ANTIBACTERIAL SUBSTANCES PRODUCED BY THE GENUS STAPHYLOCOCCUS

A Thesis

Presented to

The Department of Bacteriology and Immunology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Andy Chun-yang Hsu

October 1966

ABSTRACT

It was found that 4.9 per cent of 1,065 coagulase-positive and 8.5 per cent of 387 coagulase-negative strains of *Staphylococcus* produced antibiotics inhibiting the growth of *Staphylococcus aureus* Oxford 209P.

Antibiotics produced by four of the most active strains were investigated in detail. It was found that they inhibited growth of Gram-positive rather than Gram-negative bacteria. In addition, the activity of two was not destroyed by autoclaving or proteolytic enzymes. Activity was, however, lost after dialysis.

At the concentration employed, one antibiotic did not kill all cells of the population tested.

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. P. Warner, Dr. A. C. Maniar, Miss Claudia Kuz and staff of the Department of Bacteriology, the Winnipeg General Hospital as well as Dr. R. Martin and the staff of the Manitoba Provincial Laboratory for supplying strains of staphylococci and other microorganisms.

He is indebted to Dr. J. C. Wilt, head of the Department of Bacteriology and Immunology for arranging financial assistance from the Medical Research Council and the Faculty of Dentistry.

In particular, the author wishes to thank Dr. G. M. Wiseman for his patient guidance.

TABLE OF CONTENTS

PAGE

LIST OF TABLES vi

LIST OF FIGURES vii

INTRODUCTION 1

REVIEW OF THE LITERATURE 2

A. Action of Staphylococcal Antibiotics on Gram-Positive Bacteria 2
1. Corynebacteria 2
2. Bacilli and clostridia 6
3. Mycobacteria 7
4. Cocci 8
B. Action of Staphylococcal Antibiotics on Gram-Negative Bacteria 11
C. Action of Staphylococcal Antibiotics on Other Micro-organisms 12
D. Production and Characterization of Antibiotics from Staphylococci 13
1. Conditions required for antibiotic production 13
2. Methods of concentration and production 14
3. Properties of staphylococcal antibiotics 15

MATERIALS AND METHODS 19

A. Materials 19
1. Strains of bacteria employed 19
(a) Staphylococci 19
(b) Other kinds of bacteria 19
2. Suspending fluids 19
B. Methods 20
1. Antibiotic screening tests 20
2. Determination of inhibitory spectrum of antibiotic-producing strains of staphylococci 20
3. Preparation of small volumes of crude antibiotics . . 21
4. Quantitative titration of antibiotic 22
5. Optical density measurements 22
6. Viable bacterial counts 22
7. Agitation of cultures 24

PAGE

EXPERIMENTAL RESULTS 25

A. Incidence of Antibiotic-Producing Strains of Staphylococci Isolated from Clinical Material 25
1. Frequency of isolation 25
2. Size of inhibition zones 25
3. Sensitivity spectrum of active staphylococci to clinically-employed antibiotics 27

B. Study of Selected Antibiotic-Producing Strains of High Activity 27
1. Origin of selected strains 27
2. Stability of antibiotic-producing property of active strains 31
3. Factors influencing the quantitative production of antibiotics 31
(a) Type of medium 31
(b) Addition of carbohydrates 32
(c) Growth period 35
(d) pH of medium 35
4. Inhibitory spectra of selected active antibiotic-producing strains 38
(a) Effect on staphylococcal strains 38
(b) Effect on species of bacteria other than *Staphylococcus* 38
(c) Cross-inhibition test 43
5. Properties of crude antibiotic preparations 46
(a) Stability of the preparations 46
(b) Heat sensitivity of antibiotic preparations 46
(c) Dialyzability of antibiotic preparations 46
(d) Hemolytic activity of antibiotic preparations 49
(e) Action of proteolytic enzymes on antibiotic preparations 49
(f) Nature of the antibacterial activity of antibiotic preparations 52
6. Capability of antibiotic transfer 52

DISCUSSION 55

SUMMARY 60

BIBLIOGRAPHY 62

LIST OF TABLES

TABLE		PAGE
I.	Relationship Between Source and Active Antibiotic-Producing Strains	26
II.	Size of Inhibition Zones Produced by the Active Strains	28
III.	Sensitivity Spectrum of Active Coagulase-Positive Staphylococci to Therapeutic Antibiotics	29
IV.	Morphological and Physiological Characteristics of Four Active Antibiotic-Producing Staphylococcal Strains	30
V.	Influence of Types of Solid Media on Antibiotic Production by _Staphylococcus_	33
VI.	Effect of Carbohydrates on the Production of Staphylococcal Antibiotics	34
VII.	Effect of pH of Medium on the Production of Staphylococcal Antibiotics	37
VIII.	The Effect of Staphylococcal Antibiotics on Staphylococcal Strains	39
IX.	Inhibitory Spectrum of Staphylococcal Antibiotics	41
X.	Effect of Staphylococcal Antibiotics on Strains of _Mycobacterium_	44
XI.	Cross Inhibition Test of Four Active Antibiotic-Producing Strains	45
XII.	Stability of Crude Antibiotic Preparations Kept at $4^{o}C$	47
XIII.	Heat Sensitivity of Antibiotics 29297 and 36534	48
XIV.	Dialyzability of Staphylococcal Antibiotics 29297 and 36534	50
XV.	Action of Trypsin and Chymotrypsin on Antibiotics 29297 and 36534	51

LIST OF FIGURES

FIGURE PAGE

1. Relationship Between Antibiotic Dosage and Growth of the Indicator Strain S. aureus Oxford 209P 23

2. Influence of Time on Production of Staphylococcal Antibiotics 36

3. Influence of Antibiotic 29297 on Viable Counts of S. aureus at 20°C. 53

INTRODUCTION

The *Staphylococcus* is capable of producing a variety of distinct extracellular substances, including coagulase, hemolysins, leucocidin, staphylokinase, hyaluronidase, enterotoxin, dermonecrotic and lethal factors, as well as antibacterial factors. This investigation has been concerned entirely with antibacterial substances produced by the staphylococci, since very little is known about their properties, significance and mode of action.

These antibacterial substances can generally be divided into two categories; lytic agents, and inhibitory principles. Most lytic agents found in staphylococci actively lyse only living or dead staphylococcal cells and *Micrococcus lysodeikticus*. Other microorganisms are generally not affected by these agents. By contrast, inhibitory principles can prevent growth of a large number of Gram-positive bacteria and a limited number of Gram-negative species. Reports on the biological and chemical characteristics of these inhibitory materials vary widely in the literature.

It has been the object of the present investigation to survey the incidence of antibiotic-producing strains of *Staphylococcus* isolated from clinical material and to make an intensive investigation of two of these antibacterial substances.

REVIEW OF THE LITERATURE

Cornil and Babès (1885) showed that a *Staphylococcus* strain could inhibit the growth of *Bacillus anthracis* and another *Staphylococcus* strain. Since that time, knowledge of the antagonistic activities of staphylococci against other microorganisms has accumulated. Dujardin-Beaumetz (1934) found that a minority of staphylococci produced the antibiotic substance. Frédéricq (1946) noted that more than one antibiotic was produced by different strains of *Staphylococcus* and even a single strain might sometimes produce more than one inhibitory substance. Dujardin-Beaumetz (1932) reported that the active strains appeared to have no special cultural or biochemical features. Frédéricq and Betz (1946) also stated that the ability to produce antibiotic was not associated with pathogenicity or with any of the biological characters investigated. This was confirmed by Myers (1946), Jennings and Sharp (1947), Halbert *et al*. (1953) and Lachowicz (1963b).

A. ACTION OF STAPHYLOCOCCAL ANTIBIOTICS ON GRAM-POSITIVE BACTERIA

(1) *Corynebacteria*

Schiøtz (1909) observed that a patient with staphylococcal sore throat admitted by mistake to a diphtheria ward failed to contract diphtheria. He also noted that some chronic carriers became free of the diphtheria bacillus due to a non-specific throat infection with *Staphylococcus*.

Stovall et al. (1923) noted that if an equal amount of Staphylococcus aureus suspension was planted in liquid medium with Corynebacterium diphtheriae, the growth of the latter was suppressed or no growth occurred. When small quantities of staphylococci (corynebacteria/staphylococci 10/1) were present, the bacilli underwent well-marked morphological changes. They were reduced in size, became thinner and sometimes apparently longer, and stained as heavily banded organisms.

From staphylococcal broth cultures, Bogendoerfer (1924) was able to isolate a thermolabile substance which had the same inhibitory activity as the staphylococcal cells grown in the diphtherial cultures. This substance was soluble in alcohol, ether, acetone and benzene and could also be extracted from staphylococcal cells.

Dujardin-Beaumetz (1932) described the isolation from nasal mucus of a patient with atrophic rhinitis, and from the throat of a diphtheria carrier, of strains of staphylococci which had the power to suppress the growth of all strains of corynebacteria tested.

Duliscouët (1935) described non-pathogenic staphylococci which favoured the growth of diphtheria bacilli, and others which inhibited their growth. In later observations (1939, 1945), he stated that although inhibitory strains might be found among S. aureus and S. epidermidis, a certain rather slow-growing strain of S. epidermidis was the best. It could inhibit C. diphtheriae in vitro as well as in vivo. When this Staphylococcus strain was injected at another

place or together with diphtheria toxin, it had no effect. He called this active material "staphyline" and considered it to be produced only on solid medium.

Jennings and Sharp (1947) examined 205 strains of staphylococci which were obtained mostly from hospital environments including three strains isolated from patients with diphtherial infections, in which difficulty was experienced in isolation of diphtheria bacilli because of the action of an accompanying *Staphylococcus*. About 10 per cent of strains of *Staphylococcus* could produce an antibiotic to inhibit the growth of *diphtheriae*, *xerosis* and *pseudodiphtheriticum* (Hofmann's bacillus) species of *Corynebacterium*. They also found that there was no relationship between production of the inhibitory substance and metabolic characters or coagulase activity. Inhibition of different test organisms appeared to be a quantitative rather than a qualitative matter.

Gardner (1949) found that when the antibiotic of Jennings and Sharp had been precipitated from a cell-free culture by trichloracetic acid and reprecipitated by ammonium sulphate, complete inhibition of growth of *C. xerosis* but not *C. diphtheriae* occurred in rather high dilution. She also reported that this substance was dialyzable, thermostable at neutral or acid pH values, but was rapidly destroyed in alkaline solution. Proteolytic enzymes could inhibit this substance, as did air-bubbling and shaking.

Parker *et al*. (1955) observed that a large proportion of *Staphylococcus* strains isolated from impetigo lesions had the ability to inhibit the growth of *C. diphtheriae* and other diphtheroids on solid media. Strains from other

sources less commonly had this ability. They also found that the inhibition of "mitis" and "gravis" strains of C. diphtheriae was usually much greater than that of "intermedius" strains or of C. xerosis. This is contrary to the results of other workers, since most believed that C. xerosis was much more sensitive than C. diphtheriae to the antibiotics produced by staphylococci. Parker et al. also mentioned that inhibition could be demonstrated on serum agar and on nutrient agar, but was best seen on blood agar incubated aerobically or anaerobically. This is another contradictory result in view of the fact that others considered that serum could inhibit the production of antibiotic by Staphylococcus strains. They concluded that power to inhibit was not retained indefinitely in culture, and non-inhibiting variants, both white and golden, could be obtained by plating out old cultures.

Parker et al. (1955), Barrow (1955), Parker (1958), as well as Parker and Simmons(1959) showed that most staphylococci of bacteriophage type 71, but very few others, were able to prevent the growth of C. diphtheriae on solid media with the formation of a sharply defined zone of inhibition. These strains of Staphylococcus were closely associated with impetigo contagiosa and were rarely isolated from deeper infections. This was confirmed by Barrow (1963) who employed one hundred strains of coagulase-positive staphylococci isolated from routine bacteriological specimens. He also found that they were all penicillin-resistant, positive by the serum opacity, but negative by the egg-yolk opacity test.

(2) Bacilli and clostridia

Most investigators believed that both Bacillus and Clostridium species were moderately sensitive to the antibiotics produced by Staphylococcus. As mentioned before, Cornil and Babès (1885) first noted that a Staphylococcus could inhibit the growth of Bacillus anthracis. Tomkins (1887) confirmed this. He observed that the anthrax bacilli, when cultivated together with an ordinary Staphylococcus on the medium, were killed.

Doehle (1889) named a non-pathogenic strain of Staphylococcus which could inhibit the growth of the anthrax bacillus "Micrococcus anthracotoxicus". He considered that its inhibitory action was due to the production of some metabolic product(s) which could diffuse quickly into the medium and make it incapable of supporting the growth of B. anthracis.

Freundenreich (1888) recorded that the culture medium in which Staphylococcus had grown would not support the growth of B. mallei but would not affect the growth of B. anthracis.

On the basis of in vivo studies, Pawlowsky (1887) showed that staphylococci were quite active in protecting rabbits against infection with B. anthracis. This was also confirmed by di Mattei (1889) and Frank (1899). Beco (1895) found it true for mice also. In rats, Urbain et al. (1931) noted marked antagonism of staphylococci to B. anthracis. One ml. of a broth culture of staphylococci could destroy the infectivity of 100 to 200 lethal doses of anthrax bacilli if mixed with them before injection.

Halbert et al. (1954) studied in detail the antagonism between Staphylococcus and Clostridium septicum. They found that S. epidermidis obtained from human ocular microflora could protect the mice against at least 1,000 LD_{50} doses of C. septicum. This protection could be shown both in vivo and in vitro against spores and vegetative cells. Living staphylococci were more protective than heat-killed organisms and delay in the injection of the antibiotic-producing staphylococci until 2 to 5 hrs. after C. septicum was inoculated, greatly reduced the effectiveness of the protection but did not completely eliminate it.

(3) Mycobacteria

Roncali (1892) discovered that S. aureus and S. epidermidis inhibited the growth of tubercle bacilli in vitro. Further studies by them found that a filtrate of S. epidermidis markedly retarded the in vitro development of tubercle bacilli but when this was tried in vivo, the infection was only made worse.

Petragnani and Mazzetti (1932) found that Staphylococcus modified the growth of tubercle bacilli and killed them within 11 days. Dujardin-Beaumetz (1932) also stated that his Staphylococcus strains were active against avian tubercle bacilli.

Arena (1944) reported isolating from a patient with an infection of the respiratory tract, a strain of Staphylococcus which inhibited growth of the tubercle bacillus.

Myers (1946), in his studies on inhibition by staphylococci of mycobacteria, found that the fast-growing strains of mycobacteria were not inhibited as much

as the slow-growing strains, and often were not inhibited at all. In general, the human strains were more susceptible than fast-growing strains such as *Mycobacterium phlei*. He also noted that the inhibitory potency of *Staphylococcus* strains could not be related to their source, microscopic or colonial morphology, hemolytic activity, sugar fermentation, coagulase or lipolytic activity.

(4) *Cocci*

Gratia and Rhodes (1924) noted that a special strain of *Staphylococcus* could dissolve dead staphylococci of another strain in liquid and on solid media. Wollman (1934a,b) observed the same phenomenon and concluded that this was due to the liberation by the living organisms of "autolysins". Salmon (1949) found that if heat-killed staphylococcal cells were incorporated into nutrient agar so as to render it turbid and the same strain was grown on the surface, the colonies were surrounded by a zone of clearing. He finally isolated the substance and named it "autolysin". Welsch and Salmon (1949) found that their autolysin could also dissolve living staphylococci. Gram-negative organisms, both living and heat-killed, were unaffected. They found afterward (1950a,b) that phosphates were indispensible for production of this lysin.

Ralston *et al*. (1955, 1957a,b) were able to distinguish three types of lysins from staphylococci, "autolysin", "virolysin" and "phage-induced lysin". They found that "autolysin" activity was maximal at pH 6.0 to 6.5 with rapid loss above pH 6.5. It could lyse heat-killed or phage-treated, acetone-treated, ultraviolet-irradiated or penicillin-treated staphylococcal cells, *Micrococcus*

lysodeikticus and other Gram-positive organisms. In phage-lysed filtrates, a second lytic substance, so-called "virolysin" was obtained (Ralston and McIvor, 1964a,b). It was reported that virolysin was capable of lysing heat-killed staphylococcal cells and in conjunction with the staphylococcal phage, it could also lyse living staphylococcal cells. Unlike the "autolysin", it had no effect on M. lysodeikticus or other Gram-positive organisms. It was stable at pH 7.0 to 7.5. They were also able to isolate a third lysin, "phage-induced lysin" from strains of S. aureus. The phage-induced lysin was similar to virolysin and did not lyse any heat-killed Gram-positive or negative bacterial cells except staphylococci. It had little or no effect on heat-killed M. lysodeikticus.

In investigating the production of hyaluronidase by S. aureus, Richmond (1959) found that his special strain liberated a lytic enzyme capable of digesting intact organisms and cell-wall preparations of M. lysodeikticus. A crude preparation of this lytic substance could digest M. lysodeikticus cell walls liberating N-acetylamino sugars and a reducing sugar. Its effect was similar to the action of egg-white lysozyme. However, the crude preparation had no detectable activity on intact cells of the strain which produced this substance.

Schindler and Schuhardt (1964) isolated another lysin from a strain of S. epidermidis. This substance was named "lysostaphin". Lysostaphin lysed all living and heat-killed staphylococcal cells or cell-wall preparations, but S. epidermidis strains were lysed at a markedly slower rate than S. aureus

strains. Other bacteria, both Gram-positive and Gram-negative, including M. lysodeikticus were not lysed. Human serum reduced the rate but not the degree of lysis.

Murray and Loeb (1950) stated that their active strains of staphylococci, coagulase-positive as well as negative, could produce at least four distinct antibiotics. One of them, produced by a coagulase-positive Staphylococcus, inhibited most of the mucoid strains of Lancefield group A Streptococcus, and was active against few or none of the non-mucoid strains of same group, and none of the streptococci in Lancefield groups B, C and G. Another one from a coagulase-negative Staphylococcus inhibited the majority of streptococci tested except mucoid strains of Lancefield group C.

Hayes (1950), in studies of mixed bacterial growth in liquid media, found that the behavior of S. aureus and Str. viridans in the mixture during the first 48 hrs. of growth was influenced largely by the initial inoculum ratio of the two organisms. Only in the presence of large numbers of staphylococci was the growth of Str. viridans suppressed. No suppression of Str. viridans could be detected if the initial inoculum of staphylococci was smaller or the same as that of the staphylococci.

Of a total of 1,239 strains of staphylococci, Lachowicz (1965) was able to isolate 131 antagonistic strains (10.6%) active against S. aureus Oxford 209P. From these active strains, two similar antibiotics, called "staphylococcin" and "staphylococcin A" were isolated. These staphylococcins were bactericidal for

Gram-positive bacteria. No effect on Gram-negative organisms could be demonstrated with either of these staphylococcins.

B. ACTION OF STAPHYLOCOCCAL ANTIBIOTICS ON GRAM-NEGATIVE BACTERIA

The general opinion is that Staphylococcus cannot inhibit the growth of Gram-negative bacteria. Régnier and Lambin (1934), for example, in studying mixtures of Escherichia coli and staphylococci, found that the growth of E. coli was not altered. Gardner (1949) observed that the growth of E. coli, Shigella sonnei, Pseudomonas aeruginosa and Salmonella gaertneri was not suppressed by her purified antibiotic substance at concentrations which could inhibit the growth of mycobacteria, corynebacteria and anthrax bacilli. Murray and Loeb (1950) reported that the antibiotics produced by either S. aureus or S. epidermidis were not effective against the Gram-negative bacteria tested, such as E. coli, Salmonella schottmuelleri, Shigella sonnei, Klebsiella pneumoniae, Proteus sp., Haemophilus influenzae, Pseudomonas aeruginosa and Neisseria catarrhalis. Halbert et al.(1953) stated that all the Enterobacteriaceae, Ps. aeruginosa and Serratia mercescens were resistant to antibiotics produced by their staphylococci. Barrow (1963) found that none of his active phage-type 71 staphylococci inhibited strains of Haemophilus, Neisseria or any of the enterobacteria. Lachowicz (1965) also reported that Gram-negative bacteria were not sensitive to his staphylococcins.

In spite of this mass of data, several investigators working with staphylo-

cocci have found various degrees of inhibition with different kinds of Gram-negative bacteria tested. Freudenreich (1888), for instance, recorded that the culture medium in which staphylococci had grown could inhibit the growth of *Salm. typhi* and *Pasteurella aviseptica*. Korolev (1939) found that *S. epidermidis* inhibited the growth of *Brucella melitensis*, *Br. abortus* and *Br. suis* on solid medium. Nutini *et al*. (1946) found that although the growth of *E. coli* was not inhibited by cellular extracts of *S. aureus* either in low or in high concentrations, the growth of *Aerobacter aerogenes* and *Sh. dysenteriae* was inhibited by these extracts.

C. ACTION OF STAPHYLOCOCCAL ANTIBIOTICS ON OTHER MICRO-ORGANISMS

A few references have been encountered which discuss inhibition by *Staphylococcus* of other microorganisms such as spirilla, protozoa and fungi. Freudenreich (1888) noted that the culture medium in which *Staphylococcus* had grown would not only inhibit the growth of true bacteria but also would inhibit the growth of spirilla. Spagnolio (1912a, b) had difficulty in isolating *Leishmania* from an oriental sore. He found that the growth of *Leishmania* was inhibited by staphylococci isolated from the sore. Carvajal (1946) noted that *S. aureus* inhibited the growth of *Streptomyces griseus* when the two organisms were inoculated simultaneously, but that if *Streptomyces* was allowed to develop before the *Staphylococcus* was inoculated, the latter was inhibited.

D. PRODUCTION AND CHARACTERIZATION OF ANTIBIOTICS FROM STAPHYLOCOCCI

Little knowledge concerning the properties of these antibiotics is available and because of the variation in strains and methods employed by different workers, comparisons are difficult.

(1) Conditions required for antibiotic production

Dujardin-Beaumetz (1932) observed inhibitory activity only on solid media. Duliscouët (1939, 1945) had difficulty in detecting the active principle in liquid medium and noted that it was formed much more readily in solid medium. Marassi and Spiga (1946) found that a strain of Staphylococcus produced antibiotic substances only when oxygen was bubbled through a liquid medium or when the organism was grown on solid medium. Jennings and Sharp (1947) stated that bubbling a gas, even nitrogen, through a liquid medium could inactivate the antibiotic. Gardner (1949) reported that the antibiotic was best produced when the Staphylococcus was grown in shallow layers in an atmosphere rich in oxygen. Halbert et al. (1953) observed that antibiotic production often took place readily in liquid media. The titre of the crude antibiotic was slightly greater on solid rather than in liquid media, but differences were not striking. Barrow (1963) reported that broths varied considerably in their ability to support antibiotic production by phage-type 71 staphylococci. Among meat infusions and digests, Lemco broth, casein and lactalbumin hydrolysate media, only broth freshly prepared by tryptic digestion of meat usually gave the best yields. Decreased oxygen or

increased CO_2 tension made little difference, and shallow or deep cultures with or without intermittent shaking all gave similar results.

Barrow (1962) found that the production of antibiotic from phage-type 71 staphylococci was not affected on solid media over a pH range from 5 to 9 incubated at 37°C. under aerobic or anaerobic conditions. It also did not affect production when the organisms were grown at 25° to 30°C., even in the presence of 5 per cent (w/v) NaCl. Lachowicz (1965) stated that the maximum concentration of staphylococcin was obtained at 37°C. There was no detectable amount of staphylococcin present when the organism was incubated at 20° or 44°C. Schindler and Schuhardt (1964) found that production of lysostaphin was proportional to growth. This was also true for Lachowicz's staphylococcin.

Lachowicz and Lataczowa (1951) noted that the addition of fructose, glucose, sucrose, glycerol and mannitol to the medium caused an increase in the staphylococcin yield. Barrow (1963) also reported that the addition of glucose or mannitol at an initial pH of about 7.8, appreciably increased antibiotic production from phage-type 71 staphylococci.

(2) Methods of concentration and production

Gardner (1949) noted that inactive material could be removed from the culture fluid by adding concentrated hydrochloric acid; the antibiotic was further concentrated by evaporation in a current of air at 56°C., and precipitated by adjusting the concentrated fluid to pH 3.0. Further purification was carried out by dialysis against running water and precipitation of the remaining traces of inactive material in boiling water.

Loeb et al. (1950) stated that their antibiotic could be partially purified by addition of pyridine and ethanol to precipitate the impurities. The purified antibiotic could be precipitated by ether and dried in a vacuum without loss of activity.

Halbert et al. (1953) stated that their antibiotic from Staphylococcus was extractable with n-butanol at room temperature over pH range 2 to 7. The antibiotic was recovered from the butanol by evaporation under reduced pressure.

Barrow (1963) observed that the antibiotic from phage-type 71 staphylococci could be concentrated as follows: the culture fluid was freed from impurities by the addition of an equal volume of 10 per cent (w/v) trichloroacetic acid. After the trichloroacetic acid had been nutralized, the fluid supernatant was concentrated in a current of warm air. The copious inactive material deposited during evaporation was removed and the solution was dialysed and dried.

Schindler and Schuhardt (1965) found that their lysostaphin could be purified by repeated precipitation with solid $(NH_4)_2SO_4$, resuspending in cold 0.05 M Tris buffer (pH 7.5) and dialysing against the same kind of buffer.

(3) Properties of staphylococcal antibiotics

Bogendörfer (1924) stated that the active principle from his staphylococcal broth culture was a thermolabile, filtrable substance. It was soluble in alcohol, ether, acetone and benzene. Dujardin-Beaumetz (1932) found that his staphylococcal antibiotic was also thermolabile, being destroyed at 60°C. in one hour. Gundel and Wagner (1930) found that the antibiotic from their staphylococci was a thermostable substance.

Duliscouët (1939, 1945) stated that his active material was thermolabile, being destroyed in a few minutes at 80°C. It was water soluble but insoluble in alcohol. Myers' (1946) preparation was thermostable, being only partially inactivated by heating at 100°C. for 15 minutes. It was water- and ether-soluble and its activity was inhibited by blood serum.

Nutini et al. (1946) found that their antibiotic was filtrable through Seitz or Berkefeld filters and extractable by alcohol. There were no detectable changes in biochemical properties of test organisms exposed to this antibiotic.

Gardner (1949) showed her antibiotic from *Staphylococcus* to be heat-stable at neutral or acid pH values but very unstable under alkaline conditions. It was readily inactivated by trypsin but only partially by pepsin. It also was dialysable and filtrable through a sintered glass filter. Methanol, 80 per cent ethanol, butanol, acetone, ether and chloroform could readily inactivate it.

Loeb et al. (1950) stated that their antibiotic was unaffected by autoclaving for 20 minutes at 121°C. It was soluble in solvents which are completely miscible with water but insoluble in those solvents which are only partially or not miscible with water. It was dialysable and was adsorbed by activated charcoal. Its activity was destroyed by trypsin but not by pepsin, and was reduced when it was mixed with sheep serum. It was bactericidal for susceptible test organisms, but lysis of the bacteria was not observed. The test cells did not show any change in staining characteristics using the Gram-stain even after being suspended in a high concentration of antibiotic for four days. The test organisms became more resistant on repeated passage through media containing

gradually increasing concentrations of antibiotic but after one subculture to media without antibiotic, they were again fully sensitive.

Halbert et al. (1953) found that their antibiotics were heat-labile. They were affected by pepsin but all were destroyed by trypsin. Most of them were precipitatable with $(NH_4)_2SO_4$ and partially or fully dialysable. In the presence of plasma, they were usually slightly or moderately inactivated. Resistant mutants could be induced by serial transfers (three or more) in progressively higher concentrations of crude antibiotic in broth.

Ralston et al. (1957a, b) reported that virolysin required divalent cations, such as Ca^{++}, Mn^{++}, Co^{++}, Zn^{++} and Mg^{++} for its action. In a phage-sensitized cell preparation, an increase in virolysin concentration directly caused an increase in lytic rate but the total lysis was independent of the concentration of virolysin. The unlysed cells incubated with virolysin became Gram-negative and resistant to addition of lysin. It was different from autolysin antigenically because the antisera to autolysin had no effect on virolysin. Like autolysin, virolysin was inhibited by $CuSO_4$, $AgNO_3$, $HgCl_2$, $PbCl_2$, iodoacetate, iodine, formalin, sodium citrate and sodium desoxycholate.

Barrow (1962, 1963) discovered that the antibiotic from phage-type 71 staphylococci could withstand heating at 83°C. for three hours. It could also diffuse through cellophane. Its activity was not affected by the presence of serum, plasma or blood. It was completely destroyed by trypsin in one hour but only slightly inactivated by pepsin for the same length of time.

Schindler and Schuhardt (1965) stated that lysostaphin was completely

inactivated at 75°C. for 15 minutes but the material showed remarkable resistance to both a variety of solvents and to drying. Purified lysostaphin was sensitive to trypsin. The rate of lysis of S. aureus by lysostaphin was directly proportional to temperature over the range of 34° to 55°C. The rate of lysis was also dependent upon the amount of lysostaphin present. However, after the maximum concentration of lysostaphin had been reached, further increases in its concentration did not significantly affect either rate or extent of lysis. The optimum pH for lysis was 7.5. Lysostaphin, like lysozyme, required ions in the reaction mixture for substantial lysis of susceptible cells. An increase in the concentration of NaCl from 0 to 0.145 M. caused an increase in the rate of lysis of the staphylococcal cells. The requirement for ions could be satisfied by the substitution of KCl, K_2HPO_4, or Na_2HPO_4 for NaCl.

Lachowicz (1965) claimed that concentrated staphylococcin was thermostable, withstanding boiling for one hour. It was stable from pH 1 to 10 and was not affected by the action of pepsin or trypsin. It was dialysable and, in high voltage electrophoresis, gathered predominantly in the neutral fractions. After storage at 37°C. for 30 days, its activity fell to zero. However, at 4°C., it could be kept for at least two years. It was bactericidal for sensitive test organisms and resistant mutants could be obtained after serial transfers by gradually increasing the concentration of staphylococcin. Mutants still showed resistance to staphylococcin after two years of storage at 4°C. The activity of staphylococcin was unaltered after it had been exposed to resistant strains.

MATERIALS AND METHODS

A. MATERIALS

(1) Strains of bacteria employed

(a) Staphylococci. A total of 1,050 coagulase-positive and 387 coagulase-negative strains were obtained from the Department of Bacteriology of the Winnipeg General Hospital and the Manitoba Provincial Laboratory. They were freshly isolated from clinical specimens. Fifteen coagulase-positive laboratory strains were also available. *Staphylococcus aureus* Oxford 209P was obtained from Dr. A. C. Maniar of the hospital laboratories. Immediately on receipt, all strains were checked for purity by Gram-staining and plating-out. The strains were then subcultured on Brain-Heart Infusion (BHI) agar slants and maintained at 4°C.

(b) Other kinds of bacteria. Gram-positive and negative bacteria other than staphylococci were obtained from the stock culture collection of this department with the exception of a culture of *Neisseria gonorrheae* which was supplied by the hospital laboratory. Just before testing, all these strains were checked for purity by staining.

(2) Suspending fluids

Buffers where employed were made up according to the instructions of Cruickshank (1962).

B. METHODS

(1) Antibiotic screening tests

Preliminary investigations indicated that the stab-method was most appropriate for rapid testing of large numbers of strains for antibiotic production. The method was employed as follows: BHI plates containing 20 ml. of well dried medium were stabbed with a sealed Pasteur pipette tipped with a culture of the test strain. The plates were incubated at 37°C. for 48 hrs. The plates were now sprayed with a suspension of the indicator strains, S. aureus Oxford 209P, which had been brought to an optical density of 0.01 using a Klett filter no. 69. Spraying was done in an ultraviolet cabinet equipped with an incinerating device, using a glass atomizer of the type employed in chromatography. The plates were then re-incubated for 24 hrs. at 37°C. and the width of the zones of inhibition adjacent to the colony measured in mm. All measurements were made in duplicate and the mean was taken.

The chloroform vapour had no effect on either the production of antibiotic or on growth of the indicator strain.

(2) Determination of inhibitory spectrum of antibiotic-producing strains of staphylococci

A total of 71 strains of 32 species of Gram-positive and negative bacteria were employed. Plates were prepared as under (1) above. When special growth conditions (e.g., anaerobic incubation) were necessary, a control plate of both

indicator and antibiotic-producing strains of Staphylococcus was included.

In the testing of nutritionally fastidious gonococci, a freshly prepared chocolate plate was used. In the testing of mycobacteria, BHI plates could not be used, nor would the staphylococci grow well on solid media suited to the former. Screening of mycobacteria was therefore done in Tween-80 broth in which 1:2 and 1:4 dilutions of antibiotic were made in 6 ml. volumes. A drop (0.1 ml.) of a heavy suspension of the mycobacteria in 0.85 per cent NaCl was then added to each tube. Incubation at 37°C. was allowed to proceed for two weeks. Growth was scored visually.

(3) Preparation of small volumes of crude antibiotics

Brain-Heart Infusion agar plates were employed in the quantitative production of antibiotics. Discs of cellophane (Dennison Mfg. Co., Drummondville, Que.) cut to fit 1.5 x 10 cm. Petri-dishes and sterilized by steaming at 100°C. for 1 hr. were layered onto plates containing 20 ml. of the medium. Each plate was inoculated with 0.3 ml. of a 24 hr. BHI broth suspension of the producing strain which had been adjusted to an optical density of 0.100. A bent glass-rod was used to spread the inoculum evenly atop the cellophane. After two days' incubation at 37°C., growth was harvested by the addition of 5 ml. 0.01 M phosphate-buffered saline, pH 7.2, to each plate. The growth was loosened with a bent glass-rod, taken up with a Pasteur pipette, pooled and spun at 20,000 g. for 30 min. in a refrigerated centrifuge. The active supernatant fluid was filtered through a Millipore HA membrane, sterility

tested, and stored at $4^{o}C$. Filtration was not observed to alter the activity.

(4) Quantitative titration of antibiotic

Active filtrates were assayed as follows: a series of two-fold dilutions of antibiotic was made in 3 ml. volumes of 0.01 M phosphate-buffered saline, pH 7.2, starting at 1:2 in sterile tubes. Three ml. of a standard suspension of the indicator strain in double strength BHI broth was then added to each tube. The tubes were agitated, the optical density of each recorded, and incubated at $37^{o}C$. for 24 hrs. After incubation, the tubes were agitated on a Vortex Jr. Mixer (Scientific Industries Inc.) and the optical density again determined. The difference between the two readings was plotted on graph-paper against the $\log_2$ dilution of antibiotic (Fig. 1). The end-point was taken as the $\log_2$ dilution of the antibiotic corresponding to the optical density of the indicator strain equal to the mid-point of the linear portion of the growth curve.

(5) Optical density measurements

These were made with a Klett colorimeter employing filter no. 69 (transmission 660 to 740 mμ.).

(6) Viable bacterial counts

Viable counts were carried out according to the technique of Miles and Misra (1938). Results were expressed as the mean of 10 counts.

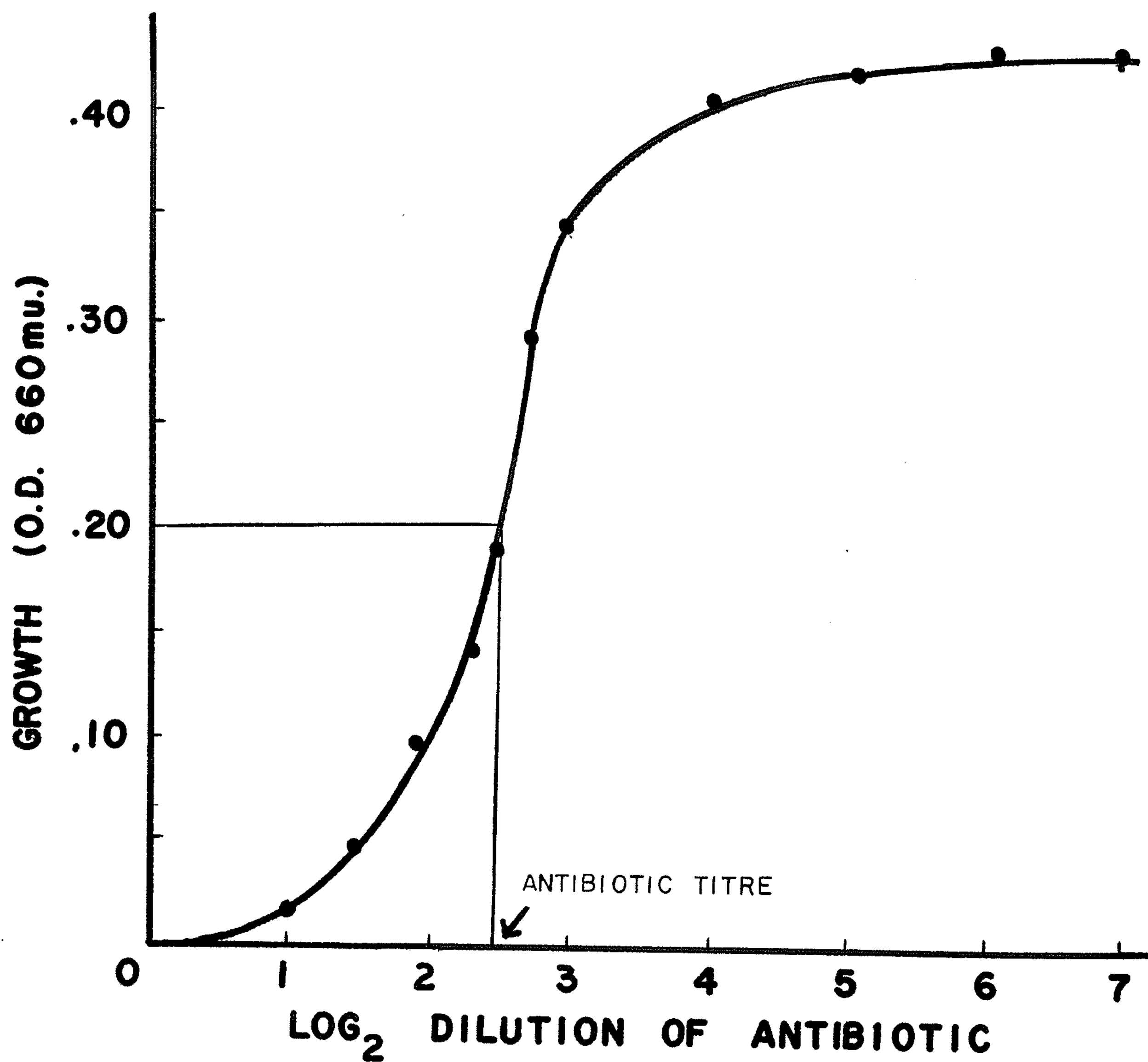

Fig. I RELATIONSHIP BETWEEN ANTIBIOTIC DOSAGE AND GROWTH OF THE INDICATOR STRAIN *S. aureus* OXFORD 209P

(7) Agitation of cultures

When it was required to agitate cultures, an Eberbach shaking water-bath (Research Specialties Co., Richmond, Calif.) was employed at a setting of 75/min.

EXPERIMENTAL RESULTS

A. INCIDENCE OF ANTIBIOTIC-PRODUCING STRAINS OF STAPHYLOCOCCI ISOLATED FROM CLINICAL MATERIAL

(1) Frequency of isolation

A total of 1,065 coagulase-positive and 387 coagulase-negative strains of staphylococci were tested for their ability to produce antibacterial substances active against S. aureus Oxford 209P. Results shown in Table I indicate that of the coagulase-positive strains tested, only 4.9 per cent inhibited growth of the indicator organism. Of the 387 coagulase-negative cultures screened, 8.5 per cent were able to inhibit the Oxford strain.

It was found that in the case of coagulase-positive staphylococci, specimens taken from the ear showed a higher incidence (10.9 per cent) of antibiotic production than those from other sources. This was also true for coagulase-negative strains; 23.5 per cent of those isolated from the ear producing antibiotic.

(2) Size of inhibition zones

The width of the zones of inhibition produced by active strains varied widely from slight (visible but not measurable) to about 6 mm. On the basis of the size of the zone, the active strains were divided arbitrarily into three groups: (a) those producing zones less than 1 mm., in width, (b) those producing zones of moderate size, 1 to 3 mm. and (c) highly active strains

TABLE I

RELATIONSHIP BETWEEN SOURCE AND ACTIVE ANTIBIOTIC-PRODUCING STRAINS

	Coagulase-positive		Coagulase-negative	
Source	Tested	Active(%)	Tested	Active(%)
Ear	55	10.9	17	23.5
Eye	35	2.9	22	13.6
Reproductive organs	74	6.8	108	11.1
Respiratory organs	405	4.2	86	8.1
Skin	410	4.2	119	5.9
Miscellaneous	86	7.0	35	0
Total	1,065	4.9%	387	8.5%

yielding zone widths above 3mm. As Table II shows, only five of 85 active strains were in category (c). Of these five, only one was coagulase-positive. Three of the five were isolated from the cervix uteri, one from the ear and one from bronchial secretion.

(3) Sensitivity spectrum of active staphylococci to clinically-employed antibiotics

Eight commonly used antibiotics, Chloromycetin, dihydrostreptomycin, erythromycin, neomycin, penicillin, sulfadiazine, terramycin and tetracycline were tested for their effect on the active antibiotic-producing strains of staphylococci. Reference to Table III shows that antibiotic production in active strains of coagulase-positive staphylococci bore no apparent relation to their resistance to these therapeutic antibiotics. Similar results were obtained with coagulase-negative strains.

B. STUDY OF SELECTED ANTIBIOTIC-PRODUCING STRAINS OF HIGH ACTIVITY

(1) Origin of selected strains

Four strains of high activity were selected for further study. These were coagulase-positive strains 35548, 36945 and coagulase-negative strains 29297 and 36534. Their physiological characteristics (Table IV) fitted the description of the genus *Staphylococcus* given in the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957). Strains 29297 and 36534 were

TABLE II

SIZE OF INHIBITION ZONES PRODUCED BY THE ACTIVE STRAINS

Zone size (mm)	Coagulase-positive	Coagulase-negative	Total
Less than 1.0	34	13	47
1.0 to 3.0	17	16	33
Larger than 3.0	1	4	5
Total	52	33	85

TABLE III

SENSITIVITY SPECTRUM OF ACTIVE COAGULASE-POSITIVE STAPHYLOCOCCI TO THERAPEUTIC ANTIBIOTICS

Size of inhibition zone with "Oxford" (mm.)	No. strains	Resistance# of strains to therapeutic antibiotics							
		C*	DS	E	N	P	SD	Te	T
Less than 1.0	32	0	1	0	0	14	26	0	1
1.0 to 3.0	16	0	5	3	2	5	14	0	1
Larger than 3.0	1	0	0	0	0	0	1	0	0

#The inhibition zone was less than 1.0 mm.

*C_{30} = Chloromycetin, 30 μg.

DS_{10} = Dihydrostreptomycin, 10 μg.

E_{15} = Erythromycin, 15 μg.

N_{30} = Neomycin, 30 μg.

P_{10} = Penicillin, 10 units

$SD_{1.0}$ = Sulfadiazine, 1.0 mg.

Te_{30} = Tetracycline, 30 μg.

T_{30} = Terramycin, 30 μg.

TABLE IV

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF FOUR ACTIVE ANTIBIOTIC-PRODUCING STAPHYLOCOCCAL STRAINS

	Active Strains			
	29297	35548	36534	36945
Colonial characters	circular, smooth, glistening, with entire margin, and of butyrous consistancy.			
Colour of colony	yellowish-white	yellowish-white	yellowish-white	milky-white
Oxygen requirement	aerobic, facultatively anaerobic	aerobic, facultatively anaerobic	aerobic, facultatively anaerobic	aerobic, facultatively anaerobic
Growth in 10% NaCl	positive	positive	positive	positive
H_2S production	negative	negative	negative	negative
Coagulase activity*	negative	positive	negative	positive
Fermentation				
Inulin	N#	N	N	N
Raffinose	N	N	N	N
Salicin	N	N	N	N
Glucose	Ag	Ag	Ag	Ag
Maltose	Ag	Ag	Ag	Ag
Mannitol	Ag	Ag	Ag	Ag
Sucrose	Ag	Ag	Ag	Ag

*Tube test.

N = no reaction; Ag = acid with small quantity gas after 24 hrs. incubation at 37°C.

isolated from the cervix uteri and 36945 from bronchial secretion. The source of 35548 is unknown.

Small quantities of active material (up to a litre) could conveniently be produced employing the procedures described under "Materials and Methods". Strains 29297 and 36945 gave yields three to four times greater than strains 35548 and 36945.

(2) Stability of the antibiotic-producing property of active strains

The ability to produce antibiotic appeared to be a stable phenomenon. As an example, 29297 was subcultured once a week for ten weeks without significant alteration of antibiotic production. One strain (30530) which initially showed promise as an active producer, lost the ability after two transfers. This, however, was the exception rather than the rule. As an added precaution, the active strains were freeze-dried.

(3) Factors influencing the quantitative production of antibiotics

(a) Type of medium. For testing the quantitative production of antibiotic by a given strain on different media, six types were chosen. These were Blood Agar, BHI Agar (BBL and Fisher), Bramann-Norlin Agar (Jackson and Little, 1957), Dolman-Wilson Agar (Dolman and Wilson, 1940), Gladstone's Synthetic Agar (Gladstone, 1938), Nutrient Agar (Difco) and Trypticase-Soy Agar (BBL). The antibiotic-producing strain was grown for 48 hrs. at 37°C. before the indicator strain was sprayed on the surface of the medium. The quantity of anti-

biotic produced in each type of medium was estimated as usual by measuring the width of the inhibition zone. The results (Table V) showed that Dolman-Wilson Agar and BHI Agar (BBL) gave the best yields amongst the eight investigated. Brain-Heart Infusion Agar prepared by two different manufacturers, BBL and Fisher, showed wide variation in antibiotic production.

Under anaerobic conditions, all the active antibiotic-producing strains tested grew poorly and there was no detectable amount of antibiotic present regardless of the type of medium used.

Dolman-Wilson broth, Brain-Heart Infusion (Difco) broth, Nutrient broth (Difco) and Trypticase-Soy broth (BBL) with or without 10 per cent calf-serum added were tested for their ability to support antibiotic production. Results obtained indicated that antibiotic production was negligible in these liquid media. Agitation of the cultures during incubation generally increased growth but was without effect on production of the antibiotics. The addition of calf-serum had a variable effect on growth but did not facilitate antibiotic production.

(b) Addition of carbohydrates. The addition of 1, 5 and 10 per cent galactose, glucose, glycerol, mannitol or sucrose to the basic BHI agar (BBL) did not enhance antibiotic production by the four strains of *Staphylococcus*, as shown in Table VI. It was noted, however, that as the concentration of glucose or sucrose in the medium increased, antibiotic production by the two coagulase-negative strains 29297 and 36534 decreased. This was not true of the two coagulase-positive producers 35548 and 36945.

TABLE V

INFLUENCE OF TYPES OF SOLID MEDIA ON ANTIBIOTIC PRODUCTION BY STAPHYLOCOCCUS

	Inhibition zone (mm.)			
	Active antibiotic-producing strains			
Type of Medium	29297(-)	36534(-)	35548(+)	36945(+)
Blood agar	3.0	3.0	0	2.0
Brain-heart infusion medium (BBL)	8.0	6.5	1.0	2.0
Brain-heart infusion medium (Fisher)	1.0	2.0	0	1.0
Bramann-Norlin medium	6.5	5.0	0	1.0
Dolman-Wilson medium	9.0	9.0	2.0	0.5
Gladstone's synthetic medium	0	0	0	0
Nutrient agar	6.0	5.5	0	0
Trypticase-soy agar	8.0	-	-	-

TABLE VI

EFFECT OF CARBOHYDRATES ON THE PRODUCTION OF STAPHYLOCOCCAL ANTIBIOTICS

Carbohydrate added		Inhibition zone (mm.) Active antibiotic-producing strain			
Type	Per Cent	29297(-)	36534(-)	35548(+)	36945(+)
Control	0	3.5*	2.5	1.0	1.0
Galactose	1	3.5	3.0	0	0
"	5	3.0	3.0	0	0
"	10	3.0	3.0	1.0	0.5
Glucose	1	3.0	3.0	0	0.5
"	5	2.5	2.0	0.5	0.5
"	10	1.0	1.0	1.0	1.0
Glycerol	1	3.0	2.5	0	0
"	5	2.0	2.0	0	0
"	10	2.0	3.0	0.5	0
Mannitol	1	3.0	2.5	0	0.5
"	5	3.0	3.0	0.5	0.5
"	10	2.5	3.0	1.0	0.5
Sucrose	1	3.0	3.0	0	0.5
"	5	0	2.5	0.5	0.5
"	10	0	2.0	1.0	1.0

*Mean of two measurements.

(c) Growth period. The time requirement for maximal antibiotic production of a given strain was determined at 37°C. on BHI Agar (BBL) plates overlain with cellophane discs. The antibiotic titre obtained revealed that the time required to reach maximum antibiotic production varied in the two strains studied (Fig. 2). Maximal antibiotic production of strain 29297 was reached after the organism had been incubated for two days. The titre of strain 29297 remained fairly constant up to the sixth day and declined slightly afterwards. Under these conditions, the amount of growth of strain 29297 did not reach a maximum until the fourth day and gradually declined afterward. After the organism had been incubated for twenty-one days, only 67 per cent of the maximal amount of antibiotic remained. In the case of strain 36534, the growth rate was similar to that of strain 29297 but maximal antibiotic production required three days of incubation at 37°C. The rate of degradation of this antibiotic resembled that of antibiotic 29297. It appears that the rate of production of these antibiotics was maximal at the time of logarithmic growth.

(d) pH of medium. The pH of BHI agar plates was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The plates were overlain with cellophane discs, inoculated with the producing strain as described in Materials and Methods. The antibiotic titre was estimated after the active strains had been incubated at 37°C. for 48 hrs. The results (Table VII) showed that the optimal pH for antibiotic 29297 was 7.0. With strain 36534, no antibiotic was detectable at pH 5.0 or 8.0. From pH 5.5 to 7.5, the antibiotic titre was fairly constant.

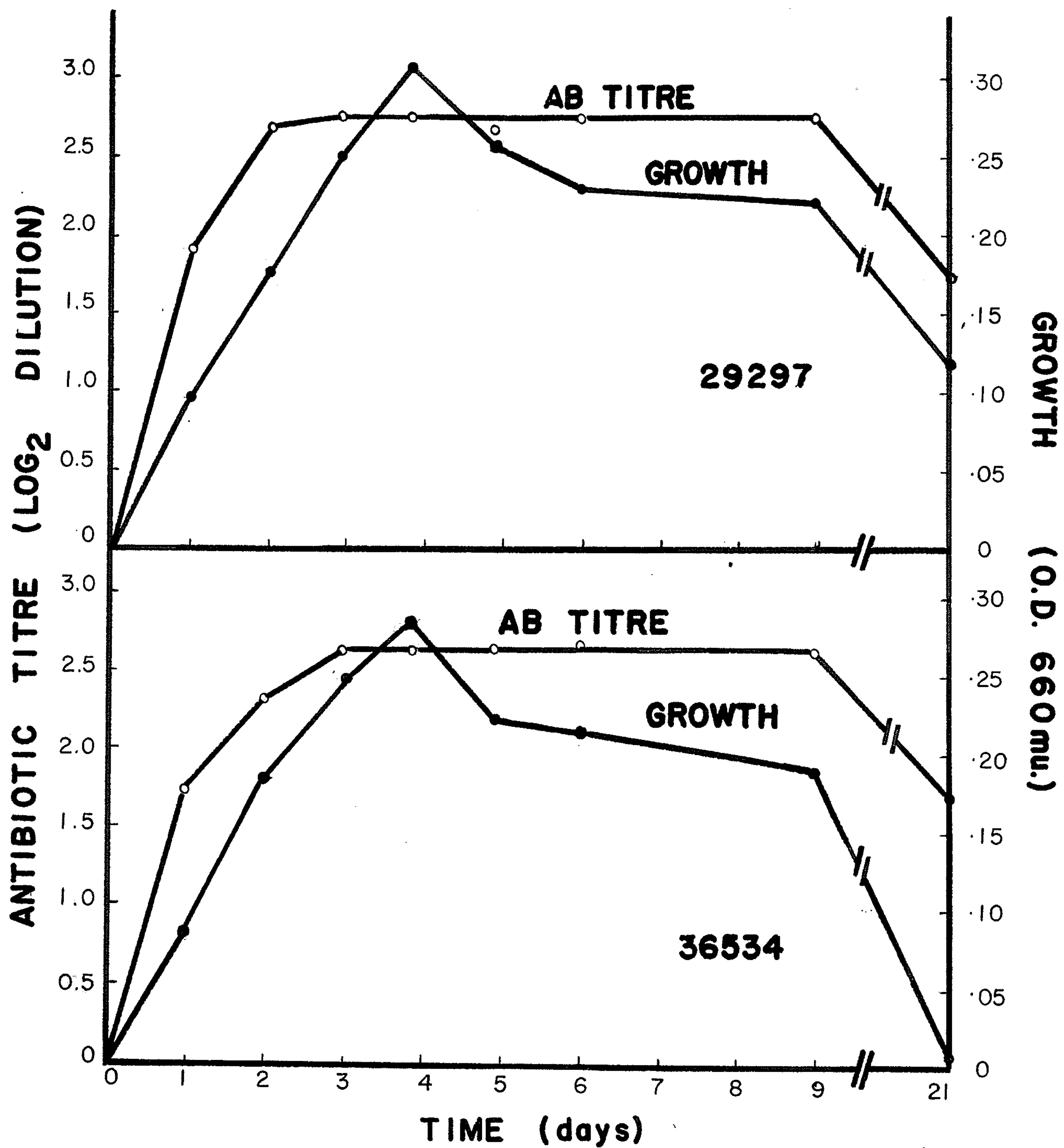

Fig. 2 INFLUENCE OF TIME ON PRODUCTION OF STAPHYLOCOCCAL ANTIBIOTICS

TABLE VII

EFFECT OF pH OF MEDIUM ON THE PRODUCTION OF STAPHYLOCOCCAL ANTIBIOTICS

pH	Antibiotic titre ($\log_2$ dilution)	
	29297(-)	36534(-)
5.0	2.25	0
5.5	2.05	2.05
6.0	2.45	2.00
6.5	2.05	2.05
7.0	2.75	2.00
7.5	2.05	2.05
8.0	1.20	0

(4) Inhibitory spectra of selected active antibiotic-producing strains

In order to test the effects of staphylococcal antibiotics on staphylococcal strains as well as other microorganisms, 25 coagulase-positive and 14 coagulase-negative staphylococci from various sources, and 16 Gram-positive and 16 Gram-negative species of bacteria other than *Staphylococcus* were selected.

(a) Effect on staphylococcal strains. It was found that most strains of *S. aureus* and all strains of *S. epidermidis* were sensitive to antibiotics produced by coagulase-negative strains 29297 and 36534 (Table VIII). Only a coagulase-positive strain belonging to phage-type 75 showed resistance to antibiotics from the four producing strains. In other cases, sensitivity to each antibiotic varied from strain to strain. Very few strains of *S. aureus* and *S. epidermidis* were sensitive to antibiotics from coagulase-positive strains 35548 and 36945, but it was noted that where sensitivity occurred, it was related to a laboratory strain rather than to one freshly-isolated from clinical material.

(b) Effect on species of bacteria other than *Staphylococcus*. It was observed that coagulase-negative strains 29297 and 36534 could inhibit the growth of most Gram-positive bacteria examined, but coagulase-positive strains 35548 and 36945 could only inhibit a few of them (Table IX). Among the Gram-positive strains employed, a non-hemolytic strain of *Str. faecalis* showed complete resistance to all four active antibiotic-producing strains. The sensitivity of the

TABLE VIII

THE EFFECT OF STAPHYLOCOCCAL ANTIBIOTICS ON STAPHYLOCOCCAL STRAINS

Indicator strain	Producing strains of staphylococci#				Source
	29297(-)	36534(-)	35548(+)	36945(+)	
S. aureus Oxford 209P	6.0	5.0	0.5	1.5	Lab strain
P-92	2.0	2.0	T*	1.5	Lab strain
G-143H	4.5	5.0	0.5	0	Lab strain
R-1	6.0	4.0	0.5	0	Lab strain
N-7428	4.5	3.5	T	2.5	Lab strain
N-9715	2.0	0	0	0.5	Lab strain
E-delta	0	2.0	0.5	T	Lab strain
Ø-55	2.0	1.5	0.5	0.5	Lab strain
Ø-75	0	0	0	0	Lab strain
Ø-77	2.5	2.5	0.5	0	Lab strain
Ø-79	3.0	4.0	0.5	1.5	Lab strain
Ø-80	3.0	3.5	T	2.0	Lab strain
Ø-81	2.0	3.0	0.5	T	Lab strain
Ø-82	3.0	3.5	T	1.5	Lab strain
38338	3.0	2.0	0	0	Urine
38249	3.0	2.0	0	0	Tracheal secretion
38390	3.0	3.0	0	0	Abscess on skin
38131	3.0	3.0	0	0	Sputum
38291	1.5	2.0	0	0	Nose
38413	3.0	2.0	0	0	Wound
2430	3.0	2.0	0	0	Eye
97321	1.5	1.0	0	0	Ear
97272	3.0	1.0	0	0	Throat
97710	3.0	2.0	0	0	Boil on skin
97781	3.0	2.0	0	0	Groin
S. epidermidis	2.0	1.0	0	0	Lab strain
38097	9.0	3.0	0	0	Cervix uteri
38394	15.0	3.0	0	0	Umbilicus
38162	6.0	4.0	0	0	Vagina
97190	6.0	6.0	0	0	Ear

TABLE VIII (Continued)

Indicator strain	Producing strains of staphylococci#				Source
	29297(-)	36534(-)	35548(+)	36945(+)	
38718	7.5	1.0	0	0	Throat
10	15.0	4.0	0	0	Scrotum
1178	12.0	4.0	0	0	Eye
100	3.0	2.0	0	0	Urine
1626	3.0	2.0	0	0	Wound
2117	9.0	4.0	0	0	Nose
1796	3.0	2.0	0	0	Bronchial secretion
750	3.5	2.0	T	2.0	Sputum
942	3.5	3.0	T	0	Vagina

Zone of inhibition in mm. employing stab-method. Average of 2 readings taken.

* Trace of inhibition.

TABLE IX

INHIBITORY SPECTRUM OF STAPHYLOCOCCAL ANTIBIOTICS

Indicator strain	Zone of inhibition (mm.)#			
	Producing strain of Staphylococcus			
	29297(-)	36534(-)	35548(+)	36945(+)
Gram-positive				
Bacillus cereus	1.0	1.0	0	2.0
B. megaterium	2.0	1.0	0	3.0
B. subtilus	6.5	4.5	0	0
Clostridium sporogenes	6.0	3.5	0	0
Cl. welchii	6.0	5.5	0	6.0
Corynebacterium diphtheriae				
gravis	9.0	5.0	0	0
intermedius	9.0	7.5	0	0
C. xerosis	9.5	8.0	T*	T
Diplococcus pneumoniae	7.0	5.0	2.0	0
Micrococcus luteus	0.5	0.5	3.0	3.0
Mycobacterium phlei	7.5	5.5	0	0
Neisseria gonorrhoeae	1.5	0	0	0
Sarcina lutea	6.0	7.0	1.0	13.5
Streptococcus faecalis				
hemolytic strain	2.5	3.5	0	0
non-hemolytic strain	0	0	0	0
Str. pyogenes	6.5	7.5	0	0
Gram-negative				
Achromobacter sp.	0	0	0	0
Aerobacter sp.	0	0	0	0
Alcaligenes sp.	0	0	0	0
Brucella abortus	2.0	3.0	0.5	1.0
Citrobacter sp.	0	0	0	0
Escherichia coli	0	0	0	0
Klebsiella pneumoniae	0	0	0	0
Proteus mirabilis	0	0	0	0
Pr. morganii	0	0	0	0

TABLE IX (Continued)

Indicator strain	Zone of inhibition (mm.)#			
	Producing strain of Staphylococcus			
	29297(-)	36534(-)	35548(+)	36945(+)
Pr. vulgaris	0.5	0.5	0.5	0.5
Pseudomonas aeruginosa	0	0	0	0
Salmonella paratyphi	0	0	0	0
S. typhimurium	0	0	0	0
S. typhosa	0	0	0	0
Serratia marcescens	0	0	0	0
Shigella flexneri	0	0	0	0

Mean of two taken.

* Trace of inhibition.

Gram-positive strains to these antibiotics varied widely from barely visible zones to zones as wide as 13.5 mm. in the case of Sarcina lutea. Gram-positive species were on the whole more sensitive to antibiotics produced by the two coagulase-negative strains 29297 and 36534.

Most Gram-negative species were completely resistant to four active antibiotic-producing strains tested. However, Brucella abortus and Proteus vulgaris showed slight sensitivity to the four. The size of the inhibition zones varied from 0.5 to 3.0 mm. with these two species.

It was found that both antibiotics 29297 and 36534 displayed inhibitory activity against some strains of mycobacteria (Table X). Most mycobacterial strains tested were more sensitive to antibiotic 36534 than to antibiotic 29297.

(c) Cross-inhibition test. Antibiotic-producing strains were also tested against themselves. Results shown in Table XI indicate that the antibiotic produced by a given active strain had little or no effect on the growth of the same strain. Even in the cases where inhibition occurred, the strain was more resistant to its own antibiotic than to the antibiotic produced by other staphylococci.

In addition to the cross-inhibition test involving the four active producers as outlined above, 35 other coagulase-positive and negative strains, which had been shown to produce no antibiotic active against Oxford 209P strain, were tested against themselves. Results were entirely negative.

TABLE X

EFFECT OF STAPHYLOCOCCAL ANTIBIOTICS ON STRAINS OF MYCOBACTERIUM

Strain	Antibiotic titre*	
	29297	36534
M. avium Sheard	< 2	< 2
M. bovis BCG	2	> 4
M. bovis 4228-2	2	2
M. kansasii Davis	< 2	> 4
M. phlei	2	2
M. tuberculosis H37Rv	2	2
" 12281	> 4	> 4
" 42815	< 2	> 4
" 48418	< 2	2
" 52243	> 4	> 4
" 55975	< 2	< 2
" 58917	< 2	> 4

*Reciprocal of highest dilution of antibiotic in Tween-80 broth inhibiting growth.

TABLE XI

CROSS INHIBITION TEST OF FOUR ACTIVE ANTIBIOTIC-PRODUCING STRAINS

Indicator strain	Inhibition zone (mm.)#			
	Active strains			
	29297(-)	36534(-)	35548(+)	36945(+)
29297 (-)	T*	1.0	0	1.5
36534 (-)	0	1.0	T	2.0
35548 (+)	3.0	2.5	0	0
36945 (+)	4.5	3.5	0	0

#Mean of two taken.

*Trace of inhibition.

(5) Properties of crude antibiotic preparations

(a) Stability of the preparations. Antibiotic 29297 was fairly stable in solution at 4°C., but this was not so in the case of antibiotic 36534. After storage at 4°C. for 60 days, the titre of antibiotic 29297 did not change significantly (Table XII). However, under the same conditions, antibiotic 36534 retained 30 per cent its original activity.

(b) Heat sensitivity of crude antibiotic preparations. Ten ml. of a crude preparation was placed in a 125 ml. Erlenmeyer flask plugged with cotton and heated in a temperature controlled water-bath for various lengths of time. After incubation, the fluid in the flask was cooled under running tap-water. The antibiotic titre of this treated fluid was then estimated. The results (Table XIII) showed that antibiotics 29297 and 36534 were relatively resistant to heat. The titre of antibiotic 29297 remained unchanged after heated at 60°C. for 2 hrs. After autoclaving (10 lb., for 15 min.), there was only a 20 per cent loss of activity of the 29297 material. In the case of antibiotic 36534, the loss was 9 per cent of the original after being heated at 60°C. for 2 hrs., and about 20 per cent after autoclaving.

(c) Dialyzability of antibiotic preparations. Ten ml. of freshly prepared antibiotic in phosphate buffered saline (0.01 M., pH 7.2) was pipetted into dialysis tubing, 1.6 cm. in diameter. The tubing was then placed in a beaker containing 500 ml. of the buffered saline. After incubation at 4°C., the anti-

TABLE XII

STABILITY OF CRUDE ANTIBIOTIC PREPARATIONS KEPT AT 4°C.

Time of storage (days)	Antibiotic titre (log_2dilution)	
	29297	36534
0	2.60	2.43
60	2.50	0.85

TABLE XIII

HEAT SENSITIVITY OF ANTIBIOTICS 29297 AND 36534

Treatment	Antibiotic titre (log_2 dilution)	
	29297	36534
Unheated	2.45	2.25
60°C., 15 min.	2.45	2.10
60°C., 2 hrs.	2.45	2.05
80°C., 15 min.	-	1.80
Autoclaved (10 lb., 15 min.)	2.05	1.80

biotic titre of the fluid inside the tubing was estimated. Results in Table XIV showed that the activity of both antibiotics 29297 and 36534 was lost after 48 hrs. The rate of activity loss of antibiotic 29297 was greater than that of antibiotic 36534. After 24 hrs., the titre of antibiotic 29297 had fallen to zero, and in the case of antibiotic 36534, 47 per cent of the activity remained.

(d) Hemolytic activity of antibiotic preparations. The hemolytic activity of the preparations was assayed by the method of Wiseman (1965). Antibiotics 29297 and 36534 were able to lyse human and rabbit red blood-cells, but not those of sheep at a 1:2 dilution. Higher dilutions of antibiotics had no hemolytic activity but inhibitory activity still remained. All lysis occurred only after incubation at 4°C. overnight.

(e) Action of proteolytic enzymes on antibiotic preparations. Antibiotics from strains 29297 and 36534 were produced on BHI agar plates layered with cellophane and harvested with 0.01 M. borate buffer, pH 8.0. To 5.8 ml. of the filtered solution was added 0.2 ml. of either trypsin (Nutritional Biochemical Co.) or chymotrypsin (Worthington Biochemical Co.) in the same buffer such that the final concentration of enzymes was 1 mg./ml. After incubation at 37°C. in a water-bath for various lengths of time, enzyme activity was stopped by the addition of calf-serum (4.5 ml. of antibiotic-enzyme mixture to 0.5 ml. of calf-serum).

It was found (Table XV) that antibiotic 36534 was relatively resistant to

TABLE XIV

DIALYZABILITY OF STAPHYLOCOCCAL ANTIBIOTICS 29297 AND 36534

Time of dialysis (hr.)	Antibiotic titre ($\log_2$ dilution)	
	29297	36534
0	1.75	2.00
24	0	0.95
48	0	0

TABLE XV

ACTION OF TRYPSIN AND CHYMOTRYPSIN ON ANTIBIOTICS 29297 AND 36534

Treatment	Incubation time (hr.)	Antibiotic titre (log_2 dilution)	
		29297	36534
Antibiotic alone	0	1.75	1.25
	2	1.75	1.25
	4	1.70	1.25
	6	1.75	1.25
Antibiotic with calf-serum	2	1.75	1.20
	4	1.75	1.25
	6	1.75	1.25
Antibiotic with trypsin	2	1.25	1.25
	4	1.25	1.25
	6	1.10	1.20
Antibiotic with chymotrypsin	2	1.65	1.10
	4	1.25	1.00
	6	1.10	1.00

the action of trypsin and chymotrypsin, there being less than a 5 per cent decrease between initial and final titres after 6 hrs. in both cases. With antibiotic 29297, loss of activity over the incubation period in presence of trypsin and chymotrypsin was about 37 per cent.

Controls employed showed that temperature of incubation alone had no influence on the titres. The two crystalline enzymes, trypsin and chymotrypsin, were active in that they removed Human Amnion cells growing on glass.

(f) Nature of the antibacterial activity of antibiotic preparations. The antibacterial activity of antibiotic preparations was examined by mixing equal volumes of antibiotic solution and a standardized suspension of the indicator strain, S. aureus Oxford 209P. At time intervals of 1, 3, 6 and 12 hrs., viable cell counts were made. It was found (Fig. 3) that in BHI broth at 20°C., the number of viable cells increased more rapidly in the absence of antibiotic 29297, than in its presence. After 12 hrs. incubation, the indicator strain multiplied more than 15-fold in the tube without antibiotic and only 3-fold in the tube with antibiotic. Antibiotic 29297 seemed to affect the multiplication rate of the bacteria, rather than their viability.

(6) Capability of antibiotic transfer

Ten clear inhibition zones produced by each of these four selected active strains were carefully cut out of the BHI agar plates and mashed with 5 ml. of phosphate buffered saline (0.01 M., pH 7.2). The fluid was filtered through an

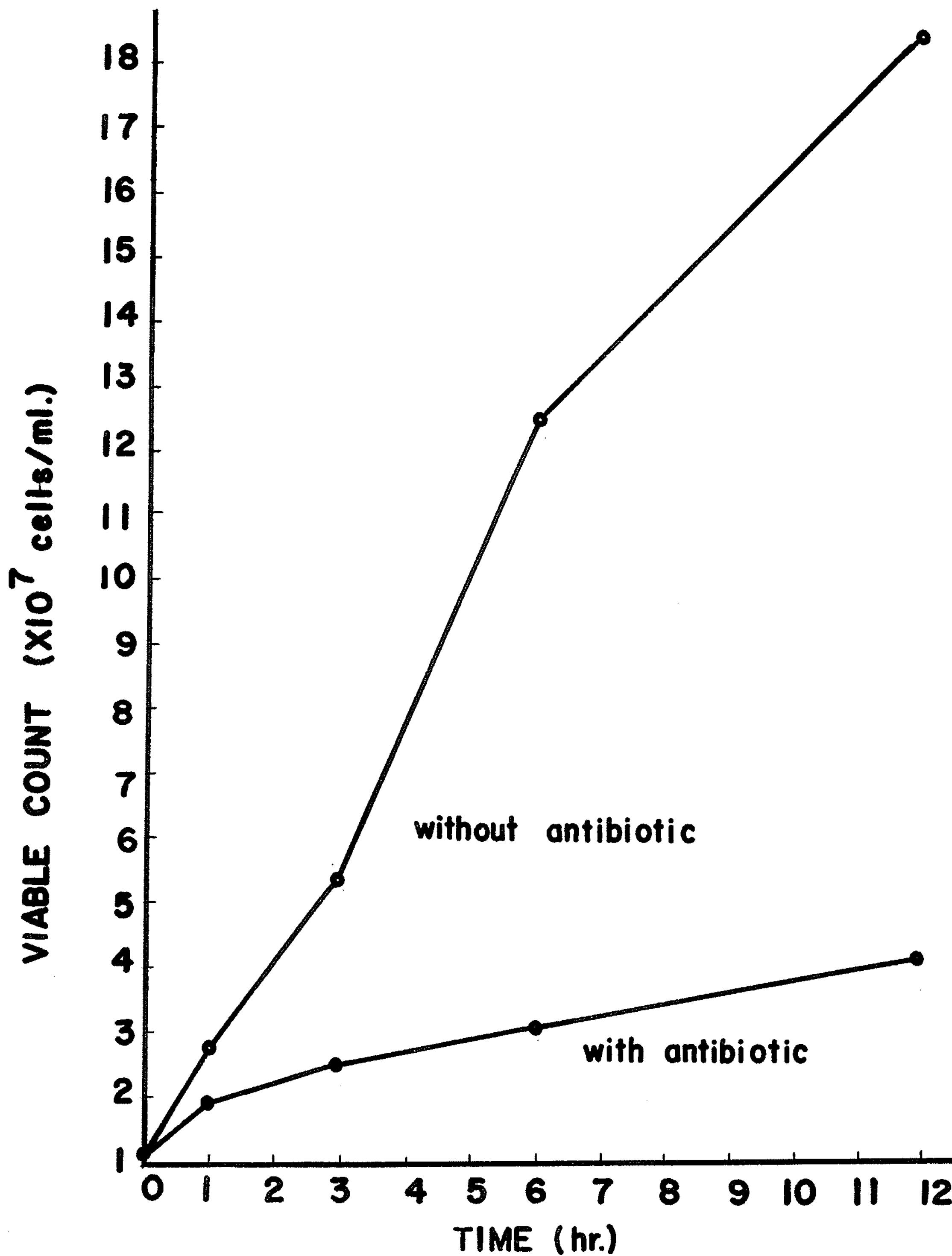

Fig. 3 INFLUENCE OF ANTIBIOTIC 29297 ON VIABLE COUNTS OF *S. aureus* AT 20°C.

HA millipore filter. A drop of this filtrate was placed on a well-dried BHI agar plate. After the fluid had been completely absorbed, a standardized suspension of the indicator strain was sprayed on by employing a glass atomizer. The plate was then incubated for 24 hrs. at 37°C. It was found that none of these four antibiotic substances could produce plaques nor could the action of these substances be transferred.

DISCUSSION

The production of antibacterial substances is an uncommon phenomenon in the genus *Staphylococcus*. The frequency of active antibiotic-producing strains isolated from clinical materials is low. Only 85 of 1,452 strains tested (about 5.9 per cent) showed inhibitory activity against the growth of the indicator strain, *Staphylococcus aureus* Oxford 209P. Among these 85 active strains, only five produced relatively large amounts of antibiotics. Similar results were obtained by Lachowicz (1965). He stated that he was only able to isolate 131 antagonistic strains from 1,239 strains of staphylococci tested against the Oxford 209P strain. Jennings and Sharp (1947) also reported that about 10 per cent of 205 strains of staphylococci inhibited the growth of corynebacteria.

There appeared to be a correlation between source of isolation and incidence of antibiotic-producing strains. Of 55 strains of coagulase-positive staphylococci isolated from the ear, 10.9 per cent were active. Of 17 coagulase-negative strains from the same source, 23.5 per cent were active. These percentages are much higher than those from other sources. It is not certain whether these figures indicate a true correlation between the ear as a source of isolation and antibiotic production, since relatively small numbers of strains from the ear were tested when compared to numbers from other areas of the body. Of a total of 529 strains isolated from various skin lesions, 4.5 per cent were active antibiotic-producing strains when the Oxford 209P

strain of S. aureus was used as indicator. When corynebacteria were employed as indicators, Parker and his colleagues (Parker et al, 1955; Barrow, 1955; Parker and Simmons, 1959) isolated a much larger proportion of active strains of staphylococci from skin lesions.

Antibiotic production by the coagulase-positive staphylococci employed in this investigation could not be related to their resistance to antibiotics in clinical use. For example, there were roughly as many antibiotic producers resistant to penicillin as there were sensitive. This finding supports that of Lachowicz (1959) but is in contrast with the work of Barrow (1963) who observed all his antagonistic strains of Staphylococcus to be resistant to penicillin. Furthermore, the present investigation has found that the physiological characteristics of the four most active antibiotic-producing staphylococci are indistinguishable from those not producing antibiotics. This confirms the report of Dujardin-Beaumetz (1932) and others.

Antibiotic production was best on solid media. Liquid media did not support production even when agitated, which suggests that oxygen tension may not have been the most important factor. It is possible that agar added to the medium might remove toxic impurities present, thus allowing formation of antibiotic.

Incubation time was also shown to be an important factor. It has been observed in this investigation that antibiotic titres of strains 29297 and 36534 on Brain-Heart Infusion agar (BBL) reached a maximum after two to three days

incubation. After 3 weeks, 70 per cent of the activity still remained. These results are not in agreement with those of Lachowicz (1963a), who found that maximum production by his strain was achieved after 24 hrs. Further incubation for eight days reduced the activity to a negligible level. Lachowicz was, of course, using a different medium and strain. Furthermore, he found (Lachowicz and Lataczowa, 1951) that carbohydrates such as fructose, glucose, sucrose, glycerol and mannitol would, when added to media, enhance antibiotic production. This observation could not be confirmed in the present study. In fact, glucose and sucrose over a range of 1 to 10 per cent progressively decreased antibiotic formation.

A wide variety of Gram-positive species of bacteria, including 24 coagulase-positive and 14 coagulase-negative strains of *Staphylococcus*, were sensitive to antibiotics 29297 and 36534. Only one coagulase-positive strain, phage-type 75, and a non-hemolytic *Str. fecalis*, were resistant. In addition, more than half of 12 strains of *Mycobacterium*, including seven human strains, were sensitive to the antibiotics. Myers (1946) claimed that the so-called "slow-growing" rather than "fast-growing" mycobacteria were inhibited by his staphylococcal antibiotics. The present investigation revealed no sensitivity differences between these two groups of organisms, in contrast with Myers' results. Resistance to antibiotics 35548 and 36945 amongst the Gram-positive species tested was more widespread. Generally, Gram-negative bacteria were insensitive to the four staphylococcal antibiotics.

The wide variation in sensitivity between Gram-positive and negative bacteria which has been observed raises questions as to the nature and mode of action of these staphylococcal antibiotics. The most obvious differences between the two groups of bacteria lie in the composition of their cell-walls. Gram-positive, but apparently not Gram-negative, cell-walls contain polyols such as ribitol and glycerol phosphoric acid esters and it could be that the staphylococcal antibiotics exert their effect by blocking the synthesis of these polyols or some other important constituent.

Both antibiotics 29297 and 36534 were relatively resistant to the action of trypsin and chymotrypsin, and both could withstand autoclaving. Also, their activity was lost after a period of dialysis. On the basis of such evidence, these antibiotics may not be proteins and therefore are not enzymes. Rather, they might be small molecular weight compounds such as peptides. This evidence, however, must be interpreted with caution, since not all proteins are hydrolyzed rapidly by proteolytic enzymes, and loss of activity after dialysis might be explained by diffusion of an essential metallic ion without which the antibiotic is inactive.

That these antibiotics are not bacteriophage seems fairly certain on the basis of the following points: (1) they possess a broad inhibitory spectrum amongst Gram-positive bacteria, (2) inhibition by these antibiotics of the strains which produced them is negligible, (3) they are resistant to autoclaving and (4) their inhibitory activity is not transferrable from plate to plate.

The relationship of antibiotics 29297 and 36534 to staphylococcal antibiotics studied by other investigators is obscure. It is unlikely that they are identical with the "lysostaphin" of Schindler and Schuhardt (1964) in that they do not lyse staphylococci and their inhibitory spectrum is not restricted to the genera Staphylococcus and Micrococcus. Nor do these antibiotics resemble bacteriocins, since bacteriocins are defined as protein antibiotics acting only on closely related strains of a given species. The two antibiotics also do not resemble the "staphylococcins" of Lachowicz (1965) in that his preparations were thermolabile, non-dialyzable, and sensitive to trypsin and chymotrypsin. He also claimed that the staphylococcins were bactericidal. Antibiotic 29297, the only one tested, appeared to be bacteriostatic rather than lethal at the concentrations employed, but it has to be admitted that the results of Fig. 3 might not reflect bacteriostasis at all. Rather, a portion of the population tested might have been killed and the rest could be resistant.

It has been assumed throughout this investigation that the inhibitory activities of the four antibiotics are due to a single entity in each case. This has, of course, not been proven. After all, these substances may be a mixture of inhibitory principles, bacteriocins, broad spectrum antibiotics and others.

In order to elucidate the nature of the antibiotics 29297 and 36534, or "spectrins" as we like to call them, it will first be necessary to undertake their purification.

SUMMARY

(1) It was found that 4.9 per cent of 1,065 coagulase-positive and 8.5 per cent of 387 coagulase-negative strains of staphylococci obtained mostly from clinical sources produced antibacterial substances inhibiting the growth of Staphylococcus aureus Oxford 209P. The size of the inhibition zone varied from a trace to 6.0 mm. A larger percentage of organisms isolated from the ear produced antibiotics than those from other areas of the body, but this difference may have been more apparent than real. Antibiotic production could not be correlated with resistance to a number of common antibiotics in clinical use. Also, antibiotic producing strains of staphylococci were indistinguishable physiologically from those not producing antibiotics.

(2) Antibiotic production by four selected strains depended upon the nature of the medium and conditions of incubation employed. Production was best on solid media overlain with cellophane and incubated aerobically. Liquid media did not support antibiotic production even when agitated. Addition of glucose, galactose, sucrose, mannitol and glycerol did not enhance production. In fact, addition of glucose and sucrose in increasing amounts progressively decreased the yield of antibiotics of 29297 and 36534. The production of antibiotics on Brain-Heart Infusion Agar was maximal after 2 to 3 days, depending on the strain employed.

(3) Antibiotics from strains 29297 and 36534 were dialysable, fairly resistant to trypsin and chymotrypsin, and could be autoclaved with slight loss of

activity. Storage for 60 days at $4^{o}C.$ reduced the activity by a factor of 70 per cent.

(4) At the concentrations employed, antibiotic 29297 was unable to kill all cells of the population tested.

(5) The antibiotics generally inhibited Gram-positive bacteria but were, with a few exceptions, unable to inhibit the growth of Gram-negative species. Of 25 coagulase-positive staphylococci tested, all but one were sensitive, and of 12 strains of mycobacteria tested, more than 80 per cent were sensitive.

BIBLIOGRAPHY

Arena, A. R. (1944). Acción inhibidora du un estafilococo sobre el cultivo del bacilo de Koch. Rev. Asoc. méd. argent. 58, 1026-1027.

Barrow, G. J. (1955). Clinical and bacteriological aspects of impetigo contaginosa. J. Hyg. (London) 53, 495-508.

Barrow, G. J. (1962). Microbial antagonism by Staphylococcus aureus. J. gen. Microbiol. 31, 471-481.

Barrow, G. J. (1963). The nature of inhibitory activity by Staphylococcus aureus type 71. J. gen. Microbiol. 32, 255-261.

Beco, L. (1895). Beitrag zum experimentellen Studium der Association des Bacillus anthracis mit dem Staphylococcus pyogenes aureus. Zbl. allg. path. Anat. 7, 641-649.

Bogendörfer, L. (1924). Hemmungsstoffe aus Bakterien und ihren Kultursubstraten Z. ges. exp. Med. 41, 620-636.

Breed, R. S., Murray, E. G. D. and Smith, R. N. (eds.) (1957). Bergey's Manual of Determinative Bacteriology, 7th edition. The Williams and Wilkins Co., Baltimore, U. S. A.

Carvajal, F. (1946). Biologic strains of Streptomyces griceus. Mycologia 38, 596-609.

Cornil, A. V. and Babès, V. (1885). Concurrence vitale des bactéries atten-uation de leurs properties dans des milieux nutritifs modefies par d'autres bacteries; tentatives de therapeutique bactériologique. J. Conn. méd. prat. (Paris) 7, 321-323.

Cruickshank, R. (ed.) (1962). Mackie and McCartney's Handbook of Bacteriology. E. & S. Livingstone Ltd. London, England.

Doehle, P. (1889). Beobachtungen ueber einen Antagonisten des milzbrandes Habilitationsschr., Kiel. Quoted by Florey et al., 1949.

Dolman, C. E. and Wilson, R. J. (1940). The kitten test for Staphylococcus enterotoxin. Canad. J. Pub. Health 31, 68-71.

Dujardin-Beaumetz, E. (1932). Action antibiotique d'une variété de staphylocoque à l'égard des bacilles Gram-positifs et acido-résistants. C. R. Soc. Biol. (Paris) 110, 1210-1213.

Dujardin-Beaumetz, E. (1934). Action antibiotique exercée in vitro par certains Streptocoques et en particulier par une Micrococque saprophyte. C. R. Soc. Biol. (Paris) 117, 1178-1180.

Duliscouët, R. (1935). Action probiotique et antibiotique des staphylocoques chez les porteurs des germs diphthéritiques. C. R. Soc. Biol. (Paris) 118, 1277-1280.

Duliscouët, R. (1939). Rechèrches expérimentales sur une substance bactériolytique dérivée des germs antagonistes. Ses application dans la thérapeutique de la diphtérie. Arch. Méd. Pharm. nav. 129, 410-443.

Elek, S.D. and Levy, E. (1950). Diffusible antigens in staphylococcal cultures. Brit. J. exp. Path. 31, 358-368.

Florey, H.W., Chain, E., Heatley, N.G., Jennings, M.A., Sanders, A.G., Abraham, E.P. and Florey, M.E. (1949). Antibiotics. A Survey of Penicillin, Streptomycin, and Other Antimicrobial Substances from Fungi, Actinomyces, Bacteria and Plants. Vol. I. Oxford University Press, London, England.

Frank, G. (1899). Ueber Mischinfection beim Milzbrand. Münch. med. Wschr. 46, 282-285.

Frédéricq, P. (1946). Sur la sensibilité et l'activité antibiotique des staphylocoques. C.R. Soc. Biol. (Paris) 140, 1167-1170.

Frédéricq, P. and Betz, M. (1946). Rapports entre les propriétés biochimiques des staphylocoques et leur sensibilité à divers antibiotiques. C. R. Soc. Biol. (Paris) 140, 1191-1192.

Freudenreich, E. de (1888). De l'antagonisme des bactéries et de l'immunité qu'il confère aux milieux de culture. Ann. Inst. Pasteur 2, 200-206.

Gardner, J.F. (1949). An antibiotic produced by Staphylococcus aureus. Brit. J. exp. Path. 30, 130-138.

Gladstone, G.P. (1938). The production of staphylococcal α-hemolysin in chemically-defined medium. Brit. J. exp. Path. 19, 208-226.

Gratia, A. and Rhodes, B. (1924). De l'action lytique des staphylocoques vivants sur les staphylocoques tues. C. R. Soc. Biol. (Paris) 90, 640-642.

Gundel, M. and Wagner, W. (1930). Weitere Studien ueber Bakterienlipoide. Ztschr. f. Immunitatsforsch u exper. Therap. 69, 63-76.

Halbert, S. P., Sonn, C. and Swick, L. (1954). Mixed bacterial infections in relation to antibiotic activities. I. Clostridium septicum-Micrococcus infections. J. Immunol. 73, 169-179.

Halbert, S. P., Swick, L. S. and Sonn, C. (1953). Characteristics of antibiotic-producing strains of the ocular bacterial flora. J. Immunol. 70, 400-410.

Hayes, L. (1950). Mixed bacterial growth. II. The growth of Staphylococcus aureus, Streptococcus pyogenes and a viridans streptococcus in mixed broth cultures. Aust. J. exp. Biol. Med. Sci. 28, 487-492.

Jackson, A. W. and Little, R. M. (1957). Staphylococcal toxins. I. Factors affecting the hemolytic activity of alpha-toxin. Canad. J. Microbiol. 3, 47-54.

Jennings, M. A. and Sharp, A. E. (1947). Antibacterial activity of the Staphylococcus. Nature, 159, 133-134.

Korolev, P. A. (1939). Symbiose und Antagonismus bei Bakterien der Gruppe Brucella mit gelber Sarcine und weissen Staphylokokken. (in Russian) Z. Mikrobiol. 22, 35-39.

Lachowicz, T. (1959). Z. badan nad antagonistycznymi szczepami gronkowcowymi. Med. Doiv. Mikrobiol. 11, 233-236.

Lachowicz, T. (1963a). Stafilokokcyna---antybiotyk pochodzenia gronkowcowego dzialajacy na gronkowce i inne Gram-dodatnie bakterie. Przeglad Lekarski 19, 1-4.

Lachowicz, T. (1963b). Badania nad izoantagonizmen wsrod gronkowcow. I. Izolacja czynnika antybiotycznego z podloza stalego. Med. Dosw. i Mikrobiol. 14, 17-25.

Lachowicz, T. (1965). Investigations on staphylococcins. Zbl. f. Bakt. I. Orig. 196, 340-351.

Lachowicz, T. and Lataczowa, W. (1951). Wpływ cukrowcow i alkoholi na antagonizm bakteryjny gronkowcow. Med. Dosw. i Mikrobiol. 2, 3-11.

Lachowicz, T. and Romanowski, T. (1962). Badania nad isoantagonizmen wsrow gronkowcow. II. Charakterystyka bakteriologiczna i wrazliwosc na stafilokokcyne A azczepow antagonistycznych. Med. Dosw. i Mikrobiol. 14, 305-309.

Loeb. L. J., Moyer, A. and Murray, R.G.E. (1950). An antibiotic produced by Micrococcus epidermidis. Canad. J. Res. (E) 28, 212-216.

Magrassi, F. and Spiga, A. (1946). Studi su un principio antibacterico d'origine coccica: estrazione, purificazione caratterizzazione chimica. Boll. Sco. ital. Biol. sper. 22, 317-319.

Mattei, C.D; (1889). Lav. Ist. Igiene Sper. Univ. Roma. Quoted by Florey et al., 1949.

Miles, A.A. and Misra, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. 38, 732-749.

Murray, R.G.E. and Loeb, L.J. (1950). Antibiotics produced by micrococci and streptococci that show selective inhibition within the genus Streptococcus. Canad. J. Res. (E). 28, 177-185.

Myers, G. (1946). The inhibition of the growth of Mycobacterium by Staphylococcus. Master Thesis of Univ. of Alberta.

Nutini, L.G., Kelly, T.A. and McDowell, M. (1946). Effect of Staphylococcus aureus extracts on various bacteria. J. Bacteriol. 51, 533-538.

Parker, M.T. (1958). Some cultural characteristics of Staphylococcus aureus strains from superficial skin infections. J. Hyg. Camb. 56, 238-253.

Parker, M.T. and Simmons, L.E. (1959). The inhibition of Corynebacterium diphtheriae and other Gram-positive organisms by Staphylococcus aureus. J. gen. Microbiol. 21, 457-476.

Parker, M.T., Tomlinson, A.J.H. and Williams, R.E.O. (1955). Impetigo contaginosa; the association of certain types of Staphylococcus aureus and of Streptococcus pyogenes in superficial skin infections. J. Hyg. Camb. 53, 458-473.

Pawlowsky, A.D. (1887). Heilung des Milzbrandes durch Bakterien und das Verhalten der Milzbrandbacillen im Organismus. Virchows Arch. path. Anat. 108, 494-521.

Petragnani, G. and Mazzetti, G. (1932). Colture secondarie nella coltura in brodo del bacillo di Koch e loro influenza sul bacillo di Koch stesso. Atti di IV Congr. maz. Microbiol., Milano, 264-268.

Ralston, D.J. and McIvor, M. (1964a). Cell-wall lysins of Staphylococcus aureus strains induced by typing phages. J. Bacteriol. 88, 667-675.

Ralston, D.J. and McIvor, M. (1964b). Lysis-from-without of Staphylococcus aureus strains by combinations of specific phages and phage-induced lytic enzymes. J. Bacteriol. 88, 676-681.

Ralston, D.J., Bear, B.S., Lieberman, M. and Krueger, A.P. (1955). Virolysin: A virus-induced lysin from staphylococcal phage lysates. Proc. Soc. exp. Biol. Med. 89, 502-507.

Ralston, D.J., Lieberman, M., Bear, B.S. and Krueger, A.P. (1957a). Staphylococcal virolysin: A phage induced lysin. Its differentiation from the autolysin of normal cells. J. gen. Physiol. 40, 791-807.

Ralston, D.J., Bear, B.S., Lieberman, M. and Krueger, A.P. (1957b). Lysis-from-without of Staphylococcus aureus K_1 by the combined action of phage and virolysin. J. gen. Physiol. 41, 343-358.

Régnier, J. and Lambin, S. (1934). Étude d'un cas d'antagonisme microbien. C. R. Acad. Sci. (Paris) 199, 1682-1684.

Richmond, M. (1959). Lytic enzymes of Staphylococcus aureus 524. Biochim. Biophys. Acta 31, 564-565.

Roncali, D.B. (1892). Ann. Igiene (sper.) 2, 87. Quoted by Florey et al., 1949.

Salmon, J. (1949). Activité bactériolytique directe des staphylocoques. C. R. Soc. Biol. (Paris) 143, 717-719.

Schindler, C.A. and Schuhardt, V.T. (1964). Lysostaphin: A new bacteriolytic agent for the Staphylococcus. Pro. nat. Acad. Sci. U.S.A. 51, 414-420.

Schindler, C.A. and Schuhardt, V.T. (1965). Purification and properties of lysostaphin. A lytic agent for Staphylococcus aureus. Biochim. Biophys. Acta 97, 242-250.

Schiøtz, A. (1909). Ugeskr. Laeg. 71, 1373. Quoted by Florey et al., 1949.

Spagnolio, G. (1912a). Sulla vitalita della Leishmania nei terreni di cultura associata ai comuni germi della cute. Malar. Mal. Paesi Caldi 3, 103.

Spagnolio, G. (1912b). Sulla vitalita della Leishmania Donovani in simbiosi con i germi delle malatteri infettive intestinali e con lo streptococco piogene. Malar. Mal. Paesi Caldi 3, 151.

Stovall, W.D., Scheid, E. and Nichols, M.S. (1923). The influence on the morphology and staining of B. diphtheriae by growth in mixed cultures with staphylococci and streptococci. Amer. J. Publ. Hlth. 13, 748-753.

Tomkins, H. (1887). Cultivation of Bacillus anthracis. Brit. med. J. 1, 328.

Urbain, A., Lanfranchi, F. and Goret, P. (1931). L'infection charbonneuse chez la rat blanc. C. R. Soc. Biol. (Paris) 107, 208-209.

Welsch, M. and Salmon, J. (1949). Bacteriolytic properties of actinomycetes and staphylococci. J. gen. Microbiol. 3, xxvii.

Welsch, M. and Salmon, J. (1950a). Action comparée de divers sels sur l'autophagie et l'autolyse de Staphylococcus aureus. C. R. Soc. Biol. (Paris) 144, 1292-1295.

Welsch, M. and Salmon, J. (1950b). Quelques aspects de la staphylolyse. Ann. Inst. Pasteur 79, 802-813.

Wiseman, G.M. (1965). Some characteristics of the beta-hemolysin of Staphylococcus aureus. J. Path. Bacteriol. 89, 187-207.

Wollman, E. (1934a). Bactériophagie et autolyse. C. R. Soc. Biol. (Paris) 115, 1616-1618.

Wollman, E. (1934b). Recherches sur l'autolyse. Les autolysins spécifiques. C. R. Acad. Sci. (Paris) 198, 1642-1644.