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Effect of Tyrothricin on Beta-Hemolytic Streptococc

Evaluation of the Effect of
Tyrothricin on β-Hemolytic
Streptococci in Saliva

by

Frank Paul Brancato

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Thesis

~~EVALUATION OF THE EFFECT OF TYROTHRICIN ON β-HEMOLYTIC STREPTOCOCCI IN SALIVA~~

Part II: Effect of Tyrothricin on β-Hemolytic Streptococci

by

Frank Paul Brancato

Part I: Evaluation of the Effect of Tyrothricin on the New York ~~5~~ V Strain of Streptococcus Pyogenes in Saliva

by

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Wilbur F. Noyes, III
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Frank P. Brancato
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Evaluation of the Effect of Tyrothricin on Beta Hemolytic Streptococci in Saliva

It is thus to the smallest of living systems, the microbes, that we must look for the solution of some of the most important problems that have faced man as well as his domesticated and friendly animals and plants.

Selman A. Waksman
(p. 268, 1947)

INTRODUCTION

Since the discovery by Dubos of a bacteriostatic and bactericidal factor in culture filtrates of Bacillus brevis, there have been many attempts, some of value and others valueless, to extend the therapeutic application of this factor.

First antibiotic of practical value, tyrothricin was early recognized to be actually a complex of two polypeptides, designated tyrocidine and gramicidin, whose mode of action against microörganisms differs. In vitro tyrocidine acts as a cationic agent causing rapid bactericidal and lytic action in high dilutions against Gram-negative and Gram-positive organisms alike. On the other hand gramicidin in vitro or in vivo interferes with the enzymatic respiratory mechanism and phosphate uptake accompanying glucose oxidation. Its detergent activity is much less than that of tyrocidine. Accordingly gramicidin activity, which is directed almost solely against Gram-positive bacteria, is primarily bacteriostatic (Dubos et al,1942).

Use of either of these components individually was contra-indicated for several reasons. While tyrocidine was found to be effective *in vitro*, it was also found to be inactivated *in vivo* by tissue exudates; gramicidin, however, retained its bacteriostatic activity *in vivo* but was discovered to be inhibited, partially or completely, by phospholipid cephalin and by mucin. Thus tyrothricin combines the bacterial potency of gramicidin in presence of tissue exudates with the greater stability and solubility of tyrocidine and is the form of the antibiotic most commonly used (Hotchkiss, 1944; Kozoll *et al*, 1946; MacKee *et al*, 1946).

Another limitation which has confined the use of tyrothricin to topical applications is its hemolytic activity when used intravenously or locally upon wounds with direct connections to the blood stream. Although it was observed that this hemolytic activity was removed by heating, the corresponding loss of bactericidal and bacteriostatic activity *in vivo* but not *in vitro* has led to an, as yet, unsuccessful search for other methods to gain this end (Herrell and Heilman, 1941).

In an effort to determine the possible value of tyrothricin in reducing significantly or eliminating completely the presence of the potentially pathogenic beta hemolytic streptococci in the buccal cavity and pharynx, despite the presence of inhibitory salivary components and, probably antagonistic, oral flora, the experimental procedures described in Part II have been carried out. As a preliminary approach to this experimental

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problem, a review of the extant literature upon the effect of tyrothricin upon beta hemolytic streptococci was also undertaken and is reported in Part I.

Part I
Effect of Tyrothricin on Beta Hemolytic Streptococci

Chapter I

Tyrothricin: Properties and Activity Influencing Clinical Use

In 1940 Dubos found that tyrothricin protected mice against 10,000 fatal doses of hemolytic streptococci. Here at last, it was thought, was the ideal agent for removing the constantly present threat of pathogenicity by an organism which enjoys practically universal distribution in or upon the human body..

Tyrothricin is actually a labile combination of two polypeptides, gramicidin and tyrocidine, with molecular weights of approximately 2500. They are present in the parent substance in a ratio of 1:4 (Hotchkiss, 1946). Gramicidin is a closed ring polypeptide containing 24 amino acid residues and no free reactive groups. On the other hand tyrocidine contains about 20 amino acid residues and has 2 free basic amino groups, 3 free amide groups, and 1 free carboxyl or hydroxyl group. The presence of these free groups probably accounts for the differences in their mode of action against microörganisms. Tyrocidine inhibits the oxidative processes of metabolism of Gram-positive and Gram-negative bacteria and is markedly bactericidal with a resultant secondary lysis: gramicidin seems to speed up oxidative metabolic processes of Gram-positive organisms and to interfere with their storage of carbohydrates and phosphates (Anderson,G.G

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1946; Hotchkiss, 1944). However tyrocidine loses practically all of its bactericidal activity in the presence of blood, serum, and pus but gramicidin retains its bacteriostatic activity in the presence of these substances. Despite the apparent ineffectiveness of tyrocidine _in vivo_, the two components are rarely separated because together they form a more stable aqueous dispersion which combines some of the most desirable properties of both and because the added care, labor, and time required to obtain absolutely pure quantities of the individual components would be prohibitive (Hotchkiss, 1944; Kozoll, 1946; MacKee,1946).

One of the first problems encountered, which handicaps the use of tyrothricin, is its insolubility in water. Neither of the components nor the parent substance are soluble in water but are dissolved by alcohol, acetone, dioxane, glacial acetic acid, and pyridine. This problem has been met in several ways. As tyrothricin is soluble in alcohol, stable aqueous suspensions are made by diluting the alcoholic solution with distilled water; in further diluting the resultant suspension with the proper volume of 5% glucose in distilled water, an isotonic solution ready for injection is obtained (Dubos and Cattaneo, 1939). However in the presence of electrolytes the antibiotic is flocculated or precipitated depending on the concentration of the electrolyte. This seems to have but little direct effect on its activity but it makes the accurate calculation of the concentration impossible and interferes with the range of activity . (Hotchkiss, 1944).

Dubos (1940) reported that tyrothricin remained suspended in water even in the presence of electrolytes when sulfated or sulfonated oils were used. He also used ox bile as a dispersing agent successfully. Then Herrell and Heilman (1941) found that the addition of glycerin to the alcoholic solution before the addition of distilled water made a stable vehicle for the dispersion of tyrothricin. In 1946 MacKee et al reported the use of a solution of tyrothricin in sodium-mixed alkyl benzene sulfonate, propylene glycol, and distilled water for the treatment of pyoderma with very satisfactory dispersion results.

A second handicap was recognized shortly after the discovery of tyrothricin. This is the inhibition of the activity of the antibiotic in the presence of certain organic substances. Therefore the consideration of the distribution of these substances, notably cephalin and mucin, is essential when the therapeutic application of tyrothricin is planned.

A serious limitation placed upon the earlier hopeful expectancy is the dangerously hemolytic activity of tyrothricin when injected intravenously or applied in areas drained by the blood stream (Dubos and Hotchkiss, 1941; Heilman and Herrell, 1941; Hotchkiss, 1944; MacLeod et al, 1940). The oral introduction of tyrothricin is also contra-indicated as it is precipitated without digestion in the presence of the digestive enzymes and is inactivated at a pH lower than 5.5 even at room temperature (Dubos, 1939 B; Hotchkiss, 1944).

Despite these serious limitations, tyrothricin has been and is being used clinically in the form of topical applications because of its stability, low tissue toxicity, activity in the presence of purulent discharges, lack of sensitizing properties, and high local concentration due to lower absorption and tissue permeability. Its rapid lethal effect on sensitive organisms makes it a valuable therapeutic aid in certain types of diseases and surgery, especially when the causative bacteria are rapidly vulnerable to its bacteriostatic and bactericidal action.

Chapter II

Activity of Tyrothricin Against Beta Hemolytic Streptococci *in Vitro*

Dubos (1939 A) reported that tyrothricin killed streptococci but did not usually lyse them. In his experimental treatment with tyrothricin of 7 strains of Group A, 3 strains of Group C, and 3 strains of Group D (Lancefield Groups), only slight lysis was observed and that did not occur uniformly. He also found that when streptococci were incubated with the antibiotic at 37°C, they lost the ability to reduce methylene blue in the presence of glucose. This inhibition of dehydrogenase activity occurred before lysis thereby indicating that lysis, when it does occur, is a secondary process (Dubos, 1939 A; Dubos and Cattaneo, 1939). Whether this antibacterial activity of tyrothricin is due to the fact that many of its amino-acid hydrolytic products are of the "unnatural" d-configuration in contrast to the l-configuration prevailing among hydrolytic products of animal proteins is a possibility which has not yet been established (Hotchkiss, 1946).

Schoenbach *et al*, (1941) reported the lethal action of tyrothricin in an eighteen hour broth culture of *Streptococcus hemolyticus* up to a final dilution of 1:1,000,000. In further *in vitro* studies on tissue culture preparations closely simulating *in vivo* conditions, 5 strains of Lancefield Group A hemolytic streptococci were inhibited by varying concentrations of tyrothricin; one strain was inhibited by 10 ug per ml. and the others

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80, 100, 100, and 120 ug per ml. respectively (Herrell and Heilman, 1941).

Crowe *et al* (1943), in an excellent comparative study of the action of tyrothricin and penicillin on cultures of organisms isolated from infections in 118 patients, found that tyrothricin in dilutions of 1:20 to 1:1,280,000, which were prepared from a stock solution of 2% tyrothricin in 94% alcohol, killed or inhibited cultures of beta hemolytic streptococci from 5 tonsil infections, 3 mastoid infections, and 11 cases of sinusitis. However against 5 cultures isolated from infections of the nasopharynx, the response was fair in 3 and poor in 2. For the treatment of diseases of this nature they reported that the action by tyrothricin *in vitro* on cultures of the causative bacteria indicated that tyrothricin should compare favorably with penicillin in most instances.

The efficacy of the action of tyrothricin *in vitro* against hemolytic streptococci is further confirmed by the observations of Hartley Jr. *et al* (1945) preliminary to the experimental clinical use of tyrothricin to influence the hemolytic streptococci carrier rate among inmates of an orphanage. Seven strains of beta hemolytic streptococci were isolated from the children's throats. One milliliter quantities of graded tyrothricin, which had been diluted to various concentrations by 10% glucose-distilled water solution, were added to tubes containing 9 milliliters of infusion broth. One tenth milliliter quantities of 18 hour infusion broth cultures of the isolated strains were

added to the charged tubes which were then incubated at 37°C for 24 hours. All strains were killed by 0.169 ug per milliliter but not 0.0169 ug per milliliter of tyrothricin.

Experimental use of tyrothricin in ointment bases against various organisms, including hemolytic streptococci, in vitro yielded poor results (Anderson, H.E., 1946).

There is no longer any doubt that even in very high dilutions tyrothricin exerts thoroughly effective bacteriostatic and bactericidal in vitro activity against Streptococcus pyogenes.

Chapter III

Activity of Tyrothricin Against Experimental Beta Hemolytic Streptococcal Infections in Laboratory Animals

The next logical step was the treatment with tyrothricin of laboratory animals artificially infected with hemolytic streptococci by various routes. Although in many instances this was done concurrently with *in vitro* experiments, for simplification and continuity the writer has transformed the usual routine into a step-wise procedure.

Dubos as early as 1940 had protected mice against ten thousand fatal doses of hemolytic streptococci by intraäbdominal injection of 0.001 - 0.002 mg of tyrothricin (gramicidin). He found the antibiotic to be uniformly curative if applied directly before infection had made headway; otherwise protection afforded was partial or nil.

Rammelkamp (1942 A) experimentally induced arthritis and empyema in rabbits by intrapleural introduction of virulent hemolytic streptococci and staphylococci. Then tyrothricin was in introduced into the pleural cavity. He found that those rabbits infected with the streptococci were protected and their pleural cavities sterilized. Dextrose solution of tyrothricin used in therapy did not prevent but inhibited in part the hemolytic activity of tyrothricin. In 1942 (B) Rammelkamp performed further similar experiments on 35 rabbits inducing streptococcal empyema

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by intrapleural injection. The tyrothricin used was an alcoholic suspension diluted to a concentration of a milligram per cubic centimeter of the antibiotic. This was further diluted on a 1:5 basis with physiological salt solution. Twenty-six of the thirty-five infected rabbits and eight uninfected rabbits were treated at intervals up to 18 hours after infection. Daily erythrocyte and leucocyte counts, hemoglobin concentration determinations, and general physical examinations were made of the test animals as a check for signs of toxicity due to tyrothricin. Twenty-two of the treated infected rabbits survived and were sacrificed in 6 to 90 days. There were no signs of toxicity among these; however, among the treated uninfected animals, adhesions, varying in severity proportionally to the dosage, were found as evidence of local tissue reaction. Of the four treated infected animals that died, one died within 24 hours after infection, with respiratory embarrassment, and the other three died from extension of the infection due to delayed therapy. All but two of the untreated infected animals died.

Neter et al (1945) introduced an inoculum of Group A beta hemolytic streptococci from an 18 hour infusion broth culture into artificially prepared wounds of rabbits. This strain had previously been proven to be susceptible *in vitro* to sulfathiazole, penicillin, streptothricin, and tyrothricin. After varying periods of time, the infected wounds were treated with the therapeutic agents. Tyrothricin and streptothricin were found to be more effective than 100 times the amount of sulfathiazole;

no significant difference in activity between equal amounts of streptothricin and tyrothricin was noted.

Chapter IV

Effect of Tyrothricin on Beta Hemolytic Streptococci Carrier Rate

In an effort to explore every possible favorable therapeutic usage for tyrothricin, clinicians began to use tyrothricin on a variety of patients where no danger due to hemolytic activity was possible.

Experimental introduction of tyrothricin as a spray into the throats of one human volunteer and two monkeys who had been shown by previous throat cultures to be carriers of Streptococcus pyogenes and Gram-negative Hemophilus hemolyticus (?) , sterilized their throats within two hours after treatment. After five days, one monkey again gave positive cultures but upon a second application of the antibiotic, it became negative and after four days was still negative. The tyrothricin used was a 1:100 saline dilution containing glycerin and needed repeated shaking during spraying. Schoenbach et al (1941) followed up these favorable results with the use of this tyrothricin spray on five persons, carriers of Streptococcus pyogenes following scarlet fever; two were chronic carriers and three were convalescents. Only in one of the chronic carriers was there an immediate reduction of bacterial count; the other cases required from four to seven days of treatment and three to four sprayings. Even then a dramatic reduction occurred only when preliminary nasal and pharyngeal cleansing was carried out.

Negative results were obtained by Hartley Jr. et al (1945) in another attempt to influence the Group A hemolyticus carrier rate. In this instance a much larger sample was used-76 children. After several throat cultures to determine the existing carrier rate, the throats of the forty boys of this group were sprayed thoroughly twice a day with a suspension of tyrothricin similar to that used by Schoenbach et al (1941). The girls of the group were used as controls, receiving no tyrothricin. After several weeks the diluent of the tyrothricin was changed from saline to 10% dextrose in distilled water resulting in a finer, more stable suspension of tyrothricin. Weekly cultures were made during the whole experiment which extended eleven weeks. Although there were no toxic effects noted during or after the sprayings, neither was there any reduction in the carrier rate. However it was noticed that those children with abnormal throat faults tended to be more consistent carriers.

Chapter V

Extension of the Use of Tyrothricin in Primary and Secondary Beta Hemolytic Streptococcal Infections

Rather irregular results were obtained by Herrell and Heilman (1941) when they used tyrothricin upon nine of their patients. Of these patients, <u>Streptococcus pyogenes</u> was the sole infecting organism in five while in three <u>Staphylococcus aureus</u> and in one a Gram-positive rod were isolated along with <u>Streptococcus pyogenes</u>. Good cures were effected in two cases with ulcers and one with maxillary sinusitis where only streptococci were present. Of three cases of eczematoid dermatitis treated, one in which the Gram-positive rod was also found responded very well; the second case, where streptococci were the sole infective agents, responded only after additional treatment; and the third case, in which staphylococci were also present, showed only partial improvement. Likewise in the cases of stasis ulcer and post-operative empyema, where staphylococci were also present, the response was incomplete and indefinite. One patient with an unclassified dermatitis, from which streptococci had been isolated, did not respond to tyrothricin. No toxicity was observed during or following treatment in any of these patients. These workers concluded that their indefinite results were due to the inadequate amounts of tyrothricin used in primary treatment.

Following the development of sulfonamide-resistant

strains of hemolytic streptococci in infections in two cases of attempted plastic-surgery, tyrothricin (gramicidin) was applied. While in one case a single application sufficed to sterilize the wound, the organisms in the second infection were untouched and apparently resistant to the antibiotic (Francis, 1942).

Rammelkamp (1942) reported the use of tyrothricin on 51 patients with 58 localized infections. Of twelve patients with sixteen ulcers, those caused by hemolytic streptococci and staphylococci were sterilized; mixed infections, especially those containing large numbers of Gram-negative organisms, did not respond. Among a group of 27 cases of hemolytic streptococcal mastoiditis on which 32 mastoidectomies were performed, 12 cavities were left untreated as controls; 15 cavities received a single application pre-operatively; and 5 cavities received multiple applications pre- and post-operatively. Following the operations, positive cultures of streptococci were obtained from 11 of the 12 controls, 8 of the 15 receiving a single application of 22 to 70 mgm. of tyrothricin, and 3 of the 5 receiving post-operative therapy. No significant difference in discharge or healing time was noted between the treated and untreated cavities. The use of tyrothricin in alcohol solution did cause some hemolysis and fresh bleeding at the time of operation. The one streptococcal empyema of 9 cases of empyema treated responded to tyrothricin following surgical drainage. Improvement followed the use of tyrothricin in a patient with sinusitis caused by a mixed infection of staphylococci and streptococci but only the

streptococci were eliminated. His negative results with a single case of hemolytic streptococcal pharyngitis, which he treated with a spray of tyrothricin in dextrose solution containing 1 mgm. of the antibiotic per ml., and the responses in the other diseases served to confirm reports of other clinicians and to establish the clinical scope of tyrothricin efficacy.

MacKee et al (1946) applied tyrothricin in 4 cases of impetigo contagiosa from 2 of which only streptococci had been isolated and from 2 streptococci and staphylococci. All cases promptly healed when treated with wet compresses of a 0.1% solution of tyrothricin in propylene glycol.

Also of considerable value in delineating the scope of tyrothricin's usefulness was the work of Kozoll et al (1946) on 77 patients, 38 of whom had beta hemolytic streptococci along with other organisms infecting their lesions. An enumeration of the types of lesions and results obtained in relation to the incidence of beta hemolytic streptococci is of interest (See Table I). It was observed that beta hemolytic streptococci were the most susceptible to tyrothricin therapy of all the infecting organisms, disappearing in 18 cases and decreasing noticeably in 5. Only in 3 instances were there any reactions to tyrothricin and these were in the nature of a mild skin reaction which disappeared in one case in spite of continuance of therapy and in the other cases after discontinuance of therapy. Neither exudate nor pus appeared to interfere with the action of the antibiotic. Dressings were kept moistened with an alcoholic solution of

Table I

Clinical Results in 77 Patients with Surgical Infections Treated with Tyrothricin (Kozoll et al, 1946)					Beta* Hemolytic Strepto- cocci
Lesion	No. of Cases	Excellent Results	Fair Results	Poor Results	
Post-op. wound	19	18		1	8
Varicose ulcer	10	7	1	2	4
Cellulitis	7	7			6
Abscess	7	5		2	4
Empyema	5	4	1		4
Burn	5	1	1	3	2
Laceration	3	2		1	1
Osteomyelitis	3			3	1
Decubitus ulcer	3	3			1
Carcinomatous ulcer	2		1	1	1
Carbuncle	2		2		1
Luetic ulcer	2	1		1	1
Infected dermatitis	2	1	1		1
Infected amputation stump	2	1	1		1
Buerger's disease	1			1	
Infected fracture	1			1	1
X-ray burn	1			1	
Tenosynovitis	1			1	1
Fistulae perinephric	1			1	
Totals	77	50	8	19	38

* Information for the incidence of beta hemolytic strepto- cocci is taken from Table III, Kozoll et al, (1946), entitled:

Type of Surgical Infections Treated in This Study and Bacterial Flora Seen with Each Lesion .

tyrothricin which was diluted with sterile distilled water so that it contained ½ to 1 mgm per ml of the antibiotic. In concluding their report, these workers listed several worthwhile criteria for the determination of lesions suitable for tyrothricin therapy.

> Tyrothricin is recommended as a nontoxic noninjurious antibiotic agent for local use in the treatment of surgical infections, if these meet the following criteria: (a) The wound is open, (b) there is adequate surgical drainage, (c) there is adequate blood supply, (d) the predominant organisms are streptococci or staphylococci or both.

Part II

Effect of Tyrothricin on the New York 5 Strain of Streptococcus Pyogenes in Saliva

Purpose of Research

Schoenbach et al (1941) reported the sterilization of the nasopharyngeal regions of 2 monkeys and 1 human volunteer from whom β-hemolytic streptococci had been isolated by a spraying with a 1:100 dilution of tyrothricin in 2.5% glycerolated physiological sodium chloride solution. Their results using a similar tyrothricin spray with 5 human β-hemolytic streptococcal postscarlatinal carriers, although regarded favorably by these workers, seem indefinite since there was an immediate reduction of streptococci without preliminary cleansing and repeated spraying in only 1 patient.

Rammelkamp (1942 A) obtained no improvement in 1 patient with streptococcal pharyngitis when he used as a spray, a dextrose solution containing 1 mg per ml of tyrothricin.

Equally negative results were obtained by Hartley Jr. et al (1945) when they attempted to influence the hemolytic streptococcal carrier rate in an orphanage. A final concentration of 0.34 mg per ml of tyrothricin, first in normal saline and glycerin and later in 10% dextrose in distilled water, was used in the form of a spray twice a day for 11 days. Yet in vitro the strains of β-hemolytic streptococci isolated were in every instance readily susceptible to the bactericidal action of

0.169 micrograms of tyrothricin per ml.

There are two apparent, significant reasons for these disappointing results in the therapeutic use of tyrothricin in nasal and pharyngeal regions: The bactericidal and bacteriostatic actions of tyrothricin are hindered if not completely blocked (1) by mucin, an important and predominant component of saliva, and (2) by the presence of mixed bacterial populations especially those containing chiefly Gram-negative organisms. The antagonistic action of the bacterial flora may be due to cephalin, a phospholipid which also inhibits tyrothricin and which has been extracted from Gram-negative organisms(Dubos, 1948; Rammelkamp, 1942 A; Waksman, 1947).

It is our intention to determine the quantities of tyrothricin which, in the presence of unaltered fresh saliva, will cause (a) an appreciable reduction and (b) complete inhibition of a strain of β - hemolytic streptococcus in relation to fixed periods of exposure to the antibiotic.

Materials

Test Organism: New York 5, a strain of Streptococcus pyogenes (Group B Lancefield), was used in these tests. This organism produced a large and well-defined zone of hemolysis when cultured on agar enriched with 0.5 ml of defibrinated human blood. It was necessary to use human blood as this bacterium yielded poorly-defined hemolysis when the medium was enriched with horse blood.

Subcultures were made each day in order to have on hand at all times a vigorously growing culture. Streaked blood agar slants, subcultured every 3 weeks, provided a seed culture in case of contamination of daily broth cultures.

Media: Of several media tried, tryptose phosphate broth (Difco), to which 0.5 gm of agar-agar per liter had been added, provided the most satisfactory fluid substrate for this fastidious organism; with the addition of 15 gm of agar-agar per liter, the same medium was made suitable for a solid substrate. In 17 hours of incubation at 37° C, an inoculum in this fluid medium of 0.1 ml (if broth unfiltered) or 1 ml (if broth filtered) from a 48 hour broth culture grew into a white cotton-like mass extending from the bottom of the tube almost to the surface; the area of medium above the growth was transparent. Upon microscopical examination, the streptococci were observed to be arranged in long chains which, when stained by Gram's method, readily gave up the gentian violet so as to appear almost Gram-negative. On the solid medium the colonies presented the characteristic pin-point morphology generally. However not infrequently, especially after 48 hours of incubation, a spreading grayish colony with a dark center and surrounded by typical hemolysis was observed.

Tyrothricin: The original concentration per ml of tyrothricin, which was provided by the White Laboratories, Inc. of Newark, NJ, was 10 mg per ml in a 50% alcohol-50% propylene glycol solution. The required concentrations were secured by diluting the stock solution, aspirated aseptically from its rubber-capped vial with

a sterile hypodermic needle and syringe, with the proper volume of 2% propylene glycol in distilled water.

<u>Saliva:</u> In order to simulate <u>in vivo</u> conditions as much as possible, the collected saliva was unfiltered or unstrained and was secured without artificial stimulation of flow. However, so as to keep antagonistic action of normal buccal flora and fauna at a minimum, the saliva with the lowest bacterial count from one of several individuals tested was used and the flow for the first 3 minutes was discarded. The freshly collected saliva was allowed to stand for several minutes in order to allow gross particles to settle, and then required amounts of the opalescent surface fluid were pipetted off when needed. Fresh saliva was supplied just previous to time for use so that the possibility of diminishing inhibitory potency due to prolonged standing was eliminated. Bacterial counts were done on each new batch of saliva along with the other tests in an effort to correlate changes of normal buccal microörganisms in number and in type with changes in the effect of the tyrothricin and the growth of the test organism. All salivary bacterial counts were done upon the same media used in the main experiment in order to detect possible presence of microörganisms whose hemolytic activity could have been confused with the hemolytic activity of the test organism.

<u>Blood</u>: Freshly drawn human blood was introduced aseptically into a sterile Erlenmeyer flask containing glass beads and defibrinated by shaking gently for several minutes.

Diluting Fluids: Physiological sodium chloride solution was used to make the necessary dilutions for plating out at the end of the exposure period.

Propylene glycol, diluted with distilled water to a concentration of 2%, was used to dilute the tyrothricin to the desired concentrations per ml.

Sterile distilled water was used to dilute bacteria cultures if necessary.

Experimental Procedures

Standardization of Inoculum Culture: It was decided tentatively to use an inoculum containing approximately 1,000,000 hemolytic streptococci per ml in the final concentration and to attain this by developing uniform procedures for the size of inoculum and for growing stock and inoculum cultures and by correlating the optical density of the inoculum culture, prior to use, with viable cell counts. Ordinary pyrex test tubes, which had been standardized on the spectrophotometer when they were filled with a colored solution, were used for growing the inoculum cultures and 1 such tube, containing medium only, was used as a blank to establish a zero reading with a 550 $m\mu$ filter. Four readings and viable cell counts were deemed sufficient to establish a dilution factor for the early experiments.

With the first lot of fluid medium, it was found necessary to introduce only 0.1 ml inoculum from a 48 hour stock culture of the test organism to obtain an abundant and vigorous growth during 17 hours of incubation at 37°C. The second lot of

fluid medium, which was prepared from newly-purchased tryptose phosphate broth powder, required 1 ml inoculum of the stock culture and even then the growth was not as abundant. This created a problem which will be discussed in the next section.

Before using the inoculum culture, the optical density was measured on a Coleman junior spectrophotometer and the dilution factor estimated by comparing the reading with values recorded in Table II. As part of each experiment, a viable cell count was made of each inoculum culture and the count and optical density supplemented the data in the standardization table.

Preparation of Concentrations of Tyrothricin: As previously mentioned, the clear, colorless stock solution contained 10 mg per ml. Working solutions, containing 1 mg per ml, were made by diluting this stock solution 1:10 with 2% propylene glycol. The resultant solution was opalescent and no precipitate could be seen; this white opacity decreased directly with the concentration of tyrothricin per ml until at a concentration of 10 μg per ml, the solution was again clear and transparent. Several negative controls for hemolytic activity of tyrothricin, which will be discussed later, would seem to indicate that there was no precipitate.

Table II

INOCULUM STANDARDIZATION TABLE

Filter	Optical Density* of Tube of Sterile Medium	Optical Density after Inoculation and Incubation for 17 hours at 37°C	Difference	Viable Cell Count (per ml)
550 mμ	.30	.43	.13	9.5×10^6
550	.30	.43	.13	$12. \times 10^6$
550	.30	.47	.17	22.2×10^6
550	.29	.51	.22	$18. \times 10^6$
550	.30	.60	.30	$62. \times 10^6$
550	.29	.60	.31	64.5×10^6

* Coleman Junior Spectrophotometer

Integration of Charge(Tyrothricin,Bacteria,and Saliva Mixture): At first the charge was worked out on the basis of a final mixture totalling 10 ml but because of the delay in collecting a sufficient quantity of saliva, in later experiments the proportions of ingredients were worked out on a basis of 5 ml of final mixture. Thus the integration of the components of each charge was such that the final mixture contained the desired concentrations per ml of streptococci and of tyrothricin:eg.-

0.5 ml of a solution of tyrothricin containing 10 times the desired concentration per ml.
0.5 ml of a bacterial culture estimated to contain 10 million bacteria per ml.
4.0 ml of a diluting fluid (saliva, saline, etc.)
5.0 ml of mixture containing tyrothricin and streptococci in the desired concentrations per ml.

Period of Exposure of Streptococci to Tyrothricin: Although it was decided tentatively to use 15 and 30 minute periods, these first experiments were carried out using 30 minute and 60 minute exposure periods in order to establish, roughly, limits of effective action. The length of time of exposure to the antibiotic also presented a difficulty which is discussed in the next section.

Controls: A control was run in every experiment to note inhibitory effect of the saliva on the test organism. The New York 5 strain of streptococci is not a normal part of the oral flora and saliva has been reported to inhibit foreign invaders of the oral cavity (Appleton, 1945; Bibby, 1937, 1938 A, 1938 B; Hine, 1936). A charge consisting of 0.5 ml of streptococci mixed with 4.5 ml of saliva was retained for 30 or 60 minutes or both before diluting and plating out. To be absolutely comparable, the charge

should have consisted of 0.5 ml of saline or water, 0.5 ml of streptococci, and 4.0 ml of saliva.

Since 2% propylene glycol was used to dilute the tyrothricin to desired concentrations, a control for possible inhibitory or bactericidal activity of this solvent was set up. For this same reason, a control was set up for a 50% alcohol- 50% propylene glycol solution without tyrothricin. During one experiment, the undiluted propylene glycol was also tested for action against New York 5.

As each concentration of tyrothricin was used, controls were set up to check the possibility of misleading hemolytic spots by precipitated tyrothricin. To do this, a ml of the proposed concentration of tyrothricin was diluted in 4 ml of normal saline to cause precipitation and then after shaking thoroughly, 1 ml of this solution was withdrawn and plated out. Such plates were incubated at 37°C and checked after 24 and 48 hour periods.

After each experiment, 1 ml of each of diluting fluids used and of blood were also plated out in order to negate too profuse contamination or contamination by non-streptococcal hemolytic microörganisms. If gross contamination of these controls was observed, especially of the blood or normal saline, the experimental results were discarded; this eliminated external factors which might have caused misleading inhibition either of the action of the tyrothricin or of the viability of the streptococci.

<u>General</u>: With each experiment, streptococcal and salivary bac-

terial population counts were made. The purpose of the test organism count has been mentioned above. Counts were made of the normal salivary bacterial population to check variability of this population from day to day chiefly in relation to the reported inhibitory effect exerted by large, mixed bacterial populations on the action of tyrothricin (Dubos, 1948).

In order to minimize the time lag between the end of exposure period and the plating out for incubation, it was found necessary to have everything in readiness; by so doing this lag period was found to range from 10 to 15 minutes.

A greater part of these experiments consisted of an original and duplicate set-up; efforts were duplicated to check accuracy of technique and to forestall loss of time because of breakage or similar accidents.

Routine use of microscopical examination of slides stained by Gram's method was performed in order to check purity of cultures and identity of organisms forming questionable colonies surrounded by a zone of β- hemolysis.

Technical Difficulties

Several problems, which arose during these experiments, were resolved although not all equally satisfactorily.

The possibility of the hemolytic activity of tyrothricin causing pseudo-streptococcal areas of hemolysis was eliminated by the controls mentioned previously. Negative controls were obtained and indicated that this potential error could be

dismissed in the case of the concentrations that were used.

The question of securing a uniform inoculum of streptococci was inadequately met. That the method was not at fault was demonstrated by the partial success attained. The large deviation, which occurred when a new lot of medium was used, served to emphasize the necessity for stringent uniformity in materials and methods. After the first lot of medium was used, the prepared standardization table was of no value and a similar table should have been prepared for the second lot of medium before proceeding with further experiments. It also is certain that greater accuracy could have been obtained if a nephelometer had been used. The spectrophotometer served very well for obtaining the optical density of cultures and the more sensitive range of the nephelometer would have aided in correlating the optical densities of high dilutions with their viable cell counts (Longsworth, 1936).

An important question arose as to whether the tyrothricin, to which approximated numbers of organisms were exposed for a definite period of time, continued its activity significantly after this period ended. That this activity continued seems certain but that it was significant is doubtful in view of 3 factors: (1) The smallness of the concentrations used, (2) the extensive dilution of the charge within 15 minutes after the end of the exposure period and (3) the precipitation of the tyrothricin by using sterile physiological sodium chloride solution as the diluting fluid. The first factor is self-explanatory as the concentrations were in terms of μg. This factor is probably not of

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considerable importance as 1 µg of tyrothricin in sterile water very effectively reduced the streptococci population after 30 minutes exposure. The dilution factor was relatively large since 1 ml of charge was diluted from 1:10 to 1:100,000 and only dilutions 1:100 to 1:100,000 were plated out and then plating out resulted in further dilution by 15 to 20 ml of agar-agar. In precipitating the tyrothricin, its bacteriostatic and bactericidal actions were further curtailed since these actions depend on intimate contact with the susceptible organisms (Bordley *et al*, 1942).

Because electrolytes are found as a component of saliva, the precipitation of tyrothricin even in the presence of traces of electrolytes was an insurmountable problem. This was partially compensated for by intermittent shaking during the exposure period. Addition of the tyrothricin component of the charge, as the last step in the preparation of the charge and after the bacteria and saliva had been thoroughly mixed, also afforded partial compensation.

Effect of the variability in numbers of normal oral flora was reduced to a negligible minimum by collecting the saliva at the same time of day- usually 2-3 hours after breakfast which is generally constant in menu- and from the same individual so that there was an uniformity in dental hygiene. Variations in consistency and lysozymic activity of saliva were not controllable (Thompson, 1940).

Experimental Results

The results obtained from experimentation are summarized in chart form to facilitate evaluation and study.

Table III

Percentage Reduction of Streptococcus Pyogenes in Saliva by Different Concentrations of Tyrothricin during 24 Hours

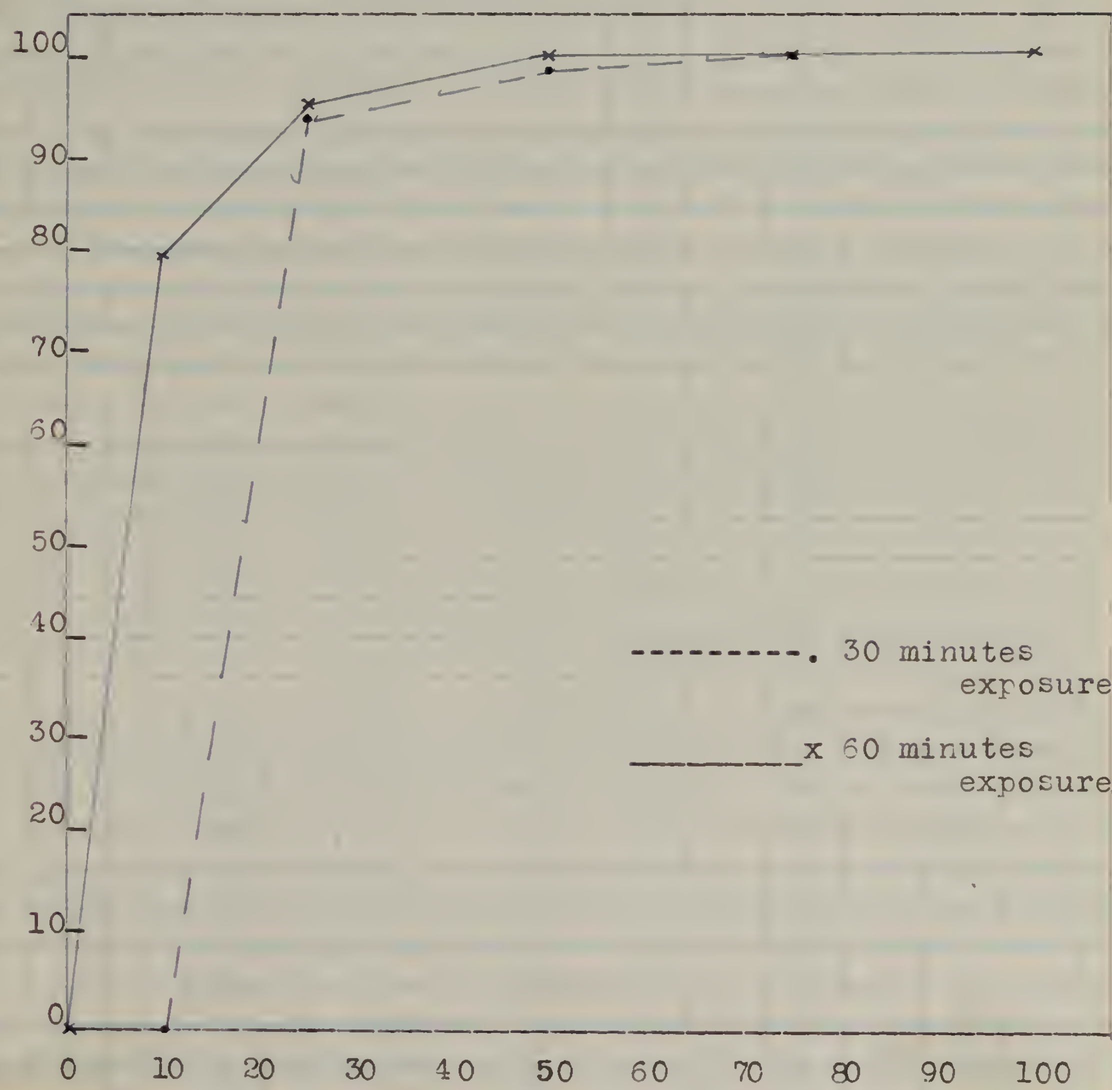
Tyrothricin Concentration per Ml	Exposure Period in Minutes	S. pyogenes Count per Ml	S. pyogenes Count in Saliva and Tyrothricin/ Ml	Percentage Reduction
10 µg	30	1.9×10^6	2.7×10^6	0
		1.0×10^6	1.8×10^6	0
	60	4.5×10^5	7.0×10^4	84.5
		2.3×10^5	4.0×10^4	75.0
25 µg	30	4.5×10^5	1.0×10^4	97.8
		2.3×10^5	1.9×10^4	91.4
	60	4.5×10^5	6.0×10^3	98.6
		2.3×10^5	1.4×10^4	91.2
50 µg	30	1.0×10^6	2.1×10^3	99.4
		4.5×10^5	0	100.0
		2.3×10^5	0	100.0
	60	4.5×10^5	0	100.0
		2.3×10^5	0	100.0
75 µg	30	2.3×10^5	0	100.0
	60	2.3×10^5	0	100.0
100 µg	1	6.1×10^5	4.0×10^3*	99.3
	30	6.1×10^5	0	100.0
	60	6.1×10^5	0	100.0

* Atypical colonies

Using tyrothricin in concentrations of 10, 25, 50, 75, and 100 µg acting for 30 and 60 minutes on Streptococcus pyogenes in saliva, the results in Table III were obtained. The percentage reduction of the test organism was calculated by using the count

Table III(A)

Percentage Reduction of Streptococcus Pyogenes in 24 Hours at Graded Concentrations of Tyrothricin per Ml.



Concentration of Tyrothricin in Saliva in µg per ml.

of viable organisms remaining after exposure to saliva as the substrate for tyrothricin action. The plates were read after 24 hours incubation at 37°C and the results calculated in percentage reduction of <u>Streptococcus pyogenes</u> . Tyrothricin in a concentration of 10 μg per ml and acting for 30 minutes had no effect. However in this concentration the count was reduced 79.8% during 60 minutes. In a concentration of 25 μg per ml, the tyro* thricin reduced the count by 95% when acting for 30 minutes and by 95% when acting for 60 minutes. 50 μg per ml of tyrothricin gave practically complete reduction in either 30 or 60 minutes. This was also true of 75 μg. There were a very small percentage of atypical (*) colonies remaining when 100 μg per ml acted for 1 minute.

Table IV

Percentage Reduction in Number of Streptococcus Pyogenes in Saliva by Different Concentrations of Tyrothricin during 48 Hours

Tyrothricin Concentration per ml	Exposure Period in Minutes	S. pyogenes Count per ml	S. pyogenes Count in Saliva and Tyrothricin (per ml)	Percentage Reduction
10 µg	30	1.9×10^6	2.7×10^6	0
		1.0×10^6	2.0×10^6	0
	60	4.5×10^5	9.0×10^4	80.0
		2.3×10^5	5.6×10^4	75.6
25 µg	30	2.3×10^5	3.8×10^4	83.5
	60	2.3×10^5	3.4×10^4	85.2
50 µg	30	1.0×10^6	1.6×10^4 *	97.4
		4.5×10^5	0	100.0*
		2.3×10^5	0	100.0*
	60	4.5×10^5	0	100.0*
		2.3×10^5	2.0×10^3 *	99.1
75 µg	30	2.3×10^5	8.0×10^2 *	99.6
	60	2.3×10^5	6.0×10^2 *	99.7
100 µg	1	6.1×10^5	9.0×10^3 *	98.5
	30	6.1×10^5	1.8×10^3 *	99.6
	60	6.1×10^5	2.7×10^3 *	99.0

* Atypical Colonies

Table IV shows the result of tyrothricin acting for 30 and 60 minutes on Streptococcus pyogenes in saliva. The percentage reduction was calculated as explained for Table III. The same plates that gave the results for Table III were counted again at 48 hours. At 48 hours there was less of an effect on Streptococcus pyogenes than at 24 hours and in the higher concentrations of tyrothricin, there appeared more of the atypical colonies of the test organism than at 24 hours. However the con-

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centrations of 50, 75, and 100 μg per ml affected practically a 100% reduction.

Table V

Effect of Saliva upon Streptococcus Pyogenes

S. pyogenes Count (per ml)	Saliva Count (per ml)	Exposure Period in Minutes	S. pyogenes Count after Treatment with Saliva at 24 Hours	48	Percentage Reduction at 24 Hours	48
1.9×10^6	1.1×10^6	30	2.8×10^6	2.8×10^6	0	0
		60	-	-	-	-
1.0×10^6	2.1×10^6	30	3.6×10^5	3.8×10^5	64.0	62.4
		60	-	-	-	-
2.3×10^5	6.0×10^6	30	2.2×10^5	2.9×10^5	4.4	0
		60	1.6×10^5	2.2×10^5	30.4	0
6.1×10^5	1.3×10^6	30	1.7×10^5	1.9×10^5	72.2	65.2
		60	3.9×10^5	3.9×10^5	46.0	46.0

The effect of saliva upon the count of *Streptococcus pyogenes* was quite variable. In some experiments there was a reduction of 65% in number of streptococci and in other instances there was no apparent effect. This effect is probably not due to the mixed bacterial population of the saliva as in one instance the saliva count was 6×10^6 per ml and there was no effect on the test organism; yet in another instance the saliva count was 1.3×10^6 and there was a 65% reduction of the test organism. The saliva count was very constant during the entire experiment and remained for the most part between 1 million and 2 million per ml with only 1 instance of a count of 6 million.

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Table VI

Effect of Tyrothricin on Saliva Count in 24 Hours

Tyrothricin Concentration (per ml)	Exposure Period in Minutes	Saliva Count without Tyrothricin (per ml)	Saliva Count with Tyrothricin (per ml)	Percentage Reduction
25 µg	30	1.1×10^6	1.2×10^6	0
	60	1.1×10^6	2.6×10^6	0
50 µg	30	1.1×10^6	5.0×10^5	54.1
	60	1.1×10^6	3.0×10^5	73.2
75 µg	30	1.1×10^6	4.2×10^4	96.2
	60	1.1×10^6	3.1×10^4	97.2
100 µg	30	1.1×10^6	3.9×10^4	96.5
	60	1.1×10^6	2.9×10^4	97.4

At higher concentrations, tyrothricin had a very considerable reducing effect on the bacterial count of the saliva, attaining reductions of 96 and 97% at concentrations of 75 and 100 µg per ml.

Controls were run in all the experiments using 2% propylene glycol mixed with saliva and these demonstrated that propylene glycol at such low concentrations exerted no significant inhibitory effect on the test organism. However one run with undiluted propylene glycol affected 100% reduction. There was no significant reduction by a charge consisting of 0.5 ml of a 50-50 mixture of alcohol and propylene glycol (2%), 0.5 ml of bacteria, and 4.0 ml of saliva.

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As a control demonstrating the effectiveness of tyrothricin *in vitro* when saliva was not present, 2 experiments, using 1 µg and 10 µg per ml of tyrothricin in sterile water, were run. 1 µg of tyrothricin per ml acting for 30 minutes reduced the number of *Streptococcus pyogenes* from 4.5×10^5 to 6.6×10^3, a reduction of 98.5%. 10 µg of tyrothricin per ml under similar conditions yielded a 100% reduction of test organisms, whereas 10 µg of tyrothricin added to the streptococci mixed in saliva and acting for 30 minutes yielded no reduction at all in 2 different experiments. There is no doubt that the presence of saliva greatly inhibits the action of tyrothricin necessitating the use of much larger concentrations per ml of the antibiotic to affect the same inhibition in corresponding exposure periods. There also seems to be a possibility that the effect obtained in saliva mixtures is not as permanent in nature, as manifested by growth of atypical colonies during 48 hours incubation.

Discussion of Experimental Results

Atypical colonies of Streptococcus pyogenes developed mostly only during 48 hours incubation and only in the higher concentrations of tyrothricin - 50 to 100 µg per ml. The percentage of these, apparently, resistant forms was very small; the reducing effect of tyrothricin on Streptococcus pyogenes was still around 78 to 99 percent including these atypical forms. At 48 hours, the count of normal Streptococcus pyogenes colonies had increased significantly from that of 24 hours. Thus 25 µg per ml of tyrothricin acting for 60 minutes decreased the count of the test organism 96% in 24 hours; but at 48 hours, the reduction was 85%. This would seem to indicate that the action of tyrothricin on Streptococcus pyogenes is chiefly bacteriostatic.

Tyrothricin in the higher concentrations appears to have a definite reducing effect on the saliva count. In concentrations of 75 µg and 100 µg per ml, it reduced the count 96%; 50 µg per ml reduced the saliva count 50 to 70%; and 25 µg per ml had no apparent effect. This tyrothricin-caused lag in the growth of normal oral flora was overcome, as demonstrated, by increased counts, during 48 hours of incubation at 37°C.

The effect of saliva on Streptococcus pyogenes was found to vary considerably on different days. Some days there was no effect while on other days there was as much as 65% reduction. This varying effect was compensated for by using the affective inoculum of streptococci (viable organisms remaining

after action of saliva on original inoculum) as the substrate for tyrothricin's activity. The difference between this count and that obtained by using the original inoculum of streptococci was so slight as to be almost negligible.

Tyrothricin acting on Streptococcus pyogenes in sterile water gave a 100% reduction at a concentration of 10 µg per ml for 30 minutes while, when the streptococci were mixed in saliva, 10 µg per ml in the same exposure period had no apparent effect. Thus it can be concluded from this and other comparable results that the action of tyrothricin is greatly reduced by saliva.

Although tyrothricin in 10 µg per ml concentration had no effect whatsoever on Streptococcus pyogenes mixed in saliva during 30 minutes exposure, there was an 80% reduction in the count when the exposure period was extended to 60 minutes. A concentration of 25 µg per ml acting for 30 minutes affected a reduction of 94% while in 60 minutes, the reduction was 96%. At a concentration of 50 µg per ml, the reduction was essentially 100% after either 30 minutes or 60 minutes exposure periods. This was also true of concentrations of 75 and 100 µg per ml. 100 µg per ml, acting for the amount of time it took to mix the contents of the tube, gave a 98% reduction indicating the rapidity of tyrothricin's antibacterial action. Thus the minimal amount of tyrothricin, necessary to produce complete inhibition of the test organism in saliva, would fall between 25 and 50 µg per ml acting for 30 minutes; this range would hold true for a 24 hour period but after this time, some growth might occur due

to resistant forms or to the overcoming of bacteriostasis.

Conclusions

1. The action of tyrothricin on <u>Streptococcus pyogenes</u> in saliva is primarily bacteriostatic.

2. Tyrothricin acts almost immediately on this test bacteria.

3. Tyrothricin above a concentration of 50 µg per ml has a definite reducing effect on the bacterial count of saliva.

4. Saliva has a variable reducing effect on the number of test bacteria.

5. The action of tyrothricin is greatly inhibited by the presence of saliva.

6. The minimal amount of tyrothricin necessary to produce complete inhibition of growth of the New York 5 strain of <u>Streptococcus pyogenes</u> in saliva is between 25 and 50 µg per ml acting for 30 minutes.

7. There is an effective reduction of <u>Streptococcus pyogenes</u> in saliva by concentrations of tyrothricin between 10 and 25 µg per ml acting for 30 minutes.

Summary

The extent of the described experiments consisted chiefly in delineating the range of concentrations of tyrothricin per ml effective against the New York 5 strain of <u>Streptococcus pyogenes</u> mixed in saliva.

The required inoculum of approximately 1 million organisms

per ml was obtained by growing cultures of the streptococci under uniform conditions and setting up a table of the absorbances and viable cell counts, from which dilution factors for further cultures could be estimated, from these cultures.

Controls were set up for determining possible inhibition of tyrothricin and/or test organisms by the various diluting fluids including saliva.

Final concentrations per ml of 10,25, 50, 75 and 100 μg per ml of tyrothricin,integrated with saliva and an approximated number of streptococci, were plated out after 30 and 60 minute exposure periods.

Whereas 1 μg per ml of tyrothricin reduced markedly the number of streptococci suspended in water after a 30 minute exposure period and 10 μg per ml,under similar conditions, caused complete inhibition, 10 μg per ml of the antibiotic was ineffective against the test organism suspended in saliva during a 30 minute exposure period but caused an 83% reduction in viable organisms during 60 minutes exposure. The length of the exposure period necessary for effective inhibition varied inversely with the concentration of tyrothricin per ml, 100 μg per ml causing a 98% reduction of viable organisms in an exposure period of 1 minute. For the 30 minute exposure period, the quantity of tyrothricin effective against this strain of streptococci mixed in saliva would fall in the 10 μg -25 μg per ml range and for shorter exposure periods, the concentration per ml would have to be

greater.

Cultures, completely negative after 24 hours incubation at 37°C, showed atypical growth after 48 hours. This is considered indicative of the bacteriostatic action of tyrothricin which, prolonged, would result in the death of large numbers of the streptococci.

The results which were obtained in these experiments chiefly serve to point out the way for further work and provide a basis for the general conclusions discussed in the previous section.

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Abstract

of

Evaluation of the Effect of Tyrothricin on
β- hemolytic Streptococci in Saliva

In 1940 Dubos reported the separation of an alcohol - soluble substance ,which was bactericidal and bacteriostatic against many Gram-positive organisms, from cultures of Bacillus brevis; second in degree of susceptibility only to Diplococcus pneumoniae, were β- hemolytic streptococci. This substance, tyrothricin, was subjected to thorough chemical analysis and purification and was found to be actually a complex of 2 polypeptides, designated gramicidin and tyrocidine. In vitro tyrocidine acts as a cationic agent causing rapid bactericidal and lytic action in high dilutions against Gram-negative and Gram-positive organisms alike; but in vivo this component is ineffectual as it quickly combines with tissue proteins and loses its antibacterial activity. Gramicidin seems to speed up oxidative metabolic processes of Gram-positive organisms only and to interfere with their storage of carbohydrates and phosphates. Early workers were quick to observe that the parent substance combined the desirable attributes of the individual components while at the same time reducing their undesirable effects. Whether the antibacterial action of tyrothricin is due to the fact that many of its amino-acid hydrolytic products are of the "unnatural" d-configuration in contrast to the l-configuration prevailing among hydrolytic products of animal proteins is a possibility yet

to be established. However tyrothricin's hemolytic action in the blood stream, very slight solubility in water, precipitation in the presence of even very slight traces of electrolytes, and inhibition by cephalin and mucin have resulted in confining the therapeutic use of tyrothricin to topical application.

In an effort to explore every possible favorable therapeutic usage for tyrothricin, clinicians have used tyrothricin in a variety of diseases where no danger due to hemolytic activity was possible. Highly effective against β-hemolytic streptococci in vitro and clinically in such primary and secondary streptococcal infections as superficial ulcers, acute sinusitis, certain forms of dermatitis, empyema, mastoiditis, purulent otitis media, abscesses of the skin and soft tissues, and wound infections, tyrothricin has not been effective in infections of the upper respiratory tract or in reducing the β-hemolytic streptococcal carrier rate.

In an attempt to delineate the range of concentration of tyrothricin per ml effective against the New York 5 strain of Streptococcus pyogenes in saliva, this experimentation, modeled after the unpublished work of Belding concerning the effect of tyrothricin on the Oxford strain of Staphylococcus aureus in saliva, was carried out.

The required inoculum of approximately 1 million organisms per ml was obtained by growing cultures of the streptococci under uniform conditions and setting up a table of the absorbances

and viable cell counts, from which dilution factors for further cultures could be estimated, from these cultures.

Controls were set up for determining possible inhibition of tyrothricin and/or test organisms by the various diluting fluids including saliva.

Final concentrations per ml of 10, 25, 50, 75, and 100 µg of tyrothricin integrated with saliva and an approximated number of streptococci were plated out after 30 and 60 minutes exposure periods and were counted after 24 and 48 hours of incubation at 37°C.

Whereas 1 µg per ml of tyrothricin reduced markedly the number of streptococci suspended in water during a 30 minute exposure period and 10 µg per ml, under similar conditions, caused complete inhibition, 10 µg per ml of the antibiotic was ineffective against this test organism suspended in saliva during a 30 minute exposure period but caused about an 80% reduction in viable organisms during 60 minutes exposure. The length of the exposure period necessary for effective inhibition varied inversely with the concentration of tyrothricin per ml, 100 µg per ml causing a 98% reduction of viable organisms during an exposure period of 1 minute. For the 30 minute exposure period, the quantity of tyrothricin effective against this strain of streptococci mixed in saliva would fall in the 10 µg - 25 µg per ml range and for shorter exposure periods, the concentration per ml would have to be greater.

Cultures completely negative during 24 hours incubation at 37°C, showed atypical growth during 48 hours. This is considered indicative of the bacteriostatic action of tyrothricin which, prolonged, resulted in the death of large numbers of the streptococci.

The results which were obtained in these experiments serve chiefly to point out the way for further work and to form a basis for the general conclusions listed below:

1. The action of tyrothricin on bacteria is inhibited by saliva to a large degree.

2. The minimal amounts of tyrothricin necessary to produce complete inhibition of growth of _Streptococcus pyogenes in_ saliva is between 25 and 50 μg per ml acting for 30 minutes.

3. There is an effective reduction of _Streptococcus pyogenes_ in saliva by concentrations of tyrothricin between 10 and 25 μg per ml acting for 30 minutes.

4. Tyrothricin acts immediately upon contact with _Streptococcus pyogenes_.

5. The action of tyrothricin on _Streptococcus pyogenes_ in saliva is apparently bacteriostatic and not of a permanent nature as manifested by growth of atypical colonies during 48 hours incubation.

6. Tyrothricin above a concentration of 50 μg per ml had a definite reducing effect on the bactericidal population of this saliva.

7. Saliva also has a bactericidal or bacteriostatic (or both) action against _Streptococcus pyogenes_.