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THE LARGE-SCALE EXTRACTION AND PURIFICATION OF SOME NEW ANTIBIOTICS PRODUCED BY BACTERIA AND A STUDY OF THEIR PROPERTIES.

CONTENTS.

ACKNOWLEDGEMENTS.

I wish to express my sincere gratitude to my supervisor, Professor Sir Ernst B. Chain, F.R.S. and to Dr. A. T. Fuller for their guidance with this work. My thanks also go to Mr. G. T. Banks and Mr. A. Fleming and members of the Pilot and Extraction Plant staff in the Department of Biochemistry, Imperial College, for their help in the large-scale production and extraction of the antibiotics.

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SUMMARY

The large-scale methods of culture (350 l. batch culture), extraction, and partial purification of two antibiotics of bacterial origin have been developed and the biological properties of the antibiotics studied.

One of the antibiotics, antibiotic 175, was produced by a strain of Pseudomonas fluorescens (str. 175). It possessed the properties of a weak acid, was stable over a wide range of pH, had a fairly wide antibacterial spectrum, low toxicity, and was bactericidal to Staphylococcus aureus (Paler) and Escherichia coli (M.R.E. 600). It was found to be haemolytic and completely inactivated by serum at concentrations of 50% and above.

The method of extraction was reasonably efficient, 75% of the units of activity in the culture were present in the product. Yields per 350 l. culture were 3-5 g. of the sodium salt having an activity of 7,900 units/mg. (0.12µg/ml.) with St. aureus (Paler) as the test organism.

The antibiotic possessed some of the properties of inhibitory substances reported to be produced by strains of Ps. fluorescens.

The other antibiotic, antibiotic 2725, was produced by Bacillus licheniformis (str. 2725). It was a peptide, molecular weight of $<10,000$, was stable over a wide pH range, had a broad spectrum, and bactericidal to St. aureus (Paler) and E. coli (M.R.E. 600) under the conditions studied. It was non-haemolytic, and its activity against St. aureus (Paler) was enhanced in the presence of serum at concentrations of 5% and above. It was found to be toxic as are other

peptide antibiotics. Extraction efficiency was of the order of 40%, with yields of 8-10g. of the hydrochloride per 350 l. culture; the product having an activity of 2,000 units/mg. (0.5µg/ml.) with St. aureus (Paler) as the test organism.

The indications were that it was not one of the known peptide antibiotics.

INTRODUCTION

1. General

Antibiotics are inhibitors of bacterial growth produced by micro-organisms. Only about five percent of the antibiotics discovered, about fifty-six out of eleven hundred, are clinically useful.

2. Historical

There have been reports of the empirical use of antibiotics in the treatment of disease as far back as Biblical times. Maurois[1] quotes a section from Psalm 51, 'Purge me with hyssop and I shall be clean.' He presumes this to be the first reference to penicillin, as one of the penicillin-producing organisms, Penicillium notatum, has been isolated from decaying hyssop. The Mayans of Mexico used a fungus in the treatment of ulcers and intestinal infections.

Lister[2] was probably the first person to investigate the antagonistic effect of one micro-organism on another. He observed the growth of Penicillium on the surface of two exposed test tubes of urine. Microscopic examination of the fluid in both tubes revealed the presence of bacteria also. He noted that there were differences in the appearance of the bacteria in these tubes. In his diary, entry dated 28th November, 1871, Lister wrote "both exhibited bacteria in abundance, but there was a marked difference between them as regards their activity". Those from the tube which had the least growth of Penicillium "exhibited the most amazing energy, not only as individuals but in the dense groups which were frequently seen and in which intense jostlings going on among the individuals was really surprising." But the bacteria from the tube with profuse growth of Penicillium "were comparatively languid, multitudes were entirely motionless and there was not the same appearance of dense groups." The experiments were incon-

clusive and subsequently abandoned. Lister assumed that there was competition for oxygen, the mould absorbing dissolved oxygen, in the urine and blocking the surface of the fluid, hence impeding further dissolution of the gas. Had Lister had a control in his experiments his conclusions might have been different.

Pasteur and Joubert (1877)[3] observed that no infection was produced when an animal was injected with an anthrax culture together with a culture of a non-pathogenic organism.

Duchesne (1897)[4] concluded in his thesis "It is to be hoped that if we were to pursue the study of biological rivalry between moulds and bacteria, we may, perhaps, succeed in discovering still other facts directly applicable to therapeutic science." This was the first reference to the possible use of the antagonism of one organism for another as a therapeutic tool.

Several instances of microbial antagonism were studied before the recent developments of antibiotics began. One arose from the discovery that the damping-off fungus Rhizoctonia solani, which kills seedlings, is itself killed by the common soil organism Gliocladium fimbriatum. This led to the isolation of gliotoxin, the first antibiotic to be prepared pure, Weindling and Emerson (1936)[5]; and Dutcher et al (1945)[6].

Some other cases involved the deliberate efforts to develop antagonistic organisms or antibiotics using masses of bacteria as substrate. From such cultures Dubos (1939)[7] isolated a strain of Bacillus brevis which inhibited the growth of pneumococci by means of a soluble excretion product; higher concentrations of the substance lysed the cocci.

Ehrlich[8] laid the foundation stone of chemotherapy, which embraces the application of natural and synthetic substances to the curing and prevention of disease. Ehrlich appreciated the selective combination of chemicals with different types of cells. He was aware of the existence of infectious disease to which aquired resistance or immunity could not be induced and invisaged chemical 'magic bullets' which could be used in the treatment of such diseases. He embarked on a programme of chemical synthesis, and modification of those compounds synthesized, and tested these 'in vivo'. He illustrated his views with reference to atoxyl (arsanilic acid). This substance had some success in the treatment of trypanosome infections and he hypothesized that its action lay in its conversion to an active substance within the body, or it influenced the body to produce a trypanoside. Ehrlich later discovered salvarson (compound 606) which was active against syphilis.

The greatest step forward in the history of antibiotic/ chemotherapeutic development came with the discovery of penicillin by Fleming (1929)[9]. Unfortunately the potential of this substance was not realised until later. In the meantime the sulpha-drugs became prominant.

Domagk (1935)[10] fulfilled Ehrlich's dream of a 'magic bullet' by discovering prontosil. This substance acted 'in vivo' only, the active substance being a metabolic product of prontosil, sulphanilamide. A programme of synthesis around sulphanilamide developed and led to a group of therapeutic substances collectively known as the sulpha-drugs which are particularly active against streptococci.

Woods (1940)[11] hypothesized that para-amino benzoic acid (PABA) acts to reverse sulphanilamide action. This is now known to be the case. Sulphanilamide is structurally similar

to PABA and competitively inhibits the incorporation of PABA into the vitamin, folic acid.

Fleming maintained that penicillin would be of great use but lacked the chemical expertise necessary to extract and purify it. He noticed the inhibitory effect was due to a specific mould and this eliminated any suggestion that the effect was due to a non-specific action, such as change of pH. He developed a crude assay based on the serial dilution of the culture broth in fresh broth, followed by the addition of a standard volume of a suspension of a test organism to each dilution of the series. His activities, by current standards were low, the broth cultures having activities of the order of 10µg/ml., whereas today commercial activities are of the order of 5000µg/ml. He showed that not all bacteria are affected by penicillin nor are susceptible ones affected to the same extent.

There was limited success with the therapeutic application of crude penicillin and before its full potential could be evaluated it had to be obtained in a purer state. Chain and Florey at Oxford suceeded in obtaining crude penicillin by freeze-drying the clarified culture fluid at pH 6.8. At this pH the salt was formed as a biologically stable brown powder. Further fractionation was achieved by extracting the powder with methanol, diluting the methanolic solution with water and re-freeze-drying. Despite the product being relatively pure its therapeutic use was limited by the lack of facilities to produce the antibiotic in sufficient quantities for repeated administration necessary with the substance at the level of purity achieved at this particular time.

Work on the industrial production of the antibiotic was conducted in the United States. There has been a phenomenal increase in the number of antibiotics discovered

and much attention has been directed towards developing derivatives of some of the useful antibiotics in order that the disadvantages of the natural forms are overcome.

3. Project

The aim of this work was to develop the semi large-scale production, by batch culture, of two antibiotics of bacterial origin, and to subsequently extract, partially purify and study the biological properties of the substances. The active strains were of Pseudomonas fluorescens and Bacillus licheniformis, denoted by the strain numbers 175 and 2725 respectively. These strains had been isolated by Dr. A. T. Fuller.

4. Previous Reports of Antibiotics Produced By the Organisms Studied.

Ps. fluorescens :- No report of an antibiotic produced specifically by a strain of this organism has been discovered since the review given by Florey et al (1949)[12]. Garre (1887) first noted the antagonistic effects of the species. When grown on gelatin it produced a diffusible substance which inhibited Vibrio cholera and Bacillus mycoides. Olitzky (1891) recorded similar results to Garre but noted that the substance had slight action on Pseudomonas pyocyanea. Laws and Andrews (1894) showed that the presence of Ps. fluorescens in sewage shortened the survival time of Salmonella typhi. Frost (1904) established that an inhibitory substance from Ps. fluorescens diffused through collodion, which was thermostable, being able to withstand 120°C. for at least 10 minutes. Lewis (1929) confirmed much of the work of Frost and in addition showed that the substance was bacteriostatic, and its action was selective, e.g. spore-forming bacteria and micrococci were very sensitive while Bacterium coli was resistant. The substance was soluble in alcohol, adsorbed onto charcoal, and remained stable to drying for long periods. It did not inhibit

the strain which produced it.

Nettche (1933) stated that 'lipoids' isolated from Ps. fluorescens were bacteriostatic. Nettche and Vogel (1937) found that in a broth culture the organism inhibited Corynebacterium diphtheriae, B. anthracis and Sarcina sp. and to a less extent staphylococci.

Kiprianova et al (1959)[13] have described an antibiotic substance from an unnamed species of Pseudomonas, strain 31, which is active mainly on gram positive and acid fast organisms.

Hence, the organism Ps. fluorescens has been shown to produce inhibitory substances although little is known of their chemical nature. This situation has probably arisen from the closeness of the identity of the organism to Ps. pyocyanea, this being so close as to lead some workers not recognizing the separate species of Ps. fluorescens.

B. licheniformis:- A strain of B. licheniformis (Weigmann) has been reported to produce an antibiotic, licheniformin, which comprises three biologically active polypeptide fractions. It was first reported by Callow and Work (1946)[14] and found particularly active against mycobacteria.

Arriagada et al (1949)[15] described a strain of B. licheniformis (str. A5) which produced an antibiotic principle which they named ayfivin. This substance was later found to be bacitracin which is almost exclusively active against gram positive organisms. There are three principal forms of bacitracin, all of which are polypeptides.

ANTIBIOTICS FROM BACTERIA

Antibiotics are the products of soil micro-organisms and of one group in particular, the Actinomycetes. About sixty antibiotics from bacteria have been described of which a mere four or five have proved useful in medicine, and then only in topical applications. The important antibiotics from bacteria will now be described, the remainder, many of which have received brief study, are given in tabular form.

The information was taken from the following principal sources: Journals of General Microbiology, 1947-70; Bacteriology, 1945-70; Biochemistry (Tokyo), 1960-69; and the American Chemical Society, 1944-69; the Biochemical Journal, 1944-69; Canadian Journal of Microbiology, 1954-70; Annals of the New York Academy of Sciences, 1948-69; Proceedings of the National Academy of Sciences (USA), 1948-69; Experientia, 1945-69; Nature, Lond., 1942-70; Antibiotics Annual, 1953-60; Antimicrobial Agents and Chemotherapy, 1960-67; Chemical Abstracts, 1940-70; Index Medicus, 1940-69; Biochemistry N.Y., 1956-69; and Applied Microbiology, 1953-70, and selected volumes of the Journal of Antibiotics, 1948-68. Additional information was taken from Antibiotics, Origin, Nature and Properties, Korzybski et al., Pergamon Press, Oxford, 1967.

Tyrothricin (Gramicidin and Tyrocidine) A crude antibiotic preparation known as tyrothricin was isolated from cultures of Bacillus brevis by Dubos (1939)[7]. It was subsequently shown to comprise two biologically active peptide fractions, gramicidin and tyrocidine, and both these fractions are inhomogenous.

Tyrothricin has been used extensively in medicine. It was produced in submerged culture in a medium composed of 1% tryptone and 0.5% sodium chloride in tap water at a pre-inoc-

ulation pH of 7.0. The medium was inoculated and heated to destroy the vegetative cells of the inoculum (70°C. for 20 minutes), and then incubated at 37°C. for 72 hours, Mitchell (1952)[16].

The antibiotic can be isolated by precipitating it, together with inactive protein, from the fermentation broth at pH 4.8. The precipitate is recovered and dissolved in alcohol at the rate of 20ml. per litre of original broth. The solution is then filtered and the filtrate evaporated to dryness 'in vacuo' and impurities removed by extraction with ether. The crude preparation is then dissolved in alcohol and 10 volumes of a 1% sodium chloride solution used to precipitate the purified antibiotic, Stokes (1946)[17]. The antibiotic is recovered by filtration and dried over phosphorus pentoxide. The yield is about 0.1g. of tyrothricin per litre of culture medium.

Tyrothricin is active against gram positive micro-organisms only e.g. Micrococcus pyogenes var aureus (20µg/ml.), Streptococcus haemolyticus (1 0µg/ml.), Streptoococus faecalis (20 µg/ml.) and is active against the yeast Candida albicans (200µg/ml.). (The figures are minimum inhibitory concentrations).

The antibacterial activity of tyrothricin is diminished by serum of whole blood. The LD_{50} of the antibiotic by the intravenous route is 1.2 mg/Kg. body weight. Owing to its gramicidin content tyrothricin haemolyses blood, hence it can only be used topically. At relatively high concentrations it is non-toxic to the skin.

Tyrothricin preparations are employed in treatment for three reasons: they contain two antibiotics with different activity against gram positive and gram negative bacteria, they are easily obtained, and the presence of tyrocidine

in preparations increases the stability of emulsions of gramicidin in solution. The mixture has been used to treat mastitis in cattle and in proprietary medicines, e.g. 'Tyrozets' (Merck, Sharpe and Dohme Ltd.).

The preparations of tyrothricin are separated into the gramicidin and tyrocidine components on the basis of their different solubilities. Tyrothricin is mixed with a 1:1 mixture of acetone and ether resulting in a solution of the neutral gramicidin fraction from which, after evaporation, crystalline preparations of gramicidin are obtained from hot acetone. The acetone/ether insoluble residue contains the tyrocidine. The tyrocidine hydrochloride is prepared by dissolving the residue in hot absolute alcohol and precipitating the hydrochloride with a 0.1 N. alcoholic solution of hydrochloric acid. The tyrocidine fraction accounts for 80% of the tyrothricin mixture.

The gramicidin fraction consists of several types of peptides, each having no free acidic or basic groups, Gregory and Craig (1948)[18]. These peptides are not mobile in an electric field and no 2,4 dinitro-fluorobenzene derivatives have been obtained signifying that they are cyclic peptides. Their mean molecular weight is about 2,800 and they contain residues of leucine, isoleucine, tryptophan, valine, alanine, glycine, ethanolamine, phenylalanine and tyrosine. The complete structure for any of these peptides has not been reported.

At least five gramicidin fractions are known, gramicidin A acounts for 80% of the whole. Ishii and Witkop (1963)[19] and (1964)[20] showed that gramicidin A is chemically inhomogenous. Isoleucine is found in the molecule of gramicidin A in variable quantities. The sum of valine and isoleucine is constant in all samples indicating a new variety of gramicidin. Gramicidin A is a mixture of 60-80% valine gramicidin and

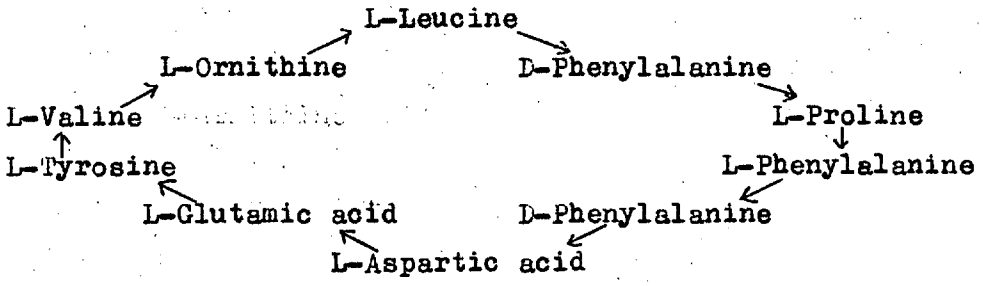
40-20% isoleucine gramicidin.

Sarges and Witkop (1964)[21] consider gramicidin A to be a linear peptide because formic acid has been found by gas chromatographic analysis of the antibiotic. Mild methanolysis of gramicidin A gave 'seco-gramicidin A', a compound with a terminal $-NH_2$ group, Ishii and Witkop (1964)[20]. This they say is evidence that the terminal amino group was blocked prior to methanolysis, by an N- formyl group.

Gramicidin is principally bacteriostatic but in high concentrations it is also bactericidal and is exclusively active against gram positive organisms. Micrococcus pyogenes var aureus and Streptococcus faecalis are inhibited by concentrations of 100 and 60µg/ml. respectively. Neisseria meningitidis and N. gonorrhoeae are both inhibited by 200 µg/ml. Resistance to this antibiotic develops easily. Extracts from gram negative bacteria, milk and serum all antagonize its action. It is only effective when brought into direct contact with the sensitive organisms. It it inactivated by digestive enzymes, hence it cannot be administered orally. The LD_{50} for mice by the intravenous route is 3.75mg/Kg. body weight. Some derivatives have been prepared with the aim of reducing its toxicity, one of note is hydroxy-methyl gramicidin obtained by treating gramicidin with formaldehyde.

The tyrocidine fraction contains mixtures of salts of similar basic peptides having free amino groups and a mean molecular weight of 1,300. Three types have been described. Palandini and Craig (1954)[22] determined the structure of tyrocidine A which is a cyclic decapeptide, see overleaf:

Tyrocidine A:



(The arrows indicate peptide linkage from CO to NH_2)

The only doubt remaining is which of the COOH groups of the glutamic and aspartic acid residues are involved in the peptide linkages. Tyrocidine A has been synthesized by Ohno and Izumiya (1966)[23].

Tyrocidine B possesses L-tryptophan in place of L-phenylalanine, King and Craig (1955)[24], and tyrocidine C has D-tryptophan and L-tryptophan in place of D and L- phenylalanine respectively, Ruttenberg et al (1965)[25].

Mach and Tatum (1964)[26] measured the incorporation of labelled tyrosine, leucine and tryptophan into Bacillus brevis cultures which were synthesizing tyrocidine. They found that the proportions of tyrocidines A, B and C were about 1:3:7 and that the amounts of phenylalanine and tryptophan available were responsible for the relative concentrations of tyrocidines A, B and C.

Tyrocidine is bacteriostatic and bacteriolytic to both gram positive and gram negative bacteria. The minimum inhibitory concentrations for the following organisms are: Micrococcus pyogenes var aureus, 140µg/ml.; Streptococcus haemolyticus, 80µg/ml.; Streptococcus faecalis, 160µg/ml. and Neisseria gonorrhoeae, 200µg/ml. Like gramicidin, tyrocidine must be brought into contact with the sensitive organisms if

it is to be effective. It is inactivated by digestive enzymes. It has not been used medicinally despite it being less toxic than gramicidin or tyrothricin. Its LD_{50} for mice intravenously is 25mg/Kg. body weight.

Tyrocidine appears to affect the lysine and glutamic acid uptake of bacterial cells, Gale and Taylor (1946)[27]. Anderson (1947)[28] considered it to act as a surface active agent. Mach and Slayman (1966)[29] found that tyrocidines affect RNA, DNA and protein synthesis in Neurospora sp. with subsequent irreversible release of K^+, nucleosides, nucleic acids and protein into the medium.

The biosynthesis of tyrocidine and gramicidin has been ivestigated in some detail particularly in cell-free systems. Mach and Tatum (1964)[26] isolated a series of enzymes from Bacillus brevis, one of which was capable of incorporating either phenylalanine or tryptophan at certain sites. They concluded that racemization of the amino acids probably occurs with a stereospecific incorporation of one of the two isomers.

Fujikawa et al (1968)[30] isolated a cell-free system which had the ability to synthesize tyrocidines and found that the formation of the antibiotics was directly influenced by the amino acid concentrations.

Okuda et al (1964)[31] found that the specificity of peptide biosynthesis resided chiefly in the soluble components of their cell-free system rather than in the ribosomal particles. D-valine was found to be incorporated directly into gramicidin rather than being derived from L-valine at a later stage in polypeptide formation. Uemura et al (1963)[32] had already concluded that gramicidin and tyrocidine biosynthesis resembles protein synthesis. Okuda et al (1964b)[33] found that L-amino

acids were activated via an adenylate which is incorporated into sRNA and transferred from amino-acyl sRNA to ribosomes. D- amino acids on the other hand are more readily activated but are poorly incorporated into sRNA and an alternative carrier is undoubtedly used.

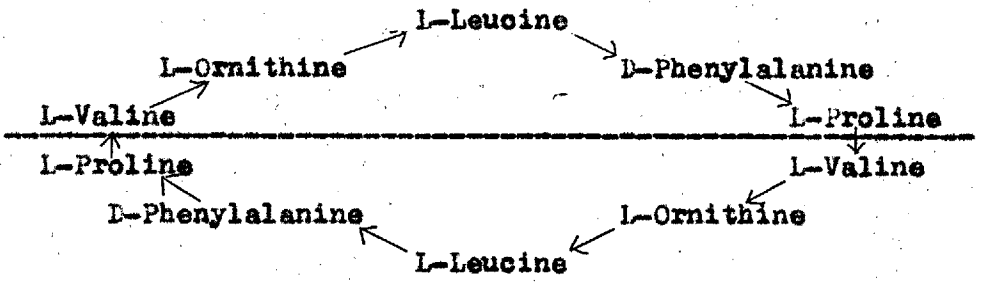
What use are the antibiotics gramicidin and tyrocidine to the bacterial cell producing them? One suggestion is that they bind to macromolecules and thus play an important rôle in the regulation of various cellular activities, Weinberg (1968)[34]. This suggestion arises from the fact that most of the antibiotic yield from a culture appears after the exponential phase prior to sporulation and hence may play an active part in spore production. Woodruff (1966)[35] concluded that antibiotics in general are waste products of the cell formed at a time when an essential metabolite becomes limiting.

Gramicidin S Gauze and Brazhnikova (1944)[36] described this antibiotic as being a product of Bacillus brevis. A suitable fermentation medium consisted of 1% glucose, 0.5% sodium chloride, 0.1% K_2HPO_4, and 1% peptone in a 1% yeast hydrolysate broth at pH 7.0. Maximum activity was achieved after 6-7 days incubation at 42°C. Only a small proportion of the antibiotic diffuses into the medium.

Isolation was achieved by precipitation of the substance from the fermentation broth at pH 4.5-4.7, the addition of 3% sodium chloride facilitating this precipitation. As precipitation of the antibiotic was not complete at this stage the process was repeated. The crude gramicidin S hydrochloride was recovered by centrifugation and dried. The lipid contaminants were removed from the resulting powder with ether and the powder extracted two or three times with ethanol. The residue resulting from the evaporation of the ethanol was

dissolved in warm acetone and the solution decolorized with charcoal. Gramicidin S crystallized from the acetone solution filtrate. Yields of 0.4-0.5g/l. of culture were typical.

Gramicidin S hydrochloride is soluble in water, acid and alkaline solutions. It is a cyclic decapeptide. Synge (1945)[37] determined its structure which is shown below:



The structure of gramicidin S resembles that of tyrocidine A (pg. 22) in that moiety above the horizontal line. In gramicidin S the polypeptide moiety is repeated below the line. Gramicidin S has been synthesized by Schwyzer and Sieber (1956)[38]. Bredesen et al (1969)[39] have determined the order in which amino acids are utilized in the biosynthesis of gramicidin S. The order is: L-phenylalanine, L-proline, L-valine, L-ornithine, then L-leucine. Kleinkauf et al (1969)[40] have shown that in the synthesis of gramicidin S, as in other protein/peptide syntheses, an amino acid has to be activated prior to it being added to the preceding amino acid.

Gevers et al (1969)[41] have extracted and purified a 100 fold two complementary fractions from Bacillus brevis (NCTC 9999) sufficient for gramicidin synthesis 'in vitro'. Fraction 1, which had a molecular weight of 280,000, is a complex responsible for activation, by pyrophosphate displacement in ATP, of four constituent amino acids, L-valine, L-ornithine, L-leucine and L-proline. Fraction 2, molecular weight 100,000, activates and interconverts L and D phenyl-

alanine. In the presence of ATP and Mg^{++}, fractions 1 and 2 form complexes with amino acids and their combination leads to antibiotic synthesis. These complexes have been isolated by gel filtration. Fraction 1 also catalyses AMP-ATP exchange in the presence of each of the four L amino acids, but at a rate twenty times slower using equivalent enzyme quantities.

Aoyagi et al (1969)[42] have obtained analogues of gramicidin S which did not differ in biological properties from the natural gramicidin. The analogues contained either D-valine or L-leucine in place of D-phenylalanine.

The cyclic structure , rather than the simultaneous presence of D and L amino acids, is considered responsible for the antibiotic properties of gramicidin S. The sequence of amino acids is not considered responsible for such properties. Erlanger and Goode (1954)[43] considered the activity of the antibiotic to be due to the inability of bacterial enzymes to break up the cyclic structure, a cyclic structure being attacked by very specific enzymes, described as 'endopeptidases'. If this theory is correct then it applies to the tyrocidinns, the polymyxin and the colistins.

Gramicidin S is bacteriostatic and bactericidal for many gram positive and acid fast organisms. Pathogenic fungi and some protozoa are sensitive to it. At relatively high concentrations the antibiotic is bactericidal for gram negative organisms of the typhoid-paratyphoid-dysentery group. The spectrum of gramicidin S is given overleaf:

Micro-organism	M.I.C.(µg/ml)
Micrococcus pyogenes var aureus	10.0-25.0
Streptococcus haemolyticus	12.0-25.0
Bacillus anthracis	25.0
Clostridium perfringens	7.0-10.0
Corynebacterium diptheriae	50.0
Mycobacterium tuberculosis	10.0-200
Paramecium caudatum	20.0-40.0
Salmonella typhi	100
Candida albicans	25.0

The antibiotic is haemolytic. Its toxicity is quite low. A subcutaneous dose of 40mg/Kg. body weight in mice is not toxic. At concentrations of 400-800µg/ml. it does not irritate the skin neither are concentrations up to 0.7mg/Kg. body weight toxic to the body cavities.

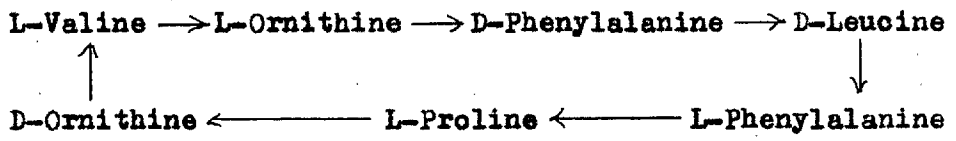
Solutions of 400-800µg/ml. in a 4% alcoholic solution have been used to swab wounds and burns. It is generally applied to surgical dressings and is used in eye and ear drops e.g. 'Sophradex' (Roussel Laboratories Ltd.).

The mode of action of gramicidin S is said to be on the cell membrane. Pressman et al (1967)[44] considers all cyclic polypeptide antibiotics, including gramicidin S, to have the ability to bind with potassium ions and protons (but not Na^+). It becomes an 'ionophore' or 'membrane carrier' because its structure is one which imparts hydrophobic properties to the periphery of the molecule and hydrophilic properties to the centre of the molecule. Hence, the molecule can pass freely through the cell membrane via the membrane lipid component. The passage of the antibiotic molecule through the cell membrane renders the latter 'leaky'.

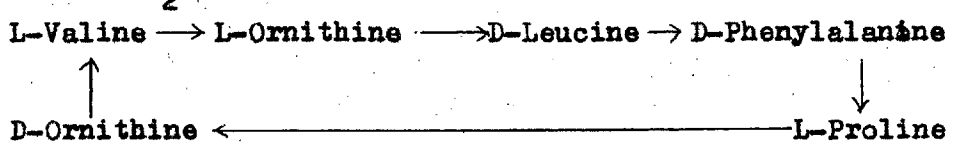
Gramicidin S is considered to act as an uncoupling agent, an inhibitor of oxidative phosphorylation. Loomis (1950)[45] showed that gramicidin S could act in this way and this could account for its toxicity at high concentrations. Bygrave and Lehninger (1966)[46] have shown that gramicidin S inhibits ADP-ATP exchange and that sensitive components of the ADP-ATP exchange activity are saturated with Mg^{++} at much lower concentrations than insensitive components. Gramicidin S is an inhibitor of photophosphorylation, Avron and Shavit (1965)[47].

Gramicidin J This additional variety of gramicidin was reported by Otani and Saito (1954)[48] as a product of a strain of Bacillus brevis. Gramicidin J is composed of two fractions which are represented structurally as follows:

Gramicidin J_1



Gramicidin J_2



The structure of gramicidin J_1, in particular, resembles that of gramicidin S. Otani and Saito (1964)[49] have reinvestigated the structure of gramicidin J_1 and as a result of amino acid analyses, infra-red absorption and X-ray diffraction studies have come to the conclusion that gramicidin J_1 and S are probably identical.

The antimicrobial spectrum of gramicidin J is given overleaf, Tomioka et al (1960)[50]. The methane-sulphonate derivatives of gramicidin J do not differ much in their biological activity from the natural variety but are about

three times less toxic. The LD_{50} of gramicidin J by the intraperitoneal route is 23mg/Kg. body weight.

Micro-organism	M.I.C.(µg/ml)
Micrococcus pyogenes var aureus	2.5
Bacillus subtilis	5.0
Pseudomonas aeroginosa	100.0
Klebsiella pneumoniae	50.0
Escherichia coli	100.0
Salmonella typhi	100.0
Mycobacterium avium	5.0
Candida albicans	25.0
Corynebacterium diptheriae	1.0
Diplococcus pneumoniae	1.0

The Polmyxins And Circulins Benedict and Langlykke (1947)[51] reported an antibiotic produced by Bacillus polymyxa which was almost exclusively active against gram negative bacteria. Ainsworth et al (1947)[52] reported aerosporin, an antibiotic isolated from Bacillus aerosporus (Greer), later found to be B. polymyxa. Stansly et al (1947)[53] discovered an antibiotic they called polmyxin which was produced by B. polymyxa. All these substances belong to the same group of peptide antibiotics, of which one, polymyxin B, is produced industrially. B. polymyxa produces several factors designated polymyxin A-E, exhibiting similar activity. Some strains produce A and C, others B or D, some just E. A further variety of polymyxin was reported by workers in the USSR., called polymyxin M, produced by B. polymyxa (Ross). Murray and Tetrault (1948)[54] described circulin which proved to be closely related to the polymyxins. Yet there is a further group of antibiotics related to the polmyxins, the colistins. In fact two of the colistins are identical with two of the polymyxins.

The production of the polymyxins has been carried out in a medium containing corn steep liquor, glucose and salts, Benedict and Langlykke (1947)[51]. Nelson et al (1950)[55] have reported a method of fermentation with B. circulans, Q 19. The fermentation was done in a medium containing 2% dextrin, 2% oatmeal, 1% ammonium sulphate, 0.4% potassium chloride, 0.5% calcium carbonate and 0.04% mono-potassium phosphate, pH 6.5-7.0. The incubation temperature was 24°C. and the duration 70 hours.

Active preparations were obtained by adsorption on charcoal, washing the charcoal with water, ethanol and anhydrous methanol, and elution with acid methanol. 10 volumes of acetone were added to the eluate, when a white precipitate formed containing polymyxin salts, Stansly et al (1951)[56]. An alternative method involved extraction with isopropyl alcohol from the fermentation broth saturated with ammonium sulphate, Porter et al (1949)[57]. Further purification was achieved by precipitating the insoluble salts of the polymyxins e.g. the picrate, which can be converted to the hydrochloride or sulphate.

The circulins can be extracted by adsorption on to an activated charcoal column under neutral conditions and eluted with an acidified 25% solution of tertiary butanol at pH 2.5-4.0, circulin B leaves the column first.

Khokhlov et al (1960)[58] reported the extraction of polymyxin M by means of ion exchange resin.

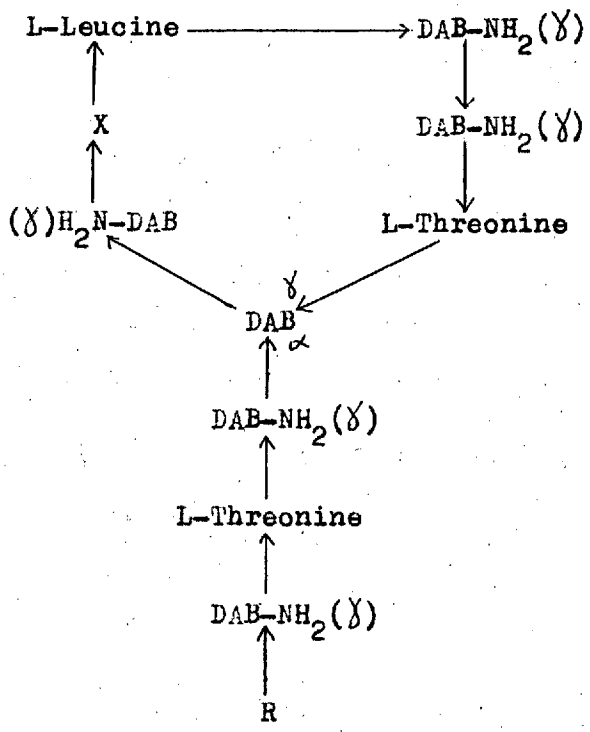
Polymyxin hydrochloride and sulphate are both soluble in water, whilst the base is insoluble. Aqueous solutions of the hydrochloride are acid (c. pH 5.0). The polymyxins are stable between pH 2 and 7, even when heated, but at more acid or alkaline pH values they lose their activity.

The polymyxins and oirculins are peptideswith molecular weights ranging between 1000 and 2000. All hydrolysates contain the basic amino acid Lαγdiamino butyric acid (DAB). Hyrolysates also contain a lipid fraction from which L(+) 6 methyl-octanoic acid has been isolated, The basic nature of the antibiotic is due to the γ amino groups of the DAB. D-leucine is found in all except polmyxin C. As no free carboxyl groups have been detected in molecules their structure is assumed to be cyclic. Polymyxin M differs from polymyxins B and C in not oontaining phenylalanine and differs from polymyxin D in not containing serine. It differs from polymyxin E in that it cannot be precipitated by ammonia or alkali. The absence of nephrotoxicity distinguishes it from polymyxin A.

Nefelova et al (1969)[59] studied the influence of cultural conditions on the growth of B. polymyxa and production of polymyxin M. They found, of the vitamins, only biotin stimulated growth and production. Kachan (1968)[60] obtained contradictory evidence, he found inositol and pantothenic acid stimulatory.

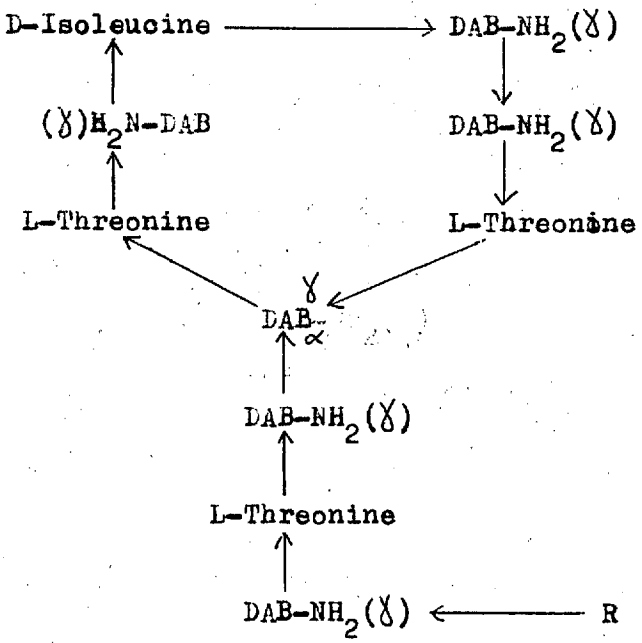
Haussmann and Craig (1954)[61] found that polymyxin B was comprised of two components, B_1 and B_2. Polymyxin E also contains two components, E_1 and E_2. The structures of polymyxin B_1, B_2, E_1, and M have been determined. Wilkinson and Lowe (1964)[62], determined the structures of B_1, B_2 and E_1; Silaev et al (1965)[63] of polymyxin M, and Hayashi et al (1968)[64] of circulins A and B. The various structures are presented overleaf.

Polymyxins B_1, B_2 and E_1.



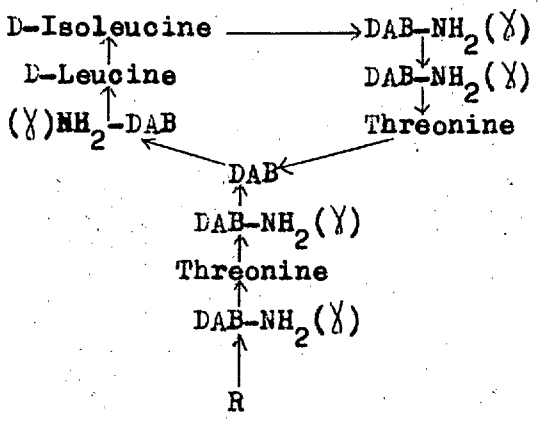
(In polymyxins B_1, R = 6-methyloctanoyl residue, X = D-phenylalanine; B_2, R = 6-methylheptanoyl residue, X = D-phenylalanine; and E_1, R = 6-methyloctanoyl residue, X = D-leucine).

Polymyxin M.



(In polymyxin M, R = 6-methyloctanoyl residue).

Circulins A and B.



(In circulin A, R = 6-methyloctanoyl residue; and in circulin B, R = iso-octanoyl residue).

The antibacterial spectrum of polymyxins A, B and D are given below:

Micro-organism	M.I.C.(µg/ml.) Polymyxin.		
	A	B	D
Salmonella typhi	0.25	0.25	0.30
Salmonella schottmeulleri	0.125	0.25	0.30
Shigella paradysenteriae	0.016	0.03	0.04
Escherichia coli	0.25	0.25	0.30
Brucella bronchiseptica	0.125	0.125	0.15
Brucella abortus	1.0	1.0	1.25
Vibrio comma	0.25	0.25	0.30
Aerobacter aerogenes	0.25	0.25	0.45
Erysipelothrix muriseptica	0.25	0.25	0.45
Pseudomonas aeruginosa	1.0	2.0	1.5
Klebsiella pneumoniae	2.0	2.0	2.5
Haemophilus pertussis	0.25	0.25	0.15

(The antibacterial spectrum of polymyxins A, B, and D are after Brownlee (1949)[65].

Concentrations of the antibiotic higher than 4µg/ml. are necessary to inhibit the growth of Streptococcus mitis (viridans), Streptococcus pyogenes, Micrococcus pyogenes var aureus and Proteus vulgaris. The sensitivity of Escherichia coli, Aerobacter aerogenes and Pseudomonas aeruginosa is very much dependent on the composition of the medium. They show more sensitivity in media rich in organic materials.

The polymyxins are quickly bactericidal under optimal conditions; the inoculum size has a great influence on the activity of polymyxin A and B, but less so on D.

The mode of action has been regarded as to be like a detergent, Anderson (1947)[28]; the polymyxins being detergents with polar amino groups and non-polar aliphatic chains of 6-methyloctanoic acid. Newton (1956)[66] obtained results which seemed to confirm this theory. He found the polymyxins increase the permeability of the cell membrane. Cells of Pseudomonas aeruginosa treated with polymyxin B, when observed under an electron microscope, exhibit marked morhological changes. The polymyxin binds to the cell membranes of sensitive cells, insensitive cells show some binding affinity. Koike et al (1969)[67] found that polymyxin B causes numerous projections to appear from the cell wall of Ps. aeruginosa leading to cytoplasmic streaming. On whole cells and protoplasts of E. coli bleb-like projections are formed and very soon lysis ensues. The blebs are derived from the outermost layer of the cell walls. The polymyxins may function as 'ionophores', Pressman et al (1967)[44].

Teuber (1969)[68] found that penicillin induced sphaeroplasts of Proteus mirabilis were susceptible to polymyxin B.

When the penicillin was removed and the cells permitted to return to their original structure, the susceptibility was lost. This is evidence for the lack of permeability of the cell wall to this antibiotic being the reason for insusceptibility.

The acute toxicity of the polymyxins varies. The LD_{50} doses of three polymyxins for mice is as follows:

Polymyxin	Subcutaneous	Intravenous	Intraperitoneal
A	87.5	6.9	13.9
B	82.5	6.1	12.1
C	160.0	11.9	24.5

Although polymyxin D is the least toxic, polymyxin B is used in preference because it is the least nephrotoxic. Polymyxin B sulphate has been used against Ps. aeruginosa, Aerobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, Haemophilus influenzae and the shigella group of organisms. It is especially useful in the treatment of urinary infections caused by Ps. aeruginosa. Polymyxin B is used together with gramicidin to give a wide spectrum of activity.

Polymyxin B has been used to suppress contaminating organisms in brewing. 0.5 ppm added to the fermenting wort is effective and does not give any off-flavour or odour, Stranekov (1964)[69].

The spectrum and toxicity of the circulins are similar to the polymyxins.

Bacitracin This is the collective name of a group of polypeptide antibiotics isolated from cultures of Bacillus subtilis. Johnston et al (1945)[70] were the first to report it.

Originally the antibiotic was produced in surface culture

in a synthetic medium composed of glucose, glutamic acid and mineral salts, by inoculating the inoculated medium at 37°C. for 3-4 days. Yields were of the order of 4-8 units per ml.* For the industrial production by submerged culture Inskep et al (1951)[71] used a medium containing soy bean meal and the yields were higher, 15-20 units/ml. At the beginning of the fermentation the medium pH was adjusted to 6.5 and rose to 8.0 at the end of the fermentation. At this pH the antibiotic is unstable with much loss within half a day of the termination of the fermentation. Snoke (1960)[72] found yields were stimulated by isoleucine, cysteine, glucose and an unidentified factor in the soy bean.

The methods of extraction have been improved. One recent method[73] is as follows: the fermented mash is clarified with celite and the antibiotic precipitated as a zinc complex with zinc sulphate. The precipitate is recovered, washed with water and dried 'in vacuo'. The recovery at this stage is 97%. The zinc complex is dissolved in a minimal amount of methanol and the solution adjusted to pH 7.5 and passed through an ion exchange column (acetate form).

The effluent is concentrated, acidified and filtered through activated charcoal. The filtrate is neutrallized, treated with zinc sulphate and the precipitate recovered by centrifugation and lyophilized. A 69.3% recovery is achieved with a product having a potency of 55 units/mg. Alternatively the methanolic solution can be percolated successively through cation active resin (H^+ form) and anion active resin (OH^- form). The effluent is then extracted with half volume butanol, the extract concentrated 'in vacuo' and finally lyophilized. The

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*The unit of bacitracin is defined as the activity exhibited by 23.8µg. of dried master standard (U.S. Pharmacoepia).

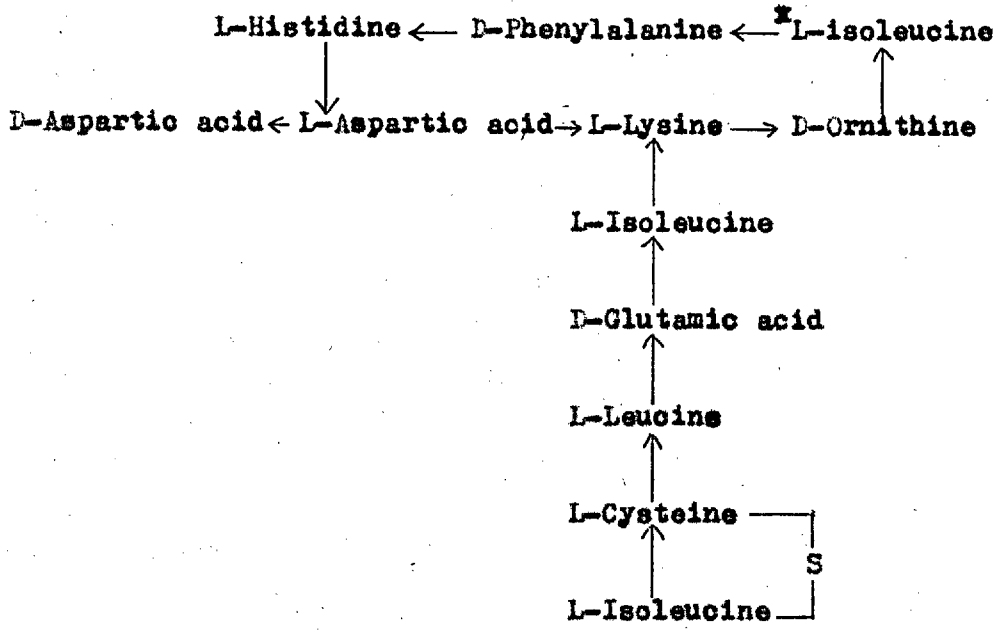
recovery is similar to the previous method but the potency is higher, 87 units/mg.

Bacitracin is a white bitter tasting powder which is very soluble in water. It is unstable above pH 9 but stable to N.HCl; it is most stable between pH 4 and 5.

The bacitracins are all peptides with a molecular weight of about 2,700. They are not decomposed by trypsin or pepsin. There are three main forms of bacitracin, A, B, and C. Others have been detected but these may result from transformations of the A, B, and C varieties, as they are by far in the minor proportion. Bacitracin E will result if bacitracin A is stored in neutral or slightly alkaline conditions.

The types of bacitracins have been separated by counter-current distribution, paper chromatography and other methods. Craig et al (1952)[74] used a countercurrent system composed of 3% acetic acid in s-butanol; Newton and Abraham (1950)[75] used one composed of amyl alcohol/n-butanol mixture against pH 7 aqueous phosphate buffer. Aida et al (1958)[76] used paper chromatography with an n-butanol/acetic acid/water system (4:1:2).

Each type of bacitracin (A, B or C) is composed of twelve amino acid residues. The A and B varieties appear to differ in that one isoleucine residue (x in the structure given below) in bacitracin A is replaced by valine in bacitracin B. Most of the amino acids are of the L configuration, except phenylalanine, glutamic acid, ornithine and aspartic acid which belong to the D series. Abraham (1957)[77] proposed that in bacitracin A the amino acids form a six-membered ring with a side chain.



One of the L-isoleucine residues is situated at the amino end of the peptide chain. This residue is connected with the adjacent cysteine residue forming a thiazolidine ring by condensation of the carboxyl group of the isoleucine with the amino and thiol groups of the cysteine, Newton and Abraham (1953)[78]. It is thought that there is a covalent bond between this terminal L-isoleucine and the D-phenylalanine residue.

Bernlohr and Novelli (1959)[79] and (1963)[80] concluded that bacitracin is produced only under cultural conditions that promote spore formation, since the greater proportion of the antibiotic occurs at the post-log phase when there is active spore formation. It is thought that a similar state of affairs occurs in the biosynthesis of the polymyxins, circulins, subtilin, and polypeptin. Bacitracin is considered to be a component of the cell wall and is released on enzymatic lysis during sporulation. Marschke and Bernlohr (1969)[81] using ^{14}C labelled D-L ornithine found that bacitracin does not constitute a quantitatively significant component of the spore coat although it has been implicated as a structural component of the spore

coat of Bacillus licheniformis. Weinberg (1967)[34] has hypothesized as to the significance of bacitracin to the organism producing it.

Snoke (1961)[82] has found that the biosynthesis of bacitracin is dependent on L-leucine, L-isoleucine, L-histidine, L-asparigine, L-ornithine and L-cysteine. Glucose was stimulatory. D-phenylalanine inhibited synthesis but this inhibition was reversed by L-phenylalanine. Stoffel and Craig (1961)[83] have suggested that the dodecapeptide is formed prior to ring formation.

Arriagada et al (1949)[15] described a strain of B. licheniformis which produced a biologically active principle called ayfivin. It was subsequently shown to be identical with bacitraoin [75]. The same strain also produces the licheniformins; the C:N ratio being decisive in the ratio of bacitraoin to licheniformin produced.

Bacitracin is principally active against gram positive and acid fast organisms. Its antimicrobial spectrum is given below:

Micro-organism	M.I.C.(µg/ml.)
Streptococcus haemolyticus	0.5-0.1
Pneumococcus sp.	2.0-0.04
Micrococcus pyogenes var aureus	100.0-1.0
Corynebacterium diptheriae	0.3-0.08
Neisseria meningitidis	0.2
" gonorrhoeae	0.12
Bacillus anthracis	80.0-250.0
Haemophilus influenzae	12.6
Clostridium perfringens (welchii)	0.5-0.04
" tetani	0.2-0.12
Actinomyces israeli	1.5-0.1
Treponema pallidum	0.08

The antibiotic is inactive against the producing organism, Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Clostridium alcaligenes and Salmonella typhi even at a concentration of 1000µg/ml. Its activity is enhanced in the presence of certain metallic ions, particularly zinc, Weinberg (1959)[84].

The bacitracins are too toxic for systemic use as they are nephrotoxic. Intravenous doses of 342mg. of bacitracin A and 500mg. of B per Kg. body weight are toxic to mice, Newton et al (1951)[85].

Bacitracin is employed topically in human and veterinary medicine. It is used as a feed supplment for pigs and poultry leading to higher conversion rates. The cobalt and the zinc salt have been added to silage to suppress undesirable organisms, Backman (1965)[86].

Paine (1951)[87] suggested that the mode of action of bacitracin might be similar to that of penicillin, namely on the cell wall. Rotta et al (1965)[88] isolated L-forms of group A streptococci which had been exposed to bacitracin. The bacterial population had the morphology and properties similar to those which had been formed by exposure to penicillin. Bacitracin does act on the protoplasts of the organism which synthesizes it. Snoke and Cornell (1965)[89] found that Bacillus licheniformis protoplasts rapidly lysed when exposed to the antibiotic. They found that the action was dependent on the amount of bacitracin present and on the amount of either cadmium or zinc ions present.

Rieber et al (1969)[90] have concluded that bacitracin interferes with cellular division and leads to membrane disorganization. Rieber and Imaeda (1969)[91] observed that Mycobacteria

exposed to bacitracin produce tubules and bacteriophage-like particles. Hence it appears that Paines' suggestion for the mode of action of bacitracin was correct.

Smith and Weinberg (1962)[92] concluded that of the clinically useful antibiotics bacitracin is unique in that it demonstrates a dual mode of action: a) it suppresses the synthesis of cell walls and b) it inhibits protein synthesis in some susceptible organisms e.g. Staphylococcus aureus.

There is an excellent review on bacitracin by Hickey in Hockenhull (1964)[93].

Nisin Rogers (1928)[94] was the first person to report an inhibitory substance produced by Streptococcus lactis. Mattick and Hirsch (1944)[95] discovered nisin, an antibiotic produced by S. lactis, which was active against gram positive microorganisms and later found it to inhibit Mycobacterium tuberculosis. Oxford (1944)[96] described the antibiotic properties of a protein substance produced by Streptococcus cremoris which he named diplococcin. Nisin and diplococcin were thought to be identical but different antimicrobial spectra showed that they were different substances.

Mattick and Hirsch grew the active strain in a medium composed of 1.5% glucose, 0.3% yeast extract, 0.3% sodium chloride, 0.6% sodium citrate, 1% dipotassium phosphate and 0.02% magnesium sulphate, pH 7.2.

Shkundova (1968)[97] found that the production of nisin was more rapid in a medium containing 6% milk hyrolysate in whey. He found that the biosynthesis of nisin corresponded with the increase in cells, reduction in pH and consumption of lactose. Baranova and Egorov (1969)[98] found that the presence

of acetate decreased the antibiotic yield on aerating the medium. Glucose, sucrose, fructose and mannose were beneficial to synthesis, sugar alcohols were not. Potassium dihydrogen phosphate was found to be the best inorganic phosphate source to use, at 2.0-3.0%.

The antibiotic accumulates in the cells and is liberated into the medium by acidifying to pH 2. In fact 60% of the nisin is in the cell walls of the organism, some in the ribosomal fraction and cellular membranes, White and Hirsch (1968)[99].

To extract the antibiotic the broth is clarified at pH 2 by centrifugation and the clear fluid extracted with 1/20 th volume of chloroform plus 2% sec. alcohol at pH 4.2-4.5. To the chloroform extract, separated in a supercentrifuge and cooled to 4^{o}C., anhydrous ethanol is added in order to precipitate the active material. The precipitate is dissolved in dilute hydrochloric acid, the pH adjusted to 2.5-3.0 with sodium hydroxide and an equal volume of ethanol added. The inactive precipitate is discarded and the solution concentrated 'in vacuo', adjusted to pH 4.0 and set aside in a refrigerator. The precipitate that forms is recovered and dissolved in dilute hydrochloric acid and lyophilized. At this stage the recovery is 20%. Further purification is carried out by dissolving the lyophilized material in dilute acid followed by precipitation of the inactive material from the solution at pH 6 and fractional precipitation with sodium chloride. The product has a potency of one million units/mg. (1mg. of standard preparation of nisin hydrochloride contains 10^{4} units, 1 unit = 0.1µg.)

Nisin is the collective name for a group of polypeptide antibiotics among which nisin A, B, C, and D may be distinguished. They can be separated by countercurrent distribution,

Berridge (1952)[100].

Hyrolysates of nisins A, B, C, and D contains the following amino acids: leucine, isoleucine, alanine, glycine, proline, aspartic acid, histidine, lysine, lanthionine and cystathionine. Nisins A, B, and C each contain two molecules of valine and methionine, which are absent in nisin D; nisin D, however, contains glutamic acid. Nisin hydrochloride has about 5% sulphur and a molecular weight of about 7,000. The structure of any of the nisins has not been reported. An isomer of cystathione, β-methyl lanthionine, is found in hyrolysates, Newton et al (1953)[101]. The ratio of this amino acid to lanthionine is 1:4. A similar ratio is found in subtilin.

Nisin when pure is a white powder, soluble in water at pH 2, but is precipitated when the solution is neutralised. A solution of nisin hydrochloride containing 0.1mg/ml. has a pH of 7.

Data obtained by Hurst (formerly Hirsch)(1966)[102] suggested that the biosynthesis of nisin resembles protein biosynthesis since they are equally sensitive to inhibitors such as chloramphenicol, puromycin and terramycin.

Nisin inhibits Streptococcus, Pseudomonas, some Neisseria, Bacillus, Clostridium, Mycobacterium, Lactobacillus, Corynebacterium and Actinomyces sp. It also inhibits Micrococcus pyogenes; Neisseria meningitidis and N. catarrhalis are insensitive. Nisin is not very toxic. When injected intravenously into rabbits in doses of 50mg. daily for 18 days it causes no loss in weight. The only change observed is the gradual obliteration of the veins in the ears, Korzybski et al.

The antibiotic is employed in cheese manufacture, partic-

ularly processed and Dutch cheeses. It is employed in canning fruit and vegetables and in chocolate manufacture. Its presence in cans of food reduces the temperature and time needed to sterilize the contents. Nisin owes its importance as a food additive to the fact that it inhibits the germination of bacterial spores. Its use has been reviewed by Tramer (1966)[103] and Hall (1966)[104].

The Licheniformins The antibiotic li cheniformin is a mixture of polypeptide antibiotics, isolated from oultures of Bacillus licheniformis (Weigmann) by Callow and Hart (1946)[14]. The strain also produces at least six different antibiotics, Callow and Work (1952)[105]. In media having a high nitrogen to carbon ratio and containing ammonium lactate, three licheniformins can be produced.

Hart and Hills (1947)[106] used the following medium: 3% glucose, 0.5% asparagine, 0.1% $MgSO_4$, 0.1% K_2HPO_4, 0.05% sodium citrate, 0.00017% ferric ammonium citrate and 0.0003% $MnSO_4$. The incubation period in this medium was 6-12 days at 37°C.

The mature fermentation broth, after the pH had been adjusted to 2.5, was autoclaved at 0.6 atmospheres pressure for 10 minutes with the object of dissolving all the licheniformins. At pH 5 the antibiotic was adsorbed onto charcoal from the filtrate and eluted with a mixture of water, n-butanol and concentrated hydrochloric acid (89:10:1). The eluate was concentrated 'in vacuo'. The adsorption on to charcoal was repeated, adsorbing at pH 6, eluting this time with butanol saturated with 3N. hydroohloric acid. The eluate was concentrated 'in vacuo' and adjusted to pH 6 and an excess of an aqueous solution of picric acid added, causing the precipitation of licheniformin picrate which was subsequently converted to

the hydrochloride and the latter precipitated by acetone.

Licheniformin hydrochloride is a colorless powder freely soluble in water and methanol. It is insoluble in butanol, acetone, ethanol and chloroform. It is stable between pH 1-9 inclusive for 10 minutes at 115°C. In 10 N. caustic soda the activity is completely lost after several days at room temperature. The licheniformins can be separated by paper chromatography or preparatively by countercurrent distribution. The molecular weights of licheniformins A, B, and C are 4,400, 3800, and 4800 respectively. These values were determined by ultracentrifugation by Ogston (1952)[107].

DeBouk (1952)[108] found that licheniformins A and B have similar amino acid compositions: aspartic acid (1), glycine (7), serine (3), proline (2), arginine (6), phenylalanine (2), valine (2) and lysine (12) on the assumption of a molecular weight of 4,400 each (figures are molecular amounts). Licheniformin C has in addition glutamic acid. As there are no free terminal amino groups it is likely they all have a cyclic structure. Owing to a predominance of basic amino acids, the licheniformins exhibit basic properties.

The unit of licheniformin is the smallest amount in 1 ml. sufficient to inhibit Mycobacterium phlei in broth for 4 days at 37°C. A preparation containing 0.2µg. was used as the standard. Its biological activity resembles that of the antibiotic subtilin but the absence of cross resistance with M. phlei is evidence for their non-identity, Hart and Moss (1950)[109].

Callow et al (1947)[110] have investigated the sensitivity of various micro-organisms to licheniformins. The antimicrobial spectrum of the varieties of licheniformin is not conspicuously different. The minimum inhibitory concentrations of preparations

of the licheniformins of potency 2.5-5.0 units/mg. for various organisms are shown below:

Micro-organism	M.I.C.(µg/ml.)
Mycobacterium phlei	0.2-0.5
" tuberculosis	1.6-6.0
Micrococcus pyogenes var aureus	0.2-0.8
Streptococcus viridescens	50.0
Diplococcus pneumeniae	6.0-50.0
Bacillus anthracis	5.0
Corynebacterium renale	0.025
" diptheriae	0.1-0.8
" equi	0.2
Escherichia coli	12.0-100.0
Pseudomonas aeruginosa (pyocyanea)	25.0
Shigella dysenteriae	6.0-25.0
" paratyphi	50.0-100.0
Brucella abortus	3.0
Vibrio cholera	50.0

50% serum was not found to affect the activity of the licheniformins. Activity is markedly increased if the medium pH is raised from 5.5 to 8.5. Intraperitoneal doses of 0.5-3.0mg. in mice infected with 0.94 of a lethal dose of Bacillus anthracis diminishes mortality by 3.6 times. The toxicity varies according to the animal. The LD_{50} for mice is 500 mg/Kg. body weight subcutaneously, 250 mg. intravenously. The antibiotic produces kidney lesions. Rabbits are not sensitive to the toxic action of the licheniformins, and mice are only moderately sensitive.

Colistin (syn. Colimycin). Koyama et al (1950)[111] described an antibiotic produced by Bacillus colistinus to which they gave the name colimycin. The name colimycin has also been ascribed to an antibiotic produced by Streptomyces fradiae var spiralis,

a substance which is, however, related to neomycin.

Colistin is a non-homogenous basic peptide, Suzuki et al (1963)[112]. It comprises two biologically active fractions, A and B, the former being the main product.

The fermentation and extraction of colistin is similar to polymyxin. Salts of colistin are relatively stable between pH 2 and 6 with loss occuring above pH 6. When dry it is stable at elevated temperatures.

Suzuki et al (1964)[113] reported the structure of colistin B and Wilkinson and Lowe (1964)[114] showed that colistin A and polymyxin E_1 were identical on physical and structural determinations. Suzuki et al (1965)[115] reported that colistin B and polymyxin E_2 are the same substance. Ruggerini and DeModica (1964)[116], however found that colistin B and polymyxin E_2 had different electrophoretic mobilities. They hypothesized that the differences may be due to different steric conformations of the two substances.

The antimicrobial spectrum of colistin is similar to polymyxin B. It is active against a wide range of gram positive and against a narrower range of gram negative micro-organisms. It is bacteriostatic and bactericidal. It inhibits pathogenic Pseudomonads, Shigellae, Salmonellae, and strains of Escherichia coli.

It is used as the sulphate or sodium methane-sulphonate, the latter being the least toxic. The LD_{50} for both salts by various routes is given in the table below:

Route of Administration	Colistin	Colistin-methane Sulphonate
Intravenous	5.46	222.33
Intraperitoneal	19.8	126.50
Subcutaneous	48.6	138.00

In contrast with other polypeptide antibiotics colistin shows low toxicity. It does over a prolonged period show lesions in the cortex of the kidney which are regeneratable, Staeminler and Lagler (1963)[117].

Its mode of action has been studied by Kawamata and Nakajima (1965)[118] and Nakajima and Kawamata (1965)[119]. They found that it affects tha permeability of cells of Escherichia coli causing release of substances absorbing at 260 mμ, mostly low molecular weight nitrogenous compounds. This release took place at 37°C. but not at 2°C. and could be prevented by bi-valent cations. It forms insoluble complexes with DNA and RNA at an optimal pH of 6.4, less antibiotic is needed to precipitate DNA. Colistin, like other cyclic polypeptide antibiotics may act as 'ionophores', Pressman et al (1967)[44].

Cobalt sulphate enhances the activity of colistin against E. coli and Pseudomonas fluorescens. The cobalt forms a complex with the antibiotic, this being shown by the lowering of the extinction curve of colistin on addition of cobalt sulphate, Elfiki and Chodat (1965)[120]. Colistin has been used with some success in the treatment of burns with septicemic complications due to Pseudomonads, Graber et al (1959-60)[121].

Subtilin Humfeld and Feustel (1943)[122] noted the antibacterial properties of cultures of certain strain of Bacillus subtilis. Janson and Hirschmann (1947)[123] studied the phenomenon more closely and named the antibacterial substance subtilin. Hassel (1948)[124] described a similar antibiotic from B. subtilis which he named subtilin C but differed in some respects to subtilin. The subtilin-producing strain has been designated B. subtilis (ATCC 6633 or NRRL B 543).

On a pilot plant scale Garibaldi and Feeney (1949)[125] used a medium composed of 10% saccharose, 1.2% citric acid, 0.4%

sodium sulphate, 0.5% malt extract, 0.42% diamino phosphate and inorganic salts, K, Mg, Mn, Fe, and Zn. The pH was adjusted to 6.8 with ammonia. Zinc (10mg/ml.) was found indispensable for subtilin production. The incubation period in submerged culture was 3-4 days at 30°C. Yields were of the order of 900µg/ml.

Lineweaver et al (1949)[126] isolated the antibiotic by extraction with half volume butanol at pH 2.0-2.5. The extract, containing 85-90% of the activity, was concentrated 'in vacuo' with the continuous addition of water to obtain the lower boiling two-constituent azeotropic system. When the volume had diminished to 1/6 the addition of water was stopped. Further concentration led to the precipitation of subtilin, which was collected and dried. Subtilin was also obtained from the unconcentrated butanolic extract by the addition of an aqueous solution of caustic soda to pH 5 and salting out the antibiotic with common salt. The crude preparation was washed with anhydrous butanol and 10% salt solution. Dimick et al (1949)[127] patented the antibiotic and its purification.

Subtilin is a polypeptide containing sulphur and a trace of phosphorus. On acid hydrolysis, 80% of the nitrogen is found in the amino nitrogen in the amino acids. It dialyses, hence its molecular weight is less than 10,000.

Between pH 2 and 7 the antibiotic is relatively stable, above pH 7 and in Normal acids it is quickly inactivated. At pH 2 it is inactivated by pepsin and at pH 7 by trypsin at 30°C. after 24 hours. Dry preparations are very stable at room temperature, Dimick et al (1947)[128].

Alderton and Fevold (1951)[129] reported the amino acid composition of subtilin: glycine (1), valine (1), leucine (4),

isoleucine (1), proline (1), phenylalanine (1), tryptophan (1), lysine (3), aspartic acid (1) glutamic acid (3), mesolanthionine (1) and some sarcosine. (Figures are molecular amounts). β-methyl lanthionine (also present in nisin) was found. The molecular weight of the antibiotic is about 3,300. The molecule is thought to be a multiloop structure owing to the large number of free carboxyl and amino groups, these being less than attributable to an open chain structure.

Stracher and Craig (1959)[130] separated preparations into three components. Preparations from broths stored for several years at low temperatures maintained their biological activity almost quantitatively. Six components were found, subtilin A accounting for 50% of the activity. There were fewer components in fresh broth, in which subtilin A was responsible for about 85% of the activity.

The unit of ~~nisin~~ subtilin is the amount of the antibiotic in 1ml. of solution which is bactericidal for Staphylococcus aureus after 10 minutes at 37°C. It is mainly active against gram positive and acid fast organisms. The table below shows the spectrum of subtilin:

Micro-organism	M.I.C. (μg/ml.)
Micrococcus conglomeratus	0.06-1.0
Streptococcus pyogenes	0.034
Lactobacillus plantarum	12.5
Treponema pallidum	2.0
Mycobacterium tuberculosis	50.0
Endamoeba histolytica	2.5

The pH influences the action of subtilin. Sacks and Pence (1958)[131] found that St. aureus was more sensitive to subtilin above pH 5.2 whilst Escheriohia coli is more resistant over the

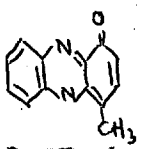
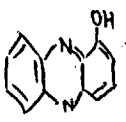
pH range 4.2-7.4, the resistance increasing as the pH approaches 7.4.

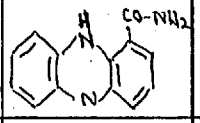
The LD_{50} of subtilin for mice is 60mg. (intravenous), 670 mg. (subcutaneous) and 5,000mg. (oral) per Kg. body weight, Anderson et al (1946)[132].

Subtilin at a concentration of 10µg/ml. irreversibly inhibits the respiration of Micrococcus pyogenes var aureus. At 30µg/ml. the organism loses considerable amounts of nitrogen and phosphorus, Sacks and Pence (1958)[131]. Anderson (1947)[28] considered subtilin to act like tyrocidine and polymyxin, namely as a surface active agent.

Subtilin has been used in canning as it suppresses the growth of Clostridium botulinum. A concentration of 20µg/ml. of subtilin destroys bacterial spores. In canning subtilin and heat are complementary in the preservation of the contents, Campbell et al (1959)[133]. Campbell and Winniarski (1959)[134] isolated a sutilin resistant strain of Cl. botulinum.

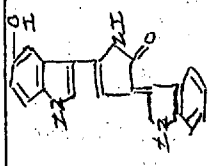
Minor Antibiotics From Bacteria.

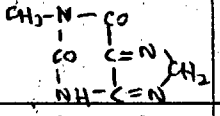
Antibiotic/ Organism/ Reference.	Production Medium.	Method of Extraction.	Chemical Nature.	Biological Activity.
Pyocyanine. Pseudomonas aeruginosa 135	Peptone broth, pH 7.4. 6 days at 37°C.	Chloroform: water partition.	Phenazine pigment. 	Active against G+ve, -ve and acid fast bacteria. Extremely toxic.
Hemipyocyanine. Ps. aeruginosa 135, 136	As above.	As above.	1-OH phenazine. 	Slightly antibacterial and inhibits fungi, less toxic than pyocyanine.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Antibiotic Px. *Pseudomonas sp.* 137	Peptone agar. pH 7.4, 25-28°C for 6 days.	Chloroform: water partition.	Similar to pyocyanine.	Active against G +ve, -ve and acid fast organisms.
Chloraphin *Pseudomonas chloraphis*, GS. 138	Asparagine, glycerol, salts, pH 7, broth. 3-4 days at 25°C.	Acetone extraction of cell homogenate.	1 amino phenazine. 	Active against G +ve and G-ve organisms.
Comirin *Bacterium antimyceticum*. 139	Peptone sucrose broth pH 7, 30°C.	BuOH extraction, precipitated by NaCl.	Peptide Differs from polymyxin and subtilin in amino acid composition.	Inhibits fungi and some yeast at $1:10^6$. Haemolytic.
Pyolipic acid *Pseudomonas aeruginosa*. 140	Peptone broth.	EtOH extract of cells; EtOH fractionated with ether.	Unknown, is a colourless oil.	Active against tubercle bacilli.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Viscosin *Pseudomonas viscosa.* 141	Peptone broth.	Adsorbed on to charcoal at pH 6; eluted with acetone, ppts. at 3°C.	Polypeptide.	'In vitro' activity against saprophytic and pathogenic Mycobacteria. G +ve and G-ve bacteria not affected.
Unamed *Pseudomonas str. 31.* 13	Glycerol, peptone agar. 30°C for 48 hours.	Ether extraction of the cells.	3 acidic substances unknown constitution 2 biologically active. Are phenol carboxyl acid derivatives.	Inhibit G +ve and acid fast organisms, and some fungi. G -ve insensitive. Inactivated by serum.
Mycobactocidin *Staphylococcus epidermidis.*[142]	Oleic albumin agar at 37°C for 7-10 days.	Cells extracted with water, then acetone and methanol.	Fractions A - M. Probably a glucoprotein.	Five fractions active against acid fast organisms. Non-toxic to mice.
Antibacterial substances from Staphylococci. 143	Brain infusion agar.	Cells removed and extracted with saline.	Unknown. Dialysable. Inhibited by EDTA.	Inhibits *Staphylococcus aureus* (Oxford 2098).

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Lactobacillin *Lactobacillus helviticus.* 144	Glucose broth enriched with tomato juice and Tween 80 30°C for 7 days.	Not extracted.	Not known.	Inhibits *Micrococcus var pyogenes.*
Unamed *Alkaligenes faecalis*, NCTC 8764 & 9220. 145	Proteose peptone broth plus glucose NaCl & trace salts. 30°C for 24 hours.	Not extracted.	Protein, a colicine.	*Proteus sp.* and many str. of *Alk. faecalis* inhibited.
Diplococcoin *Streptococcus lactis.* 96,146,147	Casein hydrolysate, salts, growth factors, sucrose. 37°C for 6 days.	Dry residue of culture extracted with acetic acid and pptd. by $Am.SO_4$.	Protein.	Inhibits G +ve organisms. Non toxic. Has been used to treat bovine mastitis.
Marcescin *Serratia marcescens.* 148	Am.citrate + glycerol 1 day in shallow surface culture at 35°C. Also in submerged culture.	Culture to 1/10 vol. pH to 1.0. Ppt. by acetone. To water. Ppt. at pH 9.	Polypeptide. 0.3 % phosphorus and 0.3% sulphur.	Active against G +ve, -ve and acid fast orgs. Very toxic, haemolytic.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Prodigiosin *Serratia marcescens*. 149,150	Peptone water pH 7.1. 2 days at 30°C.	Ether/petroleum ether mixture. Hydrochloride pptd. from concentrate.	Tri-pyrrole substance. Monovalent base.	Active against *Bacillus anthracis*, Staphylococci and fungi. Not toxic to great extent.
Iodinin *Chromobacterium iodinum*. 138,151,152	Agar medium containing beer fermentation broth.	Chloroform extraction of colony crystals.	N N'-dioxy 1, 5 di-hydroxyphenazine.	Inhibits G +ve and -ve organisms, the latter at greater concentrations.
Violacein *Chromobacterium violaceum*. 153,154,155	Lactose broth, 14 days at 22°C.	Acetone extract of cells. Then fractionated with chloroform and then ether.		Active against G +ve bacteria Not toxic, non-haemolytic.
Xerosin *Achromobacter sp.* 134. 156	Peptone, beef +yeast extract, glucose. 48 hours at 25°C.	Pptd. from medium by HCl at pH 3.2 - 3.5.	Unknown.	Inactive against G +ve and - ve bacteria + fungi. Inhibits influenza virus intracerebally.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Coliformin *Escherichia coli*. 157	Glucose, ammon. salts, phosphate + Mg^{++}. 24 hr. at 37°C.	Benzoic acid and acetone pptn.	Polypeptide. 2 or 3 bioactive substances.	Anti-fungal, inhibits fungi pathogenic to man.
Colicines *Escherichia coli*. 158, 159	Peptone water. 16 hr. at 37°C.	Charcoal adsorption, eluted glacial acetic acid	Proteins or polypeptides	Inhibits G -ve organisms. Activity enhanced in serum v. *E. coli*. Non toxic
Salmonellin *Salmonella eastbourne* 160 SCII.	Not stated Agar medium. 37°C for 3 days.	Extracted from cells with buffer.	A colicine-like antibiotic - protein or peptide.	Active against *Salmonella gallinarium*.
Toxoflavin *Bacillus cocovenans*. 161, 162	Nutrient broth. 37°C. for 3-4 days	Extracted with chloroform, the culture fluid satd. with NaCl.	Purine, isomer of 1-Methyl xanthine. 	Inhibits G +ve organisms. Very toxic. Author suggests use in treating leukemia.
Alvein *Bacillus alvei*. 163	Czapek Dox plus CSL. and glucose. 7 days at 37°C.	Charcoal adsorption, eluted acid BuOH. 20-30% recovery.	Basic polypeptide.	Inhibits G +ve and acid fast bacteria and the G -ve *E. coli* and *Ps. aeruginosa*. Haemolytic.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Bacilipin *Bacillus subtilis*. 164	Corn extract and glucose broth. 12 hr. at 34°C.	Amyl acetate at pH 5. Adsorbed on to Al_2O_3. Eluted phosphate buffer at pH 5	Two fractions separated by C.C. distribution. Unknown structure. Low M.Wt.	Inhibits some G +ve and acid fast organisms
Bacilysin *Bacillus subtilis*. 164	Same culture and prodn. medium as Bacilipin.	Charcoal adsorption, eluted PO_4 buffer and EtOH. On Al_2O_3, eluted with 25% EtOH	Peptide, has sulphur containing amino acids.	Inhibits same range of organisms as Bacilipin.
Globicin *Bacillus subtilis*. 165	Tyrothricin medium (p.18) 8 days at 30°C.	pH 4.5 ppt. to water and antibiotic pptd. by $AmSO_4$.	Peptide, contains tryptophan and sulphur.	Inhibits *Bacillus*, *Micrococcus* and *Mycobacterium sp.* Non-haemolytic
Brevin *Bacillus brevis*. 166	Peptone, salts, broth pH 6.8-7.0.	Ppt. at pH 4.0. Extracted with EtOH.	Peptide, containing sulphur.	Inhibits G +ve and acid fast organisms. Haemolytic.
Brevolin *Bacillus subtilis*. 167	Peptone and glucose broth 3-4 days at 35°C.	Charcoal adsorption, pH 2. Pptd with acetone as Hydrochloride.	Peptide.	Inhibits G +ve, -ve and acid fast organisms Toxic.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Bacillin *Bacillus subtilis*. 168, 169	Glucose, asparagine, $MnSO_4$.	Charcoal adsorption. Eluted EtOH or MeOH.	Unknown. A peptide anti-bacillin from culture antagonizes antibiotic.	Inhibits selected G +ve,-ve and acid fast organisms. Toxic.
Bacimethrin *Bacillus megatherium*. 170, 171	Glucose, soya, wheat flour, salts pH 7.	Charcoal adsorption, eluted with MeOH	Pyridine 2-methoxy-4-am. 5-OH-Me pyridine.	Inhibits G +ve, -ve, acid fast organisms, and some yeasts, low toxicity.
Edein *Bacillus brevis*, 172	Glucose, glutamate, salts. 28-30°C. for 2-3 days.	Charcoal adsorption, eluted with MeOH.	Peptide.	Inhibits G +ve, -ve, and acid fast organisms. Non-haemolytic.
Bacillomycin *Bacillus subtilis*. 173.	Glucose, glutamate, salts. 28-30°C. for 5-6 days.	Two antibiotics. One isolated. Pptd. pH 2.5 Extracted with EtOH.	Molecular Weight 960	Antifungal, toxic. Inactive against other micro-organisms.
Antifungal antibiotics *Bacillus subtilis*. 174	Potato medium, glucose, asparagine. 25°C. for 15 days.	Activity adsorbed on to Hyflo super cel, eluted with hot MeOH	Peptide.	Active against *Neurospora*, *Penicillium*, *Mucor* and *Rhizoctonia sp.*

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Bumycin *Bacillus subtilis*. 175	Yeast extract broth. 30°C. for 5 days.	Pptd. under acid conditions, ppt. extracted with EtOH at pH 7.	Unknown composition. Colourless water soluble substance.	Slightly active against G +ve, -ve bacteria. Active against fungi and acid fast organisms.
Fungistatin syn. Antibiotic XG. *Bacillus subtilis* XG. 176	Peptone yeast extract broth.	Adsorbed on to charcoal, eluted with acetone.	Polypeptide, M.Wt. 2,400 Forms inactive gel in water.	Inhibits *Monilia*, *Microsporum sp.* Haemolytic.
Mycosubtilin *Bacillus subtilis*. (subtilin str.) 177	Molasses, salts, citric acid, yeast extract. 25°C for 5 days.	EtOH extract of cells pptd at pH 2.5. Ppt. from EtOH by water.	Polypeptide having no sulphur.	Inhibits *Micrococcus lysodeikticus*, yeasts and fungi. Toxic. Inactivated by serum.
Simplexin *Bacillus simplex*. 178	Corn steep liquor broth	Charcoal adsorption, eluted with hot MeOH.	Not known	Inhibits G +ve -ve organisms, *Rhizoctonia solani*. Toxic. Inactivated by serum.
Polypeptin *Bacillus circulans*. 179	Glucose, amino acids. 30°C. for 5 days.	Inactive material pptd and super. ex. with $CHCl_3$	Basic polypeptide	Inhibits G+ve -ve, acid fast organisms and some fungi. Non-toxic. Haemolytic

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Laterosporin *Bacillus laterosporus* 180	Yeast extract glucose. 37° C. for 3-4 days.	Charcoal adsorption, eluted with acid BuOH.	Basic polypeptides	Active against G +ve and -ve bacteria.
Unamed *Bacillus natto.* 181	Glutamate, soy bean, glucose. 37° C. for 48 hr	Adsorbed on to charcoal eluted by MeOH.	Not reported Presumedly a peptide.	Inhibits G+ve organisms only.
Fluvomycin syn. Vivicil *Bacillus subtilis.* 182	Yeast extract, saccharose, citric acid, salts. 25-28°C. for 16-24 hours.	Adsorbed on to charcoal, eluted by MeOH. Purified by chromatography.	Polypeptide.	Inhibits G +ve bacteria, yeasts and fungi. Inactivated by serum. Toxic.
Pumilin *Bacillus pumilis* (subtilis). 183	Am. citrate, glucose. 30° C. for 48 hr	$AmSO_4$ pptn. Ppt. extracted with acetone. Residue extracted w. C_6H_6.	Not elucidated.	G+ve organisms inhibited. Very toxic.
Subtenolin *Bacillus subtilis.* 184	Glycerol, alanine, Mg citrate. pH 6.7-6.8. 36° C. for 3-4 days.	Adsorbed on to charcoal. Eluted with MeOH. Antibiotic pptd. from MeOH by BuOH.	Not known. sulphur content.	Weakly inhibits G+ve organisms. Toxic, excreted with urine, 50% of dose in 10hr

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Megacine Bacillus megatherium str. 216. 185	Tryptone, yeast extract, lemco. 37°C. for 16 hr.	Cells removed by centrifugation Extracted into buffer and pptd. by $AmSO_4$.	Polypeptide Considered by authors to be a colicine.	Inhibits many ystrains of B. megatherium.
Biocerin Bacillus cereus. 186	Glucose, salts, methionine. 37°C for 48 hr.	Extracted by Et_2O.	Not known. Yellow/ brown substance.	Inhibits G+ve, -ve and acid fast organisms Fairly toxic.
Colistatin Unidentified aerobic bacillus 187	Glucose, sodium nitrate, salts, tryptone. pH 6.5. 28°C for 72 hr.	Charcoal adsorption. Eluted by acid MeOH.	Not known.	Bacteriostatic only. Inhibits G+ve, to a lesser degree G -ve bacteria. Not toxic.
Mycobacillin Bacillus subtilis. 188,189	Potato extract, casein hydrolysate, glucose, beef extract.	Extracted by BuOH, BuOH residue pptd. from aq. soln of it by HCl.	Polypeptide.	Inhibits dermomycotic fungi.
Petrin Bacillus subtilis. 190	Meat and potato extract. 22°C. for 2 days.	BuOH at pH 4.5, residue of BuOH extracted with hexane.	Not known	Inhibits Neisseria, Haemophilus, and Corynebacterium sp. Toxic.

Antibiotic/ Organism/ Reference	Production Medium	Method of Extraction	Chemical Nature	Biological Properties
Micrococcin (P) Micrococcus varians. 191,192	Peptone, meat extract, salt 37°C. 7-8 days	pH2 ppt. extracted w. EtOH. Residue extr.^{d} w. $CHCl_3$.	Contains sulphur. Has thiazolidine ring M.Wt. 2,000	Inhibits G+ve and acid fast organisms. Resists pepsin and trypsin.
Staphylococcus antibiotic. Staph. sp. 193	Horse flesh trypsin digest broth +cysteine+ glucose. 37° C. for 2 day	Not extracted.	Not known.	Active against Corynebacteria.

In conclusion it is interesting to note that all the principal antibiotics from bacteria, namely tyrocidine, gramicidin S, the polymyxins, circulins, colistins and bacitracins are polypeptides. It is also significant that the antibiotics of which the structure is known, are cyclic polypeptides, either entirely or in part cyclic. There is, therefore, strong evidence for a correlation between the cyclic structure and antibiotic activity as suggested by Erlanger and Goode (1954)[43] and Pressman et al(1967)[44].

Some of the peptide antibiotics from bacteria contain unusual amino acids. Polymyxin and colistin possess residues of L$\alpha\gamma$ diaminobutyric acid. Nisin and subtilin contain lanthionine and β-methyl lanthionine in the proportions of 4:1; nisin in addition has an isomer of β-methyl lanthionine, cystathionine. Subtilin has an odd amino acid residue, that of sarcosine which is also found in the non-bacterial antibiotic actinomycin D. These amino acids are undoubtedly of significance

in respect of the antimicrobial properties of these antibiotics.

It is significant that where the steric conformation of the amino acid components of the peptide antibiotics from bacteria are known some are of the D series. In gramicidin, tyrocidine, gramicidin S and J_1, polymyxin B_1, B_2, and bacitracin A, D-phenylalanine is found. D-leucine is to be found in gramicidin J_2, E_1, circulins A anb B. Polymyxin M has D-isoleucine; bacitracin has D-ornithine and glutamic acid.

D amino acids are not normal components of proteins. They do occur in relatively small amounts in some protein hydrolysates and their presence may be attributable to inversion of the natural L isomers during hydrolysis. D amino acids probably impart some resistance to hydrolysis of the peptides containing them and their presence may account for the antimicrobial properties of the peptide antibiotics. Such properties may be imparted by both the unusual amino acids and the cyclic structure.

THE SEMI-MICRO ASSAY USED IN THIS WORK.

The method used was a micro serial dilution method. A standard inoculum was added to each tube of the dilution series. Staphylococcus aureus (Paler) was the test organism used,

Materials

1. Three borosilicate glass Pasteur pipettes were graduated to contain 6 x 10, 2 x 50 and 7 x 100 µl. of liquid. Each pipette was plugged with cotton wool and had a 2 cm. length of pressure tubing attached at the plugged end to act as a means of attachment to a plastic 1 or 2 ml. disposable syringe. Syringes were found to be superior to teats as more control could be exercised over the charging and discharging of liquid from these pipettes. The pipettes were wrapped in metal foil and sterilised at 121°C. for 15 mins. and dried at 100°C. for at least 30 mins.

2. A supply of metal sheets, each the size of a microscope slide and covered with a ¼" layer of 'Plasticine' to form a pallet.

3. A supply of acid-washed Durham tubes (35 x 8 mm.) sterilized and dried under conditions similar to the pipettes but contained in a sealed vessel e.g. crystallizing dish with a metal or glass lid.

4. A supply of sealing wax sticks*.

5. Nutrient broth consisting of nutrient broth (Oxoid CM1), 1.3%; peptone (Oxoid L37), 0.5%; neutral phosphate (a finely ground mixture of Na_2HPO_4 and KH_2PO_4 in the ratio of 9.5:9.0), 0.5%; glucose, 0.1%; in water, dispensed in 5 ml. quantities in 6" x 5/8" capped test tubes and sterilised at 121°C. for 15 mins.

6. Two sterile dry cotton wool plugged boiling tubes.

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* Obtainable in bulk from Demnson & Walkden Ltd., Verney Rd., London, S. E. 16.

7. A 5 ml., 18 hour, nutrient broth culture of the test organism incubated at 37°C.

Procedure

Production medium samples were diluted five times in a M/150 pH 7 phosphate buffer solution. Samples of organic solvent extracts were taken to dryness 'in vacuo' and the residues dissolved or suspended in the buffer. All samples for assay were sterilized by heat at the temperature and duration which would give sterility, but sustaining the minimum loss of activity.

Each sterile graduated pipette was fitted with a syringe, flamed and placed in one of the sterile boiling tubes which had been pressed firmly in a 'Plasticine' pallet such that the open end was sloping downwards. Care was taken to avoid the parts of the pipette which had been handled, from touching the inside of the sterile boiling tube storage vessel (which acted as a sterile housing for pipettes when not in use).

The remaining sterile boiling tube was half-filled with water and fitted at an angle of 30° in a retort clamp. The water was brought to the boil and allowed to simmer over a micro-burner for the duration of the assay work.

For each sample to be assayed a series of six Durham tubes were set in a 'Plasticine' pallet, each tube set at an angle of c. 30° with the closed ends embedded in the pallet. Flame sterilized forceps were used for handling these tubes, except when the closed ends of each tube was embedded in the 'Plasti-cine' by pressure from the forefinger of the free hand. The open end of each tube was flamed. The requisite number of pallets plus tubes was prepared before embarking on the next stage of the procedure.

For each series of tubes, from left to right with open

ends of the tubes facing away from the operator, using the 100µl. graduated pipette, 200µl. of sterile nutrient broth were dispensed into the first tube and 100µl. in each of the second to sixth tubes. On completing this medium dispensing stage of the assay procedure the pipette was rinsed in the simmering water and returned to the storage tube. Next, a serial dilution of each of the samples was made in nutrient broth using the 2 x 50µl. graduated pipette. 50µl. of an antibiotic sample were added to the 200µl. quantity of broth, the contents mixed by charging and discharging the pipette three times, then 100µl. of the mixture were withdrawn and discharged in the second tube containing 100 µl. of broth, the contents were mixed in a similar manner, and 100 µl. withdrawn and added to the third tube and so on to completion. Hence a 1/5, 1/10, 1/20, 1/40, 1/80, 1/160 serial dilution of a sample had been made in nutrient broth (a 1:5 then 1:2 dilution series). Before a serial dilution of the next sample was prepared the pipette was rinsed in the simmering water, flamed dry and returned to the pipette storage vessel to cool.

On completing the serial dilution of every sample for assay an approximate 1:100 dilution of the test culture in fresh nutrient broth was made by adding 50 µl. of neat 18 hour culture to 5 ml. of the fresh broth. Using the 10 µl. graduated pipette 10 µl. of culture dilution were added to each tube of every assay and on completing this culture implantation step all tubes were sealed with sealing wax. When the wax had cooled the assays were tilted to ensure the implants were mixed with the broth/antibiotic mixtures. The assays were incubated for 18 hours at 37°C.

Following incubation of the assays the tubes in each assay were observed for the presence and absence of growth. The highest dilution which suppressed growth was referred to

as the 'Titre' of the sample assayed. From the titre the activity of the sample could be ascertained and expressed as 'units per ml!, or in terms of the original volume of the production culture from which the sample was derived, as 'units' in that culture. If the weight of solute in a sample was known then the activity could be expressed in terms of 'units per mg.' or µg/ml.

The Unit was defined as the activity which in 1 ml. would inhibit the growth of a 1/100 dilution of an 18 hour culture of the test organism.

Controls were prepared to check the growth of the test organism in the absence of any antibiotic and to check the sterility of dispensed non-implanted broth.

All graduated pipettes were immersed in chromic acid until the following day when they were rinsed with water and sterilized for re-use.

Notes

1. Borosilicate glass was used in making the graduated pipettes because it has a low coefficient of expansion, hence when wet, the pipettes could be flame-dried without the risk of cracking.

2. The nutrient broth used in the assays had been reinforced with constituents found to promote profuse growth of the test organism in those tubes in which it occured, hence enabling a more definite assessment of the end-point.

3. The number of dilutions (tubes) per assay could be varied according to the predicted activity of a sample, so could the dilution of the sample be varied for the same reason also.

Advantages and Disadvantages of the Method.

A marked advantage is that small samples can be assayed. Serial dilutions in which small volumes of liquid are involved can be more accurate provided one mixes a given dilution thoroughly and accounts for the liquid retained in a pipette due to capillary action (which might be a substantial proportion in terms of the small volumes involved). An additional advantage is that only a small inoculum is used, hence reducing the risk of the possibility of resistant organisms developing during incubation.

A disadvantage may be the unorthodox aseptic technique, namely the exposure of sterile vessels and broth to the air for relatively long periods. The risk of contamination is, however, slight if one avoids touching sterile parts of the equipment e.g. tips of pipettes, open ends of the Durham tubes. Durham tubes containing broth, set in a manner in which they are set for the assays, have been left for a working day exposed to the air, sealed with wax and incubated. The broth showed no signs of contamination.

The Reproducibility of the Semi-Micro Method of Assay.

To test the reproducibility of the assay technique a total of 110 assays were done using two common antibiotics as test substances. The titres observed for each antibiotic were consistent.

Eleven assays were prepared each day for five days using a filter sterilised aqueous solution of Penicillin G* containing

*Penicillin G, 'Crystapen' brand; Glaxo Ltd.

2.0 units/ml. (1.2 µg/ml.) as the test substance. A similar series of assays were done using bacitracin* as the test substance. The solution contained 2.0 units/ml. (28.5 µg/ml.). A fresh test culture was used each day.

All 55 assays for each antibiotic gave titres of 20 and 40 for penicillin and bacitracin respectively.

*Bacitracin USP, Burroughs Wellcome Ltd.

EXPERIMENTAL - PRELIMINARY SMALL-SCALE WORK.

The methods described in this section were similar for both antibiotic-producing strains and their substances. Except where points of detail differ the relevant strains are referred to as 'the culture' or 'culture'. All cultures whether seed* or production were prepared in 100 ml. quantities contained in cotton wool plugged 500 ml. conical flasks which were incubated at the relevant temperature and agitated by rotary shaking at 200 rpm. All media prior to inoculation was sterilized at 121°C. for 15 minutes.

1. Media Development

Before the work could begin proper, a stock of master cultures was prepared from a freeze-dried culture**. An ampoule of the freeze-dried culture was opened and regenerated by incubating at 30°C. for 24 hours in nutrient broth (1.3% Oxoid CM 1), This culture was loop inoculated on to slopes of nutrient agar (1.3% Oxoid CM 1 broth plus 1.3% Oxoid No. 3 agar) contained in screw-capped universal type glass bottles. These were incubated at 30°C. for 24 hours and stored at 3°C.

A 100 ml. nutrient broth culture was prepared by loop inoculation from a master culture selected at random. Into 100 ml. amounts of media containing a variety of carbon and nitrogen sources, simple and complex, in various amounts in a basic salts solution (see Appendix 2), a 5% inoculum of the nutrient broth culture was made. The potential production cultures were incubated at 24 and 30°C. for up to 72 hours, samples being withdrawn for antibiotic assay and pH determination. The samples were sterilized by heat at a temperature

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*Nurient broth, 1.3% Oxoid CM 1; loop inoculated and incubated at 30°C. for 24 hours.

**The active strains were provided by Dr. A. T. Fuller.

and for a time considered minimum for the destruction of the types of organisms: 106°C. for 10 minutes for a non-sporing organism (strain 175), 121°C. for 5 minutes for a spore-bearing organism (strain 2725). All assay samples were diluted in M/150 phoshate buffer at pH 7* prior to sterilization. (Subsequent studies showed no loss of activity due to heat sterilization under the relevant conditions for both antibiotic substances when compared with 'Millipore' filtered samples.

The medium found suitable for production of antibiotic substance from strain 175 was 1% corn steep liquor plus ½% glucose in basic salts solution, pH to 7 prior to sterilization; for antibiotic substance from strain 2725 it was 1% soya flour, ½% fish meal and ½% glycerol in basic salts solution, with no pH adjustment prior to sterilization. A 5% nutrient broth seed culture was initially used to inoculate the respective medium and the optimum incubation temperatures for the production cultures were 24 and 30°C. for strain 175 and 2725 respectively. With the strain 175 production medium it was found immaterial whether the glucose was sterilized separately or with the rest of the constituents.

2. The Preparation of a Stock of Freeze-Dried Cultures.

a) Strain Selection. A suspension of a loopful of growth from the same stock slope used in the media development was made in a 10 ml. quantity of nutrient broth and a loopful of this suspension streaked on to plates of nutrient agar. In order to secure well isolated colonies each plate was inoculated as follows: back and forth in closely parallel lines over half of the plate. The loop was flamed, the plate turned 90° and half the uninoculated surface streaked from the original streak overlapping the latter for 1 or 2 cm. The loop was

*M/150 Phosphate Buffer, pH 7. 0.95 g. Na_2HPO_4; 0.9 gm. KH_2PO_4 per litre of water.

flamed, the plate turned 90° and the remaining surface streaked, overlapping a portion of the preceding section as before. The plates were incubated at 30°C. for 24 hours and a random selection made of 20 of the individual colonies produced. Each colony was picked off its plate and a suspension of the growth made in 10 ml. of nutrient broth. From each suspension a nutrient agar slope was prepared and a 100 ml. nutrient broth culture. These cultures were grown up at 30°C. for 24 hours, the latter agitated by rotary shaking. The slope cultures were the new master cultures and stored at 3°C.; the 100 ml. broth culture was seed for production media. 5 ml. of each broth culture was inoculated into production media and the activity determined at 24 hourly intervals up to 72 hours. It was intended that the isolate giving the highest activity would be selected for freeze-drying. All isolates, however, exhibited the same activity and one was selected at random.

b) Freeze-Drying. The selected isolate was loop inoculated into 100 ml. nutrient broth. The culture was grown at 30°C. for 24 hours, agitated by rotary shaking. The cells were recovered by centrifugation in sterile metal-capped 'Pyrex' centrifuge tubes (10 cm. x 1 cm. I.D.) at 3,600 rpm. using a 'Piccolo' centrifuge*. The cells in each centrifuge tube were resuspended in 0.1 ml. of a sterile solution of 1% glucose plus 1% peptone (Oxoid L 37) in water. Each suspension was transferred to a sterile cotton wool plugged freeze-drying ampoule (1 ml. size), care being taken to avoid the suspension wetting the barrel of the ampoule as it was dispensed from a sterile plugged Pasteur pipette. When this operation had been completed, each cotton wool plug was trimmed with a pair of scissors and pushed down the ampoule barrel and the barrel

*Griffin and George Ltd.

constricted over a fish-tail Bunsen burner, care being taken to avoid charring the cotton wool plug.

All ampoules were placed in a methylated spirits/cardice mixture and the contents allowed to freeze. Each ampoule was placed on a manifold connected via a methylated spirits/cardice water trap and a phosphorus pentoxide trap to an Edwards type ED 35 vacuum pump and freeze-dried. A minimum of 0.5 Torr was necessary to prevent thawing. When the operation had been completed (c. 16 hr.) each ampoule was sealed under vacuum at the constriction, labelled and stored at room temperature.

One ampoule was taken at random for purity and antibiotic activity assessment and preparation of a stock of agar slopes which were to be used in subsequent work.

3a) <u>The Stability of the Antibiotic Substances in their Respective Production Media: Stability at 3°C. and Room Temperature, pH 2-11 in M/10 Glycine Buffer.</u>

200 ml. of the relevant production culture were prepared and harvested at 48 hours. This culture was divided into ten portions, one portion adjusted to pH 2, another to pH 3 and so on to pH 11 using N.HCl or N.NaOH for the purpose of pH adjustment.

Each portion was diluted 1 in 2.5 with the respective glycine buffer and divided into two, one sub-portion stored at 3°C., the other at room temperature, in 'Pyrex' boiling tubes each sealed with a rubber bung. A control was prepared to check the initial activity of the culture.

At 0.5, 24, 48, 72 and 120 hours a 1 ml. sample was withdrawn from each tube, its pH adjusted to 7, diluted 1 in 2.5 with M/150 phosphate buffer (pH 7), sterilized and

assayed. The results are shown in Tables 1 (p.91) and 15 (p.108) for antibiotic substance 175 and 2725 respectively.

3b) Stability at 100°C., pH 2, 7 and 10 in M/10 Glycine Buffer.

100 ml. of production medium were harvested at 48 hours and divided into three portions and the pH of one portion adjusted to 2, another to 7 and the other to 10. A control sample was taken. Each portion was diluted 1 in 2.5 with the respective glycine buffer and 20 ml. of each of the buffered cultures dispensed into a graduated boiling tube. Each boiling tube was placed in a boiling water bath and 1 ml. samples withdrawn at 0, 0.5, 1, 2, 4 and 8 hours; compensation for reduction in volume owing to evaporation was made by adding the respective buffer before the one hour and subsequent samples were taken. Each sample was adjusted to pH 7 and diluted 2.5 times with M/150 phosphate buffer (pH 7), sterilized and assayed. The results are given in Tables 2 (p.92) and 16 (p.108) for antibiotic substances 175 and 2725 respectively.

It was determined prior to this experiment that 'double heating' namely at 100°C. and 106°C. (or 121°C. for culture 2725) at pH 7 immediately before assay, had no detrimental effect on the activity of a sample. This was found by comparing a sample which had been heated twice with a similar sample which had been heated once and sterilized by filtration.

4. The Identification of the Two Antibiotic-Producing Strains.

Taxonomic tables compiled by Cowan and Steel (1961)[194] and Bergey's Manual[195] were used as sources of reference. The features studied which led to the identification of strain 175 (Pseudomonas fluorescens) and strain 2725 (Bacillus

licheniformis) are given in Tables 3 (p.93) and 17 (p.109) for antibiotic substances 175 and 2725 respectively.

5. The Effect of Inoculum, Aeration and Antifoam on Antibiotic Yield in Shake Flask Culture.(Samples for assay were adjusted to pH 7, diluted 1 in 5 with M/150 phosphate buffer (pH 7) prior to sterilization and assay).

a) Inoculum. Quantities of nutrient broth seed culture to give an inoculum of 0.25, 0.5, 5, 10 and 15% were transferred to 100 ml. of production medium and the production cultures grown. At 3 x 24 hourly intervals samples for assay and pH determination were removed. The results are presented in Tables 4 (p.94) and 18 (p.110) for antibiotic substances 175 and 2725 respectively.

b) Aeration. Production medium volumes of 50, 75, 100 and 150 ml. were prepared in 500 ml. cotton wool plugged conical flasks and sterilised. To each was added a 0.5% inoculum of a 24 hour nutrient broth seed culture. Volumes greater than 150 ml. were not practicable in 500 ml. flasks as wetting of the cotton wool plugs resulted on rotary shaking. At 3 x 24 hourly intervals samples for assay and pH determination were withdrawn from each culture. The results are given in Tables 5 (p.94) and 19 (p.110), antibiotic substances 175 and 2725 respectively.

c) Antifoam. Two antifoam agents were selected for this experiment: silicone RD and polypropylene glycol (P2000). Various concentrations (0.1, 0.25 and 0.5% V/V) of sterile antifoam were added at a 1:2 dilution in water, to ease dispensing these rather viscous agents, to 100 ml. amounts of production medium. Each batch of medium was inoculated with 0.5% of a 24 hour nutrient broth seed culture. Samples for assay and pH determination were taken at 3 x 24 hourly intervals. The results are given in Tables 6 (p.95) and 20 (p.111) for antibiotics 175 and 2725 respectively.

6. The Changes in Antibiotic Activity, pH, Cell Count*, Glucose Concentration (str. 175)*, % Sporulation (str. 2725) and Nitrogen* in Production Medium on 100 ml. shake flask scale.(Samples for assay were adjusted to pH 7, diluted 1 in 5 with M/150 phosphate (pH 7), prior to sterilization and assay).

A series of 10 x 100 ml. quantities of production medium were each inoculated with 5% of a 24 hour nutrient broth seed culture. At eight hourly intervals a flask was removed from the shaker and the factors given above determined. The results are given in Tables 7 (p.96) and 21 (p.112) for antibiotic substances 175 and 2725 respectively.

7. The Clarification of Mature Production Medium. As it is desirable to remove cells and undissolved nutrients prior to extraction, attempts were made to ascertain if the antibiotics were soluble in the culture fluid at the pH at which optimum activity was achieved, if not, would pH adjustment render them soluble.

A 48 hour culture of the active strain was prepared in the corresponding production medium and divided into eight portions. One portion was adjusted to pH 2, another to pH 3 and so on up to pH 9 using N.HCl or N.NaOH. Each portion was centrifuged** at 4,000 rpm. for 10 minutes and the supernatant adjusted to pH 7 and diluted 1 in 5 with M/150 phosphate buffer (pH 7) and sterilized. The results are given in Tables 8 (p.97) and 22 (p.113) for antibiotic substances 175 and 2725 respectively.

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*See foot of page 78.

**Using a 'Piccolo' centrifuge.

THE FERMENTATION, METHODS OF EXTRACTION AND PARTIAL PURIFICATION OF ANTIBIOTICS 175 AND 2725, ON A 350 l. SCALE; and Properties of the Antibiotics.

The methods of extraction and partial purification were developed on a small scale using 1 l. cultures, prepared by bulking 10 x 100 ml. shake flask production medium cultures and subsequently scaled-up for work with 350 l. batch cultures. Medium. The relevant production medium was the same as that used on the 100 ml. shake flask scale namely 1% corn steep liquor plus ½% glucose in a basic salts solution (Appendix 2) for antibiotic 175 and 1% soya flour, ½% fish meal plus ½% glycerol in basic salts solution for antibiotic 2725.

For each 350 l. of production medium plus an initial 0.01% V/V silicone RD were prepared and sterilized at 121°C. for 20 mins. with rapid cooling. (The glucose of the 175 medium was sterilized separately as 60 l. of a 3.3% solution and aseptically transferred to approximately 290 l. of medium having the remaining constituents at concentrations applicable 350 l.)

Inoculum. 1 l. of a secondary nutrient broth (Oxoid CM 1) culture was used. This was prepared by inoculating 10 ml. of a nutrient broth primary culture into 1 l. of nutrient broth contained in a 3 l. conical flask. Both cultures were incubated at 30°C. for 24 hours and agitated by rotary shaking.

Antibiotic 175. a) Fermentation. The batch culture was aerated at a rate of 200 l./min. for the first eight hours and 400 l./min. thereafter with agitation at a rate of 183 rpm. from a four blade (125 x 50 mm.) impellor under a positive pressure of 15 p.s.i.g. at 24°C.

Samples for antibiotic assay, pH, total nitrogen*, glucose** and total cell count+ determination, were taken at six hourly intervals up to 48 hours when an additional final sample for dry weight estimation++ was taken.

At 48 hours the culture was clarified by centrifugation through a Sharples centrifuge at 15,000 rpm. set for liquid/solid separation, and the supernatant pumped into a suitable agitated extraction vessel. At this stage the supernatant was sampled for antibiotic assay only.

b) Extraction. 1% Sutcliffe and Speakman*+ grade 110 charcoal was added to the culture supernatant and mixed for one hour with a 4 blade (7" x 2") impellor at 400 rpm. At 55 minutes 1% w/v Hyflo super-cel filter aid was added to the mixture and the charcoal recovered by pressure filtration, using a Calmic heavy duty filter press (type E) having 12 of 42cm. diameter plates. Each plate was fitted with a C100 filter paper and precoated with a slurry of filter aid in water.

On completion of filtration the charcoal/filter aid was scraped off the filter papers into a vessel containing 70 l. of a 5:2 ethanol benzene solvent mixture and the contents mixed for one hour with an agitator having 4 of 3.5"x 2" blade impellor at 400 rpm.

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*Total nitrogen estimated by automated technique based on the Kjeldahl method.

**Glucose estimated by automated technique based on the ferricyanide method.

+Total cell counts determined using a Hawksley counting chamber (Thom), depth 0.02 mm. with 1/400 cm. squares.

++Dry weight estimation: a 40 ml. sample centrifuged 15,000 rpm., centrifugate resuspended in water, recentrifuged, centrifugate dried 110°C. overnight in a weighed dish and then reweighed.

*+Sutcliffe and Speakman Ltd., Leigh, Lancs.

The eluting solvent was recovered by filtration, using a basket filter of 50 cm. diameter having the corresponding Whatman No. 1 filter paper fitted and with a vacuum of 740 mm. provided by a Nashitor pump. Both the charcoal filtrate and the eluting solvent were sampled for assay and the latter concentrated in two stages to approximately 3 l. using the following equipment:

i) Climbing Film Evaporator (Type 1). The recovered eluting solvent was passed through this equipment which reduced its volume to approximately 12 l. The feed rate was approximately 60 l. per hour using a steam pressure of 3 p.s.i.g., vapour temperature of 30°C. and a vacuum of 710 mm.*

ii) Apex Evaporator. The climbing film evaporator concentrate was concentrated further with this equipment at a rate of 15 l./hour using a steam pressure of 2 p.s.i.g., at a water jacket temperature of 50°C., and vacuum of 740 mm.* These conditions gave a solvent temperature of approximately 22°C. The volume of the final concentrate was 1.5-3.0 l.

c) Partial Purification. This was achieved by extraction with ether. The concentrate was extracted twice with half to an equal volume of ether at pH 8.5 to remove inactive ether solubles. The activity was then extracted into ether at pH 4.5 and to water at pH 8.5, back to ether at pH 4.5, then to water at pH 8.5. At each stage the volume of solvent (ether or water) was reduced to about half that of the previous active fraction. The final aqueous extract had a volume of approximately 250ml. N.HCl was used to adjust the pH. Samples were taken for antibiotic assay from each fraction and a flow sheet constructed on each batch extracted. A typical flow sheet is given in the results section, p.98.

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*Vacuum provided by an Edwards ES 35 high capacity pump.

The final aqueous solution was freed of dissolved ether 'in vacuo', adjusted to pH 8.5, and freeze-dried using the equipment employed for freeze-drying the master culture, but with a larger manifold. A fairly high vacuum was needed to avoid thawing, < 0.1 Torr.

d) <u>The Stability of Antibiotic 175 Product at 3°C., Room Temperature and 37°C. at pH 2-11 in M/10 Glycine Buffer.</u> 300 ml. of an aqueous solution containing 0.5 mg/ml. of the product were prepared and divided into 10 x 30 ml. portions. One portion was adjusted to pH 2, another to pH 3 and so on to pH 11, using N. acid or N. alkali. Each portion was diluted 1 in 2 with the relevant glycine buffer solution and dispensed in three equal parts into 'Pyrex' test tubes and each tube sealed with a rubber bung.

One pH 2-11 series of solutions was stored in the cold at 3°C., another at room temperature (c. 20°C.) and the other stored at 37°C. At 0.5, 24, 48, 72 and 120 hours a 1 ml. aliquot was withdrawn from each tube of each series, all aliquots adjusted to pH 7 with N.HCl or N.NaOH. Each aliquot was diluted 1 in 10 with M/150 phosphate buffer (pH 7) and sterilized at 106°C. for 10 minutes and assayed up to a 1 in 320 dilution. The pattern of results were so similar to those obtained for the stability of the antibiotic in production medium that all the results are condensed into one table, Table 1 (p.91).

e) <u>The Stability of Antibiotic 175 Product at 100°C., pH 2, 7 and 10 in M/10 Glycine Buffer.</u> 15 ml. of an aqueous solution containing 1.0 mg/ml. of the product were prepared and divided into 3 x 5 ml. portions, the first adjusted to pH 2,

the second to pH 7 and the third to pH 10 using N.HCl or N. NaOH. All three were diluted 1 in 2 with the corresponding glycine buffer and each solution dispensed into a graduated boiling tube. The boiling tubes were placed in a boiling water bath and 0.5 ml. withdrawn from each tube at 0, 0.5, 1, 4, 6 and 8 hours, each aliquot adjusted to pH 7, diluted 1 in 10 with M/150 phosphate buffer (pH 7) and sterilized at 106°C. for 10 minutes. Each sample was assayed up to a 1 in 640 dilution. Compensation for loss of water from the solutions owing to evaporation were made prior to the withdrawal of all aliquots at one hour and thereafter. The results are given in Table 2 (p.92). As was the case with stability studies at 3°C., room temperature and 37°C. the results showed much similarity to those obtained with the antibiotic in production medium, hence the results of the two experiments on the stability at 100°C. were condensed into one table.

f) The Antimicrobial Spectrum of Antibiotic 175.

5 ml. of a solution containing 1 mg/ml. of the product in M/150 phosphate buffer, pH 7, were prepared and sterilized at 106°C. for 10 minutes. This solution was diluted aseptically to give solutions of 0.5, 0.25 and 10.125 mg/ml. using nutrient broth (Oxoid CM 1) as diluent. From each of the nutrient broth solutions tenefold dilutions were made in sterile broth up to 10,000.

100 µl. of each dilution of each solution were dispensed into Durham tubes set in the manner similar to the semi-micro assay. To each tube 10 µl. of a 10^{-2} dilution of a test organism culture in nutrient broth were added and each tube sealed with wax. The procedure was repeated for each test organism considered. All assays were duplicated. The incubation temperatures depended on the optimal temperature for

the growth of the organism under test. Following incubation the tubes were read for 'growth' and 'no growth' and the results expressed as minimum inhibitory concentrations (M.I.C.). Controls were prepared to check the growth of each test organism in broth in the absence of antibiotic. The results are presented in Table 9 (p.103).

g) The Effect of Serum on the Activity of Antibiotic 175 against Staphylococcus aureus (Paler).

5 ml. of a solution of the product containing 50 µg/ml. were prepared in M/150 phosphate buffer (pH 7) and sterilized at 106°C. for 10 minutes.

A series of nutrient broth solutions containing 1, 5, 10, 20, 50 and 75% unborn calf serum were prepared, the serum having been previously inactivated by heat at 56°C. for 30 minutes.

The usual semi-micro assay was done in duplicate up to a 1 in 320 dilution with each of the concentrations of serum in broth as diluent, together with a control containing no serum, and using the antibiotic solution prepared above. Growth controls were prepared to observe the effect of various concentrations of serum on the growth of the test organism. The results are given in Table 10 (p.104).

h) The Haemolytic Activity of Antibiotic 175.

A 1% solution of the antibiotic in physiological saline solution (0.85%) was prepared and diluted ten-fold with saline up to 10^{-9}. Each solution was sterilized at 106°C. for 10 minutes.

A series of eight Durham tubes were set in a 'Plastiçine' pallet as though a semi-micro assay was to be performed and 100 µl of each of the solutions dispensed into the tubes

beginning with the 10^{-9} dilution and working backwards. To each of these solutions 100 µl of fresh heparitinized rabbit blood were added and each tube sealed with wax. The tests were done in duplicate with a control having no antibiotic. The tests were stored at 3°C. for 18 hours. The procedure was repeated and the tests incubated at 37°C. for 18 hours. If haemolysis was observed the tests were repeated using dilutions of the antibiotic in saline within the range of dilutions that showed haemolysis and no haemolysis, thus enabling a more precise determination of the concentration which just gave haemolysis. The results are presented in Table 11 (p.104).

i) The Effect of Inoculum on the Activity of Antibiotic 175 against St. aureus (Paler).

A solution containing 10 µg/ml. of the antibiotic in M/150 phosphate buffer (pH 7) was prepared and sterilized at 106°C. for 10 minutes. Six semi-micro assays (up to 1 in 320 dilution) of the solution were prepared each intended to have a different implant size. 10 µl. of neat 18 hour 37°C. nutrient broth culture of St aureus (Paler) were dispensed into each of the tubes of one assay. 10 µl. of a 10^{-1} dilution of the culture into another assay and so on until a 10^{-6} dilution had been used. Total cell counts were done on each of the dilutions to give an estimate of the numbers of bacterial cells involved and to check the accuracy of the serial dilutions. The results are shown in Table 12 (p.105).

j) The Bactericidal Activity of Antibiotic 175 against St. aureus (Paler) and Escherichia coli (MRE 600).

A solution of the antibiotic containing 2 mg/ml. in M/150 phosphate buffer (pH 7) was prepared. From this solution a series of dilutions in the same buffer were made to contain 1,000, 500, 250, 25 and 2.5 µg/ml. of the antibiotic. The

solutions were sterilized at 106°C. for 10 minutes.

2 ml. of each of these solutions were diluted with 8 ml. of sterile nutrient broth. To each of these solutions in nutrient broth was added 1 ml. of a 10^{-1} dilution of a St. aureus (Paler) or E. coli (MRE 600) culture, previously incubated at 37°C. for 18 hours. A control having no antibiotic was prepared. The cultures were incubated at 37°C.

At 2, 4, 8, 24, 48 and 72 hours a 1 ml. sample was withdrawn from each culture for total and viable count estimations. The total count was determined microscopically using a bacterial counting chamber (p.78). The viable count was estimated by spread-plating, on nutrient agar plates, 0.1 ml. of each dilution of a ten-fold dilution series (up to 10^{-7}) of each sample in physiological saline solution. The plates were prepared in triplicate and incubated at 37°C. for 18 hours. Those plates which were countable were counted and the mean of triplicate plates calculated. From the data the percentage viability was ascertained. The results are given in Tables 13 and 14 (p.106).

Antibiotic 2725. a) Fermentation. The batch culture was aerated at a rate of 200 l. per minute throughout the fermentation with agitation at a rate of 183 rpm. from a four blade (125 x 50 mm.) impellor at a pressure of 5 p.s.i.g. at 30°C.

Samples for assay, pH, total nitrogen and total cell count determinations were withdrawn at six-hourly intervals up to 48 hours, when an additional final sample was taken for dry weight estimation.

At 48 hours the whole culture was adjusted to pH 3 with

concentrated hydrochloric acid and clarified by centrifugation through a Sharples MV 12 centrifuge at 15,000 rpm. A sample of the acid supernatant was taken for assay.

b) Extraction. 2% Sutcliffe and Speakman grade 339 charcoal was added to the supernatant and mixed for one hour with a four blade impellor (7" x 2" each) at 400 rpm. At 55 minutes 2% Hyflo super-cel filter aid was added to the mixture and the charcoal recovered by pressure filtration using a Calmic filter (type E) with twelve plates fitted.

The charcoal/filter aid mixture was scraped off the filter papers after the filtration had been completed and transferred to 70 l. of 5:2 industrial methylated spirits/benzene solvent mixture plus 1% conc. hyrochloric acid and the contents mixed for one hour with an agitator having four of 3.5" x 2" blade impellor at 400 rpm.

The eluting solvent was recovered by basket filtration. Both the eluting solvent and the charcoal were sampled for antibiotic assay.

Using the same conditions as in antibiotic 175 extraction (p.79), the eluting solvent was concentrated in two stages, the acid in the solvent being neutralized prior to concentration.

The Apex concentrate (c. 5 l.) was taken to dryness in a rotary evaporator with a water bath temperature of 30°C. and a vacuum of 760 mm. produced by a laboratory water pump.

c) Partial Purification. The active material was extracted from the concentrate residue with industrial methylated spirits and the solution taken to dryness in the rotary evaporator under conditions stated above for the conc-

entrate of the eluting solvent. This preliminary fractionation resulted in about 90% reduction in the dry weight.

The antibiotic was thrown out of solution as its picrate by the addition of a saturated solution of picric acid*. The quantity necessary varied from batch to batch but the volume equivalent to 12 g. of picric acid per 100 g. of pH 6.5 water soluble residue gave the best result in terms of active material recovered.

The picrate was allowed to sediment overnight at 3°C., the mother liquor was siphoned off and the picrate recovered by filtration or centrifugation. The picrate was dried at room temperature and dissolved in a minimum amount of 80% acetone in water, the solution taken to almost dryness and the antibiotic converted to its hydrochloride by the addition of 5 N. HCl at a rate of 5 ml. per 100 g. of picrate and the hydrochloride precipitated by the addition of several volumes of 100% acetone.

The hydrochloride was recovered by centrifugation and washed with acetone, recentrifuged and dissolved in water. The pH of the solution was adjusted to 8.0 with 5 N.NaOH and centrifuged. The pH of the solution was then readjusted to 7 and freeze-dried using the same equipment as was used at the corresponding stage with antibiotic 175 (p.80), although in this instance only a minimum of 0.5 Torr was necessary to prevent thawing.

At each stage in the process samples were taken for assay and a flow-sheet compiled for each batch processed. A typical flow-sheet is given in the results section, page 114.

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*0.65% w/v picric acid at 20°C.

d) The Stability of Antibiotic 2725 Product at 3°C., Room Temperature and 37°C. at pH 2-11 in M/10 Glycine Buffer.

300 ml. of a solution of the product containing 1 mg/ml. were prepared and divided into 10 x 30 ml. quantities. One quantity was adjusted to pH 2, another to pH 3 and so on up to pH 11. Each quantity was diluted 1 in 2 with the respective glycine buffer solution. The procedure from here on was similar to that adopted for antibiotic 175 (p.80) except that the aliquots were diluted 1 in 5 with M/150 phosphate buffer (pH 7) on being neutralized prior to sterilization at 121°C. for 5 mins. The results were so similar to those obtained for the experiment concerned with the stability of the antibiotic in its production medium that all the results have been condensed into one table, Table 15 (p.108).

e) The Stability of Antibiotic 2725 Product at 100°C. at pH 2, 7 and 10 in M/10 Glycine Buffer.

This was determined using a method similar to that used for antibiotic 175 (p.80) except that the solution employed contained 0.5 mg/ml. of the product and the aliquots were diluted 1 in 5 prior to sterilization and assay. The results are given in Table 16 (p.108).

f) The Antimicrobial Spectrum of Antibiotic 2725.

The method was similar to that employed for antibiotic 175 (p.81) except that the sample sterilization conditions were different. The results are given in Table 23 (p.118).

g) The Effect of Serum on the Activity of Antibiotic 2725 against Staphylococcus aureus (Paler).

The procedure differed from that used in the corresponding

experiment for antibiotic 175 (p.82) only in respect of the concentration of the solution of the product and the source of serum used. The antibiotic solution contained 100 µg/ml. and the serum was heat inactivated horse serum. The results are shown in Table 24 (p.119).

h) The Haemolytic Activity of Antibiotic 2725.

The method employed was similar to that employed for antibiotic 175 (p.82). The results are given in Table 25 (p.119).

i) The Effect of Inoculum on the Activity of Antibiotic 2725 against St. aureus (Paler).

A solution of 40 µg/ml. of the product was used, otherwise the method was the same as that used for antibiotic 175 (p.83). The results are given in Table 26 (p.120).

j) The Bactericidal Activity of Antibiotic 2725 against St. aureus (Paler) and Escherichia coli (NRL 600).

The method used was identical with that of the corresponding experiment for antibiotic 175 (p.83). The results are given in Tables 27 and 28 (p.121).

k) A Study of the Amino Acid Composition of Antibiotic 2725 by Paper Chromatography.

The product had approximately 12% total nitrogen. 10 mg. (1.2 mg. nitrogen) of the antibiotic were hydrolysed in a vacuum sealed ampoule (made from a 6" x 5/8" 'Pyrex' glass test tube) by 10 ml. of 6 N. HCl for 20 hours at 120°C.

The hydrolysate was taken to dryness over a steam bath and the residue dissolved in 1 ml. of water and the solution evaporated to dryness, thus ensuring the removal of excess HCl.

The hydrolysate was dissolved in 1 ml. of water and 10 µl of the solution spotted on to a Whatman No. 1 chromatography sheet (57 x 46cm.).

A two-dimensional chromatogram was prepared. The solvent for the first dimension comprised a 150:60 mixture of sec-butanol : 3% ammonia. This was repeated in the same dimension. Each first dimension run took approximately 24 hours.

The second dimension was performed in a 150:30:20 mixture of sec-butanol:formic acid:water and took approximately 10 hours.

The chromatogram was dried at 105°C., sprayed with a 0.25% solution of ninhydrin in acetone and developed at 105°C. for 4 minutes. The ratio of the distance travelled in the first dimension to the distance travelled in the second dimension was determined, taking measurements from as near as possible from the centre of each spot to the base lines. The ratios were compared with those obtained from a map. The method was taken from Haussman (1952)[196].

The results are given in Table 29 (p.112), and were confirmed by Thomas[197] using an amino acid analyzer. Thomas' results were quantitative and are also shown in Table 29.

RESULTS

A total of fifteen 350 l. batch cultures of Pseudomonas fluorescens, str. 175 and eleven 350 l. batch cultures of Bacillus licheniformis, str. 2725 were prepared and the respective antibiotics extracted and partially purified. A typical flow sheet for each culture and subsequent extraction is presented on page 98 for antibiotic 175 and page 114 for antibiotic 2725.

In instances where production medium samples were assayed on both the 350 l. and shake flask scale, each sample was adjusted to pH 7 and sterilized by heat (106°C./10 mins. for antibiotic 175 and 121°C./5 mins. for antibiotic 2725). All samples for assay were assayed using the 1:5 then 1:2 dilution series mentioned in the section outlining the method of assay. All assays were done in duplicate and where titres are quoted the values represent the mean of duplicated assays. All production medium samples, except those of the charcoal filtrate, obtained during preliminary extraction, were assayed at 5 times the original volume.

The results shown in Tables other than Tables 3, 17 and 29 are the means of duplicated experiments conducted on separate occasions. Explanations of the results shown in Tables 3, 17 and 29 are given at the head of the respective data.

The Stability of Antibiotic 175.

a) Stability at 3°C., Room Temperature and 37°C., pH 2-11 in M/10 Glycine Buffer. (The methods used to obtain these results are given on pages 73 and 80).

The data obtained for the stability of antibiotic 175 in its production medium over the pH range 2-11 in glycine buffer

at 3°C. and room temperature did not differ from the corresponding data obtained for the product. Neither were there any appreciable differences between the results obtained for stability at various storage temperatures, both for the antibiotic in its production medium and the product. No stability studies for antibiotic 175 in its production medium at 37°C. were done. The results are given in Table 1.

Table 1

pH	Time in Hours. (figures are assay titres)				
	0.5	24	48	72	120
2.0	100	100	100	100	50
3.0	200	200	100	100	50
4.0	200	200	200	200	100
5.0	200	200	200	200	200
6.0	200	200	200	200	200
7.0	200	200	200	200	200
8.0	200	200	200	200	200
9.0	200	200	200	200	200
10.0	200	200	200	100	100
11.0	200	200	100	100	100

These experiments showed that antibiotic 175, both in its production medium and as the product in aqueous solution is stable at temperatures up to 37°C. between pH 5 and 9 for at least five days and between pH 4 and 10 for up to two days. The antibiotic is less stable at the extremes of the pH range studied, especially at the acid pH where there is a 50% loss within half a hour whereas at the extreme alkaline pH such loss was not detected for between 24 and 48 hours.

The loss of activity at pH 2 was found to be more pronounced

at 100°C.

b) Stability at 100°C., at pH 2, 7 and 10.(Details of the experimental methods are given on pages 74 and 80).

Again no difference in the pattern of stability for the antibiotic in its production medium and as the product in aqueous solution could be detected. Table 2 shows the results of the experiments.

Table 2

pH	Time in Hours. (figures are assay titres)						
	0	0.5	1	2	4	6	8
2.0	200	25	25	<25	<25	<25	<25
7.0	200	200	200	200	100	100	100
10.0	200	200	200	200	50	50	50

At 100°C. the antibiotic appeared to be more stable at the neutral pH for up to 2 hours. The pattern of loss appeared to be greater at the acid than at the alkaline pH as was the case at the lower temperatures studied.

At 3°C., room temperature and 37°C. there was not complete loss of activity at any pH over the duration of the experiments which seems to suggest the existence of more than one antibiotic in the product. Barrow[200] found by thin-layer chromatography that there were at least five fractions in the product all of which I found to be biologically active.

The Identity of Antibiotic-Producing Strain 175.

All the biochemical tests were done in triplicate. A

summary of the taxonomic features of this organism is given Table 3.

Table 3

Feature		Feature		Feature	
Gram Reaction	-	Growth in Mac-		Mannitol(acid)	+
Shape	Rod	Conkey Broth at 37°	+	Salicin(acid)	-
Motility	+	Nitrate Reduction	+	Arabinose(acid)	-
Catalase	+	Gelatin Liquefaction	-	Xylose(acid)	-
Oxidase	+	Urease Production	+	Glycerol(acid)	-
Oxidative or		H_2S Production	-	Growth at Room	
Fermentative	Ox.	Citrate Utilization	+	Temperature	+
Pigment	+	Malonate "	+	Voges Proskauer	-
Diffusion of		Starch Hydrolysis	-	Gluconate util-	
Pigment	+			ization.	+

These features are characteristic of Pseudomonas fluorescens, particularly the feature referring to the diffusion of pigment. The gram reaction, motility, catalase and oxidase reaction and the oxidative fermentation of glucose are all characteristics of Pseudomonas sp. Most of the biochemical characteristics resemble those of Ps. pyocyanea except those of negative acid production from media where arabinose, xylose and glycerol are the sole carbon sources and the fermentation of malonate. According to Cowan and Steel (1961)[194] 21-79% of the strains of Ps. pyocyanea ferment malonate. However, my conclusions as to the identity of strain 175 were confirmed by Bascombe [198].

The Effect of Inoculum on the Yield of Antibiotic 175 in Production Medium.(Experimental details are given on p.75).

The results given in Table 4 suggest that an inoculum of 0.25% is sufficient to obtain the same yield as was previously obtained using a 5% inoculum in shake flask cultures.

Table 4

Percentage Inoculum	Time in Hours					
	24		48		72	
	pH	Titre	pH	Titre	pH	Titre
0.25	7.80	100	8.40	200	8.40	200
0.5	7.90	100	8.50	200	8.40	200
5.0	8.10	200	8.50	200	8.65	200
10.0	8.10	200	8.60	200	8.65	200
15.0	8.25	200	8.65	200	8.80	200

The Effect of Aeration on the Yield of Antibiotic 175 in Production Medium.(Experimental details are given on p.75).

The results shown in Table 5 indicate that aeration has no effect on the yield of antibiotic 175.

Table 5

Volume of Medium per Flask (ml.)	Time in Hours					
	24		48		72	
	pH	Titre	pH	Titre	pH	Titre
50	7.90	200	8.75	200	8.40	200
75	7.80	200	8.65	200	8.35	200
100	7.70	200	8.50	200	8.50	200
150	7.10	25	8.45	100	8.45	200

The Effect of Various Concentrations of Silicone RD and Polypropylene Glycol P2000 on the Yield of Antibiotic 175 in Production Medium.(Experimental details are given on p. 75).

The results are given in Table 6.

Table 6

Percentage Antifoam		Time in Hours					
		24		48		72	
		pH	Titre	pH	Titre	pH	Titre
0.1	RD	7.40	100	8.55	200	8.55	200
0.25	RD	7.40	100	8.50	200	8.55	200
0.50	RD	7.45	100	8.50	200	8.60	200
0.1	P1000	7.50	<25	8.45	<25	8.50	<25
0.25	P1000	7.45	<25	8.45	<25	8.30	<25
0.5	P1000	7.55	<25	8.35	<25	8.30	<25
Control		7.40	200	8.40	200	8.50	200

The results suggest that even the highest level of silicone RD does not alter the yield of antibiotic compared with the control. There was some delay in the achievement of optimum yield presumedly due to the depression in the dissolution of air caused by the presence of the antifoam. Polypropylene glycol P2000 does have a detrimental effect on yield although there was continued growth of the culture and the pH pattern was not appreciably different from that of the control and silicone RD supplemented cultures.

The Changes in Antibiotic Activity, pH, Cell Count, Glucose Concentration and Total Nitrogen in Antibiotic 175 Production Medium.(100 ml. shake flask scale)

Experimental details are given on page 76 . The data obtained are presented in Table 7

Table 7

	Time in Hours									
	0	8	16	24	32	40	48	56	64	72
Titre	<25	50	100	200	200	200	200	200	200	200
pH	7.00	5.40	7.15	7.25	7.85	8.15	8.30	8.35	8.45	8.55
Cell Count - ($x10^{-8}$)		15.6	106.0	251.0	302.0	290.0*	260.0	180.0	170.4	150.8
Glucose Con$^{on.}$(%)	0.49	0.18	0.15	0.13	0.09	0.03	0.02	0.015	0.015	0.015
Total N_2 (mg/ml.)	1.61	1.61	1.64	1.45	1.25	1.55	1.61	1.66	1.74	1.67

*Lysis observed from this point onwards.

Optimum yield was obtained within 24 hours as previous experiments have indicated. This peak did not correspond with the peak in the growth of the culture and antibiotic synthesis appeared to be more a function of the glucose concentration than a function of cell numbers. Synthesis ceased as soon as the glucose concentration dropped to about a quarter of its initial value. There was no loss in total nitrogen although variations in total nitrogen figures did occur and could be attributable to variations in the samples. Even at 72 hours the pH did not reach an unfavourable level.

The Clarification of the Mature Production Medium.

Experimental details are given on page 76.

The results of this minor experiment are shown in Table 8 and indicate that the antibiotic is extracellular at all the pH values tested. The relatively low titre at pH 2.0 is undoubt-

edly attributable to the instability of the antibiotic at this pH, see Table 1 (p.91).

Table 8

pH	Whole Broth	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
Titre	200*	50	200	200	200	200	200	200	200

*Assayed at pH 7.0.

A Typical Flow-Sheet for the Fermentation, Extraction and Partial Purification of Antibiotic 175.

Fermentation. (This data was obtained by the pilot plant staff at Imperial College, except the assay figures).

Sample Time (h.)	pH	Titre	Cell Count (No./ml.)	Total N_2 (mg/ml.)	Glucose Con$^{cn.}$ (%)
Pre-inoc.	7.00	<25	-	0.20	0.49
4.0	6.95	<25	4.93×10^8	0.19	0.41
10.0	4.60	<25	5.60×10^8	0.10	0.32
16.0	6.50	50	7.00×10^8	0.18	0.25
22.0	7.30	50	1.00×10^9	0.09	0.17
28.0	7.60	100	1.20×10^9	0.30	0.09
34.0	7.80	100	6.40×10^9	0.30	0.032
40.0	7.90	100	6.50×10^9	0.17	0.025
46.0	8.15	100	5.40×10^9	0.19	0.020
48.0 (Harvest)	8.30	100	-	-	-

Cell dry weight on final sample = 0.26%

Clarification, Charcoal Adsorption, Recovery and Solvent Elution.

(Titres are expressed in terms of original volume of the culture unless otherwise stated).

Titre of Culture	100(37m*)
Volume of culture (l.)	370
pH of Culture	8.30
Vol. of Culture Supernatant after Centrifugation (l.)	360
Titre of Culture Supernatant	100(36m)
Wt. of Sutcliffe and Speakman 110 Charcoal Used (Kg.)	3.7
Mixing Time (Hours)	1.0
Wt. of Hyflo Super-cel Used (Kg.)	3.7
Volume of Charcoal Filtrate (l.)	340
pH of Charcoal Filtrate	6.50
Titre of Charcoal Filtrate (at original volume)	<5(<1.5m)

*m = "million units"

Volume of Ethanol/Benzene (5:2) Used (l.)	70
Elution Time (Hours)	1.0
Volume of Solvent Recovered on Filtration (l.)	c.70
Titre of Recovered Solvent	100(36m)

Solvent Concentration

Volume of Primary Concentrate from Climbing Film Evaporator (l.)	13
Titre of Primary Concentrate	100(36m)
Volume of Secondary Concentrate from Apex Evaporator (l.)	1.65
Titre of Secondary Concentrate	100(36m)

Solvent Extraction - Ether Extraction at pH 8.50

1. Volume of Ether Added to Concentrate (ml.)	1000
Volume of Ether Recovered (ml.)	880
Titre of Ether Phase Recovered (at 1/10 original volume)	<5(<0.18m)
Volume of Concentrate Phase Recovered (ml.)	1700
Titre of Concentrate Phase Recovered	100(36m)
2. Volume of Ether Added to Concentrate Phase	500
Volume of Ether Recovered (ml.)	480
Titre of Ether Phase Recovered	<5(1.8m)
Volume of Concentrate Phase Recovered (ml.)	1700
Titre of Concentrate Phase Recovered	100(36m)

-Ether Extraction at pH 4.5

1. Volume of Ether Added to Concentrate (ml.)	1000
Volume of Ether Phase Recovered (ml.)	1000
Titre of Ether Phase Recovered	100(36m)
2. Volume of Ether Added to Concentrate (ml.)	500
Volume of Ether Phase Recovered (ml.)	495
Titre of Ether Phase Recovered	.5(1.8m)
Volume of Spent Concentrate (ml.)	1780

Titre of Spent Concentrate	<5(4.8m)

-Aqueous Extraction at pH 8.5

Volume of Water Added to Bulked Active Ether Phases (ml.)	750
Volume of Spent Ether Phase Recovered (ml.)	1210
Titre of Spent Ether Phase Recovered (at 1/10 original volume)	<5(4.8m)
Volume of Aqueous Phase Recovered (ml.)	1040
Titre of Aqueous Phase Recovered	100(36m)

-Ether Extraction at pH 4.50

Volume of Ether Added to the Aqueous Phase Recovered (ml.)	500
Volume of Ether Phase Recovered (ml.)	450
Titre of Ether Phase Recovered	50(18m)
Volume of Spent Aqueous Phase Recovered (ml.)	1050
Titre of Spent Aqueous Phase Recovered (at 1/10 original volume)	<5(4.8m)

-Aqueous Extraction at pH 8.50

Volume of Water Added to Ether Phase (ml.)	250
Volume of Aqueous Phase Recovered (ml.)	250
Volume of Spent Ether Phase Recovered (ml.)	400
Titre of Spent Ether Phase Recovered (at 1/10 original volume)	<5(4.8m)

Product.

pH of Ether-Free Aqueous Solution Adjusted to	8.50
Wt. of Freeze-Dried Product Obtained (g.)	3.55
Activity (units/mg.)	7900
" (µg/ml.)	0.12
Total Units Recovered (7900 x 3.55 x 1000)	28m
Efficiency (%)	75

The data obtained for the fermentation on the 350 l.

scale showed that practically all the glucose in the medium had been utilized when optimum activity was achieved. A similar result was obtained on the shake flask scale. Optimum activity was reached after 24 hours at a pH of about 7.5. Despite all the glucose having been used up there was an insignificant rise in the cell count and it seems, therefore, that glucose is an essential metabolite in the biosynthesis of antibiotic 175.

Differences in the cell count on the large and the small scale culture were undoubtedly due to differences in the levels of inocula, 0.25% and 5% respectively. Differences in optimum yield of the antibiotic on the two scales could not be attributed to differences in cell counts as it seemed antibiotic yield was a function of glucose concentration, besides the cell count and antibiotic activity optima did not coincide. Such differences in yield, although apparently large (100 and 200 units/ml. on the 350 l. and shake flask scale respectively) were within the limits of detection of the method of assay, since a titre of 200 differs from 100 only in there being one additional tube not exhibiting growth.

Cell dry weights were done on the final sample as an additional parameter for determining if there were any differences between batches.

The clarification of the mature culture and charcoal filtration were found to be more efficient if the fermentation was allowed to proceed for 48 hours. Continuation of the fermentation was not detrimental to the activity of the culture since the pH did not reach an unfavourable level; The clarification stage usually took about five hours using two Sharples MV 12 centrifuges.

The spent culture supernatant (or charcoal filtrate) was

always left more acid than the culture supernatant from which it was derived, pH 6.5 compared with pH 8.3. This was undoutedly due to the acidic nature of the charcoal grade employed in the preliminary extraction of the antibiotic. Sutcliffe and Speakman grade 110 charcoal had a pH of 2.5 when a 10% suspension of it was made in freshly distilled water.

Absolute ethanol was used in preference to industrial methylated spirits (IMS) as a component of the charcoal eluting solvent because grade 110 charcoal retained sufficient moisture to create a two-phase system or emulsion if IMS was used. Under these circumstances the elution was less efficient. Drying of the charcoal led to a considerable loss of activity. Hence absolute ethanol was the best source of ethanol to use and ethanol benzene was by far the most efficient eluting agent.

The final concentrate was largely aqueous and required no further concentration prior to removal of inactive ether solubles at pH 8.5. The ether/aqueous extractions were performed in a 5 l. separating funnel.

The final aqueous extract was invariably difficult to make owing to slow phase separation. Light centrifugation eased this situation. The final aqueous extract was freed of ether 'in vacuo' prior to lyophilization. Again some difficulty was encountered owing to foaming. This problem was overcome by periodic release of vacuum.

Most of the loss of activity occurred in the final stages. The efficiency of extraction was quite high, of the order of 75% yielding 3-5 g. of antibiotic per 350 l. batch having an activity of 7,900 units/mg. (0.12 µg/ml.)

The Antimicrobial Spectrum of Antibiotic 175.

Table 9 shows the antimicrobial spectrum of the antibiotic. Experimental details are given on page 81.

Table 9

Species of Micro-organism (NCTC type No. of Strain No. in parenthesis).	Temperature at which assay incubated (°C.).	Minimum Inhibitory Concn. (µg/ml.).
Aerobacter aerogenes	37	250
Bacillus alkaligenes	37	25
Bacillus subtilis	37	0.25
Bacillus thuringiensis	37	>500
Diptheria mitis (3989)	37	>500
Escherichia coli (MRE600)	37	12.5
Haemophilus bronchosepticus	37	12.5
Klebsiella pneumoniae (7242)	37	125
Lactobacillus acidophilus (1899)	30	1.25
Neisseria catarrhalis (3422)	37	1.25
Pseudomonas fluorescens (str. 175)	30	>500
Pseudomonas pyocyanea	37	>500
Proteus vulgaris	30	>500
Salmonella typhi	37	250
Shigella flexneri	37	250
Streptococcus Group C (Equi)(6177)	37	125
Streptococcus pneumoniae	37	0.25
Staphylococcus aureus (Oxford)	37	0.5
Staphylococcus aureus (Paler)	37	0.125
Vibrio cholera (8367)	37	1.25
Candida albicans	37	>500

The antibiotic was active against gram negative and gram positive bacteria. In general the gram positives were inhibited by lower concentrations than were the gram negatives, exceptions, were Vibrio cholera and Bacillus thuringiensis.

The Effect of Serum on the Activity of Antibiotic 175 Against Staphylococcus aureus (Paler) Experimental details are given on page 82.

The results shown in Table 10 suggest that the presence of serum impairs the activity of antibiotic 175 against St. aureus (Paler). At 25% serum 75% of the activity is lost whilst 75% serum suppresses activity completely.

Table 10

%Serum	Titre	Growth of the Test Culture in Serum Controls.
0	80	
1	80	Growth pellet in assay
5	40	tubes was more diffuse and
10	40	the extent of the growth
25	20	less the higher the
50	10	concentration of serum.
75	<5	

The Haemolytic Activity of Antibiotic 175.

The results of this experiment are shown in Table 11. Details of the method used are presented on page 82.

Table 11

Dilution of Antibiotic in the Presence of Blood.($2 \times 10^{-}$..)	Control	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Haemolysis at 3°C. (±)	-	+	-	-	-	-	-	--	-
" at 37°C.(±)	-	+	+	-	-	-	-	-	-

The critical dilution below which haemolysis occurs is 1 : 1,600 and 1 : 18,000 at 3 and 37°C. respectively. The product obtained was clearly haemolytic, the antibiotic itself may not, however, be haemolytic but the haemolytic property be due to impurities in the product.

The Effect of Inoculum on the Antibacterial Activity of Antibiotic 175. The test organism employed was St. aureus (Paler). The experimental method is described on p.83 . The results are shown in Table 12.

Table 12

Dilution of Culture.	Total Cell Count of Culture Dilutions Prior to Assay(No./ml.)	Dilution of Culture in Assay.	Titre
0	5.10×10^{10}	10^{-1}	40
10^{-1}	4.90×10^{9}	10^{-2}	40
10^{-2}	4.80×10^{8}	10^{-3}	40
10^{-3}	5.00×10^{7}	10^{-4}	80
10^{-4}	4.85×10^{6}	10^{-5}	80
10^{-5}	4.72×10^{5}	10^{-6}	80
10^{-6}	4.95×10^{4}	10^{-7}	160
10^{-7}	4.88×10^{3}	10^{-8}	160

This experiment was performed to test the effect of inocula at dilutions greater and smaller than the dilution used in the assay and from the results it is seen that a 1,000-fold change in the inoculum in terms of cell numbers is necessary to alter the activity by 100%, the limit of detection of the assay.

The total cell counts confirmed the accuracy with which the 10-fold dilutions were prepared as inocula for the assays.

The Bactericidal Activity of Antibiotic 175 against St. aureus(Paler) and Escherichia coli (NRE 600).

The results are shown in Tables 13 and 14 for St. aureus and E. coli respectively. Experimental details are given on page 83.

Table 13

Concentration of Antibiotic (µg/ml.)	Time in Hours					
	2	4	8	24	48	72
Control	77.0	94.0	95.1	96.0	86.0	50.0
0.5	81.9	83.0	96.0	82.0	76.5	60.0
5.0	87.3	88.0	89.0	32.0	21.0	5.7
50	80.1	86.0	87.0	27.5	19.0	5.4
100	81.3	83.0	84.0	28.0	15.1	0.56
200	81.0	84.0	71.0	15.0	10.5	0.33

The figures are expressed as percentage viability; the viability of 18 hour nutrient broth culture of St. aureus (Paler) incubated at 37°C. = 81.0%.

Table 14

Concentration of Antibiotic (µg/ml.)	Time in Hours					
	2	4	8	24	48	72
Control	80.0	95.0	97.3	98.0	65.0	52.5
0.5	83.3	90.0	95.0	87.2	90.7	59.0
5.0	84.0	91.0	94.0	53.1	44.0	35.7
50	88.0	90.0	92.3	23.0	4.9	5.0
100	80.1	86.0	50.0	20.8	4.4	2.5
200	83.0	84.0	31.0	20.5	1.6	0.33

The figures are expressed as percentage viability; the viability of 18 hour nutrient broth culture of E. coli incubated at 37°C. = 80.0%.

Antibiotic 175 appears to be bactericidal to both St. aureus (Paler) and E. coli (MRE 600). Initially (0-8 h.) there is an increase in viability of both cultures exposed to concentrations of antibiotic up to 50 µg/ml. and then a progressive loss with increase in time. At 100 and 200 µg/ml. there is a small increase in viability but not to the extent seen with lower concentrations.

The Stability of Antibiotic 2725.

a) Stability at 3°C., Room Temperature and 37°C., pH 2-11 in M/10 Glycine Buffer. (The methods used to obtain these results are given on pages 73 and 87).

The data obtained for the stability of antibiotic 2725 in its production medium over the pH range 2-11 in glycine buffer at 3°C. and room temperature did not differ from the corresponding data obtained for the product. Neither was there any significant difference between the results obtained for stability at various temperatures both for the antibiotic in its production medium and the product. No stability studies for the antibiotic in production medium at 37°C. were done. The results were compiled into one table, Table 15.

The results indicate that antibiotic 2725 is stable at temperatures up to 37°C. for at least 24 hours over the pH range 2-11 with some loss thereafter at the extremes of the range, namely 2, 10 and 11. Loss of activity was more pronounced at 100°C. at the extreme pH values studied.

Table 15

pH	Time in Hours. (figures are titres)				
	0.5	24	48	72	120
2.0	200	200	200	100	50
3.0	200	200	200	200	100
4.0	200	200	200	200	100
5.0	200	200	200	200	100
6.0	200	200	200	200	100
7.0	200	200	200	200	100
8.0	200	200	200	200	100
9.0	200	200	200	200	100
10.0	200	200	100	100	50
11.0	200	200	100	100	50

b) Stability at 100°C., at pH 2, 7 and 10. (Details of the experimental methods are given on pages 74 and 87).

The loss of activity was more rapid at the extreme pH values than at the neutral pH. The antibiotic did show a tendency to be less stable at the acid pH than at the alkaline pH as is seen in Table 16.

Table 16

pH	Time in Hours. (figures are titres)						
	0	0.5	1	2	4	6	8
2.0	100	100	100	50	25	25	<25
7.0	100	100	100	100	50	50	25
10.0	100	100	100	100	50	25	25

At all the temperatures selected for the study of stability

of the antibiotic, except 100°C., there was not total loss of activity over the period of the experiments. This suggests that there is more than one biologically active fraction in the antibiotic, some being more stable to the conditions used than others.

The Identity of the Antibiotic-Producing Strain 2725.

All the biochemical tests were done in triplicate. A summary of the taxonomic features of this organism isgiven in Table 17.

Table 17

Feature		Feature		Feature	
Gram Reaction	+	Oxidase	+	Starch Hydrolysis	+
Shape	Rod	Acid Production		NO_3^- Reduction	+
Spores (endo)	+	from Glucose	+	Urease Production	+
Motility	+	Oxidative or		Growth at 65°C.	-
Acid Fastness	-	Fermentative	Ox	Anaerobic growth	
Catalase	+	Voges Proskauer	+	in Glucose Broth	+
Gelatin Liquif$^{n.}$	+	Mannitol (gas)	+	Citrate Utiliz.$^{n.}$	+

These features are typical of Bacillus licheniformis. The organism differs from Bacillus cereus in but one feature: it can grow in glucose broth under anaerobic conditions. It is a Bacillus sp.in view of its ability to form endospores, its gram reaction, ability to liquefy gelatin and to hydrolyse starch.

The Effect of Inoculum on the Yield of Antibiotic 2725 in Production Medium.(Experimental details are given on p. 75).

The results shown in Table 18 indicate that the antibiotic yield is independent of the level of inoculum used. An inoculum of 0.25% appears to be adequate.

Table 18

Percentage Inoculum	Time in Hours 24		48		72	
	pH	Titre	pH	Titre	pH	Titre
0.25	5.60	50	7.00	200	8.00	200
0.5	5.70	50	7.15	200	8.05	200
5.0	6.00	100	7.45	200	8.25	200
10.0	6.10	100	7.90	200	8.40	200
15.0	6.40	100	7.90	200	8.45	200

The Effect of Aeration on the Yield of Antibiotic 2725 in Production Medium. (Experimental details are given on p. 75).

It appears that rapid aeration of antibiotic 2725 production medium does not affect the yield. There was not sufficient difference in titre between the highly aerated and poorly aerated flasks to suggest this. The results are shown in Table 19.

Table 19

Volume of Medium per Flask (ml.)	Time in Hours 24		48		72	
	pH	Titre	pH	Titre	pH	Titre
50	8.25	50	8.95	100	8.85	100
75	8.10	50	8.60	100	8.85	100
100	7.10	100	7.40	200	8.15	200
150	7.45	100	7.50	200	8.55	200

The Effect of Various Concentrations of Silicone RD and Polypropylene Glycol P 2000 on the Yield of Antibiotic 2725 in Production Medium.(Experimental details are given on p. 75).

The results are given in Table 20.

Table 20

Percentage Antifoam		Time in Hours					
		24		48		72	
		pH	Titre	pH	Titre	pH	Titre
0.1	RD	6.10	50	7.00	200	7.95	200
0.25	RD	6.25	50	7.00	200	7.95	200
0.50	RD	6.30	50	7.00	200	7.95	200
0.1	P2000	6.05	25	8.05	25	8.55	50
0.25	P2000	6.00	25	7.85	25	8.45	50
0.5	P2000	6.00	25	7.85	25	8.50	50
Control		6.25	50	7.00	200	7.95	200

The results indicate that polypropylene glycol does depress the yield of the antibiotic even at the smallest concentration screened whereas none of the concentrations of silicone RD affected the yield. The effect of polypropylene glycol was, however, not so drastic as its effect on the production of antibiotic 175 (p.95).

The Changes in Antibiotic Activity, pH, Cell Count, Total Nitrogen and Percentage Sporulation in Antibiotic 2725 Production Medium. (100 ml. shake flask scale)
Experimental details are given on page 76 .

The data obtained are presented in Table 21.

Table 21

	Time in Hours									
	0	8	16	24	32	40	48	56	64	72
Titre	<25	25	50	100	200	200	200	200	200	200
pH	7.10	6.10	5.80	5.90	6.80	7.05	7.10	7.70	7.90	8.10
Cell Count ($\times 10^8$/ml.)	-	10.3	40.0	44.0	47.2	52.0	36.0	25.6	22.6	21.6
%Sporulation	0	0	0	2.1	8.0	20.8	72.0	87.1	88.3	90.0
Total N_2 (mg/ml.)	0.74	0.67	0.70	0.57	0.44	0.56	0.53	0.45	0.48	0.79

The results show that optimum antibiotic activity was reached within 32 hours at a pH of 6.80. This optimum did correspond to some extent with the onset of sporulation but not with the peak in cell count and suggests that the synthesis of the antibiotic is related to the cultural conditions which promote spore formation as is implicated with the antibiotic bacitracin[79,80].

There was a small fluctuation in the total nitrogen content of the medium during fermentation and this could be attributable to sample variation. A similar state of affairs was encountered in the corresponding experiment with antibiotic 175.

The Clarification of the Mature Production Medium.

Experimental details are given on page 76.

The results obtained are shown in Table 22. It appears that antibiotic 2725 is either a component of the cell which produced it or is bound to the insoluble components of the medium, such as the soya flour and the fish meal, and is released into the culture fluid on acidification to pH 3.

Table 22

pH	Whole Broth	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
Titre	200*	200	200	100	<25	<25	<25	<25	<25

*Assayed at pH 7.0

The antibiotic was in fact found to be a component of the cell on the basis of the following observations:

a) The activity of a sample of 48 hour antibiotic 2725 production medium culture was the same (200 units/ml.) after it had been centrifuged at a rate and time sufficient to precipitate only the insoluble medium constituents (1000 x g/ 5 minutes).

b) The activity was lost when the supernatant from a) was assayed after it had been centrifuged at 25,000 x g/10 minutes.

c) The bacterial centrifugate obtained in b) on suspension in M/150 phosphate buffer (pH 7.0) exhibited the same activity as the original culture, i.e. 200 units/ml.

d) The activity was released into the suspending medium on acidification to pH 3, since activity was detected after the suspension had been centrifuged at 25,000 x g/10 minutes, and the pH adjusted to 7 prior to assay.

A Typical Flow-Sheet for the Fermentation, Extraction and Partial Purification of Antibiotic 2725.

Fermentation. (This data was obtained by the pilot plant staff at Imperial College, except the assay figures).

Sample Time (h.)	pH	Titre	Cell Count (No./ml.)	Total N_2 (mg/ml.)
Pre-inoc.	7.05	<25	-	0.91
4.3	6.90	<25	0.90×10^7	0.67
10.3	5.90	25	1.20×10^7	0.70
16.3	5.80	50	2.99×10^8	0.57
22.3	5.60	100	3.68×10^9	0.44
28.3	5.85	100	4.95×10^9	0.36
34.3	6.45	100	3.29×10^9	0.53
40.3	6.50	100	3.04×10^9	0.45
46.3	6.95	100	6.25×10^8	0.48
48.0 (Harvest)	3.10*	100	3.75×10^8	0.79

*On acidification with concentrated hydrochloric acid.
Cell dry weight on final sample = 0.54%

Clarification, Charcoal Adsorption, Recovery and Solvent Elution.

(Titres are expressed in terms of the original volume of the culture unless otherwise stated).

Titre of Culture	100(37m*)
Volume of Culture (l.)	370
Volume of pH 3 Supernatant (l.)	345
Titre of pH 3 Supernatant	100(34.5m)
Wt. of Sutcliffe and Speakman 339 Charcoal Used (Kg.)	6.5
Mixing Time (Hours)	1.0
Wt. of Hyflo Super-cel Used (Kg.)	6.5
Volume of Charcoal Filtrate (l.)	310
pH of Charcoal Filtrate	6.00
Titre of Charcoal Filtrate (at original volume)	<5(<1.5m)

*m = "million units"

Volume of Industrial Methylated Spirits/Benzene (5:2) + 1% conc. HCl. Used (l.)	70
Elution Time (Hours)	1.0
Volume of Solvent Recovered on Filtration (l.)	c.70
Titre of Solvent Recovered	100(35m)

Solvent Concentration

Volume of Primary Concentrate from Climbing Film Evaporator (l.)	12.3
Titre of Primary Concentrate	100(35m)
Volume of Secondary Concentrate from Apex Evaporator (l.)	3.3
Titre of Secondary Concentrate	100(35m)
Wt. of Residue from Secondary Concentrate (g.)	768

Methylated Spirits Extraction

1. Volume of IMS Used to Fractionate Dry Residue(ml.)	800
Volume Recovered (ml.)	765
Titre (at original volume)	50(17.5m)
2. Volume of IMS Used to Re-Fractionate Residue(ml.)	630
Volume Recovered (ml.)	600
Titre (at original volume)	10(3.5m)
3. Volume of IMS Used to Re-Fractionate Residue(ml.)	650
Volume Recovered (ml.)	648
Titre (at original volume)	5(1.75m)
Total Units Recovered by the IMS Fractionations	22.75m
Wt. of IMS Soluble Residue (g.)	35.2
Volume of Water Used to Dissolve Residue (ml.)	1750
Titre of the solution (at original volume)	50(17.5m)

Picration

Volume of Saturated Picric Acid Solution Used (ml.)	1000
Wt. of Dry Picrate Obtained (g.)	80.4
Volume of Acetone Used to Fractionate Picrates (ml.)	20

Volume of Acetone Reduced to (ml.)	14
Volume of 5N. HCl Used to form Hydrochloride (ml.)	4.0
Volume of 100% Acetone Used to Precipitate HClide(ml.)	200
Volume of Water Used to Dissolve Hyrochloride (ml.)	250
Wt. of pH Inactive Insolubles (g.)	1.4

Product

Wt. of Freeze-Dried Product Obtained (g.)	8.15
Activity (units/mg.)	2000
" (µg/ml.)	0.5
Total Units Recovered (2000 x 8.15 x 1000)	16.3m
Efficiency (%)	47

From the above flow-sheet it was noted that the optimum yield was lower than that achieved on the shake flask scale (Table 21). The difference was small. There was an unexplainable difference in the pH at the point of optimum yield, pH 5.60 and 6.80 respectively. Total cell counts differed on the two scales and were probably due to the differences in inoculum levels employed. No noticeable changes occurred in the total nitrogen content of the culture.

As was found to be the case with antibiotic 175, clarification was more efficient if the fermentation was allowed to proceed for 48 hours. Extension of the fermentation period was not detrimental to the product as the pH did not reach an unfavourable level. Clarification took about five hours using the two Sharples MV 12 centrifuges.

The pH of the charcoal filtrate was less acid than the supernatant from which it was derived, pH 6.0 compared with 3.10. This change may have been attributable to the neutral grade of charcoal employed in the preliminary extraction of

this antibiotic.

The use of industrial methylated spirits sufficed in the solvent mixture used to elute the antibiotic from the charcoal.

Prior to concentration of the solvent the hydrochloric acid was neutrallized with 880 ammonia to prevent the concentration of the acid during climbing film and Apex evaporation of the solvent. Such acid concentration would undoubtedly be unfavourable to the antibiotic.

The picration stage often proved difficult because the picrates of the antibiotic did not immediately precipitate. It was found that storage at 3°C. overnight resulted in an adequate recovery of active picrates.

At the hydrochloride preparation stage the 80% acetone used to fractionate the picrates was reduced in volume, prior to precipitation of the hydrochloride, to reduce the moisture content of the extract. Failure to do this resulted in a dark brown viscous preparation instead of an off-white particulate substance, the former having about half the activity of the latter. A combination of the use of dry picrates and relatively concentrated hydrochloric acid contributed to the avoidance of this problem.

The Antimicrobial Spectrum of Antibiotic 2725.

Table 23 shows the antimicrobial spectrum of the antibiotic. Details of the method used are given on page 87.

Table 23

Species of Micro-organism (NCTC type No. or Strain No. in parenthesis).	Temperature at which assay incubated (°C.).	Minimum Inhibitory Concn. (µg/ml.).
Aerobacter aerogenes	37	25
Bacillus coagulans (3993)	37	250
Bacillus thuringiensis	37	125
Bacillus licheniformis (str.2725)	30	25
Corynebacterium hofmanni (231)	37	5
Diptheria mitis (3989)	37	25
Escherichia coli (MRE 600)	37	5
Haemophilus bronchosepticus (452)	37	5
Klebsiella pneumoniae (7242)	37	25
Lactobacillus acidophilus (1899)	30	0.125
Neisseria catarrhalis (3622)	37	5
Pasteurella septica (2484)	37	125
Pseudomonas fluorescens (str. 175)	30	250
Proteus vulgaris	30	>500
Staphylococcus aureus (Oxford)	37	0.5
Staphylococcus aureus (Paler)	37	0.5
Staphylococcus sp. (8354)	37	1.25
Shigella dysenteriae (8375)	37	25
Streptococcus Group C (Equi)(6177)	37	0.125
Vibrio cholera (8367)	37	0.25
Candida albicans	37	250

The antibiotic was found to inhibit both gram negative and gram positive organisms. There did not appear to be any preponderance to any one of these groups. Of the organisms screened it was least active against Proteus vulgaris and most most active against Lactobacillus acidophilus and Streptococcus C (Equi).

The Effect of Serum on the Activity of Antibiotic 2725 Against Staphylococcus aureus (Paler). Experimental details are given on page 87 .

The results shown in Table 24 are interesting because concentrations of serum at and above 5% enhance the activity of the antibiotic.

Table 24

%Serum	Titre	Growth of the Test Culture in Serum Controls.
0	160	
1	160	Growth pellet in assay
5	320	tubes was more diffuse and
10	320	the extent of the growth
25	320	less the higher the
50	320	concentration of serum.
75	320	

The Haemolytic Activity of Antibiotic 2725.

The results of this experiment are shown in Table 25. Details of the method used are shown on page 88 .

Table 25

Dilution of Antibiotic in the Presence of Blood.(2 x10⁻..)	Control	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Haemolysis at 3°C.(±)	-	+*	-	-	-	-	-	-	-
Haemolysis at 37°C.(±)	-	+*	-	-	-	-	-	-	-

*Only partial haemolysis observed. Critical dilution at which partial haemolysis occurred = 4 x 10^{-2}.

The antibiotic appeared to be haemolytic at a relatively high concentration at 37°C. The antibiotic may itself be non-haemolytic but haemolysis due to impurities in the product.

The Effect of Inoculum on the Antibacterial Activity of Antibiotic 2725. The test organism employed was St. aureus (Paler). The experimental method is described on p. 88. The results are shown in Table 26.

Table 26

Dilution of Culture.	Total Cell Count of Culture Dilutions Prior to Assay(No./ml.)	Dilution of Culture in Assay.	Titre
0	4.58×10^{10}	10^{-1}	20
10^{-1}	4.60×10^{9}	10^{-2}	20
10^{-2}	4.49×10^{8}	10^{-3}	20
10^{-3}	4.60×10^{7}	10^{-4}	40
10^{-4}	4.52×10^{6}	10^{-5}	40
10^{-5}	4.30×10^{5}	10^{-6}	40
10^{-6}	4.62×10^{4}	10^{-7}	80
10^{-7}	4.60×10^{3}	10^{-8}	80

The results showed that, as was the case in the corresponding experiment with antibiotic 175, that a 1000-fold change in the inoculum altered the activity by 100%.

The Bactericidal Activity of Antibiotic 2725 against St. aureus (Paler) and Escherichia coli (MRE 600).

The results are shown in Tables 27 and 28 for St. aureus (Paler) and E. coli (MRE 600) respectively. Experimental details are given on page 88.

Table 27

Concentration of Antibiotic (µg/ml.)	Time in Hours					
	2	4	8	24	48	72
Control	58.0	74.0	95.0	96.0	86.0	42.0
0.5	50.1	70.0	93.0	95.0	23.1	20.8
5.0	58.3	67.0	95.0	50.0	6.2	4.2
50	56.0	71.0	75.0	17.3	0.89	0.71
100	56.2	66.0	62.5	12.5	0.66	0.30
200	50.0	37.5	35.0	8.3	0.57	0.30

The figures are expressed as percentage viability; the viability of 18 hour nutrient broth culture of St. aureus (Paler) incubated at 37°C. = 57.4%.

Table 28

Concentration of Antibiotic (µg/ml.)	Time in Hours					
	2	4	8	24	48	72
Control	84.0	86.0	93.1	97.0	53.0	41.0
0.5	80.0	81.0	90.0	95.1	44.0	42.0
5.0	92.0	76.0	75.3	70.3	8.5	1.3
50	69.0	67.0	65.6	10.0	<0.6	<0.01
100	67.5	62.0	60.0	8.6	<0.35	<0.06
200	56.0	37.0	25.0	8.0	<0.11	<0.02

The figures are expressed as percentage viability; the viability of 18 hour nutrient broth culture of E. coli (NRE 600) incubated at 37°C. = 80.0%. All figures with a 'less than' sign were calculated from instances where no viable count was observed. The viable count was then assumed to be $\ll$ the minimum dilution of the range of spread plates prepared e.g. for plate range of dilutions of culture 10^{-4}, 10^{-5} and 10^{-6} the viable count was considered to be $\ll 10^{4}$.

The antibiotic was bactericidal to both St. aureus (Paler) and E. coli (NRR 600). With St. aureus but not E. coli there was an increase in the viability during the first eight hours of incubation at concentrations up to 50 µg/ml. At the lowest concentration (0.5 µg/ml.) viability of both cultures continued to increase during the first 24 hours. There appeared to be no significant difference in the patterns of viability of the two organisms at 100 and 200 µg/ml. and at any concentration after 24 hours incubation.

The Amino Acid Composition of Antibiotic 2725.

The method used is described on page 88. The results are given in Table 29, which shows the mean results of triplicate assays. The mean values of total nitrogen and ammonia nitrogen determinations on the three samples used for this study were 11.003% and 1.618% . The mean total peptide was 58.65%.

Table 29

Amino Acid	mg/100 mg. Antibiotic
Arginine	26.35
Lysine	26.11
Isoleucine	13.26
Glutamic acid	6.41
Aspartic acid	6.07
Valine	4.35
Glycine	4.21
Phenylalanine	3.39
Leucine	2.85
Alanine	2.51
Histidine	1.93
Threonine	1.60
Serine	0.95

The quantitative data was obtained by Dr. A. J. Thomas and his staff, using an amino acid analyzer.

No study was made of the stereo isomeric configuration of the amino acids detected hence no knowledge was gained concerning unnatural isomers in the antibiotic. No unusual amino acids were detected. Over half the product contained residues of arginine and lysine. Some of the amino acids found might be components of peptide impurities in the product and/or the antibiotic. From the amino acid viewpoint the biologically active peptide(s) might be a known antibiotic. If its composition is the same as a known peptide antibiotic the sequence of the amino acid residues may be different and hence the antibiotic a new one.

DISCUSSION

The methods developed for the extraction and partial purification of the antibiotics were reasonably efficient. For antibiotic 175 and 2725 the efficiencies were 75% and 47% respectively. Both antibiotics require further purification. Yields of 3-5 g. and 8-10 g. of antibiotic 175 and 2725 were obtained respectively from 350 l. batch cultures with corresponding activities of 0.12 and 0.5 µg/ml. against Staphylococcus aureus (Paler).

Antibiotic 175. This antibiotic had the properties of a weak acid. It could be extracted into an organic solvent at an acid pH and into an aqueous solvent at an alkaline pH. A suitable production medium was composed of 1% corn steep liquor plus ½% w/v glucose in a basic salts solution (Appendix 2). The yield was unaffected by the level of inoculum used (Table)4). An inoculum of 0.25% and an incubation temperature of 24°C. favoured antibiotic production. The yield was uaffected by high aeration (Table 5) and by silicone RD antifoam at relatively high concentrations. Polypropylene glycol P2000 antifoam inhibited antibiotic synthesis (Table 6). Optimum yields were obtained in both the 350 l. batch culture and in the shake flask culture within 24 hours at a pH of about 7.5 (see flow-sheet p.98 and Table 7). Glucose was obviously an essential metabolite in the synthesis of the antibiotic as no further increase in activity could be detected after the glucose had been practically used up. Differences in cell count on the large and small scale cultures were probably due to differences in the level of inoculum used, 0.25 and 5% respectively. Such differences in cell count could not be implicated as being the cause of the differences in optimum yield as yield seemed to be a function of glucose concentration and , in addition to this, optimum yield did not coincide with optimum cell count.

The fluctuations in total nitrogen were insignificant and what changes that were observed could be attributed to sample variation.

The antibiotic was stable over a fairly wide range of pH (pH 5-9) both at the production medium stage and as the product at temperatures up to 37°C. Because no differences in stability of the product and the antibiotic in its production medium at temperatures up to 37°C. were apparent, the results were compiled into one table, Table 1. At the extremes of pH studied there were losses of activity. There was progressive loss, yet not total loss, even after 72 hours at pH 2 and 11.

Since there was no complete loss of activity under any of the conditions used in the compilation of the data shown in Table 1, suggests the existence of more than one biologically active component in antibiotic 175 or, alternatively, one antibiotic with more than one active centre. Each component or active centre having a different stability with respect to pH.

At 100°C. antibiotic 175 was stable at the neutral pH for between two and four hours. At pH 2 and 10 there was more rapid loss, particularly at the acid pH (Table 2).

The antibiotic appeared to be completely stable under the conditions used in the extraction and partial purification. The culture could be clarified by centrifugation at the harvest pH without loss of activity as the antibiotic was found to be extracellular (Table 8).

Sutcliffe and Speakman grade 110 charcoal was found to be the best grade to use because it not only completely adsorbed all the activity in the culture but it also released it to one of the eluting solvents employed as potential eluting agents.

The charcoal filtrate was found to be more acid than the culture supernatant from which it was derived owing to the acid nature of the charcoal, pH 6.5 oompared with pH 8.3.

Absolute ethanol was found to be superior to industrial methylated spirits (IMS) as a component of the eluting solvent because the charcoal recovered by filtration, following adsorption, retained much moisture, so much that elution with even 75 'over' IMS resulted in the development of an emulsion of benzene in alcohol/water. The emulsion was a less efficient eluting agent.

As stated earlier, the method of extraction and partial purification was reasonably efficient (75%). What losses that occurred in the extraction and partial purification were largely apparent losses due to the limitations of the method of assay.

The antibiotic had a fairly wide spectrum (Table 9). It was active against gram negative and gram positive micro-organisms. It was inactive against the organism which produced it, Ps. fluorescens, str. 175, even at relatively high concentrations. In general, gram positives were inhibited by smaller concentrations than gram negatives. There were exceptions: Vibrio cholera and Bacillus thuringiensis.

The antibiotic was inactivated by serum (Table 10). Concentrations of 50% and 75% serum rendered the antibiotic inactive against St. aureus (Paler). It was found to be haemolytic at a dilution of 1 in 1,600 and 1 in 18,000 at 3° and 37°C. respectively (Table 11). Activity was affected by 100% by a 10^3 change in the level of inoculum. (Table 12).

The antibiotic was bactericidal to both St. aureus (Paler)

and E. coli (MRE 600)(Tables 13 and 14). At concentrations up to 50 µg/ml. there was an increase in viability during the first eight hours of incubation and then a progressive loss thereafter. At concentrations of 100 and 200 µg/ml. there was an insignificant increase in viability.

Fuller[199] found that the toxicity of the substance was low. The LD_{50} for mice by the intraperitoneal route was 200 mg/Kg. body weight.

Work is in progress in the Department of Biochemistry, Imperial College, on the further purification of the antibiotic. Barrow[200] has shown by thin-layer chromatography that the substance contains at least five fractions all of which I found to be biologically active. This confirms the suspicions born from the stability studies. I found that the p-brom phenacyl esters of the antibiotic (prepared by Barrow) were inhibited to a greater degree by serum than was the parent product.

The antibiotic may be one of those antimicrobial substances reported to be produced by Ps. fluorescens (p. 16). The substance Lewis described appeared to be the one most likely to be identical with antibiotic 175, except that I found the antibiotic to be active against E. (syn. Bacterium) coli. However, antibiotic 175 appeared to be strain specific with E. coli. Beecham Research Laboratories[201] found the antibiotic active against E. coli type 61 but not against types 2 and 37. Lewis found the antibiotic he studied to be bacteriostatic, a further point of difference to antibiotic 175. The antibiotic produced by Pseudomonas str. 31 reported by Kiprianova et al (1969)[13], like antibiotic 175, is an acidic substance but differs in that it is inactive against gram negative bacteria.

Antibiotic 2725. This antibiotic was assumed to be a peptide on the basis of the amino acid composition (Table 29), the fact that it could be precipitated by ammonium sulphate and by picric acid. On the basis of the electrophoretic mobility of the antibiotic it was found to be a basic peptide with a molecular weight of less than 10^4 as it could pass through a dialysis membrane. It contained no sulphur.

A suitable medium comprised ½% glycerol, 1% soya flour and ½% fish meal in a basic salts solution. The best incubation temperature was found to be 30°C. and the antibiotic yield independent of inoculum (Table 18). Rapid aeration of the production medium culture did not affect the yield. There was insufficient difference in titre between the two highly aerated flasks and the two poorly aerated flasks to suggest this.

Silicone RD antifoam did not affect the yield of antibiotic 2725 (Table 20), however, polypropylene glycol P2000 did, although not to the extent of suppressing antibiotic activity completely as it did with antibiotic 175 (Table 6).

The optimum yield of antibiotic in the 350 l. batch culture was somewhat lower than it was on the shake flask scale. Such differences were probably due to the smaller aeration efficiency in the shake flask culture. The differences could not be attributed to differences in cell count because such differences were quite small despite the levels of inoculum being widely different (0.25% and 5% on the 350 l. batch culture and shake flask scale respectively, flow-sheet page 114 and Table 21). Optimum yield was achieved within 24-30 hours at a pH between 6 and 7.

The fluctuations in total nitrogen were negligible and continuation of the fermentation up to 48 hours assisted the

clarification of the acid culture supernatant. The antibiotic was intracellular and released only on acidification of the mature culture to pH 3 (Table 22).

No noticeable differences were observed between the stability of the antibiotic in production medium and as the final product. Also no differences were noted at temperatures up to 37°C. At no stage in the fermentation did the pH reach an unfavourable level. At the extremes of the pH range selected there was loss of activity; at pH 2 some loss occurred between 48 and 72 hours whilst at pH 10 and 11 similar losses were observed between 24 and 48 hours (Table 15). Loss of activity was more pronounced at 100°C., especially at pH 2 and 10.

In the preliminary extraction of the antibiotic with Sutcliffe and Speakman 339 charcoal the pH of the charcoal filtrate was less acid than the supernatant from which it was derived (pH 6 cf. pH 3). This change was probably due to the neutral property of the charcoal.

Industrial methylated spirits sufficed as a component of the IMS/benzene plus 1% conc. HCl eluting solvent employed.

The antibiotic had a wide antimicrobial spectrum (Table 23). It did not seem to be predomonantly active against gram negative or gram positive bacteria. It was active against the organism which produced it, B. licheniformis, str. 2725. at 25 µg/ml.

Not only was the antibiotic unaffected by serum its activity against St. aureus (Paler) was in fact enhanced in the presence of 5% serum and above (Table 24).

The antibiotic caused only partial haemolysis at a

concentration of 1 in 400 at 37°C. and failed to haemolyse at 1 in 200 at 3°C. after 18 hours. (Table 25).

Like most peptide antibiotics it was found to be toxic. Beecham Research Laboratories[201] found that the LD_{50} for mice by the intravenous route was 4 mg/Kg. body weight. This high toxicity may, however, be due to impurities in the product.

The inoculum altered the activity of the antibiotic by 100% with a 1000-fold change in cell numbers.

The antibiotic was bactericidal to both St. aureus (Paler) and E. coli (MRE 600). With St. aureus, but not E. coli, there was an increase in viability at concentrations up to 50 µg/ml. during the first eight hours of incubation. At 5 µg/ml. the viability of both cultures increased during the first 24 hours. No real differences were seen in the viability of the two organisms at 100 and 200 µg/ml. and at any concentration after 24 hours incubation.

The antibiotic contained no unusual amino acids. It was not ascertained whether the antibiotic possessed any 'unnatural' stereo-isomers. Arginine and lysine accounted for over half the total amino acid composition of the antibiotic preparation. Is the antibiotic a known one? Some of the amino acids shown in Table 29 may be derived from peptide impurities in the product. Even if the antibiotic contains the same amino acids as one of the known peptide antibiotics the sequence may be different and/or their stereo-isomeric configuration. One group of antibiotics, the licheniformins, have been isolated from a strain of Bacillus licheniformis[14]. These contained an amino acid not found in antibiotic 2725 namely proline, although like the preparation I obtained, they contained a high proportion of arginine and lysine. A feature of similarity is that the licheniformins are not affected by 50% serum.

Bacitracin (syn ayfivin) has been isolated from a strain of B. licheniformis[14]. Bacitracin A is a polypeptide antibiotic but differs from antibiotic 2725 in that it contains ornithine.

Antibiotic 2725 differs from other peptide antibiotics, except the tyrocidines, in that it exhibits fair activity against gram negative bacteria as well as positive bacteria, but differs from tyrocidine in that it is bactericidal and non-haemolytic.

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APPENDIX 1

Data Concerning Some of the Large-Scale Equipment Used.

Sharples Centrifuge. Type MV 12. Speed 15 x 10^3 rpm., set for liquid/solid separation at a rate of 50 l./ hr. Manufactured by: Sharples Centrifuges Ltd., Tower Works, Boman Road, Camberly, Surrey.

Calmic Filter. Type E. Heavy duty filter press, maximum working pressure of 30 p.s.i.g. Manufactured by: Calmic Engineering Co. Ltd., Crewe, Cheshire.

Portable Vessels (Used in solvent elution of charcoal). Jacketed 1.75' I.D., depth 1.75'. Working volume maximum 112 l. Manufactured by Stainless Steel Vessels Ltd., London.

Portable Vessel Agitator. Type II 4 blade 7" dia. impellor, speed 400 rpm. Manufactured by: Osbourne and Co. Ltd., Croydon, Surrey.

Basket Filter (Special) 50 cm. depth. Manufactured by Calmic Engineering Co. Ltd.

Climbing Film Evaporator. CFE Type 1. (Standard). 3/8" QVF units. 10' x 1' bore vertical calandra. The modifications were:

a) 1 of 20 l. and 1 of 50 l. condensate receiver to replace standard 5 l. receiver.
b) Increased cooling area i.e. condenser surface area for handling through puts of 350 lb/hr. of chloroform at 26" Hg and 140 lb/hr. of butyl acetate at 26" Hg.
c) A small additional condenser and receiver to minimize solvent loss, situated on vacuum line between the pump and solvent receivers.
d) A vacuum pump. Edwards ES 35, now replaced by a Leybold gas ballast pump Mark 62.

Manufactured by QVF Ltd., Duke St., Fenton, Stoke-on-Trent, Staffordshire.

Apex Evaporator. This has a stainless steel evaporator/crystallizer

body with an approximate maximum loading volume of 30 l. (total capacity 60 l.) with a mild steel jacket. The impellor (in the evaporator vessel) has 4 blades each carrying three teflon scrapers which ensure the heat transfer surface remains clean. The vacuum is provided by an Edwards Type ES 35 pump. There is a stainless steel condenser which recovers solvent at the rate of 15 l./hr. under the conditions used.
Manufactured by Apex Construction Ltd., Soho Square, London, W.1.

APPENDIX II

Composition of the Basic Salts Solution used in the production medium of the Preliminary Small-Scale Work.

For 5 l.

$MgSO_4,7H_2O$	1.875 g.
KH_2PO_4	2.000 g.
Na_2HPO_4	3.250 g.
KCl	2.500 g.
$MnCl_2 4H_2O$	5.0 ml. of a 0.3% solution.
Tap Water to	5.0 l.

As it is more economical to employ tap water in the preparation of large volumes of culture medium and in order to create as far as possible a standard culture medium, tap water was used in preference to distilled water in the basic salts solution.