

STUDIES ON ANTIBIOTICS.

A THESIS

submitted to the  
University of Travancore,

by

R.Raghunandana Rao, M.Sc.,

for the degree of

DOCTOR OF PHILOSOPHY.

Department of Biochemistry,  
Indian Institute of Science,  
Bangalore, 1949.

## PREFACE.

The work embodied in this thesis, was carried out by the author in the department of Biochemistry and Section of Pharmacology of the Indian Institute of Science, Bangalore, during November 1945 to October 1948.

In Part I of the thesis are described the results of investigations carried out by the author on two antibiotics "Pterygospermin" and "Allicin", the first isolated from Moringa pterygosperma and the second from Allium sativum. The medicinal properties of the plants studied have been referred to in several old Sanskrit works on medicinal plants. No systematic investigations, either on the biochemistry or pharmacology of the drugs have however been undertaken so far. Attempts have also been not made to isolate the active agents in them. The author in an attempt to prepare, highly active antibiotics from plant sources, has shown the possibility of utilising many Indian plants for the cure of diseases like those caused by M.tuberculosis. In Part II of the thesis are embodied the results of investigations aimed at evolving media for streptomycin production and the factors responsible for the observed high antibiotic formation.

Most of the work has been carried out by the author independently. Whereever any help has been taken (even of very minor nature in carrying out the experiments, or for discussing the results) due acknowledgement has been made in the publications of the author, a list of which is appended for reference. The

author however wishes to express his sincere thanks to Dr.N.W.De for the help rendered in the investigations on the Pharmacology of the two plant antibiotics, to Dr.P.R.Venkataraman, for the many suggestions and discussions which the author had with him during the early stages of the work, and to Dr.K.M.Pandalai for his interest, and to the Merck and Co.,for a standardised streptomycin sample. For the general supervision and guidance the author expresses his sincere gratitude to Prof.V.Subrahmanyam,D.Sc., F.R.I.C.,F.N.I. The author also acknowledges with gratitude the financial support of the C.S.I.R.(India).

The results embodied in the present thesis have not been used in any form by the author for any other degree. To his knowledge the results have also been not used by any of his associates.



CONTENTS.

	<u>Pages</u>
General Introduction           ...   ...   ...	1 - 14
Part I.   Studies on Plant Antibiotics   ...	15 - 120
Introduction           ...   ...   ...	15 - 18
Chapter I -Studies on pterygospermin - isolation and antibacterial properties           ...   ...	19 - 54
Chapter II-Toxicological, Pharmacological and Chemotherapeutic studies on pterygospermin   ...	55 - 74
Chapter III-Studies on the antibacterial principle of <u>Allium sativum</u> Preparation and antimicrobial properties           ...   ...	75 - 106
Chapter IV-Toxicological, and Pharmacolo- gical studies on <u>Allicin</u> .	107 - 120
Part II.   Studies on <u>Streptomycin</u> ...   ...	121 - 176
Introduction           ...   ...   ...	121 - 130
Chapter I -Utilization of <u>wheat bran</u> for streptomycin production.	131 - 145
Chapter II-Factors in wheat bran extract responsible for stimulating streptomycin formation.	146 - 154
Chapter III-Studies on the mineral requirements of <u>S. griseus</u> for streptomycin produc- tion           ...   ...   ...	155 - 165
Chapter IV-Studies on the utilization of <u>groundnut cake</u> for streptomycin production.	166 - 175
Appendix.	



GENERAL INTRODUCTION.

## GENERAL INTRODUCTION.

Antibiotic substances, are a class of naturally occurring compound having the property of inhibiting the growth of micro-organisms, which have come into prominence during recent years. The term originally used to designate chemical substances responsible for the phenomenon of 'microbial antagonism' or 'antibiosis' has however at present been extended to include similar compounds not only of microbial origin but also of plant and even animal origin. Waksman<sup>1</sup> however has suggested that the latter class of substances may be better named as 'antibiotic like'. An antibiotic may however be defined as a naturally occurring chemical compound showing pronounced antibacterial activity and having a rather heterogenous chemical nature.

The phenomenon of antagonism in mixed bacterial cultures has been studied for many years. The first demonstration of antibiotic action was made by Pasteur and Joubert<sup>2</sup> in 1877, who observed that a culture of Anthrax bacillus, in urine, contaminated by some aerial microflora were destroyed. Several similar cases of microbial antagonism have been recorded and all of have been reviewed in great detail by Waksman<sup>3</sup>. The antagonistic effect of one species on another was usually ascribed to the production of acid, to harmful enzyme systems, or to the production of hydrogen peroxide by the inhibiting organism. That organisms could produce specific toxic substances of complex nature was recognised only during recent years such an explanation developing gradually.

The remarkable development in recent years in the field of antibiotics is largely due to several investigations made during the period of 1877 - 1939, the most important of which may be summarised as follows.

1. The remarkable property of certain microbes in repressing or even destroying certain other micro-organisms, including pathogenic saprophytic forms. Mention may be made of the observations of Pasteur already referred to, of Bouchard<sup>4</sup> in 1889 on the protection afforded to rabbits from anthrax by culture fluids of Pseudomonas pyocyanea, of Lode<sup>5</sup> in 1903 on Micrococcus tetragenus and of Nicolle<sup>6</sup> in 1907 and on B. subtilis.

2. Mixed infections were found to behave differently from infections caused by single organisms, some organisms repressing the growth of others. The first attempt to treat infections by the use of less pathogenic micro-organisms was made by Cantani<sup>7</sup> in 1885, who tried to treat tuberculosis with Bacterium termo. One of the best known attempts at the replacement therapy was that suggested by Metchinkoff<sup>8</sup> in 1909 to replace harmful intestinal organisms by the Bulgarian lactobacillus.

3. Certain organisms isolated from the soil, composites, etc., and grown in artificial media were found to produce substances which had the capacity to inhibit growth of micro-organisms. These were later on designated as antibiotics. The earliest in this field was the work on P. pyocyanea notably by Bouchard<sup>4</sup> in 1889, by Honl and Bukovsky<sup>9</sup> in 1899 and the preparation of pyocyanase by



Emmerich and Low<sup>10</sup>. All of these have resulted in the recent isolation of four crystalline compounds from the organism, Pyo I, II, III and IV showing powerful anti-Gram-positive activity, by Hays et al<sup>11</sup> in 1945. Gosio<sup>12</sup> in 1896 was the first to isolate an antibiotic agent from a mould. He isolated a crystalline substance from a Penicillium. This antibiotic has been recently named as mycophenolic acid<sup>13</sup>. The observation which had the most consequence in this field was however, made by Fleming<sup>14</sup> in 1929 when he isolated and studied a chance contaminant (identified later as Penicillium notatum) having remarkable anti-bacterial properties.

The above observations and certain others such as the fact that disease producing organisms finding their way into the soil are destroyed by the soil microflora, may be said to be primarily responsible for the shaping and development of the present era of antibiotics, dating back from 1939. By that time, it became definitely recognised that several microorganisms could produce under suitable conditions, antibacterial substances. Mention may be made of penicillic acid<sup>15</sup>, penicillin and gliotoxin<sup>16</sup> as products of fungal origin, pyocyanase, pyocyanin, etc., as products of bacterial origin, which were known at that time as antibiotics.

Since 1939 however a tremendous amount of work has been carried out in the field of antibiotics which have resulted in the isolation of several antimicrobial agents. The investigations

since 1939 even though were a logical sequence to the prior findings, were however accelerated considerably by two important findings about the year 1939. First the isolation of tyrothricin by Dubos<sup>17,18</sup> and second the remarkable chemotherapeutic properties of penicillin as discovered at Oxford by Florey, Chain and co-workers<sup>19</sup>

The investigations of Dubos, culminating in the fractionation of tyrothricin into tyrocidine and gramicidin were deliberate and aimed at exploiting the phenomenon of microbial antagonism. Dubos' view point was that "the addition of living cultures of Gram-positive cocci would result in the development of a selective flora capable of attacking the living cells of these bacterial species". Experimenting on these lines he isolated the now well known antagonist Bacillus brevis and the antibiotic agents responsible for the antagonistic properties of the organisms.

As Oxford<sup>20</sup> has recently pointed out, moulds do not usually compete with bacteria but only with each other in nature. Consequently efforts at the production of antibacterial agents by moulds if successful must be in the nature of happy accidents, should the substance isolated have a very selective bacteriostatic action, without no general toxic action. The most famous of all these accidents so far is the discovery of the chemotherapeutic usefulness of penicillin a mould product, against bacterial infections.

The above facts gave a great stimulus for research work on similar lines and several planned investigations have been carried out aimed at the isolation of naturally occurring antibacterial agents from bacteria, fungi and actinomycetes. During



the ten years following the discovery of the chemotherapeutic importance of penicillin, about a hundred new antibiotic agents from different sources have been isolated and studied. Antibiotics have thus been isolated from bacteria, fungi, actinomycetes, algae, protozoa and animals. It is not attempted here to give an exhaustive list of all of them, but mention may be made of a few typical and important ones.

Thus from bacteria have been isolated besides the well known tyrocidine and gramicidin; bacitracin<sup>21</sup>, subtilin<sup>22</sup>, aerosporin<sup>23</sup> polymyxin<sup>24</sup>, and nisin<sup>25</sup>, all of which might attain chemotherapeutic status. Aerosporin<sup>23</sup> appears to be identical with polymyxin. It affords protection to animals, against several infections and is fairly non-toxic. Subtilin and bacitracin have also been found to be active in vivo.

Besides streptomycin several products have been isolated from actinomycetes. Mention may be made of Lavendulin<sup>26</sup>, Actinorubin<sup>27</sup> and streptolin<sup>28</sup>. Antiviral agents like chloromycetin<sup>29</sup>, and Aureomycin<sup>30</sup> have also been isolated. Neomycin<sup>31</sup>, an antibiotic active against streptomycin-resistant organisms has also been recently reported.

One of the most outstanding properties of the antibiotics is their selective action on bacteria and other micro-organisms, some active essentially on Gram-positive organisms, others on both Gram-positive and Gram-negative organisms and some on fungi. Antibiotics vary greatly in physical and chemical nature. Several



antibiotics are produced by a single ~~exga~~ micro-organisms and several micro-organisms may some times produce a single antibiotic.<sup>32</sup> Antibiotics also differ greatly in their toxicity, bacterial resistance, and stability in presence of tissue fluids, all of which make the chemotherapeutic efficacy of the drugs widely variant. Thus although more than a hundred antibiotics have now been described only very few of them have so far been found chemotherapeutically efficient.

The facts that many of the antibiotics of microbial origin, were not useful in vivo and also the need for agents effective in penicillin and streptomycin resistant bacterial infections have necessitated a wider serarch for similar antibiotic agents. One such approach was that of tapping the higher plants. Plants have been endowed with great therapeutic virtues from times ~~immadi~~ immemorial. After the advent of microbial antibiotics a search has been made for antibiotic agents in plants, notably by Osborne<sup>33</sup>, Pederson<sup>34</sup>, Atkinson<sup>35</sup> and Cavallito<sup>36</sup>. Even though several plants have been examined only very few of them have been studied and very few plant antibiotics isolated.

A good deal of useful information has accumulated on the investigations on the mechanism of antibiotic action. Most of the investigations appear to be either on penicillin or streptomycin. Gale et al<sup>37</sup> have found that penicillin hibits the assimilation of glutamic acid by certain pathogens. Frieden and Frasier<sup>38</sup> report that among a number of substances studied only magnesium and phosphorus affect the sensitivity of S.aureus to penicillin.



(phosphorus in the form of phosphate). Pyridoxine, but not pyridoxal or pyridoxamine acts as a penicillin antagonist. Duffrenoy and Pratt<sup>39</sup> have reported on the cytochemical mechanisms of penicillin action. Nucleic acids have been claimed to antagonise penicillin action.<sup>40</sup> Several similar observations have been reported. All of these have been summarised recently by Bailey<sup>41</sup>. The present status of the knowledge on the mode of action of antibiotics is however nebulous. Many theories have also been suggested to explain the mode of action of antibiotic substances. Mention may be made of

a) Antibiotics affect the cell by interfering with the utilisation of one of the intermediary metabolic products. Thus Gale et al<sup>37</sup> have shown that amino acid metabolism of S.aureus is affected in the presence of penicillin and Geiger<sup>42</sup> has made similar observation on E.coli in presence of streptomycin.

b) Antibiotics interfere with the sulph-hydryl enzymes of the bacterial cells, thus affecting metabolic processes.<sup>43</sup>

c) Antibiotics may act as detergents as is the case with gramicidin<sup>44</sup>.

Two of the important problems that have come up as a result of the studies on the different aspects of antibiotics are the problem of drug resistance and that of synergism. The frequency with which resistant organism are isolated from patients undergoing treatment with streptomycin, (the frequency being much less in the case of penicillin) have been an important limiting factor in its application. Two theories have been suggested to explain



the problem of resistance. One attempts to explain their occurrence as an effect of the antibiotic on the bacterium, resulting in a metabolism so altered that the organism grows in inhibitory concentrations. The other one proposed by Demerac<sup>45</sup> and Luria<sup>46</sup> is the genetic theory, which assumes the existence of highly resistant organisms, in the parent culture. A vast amount of literature has accumulated on the problem of drug resistance. It appears that resistance is widely distributed among all bacteria and is most pronounced in streptomycin. Great changes accompanying resistance have also been reported, particularly with respect to the metabolism of the organism. Nothing however, regarding the exact mechanism of development of resistance, nor regarding methods to overcome it particularly in vivo can be said.

Synergism of two antibacterial agents has been gaining more and more importance and several instances of synergism of penicillin and streptomycin with other antibacterial agents have been recorded.<sup>47-50</sup> Synergism has also been suggested as a method for overcoming drug resistance<sup>51</sup>. Two hypothesis may explain the synergism of bacteriostatic agents.

1) Critical reactions in bacterial metabolism may proceed along alternative pathways. Theoretically at least, inhibition of one pathway may not suffice for bacteriostasis, whereas multiple inhibition may suffice.

ii) Resistance of bacterial cells to an inhibiting drug may follow a normal frequency distribution. Reduction of the

ePrint@CFTRI



bacterial population by one drug, correspondingly decreases the probability of the residual population containing a cell of resistance to another drug sufficiently high to permit growth. Thus Klein and Kimmelman<sup>52</sup> by the synergistic action of penicillin, streptomycin and sulphadiazene have suppressed in vitro the emergents of resistance to all the three.

It may be pointed out that the first of the above explanations seems to indicate that synergism implies difference in the mode of action; however, lack of synergism does not imply similarity in the mode of action.

Several other new and interesting problems have also arisen in connection with the study of antibiotics. Thus the effect of components in media on antibacterial activity of antibiotics, the in vivo inactivity of some antibiotics, the development of resistance already referred to, are a few of them which have to be fully explained. Great progress has been made in the nutritional requirements of microorganisms for increased antibiotic production. Thus the role of trace elements in penicillin formation<sup>53</sup>, manganese in polymixin formation<sup>54</sup> and the role of several amino acids have been pointed out<sup>55,56</sup>. Investigations on the isolation of new strains of penicillin producing organisms have also yielded fruitful results, the yield of penicillin going upto 1,000 units per ml. from about 2 units per ml.<sup>57</sup>

It is really difficult to say anything regarding the future of antibiotics. The fact that infectious organisms could be controlled by the use of antibiotic therapy opens up a new field of medical



research which undoubtedly will bring the a brighter future for the ailing millions and the future of the antibiotic field of research may be an altogether hopeful one. When we take into consideration the fact that only very few of the micro-organisms, plants, and other natural sources have so far been tried as potential sources of antibiotics we are apt to feel that future research might bring forth antibiotics effective in all human ailments, and ideal in being devoid of the short comings of penicillin and streptomycin. The fact that micro-organisms produce different antibiotics in different media, may lend additional support to this view. On the other hand, it must be observed that of the several micro-organisms so far studied (numbering over 25,000 in Waksman's laboratory alone) only very few have been found to be antibiotic producers and of these only streptomycin and penicillin have reached recognised status in chemotherapy, we are apt to be a bit pessimistic regarding the future of antibiotics. It must however be pointed out that there is at present no recognised method for finding which micro-organism is likely to give the most useful antibiotic. It is therefore not improbable that at a later date the percentage of antibiotics attaining chemotherapeutic success may be higher. As Waksman has recently pointed out<sup>58</sup> the future research on antibiotics concentrated on the following lines might give fruitful information.

- 1) Isolation of antibiotics more potent than those known at present, but without their short comings.

ii) Isolation of antibiotics capable of exerting a synergistic effect when combined with other known antibiotics thus affording a means of overcoming drug resistance.

iii) Studies on the mode of action of antibiotic substances.

iv) A knowledge of the mechanism of development of resistance and the problem of overcoming such resistance.

v) Investigations aimed at the isolation of antibiotics active against filterable viruses and against abnormal body cells like tumours.

The search for new antibiotics must therefore continue. Perhaps only a very few will prove to be of use in chemotherapy. A better knowledge of the physiological and biochemical mechanisms adopted by various bacteria susceptible to antibiotics might help to clarify the still as obscure aspects of the mode of action of the antibiotics. The prospects for antibiotics appears to be promising and the future bright.



References.

1. Waksman S.A., (1948) *Biological reviews* 23, 452.
2. Pasteur & Joubert, (1877) *C.R.Acad.Sci.Paris*, 85, 101.
3. Waksman S.A., (1941) *Bact.Reviews*, 5, 231.
4. Bouchard C., (1889) *C.R.Acad.Sci.Paris*, 108, 713.
5. Lode A., (1903) *Zbl.Bakt.I.Abst.Original*, 33, 196.
6. Nicolle M., (1907) *Ann.Inst.Pasteur*, 21, 613.
7. Cantani A., (1885) *G.Int.Sci.Med.*, n.s.7, 493.
8. Mitchinkoff E., (1909) *Medicaments.Micrabiens.*, Paris 5.
9. Honl J., & Bukovsky J., (1899) *Zbl.Bakt.I.Abst.*, 26, 305.
10. Emmerich R., & Low O., (1899) *Z.Hyg.Infekt.Kr.*, 31, 1.
11. Hays E.E., et al (1945) *J.Biol.Chem.*, 159, 725.
12. Gosio B., (1896) *Rev.Igiene.Sanit.publ.*, 7, 825.
13. Florey, H.W., et al (1946) *Lancet*, 1, 46.
14. Fleming A., (1929) *Brit.J.Expt.Path.*, 10, 226.
15. Alsberg C.L., & Black, O.P., (1911-12) *Biochem.Balp.N.Y.* 1, 103.
16. Vaudremer A. (1913) *C.R.Soc.Biol.Paris*, 74, 278, 752.
17. Dubos, R.J. (1939) *J.Exptl.Med.*, 70, (1), 11.
18. Hotchkiss R.D., (1944) *Advances in enzymology* 4, 153.
19. Chain et al (1940) *Lancet*, (2), 226.
20. Oxford A.E., (1945) *Ann.Rev., Biochem.*
21. Johnson B.A., et al. (1945) *Science*, 102, 376.
22. Jansen E.F., & Hirschmann D.J., (1944) *Arch.Biochem*, 4, 297.
23. Ainsworth G.C. et al (1947) *Nature*, 160, 263.
24. Stansley P.G., & Schlosser M., (1947) *J.Bact*, 54, 549.
25. Mattick T.R., & Hirsch A., (1947) *Lancet*, 5th July, 5.
26. Junowieg-Kocholaty, H.E., et al. (1947) *J.Biol.Chem.*, 168, 757.

27. Kelner A. et al (1946) J.Bact.51, 591.
28. Rivett R.W., & Peterson W.H. (1947) J.Amer.Chem.Soc.69, 3006.
29. Ehrlich J., et al (1947) Science, 106, 417.
30. Broschord R.W., et al (1949) Science, 109, 199.
31. Waksman S.A., et al (1949) Science, 109, 305.
32. Carlson H.J., et al (1946) J.Bact., 52, 155.
33. Osborne E.M., (1943) Brit.J.Exptl.Path,24, 227.
34. Pederson C.S., & Fisher P., (1944) J.Bact., 47, 421.
35. Atkinson N. & Rainsford K.M., (1946) Austra.J.Expt.Biol & Med. Sci.24, 49.
36. Cavallito & Bailey (1944) J.Amer.Chem.Soc., 66, 1950.
37. Gale E.F., & Taylor E., (1946) Nature, 158, 676.
38. Frieden & Frasier (1947) Arch.Biochem., 15, 265.
39. Duffrenoy & Pratt (1947-48) J.Bact., 53, 657; 54, 127, 719; 55, 75.
40. Pandalai K.M., & George M., (1947) Brit.Med.J. II, 210.
41. Bailey J.H. and Cavallito C.J. (1948) Annual Rev.Microbiol.2, 143.
42. Geiger W.B. (1947) Arch.Biochem., 15, 227.
43. Cavallito C.J. (1946) J.Biol.Chem. 164, 29.
44. Hotchkiss R.D. & Dubos R.J. (1941) J.Biol.Chem. 141, 155.
45. Demerec M., (1945) Proc.Natl.Acad.Sci., U.S., 31, 16.
46. Luria, S.E., (1947) Bact.Rev., 11, 1.
47. Ungar J., (1943) Nature, 152, 245.
48. Bigger J.W., (1944) Lancet, 247, 142.
49. Hobby G.L., & Dawson M.H., (1946) J.Bact., 51, 447.
50. McSweeney, (1946) Lancet (2), 114.

51. Klein M., &  
Kimmelman L.J., (1946) J.Bact.,52, 245.
52. Klein M., &  
Kimmelman L.J., (1947) J.Bact.,54, 363.
53. Koffler H., Knight S.G.,  
& Frazier W.C., (1947) J.Bact.,53, 115.
- 54.
55. Woodruff H.B., &  
Ruger M., (1948) J.Bact.,56, 315.
56. Cook et al., (1947) Nature, 159, 376.
57. Raper K., (1946) Annl.New York, Acad.Sci.,48, 41.
58. Waksman S.A., (1947) 4th Internat.Congr.Microbiol.83.



PART - I.

XXXXXXXXXX

INTRODUCTION.



## INTRODUCTION.

The development in the field of antibiotics from micro-organisms resulting in the isolation of antibiotics like penicillin, and streptomycin have been summarised previously. When compared with the vast number of microbes thus studied, and the number of antibiotics isolated, the compounds found applicable in bacterial disease processes, appear to be only few in number. The search for antibiotics - naturally occurring antibacterial agents - has been hence extended to higher plants, in the hope of finding antibacterial agents which might offer chemotherapeutic application.

Plants have been, from the beginning of history and civilisation used as curatives for several diseases particularly in country<sup>ies</sup> like India, where several systems of medicine existed which made use of plants and plant products. Lists of Indian medicinal plants have been given in detail by Kirthikar and Basu<sup>1</sup> and Chopra<sup>2</sup>. While stressing on the need for systematic and detailed investigations on plants ~~antibiotics~~ they also suggest the difficulties encountered in such work in this country and also seem to think that identification of some plants is difficult.

A new impetus for research work on plants was given as a result of the investigations on micro-organisms leading to the isolation of penicillin and streptomycin. Mention has already been made of some of the attempts made to study the antibiotic contents of plants (refer page ). However, only very few plant antibiotics have been studied in detail. Crystalline compounds have been obtained

from Crepis taraxacifolia, named Crepin by Heatly<sup>3</sup> which is too toxic for in vivo experiments. The active principle of buttercups named protoanemonin by Baer et al<sup>4</sup>, even though active against a wide variety of organisms is highly toxic. Several other plant products have also been isolated and studied. Cavallito et al<sup>5</sup> isolated from Arctium minus an unsaturated lactone with weak antibacterial activity. Tomatin<sup>6,7</sup> the antibiotic principle isolated from tomato plant seems to possess antifungal properties. Puchin<sup>8</sup> is the name given by the Chinese authors to the antibiotic isolated from E.tuberosa which inhibited the growth of Gram-positive bacteria. Raphinin<sup>9</sup> is isolated from radish which appears to be highly toxic.

In view of the importance attached to Indian plants by the indigenous systems, it seem necessary to carry out investigations to find the antibiotic contents of different plants ~~expected~~ reputed to possess antibacterial properties. In the course of such a systematic survey carried out in these laboratories about 200 plants have been studied<sup>10,11</sup>. The results indicate the presence of antibiotic principles in many of them, even though all of the claims of indigenous systems could not be substantiated. Detail investigations have been carried out on a number of plants, and several agents like Caricin from Carica papaya (seeds), morellin from Garcinia morella<sup>12</sup> have been isolated which show antibacterial activities at dilutions of about 1 in 20,000 to 1 in 100,000. Similar investigations have also been carried out on two antibiotics (which appeared promising) viz., pterygospermin from Moringa pterygosperma (roots) and allidin from Allium sativum. The results of the investigations are presented in the following pages.



It may however be mentioned that pterygospermin appears to be the most active plant product isolated so far and combines high antibacterial properties and low toxicity.

Whether antibiotics from plants if found useful, can compete with antibiotics of microbial origin, economically cannot be said at present. The production of microbial products is less time consuming, since the micro-organisms are able to produce the antibiotic agents in a short time depending upon the media in which they are grown. The availability of plants or plant materials for isolation of antibiotics, is definitely much more difficult. However, the simpler chemical nature of the plant products could be interpreted as indicating that synthetic antibacterial agents identical or similar to plant products might be obtained without much difficulty or caused. Perhaps synthetic compounds having more than one "antibacterial group" might replace at least some of the present day antibiotics. The search for antibiotics in plants must hence continue in the hope that plants should also contain ideal antibiotics, similar to those existing in culture fluids of micro-organisms.

References

1. Kirthikar & Basu, (1933) Ind.Med.Plants Vol.I to VI.
2. Chopra R.N., (1933) Indigenous drugs of India.
3. Heatly N.G., (1944) Brit.J.Exptl.Path.25, 208.
5. Cavallito, C.J.,et al (1945) J.Amer.Chem.Soc.,67, 948.
4. Baer,H., Holden,M.,  
& Seegal,B.C., (1946) J.Biol.Chem. 162, 65.
6. Irving,G.W.,Jr., Fontaine,T.D.,  
& Doolittle,S.P., (1946) J.Bact.,52, 601.
7. Fontaine.,T.D.,Irving,G.W.Jr.,  
& Doolittle,S.P., (1946) Arch.Biochem.
8. Chen,S.L.,Chen,B.L.,Chen,W.K.,  
& Tang,P.S., (1945) Nature, 156, 234.
9. Ivanovicz et al (1947) Proc.Soc.Exptl.Biol.& Med,66, 625.
10. George,M.,Venkataraman,P.R.  
& Pandalai,K.M., (1947) J.Sci.& Ind.Research.6 B, 43.
11. George,M.,Venkataraman,P.R.  
& Pandalai,K.M., (1949) Ind.J.Med.Research(under publication)
12. Natarajan et al (1949) Current Science(under publication).



CHAPTER - I.

STUDIES ON PTERYGOSPERMIN - THE ANTIBIOTIC PRINCIPLE  
OF MORINGA PTERYGOSPERMA GAERTN.

Isolation and Antimicrobial properties.

Introduction.

Pterygospermin is the tentative name given to the antibiotic principle isolated from the roots of Moringa pterygosperma (drumstick). This is a fairly large and pretty deciduous tree. It belongs to the natural order Moringaceae and is very common almost in every part of India and Burma. Its bark is thick corky and grey with longitudinal cracks. The roots are pungent. The medicinal virtues of this plant have been long known in this country. It has been frequently mentioned in the 'Bhavaprakasa' and in other Sanskrit works on medicine. Almost all parts of the plant like root, leaves, seeds, and flowers have been used in the treatment of various ailments in the indigenous system.

The decoction of the root bark is recommended in inflammation abscess and calculous affections. The root of the young tree is still prescribed by the indigenous practitioners in the intermittent fever, epilepsy, hysteria, dyspepsia, etc. The root has also been used by 'hakims' in the treatment of soreness of the mouth, and throat, and pain in the gum. Several similar uses have been attributed to the other parts of the tree as well<sup>1</sup>.

Even though, the foregoing account indicates that the different parts of the tree contain medicinally useful compounds, none of the above claims seem to have been so far substantiated.



During the course of an exhaustive search for antibiotics in locally available plant materials, it was found that different parts of the tree Moringa pterygosperma showed considerable antibacterial activity<sup>2</sup>. Search in literature revealed that the only previous work done on this plant has been the isolation of two alkaloids from its bark by Chopra and co-workers<sup>3,4</sup>. The alkaloids, however, did not show any antibacterial properties. Isolation of the antibacterial principle from the root, and investigations on the nature of the antibiotic, its antibacterial properties, toxicity and chemotherapeutic efficacy, were so carried out.

#### Experimental.

Assay Method. The cup-plate method now well known and widely used for assaying several antibiotics was employed during these studies. For this the procedure recommended by Foster and Woodruff<sup>5</sup> was followed.

Test Organism. Any organism sensitive to the antibiotic can be used for the assaying. For these investigations, however, a strain of Bacillus subtilis, sensitive to the antibacterial principle of drumstick root was used. The use of this organism afforded several advantages over the use of Staphylococcus aureus, which is perhaps more widely used in routine assays. There was no appreciable difference in the 16 hours and 48 hours halo-diameters. Since a spore suspension of B. subtilis prepared as described below was used as the inoculum, the day to day preparation of the same was eliminated. The temperature of the agar at the time of seeding could be as high as 55 to 60°C. B. subtilis is non-pathogenic. The temperature of incubation was 37°C.

Inoculum. For the preparation of the inoculum, it consisted of a spore suspension of B.subtilis, nutrient broth of the following composition was used as medium.

Peptone	...	10.0 gms.
Beef extract (Difco)	...	5.0 "
Sodium chloride	...	5.0 "
Distilled water	...	1000.0 ml.
pH adjusted to $6.8 \pm 0.1$		

100 ml amounts of the above broth were distributed in 250 ml flasks plugged and sterilised by autoclaving at a pressure of 15 lbs. for 15 minutes. The contents of the flask were then inoculated with a standard loopful of the test organism, and they were incubated at 37°C. In order to prevent the formation of surface pellicles during growth Foster and Woodruff have recommended either aeration or shaking of the flasks mildly at intervals of about 15 to 30 minutes. The latter method was employed for the preparation of the spore suspension. At the end of 8 to 10 days when there was maximum sporulation the incubation was stopped and the flasks now containing the spore suspension of B.subtilis were pasteurised.

Pasteurisation for rendering most of the remaining vegetative cells non-viable, was carried out by incubating the flasks at a temperature of about 60-70°C. for about 30 to 40 minutes. During the assay about 500,000 spores were used for each petri-dish containing 20 ml of agar.



Preparing the plates. 20 ml of sterile nutrient agar seeded as indicated above was poured into each petri dish of size 100 x 25 mm. The dishes were then cooled, agar solidified and then used for assaying. The cups had an inner diameter of about 7 mm. and outer diameter of about 9 mm., the height being 10-12 mm. Before fixing the cups were kept for about 10 to 15 minutes over a hot plate, the temperature of which was approximately 45°C. The fixing of the cups and pouring of the fluids was carried out as described by Foster and Woodruff<sup>5</sup>.

At the end of about 18 hours incubation, the petri dishes were removed from the incubator, the cups removed from the petri dishes and they were washed to remove the surface growth of the test organism, care being taken not to disturb the agar in any way. The diameters of the clear haloes were then read as suggested by Foster and Woodruff<sup>5</sup>.

Unless otherwise stated assays in quadruplicates were always carried out and the mean values recorded.

Preliminary studies. It has already been stated that many parts of the plant showed antibiotic activity. Preliminary experiments were therefore carried out to ascertain whether the antibacterial activity of the different parts is due to same or different antibiotic principle. For this, the antibiotic activity of the alcoholic extracts of the different plant parts was estimated by the cylinder-plate method against three organisms, S.aureus, B.coli, and B.subtilis. The B.coli:S.aureus ratio of the activity of the different plants was taken as the criterion

to judge the nature of the antibiotic in them, difference in ratio indicating difference in the antibiotic principle also. In table I below the results of the experiments are brought out.

Table I.

Antibiotic activity of the alcoholic extracts of the different parts of Moringa pterygosperma.

Name of plant part	Dilution of the extract	Halo diameter in mm. against			Ratio of 1:3
		S.aureus 1	B.subtilis 2	B.coli 3	
Root	1/2	33.5	33.5	21.0	1.6
Stem	1/2	33.0	33.0	21.0	1.6
Leaves	1/2	22.0	22.0	32.5	0.7
Flowers	1/2	21.5	22.0	28.5	0.8

The antibacterial properties exhibited by the root and stem appear to be due mostly to same substance which has practically the same activity against Gram-positive S.aureus and B.subtilis and about two-thirds that activity against Gram-negative B.coli. The leaves and flowers, however, seem to contain another active principle which is characterised by its higher activity against Gram-negative pathogens.

The high antibacterial activity of root suggested the presence of a highly potent antibacterial agent. This can however be also interpreted as due to the high concentration of the antibiotic



was found that one unit is equal to 0.5 of the most active in the extract. The roots could be had throughout the year unlike the leaves and flowers. Taking into consideration the above facts, the root was chosen as the starting material for the isolation of the antibacterial principle. It is, however, not claimed that the antibiotic principle of the root is the same as the one present in the leaves and flowers. Preliminary investigations carried out seem to indicate that the antibiotic principle of the other plant parts are not so active as the one isolated from the roots.

The name pterygospermin has been suggested for the new compound isolated from the roots.

A pterygospermin unit. During the course of isolation of several biologically active, naturally occurring substances which cannot be had in the pure form and the properties of which are unknown, it has been found highly helpful to estimate the activity of the preparation on a unitage basis, making use of some specific but important property to define the unit. This serves as a rough guide in calculating somewhat quantitatively the percentage yield in every step and has been perhaps found most useful in the studies on antibiotics. Feeling the necessity of a similar unit for the studies on the isolation of pterygospermin it was decided to define a unit of pterygospermin as that amount of the antibiotic which when present in 1 ml of sterile nutrient broth at pH 7.0 just inhibits the growth of a given strain of S.aureus for 18 hours the amount of inoculum being 0.01 ml of a 24 hour broth culture of the same organism. After the isolation of the compound, it

was found that one unit is equal to 3.5 of the most active product.

### Isolation studies.

Preliminary experiments. A series of experiments were first carried out to find out the solvents in which pterygospermin was best extracted from the roots, and also the heat stability of the compound. It was observed that

i) the antibiotic cannot be extracted from finely cut roots, either by very dilute alkalies or acids or by buffered water.

ii) of the different organic solvents tried ethyl alcohol was the best for the extraction of the antibiotic from the roots.

iii) Steam distillation of the roots was carried out. The ether extract of the distillate did not show any antibiotic activity. Much of it could be detected in the residue left after distillation showing that the antibacterial principle is not steam distillable.

iv) preserving the root as such resulted in loss of activity whereas the alcoholic extracts retained it for a considerable length of time.

v) the active principle could be adsorbed with Norit charcoal completely from alcoholic extracts which had 70 pterygospermin units per ml when about 500 gms. of clean, fresh roots cut into very small pieces was extracted with about 750 ml of alcohol. This extract had a reddish brown colour and the characteristic smell.



vi) a second extraction of the roots with a similar volume of alcohol gave a fluid of less intense colour and smell, possessing antibacterial activity of 10 units per ml. The third extract had no activity.

The following experiments to find out a solvent for the extraction of pterygosperrin from the Norit adsorbate were then carried out. About 0.2 gms. of the charcoal (after adsorption of pterygosperrin) was shaken well with 10 ml of the different solvents as given in table II and preserved in the cold. After 24 hours, the solvents were filtered off, evaporated in the vacuum and 5 ml of alcohol added to each. The antibiotic activities exhibited by the solutions were then determined in pterygosperrin units and the percentage recoveries were calculated. In table II the results are presented. Petroleum ether and acetone stand out as the most useful solvents in eluting pterygosperrin after adsorption with Norit.

Treatment of the acetone and petroleum ether extracts. About 10 gms of the Norit after adsorption of pterygosperrin was shaken well with 200 ml of pure acetone and the acetone removed. 500 mg. of a greenish yellow oil was left behind and it inhibited the growth of S.aureus and B.subtilis at dilutions of only 1 in 20,000 when tested by the serial dilution method as described below.

The petroleum ether eluate treated similarly gave a light brown oil weighing about 200 ml. and showing antibacterial properties even at dilutions of 1 in 250,000. The acetone hence appears to elute some of the compounds as well. Purification of

the oil obtained after the removal of light petroleum was attempted. Chromatographic techniques using different adsorbents in the chromatograms without any success. The brownish oil was hence used for further work.

Table II.

Elutive capacity of different solvents.

Name of solvent	Activity of eluates	Amount of substance present in 0.2 gm. of carbon in units	Percentage eluated
Acidulated water	Nil	1500 units	About 80 per cent in the case of petroleum ether and acetone and none in other solvents
Alkaline water	"		
Neutral water	"		
Petroleum ether	120 units per ml		
Alcohol	Nil		
Methanol	"		
Acetone	120 units per ml		
Chloroform	Nil		
Ethyl ether	"		
Benzene	"		



## Table III

Preparation of pterygospermin. Based on the above experiments for the routine preparation of the antibiotic, the following method was used. About 1,000 gms. of fresh roots were cut into small pieces and extracted over-night with about 1500 ml of alcohol, in the cold. The reddish brown alcoholic extract contained 70 units of pterygospermin per ml. A second extract with an equal amount of alcohol had 10 units per ml. To 1500 ml of the first extract 10 gms. <sup>of</sup> Norit and to an equal amount of the second extract, 5 gms. of the adsorbent was added. The suspensions ~~xxx~~ were shaken well in a mechanical shaker for about 30 minutes and then filtered using celite powder as filter aid. The charcoal was then extracted with about 100 ml of light petroleum. The eluate was dried over anhydrous sodium sulphate and the solvent removed at low temperature. The oily substance left behind was dissolved in alcohol and preserved in the cold before being used for experiments. Table III gives a full picture of the attempts on the preparative aspects.

It has been a common observation that if the petroleum ether is not removed within about 48 hours of elution then needle shaped crystals separate on evaporation of the solvent. The crystals, however, did not possess any antibacterial properties. From the alcoholic solutions of pterygospermin kept for a long time also similar crystals separated resulting in loss of activity of the solutions. The antibiotic in all probability decomposes on keeping and the crystals may be one of the decomposition product.

Table III.

## Preparation of pterygospermin.

No. of experiment	Weight of roots in grams	Alcohol used in ml	Activity in units (extract I)	Activity in units (extract II)	Weight of oil isolated in mgms.	%ag yield
1	1,000	1,500	80	10	310	69.0
2	3,000	4,500	65	5	750	71.0
3	2,800	4,300	65	5	715	71.0
4	5,350	7,500	60	10	1,250	72.0
5	1,500	2,200	70	5	425	77.0
6	4,000	5,800	65	5	900	67.0
7	3,300	5,000	65	5	450	50.0
					Mean percentage yield	68.5

Differentiation of pterygospermin from Moringa alkaloids.

The

following facts point out that the present product is different from the alkaloids isolated by Ghosh et al<sup>4</sup>.

1. The antibiotic principle could not be ~~gained~~ dissolved partly or fully in dilute acids with which the alkaloids formed crystalline derivatives.
2. Alkaloidal reagents gave no derivatives with the antibiotic.
3. The alkaloids had no antibacterial properties.
4. Qualitative analysis revealed the presence of sulphur in the antibiotic principle which was absent in the alkaloids.



From the foregoing it appears that a new compound having antibacterial properties has been obtained in a very concentrated form, if not in a chemically pure state. Systematic attempts were not made to study the chemistry of the compound, since its chemical purity could not be ascertained. Qualitative analysis however, showed the presence of sulphur and nitrogen. The compound was insoluble in water. It decolourises bromine water and permanganate. Acids had no effect on the compounds whereas alkalis destroyed it.

It was observed during the investigations that the antibiotic isolated eventhough generally of the highest purity (1 mgm = 250 units) sometimes yielded products of activity as low as about 150 units per mgm. For the experiments detailed below, preparations of potency 250 units per mgm. were used. The criterion of purity was thus the antibacterial activity of the preparation measured against S.aureus and B.coli.

Stability of pterygospermin. Alcoholic solutions of pterygospermin ~~was~~ already pointed out were found to retain their activity at the room temperature for some length of time. The heat stability of the antibiotic was first studied. Solutions of pterggospermin of known activity were incubated for about 30 minutes at different temperatures ranging from 40 to 100°C. The activity of the resulting solutions were determined after making up the volume of the fluids, (ie., adjusting the loss due to evaporation). Even in tubes heated to 100°C. the activity was retained, proving the thermostable character of pterygospermin.

The effect of different Hydrogen ion concentration on the stability of the compound was next determined since this has been found to affect several antibacterial agents. For this, the following experimental procedure was adopted. Known amounts of pterygospermin were incubated after emulsifying it in buffers and broth of known pH for fixed intervals of time and the antibacterial activity of suspensions determined by the serial dilution method using S.aureus as the test organism. In table IV the results are presented.

Table IV.

Stability of pterygospermin at different Hydrogen ion concentrations.

pH	Activity in units after contact with pH for hours				
	0	3	6	8	24
In buffer					
3.5	72	72	72	72	72
4.5	72	72	72	72	71
5.5	71	71.5	72	71	71
7.0	68	69	70	70	69
8.0	71	62	48	35.5	32.5
9.0	71	38.0	32.0	25.5	20.0
In broth					
5.0	71	72	70	71	71.5
6.0	71	71.5	70	72	72
8.0	71	62.0	48	35.5	32.5
9.0	71	38	32.5	28	20



The results are indicating clearly the unstable nature of the antibiotic at alkaline reactions whereas at acid range pterygospermin is considerably stable.

Experiments of a similar nature were next carried out to find the optimum pH at which pterygospermin acts best. In table V the results are presented.

Table V.

Optimum pH at which pterygospermin acts.

pH of broth	Dilutions of the drug in broth(in thousands)					Cont
	80	160	240	320	400	
5.0	-	-	-	-	++	
6.0	-	-	-	+	++	
7.0	-	-	-	++	++	+++
8.0	-	-	+	++	++	
9.0	-	+	++	++	++	

In table - indicates absence of growth  
 + indicates slight growth  
 ++ indicates more growth  
 +++ indicates growth as much as in control

It can easily be inferred from the above results that the antibiotic acts best in acid media, the antibacterial properties being reduced considerably at alkaline range.

Antimicrobial properties of pterygospermin.

Antibacterial spectrum. For finding the minimum inhibitory concentrations of the antibiotic to different pathogenic microorganisms, alcoholic solutions of pterygospermin of known concentration (generally 100 mgm. per ml) were diluted in nutrient broth adjusted to pH 6.8. Due to the insolubility of the antibacterial agent, there was a faint turbidity upto a dilution of 1 gm. in 10,000 ml. Further dilutions gave clear broth making the use of the well known serial dilution method, for assaying the antibacterial activity of the compound possible. In regular assaying a solution of pterygospermin 100 mgm. per ml was first diluted in sterile broth to give dilutions of 1 in 2,000; 4,000; 8,000; 16,000 etc., 0.5 ml of these emulsions were added to 4.5 ml of nutrient broth at pH 6.8. The tubes were then inoculated with 0.01 ml of a 18 hour broth culture of the organisms. The tubes were then incubated at 37°C. for 18 hours, after which they were observed for growth. For acid fast organisms the following method was used. Flasks containing Long's medium (with ammonium malate instead of asparagine) containing pterygospermin in the form of emulsion, so as to give concentration of 2 mgm., 6 mgm., 12 mgm., and 18 mgm. per 100 ml of the medium were inoculated with two loopfuls of Mycobacterium tuberculosis and Mycobacterium phlei respectively. The flasks were incubated at 37°C. In the case of flasks inoculated with M. tuberculosis, no growth was observed even after three weeks, in flasks containing upto 1 unit of pterygospermin per ml whereas in the control the bacillus grew profusely. Detailed antibacterial spectrum of pterygospermin is presented in table VI.



Table VI.

## Antibacterial spectrum of pterygospermin.

No.	Name of test organism	Dilution of pterygospermin in broth x 1/1000						No. of units per ml. at max.
		1/40	1/75	1/100	1/200	1/250	1/300	
1	<u>B. subtilis</u>	-	-	-	-	+	++	1.0
2	<u>S. aureus</u> Bhat's strain virulent	-	-	-	-	+	++	1.0
3	<u>B. hyperdiastagenase</u> Lulla	-	-	-	-	+	++	1.0
4	<u>S. aureus</u> avirulent	-	-	-	-	+	++	1.0
5	<u>B. coli</u>	-	+	+	+	++	++	4.5
6	<u>Eb. typhosum</u>	-	-	+	+	++	++	3.0
7	<u>B. enteritidis</u>	-	-	+	+	++	++	3.0
8	<u>B. aerogenes</u>	-	+	+	+	++	++	4.5
9	<u>B. para typhosus B</u>	-	+	+	+	++	++	4.5
10	<u>B. paratyphosus C</u>	-	-	-	-	+	++	1.0
11	<u>B. dysenteriae</u> Shiga	-	-	-	-	+	++	1.0
12	<u>B. dysenteriae</u> Flexner	-	-	-	-	+	++	1.0
13	<u>M. phlei</u>	-	-	-	-	+	++	1.0
14	<u>M. tuberculosis</u> B.C.G.	-	-	-	-	+	++	1.0

In the table - indicates no growth

+ indicates slight growth

++ indicates growth as much as in control

Thus it will be seen that pterygospermin has a wide range of antibacterial activity; inhibiting the growth of Gram-positive, acid-fast and some Gram-negative pathogens at considerably high dilutions. Some of the Gram-negative organisms studied, however, appear resistant to the drug even at low concentrations.

Antifungal properties. In view of the remarkable antibacterial properties of the active principle isolated its effect on the growth of some of the fungi available in this laboratory were studied. The experimental methods were similar to those used for finding the antibacterial properties. After inoculation with the organisms, the tubes were however kept in a slanting position (about 10 degrees to the horizontal). The growth of the moulds was observed for a period of about three days instead of 18 hours as in the case of the bacteria. The results of these studies are recorded in table VII.

Pterygospermin thus appears to inhibit the growth of several fungi also. The amount of drug required however is about thrice that required to inhibit the growth of Gram-positive micro-organisms.



Table VII.

## Antifungal properties of pterygospermin.

No.	Name of organism	Composition of medium	Dilution of antibiotic in medium x 1/1000					No. of uni per ml. maximum inhibito dilution
			1/50	1/80	1/100	1/160	Control	
1	<u>Streptomyces griseus</u>	Nutrient broth	-	-	-	+	++	2.0
2	<u>Aspergillus fumigatus</u> Fresenius	Czapeck Dox	-	-	+	+	++	3.0
3		Nutrient broth	-	-	+	+	++	3.0
3	<u>Aspergillus fumigatus</u> No.387	Czapeck Dox	-	-	+	+	++	3.0
		Nutrient broth	-	-	+	+	++	3.0
4	<u>Penicillium notatum</u>	Czapeck Dox	-	-	+	+	++	3.0
		Nutrient broth	-	-	+	+	++	3.0
5	<u>Penicillium chrysogenum</u>	Czapeck Dox	-	-	+	+	++	3.0
		Nutrient broth	-	-	+	+	++	3.0

In tables VI and VII, dilutions may be read as 1/50,000; 1/80,000; 1/240,000 etc.

Investigations on the mode of action of pterygospermin.

As already pointed out in the introduction several interesting hypotheses have been suggested to explain the mode of action of antibiotics, even though there are no definite ideas regarding the mode of action of even a single one of them except probably notatin. However, experiments were carried out to explore the possibility of applying some of the theories suggested, to pterygospermin.

Effect of thiol compounds on the antibiotic activity of pterygospermin.

Studies with a large number of antibiotics have shown their<sup>6,7</sup> chemical reactivity towards compounds containing sulphhydryl groups. There have also been observed marked differences in reactivity of individual antibiotics towards various types of thiol compounds.<sup>8,9</sup> The antibacterial activity of the antibiotics has been explained as due to their reaction with thiol reagents. The compounds may react either with essential -SH groups of bacterial enzymes or with -SH groups in cysteinyl residues of polypeptides, preventing the formation of proteins and thus inhibiting growth<sup>10</sup>. In the present investigation, the effect of certain thiol compounds - cysteine and thiosulphate - on the antibacterial properties of pterygospermin was studied.

The reactions were carried out in phosphate buffer at pH 6.8 at a temperature of 37°C. The rate of reaction of the thiols (viz., cysteine and thiosulphate) with the antibiotic was measured



by estimating the activity of antibiotic by the serial dilution method against S.aureus. The thiols and the antibiotic were mixed in different proportion the samples incubated and removed for test at intervals upto 48 hours. The thiol concentrations in the mixtures were as given below.

Mixture I. 0.5 mgm. of cysteine or thiosulphate for every one mg. of the antibiotic.

Mixture II. 5.0 mgm. of thiols for every mg. of pterygospermin.

Mixture III 10.0 mgm. of thiols for every mg. of pterygospermin.

In tables VIII and IX below the results of these studies are presented.

Table VIII.

Effect of thiosulphate on the antibiotic activity of pterygospermin.

Ratio of thiosulphate to pterygospermin	Units of pterygospermin per ml. of mixture after incubation for - hours							
	0	2	4	6	8	10	24	48
1:2	47	47	47	47	46	47	47	47
5:1	47	47	48	46	46	47	48	47
10:1	47	46	47	48	47	48	47	47
Control	47	46	48	47	46	47	47	47

Control in the above table indicates lack of thiosulphate.

Table IX.

Effect of cysteine on the antibacterial properties of pterygospermin.

Dilution of mixtures in broth	Activity after incubating the mixtures for - hours					
	0	2	4	8	24	48
<u>Mixture I</u>						
1 in 75,000	-	-	-	-	-	-
1 in 225,000	-	-	-	-	-	-
1 in 300,000	++	++	++	++	++	++
<u>Mixture II</u>						
1 in 150,000	-	-	-	-	-	-
1 in 225,000	-	-	-	-	-	-
1 in 300,000	++	++	++	++	++	++
<u>Mixture III</u>						
1 in 80,000	-	-	-	-	-	-
1 in 200,000	-	-	-	-	-	-
1 in 300,000	++	++	++	++	++	++
<u>Control</u>						
No cysteine						
1 in 200,000	-	-	-	-	-	-
1 in 300,000	++	++	++	++	++	++



The foregoing results clearly show that both cysteine and thiosulphate do not have any effect on the antibiotic activity of pterygospermin. It has therefore to be inferred that the compound belongs to the class of antibiotics not affected by thiol reagents. This is quite possible and seems to confirm the view that different antibiotic agents function by different mechanisms.

Effect of nucleic acids on pterygospermin. Nucleic acids and related compounds have been found to antagonise<sup>11,12</sup> the antibacterial properties of many antibiotics. Based on these observations it has been postulated<sup>13,14</sup> that antibiotics probably act by side tracking nucleic acids essential for cell metabolism. Experiments were carried out to find the effect of nucleic acids on the antibacterial properties of pterygospermin.

For this antibacterial activity of pterygospermin in presence and absence of different amounts of nucleic acids were estimated by the serial dilution method. Experiments with S.aureus, however, showed that antibacterial property of pterygospermin was increased by small amounts of nucleic acid. The studies were hence extended to Gram-negative E.dysenteriae as well. The results are presented in table X and are self-explanatory. A B.D.H. sample of nucleic acid was used for these investigations.

Table X.

Effect of nucleic acid on pterygospermin .

Test organism	Activity of antibiotic	Activity of nucleic acid	Activity of nucleic acid- pterygospermin mixtures	Contro	
<u>S.aureus</u>	1/100000 -	1/1000 ++	Nucleic acid	pterygospermin	
			1/100,000 -	-	
	1/200000 -	1/2000 ++	1/1000	1/200,000 -	
			1/300,000 -	-	
	1/300000 ++			1/100,000 -	++
			1/2000	1/200,000 -	-
			1/300,000 -	-	
<u>B.dysen- teriae</u>	1/200000 -	1/1000 ++	1/200,000 -	-	
			1/300,000 -	-	
	1/300000 ++		1/400,000 -	-	++
			1/200,000 -	-	
	1/400000 ++	1/2000 ++	1/300,000 -	-	
			1/400,000 -	-	
		1/2000	1/300,000 -	-	
			1/400,000 -	-	



Effect of para-amino-benzoic acid on pterygospermin. During the course of studies undertaken to find the effect of pterygospermin and sulphapyridine together on bacterial growth (described later in this thesis) it was observed that there was no synergism between the two compounds. Lack of synergism has often times been interpreted as due to the similarity in the mode of action of drugs<sup>15,16</sup>. It seemed therefore probable that pterygospermin may have similarity towards the sulpha drugs in its mode of action on different pathogens. Effect of para-amino-benzoic acid ( a compound well known for its inhibition of sulphenamide activity) on pterygospermin was hence studied.

The experimental procedure consisted in finding out the antibacterial activity of pterygospermin in nutrient broth containing para-amino-benzoic acid in concentrations of 1 in 1000. In table XI the results are presented.

Table XI.

Effect of para-amino-benzoic acid on pterygospermin.

Concentration of para-amino-benzoic acid	Dilution of pterygospermin in broth				Control
	1/100000	1/200000	1/250000	1/300000	
Nil	-	-	-	++	++
1 mg. per ml	-	-	-	++	++
0.5 mg. per ml	-	-	-	++	++

The test organism in the above experiment was S. aureus. The absolute lack of influence is clearly brought out.

Synergism of pterygospermin with other antibacterial agents.

The problem of drug resistance has become one of the more important limiting factors in their therapeutic efficiency. It has been that bacteria develop resistance to antibacterial agents at a slow or fast rate, both in vitro<sup>17</sup> and in vivo<sup>18</sup>. Particular mention may be made of the development of resistance to streptomycin, which appears to be more frequent than that to penicillin<sup>19</sup>. Since the simplest method of getting a resistant strain of any organism is by culturing it in broth containing partially inhibitory concentrations of the drug, it has been suggested<sup>20</sup> that the development of drug resistance is due to the rapid multiplication of a few highly resistant organism present in the parent strain, which in their turn produce more and more resistant strains. By continuous culturing of the micro-organisms in sub-minimal amounts of the drug, strains resistant to very high amounts of streptomycin and penicillin have been produced<sup>21</sup>. Resistance to one drug, however, does not imply fastness to others<sup>22</sup>. Combined action of more than a single antibacterial agent has been hence found to be highly effective in suppressing the growth of micro-organisms which are resistant to one of the drugs. Synergism is a method of suppressing the growth of organisms not only of induced resistance but also of those which are naturally resistant to one of the drugs. Synergism thus plays an important role in chemotherapy. The effect of combined administration of pterygospermin and other antibacterial agents like penicillin, streptomycin and sulphapyridine were hence carried out.



The synergistic effect of penicillin with several other compounds like the sulpha drugs<sup>23</sup>, basic dyes<sup>24</sup>, streptomycin<sup>25</sup>, certain metallic ions<sup>26</sup> and urea<sup>27</sup> have been demonstrated in vitro. Some of these findings have been extended to the in vivo treatment of diseases like typhoid<sup>28</sup> and meningitis<sup>29</sup>, by the combined administration of penicillin and sulphathiazole. Streptomycin penicillin mixtures have also been of great value against resistant organisms.<sup>30</sup>

For finding out the synergistic effect of pterygospermin with other antibacterial agents the samples used are given below.

**Penicillin:** A sample of crystalline sodium salt of penicillin G.

**Streptomycin:** Crystalline streptomycin hydrochloride supplied by Merck and Company, and having an activity of 655 per mg.

**Pterygospermin:** Most active samples prepared by the method given previously.

**Sulpha drug:** A crystalline sample of sodium sulpha-pyridine.

The experimental procedure consisted in first finding the minimum inhibitory concentrations of the antibacterial agents separately, in nutrient broth by the serial dilution method against S. aureus and B. coli, typifying Gram-positive and Gram-negative pathogenic micro-organisms. In table XII, the results are given.

Table XII.

Antibacterial properties of drugs when present individually.

Organisms	Pterygospermin in units	Growth in broth	Penicillin in units	Growth in broth	Strepto- mycin in units	Growth in broth
<u>S. aureus</u>	2.0	-	0.10	-	5.0	-
	1.5	-	0.075	-	2.0	-
	1.0	-	0.030	-	1.0	-
	0.75	++	0.025	+	0.5	++
	0.50	++	0.010	++	0.2	++
<u>B. coli</u>	8.0	-	10.0	-	5.0	-
	6.0	-	5.0	-	2.0	-
	4.0	-	4.0	++	1.5	-
	3.5	++	3.0	++	1.0	-
	3.0	++	2.0	++	0.5	++

In the above table figures for penicillin and streptomycin are given in international units and for pterygospermin in the units defined previously.

In a second series of experiments amounts of pterygospermin lower than the minimum inhibitory concentrations were taken in broth and the effect of adding subminimal concentrations of penicillin and streptomycin on the growth of the test organisms were observed.



In table XIII the results are presented.

Table XIII.

Antibacterial activity of pterygospermin in presence of penicillin and streptomycin.

Organism	Pterygospermin in units per ml	Growth in broth	Pterygospermin along with	
			Penicillin 0.025 units	Streptomycin 0.5 units
<u>S.aureus</u>	0.80	++	-	-
	0.60	++	-	-
	0.50	++	-	-
	0.10	++	++	++
<u>B.coli</u>			4.0 units per ml	0.5 units per ml
	3.50	++	-	-
	2.00	++	-	-
	1.75	++	-	-
	1.00	++	++	++

It can be concluded from the above results that pterygospermin can reinforce the antibacterial activity of drugs like penicillin and streptomycin. The results are, however, not very elaborate since differing concentrations of penicillin and streptomycin were not used.

In a third series of experiments, the effect of different sub-minimal concentrations of penicillin and streptomycin in presence of several non-inhibitory concentrations of pterygospermin, on the growth of S.aureus and B.coli were studied. In table XIV the results of these experiments are presented.

Table XIV.

Combined action of pterygospermin with other antibiotics.

Name of organism	Pterygospermin in units per ml	Penicillin in units per ml				Streptomycin in units per ml			
		0.025	0.02	0.015	0.010	0.50	0.25	0.15	0.05
<u>S.aureus</u>	0.75	-	-	-	++	+	-	-	+
	0.50	-	-	+	++	-	+	++	++
	0.25	-	+	++	++	-	+	++	++
	0.10	+	++	++	++	+	++	++	++
	0.00	+	++	++	++	+	++	++	++
<u>B.coli</u>		4.0	3.0	2.0	1.0	0.50	0.25	0.15	0.05
	3.0	-	-	-	-	-	+	+	++
	2.0	-	-	-	+	-	+	+	++
	1.5	-	+	+	+	-	+	++	++
	1.0	+	++	++	++	+	+	++	++
	0.5	++	++	++	++	+	++	++	++
	0.00	++	++	++	++	++	++	++	++



The above results reveal a high degree of synergism. The concentration of penicillin required to inhibit S.aureus is reduced to about one half when 0.75 units of pterygospermin are present in one ml. In presence of 0.5 units of streptomycin (non-inhibitory) the amount of pterygospermin required to inhibit S.aureus goes down from 1.0 to 0.25 units per ml. The effect of penicillin-ptyerygospermin mixtures against Gram-negative B.coli is more outstanding, the amount of penicillin required ~~going~~ going down by about four to five times, when 3 units per ml (non-inhibitory) of pterygospermin are present. These findings are very interesting from point of view of the mechanism of action of different antibiotic agents as well as from its possible application in clinical practice. Indeed it may be remembered that Prof.Fleming in his recent Linacre lecture<sup>31</sup> has given expression to the possible use of "antibacterial chemical combination".

Sulphapyridine - pterygospermin mixtures. Experiments similar to those described above for pterygospermin - penicillin and streptomycin pterygospermin mixtures have been done for sulphapyridine-ptyerygospermin mixtures also. Investigations with S.aureus however, failed to show any synergistic effect. Experiments were hence not extended to Gram-negative B.coli, and the summary of the investigations with S.aureus are brought out in table XV.

Table XV.

Combined action of pterygospermin and sulphapyridine  
against Staphylococcus aureus.

Pterygospermin in units per ml	Sulphapyridine <sup>3</sup> dilution - x 10 <sup>3</sup>	Growth in broth
N11	N11	+++
"	0.2	+
0.75	N11	+
0.375	0.02	++
0.375	0.10	++
0.375	0.20	+
0.75	0.10	+
0.75	0.20	+
N11	0.25	+
"	0.30	-
1.0	N11	-

### DISCUSSION.

Several interesting facts regarding the antibiotic principle of Moringa pterygosperma are brought out in the above studies. The isolation of the antibiotic principle from the roots has been possible by a simple process, the yield being about 65%. The amount of the drug that could be isolated from the roots, however,



is very little. About 1,000 gms. of the roots contained only 500 mgm. of the drug and the yield is about 310 mgm. The compound isolated appears to be thermostable. Long keeping, however, results in slow loss in activity. Alkalies instantaneously inactivates the compound.

The optimum pH for its activity is on the acidic side. During these investigations, however, all assays for its antibiotic potencies were measured at pH 6.8. At this pH about 3.5 of the compound inhibits the growth of many pathogenic microbes including M.tuberculosis. In its action against Gram-negative organisms it behaves in a unique manner. Thus organism like B.dysenteriae are inhibited at concentrations of 3.5 per ml whereas B.coli and a few other Gram-negative organisms demand the presence of about three times the amount of the drug in the medium for their inhibition. No exact reason for this observed fastness of certain Gram-negative pathogens to the drug could be given. Whether it is due to the peculiarities of the strains used or due to the inherent inability of pterygospermin to inhibit these organisms cannot be said. The effect of the antibiotic on fungi has also been clearly brought out.

The marked antibacterial activity of pterygospermin against several Gram-positive, Gram-negative and acid-fast pathogens would suggest the possibility of its therapeutic application in human infections. In view of the facts that the fungi used in these experiments are not typical pathogenic ones (even though certain Aspergilli and Actinomycetes are known to be pathogenic)

nothing regarding the applicability of pterygospermin in fungal infections of mankind is suggested. The synergistic effect of pterygospermin - penicillin and pterygospermin - streptomycin mixtures which are reported, are likely to assume great importance in the control of infections which are resistant to one or both the drugs.

Experiments carried out with a view to finding the mode of action of the drug have given no useful information. Its non-inactivation by thiols in all probability indicates the inability of the drug to inactivate the enzymes (having sulphhydryl groups) as well. The inability of para-amino-benzoic acid to antagonise its action reveals its difference from sulpha drugs in its mode of action.

#### Summary and Conclusions.

1. The antibacterial principle of Moringa pterygosperma has been isolated. ~~The~~ A convenient method for its isolation has been developed. It has been named "pterygospermin".

2. The antibiotic has been shown to be inactivated by alkalies. It is thermostable, but loses its activity on long preservation.

3. Pterygospermin in very low concentrations inhibits several pathogenic bacteria including ~~myxa~~ Mycobacterium tuberculosis. It inhibits also the growth of many fungi.



4. It is not inactivated by thiol reagents, nucleic acids, and para-amino-benzoic acid. (1938) "Indigenous Flora of India"

5. Sub-minimal concentrations of penicillin and streptomycin enhance the antibiotic properties of pterygospermin against Gram-negative and Gram-positive micro-organisms. (1938) Ind. J. Med. Research 25, 233

## References.

1. Chopra, R.N. (1933) "Indigenous Drugs of India"
- Kirthikar, K.R. and  
Basu B.D. (1933) "Indian Medicinal Plants"
2. George, M. et al (1947) J.Sci.Ind.Research., 6B, p 42.
3. Chopra, R.N., De, P.,  
and De, N.N. (1932) Ind.J.Med.Research, 20, 533.
4. Ghosh, S., Chopra R.N.,  
and Dutt, A. (1935) Ind.J.Med.Research, 22, 785.
5. Foster, and Woodruff (1944) J.Bact. 47, 43.
6. Cavallito C.J. et al (1944) Science, 100, 390.
7. Atkinson and Stanley (1943) Australian.J.Exptl.Biol & Med.  
Sciences, 21, 255.
8. Cavallito C.J., et al (1945) J.Bact., 50, 61.
9. Bondi A., et al (1946) Science, 103, 399.
10. Cavallito C.J., et al (1946) J.Biol.Chem., 165, 29.
11. Pandalai K.M., et al (1947) Brit.Med.J., 2, 210.
12. Faguet M. (1948) Ann.Inst.Pasteur, 74, 75.
13. Pandalai K.M., et al (1948) Ind.J.Med.Research, 36, 197.
14. Duffrenoy J. & Pratt R. (1949) J.Bact. 57, 13.
- 15.
16. Fildes P. (1940) Lancet, 1, 955.
17. Klein M. (1947) J.Bact, 53, 463.
18. Findland M., et al (1946) J.Amer.Med.Assn., 132, 16.
19. Graessle, O.E., and  
Frost B.M. (1946) Proc.Soc.Exptl.Biol & Med, 63, 171.
20. Klein M., and  
Kimmelman L.J. (1946) J.Bact., 52, 471.
21. Ungar J. (1943) Nature, 152, 245.



22. Bigger J.W. (1944) Lancet, 247, 142.
23. Hobby G.L., & Dawson M.H. (1946) J.Bact., 51, 447.
24. George M., & Pandalai  
K.M. (1946) Nature, 158, 709.
25. Klein M. and  
Kimmelman L.J. (1947) J.Bact, 54, 363.
26. Stratt L.A. et al (1948) J.Amer.Pharm.Assn., 37, 133.
27. Kirby W.M.M. (1944) Proc.Soc., Exptl. Biol. & Med. 57, 14
28. Moore, B.P., et al (1948) Lancet, 254, 476.
29. Mc Sweeney (1946) Lancet II, 114.
- 30.
31. Fleming A. (1946) Linacre lecture (Camb.Univ.Press)

CHAPTER - II.



TOXICOLOGICAL, PHARMACOLOGICAL, AND CHEMOTHERAPEUTIC  
STUDIES ON PTERYGOSPERMIN

Introduction.

Results have been presented in the previous chapter, which have shown the high in vitro activity of pterygospermin. Gram-positive, Gram-negative and acid-fast pathogens do not grow when 3.5 of the drug are present in 1 ml. Thus its activity against Gram-negative and acid-fast pathogens is more pronounced than that of penicillin, which is rather inefficient in the cure of the infections caused by such organisms. Streptomycin the antibiotic which has now achieved considerable importance (next only to penicillin), has got several limitations, such as inability to act at the pH of certain bacterial infections (acidic), delayed toxicity, and perhaps the most important, development of streptomycin-fastness by several micro-organisms, particularly in vivo, thereby making prolonged use of drug streptomycin almost impossible. The search for new antibiotic agents hence continues with the hope of isolating naturally occurring compounds which may have all the advantages of streptomycin and penicillin but not their weaknesses.

In order that an antibiotic should attain chemotherapeutic importance it should satisfy several important conditions. Thus, it should be capable of exerting a bacteriostatic or bactericidal effect upon pathogenic bacteria, not only in the test tube but also in the animal body, but not very toxic nor exerting any otherwise

ePrint@CFTRI

undesirable effects on the body, not inactivated by body weight (the most important of which is blood) and preferably stable even at room temperature for a considerable length of time.

Florey has remarked<sup>1</sup> "..... after the substance has been extracted in a highly concentrated on pure form, the range of organism against which it is active - usually pathogenic organisms are considered first - is determined, together with such data as whether it is inactivated by serum or body fluids. The toxicity to whole animals, usually mice, is determined and it is particularly important to determine the effect of repeated injections, for in chemotherapeutic practice repeated doses will always be necessary. For example, some antibiotics of which a single injection is well tolerated produce live damage when the injection is repeated often. Toxicity to individual tissues is determined by in vitro observations on leucocytes or tissue cultures and by injection <sup>into</sup> the cerebrospinal cavity or other selected sites in animals. Pharmacological investigations into the effect of the substance on the most important physiological functions follow. Of these, observations on the heart, blood pressure and respiration are most important. If, at the end of these investigations, a substance appears to be both sufficiently powerful against bacteria and sufficiently non-toxic, protection experiments on appropriate animals, such as, mice or for the tubercle bacillus, guinea pigs become justifiable".

Investigations reported already have shown the antibiotic principle of drumstick root to possess considerably high antibacterial properties, thus making it necessary to investigate regarding its,



activity in presence of blood and other tissue fluids, toxicity to laboratory animals like mice, (both acute and cumulative), pharmacological experiments and its efficacy in curing in vivo infections. The results of these investigations are presented below.

#### Experimental.

Stability of pterygospermin in presence of blood. The stability and activity of an antibiotic in presence of tissue fluids is of considerable importance. Thus the oral administration of penicillin is practically impossible in view of its inactivation by the high acid reaction of the gastric juice<sup>2</sup>. Certain other antibacterial agents like notatin<sup>3</sup> do not find applicability in bacterial infections due to their inactivation by blood. This may be due to the enzymes in blood<sup>4</sup>, or may be due to the high adsorptive capacity of blood as in the case of penicillin K<sup>5</sup>, which appears to lose much of its in vitro effect due to its loose complex formation with serum proteins, or it may be due to the alkaline pH of the blood. Other constituents of blood like the trace elements and glucose may also inhibit or inactivate the antibacterial properties of drugs. Experiments were carried out to find the effect of incubating pterygospermin with blood and serum.

Fresh rabbit's blood and human serum were used as media. Pterygospermin was mixed with these, sterile citrate, and sterile water in such a way as to give a final concentration of about 18 units of pterygospermin per ml. The mixtures were incubated

at 37°C. and their antibiotic activity determined by the serial dilution method against S. aureus, at fixed intervals of time. In table XVI below the results are presented.

Table XVI.

Stability of pterygospermin in presence of rabbit's blood and human serum.

Time after mixing (in hours)	Activity in pterygospermin units per ml when mixed with			
	Sterile water	Sterile citrate	Citrated blood	Human serum
0	18	18	18	18
2	18	18	18	18
4	18	18	17	18
6	17	18	17	17
8	18	17	18	17
10	17	17	18	17
24	17	17	17	17

That pterygospermin is stable and active in presence of blood is very clearly brought out in the table.

Stability in presence of gastric and pancreatic fluids. Before studying the toxicity to animals, it seemed essential to find out the stability of the drug in presence of gastric and pancreatic juices. This is of great importance in finding the oral toxicity of the drug and since it has been stated that the alkaline reactions inactivate pterygospermin.



The experimental procedure was similar to the one described for finding the effect of pterygospermin activity in presence of blood. Artificial gastric and pancreatic fluid prepared according to Maney and Kuever<sup>6</sup> and of the following composition were used, for incubating the antibiotic.

Artificial gastric fluid:-

Sodium chloride	...	1.400	gms.
Potassium chloride	...	0.500	"
Calcium chloride	...	0.060	"
Hydrochloric acid 36%	...	6.944	"
Pepsin	...	3.2	"
Distilled water to make 1,000 ml.			

Artificial pancreatic fluid:-

Pancreatin	...	2.8	gms.
Sodium carb bicarbonate	...	15.0	"
Distilled water to make 1,000 ml.			

The results of these investigations are presented in table XVII.

It may be seen from the table that pancreatic juice inactivates the drug considerably, within about eight hours whereas the drug is active in presence of gastric juice even after 24 hours incubation. In view of the inactivation of the drug in presence of pancreatic juice most probably due to its alkaline pH the toxicity of the drug when orally administered was not studied.

Table XVII.

**Stability of pterygospermin in presence of  
gastric and pancreatic fluids.**

Time after mixing (in hours)	Antibacterial activity of pterygospermin in units per ml when mixed with		
	Sterile buffer pH 6.8	Gastric juice pH 1.7	Pancreatic juice pH 9.3
0	18	18	18
2	18	18	17
4	18	18	16
6	18	18	15
8	17	17	11
10	18	17	8
24	18	18	4

Studies on the toxicity of pterygospermin.

Data presented above have shown pterygospermin to possess a wide range of antibacterial activity and stability in presence of blood. Experiments were next carried out to find out the effect of parenteral administration of the drug to laboratory animals like mice and rats. The following experimental procedure was used.



Animals. Young healthy mice weighing about 20 to 25 gms. were used. As far as possible animals of approximately the same age were taken for the same experiments. <sup>Throughout</sup> ~~Through~~ the experiments special care was taken to maintain the animals on a balanced diet, water being available at all times.

Pterygospermin. Prepared according to the method previously described was used. It had an activity of about 250-300 units per mg. Since the drug was insoluble in water, an emulsion in water was used for the experiments.

Acute toxicity. Acute subcutaneous toxicity was determined as follows. The experimental animals were divided into several groups, each group containing six animals. Different but known amounts of pterygospermin were injected to animals of different groups, the quantity received by members of the same group being equal. In all cases, the strength of the emulsion were so adjusted that a mouse received not more than 0.20 ml. The acute toxicity was determined by giving large quantities of the drug in one injection. After the injection the animals were kept under close observation for a period of ten days.

In those groups where the mortality rate was high the <sup>a</sup> experiments were repeated using ~~the~~ larger number of animals. Mice were used for the subcutaneous toxicity studies. In table XVIII below the results of acute subcutaneous toxicity of pterygospermin are presented. It may be seen that 50% mortality occurs when the dose administered is 300 mg. per Kg of body weight.

Table XVIII.

## Acute subcutaneous toxicity of pterygospermin.

Dose in mg/Kg.	No. of mice tested for each dose	Total number of animals dead after - days											
		1	2	3	4	5	6	7	8	9	10	21	
5	6	0	0	0	0	0	0	0	0	0	0	0	0
10	6	0	0	0	0	0	0	0	0	0	0	0	0
50	6	0	0	0	0	0	0	0	0	0	0	0	0
100	6	0	1	1	1	1	1	1	1	1	1	1	1
200	10	0	1	2	2	2	2	2	2	2	2	2	2
300	30	9	13	15	15	15	15	15	15	15	15	15	15
400	15	5	8	11	12	12	12	12	12	12	12	12	12
500	15	7	9	13	14	15	15	15	15	15	15	15	15
No drug	6	0	0	0	0	0	0	0	0	0	0	0	0

Cumulative toxicity. The effect of daily administration of the drug was determined on a group of mice. Small amounts of pterygospermin were injected subcutaneously for a period of 15 days daily and on alternate date for the next 7 days. Other experimental details were as for acute toxicity experiments. The results are given in table XIX below. 50% mortality occurred as indicated therein when the dose administered was 50 mg. per Kg.



Table XIX.

Cumulative subcutaneous toxicity.

Daily dose in mg/Kg.	No. of mice used	Total number of mice dead after days												
		1	2	3	4	5	6	7	8	9	12	14	21	
35	6	0	0	0	0	0	0	0	0	0	0	0	0	0
45	6	0	0	0	0	0	0	0	0	0	0	0	0	0
50	10	0	0	3	3	3	3	3	4	4	4	5	5	5
55	6	0	2	3	4	5	6	6	6	6	6	6	6	6
85	6	0	2	3	4	5	6	6	6	6	6	6	6	6
115	6	2	3	4	5	6	6	6	6	6	6	6	6	6
Nil	6	0	0	0	0	0	0	0	0	0	0	0	0	0

Toxicity of different samples of pterygospermin. Pterygospermin prepared by the method presented above often possesses activity of 250 to 300 units per mg. However, it has been observed that in some instances, the preparations have very low activity about 150 units per mg. Toxicity of different specimens having different activities were hence studied. It may be mentioned in this connection that similar experiments with preparations of widely differing activity have been determined by Reke *et al*<sup>7</sup> for streptothricin and Salle *et al*<sup>8</sup> for subtilin. In table XX the results of these experiments are presented.

Table XX.

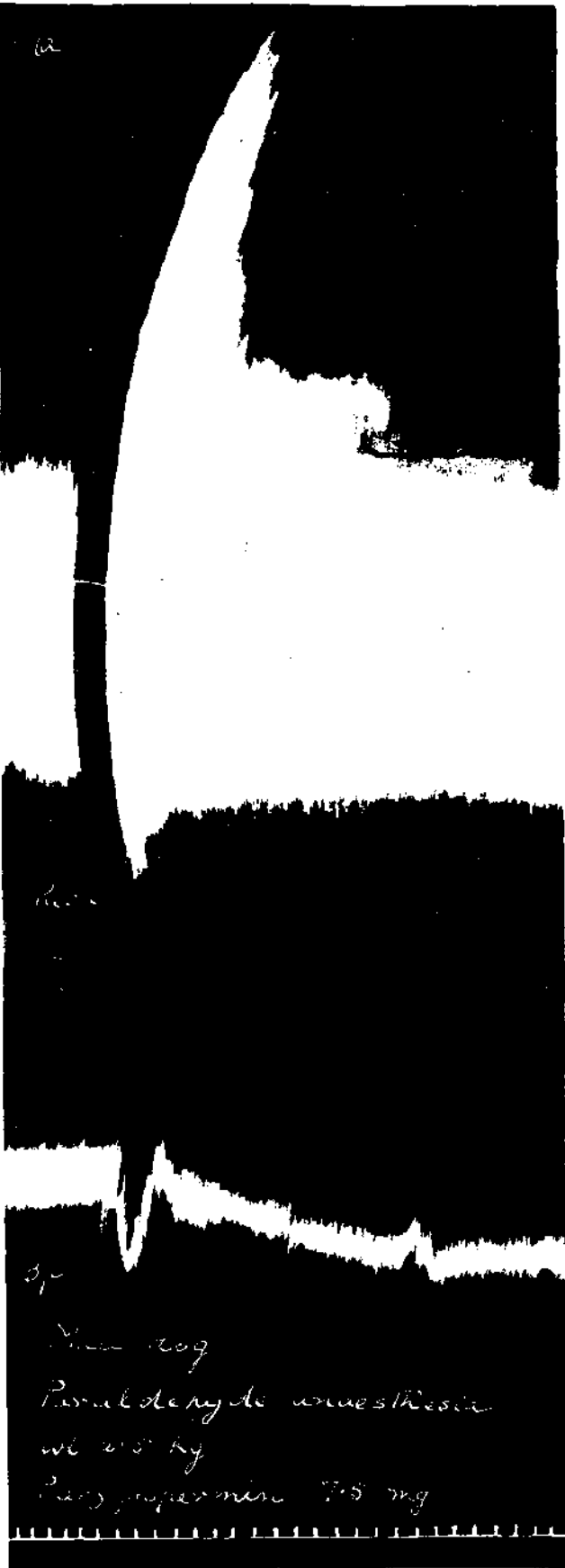
Toxicity of samples of pterygospermin of different activities.

Batch number	Dose in mg/Kg.	Dose in units per Kg.	Units per mg.	Total No. of mice used	Total mice dead after 15 days	Percentage mortality
3	300	75,000	250	12	6	50
13	400	80,000	200	8	5	62.5
17	500	75,000	150	9	5	55.5
26	375	75,000	200	9	5	55.5
31	450	72,000	160	8	4	50.0

In the above experiments, the effect of approximately the same units of the pterygospermin samples (estimated according to the pterygospermin unitage defined previously in this thesis) on mice were determined, the total amount of the drug injected varying depending upon the strength of the sample used, as indicated in table XX. As can be seen therefrom, the results suggest that the foreign materials associated with pterygospermin preparations of different antibacterial potencies do not possess much of a toxic effect on the animals used.



12



Male dog

3p

Male dog  
 Paraldehyde anaesthesia  
 wt 10 kg  
 Pilocarpine 7.5 mg

13



Female dog

3p

Female dog  
 Paraldehyde anaesthesia  
 wt 3.5 kg  
 Pilocarpine 7.5 mg

## PHARMACOLOGICAL INVESTIGATIONS ON PTERYGOSPERMIN.

Studies essentially of a preliminary nature were performed in order to obtain some insight regarding the effect pterygospermin might have on some of the more important bodily functions, if employed in the treatment of bacterial infections. They consisted chiefly in finding the effect of pterygospermin on blood pressure, respiration and heart.

Effect on blood pressure. This was studied in cats and dogs under paraldehyde anaesthesia, using the usual methods of manometric recording, and canulation of the carotid artery. Injections of pterygospermin were made into the femoral vein and the effect on blood pressure studied and recorded. In view of the insoluble nature of the drug, all injections of the drug (as an emulsion in 50 per cent alcohol) were made slowly.

It was observed that in practically all the animals even small doses of pterygospermin reduced the blood pressure. Results of two typical experiments are given in plate 1. They show the effect of injecting 7.5 mg. of pterygospermin in two dogs. The marked fall in blood pressure (about 20 mm.) appears to be very persistent.

### Effect of respiratory system.

For these studies the tracheal pressure, in dogs and cats under paraldehyde anaesthesia was recorded with a Marey's tambour. Injections of pterygospermin were made as already indicated.



Chemotherapeutic investigations on pterygospermin.

It was observed that in most of the animals, there was depression of respiratory movements, immediately after injection when the dose was even 1 mg. per Kg. When, however, the dose was increased, the depression was complete and pronounced. The results of a typical experiment are shown in plate 1. It may be seen that when 7.5 mg. of pterygospermin were injected into a dog weighing 3.5 Kg. there was complete depression in the respiratory movements.

Effect on heart. Miocardiograph experiments.

The effect of the drug on the auricles and ventricles were investigated by the usual methods.

Injections of about 5 mg. of pterygospermin for animals weighing 3 Kg., it was observed in most cases depressed both auricles and ventricles. In plate 1 the results of one typical experiment are shown and it indicates clearly that 7.5 mg. of the drug depress both the auricles and ventricles.

Whether these observed effects are due either to the direct effect of the drug on cardiac muscles or are controlled by the vagus, could not be inferred from the data presented above.

Chemotherapeutic investigations on pterygospermin.

The results presented above on the properties of pterygospermin show it to be considerably active against a variety of micro-organisms, to be highly non-toxic and stable in presence of tissue fluids like blood. All these along with the stable nature of the antibiotic principle suggested, that it may be efficient in curing infections in animals. Experiments primarily of a preliminary nature were carried out to find the in vivo efficacy of the drug. These have given sufficient indications of its chemotherapeutic usefulness, and are recorded below.

Literature investigation revealed that there is practically no clearly defined method to find out the in vivo efficacy of any antibiotic principle. Broadly it may be said that the general principle outlining all the methods followed is the same. It consisted in finding the capacity of the drug to protect animals (usually mice) infected with virulent pathogenic micro-organisms. In details the methods have however differed considerably. Thus Robinson<sup>9</sup> has used considerably very small amounts of the organism for infection, viz.,  $0.5 \times 10^{-4}$  ml of a six hour broth culture for mice, whereas Salle et al<sup>10</sup> have used 0.1 to 0.2 ml of a 24 hour broth culture. The use of very small amounts for causing infection, by suspending the organism in resistance lowering agents like gastric mucin as is used by Robinson et al<sup>9</sup> in their investigations with S.aureus is perhaps apt to give exaggerated results regarding the in vivo efficacy of the drugs under investigation. The use of large inocula also, however, have got several inherent disadvantages like the effect of endotoxins on the animals.



For the investigations carried out on pterygospermin the following experimental procedure was adopted. A mouse virulent strain of S.aureus, inhibited by pterygospermin was used as the test organism. Lethal dose was defined as that amount of the organism which when injected into animals intraperitoneally, kills them within a period of 48 hours of injection.

To find the Lethal dose the following simple experiments were first carried out. To different groups of mice (ten in each group) differing amounts of a 6 hour broth culture of S.aureus grown in nutrient broth was injected intraperitoneally, and the time for 100% mortality determined. It was found that mice injected with 0.5 ml of the broth culture died within 24 hours, injected with 0.2 ml died within 48 hours and injected with 0.1 ml took 4 to 8 days for complete mortality. The lethal dose of the organism was hence taken as 0.2 ml of a 6 hour broth culture of the organism for the subsequent experiments.

Pterygospermin being not highly soluble the pH of blood it was thought that its absorption and excretion when injected subcutaneously in animals, may be slow. The effect of single doses of the drug in protecting mice from Staphylococcal infections in mice were hence first studied as described for bacitracin<sup>11</sup>. The following experimental procedure was followed.

White mice weighing approximately 25 to 30 gms. were taken and divided into groups of ten. All the mice were injected

intraperitoneally with 0.2 ml of a 6 hour broth culture of the organism. One group was kept as control, while members of the other groups received differing amounts of pterygospermin, subcutaneously, emulsified in water 0.2 ml being used for each mouse. The animals were all kept under observations for a period of ten days. The percentage surviving after that period were determined. The results of the investigations are given in table XXI.

Table XXI.

In vivo efficacy of pterygospermin (Single injection).

Group Number	Dose in per mouse	Dose in units per mouse	No. of mice	Total No. of animals dead after - days					Percentage mortality	
				1	2	3	4	6		8
1	Nil	Nil	10	6	10	10	10	10	10	100
2	10	3.0	10	1	2	4	4	4	4	40
3	25	8.0	10	1	2	2	2	2	2	20
4	50	16.0	10	1	1	1	1	1	1	10
5	75	25.0	10	-	-	-	-	-	-	Nil

The results presented in the above table clearly show that even single injections of the drug in small amounts is sufficient to protect mice from lethal infections of S.aureus.



In a second series of experiments the effect of repeated injections of small amounts of pterygospermin, given at intervals of 10 hours subcutaneously were studied. The total period of injections extended over about 60 hours. The experimental technique used for these investigations was the same as that used for finding the effect of single injections. In table XXII the results are presented.

Table XXII.

In vivo efficacy of pterygospermin  
(Repeated injections).

Group number	Dose in per injection	Dose in units per injection	Number of animals per group	No. of animals dead after ten days	Percentage survival
1	Nil	Nil	10	10	Nil
2	5	1.5	10	Nil	100
3	10	3.0	10	"	100
4	15	4.5	10	"	100

The results show clearly the remarkable in vivo activity of pterygospermin. Even 5 micrograms of the drug are sufficient to protect mice from lethal infections of S. aureus.

### Discussion.

A systematic investigation as suggested by Florey<sup>1</sup> has been carried out on pterygospermin the antibiotic principle isolated from the roots of Moringa pterygosperma.

It possesses considerably high antibiotic properties even against acid-fast organisms, and appears to be one of the most active plant products isolated so far. The antifungal properties of pterygospermin compare favourably with those of some of the most active naturally occurring antifungal agents so far isolated<sup>12, 13</sup>.

The remarkable antibacterial activity exhibited by pterygospermin in vitro coupled with its low toxicity necessitated investigations on the in vivo activity of the drug. The toxicity of the compound studied appears to be one of the lowest so far recorded for naturally occurring antibacterial agents of both microbial and plant origin. From the data compiled by Kavanagh<sup>14</sup> on the toxicity of antibiotics, it is found that other than penicillin and streptomycin only helvolic acid<sup>15</sup> and mycophenolic acid appear to have toxicity comparable to that of pterygospermin. Helvolic acid however, did not protect mice against experimental infections of pathogenic micro-organisms and damaged the liver tissue. Mycophenolic acid is not very highly active against bacteria even in vitro.

Thus it may be seen, that pterygospermin combines two of the most essential properties, viz., a high antibiotic activity



and low toxicity, required of antibiotic substances which may offer chemotherapeutic possibilities. The results of in vivo experiments carried out have given highly encouraging results. Thus  $2 \times 5$  to 10 of the drug injected at 10 hour intervals sufficed to protect animals from lethal staphylococcal infections. A rough estimate may be made of the chemotherapeutic index, based on the observations that the  $L.D_{50}$  by single injections of the drug is 300 mg per Kg. and the protecting dose for about 80% animals is about 25 micrograms per animal of approximately 25 gms. weight. The value of nearly 300 obtained for the chemotherapeutic index shows that pterygospermin in all probability should achieve chemotherapeutic importance.

Detailed investigations on the in vivo efficacy of the drug in curing infections of M.tuberculosis could not be undertaken. Nothing definite, could therefore be said regarding the possible role, pterygospermin might play in future, in the chemotherapy of acid-fast pathogens. From the available data on the in vivo efficacy of antibiotics in general, it may not be altogether wrong to assume that, pterygospermin might prove useful in curing such diseases as well. The fact that the cumulative toxicity of the drug is as high as 50 mg. per Kg. suggests the possibility of using high doses of the drug in cases where smaller ones prove inafficacious in the cure of acid-fast infections.

The remarkable synergistic effect of pterygospermin and penicillin mixtures, or mixtures of pterygospermin and streptomycin are also likely to be of importance. Combined

administration of two drugs, has been suggested and has been found useful as a method of over-coming the problem of drug resistance, particularly when continued administration of the drugs are essential. In a recent review Waksman<sup>17</sup> has suggested that antibiotic acting synergistically with drugs of proved value are also likely to achieve importance in future. Bearing in mind these facts it might be perhaps justifiable to assume that the combined administration of drugs like pterygospermin and streptomycin would be useful particularly in curing infections like M.tuberculosis.

#### Summary.

1. Pterygospermin is active in presence of blood and gastric juice, but loses its antibacterial properties in presence of pancreatic juice. This appears to be due to the alkalinity of the pancreatic juice.
2. The cumulative subcutaneous toxicity of pterygospermin has been studied. 100% mortality occurred with when the dose was 55 mg.per Kg. and the L.D.<sub>50</sub> was found to be 50 mg.per Kg.
3. The acute subcutaneous toxic dose of pterygospermin at which 50% of the animals die appears to be 300 mg.per Kg.
4. In vivo studies with S.aureus as the test organisms show that pterygospermin protects mice when the dose of the drug is about 5 per mouse and when it is administered at intervals of about 10 hours for a period of 60 hours.



References.

1. Florey H.W. (1946) Brit.Med.Bull.,4, 248.
2. Benedict R.G.,et al (1946) J.Bact.,51, 291.
3. Coulthard C.E.,et al (1945) Biochem.J. 39, 24.
- 4.
5. Tompsett R.,et al (1947) J.Bact.,53, 581.
6. Maney & Kuever (1941) J.Amer.Pharm.Assn.,30, 276.
7. Rake, G.et al. (1945) Amer.J.Med.Sci.,210, 61.
8. Salle A.J & Jann G.J. (1945) Proc.Soc.Exptl.Biol.& Med.,60,60.
9. Robnson, H.J.,et al (1942) J.Pharm & Exptl.Therap.76, 316.
10. Salle, A.J & Jann G.J. (1946) Proc.Soc.Exptl.Biol.& Med.63, 519
11. Johnson, B.A.,et al (1945) Science, 102, 376.
- 12.
13. Irving G.W. Jr.,et al (1946) J.Bact.,52, 601.
14. Kavanagh F. (1948) "Recent Advances in Enzymology"  
Vol.VIII, p 461.

CHAPTER - III.



STUDIES ON THE ANTIBACTERIAL PRINCIPLE OF ALLIUM SATIVUM.

Introduction.

Allium sativum (garlic) is very commonly found all over India. It either grows wild or is extensively cultivated, particularly due to its use as spice. Garlic has been held in great repute by the ancient physicians of India. It is considered to be hot and stimulant and is administered in fevers, coughs and other debilitating conditions. It has also a reputation as a febrifuge~~z~~ in intermittent fevers.

The juice of the cloves of garlic has been employed by several native physicians of this country, as an antiseptic in ulcerated surfaces and wounds with very satisfactory results. Definite improvement in the condition of infected wounds can be observed within 24 hours of washing with garlic juice which has been diluted four times with water. Minchin<sup>1</sup> has stated that he has used garlic preparations in the treatment of wounds and in foul ulcers for a period of about fifteen years and obtained very satisfactory results.

The internal administration of the juice of Allium sativum is also very common among several indigenous systems of medicine in this country, particularly in the treatment of coliform and tubercular infections. According to Minchin<sup>1</sup> garlic is a remedy for several diseased conditions. He considers it a prophylactic for typhus, typhoid and diphtheria. Crossman<sup>2</sup> has suggested that if given in sufficient doses, garlic is an invaluable remedy in

the treatment of pneumonia. He has used it for two years in the treatment of lobar pneumonia and according to his published reports<sup>3</sup> in no instance has it failed to bring down the temperature, pulse and respiration to normal in about forty-eight hours. In tubercular infections Minchin advocates the use of garlic, which often diminishes cough and expectoration.

Kitagawa and Amano<sup>4</sup> have carried out experiments to find out the action of garlic on B.coli in vitro and E.typhosus in vivo in mice. Their results indicate that garlic contains a strong antiseptic substance. They concluded that the antiseptic action of garlic is due to the unstable sulphur in the alkylpolysulphides from glucosides by enzyme action. Umeroi<sup>5</sup> has claimed that the active principle in the ether extracted oil of Allium sativum is allyl polysulphide. Jacobsen<sup>6</sup> has suggested that the antibacterial activity of garlic can be explained as due to the action of a bacteriophage present in it. Vollarth et al<sup>7</sup> have reported that acrolein present in garlic is responsible for its high bactericidal properties. Lindgreen and co-workers<sup>8,9</sup> have found that vapours of crushed garlic are germicidal to several micro-organisms, including acid-fast and non-acid-fast Mycobacterium leprae. Tokin<sup>10</sup> has claimed the therapeutic virtues of garlic to chemically unidentified substances designated as phytanocides. Cavallito and Bailey<sup>11</sup> have shown, that the oil of garlic obtained by steam distillation of the crushed cloves and subjected to subsequent fractional distillation according to Semmler<sup>12</sup> failed to show any antibiotic activity when tested



by the cylinder-plate method. It was thus established that the natural diallyl sulphides and diallyl polysulphides of the oil of garlic have no appreciable antibacterial properties. Kedling<sup>13</sup>, Bocker<sup>14</sup> and Allweiss<sup>15</sup> have suggested that the aromatic oils of garlic are responsible for its antiseptic properties.

Cavallito and Bailey<sup>11</sup> have isolated a new compound and named it allicin. (Even though they have subsequently withdrawn this name<sup>16</sup>, the same is retained here to indicate the antibacterial principle of Allium sativum). Their product was a colourless oil obtained by the steam distillation of the alcohol concentrates of garlic, the temperature and pressure being kept low during the preparation. Based essentially on the alkaline hydrolysis, reaction with cysteine, polarographic measurements and absorption spectrum they suggested<sup>17</sup> to the compound the structure

$$R - S - S - R$$

The alternative structure  $R - S - O - S - R$  was however not eliminated (R in the above formulae stands for allyl grouping).

From the foregoing, it is easily seen that with the possible exception of the work of Cavallito and Bailey<sup>11</sup> none of the other reports explain correctly the therapeutic virtues attributed to garlic, particularly against Gram-positive and Gram-negative pathogens. They did not extend their investigations to acid-fast micro-organisms. They also do not seem to have studied the in vivo effect of allicin. Since preliminary investigations carried out in this laboratory showed the presence of a highly active antibacterial agent in garlic systematic investigations

on the in vitro and in vivo aspects of the antibiotic were carried out and the results are presented. (After completion of this work Machado et al<sup>18</sup> have published their investigations on garlic in which they claim to have isolated a new antibiotic extract from garlic, which they have named as garlicin, and which they find active both in vitro and in vivo against bacteria of the colon group. Their compound is also highly non-toxic).

### Experimental.

Preliminary investigations. A series of simple experiments were first carried out to find the solubility and stability of the active principle in different solvents, its stability etc., in order to evolve out a simple method for the preparation of the active material. Cloves of garlic cleared of root stumps and dry outer covering was always used for these experiments.

Garlic juice obtained by pressing, crushed garlic had when fresh, an antibacterial activity of 30 mm. when tested by the cylinder-plate method already described (refer page ). This activity of the juice was however partly lost either when kept at the room temperature or <sup>when</sup> heated even to about 50°C. Experiments like hot extraction and steam distillation of garlic were not therefore tried, to isolate the antibiotic principle.

It was found during the preliminary investigation that the antibacterial principle of garlic, was best extracted from the cloves by alcohol, even though it was soluble in several solvents like benzene, chloroform and ether. The alcoholic extract



of garlic when diluted one in ten showed a halo of diameter 18 mm.

Preparation of Allicin. For the purification and concentration of the antibiotic, therefore, experiments based essentially on the fractionation of the garlic juice or purification of the alcoholic extract were carried out. All extracts showing antibacterial potencies of less than 13 mm. when tested by the cylinder-plate method were rejected, during these experiments.

Method I. It has already been stated that the crushed juice of garlic showed considerable antibacterial activity. The present method is based essentially on the fractionation of the juice of garlic.

500 gms. of garlic were crushed in an end runner and pressed in a claver press at a pressure of 2,000 lbs. per square inch. The juice so obtained showed a high antibiotic activity, whereas the alcoholic extract of the crushed residue showed only very slight activity. The juice was mixed with ten volumes of alcohol and left over-night in the cold and then filtered. The filtrate was evaporated to a very small volume under reduced pressure at a temperature not exceeding 30°C. The residue after the removal of alcohol consisted of a mixture of an oily substance and a sticky gum. This residue was extracted with 200 ml of chloroform in which the gummy material was insoluble. After drying the chloroform extract over anhydrous sodium sulphate for one hour in the cold and removal of the solvent an oil weighing 677.5 mgm. was obtained. This oil in 1 mgm. concentrations

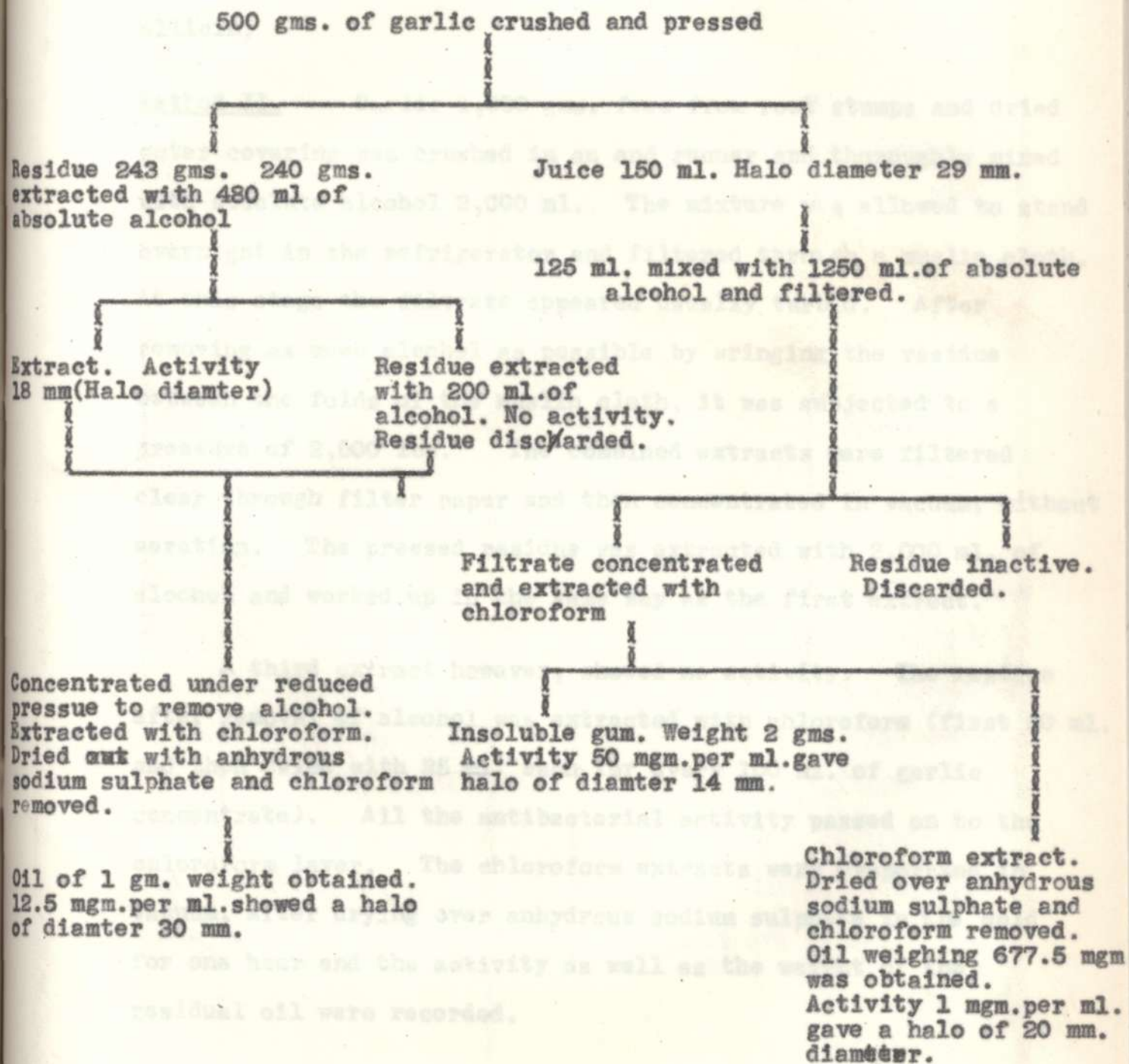
showed a diameter of 20 mm. by the cylinder-plate method. The gummy material weighed about 2 gms. and was soluble in water. It, however, showed only very slight activity 50 mgm. per ml. giving a halo of diameter 14 mm.

The residue after pressing the cloves, was extracted with 420 ml. of alcohol overnight in the cold. This alcoholic extract showed slight antibacterial activity. A ~~second~~ second extract of the residue with about 300 ml. of alcohol failed to show any activity. The combined alcoholic extracts were distilled under reduced pressure and the concentrate taken up in about 75 ml. of chloroform. After drying the chloroform extract and removing the solvent, furnished an oil weighing 0.5 gms. which showed considerable antibacterial activity. (1 mg. per ml gave 20 mm.). The residue left behind after chloroform extraction was found to have no activity at all, indicating thereby that all the antibiotic passes into the chloroform layer. A schematic representation of the method of fractionation of garlic and concentration of the antibacterial principle are brought out clearly in figure I below.

During the course of these experiments for finding out the activity by the cup-plate method, all solvents were removed and the residues taken up in equal amount of water or alcohol before they were poured in the cups. Cups with alcohol did not show any activity and hence the above procedure.



Figure I.



From the foregoing experiments it is clear that a great part of the antibiotic activity comes off in the juice. Further it has been found possible to extract all the antibiotic principle from

the aqueous layer by chloroform. Based on these observations the following procedure was employed in routine preparation of allicin.

Method II. Garlic 1,000 gms. free from root stumps and dried outer covering was crushed in an end runner and thoroughly mixed with absolute alcohol 2,000 ml. The mixture was allowed to stand overnight in the refrigerator and filtered through a muslin cloth. At this stage the filtrate appeared usually turbid. After removing as much alcohol as possible by wringing the residue between the folds of the muslin cloth, it was subjected to a pressure of 2,000 lbs. The combined extracts were filtered clear through filter paper and then concentrated in vacuum, without aeration. The pressed residue was extracted with 2,000 ml. of alcohol and worked up in the same way as the first extract.

A third extract however, showed no activity. The residue after removal of alcohol was extracted with chloroform (first 50 ml. and then twice with 25 ml. each for every 100 ml. of garlic concentrate). All the antibacterial activity passed on to the chloroform layer. The chloroform extracts were evaporated in vacuum, after drying over anhydrous sodium sulphate in the cold for one hour and the activity as well as the weight of the residual oil were recorded.

It has been a common observation that when the oil was allowed to stand in the refrigerator for some days, there was always a separation of solid particles with impairment in



potency. If, however, the oil was dissolved in alcohol, such that 1 ml. of the solution contained about 100 mgm. of oil, it was observed that separation of the solid resulting in loss in activity of the compound was considerably delayed. Alcohol seems to exert a stabilising influence on the antibiotic. In table XXIII results of three typical experiments are given.

In general routine work, when allicin was prepared for experimental work, garlic was extracted twice with absolute alcohol in the proportion given above, filtered, pressed and the combined filtrate after clarification, concentrated in vacuum and the ~~residue~~ concentrate extracted with chloroform thrice as indicated above.

In order to ascertain the activity ~~of~~ and purity of samples prepared by the method given above a curve similar to the standard curve used for penicillin assays was drawn, by plotting amount of the antibiotic against halo diameter. The samples obtained by the first method described above was used for the standard curve which is shown in Figure II. In table XXIV, the halo diameters of emulsions containing differing amounts of the drug against S.aureus and B.subtilis are given.

Table XXIII.

Preparation of Alliecin.

Weight of garlic taken	Volume of alcohol for extract I	Volume of chloroform used	Weight of oil in gms.	Vol. of alcohol for extract II	Wt. of oil from extract II in gms.	Total yield in gms.	Activity of the oil
500 gms.	1000 ml. Extract had a volume of 860 ml. and activity of 31 mm. Volume after removal of alcohol 180 ml.	Extracted with 180 ml. The residue showed activity of 12 mm.	1.027	1000 ml. Volume of extract 870 ml. Activity 18.5 mm.	0.79	1.817	mg. per ml had and activity of 20 mm.
500 gms.	1000 ml. Volume of extract 960 ml. Activity 31 mm.	160 ml. Aqueous phase after extraction had activity 12 mm.	1.10	1000 ml. Volume of extract 850 ml. Activity 18.0 mm.	0.68	1.78	- do -
500 gms.	1000 ml. Volume of extract 900 ml. Activity 31 mm.	200 ml. Activity of aqueous phase after extraction 13 mm.	1.18	1000 ml. Volume of extract 870 ml. Activity 18.0 mm.	0.54	1.72	- do -



Table XXIV.

Antibiotic activity of allicin by cup-plate method.

Amount of allicin per ml.	Halo diameter in mm.	
	B. subtilis	S. aureus
5.0 mg.	35.5	37.5
2.0 "	27.5	32.5
1.0 "	21.0	27.5
0.5 "	17.5	23.5
0.25 "	15.0	21.5
0.10 "	13.5	19.5
0.05 "	12.0	17.5

Some properties of allicin.

Before taking up investigations on the antibacterial, and biological aspect of allicin isolated, studies of lesser importance, viz., on certain physical properties of the compound, were carried out primarily to ascertain the identify of the compound with that of the preparation of Cavallito et al<sup>11</sup>.

In table XXV a short summary of the properties of the sample prepared and used in these investigations are compared with those of Cavallito and Bailey<sup>11</sup>.

Table XXV.

## Properties of Alliecin.

Property	Alliecin prepared as given above	Cavallito's preparation
Colour	Pale yellow oil	Colourless oil
Odour	Of garlic	Of garlic
Refractive index	1.556	1.561
Irritability	A skin irritant	A skin irritant
Stability in alkaline reactions	Unstable	Unstable
Elements present other than carbon, hydrogen and oxygen	Sulphur	Sulphur
Amount of sulphur	39.6	39.69 to 40.90
Solubility	Slightly soluble in water. Highly soluble in alcohol, ether, benzene and chloroform	Soluble in water about 2.5%. Fairly soluble in alcohol, benzene, chloroform and ether



Effect of pH on the antibacterial activity of allicin. The reaction of the medium has been often times found to affect the antibacterial activity and stability of antibiotic agents. Thus, streptomycin acts best at an alkaline pH and penicillin is destroyed at acidic and alkaline ranges. Before finding the antibacterial properties of allicin experiments were carried out to find out the stability of allicin at different pH ranges and also to find out the optimum hydrogen ion concentration required for it to act best.

To find out the stability of allicin in different hydrogen ion concentration following procedure was adopted. Known amounts of allicin were incubated after emulsifying it, in buffers and broth of known pH for fixed intervals of time and the antibacterial activity of the emulsions determined by the serial dilution method using S.aureus as the test organism. In table XXVI, the results of these experiments are given.

From the table it is easily seen that the antibacterial principle of garlic is inactivated at alkaline pH ranges, 24 hours incubation resulting in complete inactivation of the compound.

Similar experiments were also carried out to ascertain the optimum pH at which allicin inhibits the growth of micro-organism. Broth, the pH of which was adjusted to 6.0, 7.0 and 8.0 were used as media. The antibacterial properties of the drug were studied against two organisms; S.aureus and E.coli. The results of the experiments are given in table XXVII.

Table XXVI.

Effect of pH on the antibacterial activity of alliin.

pH	Period of incubation with given pH											
	0 hours Dilution of alliin (in thousands)				3 hours Dilution of alliin (in thousands)				24 hours Dilution of alliin (in thousands)			
	5	10	20	50	5	10	20	50	5	10	20	50
In buffer												
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	+	+	+	+	+
In broth												
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	+	+	+	+	+



Table XXVII.

## Antibacterial activity of allicin at different pH.

pH of broth	S. aureus				B. coli			
	Dilution of allicin (in thousands)				Dilution of allicin (in thousands)			
	5	10	20	50	5	10	20	50
5.5	-	-	-	-	-	-	-	-
6.5	-	-	-	-	-	-	-	-
7.0	-	-	-	-	-	-	-	-
8.0	+	+	+	+	+	+	+	+

The above results show clearly that allicin exhibits its activity against gram-positive and gram-negative micro-organisms, best in broth when it is acidic, whereas it seems to be inactivated to a considerable extent at alkaline ranges. In experiments carried out to find the antibacterial spectrum of allicin the pH of broth was therefore adjusted to  $6.8 \pm 0.1$ .

concentration of 1 in 30,000 of allicin which resulted in growth

Antibacterial spectrum of allicin. The relative effects upon different bacteria of any antibiotic agent is called its antibacterial spectrum. The minimum amount of the antibacterial principle necessary to inhibit different micro-organisms is generally estimated by the serial dilution method.

Different amounts of the antibiotic principle are taken in known amounts of sterile nutrient broth and the tubes inoculated with a 24 hour broth culture of the strain whose susceptibility to the antibiotic is to be studied. At the end of 18 or 24 hours the tubes are observed, for growth. In general, there will be a region where there is complete absence of growth, a region where the growth is partial and a region in which the growth in tubes will be comparable to those in the control.

For finding the antibacterial activity of allicin, to tubes containing sterile nutrient broth 4.5 ml each, was added 0.5 ml of allicin solution. The amount of allicin in 0.5 ml of sterile water was so adjusted to get a final dilution of 1 in 20; 40; 80 and ~~100x000~~ 100 thousands. The tubes were then inoculated with 0.01 ml of an 18 hour broth culture of the test organisms. All the tubes were incubated the temperature of 37°C. and observed after 18 hour incubation for growth of the test organisms. A concentration of 1 in 30,000 of allicin which permitted no growth was tested for stasis by removing 0.1 ml from the tube and adding it to 5 ml of fresh broth. All organisms thus tested, grew well proving that allicin is essentially bacteriostatic and not bactericidal.



For acid-fast organisms, however, the following procedure was adopted. Flasks containing Long's media (with ammonium malate instead of asparagine) containing allicin in the form of an emulsion, so as to give concentrations of 2mgms, 6 mgms., 12 mgms and 18 mgms. per 100 ml of media were inoculated with 2 loopfuls of M.tuberculosis (human strain B 52 H<sub>1</sub> Kasauli) and M.Phlei respectively. The flasks were incubated at 37°C. In the case of flasks inoculated with M.tuberculosis, no growth was observed in the presence of allicin, after three weeks, whereas in the control the bacillus grew profusely. In the 2 mgm. flask, a slight curdy submerged growth was observed from the fourth week onwards. In the case of M.phlei, there was no growth even after five days whereas in the control there was rapid growth from the third day onwards.

The results of experiments with different micro-organisms, ie., the antibacterial spectrum of allicin is brought out in table XXVIII.

With a view to ascertain the effect of higher concentrations of allicin on M.tuberculosis, 17 ml of Long's medium containing 6.5 mgm. of allicin in the form of emulsion, was inoculated with three loopfuls of the bacillus from a slant and incubated for 24 hours, along with a control similarly prepared but without the antibiotic. Subcultures were then made from the two flasks on Dorset's egg medium. After four weeks no growth was observed in the subcultures from the allicin flask, while there was good growth in the subcultures from the control.

Table XXVIII.

## Antibacterial spectrum of allixin.

Name of organism	Dilution of allixin in medium in thousands					Control
	1/20	1/40	1/50	1/60	1/80	
<u>B. subtilis</u>	-	-	-	+	++	++
<u>S. aureus</u>	-	-	-	+	++	++
<u>B. aerogenes</u>	-	-	-	+	++	++
<u>B. coli</u>	-	-	-	+	++	++
<u>B. paratyphosus A</u>	-	-	-	+	++	++
<u>B. paratyphosus C</u>	-	-	-	+	++	++
<u>B. enterditis</u>	-	-	-	+	++	++
<u>B. dysenteriae Flexner</u>	-	-	-	+	++	++
<u>M. phlei</u>	-	-	-	+	++	++
<u>M. tuberculosis</u>	-	-	-	+	++	++

It is clear from table XXVIII that allixin is able to inhibit the growth of many of the Gram-positive, Gram-negative, and acid-fast bacilli at a dilution of 1 in 50,000.



## Table XXIX

The antibacterial properties of allicin given above, are found however to be considerably lower than those recorded by Cavallito and Bailey<sup>11</sup>, even though in several other properties like sulphur content and refractive index both resemble closely. The difference in the antibacterial properties of the two preparations may be due, either to the resistance of the micro-organisms used in the present studies, to allicin as compared with those used by Cavallito et al<sup>11</sup> or due to the difference in the inoculum, since their inoculum in finding the antibacterial activity of allicin was smaller than the one used in the present experiments. The latter explanation becomes important, when we take into consideration the fact that the activity of several antibiotics is dependent on the amount and age of the inoculum. Experiments were hence undertaken to find the effect of differing amounts of inocula on the antibiotic activity of allicin against S.aureus and B.coli.

The usual serial dilution method was employed. The results are given in table XXIX. They show clearly that the amount of inoculum does have a definite effect on the antibacterial activity of allicin. It is therefore highly probable that the low antibacterial activity of the present preparation may be due to the comparatively high inoculum used in finding out the antibiotic properties of the compound. It may be mentioned that Cavallito and Bailey<sup>11</sup> have recorded for their preparation antibacterial property about 2.5 times that of the present preparation, viz., inhibiting micro-organisms at dilutions of 1 in 125, 000.

Table XXIX.

Effect of inoculum on the antibiotic activity of alliin.

Amount of inoculum in ml. per 5 ml.	<u>S.aureus</u>				<u>B.coli</u>			
	Dilution of alliin in thousands				Dilution of alliin in thousands			
	10	20	40	80	10	20	40	80
0.05	-	+	+	++	-	+	+	++
0.02	-	-	+	++	-	-	+	++
0.01	-	-	-	++	-	-	-	++
0.005	-	-	-	+	-	-	-	+

Antifungal properties of alliin.

In view of the high antibacterial properties of the active principle of garlic, its effect on the growth of some of the more common fungi available in this laboratory was studied. The experimental methods were similar to those used for finding the antibacterial activity. After inoculation, the tubes were however, kept in a slanting position (about  $10^\circ$  to the horizontal). The growth of the moulds was observed for a period of about three days instead of 24 hours as in the case of the bacteria. The results of these studies are brought out in table XXX. They show that alliin inhibits the growth of the fungi studied, at dilutions of about 1 in 25,000.



Table XXX.

## Antifungal properties of allicin.

Name of organism	Name of medium	Dilution of antibiotic in medium (in thousands)				Control
		1/10	1/25	1/50	1/75	
<u>Aspergillus fumigatus</u> Fresenius	Czapeck Dox at pH 5	-	-	++	++	++
- do -	Nutrient broth at pH 7	-	-	++	++	++
<u>A. ustus</u>	Czapeck Dox	-	-	++	++	++
<u>P. cyclopium</u>	- do -	-	-	++	++	++
<u>P. notatum</u>	= do -	-	-	++	++	++
<u>A. griseus</u>	Nutrient broth at pH 7	-	++	++	++	++

### Studies on the mode of action of allicin.

As already indicated earlier in this thesis, of the many theories suggested to explain the mode of action of antibiotic, two stand out as most prominent. The first of these suggested by Cavallito et al<sup>18</sup>, based on the observation that several antibacterial agents are inactivated by compounds having reactive sulph-hydryl groups. The second a more recent one by Gale<sup>19</sup> based on the observation that amino acid assimilation by microbes is prevented in presence of antibacterial agents like penicillin.

Based on the specificity and speed of the reaction with thiols, the antibiotic agents have been divided into three groups by Cavallito<sup>20</sup>. (i) which are rapidly reactive towards many sulph-hydryl compounds, eg., gliotoxin and allicin; (ii) which react with most sulph-hydryl compounds, but with some difference in rate eg., pyocyanine; (iii) which react slowly with more specific thiol groups eg., penicillin and streptomycin.

The antibacterial activity of the antibiotics has been explained as due to their reaction with thiols. The compounds may react with essential sulph-hydryl groups of bacterial enzymes or with sulph-hydryl groups in cysteinyl residues of polypeptides, preventing the formation of protein and thus inhibiting growth<sup>20</sup>. Experiments to find out the effect of allicin on some enzymes having thiol groups as active groupings were hence carried out. Papain the proteolytic enzyme from Carica papaya, beeta amylase from sweet potatoes and ficus rennet from Ficus carica were used as sulph-hydryl enzymes.



Effect of allicin on the milk-clotting activity of papain.

Papain isolated from the fresh latex of Carica papaya was used. Balls and Lineweaver<sup>21</sup> and Ganapati and Sastri<sup>22</sup> have shown that the milk-clotting activity of this enzyme is closely associated with the presence of sulph-hydryl groups in it.

One ml. of a solution of papain was mixed with an equal amount of allicin emulsion. Thus in different tubes the same amount of papain was mixed with different amounts of allicin. The tubes were then incubated in a water bath at a temperature of 37°C. At the end of five, fifteen, and thirty minutes, the enzyme activity was estimated. For this 0.2 ml. of the reaction mixtures were allowed to act on 2 ml. of a 10 per cent solution of Kilm milk powder which was also incubated at 37°C. and pH of which was adjusted to 4.6 by acetate buffer. The clotting time was noted according to Balls and Hoover<sup>23</sup>. Controls without papain and without allicin were also simultaneous studied. In table XXXI the results of the studies on the effect of allicin on the milk-clotting activity of papain are given.

The inhibitory action of allicin on papain is seen from the results presented in table. Even in dilutions of 1 in 300,000 after thirty minutes incubation about 50 per cent of the enzyme activity is lost, whereas at concentrations of about 1 in 20,000 the enzyme is almost completely inactivated by the antibiotic within five minutes.

Table XXXI.

Effect of allicin on the milk-clotting activity of papain.

Dilution of allicin in allicin - enzyme mixture	Time in seconds for clotting after incubation for			
	0 minutes	5 minutes	15 minutes	30 minutes
Control without allicin	29	29	29	29
1 in 1,000,000	29	29	29	29
1 in 300,000	29	48	50	53
1 in 200,000	29	50	52	57
1 in 160,000	29	53	57	58
1 in 100,000	29	85	90	100
1 in 80,000	29	100	117	167
1 in 40,000	29	197	240	273
1 in 20,000	29	260	290	343
1 in 10,000	29	290	340	370
No enzyme	No clotting even after 500 seconds			

Effect of allicin on beata amylase. Beeta amylase the amyolytic enzyme from sweet potatoes was the next to be studied. Weill and Cladwell<sup>24</sup> have adduced evidence for the presence of sulph-hydril groups in the enzyme, which are essential for maltose formation from amylose by its action.



## Table XXXI.

5 ml portions of the enzyme solution containing 1 mgm. of beeta amylase per ml mixed with 2.5 ml of acetate-acetic acid buffer at pH 5.7 were taken in different tubes. 2.5 ml of allacin emulsion containing different amounts of allacin was then added to the tubes. Thus different (but known) concentration of allacin in 10 ml of the enzyme solutions was obtained. The tubes containing the mixtures of antibiotic and enzyme were then incubated at 37°C. At the end of fixed intervals 1 ml. of the mixture was allowed to act on 4 ml. of an amylose solution, for 20 minutes at 37°C. At the end of this period 5 ml. of alkaline ferricyanide was added to 1 ml. of the above mixture and the amount of maltose formed estimated by the modified Hagedorn and Jenson method<sup>25</sup>. Two controls were also run simultaneously. In one allacin was not added. In the second the enzyme was inactivated by boiling it for fifteen minutes before the addition of allacin. The results of these investigations are presented in table XXXII.

The inactivation of beeta amylase by allacin does take place. However, it appears to be considerably lesser than what was observed in the case of the proteolytic enzyme papain. In order to ascertain the above findings the effect of allacin on yet another enzyme, having sulph-hydryl groups was studied.

Table XXXII.

Effect of alligin on the amylolytic activity of beeta amylase.

Dilution of antibiotic in alligin - beeta amylase mixture	Amount of maltose formed in mgm. after incubating the mixtures for - minutes			
	1	5	15	30
No alligin	3.047	3.047	3.047	3.047
1 in 200,000	2.85	2.815	2.766	2.669
1 in 40,000	2.789	2.749	2.676	2.469
1 in 20,000	2.749	2.667	2.275	2.269
No enzyme	Nil	Nil	Nil	Nil

Effect of alligin on Ficus-enzyme. This enzyme is isolated from the latex of Ficus carica (common fig) and has been found to possess high milk-clotting properties. Activation studies using glutathione, cysteine and other thiol reagents and reversible inhibition by Copper and maleic acid support the sulph-hydryl nature of the enzyme<sup>26,27</sup>. Experiments were hence carried out to find the effect of alligin on the milk-clotting activity of Ficus carica enzyme. The method similar to that previously described for the alligin-papain system was used, and the results are presented in table XXXIII.

The effect of alligin on this enzyme is very similar to that on papain, its milk-clotting activity being inhibited considerably even at very high dilutions.



Table XXXIII.

Effect of allicin on Ficus - enzyme.

Dilution of allicin in mixture	Time for clotting in seconds after incubation for - minutes			
	0	5	15	30
Control without allicin	27	27	27	27
1 in 500,000	27	27	27	29
1 in 250,000	27	38	48	55
1 in 150,000	27	45	55	61
1 in 50,000	27	137	187	211
No enzyme	No clotting even after ten minutes			

The reactive group in allicin, therefore, is all probability  
The reactive group of allicin. With a view to ascertain the  
 reactive groups in allicin  $R - S - S - R$  where R stands for allyl,  
 where there are two reactive groups viz., the double bonds of the  
 allyl groups and the reactive oxygen, the effect of two allyl  
 compounds (allyl alcohol and allyl bromides) when present in  
 small amounts, on the enzymes, papain and ficus-enzyme was  
 investigated. Known amounts of the two compounds were mixed  
 with papain and ficus-enzyme, incubated for one hour and the milk-  
 clotting activity of the mixtures determined. The results are  
 presented in table XXXIV.

Table XXXIV.

The effect of allyl alcohol and bromide on papain and Ficus-rennet.

Name of compound	Dilution	Clotting time in seconds for papain	Clotting time in seconds for Ficus-enzyme
Allyl alcohol	N11	30	29
	1 in 5,000	30	29
	1 in 1,000	30	29
Allyl bromide	N11	30	29
	1 in 5,000	30	29
	1 in 1,000	30	29

The reactive group in allicin, therefore, in all probability is - S - S - which acts by virtue of its ability to inhibit the sulph-hydryl enzymes of bacteria.

#### Discussion.

The antibiotic principle of garlic, the preparation and properties of which have been described, appears to be the same as the one isolated and studied by Cavallito et al<sup>11</sup>. The sulphur content, and refractive index of both the preparations are same. The antibacterial activity (as estimated by the cup-plate method) of the two compounds also appears to be the same. It may thus be safely assumed that the antibacterial principle of garlic isolated



during these investigations is the same as the one described by the American workers. However, the antibacterial activity of the two preparations, when studied by the serial dilution method appears to be highly different, the present preparation ~~method~~ inhibiting organism at dilutions of 1 in 50,000, whereas the preparation of Cavallito et al<sup>11</sup> inhibited pathogens even at dilutions of 1 in 125,000. The results presented, after some explanation for these facts. Thus it may be pointed out that the antibacterial properties of allicin seem to depend on the amount of inoculum, and Cavallito's inoculum when testing the antibacterial properties by the serial dilution method, was considerably smaller than the one used in the present investigations.

The yield obtained by the present method is much higher than that of the American workers. They isolated only 1.5 gms. of the oil from 1 Kg. of garlic, whereas by the methods described herein, the yield of the oil is as high as about 3.5 to 4 gms. per Kg. It is worthwhile mentioning in this connection that the theoretical amount of allicin present in garlic is about 4.6 gms. per Kg. when calculated from the data presented by Cavallito (viz., 2.5 to 4 mgm. per ml. for the alcoholic extract from 4 Kg. of garlic in 5,200 ml.) and about 5 gms. per Kg. when calculated according to the present findings. The percentage yield by the method described above, is approximately 70 to 80.

Investigations on the mode of action of the drug have also given some valuable results. Allicin inactivates the enzymes,

papain, beeta amylase and ficus-rennet, all having sulph-hydryl as prosthetic grouping. It is hence highly probable that the bacterial growth is prevented by this compound, by inhibiting sulph-hydryl enzymes in them. The reactive group in alliin as already pointed out appears to be - S - S - and not the double bond of allyl groupings.

#### Summary.

1. An easy method for the preparation of alliin, the antibiotic agent of garlic has been evolved out, the yield being about 70 to 80 per cent.

2. It is easily destroyed at alkaline pH, and is highly unstable. It can best be preserved in the cold as alcoholic solution.

3. Alliin inhibits the growth of several Gram-positive, Gram-negative and acid-fast pathogens at concentrations of about 20 per ml. It has got fairly high antifungal properties as well.

4. Alliin inhibits the activity of several sulph-hydryl enzyme even at dilutions of about 1 in 300,000 and in all probability acts by inhibiting similar enzymes, which play important roles in cellular metabolism. This activity of alliin appears to be due essentially to the - S - S - group in it.



## References.

1. Minchin (1916) Med.Press and Circ. June 1913.
2. Crossman (1918) Quoted in Med.annual 1918.
3. Crossman (1927) J.Biol.Chem., 102, 737.
4. Kitagawa & Amano (1936) Amer.Chem., Abstracts, 30, 3019.
5. Umeroi (1936) Chem. Abstracts, 24, 2191.
6. Jacobsen (1937) Chem. Abstracts, 31, 6689.
7. Vollarth et al (1937) Proc.Soc.Exptl.Biol & Med., 36, 55.
8. Lindgreen et al (1936) Proc.Soc.Exptl.Biol & Med., 35, 477.
9. Lindgreen et al (1937) Food Research, 1, 163.
10. Tokin (1944) Amer.Rev.Sov.Med., 1, 237.
11. Cavallito C.J. & Bailey J.H. (1944) J.Amer.Chem.Soc., 66, 1950.
12. Semmler (1893) Archiv.der Pharmazie 230,434..
13. Kedhing F. (1939) Angew Botan. 21, 1-45.
14. Bocker O.E. (1939) Z.Hyg.Infektious.Krankh, 121, 166.
15. Allweiss P. (1940) Z.Hyg.Infektious.Krankh, 122, 383.
16. Cavallito et al (1945) J.Amer.Chem.Soc., 67, 1032.
17. Cavallito et al (1945) J.Amer.Chem.Soc., 66, 1952.
18. Machado et al (1948) Anais.Paulistas de Medicina e Cirurgia, 55, 93.
- 19(a) Cavallito C.J., et al (1945) J.Bact. 50, 61.
19. Gale E.F. (1946) Nature 158, 676.
20. Cavallito C.J., et al (1946) J.Biol.Chem., 165, 29.
21. Balls & Lineweaver (1939) J.Biol.Chem., 130, 669.

22. Ganapathi & Sastri (1940) *Current Science*, 9, 413.
23. Weil C.E. & Cladwell  
M.L. (1945) *J.Amer.Chem.Soc.*, 67, 214.
24. Balls and Hoover (1937) *J.Biol.Chem.* 121, 737.
25. Hanes C.S. (1929) *Biochem.J.* 23, 99.
26. Krishnamurti C.R.et al (1947) *Science & Culture*, 13, 204.
27. Krishnamurti C.R.et al (1949) *Ind.J.Dairy Sci.*, 2, 19.



CHAPTER - IV.

PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES ON ALLICIN.

Sodium chloride	1.400 gms.
Potassium <u>Introduction.</u>	0.500 "
Calcium chloride	0.500 "

Data presented in the previous chapter have shown that alliin the antibacterial principle of garlic exhibits pronounced antibacterial properties towards several pathogenic micro-organisms, including acid-fast ones. These properties encourage speculation concerning its possible therapeutic applicability in human and animal infections particularly of Gram-negative pathogens which are resistant to many of the present day drugs, including penicillin. A series of experiments were carried out to explore the chemotherapeutic applicability of alliin.

Experimental.

Effect of artificial gastric and pancreatic juice on alliin. Before finding the toxicity of alliin when administered orally, it seemed essential to investigate regarding the stability of the drug in presence of gastric juice and pancreatic juice. Studies described previously have shown that the antibiotic is inactivated at alkaline pH and hence it is very likely that it may be inactivated in presence of pancreatic juice. For confirmation however, the stability of alliin in presence of artificial gastric and pancreatic fluids prepared according to the Maney and Kuever<sup>1</sup> and of the following composition was studied.



## Artificial gastric fluid.

Sodium chloride	...	1.400	gms.
Potassium chloride	...	0.500	"
Calcium chloride	...	0.060	"
Hydrochloric acid 36%	...	6.944	"
Pepsin	...	3.2	"
Distilled water to make 1,000 ml.			

## Artificial pancreatic fluid.

Pancreatin	...	2.8	gms.
Sodium bicarbonate	...	15.0	"
Distilled water to make 1,000 ml.			

The experiments were done as follows. An aqueous emulsion of allucin was added to three sets of tubes (duplicates in each) containing six ml. amounts of sterile water, gastric juice and pancreatic juice, in such a way as to make the final concentration of allucin in each tube 3  $\frac{\text{mgm}}{\text{gm}}$  per ml. The tubes were then incubated at 37°C. and the activity of the mixtures assayed by the xi cylinder-plate method, at definite intervals. The antibacterial properties of the pure gastric and pancreatic fluids were also determined. The results of these investigations are given in tables XXXV and XXXVI.

They show clearly that allucin is not destroyed by gastric juice even after 24 hours of incubation. In presence of pancreatic fluid, however, the antibiotic is destroyed about 30 to 40% in three hours and being complete in 24 hours.

Table XXXV.

Stability of alliin in presence of gastric juice.

Time in minutes after mixing	Mean diameters of haloes in mm.		
	Control in presence of sterile water	Gastric juice with alliin at pH 1.7	Gastric juice without alliin at pH 1.7
0	31.0	31.0	12.5
15	30.0	32.0	-
30	28.5	31.0	-
60	31.0	31.0	12.5
120	31.0	30.5	-
180	30.0	30.0	12.5
24 x 60	29.5	29.0	12.5

Table XXXVI.

Stability of alliin in presence of pancreatic juice.

Time in minutes after mixing	Mean halo diameters in mm.		
	Control in presence of sterile water	Alliin in pancreatic juice pH 9.3	Pancreatic juice alone pH 9.3
0	31.0	31.0	
15	30.0	29.0	
60	31.0	29.0	
120	30.5	28.0	
180	30.0	26.0	
24 x 60	29.0	Nil	Nil



Oral administration of allicin may be so of no great applicability in chemotherapy and hence the toxicity of the drug when fed orally was not studied.

Effect of allicin in presence of blood. Several antibacterial agents in presence of blood are inactivated greatly either due to enzymes present in the blood or due to the proteins in blood which adsorb the antibacterial agents<sup>2</sup> or in some other cases due to the alkaline reaction of blood<sup>3</sup>. Thus many of the indicator dyes like methylene blue are inactivated by the enzymes in blood. The inactivation of penicillin K by blood is probably due to its adsorption by blood proteins.<sup>2</sup> Notatin<sup>4</sup> is another outstanding example of an antibiotic losing much of its activity in presence of blood. It seems therefore of primary importance to investigate regarding the stability or activity of a compound in presence of blood. The activity of allicin, when incubated with blood for about 48 hours was hence studied.

Allicin was mixed with sterile citrated rabbit's blood, sterile citrate, and sterile water, in such a way as to give a final concentration of 3 mgm. per ml of allicin, and incubated at 37°C. The antibiotic activity of the mixtures was determined at fixed intervals of time. The results are given in table XXXVII and they show that blood does not seem to have any effect on the antibiotic properties of allicin.

Table XXXVII.

**Stability of allicin in presence of rabbit's blood at 37°C.**

Time in hours	Halo diameter. Mean values in mm.		
	Blood, citrate and allicin	Sterile water citrate & allicin	Sterile water and allicin
0	31	31	31
5	31	31	31
15	31	31	30
20	30	31	31
30	29	30	29
40	28	30	31

Studies on the toxicity of allicin.

Data presented above have shown allicin to possess a wide range of antibacterial activity and stability in presence of blood. Experiments were next carried out to find out the effect of parenteral administration of the drug to laboratory animals like mice and rats.

Animals. Young healthy mice weighing about 20 to 25 gms. and rats of about 90 gms. were used. As far as possible animals of the same age were taken for the same experiments. Throughout the duration, special care was taken to maintain the animals on a balanced diet, water being available at all times.



Allicin. This was prepared as described previously, the purest preparations being used in all the experiments. In view of the sparingly soluble nature of the drug in water, the alcoholic solution of the compound emulsified by diluting in water was injected. The acute and cumulative toxicity <sup>by</sup> subcutaneous administration, and the intraperitoneal acute toxicity were determined. Cavallito and Bailey<sup>5</sup> have described the toxicity of their preparation as intravenous L.D<sub>50</sub> to be 60 mgm.per Kg. and subcutaneous L.D<sub>50</sub> to be 120 mgm.per Kg.

Acute toxicity. This was determined as follows. The experimental animals were divided into several groups, each group containing six animals. Different but known amounts of allicin were injected to animals of different groups, the quantity received by members of the same group being equal. In all cases the strength of the emulsion were so adjusted that a mouse received not more than 0.20 ml and a rat 0.25 ml. The acute toxicity was determined by giving large quantities of the drug in one injection. After the injection the animals were kept under observation for a period of ten days.

Animals dying during the period of observation were dissected and a gross examination of the more important body organs like liver, lungs and kidneys was carried out. At the termination of the period (viz., 10 days after injection) a few of the surviving animals were also dissected for examination of body organs.

Dissected animals showed nothing abnormal, other than slight necrosis at the site of injection.

In these groups where the mortality rate was high the experiment was repeated using a larger number of animals. Rats were used for the subcutaneous toxicity studies and mice for intraperitoneal toxicity. In table XXXVIII the results of experiments on rats to find the subcutaneous toxicity of allidin are given.

Table XXXVIII.

Acute subcutaneous toxicity of allidin to rats.

Dose in mg./Kg.	No. of animals in group.	Total number of rats dead after - days										
		1	2	3	4	5	6	7	8	9	10	
10	6	0	0	0	0	0	0	0	0	0	0	0
20	6	0	0	0	0	0	0	0	0	0	0	0
30	6	1	1	2	2	2	2	2	2	2	2	2
50	6	1	2	3	3	3	3	3	3	3	3	3
75	12	4	5	7	8	8	8	8	8	8	8	8
100	12	6	6	8	10	12	12	12	12	12	12	12
Nil control	control 6	0	0	0	0	0	0	0	0	0	0	0

From the results presented it can be seen that 50 per cent mortality occurred when 50 mg. per Kg. was injected. The acute subcutaneous L.D. 50 is therefore 50 mgm. per Kg.



In table XXXIX the acute intraperitoneal toxicity of allicin to mice is brought out.

Table XXXIX.

Acute intraperitoneal toxicity of allicin.

Number of mice in group	Dose in mg/Kg.	Total number of mice dead after - days									
		1	2	3	4	5	6	8	9	10	
6	15	0	0	0	0	0	0	0	0	0	
6	25	1	2	2	2	3	3	3	3	3	
6	30	1	3	3	5	5	5	5	5	5	
6	Nil control	0	0	0	0	0	0	0	0	0	

The intraperitoneal acute toxicity of allicin is considerably higher than the subcutaneous toxicity. 50 per cent of the animals being killed by 25 mgm. of the drug for every Kg. of body weight.

Cumulative toxicity. The effect of daily administration of the drug was determined on a group of mice. Small amounts of allicin were injected subcutaneously daily for a period of ten days, and on alternate days for the next seven days. Other experimental details were as given for acute toxicity. From the tenth day onwards, dissection of one animal from each group was carried out and important organs examined. This, however, revealed nothing abnormal. The results of cumulative toxicity studies are given

Table XL.

Cumulative toxicity of alliin.

Dose in mg./Kg.	Number of mice in each group	Total number of mice dead after - days								
		1	2	3	4	6	8	10	14	21
0.75	6	0	0	0	0	0	0	0	0	0
1.50	6	0	0	0	0	0	0	0	0	0
3.00	6	0	0	1	1	1	1	1	1	1
4.00	6	0	1	2	2	2	2	2	2	2
5.00	6	0	1	2	3	3	3	3	3	3
6.25	6	2	3	4	5	5	5	5	5	5
Nil	6	0	0	0	0	0	0	0	0	0

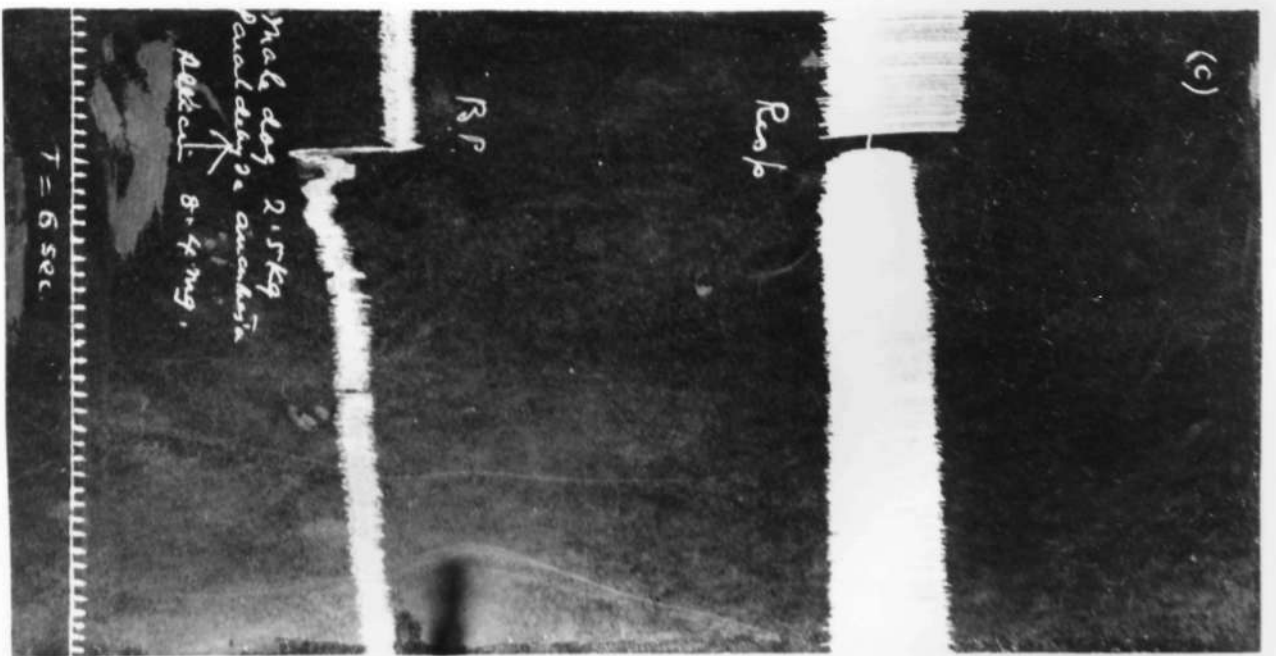
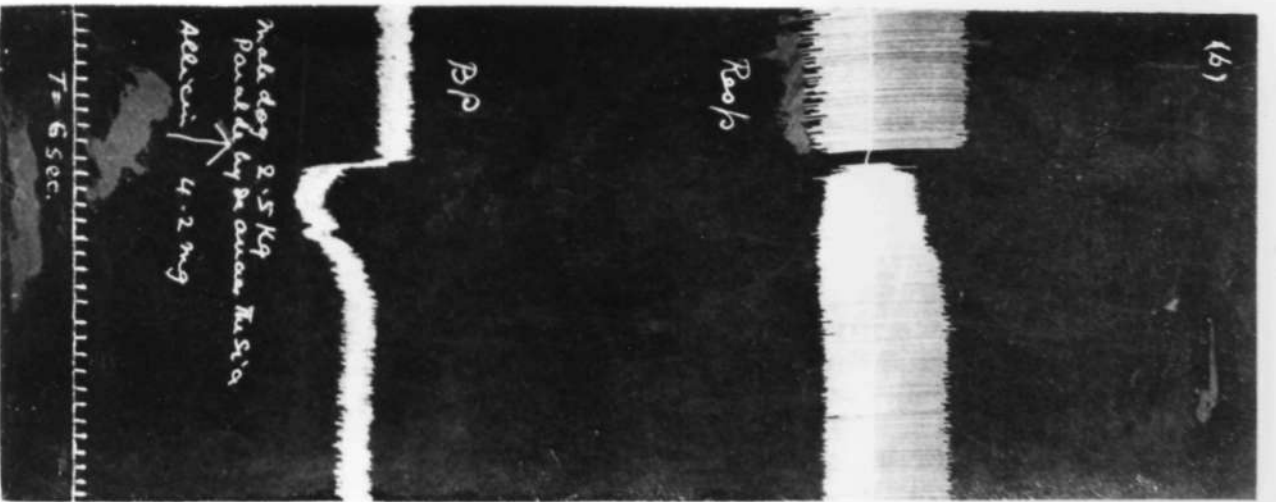
The cumulative toxicity of alliin appears to be about 5 mgm. per Kg. for mice.

#### Pharmacological investigations on alliin.

Studies essentially of a preliminary nature were carried out to find the effect of alliin on some of the important bodily functions.

Respiratory system. Plate 2 shows the record of tracheal pressure with a Marey's tambour in a dog under paraldehyde anaesthesia. An injection of 0.25 mg. of alliin usually produced no change in respiratory excursions. With a bigger dose, such as 1 to 2 mg.





there was a tendency for the respiration to become slightly slower, but it seems to be only a temporary effect due to the central vagal inhibition. This effect is not observed after section of the vagi in the neck. With toxic doses the respiratory tracings show a good deal of variations; probably secondary to the circulatory disturbances brought about by the drug.

The action of the drug on the bronchioles was tested by recording the intra-pleural pressure by means of a canula introduced through the ribs into the pleural cavity. The animals were kept under artificial respiration from a mechanical pump with regulated number of strokes, in order to maintain the air pumped into the lungs constant. Allicin in small doses did not produce any appreciable change in the ~~intrape~~ intra-pleural pressure. When, however, a toxic dose of the injections were made, certain changes at the late stages were observed which appear to be entirely due to the circulatory disturbances.

Circulatory system.

Effect of systemic blood pressure.

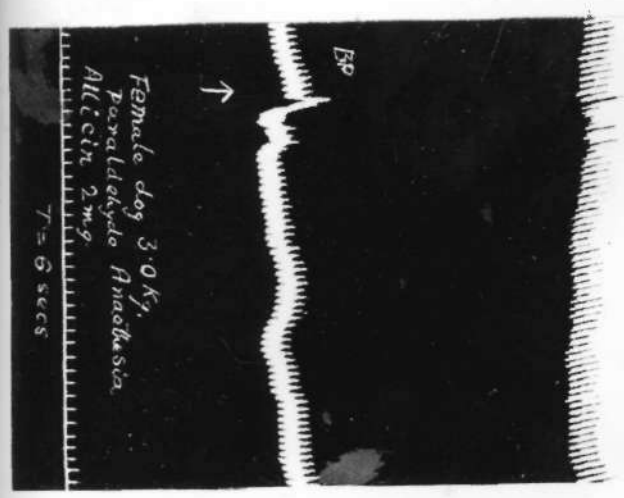
Injections of 2 mgm. of allicin into the femoral vein, produced in the majority of animals, a fall of the ~~arteri~~ carotid blood pressure, varying from 10 to 15 mm. of mercury, within five seconds of administration. The fall of blood pressure is maintained for a good length of time and it rarely resumes its former level. With increasing dosage, viz., 4 to 10 mg. this fall in pressure became marked, and there was distinct slowing of the heart beats (Plates 2 and 3). When larger doses were injected the fall in pressure was about 15 mm. of mercury before collapse.



(a)

Auricle

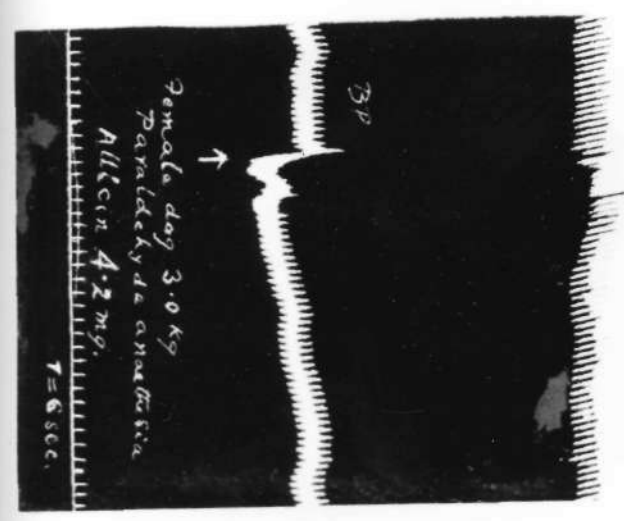
Ventricle



(b)

Auricle

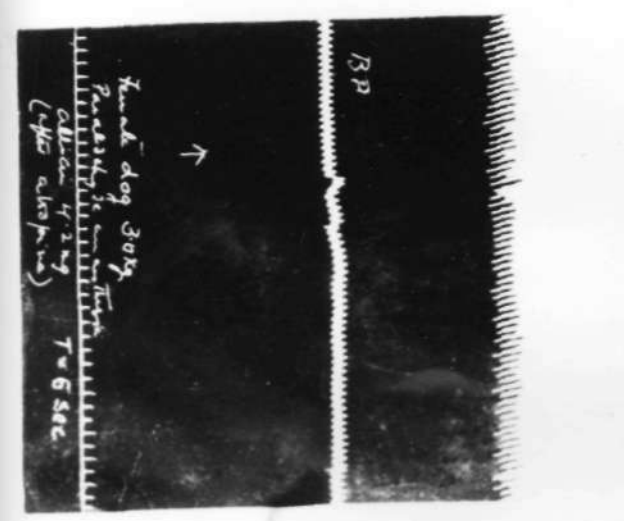
Ventricle



(c)

Auricle

Ventricle



The fall in blood pressure was not observed after the termination of the vagi were paralysed with atropine, showing that the vagal inhibition does play an important part in the fall produced.

Effect on heart.      Mio-cardiograph experiments.

As shown in plate 3 with 2 mgm. doses, there was a slight diminution in the amplitude of the auricular and ventricular systole. With larger doses viz., 4 to 10 mg. a distinct slowing of the rhythm was evident. The auricles and ventricles became markedly depressed (plate 3), the effect on the auricles being more pronounced. With still larger doses, the amplitude of auricular contractions, was greatly diminished and there was rapid weakening, leading to fibrillary twitchings of the auricle and ultimate paralysis.

In order to determine whether the effect of allicin was due to an action on the nervous mechanisms or on the myocardium, the nervous connections of the heart with the central nervous system were severed. It was, however, observed that there were very little alterations in the effects produced by the drug, showing that the centre is not entirely responsible for the cardiac changes. When the terminations of the vagi were paralysed by atropine, the effects of the drug on the auricles and ventricles disappeared to a great extent (plate 3). It was hence concluded that stimulation of the inhibitory nervous mechanism was responsible to a great extent in slowing the auricular and ventricular movements.



### Discussion.

The stability of allicin is greatly impaired, when incubated with pancreatic juice, whereas gastric fluid and blood do not have an injurious effect on the antibacterial properties of the drug. These facts seem to point that if fed orally, allicin may not find much use. Oral toxicity of the drug was hence not investigated.

The subcutaneous and intraperitoneal toxicity of the drug appears to be fairly high, particularly when compared with other drugs, which have found application in bacterial infections. These facts, along with the unstable nature of the drug seem to indicate that allicin may not be of much importance in chemotherapy. Experiments to find the in vivo efficacy of the drug were hence not undertaken.

A comparison of the two compounds, pterygospermin and allicin may be made. Both appear to be oils sparingly soluble in water. Besides, carbon, hydrogen and oxygen both contain sulphur. Pterygospermin however contains nitrogen. In their stability in presence of tissue fluids also they resemble each other. This inactivation seems to be due to the alkalinity of the fluid rather than to its other constituents.

In their ability to inhibit the growth microorganisms and toxicity they differ to a great extent. Pterygospermin inhibits several microorganisms when present in concentrations about 5 to 6 times as low as that at which allicin acts. The toxicity of pterygospermin also appears to be much less, the cumulative L.D<sub>50</sub> being about 10 times that of allicin. Investigations on the

effect of the drugs on the important bodily functions have shown that both depress the respiration, blood pressure and the auricles and ventricles.

The mode of action of the two compounds seems to be different. Allicin in all probability acts by inactivating the sulph-hydryl enzymes by virtue of its - S - S - group. Pterygospermin has  
0  
no effect on sulph-hydryl compounds and hence does not seem to act in a way similar to that of allicin.

#### Summary.

1. Allicin, is active in presence of blood and gastric juice. It loses its antibacterial properties in presence of pancreatic juice. In all probability, this is due to the alkalinity of the pancreatic juice.
2. The cumulative subcutaneous toxicity of allicin has been studied. 100% mortality occurred when the dose injected was more than 6.25 mg.per Kg. The L.D<sub>50</sub> was about 5 mg.per Kg.
3. The acute subcutaneous L.D<sub>50</sub> appears to be about 50 mg. per Kg. and the acute intraperitoneal L.D<sub>50</sub> 25 mgm.per Kg.
4. Allicin in small concentrations depresses the lungs and heart.



References.

1. Maney and Kuever (1941) J.Amer.Pharm.Assn.,30, 276.
2. Tompsett R. et al (1947) J.Bact.,53, 581.
3. Abraham et al (1941) Lancet (2), 178.
4. Coulthard C.E. et al (1945) Biochem.J.,39, 24.
5. Cavallito C.J. & Bailey J.H. (1944) J.Amer.Chem.Soc.,66, 1950.

27/08/2019

PART - II.



INTRODUCTION.

## STUDIES ON STREPTOMYCIN.

### Introduction.

In view of the clinical attractiveness of antibiotic agents as typified by penicillin, experiments have been carried out in several laboratories, essentially with a view to isolating, similar substances which may of use in chemotherapy. The importance of these compounds lie in their ability to effect striking and specific bacteriostatic action in vivo, without the simultaneous production of severe toxic symptoms. Of the several attempts thus made one of the most successful was that of S.Waksman and his associates at the Rutgers University, in the isolation of streptomycin from Streptomyces griseus.<sup>1</sup>

In a search for antagonistic micro-organisms, especially organisms that are active against Gram-negative bacteria, the actinomycetes<sup>2,3,4</sup> were found to offer extensive potentialities, 20 to 30% of all the organisms tested possessing marked anti-bacterial properties. These cultures were either isolated and tested for their ability to inhibit the growth of bacteria, from different soils and composites, or were taken from the varied type collections. Search for micro-organisms, antagonistic to acid-fast bacteria also resulted in the isolation of an actinomyces<sup>5,6</sup> which was found capable of producing in certain media an antibiotic that seemed to possess many desirable anti-bacterial and pharmacological properties. This organism has



been found to be similar in appearance<sup>7</sup>, cultural characteristics and morphology to Actinomyces griseus isolated from the soil previously<sup>8</sup>.

According to Waksman<sup>9</sup> the composition of the medium has a marked effect on the production of streptomycin. He states that streptomycin formation depends largely on the presence of certain organic constituents in the medium. These substances are according to the Rutgers workers<sup>9</sup> present in meat extract or corn steep liquor. In a more recent communication<sup>10</sup> Waksman has designated this substance as an 'activity factor' either serving as a precursor of the streptomycin molecule as a whole or of an important group in the molecule or functioning as a prosthetic group in the mechanism essential for the biosynthesis of streptomycin.

The strains of S. griseus that produce streptomycin are not absolutely stable and undergo marked variations<sup>9</sup> as is the case with several actinomycetes. Waksman<sup>8</sup> has directed attention as far back as 1919, to the variability in the proteolytic mechanism of Actinomyces griseus. In more recent investigations recorded by him<sup>11</sup> strains of the organisms varying considerably in streptomycin producing capacity have been described. They varied in their morphology, viz., formation of an aerial mycelium, and in their physiology, such as production of antibiotic substance, formation of acid, rate of glucose utilisation, autolysis, and production of a gummy material. Between these two extremes, intermediary strains were obtained from which either of the first

two types could be readily isolated. Both the active and inactive cultures are alike in many of their cultural characteristics, such as lack of dark pigmentation on organic media, proteolytic action, and haemolytic capacity. Waksman<sup>9</sup> has also stated that in practical production of streptomycin the formation of inactive variants of S.griseus need not be feared; conditions for obtaining the inactive variants are so completely different from those commonly employed in the laboratory or in the industrial production of the antibiotic agent, that the possibility of a strain degeneration is very small. Investigations carried out recently by Waksman<sup>12</sup> with a view to isolate streptomycin producing strains have resulted in the following conclusions.

1. Not all strains of S.griseus are capable of producing streptomycin.
2. Streptomycin producing strains of S.griseus form active and inactive variants.
3. The inactive variants comprise two types, one being free from aerial mycelium and the other producing a pink tinge, in the vegetative growth, the aerial mycelium being typical of S.griseus.
4. A medium enriched with streptomycin can be utilised for the isolation of fresh strains,
- and 5. A streptomycin enriched medium can also be utilised for purifying active cultures of S.griseus from inactive variants.

For the production of the antibiotic, several media besides those suggested by Waksman have been however found useful.



Le Page and E.Campbell<sup>13</sup> have carried out investigations using yeast extract, in media for formation of streptomycin and observed that a medium containing yeast extract 1% produced more of the antibiotic than in Waksman's medium. Rake and Donovick<sup>14</sup> report that beef extract is not required in a medium containing about 1% soyabean meal, in which antibiotic production has been estimated to be about 240 per ml. They have also stated that addition of beef extract to their medium in shake flasks delays to some extent production of streptomycin. Vanderbrook and associates<sup>15</sup> have found that in a medium of composition dextrose 10 gms., Curbay B.G. 1 gm. sodium chloride 5 gms. basic potassium phosphate 1 gm. ammonium sulphate 2.5 gms. magnesium sulphate 0.25 gms. and calcium carbonate 3.5 gms. tap water to make a litre, submerged cultures of S.griseus can be harvested at a potency of 170 per ml. The fermentation was carried out in iron flask tanks. Using straw infusion liquor as a content of the medium Dey<sup>16</sup> has reported successful antibiotic production by S.griseus. Bennett<sup>17</sup> has observed that acid hydrolysed casein, acid hydrolysed wheat gluten, acid hydrolysed stillage from wheat mash yeast alcohol fermentation, asparagus juice and acid hydrolysed rabbit fur gave streptomycin titres comparable with media containing corn steep liquor. A medium containing rice bran has been reported by Woodruff et al<sup>18</sup> and a synthetic medium by Ainsworth et al<sup>19</sup>.

The growth requirements of S.griseus have also been investigated in recent times, in great detail. Of the several suggestions put forward, mention may be made of those by of Rake et al<sup>14</sup> who

stress on the importance of sodium ions; McFarlane and Feiser<sup>20</sup> who studied the amino acid requirements of the organism and concluded that valine, histidine and arginine to be the most important; and the conclusions of Dulaney<sup>21</sup> that yields of 800 per ml were obtained by the use of L.proline as the sole nitrogen source.

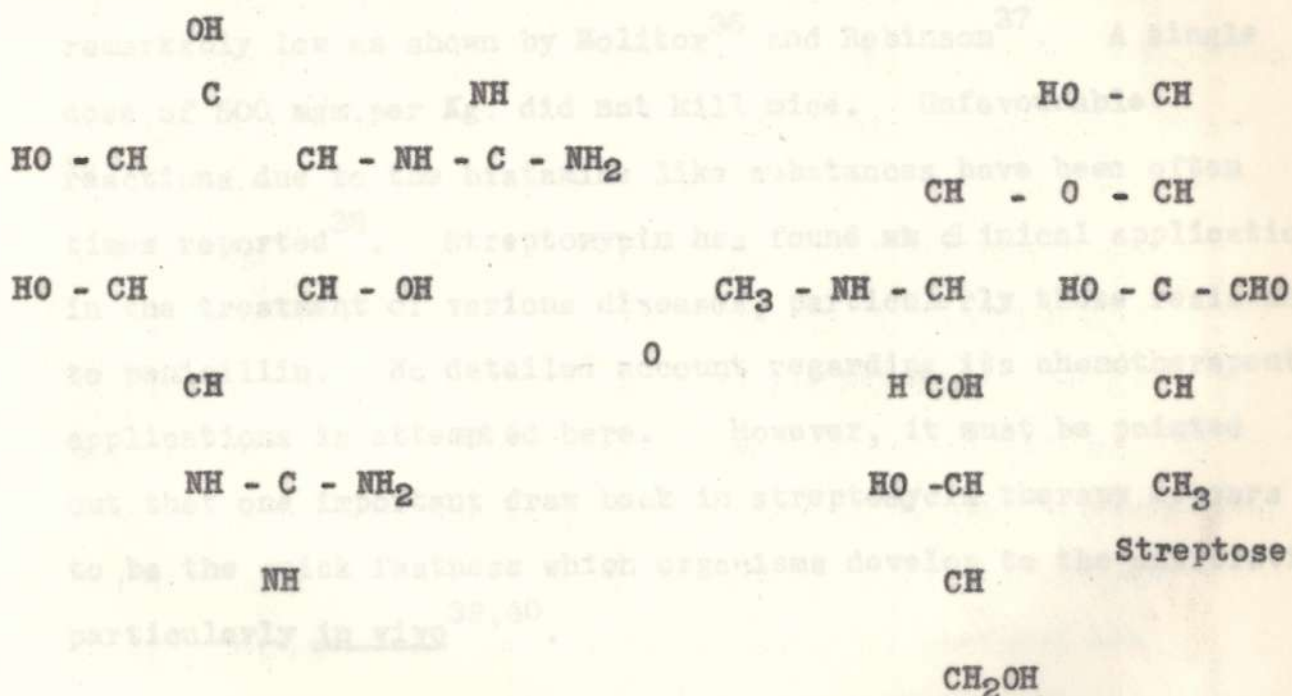
Several methods have been described for the assaying of streptomycin. In view of the fact that the chemical characteristics of the drug are being elucidated only recently, methods for estimating streptomycin chemically are few. Boxer and co-workers<sup>22</sup> have however reported chemical methods based on the formation of maltol from streptomycin by heating with alkali, and on the formation of a fluorescent hydrazone of streptomycin<sup>23</sup>, the latter one being the more sensitive. A colorimetric method based on the aldehyde group of streptomycin has been reported by Marshall and co-workers<sup>24</sup>. Several modifications of the agar plate method have also appeared. Mention may be made of the paper disc-plate method of Loo et al<sup>25</sup>. Donovick et al<sup>26</sup> have reported a broth dilution method for assaying streptomycin. Heilman<sup>27</sup> has described a slide cell technique for the assay of streptomycin in body fluids.

Several methods for the purification of streptomycin and preparation of crystalline derivatives have also been described. Thus crystalline helianthates<sup>28</sup> and Reinecketes<sup>29</sup> were the first to be obtained. The sulphates and chlorides were next



isolated<sup>30</sup> in the pure form. Peck et al<sup>31</sup> have also reported the preparation of a trihydrochloride, calcium chloride double salt of streptomycin. remaining oxygen groups are still not definitely

Crystalline streptomycin has the probable formula  $C_{21}H_{39}N_7O_{12}$  and is compounded of the following entities. Streptidine and streptobiosamine consisting of N-methyl-1-glucosamine and the streptose moiety.



n-methyl-1-glucosamine

**STREPTIDINE**

**STREPTOBIOSAMINE**

The structure of streptidine and of N-methyl glucosamine have been established by the preparation of many derivatives. The molecular arrangement of streptobiosamine shown above is due to Kuehl<sup>32</sup>. Fried and Wintersteiner<sup>33</sup> have shown that streptomycin possesses an aldehyde group, which resides in the maltol forming

moiety. The position of the carbonyl groups, the point of attachment of streptidine and N.methyl-1-glucosamine and the function of the remaining oxygen groups are still not definitely known.

Streptomycin has been found to have antibacterial activity against a wide variety of organisms which include Gram-positive, Gram-negative and acid-fast pathogens.<sup>34,35</sup> Its toxicity is remarkably low as shown by Molitor<sup>36</sup> and Robinson<sup>37</sup>. A single dose of 500 mgm.per Kg. did not kill mice. Unfavourable reactions due to the histamine like substances have been often times reported<sup>38</sup>. Streptomycin has found ~~an~~ clinical application in the treatment of various diseases, particularly those resistant to penicillin. No detailed account regarding its chemotherapeutic applications is attempted here. However, it must be pointed out that one important draw back in streptomycin therapy appears to be the quick fastness which organisms develop to the antibiotic particularly in vivo<sup>39,40</sup>.

During recent years some other antibiotics have also been isolated from S.griseus. Mention may be made of Grisein isolated by Waksman<sup>41</sup> and Actidone isolated by Whiffen<sup>42</sup>. Their properties are however different from those of streptomycin as has been recently pointed out by Waksman<sup>43</sup>. Even streptomycin during recent times has come to be known as the streptomycin complex<sup>44</sup> and appears to be a mixture of more than a single compound.<sup>45</sup> A new streptomycin producing strain has also been recently reported<sup>46</sup>.



Thus it may be seen that investigations of a wide and varied nature have been, and is being carried out on streptomycin. It must however be pointed out that, streptomycin has got several weaknesses like the resistance which organisms develop to it particularly in vivo and delayed toxicity in a few cases. At the same time it is true that next to penicillin, streptomycin is the only antibiotic that has gained wide chemotherapeutic applications. It is effective in curing infections like those caused by K.pneumoniae and M.tuberculosis, which are resistant to penicillin. Perhaps at a future date combined administration of streptomycin along with other antibacterial agents, might attain importance since such a method may be of use in overcoming the streptomycin-fastness developed by microorganisms. Investigations on the nutritional requirements of the organisms, media and cultural conditions, for high antibiotic production, as also investigations on the methods for the isolation of the drug and allied problems must hence continue in the hope of making the availability of this important drug easier and its production cheaper. Some attempts made in such directions are given in the following pages.

References.

1. Schatz, A., & Waksman, S.A. (1944) Proc. Soc. Exptl. Biol. & Med, 57, 244.
2. Waksman, S.A. et al (1941) J. Bact., 42, 816.
3. Waksman, S.A., et al (1942) Soil. Science, 54, 281.
4. Welsch, M., (1942) J. Bact., 44, 571.
5. Schatz, A., et al (1944) Quart. Bull., North Western Univer. Med. School, 19, 207.
6. Waksman, S.A., et al (1944) Proc. Staff. Meet. Mayo Clinic, 19, 537.
7. Waksman, S.A., (1917) J. Bact.,
8. Waksman, S.A., et al. (1945) J. Amer. Pharm. Assn., 34, 273.
9. Schatz, A., & Waksman, S.A. (1945) Proc. Nat. Acad. Sci., 31, 129.
10. Waksman, S.A. et al (1946) J. Bact., 51, 753.
11. Waksman, S.A. et al (1946) J. Bact., 52, 393.
12. Waksman, S.A., et al (1947) Proc. Soc., Exptl., Biol & Med, 66, 617.
13. Le Page, G.A., & Campbell, E. (1946) J. Biol. Chem., 162, 163.
14. Rake, G., & Donovick, R., (1946) J. Bact. 52, 223.
15. Vanderbrook, M.J. et al (1946) J. Biol. Chem., 165, 463.
16. Dey, N.C. (1946) Science & Cult., 12, 100.
17. Bennett, (1946) J. Bact
18. Woodruff, et al (1946) J. Bact.
19. Ainsworth, G.C., et al (1947) J. Microbiol. 1, 335.
20. Mc Farlane, & Eiser, (1948) Cand. J. Research, 26, 164.
21. Dulaney, E.L., (1948) J. Bact., 56, 308.
22. Boxer, G.E., et al (1947) J. Biol. Chem., 169, 153.
23. Boxer, G.E., et al (1947) J. Biol. Chem., 170, 491.



24. Marshall, E.K., et al (1947) J.Pharmacol & Exptl.Therap, 90, 367.
25. Loo, Y.H., et al (1945) J.Bact. 50, 701.
26. Donovanick, R., et al (1945) J.Bact, 50, 623.
27. Heilman, D.H., (1945) Proc.Staff.Meet.Mayo.Clinic. 20, 145.
28. Kuehl, F.A., et al (1945) Science, 102, 34.
29. Fried, J. & Wintersteiner, O., (1945) Science, 101, ~~63~~ 613.
30. Denkelwater, R.G., et al (1945) Science, 102, 9.
31. Peck, et al. (1945) J.Amer.Chem., Soc., 67, 1866.
32. Kuehl, F.A., Jr. et al (1946) J.Amer.Chem., Soc., 68, 2679.
33. Fried, J. & Wintersteiner, O., (1947) J.Amer.Chem.Soc., 69, 79.
34. Youmans, G.P., & Feldman, W.H., (1946) J.Bact. 51, 608.
35. Feldman, W.H., & Hinshaw, H.C. (1945) Amer.Rev.Tuberculosis, 52, 269.
36. Molitor, H. ¶ (1946) Annl.N.Y., Acad.Sci., 48, 101;
37. Robinson, H.J., (1946) Annl.N.Y., Acad.Sci., 48, 119.
38. Molitor, H., (1945) J.Pharmacol. & Exptl.Therap.
39. Keefer, C.S., et al (1946) J.Amer.Med.Sci., 132, 70.
40. Waksman, S.A., (1949) Science, 109, 305.
41. Waksman, S.A., et al (1946) J.Bact. 51, 753.
42. Leach, B.E., Ford, J.H.,  
& Whiffen, A.J., (1947) J.Bact., 53, 769.
43. Waksman, S.A., (1948) J.Bact., 56, 259.
44. Waksman, S.A., (1948) Biol.Reviews, 23, 471.
45. Trussell, P., et al (1947) J.Bact., 53, 769.
46. Johnstone, D.B.,  
& Waksman, S.A., (1947) Proc.Soc.Exptl., Biol. & Med, 65, 294.

CHAPTER - I.



## UTILIZATION OF WHEAT BRAN FOR STREPTOMYCIN FORMATION.

### Introduction.

The remarkable properties of streptomycin, viz., a wide range of activity and low toxicity, responsible for its chemotherapeutic success have been detailed in the introduction. The results of some of the more important attempts made at finding cultural conditions for good streptomycin production such as those of Le Page and E. Campbell<sup>1</sup>, Rake and Donovan<sup>2</sup>, Vanderbrook et al<sup>3</sup>, Woodruff<sup>4</sup>, Ainsworth et al<sup>5</sup>, Mac Farlane and Feiser<sup>6</sup>, Dulaney<sup>7</sup>, have also been referred to there.

In the course of pursuing a programme of work aimed at the utilization of locally available cheap materials as media for streptomycin production, wheat bran was one of the first to be tried and one of the most useful. Wheat bran has been reported often times to be useful in culturing microorganisms and production of commercially important product like enzymes, and antibiotics. Thus Bindall and Sreenivasayya<sup>8</sup> have, in extensive investigations carried out to design media for enzyme production by A. oryzae, reported the advantageous results of culturing the microorganisms in wheat bran. The results of using moist wheat bran as medium for penicillin production by P. notatum have been reported by Sreenivasa Rao<sup>9</sup>. In all these solid wheat bran has been employed, which however possesses several undesirable qualities. Coghill has pointed out<sup>10</sup>, the inherent disadvantages in using solid bran. These consist essentially in the difficulty encountered in sterilizing solid bran, and the heat accumulation

and consequent temperature increase in certain parts of the medium, during fermentation which entails loss of products like antibiotic and enzymes. Both these are due mainly to the poor heat conductivity of the bran.

For the investigations recorded below an aqueous extract of the bran was used. The method of preparing this extract and its composition are also described.

### Experimental.

Wheat bran - the broken coat of the grain which separates during sifting - available in local market was used. Representative samples were analysed for moisture, protein, carbohydrates, fats, ash and fibre, before using the bran for preparing the extract. In table XLI the results are given.

Table XLI.

#### Composition of wheat bran.

Form	Samples			Mean values
	1	2	3	
Moisture	12.5	12.7	12.9	12.7
Protein	15.2	15.7	15.0	15.3
Ether extratives	1.89	1.91	2.01	1.93
Carbo- hydrates	54.8	55.5	55.1	55.13
Ash	5.74	5.84	5.85	5.81

Values are in grams per 100 gms of dry wheat bran.



The following methods were used for the analysis of the bran.

Moisture: A known amount of the wheat bran was dried to constant weight in an incubator at 110°C, and the loss of moisture determined and the percentage calculated.

Proteins: A known amount of the bran was digested, using the digestion mixture recommended by Chibnall *et al*<sup>11</sup>, and the amount of nitrogen determined by the micro-Kjeldahl method. The percentage of proteins was then calculated by using the factor 6.05

Carbohydrates: were estimated by the method of Hassid *et al*<sup>12</sup> with the following modifications. Hydrolysis with the enzyme was carried out at 37°C for four hours, instead of two. Alkaline ferricyanide was titrated against sodium thiosulphate, in the place of ceric sulphate.

Ether extractives: A known weight of the bran was extracted with ether in a Soxhlet's apparatus and the loss in weight of the material after drying was found out. The weight of the oil obtained after complete removal of ether was also determined. The mean of the two values was used to calculate the percentage fatty material in the bran.

Ash: This was estimated by a muffle furnace, for a period of about 8 hours, the temperature of the muffle being about 800°C.

Fibre: was computed by difference.

Preparation of the extract. After a series of preliminary experiments, the following method was used for the preparation of the aqueous extract of bran. Commercial bran (1000 gms) was mixed thoroughly in tall bottles with (6000 ml) water. The suspension was then aerated, by sucking air through a water pump.

for a period of about 48 to 60 hours, the aeration serving primarily as agitation. At the end of the period the suspension was filtered, the filtrate distributed in conical flasks of about a litre capacity (500 ml into each flask) plugged and autoclaved at 15 lbs pressure for half an hour. A thick white coagulum settled down during autoclaving. This was removed either by filtration or by centrifugation. A thick brownish liquid was obtained. Its total solid contents varied from 3.6 to 4.3 gms per 100 ml. About 30% of the nitrogen of the bran was solubilized by the above process. In table 42 the composition of the extract is brought out.

Chemical methods of analysis used.

Total solids: The material dried to constant weight in a hot air oven at 110°C.

Ash: Dry material was ashed at 800°C in an electric muffle for about 8 to 10 hours.

Total nitrogen: A known amount of the material was digested by using the mixture recommended by Chibnall, Rees and Williams<sup>11</sup> and nitrogen determined by the micro-Kjeldahl method.

Non-protein nitrogen: was determined on the 15% trichloroacetic acid filtrate.

Basic nitrogen: The phosphotungstic acid precipitate after decomposition with barium hydroxide was used for estimation of basic nitrogen.

Amino nitrogen: was estimated by the formal titration method due to Sorensen.



Amide nitrogen: The method of Damodaran<sup>13</sup> was used. 2 ml aliquots of the extracts were heated with 5 ml portions of alkali and distilled in a micro distillation apparatus for 8 minutes.

Carbohydrates: The amount of carbohydrates after hydrolysis with 2 N HCl, for 4 hours were estimated by the Hagedorn and Jenson<sup>14</sup> method.

Table XLII

Composition of the aqueous extract of bran

From	Mean value in mg per 100 ml.
Total solids	3800
Total nitrogen	134.9
Amino nitrogen	15.01
Amide nitrogen	18.50
Basic nitrogen	81.70
Protein nitrogen	24.13
Carbohydrates	475.0
Ash	860.7

Bacteriological methods.

Strain of organism. A Waksman's strain of Streptomyces griseus obtained through the N.C.T.C. America was used for the investigations. They were maintained on Waksman's meat extract medium.

Streptomycin assay. Several modifications of the cup-plate method described previously (refer page 15-17) have been suggested for the assaying of streptomycin.

In all these, the diameters of the haloes of the unknown samples, are determined by a standard curve obtained by plotting values of known concentrations, the reaction of the agar used being alkaline. In the present investigations 20 ml of agar (composition, beef extract 1.5 gm, difco peptone 6 gms, yeast extract 3 gms, agar 20 gms per 1000 ml of distilled water pH  $7.6 \pm 0.1$ ) was used for each petri dish of diameter 15 cms and height 2 cms. A spore suspension of Bacillus subtilis prepared according to Foster and Woodruff<sup>18</sup> and susceptible to streptomycin was the test organism. A crystalline sample of streptomycin hydrochloride (supplied by the Merck and Co., Rahway) possessing an activity of 655 per mg was used as the standard. Solutions of the standard were prepared in 0.1 M phosphate buffer at pH 7.6 and assayed to get a standard curve. Such curves were prepared almost daily for calculating values of the unknown samples.

For assaying, the samples were prepared by clarifying the culture fluids, by centrifugation and diluting them suitably, in phosphate buffer 0.1 M at pH 7.6, in such a way that the values of the halo-diameters may fall in the standard curve.

Measurement of growth. This was determined only for surface cultures. The test tubes (refer below) were centrifuged, the growth collected and dried at 40°C overnight and weighed.

Stationary cultures.

For studying the day to day changes in pH, Streptomycin titre, and growth, bacteriological test tubes of uniform size (15 cms long and 1.7 cm in diameter) containing 5 ml of sterile medium were inoculated with a spore suspension of the organism. The tubes



were then placed in a slanting position (at an angle of about 10° to 15° to the horizontal) so as to furnish a large surface for growth, and incubated at a temperature of 25°C. Two tubes from each group were removed at intervals of 24 hours centrifuged, fluids used for assay (as described previously) and pH determination, and the residue weighed as given above for finding the growth.

Preliminary investigations, aimed at finding the effect of growing S. griseus in unsupplemented bran extract were first carried out. Aqueous extract of wheat bran, diluted to contain total solids ranging from 0.5 gms to 3.0 gms per 100 ml were used as media. The reaction before autoclaving was adjusted to  $6.8 \pm 0.1$ . The results of the investigations are given in table XLIII. Since they indicated, that high streptomycin production took place even when the total solids were reduced to 1 gram per 100 ml. extract of this composition was used as the basal medium, in detailed studies.

To the basal medium, several additions in the form of carbohydrate materials, minerals etc were made in different amounts either individually or together, and streptomycin formation studied. In table XLIV the composition of the media and in XLV the day to day changes in pH, streptomycin titre and growth are given.

In stationary cultures surface mats typical of Streptomyces griseus appeared in many media from the 3rd day onwards. In medium containing sodium acetate growth did not take place. It has been a common observation that the antibiotic activity of culture fluids reached a maximum during or immediately after sporulation.

Table XLIIISTREPTOMYCIN PRODUCTION IN BRAN EXTRACT OF DIFFERENT TOTAL SOLID CONTENT.

Total solids in medium in gms/100 ml	Reaction of the medium.						Streptomycin titre in per ml.					
	3	4	5	6	7	8	3	4	5	6	7	8
0.5	6.9	7.3	7.4	7.6	7.9	7.9	29	36	98	89	85	78
1.0	7.1	7.4	7.6	7.7	7.9	7.9	29	35	108	110	108	99
2.0	7.1	7.2	7.6	7.7	7.9	7.9	32	69	111	114	109	92
3.0	6.9	7.2	7.4	7.6	7.8	7.9	25	32	69	93	111	103

Table XLIVComposition of media

No. of medium	Weight in grams of constituents added per 100 ml of basal medium, bran extract of total solids 1 gm per 100 ml.				
	Sodium chloride	Glucose	Brown sugar	Sodium acetate	Minerals
1	Nil	Nil	Nil	Nil	Nil
2	0.5	"	"	"	"
3	Nil	2.0	"	"	"
4	"	Nil	2.0	"	KH <sub>2</sub> PO <sub>4</sub> 1% FeSO <sub>4</sub> and MgSO <sub>4</sub> in traces.
5	"	"	Nil	"	"
6	"	2.0	"	"	"



Table XLV

Streptomycin formation in media.

No. of medium		Days after inoculation						
		3	4	5	6	7	8	9
1	pH	7.1	7.4	7.6	7.7	7.9	7.9	7.9
	Streptomycin in /ml.	29.0	35.0	108	110	108	99	95
	Weight of growth in mg/10 ml.	20.0	21.0	21.0	18.0	17.0	16.0	17.0
2	pH	7.1	7.4	7.6	7.7	7.9	7.9	7.9
	Streptomycin in /ml.	126	160	95	73	68	63	60
	Weight of growth in mg/10 ml.	24	22	22	21	20	16	14
3	pH	6.9	7.1	7.2	7.4	7.4	7.6	7.7
	Streptomycin	20	30	28	28	27	25	23
	Weight of growth	10	10	12	14	20	20	18
4	pH	7.1	7.2	7.3	7.4	7.4	7.4	7.4
	Streptomycin	30	45	75	73	73	65	55
	Weight of growth	-	-	10	10	12	18	16
5	pH	7.2	7.3	7.6	7.7	7.9	7.9	7.9
	Streptomycin	65	85	85	65	65	45	30
	Weight of growth	20	16	14	15	15	15	15
6	pH	7.0	7.1	7.4	7.4	7.4	7.4	7.4
	Streptomycin	20	33	30	15	12	-	-
	Weight of growth	10	8	8	8	8	8	8

The effect of sodium chloride. From the data presented in table XLV it can be seen that of the different additions made, only sodium chloride increased the streptomycin titre considerably. Detailed investigations to find the effect of different amounts of sodium chloride on antibiotic production were undertaken, and the results are given in table XLVI. The remarkable effect of sodium chloride is obvious.

Table XLVI

Effect of sodium chloride on streptomycin production.

Composition of medium		Streptomycin in .gm.per ml (days after seeding)						
Wheat bran extract	Sodium chloride	2	3	4	5	6	7	8
100 cc containing 1 gm of total solids.	0.5 gm.	75	126	160	95	73	68	63
	1.0 "	66	148	178	93	88	82	77
	2.0 "	46	161	163	98	98	83	82
	4.0 "	15	73	78	58	53	43	32
	5.0 "	15	40	78	70	65	45	33
100 cc containing 2 gm. of total solids	0.5 gm	40	189	159	137	108	95	86
	1.0 "	52	179	203	138	123	86	76
	2.0 "	45	169	98	89	75	68	58
	4.0 "	23	85	93	93	60	54	33
	5.0 "	15	18	45	52	53	33	35



Shaken cultures. The antibiotic production by S. griseus has been reported to be quickened and increased, by shaken culturing. Effect of shaken culturing, was hence studied in those media which gave encouraging results by stationary culturing. The experimental procedure for the same consisted in the following. Six bottles of 100 ml capacity containing 30 ml of medium, were inoculated after sterilization with a spore suspension of the organism grown in Waksman's medium. They were then shaken in a mechanical shaker. One bottle from each group, was removed daily and the antibiotic activity estimated. The results of the experiments are presented in table XLVII and they indicate that shaken culturing does not have much advantage over stationary ones.

Table XLVII

Antibiotic production by shaken cultures.

Age of culture in days.	Streptomycin titre in /ml in medium		
	I	A	II
2	20	20	20
3	70	75	75
4	80	150	110
5	80	120	100
6	75	110	90

Medium A in table has the composition:- Wheat bran extract of total solids 2%, and sodium chloride 1%. Media I and II are of the composition given previously in table XLIV.

Flooded cultures. Flooding of cultures has been in several instances found to increase the antibiotic production considerably. In some cases even flooding with distilled water, has been reported<sup>19</sup> to be very effective. The effect of flooding was hence studied in one medium viz. the one which gave best streptomycin titre by stationary culturing.

Culture fluids from 10 day old cultures Streptomyces griseus grown in the same medium were removed carefully, without disturbing the surface mat. 5 ml of fresh medium or sterile water were then poured into the tubes exercising great care not to disturb the surface mat. The tubes were again incubated and the antibiotic activity determined as already described. In table XLVIII the results of the investigations are presented.

Table XLVIII  
Effect of flooding on streptomycin production.

Age of culture in days	Streptomycin titre in per ml in medium		
	Before flooding	After flooding	Flooding with sterile water
2	25	300	80
3	90	650	90
4	203	600	70
5	80	500	50

The medium used in the above experiment had the following composition: Wheat bran extract of total solids 2%, and sodium chloride 1%.



It may be noted from the results given that flooding with the medium increases the antibiotic production by about 4 to 5 times, whereas flooding with sterilised water is not of much advantage.

#### Discussion.

Some of the media described above, can be used with advantage in streptomycin production. The antibiotic formation is as high as 1500 to 2000 per gram of the wheat bran used.

Certain aspects regarding the nutritional requirements of the organism, for successful streptomycin production, are also revealed. Excess of carbohydrates given as particularly glucose, seems harmful for streptomycin formation. It must however be mentioned that the observed low streptomycin titre, in media containing glucose may perhaps be due to the inhibition of streptomycin activity by glucose.<sup>20</sup> These remarks seem justifiable since, in brown sugar medium also streptomycin formation is decreased, but not to such an extent as is observed in media containing glucose.

Of the different minerals investigated, sodium chloride seems to have a very great influence. Its presence in 1% concentrations appears to be optimal. Higher ones are definitely harmful. In the presence of sodium chloride the antibiotic production is not only increased but is also quickened. Observations of a similar nature regarding the role of sodium chloride in antibiotic formation, by Aspergillus fumigatus,<sup>21</sup> Penicillium notatum<sup>22</sup> and S. griseus<sup>23</sup> have been previously recorded. The exact nature in which sodium chloride acts is however not clear. The observations of

Mc Farlane et al,<sup>24</sup> that sodium chloride extracts streptomycin from the mycelium, have thrown some light on the problem. But the presence in the mycelium of another antibiotic grisen,<sup>25</sup> which is soluble in water and probably also in saline appears to suggest that Mc Farlane's conclusions regarding the role of sodium chloride might demand further confirmation.

Since the extract used contained appreciable quantities of minerals, nothing could be inferred regarding the role of the added minerals. The importance of the minerals in streptomycin formation has however been discussed later.

#### Summary

1. A method for the production of aqueous extract of wheat bran, and its composition are given.
2. The extract as such, has been found to be useful for streptomycin production by S. griseus. The presence of sodium chloride up to 1% has been found to increase the streptomycin production considerably.
3. Shaken cultures in the media studied, had no advantage over stationary ones.
4. Flooding of cultures with media, increased antibiotic production by about 4 to 5 times.



### References

1. Le Page, G.A. & Campbell, E. (1946) *J. Biol. Chem.*, 162, 163.
2. Rake, G. & Donovan, R., (1946) *J. Bact.*, 52, 223.
3. Vanderbrook, M.J., et al (1946) *J. Biol. Chem.*, 165, 463.
4. Woodruff et al (1946) *J. Bact.*, 5
5. Ainsworth, G.E., et al (1948) *J. Genl. Microbiol.* 1, 335.
6. McFarlane, & Eiser (1948) *Cand. J., Research*, 26, 164.
7. Dulaney, E.L., (1948) *J. Bact.* 56, 308.
8. Bindall, A.N., & Sreenivasaya, M., (1944) *J. Sci. Ind. Research*, 3.
9. Sreenivasa Rao, S., (1944) *Nature*, 154, 83.
10. Coghill, R.D., (1944) *Chem. Eng. News*, 22, 588.
11. Chibnal, Rees & Williams (1943) *Biochem. J.*, 37, 354.
12. Hassid et al (1940) *Ind. Eng. Chem. Anal. Edn.*, 12, 142.
13. Damodaran, M., (1931) *Biochem. J.*, 25, ~~21~~<sup>2123</sup> 2123.
14. Hanes, C.S., (1929) *Biochem. J.*, 23, 99.
15. Loo, Y.H., et al (1945) *J. Bact.*, 50, 701.
16. Donovan, R., et al (1945) *J. Bact.*, 50, 623.
17. Marshall, E.K., et al (1947) *J. Pharmacol. & Expt. Therap.* 90, 367.
18. Foster & Woodruff. (1944) *J. Bact.*, 47, 43.
19. Waksman, S.A., et al (1946) *J. Bact.*, 53
20. Rake, et al (1946) *J. Biol. Chem.*
21. Venkataraman et al (1946) *Nature*, 158, 241.
22. Cook, et al (1945) *Biochem. J.*, 39,
23. Waksman, S.A., et al (1948) *J. Bact.*, 55, 739.

CHAPTER - II.



FACTORS IN WHEAT BRAN EXTRACT RESPONSIBLE FOR STIMULATING  
STREPTOMYCIN FORMATION.

Introduction.

Results presented in the previous chapter have shown that wheat bran, used in the form of its extract, stimulates streptomycin production to a great extent, when S. griseus is grown in it. It has been shown that about 1500 to 2000 of streptomycin could be produced per gram of the solid bran. Investigations aimed at finding the factors in the bran extract responsible for its advantageous effects, were next carried out. No such attempt seem to have been made in spite of the fact already pointed out, that wheat bran is a good medium for antibiotic and enzyme production by several microorganism. In a recent communication a suggestion has been made, that the high antibiotic activity observed when microorganisms are grown in the bran, might be due to the antibacterial fatty acids present in the bran<sup>1</sup>. This suggestion however does not offer a complete explanation for the observed phenomenon, particularly after taking into consideration the results described below.

The investigations broadly consisted in fractionating the bran extract and finding the antibiotic production, when the organism was grown in individual fractions or mixtures. A similar series of experiments carried out by Cook et al<sup>2</sup>, to find the factors in pea extract responsible for penicillin formation, when

P.notatum was grown in it, showed that the stimulating factors were in the 80% ethanol soluble fraction. The present investigation have shown the importance of minerals.

### Experimental

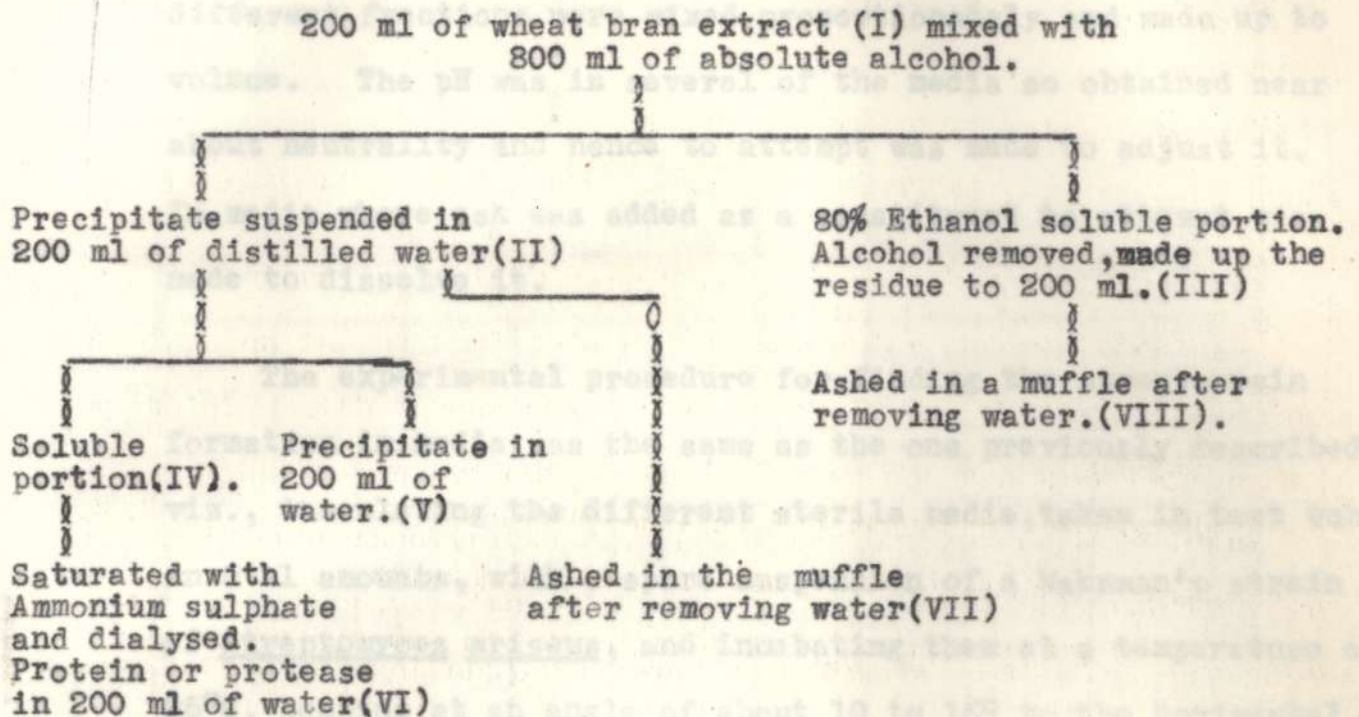
#### Preparation of the fractions and their composition.

The method for the preparation of the aqueous extract of bran has been detailed previously (refer page ). The extract was fractionated after adjusting the total solids to 2%. For this the following procedure was adopted. It was first fractionated into the 80% ethanol soluble portion and the precipitate obtained therein. Growth and antibiotic production by S.griseus in the two fractions were then studied by the methods already described. It was found that eventhough both of the fractions supported growth, streptomycin formation was poor. A mixture of the two fractions however behaved as the original extracts. The 80% ethanol insoluble portion was hence further fractionated as shown schematically in figure II. When however mixtures of two fractions were used the volume of water added was maintained as the same as that of the original extract taken for fractionation without effecting the total solids of the individual fractions.

All the fractions were analysed for total solids, total nitrogen and ash. Methods for the analyses have been already indicated



Figure II.

Scheme of fractionation of wheat bran extract.

(The numbers in brackets indicate the number of the media.)

Table XLIX

Composition of the fractions.

No. of the fraction as in Fig. II	Total solids in mg per 100 ml.	Total nitrogen in mg per 100 ml.	Ash as percentage of total solids.
I	2000	71.6	22.6
II	460	7.06	14.2
III	1540	64.55	25.2
IV	383	4.96	9.34
V	76	2.1	-
VI	25	4.72	-

Preparation of media and antibiotic formation in them.

For the preparation of the media the total solids of the different fractions were mixed proportionately and made up to volume. The pH was in several of the media so obtained near about neutrality and hence to attempt was made to adjust it. In media where ash was added as a constituent no attempt was made to dissolve it.

The experimental procedure for finding the streptomycin formation in media was the same as the one previously described viz., inoculating the different sterile media, taken in test tubes in 5 ml amounts, with a spore suspension of a Waksman's strain of Streptomyces griseus, and incubating them at a temperature of 25°C, keeping at an angle of about 10 to 15° to the horizontal. Two tubes from each group were removed daily and the reaction of the culture fluids, their antibiotic activities and the weight of mycelial growth were determined. It was found that in many of the media thus studied, there was practically very little growth and streptomycin formation. A summary of the results of the above studies in media where there was streptomycin production are given in table L.

The results presented in Table L clearly indicate that inorganic constituents of the bran extract play an extremely important role in streptomycin formation. Since however neither the minerals of fraction II nor those in fraction III stimulated growth and streptomycin formation it seems at least two elements each present in one of the fractions are essential. The



possibility, the optimum concentrations of the important element or elements is attained only on mixing the ash of both the fractions cannot however be overruled, their number being of lesser importance.

Table L.

Streptomycin formation in different media

Composition of media as the number of the fractions mixed	Reaction of culture fluids after days.						Streptomycin titre in per ml after days.					
	4	5	6	7	8	9	4	5	6	7	8	9
I	7.1	7.2	7.4	7.6	7.9	7.9	32	69	111	114	109	92
II	6.9	7.0	7.1	7.1	7.3	7.3	N11	15	15	12	N11	N11
III	6.9	7.3	7.4	7.4	7.6	7.6	"	N11	13	N11	"	"
II + III	7.0	7.1	7.3	7.6	7.9	7.9	30	65	98	112	106	91
II + VIII	7.1	7.2	7.3	7.4	7.8	7.9	30	49	54	78	83	75
III + VII	7.3	7.6	7.8	7.9	7.9	7.9	28	53	108	110	108	98

Experiments with complete ash of wheat bran extract.

In order to confirm the findings detailed above, viz., the stimulating effects of the mineral constituents present in the aqueous extract of bran, on streptomycin formation, the following experiments were carried out. Complete wheat bran extract was ashed and differing amounts of this ash were added to a basal medium which by itself supported very little growth and stimulated very slight streptomycin formation. This medium had the composition,



Difco peptone..... 0.5 gms

Glucose ..... 1.0 "

Sodium chloride..... 0.5 "

Distilled water..... 1000 ml.

pH adjusted to..... 6.8

The usual experimental procedure was followed. The results of these experiments are given in table LI.

Table LI

Effect of wheat bran extract ash on streptomycin production.

Composition of medium	Ash in medium mg per 100 ml.	Reaction of medium after days.						Streptomycin in per ml after days.					
		4	5	6	7	8	9	5	6	7	8	9	10
Whole bran extract		7.1	7.2	7.6	7.7	7.7	7.9	69	111	114	109	92	91
	Nil	6.9	7.4	7.4	7.5	7.6	7.6	24	24	25	28	24	24
	100 mg	7.1	7.2	7.3	7.4	7.6	7.9	24	28	36	64	64	48
Basal medium 100 ml.	200 mg	7.3	7.3	7.4	7.6	7.8	7.9	22	28	45	78	76	78
	400 mg	7.4	7.5	7.6	7.8	7.9	7.9	28	28	54	98	85	88
	500 mg	7.5	7.6	7.8	7.9	7.9	7.9	25	28	61	108	93	92

It will be seen that addition of about 500 mg of ash to the basal medium increased the streptomycin production to about 100 per ml. Since the ash content of wheat bran extract is about 450 mg per 100 ml when the total solids are adjusted to 2% these results prove conclusively that its minerals are mainly responsible for the good antibiotic production observed when S. griseus is grown in it.



Table III

It has been claimed<sup>1</sup> that the fatty acid like components in wheat bran extract which show antibacterial properties are responsible for the high antibiotic formation observed when micro-organisms like S.griseus, P.notatum<sup>3</sup> and A.fumigatus<sup>4</sup> are grown in it. Such a possibility cannot however be entertained in the case of the aqueous extract of the bran used in these investigations, in view of the insolubility of the fatty materials of the bran in water. Further the extract prepared by us did not show any antibacterial activity against strains of B.subtilis and S.aureus susceptible to streptomycin. All these facts along with the data presented in Tables L and LI above conclusively prove that for streptomycin formation by S.griseus much more important than the fatty component of the bran, are its mineral constituents which seem most essential for successful antibiotic production.

The role of meat extract ash. In view of the encouraging results obtained with the ash of wheat bran extract, similar experiments were carried out with meat extract ash to find out whether that can replace the whole extract in Waksman's medium, commonly employed in streptomycin production. The results of these studies along with those obtained, when the organism was grown in Waksman's medium are brought out in Table LII. The results presented there are self explanatory.

Table LII

The role of meat extract ash in streptomycin formation.

Number of days after seeding.	Wakman's medium		Meat extract ash medium	
	pH	Streptomycin in /ml.	pH	Streptomycin in /ml.
4	6.9	18	6.9	23
5	7.4	34	7.2	32
6	7.4	65	7.5	54
7	7.6	75	7.6	73
8	7.9	82	7.9	76
9	7.9	84	7.9	72

Discussion

The results presented above have shown the pronounced effect of the minerals in the bran extract. The source of nitrogen and carbohydrates seems to be of minor importance. The nitrogenous materials present in the bran extract are practically as efficient and useful as the difco peptone used. The carbohydrates of the bran extract and glucose are also having the same influence on growth and streptomycin formation. The pH of the medium as usual became alkaline during the streptomycin formation.

However the fact that the antibiotic production in medium II + VIII (in table L) was less than that in medium III + VII, eventhough the mineral contents of both were the same (the total nitrogen and total solids of the two differing) may perhaps be interpreted as indicating the necessity of a certain minimum amounts of nitrogenous and carbonaceous materials, without ~~which~~



which streptomycin production may be either reduced or completely absent.

As already pointed out two explanations may be offered for the observation that fractions II and III do not independently stimulate antibiotic formation. Thus, it may be that there are two essential elements in the bran extract which on fractionation pass on, one into each fraction, or it may be due to the lack of optimal concentration of the element or elements in the fractions.

#### Summary

1. Fractionation of wheat bran extract has been carried out. These fractions have been analysed for nitrogen, ash and total solids.
2. Streptomycin production in these fractions has been studied. It has been found that minerals present in the bran extract are mainly responsible for the antibiotic production by S. griseus. Results of a similar nature have been obtained for meat extract ash.

#### References.

1. Humfeld, H., (1947) J.Bact., 54, 573.
2. Cook et al (1945) Biochem.J., 39, 314.
3. Srinivasa Rao, S., (1944) Nature, 154, 83.
4. Venkataraman, P.R. et al (1946) Nature, 158, 241.

CHAPTER - III.



STUDIES ON THE MINERAL REQUIREMENTS OF S.GRISEUS FOR  
STREPTOMYCIN PRODUCTION.

Introduction

been

Minerals have often times/found to be of primary importance in antibiotic production by microorganisms. Several instances where small amounts of inorganic ingredients present in the media increasing antibiotic production have been reported. Thus for Penicillin notatum Foster et al<sup>1</sup> have shown the influence of small amounts of zinc. Importance of mineral constituents in penicillin production have also been emphasized by Moyer et al<sup>2</sup> and Pratt et al<sup>3</sup>. In detailed investigations carried out by Keffler et al<sup>4</sup> they find that traces of iron are necessary for antibiotic production by P.notatum and that effect of iron could be completely prevented by small quantities of copper. The advantageous effects of sodium chloride in antibiotic production have been reported by Cook et al<sup>5</sup>. Similar investigations on pyocynin production, undertaken by Burlon et al<sup>6</sup> show that ions of Mg, SO<sub>4</sub>, K, PO<sub>4</sub> and Fe to be essential.

Investigations presented in the previous chapters on streptomycin formation by S.griseus have shown that the ash content of the wheat bran extract used is mainly responsible for the high antibiotic production observed. It was shown therein that addition of about 500 mg of the ash to 100 ml of a basal medium resulted in antibiotic production, comparable to that in the aqueous extract of the bran. Experiments were hence carried out to find the minerals of the ash that are responsible for its advantageous effects.



(After the completion of the present work, a few reports regarding the nutritional requirements of S. griseus for successful antibiotic production have appeared. Most important of these from the point of view of the investigations to be detailed below are those of Thornberry and Anderson<sup>7</sup>. While investigating on a synthetic medium for streptomycin production they found that manganese, potassium, zinc, iron and magnesium are necessary for good streptomycin formation. Mc Farlane et al<sup>8</sup> have shown the advantages of using amino acids, particularly valine, arginine and histidine and among these the authors report, valine to be of primary importance. Among the amino acids proline has also been reported to ~~be~~ increase antibiotic production enormously<sup>9</sup>.)

The investigations of Rake and Donovan<sup>10</sup> on soyabean meal and sodium chloride; as also the effect of potassium<sup>11</sup> have been previously referred to.

The present investigations were however a logical sequence of the results recorded in the previous chapters viz., the antibiotic production by S. griseus in the aqueous extracts of bran, the results of investigations on the fractionation of bran which revealed the importance of the mineral constituents and the results of studies on meat extract ash.

#### Experimental

The culture of S. griseus used, and the method of estimating streptomycin potency, of the culture fluids were the same as those previously described. The nature of culturing, the temperature of incubation etc have also been previously outlined.



The investigations to be described, broadly consisted in adding minerals (known both qualitatively and quantitatively) to a basal medium of known mineral content, and studying the streptomycin formation in the media so obtained. The minerals used however were those present in the ash of bran extract.

Composition of wheat bran extract ash. A semi quantitative analysis of the wheat bran extract ash was first carried out and the results are presented in table LIII below.

Table LIII

Composition of the ash of wheat bran extract and meat extract.

Name of element.	Wheat bran extract ash	Meat extract ash
Sodium	++	+++
Calcium	+++	+++
Magnesium	+++	+++
Aluminium	+++	+++
Iron	++	++
Phosphorus	++	++
Potassium	++	++
Copper	+	+
Manganese	+	+

In the table +++ indicates abundance,  
 ++ indicates presence, and  
 + indicates traces of the elements.

From the data presented it appears, that there is a similarity in the composition of the two ashes.

The following minerals were estimated quantitatively: Iron, Potassium, Phosphorus, Calcium and Sodium, by the methods given below and the results are presented in table LIV.

Sodium. Zinc uranyl acetate method was used.

Iron. The colorimetric method of Kennedy and Smith<sup>12</sup> was used.

Potassium was estimated by the cobaltinitrite method.

Phosphorus was estimated by the modified Fiske and Subbarow method.<sup>13 & 14</sup>

Calcium was estimated acidometrically.<sup>15</sup>

Table LIV

Composition of wheat bran extract ash.

Mineral	Amount of element present in 100 gms of ash.
Sodium	21.32 gms
Potassium	33.23 "
Calcium	24.92 "
Iron	137.3 mg
Phosphorus	463.7 "

Preliminary investigations. These experiments were aimed at finding the elements in wheat bran extract, essential for production of streptomycin. The investigations essentially were qualitative in nature. Since the importance of sodium has already been dealt with in a previous chapter it was not investigated. The effect when sodium chloride was present in



0.5% and 2% were studied. A series of 12 media were prepared by adding different minerals. To find the effect of a particular element that one was omitted from the media and all the others mixed. Peptone was used as nitrogen source and glucose as source of carbon. Phosphorus was supplied in the form of  $\text{Na}_2\text{HPO}_4$ .

All the media were adjusted before tubing and inoculating to pH  $7.0 \pm 0.2$  and autoclaved as usual. In table LV below minerals used, their relative amounts and composition of the different media are given.

Of the elements detected to be present in the ash all have been used in the media. Sodium has been used in considerably large quantities since its advantageous effects were observed even in concentrations of about 2%. Other elements have been used in rough proportionality to their existence in the ash. Manganese was not tried primarily because it was present only in very small traces.

The antibiotic titre and pH of the culture fluids were measured daily for a period of about 14 days, after the inoculation. It was observed that in media I and II and in media containing aluminium growth was poor. In others there was good growth. The results of antibiotic activity and pH in different media are summarised in table LVI.

Of the different elements tried iron is the only one which appears to have profound influence on streptomycin production. Thus absence of iron brought down streptomycin formation to 15 units

from about 70 units per ml. The effect of other elements like potassium and magnesium is negligible even though data presented seem to indicate that they too may have a slight influence on streptomycin formation.

Table LV  
Composition of media

No. of medium	Pep- tone	Glu- cose	KNO <sub>3</sub>	Sodium phosph- ate	Sodium chlor- ide	Copper sulph- ate	Ferric chlor- ide	Magne- sium sulph- ate	Alum	CaCl <sub>2</sub>
I	1.0	2.0	-	-	-	-	-	-	-	-
II	1.0	2.0	-	-	2.0	-	-	-	-	-
III	1.0	2.0	0.05	0.2	0.5	0.001	0.003	0.05	-	-
IV	1.0	2.0	0.05	0.2	0.5	-	0.003	0.05	-	-
V	1.0	2.0	0.05	0.2	0.5	0.001	-	0.05	-	-
VI	1.0	2.0	0.05	0.2	0.5	0.001	0.003	-	-	-
VII	1.0	2.0	0.05	0.2	0.5	0.001	0.003	0.05	0.05	-
VIII	1.0	2.0	0.05	0.2	2.0	0.001	0.003	0.05	-	-
IX	1.0	2.0	0.05	0.2	2.0	0.001	0.003	0.05	0.05	-
X	1.0	2.0	0.05	0.2	0.5	0.001	0.003	0.05	0.05	0.05
XI	1.0	2.0	0.05	0.2	2.0	0.001	0.003	0.05	-	0.05
XII	1.0	2.0	-	0.4	0.5	0.001	0.003	0.05	-	-

In the above table the figures give the amount of substances added in gms per 100 ml of the medium.



Table LVI  
Antibiotic titre in present of different elements.

No. of medium	Maximum antibiotic activity		pH	
	Streptomycin in per ml.	Days after inoculation	Maximum pH	Days after inoculation
I	20	6	7.2	6
II	35	6	7.2	6
III	72	7,8.	7.7	9
IV	68	7,8.	7.7	9
V	15	7,8.	7.8	10
VI	59	7,8.	7.8	9
VII	71	7,8.	7.7	9
VIII	92	7,8.	7.8	8,9,10
IX	91	7,8.	7.7	9
X	73	7,8.	7.7	9
XI	88	7,8.	7.7	9
XII	65	7,8.	7.9	9

Optimum concentrations of iron.

Investigations were next carried out to find out the effect of differing concentrations of iron on streptomycin formation. A basal media without iron and of the following composition was used for this work.

Peptone	1.0 gm
Glucose	2.0 "
Sodium chloride	0.5 "
$\text{KH}_2\text{PO}_4$	0.5 " per 100 ml.
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.001 Gm.
$\text{MgSO}_4$	0.05 "

To this basal medium, small amounts of iron was added in the form of ferric chloride. The experimental procedure for finding the antibacterial activity of the culture fluids, were the same as those already described, (refer page ). A summary of the results is given in table LVII.

Table LVII.

The effect of iron on streptomycin production.

Amount of iron in mgm. per 100 ml.	Maximum streptomycin titre	Date after inoculation	Maximum pH	Date after inoculation
Nil	15	-	7.8	10
0.125	68	9	7.8	9
0.250	75	8	7.9	9
0.500	93	8	7.9	9
0.600	98	8	7.9	9
0.700	108	8	7.9	9
1.000	110	8	7.9	8

The remarkable effect of small concentrations of iron are very clearly brought out, about 0.7 mg. per 100 ml being the optimum. The higher concentration studied did not have much more effect, than the above concentration. It may be pointed out that the amount of iron present in wheat bran extract when its total solids are adjusted to 2% is about 0.62 mg. per 100 ml.



### Discussion

An attempt has been made to find the minerals (essentially from those present in the bran extract) which stimulate production of streptomycin. Investigations recorded have shown the importance of iron in streptomycin formation. Streptomycin production in the media of known composition, appears to suggest that the claim, that streptomycin production depends largely on the presence of certain growth promoting agents, organic in nature may not altogether be correct. Further, the claim, that the high antibiotic production observed when microorganisms are grown in it is due to the antibacterial fatty materials in the bran also appear to be unjustified.

Results presented in the previous chapter suggested, the possibility that more than a single element was essential for good streptomycin production. The investigations carried out on the mineral requirements of the organism have shown however the necessity and the importance only one element viz., iron. The explanation for this observation appears to be difficult, based on the available data regarding the metabolism of S. griseus. The rough quantitative relationship observed between the iron content and antibiotic production, disclaims the alternative explanation, that the very low antibiotic titre observed in the two fractions of wheat bran extract, are due to the lack of an optimal concentration of the essential element in them (refer page       ).

A correct explanation of this phenomena, may be possible perhaps only after a thorough understanding of the mechanism by which the



the minerals exert the observed effects. Thus if it may be assumed that the minerals play the role of bio-catalysts in the biosynthesis of the antibiotic, it may be that they act at some particular stage in the step wise synthesis of the antibiotic, from the compounds already existing in the medium, and hence the minerals required for the formation of antibiotics may also vary, depending upon the other ingredients present in the medium. This suggestion might offer an explanation for the facts described above. The fact that several elements, amino-acids etc. have all been found to affect the antibiotic production of different microorganism might perhaps lend support to the above suggestion. While, it is true that each element or amino-acid is essential and steps up streptomycin formation in one particular medium, it may be that the particular activating factor may lose much of its influence if placed in an entirely different medium, whose composition is different from the one where the particular compound is indispensable. It may not hence be correct in the strictest sense <sup>to say</sup> that iron is essential for streptomycin production. However, the high antibiotic production observed when S. griseus is grown in the bran extract is due to the minerals particularly the iron and in all probability not due to the antibacterial properties exhibited by the fatty material in the bran.

#### Summary.

Investigations carried out to find the role of minerals in streptomycin formation have shown the importance of iron, which even in small concentrations stimulates the formation of the antibiotic.



References

1. Foster, J.W., Woodruff, H.B.,  
& McDaniel, L.E. (1943) J. Bact. 46, 421.
2. Moyer, A.J., Coghill, R.D., (1946) J. Bact. 51, 79.
3. Pratt, R., (1945) Amer. J. Botany, 32, 528.
4. Koffler, H., Knight, S.G.,  
& Frazier, W.C., (1947) J. Bact., 53, 115.
5. Cook, et al (1945) Biochem., J., 39, 314.
6. Bourton, M.O., et al (1948) Cand., J. Research, 26, 15.
7. Thornberry & Anderson., (1948) Arch. Biochem., 16, 389.
8. McFarlane and Feizer, (1948) Cand., J. Research, 26, 164.
9. Woodruff, H.B. & Ruger, M., (1948) J. Bact. 56, 315.
10. Rake, G., and Donovick, R., (1946) J. Bact., 52, 223.
11. Bennett et al (1946) J. Bact. 51,
12. Kennedy, R.P., (1927) J. Biol. Chem. 74, 385.
13. Fiske & Subba Row J. Biol. Chem.
- 14.
15. (1937) J. Biol. Chem. 122, 665.
16. Bailey, J., & Cavallito, C.J., (1943) J. Bact. 45, 30.
17. Jawsen, E.F. & Hirschmann, D.J., (1944) Arch. Biochem. 4, 297.
18. Lewis, J.C., Dimmick, K.P.,  
& Feustel, I.C., (1945) Ind. Eng. Chem., 37, 996.

CHAPTER - IV.



## STUDIES ON THE UTILISATION OF GROUNDNUT CAKE FOR STREPTOMYCIN

### PRODUCTION.

### Introduction.

It has been widely accepted that groundnut cake may be considered as a fairly complete food, from the stand point of chemical composition and ready digestibility<sup>1</sup>. It contains an abundance of energy producing materials in the form of carbohydrates and fats to meet the demands for heat and activities of the bodily machine. The work of Johns and Jones<sup>2,3,4</sup> and of Brown<sup>5</sup> has shown that the two proteins occurring in groundnut, arachin and conarachin, contain significant proportions of indispensable amino acids<sup>6</sup>, thus furnishing fairly good quantities of tissue building components in the form of proteins to meet the requirements of the growing young organisms, and the wear and tear of the fully developed organisms. It contains several vitamins of the B-complex group<sup>7-11</sup> and is also a very rich source of vitamin E<sup>12</sup>.

Groundnut cake is the residual meal left behind when the former has been freed from oil by mechanical pressure. In India the cake is largely used as a fodder or fertiliser, even though it has been widely used for the production of adhesives, plastics, water paints and allied materials. Fontaine and Burnett<sup>13</sup> have studied in great detail the peptisation of nitrogenous materials of solvent extracted and hydraulic pressed pea-nut meal under varied conditions of pH and salt concentration. Peptones of peanut meal have been prepared<sup>14</sup> by the peptic and pancreatic digestion, with

a view to utilise them for culturing of pathogenic micro-organisms. Vegetable media containing papain hydrolysed peanut meal have been found<sup>15</sup> to support growth and good toxin production as well. Basu and Sengupta<sup>16-18</sup> have also recorded the results of their investigation on groundnut cake and its use as media for culturing of pathogenic organisms.

It is clear from the foregoing that groundnut cake suitably processed can be utilised for supporting the growth of micro-organism with toxin production in many cases. Investigations to use the cake after digestion, in media for production of streptomycin have been carried out and the results of the investigations are recorded below.

#### Experimental.

##### Preparation of the material and proximate chemical composition of the cake.

Groundnut cake available in the local markets was used. The cake was first powdered and passed through a 60 mesh seive. The powdered material was analysed for proteins, fats, carbohydrates, ash and moisture. The results of these investigations are presented in table LVIII.

Table LVIII.

Proximate composition of groundnut cake.

Form	Mean value of present work	Basu and Sengupta's values.
Moisture	5.01%	8.10%
Protein	51.88%	52.93%
Fat	6.82%	5.43%
Carbohydrates	26.63%	22.08%
Ash	5.50%	5.75%
Fibre	4.16%	5.71%



## Table LIX.

Preparation of the groundnut cake hydrolysate. For using it as medium the groundnut cake was hydrolysed, by using papain. The following method was used. Finely powdered groundnut cake (50 gms.) was dispersed in 300 ml of water in a 500 ml round bottomed flask, carrying in a condensor. Papain (crude) 1 gm. was added to the mixture and the flask shaken for about ten minutes. It was then heated in a water-bath, the temperature of which was maintained at about  $75 \pm 2^{\circ}\text{C}$ ., for a period of five hours; the mixture being shaken frequently. At the end of five hours, 20 ml of 0.5 N sulphuric acid was added and the digestion mixture boiled for about fifteen minutes under reflex and filtered hot or centrifuged. The residue was washed twice with hot water (75 and 50 ml). The combined filtrate or centrifugates and the washings were clarified if necessary, by ~~filtration~~ refiltration through ~~the~~ filter paper and ~~the~~ made up to a total volume of 400 ml containing 1 ml of chloroform.

The hydrolysate so obtained gave positive test for, ~~by~~ biuret, Mellon's, Xanthoproteic and Hopkins - Cold reactions. Iodine gave the characteristic blue colour with the hydrolysate, which reacted very feebly with reducing agents. In table LIX the mean values for the percentage of nitrogen and carbohydrates solubilised under the conditions of the preparation of the hydrolysate are presented. The mean pH of the hydrolysate was usually 5.3 and it gave only a faint turbidity with 5% trichloroacetic acid. Cysteine was present in the hydrolysate in very small quantities as indicated by its faint reaction with uric acid reagent.

Table LIX.

Percentage of carbohydrates and nitrogen solubilised.

Form	Groundnut cake 50 gms.	Hydrolysate 400 ml	Percentage solubilised
Carbohydrates	13.32 gms.	1.66 gms.	12.49
Nitrogen	4.0 "	2.72 "	66.3

The residue after the hydrolysis was dried and weighed. The mean value for a series of experiments was 20 gms.

The main papain digest of groundnut was analysed for total solids, total nitrogen, amino nitrogen, amide nitrogen, basic nitrogen, ~~in~~ tyrosine, tryptophane, ash and carbohydrates. The methods of analysis for the above, other than tyrosine and tryptophane have been given previously (Refer page      ). For the amino acids the following methods were used. They were estimated according to the method of Folin and Ciocalteu<sup>19</sup>, after hydrolysing 2 ml of the hydrolysate with 2 ml of 40% sodium hydroxide in a boiling water-bath for eight hours. The results of the analysis are given in table LX.

In table LXI the proportions of various forms of nitrogen expressed as percentages of the total nitrogen of the hydrolysate are brought out.



Table LX.

Composition of the groundnut cake hydrolysate.

Form	Digest number					Mean
	I	II	III	IV	V	
Total solids	57.7	52.2	57.6	55.5	55.7	55.7
Total nitrogen	6.80	6.85	6.89	6.80	6.66	6.80
Amino nitrogen	1.49	1.49	1.52	1.39	1.55	1.49
Amide nitrogen	0.73	0.70	0.73	0.77	0.73	0.73
Basic nitrogen	4.25	4.27	4.12	4.38	4.33	4.27
Tyrosine	2.18	2.04	1.86	2.04	2.03	2.04
Tryptophane	0.51	0.53	0.51	0.51	0.49	0.51
Carbohydrates	-	-	-	-	-	4.16
Ash	-	-	-	-	-	3.00

Values are given in gms. per 1,000 ml.

Table LXI.

Various forms of nitrogen expressed as percentages of the total nitrogen.

Form of nitrogen	Mean value expressed as percentage of the total nitrogen
Amino nitrogen	21.09
Amide nitrogen	10.76
Basic nitrogen	62.79
Tyrosine nitrogen	2.23
Tryptophane nitrogen	1.03
Mean complexity of the peptone	4.75

Stationary cultures. To study the antibiotic formation in stationary cultures, in media containing the papain digest of groundnut cake, the experimental procedure already described (refer page ) were used. The composition of the media is given in table LXII, and the results of investigations on antibiotic production, pH of culture fluids, and weight of growth are recorded in table LXIV.

Table LXII.

Composition of the different media used.

Number of medium	Weight in gms. of the components added					Basal medium
	Sodium chloride	Glucose	Brown sugar	Sodium acetate	Minerals *	
1	Nil	Nil	Nil	Nil	Nil	100 ml. of 2% total solids
2	0.5	"	"	"	"	- do -
3	2.0	"	"	"	"	- do -
4	Nil	2.0	"	"	"	- do -
5	"	Nil	2.0	"	+	- do -
6	"	2.0	Nil	"	+	- do -
7	"	Nil	"	"	+	= do -
8	"	"	"	2.0	+	- do -
9	2.0	2.0	"	Nil	+	- do -
10	2.0	2.0	"	"	Nil	= do -

In the above table the minerals had the composition Potassium dihydrogen phosphate 1%, ferrous and magnesium sulphates in traces.  
+ indicates the presence of the minerals.



Table LXIII.

Streptomycin formation in media containing groundnut cake  
hydrolysate.

No. of medium as in table LXII		Days after inoculation								
		4	5	6	7	8	9	10	11	12
1	A	15	25	25	25	25	25	25	25	25
	B	7.3	7.6	7.8	7.9	7.9	8.0	8.1	8.1	8.1
	C	2	3	3	3	2	2	2	2	2
2	A	15	22	23	25	25	25	25	23	22
	B	7.4	7.9	7.9	7.9	7.9	7.9	7.9	7.9	7.9
	C	2	3	3	4	3	4	2	2	2
3	A	25	48	68	85	82	77	75	73	68
	B	7.2	7.7	7.9	7.9	7.9	7.9	7.9	7.9	7.9
	C	-	5	7	10	8	7	6	6	5
4	A	18	22	25	25	30	28	27	25	20
	B	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
	C	-	2	3	2	2	3	3	2	2
5	A	-	-	15	20	20	18	17	15	12
	B	5.4	6.0	6.0	6.2	6.4	6.4	6.4	6.4	6.4
	C	8	12	14	16	14	12	13	12	12

Table LXIII (continued)

No. of medium as in table LXII		Days after inoculation								
		4	5	6	7	8	9	10	11	12
6	A	15	25	50	50	45	47	45	47	48
	B	7.0	7.4	7.6	7.8	7.9	7.9	7.9	7.9	7.9
	C	4	8	13	14	14	13	12	12	12
7	A	-	-	15	20	20	20	18	17	15
	B	7.0	7.4	7.6	7.7	7.8	7.9	7.9	7.9	7.9
	C	Negligible								
8	No growth									
9	A	65	85	110	130	120	110	115	109	108
	B	7.0	7.4	7.9	7.9	7.9	7.9	7.9	7.9	7.9
	C	4	8	13	14	14	12	12	13	13
10	A	63	85	110	110	110	106	103	100	102
	B	7.2	7.5	7.6	7.9	7.9	7.9	7.9	7.9	7.9
	C	Negligible								

In the above table A represents Streptomycin units per ml

B " pH of the culture fluids

C " the growth for 10 ml of medium.

ePrint@CFTRI



Shaken culturing. The importance of shaken culturing has been dealt with previously. The antibiotic production by S. griseus when grown in some media containing groundnut cake hydrolysate was studied by the method already described (refer page ). The results are given in table LXIV.

Table LXIV.

Streptomycin formation in shaken cultures.

Days after inoculation	Streptomycin titre in per ml. in medium.		
	3	9	10
2	20	20	20
3	30	35	40
4	40	55	80
6	110	120	130
7	110	115	125

The results presented in the above table clearly indicate that streptomycin formation is not enhanced but is only quickened when S. griseus is grown in groundnut cake hydrolysate media by shaken culturing.

### Discussion.

The groundnut cake hydrolysate is marked by its high nitrogen content and low carbohydrate and mineral contents. This might explain the low antibiotic formation in it, as also the increased streptomycin formation when glucose was added to it. Eventhough, many media used stimulated streptomycin formation, yet the media containing the aqueous extracts of wheat bran appear to be superior in several ways to those containing the papain digest of groundnut cake.

Sodium chloride increased the antibiotic production considerably. The other minerals added did not have any noticeable effect on streptomycin production. In view of the inferiority of the papain digest to wheat bran extract, the studies on groundnut cake hydrolysate were not pursued further.

### Summary

1. A method for the preparation of a papain digest of groundnut cake has been described. The analysis of the digest has also been carried out.
2. Antibiotic production by S. griseus in the unsupplemented and supplemented media has been studied. Good antibiotic formation was observed in some of them.
3. Shaken cultures did not have much advantage over stationary ones.



References.

1. Holmes, A.D., (1918) U.S.Dept.Agric.Bull.,28, 717.
2. Johns, C.O.,& Jones,D.B.,(1916) J.Biol.Chem.,28, 77.
3. Johns, C.O.,& Jones,D.B.,(1917) J.Biol.Chem.,30, 33.
4. Johns,C.O.,& Jones,D.B.,(1918) J.Biol.Chem.,36, 491.
5. Brown,W.L., (1942) J.Biol.Chem.,142,299.
6. Rose,W.C., (1937) Science, 86, 298.
7. Kline, O.L.,et al. (1936) J.Nutrition,12, 455.
8. Sherwood,F.W.,&  
Halverson,J.O., (1932) J.Agric.Research,44, 849.
9. Sherwood,F.W.,&  
Halverson,J.O., (1938) J.Agric.Research,56, 927.
10. Pickett,T.A., (1942) Georgia Agric.Expt.Sta.,  
Circ,128, 10.
11. Fontaine and Burnett, (1944) Ind.Eng.Chem.,36, 164,284.
12. Bertilot, A.,et al., (1930) Bull.Soc.Chem.Biol, 12, 1029.
13. Basu and Sen Gupta (1945) Ind.Med.Gaz.
14. Folin and Ciocalteu (1927) J.Biol.Chem.,73,627.

## APPENDIX

List of Publications.

1. Investigations on plant antibiotics. Part I, Studies on Allicin, the antibacterial principle of Allium sativum  
R.Raghunandana Rao., S.Sreenivasa Rao., and P.R.Venkataraman.  
Journal of Scientific & Industrial Research, 5B, 31, (1946)
2. Investigations on plant antibiotics. Part III, Studies on pterygospermin the antibacterial principle of M.pterygosperma  
R.Raghunandana Rao, and Mariam George.  
Ind.J.Med.Research, 37, (April), 1949.
3. Toxicity of Allicin and Pterygospermin.  
R.Raghunandana Rao and S.Natarajan.  
Proc.Ind.Acad.Science, 29, 148, (1949)
4. Studies on the pharmacology of Allicin and pterygospermin.  
R.Raghunandana Rao, N.N.De., and S.Natarajan.  
(Under publication).
5. Inhibition of Mycobacterium tuberculosis by garlic extract.  
R.Raghunandana Rao., S.Sreenivasa Rao., S.Natarajan and P.R.Venkataraman.  
Nature, 157, 441, (1946).
6. Pterygospermin the antibacterial principle of M.pterygosperma.  
R.Raghunandana Rao., Mariam George., and K.M.Pandalai.  
Nature, 158, 745, (1946).
7. Synergism of pterygospermin with other antibacterial agents.  
R.Raghunandana Rao., and Mariam George.  
J.Sci.Ind.Research, 8, 111, (1949)
8. Effect of antibiotics on the milk clotting activity of the enzymes of Carica papaya and Ficus carica.  
R.Raghunandana Rao., and C.R.Krishnamurthi.  
Current Science, 17, 23, (1948).
9. On Morellin the antibacterial principle of Garcinia morella.  
R.Raghunandana Rao., and S.Natarajan.  
Current Science, (under publication).
10. Utilization of groundnut cake for streptomycin production.  
R.Raghunandana Rao., S.Sreenivasa Rao., and P.R.Venkataraman.  
Nature, 158, 23, (1946).



11. Utilization of Wheat bran for streptomycin production.  
R.Raghunandana Rao.,  
Nature, 162, 820, (1948).
12. The role of inorganic constituents of wheat bran extract in streptomycin production.  
R.Raghunandana Rao.  
Nature, 162, 850, (1948).
13. Preparation and composition of groundnut cake hydrolysate.  
R.Raghunandana Rao., and P.R.Venkataraman.  
J.Amer.Pharm.Assn., p 184, (April 1949).
14. Studies on streptomycin Part I. Wheat bran extract as medium for streptomycin production.  
R.Raghunandana Rao.  
(under publication).
15. Studies on streptomycin Part II. The factors in the bran extract responsible for stimulating streptomycin production.  
R.Raghunandana Rao.  
(under publication).
16. Studies on streptomycin Part III. The role of certain minerals in streptomycin formation.  
R.Raghunandana Rao.  
(under publication).
17. Studies on streptomycin Part IV. The nitrogen metabolism of S.griseus during streptomycin formation.  
R.Raghunandana Rao.  
(under publication).
18. Studies on streptomycin Part V. Utilization of groundnut cake for streptomycin production.  
R.Raghunandana Rao.  
(under publication).
19. Relation between the source of nitrogen and antibiotic production by Aspergillus fumigatus Fresenius.  
R.Raghunandana Rao., and P.R.Venkataraman.  
Nature, 158, 241, (1946).

(Reprints of publications available at present are attached)