

present in normal skin and other tissues.¹⁰ This recent work has been reviewed^{10, 11} and will not be discussed here.

Conclusions. The experiments described indicate that testicular extracts have the property of increasing capillary permeability. No evidence was found that they could increase absorption from the skin, nor were pharmacological effects on smooth muscle or blood pressure demonstrable.

13559

Observations on Resistance of *Staphylococcus aureus* to Action of Tyrothricin.

CHARLES H. RAMMELKAMP. (Introduced by Chester S. Keefer.)

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.

Tyrothricin, the crude substance isolated by Dubos from a spore-bearing soil bacillus has been found to kill gram-positive micro-organisms both *in vitro* and in the experimental animal.^{1, 2} It is now known that tyrothricin contains 2 crystalline fractions, tyrocidine and gramicidin, the latter exhibiting the greater bactericidal action *in vitro* and *in vivo*.³ Because of the difficulties encountered in preparing crystalline gramicidin, tyrothricin has been used almost exclusively in the treatment of infections.

Although tyrothricin is active against all gram-positive organisms, the different genera vary considerably in their susceptibility to its action. It is now well established that pneumococci and streptococci are most susceptible *in vitro* whereas staphylococci require somewhat greater concentrations of tyrothricin before a definite killing effect is observed.

During the past 2 years we have used tyrothricin in the treatment of experimental and clinical infections^{2, 4} caused by the pneumococcus, streptococcus, and staphylococcus. Early in these studies the observation was made that it was difficult to predict from the *in*

¹⁰ Meyer and Palmer, *J. Biol. Chem.*, 1936, **14**, 689.

¹¹ Madinaveitia and Quibell, *Biochem. J.*, 1940, **34**, 625.

¹² McClean and Hale, *Biochem. J.*, 1941, **35**, 159.

¹ Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 11.

² Rammelkamp, C. H., to be published.

³ Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

⁴ Rammelkamp, C. H., and Keefer, C. S. (abstract), *J. Clin. Invest.*, 1941, **20**, 433.

vitro susceptibility of staphylococci in general the amount of tyrothricin required for the sterilization of a local infection caused by this organism. There appeared to be 2 possible explanations for this difficulty; either (1) the strains were resistant to tyrothricin or (2) certain inhibitory substances were present in the infected area which prevented the action of tyrothricin on staphylococci. Both of these factors were found to play an important rôle in determining the effectiveness of tyrothricin therapy in staphylococcic infections. The present report deals with the observations made on the resistance of staphylococci to the action of tyrothricin.

Methods. With the exception of one strain (Lederle) all strains of *Staphylococcus aureus* used in this study were recently isolated from patients infected with this microorganism. The strains were stored on blood-agar slants until needed.

A stock solution of tyrothricin* in 95% alcohol was prepared in a concentration of 50 mg per cc. This solution of tyrothricin was added to sterile 10% glucose in distilled water so that the desired concentration was contained in 0.5 cc. The glucose tyrothricin suspension was then added to eleven tubes containing 4.5 cc of veal-infusion broth. The final concentration of tyrothricin in these tubes varied from 0.001 to 1.6 mg per cc. To each series of tubes 0.1 cc of a 12-hour broth culture of the test strain of *Staphylococcus aureus* was added. The final number of organisms present in the inoculated tubes was found to be 10^6 per cc.

The series of tubes were incubated at 37°C for 24 hours. The occurrence of visible growth was noted after 24 hours' incubation. At 24 hours 0.1 cc of each culture was transferred to 5 cc of veal-infusion broth. Following this 0.1 cc was removed from the subcultures in a second transfer. Both series of tubes were incubated for 24 hours and visible growth noted.

Development of Resistant Strains in vitro. Resistant strains were developed *in vitro* by repeated transfers in increasing concentrations of tyrothricin in veal-infusion broth. A series of 11 culture tubes as described above was inoculated with 0.1 cc of a 12-hour broth culture of one of the 3 pathogenic strains of *Staphylococcus aureus*. At 24-hour intervals transfers were made from the tube containing the greatest concentration of tyrothricin and showing full growth to a fresh series of 11 tubes containing tyrothricin. Comparative tests using the original strains and the strains passed through tyrothricin were run simultaneously as described above.

* Tyrothricin was supplied by Dr. John Henderson, Sharp and Dohme Laboratories, and by Dr. Weed Malcolm of the Lederle Laboratories, Inc.

Results. The lowest concentration of tyrothricin required to cause inhibition of growth and killing of each of the 19 strains of *Staphylococcus aureus* was determined. Inhibition was determined by the absence of visible growth in the culture medium containing tyrothricin which had incubated for 24 hours. Killing was determined by absence of growth in both series of subcultures of the medium containing tyrothricin.

In these tests inhibition of growth was produced by a concentration of from 0.024 to 0.2 mg per cc. Twelve of the 19 strains were inhibited by a concentration of less than 0.1 mg. A bactericidal effect was exhibited by a concentration of from 0.024 to 0.8 mg of tyrothricin per cc. Nine strains of staphylococci were both inhibited and killed by identical concentrations of the bactericidal substance, in the remaining 10 strains a somewhat greater concentration of tyrothricin than that causing inhibition was required to produce killing.

These results demonstrate, then, that there is roughly a 10-fold difference in the concentration of tyrothricin necessary to produce inhibition or killing of the various strains of *Staphylococcus aureus*.

Production of Tyrothricin-resistant Strains. Although different strains of *Staphylococcus aureus* vary in their susceptibility to the action of tyrothricin, this variation did not appear to explain entirely the failures encountered in the treatment of local infections caused by this organism. Such a failure occurred in a male, aged 65 years, who had a large ulcer of the lower leg secondary to an inadequate circulation. On institution of tyrothricin therapy the ulcer became sterile and rapid healing took place. After 25 daily applications of tyrothricin in amounts varying from 20 to 100 mg the patient was discharged from the hospital. At this time the ulcer was practically healed, so that it seemed reasonable to expect that ambulatory treatment with tyrothricin would keep the lesion sterile. During the next several months tyrothricin was applied at irregular intervals. Cultures of the ulcer during this period frequently yielded *Staphylococcus aureus* and the ulcer gradually enlarged. At this time the organism was isolated and stored on a plain agar slant for future study. The application of tyrothricin was gradually increased in frequency until it was applied twice daily. In spite of frequent treatment with the bactericidal substance, the cultures remained positive. After 74 days of therapy the organism was again isolated and its resistance to tyrothricin compared to the strain previously isolated from the same patient.

In Table I the results obtained with the 2 strains isolated from the ulcer (Sweeney) are recorded. The original strain showed

The above observations led to the attempt to produce *in vitro* strains of *Staphylococcus aureus* which resisted the action of tyrothricin. The results of these studies are included in Table I. Markedly resistant strains were produced after 20 to 28 days' exposure to increasing concentrations of tyrothricin. The original strain (Sweeney) isolated from the patient mentioned above became very resistant to tyrothricin after 20 days' growth in broth containing tyrothricin. Strain Maher showed an increased tolerance to tyrothricin of about 160-fold as determined by bactericidal action. These observations demonstrate, then, that staphylococci may become resistant to the action of tyrothricin both *in vitro* and *in vivo*.

TABLE I.

[illegible]

Discussion. The different strains of *Staphylococcus aureus* associated with clinical infections showed a definite variation in their susceptibility to the action of tyrothricin when measured by either inhibition of growth or actual killing. Although the majority of strains were killed by concentrations of 0.2 mg of tyrothricin per cc, two organisms required a concentration of 0.4 and 0.8 mg of tyrothricin to cause the same effect. This variation in susceptibility of the staphylococcus to the action of tyrothricin is believed to be a factor in determining the successful sterilization of local lesions treated with the bactericidal substance. The organism (Strain 10) isolated from a patient whose infection responded most readily to tyrothricin-therapy was found to be inhibited and killed by a concentration of 0.024 mg per cc. Streptococcal infections in man respond more readily than staphylococcal infections,⁵ and by determining the relative susceptibility of numerous streptococcal strains we found that a concentration of tyrothricin as small as 0.0002 mg per cc caused both inhibition of growth and actual killing.

It has been demonstrated that staphylococci which were exposed in broth to increasing concentrations of tyrothricin soon developed a definite increased resistance to the action of the bactericidal substance. That such acquired tolerance may play a rôle in clinical infections was demonstrated in one patient. In this instance large amounts of tyrothricin applied to the local lesion after several months of treatment failed to sterilize whereas early in the course of therapy sterilization was effected readily.

These two factors, then, should be considered in explaining the failures encountered in staphylococcal infections treated with tyrothricin. Other factors believed to be of significance in determining the effectiveness of this form of therapy are the presence of inhibiting substances such as pus, serum, or gram-negative bacteria⁵ and the accessibility of the staphylococci to the particles of tyrothricin which are applied.

Conclusions. Various strains of *Staphylococcus aureus* differ in their susceptibility to the action of tyrothricin. It is possible to increase the resistance of staphylococci to tyrothricin by growing the organism in increasing concentrations of the bactericidal substance. In one patient treated with tyrothricin there was a marked increased resistance of the infecting organism after several weeks of therapy.

This work was supported in part by Lederle Laboratories, Inc. Miss Marjorie Jewell and Thelma Maxon gave assistance in these studies.

⁵ Rammelkamp, C. H., and Keefer, C. S., unpublished observations.