was added.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TIME (hrs)

Figure 2

Purification of R-type pyocin (611 131) by DEAE-cellulose chromatography.

Fractions containing inhibitory activity are indicated by A and B. Insert: Induction of pyocin production in Pseudomonas aeruginosa PA-103 by mitomycin C (1 ug/ml). The arrow indicates time of mitomycin C addition.

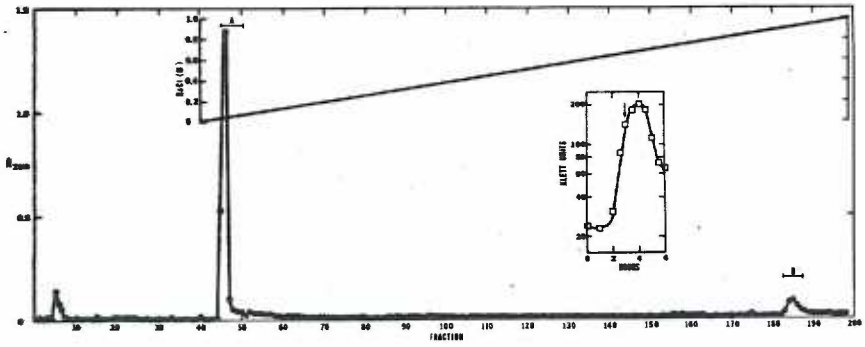

A
B
FRACTION

Figure 3

Electron micrograph of the R-type pyocin 611 131.

Pyocin 611 131 was purified as listed in Materials and Methods. A 1:10 dilution of the pyocin suspension was made in 0.1 M ammonium acetate. The pyocin was negative stained with 1.5% sodium phosphotungstate (pH=6.5). Symbols: uc, uncontracted pyocin; c, contracted pyocin. Bar= 0.1 um. Specimen examined with a Phillips 200 electron microscope.

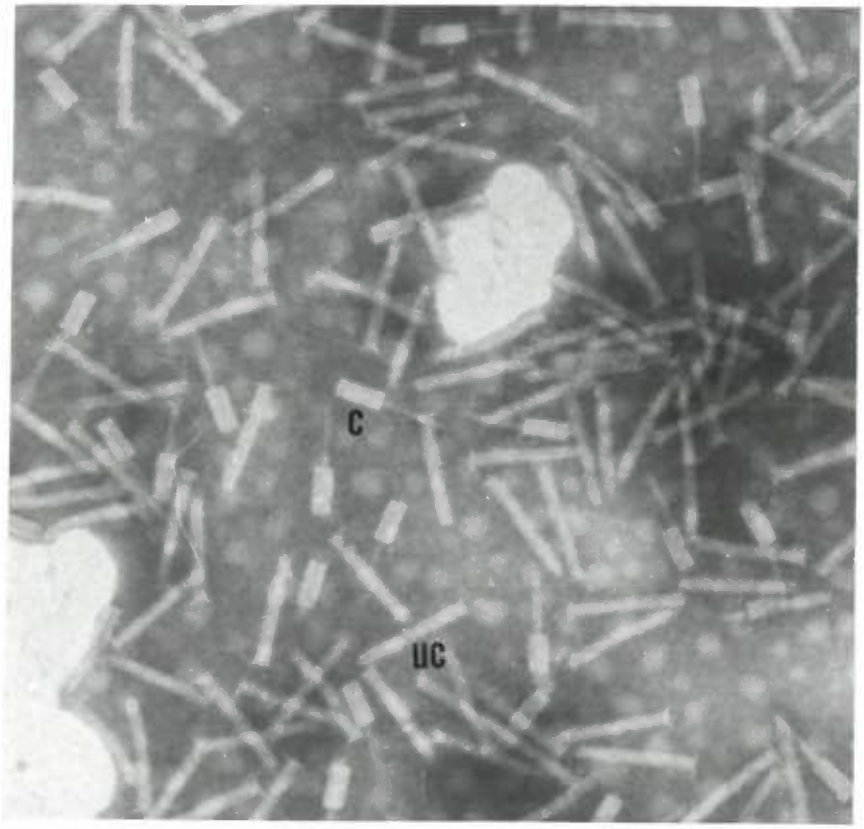

C
UC

Figure 4

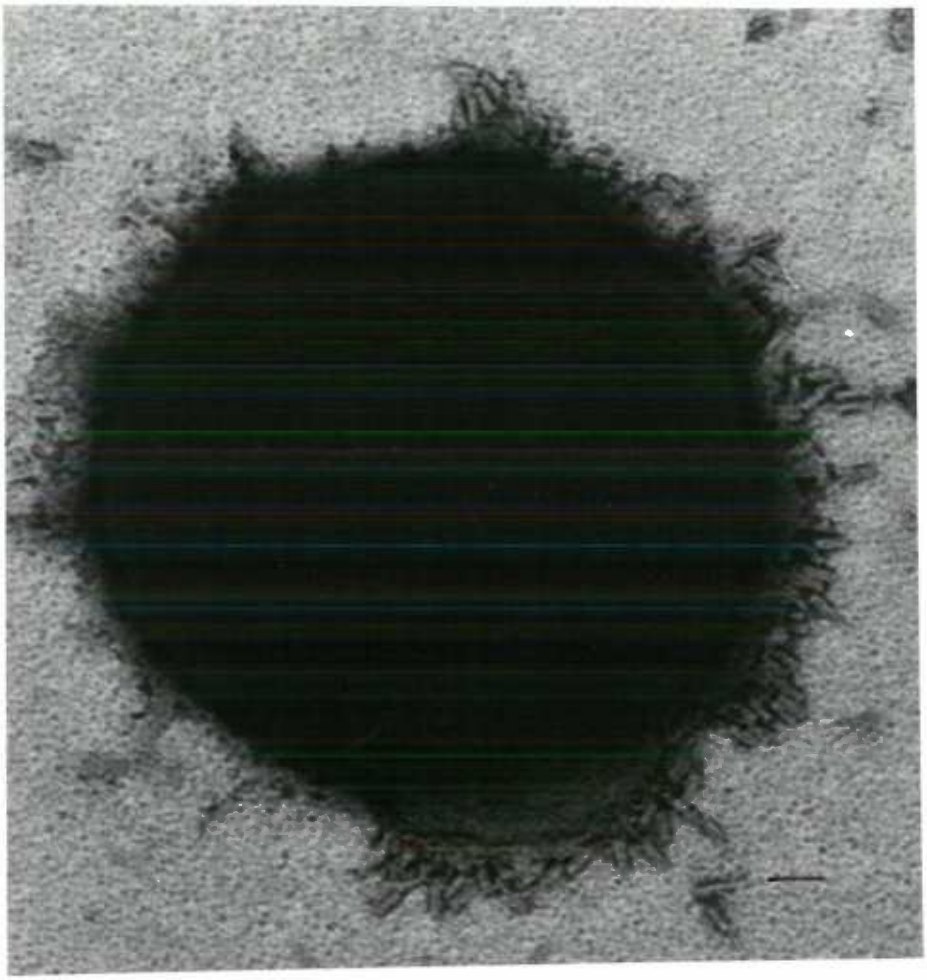
Interraction of R-type pyocin 611 131 with cells of N. gonorrhoeae strain 72H870.

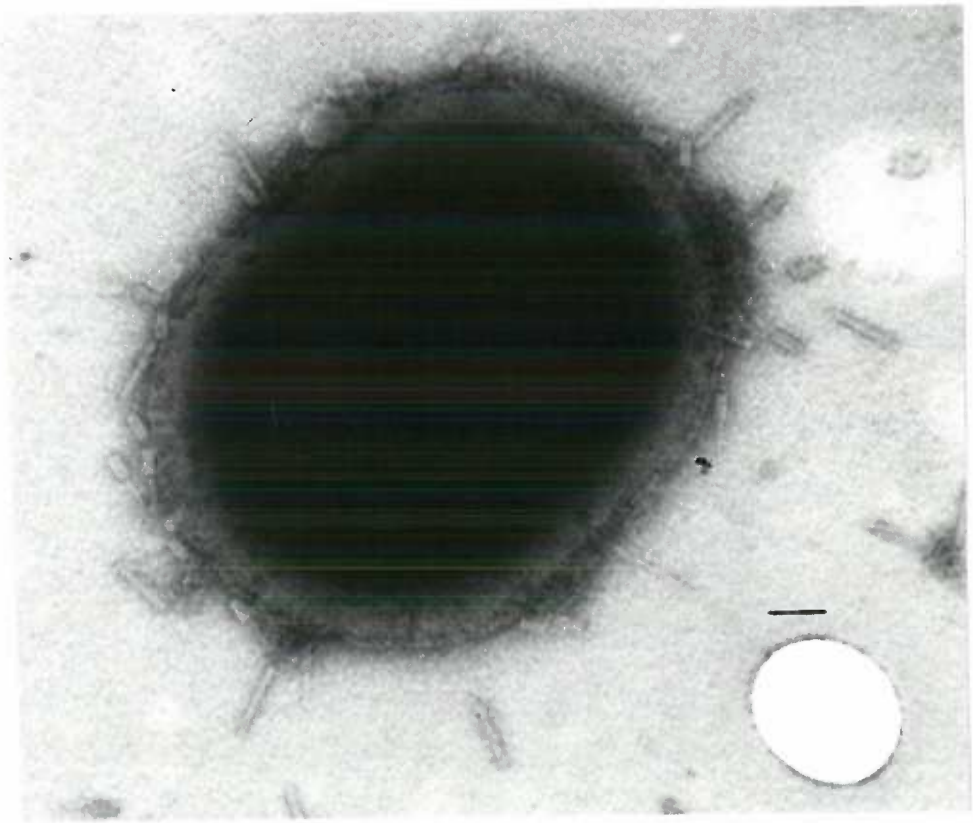
Sensitive cells of strain 72H870 were incubated with the pyocin for 30 minutes at 37°C. 0.1 ml of this suspension was negative-stained with sodium phosphotungstate and examined with the RCA EMU-3G electron microscope. Bar=0.1 um.

Figure 5

Interraction of R-type pyocin 611 131 with N. ovis

Resistant cells of N. ovis strain T2B (CDC Atlanta, Ga.) were prepared and stained as above. Bar=0.1 um.





Enzyme	Concentration (mg/ml)	Incubation time (hours)	Pyocin (units/ml) Before	After
Trypsin	0.5	3	$2x10^6$	$2x10^6$
Protease (strep. griseus)	1.0	3	$2x10^6$	$2x10^6$
Protease (B. subtilis)	1.0	3	$2x10^6$	$2x10^6$

Table 2. The effect of various proteolytic enzymes on pyocin 611 131.

The purified pyocin was incubated with the various enzymes at 37°C. Following incubation, these preparations were serially diluted and titered on P. aeruginosa strain PS-7 as listed in Materials and Methods.

Additions	Protein (ug)	Acid insoluble radioactivity (cpm)
Control	0	121
Crude R-type pyocin	7.0	73
Purified R-type pyocin	5.0	96
Exotoxin A	0.01	2788
Diptheria toxin fragment A	0.01	2839

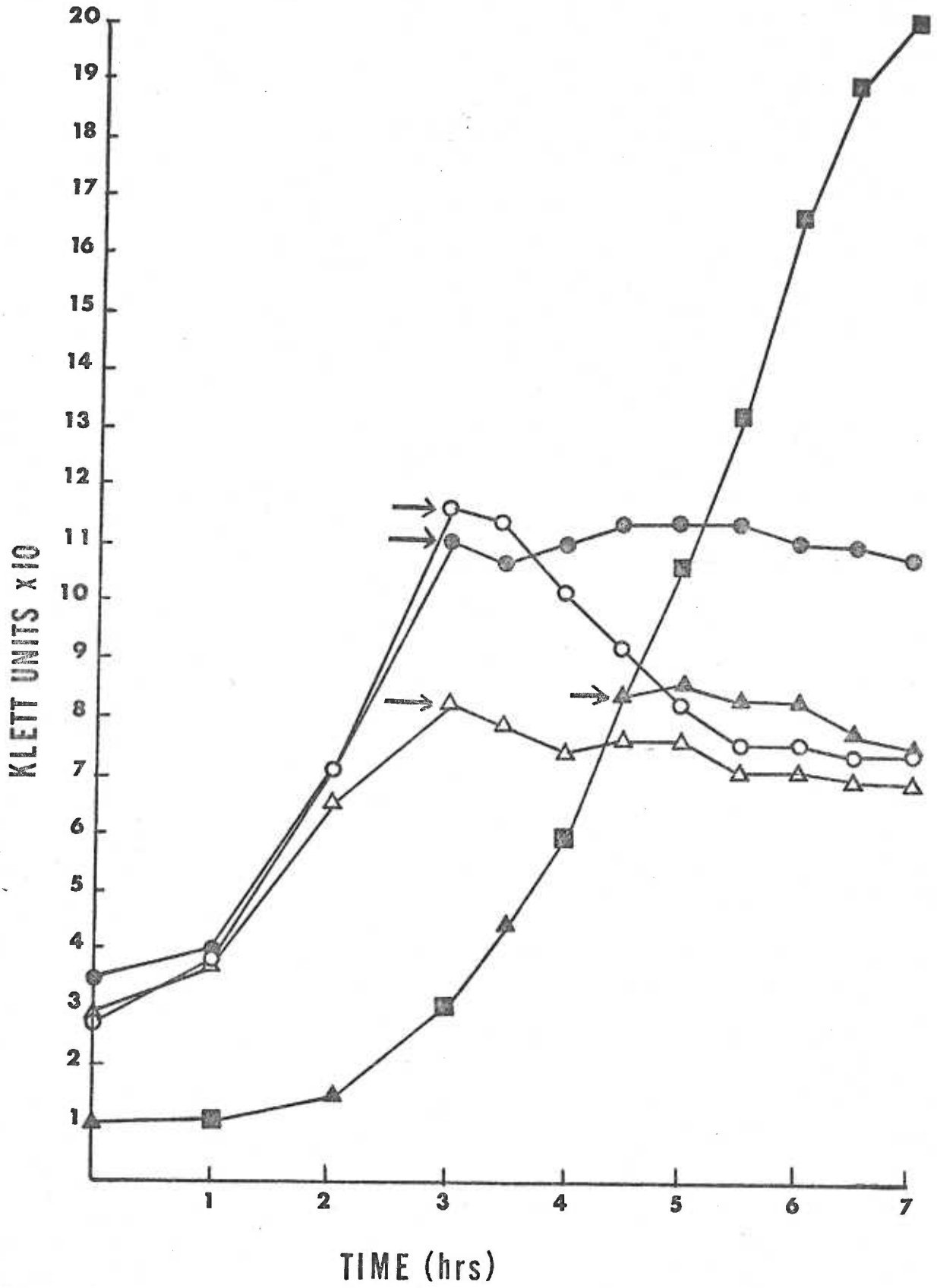
Table 3. Adenosine diphosphate-ribosyl transferase activity in preparations of R-type pyocin 611 131.

The assay was performed as listed in Materials and Methods. The crude R-type pyocin represents the pyocin 611 131 prior to purification. The control contained H_2O in place of toxin or pyocin preparation.

Figure 6

Inhibition of Neisseria gonorrhoeae by an R-type pyocin (611 131).

Cells of N. gonorrhoeae were grown in GC broth with added supplements. Pyocin 611 131 (2×10^5 units/ml) was added at the times indicated by arrows. Symbols: △, N. gonorrhoeae strain JW-31; ○, N. gonorrhoeae strain 72H870; ●, N. gonorrhoeae strain 72H873; ▲, N. gonorrhoeae strain Cs-7; ■, P. aeruginosa strain PA-103.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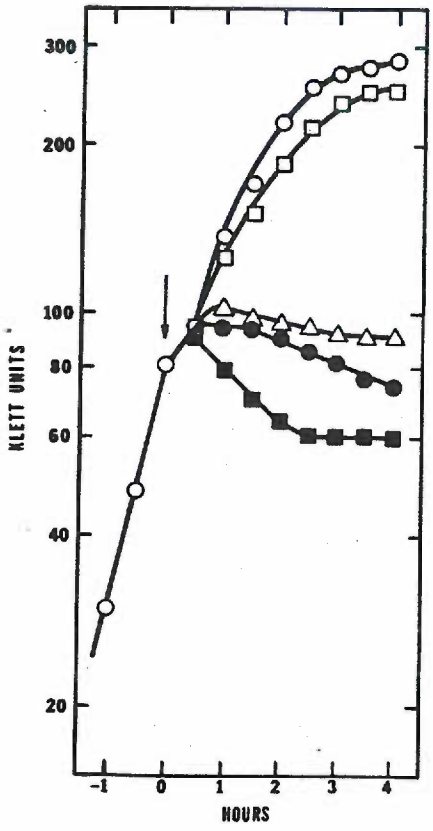
TIME (hrs)

Figure 6

Figure 7

Effect of R-type pyocin (611 131) on the growth of N. gonorrhoeae strain 72H870.

Cells of N. gonorrhoeae strain 72H870 were grown in one flask until growth reached approximately 100 Klett units (1.4×10^8 cfu/ml). The exponentially growing culture was split into 5 flasks followed by addition of the purified pyocin. Symbols: ○, no addition; □, 400 units pyocin/ml; △, 1000 units pyocin/ml; ●, 4000 units pyocin/ml; ■, 20,000 units pyocin/ml.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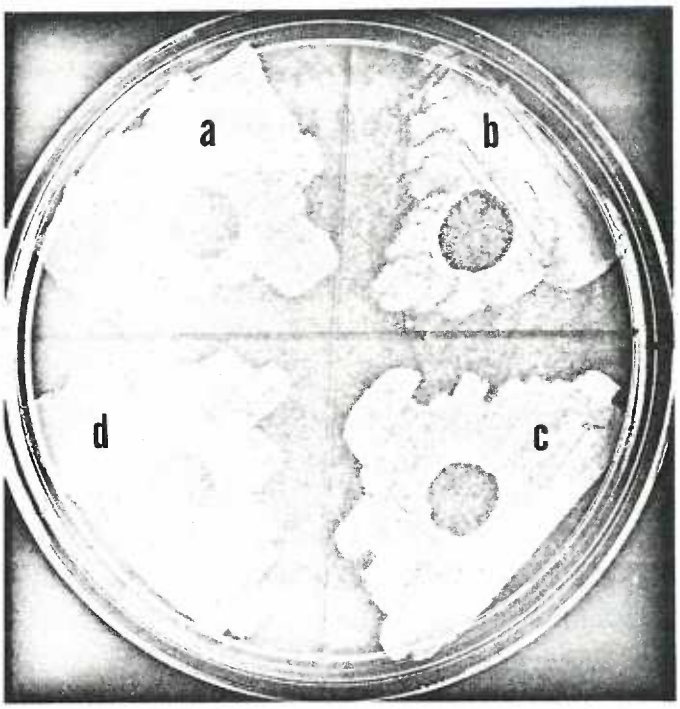
HOURS

Figure 8

Inhibition of the four colony types of N. gonorrhoeae strain 1138.

Colony types T-1, T-2, T-3 and T-4 of N. gonorrhoeae strain 1138 were maintained by selective subculture. The spot test was performed as listed in Materials and Methods. Symbols: a, colony type T-1; b, T-2; c, T-3; d, T-4.


a
b
d
c

Species	Sero-group	Number of strains tested	Number of strains sensitive
N. gonorrhoeae	-	56	56
N. meningitidis	A	4	0
	B	5	1
	C	3	0
	X	3	0
	Y	1	1
	Z	2	1
	135	2	0
N. lactamica	-	16	5
N. mucosa	-	1	0
N. flava	-	1	0
N. subflava	-	1	0
N. ovis	-	1	0
N. flavescens	-	1	0

Table 4. Sensitivity of various Neisseria species to purified R-type pyocin (611 131) from P. aeruginosa strain PA-103

Strains were listed as being sensitive to pyocin 611 131 only if clear zones of inhibition were observed by the spot test as listed in Materials and Methods.

Pyocin (611 131) (Added to)	Indicator strain	Pyocin (units/ml) Before	After inc.
N. gonorrhoeae JW-31	*P. aeruginosa* PS-7	$2x10^6$	$1x10^2$
P. aeruginosa PS-7	*N. gonorrhoeae* JW-31	$2x10^6$	0

Table 5. Assay for residual activity after incubation of the pyocin with sensitive bacteria.

Overnight inocula were resuspended in fresh GC broth as listed in Materials and Methods. 1.0 ml of the pyocin was combined with 6.0 ml of cells (approx. 100 Klett units). Incubation was at 37°C for 45 min. After removal of the cells by centrifugation residual activity was assayed by the standard titering procedure.

Bacteria	Incubation Time (min)	cpm (supernatants)	% Bound
N. gonorrhoeae Cs-7	0	249	0
"	2	211	15
"	15	144	43
"	30	155	38
N. gonorrhoeae 72H870	0	154	0
"	2	100	35
"	15	54	65
"	30	47	69
N. ovis T2 B	0	213	0
"	2	212	0
"	15	200	1
"	30	216	0

Table 6. Adsorption of ^{3}H-labeled R-type pyocin 611 131 to bacteria.

0.5ml of the ^{3}H-labeled pyocin was mixed with 4.5 ml of the cell suspension (approx. 1.4×10^{8} cfu/ml). Radioactivity of the supernatant was measured after incubation. Incubation was at room temperature.

DISCUSSION

Bacteriocins have been defined as antibiotic substances of protein nature produced by certain strains of bacteria which are active against strains of the same or closely related species (38). Their species specificity distinguish them from classical antibiotics (1). The consistency with which bacteriocins are so defined (38, 79, 80) suggests either that the bacteriocins tested thus far are indeed species specific or that no attempt was made to define antibacterial spectra outside of the producing species. There have been other reports however, of inhibition outside of the producer species among closely related bacteria (77). Negative results have been obtained when bacteriocins of gram negative species have been tested on gram positive organisms (78).

Geizer (61) observed that the growth of N. gonorrhoeae was inhibited by unknown substance(s) produced during the growth of strains of Vibrio cholerae, Escherischia coli, Aeromonas hydr philia, Micrococcus species and P. aeruginosa. I have confirmed this observation with P. aeruginosa and further have sought to isolate and identify the inhibitory substance.

It was necessary first to determine under what

growth conditions the inhibitory factor was produced. Spontaneous production was noted in growing cells both in broth and by the cross-streak method of Fredericq (1). Glycerol was used as the primary energy source for P. aeruginosa in these studies. However, inhibitory activity was still observed when glucose was substituted in the agar plates. This is in contrast to the repression of colicin activity by glucose which has been noted elsewhere (64).

The inducible nature of the inhibitor was first shown using mitomycin C, an agent which selectively inhibits the synthesis of DNA and which requires only a brief exposure to exert its maximum activity (66). For consistency GC broth was utilized for experiments involving both N. gonorrhoeae and P. aeruginosa. In both trypticase soy and GC broths it was found that the induction patterns were essentially identical. Addition of mitomycin C to cells growing in both types of media caused cell lysis within approximately 3 hours and routinely led to inhibitory titers of 2×10^5 units/ml in the partially purified preparations. The induction pattern was similar to that obtained by Kageyama (36) with U.V. induction R-type pyocins.

The inducible nature of the inhibitor suggested the

involvement of a bacteriophage or bacteriophage product. No plaques were present but a broad zone of inhibition was seen. These zones became more turbid as the dilution was increased until all activity was abolished. This finding would indicate either that a bacteriocin or another extracellular product was responsible for the inhibition. An attempt was made to transfer the inhibitory activity using an agar plug taken out of the center of the zone of inhibition. Since bacteriophages may be propagated on sensitive strains with a subsequent increase in titer, a similar finding would at least indicate the presence of an inhibitor capable of replicating within the infected host. However, in this case, no increase in titer was observed.

Since the above data suggested the presence of a bacteriocin, it became necessary to identify what kind of bacteriocin was involved. The situation is more complex in the case of P. aeruginosa because it is known that some strains produce both the S and R-type pyocins. These bacteriocins may exert their effect independently or may combine to give an increase size of the zone of inhibition (9). Ito et al. presented data that S-type pyocins alone or S with R-type pyocins gave much larger zones of inhibition than did the R-type pyocin alone. Since the

preparation used in these studies gave larger zones than those presented for the R-type in the above paper, this suggested that some S pyocin may be present. To investigate this possibility Govan's modification (21) of Kageyamas (13) purification procedure was used. This method was chosen because it results in higher titer R-type preparation and because it incorporates a method of removing S pyocin activity (9). During chromatography on DEAE-cellulose, S-type pyocins will pass through the column whereas the R-type will adsorb and be retained by the column. Post column pooled-pyocin fractions still gave the same size for the zone of inhibition. Therefore it was concluded that an S-type pyocin did not contribute to the initial observed inhibition. No activity was found in the eluent.

R-type pyocins are known to be resistant to proteolytic enzymes whereas the S or soluble type are readily degraded (7). The purified post-column pyocin preparation was subjected to both trypsin and pronase digestion. There was no loss of activity after extended incubation of the pyocin with these enzymes. Moreover, the size of the zone of inhibition remained the same as it was prior to enzymatic treatment which further suggested that the activity shown by this preparation was not due to S-type pyocins.

The difference in titer observed when pyocin 611 131 was assayed on N. gonorrhoeae and P. aeruginosa is still unexplained. The inhibitory titer was consistently higher when assayed on the P. aeruginosa strain PS-7. The data for the inhibition of N. gonorrhoeae strain JW-31 in broth, showed a typical concentration dependence. More information concerning this was obtained from the adsorption experiment. The finding that there was residual activity remaining after incubation of the pyocin with the JW-31 suggests either that there are fewer binding sites on N. gonorrhoeae or that more than one pyocin type was present in the purified preparation. The later choice is unlikely since the pyocin typing revealed only the one type which did not change throughout the purification procedure.

The broad spectrum of activity within N. gonorrhoeae is highly unusual even for bacteria which are very closely related. Every isolate tested thus far has been sensitive to the pyocin 611 131. One could speculate that strain differences within N. gonorrhoeae are much more subtle than are found in other genera. Unfortunately, there is no adequate method for typing the gonococci at this time so information concerning strain differences, no matter how subtle, are lacking. Nevertheless the isolates tested have been obtained from clinical cases on

both the east and west coasts of the U.S.

Since the sensitivity to the pyocin is so broad than at least, two possibilities exist. The first is that all strains tested possess the same pyocin receptor or secondly, the receptor which is possessed by the gonococci is non-specific and chemically distinct from those receptors which have been analyzed to date. As a possible insight into this question, the four colony types of N. gonorrhoeae strain 1138 were tested for their sensitivity to pyocin 611 131. Since it is known that colony types 1 and 4 at least, have certain differences in their lipopolysaccharide (62), it was hoped that there might also be differences in pyocin sensitivity. However, when assayed, all four colony types were found to be sensitive.

The spectrum of activity became more restricted outside of N. gonorrhoeae. Strains of N. lactamica showed a greater sensitivity to the pyocin than did the strains of N. meningitidis tested. Early results with the meningococci suggested that those strains which possess a capsule might be protected from the pyocin inhibition.

However, with subsequent assays, no correlation between the capsule and protection was found to exist since

both capsulated and non-capsulated strains were inhibited.

Electron microscopy revealed the presence of rod-shaped particles whose dimensions corresponded very closely to other reported pyocins. The low percentage of contracted particles suggested that this purification procedure was successful in preserving most of the inhibitory activity. The uncontracted pyocins could be seen to adsorb to sensitive cells by the tail-end of the particle. However, in none of the preparations were base plate and tail fibers seen as have been described by other authors (18). When the pyocins were combined with resistant cells, some particles could be seen in proximity to the cell. However, no massive adsorption of the pyocins to the cell was evident.

SUMMARY AND CONCLUSIONS

P. aeruginosa strain PA-103 produces a bacteriocin which inhibits the growth of N. gonorrhoeae. These particles were shown to be inducible with mitomycin C, resistant to proteolytic enzymes and incapable of self-replication. Electron microscopy revealed the presence of rod-shaped particles resembling bacteriophage tails which is characteristic of the R-type pyocins. Pyocin typing classified these bacteriocins as pyocin type 611 131.

The inhibitory spectrum of these pyocins is very broad. All strains of N. gonorrhoeae tested were inhibited. However, outside of this species, the activity of the pyocins was more restricted. Broth inhibition studies showed that cell death begins within 30 minutes after the addition of the pyocins. Adsorption studies using ^{3}H -labeled pyocins revealed that the adsorption of pyocin to sensitive bacteria is virtually complete within 15 minutes. In addition there appears to be some quanitative difference in the number of binding sites between N. gonorrhoeae strain JW-31 and P. aeruginosa strain PS-7 since there are residual pyocin particles remaining after incubation with the JW-31 which can bind to the PS-7.

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CORRECTIONS

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