THE UNIVERSITY OF MANITOBA

THE NATURE OF ANTIBIOTIC SUBSTANCES FROM STAPHYLOCOCCI

by

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TO MY PARENTS AND WEI-JEN

ABSTRACT

THE NATURE OF ANTIBIOTIC SUBSTANCES FROM STAPHYLOCOCCI

Antibiotics from the 29297 and 36534 strains of Staphylococcus epidermidis were purified by acid and Ba(OH)$_2$, followed by ZnCl$_2$ which separated the active principles into two fractions. These were designated the zinc-precipitable and zinc-soluble fractions. The use of Dowex and/or Sephadex further increased the specific activity of these antibiotic components. All fractions of both antibiotics were crystallized from aqueous solutions. Recrystallization and methanol precipitation of the antibiotics brought the specific activity to a relatively constant value.

These purified antibiotic fractions contained no carbohydrates, proteins, lipids, nucleic acids or heavy metal ions. All fractions each contained 11 amino acid residues, eight of which they shared in common. Molecular weights were about 1200. No C- or N-terminal amino acid residues were detected. The ratio of L- to D-amino acids for 29297 zinc-soluble and 36534 zinc-precipitable fractions was 7:4, while that for 29297 zinc-precipitable and 36534 zinc-soluble fractions was 5:6. Thus the antibiotics were low molecular weight cyclic peptides of which about half the amino acids were D-isomers.

The peptide sequence of the 29297 zinc-precipitable fraction was tentatively determined. It had the structure:

    cyclo-Lys-Ala-Asp-Glu-Ser-Leu-Thr-Gly-Val-Gly-Arg, or

    cyclo-Lys-Ala-Asp-Glu-Ser-Leu-Thr-Val-Gly-Gly-Arg.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

It has been known for many years that the staphylococci produce antibiotics. Most investigations, however, have been mainly concerned with the isolation of these growth-inhibiting strains and the characterization of their inhibitory spectra. Consequently, little was known of their chemical nature.

Hsu and Wiseman (1967) made a study of large numbers of coagulase-positive and negative staphylococci. They found that 4.9% of 1065 coagulase-positive and 8.5% of 387 coagulase-negative strains inhibited growth of the Oxford 209P indicator strain of <u>Staphylococcus</u>. Preliminary work completed on two active strains, 29297 and 36534, showed that the antibiotics were resistant to heat, acid and alkaline conditions, and that they were partially sensitive to trypsin and chymotrypsin. The spectrum of these substances was similar in that growth of Gram-positive but generally not Gram-negative bacteria was inhibited. The available evidence indicated that these substances were not bacteriocins.

This investigation has been continued with a view to the development of satisfactory procedures for the purification of these two antibiotics so that their chemical characterization could be effected.

REVIEW OF THE LITERATURE

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PEPTIDE ANTIBIOTICS FROM EUBACTERIALES

The production of antibiotics by microorganisms, which is closely
associated with survival of species in nature, is a common phenomenon in
the microbial world. The action of these agents is highly selective; each
is only active against certain groups of sensitive organisms. Since the
discovery of penicillin by Fleming (1929), many such antimicrobial agents
have been found and isolated, and some have been utilized for clinical
and industrial purposes.

Antibiotics produced by members of Eubacteriales can generally
be grouped into three categories according to their chemical nature. They
are: (1) protein or lipoprotein polysaccharide complexes, (2) simple
pigments, and (3) peptide antibiotics.

Jacob et al. (1953) named the protein or lipoprotein polysaccha-
ride complexes bacteriocins. In contrast to all other antibiotics, bacter-
iocins act only on strains of the same or closely related species by means
of specific receptors, and they are bactericidal. In some ways they closely
resemble bacteriophages, except that they can not multiply in the sensitive
cells as phages do.

Pigmented antibiotics are simple chemical substances produced by
chromogenic species. Their chemical nature is diversified and generally
they are highly toxic to higher animals.

Peptide antibiotics differ from normal peptides or proteins not only by their unique biological activity, but by the possession of several other characteristics. Abraham (1957) first stressed that most peptide antibiotics are cyclic and contain both D- and L- amino acid residues. Bodanszky and Perlman (1969) further pointed out the following specific features of peptide antibiotics: (1) Certain amino acids commonly found in proteins, for example, arginine, histidine, and methionine, occur rarely in peptide antibiotics. (2) Not only is the occurrence of D-amino acids quite general, but D- and L-isomers of the same amino acids occur in the same compound, often as next neighbors. (3) Cyclic peptide antibiotics include both heterodetic and homodetic structures. (4) Proline, N-methylated amino acids, and imino acids which probably preclude helix formation, and hence facilitate cyclization, often are found in peptide antibiotics. (5) The formation of small cyclic areas within the macrocyclic structure of the peptide molecule is a common feature. (6) Unusual amino acids and/or non-amino acid moieties are frequently present.

From the survey of known peptide antibiotics from Eubacteriales, one finds that the majority of them are produced by Gram-positive strains. One also discovers that different strains of the same species usually produce different kinds of peptide antibiotics and that strains of completely different species may produce a similar agent. Furthermore, a single strain usually produces a mixture of chemically closely related peptides. Each differs from the other only by one or two residues in the molecule.

Most bacterial peptide antibiotics are stable in heat, resistant to acid hydrolysis, and relatively insensitive to the action of proteolytic

enzymes. Their solubilities are highly dependent on the content of hydro-
philic and hydrophobic groups in the molecule. Their cyclic nature is
shown by failure to detect α-amino and α-carboxyl groups. Unlike the
peptide antibiotics isolated from other sources, the non-amino acid moieties
so far isolated from peptide antibiotics from Eubacteriales are saturated
or unsaturated aliphatic acids and amines. The molecular weight of these
peptides varies widely in different cases, from less than 300 for bacilysin
to 7000 for nisin.

Some of these peptides, such as the bacitracins, gramicidins,
and polymyxins have been used for chemotherapeutic purposes; others, such
as nisin and subtilin, have been used in the preservation of food. However
most of them have had no practical value because of their high toxicity to
animals.

Despite the large numbers of peptide antibiotics that have been
shown to be produced by strains of Eubacteriales, only a small proportion
of them has been investigated systematically. In this review, only those
whose chemical structures are known shall be discussed. Emphasis has
been placed on the properties, isolation and structure of the antibiotic.

I. BACILYSIN.

(1). Producing strains and properties of bacilysin:

Abraham et al. (1946) isolated a strain of Bacillus subtilis
(NCTC 7197) from the soil at Oxford which produced a water-soluble anti-
biotic. Florey et al. (1949) named this antibiotic bacilysin. Newton (1949)

reported another strain, B. subtilis A-14 obtained from a contaminated culture of Mycobacterium tuberculosis from Chile which produced bacilysin and several other antibiotics. Gilliver (1949) discovered several other bacilysin-producing strains in the National Collection of Type Cultures and in the collection of Dr. T. Gibson of Edinburgh. They were B. subtilis and B. pumilus.

Newton (1949) found a bacilysin which contained nitrogen and sulphur and showed that it was insoluble in all dry organic solvents except methanol. An aqueous solution of bacilysin did not form a precipitate with picric, phosphotungstic, or trichloroacetic acids or lead acetate. It gave a positive ninhydrin test and Pauly diazo reaction (Pauly, 1904), and the Molisch test was negative. The bacilysin was active against staphylococci and corynebacteria, but B. anthracis and Streptococcus pyogenes were not affected. Florey et al (1949) showed that bacilysin was easily extracted by phenol from broth. The substance gave a negative biuret test. It was stable in aqueous solution at $100^{\circ}C$ for five minutes at pH 7, was largely inactivated under these conditions at pH 2 or pH 9, and was inactivated by trypsin but not by pepsin. Bacilysin caused a rapid lysis of cultures of staphylococci growing in a liquid medium, but a small proportion of the cells survived and soon multiplied to yield a highly resistant strain. Rogers et al (1965a) showed that highly purified bacilysin had no sulphur content and gave a negative result for the diazo reaction.

(2). Isolation:

Newton (1949) isolated bacilysin by acidification of the culture

fluid, adsorption of bacilysin to activated charcoal, and elution with a mixture of ethanol and potassium phosphate solution. The eluate was percolated rapidly through a column of alumina, and was concentrated and lyophilized.

Rogers et al. (1965a) obtained crude bacilysin by acidification of the whole culture with acetic acid and removal of bacterial cells. The fluid was neutralized and passed through a Zeo-Karb (SRC-5, H^+ form) ion exchange resin column. Bacilysin was eluted from the resin with an aqueous pyridine solution and concentrated. Further purification was achieved by chromatography in pyridine-acetate buffers on columns of Dowex 50-X2 and Dowex 50-X8, respectively, and by gel filtration in aqueous propanol on Sephadex G-25. The purified bacilysin behaved as a single ninhydrin-positive substance when it was subjected to paper chromatography and to paper electrophoresis.

(3). Structural studies:

Newton (1949) found alanine, tyrosine, leucine and phenylalanine residues in the acid hydrolysates of bacilysin. Rogers et al. (1965b) noted that bacilysin contained C, H and N but no Cl or S. A possible empirical formula was $C_{12}H_{18}N_2O_5$ with a molecular weight of 270. Acid hydrolysates of bacilysin contained two ninhydrin-positive compounds which behaved like alanine and tyrosine respectively on paper chromatography and electrophoresis. Quantitative analysis indicated that both compounds had an equal molar ratio. Enzymatic studies showed that each had the L-configuration. However, the ultraviolet spectrum of bacilysin showed that no

tyrosine residue was present in the molecule and a nuclear-magnetic-resonance

analysis indicated that olefinic and aromatic protons were absent. Hydro-

lyzed by leucine aminopeptidase and by pronase, bacilysin gave alanine and

an uncharacterized amino acid. This substance might be a cyclohexanone

with a 2,3-epoxy-1-oxo group. Since the results of electrometric titration

showed that bacilysin was a peptide containing one free α-amino group and

one free carboxyl group, and since the dinitrophenyl (DNP) derivative of

bacilysin yielded DNP-alanine on acid hydrolysis, the tentative structure

as shown below (1) was suggested by Rogers et al. (1965b).

$$CH_3 \qquad CH_2$$
$$H_3N.CH.CO.NH.CH.CO_2$$
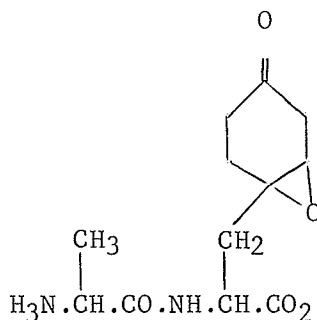
(1)

II. BACITRACINS.

(1). Producing strains:

Johnson et al. (1945) isolated a Bacillus strain with strong

antibiotic activity from a patient called Tracy. The culture was first

identified as B. subtilis and called "Tracy I strain", the active principle

being named "bacitracin". Burden (cited by Meleney and Johnson, 1949)

reclassified it as B. licheniformis. This classification was confirmed by Smith (cited by Welch and Lewis, 1951) and was also supported by the seventh edition of Bergey's Manual of Determinative Bacteriology (1957). This strain has lately been designated ATCC 10716.

Magarao et al. (1944) found a Bacillus which lysed Mycobacterium tuberculosis. This strain was later separated into two species, B. subtilis, and B. licheniformis. Antibiotics produced by the B. subtilis strain were described by Newton (1949) (see page 4). The B. licheniformis strain was known as "A-5" and the antibiotic produced was called "ayfivin" (Arriagada et al., 1949). Ayfivin was found to be identical with bacitracin by Newton and Abraham (1950) who used counter-current distribution. Consequently the name "ayfivin" was abandoned.

(2). Properties of bacitracin:

Anker et al. (1948) reported that bacitracin was stable at acid or neutral pH but was rapidly inactivated above pH 9. Hydrogen peroxide destroyed its activity rapidly. It was soluble in water and polar organic solvents but insoluble in non-polar solvents. In aqueous solution, its activity passed through a nitrocellulose membrane which held back particles of molecular weight 2000 or more. The antibiotic was precipitated by salts of heavy metals. The precipitate was inactivated if heavy metal ions low in the electromotive series were used. On the other hand, heavy metal ions high in the electromotive series such as zinc, did not result in inactivation, but precipitation of the active material was incomplete. Besides the heavy metals, bacitracin was precipitated from aqueous solution by organic acids

and other protein precipitants.

Arriagada et al. (1949) noted that bacitracin behaved as a weak base. It gave positive results with the ninhydrin test and with Knoop's test for histidine (Knoop and Windaus, 1905, Windaus and Knoop, 1906). It was stable for 10 min at 100°C in aqueous solutions at pH 2, 7 and 10. Bacitracin was not affected by trypsin and pepsin but traces of Cu^{++} ions diminished its biological activity. The agent acted mainly against Gram-positive strains including mycobacteria and Gram-negative species were relatively insensitive.

Craig et al. (1952a) found that bacitracins A, B, D, and E all exhibited low intensity ultraviolet absorption maxima at 253 nm[1], whereas bacitracin C absorbed more strongly at 258 nm with a shoulder at 253 nm.

(3). Isolation:

(a). By extraction and precipitation methods:

Johnson et al. (1945) extracted bacitracin from crude fluid with n-butanol and concentrated the resulting extract in vacuo. Anker et al. (1948) effected further purification by mixing the concentrated extract with an ether-butanol mixture and then acidified this with HCl. After separation of two phases, the aqueous layer was extracted with the nutanol-ether mixture and then ether alone. The final aqueous solution was distilled, neutralized, and lyophilized. For commercial purposes, Anker et al. (1948) suggested further purification of the butanol extract with MgO to remove pigments at neutral pH and in the cold or by precipitating bacitracin with salicylate.

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(1) nanometer: One nanometer equals 10^{-9} meter.

Sharp et al. (1949) adsorbed bacitracin to charcoal and eluted it with acidified n-butanol. Bacitracin was precipitated from butanol as the picrate which was dried and redissolved in acidified ethanol. The resulting bacitracin hydrochloride was precipitated with dry ether and dissolved in water before lyophilization.

Johnson and Meleney (1950) used ammonium molybdate in the isolation of bacitracin. Bacitracin was precipitated as the molybdate from broth. This was extracted with water at neutral pH and the extract was then repeatedly treated with n-butanol.

Regna and Solomons (1951) precipitated bacitracin from broth by the monosodium salt of Polar Yellow 5G. Methylene disalicylic acid (Baron, 1956) and lignins (Ziffer and Cairney, 1962) were also used as precipitants of bacitracin for commercial use.

(b). By ion-exchange chromatography:

Many attempts have been made to use ion-exchange resins as a means of isolating bacitracin. Usually, the use of ion-exchange chromatography alone failed to achieve the goal of high purity. Other processes, such as extraction and precipitation, are required for prior concentration or for further purification. Important work concerning the use of ion-exchangers for bacitracin purification is summarized in Table I.

TABLE I

ION-EXCHANGE METHODS IN BACITRACIN PURIFICATION

investigators	starting material	type of resin used	eluting fluid	note
Shortridge (1957)	culture fluid, pH 5.0	Amberlite IRC-50 (ammonium form)	2% NH_4OH	eluting fluid active
Hodds (1958)	culture fluid, pH 4.5	Amberlite IRC-50 (acid form)	2N NaOH	" "
Zinn and Chornock (1958)	Zn-bacitracin, pH 4.0	Amberlite MB-4	--	effluent active
Chaiet and Cochrane (1959)	(1) culture fluid, pH 4.0	acid form of Duolite C-10, Duolite C-25, Amberlite IRC-50, Amberlite XE-89, or Dowex 50 X1	dilute NH_4OH	eluting fluid active
	(2) concentrated eluate from (1)	Duolite A-7 (acetate form)	--	effluent active
Konigsberg and Craig (1959)	commercially purified sample, pH 4.5 or further purified by countercurrent dis-tribution at pH 4.5	carboxymethyl cellulose	(1) 0.05M sodium acetate buffer, pH 4.5 (2) pyridine-acetate buffer, pH 4.9	eluting fluid active

(c). By countercurrent distribution methods:

Barry et al. (1948) studied commercial bacitracin by counter-current distribution in the system sec-butanol-1.7% acetic acid, and found that bacitracin resolved into a major band (83% of sample) and two smaller bands. Craig et al. (1949a,b) obtained similar results in their distribution studies.

Sharp et al. (1949) studied the purity of bacitracin hydrochloride by the countercurrent distribution technique in the system amyl alcohol-n-butanol-0.1M phosphate buffer at pH 7, and found that bacitracin contained three active constituents. Newton and Abraham (1950) used the same system for bacitracin separation by countercurrent distribution. They also obtained three active components referring to them as bacitracins A, B, and C. After redistribution in the same system, bacitracin A appeared to be homogeneous but the homogeneity of bacitracins B and C was still uncertain after the same treatment.

Craig et al. (1952a) found five active components in commercial bacitracin by countercurrent distribution. Bacitracin A was a major con-stituent (60% of the total), whereas the other components, B, D, E and F occurred only in small amounts.

Newton and Abraham (1953a) resolved bacitracin into at least 10 active polypeptides using countercurrent distribution. These were called bacitracins E, D, B, A', A, C, G, F_1, F_2, and F_3 according to the order of their partition coefficients.

(d). Other methods:

Porath (1952) showed that "pure" bacitracin preparations obtained by countercurrent distribution were still heterogeneous. He proposed the use of charcoal and electrophoresis for further purification. The bacitracin preparations were adsorbed to active charcoal and eluted with acidic hexanol-ethyl acetate. Zone electrophoresis was performed on columns filled with a suspension of cellulose powder in sodium acetate buffer. As the result of this improved purification, Porath (1954) obtained two new bacitracins, H and I.

(4). Structural studies:

(a). Structure of bacitracin A:

Barry et al. (1948) found that the purified major component of bacitracin (bacitracin A) contained nine amino acid residues, one of which was not identified. The known amino acids were phenylalanine, leucine, isoleucine, glutamic acid, aspartic acid, lysine, histidine, and cystine and the unknown substance might be ornithine or hydroxylysine. Ammonia was also present while methionine, valine, threonine, serine, proline and arginine were absent. Sharp et al. (1949) noted the presence of ornithine. They also showed that bacitracin A had three ionized groups which could be detected by titration at pH 2 - 5, pH 5.5, and pH 10 - 12.

Craig et al. (1949b) believed that the major component of bacitracin (bacitracin A) was a polypeptide consisting of L-histidine, L-leucine, L-cysteine, L-glutamic acid, DL-phenylalanine, DL-aspartic acid, D-isoleucine, L-valine and ornithine whose configuration they were unable to

determine.

Porath (1952) reported that bacitracin A contained two aspartic
acid, one glutamic acid, one leucine, one isoleucine, two or three phenyl-
alanine, one cysteine, one histidine, one lysine and one ornithine residue
in the molecule with a molecular weight of 1460. Similar molecular weight
figures for bacitracin A were also reported by Craig et al (1952b, 1953)
and by Newton and Abraham (1953b).

Craig et al (1952b) revealed that acid hydrolysates of bacitracin
A contained two DL-aspartic acid, one D-glutamic acid, one L-leucine, two
L-isoleucine, one D-phenylalanine, one L-cysteine, one L-histidine, one
L-lysine, one D-ornithine, on NH_3 and in addition, two unknown ninhydrin-
positive residues. The unknown substances behaved as methionine and trypto-
phan on chromatography but neither of these two amino acids was involved
as was shown by their failure in isolation experiments and chemical quali-
tative determinations. Craig et al (1953) found one of the unknown subs-
tances to have the empirical formula C_5H_8O whereas the empirical formula
of bacitracin A was $C_{65}H_{102}O_{17}N_{16}S$. Bacitracin A was believed to be cyclic
since only δ-DNP-ornithine and DNP-histidine were isolated from the hydro-
lysates of DNP-bacitracin A. A second ring involving the sulphur group
was also proposed by Craig et al (1953) because the thiol group could be
detected only after hydrolysis with acid. Similar proposals were made by
Newton and Abraham (1953b). The ring structure was also supported by
Ingram (1953) who examined bacitracin A with the methylation technique.

Based on his own findings, Porath (1953) proposed a possible

arrangement of amino acid residues in the bacitracin A molecule as shown

below (2). He believed that the distribution of amino acids in bacitracin

$$
\begin{array}{cccccc}
 & & & -S & & \\
 & & & | & & \\
R\text{---}Lys & \text{---} & Glu\text{---}Cys & \text{---}Ileu & \text{---}Leu \\
 & & | & & | \\
 & & Asp & & Phe \\
 & & | & & | \\
 & & Asp\text{---}His\text{---}Orn & \text{---} & Ileu
\end{array}
$$
$$(2)$$
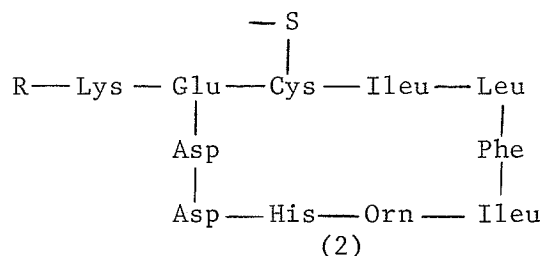
A was highly asymmetrical. The molecule consisted of a lipophilic and a

lipophobic part. The uneven distribution of basic and acidic amino acids

made the lipophilic part of bacitracin A strongly polar.

Lockhart et al. (1954a) suggested that the sequence shown below (3)

$$
\begin{array}{cccc}
 & -S & & \\
 & | & & \\
Ileu\text{---} & Cy\text{---} & Leu \text{---} & Glu
\end{array}
$$
$$(3)$$
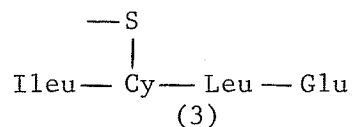
was present in bacitracin A. They also believed that the Ileu side of this

sequence was connected to a peptide containing ornithine, phenylalanine

and isoleucine residues with ornithine as the N-terminal amino acid residue.

The Glu residue on the right hand side was connected to aspartic acid. In

this cyclic structure, most of the polar side chain was grouped together

on one part of the ring and most of the non-polar side chain on the other.

Porath (1954) found a small amount of DNP-isoleucine in the acid

hydrolysates of bacitracin A but thought this was a contaminant. Lockhart

et al. (1954) also reported the isolation of DNP-isoleucine from the acid

hydrolysate. However, an attempt to degrade bacitracin A by the Edman

method yielded only 5% of the theoretical amount of 3-phenyl-2-thiohydantoin

expected from an N-terminal isoleucine.

After careful study Piez (1954) suggested that the unknown sub-

stance in the position occupied by methionine found by Craig et al. (1952b)

might be an alloisoleucine.

Craig et al. (1954) restudied the partial acid hydrolysates of

bacitracin A and concluded that bacitracin A contained three isoleucine

residues rather than two as reported earlier (Craig et al., 1952). They

also confirmed the existence of alloisoleucine and noted that it was the

N-terminal residue of bacitracin. Elementary analysis showed: C, 55.2;

H, 7.3; N, 15.8; and S, 2.2% Thus the empirical formula was $C_{68}H_{107}O_{18}N_{17}S$

for bacitracin A. The results of partial hydrolysis suggested the following

sequence (4).

(2)
$$Ileu \rightarrow Cys \rightarrow Leu \rightarrow Glu \rightarrow Ileu \rightarrow Lys \rightarrow Orn \rightarrow Ileu$$
$$Asp \leftarrow His \leftarrow Phe$$
$$Asp$$
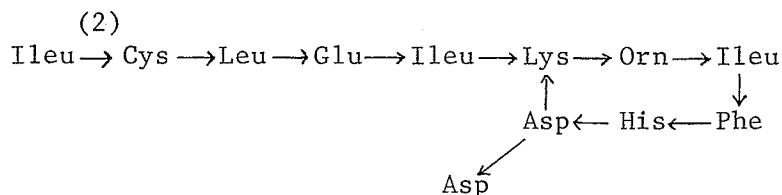
(4)

The thiazoline ring should be as follows (5):
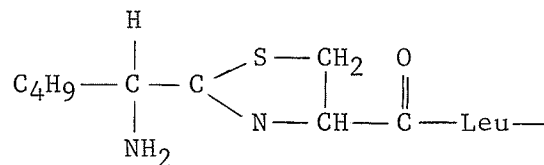
(5)

_____

(2) $\longrightarrow$ means a peptide bond from a carboxyl group to an amino group.

However, this structure failed to account for the occurrence of a peptide

fragment of Cys-SH⟶Ileu⟶Phe which was found in partial acid hydrolysate.

Also the position of an amide group still remained to be determined.

Lockhart and Abraham (1954a,b) also found D-alloisoleucine to be

the N-terminal group of bacitracin A. Based on their partial acid hydrolysis

studies, they proposed a tentative structure for bacitracin A (see below, 6)

in which the α-amino group of lysine appeared to be joined to an isoleucine

residue and the ε-amino group to an aspartic acid residue. Aspartic acid

linked to lysine had the L-configuration and the second aspartic acid residue,

the D-configuration.

$$Ileu \rightarrow Cys\text{-}SH \rightarrow Leu \rightarrow Glu \rightarrow Ileu \xrightarrow{\alpha} Lys \xleftarrow{\epsilon}$$

$$L\text{-}Asp \leftarrow (D\text{-}Asp) \leftarrow His$$

$$Orn \longrightarrow Ileu \longrightarrow Phe$$
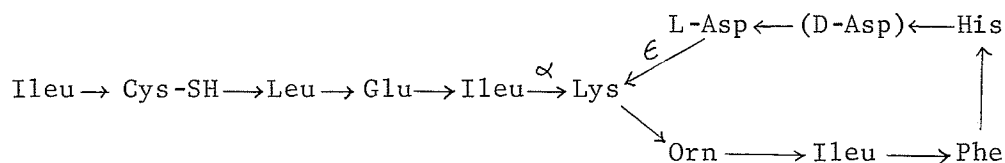
(6)

These authors modified this structure to another (shown below, 7)

in which the dotted line represented a tentative unknown linkage. They

also believed that the glutamic acid or one of the aspartic acid residues

in this structure could be present as glutamine or asparagine respectively.

A thiazoline ring which involved the carboxyl group of the isoleucine

residue and the adjacent cysteine was also present.

$$Asp \searrow$$
$$Asp \leftarrow His \leftarrow$$
$$Lys \swarrow \qquad Phe$$
$$\uparrow \searrow Orn \rightarrow Ileu \nearrow \qquad Ileu$$
$$Ileu \leftarrow Glu \leftarrow Leu \leftarrow Cys \swarrow$$
$$S —$$
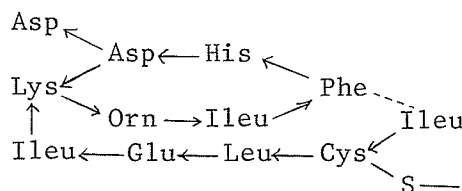
(7)

Lockhart et al. (1955) and Hausmann et al. (1955a) reconfirmed the
condensation of the N-terminal isoleucine with the cysteine residue to form
a thiazoline ring. They also found that there were three of the isoleucine
residues in bacitracin A, which supports the work of others. After studying
their partial acid hydrolysis data, Hausmann et al. (1955b) reported two
possible amino acid sequence formulae for bacitracin A (8 and 9). Structure

$$Ileu \rightarrow Cys\text{-}SH \rightarrow Leu \rightarrow Glu \rightarrow Ileu \rightarrow Lys \begin{array}{c} Orn \rightarrow Ileu \\ \\ Asp \leftarrow His \\ \\ Asp \end{array} Phe$$

(8)

$$Ileu \rightarrow Cys\text{-}SH \rightarrow Leu \rightarrow Glu \rightarrow Ileu \rightarrow Lys \begin{array}{c} Orn \rightarrow Ileu \rightarrow Phe \\ \\ Asp \leftarrow Asp \leftarrow His \end{array}$$
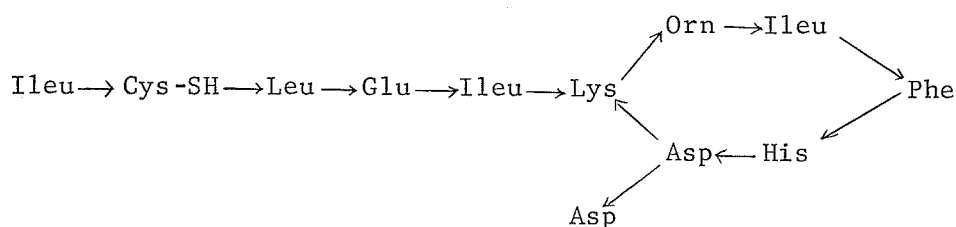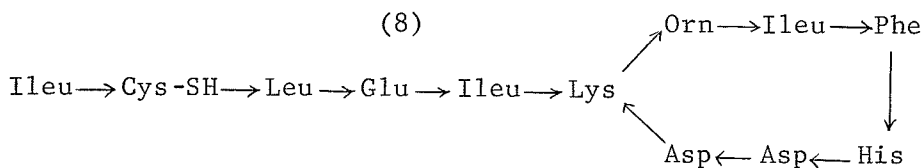
(9)

(8), which was similar to the one proposed by Lockhart and Abraham (1954b),
was preferred because it accounted for all the partial hydrolysis data
obtained with the exception of peptide fragments of DNP—Phe→Ileu→Leu
and Phe→Ileu.

Weisiger et al. (1955a) rejected structure (2) proposed by Porath
on the grounds that a peptide fragment containing Lys, Asp, Orn, and Ileu
found in their acid hydrolysate of bacitracin A could not arise from this
formula.

Lockhart and Abraham (1956) reconfirmed the correctness of their
proposed structure for bacitracin A (7) and pointed out that an isoleucine

residue was joined to the α-amino group of lysine and an aspartic acid residue to the ϵ-amino group. The arrangement around the lysine residue in bacitracin A would appear to be as shown in (10).

$$D\text{-Asp} \overset{\beta}{\leftarrow} L\text{-Asp} \overset{\epsilon}{\longrightarrow} L\text{-Lys} \overset{\alpha}{\longleftarrow} L\text{-Ileu} \longleftarrow$$
$$\downarrow$$
$$\longleftarrow D\text{-Orn}$$

(10)
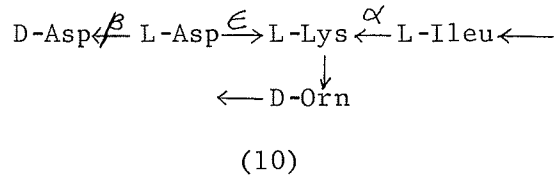
Wrinch (1957) proposed a cyclol theory to explain the formation of Phe $\longrightarrow$ Ileu $\longrightarrow$ Cys-SH and Phe $\longrightarrow$ His fragments. According to this theory, the α-carboxyl carbon atom of phenylalanine was bound to the α-amino groups of histidine and isoleucine. The structure of bacitracin A would be as shown below (11).

$$\begin{array}{l} \ulcorner ------------NH\text{-}CH\text{-}R_{Phe}\text{---}C^*(OH)\text{-}NH\text{-}CHR_{His}\text{-}CO\text{------} \\ \vert \qquad\qquad\qquad\qquad\qquad\qquad\qquad \vert \\ \llcorner --CO\text{-}CHR_{Cys}\text{-}NH\text{---}CO\text{-}CHR_{Ileu}\text{-}NH \end{array}$$

(11)
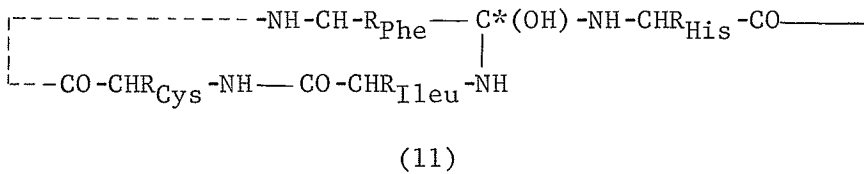
This, however, still remains to be proved.

Craig et al. (1958) summarized all the findings in the literature and concluded that the sequence formula of bacitracin A should be as shown in (12).

(12)
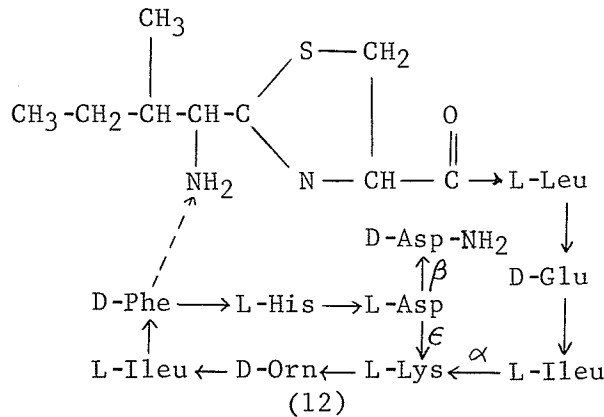
(b). Structural studies on bacitracin B:

Craig et al. (1952a) showed that bacitracin B had the same amino acid composition as bacitracin A with an additional spot whose position on the paper chromatogram corresponded to that of valine. However, they believed that it was not valine. The existence of a valine-like substance in bacitracin B was also reported by Newton and Abraham (1953a) and by Lockhart et al. (1954) as well as by Lockhart and Abraham (1954).

By comparison of the results obtained from hydrolysates of bacitracins A and B, Lockhart and Abraham (1954) thought that the difference between the bacitracin B molecule and bacitracin A molecule was that B contained the sequence Lys—→Orn—→Val—→Phe and A had the sequence Lys—→ Orn—→Ileu—→Phe.

However, the structure of bacitracin B was not really settled because Craig et al. (1958) showed that Orn—→Val—→Phe, Orn—→Ileu—→Phe, Leu—→Ileu, Phe—→His, Asp—→Lys—→Orn sequences were isolated from bacitracin B hydrolysates. The contradiction here might be due to lack of homogeneity since both Porath (1954) and Konigsberg and Craig (1959) found subtle heterogeneity still persisting in bacitracin preparations, even in bacitracin A. Further work is still required.

(c). Structure of bacitracin F:

Craig et al. (1953) noted that bacitracin A was not stable in neutral or alkaline pH and was slowly transformed to give mainly bacitracin F. Newton and Abraham (1953a,b) found bacitracin F to be a mixture of bacitracins F_1, F_2 and F_3, separable by countercurrent distribution methods.

Hausmann et al. (1955b) showed that transformation from bacitracin

A to bacitracin F involved the loss of ammonia. Quantitative amino acid analysis revealed all the amino acid residues normally found in bacitracin A to be present in bacitracin F except cysteine and alloisoleucine. Hydrolysis of fully DNP-substituted bacitracin F gave only one DNP-amino acid, δ-DNP-ornithine. A crystalline acid which had sulphur and nitrogen in a 1:1 ratio, a molecular weight of 207 and the formula $C_9H_{11}O_3NS$, was found in hydrolysates of bacitracin F.

Weisiger et al. (1955b) believed that the acid substance $C_9H_{11}O_3NS$ had structure (13).

(13)
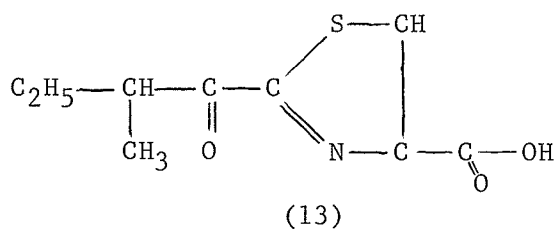
With the loss of two moles of ammonia which was thought to be liberated from isoleucine and asparagine residues of bacitracin A, the formula of bacitracin F might be (14).

(14)
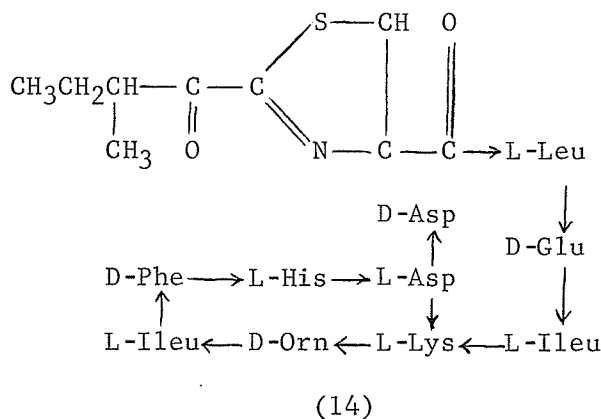
However, Craig et al (1958) pointed out that bacitracin F behaved as a neutral substance and gave a strong ninhydrin color which would not occur with a substance having merely the free δ-amino group of ornithine. Furthermore, a mole of ammonia was still present and unaccounted for in the amide determination which could not be explained by the proposed formula. Konigsberg and Craig (1962) showed that bacitracin F had the empirical formula $C_{66}H_{99}O_{17}N_{16}S$. Bacitracin A was transformed to bacitracin F actually by the loss of one mole of ammonia, two hydrogens and a gain of one oxygen atom. Amide determination by Conway's procedure (Conway, 1933) indicated that the amide group of bacitracin A still to be present in bacitracin F. Therefore, the correct formula of bacitracin F should be (15):

$$
\begin{array}{c}
\text{S}\!-\!\text{CH} \\
\text{CH}_3\text{-CH}_2\text{-CH}\!-\!\text{CH}\!-\!\text{C} \\
\quad\quad\quad\text{CH}_3\quad\text{O} \\
\text{N}\!-\!\text{C}\!-\!\text{C}\!\rightarrow\!\text{L-Leu} \\
\text{D-Asp-NH}_2\quad\quad\text{D-Glu} \\
\text{D-Phe}\!\rightarrow\!\text{L-His}\!\rightarrow\!\text{L-Asp} \\
\text{L-Ileu}\!\leftarrow\!\text{D-Orn}\!\leftarrow\!\text{L-Lys}\!\leftarrow\!\text{L-Ileu}
\end{array}
$$

(15)
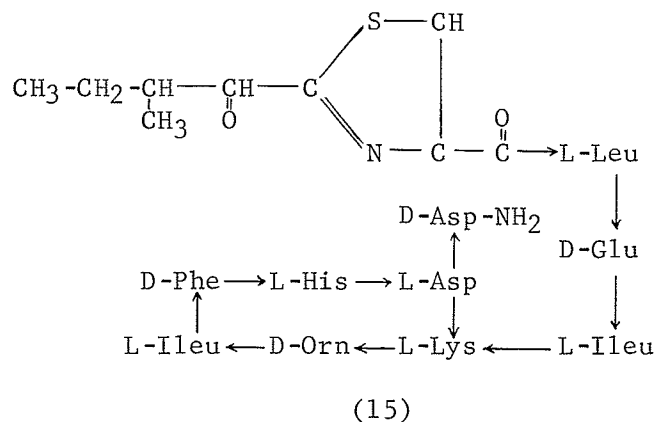
(d). Structural studies of other bacitracins:

Much less work has been done on the structures of other bacitracins. The only information available is from Newton and Abraham (1953b) who pointed out that all bacitracins contained cystein, orithine, lysine, histidine, aspartic acid, glutamic acid, leucine and/or isoleucine, and phenylalanine residues. Bacitracin D and bacitracin E differed from bacitracin A by having

a substance behaving like valine on a paper chromatogram. Bacitracin C

differed from A by having a glycine-like substance. Smaller amounts of the

valine-like substance were also obtained from bacitracins C, G, F_1, F_2, and

F_3. Furthermore, all bacitracins had a masked thiol group which appeared

in mild acid hydrolysis. All bacitracins except bacitracins E and D contained

an amide group.

III. EDEINE.

(1). Producing strains and properties of edeine:

Kurylo-Borowska (1959a) isolated a strain of B. brevis Vm 4 from

soil which produced an antibiotic that he named "edeine". The yield of

edeine from this strain was low. Chmara and Borowski (1966) obtained a

highly antibiotic-producing mutant by induction with ultraviolet light. The

strain was identified as B. brevis Vm 4-no. 587.

Kurylo-Borowska (1959b) noted that edeine was a strongly basic

material which was soluble in water, water-saturated phenol, ethylene glycol,

and acid methanol but insoluble in other organic solvents. It gave positive

reactions in biuret, ninhydrin, Millon and Sagakuchi tests and formed pre-

cipitates when picric and flavianic acids, sodium tungstate or reinecke salt

were added to edeine aqueous solutions. The antibiotic was more stable in

acid than in alkaline medium. However, heating to $60^{\circ}C$ for 30 min. at

acid pH caused 75% inactivation. Edeine was affected by the presence of

serum or blood and was inactivated slowly by trypsin. It was active against

Gram-positive and Gram-negative microorganisms and mycobacteria were also

sensitive. Kurylo-Borowska (1962) further showed that the growth of some fungi and yeasts was inhibited by edeine, and that it also had a high toxicity to mammalian neoplastic cell cultures and to experimental animals.

Roncari et al. (1966) showed that edeine solutions absorbed maximally at 272 nm. Chmara and Borowski (1968) noted that both edeines A and B exhibited the same inhibitory spectrum but that edeine A was more active than edeine B. Some, but not all, strains of bacteria were resistant to both edeines A and B.

(2). Isolation:

Kurylo-Borowska (1959b) isolated edeine by adsorption to activated charcoal and elution with acidified methanol. Edeine also was isolated by direct extraction from neutralized broth with phenol. The phenol was removed by extraction with ether, and the isolated material was dried in vacuo. Further purification was obtained by partition chromatography on silica gel using isopropanol-methanol-water mixture as a developing fluid.

Roncari et al. (1966) separated edeine from the culture broth by extraction with phenol, and further extraction of the phenolic solution with 5% acetic acid. Further purification after concentration was effected by gel-filtration on Sephadex G-25 and countercurrent distribution. Roncari et al. (1966) found only one active component in edeine preparations.

Borowski and Chmara (1967a,b) adsorbed edeine to a cation exchange resin, Wofatit CP-300 (Na^+ form), from diluted culture broth. The edeine was eluted with H_2SO_4 solution. Further purification was effected on a carboxymethyl cellulose 70 (Na^+ form) column in a NaCl gradient with sub-

sequent desalting on Sephadex G-25. By this method, they found that edeine
was a mixture of four biologically active compounds which they named edeine
A, B, C and D. Edeines A and B were major components of the complex, while
edeine C was present in very small quantities and edeine D in traces.
Borowski et al. (1966, 1967) found that these four components could also be
separated by paper chromatography.

Hettinger et al. (1968) pointed out that edeine B was very solu-
ble in phenol. With the method of Roncari et al. (1966), edeine B was lost
during the step in which edeine was extracted from phenol in aqueous acetic
acid. They modified their earlier procedure (Roncari et al., 1966) through
adsorption of edeine on Dowex 50-X4 (H^+ form) resin and elution with NH_4OH
solution. The eluate was neutralized with acetic acid and concentrated.
Further purification was achieved on Sephadex G-25 and with countercurrent
distribution in the system phenol-ammonium acetate-acetic acid.

(3). Structural studies:

Kurylo-Borowska (1959b) found that acid hydrolysates of edeine
showed six ninhydrin-reacting spots on paper chromatograms. Four of these
spots had the same R_f values as glycine, arginine, tyrosine and glutamic
acid. Roncari et al. (1966) showed that edeine contained glycine, isoserine,
α,β-diaminopropionic acid, isotyrosine and spermidine residues as well as
an unknown ninhydrin-positive substance (16). Roncari et al. (1966)

$$CH_3-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2$$
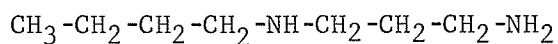
(16)

believed edeine to have a molecular weight of 1500.

Hettinger and Craig (1968) reported that when edeine A acid hydro-
lysates were subjected to paper chromatography and electrophoresis, seven
ninhydrin-positive compounds were present. These were identified as glycine,
isoserine, β-tyrosine, α,β-diaminopropionic acid, spermidine, 2,6-diamino-
7-hydroxyazelaic acid (DAHA, 17) and 2,6-diamino-7-nonenedioic acid (DAND,
18). Hettinger and Craig (1968) found that DAND was an artifact which arose

$$\begin{array}{cc} NH_2 & NH_2\ OH \\ | & |\ | \\ HOOC-CH-CH_2-CH_2-CH_2-CH-CH-CH_2-COOH \end{array}$$

(17)

$$\begin{array}{cc} NH_2 & NH_2 \\ | & | \\ HOOC-CH-CH_2-CH_2-CH_2-CH-CH=CH-COOH \end{array}$$

(18)
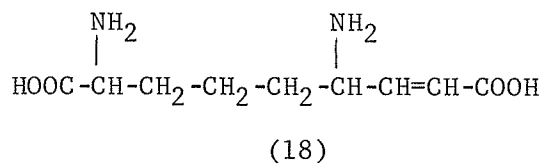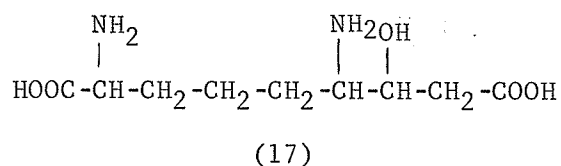
under acid hydrolysis conditions by dehydration of DAHA. All the six natural
amino acid residues present in edeine A were in an equal molecular ratio.
The molecular weight of the free base of edeine A was 775 and the acetate,
995.

Hettinger et al. (1968) noted that edeine B contained the amino
acids, glycine, isoserine, β-tyrosine, α,β-diaminopropionic acid and DAHA
as did edeine A but spermidine in edeine A was substituted by N-guanyl-N'-
(3-aminopropyl)-1,4-diaminobutane (guanylspermidine, 19) in edeine B. All

$$\begin{array}{c} NH \\ \| \\ NH_2-C-NH-CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2 \end{array}$$

(19)
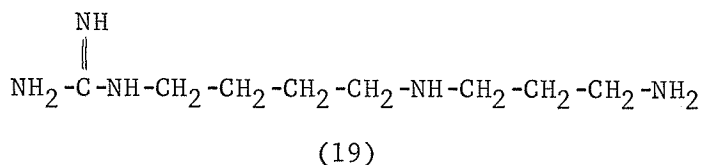
six compounds had also an equal molecular ratio. Acidimetric titration of edeine A indicated that a total of seven groups were titratable between pH 1.5 and 12.0 and with edeine B, only a total of six were detected at the same pH range. In the case of edeine A, five could be accounted for as free amino groups, one as the hydroxyl group of β-tyrosine and one a free carboxyl group. In the case of edeine B, the missing one might be a guanidino group whose pK was greater than pH 12.

Hettinger and Craig (1970) noted that edeine A was a mixture of the antibiotic peptide edeine A_1 and its inactive isomer, edeine A_2. Likewise, edeine B was a mixture of active edeine B_1 and inactive edeine B_2. Determined by examination of fragments obtained by partial acid hydrolysis, the amino acid sequence of edeine A_1 was N-(β-tyrosyl)-isoseryl-($\alpha \rightarrow \alpha$)-2,3-diamino propionyl-($\alpha \rightarrow \alpha$)-2,6-diamino-7-hydroxyazelayl-($\omega \rightarrow \alpha$)-glycyl-$NH(CH_2)_3NH(CH_2)_4NH_2$ (20). In edeine B_1, the sequence was identical except that the terminal amino group of the spermidine residue was replaced by a
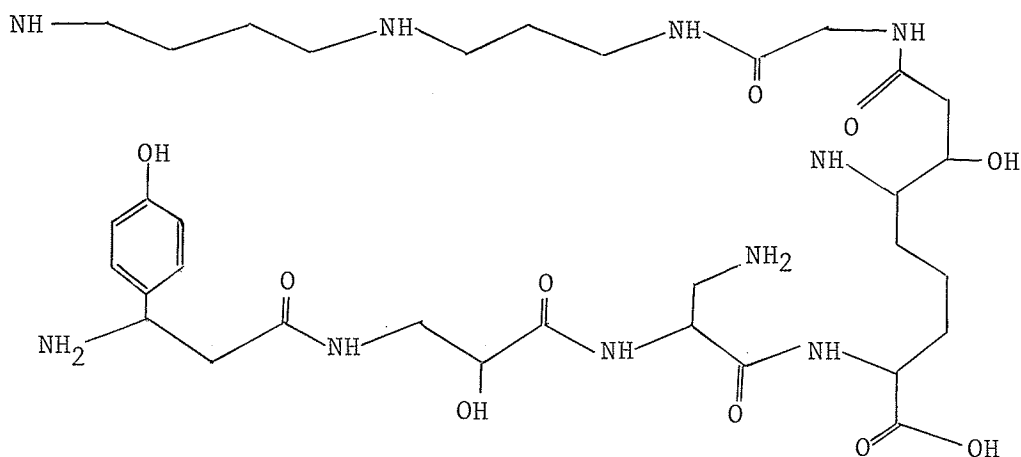
(20)

guanidino group of guanyl spermidine. Edeines A_2 and B_2 had identical

sequences with edeines A_1 and B_1 respectively except that the isoseryl-

2,3-diaminopropionyl linkage was $\alpha \rightarrow \beta$ instead of $\alpha \rightarrow \alpha$.

IV. LICHENIFORMINS:

(1). Producing strain and properties of licheniformins:

Licheniformin was isolated from a stock strain of B. licheniformis

by Callow and Hart (1946). It was active against Gram-positive bacteria

and the mycobacteria but had little effect on Gram-negative species (Callow

and Hart, 1946; and Callow et al., 1947a,b).

These authors (1947b) also noted that licheniformin could be

autoclaved at 10 lb. pressure for 10 min. over the pH range 1 - 9. Licheni-

formin hydrochloride was very soluble in methanol, soluble in water, sparingly

soluble in ethanol and insoluble in dry n-butanol, acetone, or non-polar

organic solvents. It was charred on heating strongly, and was positive with

biuret and Sakaguchi tests but negative with ninhydrin, Molisch and glucosamine

tests. The antibiotic activity of licheniformin was not altered by serum,

and it was moderately toxic to mice.

(2). Isolation:

Callow and Hart (1946) proposed the following method for licheni-

formin isolation: The whole culture was first acidified to pH 2 with HCl

and then treated with ethanol. The precipitate resulting was extracted

with diluted acid and heat. The active principle was then precipitated out

from the solution by mixing with a large volume of ethanol. This method was
later modified by Callow et al. (1947a,b). After boiling the whole culture
at pH 2, they then removed the solid and the liquid was neutralized to pH 5
with filtration through a bed of active charcoal. Elution was effected by
passage through acidified n-butanol saturated with water. The active eluate
was concentrated and passed through a column of activated charcoal. The
effluent was adjusted to pH 10, clarified, and neutralized, the licheniformin
being then precipitated from solution as the picrate. This was converted
to hydrochloride with concentrated HCl and the hydrochloride was precipitated
by the addition of acetone.

Callow and Work (1952) separated licheniformin into licheniformins
A, B, and C by countercurrent distribution.

(3). Structural studies:

Callow and Work (1952) showed that licheniformin A and licheniformin
B hydrolysates gave glycine, serine, proline, valine, phenylalanine, lysine,
arginine and aspartic acid residues and licheniformin C contained an addi-
tional glutamic acid residue. Licheniformin A hydrochloride had C, 44.4;
H, 7.0; N, 18.1 and Cl, 11.9%; licheniformin B hydrochloride had C, 44.7;
H, 7.2; N, 18.4 and Cl, 10.4% and licheniformin C hydrochloride had C, 44.7;
H, 8.1; N, 17.8; and Cl, 11.5% Licheniformins A and B were all composed of
seven glycine, six arginine, three serine, two proline, two phenylalanine,
two valine, 12 lysine and one aspartic acid residues. Since all licheni-
formins failed to react with 1-fluoro-2,4-dinitrobenzene (FDNB) reagent, it
was believed that they were all cyclic peptides.

Ogston (1952) determined the molecular weights of licheniformins by sedimentation and diffusion methods, and found that licheniformin A was 4400, B, 3800 and C, 4800.

V. MICROCOCCIN AND MICROCOCCIN P.

(1). Producing strains and properties of micrococcins:

Su (1948a) isolated a strain of Micrococcus from sewage which was inhibitory to many Gram-positive microorganisms and mycobacteria but inactive against Gram-negative species. The substance responsible for this effect was designated micrococcin.

Fuller (1955) found a strain of B. pumilus in soil collected in East Africa which was an active antibiotic-producer. Abraham et al. (1956) showed both micrococcin and the antibiotic from B. pumilus to have very similar biological, chemical and physical properties and believed the antibiotic from B. pumilus was a substance closely related to micrococcin. Therefore, they proposed the name "micrococcin P" for the antibiotic produced by this strain.

Both micrococcins were only slightly soluble in water, but readily soluble in polar organic solvents and insoluble in non-polar organic solvents. Their aqueous solutions appeared to be neutral in reaction, dextrorotatory, and fluoresced in ultraviolet light. They were freely dialysable. Crystalline preparations of micrococcins melted at 220 - 230°C with decomposition. Both were thermostable, withstanding 115°C for at least 10 min. Their antibacterial activity was not affected by incubation with pepsin, trypsin,

cysteine or serum. However, they were rapidly destroyed by alkali, light

and copper ions. Qualitative tests showed that they contained nitrogen and

sulphur but no halogens. The ninhydrin reaction was negative with both

micrococcins (Su, 1948b; Heatley and Doery, 1951; Fuller, 1955; and Abraham

et al., 1956).

(2). Isolation:

Su (1948b) brought the whole culture to pH 2 with concentrated HCl

and repeatedly extracted the resulting precipitate with ethanol after which

the ethanolic solution was concentrated and extracted with chloroform. The

active principle was precipitated from chloroform and the solution was passed

through an alumina column with ethanol as an eluting fluid. The same process

was used by Heatley and Doery (1951), but they managed to crystallize the

micrococcin from its ethanolic solution. The crystalline product was homo-

geneous as judged by countercurrent distribution.

Fuller (1955) used acidification of the culture with HCl and

precipitation with ammonium sulphate in the isolation of micrococcin P.

The precipitates were extracted with ethanol and the ethanolic solution was

evaporated to dryness. The residue, after being washed with ether and water,

was dissolved in ethanol. This ethanolic solution was further purified by

passage through an alumina column. The effluent was then dried and the

residue was crystallized from a mixture of acetic acid and ethyl acetate.

(3). Structural studies:

Heatley and Doery (1951) showed by elementary analysis that micro-

coccin was C-methyl, 6.9; C, 49.0 - 49.5; H, 4.6; N, 13.9; and S, 15.9% with an empirical formula $C_{25}H_{28}O_6N_6S_3$. Ammonia present in acid hydrolysates amounted to 24.2% of the total nitrogen in micrococcin. The molecular weight of micrococcin appeared to be between 2170 and 2720.

Fuller (1955) noted that micrococcin P was composed of C, 48.9; H, 4.9; N, 13.7; S, 16.0 and O, 16.7% which corresponded to a minimal formula of $C_8H_9N_2O_2S$.

Abraham et al. (1956) showed that on acid hydrolysis, both micrococcins broke down to give a series of compounds which could be separated into soluble and insoluble components in cold diluted hydrolysate mixture. The acid soluble fraction was composed of L-threonine, propionic acid, ammonia, an ether-soluble substance $C_7H_7O_3NS$, a substance with the formula $C_8H_{12}O_2N_2S$, and two unknown ninhydrin-positive compounds. The acid-insoluble fraction contained a substance with the formula $C_{24}H_{23}O_5N_5S_4$ and another of composition $C_{16}H_{19}O_3N_3S_3$. Brookes et al. (1957), by degradation and synthesis, identified the substance $C_7H_7O_3NS$ as 2-propionyl-thiazole-4-carboxylic acid (PCTA, 21), and the substance $C_8H_{12}O_2N_2S$ as (+)-2-(1-amino-2-methypropyl)-thiazole-4-carboxylic acid (AMPTCA, 22). The molecular weight of micrococcin

$$CH_3-CH_2-CO \underset{\underset{N}{\diagdown \diagup}}{\overset{S \underline{\quad} CH}{\underset{C}{\big| \quad \big|}}} C \underline{\quad} COOH$$
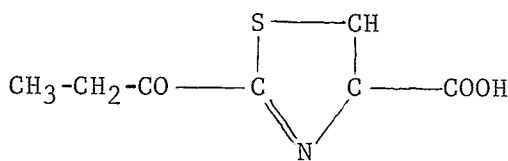
(21)

P was estimated to be about 2200.

Mijovic and Walker (1960) identified the two unknown ninhydrin-

```
                    S ——— CH
                    |      |
CH3— CH—CH-C        C ——— COOH
      |   |    \\   /
      CH3 NH2   N
```

(22)
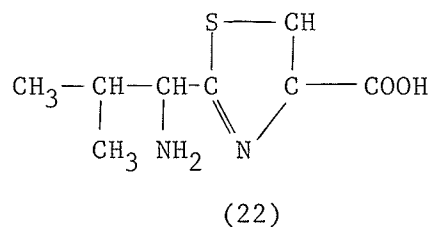
reacting substances reported by Abraham et al. (1956) as aminoacetone and

2-amino-propan-1-ol respectively. These authors also reported that there

were two threonine, four aminopropanol, two propionic acid and two AMPTCA

present in one molecule of antibiotic. Aminoacetone was not stable in

acid hydrolysis and only 0.51 mole could be isolated from every mole of

micrococcin P. Micrococcin P also had four acetylable and two free carbo-

xyl groups in the molecule.

Brookes et al. (1960a,b) believed that the substance $C_{24}H_{23}O_5N_5S_4$

found by Abraham et al.(1956) on acid hydrolysis might have a polythiazole

structure (23) which arose by cyclisation and dehydrogenation of a polycy-

steine peptide.

```
  {        C8H13ON          CO2CH3
  {  {   S —— CH  }
  {  {   |     ||  }     S — CH        S — CH
  ? {  { —C    C —}     |     ||      |     ||
  {  {    \\   /   }2   —C     C ——— C      C ——— CO2CH3
  {  {     N        }    \\   /        \\   /
  {                       N             N
```

(23)
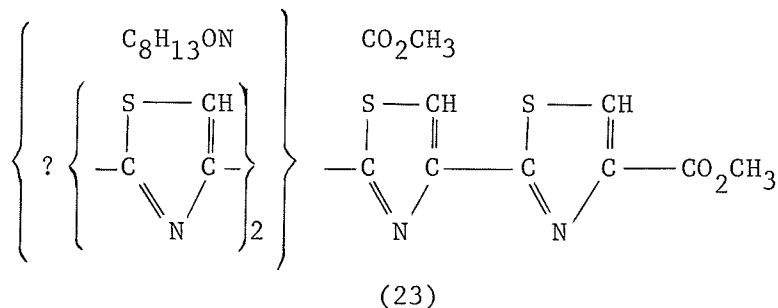
Dean et al. (1961) noted that the AMPTCA had the D-configuration

in micrococcin P.

The detailed structure for both micrococcins is still unknown.

VI. MYCOBACILLIN.

Majumdar and Bose (1958) isolated a strain of B. subtilis in India which produced a highly active antifungal substance which they called "mycobacillin". They noted that mycobacillin was soluble in lower alcohols, acetone, acetic acid, NaOH, HCl, pyridine and water above pH 7, but insoluble in ether and chloroform. It was thermostable but the stability was greater at acid than at alkaline pH. Mycobacillin gave positive reactions to biuret, xanthoproteic and Millon's tests, but was ninhydrin negative. It had no effect on the growth of Gram-positive or Gram-negative bacteria, inhibiting only fungi.

Majumdar and Bose isolated mycobacillin by extraction of the culture with n-butanol and precipitation from the butanolic solution with HCl. The active precipitates were dissolved in NaOH solution and reprecipitated with HCl. These were dissolved in ethanol and reprecipitated by ether. The last step was repeated until ultimately mycobacillin was crystallized from the ethanolic solution.

The authors showed mycobacillin to have a molecular weight of 1775 by their diffusion method (Majumdar and Bose, 1958). Majumdar and Bose (1960) reported that the molecule of mycobacillin contained five aspartic acid, two glutamic acid, two tyrosine, one serine, one alanine, one leucine and one proline residue, as shown below (24).

```
       ,Glu—→Asp—→Ala—→Asp—→Pro
      /                        \
  Leu                           Asp
      ↖                          ↓
       Asp←—Ser←—Tyr←—Asp←—Tyr← Glu
```
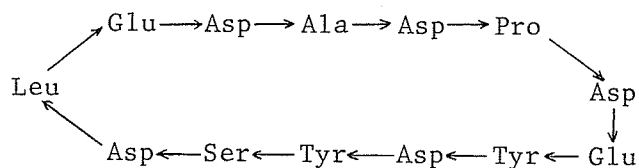
(24)

Banerjee and Bose (1963) further found that four out of five

aspartic acid and two glutamic acid residues were D-isomers and the rest

were L-isomers. However, the position of L-aspartic acid in the mycobacillin

molecule still must be decided.

VII. NISINS.

(1). Producing strains and properties of nisin:

Mattick and Hirsch (1944) isolated several strains of group N

streptococci which produced an inhibitory substance named nisin from old

milk. Nisin was found to be dialysable, and it was active against Gram-

positive but inactive against Gram-negative bacteria. Mattick and Hirsch

(1947) identified the nisin-producing strains as Streptococcus lactis and

found mycobacteria also to be sensitive to nisin. They also noted that

nisin was stable when boiled in acid solutions or stored for months as a

dry powder. Berridge (1949) found that although nisin solutions could

withstand boiling for 10 min., they were not autoclavable. Nisin was also

quickly destroyed under alkaline conditions or in an enzyme-rich medium

such as blood. Except for ethanol and formamide, those organic solvents

that dissolved nisin usually destroyed its biological activity. Nisin was

very soluble in water at low pH.

(2). Isolation:

Mattick and Hirsch (1947) showed that nisin could be isolated by

acidification of the culture broth, extraction with a mixture of chloroform

and sec-octanol followed by precipitation with ethanol. Berridge (1949) found that nisin could be further purified by precipitation from phosphate buffer with NaCl and crystallized from acetone.

Hirsch (1951) suggested the nisin-producing strain actually synthesized several antibiotics. Berridge (1952) isolated nisins A and B from a preparation by countercurrent distribution, and Bavin et al. (1952) obtained similar results. Berridge et al. (1952) showed that the crystalline nisin was a mixture of 49% nisin A, 32% B, 12% C, 5% D and possibly 3% of nisin E.

Cheeseman and Berridge (1957) proposed an improved method for nisin isolation which involved salting out, solvent extraction and fractional precipitation with acetone as well as countercurrent distribution. This method was found to give a better percentage recovery and lower amounts of impurities in the final products.

(3). Structural studies:

Berridge (1949) showed that the acid hydrolysates of nisin contained alanine, valine, leucine, isoleucine, cysteine, and aspartic acid residues together with several unidentified substances.

Berridge et al. (1952) noted that the chromatogram of acid hydrolysates of nisins A, B and C were strikingly similar and only slight differences were apparent in that of nisin D. The results showed that all four nisins contained leucine and/or isoleucine, alanine, glycine, proline, aspartic acid, histidine and lysine. Valine and methionine were present in nisins A, B and C but absent from D. There were two other amino acids, lanthionine and a cystathionine-like substance present in all four nisin

molecules. Quantitatively, hydrolysates of mixed nisins consisted of four leucine, two valine, two alanine, two glycine, two proline, one serine, two lysine, two histidine, one aspartic acid, two methionine, one lanthionine, and four cystathionine-like residues. Newton and Abraham (1953c) believed the cystathionine-like substance found by Berridge et al. (1952) actually was one of the β-methyllanthionines, $C_7H_{14}O_4N_2S$ (25) which might be identical to the C_7 amino acid isolated from subtilin (see page 67).

$$\underset{\substack{| \\ NH_2}}{HOOC-CH-CH_2}-S-\underset{\substack{| \\ NH_2}}{\overset{\overset{\textstyle CH_3}{|}}{CH}-CH-COOH}$$
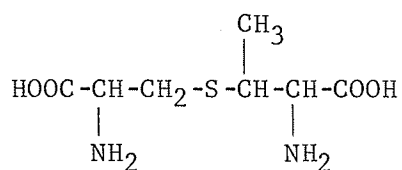
β-methyl-lanthionine

(25)

Cheeseman and Berridge (1959) showed that nisin A contained nine leucine and isoleucine, three methionine, three valine, two proline, four alanine, six glycine, one serine, three aspartic acid, three histidine, nine lysine, nine β-methyllanthionine, and three lanthionine residues with a molecular weight of 6826. They also reported that there were no free α-amino or α-carboxyl groups in nisin A and the only free groups found were the ϵ-amino group of lysine and the imino group of histidine. Thus, they believed that nisin A was a cyclopeptide.

Bodanszky and Perlman (1964) believed that the number of amino acid residues and the molecular weight of nisin A reported by Cheeseman and Berridge (1959) were too high. According to their proposal, nisin A

might have only 18 amino acid residues with a molecular weight around 2500

(Table II). This was partially confirmed by Gross and Morell (1967) who

found a value of 3290 by amino acid analysis and a value of 3510 by the

method of partial substitution for the molecular weight of nisin A.

Gross and Morell (1967) discovered a new amino acid, dehydroalanine,

(26) present in the nisin molecule. The biological activity of nisin was

believed to be directly related to the presence of dehydroalanine.

$$CH_2 = \underset{\underset{(26)}{\overset{|}{NH_2}}}{C} - COOH$$
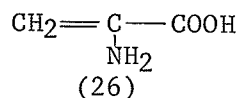
After studying the migration pattern of nisin with polyacrylamide-

gel electrophoresis, Ingram et al. (1967) disagreed with the proposal of

Bodanszky and Perlman (1964). They believed the molecular weight of nisin

A should be 7000. Jarvis et al. (1968) held a similar point of view accor-

ding to their ultracentrifugation analysis. To summarize, detailed structures

of the nisins have yet to be elucidated.

VIII. POLYMYXIN GROUP.

A. POLYMYXINS A, B, C, D, AND E.

(1). Producing strains and nomenclature:

Ainsworth et al. (1947) isolated B. aerosporus (synonymous with

B. polymyxa) from the soil and found that it produced an antibiotic which

they named "aerosporin". Simultaneously, Stansly et al. (1947) found a

TABLE II

AMINO ACID COMPOSITION OF NISIN A

amino acid	reported by Cheeseman & Berridge (1959)	reported by Bodanszky & Perlman (1964)
Isoleucine and Leucine	9	3
Methionine	3	1
Valine	3	1
Proline	2	1
Alanine	4	1
Glycine	6	2
Serine	1	-
Aspartic acid	3	1
Histidine	3	1
Lysine	9	3
β-Methyllanthionine	9	3
Lanthionine	3	1
Total	55	18

strain of B. polymyxa in soil and called the antibiotic substance it pro-
duced, "polymyxin". Benedict and Langlykke (1947) also noted the existence
of antibiotic-producing strains of B. polymyxa in the soil.

Brownlee and Jones (1948) noted that both aerosporin and polymyxin
had an identical inhibitory spectrum and similar chemical composition. They
also found that strains of B. aerosporus and B. polymyxa were identical.
For these reasons, they proposed that polymyxin be the generic name for this
group of antibiotics and suggested that aerosporin designate polymyxin A
and that the original polymyxin designate polymyxin D.

Jones (1948c) reported the isolation of polymyxins B, C, E from
different strains of B. polymyxa. He also noted that various strains of
B. polymyxa could produce the same polymyxin but that a given strain produced
only the one type of polymyxin.

(2). Properties of polymyxins:

Ainsworth et al. (1947) found that polymyxin A was active against
Gram-negative but inactive against Gram-positive bacteria. Stansly et al.
(1947) reported a similar activity for polymyxin D. These authors showed
that polymyxin D was inactivated by pepsin, trypsin, pancreatin and erepsin
with prolonged incubation and that it was dialyzable through cellulose mem-
branes. Jones (1948a) showed that polymyxin A was a basic peptide having
pK values of 5.8 and 10.5 and also that it would form salts with mineral
acids. Catch and Friedmann (1948) noted that polymyxin A was labile in
alkaline but stable in acid conditions, freely soluble in water and methanol
and precipitated by many base precipitants and acid azo-dyes. It had no

characteristic absorption or fluorescence in the ultraviolet region of the spectrum. Similar properties were also reported for polymyxin D.

Although there is no information available regarding the properties of polymyxins B, C and E, it is generally believed that they are similar to polymyxins A and D.

(3). Isolation:

Ainsworth et al. (1947) isolated polymyxin A by adsorption from broth with activated charcoal and elution with aqueous acetone. The polymyxin was precipitated by the addition of dry acetone. A similar method was used by Stansley et al. (1947) to obtain polymyxin D. However, they further purified polymyxin D to the hydrochloride salt from the water-insoluble picrate. Catch and Friedmann (1948) further purified the acetone precipitates of polymyxin A by fractional precipitation as the helianthate and then converted the helianthate salt to hydrochloride or sulfate salts by treatment of methanolic suspensions of polymyxin A helianthate with the appropriate mineral acid. The hydrochloride was then repeatedly precipitated by acetone.

Porter et al. (1949) proposed the use of isopropanol extraction under acid conditions to separate polymyxin from the culture broth. The extract was concentrated and the concentrate was adsorbed on acid-washed aluminum oxide columns. Elution was accomplished by water and NaOH solution. After the eluate was neutralized, the active principle was precipitated as picrate which was subsequently converted to hydrochloride with the addition of HCl to the acetone solution of polymyxin picrate. The

polymyxin hydrochloride was crystallized from aqueous solution with the aid
of ammonia.

Regna et al. (1949) purified crude polymyxin B by chromatography
on cotton sodium succinate followed by precipitation as the salt of the dye,
Polar Yellow, and crystallization as the salt of naphthalene-β-sulfonate.
Wilkinson (1949) also crystallized polymyxins B and E as naphthalene-β-sul-
fonates.

(4). Structural studies:

Catch and Friedmann (1948) found polymyxin A hydrochloride to have
the analysis: C, 45.5; H, 7.7; N, 15.8; and Cl, 14.0%. Sulphur and phos-
phorus were absent. Jones (1948a) found that acid hydrolysates of polymyxin
A contained leucine and threonine with a molar ratio of 1:3 and an unknown
acid residue. This unknown basic amino acid was identified by Catch and
Jones (1948) as α,γ-diaminobutyric acid (DAB). Jones (1948b) showed that
leucine in the polymyxin A molecule had a D-configuration, and that threonine
was an L-isomer but was not able to determine the configuration of DAB.
Brownlee and Jones (1948) reported the existence of an unknown, optically
active fatty acid with a formula of $C_9H_{18}O_2$ and determined that the DAB in
polymyxin A was an L-isomer. Catch et al. (1948) confirmed the presence of
D-leucine, L-threonine and L-DAB by isolation. They also noted that DAB
was a mixture of L-DAB and D-DAB in a proportion of 3:1 but believed that
the D-DAB was an artifact. They found the same unknown fatty acid also to
be present in polymyxin B.

Jones (1948b) noted that all polymyxins appeared to have in the

molecule threonine, DAB and a saturated optically active fatty acid, but

they differed from each other qualitatively in composition of other amino

acids (Table III).

Short (1948) showed that polymyxin B contained two molecules of

phenyl-alanine and three of threonine in the peptide component and had a

minimum molecular weight of 2500. She also found that polymyxin E had a

threonine content of 14.36% and the molecular weight corresponding to this

figure would be of the order of $(835)_n$.

Catch et al. (1949) found that the unknown fatty acid was similar

to pelargonic acid and was present as an N-acyl grouping. Also, they noted

that the fatty acids found in polymyxins A, B and D were identical.

Gore and Peterson (1949) examined polymyxins A and D with infra-

red spectrophotometric methods and noted that polymyxins A and D were dif-

ferent antibiotics. The unknown fatty acid constituent was similar to but

not identical with pelargonic acid. The unknown fatty acid constituent

had more methyl groups than the natural pelargonic acid. This led to the

identification of the unknown acid as D-6-methyl-octan-1-oic acid (MOA)

by Wilkinson (1949).

Bell et al. (1949) noted that polymyxin D hydrochloride consisted

of: C, 43.5; H, 7.8; N, 15.2; O, 23.2; and Cl, 10.3% with a molecular weight

of 1144 and an empirical formula $C_{50}H_{97}N_{15}O_{15}Cl_4$. It had one D-leucine,

three L-threonine, one D-serine, and five L-DAB. After reaction of the poly-

myxin D with FDNB reagent, four γ-DNP-α-amino-butyric acid and a free DAB

were isolated. This indicated that the molecule had only four free γ-amino

groups present.

TABLE III

THE AMINO ACID COMPOSITION OF POLYMYXINS
(Jones, 1948b)

Polymyxin	Leucine	Phenylalanine	Threonine	Serine	DAB
A	+	-	+	-	+
B	+	+	+	-	+
C	-	+	+	-	+
D	+	-	+	+	-
E	+	-	+	-	+

From pressure-area measurements, Few and Schulman (1953) concluded that there was no significant difference in the molecular weights of polymyxins A, B, D and E. The molecular weight of the polymyxins had a mean value of 1250.

(i). Structures of polymyxin B:

Hausmann and Craig (1954) revealed the presence of more than one peptide in the crystalline polymyxin B preparation purified by Regna et al. (1949) by countercurrent distribution. The major component was designated polymyxin B_1 and the minor, polymyxin B_2. Polymyxin B_1 contained a fatty acid identical to MOA but the B_2 had an unknown C_8 fatty acid which was not optically active. Polymyxin B_1 consisted of five L-DAB, two L-threonine, one L-leucine, one D-phenylalanine, one D-DAB, and one (+)-MOA, with an empirical formula $C_{56}H_{99}O_{14}N_{16}$ and a molecular weight of 1220.

After carefully studying the results of partial acid hydrolysis, Hausmann (1956) proposed two tentative structures (27a and 27b). He believed either of these two structures fulfilled all the requirements of the data.

```
                        rNH2
                         |
         rNH2-DAB ——→DAB ——→Thr-OH
               ↑                ↓
             Leu            DAB-rNH2
               ↑                ↓
         Phe←— DAB ←— DAB←— Thr←— DAB←—MOA
               |          |        |
             rNH2        OH      rNH2
```

(27a)

```
                        rNH₂
                         |
        rNH-DAB ——→DAB ——→Thr-OH
                ↑                 |
              Leu                 |
                ↑                 ↓
        Phe←— DAB ←—DAB←— DAB←— Thr←—DAB←— MOA
               |        |        |         |
              rNH₂     rNH₂     OH        rNH₂
```
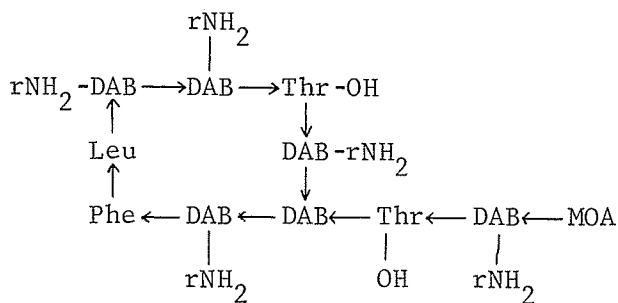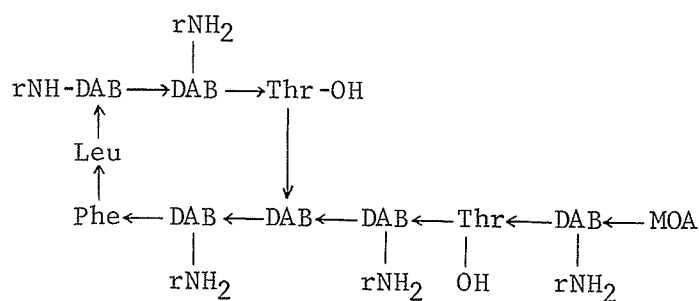
$$
\text{(27b)}
$$

Independently, Biserte and Dautrevaux (1957a) also came to the
same conclusion from their structural studies of polymyxin B. They believed
that polymyxin B₁ could have either an octacyclopeptide (28a) or heptacyclo-
peptide structure (28b) with a side chain formed of two or three amino acids
attached to a DAB residue. The ⍺-amino group of DAB terminating this side
chain was the D-isomer and was joined to the carboxyl group of MOA.

```
                          rNH₂
                           |
      rNH₂-L-DAB ——→ L-DAB ——→ L-Thr-OH
                  ↑                   |
              L-Leu                   ↓
                  ↑             L-DAB-rNH₂
                                      |
                                      ↓
      D-Phe←——L-DAB←— L-DAB←— L-Thr←— D-DAB←— MOA
                 |               |        |
                rNH₂            OH       rNH₂
```

$$
\text{(28a)}
$$

```
                          rNH₂
                           |
      rNH₂-L-DAB ——→ L-DAB ——→L-Thr-OH
                  ↑                   |
              L-Leu                   |
                  ↑                   ↓
      D-Phe←——L-DAB←— L-DAB←— L-DAB←— L-Thr←— D-DAB←—MOA
                 |              |        |         |
                rNH₂           rNH₂     OH        rNH₂
```
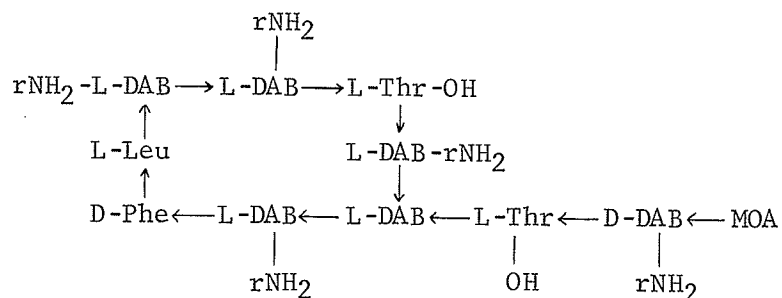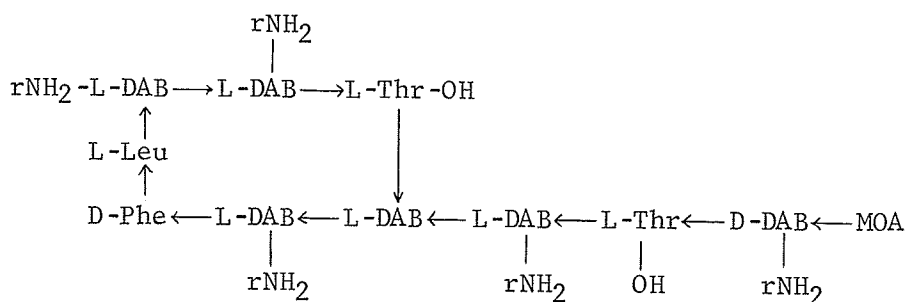
$$
\text{(28b)}
$$

Furthermore, the carboxyl group of threonine in the octacyclopeptide ring could join with either an α-amino (to be called 8α) (28c) or Γ-amino group (to be called 8Γ) (28d) of the DAB. In the heptacyclopeptide ring, the carboxyl group of DAB could attach to either α-amino (7α) or Γ-amino groups (7Γ) of the DAB in the ring (28c,d).
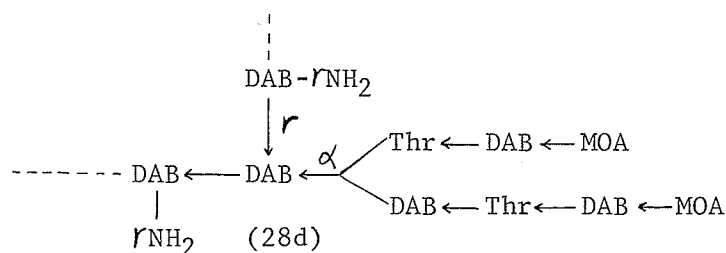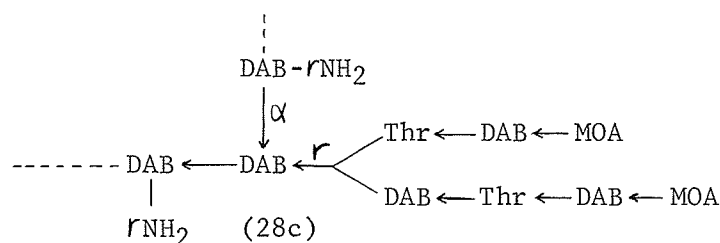
Thus one of the four possible alternatives, 7α, 7Γ, 8α or 8Γ might be the structure of polymyxin B_1. These four cyclic peptides were synthesized by Vogler et al. (1959, 1960, 1961) and by Studer et al. (1961, 1963). Vogler et al. (1963) reported that both synthetic 8α and 8Γ were not identical with the natural product in view of their low in vitro antibacterial activity against Brucella bronchiseptica. Both cyclic variants 7α and 7Γ were highly active. However, on the basis of microbiological experiments, 7Γ was more favored than 7α, although other properties of 7α more closely resembled the natural polymyxin B_1. The difference between the natural

product and synthetic 7α was that the latter had optical activity. There-
fore, it was concluded that none of the four synthesized substances was
identical with natural polymyxin B_1.

Suzuki et al. (1963d), by means of enzymatic hydrolysis studies,
pointed out that D-DAB was not present in the polymyxin B_1 molecule and the
DAB next to the fatty acid was an L-isomer. Furthermore, the side chain of
polymyxin B_1 was linked to the α-amino group of DAB in the cyclic portion
and the γ-amino group was involved in the ring formation (7α). Wilkinson
and Lowe (1964a) also believed that polymyxin B_1 had no D-DAB residue pre-
sent and the structure of polymyxin B_1 might be either 7α or 8γ.

Suzuki et al. (1964) further proved that the polymyxin B_1 had a
7α structure with all L-DAB. This was confirmed by Vogler et al. (1965)
by synthesis.

For polymyxin B_2, Wilkinson and Lowe (1963) noted that it had the
same amino acid residues as polymyxin B_1 but with an unknown optically in-
active fatty acid, $C_8H_{16}O_2$, rather than an optically active methyloctanoic
acid. This unknown acid was identified as isooctanoic[3] (6-methylheptanoic)
acid by Wilkinson and Lowe (1963). Wilkinson and Lowe (1964b) showed that
polymyxin B_2 had a similar structure to B_1 except the (+)-MOA was substi-
tuted by IOA. Wilkinson and Lowe (1964c) finally decided that polymyxin
B_2 should have a 7α structure similar to other polymyxins.

(ii). Structure of polymyxin E:

Suzuki et al. (1965) fractionated polymyxin E into polymyxin E_1
and polymyxin E_2 by countercurrent distribution. Amino acid composition
of polymyxins E_1 and E_2 was the same as those of unfractionated polymyxin

(3) Isooctanoic acid is also known as IOA.

E, namely, six L-DAB, two L-threonine, one L-leucine and one D-leucine.

The fatty acids obtained from the acid hydrolysates of polymyxins E_1 and E_2

were identified as MOA and IOA respectively. Partial hydrolysis studies

revealed that both antibiotics had the 7α structure similar to polymyxin B

except the position of D-phenyl-alanine in the ring of polymyxin B was sub-

stituted by D-leucine in polymyxin E (29).

```
                          rNH2
                           |
      rNH2-L-DAB ──→L-DAB──→L-Thr-OH
               ↑                |
            L-Leu               |
               ↑                ↓                              MOA
         D-Leu←─L-DAB←──L-DAB←──L-DAB←──L-Thr←──L-DAB←<
               |                |        |        |            IOA
             rNH2             rNH2      OH      rNH2
```

<div align="center">(29)</div>

(iii). Structure of polymyxin D:

   Hayashi <u>et al</u>. (1966) found that polymyxin D also was a mixture

of two components separable by the countercurrent distribution method. Both

polymyxins D_1 and D_2 had the same amino acid compositions, five L-DAB, three

L-threonine, one D-leucine and one D-serine. The fatty acid found in poly-

myxin D_1 was identified as MOA and in polymyxin D_2, IOA. Based on partial

acid and enzymatic hydrolyses, the structure of polymyxins D were identified

as the 7α-type like other polymyxins (30).

   (iv). Structures of polymyxin A:

   Wilkinson and Lowe (1966) reported that polymyxin A could be sepa-

rated into two components, polymyxins A_1 and A_2 by countercurrent distri-

bution. Both polymyxins A had five L-DAB, three L-threonine, one D-leucine,

```
                          rNH2
                           |
       rNH2 -L -DAB ──→ D -DAB ──→ L -Thr -OH
                ↑                    |
       HO -L -Thr                    | r
                ↑                    ↓   α
         D -Leu ←── L -DAB ←── L -DAB ←── D -Ser ←── L -Thr ←── L -DAB ←   MOA
                           |                          |         |        ↘ IOA
                         rNH2                         OH       rNH2
                                    (30)
```

and one D-DAB. Just like other polymyxins, polymyxin A_1 contained MOA, and

polymyxin A_2, IOA. Both A_1 and A_2 were found to be of the 7α-type (31).

```
                          rNH
                           | 2
       rNH2 -L -DAB ──→ L -DAB ──→ L -Thr -OH
                ↑                    |
       HO -L -Thr                    |
                ↑                    ↓
         D -Leu ←── L -DAB ←── L -DAB ←── D -DAB ←── L -Thr ←── L -DAB ←   MOA
                           |                 |        |         |        ↘ IOA
                         rNH2              rNH2      OH        rNH2
                                    (31)
```

(v). Structure of polymyxin C:

Nothing is known about the fatty acid content and detailed struc-

ture of polymyxin C.

B. CIRCULINS.

(1). Producing strain and properties of circulins:

Murray and Tetrault (1948) isolated a strain of B. circulans (Q-19) from a soil sample which produced an antibiotic they called "circulin". Circulin was more active against Gram-negative than Gram-positive micro-organisms. Murray et al. (1949) noted that circulin sulphate was very soluble in water, less soluble in lower alcohols and insoluble in non-polar organic solvents. In acid solutions, it withstood autoclaving for 15 min. at 15 lbs. of pressure, but stability decreased if the pH of the solution increased above 7. It was not inactivated by pepsin but the activity was destroyed by trypsin within seven days. Circulin was dialysable, and it showed levorotation in aqueous solutions. The antibiotic gave positive biuret and ninhydrin tests and was toxic to mice, the LD_{50} being 23 mg. per kg. of body weight when injected intravenously. Peterson and Reineke (1949 a,b) found that circulin was inactivated by lipase. Howell (1949) found crystalline circulin sulphate to have a melting point of $235^{\circ}C$ and absorption bands at 252, 258 and 264 nm. The isoelectric point of circulin in 0.02M citrate buffer was at pH 7.

(2). Isolation:

Murray et al. (1949) isolated circulin as the sulphate from clari-fied culture fluids by adsorption on active charcoal and elution with aqueous tertiary butanol acidified with H_2SO_4. The eluate was concentrated in vacuo and neutralized with $Ba(OH)_2$. Peterson and Reineke (1949a,b) further puri-

fied circulin sulphate by a chromatographic method in which the antibiotic
was subjected to chromatography on a column of a mixture of Darco-50 and
Celite-545 and eluted with aqueous tertiary butanol acidified with H_2SO_4.
By these methods, they were able to separate circulin into two active compo-
nents, circulins A and B.

Howell (1949) purified circulin by extraction with an acetone-
H_2SO_4-ammonium sulphate mixture. The active principle was then precipitated
from the acetone solution by the addition of chloroform. The precipitate
was dissolved in water and reprecipitated with the addition of ammonium
sulphate. Finally, the circulin sulphate was crystallized from a diluted
ethanolic solution. Recrystallization could be performed in methanol,
ethanol, isopropanol, acetone or molar sodium acetate solutions.

(3). Structural studies:

Peterson and Reineke (1949a,b) found that circulin contained five
L-DAB residues, one L-threonine, one D-leucine and a fatty acid residue with
a minimum molecular weight of 854. The carboxyl groups of all amino acid
residues were bound, the only free groups in the molecule being the Γ-amino
groups of DAB. It was believed that the fatty acid was attached to the
hydroxyl group of threonine and this was the reason circulin was sensitive
to lipase. Howell (1949) found that circulin sulphate had a molecular weight
of 1050 and the empirical formula $C_{41}H_{55}O_{15}N_9SO_4$. Dowling et al. (1952)
showed that both circulins A and B contained isoleucine in addition to the
constituents reported by Peterson and Reineke.

Grady et al. (1958) showed that circulin A consisted of L-DAB,

L-threonine, D-leucine, L-isoleucine and MOA in the molar ratios 6:2:1:1:1.
From the data obtained by partial acid hydrolysis, Koffler (1959) proposed
a tentative formula for circulin A (32) which was a cyclodecapeptide with
no side chain. He also believed that circulin B had an identical structure

$$
\begin{array}{c}
rNH_2 \\
| \\
D\text{-}Leu \longrightarrow L\text{-}Ileu \rightarrow L\text{-}DAB \longrightarrow L\text{-}DAB\text{-}rNH_2 \longleftarrow MOA \\
\uparrow \qquad\qquad\qquad\qquad\qquad \downarrow \\
rNH_2\text{-}L\text{-}DAB \qquad\qquad\qquad L\text{-}DAB\text{-}rNH_2* \\
\uparrow \qquad\qquad\qquad\qquad\qquad \downarrow \\
rNH_2\text{-}L\text{-}DAB \longleftarrow L\text{-}Thr \longleftarrow L\text{-}DAB \longleftarrow L\text{-}Thr\text{-}OH \\
| \qquad\qquad | \\
OH \qquad\quad rNH_2
\end{array}
$$
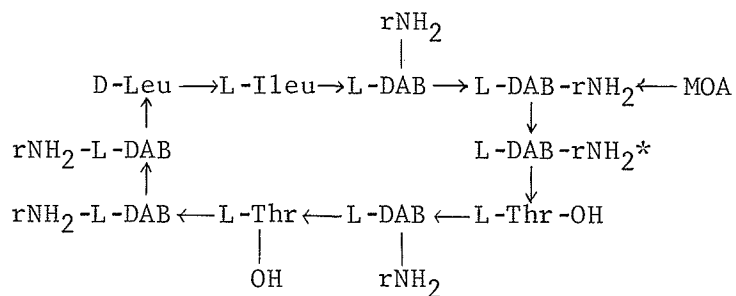
(32)

to circulin A with the exception that the MOA in circulin B was attached
to the r-amino group of DAB as marked by an asterisk in the formula (32).

Since circulins and polymyxins had close similarity in biological
and chemical properties, the correctness of the structure of circulins A
and B proposed by Koffler (1959) aroused the concern of Fujikawa et al.
(1965). They confirmed the constituents of circulin A reported by Koffler
(1959), but from their partial acid hydrolysis and enzymatic hydrolysis
studies, they found that circulin had a different structure than that pro-
posed by Koffler. The structure of circulin A was similar to polymyxin B_1,
differing from B_1 only in replacement of D-Phe and L-Leu of the B_1 with
D-Leu and L-Ileu in circulin A (33).

$$
\begin{array}{c}
rNH_2 \\
| \\
rNH_2\text{-}L\text{-}DAB \longrightarrow L\text{-}DAB \longrightarrow L\text{-}Thr\text{-}OH
\end{array}
$$

rNH$_2$-L-DAB $\longrightarrow$ L-DAB $\longrightarrow$ L-Thr-OH
    $\uparrow$ $\downarrow$ r

L-Ileu
  $\uparrow$
                           α MOA

D-Leu $\longleftarrow$ L-DAB $\longleftarrow$ L-DAB $\longleftarrow$ L-DAB $\longleftarrow$ L-Thr $\longleftarrow$ L-DAB $<$
            | | | | IOA
           rNH$_2$ rNH$_2$ OH   rNH$_2$
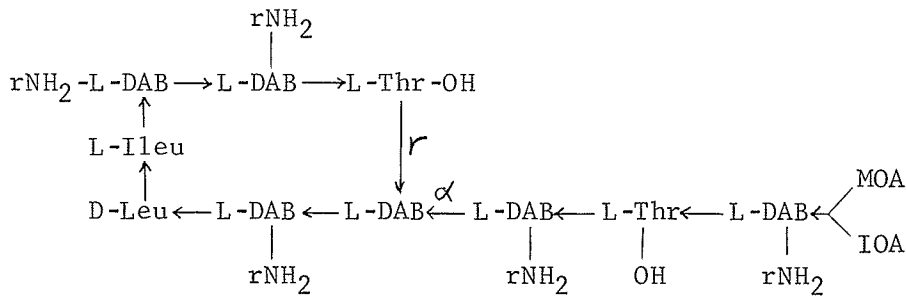
(33)

Hayashi *et al.* (1968) found that circulin B had a similar struc-
ture to circulin A and only differed from A in having IOA in place of MOA
(see 33 above).

C. COLISTINS.

(1). Producing strain and properties of colistins:

Koyama *et al.* (1950) isolated colistin from cultures of B. colis-
tinus, which was believed to be closely related to B. polymyxa. Colistin
also is known as colomycin, colimycine or colo-mycin but it differs from
the Soviet antibiotic colimycin (Gause *et al.*, 1956), which is produced by
Streptomyces and is related to neomycin.

Wright and Welch (1960) reported that colistin sulphate absorbed
only in the 210-250 nm region in the ultraviolet spectrum. Schwartz *et al.*
(1960) noted that colistin was a basic peptide. The base itself was in-
soluble but the salt was soluble in water. Solutions of colistin were levo-
rotatory and stable at pH 2 to 6 but the stability decreased above pH 6.
The presence of serum in colistin solutions did not alter antibiotic acti-
vity, and it was active against Gram-negative but much less so against Gram-

positive bacteria and fungi.

(2). Isolation:

Koyama (1952) isolated colistin sulphate from broth by treatment with sulphuric acid and heat. Colistin could also be isolated as the picrate. Kurihara and Suzuki (1953) converted colistin picrate to hydrochloride by dissolving picrate in concentrated HCl with crystallization from acetone. Fujiko and Namiki (1957) obtained crystalline colistin phosphate from an alcoholic solution of colistin by mixing with concentrated H_2SO_4.

Fujimasa (1956) absorbed colistin to a weak acidic cation exchanger and liberated it with acid solution with further purification by anion exchange. A similar procedure was used by Morito (1962a) in which he first acidified the culture fluid with HCl, adsorbed the colistin to a column of Amberlite IRC-50 (Na^+ form) and then eluted it with HCl solution. The active principle was precipitated by adjusting the pH so that it was alkaline and the procedure was repeated until a pure substance was obtained.

(3). Structural studies:

Oda <u>et</u> <u>al</u>. (1954) found that colistin could be separated into three active components, colistins A, B and C by paper chromatography using 0.2M glycine buffer (pH 2.5) as a developing fluid. Oda and Ueda (1954) showed that the hydrolysates of colistin contained leucine, threonine, DAB, and the fatty acid, MOA.

Suzuki (1956) noted that colistin had free OH and r-amino groups, the OH group of which was essential to the antibiotic activity. Suzuki (1957a) believed that colistin was composed of L-threonine, L-leucine, L-DAB and MOA in a molar ratio of 1:1:1:5:1. Its hydrochloride salt had a molecular weight of 1200 $\pm$ 50 and the empirical formula $C_{45}H_{85}O_{10}N_{13} \cdot 4HCl$. Suzuki (1957b) further showed that colistin had a threonine OH group and four r-amino groups of DAB free, but there were no free α-amino or carboxyl groups in the molecule. From these data together with the information obtained from partial hydrolysis, Suzuki (1957c) proposed a tentative structure for colistin (34).

$$
\begin{array}{ccccc}
 & \text{L-DAB} \longrightarrow \text{L-DAB} \longrightarrow \text{D-Leu} & \\
\text{L-DAB} & & & \text{L-DAB} \\
 & \text{L-Thr} \longleftarrow \text{L-DAB} \longleftarrow \text{L-Leu} & \\
 & \uparrow & \\
 & \text{MOA} &
\end{array}
$$

(34)

Biserte and Dautrevaux (1957b) confirmed that the colistin possessed neither terminal α-amino nor α-carboxyl groups but they found only one threonine, one leucine and four DAB residues in the colistin molecule. Dautrevaux and Biserte (1961) showed that colistin was an heterometric

decapeptide consisting of six L-DAB, two L-threonine, one L-leucine, one D-leucine and one MOA residue. It had the structure of a cyclopentapeptide with a side chain of five amino acids attached to a residue of L-DAB in the ring. The α-amino group of the DAB which terminated this side chain was bound to the carboxyl group of the fatty acid, MOA. Two possible arrangements of the cyclopentapeptide ring were proposed by them (35a and 35b).

```
L-DAB ──→L-Thr
  ↑             ╲  r
  │              L-DAB ←─α─ L-DAB ←── L-DAB ←── L-Leu ←── D-Leu ←── L-DAB ←── MOA
  │             ╱
L-Thr ←── L-DAB
```

(35a)

```
L-Thr ──→L-DAB
  ↑             ╲  r
  │              L-DAB ←─α─ L-DAB ←── L-DAB ←── L-Leu ←── D-Leu ←── L-DAB ←── MOA
  │             ╱
L-Thr ←── L-DAB
```

(35b)

In 1961, Suzuki et al. (cited by Suzuki et al., 1963a) found that commercial colistin could be separated into three components corresponding respectively to the colistins A, B and C designated by Oda et al. (1954). Colistins A and B had much stronger antibacterial activity than colistin C. The concentration of colistin C in commercial preparations was small and was frequently absent.

Morito (1962a) separated colistin A into colistins A_1 and A_2 by ion-exchange and countercurrent distribution methods. Colistins A_1 and A_2 were similar in all respects but differed in fatty acid content. Colistin A_1 contained MOA and colistin A_2, IOA. Morito (1962b) further showed that

both colistins had two leucine, one threonine, and five DAB residues and he proposed an identical structure to that reported by Suzuki (1957c).

Suzuki et al. (1963a) showed that both colistins A and B contained six L-DAB, two L-threonine, one L-leucine and one D-leucine. Furthermore, colistin A contained MOA and colistin B, IOA. However, they could not separate colistin A into colistins A_1 and A_2 as claimed by Morito (1962a). The molecular weight of the hydrochloride of both colistins A and B was about 1360.

Suzuki et al. (1963b) confirmed that colistin A had no free α-amino or carboxyl groups in its molecule. Based on the earlier data and on the results from partial acid hydrolysis, he proposed two possible structures (36a and 36b). Later, from information obtained from enzymatic studies on colistin A, the same authors (1963c) eliminated structure 36b.

$$
\begin{array}{c}
rNH_2 \\
| \\
rNH_2\text{-}L\text{-}DAB \longrightarrow L\text{-}DAB \longrightarrow L\text{-}Thr\text{-}OH \\
\uparrow \qquad\qquad\qquad \downarrow r \\
L\text{-}Leu \\
\uparrow \\
D\text{-}Leu \leftarrow L\text{-}DAB \leftarrow L\text{-}DAB \overset{\alpha}{\leftarrow} L\text{-}DAB \leftarrow L\text{-}Thr \leftarrow L\text{-}DAB \leftarrow MOA \\
| \qquad\qquad | \qquad | \qquad | \\
rNH_2 \qquad\qquad rNH_2 \quad OH \quad rNH_2
\end{array}
$$

(36a)

$$
\begin{array}{c}
rNH_2 \\
| \\
rNH_2\text{-}L\text{-}DAB \longrightarrow L\text{-}DAB \longrightarrow L\text{-}Thr\text{-}OH \\
\uparrow \qquad\qquad\qquad \downarrow \alpha \\
L\text{-}Leu \\
\uparrow \\
D\text{-}Leu \leftarrow L\text{-}DAB \leftarrow L\text{-}DAB \overset{r}{\leftarrow} L\text{-}DAB \leftarrow L\text{-}Thr \leftarrow L\text{-}DAB \leftarrow MOA \\
| \qquad\qquad | \qquad | \qquad | \\
rNH_2 \qquad\qquad rNH_2 \quad OH \quad rNH_2
\end{array}
$$

(36b)

Suzuki and Fujikawa (1964) further proved that colistin B had an identical structure to colistin A except that colistin B contained IOA instead of MOA.

Wilkinson (1963) made a comparison of polymyxin E and colistin and found that both antibiotics were indistinguishable on all the chemical and physical grounds. Wilkinson and Lowe (1963) found that polymyxins B_1, E_1 and colistin A had the same fatty acid residue and MOA, and that polymyxins B_2, E_2 and colistin B contained IOA. Wilkinson and Lowe (1964c) confirmed the structure of colistin A proposed by Suzuki (1963) and pointed out that both polymyxin E_1 and colistin A were identical. Wilkinson and Lowe (1964d) showed that polymyxin E_2 also had the same structure as colistin B. This was confirmed by Suzuki et al. (1965), and thus it is believed that poly- myxins E and colistin are identical substances.

D. <u>POLYMYXINS M</u>.

From a soil sample obtained in the vicinity of Moscow, Ilinskoya

and Rossovskaya (1958) isolated a strain of <u>B</u>. <u>polymyxa</u> which produced an

antibiotic substance similar to the polymyxins. This antibiotic was desig-

nated polymyxin M.

Khokhlov <u>et</u> <u>al</u>. (1960) noted that polymyxin M could be purified

by means of ion-exchange methods and its properties were similar to those

of other polymyxins. It melted with decomposition at 225 - 228°C, had a

rotation power $\left[\alpha\right]_D^{25}$= -48.1 (in water) and a molecular weight of 1185.

The hydrolysates of polymyxin M contained DAB, threonine, leucine, and fatty

acid residues, the fatty acid being identified as MOA. Stepanov <u>et</u> <u>al</u>. (1960)

showed that polymyxin M contained five free amino groups. Khokhlov and

Chih (1961) and Silaev <u>et</u> <u>al</u>. (1961a,b) showed that polymyxin M contained

one leucine, three threonine, six DAB and one MOA residue. No free α-amino

or α-carboxyl groups were detected.

Silaev <u>et</u> <u>al</u>. (1962) found that purified polymyxin M was homogeneous

as judged by countercurrent extraction. Yulikova <u>et</u> <u>al</u>. (1965) and Silaev

<u>et</u> <u>al</u>. (1965) proposed an identical structure for polymyxin M (37).

Leu $\longrightarrow$ DAB $\longrightarrow$ Thr
$\uparrow$ $\downarrow r$
DAB
$\uparrow$
Thr $\longleftarrow$ DAB $\longleftarrow$ DAB $\overset{\alpha}{\longleftarrow}$ DAB $\longleftarrow$ Thr $\longleftarrow$ DAB $\longleftarrow$ MOA
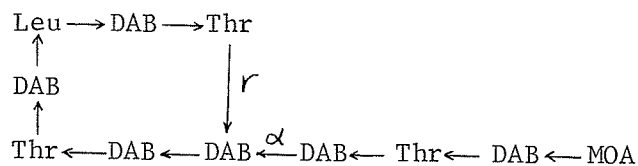
(37)

However, Wilkinson and Lowe (1966) found that purified polymyxin M, just like other polymyxins, was a mixture of closely related antibiotics and was separable into M_1 and M_2 by countercurrent distribution. Both had the same amino acid composition but differed from each other in fatty acid content. Polymyxin M_1 contained MOA and polymyxin M_2, IOA. They also noted that the amino acid composition, electrophoretic and chromatographic behavior, optical rotation and nephrotoxicity of polymyxins M were paralleled by those of polymyxins A. They believed that polymyxins M were identical with polymyxins A and should have the structure shown below (38).
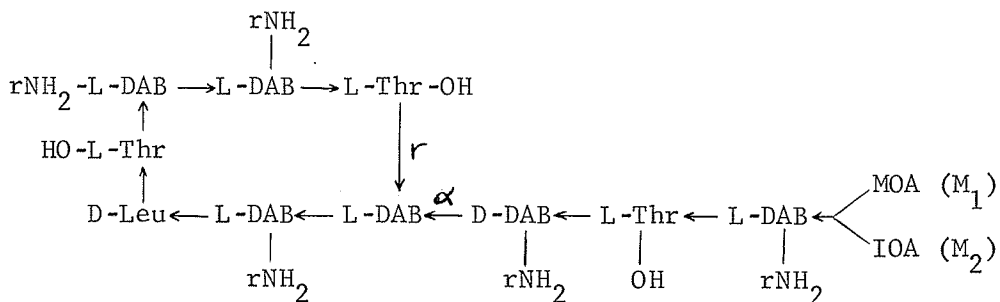
(38)

E. SUMMARY

All members of the polymyxin group of antibiotics are basic pep-
tides. They can be classified into two groups according to the solubilities
of their free bases in water: the water-soluble group containing polymyxins
A, C, D and M; and the water-insoluble group, polymyxins B and E, colistins
A and B, and circulins A and B.

Except for polymyxin C whose characterization is incomplete, each
antibiotic consists essentially of two closely related peptides. Each member
of the pair differs from the other only in the content of fatty acid,
having either (+)-6-methyloctanoic acid (MOA) or an isooctanoic acid (IOA).
All polymyxins share common amino acid residues, L-α,γ-diaminobutyric acid
(DAB) and L-threonine, but different individual pairs have their own specific
amino acids (Table IV).

Furthermore, all polymyxins have a common type of structure (39)
in which a chain of three amino acids is attached to a cyclic heptapeptide
ring via the α-amino group of the DAB residue. The terminal α-amino group
of the chain is acylated with one or the other of the two fatty acids (R).
Differences in individual polymyxins reside in the nature of the three amino
acid residues X, Y, and Z (Table V).

TABLE IV

SUMMARY OF THE AMINO ACID AND FATTY ACID COMPOSITION OF POLYMYXINS

polymyxin	DAB	Leu	Phe	Ileu	Thr	Ser	fatty acid
WATER-SOLUBLE GROUP							
$A_1 = M_1$	5L & 1D*	1D	-	-	3L	-	MOA
$A_2 = M_2$	5L & 1D	1D	-	-	3L	-	IOA
C	+**	-	-	-	+	-	MOA
D_1	5L	1D	-	-	3L	1D	MOA
D_2	5L	1D	-	-	3L	1D	IOA
WATER-INSOLUBLE GROUP							
B_1	6L	1L	1D	-	2L	-	MOA
B_2	6L	1L	1D	-	2L	-	IOA
E_1 = colistin A	6L	1L & 1D	-	-	2L	-	MOA
E_2 = colistin B	6L	1L & 1D	-	-	2L	-	IOA
circulin A	6L	1D	-	1L	2L	-	MOA
circulin B	6L	1D	-	1L	2L	-	IOA

* 5L & 1D means five L-isomers and one D-isomer.

** + signifies present, and -, absent.

$$
\begin{array}{c}
\overset{\text{rNH}_2}{\underset{|}{}} \\
\text{rNH}_2\text{-L-DAB} \longrightarrow \text{L-DAB} \longrightarrow \text{L-Thr-OH}
\end{array}
$$
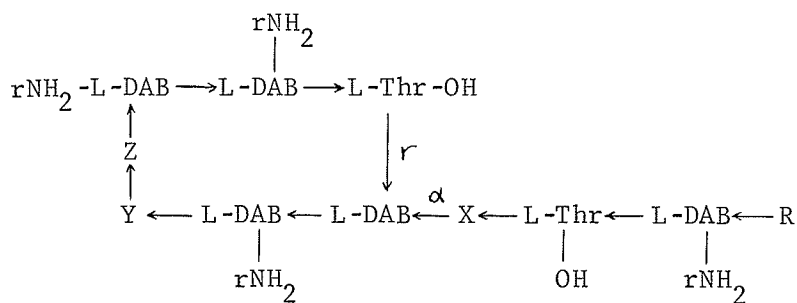
(39)

TABLE V

STRUCTURE OF THE MEMBERS OF THE POLYMYXIN GROUP OF ANTIBIOTICS

Polymyxin	R	X	Y	Z
$A_1 = M_1$	MOA	D-DAB	D-Leu	L-Thr
$A_2 = M_2$	IOA	D-DAB	D-Leu	L-Thr
B_1	MOA	L-DAB	D-Phe	L-Leu
B_2	IOA	L-DAB	D-Phe	L-Leu
D_1	MOA	D-Ser	D-Leu	L-Thr
D_2	IOA	D-Ser	D-Leu	L-Thr
E_1 = colistin A	MOA	L-DAB	D-Leu	L-Leu
E_2 = colistin B	IOA	L-DAB	D-Leu	L-Leu
Circulin A	MOA	L-DAB	D-Leu	L-Ileu
Circulin B	IOA	L-DAB	D-Leu	L-Ileu

IX. <u>POLYPEPTIN</u>.

(1). Producing strain, nomenclature and properties of polypeptin:

McLeod (1948) isolated a bacterium, <u>B</u>. <u>krzemieniewski</u> M-14 from

soil, which produced an antibiotic. The antibiotic was named "circulin".

Since this name was also applied to another antibiotic produced by the

<u>B</u>. <u>circulans</u> Q-19 strain discovered by Murray and Tatraut (1948), this

antibiotic was renamed "polypeptin" (Garson <u>et</u> <u>al</u>., 1949).

McLeod (1948) showed that polypeptin could be autoclaved for 30

min. in acid or neutral solutions but not in alkaline without denaturation.

It was active against Gram-positive and Gram-negative bacteria as well as

fungi and was extremely toxic to mice. Polypeptin also caused hemolysis

of the erythrocytes of man, rabbit, sheep and mouse.

Howell (1950) noted that polypeptin sulphate was soluble in water,

alcohols, and pyridine but insoluble in organic solvents, with polypeptin

acetate having higher solubility than the sulphate. The sulphate melted

at 230°C with decomposition, showed specific rotation $\left[\alpha\right]_{D}^{20}$= -93.3° (c, 3%

in 70% isopropanol) and had an isoelectric point at pH 11. It was not in-

activated by pepsin, trypsin, hydrogen sulfide or ferric chloride.

(2). Isolation and structural studies:

Howell (1950) isolated polypeptin by acetone extraction from

acidified culture broth, removal of impurities from the extract by ammonium

sulphate precipitation and reprecipitation by chloroform. This precipitate

was dissolved in warm ethanol and crystalline polypeptin sulphate was obtained

from this solution when it cooled. Recrystallization was performed in either methanolic or ethanolic solutions. The crystalline polypeptin sulphate still appeared to be a mixture with the major component approaching 90%.

In countercurrent distribution studies, Hausmann and Craig (1952) found that crystalline polypeptin sulphate contained at least two components, the major one consisting of 80% of the mixture in contrast with the results of Howell (1950). The major component contained three L-DAB, one L-threonine, one D-valine, one L-isoleucine, two L-leucine, one L-phenylalanine and one unknown fatty acid with the formula $C_8H_{13}O_2$. The molecular weight of the free base was 1145 with the empirical formula $C_{56}H_{96}O_{13}N_{12}$.

The detailed structure of polypeptin still remains unknown.

X. SERRATAMOLIDE.

On media containing glycerol and peptone, Wasserman et al. (1961, 1962) noted that certain strains of Serratia marcescens produced a neutral colorless compound, which they named "serratamolide". The antibiotic activity of serratamolide against Gram-positive organisms was similar to that of depsipeptides isolated from Fusarium. Hydrolyzed under mild alkaline conditions, serratamolide converted to serratamic acid (40) which had been isolated from several members of the Serratia group by Cartwright (1955).

Serratamolide was isolated from liquid cultures by methylene chloride extraction or from solid media by acetone extraction. After the removal of the solvent by distillation in vacuo, the active residue was

$$\underset{\substack{| \\ \text{OH}}}{} CH_3(CH_2)_6CH\text{-}CH_2CONHCH \overset{\text{COOH}}{\underset{\text{CH}_2\text{OH}}{<}}$$
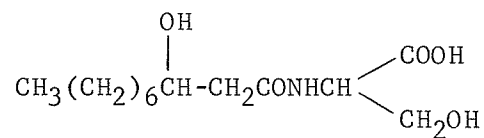
(40)

extracted with boiling ethanol. By a process of fractional crystallization in hot and cold ethanol, crystalline serratamolide was obtained. Recrystallization was effected from absolute ethanol (Wasserman et al., 1961, 1962).

Wasserman et al. (1961, 1962) showed that serratamolide was optically active where $[\alpha]_D^{25} = +4.8$ (ethanol), containing two free hydroxyl and two C-methyl groups but no amino nitrogen or methoxyl. It had a melting point 159 - 160°C. No absorption above 200 nm was found except for end-absorption above 250 nm. Elementary analysis showed that serratamolide's molecular formula was $C_{26}H_{46}O_8N_2$. In acid hydrolysis, serine and an oily acid-insoluble component, 3-hydrodecanoic acid, were found.

Based on the information obtained from infrared spectrum analysis, and partial and total hydrolyses, Wasserman et al. (1962) suggested the structure of serratamolide was as shown below (41). Shemyakin et al. (1964) confirmed this by synthesis of the diacetyl derivative.

```
  ┌─→ L-Serine ──→ D-3-hydroxydecanoic acid ──┐
  │                                            │
  └── D-3-hydroxydecanoic acid ←── L-Serine ←──┘
```
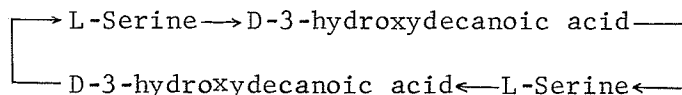
(41)

XI. <u>SUBTILINS</u>.

(1). Producing strain and properties of subtilins:

Jansen and Hirschmann (1944) found a strain of <u>B</u>. <u>subtilis</u> which produced a polypeptide antibacterial substance which they designated "subtilin". Subtilin was suspected to be a mixture of antibiotics rather than a single active entity. These authors showed that subtilin was stable in acid but easily inactivated by alkali. At pH 2.5, it withstood autoclaving at 115°C for 10 min. The antibiotic was sensitive to light and was freely dialysable.

Salle and Jann (1945) found that subtilin was active against Gram-positive bacteria, fungi and <u>Neisseria</u>. Anderson <u>et</u> <u>al</u>. (1946) stated that subtilin had no immediate effect on erythrocytes but caused hemolysis after 24 hr. incubation at 4°C. However, Salle and Jann (1947) showed that subtilin was not toxic to experimental animals.

Dimick <u>et</u> <u>al</u>. (1947a,b) showed that subtilin was soluble in acid and wet alcohols but insoluble in alkali and all dry organic solvents. Its solubility in acid solutions decreased markedly in the presence of salts. Both trypsin and pepsin inactivated the antibiotic.

Hassell(1948) noted that subtilin gave positive responses to the ninhydrin reagent, Ehrlich's reagent for indole derivatives and Folin-Denis reagent for phenol. Brink <u>et</u> <u>al</u>. (1951) found that an aqueous solution of subtilin possessed no maximum absorption in the ultraviolet region but had a shoulder at 275 - 280 nm and an inflection point at 285 nm. Specific rotation was $[\alpha]_D^{25} = -36°$ (c, 0.865 in water).

(2). Isolation:

Jansen and Hirschmann (1944) acidified whole culture fluid with HCl and the resulting precipitate was ground and extracted with warm ethanol. The active principle was then salted out from ethanolic solution by the addition of NaCl. The antibiotic was crystallized from the ethanol and NaCl solution.

Dimick et al. (1947b) isolated subtilin through n-butanol extraction from acidified culture fluid. After the addition of petroleum ether, the n-butanolic solution was extracted with acetic acid. The active principle in the acetic acid was salted out by NaCl. Impurities present were removed by ethanol and the active residue left was dissolved in acetic acid repeatedly salted out by NaCl, deionized by ion-exchangers and finally dried. A similar but simplified method was used by Fevold et al. (1948) for the isolation of the antibiotic.

Brink et al. (1951) found that subtilin concentrates from the above methods were essentially homogeneous when they were subjected to countercurrent distribution. Stracher and Craig (1959), however, found subtilin to be a mixture of peptide antibiotics, and named the major component subtilin A. Alderton and Snell (1959) noted that subtilin contained at least six active components separable by electrophoretic methods. The major one was identical to Stracher and Craig's subtilin A.

(3). Structural studies:

Jansen and Hirschmann (1944) showed crystalline subtilin to have an elemental analysis of: C, 54.4; H, 7.7; and N, 14.5%. It was predicted

that tyrosine would be present. Dimick et al. (1947b) found that subtilin

contained 15.8% total N or 11.0% amino-N and 4.2% sulphur. Titrating with

lithium hydroxide, Brink et al. (1951) gave a value of 3340 for the molecu-

lar weight of subtilin.

Lewis and Snell (1951) showed subtilin was composed of two gly-

cine, one alanine, one leucine, one isoleucine, one proline, one phenyl-

alanine, one tryptophan, three lysine, three glutamic acid, three lanthio-

nine-like substances, four unknown $C_7H_{12}O_3N_2S$, and five amide residues.

The average minimum molecular weight was 3420 but might possibly be 7000.

Aspartic acid and glycine were not in D-configuration and aliphatic acids

were not present. Furthermore, the subtilin molecule might be a ring struc-

ture. By isolation, Alderton and Fevold (1951) showed the lanthionine-like

substance reported by Lewis and Snell (1951) to be mesolanthionine. Carson

(1952) noted that the unknown C_7 substance referred to above was an unknown

diamino-dicarboxyl sulfur acid with the formula $C_5H_8S(NH_2)_2(COOH)_2$. He

also detected the presence of another unknown unnatural amino acid which

contributed a free α-amino group to the molecule. Other free amino groups

found in the subtilin molecule were contributed by the δ-amino group of

lysine and one of the two amino groups of the diamino-dicarboxyl sulfur

amino acids. Alderton (1953) identified this C_7 unknown compound as methyl-

$$\text{HOOC-CH-CH}_2\text{-S-CH-CH-COOH}$$

with CH_3 branch on the third carbon and NH_2 groups below the first and fourth carbons
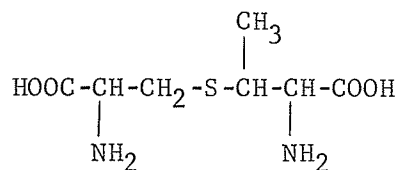
(42)

lanthionine (42).

Stracher and Craig (1959) found that subtilin A contained all the amino acid residues reported by Lewis and Snell (1951) with an additional one discovered by Carson (1952) which was identified as sarcosine (N-methyl-glycine). Subtilin possessed three free lysine δ-amino groups and one sarcosine α-amino group. The subtilin A molecule contained rings consisting in part of five sulphur atoms with a side chain comprised of Sar-(Glu.Leu. Sar.Asp)-Lan-. Sarcosine was the N-terminal substance and lanthionine served as the bridge-head for attachment of the side chain. One of the five rings in the molecule must be completely peptidic where the rest might be peptide chains joined by the thioester linkages of the four β-methyl-lanthionines present. Subtilin A was believed to have an empirical formula $C_{144}H_{226}N_{38}O_{37}S_5$ and a molecular weight 3242. The detailed structure of subtilin A remains unknown and little information regarding other subtilins is available at the present time.

XII. TYROTHRICIN, TYROCIDINES AND GRAMICIDINS.

A. TYROTHRICIN.

(1). Producing strains:

Dubos (1939a,b) isolated a Gram-positive Bacillus, capable of lysing living staphylococci, from soil to which he had repeatedly added mineral media and suspensions of staphylococci, pneumococci and group A hemolytic streptococci over a period of two years. Dubos and Hotchkiss (1941) identified this organism as B. brevis BG strain. They also isolated several other strains of the same species from cheese, soil, manure and sewage all strains of which were capable of producing varying amounts of the same antibacterial agent.

Hoogerheide (1940a,b) as well as Strokes and Woodward (1942) also independently isolated from soil other strains of B. brevis which produced a similar substance.

(2). Isolation:

Dubos (1939b) found that the active principle in the culture medium was precipitable at pH 4.5 and the precipitate was soluble in neutral buffers. Dubos and Cattaneo (1939) noted that acidic acetone removed the impurities from the resulting solution. The active material left in the acetone solution was precipitable by the addition of a large volume of salt solution. This precipitate was no longer soluble in water but readily so in acetone or ethanol. Hotchkiss and Dubos (1940a) obtained three crystal-

line preparations from this precipitate with further treatment. Two acid substances were isolated from the alcohol solution of the precipitate by precipitation with ether and fractional crystallization of the dried precipitate from hot ethanol. One of these two acids which was least soluble in ethanol was designated graminic acid, and the other, larger in amount and more soluble in alcohol, was called gramidic acid. The third substance, named gramicidin, which was soluble in alcohol but insoluble in ether, was obtained by repeated precipitation from alcohol through the addition of ether. Later, however, Hotchkiss and Dubos (1940c) discovered that graminic acid was actually a hydrochloride salt of a weak base and renamed it tyrocidine hydrochloride. At the same time, they also noted that gramidic acid was a mixture of free tyrocidine and its hydrochloride salt. Therefore, the name gramidic acid was abandoned. They also used the name "tyrothricin" for the crude bactericidal agent (a mixture of tyrocidine and gramicidin) which had been isolated from B. brevis.

B. TYROCIDINES.

    (1). Isolation:

        Hotchkiss and Dubos (1940b) obtained a highly purified crystalline tyrocidine preparation by repeated recrystallization from acidified absolute methanol. In countercurrent distribution studies, Battersby and Craig (1952a) found that crystalline tyrocidine was a mixture of three active major components and a number of minor peptides. They named the three major ones tyrocidine A, B and C according to the order of their partition ratio in countercurrent distribution.

(2). Properties:

Hotchkiss and Dubos (1940c) showed that crystalline tyrocidine hydrochloride melted at 237 - 239°C with decomposition and had an optical rotation $[\alpha]_D^{25} = -102°$ (c, 1.00, 95% ethanol). Hotchkiss and Dubos (1941) noted that tyrocidine was resistant to the action of common proteolytic enzymes. It was soluble in methanol, ethanol, acetic acid and pyridine, sparingly soluble in water, acetone and dioxane, and insoluble in ether and hydrocarbon solvents. Aqueous solutions of tyrocidine had low surface tension and behaved as a detergent solution. Tyrocidine precipitated a number of soluble proteins as do some of the cationic detergents.

Hotchkiss (1941) noted tyrocidine reacted slowly with nitrous acid, and that it had a major dissociation point at pH 8.6 and a secondary one above pH 10.

Dubos (1941) found that tyrocidine was active against both Gram-positive and Gram-negative species, and that it lysed cells which were sensitive to it. Its action, however, was inhibited by many tissue constituents.

(3). Structural studies:

Hotchkiss (1941) found that the hydrolysates of tyrocidine contained tyrosine, tryptophan, aspartic acid and other dibasic amino acid residues. From determination of the chloride content, the minimal molecular weight for tyrocidine hydrochloride was 1285 $\pm$ 50 and the empirical formula might be $C_{63}H_{83}N_{13}O_{13}HCl$. He believed that tyrocidine was a peptide constructed in such a way that two basic amino, three amide and one

carboxyl or acidic phenolic group were free.

Christensen et al. (1941) reported that tyrocidine's elementary analysis was: C, 59.7; H, 6.9; N, 14.5; and Cl, 2.52% with dissociating groups at pH 9.0 and above pH 11. Its minimum molecular weight was 2700 and it contained tryptophan, tyrosine, alanine, phenylalanine, ammonia and dibasic amino acid residues.

Gorden et al. (1943b) found phenylalanine, leucine, proline, valine, tyrosine, ornithine, glutamic acid, tryptophan and aspartic acid residues in acid hydrolysates of tyrocidine. The phenylalanine isolated from hydrolysates had mainly the D-configuration and the other amino acids were all L-isomers. Christensen et al. (1945) confirmed the presence of L-aspartic acid, L-valine, L-leucine, and L-tryptophan by microbiological determination assay.

(i). Structure of tyrocidine A:

Battersby and Craig (1952a) separated three major components of tyrocidine by countercurrent distribution and found that all three peptides contained phenylalanine, leucine, tyrosine, valine, proline, ornithine, glutamic acid, and aspartic acid as well as ammonia residues in their hydrolysates. In addition, tyrocidines B and C contained tryptophan. There were no free α-amino or α-carboxyl groups in any of the three components. The γ-carboxyl group of aspartic acid and the δ-carboxyl group of glutamic acid were conjugated with amide groups which converted to ammonia during acid hydrolysis. The only free functional groups in the molecule were the ϵ-amino group of ornithine and the hydroxyl group of tyrosine.

In tyrocidine A, Battersby and Craig (1952a) found that all

amino acid residues were L-isomers except that of phenylalanine which appeared to be a mixture of two moles of the D- with one mole of the L-isomers. They believed that the amino acid residue formula of tyrocidine A could be written as:

(D-Phe)$_2$(L-Phe)(L-Val)(L-Tyr)(L-Leu)(L-Pro)(L-Orn)(L-Asp-NH$_2$)(L-Glu-NH$_2$)

which corresponded to the empirical formula $C_{66}H_{86}O_{13}N_{13}$. Battersby and Craig (1952b) found tyrocidine A to have a minimal molecular weight of 1270.

Paladini and Craig (1954) proposed a structural formula for tyrocidine A (43) which was confirmed by Ohno and Izumiya (1966) by synthesis.

$$L\text{-Val} \longrightarrow L\text{-Orn} \longrightarrow L\text{-Leu} \longrightarrow D\text{-Phe} \longrightarrow L\text{-Pro}$$
$$\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow$$
$$L\text{-Tyr} \longleftarrow L\text{-Glu} \longleftarrow L\text{-Asp} \longleftarrow D\text{-Phe} \longleftarrow L\text{-Phe}$$
$$\vert \qquad\qquad \vert$$
$$\delta\,NH_2 \qquad rNH_2$$

(43)

(ii). Structure of tyrocidine B:

King and Craig (1955a) found that tyrocidine B was also a cyclopeptide having an amino acid formula:

(L-Orn)(L-Leu)(D-Phe)$_2$(L-Pro)(L-Try)(L-Asp-NH$_2$)(L-Glu-NH$_2$)(L-Tyr)(L-Val).

Its empirical formula was $C_{68}H_{88}O_{13}N_{14}$ with a molecular weight of 1346. King and Craig (1955b) proposed a structural formula for tyrocidine B which differed from tyrocidine A only in that L-phenylalanine was replaced with an L-tryptophan residue (44).

```
L-Val ——→ L-Orn ——→ L-Leu ——→ D-Phe ——→ L-Pro
   ↑                                        │
   │                                        ↓
L-Tyr ←—— L-Glu ←—— L-Asp ←—— D-Phe ←—— L-Try
           │           │
          δNH₂        rNH₂
```

$$\delta NH_2 \qquad rNH_2$$

(44)

(iii). Structure of tyrocidine C:

Ruttenberg et al. (1965a) noted that tyrocidine C was also a cyclic decapeptide with a structure similar to the other tyrocidines. Tyrocidine C differed from tyrocidine B by substitution of one of the D-phenylalanine residues with a D-tryptophan (45).


```
L-Val ——→ L-Orn ——→ L-Leu ——→ D-Phe ——→ L-Pro
   ↑                                        │
   │                                        ↓
L-Tyr ←—— L-Glu ←—— L-Asp ←—— D-Try ←—— L-Try
           │           │
          δNH₂        rNH₂
```
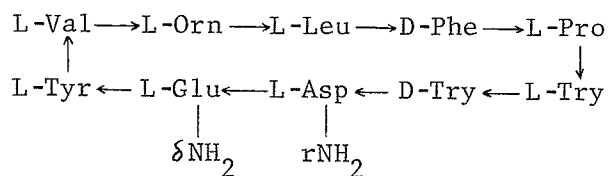
(45).

C. GRAMICIDINS.

   (1). Purification:

   Hotchkiss and Dubos (1940c) showed that gramicidin could be obtained directly by extraction of the alcoholic solution of tyrothricin with acetone. Further purification was effected by repeated crystallization from acetone.

   Gregory and Craig (1948) found that crystalline gramicidin was a mixture of chemically related substances. They obtained two crystalline components, together with a mixture, after countercurrent distribution. The first component, which was less soluble in organic solvents, was named gramicidin A and the other, gramicidin B. Craig et al. (1949a) showed by countercurrent distribution that gramicidin contained at least three major components, gramicidin A, B and C. Ramachandran (1963) separated a new component, gramicidin D, from gramicidin A.

   (2). Properties of gramicidins:

   Hotchkiss and Dubos (1940a) found that crystalline gramicidin hydrochloride had a melting point of 238 - 239°C and an optical rotation $\left[\alpha\right]_D^{25} = +5°$ (95% ethanol). Hotchkiss and Dubos (1941) noted that, like tyrocidine hydrochloride, gramicidin was also resistant to the action of common proteolytic enzymes. It was soluble in lower alcohols, acetic acid and pyridine, moderately soluble in dry acetone and dioxane and insoluble in water, ether, hydrocarbons and chlorinated hydrocarbons.

   Christensen et al. (1941) showed that gramicidin lost no activity

during two hrs. of boiling in alcohol, little during 15 hrs. of standing

in a 50% alcoholic solution of 1N NaOH. However, heat treatment in the

presence of diluted HCl or NaOH alcoholic solutions completely destroyed

its activity within two hrs.

Tishler et al.(1941) showed that gramicidin had principle absorp-

tion maxima at 281.5, 290 and 269 nm. It responded positively to common

protein tests such as the biuret, xanthoproteic and the Hopkin-Cole reaction.

Gramicidin was precipitated by some protein precipitants such as flavinic,

sifiamic and picric acids.

Dubos (1941) showed that gramicidin was completely inactive

against Gram-negative bacilli, moderately active against meningococci and

gonococci, and highly inhibitory to all Gram-positive strains. It never

caused lysis even in the most susceptible strains. The effect of gramici-

din was slightly inhibited by serum, tissue extracts or peptone but the

phospholipid, phosphatidyl serine was strongly inhibitory.

Dubos and Hotchkiss (1941) found that gramicidin caused a slow

hemolysis of erythrocytes but small quantities of glucose prevented this

action.

(3). Structural studies:

(i). Preliminary studies:

Hotchkiss and Dubos (1940b) showed that gramicidin consisted of: C, 62.7; H, 7.5; and N, 13.9% with an empirical formula $C_{74}H_{106}N_{14}O_{14}$. The molecular weight was about 1400. Gramicidin contained neither free basic nor acidic groups. However, after hydrolysis, it liberated a total of 11 moles non-volatile acid, 11 moles of basic groups, 10 moles of amino acids (including 2.4 moles of tryptophan) and a mole of 14-16 carbon-saturated aliphatic acid. Methoxyl and acetyl groups, histidine, arginine, tyrosine and ammonia were all absent in gramicidin. Furthermore, nearly one-half of the amino acid residues in gramicidin were D-isomers. Lipmann et al. (1941) indicated that 45% of the amino acids in gramicidin hydrolysates had the D-configuration.

Hotchkiss and Dubos (1941) further showed that gramicidin contained no sulphur, halogen or phosphorus in its molecule. Hotchkiss (1941) showed that gramicidin was a polypeptide containing 14.8% nitrogen. L-tryptophan, D-leucine, alanine, and an unknown hydroxyamino acid residue were present. The molecular weight of gramicidin was in the range of 1250 to 1550 and the empirical formula might either be $C_{55}H_{79}N_{11}O_{10}$ or $C_{74}H_{105}N_{15}O_{13}$. Independently, Christensen et al. (1941) showed that gramicidin's elementary analysis was C, 62.5; H, 7.5 and N, 14.6%, similar to the results of Hotchkiss and Dubos above. No sulphur or phosphorus was present and they showed that tryptophan, leucine, alanine and the unknown hydroxyl amino acid content of gramicidin had an approximate molecular ratio of 2:2;(2 or 3):1.

Christensen (1943) showed the presence of DL-valine residues in

all gramicidins. Gordon et al. (1943a) suggested that the gramicidin

molecule contained 24 amino acid residues distributed as: six D-leucine,

five L-tryptophan, five DL-valine, two L-alanine, two glycine and two of

the unknown hydroxylamino residues. Synge (1944a) showed that the five

DL-valine residues were distributed as two L-valine and three D-valine.

Synge (1944b) identified the unknown hydroxylamino compound as 2-amino-

ethan-1-ol, $(NH_2-CH_2-CH_2-OH)$.

Synge (1945a) believed that the gramicidin molecule contained

six L-tryptophan, six D-leucine, three D-valine, three L-alanine, two L-

valine, two glycine and two ethanolamine residues in contrast with the

previous results. The ethanolamine residues were involved in a peptide

linkage through their amino groups and their hydroxyl groups were free.

However, James and Synge (1951) later suggested that the hydroxyl groups

of ethanolamine might not be free. They also said that gramicidin con-

tained neither volatile and non-volatile fatty acid nor ether-extractable

carboxylic acid. The ureido structure was also thought to be absent.

Craig et al. (1949a) found that all three major components,

gramicidins A, B and C contained glycine, alanine, leucine and tryptophan,

but gramicidin B had phenylalanine and gramicidin C, tyrosine in addition.

Okuda et al. (1962) showed that gramicidin A contained one glycine, two

L-alanine, four DL-valine, four D-leucine, four L-tryptophan and one

ethanolamine. Gramicidins B and C differed from gramicidin A in containing

L-phenylalanine and L-tyrosine respectively in place of one or more L-tryp-

tophan.

Ramachandran (1963) showed that all gramicidins contained glycine,

alanine, valine, leucine, tryptophan, and ethanolamine residues. However, gramicidin A and gramicidin B differed from each other in tryptophan content. Gramicidin B contained additional tyrosine and gramicidin C, additional phenylalanine. Gramicidin D had one or two isoleucine residues which replaced one or two of four residues of valine present in gramicidin A (Table VI).

TABLE VI

AMINO ACID COMPOSITION OF GRAMICIDINS PROPOSED BY RAMACHANDRAN (1963)

Gramicidin	K^*	Gly	Ala	Val	Leu	Try	Ileu	Phe	Tyr	Ethanolamine
A	0.70	1	2	4	4	6	0	0	0	1
B	0.32	1	2	4	4	3-4	1	1	0	1
C	1.41	1	2	4	4	6	0	0	1	1
D	0.59	1	2	3	4	6	1	0	0	1

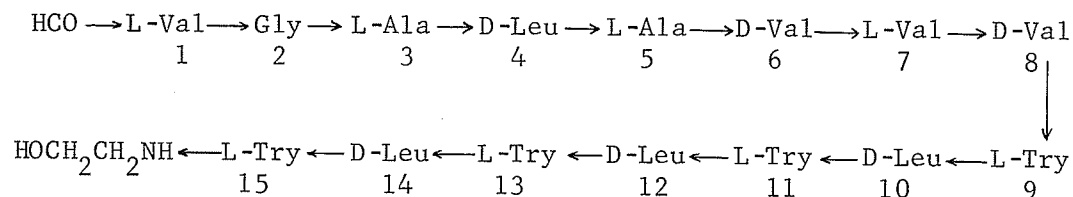K^* value is the partition ratio in countercurrent distribution.

(ii). Present concepts of the structure of gramicidin A:

Ishii and Witkop (1963) showed that gramicidin A was heterogeneous by means of their isolation of valine-gramicidin A and isoleucine-gramicidin A. Valine-gramicidin A had four L-tryptophan, four D-leucine, two D-valine, two L-valine, one glycin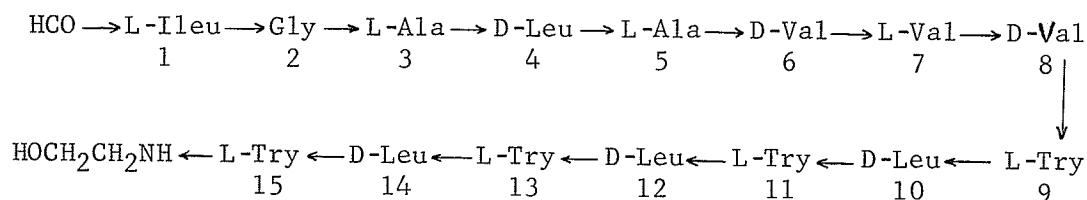e and one ethanolamine residue. Isoleucine-gramicidin A had similar constituents except that one of the two L-valines in valine-gramicidin A was substituted by one L-isoleucine.

Sarges and Witkop (1964a) discovered that the gramicidins A con-

tained an N-formyl group. They later showed (1964b) both gramicidins A

to be linear N-acylated decapentapeptide ethanolamines. Sarges and Witkop

(1965a) proposed the following structures for gramicidins A (46a,b) which

were found to be correct by chemical synthesis (Sarges and Witkop, 1965b).
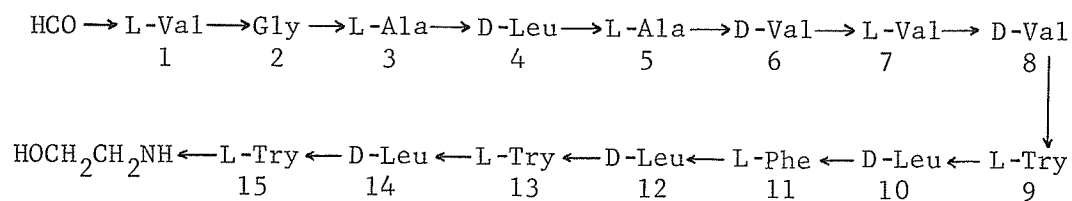
$$
\begin{array}{ccccccccc}
\text{HCO} \longrightarrow \text{L-Val} \longrightarrow \text{Gly} \longrightarrow \text{L-Ala} \longrightarrow \text{D-Leu} \longrightarrow \text{L-Ala} \longrightarrow \text{D-Val} \longrightarrow \text{L-Val} \longrightarrow \text{D-Val} \\
1 \qquad\quad 2 \qquad\quad 3 \qquad\quad 4 \qquad\quad 5 \qquad\quad 6 \qquad\quad 7 \qquad\quad 8 \\
\downarrow \\
\text{HOCH}_2\text{CH}_2\text{NH} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \\
15 \qquad\quad 14 \qquad\quad 13 \qquad\quad 12 \qquad\quad 11 \qquad\quad 10 \qquad\quad 9
\end{array}
$$

(46a valine-gramicidin A)

$$
\begin{array}{ccccccccc}
\text{HCO} \longrightarrow \text{L-Ileu} \longrightarrow \text{Gly} \longrightarrow \text{L-Ala} \longrightarrow \text{D-Leu} \longrightarrow \text{L-Ala} \longrightarrow \text{D-Val} \longrightarrow \text{L-Val} \longrightarrow \text{D-Val} \\
1 \qquad\quad 2 \qquad\quad 3 \qquad\quad 4 \qquad\quad 5 \qquad\quad 6 \qquad\quad 7 \qquad\quad 8 \\
\downarrow \\
\text{HOCH}_2\text{CH}_2\text{NH} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \longleftarrow \text{D-Leu} \longleftarrow \text{L-Try} \\
15 \qquad\quad 14 \qquad\quad 13 \qquad\quad 12 \qquad\quad 11 \qquad\quad 10 \qquad\quad 9
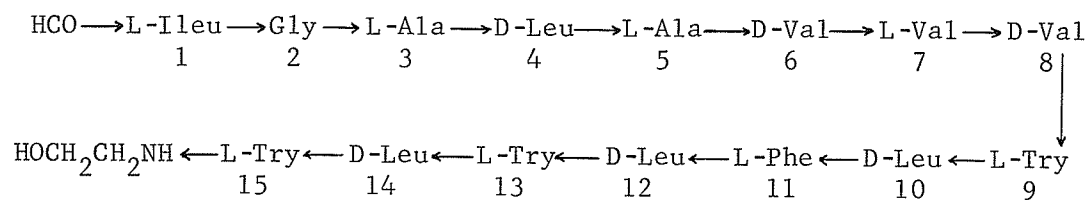\end{array}
$$

(46b isoleucine-gramicidin A)

(iii). Present concepts of structure of gramicidin B:

Sarges and Witkop (1965c) showed that gramicidin B, like gramici-

din A, was mixture of valine- and isoleucine-gramicidins B. The N-formyl

group was also present. Gramicidins B were linear N-acylated decapenta-

peptide ethanolamines with similar amino acid compositions except that

tryptophan at position 11 of gramicidin A was replaced by L-phenylalanine
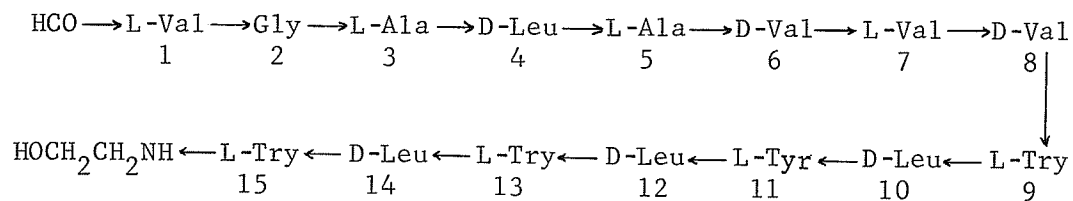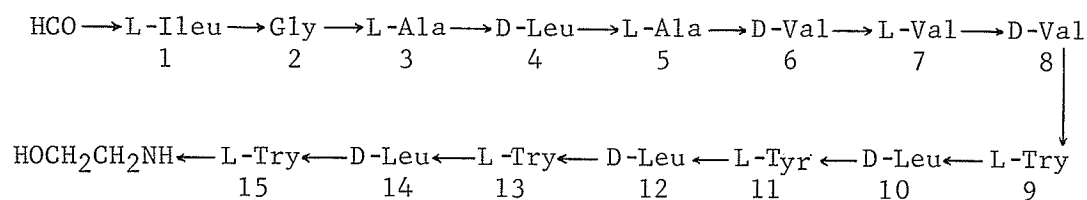
(47a,b).

HCO → L-Val → Gly → L-Ala → D-Leu → L-Ala → D-Val → L-Val → D-Val
        1   2   3   4   5   6   7   8

HOCH$_2$CH$_2$NH ← L-Try ← D-Leu ← L-Try ← D-Leu ← L-Phe ← D-Leu ← L-Try
                  15   14   13   12   11   10   9

(47a valine-gramicidin B)

HCO → L-Ileu → Gly → L-Ala → D-Leu → L-Ala → D-Val → L-Val → D-Val
        1   2   3   4   5   6   7   8

HOCH$_2$CH$_2$NH ← L-Try ← D-Leu ← L-Try ← D-Leu ← L-Phe ← D-Leu ← L-Try
                  15   14   13   12   11   10   9

(47b isoleucine-gramicidin B)

(iv). Structure of gramicidin C:

    Sarges and Witkop (1965d) went on to show that gramicidin C was also a mixture of valine and isoleucine-gramicidins C. Gramicidins C were analogous to gramicidins A and B, except for the amino acid at position 11 being replaced by L-tyrosine instead of tryptophan or phenylalanine (48a,b).

HCO → L-Val → Gly → L-Ala → D-Leu → L-Ala → D-Val → L-Val → D-Val
        1   2   3   4   5   6   7   8

HOCH$_2$CH$_2$NH ← L-Try ← D-Leu ← L-Try ← D-Leu ← L-Tyr ← D-Leu ← L-Try
                  15   14   13   12   11   10   9

(48a valine-gramicidin C)

HCO →L-Ileu →Gly →L-Ala →D-Leu →L-Ala →D-Val →L-Val →D-Val
        1   2   3   4   5   6   7   8

HOCH$_2$CH$_2$NH ← L-Try ← D-Leu ← L-Try ← D-Leu ← L-Tyr ← D-Leu ← L-Try
                15   14   13   12   11   10   9

(48b isoleucine-gramicidin C)

D. GRAMICIDIN S.

(1). Producing strain and properties of the antibiotic:

Gause and Brazhnikova (1944a) isolated a bacterium, B. brevis

which they labelled the "Gause-Brazhnikova" strain, from Russian soil. It

produced a bactericidal polypeptide which was called "Soviet gramicidin"

or gramicidin S.

Gause and Brazhnikova (1944b) showed that gramicidin S was thermo-

stable, withstanding heating to 160°C in dry crystalline form or autoclaving

at 120°C for 30 min. in solution or boiling in 1% alcoholic HCl. Belozersky

and Paschina (1944) noted that gramicidin was insoluble in water, acids,

or alkali, but very soluble in alcohol or acetone. It was slowly dialyzable.

The crystalline form of gramicidin S melted at 268 - 270°C and contained

13% of nitrogen. The biuret reaction was positive but the xanthoproteic

and Millon's reactions were negative. Its activity was directed against

both Gram-positive and Gram-negative bacteria.

(2). Isolation:

Gause and Brazhnikova (1944a) showed that gramicidin S could be isolated by acidification of the culture fluid. The resulting precipitate was dissolved in ethanol and the impurities were removed by ether extraction. The alcoholic solution was then concentrated, mixed with activated charcoal, and filtered. The pure gramicidin S would crystallize from this filtrate in the cold. Recrystallization was effected in an acetone-water mixture in the presence of charcoal.

(3). Structural studies:

Belozersky and Paschina (1944) noted the presence of ornithine, proline, and leucine in the hydrolysates of gramicidin S. In the molecule, the free amino group was contributed by an α-amino residue and the δ-amino group of ornithine was bound.

Synge (1945b) showed that gramicidin S was a polypeptide compound with an equal molar ratio of L-valine, L-proline, L-leucine, L-ornithine and D-phenylalanine. It possessed only one basic amino group and no free carboxyl group. In light of the evidence of a high optical rotation value of the molecule, Synge (1945b) believed that gramicidin S was a cyclopeptide. Sanger (1946) also expressed the same view because he found only the δ-amino group of ornithine with the FDNB method.

Consden et al. (1946, 1947) found that gramicidin S had the sequence -α-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl- which occurred once or repeatedly in a cyclic peptide chain. According to Crowfoet and Schmidt (cited by Consden et al., 1946), who examined a series

of derivatives of gramicidin S by X-ray crystallography, gramicidin S had either a simple pentapeptide structure or was a decapeptide which had crystallographic two-fold symmetry. The two-fold symmetry could arise as a result of the repetition of the peptide sequence in a cyclic decapeptide.

The cyclodecapeptide structure for gramicidin S (49) was established through molecular weight determination by the partition substitution method (Battersby and Craig, 1951), chemical synthesis (Schwyzer and Sieber, 1956) and X-ray studies (Schmidt et al., 1957).

$$
\begin{array}{ccccc}
\text{L-Val} \longrightarrow & \text{L-Orn} \longrightarrow & \text{L-Leu} \longrightarrow & \text{D-Phe} \longrightarrow & \text{L-Pro} \\
\uparrow & & & & \downarrow \\
\text{L-Pro} \longleftarrow & \text{D-Phe} \longleftarrow & \text{L-Leu} \longleftarrow & \text{L-Orn} \longleftarrow & \text{L-Val}
\end{array}
$$
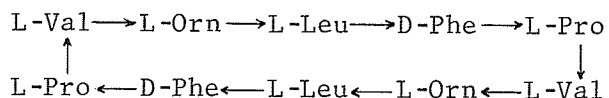
(49)

Since gramicidin S is a basic cyclodecapeptide as are the tyrocidines and has a pentapeptide sequence Val-Orn-Leu-Phe-Pro identical to them, and since the gramicidins are linear pentadecapeptides and structurally unrelated to gramicidin S, Ruttenberg et al. (1965b) feel that the term gramicidin S is a misnomer.

E. GRAMICIDINS J₁ AND J₂.

    (1). Producing strains and properties of gramicidins J:

        Otani and Saito (1954) isolated a strain of B. brevis from soil near Osaka, which produced an antibiotic related to gramicidin S. This antibiotic was named "gramicidin J". Several years later, another strain of B. brevis which also produced a similar substance was isolated in Japan by Okuda (1959). This antibiotic was named "gramicidin J_2". For clarity, Okuda (1959) proposed renaming the former as gramicidin J_1.

        Toki (1955) showed that gramicidin J_1 had absorption maxima at 264, 258, 252, and 250 nm in alcoholic solution. Otani et al. (1958) showed that crystalline gramicidin J_1 contained only one active entity judged by the countercurrent distribution method. It was very soluble in ethanol, methanol, and chloroform, slightly soluble in acetone and water, and insoluble in ether. Gramicidin J_1 melted at 292 - 293°C with decomposition. Its optical rotation was $[\alpha]_D^{18} = -308.9°$ (96% ethanol), and it gave positive responses to biuret and ninhydrin tests. Gram-positive but not Gram-negative bacteria were sensitive to the antibiotic. It was hemolytic to goat erythrocytes and toxic to mice, having an intraperitoneal $LD_{50} = 12.5$ mg/kg of body weight.

        Okuda (1959) noted that gramicidin J_2 had identical physico-chemical and biological activities to gramicidin J_1 but the two were distinguishable by countercurrent distribution. Gramicidin J_2 showed absorption maxima at 265, 258 and 251 nm.

-89-

(2). Isolation:

Otani (1957) purified gramicidin J_1 by acidification of the culture fluid with HCl and adsorption of the antibiotic with diatomaceous earth. The active principle was removed from the earth by extraction with warm methanol. Gramicidin J_1 was then salted out from the methanol by NaCl and further purification was effected by repeated crystallization from acidified methanol. Otani isolated gramicidin J_2 with similar treatment. The impurities left were further removed by precipitation with water and adsorption to Al_2O_3. The purified antibiotic was then crystallized from methanolic solution.

(3). Structural studies:

Otani and Saito (1954) and Saito (1958a) showed that gramicidin J_1 contained one D-phenylalanine, one L-phenylalanine, one D-leucine, one L-ornithine, one D-ornithine, one L-valine and one L-proline residue. Elementary analysis showed its hydrochloride to have C, 58.12; H, 8.03; N, 13.7 and Cl, 5.4%. After reaction with FDNB reagent, the only DNP-derivative found in the hydrolysates of DNP-gramicidin J_1 was DNP-ornithine. Since it contained no free α-amino or α-carboxyl groups in the molecule, a cyclic peptide structure was proposed. The molecular weight would be about 800 as determined by the partial substitution method.

Based on the analyses of partial acid hydrolysates, Saito (1958b) showed gramicidin J_1 to be a cycloheptapeptide with a structure as shown below (50).

```
      ┌─→D-Phe──→D-Leu──→L-Phe ──────┐
      │                              │
   L-Orn←──── L-Val←──── D-Orn ←── L-Pro
```
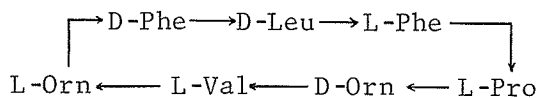
(50)

Otani (1957) and Okuda (1959) showed that gramicidin J_2 contained one D-ornithine, one L-ornithine, one D-leucine, one L-valine, one D-phenyl-alanine and one L-proline residue. Elementary analysis showed it had C, 55.07; H, 8.17; and N, 14.54%.

Noda (1959) found that the synthetic cyclo-D-phenylalanyl-D-leucyl-L-ornithyl-L-valyl-D-ornithyl-L-prolyl.2HCl had the same antibiotic activity, paper chromatographic behavior and X-ray diffraction pattern as natural gramicidin J_2. Therefore, gramicidin J_2 must have the structure shown in (51).

```
   D-Phe──→ D-Leu──→L-Orn
      ↑                │
   L-Pro←──D-Orn←── L-Val
```
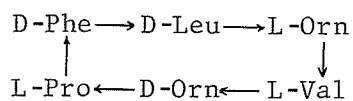
(51)

XIII. STAPHYLOCOCCAL ANTIBIOTICS.

Over the years, various investigators have shown that both
S. aureus and S. epidermidis produce antibacterial agents which appear to
have spectra similar to peptide antibiotics produced by other bacteria.
Apart from this, knowledge regarding the nature and properties of these
antibiotics is rather fragmentary.

One of the earliest reports concerning antibiotics from staphylo-
cocci is that of Cornil and Babes (1885) who showed that a staphylococcal
strain inhibited growth of the anthrax bacillus and other strains of
Staphylococcus. The most recent reports are those of Lachowicz (1965)
and Hsu and Wiseman (1967).

The major features of this poorly characterized group of anti-
bacterial agents, so far as they are known, are summarized in Table VII.
Noteworthy characteristics of the group as a whole are that the agents are
relatively stable over a wide range of pH, and are quite heat resistant.
Their sensitivity to trypsin and other proteolytic enzymes is variable.
Generally speaking, their inhibitory spectrum is restricted to Gram-
positive bacteria.

Little is known about the chemical nature of these antibiotics,
but it does appear that those at least which are mentioned in the table
are peptides, large and small.

TABLE VII

PROPERTIES OF STAPHYLOCOCCAL ANTIBIOTICS

authors	heat stable at	effects of			dialy-zability	soluble in	inhibitory spectrum	chemical nature
		pH	enzyme	serum				
Jennings and Sharp (1947)	100°C for 5 min	stable at 4.5 - 8.0	?	?	no	water	Gram +	?
Gardner (1949)	acid but not alkaline pH	stable in acid	inactivated by trypsin	?	yes	water and alcohol	Gram +	protein
Loeb et al. (1950)	121°C for 20 min	stable at 2 - 12	inactivated by trypsin, but not by pepsin	partially affected	yes	water and hydrophilic solvents	Gram +	poly-peptide
Fregnan and Smith (1962)	100°C for few min	stable at 2 - 8	inactivated by trypsin & α-amylase	?	no	water	mycoba-cteria	polypep-tide or glyco-protein
Barrow (1962, 1963)	83°C for few min	stable in acid or neu-tral pH	inactivated by trypsin not by pep-sin	not affected	yes	water	Gram +	peptide
Lachowicz (1965)	100°C for 1 hr	stable at 1 - 10	not affected by pepsin or trypsin	?	no	water	Gram +	poly-peptide
Hsu and Wise-man (1967)	115°C for 10 min	stable at 2 - 11	partially inactivated by trypsin & chymotrypsin	not affected	yes	water	Gram + & myco-bacteria	peptide

MATERIALS AND METHODS

MATERIALS AND METHODS

I. MATERIALS

    (1). <u>Strains of bacteria employed</u>:

        Two active antibiotic-producing strains, 29297 and 36534, isolated by Hsu and Wiseman (1967) were selected. Both strains were coagulase-negative staphylococci isolated from the cervix-uteri of two different patients. Their physiological characteristics (see Appendix I) fitted the description of the genus <u>Staphylococcus</u> in the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed <u>et al</u>., 1957). <u>Staphylococcus aureus</u> Oxford 209P, which was used as the standard indicator strain, was originally obtained from Dr. A.C. Maniar of the Provincial Laboratories in Winnipeg.

        All bacterial strains were kept in the lyophilized state at room temperature. Before use, the cells were revived by suspension in Brain-Heart Infusion (BHI) broth and 18-hr subculture on BHI agar slants at 37°C. The purity of the culture was checked by the Gram-staining reaction.

    (2). <u>Media and chemicals</u>:

        Proteose peptone, BHI broth and BHI agar media were obtained as the dehydrated form from Difco Laboratories, Detroit, Michigan. Whenever possible, all reagents used in this study were of the analytical grade. Unless otherwise indicated, buffers used were made up according to the instructions of Cruickshank (1965).

(3). Glassware:

The glassware used for chemical tests was first boiled with dilu-ted HCl solution for two hr, washed with detergent, rinsed thoroughly with tap-water and then with distilled water.

II. METHODS

(1). Quantitative assay of antibiotic activity:

Fluids containing antibiotic were assayed by the methods of Hsu and Wiseman (1967). A series of two fold dilutions of antibiotic was made in three ml volumes of distilled water, starting at 1:2 in calibrated Klett tubes. The diluted antibiotic was sterilized by autoclaving at 10 lb pres-sure (110°C) for 10 min. After cooling, three ml of a standard suspension of the Oxford indicator strain in double strength BHI broth was added to each tube. The tubes were agitated and the optical density (O.D.) of each tube was recorded in a Klett colorimeter equipped with filter no. 69 (trans-mission 660 - 740 nm). The tubes were incubated at 37°C for 18 hr. At the end of the incubation, tubes were shaken on a Vortex Jr. Mixer (Scientific Industries Inc., New York) for 15 sec and the O.D. was again determined. The difference between the two O.D. readings was plotted on a graph paper against the reciprocal value of the $\log_2$ dilution of the antibiotic. The end point was taken as the reciprocal value of the $\log_2$ dilution of the antibiotic corresponding to the O.D. of the indicator strain equal to the mid-point of the linear portion of the growth curve. The $\log_2$ value was then treated as given in the following example:

$\underset{\text{antibiotic titre}}{\log_2}$	total units (reciprocal of antilog_2)	units antibiotic/ml
4	16	5.3

The total antibiotic units were divided by three to give units/ml, since the volume of fluid in the titration was 3 ml.

(2). Production of crude antibiotic:

Dolman-Wilson liquid medium (Dolman and Wilson, 1940) supplemented with ferrous ammonium sulphate, manganese chloride and sodium citrate (see Appendix II) was employed in antibiotic production. The medium was divided into two parts and prepared as follows: Part I consisted of proteose peptone and 900 ml of distilled water. This solution was adjusted to pH 7.8 and autoclaved at 15 lb pressure (121°C) for 25 min. Part II was composed of the salt mixture and ammonium lactate which were dissolved in 100 ml of distilled water. The pH of this solution was adjusted to 5.5 and the fluid was sterilized by passage through a Millipore HA membrane (pore size = 450 nm). Part I was cooled to room temperature and added to Part II with thorough mixing. The pH of the mixture was 7.2. Five hundred ml of this medium was dispensed into a sterile one litre Erlenmeyer flask which was then plugged with cotton.

A heavy suspension of the antibiotic-producing strain adjusted to O.D. = 0.500 was prepared in distilled water. Each flask was seeded with 0.5 ml of this suspension and then incubated at 32°C with constant shaking in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The shaking speed was adjusted to 110 strokes per min;

the length of incubation period being three days for strain 29297 and four

day.s for strain 36534. After incubation, the purity of the culture was

checked by staining and the bacterial cells were removed by centrifugation

at 10,000 g for 15 min in a refrigerated centrifuge. The active superna-

tant was referred to as crude antibiotic fluid.

Each operation produced about 7.5 litres of active liquid with a

titre varying from three to six units per ml.

(3). Concentration of the antibiotic fluids:

Flash evaporation was used in the concentration of crude and

purified antibiotic fluids. The flash-evaporator was obtained from the

Buchler Instrument Co., Fort Lee, N.J. The temperature of the water bath

in which the evaporating flask was kept, was 50 ± 1^oC, a temperature which

did not affect antibiotic activity.

(4). Ion-exchange chromatography:

A strong cationic ion-exchanger, Dowex 50-X2 (H^+ form, 50 - 100

mesh, J.T. Baker Chemical Co., Philipsburg, N.J.) was selected for chromato-

graphy. The resin was washed and converted to the Na^+ form according to

the procedure suggested by Moore and Stein (1951). After treatment, the

resin was suspended in 0.1M NaCl solution, pH 7.0, poured into a 4.5 X 60cm

column and packed by gravity. The column was equilibrated with a continuous

flow of 0.1M NaCl solution, pH 7.0, at a rate of 200 ml/hr for two days. The

material to be chromatographed was adjusted to pH 7.0 before application to

the column and the sample size was limited to 100 ml per column per run.

Antibiotic was eluted in 100 ml volumes from the column with 0.1M NaCl at pH 7.0 at a rate of 200 ml/hr. At the end of each run, the resin was cleaned with 200 ml of 1N HCl and regenerated by addition of 400 ml of 2N NaOH solution. The excess NaOH was removed with 0.1M NaCl.

(5). Gel filtration:

Sephadex gels and columns (5 X 100 cm in size) were supplied by Pharmacia of Canada Ltd., Montreal. The procedures used for preparation of the gel and column packing were those recommended by the manufacturer (Pharmacia, 1963). After packing, distilled water at a rate of 160 ml/hr was passed through the columns from top to bottom for two days. The void volume (V_o) of each column was determined with blue dextran (Sigma Chemical Co., St. Louis, Mo.). Forty ml 0f 0.01% dye was passed through the column and V_o was taken as that volume which corresponded to elution of the dye from the column at maximum O.D. 540 nm.

Samples of antibiotic, 40 ml in volume, were neutralized before being added to the columns, and the flow rate was adjusted to 160 ml/hr, distilled water being used as the eluent. Twenty-eight fractions of 40 ml each, commencing 120 ml before the appearance of V_o, were collected from the column.

After each use, columns were cleaned by the application of 3 - 4 litres of distilled water. Since the gel filtrations were performed at room temperature, growth of microorganisms could occasionally have been a problem. This was controlled by forcing 100 ml of 0.1% (w/v) sodium azide in water through the column from bottom to top. Azide was cleared from the column by the application of several void volumes of distilled water.

(6). <u>Mobility of staphylococcal antibiotics in an electric field</u>:

The mobility of the antibiotics produced by these two staphylococcal strains in an electric field at different pH values was detected by the following processes. Twenty-five ml of BHI broth with 2% Noble agar (Difco) added was poured onto a 102 X 83 mm glass plate which was placed in a large Petri-dish (14 cm in diameter). After the agar solidified, the plate was seeded with the producing strain by stabbing the centre of the plate with an end-sealed Pasteur pipette which had previously been dipped into the suspension of the producing strain. After being incubated at 37°C for two days, the plate was put into the electrophoresis chamber with filter paper wicks dipped into the buffer reservoirs. The plate was allowed to stand in such a manner for two hr at room temperature to facilitate equilibration of the buffer. The buffers used were 0.05M citrate-phosphate buffer (pH 5.0 - 5.5), phosphate buffer (pH 6.0 - 7.5), and barbitone buffer (pH 8.0 - 9.0). Then, electric current (20 v/cm and 50 mA/plate) was applied for various lengths of time. Immediately after the electrophoresis, the plate was sterilized with chloroform vapor for one hr at room temperature in a closed metal chamber and sprayed with the standard suspension of the indicator strain, <u>S</u>. <u>aureus</u> 209P, which was suspended in BHI broth concentrated four-fold. The sprayed plate was incubated at 37°C for 18 hr and the distance of the clear inhibition zone from the origin was determined in mm.

(7). Chemical analyses:

(a). Qualitative:

Qualitative chemical tests of antibiotic preparations were based on the methods outlined in Shriner et al. (1964), Oser (1965) or Kabat and Mayer (1961) except that phenolic groups were determined by the method of Inglett and Lodge (1959) and sulphur groups were assayed by the method of Klipp and Barney (1959).

(b). Quantitative determination:

Protein content was determined either by the method of Lowry et al. (1951), with egg albumin as the standard, or by the quantitative ninhydrin method of Moore and Stein (1954) with leucine as the standard. Total nitrogen content was assayed by the modified micro-Kjeldahl technique of Markham (1942) with $(NH_4)_2SO_4$ as the standard.

(c). Amino acid analyses:

Amino acid content of the purified antibiotic products was assayed with Technicon Auto Analyzer (Technicon Corp., Ardsley, New York) through the courtesy of Mr. Yong-Soo Park of the Department of Pharmacology, University of Manitoba.

(d). N- and C-terminal amino acid determination:

The N-terminal amino acid groups of the highly purified materials were determined by Sanger's method (Sanger, 1945, 1949). One ml of highly purified antibiotic solution (about 10 mg in dry weight) was conjugated with an excess amount of Sanger's 1-fluoro-2,4-dinitro-benzene (FDNB) reagent (Sanger, 1949). After the conjugation reaction was complete, free FDNB reagent was removed by extraction with chloroform and the treated material

was treated with 6N HCl at 110°C for 12 hr in a sealed evacuated ampoule.
This fluid, freed from the acid by flash-evaporation, was extracted three
times with an equal volume of ether. The ether extracts were pooled and
concentrated. Then, both the ether concentrate and the aqueous solution
of the treated fluid, together with standard N-2,4-dinitrophenyl (DNP)
amino acid (Sigma Chemical Co.), were applied to Whatman no. 1 filter paper
which had previously been saturated in 0.1M phthalate buffer, pH 6.0 (Bla-
ckburn and Lowther, 1951). The paper was developed in a solvent system
of tertiary amyl alcohol saturated with phthalate buffer, pH 6.0.

The C-terminal amino acid groups of the purified antibiotics were
detected by the Dakin-West degradation as given by Vanderhaeghe and Par-
mentier (1960). One ml of highly purified antibiotic solution (about 10
mg dry weight) was heated with 0.5 ml of pyridine and 1.25 ml of acetic
anhydride in a sealed evacuated tube at 130°C for 6 hr. The fluid was sub-
jected to flash-evaporation to dryness and the residue was further hydro-
lyzed in 1.5 ml of 6N HCl at 110°C for 18 hr. After the acid was removed
by further flash-evaporation, the residue was dissolved in distilled water
and the solution was chromatographed on Whatman no. 1 paper in the solvent
system n-butanol/acetic acid/water, 4:1:5 by volume.

(e). Determination of the amino acid sequence of the antibiotic
peptide:

The highly purified antibiotic 29297 zinc-precipitable fraction
was partially hydrolyzed by heating at 110°C for two hr with 6N HCl in a
sealed evacuated tube. After the HCl was completely removed by flash-eva-
poration, the peptide fragments in the residue were conjugated with FDNB

reagent by Sanger's method as previously described. The resulting DNP-peptides were extracted by chloroform and the chloroform-extracted material was concentrated by evaporation. This material was chromatographed as described previously except that it was applied as a band one cm in width to Whatman no. 4 filter paper. The resulting separated bands were cut off and the DNP-peptide on each band was eluted by the same solvent. The eluent was concentrated and its purity was checked by thin-layer chromatography on silica gel G (E. Merck Co., Darmstadt, Germany) plates with tertiary amyl alcohol saturated with 0.1M phthalate buffer, pH 6.0, as the developing solvent. The free amino acids left in the aqueous fraction were also identified on paper with n-butanol/acetic acid/water, 4:1:5 by volume, as the developing agent.

(8). Acid hydrolysis:

Hydrolysis of antibiotic peptide with acid was performed in the following manner: An equal volume (one ml) of concentrated HCl (22° Be', J.T. Baker Chemical Co., Philipsburg, N.J.) and aqueous antibiotic solution were mixed in a five ml ampoule. This was evacuated with a water-pump and the ampoule sealed. The sealed ampoule was autoclaved at 10 lb pressure (110°C) for 18 hr. After heating, the liquid was transferred to the flash-evaporator and evaporated to dryness. The sample was freed of HCl by repeated dissolution in distilled water and evaporation to dryness.

(9). Enzymatic oxidative deaminations:

Deamination of the acid hydrolysates of highly purified antibiotics

by L-amino acid oxidase was investigated according to the method of Wellner
and Meister (1960). The enzyme was purchased from Sigma Chemical Co. (St.
Louis, Mo.), the source of which was Crotalus adamanteus venom.

Deamination by D-amino acid oxidase was performed by the method
of Burton (1955). The enzyme, which was obtained from hog kidney, was
purchased from Sigma. Catalase from beef liver and other co-factors were
purchased from the same source.

Standards used with both methods were solutions which contained
100 mM L-leucine and 300 mM D-alanine.

In both procedures, the Warburg flask was incubated at 37°C in a
water bath and equilibrated in air for 15 min prior to the addition of enzyme.
The manometric apparatus was supplied by Bronwill Scientific Inc. (Rochester,
N.Y.). Readings were taken at 10 min intervals for 30 min and then at 30
min intervals for 5½ hr. At the end of this time, total O_2 uptake was cal-
culated by the method of Umbreit et al. (1957).

EXPERIMENTAL RESULTS

RESULTS

PART I. PRODUCTION AND TITRATION OF THE ANTIBIOTICS

I. DEVELOPMENT OF LIQUID MEDIUM SUITABLE FOR ANTIBIOTIC PRODUCTION

Hsu and Wiseman (1967) had produced antibiotics 29297 and 36534 on solid media in large trays overlain with cellophane. While their media gave reasonably good yields, quantities obtained, however, were limited and handling of the large trays was awkward. It was therefore felt that development of a liquid medium ought to be investigated.

Hendlin (1949) had shown that ferrous and manganese ions were essential for good production of bacitracin. Other investigators have found this to apply to certain other peptide antibiotics. Consequently, 0.01 gm/L of ferrous ammonium sulphate and 0.2 gm/L of $MnCl_2$ were added to the basic Dolman-Wilson medium (Dolman and Wilson, 1940). Sodium citrate at a concentration of 0.2 gm/L was also added to prevent precipitation of salts. This medium was referred to as supplemented Dolman-Wilson medium. The preparation of the medium has been described in Materials and Methods as well as in Appendix II. After sterilization, the supplemented medium was dispensed in 100 ml volumes into 250 Erlenmeyer flasks. Each flask was inoculated with 0.1 ml of the producing strain suspended in distilled water to O.D. = 0.500. Some flasks were shaken at 110 strokes/min and others remained stationary. Incubation proceeded for three days at $37^{\circ}C$ at which time pH, growth and antibiotic activity were measured.

The results (Table VIII) showed that agitation of the cultures during the incubation period generally increased the growth in both supplemented and unsupplemented Dolman-Wilson media. As the result of abundant growth, the pH of the agitated cultures was much higher than that in stationary media. Under stationary conditions, both antibiotic-producing strains grew poorly and produced no detectable antibiotics regardless of the type of medium employed. With agitation, however, antibiotics were produced by both strains in the supplemented medium only, although the growth of either strain in the unsupplemented medium was very similar to the supplemented one. Therefore, it was concluded that suitable aeration and supplementation of the medium were both necessary for the facilitation of antibiotic production for strains 29297 and 36534. Subsequent experiments thus employed Dolman-Wilson medium supplemented with ferrous ammonium sulphate, $MnCl_2$, and sodium citrate.

An attempt was made to substitute the proteose-peptone of Dolman-Wilson medium with casamino acids (Difco) and growth factors, or with corn steep liquor obtained from the A.E. Staley Mfg. Co. of Illinois, in order to facilitate antibiotic production. The results (Table IX) indicated that although the growth of either strain was not affected by the substitution, antibiotic production was partially or completely eliminated.

TABLE VIII

EFFECT OF AGITATION AND SUPPLEMENTATION OF DOLMAN-WILSON MEDIUM ON ANTIBIOTIC PRODUCTION IN STAPHYLOCOCCI

		strain 29297			strain 36534		
treatment		growth*	pH	antibiotic activity (units/ml)	growth	pH	antibiotic activity (units/ml)
stationary	unsupplemented	0.100	7.1	0	0.090	7.1	0
	supplemented	0.100	6.5	0	0.100	6.5	0
shaken**	unsupplemented	0.760	8.3	0	0.750	8.4	0
	supplemented	0.800	8.7	3.9	0.800	8.7	4.0

* O.D. 660 nm

** shaken at 110 strokes/min

TABLE IX

EFFECT ON ANTIBIOTIC PRODUCTION OF PROTEOSE PEPTONE REPLACEMENT IN
SUPPLEMENTED DOLMAN-WILSON MEDIUM BY CASAMINO ACIDS OR CORN STEEP LIQUOR

proteose peptone subtituent	conc. (%)	strain 29297		strain 36534	
		growth*	antibiotic activity (units/ml)	growth	antibiotic activity (units/ml)
none (control)	-	0.600	4.2	0.500	4.1
casamino acids**	2	0.700	0	0.800	0
corn-steep liquor	1	0.400	0.7	0.400	0
	2	0.800	2.1	0.800	0
	5	1.250	0.7	0.900	0

* O.D. 660 nm

** growth factors added as follows:
nicotinic acid, 2×10^{-7}M; pyridine, 2×10^{-7}M;
biotin, 2.5×10^{-6}M; thiamine, 2×10^{-7}M; and
riboflavin 5×10^{-6}M

II. FACTORS INFLUENCING PRODUCTION OF THE ANTIBIOTICS

(1). Effect of temperature:

A 1000 ml Erlenmeyer flask which contained 500 ml of supplemented Dolman-Wilson medium was inoculated with 0.5 ml (0.1 ml/100 ml medium) of standardized culture where O.D. = 0.500. Three flasks were incubated over a temperature range 24 - 40°C for three days during which they were shaken at the rate of 110 strokes/min.

Results are shown in Fig. 1 in which it is observed that the optimal temperatures for growth and for production of antibiotics do not coincide. The highest titres of antibiotics 29297 and 36534 were attained at a temperature of 32°C whilst maximal growth in both cases took place at 37°C.

(2). Effect of incubation time:

Conditions of this experiment were similar to those given in (1) except that incubation proceeded at 32°C while incubation time was varied. Results are given in Fig. 2 and show that with strain 36534, maximal antibiotic production was attained after four days of incubation and with 29297, after three days. Differences in both cases over the three to five days range were not large. With both strains, the O.D. rose to a maximum at two to three days, falling off thereafter. In view of the small differences in antibiotic titre over the three to five days ranges, cultures were subsequently incubated for three to four days.

In summary, quantitative antibiotic production was facilitated

FIGURE 1

EFFECT OF TEMPERATURE OF INCUBATION ON THE ANTIBIOTIC PRODUCTION
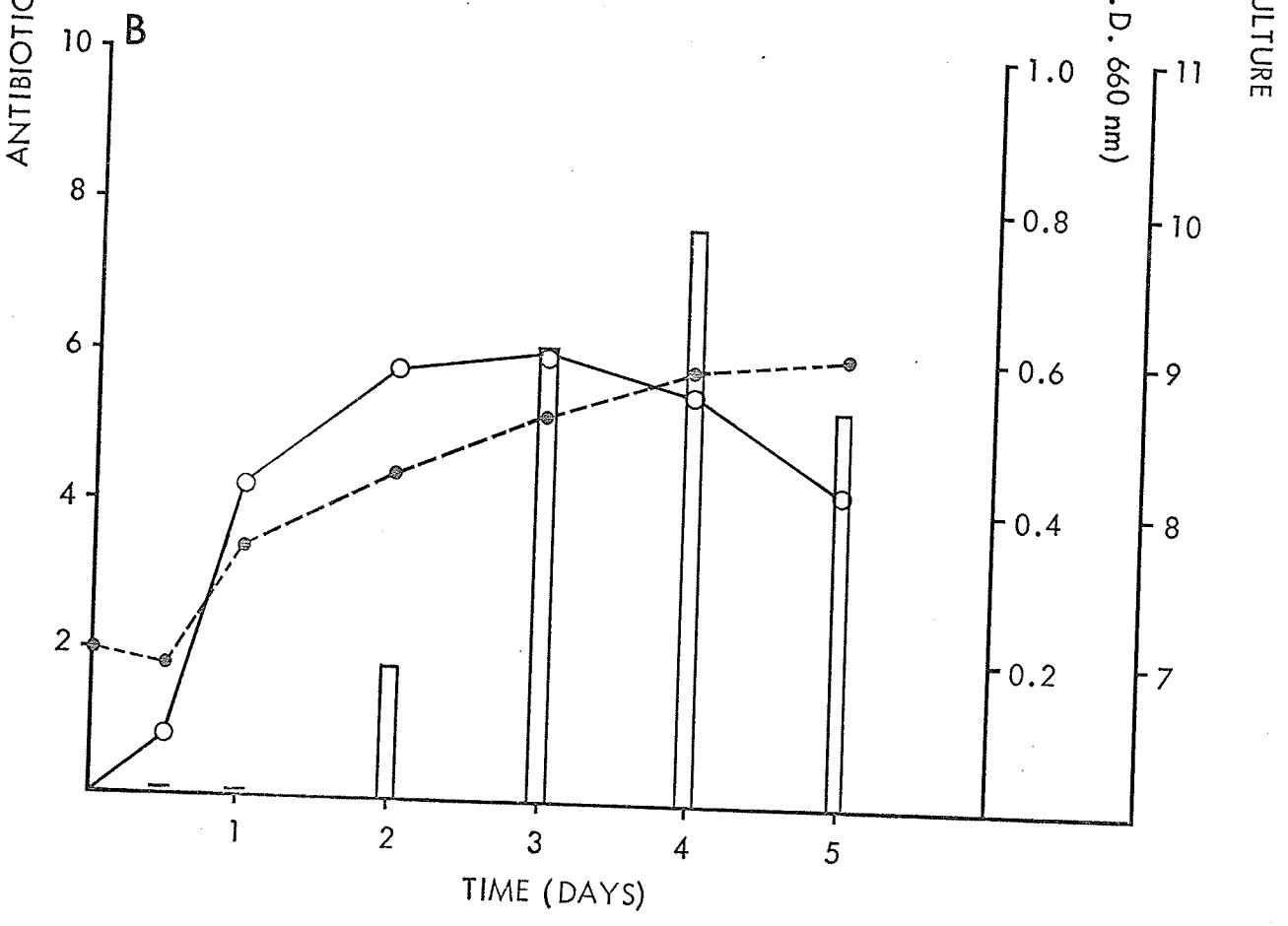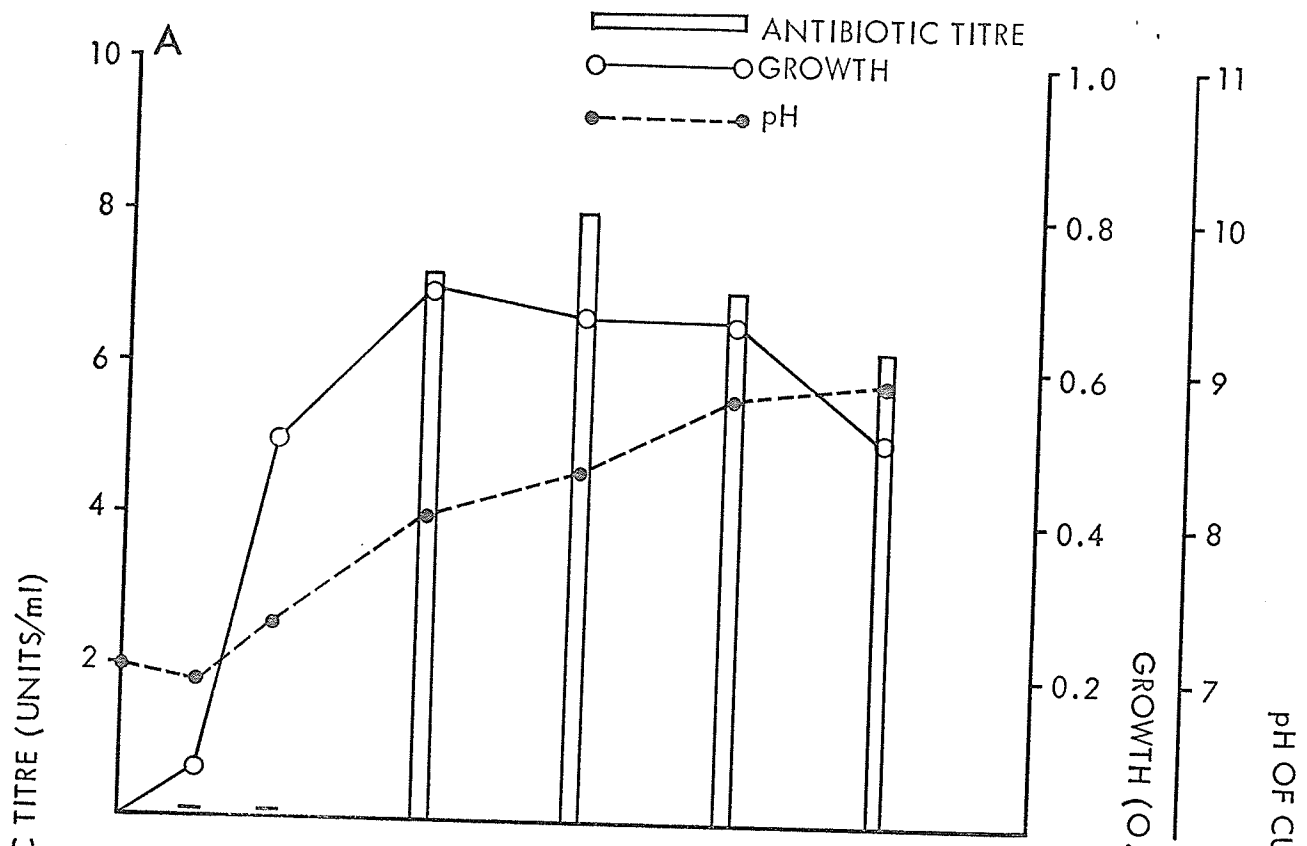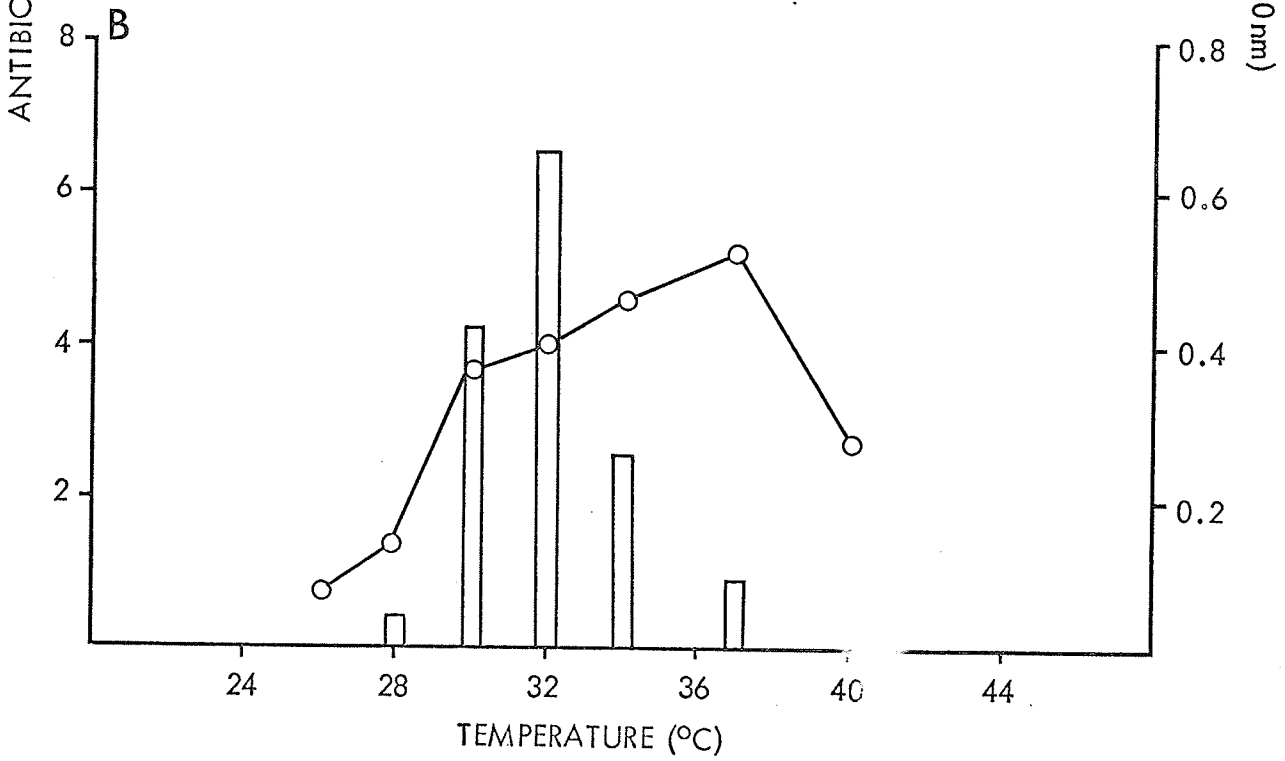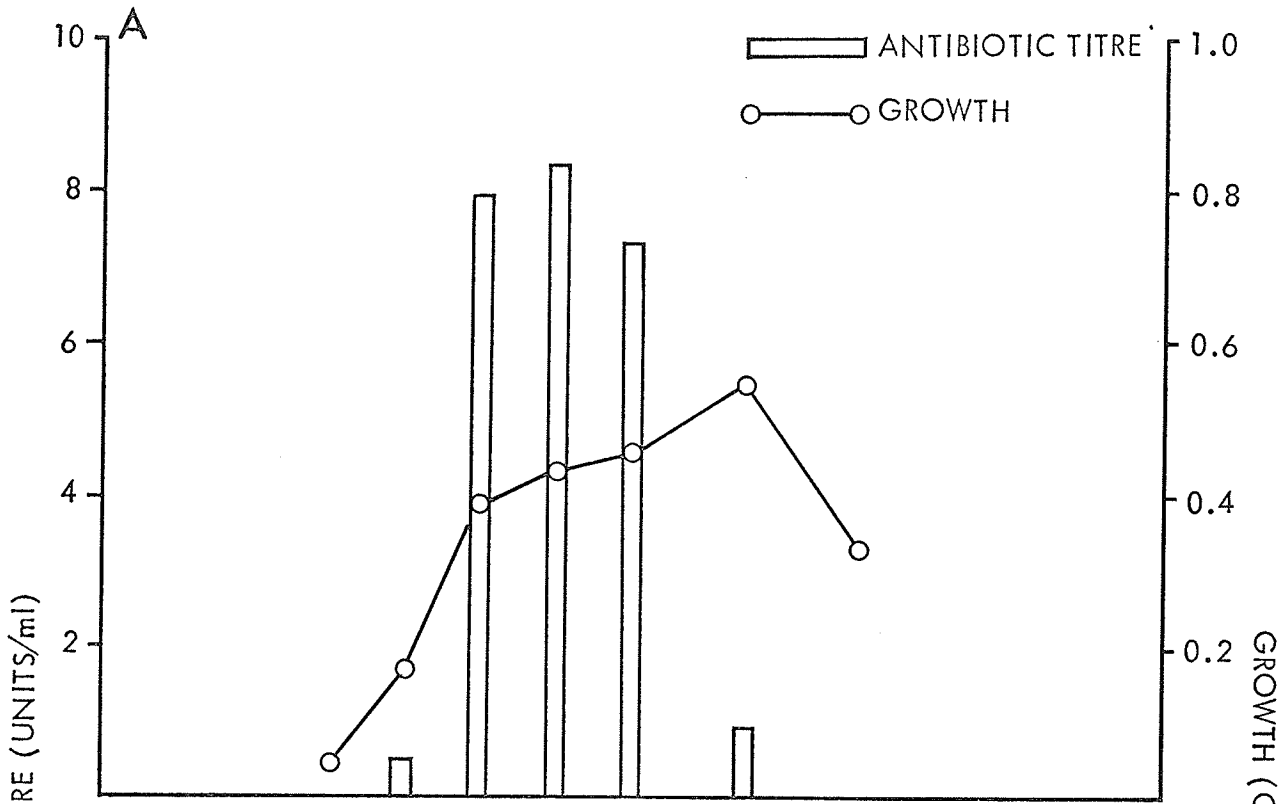
(A). STRAIN 29297

(B). STRAIN 36534

FIGURE 2

EFFECT OF LENGTH OF INCUBATION ON THE ANTIBIOTIC PRODUCTION

(A). STRAIN 29297

(B). STRAIN 36534

by the inoculation of flasks containing 500 ml volumes of supplemented

Dolman-Wilson medium. Each flask received 0.1 ml inoculum/100 ml medium

as described previously. Incubation proceeded at 32°C for three to four

days in the Brunswick shaker with an agitation rate of 110 strokes/min.

III. FACTORS INFLUENCING ANTIBIOTIC ASSAY

(1). <u>Sterilization of the antibiotics</u>:

The stability of crude antibiotic fluids and solutions of highly

purified product obtained after double crystallization and double methanol

precipitation (ref. Part II) was tested. The antibiotic liquid was divided

into two parts. One was sterilized by autoclaving at 10 lb pressure (110°C)

for 10 min and the other was filtered through a Millipore HA membrane. Both

parts were then titrated separately under the same conditions.

It was noted that both methods of sterilization gave identical

results (Table X). Crude and highly purified materials resisted heat

equally well. For the sake of convenience, autoclaving was the method of

choice when sterile active fluids were required.

(2). <u>Medium</u>:

Antibiotics were titrated as described in Materials and Methods

but it was decided to test the effect of the assay medium on antibiotic

titres. Media tested were Brain Heart Infusion broth (Difco), Veal Infu-

sion broth (BBL), Trypticase-Soy broth (Difco), and Nutrient broth (BBL).

TABLE X

COMPARISON OF ANTIBIOTIC TITRES OF ACTIVE FLUIDS STERILIZED BY TWO METHODS

material	concentration (mg N/ml)	antibiotic titre (units/ml)	
		sterilized by filtration*	sterilized by autoclaving**
produced by strain 29297			
crude fluid	1.98	4.2	4.1
highly purified zinc-precipitable fraction***	0.17	16.6	16.2
highly purified zinc-soluble fraction	0.20	8.0	8.0
produced by strain 36534			
crude fluid	5.11	4.4	4.5
highly purified zinc-precipitable fraction	0.05	7.5	7.6
highly purified zinc-soluble fraction	0.09	6.5	6.4

* passage through a Millipore membrane (pore size = 450 nm)

** autoclaved at 10 lb pressure (110°C) for 10 min

*** twice crystallized and twice methanol precipitated

As shown in Table XI, different end points were obtained with the various media. Brain Heart Infusion broth caused the least inhibition and was used subsequently.

(3). Incubation period:

The antibiotic titre, as affected by the length of incubation of the indicator strain, was also investigated. Antibiotic titrations of crude and concentrated, partially purified material (HCl-treated fraction, ref. Part II) were performed as described under Materials and Methods. Titres were read over the time interval 8 - 48 hr. Results (Table XII) showed that titres of a given antibiotic fluid remained approximately constant between eight and 24 hr with deterioration beyond 24 hr. This was particularly notable with the partially purified, concentrated materials. Subsequent titrations were incubated for 18 hr for the sake of convenience since titres were relatively stable during the 8 - 24 hr time interval.

TABLE XI

EFFECT OF ASSAY MEDIUM ON THE ANTIBIOTIC TITRES

broth	growth of indicator strain*	antibiotic activity (units/ml)	
		29297	36534
Brain Heart Infusion	0.370	4.0	5.3
Veal Infusion	0.375	2.1	2.7
Trypticase-Soy	0.380	1.8	2.0
Nutrient	0.370	1.2	0

* O.D. 660 nm

TABLE XII

RELATIONSHIP BETWEEN ANTIBIOTIC TITRE AND LENGTH OF
INCUBATION PERIOD OF THE INDICATOR STRAIN

length of incubation (hr)	growth of indicator strain*	antibiotic titre (units/ml)	
		29297	36534
crude material			
8	0.300	3.5	4.0
12	0.340	3.3	3.2
24	0.370	3.2	3.2
48	0.370	2.9	2.1
partially purified and concentrated material			
8	0.300	16.0	33.0
12	0.310	14.9	33.0
24	0.370	14.9	32.0
48	0.360	6.9	14.9

* O.D. 660 nm

PART II. PURIFICATION

I. EFFECT OF MINERAL ACIDS ON THE ANTIBIOTIC ACTIVITY

The use of hydrochloric acid in the purification of antibiotic was tested. Twenty-five ml of the crude antibiotic fluid was acidified to pH 2 with concentrated acid. After the acidified fluid had stood overnight at 4°C, considerable precipitation resulted which was separated from the fluid by centrifugation and then suspended in nine ml of distilled water. Both the suspension and supernatant fluid were neutralized with 4N NaOH and diluted to 10 and 30 ml respectively with distilled water. All original antibiotic activity was found in the supernatant fraction and none was associated with the precipitate (Table XIII). Specific activity of 29297 and 36534 preparations increased about 1.5 - 2-fold. Acid controls which were neutralized by the addition of alkali were not toxic to the indicator strain. Sulphuric, phosphoric and nitric acids were also tested but offered no advantages over hydrochloric acid.

II. FURTHER PURIFICATION OF THE HCl-TREATED ANTIBIOTICS

(1). Precipitation by metal ions:

(a). Preliminary studies:

The object of these studies was to see if metal ions would cause precipitation in the HCl-treated antibiotic fluid and whether the precipitate was soluble at acid and alkaline pH. It would then be possible to

TABLE XIII

EFFECT OF HYDROCHLORIC ACID ON THE ACTIVITY OF STAPHYLOCOCCAL ANTIBIOTICS

treatment	fraction	antibiotic 29297			antibiotic 36534		
		activity (total units)	recovery (%)	specific activity*	activity (total units)	recovery (%)	specific activity
crude		96	100	2.2	90	100	0.9
crude with HCl	precipitate	0	0	-	0	0	-
	supernatant	96	100	3.1	90	100	1.6

* units of antibiotic activity per mg of protein

select those ions which caused heavy precipitation and determine how they affected the antibiotic activity.

Sixteen mono-, di-, and tri-valent metal salts were tested. Each salt was added to 25 ml of the antibiotic fluid to give a final concentration of 0.1M. The resulting precipitate was suspended in distilled water and the suspension was equally divided into three parts. Part I, II, and III were carefully adjusted to pH 3, 7, and 10 respectively.

The results (Table XIV) showed that the metal ions could be classified into three groups according to the amount of precipitate formed. Group I, which included Al^{+++}, Ba^{++}, Fe^{+++}, Fe^{++}, Hg^{++}, Mg^{++}, Mn^{++}, Pb^{++}, and Zn^{++}, caused heavy precipitation in the HCl-treated antibiotic fluid. Group II consisted of Ca^{++}, Cd^{++}, Cu^{++} and Ag^{+} ions which formed moderate amounts of precipitates. Little or no precipitation occurred in Group III which contained Mg^{++}, Co^{++}, Li^{+} and Ni^{++}. Most precipitates were soluble at pH 3 but became insoluble at pH 7 and 10. An exception was the precipitate caused by Pb^{++} ions, which was insoluble at any pH.

With the exception of lead acetate, salts which caused moderate to heavy precipitation in the HCl-treated antibiotic fluids were further investigated for their effects on antibiotic activity. Except for Ba^{++}, Mn^{++}, Zn^{++}, Ca^{++} and Ag^{+}, the other salts were too toxic for use as precipitants. Silver and calcium ions were not considered since it was found that they afforded low recoveries of the antibiotics.

(b). Effect of $Ba(OH)_2$ on the antibiotic activity:

After the removal of precipitate from the HCl-treated antibiotic fluid, the supernatant required neutralization before an assay could be

TABLE XIV

PRECIPITABILITY OF ANTIBIOTIC FLUIDS WITH METAL SALTS AT NEUTRAL pH
AND SOLUBILITY OF THE RESULTING PRECIPITATE AT DIFFERENT pH LEVELS

metal salt	amount of precipitate		solubility of precipitate		
	29297	36534	pH 3	pH 7	pH 10
GROUP I					
$Al_2(SO_4)_3(NH_4)_2SO_4$	heavy	heavy	+*	-	-
$BaCl_2$	"	"	+	-	-
$FeSO_4$	"	"	+	-	-
$FeCl_3$	"	"	+	-	-
$HgCl_2$	"	"	+	-	-
$MnCl_2$	"	"	+	-	-
$Pb-(CH_3COO)_2$	"	"	-	-	-
$ZnCl_2$	"	"	+	-	-
GROUP II					
$CaCl_2$	moderate	moderate	+	-	-
$Cd(NO_3)_2$	"	"	+	-	-
$CuSO_4$	"	"	+	-	-
$AgNO_3$	"	"	+	-	-
GROUP III					
$MgSO_4$	light	none			
$Co(NO_3)_2$	none	"			
$LiCl$	"	"			
$NiCl_2$	"	"			

* + indicates soluble, and - indicates insoluble

performed. Since Ba^{++} ion was an excellent non-toxic precipitant, its use
as a neutralization agent was considered. For this purpose, highly alka-
line $Ba(OH)_2$ was investigated; the HCl-treated antibiotic fluid (pH 2) was
mixed with 0.1M salt and the resulting precipitate from 30 ml of the anti-
biotic fluid was separated from the supernatant by centrifugation. The
precipitate was dissolved in eight ml of 0.01N HCl solution, neutralized
and clarified. The supernatant at pH 9.0 was neutralized to pH 7.0. In
order to avoid the formation of metal-protein precipitates in the BHI titra-
tion medium, the antibiotic activity of the fluids, in this experiment only,
was assayed in Dolman-Wilson medium. This medium did not precipitate in
the presence of the metal ions as did BHI.

The results (Table XV) showed that the precipitate caused by Ba^{++}
ions was completely inert whereas the total activity was preserved in the
supernatant fraction. Specific activity of 36534 and 29297 increased three
to eight fold respectively when compared with the control. The $Ba(OH)_2$ salt
was used for further purification of the HCl-treated material.

(c). Effect of $MnCl_2$ and $ZnCl_2$ on the activity:

After the $Ba(OH)_2$-treated antibiotic fluid was clarified, the
effect of further treatment with 0.1M $MnCl_2$ or $ZnCl_2$ was investigated. The
resulting precipitates were dissolved in 0.01N HCl, neutralized with NaOH,
clarified and tested. The supernatant which contained the soluble Mn^{++} or
Zn^{++} ions was saturated with H_2S. The resulting precipitate was inert and
was discarded before the supernatant was neutralized and tested.

The results (Table XVI) showed that both metal ions could precipi-
tate certain amounts of antibiotic out of solution. However, Zn^{++} ions were

TABLE XV

EFFECT OF BARIUM HYDROXIDE ON THE ACTIVITY OF STAPHYLOCOCCAL ANTIBIOTICS

| treatment* | fraction | antibiotic 29297 | | | antibiotic 36534 | | |
		activity (total units)	recovery (%)	specific activity**	activity (total units)	recovery (%)	specific activity
none		3725	100	1.9	8093	100	1.3
treated with Ba(OH)$_2$	precipitate	0	0	-	0	0	-
	supernatant	3600	97	16.5	8008	99	3.7

* antibiotic assays performed after neutralization to pH 7.0

** units of antibiotic activity per mg of protein

TABLE XVI

COMPARISON OF THE EFFECTS OF $MnCl_2$ AND $ZnCl_2$ ON THE ACTIVITY OF STAPHYLOCOCCAL ANTIBIOTICS

treatment	fraction	antibiotic 29297			antibiotic 36534		
		activity (total units)	recovery (%)	specific activity*	activity (total units)	recovery (%)	specific activity
Ba(OH)$_2$-treated control		13254	100	15.5	13728	100	4.0
further treated with $MnCl_2$	precipitate**	2386	18	14.5	1922	14	12.0
	supernatant***	5037	38	17.4	3569	26	7.8
further treated with $ZnCl_2$	precipitate	4374	33	23.4	4805	35	26.6
	supernatant	7687	58	10.1	8374	61	11.2

* units of antibiotic activity per mg of protein

** precipitate dissolved in 0.01N HCl, neutralized with NaOH and tested

*** supernatant treated with H_2S and neutralized with NaOH before testing

superior to Mn^{++} ions when percent recovery and specific activity were considered. Thus, the fraction was treated further with zinc and was referred to as the zinc-precipitable fraction. The remaining activity in the supernatant was referred to as the zinc-soluble fraction. Other zinc salts were as effective as $ZnCl_2$ in precipitating the antibiotics, but the chloride was subsequently used.

(d). Influence of pH on precipitation of antibiotics by zinc ions:

Since zinc ions caused precipitation only in neutral and alkaline conditions (see Table XIV), two conditions, pH 7 and 9, were selected for an assessment of their effects on quantitative recovery of zinc-precipitated antibiotics. Thirty-five ml of $Ba(OH)_2$-treated antibiotic fluid at pH 9.0 was adjusted to pH 7.0 with HCl. This was was then mixed with 5.0 ml of aqueous zinc chloride solution to give a final concentration of the salt from 0.01 to 0.2M. The precipitate was collected, dissolved and assayed as described previously.

The results (Table XVII) showed that at pH 7 and 9 a 0.1M concentration of zinc ions was the most effective in precipitating antibiotic activity with slightly better recovery at pH 9.0. Under these conditions, precipitation was complete since additional zinc added to the clarified supernatant produced no further precipitate even though some activity still remained.

In summary then, HCl-treated antibiotic fluid was brought to pH 9.0 with 0.1M $Ba(OH)_2$. The precipitate was discarded and antibiotic in the supernatant was further precipitated by the addition of 0.1M $ZnCl_2$.

TABLE XVII

RELATIONSHIP BETWEEN pH OF THE ANTIBIOTIC FLUIDS, CONCENTRATION OF ZINC
IONS AND RECOVERY OF ZINC-PRECIPITATED ANTIBIOTICS

pH of the Ba(OH)$_2$-treated antibiotic fluid	concentration of zinc ions (Moles)	antibiotic 29297 activity (total units)	recovery (%)	antibiotic 36534 activity (total units)	recovery (%)
7*	0	148	100	144	100
	0.01	0	0	0	0
	0.05	18	12	0	0
	0.10	54	37	34	24
	0.20	36	24	22	15
9	0	148	100	144	100
	0.01	0	0	0	0
	0.05	22	15	24	17
	0.10	62	42	48	33
	0.20	38	26	26	18

* adjusted to this pH by the addition of HCl

(2). Precipitation by organic compounds:

Neutralized HCl-treated antibiotic fluids were treated with various organic compounds and the precipitation which occurred was noted as shown in Table XVIII. It was found that with the exception of urea, all substances added caused a moderate to heavy precipitation. Most precipitates were soluble in distilled water at neutral pH. An exception was the precipitate caused by tannic acid, which was insoluble in water between pH 3 - 11.

Four of the compounds, picric and trichloroacetic acids, methanol and ethanol, were further investigated since they appeared to be good precipitants. All permitted relatively good recoveries of antibiotics, but removal of picric and trichloroacetic acids became a problem when it was realized that the antibiotics were small dialyzable peptides. Recoveries with methanol were slightly better than with ethanol although results were similar. In Table XIX, it was shown that about 77% of the antibiotics was removed as a precipitate with a two fold increase in specific activity. Of interest here is the fact that methanol precipitations had to be performed at 4°C with subsequent removal of the alcohol at that temperature. Precipitations at room temperature destroyed the activity.

(3). Extraction with water immiscible organic solvents:

Water immiscible organic solvents such as benzene, chloroform, ethyl ether, ethyl acetate, hexane and petroleum ether were tested for their ability to extract the antibiotic activity from neutralized HCl-treated material. The ratio of solvent to the antibiotic fluid was 4:1. It was

TABLE XVIII

PRECIPITABILITY OF ANTIBIOTIC FLUID IN THE PRESENCE OF SOME
ORGANIC COMPOUNDS

substance	amount added	amount of precipitate 29297	36534	solubility at pH 7
picric acid	0.1M	heavy	heavy	soluble
tannic acid	0.01M	"	"	insoluble
methanol	4:1 (v/v)	"	"	soluble
ethanol	"	"	"	"
isopropanol	"	"	"	"
acetone	"	"	"	"
trichloroacetic acid	0.1M	moderate	"	"
n-butanol	4:1 (v/v)	"	moderate	"
tert-amyl alcohol	"	"	"	"
propylene glycol	"	"	"	"
ethylene chloride	"	"	"	"
urea	0.1M	none	none	-

TABLE XIX

EFFECT OF METHANOL ON THE ACTIVITY OF STAPHYLOCOCCAL ANTIBIOTICS*

treatment	fraction	antibiotic 29297			antibiotic 36534		
		activity (total units)	recovery (%)	specific activity**	activity (total units)	recovery (%)	specific activity
none		1660	100	4.9	3940	100	10.3
methanol	precipitate	927	56	8.0	3043	77	22.9
	supernatant	547	33	2.5	827	21	3.3

* performed at 4°C

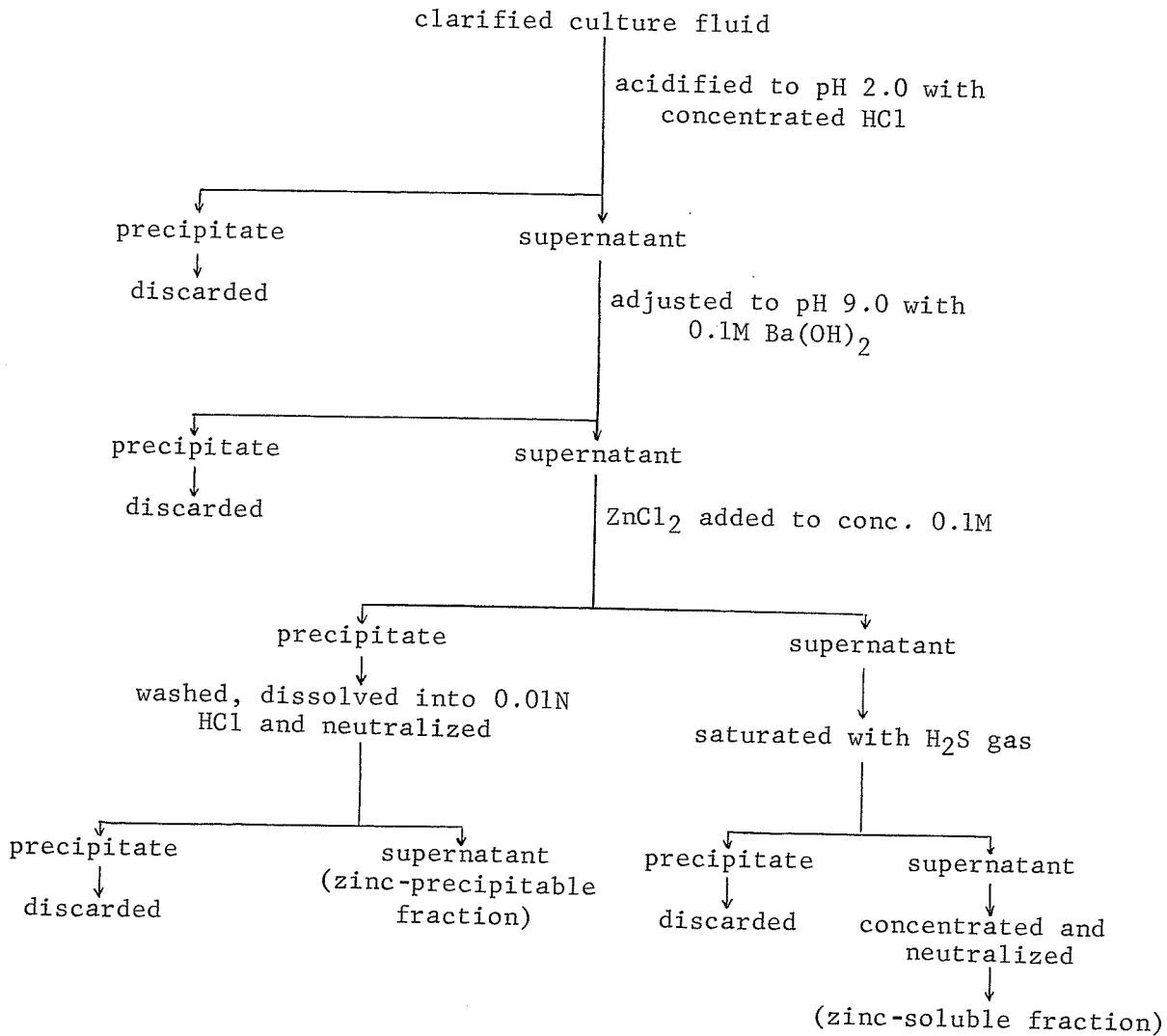** units of antibiotic activity per mg of protein

found that solvent extract, evaporated to dryness and resuspended in dis-
tilled water, contained no activity in contrast with the aqueous fraction
which retained full activity.

III. OUTLINE OF INITIAL SCHEME FOR PARTIAL PURIFICATION

According to the evidence presented in these studies, an initial
scheme (Table XX) suitable for partial purification of staphylococcal anti-
biotics was arranged. The clarified culture fluid, which was derived from
the supplemented Dolman-Wilson liquid medium, was first acidified to pH 2
with concentrated HCl and then allowed to stand at 4°C overnight. The inert
precipitate formed due to acidification was removed by centrifugation and
discarded. The supernatant was adjusted to pH 9 by the direct addition of
solid $Ba(OH)_2$ to the fluid, to a concentration of approximately 0.1M. This
treatment had two purposes: These were the further removal of inert impu-
rities as precipitate and provision of alkaline conditions for the $ZnCl_2$
treatment. After the fluid was clarified, 0.1M $ZnCl_2$ was added. At this
stage, a portion of the antibiotic from either strain precipitated out of
the solution. These precipitates were soluble only in acid conditions.
That portion precipitable by zinc is designated the zinc-precipitable frac-
tion, and that in the supernatant, the zinc-soluble fraction. Since a por-
tion of the antibiotic from both strains remained in the supernatant fluid,
it was considered that the crude antibiotics were mixtures of closely re-
lated compounds.

TABLE XX

INITIAL SCHEME USED FOR PARTIAL PURIFICATION OF STAPHYLOCOCCAL
ANTIBIOTICS

clarified culture fluid

acidified to pH 2.0 with
concentrated HCl

precipitate   supernatant

discarded

adjusted to pH 9.0 with
0.1M $Ba(OH)_2$

precipitate   supernatant

discarded

$ZnCl_2$ added to conc. 0.1M

precipitate   supernatant

washed, dissolved into 0.01N   saturated with H_2S gas
HCl and neutralized

precipitate   supernatant   precipitate   supernatant

discarded   (zinc-precipitable   discarded   concentrated and
                fraction)   neutralized

(zinc-soluble fraction)

For the zinc-precipitable fraction, the precipitate was collected by centrifugation, resuspended in distilled water and recentrifuged. The latter processes were repeated twice. The washed precipitate was dissolved in 0.01M HCl, immediately neutralized and the insoluble material discarded. As to the zinc-soluble fraction, it is obvious that the supernatant fluid after treatment with 0.1M ZnCl , contains Zn^{++} and Ba^{++} salts. These were removed by precipitation with H_2S gas which was generated from HCl and Na_2S and bubbled through the fluid. It was noted after clarification and neutralization that the zinc-soluble and zinc-precipitable fractions had a yellow color.

Table XXI gives the percent recovery of antibiotics and specific activity at different stages in the initial scheme. As shown in the table, recoveries of zinc-precipitable and soluble fractions of 29297 were 33 and 41% respectively with about a 27 fold increase in specific activity. With respect to antibiotic 36534, percent recoveries were about the same, and specific activities showed an increase of 17 fold when compared to crude. If one assumes the total recovery in both cases from IV a and b to be about 75% (see Table XXI), it is evident that about 15 - 20% of the activity of the Ba^{++}-treated material is unrecoverable.

TABLE XXI

SPECIFIC ACTIVITY AND PERCENT RECOVERY OF STAPHYLOCOCCAL ANTIBIOTICS 29297
AND 36534 AT DIFFERENT PURIFICATION STAGES

stage of purification	amount (ml)	activity (total units)	recovery (%)	total protein (mg)	specific activity*
ANTIBIOTIC 29297					
I. crude	940	3760	100	4559.0	0.8
II. HCl-treatment	955	3725	96	1989.0	1.9
III. Ba(OH)$_2$-treatment	900	3600	96	217.8	16.5
IV. ZnCl$_2$-treatment					
a. Zn-precipitable fraction	150	1245	33	52.5	23.7
b. Zn-soluble fraction	850	1530	41	74.0	20.7
ANTIBIOTIC 36534					
I. crude	960	8373	100	13680.0	0.6
II. HCl-treatment	975	8093	97	6332.5	1.3
III. Ba(OH)$_2$-treatment	910	8008	96	2138.0	3.7
IV. ZnCl$_2$-treatment					
a. Zn-precipitable fraction	150	2955	35	288.0	10.3
b. Zn-soluble fraction	390	3393	41	322.8	10.5

* units of antibiotic activity per mg of protein

IV. FURTHER PURIFICATION OF ZINC-PRECIPITABLE AND ZINC-SOLUBLE FRACTIONS

Further purification was felt to be necessary since the specific activity of final products from fractions of both antibiotics showed no sign of reaching a plateau.

(1). Ion-exchange chromatography:

A cation-exchanger, Dowex 50-X2, was selected and was prepared and operated as described in Materials and Methods. Results are shown in Fig. 3. It was noted that all fractions showed only one antibiotic activity peak which came off the column about 100 to 200 ml after the void volume (V_o = 300 ml). The peak of activity, in all cases, was not coordinated with the peak absorption at 280 nm. Table XXII shows that activity levels for zinc-precipitable and zinc-soluble 29297 in the effluent were low, about 80% of the activity of both fractions being irretrievably lost on the column. Specific activity decreased with the zinc-precipitable fraction; the increase shown by the zinc-soluble fraction was negligible. Results obtained with 36534 were different. Only about 5 - 10 % of the antibiotic activity was lost on the column, and specific activity of both fractions increased about two to three fold.

(2). Gel filtration:

Gel filtration was conducted with Sephadex G-15 columns which were prepared and operated as described in Materials and Methods. The partially purified antibiotic fluid, after zinc treatment, was subjected

FIGURE 3

DOWEX 50-X2 ELUTION PATTERNS OF DIFFERENT FRACTIONS OF
ANTIBIOTICS 29297 AND 36534

(A). 29297 ZINC-PRECIPITABLE FRACTION

(B). 29297 ZINC-SOLUBLE FRACTION

(C). 36534 ZINC-PRECIPITABLE FRACTION

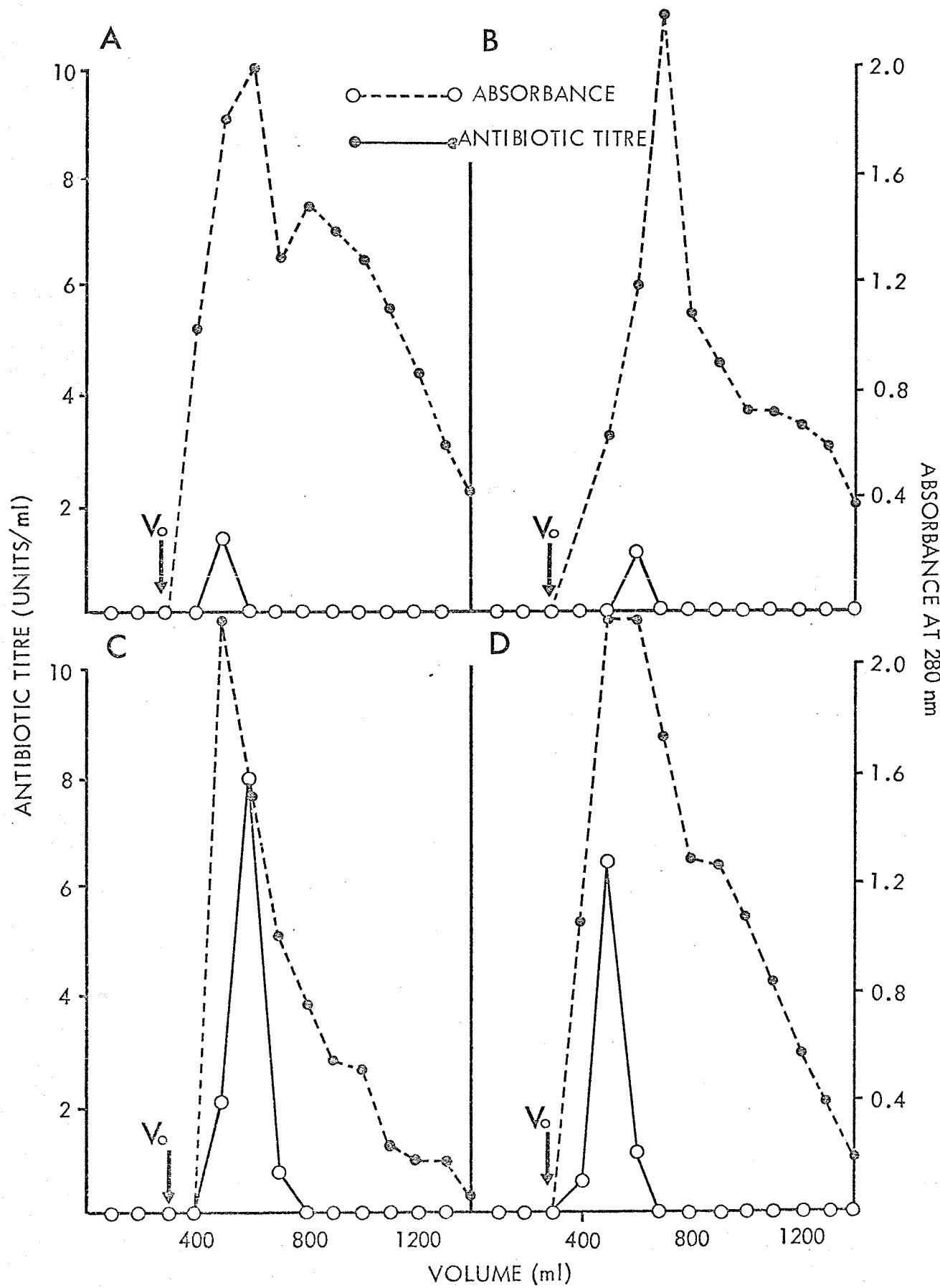(D). 36534 ZINC-SOLUBLE FRACTION

V_o = void volume

TABLE XXII

EFFECT OF DOWEX 50-X2 PASSAGE ON SPECIFIC ACTIVITY OF
ZINC-PRECIPITABLE AND SOLUBLE FRACTIONS OF ANTIBIOTICS 29297 AND 36534

fraction	antibiotic 29297			antibiotic 36534		
	activity (total units)	recovery (%)	specific activity*	activity (total units)	recovery (%)	specific activity
Zn-precipitable						
before	670	100	31.2	1330	100	32.4
after	130	19	18.1	1100	83	72.8
Zn-soluble						
before	730	100	18.7	900	100	25.0
after	.110	15	22.7	890	99	79.5

* units of antibiotic activity per mg of protein

to gel filtration on Sephadex G-15. The elution patterns from the G-15 columns are illustrated in Figs. 4 and 5. In all cases, antibiotic activity was partially or completely separable from material which absorbed at 280 nm. Antibiotic activity itself never showed more than one peak regardless of treatment.

Concentrated active effluents of antibiotic 29297 obtained from Sephadex G-15 were usually colorless. Similar fractions of antibiotic 36534 were light yellow in color. Rechromatography on Sephadex G-10 (data not shown) did not remove this color.

As indicated in Table XXIII, percent recoveries of all fractions were close to unity. Furthermore, specific activity was increased in all cases, the 29297 zinc-soluble fraction showing the largest increase and the 36534 zinc-soluble fraction the smallest.

(3). Crystallization:

After Sephadex gel-filtration, both fractions of antibiotic 29297 could be crystallized at 4°C if the active fluids were previously concentrated at 55°C to a point of saturation or near saturation. However, antibiotic 36534 fractions could not be crystallized under the same conditions if only Sephadex gel-filtration had been previously used for further purification. It was found that the concentrated liquid of the zinc-precipitable and soluble fractions of antibiotic 36534 must be passed through Dowex 50-X2 and then desalted on Sephadex G-15 before crystallization could be effected. Recrystallization of both 29297 and 36534 fractions was performed by dissolving the crystals in distilled water at 55°C until

FIGURE 4

ELUTION PATTERNS OF ANTIBIOTIC 29297 FROM SEPHADEX G-15 COLUMNS

(A). ZINC-PRECIPITABLE FRACTION

(B). ZINC-SOLUBLE FRACTION
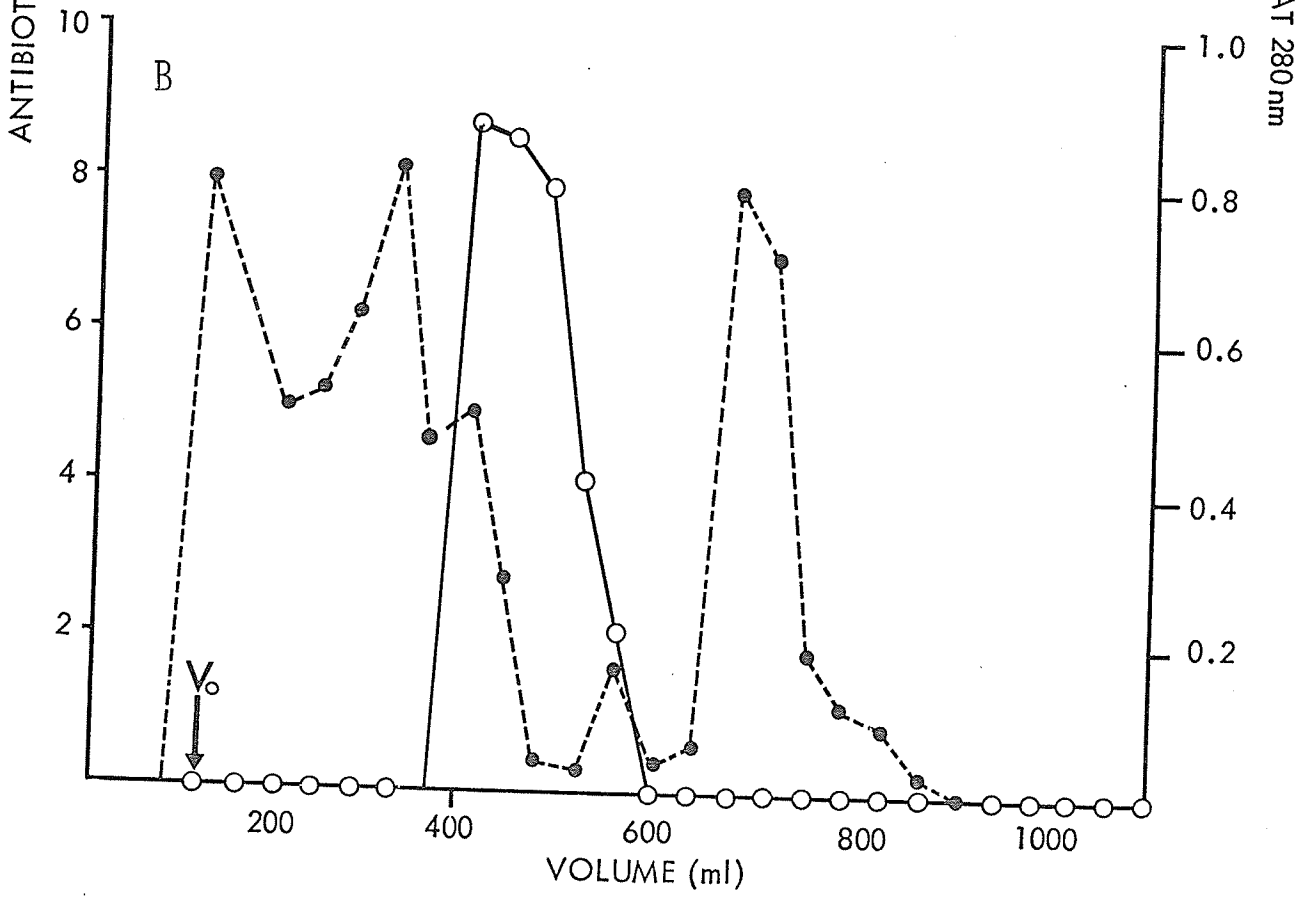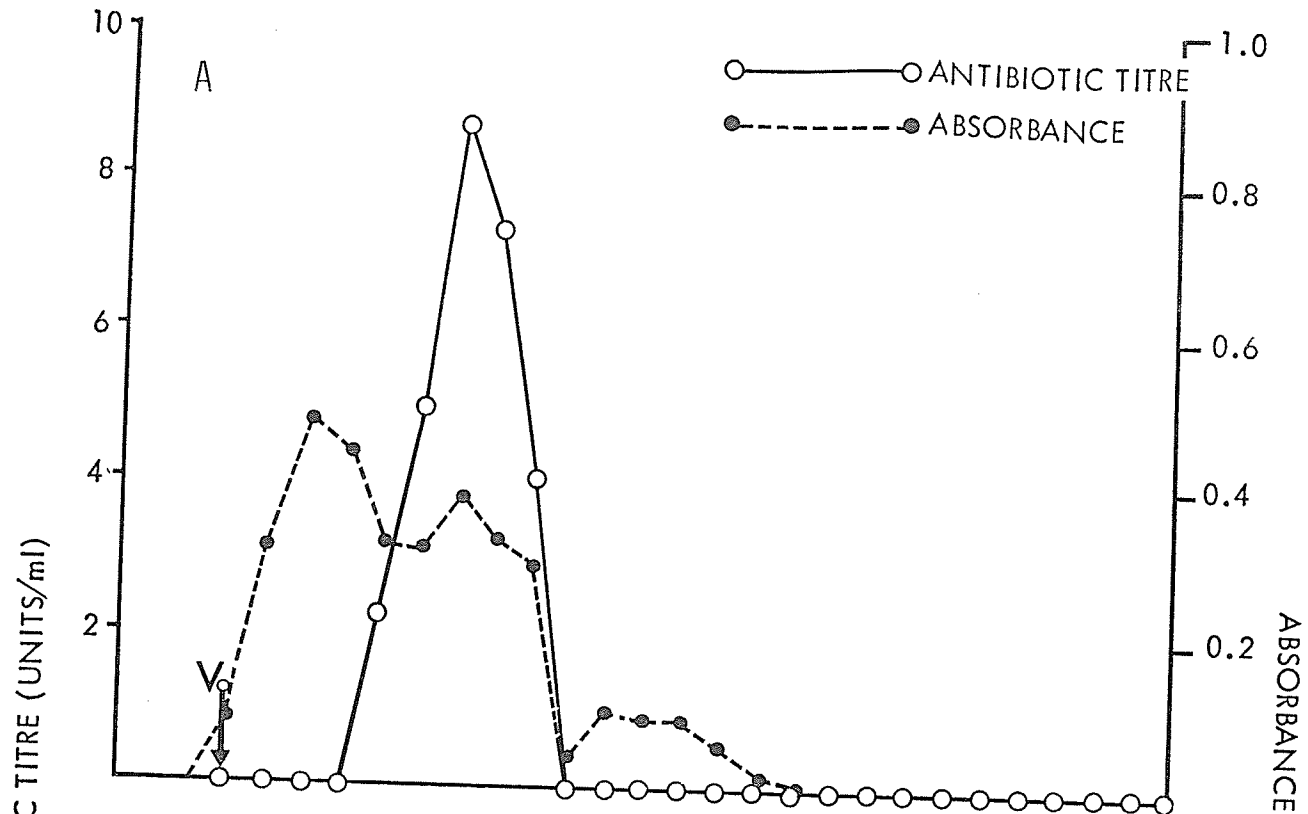
V_o = void volume

FIGURE 5

ELUTION PATTERNS OF ANTIBIOTIC 36534 FROM SEPHADEX G-15 COLUMNS

(A). ZINC-PRECIPITABLE FRACTION

(B). ZINC-SOLUBLE FRACTION

V_o = void volume
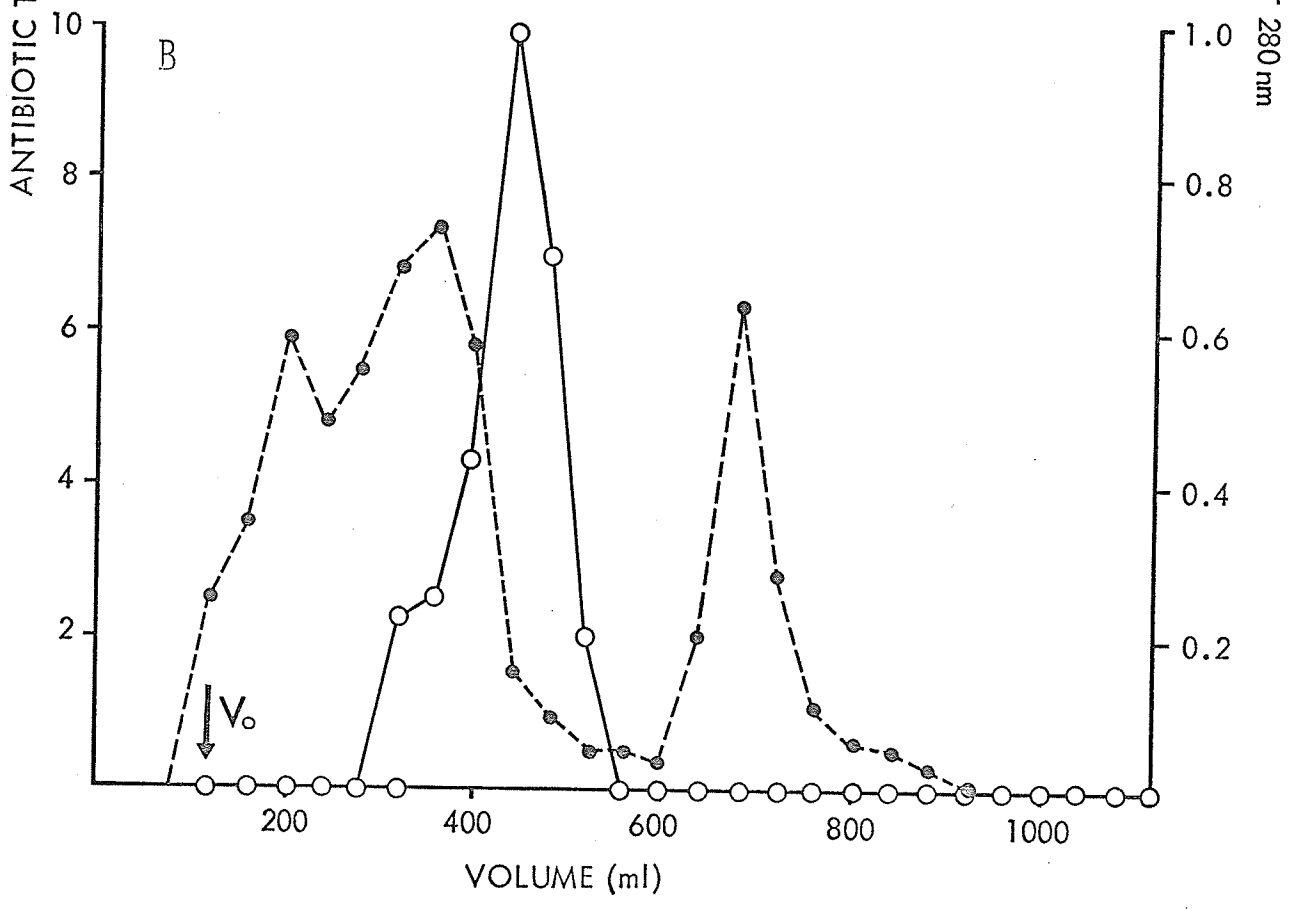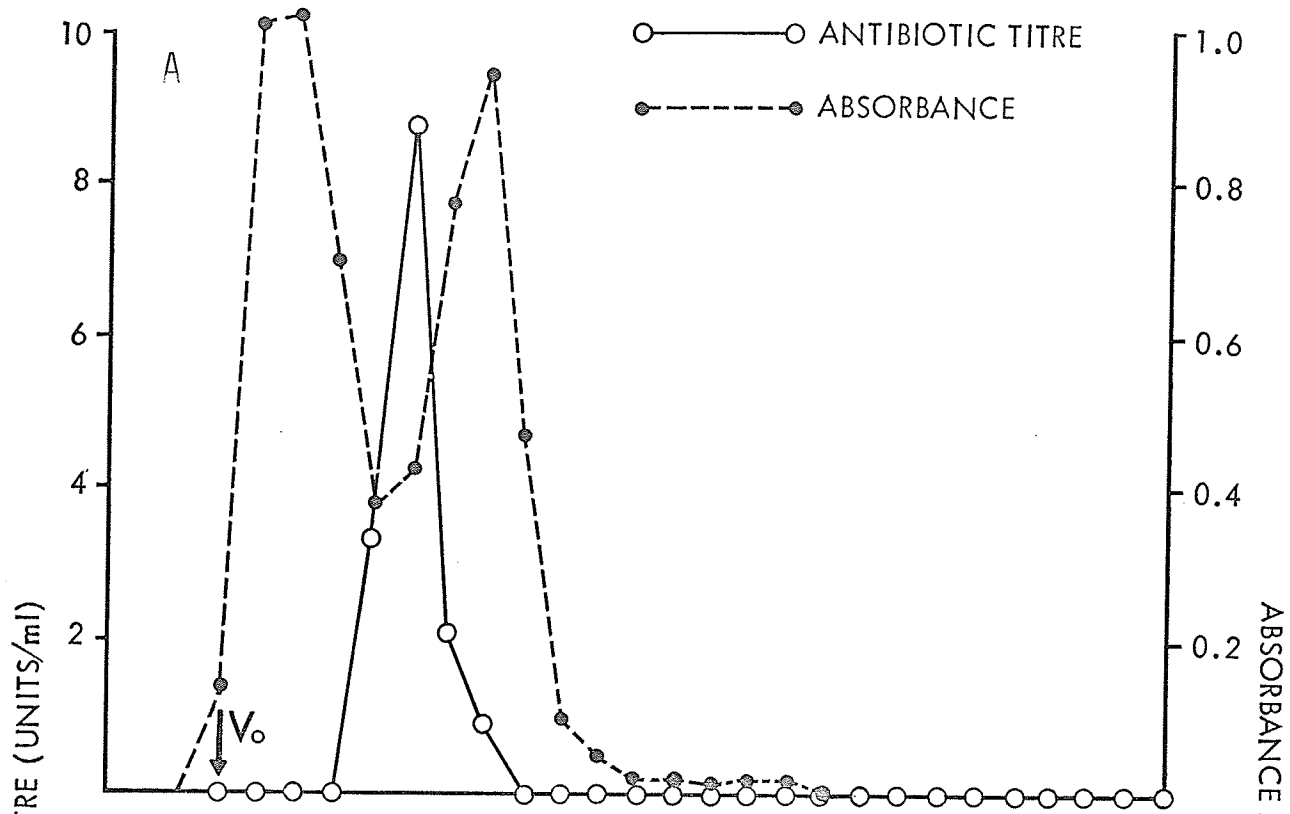
TABLE XXIII

EFFICIENCY OF SEPHADEX G-15 GEL FILTRATION IN THE FURTHER
PURIFICATION OF ANTIBIOTICS 29297 AND 36534

antibiotic fraction	antibiotic activity (total units)		recovery (%)	specific activity*	
	before	after		before	after
29297 zinc-precipitable	620	595	96	36.9	135.2
29297 zinc-soluble	210	205	98	16.8	117.1
36534 zinc-precipitable	190	187	98	20.7	33.4
36534 zinc-soluble	97	94	97	12.9	19.6

* antibiotic units per mg of protein

the liquid was saturated by the antibiotic, then filtration through a Milli-pore HA membrane (pore size = 450 nm) and rapid cooling to 4°C. Little or no further increase in specific activity after recrystallization was noted in all fractions (see Tables XXVI and XXVII). Crystals of the fractions were colorless, odorless and octahedron-shaped, but after standing soon assumed an irregular appearance (Fig. 6). Loss of water may have contributed to this change.

V. FINAL PURIFICATION SCHEMES FOR STAPHYLOCOCCAL ANTIBIOTICS 29297 AND 36534

Final purification schemes for 29297 and 36534 are shown in Tables XXIV and XXV respectively. It will be noted that purification of 29297 (Table XXIV) does not require Dowex 50-X2. That given in Table XXV for 36534 requires that the material must be passed through Dowex 50-X2 followed by desalting on Sephadex G-15.

Recrystallized fractions of both antibiotics were occasionally weakly positive when tested by the Lowry and ninhydrin reagents. Since methanol was effective as a precipitant of antibiotics from partially puri-fied material (see Table XIX), recrystallized fractions were subjected to methanol treatment at 4°C as described previously. It was found that the antibiotics, which were precipitated by this procedure, were generally freed from ninhydrin and Lowry positive material.

Since the antibiotics now contained no Lowry or ninhydrin-posi-tive material, neither of these reagents was of value in specific activity determinations. It was therefore necessary that specific activity be

FIGURE 6

CRYSTAL FORMS OF STAPHYLOCOCCAL ANTIBIOTICS*

   (A). 29297 ZINC-PRECIPITABLE FRACTION

   (B). 29297 ZINC-SOLUBLE FRACTION

   (C). 36534 ZINC-PRECIPITABLE FRACTION

   (D). 36534 ZINC-SOLUBLE FRACTION

      * Mag. = 400X
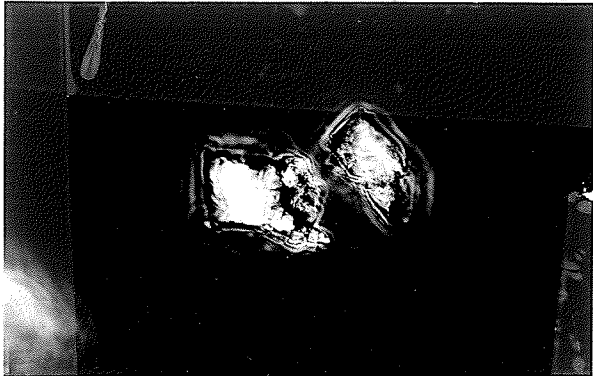
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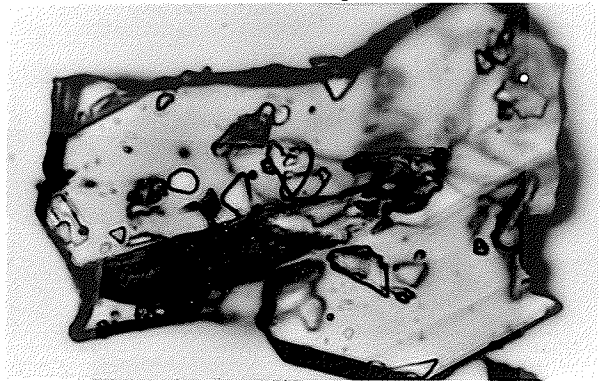
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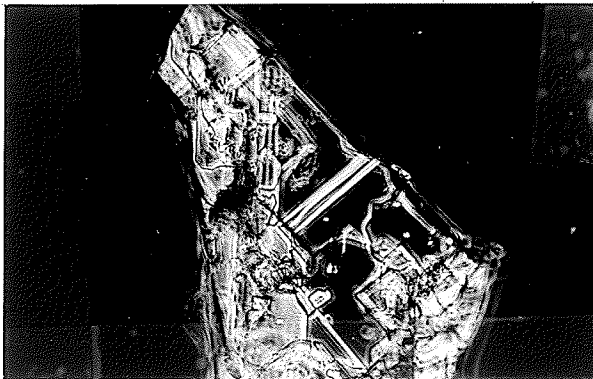
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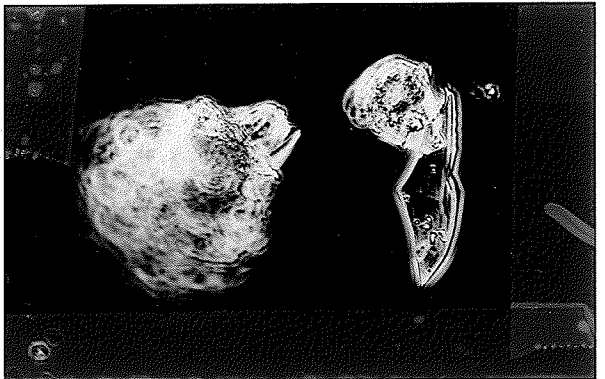
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TABLE XXIV

PURIFICATION SCHEME OF STAPHYLOCOCCAL ANTIBIOTIC 29297

clarified culture fluid
| HCl to pH 2.0
↓
supernatant
| Ba(OH)$_2$ to pH 9.0
↓
supernatant
| ZnCl$_2$ to 0.1M

precipitate (zinc-precipitable fraction)
↓
washed, dissolved into 0.01N HCl solution and neutralized
↓
supernatant
↓
Sephadex G-15 column
↓
concentrated and crystallized
↓
recrystallized
↓
methanol-precipitation (final product)

supernatant (zinc-soluble fraction)
↓
saturated with H$_2$S gas
↓
supernatant
↓
concentrated and neutralized
↓
Sephadex G-15 column
↓
concentrated and crystallized
↓
recrystallized
↓
methanol-precipitation (final product)

TABLE XXV

PURIFICATION SCHEME OF STAPHYLOCOCCAL ANTIBIOTIC 36534

clarified culture fluid

| HCl to pH 2.0

supernatant

| Ba(OH)$_2$ to pH 9.0

supernatant

| ZnCl$_2$ to 0.1M

precipitate
(zinc-precipitable fraction)

washed, dissolved into 0.01N
HCl solution and neutralized

supernatant

Dowex 50-X2 column

Sephadex G-15 column

concentrated and crystallized

recrystallized

methanol-precipitation
(final product)

supernatant
(zinc-soluble fraction)

saturated with H$_2$S gas

supernatant

concentrated and neutralized

Dowex 50-X2 column

Sephadex G-15 column

concentrated and crystallized

recrystallized

methanol-precipitation
(final product)

redetermined by nitrogen analysis, as performed by Markham (1942) micro-

Kjeldahl method (see Table XXVI and XXVII). Recovery of antibiotic 29297

zinc-precipitable fraction after the second methanol precipitation was 3%;

that for the zinc-soluble fraction was 6 %. Percent recoveries for the

zinc-precipitable and soluble fractions of antibiotic 36534 were similar.

TABLE XXVI

SPECIFIC ACTIVITY AND PERCENT RECOVERY OF STAPHYLOCOCCAL ANTIBIOTIC
29297 AT DIFFERENT STAGES OF PURIFICATION

stage of purification	total volume (ml)	total activity (units)	recovery (%)	total nitrogen (mg)	specific activity*
I. crude	955	13084	100	6178.9	2.2
II. HCl-treatment	900	12822	98	4220.0	3.1
III. Ba(OH)$_2$-treatment	770	12691	97	2641.1	4.8
IV. ZnCl$_2$-treatment					
a. zinc-precipitable fraction	520	4318	33	561.6	7.7
1. Sephadex G-15	320	4187	32	144.9	28.9
2. crystallization	55	2094	16	22.1	124.2
3. recrystallization	38	1047	8	12.9	114.2
4. methanol-precipitation	24	654	5	6.7	152.8
5. 2nd methanol-precipitation	25	390	3	2.5	156.0
b. zinc-soluble fraction	735	7850	60	917.5	7.4
1. Sephadex G-15	435	7720	59	440.4	17.5
2. crystallization	135	3664	28	87.0	42.2
3. recrystallization	65	1832	14	35.1	52.0
4. methanol-precipitation	47	1308	10	21.6	60.9
5. 2nd methanol-precipitation	40	785	6	12.8	61.3

* units of antibiotic activity per mg of nitrogen

TABLE XXVII

SPECIFIC ACTIVITY AND PERCENT RECOVERY OF STAPHYLOCOCCAL ANTIBIOTIC
36534 AT DIFFERENT STAGES OF PURIFICATION

stage of purification	total volume (ml)	total activity (units)	recovery (%)	total nitrogen (mg)	specific activity*
I. crude	950	7600	100	8825.5	0.9
II. HCl-treatment	910	7372	97	5427.0	1.4
III. Ba(OH)$_2$-treatment	800	7370	97	3006.9	2.5
IV. ZnCl$_2$-treatment					
a. zinc-precipitable fraction	305	2660	35	408.7	6.5
1. Dowex 50-X2	165	2204	29	123.8	17.9
2. Sephadex G-15	125	2128	28	86.3	24.8
3. crystallization	60	1564	14	13.9	112.5
4. recrystallization	30	782	7	5.0	155.6
5. methanol-precipitation	17	456	6	2.7	165.6
6. 2nd methanol-precipitation	20	304	4	1.8	168.9
b. zinc-soluble fraction	400	3876	51	1328.0	2.9
1. Dowex 50-X2	495	3800	50	405.9	9.4
2. Sephadex G-15	450	3790	50	252.0	15.2
3. crystallization	60	1900	25	27.6	69.6
4. recrystallization	35	988	13	14.4	68.3
5. methanol-precipitation	27	760	10	10.0	76.0
6. 2nd methanol-precipitation	25	530	7	6.9	76.8

* units of antibiotic activity per mg of nitrogen

PART III. CHARACTERIZATION OF ANTIBIOTIC FRACTIONS

I. BIOLOGICAL PROPERTIES

Effect of 29297 zinc-precipitable fraction on viability of S. aureus

209P:

The effect of highly purified [4] antibiotic preparations on the

viability of indicator cells was studied by mixing equal volumes of anti-

biotic solution (85.4 units/ml) and a standardized suspension of the indi-

cator strain, S. aureus 209P, (O.D. = 0.100) in double strength BHI broth.

The mixture was incubated at 37°C. At different intervals, samples (0.5

ml each) were taken and viable counts were made according to the technique

of Miles and Misra (1938). Results were expressed as the mean of 10 counts.

Table XXVIII shows that the antibiotic had a bactericidal effect

upon the indicator strain, about 5% of the initial inoculum alive after 16

hr incubation. Most of the cells were killed in the first hour with the

rate of killing decreased thereafter.

Some observations were made on the appearance of treated indica-

tor strain under the microscope. No evidence of unusual shape or form was

observed in Gram-stained cells, but it was noted that many cells became

Gram-negative after exposure to the antibiotic. The antibiotic did not

cause lysis of the cells as reflected in the absence of a reduction in

optical density (660 nm) of a test suspension.

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(4). twice crystallized and twice methanol precipitated.

TABLE XXVIII

EFFECT OF ANTIBIOTIC 29297 ZINC-PRECIPITABLE FRACTION ON
VIABILITY OF INDICATOR STRAIN OF STAPHYLOCOCCUS AUREUS 209P

time (hr)	viable counts ($\times 10^8$ cells/ml)	
	control	antibiotic*
0	1.16	1.16
$\frac{1}{4}$	0.87	0.44
$\frac{1}{2}$	1.02	0.51
1	1.47	0.19
2	2.34	0.19
4	3.79	0.19
8	11.20	0.18
16	19.50	0.06

* antibiotic concentration = 42.7 units/ml

II. NATURE OF THE ANTIBIOTICS

(1). <u>Stability of antibiotic fluids under ultraviolet light irradiation</u>:

Stability of antibiotic under ultraviolet light irradiation was studied with both partially (HCl-treated) and highly purified (twice crystallized and twice methanol-precipitated) materials. Ten ml of the antibiotic fluid was placed in a disposable plastic Petri-dish, 8.5 cm in diameter. The thickness of the liquid layer in the dish was about 1 mm. The plate was placed under an ultraviolet light lamp (General Electric Co., TB tube, 30 watts, 115 V, 91.5 cm in length) and was irradiated for various lengths of time at room temperature. The distance from the lamp to the center of the plate was 20 cm. After irradiation, the liquid was sterilized by filtration and then assayed for activity.

The results are shown in Table XXIX. It was noted that both the partially and highly purified antibiotic fluids were equally resistant to the irradiation. After 10 min of irradiation, the loss of activity was never higher than 10% of the original in all cases.

(2). <u>Absorption spectra in ultraviolet and visible light</u>:

Aqueous solutions of highly purified material which contained about 2 mg of ninhydrin-positive material[5] per ml were scanned in the Unicam SP-800B spectrophotometer from 200 to 800 nm. It was found that the antibiotics did not show any absorption in the visible light region (350 -

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(5). determined after hydrolysis. Unhydrolyzed material is usually ninhydrin-negative

TABLE XXIX

STABILITY OF PARTIALLY AND HIGHLY PURIFIED STAPHYLOCOCCAL ANTIBIOTICS
UNDER ULTRAVIOLET LIGHT IRRADIATION

material	irradiation time (min)	antibiotic 29297 activity (total units)	recovery (%)	antibiotic 36534 activity (total units)	recovery (%)
partially purified*	0	170	100	190	100
	1	160	94	170	90
	5	165	97	180	94
	10	165	97	170	90
highly purified**					
a. zinc-precipitable fraction	0	400	100	730	100
	1	400	100	720	98
	5	390	98	730	100
	10	400	100	720	98
b. zinc-soluble fraction	0	450	100	870	100
	1	440	98	860	99
	5	440	98	850	98
	10	430	96	850	98

* HCl-treated antibiotic fluids

** Twice crystallized and twice methanol precipitated preparations

800 nm). In the ultraviolet region (200 - 350 nm), however, a small shoulder at about 270 - 280 nm was observed with the 29297 zinc-precipitable fraction (Fig. 7). A similar shoulder at 255 - 265 nm was observed with the 29297 zinc-soluble fraction. Shoulders were observed in the same wavelength regions for the fractions of the antibiotic 36534 but they were very much flattened.

(3). <u>Mobility of crude antibiotics in an electric field at various</u>

<u>pH values</u>:

The mobility of antibiotics 29297 and 36534 in an electric field at different pH values was investigated as reported in Materials and Methods. It was found that antibiotic 36534 showed slight movement towards the anode if the pH of the buffer was above 5.5 and the duration of electrophoresis was longer than 45 min. Antibiotic 29297 on the other hand did not move at any pH regardless of the length of time applied.

(4). <u>Melting points</u>:

The melting point of all four highly purified antibiotic preparations was determined by the method of Shriner <u>et</u> <u>al</u>. (1964). Twice crystallized and twice methanol-precipitated material was first dried at 37°C and further dried at 160°C for two hr. The dried material was immediately ground to a fine powder. The experiment was conducted in a concentrated sulphuric acid bath heated such that the temperature rose at the rate of 1°C per min. It was noted that all fractions tested did not show any sign of melting up to 320°C. Rather, the antibiotics decomposed with charring

FIGURE 7

THE ULTRAVIOLET LIGHT ABSORPTION SPECTRA OF STAPHYLOCOCCAL ANTIBIOTICS

(A). 29297 ZINC-PRECIPITABLE FRACTION

(B). 29297 ZINC-SOLUBLE FRACTION

(C). 36534 ZINC-PRECIPITABLE FRACTION

(D). 36534 ZINC-SOLUBLE FRACTION
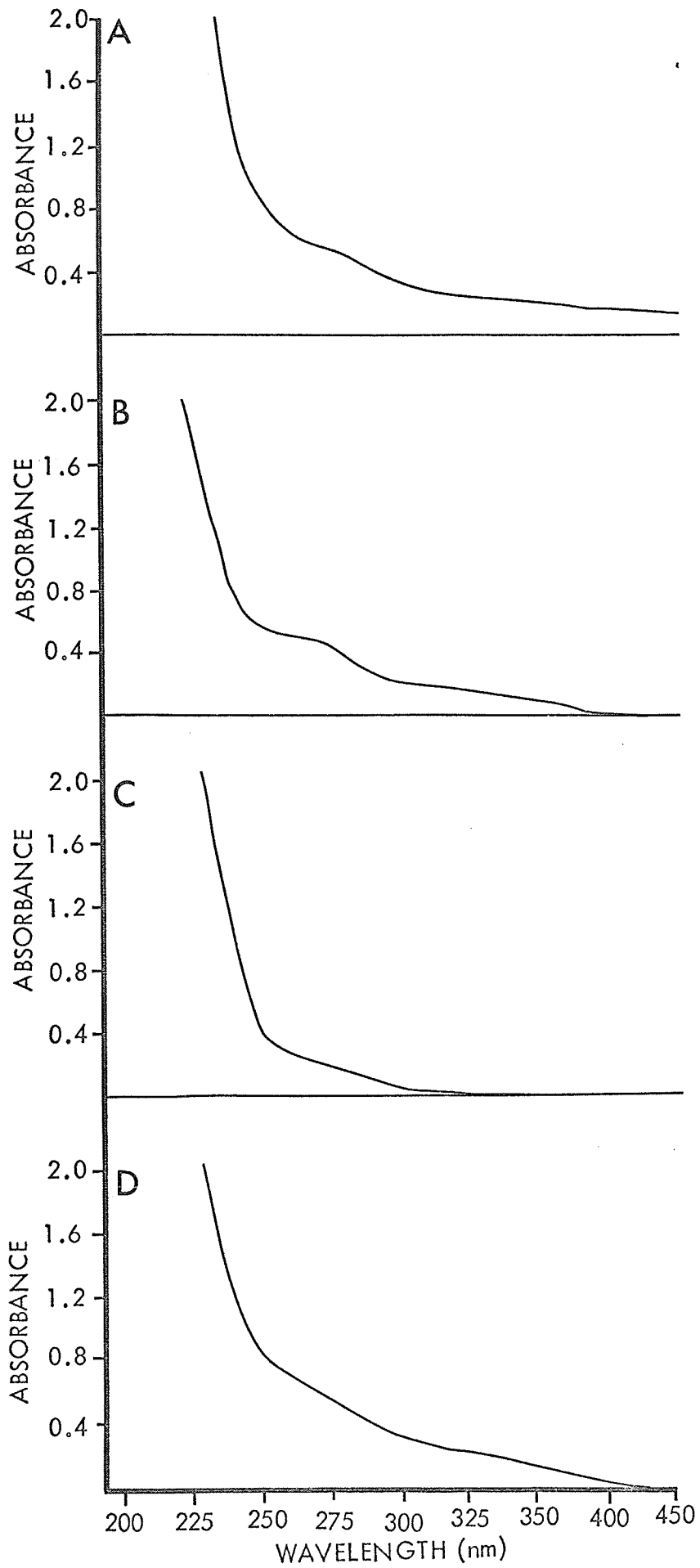
when the temperature reached 278 - 280°C.

III. CHEMICAL ANALYSES

(1). Qualitative:

A number of qualitative chemical analyses was performed on all four highly purified preparations to assess their degree of purity and to help determine their chemical nature. For comparison, a pure substance known to give a positive reaction with the reagent in question was used as a reference. Reagent blanks were also included as negative controls and all work was done in duplicate.

The antibiotics gave positive results with the biuret, periodic acid oxidation, ferrous chloride and Dubois' tests (Table XXX). All other chemical tests conducted (Table XXXI) were negative. The exceptions were Lowry and ninhydrin tests which occasionally showed a weakly positive result with some batches of purified material. These results were thought to be caused by traces of contamination rather than the antibiotics themselves. Such material was further treated with methanol at 4°C until all ninhydrin-positive and Lowry-positive substances were removed.

These analyses clearly indicate that the four purified antibiotics are free of carbohydrates, proteins, lipids, and nucleic acids. The nature of the positive reactions indicates that they may be peptides with some hydroxyl functional groups.

TABLE XXX

POSITIVE CHEMICAL TESTS ON ANTIBIOTICS 29297 AND 36534*

name of test	reference chemical	responsible groups
Biuret	urea	$RCONH_2$, $RCSNH_2$, RCH_2NH_2 or $RCH(NH)NH_2$
Periodic acid oxidation	hydroxyamine-HCl	$RCH(OH)-(OH)CHR'$, $RCH(OH)-COR'$, $RCO-COR'$ or $RCH(OH)-CH(NH_2)R'$
Ferric chloride	phenol	enols
Dubois	glucose	reducing groups

* zinc-precipitable and zinc-soluble fractions from each antibiotic

TABLE XXXI

NEGATIVE CHEMICAL TESTS ON ANTIBIOTICS 29297 AND 36534*

name of test	reference chemical	responsible groups
Molisch	glucose	carbohydrates
Benedict	glucose	reducing sugars
Fehling	glucose	reducing sugars
Silver nitrate	NaCl	halogens
Sodium iodide	NaCl	chlorides
Millon-Nasse	phenol	tyrosine or 3,5-unsaturated phenol
Sulphur	cystine	cysteine or cystine
Barium chloride	cystine	sulphur
Diazotized sulfanilic acid	phenol	phenolic
Formaldehyde-sulphuric acid	phenol	phenolic
Sodium bisulfite	formaldehyde	aldehyde
Tollen	glucose	aldehyde
Potassium permanganate	toluene	unsaturated aliphatic
Fiske-Subbarow	KH_2PO_4	phosphorus
Ninhydrin**	egg albumin	α-NH_2
Lowry**	egg albumin	aromatic amino acid
Xanthoproteic	egg albumin	aromatic

* zinc-precipitable and zinc-soluble fractions from each antibiotic

** occasionally weakly positive

(2). <u>Combustion:</u>

It was felt necessary to ensure that the antibiotic activity of the preparations was not due to the presence of a toxic undetected inorganic compound or metal ion. Solutions of the highly purified materials were first gently heated to dryness in crucibles and then strongly heated until all organic substances in the residues were completely vapourized. The ash left was dissolved in distilled water and the liquid was assayed.

Only a trace of ash was left after burning. This was white in color and showed a yellowish flame in the Bunsen burner. The ash could be dissolved in distilled water and when tested this solution was alkaline (about pH 9). The solution showed no toxic effects on the indicator strain, although the original before burning was highly active.

(3). <u>Pronase hydrolysis:</u>

Since the qualitative chemical analyses led one to believe that these antibiotics were peptide in nature, highly purified antibiotics were incubated with the non-specific proteolytic enzyme, pronase. The experiment was performed as follows: Antibiotic solution was mixed with pronase in a ratio of substrate to enzyme = 1:2 (w/w). The mixture was incubated at $37^{\circ}C$ for various lengths of time. After incubation was complete, the reaction was stopped by autoclaving at 10 lb pressure ($110^{\circ}C$) for 10 min and the antibiotic activity of this heated solution was assayed. Also, pronase-hydrolyzed material was applied to Whatman filter paper no. 1 together with the untreated solution. The paper was developed by a solvent system which contained n-butanol/acetic acid/water, 4:1:5 by volume.

Distilled water in the presence and absence of pronase was tested along with the samples as controls.

The results (Table XXXII) showed that the activity of antibiotic 29297 was completely destroyed after it had been incubated with pronase for 24 hr. In the same length of time, an average of 90% of the activity of antibiotic 36534 was diminished. A longer incubation period with the enzyme up to four days had little effect on the remaining activity of antibiotic 36534. Pronase itself did not have any toxic effects on the indicator strain.

Paper chromatograms revealed that although the untreated material did not show any ninhydrin-positive spots, those treated with pronase had six to seven ninhydrin-positive spots. One of these spots had an identical R_f value with the pronase which had been incubated under the same conditions.

(4). Oxidation by nitrous acid:

Since both qualitative chemical analyses and pronase hydrolysis had demonstrated that the antibiotics likely were peptides, the presence of free α-amino groups and their relationship to antibiotic activity were investigated. Oxidation of the antibiotics with nitrous acid was performed since it is known that nitrous acid reacts specifically with free amino groups by converting these groups to hydroxyl groups with the liberation of nitrogen gas (Oser et al., 1965). Nitrous acid was prepared according to the method of Shriner et al. (1964). Three ml of highly purified antibiotic fluid was mixed with three ml of freshly prepared HNO_2 solution. The mixture was incubated at 55°C for one hr. Then the reaction was

TABLE XXXII

THE EFFECT OF PRONASE ON HIGHLY PURIFIED ANTIBIOTIC PREPARATIONS

material	incubation (days)	antibiotic activity (units/ml) without pronase	with pronase	activity lost (%)
29297 zinc-precipitable	0	6.9	-	0
	1	3.2	0	100
29297 zinc-soluble	0	10.5	-	0
	1	7.2	0	100
36534 zinc-precipitable	0	10.7	-	0
	1	9.5	0.5	95
	4	9.4	0.4	96
36534 zinc-soluble	0	15.5	-	0
	1	14.4	2.3	84
	4	10.7	1.0	91
water	0	0	0	-
	1	0	0	-
	4	0	0	-

stopped by neutralization of the mixture with 4N NaOH. The neutralized
fluid was sterilized by filtration and assayed for antibiotic activity.

It was observed that under the conditions used, the controls which
consisted of a mixture of cysteine and tyrosine, reacted vigorously with the
nitrous acid. On the other hand, little or no reaction was observed in the
mixture of antibiotic fluid and acid.

The results of antibiotic assay (Table XXXIII) showed that after
nitrous acid treatment, antibiotic solutions increased their activity great-
ly. Since sodium nitrite (a product of neutralization of nitrous acid with
NaOH) itself had a toxic effect on the growth of the indicator strain as
shown in the controls, the increase in activity was interpreted as evidence
of a synergistic reaction between antibiotic and sodium nitrite.

(5). Amino acid content:

The amino acid content of highly purified antibiotic preparations
was assayed in the Technicon Auto Analyzer (Technicon Corp.). Before assay,
the material was hydrolyzed by 6N HCl and the acid removed by flash-eva-
poration. Results of the assays (Table XXXIV) revealed that the four frac-
tions of the two staphylococcal antibiotics had an identical numbers of
amino acid residues. Each fraction contained 11 amino acid residues, eight
of which were commonly shared and the remaining three of which were differ-
ent in each of the four fractions. Those shared in common were two glycine,
one aspartic acid (or asparagine), one glutamic acid (or glutamine), one
alanine, one arginine, one lysine and one serine. In addition, the 29297
zinc-precipitable fraction contained one threonine, one valine and one

TABLE XXXIII

EFFECT OF NITROUS ACID OXIDATION ON THE TITRE OF HIGHLY
PURIFIED ANTIBIOTICS

material	antibiotic activity (units/ml)	
	untreated	treated
29297 zinc-precipitable	4.8	8.0
29297 zinc-soluble	7.0	12.0
36534 zinc-precipitable	1.9	2.7
36534 zinc-soluble	5.0	8.0
cysteine and tyrosine mixture (control 1)	0	1.6
water (control 2)	0	0.7

TABLE XXXIV

AMINO ACID COMPOSITION OF STAPHYLOCOCCAL ANTIBIOTICS

| amino acid | minimal number of residues | | | |
| | 29297 | | 36534 | |
	zinc-precipitable	zinc-soluble	zinc-precipitable	zinc-soluble
Glycine	2	2	3	2
Aspartic acid	1	2	1	2
Glutamic acid	1	2	1	1
Alanine	1	1	1	1
Arginine	1	1	1	1
Lysine	1	1	1	1
Serine	1	1	1	1
Threonine	1	0	1	1
Proline	0	1	1	0
Valine	1	0	0	0
Leucine	1	0	0	0
Histidine	0	0	0	1
Ammonia	2	4	2	2
Total amino acid residues	11	11	11	11
Minimal molecular weight	1147	1206	1088	1186

leucine; the 29297 zinc-soluble fraction contained one aspartic acid (or
asparagine), one glutamic acid (or glutamine) and one proline; the 36534
zinc-precipitable fraction had one glycine, one threonine and one proline;
and the 36534 zinc-soluble fraction possessed one aspartic acid (or aspara-
gine), one threonine and one histidine. Based on the sum of the molecular
weight of the amino acid residues, the minimal molecular weight of these
antibiotics would be approximately 1200. This figure coincided with the
elution pattern of Sephadex G-15.

(6). Analyses of terminal amino acid residues:

Although the experiment on oxidation with nitrous acid had re-
vealed that these peptide antibiotics contained no free α-amino groups,
further evidence was sought to support this finding by means of the analy-
sis of terminal amino acid residues. The N-terminal residues in the highly
purified antibiotics were determined by Sanger's method (Sanger, 1945, 1949)
and the C-terminal by the Dakin-West degradation (Vanderhaeghe and Parmen-
tier, 1960). Detailed procedures are given in Materials and Methods.

(a). N-terminal residues:

It was found that either extracts of the FDNB-treated hydrolysates
of the four antibiotic fractions were colorless and showed no yellow spot
on paper chromatograms. The only DNP-amino acid found in the acid hydro-
lysates of the four antibiotic fractions was water soluble ϵ-DNP-lysine.
Based on this evidence, it was concluded that these peptides had no free
α-amino groups in their molecules.

(b). C-terminal residues:

The material treated with acetic anhydride gave the same number

of ninhydrin-positive spots as the untreated control. Also, there were no
apparent differences in the color density of the spots when treated as
compared to the untreated material. The acetic anhydride would react with
any free C-terminal amino acid and convert it to the corresponding amino
alcohol which would not give a positive ninhydrin test. Failure to change
the number of ninhydrin-positive spots meant that there were no free C-
terminal groups present in the antibiotic fractions.

(7). Oxidative deamination of antibiotics by L- and D-amino acid

   oxidases:

Information on L- and D-amino acid residues and their ratio to
one another in the antibiotic peptides was provided by oxidative deamination
in the presence of L- and D-amino oxidases. The experiments were performed
as described in Materials and Methods. Results were expressed as the total
O_2 uptake after incubation for six hr at 37°C which was calculated according
to the formula given by Umbreit et al. (1957).

In Table XXXV, oxygen uptake in μl is expressed as an equivalent
to L-leucine and D-alanine. It is evident from the table that L- and D-
amino acid oxidases deaminated the antibiotic hydrolysates in all cases,
as reflected by O_2 uptake. From the data presented, it has been determined
that some of the amino acids are in the D-form. With respect to 29297
zinc-soluble and 36534 zinc-precipitable fractions, four of the 11 amino
acids are D-form. In the case of 29297 zinc-precipitable and 36534 zinc-
soluble fractions, six of the 11 amino acids occur as the D-isomer.

TABLE XXXV

OXIDATIVE DEAMINATION OF ACID HYDROLYSATES OF PURIFIED ANTIBIOTICS BY L AND D-AMINO ACID OXIDASES

substrate	O_2 uptake in presence of L-amino acid oxidase (μl)	equivalent to L-leucine (mM)	O_2 uptake in presence of D-amino acid oxidase (μl)	equivalent to D-alanine (mM)	ratio (L/D)
L-leucine standard	296.38	100.00	-	-	
D-alanine standard	-	-	116.18	300.00	
29297 zinc-soluble	23.04	7.77	16.05	4.60	7/4
29297 zinc-precipitable	16.15	5.44	20.40	5.85	5/6
36534 zinc-soluble	40.71	13.73	54.67	15.68	5/6
36534 zinc-precipitable	28.50	9.61	20.16	5.78	7/4

* the ratio of equivalents to L-leucine and D-alanine in the same antibiotic preparation

IV. STRUCTURAL STUDIES

Highly purified 29297 zinc-precipitable fraction was used for structural studies. The material was first subjected to partial acid hydrolysis which was conducted as described in Materials and Methods. After completion of hydrolysis, peptide fragments were conjugated with Sanger's FDNB reagent. Nine DNP-peptides were thus isolated and analyzed. The results (Table XXXVI) showed that four contained lysine, two contained valine and the rest leucine, serine or aspartic acid (or asparagine) as the N-terminal amino acid residues. The amino acid content of each peptide fragment is also given in the Table. From these results, a tentative sequence for each peptide fragment was deduced and these are compiled in Table XXXVII.

Since this antibiotic fraction contained two glycine residues per molecule of the antibiotic, and since glycine would only show one ninhydrin-positive spot on paper chromatograms, two alternative sequences are possible for fragments 4 - 8. All the peptide sequence overlapped one another, the overall arrangement of these fragments giving

Lys-Ala-Asp-Glu-Ser-Leu-Thr-Gly-Val-Gly-Arg-Lys-Ala

or

Lys-Ala-Asp-Glu-Ser-Leu-Thr-Val-Gly-Gly-Arg-Lys-Ala

TABLE XXXVI

AMINO ACID CONTENT OF PEPTIDE FRAGMENTS ISOLATED FROM PARTIAL
ACID HYDROLYSATES OF THE 29297 ZINC-PRECIPITABLE FRACTION

peptide fragment	DNP-amino acid	free amino acid content
1	di-DNP-Lysine	Ala, Asp, Glu
2	di-DNP-Lysine	Ala, Asp, Glu, Ser
3	di-DNP-Lysine	Ala, Asp, Glu, Ser, Leu, Thr
4	di-DNP-Lysine	Ala, Asp, Glu, Ser, Leu, Thr, Gly, Val
5	DNP-Valine	Gly
6	DNP-Valine	Gly, Arg, Lys, Ala
7	DNP-Leucine	Thr, Val, Gly, Arg, Lys, Ala
8	DNP-Serine	Leu, Thr, Val, Gly, Arg
9	DNP-Aspartic acid	Glu, Ser

TABLE XXXVII

TENTATIVE PEPTIDE SEQUENCES OF FRAGMENTS ISOLATED FROM PARTIAL ACID
HYDROLYSATES OF ANTIBIOTIC 29297 ZINC-PRECIPITABLE FRACTION

peptide fragment	tentative peptide sequence
1	Lys-Ala-Asp-Glu
2	Lys-Ala-Asp-Glu-Ser
3	Lys-Ala-Asp-Glu-Ser-Leu-Thr
4	Lys-Ala-Asp-Glu-Ser-Leu-Thr-<u>Gly-Val-Gly</u> or Lys-Ala-Asp-Glu-Ser-Leu-Thr-<u>Val-Gly-Gly</u>
5	<u>Val-Gly</u> or <u>Val-Gly-Gly</u>
6	<u>Val-Gly</u>-Arg-Lys-Ala or <u>Val-Gly-Gly</u>-Arg-Lys-Ala
7	Leu-Thr-<u>Gly-Val-Gly</u>-Arg-Lys-Ala or Leu-Thr-<u>Val-Gly-Gly</u>-Arg-Lys-Ala
8	Ser-Leu-Thr-<u>Gly-Val-Gly</u>-Arg or Ser-Leu-Thr-<u>Val-Gly-Gly</u>-Arg
9	Asp-Glu-Ser

overall   Lys-Ala-Asp-Glu-Ser-Leu-Thr-<u>Gly-Val-Gly</u>-Arg-Lys-Ala
or
Lys-Ala-Asp-Glu-Ser-Leu-Thr-<u>Val-Gly-Gly</u>-Arg-Lys-Ala

Because there were 11 amino acid residues, this sequence should be

Cyclo-Lys-Ala-Asp-Glu-Ser-Leu-Thr-Gly-Val-Gly-Arg

or

Cyclo-Lys-Ala-Asp-Glu-Ser-Leu-Thr-Val-Gly-Gly-Arg.

The aspartic and glutamic acid residues might be present as asparagine and glutamine respectively.

A tentative structural formula for 29297 zinc-precipitable fraction is given in Fig. 8.

FIGURE 8

TENTATIVE STRUCTURE OF 29297 ZINC-PRECIPITABLE FRACTION
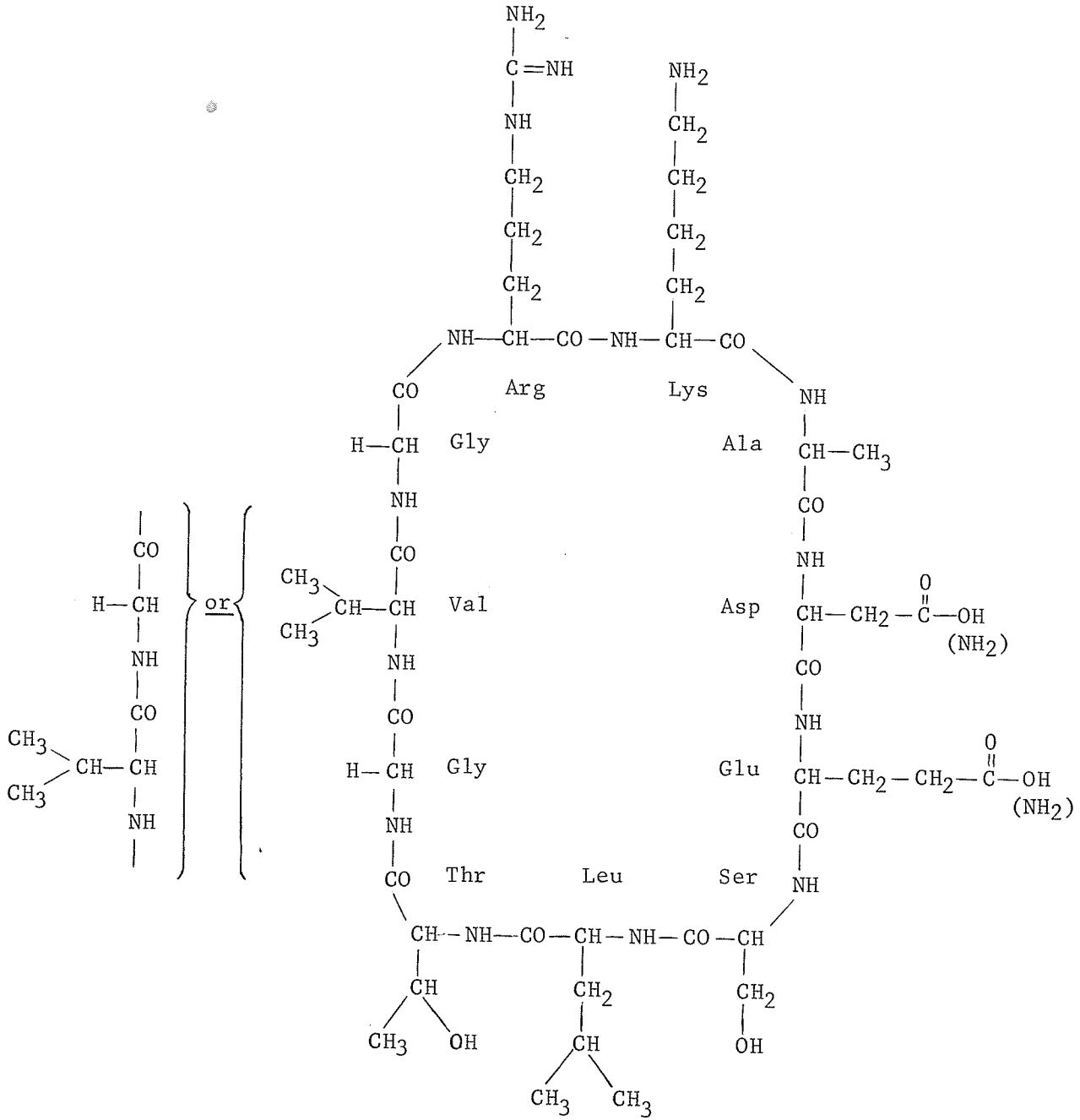
DISCUSSION

DISCUSSION

A method has been developed which permits production of staphylo-
coccal antibiotics in a liquid medium. Quantities of antibiotics produced
in the supplemented Dolman-Wilson medium match those produced on solid
media. Adequate oxygen tension and the presence of Fe^{+++} and Mn^{++} ions
were both necessary for antibiotic production. Other investigators (Dujar-
din-Beaumetz, 1932; Duliscouet, 1939, 1945; Jennings and Sharp, 1949) clai-
med that their strains of Staphylococcus would produce antibiotics only
upon solid media. It is difficult to compare their results with those
presented here in view of the facts that different strains and different
media were used by these investigators. Furthermore, the genus Staphylo-
coccus appears to produce a large family of closely related antibacterial
agents, many of which might conceivably require different conditions for
production. Evidence has been put forward in this investigation that
several fractions with antibacterial activity are produced by Staphylococcus
strains 29297 and 36534.

Optimal antibiotic production by both strains in the supplemented
liquid medium required an incubation temperature of 32°C. A temperature
of 37°C, however, was required for maximal growth. The appearance of anti-
biotic coincided with commencement of the stationary phase of growth with
respect to strain 36534, but Fig. 2 indicates that for strain 29297, maxi-
mum titres were reached at the end of the logarithmic phase of growth. No
data are available which would indicate unequivocally that the antibiotics
are truly extracellular products,.but the results of Fig. 3 are not incon-

sistent with this. In view of the amino acid composition of the antibiotics, it is unlikely that they would be a component of the cell wall of the producing strain.

Initial observations of Hsu and Wiseman (1967) indicated that antibiotics of both strains 29297 and 36534 remained in the supernatant fluid after precipitation of macromolecular impurities by HCl. This has been confirmed and extended to include precipitation of additional impurities with $Ba(OH)_2$. The resulting precipitate was inert, the activity being found entirely in the supernatant solution. The presence of $Ba(OH)_2$ created optimal conditions for zinc treatment. It is obvious that antibiotic activity precipitated directly from crude material by zinc would be heavily contaminated with proteins, polysaccharides and possibly nucleic acids. Thus it was felt that use of a heavy metal salt in purification must be preceded by removal of such substances by acid or other treatment.

The addition of $ZnCl_2$ to $Ba(OH)_2$-treated antibiotics separated the material into two active fractions. Since a part of the activity is precipitable by zinc ions, it seems feasible to suggest that Zn^{++} combines with one of the components of the mixture. The zinc-antibiotic complex is soluble at pH 2 or lower, which indicates that dissociation of the complex has probably taken place. Since it is impossible to test the antibiotic activity of this solution at such low pH, it is necessary to neutralize with NaOH. It was observed that a precipitate was formed after the pH of the solution was brought to 7. It is very likely that this precipitate is $Zn(OH)_2$ since Na^+ ions are higher in the electromotive series than Zn^{++}. Consequently, Na^+ would replace Zn^{++} in the zinc-antibiotic complex. The

overall reaction which taken place is thought to be represented by the
following equations:

(1) $Zn^{++} + AB^{--} \xrightarrow{\text{pH 9}} ZnAB\downarrow$ (precipitation)

(2) $ZnAB \xrightarrow{\text{pH 2}} Zn^{++} + AB^{--}$ (dissociation)

(3) $Zn^{++} + AB^{--} + 2NaOH \xrightarrow{\text{pH 7}} Zn(OH)_2\downarrow + Na_2AB$ (replacement)
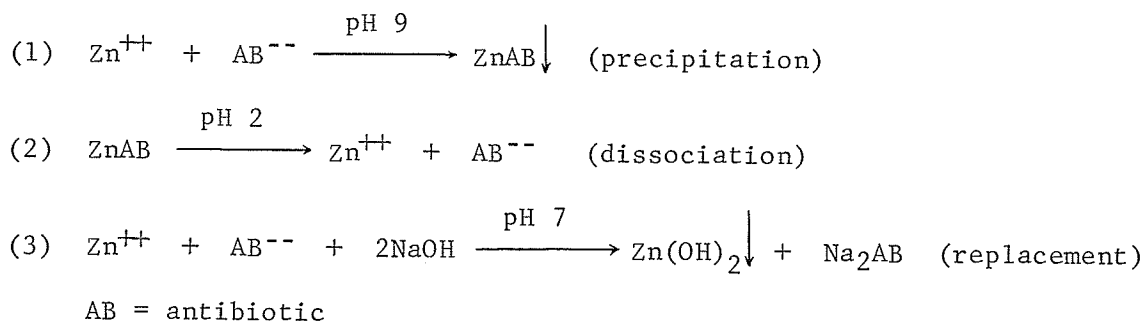
AB = antibiotic

With regard to the supernatant fraction of the zinc-treated anti-
biotics, soluble Ba^{++} and Zn^{++} ions were removed by the application of H_2S
gas. Hydrogen sulphide will precipitate these ions as the insoluble sul-
phides. The solution at this point can be neutralized and concentrated
without loss of activity.

Anker et al. (1948) reported that bacitracin could be precipitated
with inactivation by heavy metal ions low in the electromotive series. How-
ever, they found that heavy metals high in the series precipitated the anti-
biotic without loss of activity. With regard to staphylococcal antibiotics
29297 and 36534, the opposite situation applies. As already mentioned Ca^{++}
and Mn^{++}, in contrast with Zn^{++} ions afford low recovery of activity.

Precipitation of 29297 and 36534 by Zn^{++} separates the antibio-
tics into a zinc-precipitable and a zinc-soluble fraction. As already
stated, the addition of extra Zn^{++} to supernatant permits no further reco-
very from that source. It is not, therefore, correct to state that the
precipitation caused by Zn^{++} ions is incomplete. Rather it suggests that

Zn^{++} has precipitated one of two antibiotics present in crude culture.

In connection with the further purification of these antibiotic fractions, Sephadex G-15 was used to remove impurities from both zinc-precipitable and zinc-soluble components of 29297 and 36534. However, as Table XXIII shows, this treatment increased the specific activity of the two components of 29297 four to seven-fold. With regard to 36534, the increase was much less, but Sephadex G-15 was nevertheless of value in desalting the solutions. Passage of the components of 36534 through Dowex 50-X2 also increased specific activity two to three-fold with good recoveries. Its use with 29297 caused little or no increase in specific activity withlow recovery. Consequently it was felt that Dowex was of no value in the purification of 29297.

The evidence presented in this study strongly indicates that the zinc-precipitable and zinc-soluble antibiotic fractions of strains 29297 and 36534 are low molecular weight peptides. The positive periodic acid oxidation test suggests that these fractions do contain free hydroxyl groups. These are characteristic of the amino acids threonine and serine, either of which or both are found in all fractions. Aromatic amino acids are not present in any fraction as indicated by amino acid analysis, negative Lowry and xanthoproteic tests. However, a small shoulder was observed in the ultraviolet light absorption spectra of all fractions. This would indicate that the antibiotic molecules probably possess a ring structure. The fractions did not give a color with ninhydrin reagent and little or no reaction was observed with nitrous acid. These responses indicate that the antibiotics contain no free α-amino groups as do normal peptides or proteins.

This strongly suggests that the antibiotics are cyclic like numerous other

microbial peptide antibiotics, or that the α-amino groups are masked by

acidic groups as one finds in the polymyxins and gramicidins A, B, and C.

Furthermore, no C- or N-terminal amino groups were detected in any of the

antibiotic fractions. Masking of these groups by fatty acids seemed un-

likely in view of the fact that none were detected in the hydrolysates of

the antibiotics. By contrast, N-terminal amino acid residues are not found

in polymyxins. It has been shown that their N-terminal groups are masked

by linkage with 6-methyl-octan-1-oic acid (MOA) or isooctanoic acid (IOA)

as mentioned in the Review of the Literature. The N- and C-terminal groups

of gramicidins A, B, and C are conjugated with formic acid and ethanolamine

respectively.

In summary, evidence which indicates cyclization of the four

antibiotic fractions is listed as follows:

(1) ninhydrin test and nitrous acid oxidation negative

(2) no detectable C- and N-terminal amino groups

(3) small shoulder at 260 - 280 nm in UV spectra

(4) no oily (fatty acid) substances detected in acid hydrolysates

All fractions are very soluble in water but insoluble in the

usual organic solvents. According to the amino acid analyses, none of the

amino acids found in the fractions contain hydrophobic side chains.

None of the fractions proved to be particularly mobile in an

electric field. The isoelectric point (pI) depends upon the pK_a values of

the ionic groups of the side chains. The average of the total pK_a values

should give an approximate value of the pI. Ionic groups found in the

antibiotic fractions are β-carboxyl of aspartic acid (pK_a = 3.0 - 4.7),

γ-carboxyl of glutamic acid (pK_a = 4.4), ϵ-amino of lysine (pK_a = 9.4 -

10.6), guanidinium of arginine (pK_a = 11.6 - 12.6) and imidazolium of

histidine (pK_a = 5.6 - 7.0). The numbers of acidic and basic groups in

the antibiotic molecules are roughly the same. Therefore, the pI of the

antibiotics should approximate 7.

The structure of the 29297 zinc-precipitable fraction has been

tentatively determined as shown below:

$$\text{Lys} \longrightarrow \text{Ala} \longrightarrow \text{Asp} \longrightarrow \text{Glu} \longrightarrow \text{Ser} \longrightarrow \text{Leu}$$
$$\uparrow\quad\text{Arg} \longleftarrow \text{Gly} \longleftarrow \text{Val} \longleftarrow \text{Gly} \longleftarrow \text{Thr} \longleftarrow\quad$$

However, the region -Gly$\longrightarrow$Val$\longrightarrow$Gly- is in dispute. Reference to Table

XXXVII of the Results indicates that this region of peptide fragments 4 -

8 may possibly be -Val$\longrightarrow$Gly$\longrightarrow$Gly-. The problem here is reflected in

the fact that two glycine have been detected in each mole of the antibiotic.

Also, no peptide fragments have been isolated which contain only glycine

or valine. Two peptide fragments isolated contained the repeating units

-Lys$\longrightarrow$Ala-. The same repeating unit is found in fragments 1 - 4. This

provides further strong evidence of cyclization.

Although the structure of the other three antibiotic peptides

was not determined, it is very likely, in view of their chemical similari-

ties, that they are also cyclic in structure. Eight amino acids are shared

in common by all fractions, the exceptions being valine, leucine and serine.

If these amino acids are absent, they may be replaced by others such as

aspartic and glutamic acids, proline or histidine. In the polymyxins, tyrocidines and gramicidins, a number of amino acids likewise are shared in common by members of the groups, and certain individual amino acids may or may not be present and therefore substituted by others.

The staphylococcal antibiotics show some of the specific features of peptide antibiotics as compiled by Bodanszky and Perlman (1969). These are (1) the occurrence of D-amino acids, (2) the existence of proline and imino acid, (3) the formation of small cyclic areas within the macrocyclic structure of the peptide molecule, and (4) stability to heat and relative insensitivity to the action of proteolytic enzymes (see Hsu and Wiseman, 1967). However, they felt that antibiotics which contain arginine or arginine and histidine are relatively uncommon.

The unique features of the staphylococcal antibiotics investigated in this study are that they do not contain sulphur, aromatic or unusual amino acids and they have a high ratio of D- to L-amino acids. Non-amino acid moieties are also not found in their molecules.

The relationship between other staphylococcal antibiotics and the antibacterial peptides investigated in this study is unclear. However, it is evident that 29297 and 36534 peptides are not enzymes in contrast with the lysostaphin group of Schindler and Schuhardt (1964). None of the 29297 and 36534 peptides causes lysis of sensitive strains at any concentration and they cannot therefore be identical with lysostaphins or with the staphylolytic agent described by Suginaka et al. (1967). Since so little is known of the antibacterial substances isolated from staphylococci by Myers (1946), Jennings and Sharp (1947), Gardner (1949) and Loeb et al. (1950), it is

impossible to compare the preparations with those described by these investigators. It is likely, however, that the agents described are peptides. Fregnan and Smith (1962), Barrow (1962, 1963) and Lachowicz (1965) described staphylcoccal antibacterial agents which probably were peptides, as well, but again, too little is known of these substances to make a proper comparison of them with the 29297 and 36534 peptides.

Another group of antibacterial agents of interest is that described by Fredericq (1946) which he called "staphylococcin". Fredericq thought that such substances should be classified as bacteriocins. Hamon and Peron (1963) studied 20 staphylococcins and found that Listeria, Bacillus and Corynebacterium were often sensitive to the agents. Obviously the staphylococcins described are not true bacteriocins as defined by Jacob et al. (1953), nor are the 29297 and 36534 peptides properly called bacteriocins, sincetheir inhibitory spectrum extends through the range of Gram-positive species.

Another antibiotic isolated from Gram-positive cocci worthy of mention is micrococcin, from Micrococcus spp. It has been investigated by Su (1948a,b) and by Heatley and Doery (1951). They have shown that it is a polythiazole which has arisen from cyclization of polycysteine. It is a close relative of micrococcin P isolated from Bacillus pumilus by Fuller (1955). Peptides 29297 and 36534 are unrelated to micrococcin in that they contain no cysteine, or sulphur for that matter. Nisins of streptococci (Mattick and Hirsch, 1944) also contain sulphur amino acids in contrast with the antibiotics described in this study.

Nothing is known of the mode of action of the antibiotics 29297

and 36534. It is known that they are bactericidal (see Part III of Results), and that they are not lytic. Earlier observations by Hsu and Wiseman (1967) indicate that Gram-positive bacteria are sensitive to the agents. Gram-negative species were generally resistant, the only exception being <u>Brucella</u> <u>abortus</u>. The major differences between Gram-positve and Gram-negative bacteria reside in the cell wall structure. It thus seems reasonable to propose that peptides 29297 and 36534 act by blocking cell wall synthesis. Speculation beyond this is not possible at the present time.

SUMMARY

SUMMARY

(1) Antibiotics from <u>Staphylococcus epidermidis</u> 29297 and 36534 were produced in supplemented Dolman-Wilson medium in the absence of agar. Aeration (as reflected by agitation) and temperature were important in the production of maximum amounts of antibiotics. Length of incubation was also a factor.

(2) Antibiotic assays were affected by the nature of the suspending broth and length of incubation.

(3) Initial purification of crude antibiotics involved treatment with HCl to pH 2.0. The supernatant fluid, which contained the activity, was adjusted to pH 9.0 with $Ba(OH)_2$. The Ba^{++} ions precipitated further impurities and brought the pH to optimal conditions for treatment with $ZnCl_2$.

(4) Precipitation of the active principles with Zn^{++} ions separated the 29297 and 36534 antibiotics into two fractions designated zinc-precipitable and zinc-soluble.

The zinc-precipitable and zinc-soluble fractions of 36534 were further purified on columns of Dowex 50-X2 and Sephadex G-15. The two fractions of 29297 were subjected to Sephadex G-15 gel filtration only, since low recoveries were obtained with Dowex 50-X2.

(5) All fractions of antibiotics were twice crystallized from aqueous solutions. Traces of ninhydrin-positive or Lowry-positive material in the crystallized fractions were removed by precipitation with methanol at $4^{o}C$. The final products decomposed without melting when heated to 278
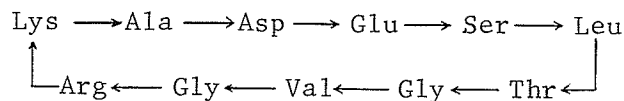
- 280°C to leave a trace of non-toxic white ash.

(6) Recrystallized and twice methanol-precipitated fractions of the antibiotics did not absorb in the visible spectrum (350 - 800 nm) and showed only a small shoulder at 260 - 280 nm in the UV region. The antibiotics were immobile in an electric field.

(7) Qualitative tests showed that the antibiotics were free of carbohydrates, lipids, proteins and nucleic acids. They were sensitive to pronase and could be autoclaved without loss of activity. No N- or C-terminal amino acid residues were detected. The fractions were ninhydrin-negative and did not react with nitrous acid.

(8) Amino acid analyses of the highly purified fractions showed that they contained 11 amino acids, eight of which they shared in common. The three remaining residues varied from fraction to fraction. The minimal molecular weights were approximately 1200 in each case.

(9) The evidence suggests that the 29297 and 36534 zinc-soluble and zinc-precipitable fractions are cyclic peptides, about half of their amino acids occurring as the D-isomers.

(10) Sequence studies of the 29297 zinc-precipitable fraction showed that it probably had the structure:

$$Lys \longrightarrow Ala \longrightarrow Asp \longrightarrow Glu \longrightarrow Ser \longrightarrow Leu$$
$$\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow$$
$$Arg \longleftarrow Gly \longleftarrow Val \longleftarrow Gly \longleftarrow Thr \longleftarrow$$

The -Gly$\longrightarrow$Val$\longrightarrow$Gly- sequence is in dispute and could be -Val$\longrightarrow$Gly$\longrightarrow$Gly-.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abraham, E.P. (1957). The Bacitracins. In: Biochemistry of Some Peptide
    and Steroid Antibiotics. Ciba Lectures in Microbial Biochemistry,
    John Wiley and Sons, Inc., New York, U.S.A., pp. 1 - 29, 1957

Abraham, E.P., Callow, D. and Giller, K. (1946). Adaptation of Staphylo-
    coccus aureus to growth in the presence of certain antibiotics.
    Nature 158, 818, 1946

Abraham, E.P., Heatley, N.G., Brookes, P., Fuller, A.T. and Walker, J.
    (1956). Probable identity of an antibiotic produced by a spore-
    bearing bacillus of Bacillus pumilus group with micrococcin. Nature
    178, 44, 1956

Ainsworth, G.C., Brown, A.M. and Brownlee, G. (1947). Aerosporin. An
    antibiotic produced by Bacillus aerosporus Greer. Nature 160, 263,
    1947

Alderton, G. (1953). A new sulfur-containing amino acid from subtilin.
    J. Amer. Chem. Soc. 75, 2391, 1953

Alderton, G. and Fevold, H.L. (1951). Lanthionine in subtilin. J. Amer.
    Chem. Soc. 73, 463, 1951

Alderton, G. and Snell, N. (1959). Isolation of subtilin A. J. Amer.
    Chem. Soc. 81, 701, 1959

Anderson, H.H., Villela, G.G., Hansen, E.L. and Reed, R. (1946). Some
    physical and biological properties of subtilin and other antibiotics.
    Science 103, 419, 1946

Anker, H.S., Johnson, B.A., Goldberg, J. and Meleney, F.L. (1948). Baci-
    tracin: Methods of production, concentration and partial purification
    with a summary of the chemical properties of crude bacitracin. J.
    Bacteriol. 55, 249, 1948

Arriagada, A., Florey, H.W., Jennings, M.A., and Wallmark, I.G. (1949).
    Biological observations on ayfivin. Brit. J. Exp. Pathol. 30, 458,
    1949

Banerjee, A.B. and Bose, S.K. (1963). Amino acid configuration of myco-
    bacillin. Nature 200, 471, 1963

Baron, A.L. (1956). Bacitracin compounds and bacitracin recovery from
    crude fermentation liquors. U.S. Patent 2,774,712 (1956), Chem.
    Abstr. 51, 4657, 1957

Barrow, G.I. (1962). Microbial antagonism by Staphylococcus aureus. J. Gen. Microbiol. 31, 471, 1962

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

Barry, G.T., Gregory, J.D. and Craig, L.C. (1948). The nature of bacitracin. J. Biol. Chem. 175, 485, 1948

Battersby, A.R. and Craig, L.C. (1951). The molecular weight determination of polypeptides. J. Amer. Chem. Soc. 73, 1887, 1951

Battersby, A.R. and Craig, L.C. (1952a). The chemistry of tyrocidine. I. Isolation and characterization of a single peptide. J. Amer. Chem. Soc. 74, 4019, 1952

Battersby, A.R. and Craig, L.C. (1952b). The chemistry of tyrocidine. II. Molecular weight studies. J. Amer. Chem. Soc. 74, 4023, 1952

Bavin, E.M., Beach, A.S., Falconer, R. and Friedmann, R. (1952). Nisin in experimental tuberculosis. Lancet 1952(1), 127, 1952

Bell, P.H., Bone, J.F., English, J.P., Fellows, C.E., Howard, K.S., Rogers, M.M., Shepherd, D.G. and Winterbottom, R. (1949). Chemical studies on polymyxin: Comparison with aerosporin. Ann. N.Y. Acad. Sci. 51, 897, 1949

Belozersky, A.N. and Paschina, T.S. (1944). Chemistry of gramicidin S. Lancet 1944(2), 716, 1944

Benedict, R.G. and Langlykke, A.F. (1947). The antibiotic activity of Bacillus polymyxa. J. Bacteriol. 54, 24, 1947

Berridge, N.J. (1949). Preparation of the antibiotic nisin. Biochem. J. 45, 486, 1949

Berridge, N.J. (1952). Countercurrent distribution of nisins. Nature 169, 707, 1952

Berridge, N.J., Newton, G.G.F. and Abraham, E.P. (1952). Purification and nature of the antibiotic nisin. Biochem. J. 52, 529, 1952

Biserte, G. and Dautrevaux, M. (1957a). Structure de la polymyxine B. Bull. Soc. Chim. Biol. 39, 795, 1957

Biserte, G. and Dautrevaux, M. (1957b). A propos de la composition de la colimycine. Compt. Red. Soc. de Biol. 151, 1888, 1957

Blackburn, S. and Lowther, A.G. (1951). The separation of N-2:4-dinitro-

phenyl amino acids on paper chromatograms. Biochem. J. 48, 126, 1951

Bodanszky, M. and Perlman, D. (1964). Are peptide antibiotics small proteins. Nature 204, 840, 1964

Bodanszky, M. and Perlman, D. (1969). Peptide antibiotics. Science 163, 352, 1969

Borowski, E. and Chmara, H. (1967a). Antibiotic edeine. III. Pilot plant scale fermentation and isolation of edeine complex. Acta Microbiol. Pol. 16, 159, 1967

Borowski, E. and Chmara, H. (1967b). Antibiotic edeine. IV. Desalting of edeine preparations by gel filtration technique. Acta Microbiol. Pol. 16, 173, 1967

Borowski, E., Chmara, H. and Jareczek-Morawska, E. (1966). The antibiotic edeine. VI. Paper and thin-layer chromatography of components of the edeine complex. Biochim. Biophys. Acta 130, 560, 1966

Borowski, E., Chmara, H. and Jareczek-Morawska, E. (1967). Antibiotic edeine. V. Isolation and purification of edeine A and B. Chemotherapia 12, 12, 1967

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. 1957

Brink, N.G., Mayfield, J. and Folkers, K. (1951). The purification of subtilin concentrates by countercurrent distribution. J. Amer. Chem. Soc. 73, 330, 1951

Brookes, P., Clark, R.J., Fuller, A.T., Mijovic, M.P.V. and Walker, J. (1960a). Chemistry of micrococcin P. Part III. J. Chem. Soc. 1960, 916, 1960

Brookes, P., Clark, R.J., Majhofer, B., Mijovic, M.P.V. and Walker, J. (1960b). Chemistry of micrococcin P. Part IV. A method for the structural study of thiazoles. J. Chem. Soc. 1960, 925, 1960

Brookes, P., Fuller, A.T. and Walker, J. (1957). Chemistry of micrococcin P. Part I. J. Chem. Soc. 1957, 689, 1957

Brownlee, G. and Jones, T.S.G. (1948). The polymyxins: A related series of antibiotics derived from Bacillus polymyxa. Biochem. J. 43, xxv, 1948

Burton, K. (1955). D-amino acid oxidase from kidney. In: Methods in Enzymology. Vol. II. (Colowick, S.P. and Kaplan, N.O. eds.). Acade-

mic Press Inc., New York, U.S.A., pp. 199 - 204, 1955

Callow, R.K., Glover, R.E. and Hart, P.D. (1947b). Lichenifomin. The anti-
    biotic material from Bacillus licheniformis, concentration and some
    chemical and biological properties. Biochem. J. 41, xxvii, 1947

Callow, R.K., Glover, R.E., Hart, P.D. and Hills, G.M. (1947a). Licheni-
    formin. An antibiotic substance from Bacillus licheniformis, active
    against Mycobacterium tuberculosis. Brit. J. Exp. Pathol. 28, 418,
    1947

Callow, R.K. and Hart, P.D. (1946). Antibiotic material from Bacillus
    licheniformis active against species of mycobacteria. Nature 157,
    334, 1946

Callow, R.K. and Work, T.S. (1952). Antibiotic peptides from Bacillus
    licheniformis. Licheniformins A, B and C. Biochem. J. 51, 558, 1952

Carson, J.F. (1952). The free amino groups of subtilin. J. Amer. Chem.
    Soc. 74, 1480, 1952

Cartwright, N.J. (1955). Serratamic acid, a derivative of L-serine pro-
    duced by organisms of the Serratia group. Biochem. J. 60, 238, 1955

Catch, J.R. and Friedmann, R. (1948). The isolation and partial purifica-
    tion of aerosporin. Biochem. J. 42, lii, 1948

Catch, J.R. and Jones, T.S.G. (1948). The chemical nature of aerosporin.
    Part II. Isolation of a new natural amino acid. Biochem. J. 42, lii,
    1948

Catch, J.R., Jones, T.S.G. and Wilkinson, S. (1948). The chemistry of
    polymyxin A. Isolation of the amino acids D-leucine, L-threonine,
    L-α,γ-diaminobutyric acid and an unknown fatty acid. Biochem. J.
    43, xxvii, 1948

Catch, J.R., Jones, T.S.G. and Wilkinson, S. (1949). The chemistry of
    polymyxin A. Ann. N.Y. Acad. Sci. 51, 917, 1949

Chaiet, L. and Cochrane, T.J.Jr. (1959). Bacitracin. U.S. Patent 2,915,432
    (1959), Chem. Abstr. 55, 27790, 1960

Cheeseman, G.C. and Berridge, N.J. (1957). An improved method of prepar-
    ing nisin. Biochem. J. 65, 603, 1957

Cheeseman, G.C. and Berridge, N.J. (1959). Observations on the molecular
    weight and chemical composition of nisin A. Biochem. J. 71, 185, 1959

Chmara, H. and Borowski, E. (1966). Antibiotic edeine. I. Isolation of

induced high activity mutant of <u>Bacillus</u> <u>brevis</u> Vm-4. Acta Microbiol. Pol. <u>15</u>, 223, 1966

Chmara, H. and Borowski, E. (1968). Antibiotic edeine. VII. Biological activity of edeine A and B. Acta Microbiol. Pol. <u>17</u>, 59, 1968

Christensen, H.N. (1943). Isolation of valylvaline from gramicidin hydrolysate. J. Biol. Chem. <u>151</u>, 319, 1943

Christensen, H.N., Edward, R.R. and Piersma, H.D. (1941). Composition of gramicidin and tyrocidine. J. Biol. Chem. <u>141</u>, 187, 1941

Christensen, H.N., Uzman, L. and Hegsted, D.M. (1945). A note on the amino acid composition of tyrocidine. J. Biol. Chem. <u>158</u>, 279, 1945

Consden, R., Gordon, A.H., Martin, A.J. and Synge, R.L. (1946). Gramicidin S. The sequence of the amino acid residues. Biochem. J. <u>40</u>, xliii, 1946

Consden, R., Gordon, A.H., Martin, A.J. and Synge, R.L. (1947). Gramicidin S. The sequence of the amino acid residues. Biochem. J. <u>41</u>, 596, 1947

Conway, E.J. (1933). An absorption apparatus for the micro-determination of certain volatile substances. II. The determination of urea and ammonia in body fluid. Biochem. J. <u>27</u>, 430, 1933

Cornil, A.V. and Babés, V. (1885). Concurrence vitale des bactéries attenuation de leurs properties dans des milieux nutritifs modifies par d'autres bacteries; tentatives de therapeutique bactériologique. F. Conn. Méd. Prat. (Paris) <u>3 ser. 7</u>, 321, 1885

Craig, L.C., Gregory, J.D. and Barry, G.T. (1949a). Studies on polypeptides and amino acids by countercurrent distribution. Cold Spring Harbour Symp. Quant. Biol. <u>14</u>, 24, 1949

Craig, L.C., Gregory, J.D. and Barry, G.T. (1949b). Purity studies on polypeptide antibiotics. Bacitracin. J. Clin. Invest. <u>28</u>, 1014, 1949

Craig, L.C., Hausmann, W. and Weisiger, J.R. (1952b). The qualitative and quantitative amino acid content of bacitracin A. J. Biol. Chem. <u>199</u>, 865, 1952

Craig, L.C., Hausmann, W. and Weisiger, J.R. (1953). The molecular weight of bacitracin A. J. Biol. Chem. <u>200</u>, 765, 1953

Craig, L.C., Hausmann, W. and Weisiger, J.R. (1954). Structural studies with bacitracin A. J. Amer Chem. Soc. <u>76</u>, 2839, 1954

Craig, L.C., Konigsberg, W. and Hill, R.J. (1958). Bacitracin. Ciba

Found. Symp. on Amino Acids and Peptides with Antimetabolic Activity. (Edited by Wolstenholme, G.E.W. and O'Connor, C.M.) J. and A. Churchill Ltd., London, England, pp. 226 - 246, 1958

Craig, L.C., Weisiger, J.R., Hausmann, W. and Harfenist, E.J. (1952a). The separation and characterization of bacitracin polypeptides. J. Biol. Chem. 199, 259, 1952

Cruickshank, R. (ed.) (1965). Medical Microbiology, 11th edition. E. and S. Livingstone Ltd., London, England, 1965

Dautrevaux, M. and Biserte, G. (1961). Structure de la colimycine. Buil. Soc. Chim. Biol. 43,495, 1961

Dean, B.M., Mijovic, M.P.V. and Walker, J. (1961). Chemistry of micrococcin P. Part VI. Racemisation of 2-(1-amino-2-methyl-propyl)thiazole-4-carboxylic acid, and related studies. J. Chem. Soc. 1961, 3394, 1961

Dimick, K.P., Alderton, G., Lewis, J.C., Lightbody, H.D. and Fevold, H.L. (1947b). Purification and some properties of subtilin. Arch Biochem. 15, 1, 1947

Dimick, K.P., Alderton, G., Lightbody, H.D. and Fevold, H.L. (1947a). A method for purification of subtilin. Federation Proc. 6, 247, 1947

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

Dowling, J.H., Koffler, H., Reitz, H.C., Peterson, D.H. and Tetrault, P.A. (1952). Differentiation between circulin A and B and polymyxin A and E by paper chromatography. Science 116, 147, 1952

Dubos, R.J. (1939a). Studies on a bactericidal agent extracted from a soil bacillus. I. Preparation of the agent. Its activity in vitro. J. Exp. Med. 70, 1, 1939

Dubos, R.J. (1939b). Studies on a bactericidal agent extracted from a soil bacillus. II. Protective effect of the bactericidal agent against experimental pneumococcus infections in mice. J. Exp. Med. 70, 11, 1939

Dubos, R.J. (1941). Bacteriostatic and bactericidal agents obtained from saprophytic microorganisms. J. Pediat. 19, 588, 1941

Dubos, R.J. and Cattaneo, C. (1939). Studies on a bactericidal agent extracted from a soil bacillus. III. Preparation and activity of a protein-free fraction. J. Exp. Med. 70, 249, 1939

Dubos, R.J. and Hotchkiss, R.D. (1941). The production of bactericidal substances by aerobic sporulating bacilli. J. Exp. Med. 73, 629, 1941

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

Duliscouët, R. (1935). Action probiotique et antibiotique des staphylocoques ches les porteurs des germes diphthéritiques. C. R. Soc. Biol. (Paris) 118, 1277, 1935

Duliscouët, R. (1939). Rechérches expérimentales sur une substance bactériolytique dérivéc des germes antagonistes. Ses application dans la thérapeutique de la diphthérie. Arch. Méd. Pharm. Nav. 129, 410, 1939

Fevold, H.L., Dimick, K.P. and Klose, A.A. (1948). Isolation of subtilin from submerged cultures. Arch. Biochem. 18, 27, 1948

Few, A.V. and Schulman, J.H. (1953). Unimolecular films of polymyxins A, B, D, and E at the air-water interface. Biochem. J. 54, 171, 1953

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of Bacillus influenzae. Brit. J. Exp. Pathol. 10, 226, 1929

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, actinomycetes, bacteria and plants. Vol. I. Oxford Univ. Press, London, England, pp. 457 - 459, 1949

Fredericq, P. (1946). Sur la sensibilite et l'activite antibiotique des staphylocoques. Compt. Rend. 140, 1167, 1946

Fregnan, G.B. and Smith, D.W. (1962). Mycobactocidin. A new antibiotic active against mycobacteria. J. Bacteriol. 83, 1069, 1962

Fujikawa, K., Suketa, Y., Hayashi, K. and Suzuki, T. (1965). Chemical structure of circulin A. Experientia 21, 307, 1965

Fujiko, K. and Namiki, S. (1957). Crystalline colistin phosphate. Japanese Patent 6624 (1957), Chem. Abstr. 52, 10510, 1958

Fujimasa, M. (1956). Antibiotic substance, colistin. Japanese Patent 1346 (1956), Chem. Abstr. 51, 6096, 1957

Fuller, A.T. (1955). A new antibiotic of bacterial origin. Nature 175, 722, 1955

Gardner, J.F. (1949). An antibiotic produced by Staphylococcus aureus. Brit. J. Exp. Pathol. 30, 130, 1949

Garson, W., McLeod, C., Tetrault, P.A., Koffler, H., Peterson, D.H. and Colincsworth, D.R. (1949). On the naming of two antibiotics from members of Bacillus circulans group: Circulin and polypeptin. J. Bacteriol. 58, 115, 1949

Gauze, G.F. and Brazhnikova, M.G. (1944a). Gramicidin S and its use in the treatment of infected wounds. Nature 154, 703, 1944

Gauze, G.F. and Brazhnikova, M.G. (1944b). Gramicidin S: Origin and mode of action. Lancet 1944(2), 715, 1944

Gauze, G.F., Kotchetkova, G.V., Freobrazhenskaya, T.P. and Pévzner, N.S. (1956). Production of colimycin in cultures of Streptomyces fradiae var. spiralis. Antibiotiki 1, 4, 1956

Gillver, K. (1949). The antibacterial properties of some species of aerobic spore-forming bacilli. Brit. J. Exp. Pathol. 30, 214, 1949

Gordon, A.H., Martin, A.J.P. and Synge, R.L.M. (1943a). The amino acid composition of gramicidin. Biochem. J. 37, 86, 1943

Gordon, A.H., Martin, A.J.P. and Synge, R.L.M. (1943b). Amino acid composition of tyrocidine. Biochem. J. 37, 313, 1943

Gore, R.G. and Peterson, E.M. (1949). Infrared studies. Ann. N.Y. Acad. Sci. 51, 924, 1949

Grady, J.E., Koffler, H., Parsons, J.L., Kobayashi, T. and Tetrault, P.A. (1958). Linkage between 6-methyloctanoic acid and cyclopeptide in circulin A. Federation Proc. 17, 233, 258

Gregory, J.D. and Craig, L.C. (1948). Countercurrent distribution of gramicidin. J. Biol. Chem. 172, 839, 1948

Gross, E. and Morell, J.L. (1967). The presence of dehydroalanine in the antibiotic nisin and its relationship to activity. J. Amer. Chem. Soc. 89, 2791, 1967

Hamon, Y. and Peron, Y. (1963). Quelques remarques sur les bacteriocins produites par les microbes gram-positifs. Compt. Rend. 257, 1191, 1963

Hassall, C.H. (1948). Subtilin C. An antibiotic concentrate from Bacillus subtilis. Nature 161, 317, 1948

Hausmann, W. (1956). The amino acid sequence of polymyxin B. J. Amer. Chem. Soc. 78, 3663, 1956

Hausmann, W. and Craig, L.C. (1952). Polypeptin. Purification, molecular weight determination and amino acid composition. J. Biol. Chem. 198, 405, 1952

Hausmann, W. and Craig, L.C. (1954). Polymyxin B_1. Fractionation, molecular weight determination, amino acid and fatty acid composition. J. Amer. Chem. Soc. 76, 4892, 1954

Hausmann, W., Weisiger, J.R. and Craig, L.C. (1955a). Bacitracin A. Further studies on the composition. J. Amer. Chem. Soc. 77, 721, 1955

Hausmann, W., Weisiger, J.R. and Craig, L.C. (1955b). Partially hydrolysis studies with bacitracin A. J. Amer. Chem. Soc. 77, 723, 1955

Hayashi, K., Suketa, Y. and Suzuki, T. (1968). Chemical structure of circulin B. Experientia 24, 656, 1968

Hayashi, K., Suketa, Y., Tsukamota, K. and Suzuki, T. (1966). Chemical structure of polymyxin D_1. Experientia 22, 354, 1966

Heatley, N.G. and Doery, H.M. (1951). The preparation and some properties of purified micrococcin. Biochem. J. 50, 247, 1951

Hendlin, D. (1949). The nutritional requirements of a bacitracin-producing strain of Bacillus subtilis. Arch. Biochem. 24, 435, 1949

Hettinger, T.P. and Craig, L.C. (1968). Edeine II. The composition of the antibiotic peptide edeine A. Biochemistry 7, 4147, 1968

Hettinger, T.P. and Craig, L.C. (1970). Edeine IV. Structures of the antibiotic peptides A_1 and B_1. Biochemistry 9, 1224, 1970

Hettinger, T.P., Kurylo-Borowska, Z. and Craig, L.C. (1968). Edeine III. The composition of the antibiotic peptide edeine B. Biochemistry 7, 4153, 1968

Hirsch, A. (1951). Various antibiotics from one strain of Streptococcus lactis. Nature 167, 1031, 1951

Hodds, B.W.G. (1958). Bacitracin. German Patent 1,026,484 (1958), Chem. Abstr. 54, 18879, 1960

Hoogerheide, J.C. (1940a). Studies on capsule formation. III. Inhibition of capsule formation of Klebsiella pneumoniae (Friedlaender's bacterium) by an agent produced by a soil bacillus. J. Bacteriol. 40, 415, 1940

Hoogerheide, J.C. (1940b). An agent isolated from a soil bacillus which inhibits encapsulation of Friedlaender's bacterium and is highly bactericidal for Gram-positive microorganisms. J. Franklin Inst. 229, 677, 1940

Hotchkiss, R.D. (1941). Chemical nature of gramicidin and tyrocidine. J. Biol. Chem. 141, 171, 1941

Hotchkiss, R.D. and Dubos, R.J. (1940a). Fractionation of the bactericidal agent from cultures of a soil bacillus. J. Biol. Chem. 132, 791, 1940

Hotchkiss, R.D. and Dubos, R.J. (1940b). Chemical properties of bactericidal substances isolated from cultures of a soil bacillus. J. Biol. Chem. 132, 793, 1940

Hotchkiss, R.D. and Dubos, R.J. (1940c). Bactericidal fractions from an aerobic sporulating bacillus. J. Biol. Chem. 136, 803, 1940

Hotchkiss, R.D. and Dubos, R.J. (1941). The isolation of bactericidal substances from cultures of Bacillus brevis. J. Biol. Chem. 141, 155, 1941

Howell, S.F. (1949). Purification, crystallization and properties of circulin. An antibiotic from Bacillus circulans group. Federation Proc. 8, 208, 1949

Howell, S.F. (1950). Polypeptin. An antibiotic from a member of the Bacillus circulans group. II. Purification, characterization and properties of polypeptin. J. Biol. Chem. 186, 863, 1950

Hsu, C.Y. and Wiseman, G.M. (1967). Antibacterial substances from staphylococci. Canad. J. Microbiol. 13, 947, 1967

Ilinskoya, S.A. and Rossovskaya, V.S. (1958). Biological peculiarities of Bacillus polymyxa Ross and conditions of submerged biosynthesis of polymyxin. Antibiotiki 3, 10, 1958

Inglett, G.E. and Lodge, J.P. (1959). Comparative phenolic spot tests. Application to some air particulate fractions. Anal. Chem. 31, 248, 1959

Ingram, V.M. (1953). The identification of peptide end-groups as dimethyl-amino acids. J. Biol. Chem. 202, 193, 1953

Ingram, L., Tombs, M.P. and Hurst, A. (1967). Mobility-molecular weight relationships of small proteins and peptides in acrylamide gel electrophoresis. Anal. Chem. 20, 24, 1967

Ishii, S.I. and Witkop, B. (1963). Gramicidin A. I. Determination of composition and amino acid configuration by enzymatic and gas chromatographic methods. J. Amer. Chem. Soc. 85, 1832, 1963

Jacob, F., Lwoff, A., Siminovitch, L. and Wollman, E. (1953). Définition de quelques termes relatifs à la lysogénie. Ann. Inst. Pasteur 84, 222, 1953

James, A.T. and Synge, R.L. (1951). Non-peptide linkages in gramicidin. Biochem. J. 50, 109, 1951

Jansen, E.F. and Hirschmann, D.J. (1944). Subtilin. An antibacterial product of Bacillus subtilis. Culturing conditions and properties. Arch. Biochem. 4, 297, 1944

Jarvis, B., Jeffcoat, J. and Cheeseman, G.C. (1968). Molecular weight distribution of nisin. Biochim. Biophys. Acta 168, 153, 1968

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

Johnson, B.A., Anker, H. and Meleney, F.L. (1945). Bacitracin. A new antibiotic produced by a member of the Bacillus subtilis group. Science 102, 376, 1945

Johnson, B.A. and Meleney, F.L. (1950). Bacitracin. U.S. Patent 2,498,165 (1950), Chem. Abstr. 44, 6088, 1950

Jones, T.S.G. (1948a). The chemical nature of aerosporin. Biochem. J. 42, xxxv, 1948

Jones, T.S.G. (1948b). The chemical nature of aerosporin. III. The optical configuration of the leucine and threonine components. Biochem. J. 42, lix, 1948

Jones, T.S.G. (1948c). The chemistry basis for the classification of the polymyxins. Biochem. J. 43, xxvi, 1948

Kabat, E.A. and Mayer, M.M. (1961). Experimental Immunochemistry. Charles C. Thomas, Publisher, Springfield, Illinois, U.S.A., 1961

Khokhlov, A.S. and Chih Chang-Ch'ing (1961). Determination of the amino acid composition of polymyxin M by the method of countercurrent distribution. Biokhimiya 26, 296, 1961 (English Translation, 26, 267, 1961)

Khokhlov, A.S., Silaev, A.B., Stepanov, V.M., Yulikova, E.P., Troshko, E.V., Levin, E.D., Mamiofe, S.M., Sinitsyna, Z.T., Chi, Chan-Tsin, Solov'eva, N.K., Il'inskaya, S.A., Rossovskaya, V.S., Dmitrieva, V.S., Semenov, S.M., Veis, R.A., Berezina, E.K., and Rubtsova, L.K. (1960). A new type of polymyxin-Polymyxin M. Antibiotiki 5, 3, 1960

King, T.P. and Craig, L.C. (1955a). The chemistry of tyrocidine. IV. Purification and characterization of tyrocidine B. J. Amer. Chem. Soc. 77, 6624, 1955

King, T.P. and Craig, L.C. (1955b). The chemistry of tyrocidine. V. The amino acid sequence of tyrocidine B. J. Amer. Chem. Soc. 77, 6627, 1955

Klipp, R.W. and Barney, J.E.II. (1959). Determination of sulfur traces in naphthas by lamp combustion and spectrophotometry. Anal. Chem. 31, 596, 1959

Knoop, F. and Windaus, A. (1905). Die Konstitution des Histidins. Beitr. Chem. Physiol. Pathol. 7, 144, 1905

Koffler, H. (1959). Chemistry, site of action and biosynthesis of the circulins. Science 130, 1419, 1959

Konigsberg, W. and Craig, L.C. (1959). Cellulose ion-exchange and rotatory dispersion studies with the bacitracin polypeptides. J. Amer. Chem. Soc. 81, 3452, 1959

Konigsberg, W. and Craig, L.C. (1962). On bacitracin F. J. Org. Chem. 27, 934, 1962

Koyama, Y. (1952). A new antibiotic substance, colistin. Japanese Patent 1546 (1952), Chem Abstr. 47, 6097, 1953

Koyama, Y., Kurosawa, A., Tsuchiya, A. and Takakuta, K. (1950). A new antibiotic colistin produced by spore-forming soil bacteria. J. Antibiotics 3, 457, 1950

Kurihara, T. and Suzuki, K. (1953). Colistin. J. Pharm. Soc. Japan 73, 414, 1953

Kurylo-Borowska, Z. (1959a). Antibiotic properties of the strain Bacillus brevis Vm-4. Biul. Inst. Med. Morskiej. 10, 83, 1959

Kurylo-Borowska, Z. (1959b). Isolation and properties of pure edeine. An antibiotic of the strain Bacillus brevis Vm-4. Biul. Inst. Med. Morskiej. 10, 151, 1959

Kurylo-Borowska, Z. (1962). On the mode of action of edeine. Biochim. Biophys. Acta 61, 897, 1962

Lachowicz, T. (1965). Investigations on staphylococcins. Z. Bakteriol. Parasiteuk. Infektionskr. Hyg. Abt. Orig. 196, 340, 1965

Lewis, J.C. and Snell, N.S. (1951). The amino acid composition of subtilin. J. Amer. Chem. Soc. 73, 4812, 1951

Lipmann, F., Hotchkiss, R.D. and Dubos, R.J. (1941). The occurrence of D-amino acids in gramicidin and tyrocidine. J. Biol. Chem. 141, 163, 1941

Lockhart, I.M. and Abraham, E.P. (1954a). The amino acid sequence in bacitracin A. Biochem. J. 58, 633, 1954

Lockhart, I.M. and Abraham, E.P. (1954b). Observations on the structure of bacitracin A. Biochem. J. 58, xlvii, 1954

Lockhart, I.M. and Abraham, E.P. (1956). Experiments on the lysine and aspartic acid residues in bacitracin A. Biochem. J. 62, 645, 1956

Lockhart, I.M., Abraham, E.P. and Newton, G.G.F. (1955). The N-terminal and sulphur-containing residues of bacitracin A. Biochem. J. 61, 534, 1955

Lockhart, I.M., Newton, G.G.F. and Abraham, E.P. (1954). Structure of bacitracin A. Nature 173, 536, 1954

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

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193, 265, 1951

Magarão, M.F., Arriagada, V.A. and Thales, S. (1944). Lytic action of Bacillus subtilis on Mycobacterium tuberculosis. Rev. Brasil de Med. 1944, 556, 1944, Abstracted in Amer. Rev. Tuberc. Abstr. 51, 59, 1955

Majumdar, S.K. and Bose, S.K. (1958). Mycobacillin. A new antifungal antibiotic produced by Bacillus subtilis. Nature 181, 134, 1958

Majumdar, S.K. and Bose, S.K. (1960). Amino acid sequence in mycobacillin. Biochem. J. 74, 596, 1960

Markham, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. 36, 790, 1942

Mattick, A.T.R. and Hirsch, A. (1944). A powerful inhibitory substance produced by group N streptococci. Nature 154, 551, 1944

Mattick, A.T.R. and Hirsch, A. (1947). Further observations on an inhibitory substance (nisin) from lactic streptococci. Lancet 1947(2), 5, 1947

McLeod, C. (1948). Circulin. An antibiotic from a member of the Bacillus circulans group. I. Bacteriological studies. J. Bacteriol. 56, 749, 1948

Meleney, F.L. and Johnson, B.A. (1949). Bacitracin. Amer. J. Med. 7, 794, 1949

Mijovic, M.P.V. and Walker, J. (1960). Chemistry of micrococcin P. Part II. J. Chem. Soc. 1960, 909, 1960

Miles, A.A. and Misra, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. 88, 732, 1938

Moore, S. and Stein, W.H. (1951). Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem. 192, 663, 1951

Moore, S. and Stein, W.H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211, 907, 1954

Morito, T. (1962a). The antibiotic colistin from Bacillus colistinus. III. Isolation of colistin A_1 and A_2. Nippon Nogei Kagaku Kaishi 36, 393, 1962

Morito, T. (1962b). The antibiotic colistin from Bacillus colistinus. IV. The amino acid sequence of colistin A_1. Nippon Nogei Kagaku Kaishi 36, 398, 1962

Murray, F.J. and Tetrault, P.A. (1948). A new antibiotic effective against Gram-negative organisms. Proc. Soc. Amer. Bacteriol. 1, 20, 1948

Murray, F.J., Tetrault, P.A., Kaufmann, O.W., Koffler, H., Peterson, D.H. and Colingsworth, D.R. (1949). Circulin. A new antibiotic from an organism resembling Bacillus circulans. J. Bacteriol. 57, 305, 1949

Myers, G. (1946). The inhibition of the growth of Mycobacterium by Staphylococcus. M.Sc. Thesis, University of Alberta, Edmonton, Alberta, 1946

Newton, G.G.F. (1949). Antibiotics from a strain of Bacillus subtilis. Bacilipin A and B, and bacilysin. Brit. J. Exp. Pathol. 30, 306, 1949

Newton, G.G.F. and Abraham, E.P. (1950). Ayfivin and bacitracin. Resolution of crude products into similar series of peptides. Biochem. J. 47, 257, 1950

Newton, G.G.F. and Abraham, E.P. (1953a). Some properties of the bacitracin polypeptides. Biochem. J. 53, 597, 1953

Newton, G.G.F. and Abraham, E.P. (1953b). Observations on the nature of bacitracin A. Biochem. J. 53, 605, 1953

Newton, G.G.F. and Abraham, E.P. (1953c). Sulphur-containing amino acids of nisin. Nature 171, 606, 1953

Noda, Y. (1959). Total synthesis of gramicidin J_2. Nippon Kagaku Zasshi 80, 411, 1959

Oda, T., Kinoshita, M., Yamanaka, O. and Ueda, F. (1954). Colistin. I. Paper chromatography of colistin. J. Pharm. Soc. Japan 74, 1243, 1954

Oda, T. and Ueda, F. (1954). Colistin. II. Leucine, threonine, α,γ-diaminobutyric acid and fatty acid isolated from colistin hydrolysate. J. Pharm. Soc. Japan 74, 1246, 1954

Ogston, A.G. (1952). Sedimentation and diffusion of licheniformins A, B and C. Biochem. J. 51, 569, 1952

Ohno, M. and Izumiya, N. (1966). Synthesis of tyrocidine A. J. Amer. Chem. Soc. 88, 376, 1966

Okuda, K. (1959). Gramicidin J_2. Separation and identification of gramicidin J_2. Osaka Shiritsu Daigaku Igaku Zasshi 8, 1443, 1959

Okuda, K., Lin, C.S. and Winnick, T. (1962). Amino acid composition of gramicidin. Nature 195, 1067, 1962

Oser, B.L. (ed.) (1965). Hawk's Physiological Chemistry. 14th edition, The Blakiston Division, McGraw-Hill Book Co., New York, U.S.A., 1965

Otani, S. (1957). Gramicidin J. A new antibiotic. Japanese Patent 3997 (1957), Chem. Abstr. 52, 16704, 1958

Otani, S., Nagano, H. and Saito, Y. (1958). A new antibiotic polypeptide produced by a strain of Bacillus brevis Nagano. Osaka Shiritsu Diagaku Igaku Zasshi 7, 640, 1958

Otani, S. and Saito, Y. (1954). Chemistry of gramicidin J. Proc. Japan. Acad. 30, 991, 1954

Paladini, A. and Craig, L.C. (1954). The chemistry of tyrocidine. III. The structure of tyrocidine A. J. Amer. Chem. Soc. 76, 688, 1954

Pauly, H. (1904). Ueber die Konstitution des Histidins. Z. Physiol. Chem. 42, 508, 1904

Peterson, D.H. and Reineke, L.M. (1949a). The chemistry of circulin. Cellulose chromatographic separation of amino acid constituents with powdered cellulose. J. Biol. Chem. 181, 95, 1949

Peterson, D.H. and Reineke, L.M. (1949b). Chemical studies of circulin. J. Clin. Invest. 28, 1053, 1949

Pharmacia Co. (1963). Sephadex in Gel Filtration. Theory and Experimental Technique. Pharmacia Fine Chemicals Inc., New York, U.S.A., 1963

Piez, K.A. (1954). The separation of the diastereoisomers of isoleucine and hydroxylysine by ion exchange chromatography. J. Biol. Chem. 207, 77, 1954

Porath, J. (1952). Purification of bacitracin and some properties of purified bacitracin. Acta Chem. Scand. 6, 1237, 1952

Porath, J. (1953). Structure of bacitracin A. Nature 172, 871, 1953

Porath, J. (1954). Purification of bacitracin polypeptides by charcoal chromatography and zone electrophoresis. Acta Chem. Scand. 8, 1813, 1954

Porter, J.N., Broschard, R., Krupka, G., Little, P. and Zellat, J.S. (1949) Isolation and production of polymyxin. Ann. N.Y. Acad. Sci. 51, 857, 1949

Ramachandran, L.K. (1963). On the heterogeneity of gramicidin. Biochemistry 2, 1138, 1963

Regna, P.P. and Solomons, I.A. (1951). Recovery of bacitracin from fermentation broths. U.S. Patent 2,556,375 (1951), Chem. Abstr. 45, 7753, 1951

Regna, P.P., Solomons, I.A., Forscher, B.K. and Timereck, A.E. (1949). Chemical studies on polymyxin B. J. Clin. Invest. 28, 1022, 1949

Rogers, H.J., Lomakina, N. and Abraham, E.P. (1965b). Observations on the structure of bacilysin. Biochem. J. 97, 579, 1965

Rogers, H.J., Newton, G.G.F. and Abraham, E.P. (1965a). Production and purification of bacilysin. Biochem. J. 97, 573, 1965

Roncari, G., Kurylo-Borowska, Z. and Craig, L.C. (1966). On the chemical nature of the antibiotic edeine. Biochemistry 5, 2153, 1966

Ruttenberg, M.A., King, T.P. and Craig, L.C. (1965a). The chemistry of
     tyrocidine. VI. The amino acid sequence of tyrocidine C. Biochemistry
     <u>4</u>, 11, 1965

Ruttenberg, M.A., King, T.P. and Craig, L.C. (1965b). The use of the tyro-
     cidines for the study of conformation and aggregation behavior.
     J. Amer. Chem. Soc. <u>87</u>, 4196, 1965

Saito, Y. (1958a). Structure of gramicidin J. I. Dinitrophenyl derivatives
     of gramicidin J. Osaka Shiritsu Daigaku Igaku Zasshi <u>7</u>, 499, 1958

Saito, Y. (1958b). Structure of gramicidin J. II. Amino acid sequence of
     gramicidin J. Osaka Shiritsu Daigaku Igaku Zasshi <u>7</u>, 503, 1958

Salle, A.J. and Jann, G.J. (1945). Subtilin. An antibiotic produced by
     <u>Bacillus</u> <u>subtilis</u>. I. Action on various organisms. Proc. Soc. Exp.
     Biol. Med. <u>60</u>, 60, 1945

Salle, A.J. and Jann, G.J. (1947). The nature, properties and toxicity of
     subtilin and its chemotherapeutic effect on the course of experimental
     infections in animals. J. Bacteriol. <u>54</u>, 269, 1947

Sanger, F. (1945). The free amino groups of insulin. Biochem. J. <u>39</u>, 507,
     1945

Sanger, F. (1946). The free amino groups of gramicidin S. Biochem. J. <u>40</u>,
     261, 1946

Sanger, F. (1949). The terminal peptides of insulin. Biochem. J. <u>45</u>, 563,
     1949

Sarges, R. and Witkop, B. (1964a). Formyl, a noval NH_2-terminal brocking
     group in a naturally occurring peptide. The identity of seco-gramicidin
     with desformylgramicidin. J. Amer. Chem. Soc. <u>86</u>, 1861, 1964

Sarges, R. and Witkop, B. (1964b). Gramicidin A. IV. Premary sequence of
     valine and isoleucine-gramicidin A. J. Amer. Chem. Soc. <u>86</u>, 1862, 1964

Sarges, R. and Witkop, B. (1965a). Gramicidin A. V. The structure of valine
     and isoleucine-gramicidin A. J. Amer. Chem. Soc.<u>87</u>, 2011, 1965

Sarges, R. and Witkop, B. (1965b). Gramicidin A. VI. The synthesis of
     valine and isoleucine-gramicidin A. J. Amer. Chem. Soc. <u>87</u>, 2020,
     1965

Sarges, R. and Witkop, B. (1965c). Gramicidin. VII. The structure of valine
     and isoleucine-gramicidin B. J. Amer. Chem. Soc. <u>87</u>, 2027, 1965

Sarges, R. and Witkop, B. (1965d). Gramicidin. VIII. The structure of
     valine and isoleucine-gramicidin C. Biochemistry <u>4</u>, 2491, 1965

Schindler, C.A. and Schuhardt, V.T. (1964). Lysostaphin. A new bacterio-
    lytic agent for the Staphylococcus. Proc. Nat. Acad. Sci. U.S. 51,
    414, 1964

Schmidt, G.M.J., Hodgkin, D.C. and Oughton, B.M. (1957). A crystallographic
    study of some derivatives of gramicidin S. Biochem. J. 65, 744, 1957

Schwartz, B.S., Warren, M.R., Barkley, F.A. and Landis, L. (1960). Micro-
    biological and pharmacological studies of colistin sulfate and sodium
    colistin methanesulfonate. Antibiotics Annu. 1959-1960, 41, 1960

Schwyzer, R. and Sieber, P. (1956). New synthesis in the peptide field.
    Chimia 10, 265, 1956

Sharp, V.E., Arriagada, A., Newton, G.G.F. and Abraham, E.P. (1949).
    Ayfivin. Extraction, purification and chemical properties. Brit. J.
    Exp. Pathol. 30, 444, 1949

Shemyakin, M.M., Ovchinnikov, Y.A., Antonov, V.K., Kiryushkin, A.A., Ivanov,
    V.T., Shehelokov, V.I. and Shkrob, A.M. (1964). Total synthesis of
    serratamolide. I. Synthesis of 0,0'-diacetyl-serratamolide.
    Tetrahedron Letters 1964, 47, 1964

Short, E.I. (1948). Quantitative estimation of threonine and phenylalanine
    content of the polymyxins. Biochem. J. 43, lxii, 1948

Shortridge, R.W. (1957). Recovery and purification of bacitracin. U.S.
    Patent 2,776,240 (1957), Chem. Abstr. 51, 4657, 1957

Shriner, R.L., Fuson, R.C. and Curtin, D.Y. (1964). The Systematic Identi-
    fication of Organic Compounds. A Laboratory Manual. 5th edition, John
    Wiley and Sons, Inc., New York, U.S.A., 1964

Silaev, A.B., Stepanov, V.M., Yulikova, E.P. and Muratova, G.L. (1961b).
    Chemistry of polymyxin M. II. Quantitative amino acid composition.
    Zh. Obshch. Khim. 31, 1023, 1961

Silaev, A.B., Stepanov, V.M., Yulikova, E.P., Troshko, E.V. and Levin, E.D.
    (1961a). Chemistry of polymyxin M. I. Qualitative amino acid composi-
    tion. Analysis of the end groups. Zh. Obshch. Khim. 31, 297, 1961

Silaev, A.B., Yulikova, E.P. and Baratova, L.A. (1962). Chemistry of poly-
    myxin M. V. Identification of the fatty acid. Zh. Obshch, Khim. 32,
    818, 1962

Silaev, A.B., Yulikova, E.P. and Baratova, L.A. (1965). Chemistry of poly-
    myxin M. IX. Structure of polymyxin M. Sintez. Prirodn. Soedin. ikh.
    Analogov i Fragmentov Akad. Nauk. SSSR Otd. Obshch. i. Tekhn. Khim.
    1965, 198, 1965

Stansly, P.G., Shepherd, R.G. and White, H.J. (1947). Polymyxin: a new chemotherapeutic agent. Bull. Johns Hopkins Hosp. <u>81</u>, 43, 1947

Stepanov, V.M., Silaev, A.B. and Katrukha, G.S. (1960). The molecular weight of polymyxin M. Biokhimiya <u>25</u>, 749, 1960, (English Translation <u>25</u>, 573, 1960)

Stracher, A. and Craig, L.C. (1959). Characterization studies with subtilin. J. Amer. Chem. Soc. <u>81</u>, 696, 1959

Strokes, J.L. and Woodward, C.R.Jr. (1942). The isolation from soil of spore-forming bacteria which produce bactericidal substances. J. Bacteriol. <u>43</u>, 253, 1942

Studer, R.O., Lergier, W. and Vogler, K. (1963). Synthesen in der Polymyxin-Reihe 7. Synthese des hochaktiven cyclischen Decapeptids 7α. Helv. Chim. Acta <u>46</u>, 612, 1963

Studer, R.O., Vogler, K. and Lergier, W. (1961). Synthesen in der Polymyxin-Reihe 5. Synthese des cyclischen Decapeptids 8α. Helv. Chim. Acta <u>44</u>, 131, 1961

Su, T.L. (1948a). Antibiotic-producing organisms in faeces and sewage. Brit. J. Exp. Pathol. <u>29</u>, 466, 1948

Su, T.L. (1948b). Micrococcin. An antibiotic substance formed by a strain of <u>Micrococcus</u>. Brit. J. Exp. Pathol. <u>29</u>, 473, 1948

Suginaka, H., Kashiba, S., Amano, T., Kotani, S. and Imanishi, T. (1967). Purification and properties of a staphylolytic factor produced by a strain of <u>Staphylococcus epidermidis</u>. Biken J. <u>10</u>, 109, 1967

Suzuki, K. (1956). Relation between the antibiotic activity of the colistin and the reactive group. Ann. Rept. Tohoku Coll. Pharm. <u>3</u>, 59, 1956

Suzuki, K. (1957a). Colistin, an antibiotic peptide. III. Determination of amino acids in colistin. Tohoku Yakka Daigaku Kiyo <u>4</u>, 117, 1957

Suzuki, K. (1957b). Colistin, an antibiotic peptide. IV. Terminal groups of colistin. Tohoku Yakka Daigaku Kiyo <u>4</u>, 127, 1957

Suzuki, K. (1957c). Colistin, an antibiotic peptide. V. Partial hydrolysis of colistin. Tohoku Yakka Daigaku Kiyo <u>4</u>, 135, 1957

Suzuki, T. and Fujikawa, K. (1964). Studies on the chemical structure of colistin. IV. Chemical structure of colistin B. J. Biochem. (Japan) <u>56</u>, 182, 1964

Suzuki, T., Hayashi, K. and Fujikawa, K. (1963c). Studies on the chemical structure of colistin. III. Enzymatic hydrolysis of colistin A. J. Biochem. (Japan) $\underline{54}$, 412, 1963

Suzuki, T., Hayashi, K., Fujikawa, K. and Tsukamoto, K. (1963d). Contribution to the elucidation of the structure of polymyxin B_1. J. Biochem. (Japan) $\underline{54}$, 555, 1963

Suzuki, T., Hayashi, K., Fujikawa, K. and Tsukamoto, K. (1964). Contribution to the elucidation of the chemical structure of polymyxin B. J. Biochem. (Japan) $\underline{56}$, 335, 1964

Suzuki, T., Hayashi, K., Fujikawa, K. and Tsukamoto, K. (1965). The chemical structure of polymyxin E. The identities of polymyxin E_1 with colistin A and of polymyxin E_2 with colistin B. J. Biochem. (Japan) $\underline{57}$, 226, 1965

Suzuki, T., Inouye, H., Fujikawa, K. and Nagasawa, S. (1963b). Studies on the chemical structure of colistin. II. Amino acid sequence of colistin A. J. Biochem. (Japan) $\underline{54}$, 173, 1963

Suzuki, T., Inouye, H., Fujikawa, K. and Suketa, Y. (1963a). Studies on the chemical structure of colistin. I. Fractionation, molecular weight determination, amino acid and fatty acid composition. J. Biochem. (Japan) $\underline{54}$, 25, 1963

Synge, R.L.M. (1944a). Analysis of a partial hydrolysate of gramicidin by partition chromatography with starch. Biochem. J. $\underline{38}$, 285, 1944

Synge, R.L.M. (1944b). Isolation of ethanolamine from acid hydrolysates of gramicidin. Biochem. J. $\underline{38}$, xxxi, 1944

Synge, R.L.M. (1945a). The hydroxyamino component of gramicidin hydrolysate. Biochem. J. $\underline{39}$, 355, 1945

Synge, R.L.M. (1945b). Gramicidin S. Overall chemical characteristic and amino acid composition. Biochem. J. $\underline{39}$, 363, 1945

Tishler, M., Strokes, J.L., Trenner, N.R. and Conn, J.B. (1941). Some properties of gramicidin. J. Biol. Chem. $\underline{141}$, 197, 1941

Toki, T. (1955). Optical determination of gramicidin J. J. Osaka City Med. Centre $\underline{4}$, 266, 1955

Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1957). Manometric Techniques. Burgess Publishing Co., Minneapolis, U.S.A. 1957

Vanderhaeghe, H. and Parmentier, G. (1960). The structure of factor S of staphylomycin. J. Amer. Chem. Soc. $\underline{82}$, 4414, 1960

Vogler, K., Lanz, P. and Lergier, W. (1959). Synthesis of a decapeptide from the polymyxin series. Experientia 15, 334, 1959

Vogler, K., Studer, R.O., Lanz, P., Lergier, W. and Bohni, E. (1961). Total synthesis of two cyclodecapeptides exerting polymyxin-like activity. Experientia 17, 233, 1961

Vogler, K., Studer, R.O., Lanz, P., Lergier, W. and Bohni, E. (1965). Synthesis in the polymyxin series. IX. Synthesis of polymyxin B_1. Helv. Chim. Acta 48, 1161, 1965

Vogler, K., Studer, R.O., Lanz, P., Lergier, W., Bohni, E. and Fust, B. (1963). Synthesen in der Polymyxin-Reihe 8. Synthese des hochakativen cyclischen Decapeptids 7 gamma sein Vergleich mit naturlischem Poly-myxin B. Helv. Chim. Acta 66, 2823, 1963

Volger, K., Studer, R.O., Lergier, W. and Lanz, P. (1960). Synthesen in der Polymyxin-Reihe 4. Synthese des cyclischen Decapeptids 8 gamma. Helv. Chim. Acta 43, 1751, 1960

Wasserman, H.H., Keggi, J.J. and McKeon, J.E. (1961). Serratamolide, a metabolic product of Serratia. J. Amer. Chem. Soc. 83, 4107, 1961

Wasserman, H.H., Keggi, J.J. and McKeon, J.E. (1962). The structure of serratamolide. J. Amer. Chem. Soc. 84, 2978, 1962

Weisiger, J.R., Hausmann, W. and Craig, L.C. (1955a). On the partial hydro-lysis of DNP-bacitracin A. J. Amer. Chem. Soc. 77, 731, 1955

Weisiger, J.R., Hausmann, W. and Craig, L.C. (1955b). Bacitracin A. The nature of the linkage surrounding the sulfur. J. Amer. Chem. Soc. 77, 3123, 1955

Welch, H. and Lewis, C.N. (1951). Antibiotic Therapy. Arundel Press, Washington D.C., U.S.A., 1951

Wellner, D. and Meister, A. (1960). Crystalline L-amino acid oxidase of Crotalus adamanteus. J. Biol. Chem. 235, 2013, 1960

Wilkinson, S. (1949). Crystalline derivatives of the polymyxins and the purification of the fatty acid component. Nature 164, 622, 1949

Wilkinson, S. (1963). Identity of colistin and polymyxin E. Lancet 1963(1), 922, 1963

Wilkinson, S. and Lowe, L.A. (1963). Identities of the fatty acids derived from the polymyxins and colistin. Nature 200, 1008, 1963

Wilkinson, S. and Lowe, L.A. (1964a). Structure of polymyxin B_1. Nature 202, 1211, 1964

Wilkinson, S. and Lowe, L.A. (1964b). Structures of polymyxin B_2 and polymyxin E_1. Nature <u>204</u>, 185, 1964

Wilkinson, S. and Lowe, L.A. (1964c). Structures of polymyxin B_2 and polymyxin E_1. Nature <u>204</u>, 993, 1964

Wilkinson, S. and Lowe, L.A. (1964d). The identities of the antibiotics colistin and polymyxin E. J. Chem. Soc. <u>1964</u>, 4107, 1964

Wilkinson, S. and Lowe, L.A. (1966). Structures of the polymyxin A and the question of identity with the polymyxin M. Nature <u>212</u>, 311, 1966

Windaus, A. and Knoop, F. (1906). Zur Konstitution des Histidins. Beitr. Chem. Physiol. Pathol. <u>8</u>, 406, 1908

Wright, W.W. and Welch, H. (1960). Chemical, biological and clinical observations on colistin. Antibiotics Annu. <u>1959-1960</u>, 61, 1960

Wrinch, D. (1957). Structure of bacitracin A. Nature <u>179</u>, 536, 1957

Yulikova, E.P., Silaev, A.B., Muratova, G.L., Shmeleva, G.A. and Sokolova, L.J. (1965). Chemistry of polymyxin M. VIII. Investigation of the products of partial hydrolysis of polymyxin M. Sintez. Prirodn. Soedin. ikh Analogov i Fragmentov. Akad. Nauk. SSSR Otd. Obshch i Tekhn. Khim. <u>1965</u>, 193, 1965

Ziffer, J. and Cairney, T.L. (1962). Lignin-bacitracin complex as growth stimulant and bacitracin purifier. U.S. Patent 3,035,919 (1962), Chem. Abstr. <u>57</u>, 5128, 1963

Zinn, E. and Chornock, F.W. (1958). Bacitracin. U.S. Patent 2,834,711 (1958), Chem. Abstr. <u>52</u>, 14096, 1958

APPENDICES

APPENDIX I

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE ANTIBIOTIC-
PRODUCING STAPHYLOCOCCAL STRAINS 29297 AND 36534

	characteristics
colonial characters	circular, smooth, glistening, with entire margin, and of butyrous consistency
color of colony	yellowish white
oxygen requirement	aerobic, facultatively anaerobic
growth in 10% NaCl	positive
H_2S production	negative
coagulase-activity*	negative
fermentation	
inulin	N**
raffinose	N
salicin	N
glucose	Ag
maltose	Ag
mannitol	Ag
sucrose	Ag

* tube and slide test

** N = no fermentation
   Ag = acid with small quantity gas after 24 hr incubation at 37°C

APPENDIX II

CONSTITUENTS OF SUPPLEMENTED DOLMAN-WILSON LIQUID MEDIUM USED
FOR ANTIBIOTIC PRODUCTION

PART I:

     Difco proteose peptone .... 20.0 gm

     Distilled water .... 900 ml

  Part I is adjusted to pH 7.8 and autoclaved at 15 lb pressure for 25 min.

PART II:

     NaCl .... 5.0 gm

     Ammonium lactate (80% syrup) .... 8.0 ml

     K_2HPO_4 .... 1.0 gm

     $NaH_2PO_4 \cdot H_2O$ .... 1.0 gm

     $MgSO_4 \cdot 7H_2O$ .... 0.2 gm

     $CaCl_2$ .... 0.1 gm

     Ferrous ammonium sulphate (1% solution in 0.1N HCl) .. 1.0 ml

     $MnCl_2 \cdot 4H_2O$ .... 0.2 gm

     Sodium citrate .... 0.2 gm

     Distilled water .... 100 ml

  Part II is sterilized by passage through a Millipore filter. pH is
adjusted to 5.5.

  After Part I is cooled to room temperature, Part II is added to Part I
and mixed well. Final pH is 7.2.