Shakuntala H. Rawani.

"BACTERIAL DECOMPOSITION OF STREPTOMYCIN".

ABSTRACT

The aim of the project was to elucidate the kinetics of streptomycin decomposition by soil bacteria. Primary investigations involved experiments such as adding 1000 µg of streptomycin/gm of sterile and non-sterile soil and studying its stability and both, the cause and the course of its disappearance. It was found that most of the added streptomycin was strongly adsorbed on soil colloids. The evidence that the disappearance of added streptomycin from normal soil as compared to its constant recovery from sterile soil, and an increase in the microbial population. Simultaneous to decomposition of the antibiotic from the normal soil, suggested that streptomycin was microbiologically decomposed. Soil was repeatedly treated to enrich it with the streptomycin decomposing organism, and the course of decomposition of succdssive additions of streptomycin was followed.

Samples of "Soil extract" (mixed soil population) from the above "active Soil" in which streptomycin had been decomposed were introduced into bentonite containing laboratory synthetic media with streptomycin as the sole source of carbon and nitrogen and the course of degradation of streptomycin was studied; bentonite was added to the medium to make conditions similar to thosein soil. In

these media all except 20 µg of streptomycin/ml of medium was adsorbed on the clay, and the free streptomycin was decomposed in 4-5 days after an induction period in which no decomposition took place. Parallel experiments were performed with synthetic media containing 20 µg/ml of antibiotic and no bentonite; the streptomycin in this case, too, was decomposed in similar manner. Degradation of streptomycin in both these types of media was studied under different environmental conditions.

Mixed cultures which decomposed streptomycin were isolated and isolates obtained were studied for their ability to decompose streptomycin in synthetic media containing streptomycin as the sole source of carbon and nitrogen (streptomycin-synthetic-medium). A detail characterization and identification of one of the prominent and active isolates was carried out; the isolate was identified as a member of the family Corynebacteriaceae, belonging to the genus Cellulomonas, Viz. Cellulomonas fimi. Studies similar to those done with mixed cultures were carried out to investigate the kinetics of streptomycin decomposition by this organism.

The above experiments showed that the organism decomposed streptomycin (20 µg/ml) incorporated in the streptomycin-synthetic-medium in 21 days with an induction

period of 18 days in which no decomposition took place. An important observation was, that though there was no decrease in the streptomycin content during the induction period, there was a hundred-fold increase in the total viable cell numbers of the organism within 24 hours. It is interesting to note that decomposition of streptomycin was always associated with an amine-like odour. Successive additions of antibiotic (20 µg/ml) made in the same medium after the previous dose had disappeared, were decomposed without any induction period. Washed cell suspensions taken from the culture after decomposition were able to degrade streptomycin in fresh streptomycin-synthetic-medium in 7 days with an induction period of 4 days. In this case the maximum viable cell numbers were obtained in 7 days, which suggested a competition between the enzymes involved in the synthesis of cell building material and the streptomycin decomposing enzymes.

Of considerable importance were the experiments performed to investigate the energy sources for the multiplication of the organism during the induction period, which showed that ANALAR salts such as $MgSO_4.7H_2O$ and $CaCl_2$ were responsible in part, if not entirely, for growth.

BACTERIAL DECOMPOSITION
OF
STREPTOMYCIN

by

SHAKUNTALA HANSRAJ RAVANI

THESIS

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SECTION IV

Decomposition of streptomycin by Cellulomonas fimi.

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INTRODUCTION

FOREWORD

The natural environments known as soils are systems comprising numerous living forms ranging from submicroscopic viruses and prages, through the microscopic bacteria, actinomycetes, fungi, algae and protozoa to the lower animals forms which can be seen with the naked eye. The micro-organisms make the soil a living system rather than a dead mass of mineral matter and organic residues. Although the enormous population of micro-organisms forms only a small part of the soil mass; it is responsible for the many of the chemical transformations and even for some of the physical changes that take place there in. They affect the life and economy of man in many ways.

The qualitative and quantitative composition of soil population is controlled by the nature and availability of nutrients; the physical chemical and biological nature of the habitat; as well as the environmental conditions such as aeration, temperature, hydrogen-ion concentration and the available moisture. Associative and antagonistic relationships between the members of soil population are very common and many micro-organisms are capable of producing antagonistic substances. Though found to be controversial aspect, it may however, be regarded as merely one of the aspects of a more general problem, the "struggle for existance".

In other words, the general interrelationships in a living system, containing a wide varity of micro-organisms growing side by side in a complex substrate - the soil.

Since the majority of antibiotic-producing organisms are of soil origin, the question of their significance in nature and relative importance in the soil fertility has naturally been raised. Also, apart from their medical uses in the restoration of health in men and animals, they also found use in agriculture, for the control of soil-borne plant pathogens. In the light of aforesaid it is important both from the academic as well as practical point of view to study these antibiotics (antagonistic substances) and their fate in soil economy.

The work to be described in this thesis is concerned with the fate and effect of any streptomycin that may reach the soil, a problem related to many investigations on the metabolism of substances, of both biological and non-biological origin, either normally present in the soil, produced there in, or introduced into it. Hence the first section of introduction deals with the production and role of antibiotics in the soil; and the second with the use of antibiotics for the control of plant diseases pathogens. In the third section the literature concerning the stability of antibiotics in the soil will be reviewed, finally the microbiological decomposition of antibiotics with special reference to

streptomycin will be discussed.

I) The production and role of antibiotics in the soil.

The abundant microbial population of soil is very complex; the members of which depend upon one another for their nutrients. This results in formation of associative and antagonistic relationship between the soil micro-organisms. Many cases are known of antagonism between the soil micro-organisms. Experiments in vitro demonstrate that microbial antagonists can decrease the virulance of phytopathogenic agents and protect the plant from the infection. Also, there is a tremendous amount of evidence available in the literature that by manure treatment of the soil alone or together with the antagonists certain plant diseases could be controlled. The mechanism in all these cases of antagonism, is little understood, and antibiotic production suggests a possible mechanism. This assumption is based on the evidences that antibiotic-like substances are present in the natural soil. Also, the majority of antibiotic-producing organisms are of soil origin; which may produce antibiotics in the soil under suitable conditions. Indeed, progress of research in this field showed that antibiotics can be produced in the soil.

Thus according to the development of the problem of 'production and role of antibiotics in the soil' the relevant literature now follows which is sub-divided into four groups

namely, 1) Microbial antagonism in soil, 2) Occurance of "toxic" and antibiotic substances in the natural soil, 3) Antibiotic producing soil-micro-organisms, and 4) Production of antibiotics in the soil.

1) Microbial antagonism in soil.

The phenomenon of antagonism between soil-micro-organisms is very common and many bacteria, actinomycetes and fungi, isolated from the soil can act on phytopathogenic fungi.

Sanford (1926) and Millard and Taylor (1927) began the study of relations existing between soil micro-organisms and the pathogen-host complex. Sanford proposed that the beneficial action of certain manuring crops in reducing the common scab of potato was indirect and resulted from the effects of bacteria antagonist to Streptomyces scabies. Millard and Taylor (1927) suggested that under certain conditions attacks by S.scabies, was reduced by an antagonist, S.praecox. Sanford and Broadfoot (1931) studied the antagonistic effect of a number of fungi and bacteria on Ophiobolous graminis; and Lal (1939) and Winter (1940) showed the antagonistic effect of Penicillium, Trichoderma and Absidia species on Helminthosporium sativum.

Phytium, a root parasite of sugar cane was inhibited by antagonistic actinomycetes (Tims, 1932); and Hypochnus, Sclerotium and Phytophthora were supressed by numerous soil

organisms (Endo, 1931-33).

Thus there are many cases showing antagonism between micro-organisms and this phenomenon has been utilized by many soil microbiologists for the control of plant diseases by manurial treatment of soil alone or together with the inoculation of antagonists. Also there are evidences that disease incidence could be reduced by inoculation of infested soil with antagonists. The examples are given below.

Millard and Taylor (1927) made one of the first experiments in which inoculation with an antagonist and organic manuring were combined, they showed that Streptomyces praecox, an antagonist to S. scabies, controlled the pathogen in sterilised soil, the addition of green manures enhanced the action. Grossbard (1952) conducted a series of experiments (1945-48) to designed to control the "damping-off" diseases of tomato seedling due to Phytium cryptogea and Phytium parasitica by inoculating sterile soil supplemented with glucose, sugar beet pulp or wheat straw, with either Aspergillus clavatus or Penicillium patalum. Wood (1951) showed that Penicillium claviforme alone did not protect the pathogen Rhizoctonia in infested soil, but a combination of soil amendment with inoculation of antagonists resulted in the protection of lettuce plant, even in the field.

Control of diseases by soil supplementation without inoculation, have been reported by many workers from 1923 up to to-day. Thus Millard (1923) demonstrated the control of

potato scab by heavy manuaring with the green manure and organic manure. Fellows (1929) successfully used chicken manure to control "take-all" diseases of wheat. The root-rot disease of cotton, caused by Phymatotrichum omnivorum, was reported to be checked by the addition of organic manure to the soil before crop season (Eaton, 1934; and King 1934). The Sclerotium rot of sugar beets was found by Leach (1942) to be reduced by the use of nitrogenous fertilizers. Novogradudsky (1936) suggested that various bacteria and actinomycetes have a marked selective fungistatic effect, and believed that abundance of these inhibitors in soil would check the multiplication of pathogenic fungi. Similarly, some other diseases caused by soil-borne fungi have been ameliorated by adding organic materials to infested soil (Hildebrand and West, 1941; Nandi 1948; Sinha 1950; Davey and Papaviza 1959, 1960; Synder, Schroth and Christon, 1959; Zentmyer, 1964; Singh, Vasudeva and Bajaj, 1965; and Buxton, Khalifa and Ward 1965).

Disease incidence could sometimes be reduced by inoculation of the infested soil with saprophytic organisms known to be antagonistic to the pathogen in synthetic media (Sanford and Broadfoot, 1931; Tims 1932; Garrett 1934; Winter 1940; Subramanian 1946; Slagg and Fellows 1947; Wood and Tevit 1955; and Sanford 1959).

Thus, the role of microbial antagonism in the biological control of soil-borne plant diseases has been well emphasized,

and a possible practical application of this, which would consist in treating soil so as to encourage the development of the antagonist, is not without justification. The mechanism by which the control is brought about is probably complex and many factors are known to contribute to the effect, but it may be surmised that in part at least it is due to the action of antibiotic formed by organisms developing freely on the added nutrients.

It is relevant to point out that the rhizosphere contains a different flora from soil not occupied by plant roots (Garrett 1939; Lochhead and Thaxton 1947; Weindling 1946), and that this root-occupied zone is richer in nutrients, not only due to the accumulation of degenerated tissue cells but through active excretion from the roots. According to Linford (1942) bacteria grow in dense masses on the surface of even quite young roots where dead cells would be absent. The abundant growth may perhaps lead to antibiotic formation, and if so the substance produced would be located just where they would best exert their effect against root pathogens. Indeed, Rangasawami and Vidyasekaran (1963) showed that the amount of antibiotic produced by a Streptomyces species (see Rangasawami and Ethiraj, 1962) in the rhizosphere of corn was relatively higher than in the soil. This they thought was due to more nutrients available in the rhizosphere region. Similar results were obtained by

Nikitina (1958) who reported that a mycolytic bacterium, when present in the rhizosphere of three-year-old lucerna; practically destroyed Fusarium culmorum.

2) Occurance of toxic and antagonistic substances in the soil.

It has been known for a long time that soil may contain substances which affect adversely the growth of both plants and micro-organisms. In 1904 Frost attempted to demonstrate the presence in soil of substances inhibitory to Bacillus typhosus, but without success. Whereas Greig-Smith (1912-15) was more successful in finding thermolabile substances active against B.prodigiosus Hutchinson and Thayson (1918) proved that different soils may contain different toxins, and Lewis (1928) suggested that these toxic substances might be the product of the growth of micro-organisms. To these antagonistic substances of microbial origin, the name "antibiotics" is now applied.

In 1932 Weindling noted that antagonism of Gliocladium fimbriatum to a number of fungi. In 1934 he studied a lethal principle produced by this organism and found that it was responsible for parasitic action of G.fimbriatum on Rhizoctonia solani and other soil fungi; and two years later Weindling and Emerson (1936) isolated the lethal substance in crystalline form and named gliotoxin; this antibiotic first isolated by Weindling and Emerson in 1936, is now known to be produced by a number of fungi-Aspergillus fumigatus,

Penicillium terlikowzkii, P. cinerascens and Trichoderma viride, which suggests that it may have special significance in natural processes.

Another example is the research work of Van Luijk (1938) who isolated a number of fungi antagonist to Phytium spp. causing damping off of seedling of lucerne and various grasses. Penicillium expansum was particularly effective and, like Trichoderma viride, it was found to be associated with the production of a diffusible toxic substance; which was finally isolated in pure form and named expansine (Nauta et al, 1946).

A particular interesting example has been observed for soil of Wareham, Heath, Dorset, which is markedly inhibitory to mycorrhiza fungi. This had been the subject of a series of investigations by Rayner (1934; 1936; 1939; 1941), and Neilson and Jones (1941). In this case of soil toxicity, clear proof was obtained that a diffusible fungal substance was present in all soils showing symptoms of toxicity. Later Brian, Hemming and McGowan (1945) conformed the earlier observations and ascribed the soil toxicity to the activity of the predominating fungal flora namely Penicillium janczewskii, P. terlikowskii and P. nigricans-janczewskii species, which all are regarded as typical soil penicillia and are capable of producing antibiotics namely griseofulvin (Brian, Curtis and Hemming, 1946; McGowan 1946), gliotoxin (Brian, 1946), and a fungistatic bacterial red pigment

(Curtis and Grove, 1947) respectively.

Without considering further examples we may conclude that there is evidence that the well known antagonism between certain soil saprophytes (fungi and bacteria) and soil-borne plant pathogenic fungi is associated with the production, by the saprophytes, of antibiotic substances.

It is, however, true that instances of the production of antibiotics in natural soil as a result of microbiological activity are very few which are now discussed. The case of Wareham Heath soil already mentioned is a clear example of presence of antibiotic (gliotoxin) in natural soil. Other less satisfactory examples are also available. Thus, Waksman and Woodruff (1942) were able to extract material having an activity resembling that of actinomycin, from the natural soils. Of the two soils examined, the larger was yielded by the least fertile soil. Newman and Norman (1943) also considered that natural soils might contain antibiotics in an active functioning state. They attributed to this cause the failure of organisms from surface soil to establish themselves in the subsoil. Grossbard (1948 b) showed that centrifugates from certain untreated soils occasionally gave narrow zones of partial inhibition of both Bacterium coli and Bact. carotovorum, while with Sarcina lutea there was a zone of complete inhibition. Sinha (1950) found that soil extract from unsupplemented soil with acid alcohol gave a

zone of partial inhibition of Bact.coli.

Thus, it is evident that soil may contain inhibitory substances or antibiotic like substances. Also, considering that the majority of antibiotic producing organisms are soil saprophytes, it should not be experimentally difficult, as could be concluded from above cases, to produce antibiotics in soil. The last two points are now discussed.

3) Soil micro organisms producing antibiotics.

The discovery that certain soil micro-organisms which produced antibiotics when grown in pure cultures in laboratory served to focus attention more than ever before on the subject of soil microbiology. The present concept of antibiotics only slowly developed until penicillin (Fleming 1927) appeared on the scene. The therapeutic uses of this and other antibiotic substances were chiefly respinsible for the extensive work in this field. Each year hundreds of new antibiotic substances are discovered ranging from amphomycin through hygromycin and pyridomycin to vinacetin. The search for new antibiotics continue. The list of these antibiotics has become very long by now, it is not proposed to discuss them here in detail and a full account of these antibiotics, their nature and properties has been given by workers namely Florey et al (1949) Baron (1950) and Brian (1951). Only few examples are given here to emphasize that many of the organisms under discussion were isolated from the soil;

particular emphasize is laid on the affect of antibiotics on plant-pathogenic micro-organisms.

The known antibiotic producing micro-organisms of soil origin include bacteria, actinomycetes and fungi. Among bacteria, subtilin is produced by Bacillus subtilis and is found to be active against gram-positive and gram-negative bacteria including Xanthomonas translucens which attacks barley seedlings (Goodman and Henry, 1947). Other strains of B.subtilis form eumycin and bacillomycin which are active against many fungi. As shown by Gilliver (1946) tyrothricin is active against gram-positive and gram-negative bacteria, including many pathogens. It is produced by Bacillus brevis (Dubos, 1939). Licheniformin from Bacillus licheniformis was found to be effective against Mycobacterium tuberculosis (Callow and Hart, 1946). Cordon and Henseler (1939) controlled the virulence of an important plant pathogen Rhizoctonia solani by simplexin produced by Bacillus simplex. Colistain is formed by a soil bacillus as shown by Gause (1946), violacein produced by Chromobacterium violaceum (Leichstein and Van de Sand, 1945; 1946); and iodinin produced by Chromobacterium iodinum (Weinendling, 1945) are other useful antimicrobial substances.

The ability to repress the development of other micro-organisms appears to be wide spread among the actinomycetes. Proactinomycin, from Nocardia gardeneri was found to be active against several phytopathogenic bacteria including Bacillus

polymyxa and O.michiganeuse (Gilliver, 1946). Streptomycin from Streptomyces griseus is the most important member of this group, for its activity against many gram-positive bacteria pathogenic to man as well as plant, and for its relatively low toxicity to the human or animal body (Waksman, 1944), chloromycetin (Smith et al, 1948), neomycin (Waksman and Lechevalier 1949), actinomycin, actinomycetin, mycetin and streptothricin etc are other useful antibiotics produced by soil actinomycetes.

Although the antibacterial effects of fungi were reported by early workers (Duchesne, 1897; Vandremer 1913) the real stimulus to the search for antibiotics was not forthcoming until Fleming (1929) discovered penicillin, produced by a strain of Penicillium notatum. Later, Waksman and Horning (1943) studied systematically the whole group of antibiotic-producing soil fungi and put them into eleven groups e.g. Absidia, Alternaria, Aspergillus, Botrytis, Cladosporium, Fusarium, Mucor, Penicillium, Rhizopus, Trichoderma and Verticillum. Species from many of these genera are known to form antibiotics namely Aspergillus, Penicillium, Trichoderma, and Fusarium, useful for both plant and human pathogens. Penicillin and notatin from Penicillium notatum, citrinin from Penicillium citrinun (Raistrick et al, 1941) and patulin (Waksman et al, 1943) produced by Penicillium patulin are active against many plant pathogenic fungi like Phytium (Anslow et al, 1943) and Ceratostomelta ulmi (Waksman and

Buige, 1943). Two other antibiotics from Penicillia are penicillic acid and mycophenolic acid. Mycophenolic acid found to be active against Rhizoctina and Phytophthora (Gilliver 1946).

Among antibiotics produced by Aspergilli are fumigatin and fumigain by A. fumigatus and flavacin and aspergillic acid formed by A. flavus. Five antimicrobial substances have been obtained from genus Fusaria, namely, javanicin from F. javanicum, avenacein from F. avenaceum, fructigenin from F. fructiginum, lateritinin from F. lateritium and sambucinin from F. sambucinum. Gliotoxin and viridin produced by Trichoderma viride are also active against several pathogenic fungi. The report of the antagonistic effects of fungi either on the same species or on the other fungi are numerous. This phenomenon was considered to be of great value in connection with plant diseases. Thus there are good reasons to believe that the capacity to produce antibiotic is frequent among, and characteristic, of the soil micro-organisms.

4) Production of antibiotics (Antagonistic substances) in soil.

It is difficult to decide whether the soil organisms reported above are also capable of synthesizing their antibiotic substances in their natural habitat-the soil. The main difficulty being that the soil is poor medium (Waksman, 1948), where as a high level of nutrition and the

use of complex media are necessary for the abundant antibiotic production in the laboratory. Indeed, with the progress in this field it is now well established that the antibiotic substances can be produced in the soil at least under certain conditions.

The first experimental work on the production of antibiotic substances in the soil was reported by Grossbard (1948) who showed that in a sterilized soil enriched with glucose, wheat, straw, or beet pulp, Penicillium patulum produced an antimicrobial substance, patulin. Later studies (Grossbard 1952) showed that the patulin is produced by other species of Penicillium and by Aspergillus clavatus and A. terreus as well when grown in sterilized soil, enriched with carbohydrates or other assimilable organic substances. It has also been shown by Hessayon (1951) that an antibiotic substance could be produced in unsupplemented but autoclaved soil inoculated with Trichothecium roseum (an organism which produces the antibiotic trichothecin). Similarly, Gregory, Allen, Riker and Peterson (1952) demonstrated the production of antibiotic activity in unsterile soil which had been supplemented with soybean meal, glucose and corn-steep liquor and inoculated with P. patulum. In these cases it was assumed, but not conclusively demonstrated, that the antibiotic formed in the soil was identical with that produced in synthetic media.

Gottlib and Siminoff (1952), clearly demonstrated the production of a specific antibiotic by Streptomyces venezuelae in sterilized but unsupplemented soil, and identified it as chloromycetin by its chemical reactions and by paper chromatography. Also Wright (1954) showed the production of gliotoxin by Trichoderma viride in autoclaved soil either unsupplemented or normal soil supplemented with as little as 1% of organic matter. Similar data on the ability of some micro-organisms including fungi, to form antibiotic substances in soil have been presented by many workers (Grossbard 1952; Stevenson and Lockhead 1953; Wright 1954, 1955, 1956; Wright and Grove 1957; Krasil'nikov 1954; Koreniyako et al, 1955; Kalyansundaram 1955 and Brian 1957).

Failure to detect antibiotics in soil cultures of organisms known to be capable of their production in the laboratory, may be due to the lack of selectivity in the methods used for their detection, most of which have involved extraction from the soil. Stevenson (1956) developed a sensitive method, other than soil extraction, for detection of antibiotics in the soil; using agar-coated, fungal spores seeded, microscopic slide, buried in the soil culture and studying the degree of inhibition of germination of spores. Since then many workers have used this sensitive method and showed antibiotic production in soils. Thus Mirchink and Greshnykh(1961) presented data indicating that Penicillium cyclopium and P. pupuogenum were capable of producing

antibiotics when introduced into non-sterile soil; the amount increasing upon the addition of sucrose. Similar evidence of antibiotic substance production by Streptomyces species in unamended soil was provided by Rangasawami and Ethiraj (1962). Also Rangasawami and Vidyasekaran (1963) showed the antibiotic production by using the above Streptomycetes (Rangasawami and Ethiraj 1962) in the rhizosphere of corn (Zea mays). Vasudeva, Singh and Iyengar (1962) reported the production of bulbiformin by Bacillus subtilis in the soil. Antibiotic production was considerably enhanced when the soil was sterile and enriched with nutrients such as aspartic acid or aspparagin, and dextrose or root residues of certain plants. Recently Singh, Vasudeva and Bajaj (1965) have shown the production of bulbiformin by Bacillus subtilis on the seed coats, spermatosphere and rhizosphere of bacterized pigeon-pea seeds sown in sterile and unsterile soil. The antibiotic produced as a result of bacterization became systemic in plant tissue and provided a protective zone around the roots of pigeon-pea seedlings. Also appreciable reduction in the incidence of pea wilt has been obtained through seed bacterization.

Although, the synthesis of bulbiformin on the seed coat, spermatosphere, and rhizosphere in unsterilized was comparatively lower than that in the sterilized soil, the fact that bulbiformin was produced in unsterilized soil in

appreciable quantities is important from the practical point of view, as most of the organisms which produced antibiotics in sterilized soil fail to do so under normal unsterilized condition.

II) Antibiotics in the control of plant diseases with special reference to Streptomycin.

The use of antibiotics as protectants or as eradicants of plants diseases is still a comparitively newer approach to control plant diseases; though antibiotics, or antagonistic phenomenon among micro-organisms, and its relation to plant diseases had been recognised for more than 80 years. In the near future, however, antibiotics may possibly be used as widely for this purpose as they now are in control of human diseases.

Gliotoxin, the first antibiotic to be used for plant disease control, was isolated and purified by a plant pathologist (Weindling and Emerson 1936) even before the discovery of penicillin. Because of its antifungal properties, it was studied as early as 1933 as a protectant against certain plant pathogens. Shortly after penicillin and streptomycin appeared on the market for medical uses, several plant pathologists investigated them for control of phtopathogenic bacteria; but only limited experiments were done at that time due to short supply and extremely high cost of these antibiotics

Recently from 15 years widespread and keen interest has developed in this type of research and many improved compounds having bactericidal and fungicidal properties have been marketed by many pharmaceutical firms which produces antibiotics.

Major investigations before 1951, consisted of screening plant pathogen *in vitro* to determine their sensitivity to antibiotics (Weindling and Emerson 1936; Brown and Boyle 1944; Brian and Hemming 1945). It was not until 1952, however that an antibiotic was used under field conditions for the control of plant diseases. Thus Zaumeyer *et al* (1953) showed that beans were protected from infection by halo blight organisms (*Pseudomonas phaseolicola*) with a streptomycin dust.

Recognisation of this fact led the attention of soil microbiologists and plant pathologists towards the use of special group of chemical compounds known as antibiotics for the control of plant diseases, although there are many chemical compounds in current use to prevent or eradicate diseases of plant. A major reason to support this view is the hope of discovering a compound that will exhibit a minimum toxicity to plants, maximum toxicity against parasites, and the property of penetrating into plant cells and accumulating there in amounts detrimental to the parasites located deep inside the plant tissues. Also the compound and quantity used should be ineffective to men and animals, other advantages of such agents would be less washing-off by

rain and upon translocation in the plant, protection of untreated plants or new growth. Also antibiotics might be of value in cases where the commonly used chemicals may produce injurious side effects on the crop or may be toxic for human beings or animals.

An extensive literature is avaible on the successful use of antibiotics for the control of plant disease; and on the efficacy of antibiotics as chemical agents for plant disease (Anderson and Gottlieb 1952; Dunegan 1954; Hopwood 1957; Klinkowski 1948;1954; Zaumeyer 1955;1958; and Dekker 1963). An attempt to present a review of this type will be out of place and only few examples are given below.

Streptomycin

Streptomycin

Streptomycin, discovered in 1944, is produced by Streptomyces griseus. Although penicillin is the oldest known antibiotic against bacterial diseases of both men and animals, streptomycin is the first antibiotic to function as a reliable remedy against many bacterial diseases of plants. It is very active against a broad range of bacterial plant pathogens, in vitro as well as on the plants. Originally thought to be solely an antibacterial agent, it has lately been shown to have anti-fungal properties under in vivo conditions.

The most important application of streptomycin has been against fire blight diseases, caused by Erwinia amylovora in many rosaceous plants. Because of the devastating

TABLE 1

Antibiotics in the control of Plant Diseases.

Name of Antibiotics	Year of Discovery	Antib. Prodg. Organism	Disease Controlled	References.
1) Griseofulvin	1939	Penicillum griseofulvum	1)Powdery mildews & Botrytis disease	Brian (1960)
			2)Bean rust by uromyces appendiculatus	
			3)Fungal diseases, i)on coffee by Phyllosticta coffeicola	Rodriquez et al (1957)
			ii)on lettuce by Botrytis cinera	Webb(1958)
			iii)on tulips by B.tulipae	Valaskova (1958)
			iv)on apple trees by sclerotina fructigena & S.mali	Byrde(1959) & Rhodes (1962)
			v) on beets by Cercospora beticola	Ghillini & Alguisi (1960)
2) Streptomycin	1944	Streptomyces griseus	- see below -	
3) Cyclohexamide (Actidione)	1946	Streptomyces (other species)	1)Blister rust of white pine tree	Moss(1957, 1958,1960 & 1961)
			2)Turf diseases cuased by various Fungi	Ford(1958 & Smith & Guyer 196
			3)Powdery mildew disease	Ford(1958

Antibiotics in the control of plant disease - contd.

			i)on fruits	Szirmai & Voras (1960)
			ii)on roses	Palmer et al (1959;1960) Deep & Bartlett (1961)
			iii)on tuberous begonia	Thompson (1961)
4) Tetracyclines includes. i)oxy tetra cycline ii)chlortetra cycline iii)tetra cycline	1948-53	Strepto myces. species	1)Crown gall on pear & cherry nursery trees	Deep (1958) Cole (1959)
			2)Tumoreus on tomatoes	Koremyako & Koveshnitov(1960)
5)Polypeptides includes i)Tyrothricin ii)Subtilin iii)grewmicidin iv)polymyxin v)phytoactin	1939 onw.	Bacillus brevis others by Strepto myces & Bacillus species	Active on gm-positive i)& some gm-negative bacteria as well as fungi	
			ii)Downy mildew of cucumber	Ark & Thompson (1957)
			iii)Powdery mildew on apple	Sprague(1958)
			iv)Controlled Sclerotina fructicola & S.laxa on Cherries	Grover (1960)
			iv)White pine blister rust canker	Van Arsotel (1962) Moss(1961)
			v)Cotton seed infected with Xanthomonas Malvacessrum	Rangasawami (1957) Babayan, Karapety & Sarksyan(1958)
6)Polynes (Antibiotics containing conjugated polyne chromophere)includes i)Filipin ii)Pimricin iii)Nystatin etc.	Sin 1950	Strepto myces Spc	i)Downy mildew on hop	Stanek et al (1959)
			ii)Fungal spotting on orchids by Botrytis, Alternaria & Fusarium Spp.	Frank et al (1959)
			iii)Botrytis Tulipae on Tulips.	Valaskova (1958,1961)

Antibiotics in the control of Plant Disease - contd.

7)Macrolides (Basic antibiotics which have tactone ring in common includes. i)Spiramycin ii)Crythromycin	Last 10-12 years	Strepto myces Spp	i)Active on gm-positive bacteria ii)Slight reduction of tomato canker.	Logsdon (1961) & Keyworth & Howell (1961) Kruger (1961)

characters of this disease, the cultivation of a highly susceptible Bartlett pear had to be abandoned in the eastern part of the United States. Very promising results have now been obtained with various streptomycin formulation sprays by Goodman (1953,1954) in Missouri, Young and Winter (1953) in Ohio, Heuberger (1953) in Delaware, Ark (1954,1954 1955) and Dunegan et al (1954) in California, Kienholz (1955) in Oregan, Clayton (1955) in North Carolina, Kirby (1954) in Pennsylvania, and Mills (1955) in New York. These sprays have remarkably reduced the blight infections on pears and apples wherever they were used. Goodman (1953) in Missouri obtained a 100% control of fire blight of apples by using streptomycin, either in combination with Terramycin or alone. It was suggested that the therapeutic practice of application of streptomycin close to 50 days before harvest, combined with careful pruning of infected twigs, should again make possible the growing of Bartlett pears in the Eastern areas of the United States (Keil and Wilson 1962).

Streptomycin is also active against walnut blight caused by Xanthomonas junglandis (Ark, 1955; Miller 1959). Indeed, streptomycin sprays and dust are now part of a routine disease control programme in the United States and are officially recommended against fireblight on apples, pears and some ornamental shrubs of rose family, and against walnut blight. Dye and Dye (1954) showed that under green-house conditions seedling peach trees could be protected from bacterial canker (pseudomonas syringae) with streptomycin sulphate sprays. Brown and Heep (1946) reported elimination of Xanthomonas pruni from cankers on plum twigs with streptomycin.

Anderson (1957) showed that beans could be protected against both common blight and fuscous blight caused by X. phaseoli var. fuscans by streptomycin sprays. Recently streptomycin has been reported to be more active against fire blight than are Cooper (Parker 1961) and Brodeaux mixture (Luepschen, 1960), with the additional advantage that fruit russetting is avoided.

Bacterial blight of celery, caused by Pseudomonas apii were found to be controlled by application of Agrimycin (Cox 1955). In England, Crosse and Bennet (1957) reduced infection of bacterial canker of cherry caused by Pseudomonas syringae with five spray applications of streptomycin. Bacterial spots of tomato and pepper, caused by Xanthomonas vesicatoria have been reported to be controlled

by an Agri-mycin spray (containing 200 p.p.m. of streptomycin) by Conover (1954).

The use of streptomycin as a seed disinfectant does have its advantages for eradication of deep-seated infection. Streptomycin was found to be an effective treatment of seed infected with the halo blight organism - Pseudomonas phaseolicola. (Smith, 1949; Hildreth and Starr 1950; Nultsch 1958; Afanasief & Snarp, 1958). Results of extensive field experiments were so convincing that much of the seed for the 1960 crop in England was treated with a streptomycin formulation. Recent reports on seed disinfection with streptomycin include Xanthomonas malvacearum on cotton (Babyan et al 1958), X. campestris on crucifers (Klisiewicz and Pound 1961), Corynebacterium michiganense on tomatoes (Kruger 1959), Pseudomonas coronataciens on oats (Griffiths and Peregrine 1960), and P. atrofaciens on wheat and barley (Stanek and Ujevic 1959).

Almost every conceivable method of administering antibiotics to plant has been exploited according to the nature of the disease and the characteristics of the plant. Sprays, dusts, dips and soil treatments have been used predominantly. However, several other unique methods of administration have also been employed.

There are numerous reports on more or less successful experiments with foliar application of streptomycin against bacterial disease, caused by species of genera Erwinia,

Pseudomonas, Xanthomonas, and Bacterium; black leg of potatoes, Stewart's disease of sweet corn, bacterial wilt of cucumber, bacterial blight of celery, common and fuscous blight of beans, bacterial blight of soybeans, black chaff of wheat, leaf rot of philodendron (Goodman 1959; Tanner 1958) blast and bacterial canker of stone fruit (Boyd 1958), bacterial spot of peach, plum and apricot (Verneau, 1958; Klos 1958), citrus canker (Rangasawami, Rao & Lakshmanan 1959), angular leaf spot of cucumber (Stanek, 1958; 1960), halo-blight of beans (Afanasief, 1958), soft rot of lettuce (Winfree, 1958), black rot of crucifers (Klisiewicz 1961), bacterial spot of pepper and tomato (Chandler 1958; Sowell 1959; Stall 1959; Thayner 1961; Stall 1962), bacterial leaf spot of wheat and barley (Stanek 1959), black arm of cotton (Balanchandran et al 1960), leaf blight of corn (Sabet 1956), and wildfire on tobacco (Hitier 1960; Shaw 1959).

Dipping potato seed pieces in streptomycin provides a substantial control of ring rot caused by Corynebacterium spedonicum (Logsdon 1961). Also streptomycin sulphate shows an additive effect on different seed potato disinfectants used for the control of this disease (Bonde and Johnson 1958). Control of olive knot (Pseudomonas savastanoi) and crown gall (Agrobacterium tumefaciens) of apricot and peach in green-house experiments, was obtained by brushing streptomycin in an isoamyl-kerosine-lanolin-vaseline mixture on the galls (Ark and Thomson 1960), and a reduction of steam

and leaf rot of Diffenbachia (Erwinia diffenbachiae) by a preplanting dip in a streptomycin solution (McFadden 1961).

A soil drench with combination of streptomycin and 8-quinolinal sulfate cured geranium plant infected with foot fot caused by a Fuserium and a Xanthomonas species (Stoddard 1957). This technique is now being used commercially by florists in the northeastern part of the United States.

It is obvious, therefore, that streptomycin is one of the important antibiotics used for the control of plant diseases. Although phtotoxicity may occur, it usually does not present the real problem at the concentrations usually used in the control of many plant diseases. In addition, a stimulation of plant growth by streptomycin has been reported (Stanek & Preslicka 1960). More serious complications may arise by the development of streptomycin-resistant pathogens as a result of streptomycin treatment. To avoid the build-up resistance, Agrimycin 100, containing streptomycin together with some Oxytetracycline, has been developed (Dekker 1963).

Streptomycin is readily absorbed by some plants (Anderson & Nienow, 1947; Pramer 1953; Crowdy & Pramer 1955); and exerts a marked systemic action (Napier et al 1956); it is readily absorbed and translocated in chrysantheum cuttings and persisted in sufficient amounts to protect the newly developing plants from infection (Robin et al 1954), its actual translocation in the hop plant was demonstrated by Maier (1960). It has a direct action on the pathogens inside

the plant tissue (Pramer et al 1956); and has direct action on the host (Müller, Mackay and Fried 1954; and Crosse et al 1960).

Streptomycin is now on the market in various formulations namely Agrimycin 100 and 500; Agristrep, a solution of streptomycin sulphate in a mixture of water and glycerin developed in Holland (Kock 1958); and Fytostrep, a Czechoslovak preparation containing 15% streptomycin together with Oxytetracycline in a ratio of 10:1 (Stanek & Wasserbauer 1960).

The development of an antibiotic that can be applied to the soil for control of root parasities is greatly needed. Species of Phytium, alone, constitute one of the most destructive groups of plant pathogens and many species of this group cause root rots and damping-off of seedling. Zaumeyer suggested in 1956 (First Internal Conference on Antibiotics in Agriculture) that disease of root and damping-off of seedlings could be controlled by streptomycin compounds if they retain their activity when applied to the soil. This belief was based upon the fact that streptomycin formulations have effectively controlled two species of Phytophthora causing mildew of lima bean and late blight of tomato and one species of Peronospora causing blue mold of tobacco, both of which genera are closely related to the genus Phytium.

Production of an antibiotic, able to control Phytium species and other soil inhibiting organisms such as Fusarium

and Rhizoctinia that are usually unhampered by the older methods is a real challenge to the industry. Also new compound of antibiotics should be commercially produced for only "agricultural uses" bearing at least the following properties.

1) They must have a composition with the soil constituents.

2) The preparation should retain its natural stability, antagonistic activity and chemical composition for long periods of time. For this purpose salts of antibiotics may be prepared which are more easily soluble and stable in soil.

3) It should preferably be of low molecular size so that there should not be any adverse effect on the translocation properties.

4) Naturally, it must be harmless for plants, animals and human being.

5) The chemical compound of the antibiotic should preferably be a strong buffer also.

6) From economical point of view it should be cheap enough to be used in agriculture in large amounts. For this purpose antibiotics less pure than used in medicine can be prepared and which would reduce the price of the compound.

Thus, as mentioned above stability, chemical composition, antagonistic activitic i.e. degradation of antibiotic etc., factors should be considered before using any antibiotic compounds for agricultural purposes. The relevent literature on these aspects now follows.

III) Stability of Antibiotics in Soil.

As already mentioned many of the antibiotic producing micro-organisms are found in soil. It is expected that since natural soils being poor in microbial nutrients, these would not produce large amounts of antibiotics, yet continuous production over large periods even at a very low rate would lead to the accumulation of significant amounts. But as it is evident, instances of obvious toxicity in natural soils are rare. Although certain workers have shown that in soils (After plant or animal matter has been decomposed) there are many substances bearing properties resembling to those of antibiotics (Newman & Norman 1943; Nandi, 1948; Sinha 1950; and Monib 1953). Even then, it is quite rare that these substances accumulate to such an extent to make the soil unsuitable for microbial development. The non-persistance of these toxins in normal soils was also visualised by Hutchinson and Thaysen (1918). These evidences indicate that the mechanism of their destruction, inactivation of neutralisation must exit in the soil. It is therefore natural to investigate their fate in the soil, whether introduced into the soil or produced by inoculation of micro-organisms capable of producing the antibiotics. The investigation would help in the understanding of the dynamics of biological activity in soil, since it has, for instance, been shown by Quastel

and Scholfield (1951) that chloramphenical is a potent inhibitor of an important step in Nitrogen fixation cycle, namely nitrification. Also it would indicate the potential usefulness of antibiotics for the control of soil-borne plant pathogens. As has been suggested by Lochhead & Landerkin (1949) that before embarking on extensive trials with known antibiotics or antibiotic producing organisms, it would seem desirable to know which antibiotics are most likely to prove useful. Factors such as (a) stability against both chemical and biological decomposition, (b) retention of activity in the presence of soil constituents, (c) firmness of absorption on soil colloids, which may, control both activity in soil and retention in the fact of leaching, and also, (d) specific activity against the pathogens to be controlled and (e) if possible be harmless to desirable soil organisms, are all important.

Earlier workers were of the opinion that soil toxins could be destroyed in various ways, such as by the application of heat, oxidation or adsorption by means of certain adsorbing agents such as carbon black etc. It is now known that most antibiotics, when added to the normal soil, disappear with remarkable rapidity. Antibiotics may undergo physical inactivation, chemical decomposition or biological decomposition; and depending upon the nature of the antibiotic, the type of the soil and nature of soil

microflora, one or even all of the three processes may operate. A summary of these processes is given here.

Physical inactivation:-

Soil contains many colloids with a tremendous surface area and therefore high adsorption capacity, so this process is of the utmost importance. Antibiotics when added to the soil are readily inactivated by soil colloids, minerals and organic matter. A comprehensive investigation was carried out by Gottlib and his colleague (1952) who studied the production and role of antibiotics in soil and dealt mainly with Streptomycin, aureomycin, terramycin chloramphenical, actidione and clavacin. They divided antibiotics into three general groups according to their dissocation i.e. basic, neutral and acidic.

The soil colloids are predominantly acidic in nature. Hence, basic antibiotics are strongly bound and inactivated by soil complexes. Streptomycin, a compound containing the strongly basic quanidine group is rapidly removed from the soil by soil colloidial complex, including clay and the organic matter (Siminoff & Gottlib 1951). These workers further reported that clay colloids adsorb large quantities of antibiotic, in particular a bentonite or illite clay was very active and removed the greatest amount from solution. Ionic substitution was much more pronounced in bentonite than in illite, since the former had

a greater number spots to which cations were attracted and thus a higher base exchange capacity. The adsorption of antibiotics by clay minerals results in expansion of the crystal lattice and flocculation of the clay. The higher the concentration of streptomycin the higher the degree of flucculation. The adsorption phenomenon involved an equilibrium between the antibiotic and cations already adsorbed onto the exchange spots of clay (Siminoff and Gottlieb, 1951).

Although it has been established that streptomycin could be adsorbed, it is not clear whether such adsorbed antibiotic could enter into normal base-exchange reactions. If such a dynamic situation exists in the soil, any amount of streptomycin produced there could eventually act upon the susceptible flora after being replaced from the collodial complex by other cations. Experiments show that the hydrogen-ion from hydrochloric acid did not exchange with streptomycin, and very little could be removed by base-exchange agents. For example methylene blue and janus green were able to replace adsorbed antibiotic from a sandy soil; but did not effect removal from heavier slit loam soils. No streptomycin could be recovered from the clay by electrolysis. Ark and Alcorn (1956) reported that when streptomycin was formulated with bentonite clay, a strong physical union resulted and streptomycin was not

detected from the bentonite particles in sufficient quantities to be useful at the locus of application. However, addition of dipotassium phosphate, peptone or certain other substances to the bentonite-streptomycin adsorption complex released adequate amount of the antibiotics for use as bactericide. Exchange reactions may very well be the basis of a soil slurry technic used successfully by Pramer and Starkey (1950a.1950b.1951) and Starkey and Pramer (1953) for the assay of streptomycin, which is strongly adsorbed on soil particles and not extractable by solvents. Streptomycin, another basic substance, is similarly adsorbed by soil colloids (Gottlieb 1952), viomycin, circulin, subtilin and actinomycin, all of which contain aminoacids in peptide linkage as part of the molecule, are also absorbed by soil colloids (Martin and Gottlieb 1955).

As regards Amphoteric antibiotics have a variable behaviour depending upon the balance of ionization of the basic and acidic group i.e. on the pH (Martin and Gottlieb 1952). Aureomycin and Terramycin, which are amphoteric antibiotics, behaved as basic in acid conditions and were inactivated by clay in both its forms. Aureomycin could only be tested in acidic condition as being unstable in alkaline condition.

The natural antibiotics, as would be expected, are not readily adsorbed. They are weekly bound by soil colloids

and retain a relatively high degree of activity in the soil. Chloromycetin (one of the neutral antibiotics) is not adsorbed by clay and easily be recovered completely shortly after its addition to the soil and its activity persists in the soil. If produced in the soil in sufficient quantity, it would be able to exert its antagonistic action in a free or in a weakly bound state. Similarly Actidione, another neutral antibiotic, is not removed from aqueous solution by normal soil and could be removed from sterile soil (Gottlieb, Siminoff and Martin 1952).

Acidic antibiotics, especially in the ionized form, fall into (an activity) range in the soil between the neutral and the basic antibiotics. Clavacin, an acidic antibiotic is relatively indifferent to the presence of clay. It is stable for long time in a sterile soil (Gottlieb 1952; Gottlieb, Siminoff and Martin 1952). Gottlieb (1952) reported that clavacin did not flocculate either illite or bentonite, and only a small but a constant amount was removed from solution. Also X-ray diffraction studies did not show any spreading effect. The solution of clavacin compounds lost very little activity during the first few hours after addition to the sterile soil, indicating that adsorption was not important (Gregory, Allen, Riker and Peterson 1952). Similarly Griseofulvin was not inactivated

in sterile soil (Brian et al 1951). As no direct evidence on the adsorption of gliotoxin is available and the fact that it is taken up by plants from soil containing it may be expected to indicate that it is not removed by colloids from the soil solution.

Chemical decomposition due to intrinsic instability of the antibiotic molecule.

Antibiotics are subject to undefined chemical transformation in the soil. It depends upon various factors such as pH of the soil, as mentioned earlier penicillin is less stable in acidic than in basic solution and correspondingly less stable in acidic than in basic soils. Similarly inactivation of antibiotics like viridin, gliotoxin, frequentin, albidin etc., can be explained by their intrinsic chemical instability in aqueous solution at pH of the soil. Inactivation of antibiotics in soil also depends upon their capacity to form complexes with other constituents of the soil. Streptomycin was inactivated even in a muck soil which contains a negligible clay colloid content (Gottlieb and Siminoff 1950). In such circumstances many possibilities exist for the alteration of the original compound. Actidione which is not adsorbed seems to undergo change even in sterile soil and its activity in the soil is gradually decreased (Gottlieb, Siminoff and Martin 1952). This could be attributed to the unfavourable pH of the soil

(Gregory, Allen, Riker and Peterson 1951). Some antibiotics are unstable in the presence of the atmosphere, while others are liable to decompose at an acidic or alkaline pH. Patuline is rapidly decomposed in alkaline soil (Gregory, Allen, Riker and Peterson 1952). At any one time other innumerable decomposition products present in the soil may react with the antibiotic and inactivate them. The rapid inactivation of gladiolic acid, penicillin and cyclohexamide in partially sterile garden soil, and some other similar observations under pH conditions favourable to stability, strongly suggest that some other form of inactivation may occur. It is conceivable that the antibiotic in such cases is reacting chemically with a soil constituent, or that some soil constituent acts as a catalyst.

IV. Microbial decomposition of Antibiotics with Special reference to Streptomycin.

Soil, at any time, is in dynamic equilibrium and this equilibrium is likely to be disturbed when there is any alteration in the conditions, either natural or unnatural. Furthermore, the growth of new cells of micro-organisms in the soil permits the occurrance of adaptation and mutation processes, which results in the change of enzymatic equipment of the individual organism and of the equilibrium system of the soil as a whole. But the soil is usually in a state of dynamic equilibrium and if it is changed its

recovered very soon or new equilibrium is brought about by the microbial activity. It shows that soil microflora has an almost infinite capacity of life for adapting itself to a practically endless variety of external conditions. The soil organisms have great potentialities for synthesis and decomposition. Soil micro-organisms have been isolated which are capable of oxidizing and utilizing such unpromising substances as phenol, benzene, xylene, and many polyphenols. Gray and Thronton (1928) found bacteria oxidizing cresols, toluence and naphthelene. Johnson, Goodale and Turkevitch (1942) isolated micro-organisms oxidizing n-pentane, n-hexane, n-octane and n-nonane. Claus and Walker (1964) isolated a Pseudomonas and an Achromofacter capable of decomposing toluence, benzyne and certain other related compounds while Sundman (1964) isolated some lignandylic soil bacteria capable of oxidizing simple phenolic compounds such as -conidendrin. Also Aaslested and Larson (1964) isolated a bacterium from the soil which was found to oxidize 2-methylalamine.

The microbial breakdown of aeromatic chloro-compounds by soil micro organisms has been studied in detail by Erikson (1941), Andus (1950), Vlitos (1959), Walker (1954) and Walker & Wilshire (1955). Pseudomonas species isolated from the soil which are capable of breaking down many organic compounds in soil, including phenol and benzoic acid (Evans,

Smith, Linstead and Elvidge 1951), and can also decompose 1-chloro-naphthalene (Walker and Wilshire 1955).

Since antibiotics are natural products, it is highly probable that all are susceptible to microbial attack and degradation in soil.

The rate of decomposition is dependent upon the nature of the antibiotics, and is influenced by the physical chemical and biological characteristics of the soil (Winter and Willeke 1951; Jeffreys 1952; Wright and Grove 1957). Evidence that antibiotics disappear more rapidly from natural than from sterile soil, in which they may be stable for longer periods even though absorption may have occurred, suggests that it is microbial decomposition of antibiotics which take place in natural soils.

Waksman and Woodruff (1942-) were the first to introduce an antibiotic, namely actinomycin, into both sterile and non-sterile soil. In both cases the antibiotic was largely inactivated. They attributed this to absorption by clay particles in the natural soil where the greater inactivation in non-sterile soil was ascribed as destruction by micro organisms.

Jeffreys (1952) used eight metabolic products of soil fungi, together with penicillin and streptomycin, and studied the conditions affecting the inactivation of these antibiotics in the soil. He expressed biological inactivation by

considering more rapid inactivation of antibiotic in fresh than in partially sterile soil. Biological inactivation was most notable with antibiotics namely griseafulvin, mycophenolic acid, patutin, albidin and frequentine; but it was a contributory factor to the inactivation of several others. Jefferys also found that it occurred only in garden soils which have highest organic matter content and the most abundant microflora. Similar report for microbial decomposition of antibiotic in soil were provided by many other workers namely, Nandi (1948) with tyrothricin; Brian Wright, Stubbs and Way (1951) and Jefferys (1952) with griseofulvin, Gottlieb and Siminoff (1951) Gottlieb (1952) Barlich et al (1952) and Monib (1953) with Chloramphenicol; and Siha (1950) and Krasilnikov (1954) with streptomycin; Krasilnikov (1954) with chloretetracycline, Globisporin and oxytetracycline; and Siminoff and Martin (1952) with gliotoxin; Shirodkar (1953) with tyrothricin, gliotoxin, patutin and streptomycin.

Nissen (1954) studied the effects of high concentrations of antibiotics on the complex micropopulations of the soil. He used five antibiotics namely penicillin, chloromycelin, terramycin, streptomycin and aureomycin; and studied their effect on the evalution of carbon dioxide as a measure of the rate and extent of biological activity in soil. Penicillin, chloromycetin and terramycin brought about a slight

inhibition in the evolution of carbon dioxide in the first 24-72 hours, perhaps corresponding to a lag phase, followed by a strong rise in the production of carbondioxide (10-20%) as compared to that of the control soil. With aureomycin and streptomycin there was a slight but persistent inhibition during the first 14 days period, perhaps on the account of the amount added which probably exceeded the absorption capacity of soil. This indicates that there was no decomposition of these two substances within the experimental period.

Pramer and Starkey (1951) used different methods of antibiotic assay from that of Gottlieb and his colleagues (1951) and found that streptomycin when added to the sterile soil, lost no activity in a period of 3 weeks where as in non-sterile soil, more than half of the antibiotic disappeared in one week and complete loss occurred within 2 weeks which suggests biological decomposition of antibiotic.

In all the above studies proof of inactivation of antibiotics in the soil, and hence importance of micro organisms in the inactivation of antibiotics in the soil, has been provided by showing more rapid inactivation of antibiotics from non-sterile than from sterile soil. In a very few cases definite proof of microbial action has been provided by isolating the organisms responsible for antibiotic decomposition.

Katz and Pienta (1957) studied the decomposition of actinomycin in soil and isolated an Achromobacter Sp capable of decomposing the antibiotic. They also presented the observations concerning the nature and the optimum conditions for its inactivation. Moreover they showed that non-specific adsorption of the antibiotic by the cells of the organism did not appear to be involved since only trace quantities of the antibiotic can be detected after extraction of resting cells which have attacked actinomycin. This strain also decomposed actinomycin A & B.

Wright (1955) observed the production of griseofulvin in soil by Penicillium nigricans. Also its persistance in sterile soil was observed by Jefferys (1952) and Wright (195
Wright and Grove (1957) studied the disappearance of griseofulvin from a natural soil. They thought that under normal conditions microbial breakdown would play a longer part in preventing accumulation of griseofulvin in soil, as antibiotics and most organic compounds are readily attacked in soil by micro-organisms. Indeed they observed that successive additions of griseofulvin (after the previous dose had disappeared) to the soil disappeared with increasing rate (which indicated microbial inactivation of antibiotic). They isolated a dermatiaceous fungus and a bacterium (Pseudomonas sp.) which could breakdown griseofulvin in liquid culture. The pH of the solution

determined which organism was active. The pH of the soil was such that both organisms probably played some part in breaking down griseofulvin.

Nissen (1954) studied the decomposition of antibiotics in the soil, and plated his cultures on media containing penicillin, chloromycetin or terramycin as the only source of carbon. He was able to isolate Penicillium and a Fusarium sp., and suggested that fungi play an important part in the decomposition of antibiotics in soil.

Abram (1957) isolated a dominant organism - a Streptomyces sp., from chloramphenicol-treated soil and showed that it is able to inactivate the antibiotic in cell substrate mixture and observed three distinct reactions; 1) abolition of antibiotic activity, 2) destruction of nitrophenyl group, and 3) reduction to aminophenyl.

Previous workers were of the opinion that streptomycin is very resistant to decomposition. So much so that there was a prevailing opinion that it could not be decomposed biologically (see Pramer and Starkey 1951). But Pramer and Starkey (1951) considered that as it is a natural product it must be susceptible to microbial attack. They demonstrated the disappearance of streptomycin from natural soil; and isolated a gram-negative bacterium resembling Pseudomonas fluorescence capable of decomposing streptomycin contained in the mineral salts medium as the sole source of

energy and organic source of carbon for growth. Studies on the biochemical pathways of antibiotic degradation were not carried out at that time. Later Klien and Pramer (1962) studied the decomposition of streptomycin in detail with the above pseudomonad and analysed the culture filtrate. They showed that each of the three moieties of the streptomycin molecule underwent simultaneous change when the antibiotic served as a sole source of energy, organic carbon and nitrogen for growth of the pseudomonad Guanido groups of streptidine were altered as shown by oxidized nitroprusside test; streptose moiety of streptomycin was modified as evident by failure of streptomycin to undergo rearrangement and formation of maltol during alkaline hydrolysis; and the N-methyl-L-glucosamine moiety of streptomycin was transformed during the growth as measured by oxidized nitroprusside or maltol method. Washed cell suspensions of the pseudomonad were capable of dehydrogenating streptomycin when methylene blue was employed as hydrogen acceptor. Where as cell-free sonicates did not demonstrate the similar activity. This showed that bacterial dissimilation of streptomycin involved an oxidation system in which methylene blue can act as hydrogen acceptor. They suggested the name streptomycin dehydrogenase for the enzyme which catalyze the reaction.

Ghadialy (1961) studied decomposition of streptomycin in soil and demonstrated a rapid disappearance of antibiotic from

non-sterile soil as compared to that of the constant recovery from sterile soil, and the increase in the rate of disappearance with successive additions of streptomycin to soil which indicated microbial breakdown of the antibiotic in the soil. Moreover she isolated two gram-negative motile organisms, provisionally identified as members of the genus Pseudomonas; these organisms were capable of decomposing streptomycin in the mineral-salts medium containing it as only source of carbon and nitrogen for growth and energy.

Although the decomposition of many natural products and chemicals in the soil has been studied little attention (except for a few cases) has been paid to the isolation of organisms responsible for the decomposition of antibiotics and to detailed studies regarding biochemical pathways of antibiotics degradation. Mention may be made of the work of Katz & Pienta (1957) with regards to actinomycin decomposition; of Perlman (1952), Ghadialy (1961), and Klien & Pramer (1962) regarding streptomycin; and of Abram (1957) and Smith & his co-workers (1950) who studied the degradation of chloramphenicol. In all these studies organisms responsible for the decomposition of these antibiotics were isolated.

Also, Sakakibara (1951) studied streptomycin decomposition by Bacterium coli var communior and Staphylococcus aureus and proved that it was due to the

action of enzymes streptomycinase and Zymase. He also studied the streptomycin resistant varient of certain bacteria and suggested that all these bacteria produce streptomycin-decomposing enzyme (streptomycinase). When these bacteria were exposed to the sub-lethal concentration they first decompose streptomycin by the action of streptomycinase (the first or glucosidase-like decomposition) and then decompose and assimilate its decomposition products by fermentation (the second or Zymase-like decomposition). Further continuous repetition of these two processes at the same concentration of streptomycin strengthens these both reactions.

Perlman (1952) studied the chemical changes occuring during growth of a streptomycin-decomposing pseudomonad on a streptomycin-NH_4Cl- salts medium and suggested that streptomycin-oxime and one other substance giving a positive Sakaguchi test (which was not due to streptomycin) were formed as indicated by paper chromatography.

TABLE 2

Antibiotics degraded microbiologically in the soil.

Antibiotics degraded	References
Actinomycin	Waksman and Woodruff 1942, Katz & Pienta 1952.
Albidin	Jefferys, 1957.
Chloramphenicol	Erlich et al 1952; Gottlieb & Siminoff 1952; Nissen 1954; Monib 1953; Abram 1957.
Chloretetracycline	Krasilnikiv 1954.
Cycloheximide	Gottlieb et al, 1952.

TABLE 2 - Contd.

Antibiotics degraded	References
Frequentine	Jefferys 1952.
Globisporin	Krasilnikov 1954.
Griseofulvin	Brian et al 1951; Jefferys 1952; Wright and Grove 1957.
Mycophenolic acid	Jeffreys 1952.
Oxytetracycline	Krasilnikov 1954; Nissen 1954.
Streptomycin	Pramer and Starky 1951; Sinha 1950; Winter and Willeke 1951; Krasilnikov 1954; Ghadially 1961.

Aim of the Investigations

As shown in the literature review, antagonism among the complex microbial population of soil is very common and many evidences of presence of antagonistic substances in the soil are available. Moreover many soil-micro-organisms are known to produce antibiotics in the laboratory media and it is possible that they may do so in their natural habitat - the soil. Also antibiotics may reach to soil through their use in the control of plant diseases. But evidences show that such antibiotics do not persist in the soil for a long time. This suggests that mechanism for their inactivation or destruction in the soil must be present. But very little information is available regarding such mechanism and other important soil processes; still less is understood about them. As suggested by one of the great soil microbiologist Winogradsky (1949) soil microbiology is only at its "introductory Stage". Much more remains to be done before soil microbiology is understood.

One of the important aspect to be studied in the soil microbiology is the decomposition of antibiotics, the fate of the antibiotic that may reach the soil and its effect on soil-micro-organisms and thus on soil fertility and soil economy. Many investigations indicate the importance of micro-organisms in the decomposition of antibiotics in the soil

but in very few cases have the organisms responsible for such processes been isolated and studied in detail. Information regarding the nature of such isolates may help in eliminating such organisms by use of conditions antagonistic to them. Also such studies will be useful in understanding the biochemical pathways of antibiotic degradation (Smith and Worrel 1950; Perlman 1952; Katz and Pienta 1957; Wright and Grove 1957). If such information is available then the antibiotics which are biologically decomposed in the soil in few days or weeks could be made useful in agriculture if the ways are known to control the specific organisms responsible for its breakdown and/or the enzyme causing the decomposition of antibiotic could be eliminated.

To increase our knowledge in the aforesaid aspects an attempt has been made to study the degradation of streptomycin in the soil. In this thesis a detailed report of the experiments carried out is given and it is shown that streptomycin may be microbiologically degraded in the soil.

Also the specific organisms responsible for streptomycin degradation in the soil were isolated identified characterized and studied in detailed. These studies consisted of elucidating the effect of environmental factors on the growth kinetics of the organism and thus on the degradation of streptomycin.

METHODS TECHNIQUE AND MATERIALS

Section I.

I. The estimation of streptomycin.

Streptomycin sulphate B.P. (Glaxo Laboratories Ltd.) of potency 745 units/mg was used throughout the present investigation.

The potency of Streptomycin was determined by biological and chemical methods.

(a) Biological Method:-

The microbiological assay technique employed was an agar-plate diffusion method as modified by Wilkins (1949). In this method a standard graph showing relationship between the diameter of zone of inhibition of a susceptible micro-organism and the concentration of antibiotic is prepared. The concentration of an unknown sample is determined by interpolating the diameter of zone of inhibition it produces, on the standard graph.

Organism used:- The organism used was a strain of Bacillus Subtilis (R.C.S.T. No.60). It was maintained on slopes of nutrient agar (Oxoid). The organism was sub-cultured every week at 37°C.

Inoculum:- For the experimental purpose the organism was sub-cultured in the medium of the following composition:-

Peptone (Oxoid) - 5.0g, Nacl (Analar grade)-3.5g.

Yeast-extract (Oxoid)-1.5g, Glucose(Analar grade)-1.0g.

Lab-lemco (Oxoid)-1.5g, K_2HPO_4 B.D.H - 3.68g.

KH_2PO_4 B.D.H - 1.32g.

de-ionised water - 1000ml.

pH adjusted so as to be 7 after sterillization.

After 4-5 sub-cultures at 37°C at 24 hours interval, in the above medium, a uniformally sensitive culture (to streptomycin) and a relatively constant viable population was obtained. A 24 hours old (uniformly sensitive) culture was used to seed the assay medium.

The assay medium contained Peptone-5.0g; Lab-lemco-3.0g: Agar-15.0g, de-ionised water - 1000ml, pH 7.8 - 8.0 after sterilization.

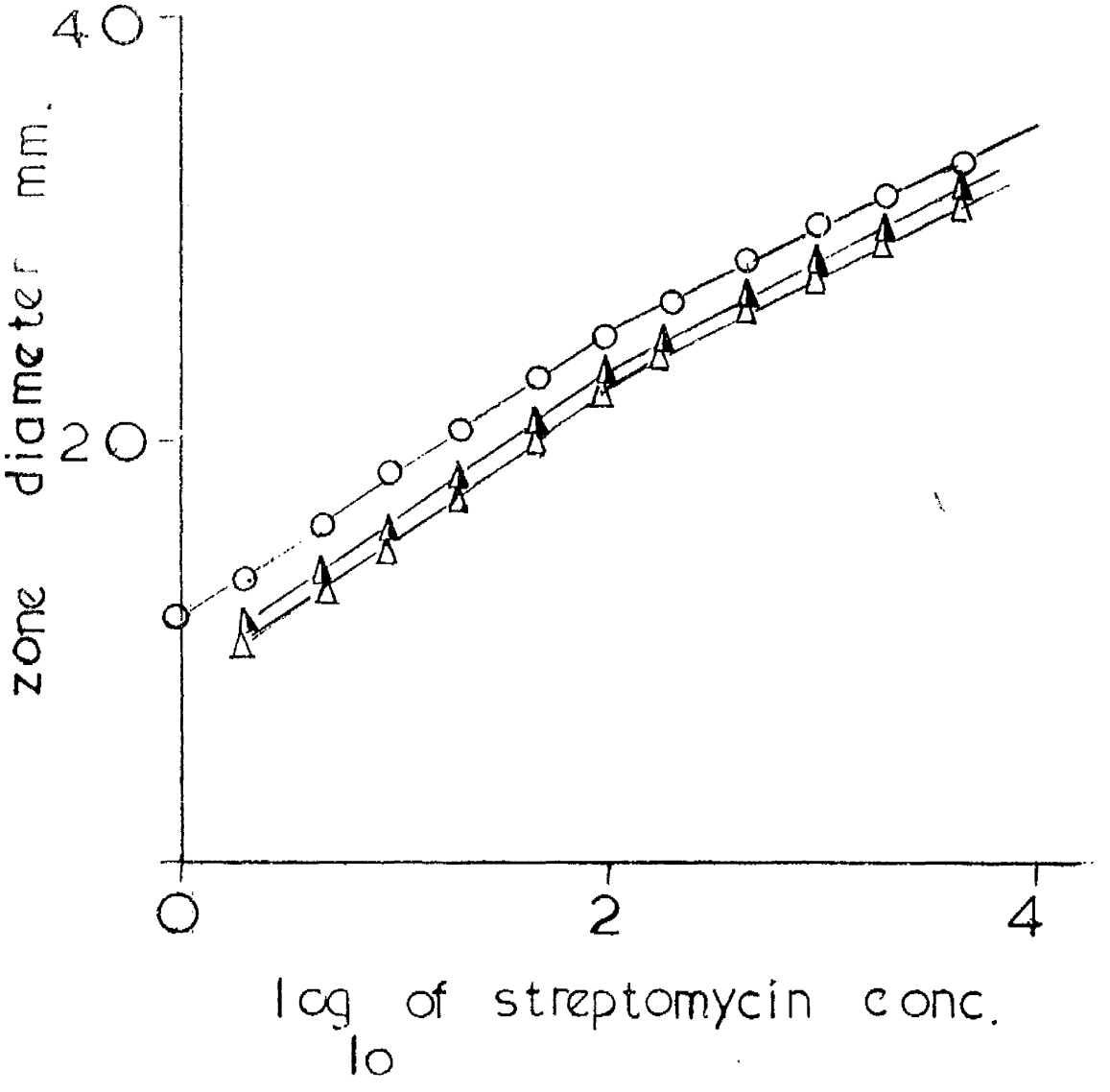
Procedure:- 20 ml portion of the above assay medium, seeded with 1.0% (V/V) of a 24 hour old broth-culture, was used per plate of uniform size (4 inch diameter). After the medium had solidified, cavities were cut in the agar with a sterile 10 m.m. cork-borer. Into the cups 0.2ml of the solution to be tested was placed from a pipette, the cups were thereby completely filled. The plates were kept at room temperature for 15 minutes before being transferred to the incubactor (37°C), this time lapse was desirable to allow some of the fluid to be absorbed into the agar and hence, the risk of spilling upon moving the plates was reduced. After 24 hours at 37°C the diameters of the zones of inhibition were measured to the nearest 0.5m.m.

Each concentration was tested in triplicate on separate plates.

The standard curve

From a stock solution of streptomycin, two sets of dilution were prepared, one in sterile de-ionised water and the other in phosphate buffer of pH 8.0, covering the range 5,000 to 0.1 µg/ml. These were assayed as described above, using two cavities on each of 3 plates for each concentration. The remaining two cavities in each plate received the solution containing 20 ug of streptomycin/ml, to enable a correction to be made for variability between the plates. There were thus 6 readings for each concentration except that of 20 µg/ml for which there were 84. After the mean zone diameter had been obtained, the value for each concentration, other than 20 µg/ml, was corrected to allow for the difference between the mean zone diameter given by 20 µg/ml on that particular set of plates and the grand mean (correction point) value for 20 µg/ml. Thus, if the 20µg/ml gave a smaller mean zone diameter than the correction point value, the difference was added to the mean zone diameter for the concentration in question, to allow for the smaller response, and vice versa. The corrected mean zone diameter were then plotted against log streptomycin concentration (Table 3 and Fig.1). The mean of 84 values for 20 µg/ml formed the "correction point" for

Biological Assay



○ in water

△ in sterile soil extract

▲ in non-sterile soil extract

Fig 1 : Standard graph for the assay of streptomycin in water, and in acid-methanol extracts of sterile and non-sterile soil.

the assay of unknown solutions.

TABLE 3

The relation between Log 10 streptomycin concentration and diameter of zone of inhibition

Test Organisms - Bacillus Subtilis R.C.S.T. 60.

Streptomycin Concentration (µg/ml)	Log 10 of Streptomycin Concentration	Mean corrected zone diameter in	
		Water(pH 7.0)	Buffer(pH 8)
5.000	3.699	33	33
2,000	3.301	31.5	31.5
1,000	3.000	30	30
500	2.699	28.5	28.5
200	2.301	26.5	26.5
100	2.000	25	25
50	1.699	23	23
20	1.301	20.5	21
10	1.000	18.5	19
5	0.699	16	16.5
2	0.301	13.5	14.5
1	0.000	12	12
0.5	-0.301	negligible	11
0.2	-0.699	no zone	no zone
0.1	-1.000	no zone	no zone

It is clear from the results obtained (See Table 3) that it made little difference whether the streptomycin was dissolved in de-ionised water or in phosphate buffer pH 8.0

(See also Waksman 1949). For convenience de-ionised water and the corresponding graph was used in future work when streptomycin assays were carried out. This graph was treated as "bilinear". However, as shown later this graph could not be used for the assay of streptomycin extracted from the soil, as substances extracted with the antibiotic interferred with the results and therefore another curve had to be constructed.

Assay of unknown Solutions.

The usual procedure of assay as above was carried out in which two cavities in each 3 plates received the unknown solution (dilutions prepared if necessary), while the other two in each plate received the reference solution containing 20 µg/ml.

When the assay value of the reference solution was larger than the correction point, the difference between these was substracted from the mean zone diameter value of the unknown solution, and when it was smaller it was added. The value of thus corrected zone diameter of unknown solution was then read off, from the standard curve.

(b) Chemical Method - The maltol Method.

This method depends upon the fact that streptomycin, when heated in the presence of dilute alkali, forms maltol (2-methoxy-3-hydroxy-gamma-pyrone; Schenk and Spielman 1945), from the streptose portion of the intact streptomycin

molecule, (the biologically active portion). It is stable in acid solutions and gives colour with ferric ions. When ferric ammonium sulphate is used to develope the colour, the amount of streptomycin estimated ranges from 500 to 2,500 µg. When the phenol reagent of Folin and Ciocalteau (1927) is substituted, as in the method described by Boxer, Jelinek and Leghorn (1947), the test becomes more sensitive and the range of 20 to 250 µg of streptomycin could be estimated. In the present work the phenol reagent of Folin and Ciocalteau, was used as it was more suited to the concentrations of streptomycin employed.

Procedure.

5.0 ml of streptomycin solution to be tested was put in a test tube and after adding 1.0 ml of 2n NaOH to it, it was kept in a boiling water bath for 3 minutes. It was then allowed to cool for 3 minutes in a cold water bath. To this cooled alkaline solution 1.0 ml of the phenol reagent was added drop-wise and mixed thoroughly. After 1 to 2 minutes 3.0 ml of (20n) sodium carbonate solution was added, the tube shaken thoroughly, and allowed to stand for 10 minutes. The light transmission was then determined by using a photoelectric colorimeter with a 6,600A° filter (red filter). A blank without streptomycin served as control and its transmission recorded as zero point.

Standard Curve. The relationship between streptomycin concentration and light transmission was established by running triplicate estimations (as above) on aqueous streptomycin solution prepared from the stock solution and ranging in concentration between 4 to 48 µg/ml in steps of 4 µg/ml, so that the amounts of 20 to 240 ug were being estimated. The mean values are shown in Table 2 and relationship is shown graphically in Fig.2.

A similar experiment with streptomycin dissolved in the synthetic medium used for the decomposition studies were carried out. The results were substantially the same as in aqueous solution (see table 4).

Assay of unknown Solution. The amounts of streptomycin in the culture fluids were obtained by interpolating the transmission reading on the standard curve.

EE. Soil

The freshly dug soil (to 6" deep but excluding the surface 1") from the Chelsea Physics Gardens, London, was used for all the work. This soil is considered to be rich in organic matter and is dressed with compost every year.

(a) Handling and Storage.

The soil was partly air dried to facilitate handling. It was first passed through a 3 mm sieve and later

Chemical Assay

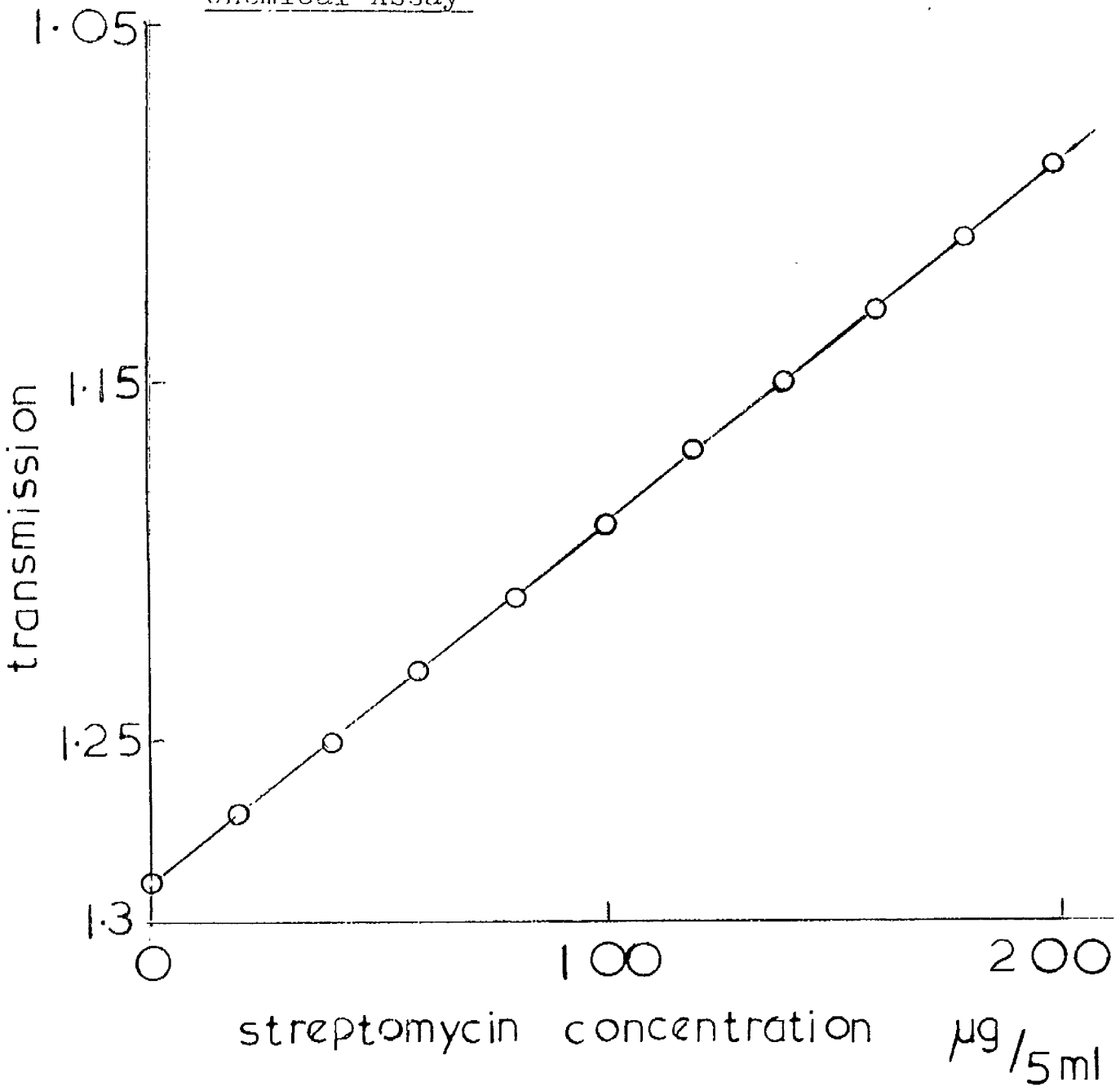


Fig 2: The relationship between the amount of streptomycin and transmission in the calorimeter (using red filter) after the colour developement.

TABLE 4

Chemical Method

The relationship between amount of streptomycin concentration and light transmission at 6,600A°

Streptomycin Concentration µg/ml	Streptomycin Amount µg.	Mean transmission at 6,600A° (red filter).
0	0	1.29
4	20	1.27
8	40	1.25
12	60	1.23
16	80	1.21
20	100	1.19
24	120	1.17
28	140	1.15
32	160	1.13
36	180	1.11
40	200	1.9
44	220	1.07
48	240	1.06

through a 2 mm sieve and stored in tightly closed glass jars at room temperature.

(b) Water Content

This was determined by drying a weighed soil sample to constant weight in an oven at 105°C over night, and reweighing. An average value was calculated from the results of three determinations.

(c) Water holding Capacity

The maximum water-holding capacity of the soil was determined by Hilgard's method (Freed and Waksman, 1928), using copper trays 1 cm deep with perforated bottom. It was calculated on the dry soil basis, i.e. if a certain amount of soil was needed, the equivalent amount of moist soil was weighed out and water was added to the desired level. All the experiments were conducted at 50% the maximum water holding capacity. The maximum water holding capacity of the Chelsea Soil was 43.4%.

(d) Determination of pH

A portion of soil was shaken for 10 minutes with 4 parts of distilled water per unit dry weight of soil and allowed to stand at room temperature for 4 hours. It was then filtered and the pH of the clear filtrate was determined by means of a glass electrode. It was 6.75.

III. Addition and Extraction of Streptomycin

(a) Soil treatment.

The experiments were usually conducted in 100 g portions of sieved and partially dried soil contained in 250 ml conical flasks plugged with cotton wool. Streptomycin solution was prepared by dissolving the antibiotic in distilled water and sterilizing by bacteria-proof filtration (Seitz's filter). This stock (antibiotic) solution was stored at +4°C. Sufficient streptomycin solution to bring the dosage to 1,000 µg/g of soil was added dropwise to the sieved partially dried unsterile or sterile soil which had been autoclavin at 15 lbs pressure for 2 hours. The flasks were shaken thoroughly to distribute the antibiotic as evenly as possible, and the moisture content adjusted to the necessary level by adding sterile distilled water dropwise from a pipette to avoid the formation of muddy lumps. The flasks were then shaken again, samples taken to give zero time data, and the remainder was incubated at 25°C. At suitable intervals samples were taken as required and the remainder returned to the incubator. The loss of water by evaporation was made good by the addition of sterile distilled water (after weighing the flasks) every third or fourth day. Controls of unsterile and sterile soils were set up as required and incubated. In the former case the necessary

volume of sterile distilled water was added and the soil incubated. In the latter case 100 ml portions of soil contained in 250 ml flasks was autoclaved at 15 lbs pressure for 2 hours. The flasks were weighed before and after autoclaving to know the loss of water during autoclaving, which was taken in to account later while adjusting the moisture content. To avoid excessive loss of water by autoclaving the average amount (3ml) lost during an autoclaving was added to the soil before autoclaving. No further addition of water was usually required, apart from the usual loss during incubation due to evaporation.

(b) Extraction of Streptomycin from Soil

The next step, after addition of streptomycin to soil, was to find out a proper quantitative procedure to determine the amount of streptomycin present at any time in the treated soil.

Streptomycin is water soluble. It is also soluble in alcohols and acidified alcohols, but is insoluble in solvents such as chloroform, ether and ethyl acetate. Since streptomycin is water soluble, it is natural to try its extraction first with water. However, it has been shown by Sinha (1950) and Ghadialy (1961) that extraction of streptomycin with water, from the treated soil was very low. Moreover, Woodthrope and Ireland (1947), Sinha (1950) and Ghadialy (1961) concluded that acid methanol was the best

solvent for extraction of streptomycin from the treated soil. The following experiments were therefore performed, to varify those findings.

(i) Extraction with water:-

For this purpose, two, 5 gms samples were weighed out from the flask containing sterile soil which had just been treated with 1000 ug of streptomycin/g, into 1 oz. sterile screw-capped bottles, and 10 ml of water was added to each. One bottle was then shaken for 2 hours at R.T. and another was shaken at 45°C for 10 minutes. Similar duplicate samples were also derived from the normal soil (i.e. non-sterile soil) which was similarly treated with streptomycin. The soils were allowed to settle down and the clear supernatants were decanted. The sediments were shaken with water, centrifuged and the final portions of supernatants were combined and then assayed, to find out streptomycin content, in the usual manner.

TABLE 5

The immediate recovery of streptomycin from soil treated with 1,000 µg/g by water extraction.

Type of Soil	Mean recovery of streptomycin (µg/g of soil) by	
	Shaking for 2hrs at R.T.	Shaking at 45°C for 10 mins.
Non-sterile	1.5	1.5
Sterile	1.8	1.8

As seen from the results (Table 5), The recovery of streptomycin, with water, from the treated soil was extremely low. This may, either, have been due to the antibiotic being adsorbed strongly to the soil colloids, or the antibiotic may have been destructed by chemical reaction with some constituents of the soil. Biological inactivation was unlikely cause here, as the time interval between addition and extraction of streptomycin to soil was very short. Also, almost the same recovery of streptomycin from both sterile and unsterile treated soil, further supports the view.

ii) Extraction with Organic Solvent

As mentioned above, streptomycin is also soluble in alcohols and acidified alcohols. Solvents such as methanol, ethanol, acid methanol and acid ethanol (2.0 ml of concentrated Hcl/100 ml of each alcohol) were therefore tried this time, to extract streptomycin from the treated soil, which was probably strongly adsorbed by soil colloids. The procedure was as follows:-

100 gm portions of sterile and un-sterile soil contained in 250 ml flasks, were treated with 1000 µg of streptomycin/g of soil, in usual manner. The flasks were incubated, together with untreated sterile and unsterile soil controls, at 25°C for 2 hours. Enough number of 5 gm samples were then withdrawn from each of the four types of soil and were

treated with each of the four solvents in 1 oz. screw-capped bottles; amount of solvent being 10 ml for each of 5 g portion. The bottles were shaken well and allowed to stand at R.T. for 24 hours with occasional shaking. The samples were centrifuged and supernatant decanted. This procedure was repeated twice. The final portions of supernatant were combined and neutralized using 2N NaOH for the alcohol solvent extracts and 80% NaOH for the acidified alcohol solvent extracts. Usually little reddish-brown precipitate was formed but this was neglected. The fluid was evaporated to dryness (by means of a vaccum pump) under the reduce pressure over $CaCl_2$ at R.T. The residue was taken up in a measured quantity of water, equivalent to the weight of moist soil in grammes, and shaken well. After preparing proper dilutions in sterile distilled water the suspension was assayed, using the appropriate standard curve. The extraction and assay of antibiotic were done on the same day to avoid loss due to hydrolysis.

TABLE 6

The recovery of streptomycin from soil treated with 1000 µg/g, by organic solvents.

Type of Soil	Streptomycin added (µg/g) of soil	Mean recovery of streptomycin (µg/g of soil) with Methanol µg/g	Ethanol µg/g	Acid Methanol µg/g	Acid Ethanol µg/g
Non-sterile	1000	110	75	282	186
"	0	0	0	0	0
Sterile	1000	175	120	435	256
"	0	0	0	0	0

From the results (Table 6) it is clear that acid methanol was the best solvent, of the solvents tried, for extraction of streptomycin from the treated soil. Further experiments were, therefore, performed with acid methanol only.

Three sets of 5 g samples were withdrawn from both sterile and unsterile soil, treated with 1000 µg of streptomycin/g of soil, in usual ways into 1 oz. screw-capped bottles. To each sample bottle, 10 ml of acid methanol was added, and bottles were shaken well; one set was shaken for 2 hours at R.T. another was shaken and allowed to stand at R.T. for 24 hours, and third set was shaken for 10 minutes at 45°C in a constant temperature bath. The treatment of supernatants and rest of the procedure followed was as described above.

TABLE 7

The recovery of streptomycin, from soil treated with 1000 µg/g with acid methanol.

Type of Soil	Mean recovery of streptomycin (µg/g) by		
	Shaking for 2hr at R.T.	Shaking for 24hr at R.T.	Shaking for 10mins at 45°C
Non-sterile	310	282	310
Sterile	460	435	460

From the results (Table 7) it is evident that both the methods, extraction for 2 hr at R.T, and extraction for 10 mins. at 45°C, were equally effective and were better than the method of extraction for 24 hr. at R.T. From the time saving point of view, it was decided to use the method of shaking for 10 minutes at 45°C in the future work.

All the above results (Table 5,6,7) show that the initial recovery of streptomycin, from both types of soil, was very low, which suggests that the antibiotic was strongly adsorbed and bound by soil colloids. It is well established that basic antibiotics like streptomycin, streptothricin etc, are strongly absorbed by the soil colloids. Another striking point was that the recovery from sterile soil was always (See tables 5,6,7) greater than that from the non-sterile soil. It is possible that autoclaving had either lowered the absorption capacity or strength of the soil colloids, or had destroyed the substances normally present in the soil; these substances are extracted by acid methanol, and antagonise streptomycin. As mentioned earlier, the possibility of microbial degradation of antibiotic, could be ruled out as the time period was very short between addition and extraction of streptomycin from both types of soil samples.

(c) Assay of Streptomycin in Soil Extracts:-

To decide whether the recovery of streptomycin by

acid methanol was in any way affected by presence of substances extracted together with the streptomycin from the soil, an assay of antibiotic in soil extract was carried out.

For this purpose 10 gm portions of untreated sterile and non-sterile, soil were weighed out in 1 oz. screw-capped bottles. To each bottle 20 ml of acid methanol was added and bottle shaken for 10 minutes at 45°C; the supernatants were centrifuged. After centrifugation, supernatants were decanted and the volume was made up so as to have final volume (after addition of antibiotic solution) of 20 ml. To twelve such portions (20 ml) of extracts from each type of soil, streptomycin was added to give concentrations of 5,000, 2000, 1000, 500, 200, 100, 50, 20, 10, 5, 2 and 1 µg/2ml. The portions were allowed to stand for 2 hours at R.T. to stimulate procedure of extracting streptomycin from soil. These portions were then treated exactly as in the extraction procedure (see previous experiment), the residue being finally taken up in 10 ml water and assayed biologically. The results obtained are shown in Table 8.

From a given concentration wider zones were obtained by streptomycin dissolved in extract of non-sterile soil then in that from the extract of sterile soil, but in both the cases the zones were smaller than those obtained by

TABLE 8

The relationship between mean zone diameter and $\log_{10}$ Streptomycin concentration in the agar cup assay of streptomycin dissolved in acid methanol extracts of sterile and non-sterile soil.

Streptomycin concentration (µg/ml)	Log 10 strepto-mycin concent.	Mean zone diameter (mm) in the extract of non-sterile soil	extract of sterile soil
5000	3.6999	32	31.5
2000	3.301	30	29.5
1000	3.000	28.5	28
500	2.699	27	26.5
200	2.301	25	24.5
100	2.000	23.5	23.
50	1.699	21	20.5
20	1.301	18.5	17.5
10	1.000	16	15
5	0.699	14	13
2	0.301	11.5	10.5
1	0.000	negligible	negligible
0.5	-0.301	no zone	no zone

corresponding concentrations in water (see Table 3.8 & Fig.1.). This shows that acid methanol did extract substances which lowered the activity of streptomycin. Furthermore it suggests

That the processes of soil sterilization by autoclaving led to the extraction by acid methanol of a greater concentration of these substances, possibly soluble salts, which are known to reduce the activity of streptomycin.

IV. The enumeration of micro-organisms

The normal dilution plate technique was used for estimating the numbers of micro-organisms in the soil. 1ml portions of diluted soil samples were either added to Thronton's agar (Thronton 1922) or to modified soil extract agar (Jacobs and Dadd, 1959). The plates being incubated at 25°C for 8-10 days and colonies counted.

V. Determination of Resistance to Streptomycin

To study the degree of resistance of isolates to streptomycin, the 'gradient plate technique' as described by Szybalski (1952) was used; in which a standardized bacterial suspension was streaked parallel to the concentration gradient axis and inhibitory concentration was read directly from the length of the growth along the streak.

Degradation of streptomycin in laboratory media

The following media were employed in the studies concerned with streptomycin decomposition by and growth kinetics of bacteria.

(A) Liquid media.

(a) Nutrient broth containing 20 µg g streptomycin/ml.
(b) Yeast-extent broth containing 20 µg g streptomycin/ml.
(c) Synthetic medium.

A synthetic media composed of mineral salts were used to study the decomposition of streptomycin by mixed cultures as well as pure culture isolates. Also a detailed study of growth characters was performed in these media. The composition as the media is as follows.

Medium SM: K_2HPO_4 - 1.0g, $MgSO_4.7H_2O$ - 0.2g; $CaCl_2$ - 0.1g; NaCl - 0.1g; $FeCl_3$ - 0.002g; and de-ionised water - 1000ml. pH 7 after sterilization.

Medium SSM: To above basal-salts medium - medium 'SM' Streptomycin is added as the sole source of carbon and nitrogen. The concentrations of streptomycin are specified in the experiments.

Medium GNSM: Glucose and $(NH_4)_2SO_4$ are added to SM medium.

(1) Usually, unless otherwise specified 50 ml portion of synthetic medium contained in 150 ml 'pyrex Glass' conical flasks was used. Before use the flasks were soaked in 7% Hcl (over-night), rinsed with tap water (7 times) and de-ionised water (3 times), plugged with cotton wool and sterilized at 15 lbs pressure for 15-20 minutes.

(2) The temperature of incubation in all the experiments unless specified was $25^{\circ}C$.

(3) The usual method of biological and chemical assays of streptomycin (described earlier) were carried out to estimate the amount present at any particular time in the culture medium.

(4) Because one of the strain studied does not give measurable turbidity in the medium growth was estimated by the 'plate count method' as described by (Miles and Misra The plates were incubated at 25°C for 2-3 days and the colonies counted.

(5) Inoculum.

The bacterial cells were harvested from the slants with sterile de-ionised water, the cells concentrated and washed three times with sterile de-ionised water. The washed pallet was finally suspended in sterile de-ionised water, well shaken and the suspension transferred into streptomycin-synthetic medium flask (SSM) and the flasks incubated at 25°C. After 48 hours 1ml of culture was transferred to fresh 'SSM' medium and thus sub-culturing done twice more. For studies on growth kinetics and streptomycin decomposing ability, 48 hour old cell material of the 3rd sub-culture was used for incubation of various media after being washed 3 times with water by centrifugation.

(7) The culture flask was always shaken on a mechanical shaker (42 occilations/min.) in the incubator at 25°C.

(8) In Solid Media - 'Cut out technique'.

Agar media (in petridish), either nutrients or synthetic containing 20 µg of streptomycin/ml were streaked by means of platinum loop, with the isolates to be studied. The

plates were placed in a polythene bag with a beaker of water to maintain the humidity and thereby preventing the evaporation and were incubated at 25°C for 8-10 days. At intervals two rectangular portions of 1" x 1.5" were cut from each plate with one piece closer to the streak (with the shorter side parallel to the streak); the other portion has been cut out of the agar as far as away from the streak as possible. The latter served as control piece. Both pieces were placed carefully on a nutrient agar plate seeded with B. subtilis (in a similar way as for the biological assay of streptomycin) and being incubated at 37°C for 18-24 hours. After incubation plates were examined for the presence of zone of inhibition around the pieces. The control piece usually showed equal zones of inhibition on all the sides, whereas the other piece which had been nearer to the streak did not show any zone inhibition at all or show smaller zones according to the amount of streptomycin decomposed.

SECTION II

Characterization and Identification of the Streptomycin decomposing Organisms.

Characterization of the isolates of streptomycin decomposing organisms was done according to Berger's Manuel 7th edition, 1957.

Media used were nutrient agar (oxoid) nutrient broth (oxoid) and synthetic medium (SM).

(a) Morphology and Staining properties

Morphology, gram-reaction and size:- Air-dried films of young culture from nutrient agar, nutrient broth and from solid and liquid synthetic medium were stained by modified gram-stain method as described by Kopeloff and Beerman (1922). The preparation was examined microscopically using oil-immersion-lens (92 x Also the measurements of size of the stained organisms were made by using a calibrated eye-piece graticule.

Metachromatic granules staining:-

Organisms were grown on nutrient agar, nutrient broth and potassium tellurite (0.6, 2%) medium and then examined for the presence of the metachromatic granules by Albert's method (1920).

Acid fast staining

The Ziel-Neelson method was used for studying the acid-fastness of the organism.

Staining of Fatty material

The organisms were grown on peptone agar containing carbohydrates namely glucose or glycerol (2%) (Forsyth, Hayward and Robert 1958) and then stained by Burdon's method (Burdon 1946). Also similar slides were prepared from the growth on peptone agar without carbohydrates, and also from nutrient agar and presence of fatty material was studied as above.

Motility and Flagella staining

Hanging-drop preparation of young cultures were made to study motility. Flagella staining was done by Leifson's Method (Leifson 1950) and a modification of Fontanan's silver plating technique (Rhodes 1958).

(b) Growth Characteristics.

The growth characters were determined (i) in liquid media (ii) on solid media.

In liquid media:- Nutrient broth undiluted and in concentration of 1:1, 1:2, 1:3, 1:4, 1:5 (to dilute the nitrogen source) was used, also yeast-extracte broth, thioglycocollate medium, and synthetic-medium, were used. Inoculated media were incubated at 10°, 15°, 25°, and 37°C and growth characters studied.

On Solid media:- Nutrient agar, nutrient agar diluted to have concentration of 1:1, 1:2, 1:3, 1:4, 1:5 and slopes

prepared, MacConkey's agar (oxoid), Eosion-methylene blue agar (Oxoid), Wilson and Blair's medium (Oxoid), 1% starch agar, potassium tellurite medium (0.6%, 2%) and synthetic-medium solidified with agar were used for the study.

(c) Physiological characteristics

Anaerobic conditions Nutrient agar (Slopes & plates) and nutrient broth were inoculated and incubated at 25°C in a McIntosh and Filde's jar containing hydrogen.

Temperature

Inoculated nutrient agar and broth tubes together with diluted media (1:1, 1:2, 1:3, 1:4, 1:5) were incubated at 5, 10°, 15°, 20°, 25°, 30°, 33.5°, 37°C and 55°C and growth obtained studied every 24 hours.

pH Value of the Medium

Nutrients broth adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 respectively were inoculated and growth studied.

(d) Biochemical reactions

All the following tests were carried out at 10°, 15°, 25° and 37°C temperature.

To peptone water carbohydrates, alcohols and glucosides etc, as mentioned below, were added and media were used to study the fermentation ability of the organisms. They were as follows:- Monosaccharides - Arabinose (0.5%) Rhamnose (0.25%), Xylose (0.5%), glucose (1.0%), Fructose

(1.0%), mannose (0.5%).

Disaccharides - Sucrose (1.0%), Lactose (1.0%), Maltose (1.0%), Trehalose (0.25%), Melibiose (0.25%) and Cellobiose (0.5%).

Polysaccharides - Raffinose (0.5%), Inulin (1.0%), Dextrin (1.0%) and glycogen (0.25%).

Alcohols - Glycerol (1%), Erythritol (1.0%), Adonitol (1.0%), Mannitol (1.0%), Sorbitol (1.0%), and dulcitol (1.0%).

Glucosides - Salicin (1.0%), Aesculin (0.25%), and -methyl glucoside (0.5%).

Tubes containing 5-7 ml of each of the above mentioned media with inverted Durham tubes were inoculated.

Methyl red (M.R.) and Voger Proskauer (V.P) tests.

Glucose-phosphate-peptone broths (oxoid) were inoculated, incubated and M.R, V.P, tests performed after every 2 days. V.P, test was performed by coblent$_3$ modification (Coblent$_3$ 1943).

Cellulase test

To study the ability of the bacteria to attack (decompose) cellulose, the following methods were employed:

i) Filter paper strip Method.

Test tubes containing 0.5% of the peptone and a filter paper strip were inoculated with the culture to be tested.

Tubes were incubated with uninoculated control medium tubes. If the organism was able to attack cellulose then the filter paper strip turned to pulpy mass or will be weakened so on slight agitation of the culture tube, fibres start separating from the strip.

ii) Carboxy-methyl-cellulose test (C.M.C. method):

Substrate:- In this method the substrate used for the test is a semi-synthetic form of cellulose namely carboxy-methyl-cellulose (C.M.C.).

Enzymes:- Tubes containing peptone water (0.5%) were inoculated with the culture and were incubated with uninoculated control medium at 25°C, for 2-3 weeks. After 2 weeks the culture medium was filtered through a Seitz's filter and the (cell-free) filtrate obtained was used as enzyme.

Procedure:-

To the mixture of 0.35 ml of substrate (1% C.M.C. solution) and 0.15 ml of (M/5 PO_4-PO_4) buffer pH 7.6, 0.1 ml of enzyme was added and the mixture was incubated at 37°C for 1 hour. After one hour incubation the total reaction mixture was subjected to the estimation of reducing sugar by the method described below.

Calorimetric estimation of reducing sugars with $K_3Fe(CN)_6$

Ferric alum-Duponal-H_3PO_4 reagent.

The procedure followed in this method is a modified form of Park & Johnson (1949) method by Halliwell (1961).

Reagents:-

Solution (a) - 0.1 g of Ferric aluminium sulphate (analar) was dissolved in 1.5 ml of conne. phosphoric acid (ANALAR: 90%) and 48.5 ml of water.

Solution (b):- To solution (a) a mixture containing 0.15g of Duponal (Duponal ME, dry, kindly given by E.I. du Pont de Memours and Co. Inc., Wilmington, Del., U.S.A.) in 1.0 ml of conne. phosphoric acid and 49 ml of water, was added.

Procedure:-

The volume of the sample containing reducing sugars (mentioned above) was made up to 3.0 ml with distilled water and 1 ml of, each of, Cyanide-carbonate solution (0.06% KCN + 0.52% Na_2Co_3 anhydrous) and $K_3Fe(CN)_6$ solution was added. The reaction mixture was shaken thoroughly and was incubated at 99°C in water-bath for 25 minutes. It was cooled for 5 minutes in cold-water-bath and was then treated with 2 ml of ferric alum Duponal-phosphoric acid-reagent. Colours were allowed to

develop at room temperature for exactly 15 minutes and were read on Spectrophometer SP 600 at mu 700.

Nitrate reduction test, catalase production, hydrogen-sulphide production, hydrolysis of urea, action on milk, nitrite production, citrate utilization, starch hydrolysis cellulose test and haemolysis etc tests were performed according to Bergy's Manmual.

Detailed study of environmental factors on growth of organisms were performed in synthetic media, which are discussed separately.

The effect of environmental factors on growth of Organisms

The effect of environmental factors on growth of Organisms

In all the following experiments, unless specified 50 ml portions of medium (media) contained in 150 ml conical flasks of pyrex glass were used. 'SSM' medium was inoculated with 1 ml of a 48 hour old growth from 'SSM' medium, and similarly 'GNSM' medium was inoculated with 1 ml of a 48 hour from 'GNSM' medium. When slant cultures and washed cell suspension inoculum were used they are specified. Unless specifically mentioned the temperature of incubation was $25^{o}C$. Cultures were shaken on a mechanical horizontal shaker (Occilation 42/min.). Sample dilutions of the culture were prepared by serial tenfold dilution procedure as described earlier and growth measured in terms of viable cells as obtained by plate counts.

Experiments performed in this study were as follows:-

(a) Growth of organism in 'SSM' medium and 'GNSM' medium was studied.

(b) Different carbon and nitrogen sources, their concentrations and additional carbon and nitrogen sources in the medium, were used and their effect on growth of organisms was studied.

(c) Effect of size of inoculum and source of inoculum on growth was studied. In this set of experiments sizes of

inocula used were 4×10^3 cells/50 ml, or $3-8 \times 10^1$ cells/50 ml of medium, size being estimated by plate counts. Experiments were performed with 'SSM' and 'GNSM' media. Sources of inoculum used were from the same medium as well as from the different medium i.e. 'SSM' medium was inoculated with inoculum taken from 'GNSM' medium and 'GNSM' medium was inoculated with inoculum from 'SSM' medium. Inoculum was prepared by concentrating the 48 hour old growth in 'SSM' or 'GNSM' medium (as required) by centrifugation and washing three times with sterile de-ionised water. Parallel experiments with inocula from same medium were performed for comparison.

(d) Experiments were performed in anaerobic conditions by placing the inoculated 'SSM' medium flasks in McIntosh and Filde's jar in which oxygen was then replaced by hydrogen.

(e) Experiments with 'SSM' medium adjusted to different pH values (4,5,6,7 or 8 pH) were performed and growth studied in usual manner.

(f) In all the above experiments maximum growth as estimated by plate counts was between 5×10^6-10^7 cell/ml irrespective of concentration or presence of additional carbon and nitrogen sources in the medium. Also when experiment was performed with basal-salts synthetic medium (SM) which does not contain any carbon and nitrogen sources,

more or less same growth was obtained. Experiments were therefore performed to study the effect of impurities present in the medium on growth of organism. They were as follows:-

i) A set of experiments was performed in which growth of organisms was studied in 'SM' medium prepared with double glass distilled water against the growth in 'SM' medium prepared with de-ionised water.

ii) Experiments were performed with 'SM' medium deffficient in either of its salt constituents.

iii) Similar results as before were obtained in the above sets of experiments. So further experiments were performed with media prepared with individual constituent (salts) of the 'SM' medium. They were as follows:-

Synthetic medium 'SM' medium

K_2HPO_4	-	1.0g
$Mgso_4\ 7H_2O$	-	0.2g
$Cacl_2$	-	0.1g
Nacl	-	0.1g
$Fecl_3$	-	0.002g
de-ionised water	-	1000ml.

pH 7 after sterilization.

Above is the composition of synthetic (medium SM). It's each constituent was used to prepare the medium for e.g. K_2HPO_4 - 1g dissolved in 1000 ml of de-ionised water

and pH 7 after sterilization.

Similarly other salts such as $MgSO_4$, $7H_2O$ - 0.2g; $CaCl_2$ - 0.1g, NaCl - 0.1g, or $FeCl_3$ - 0.002g was dissolved in 1000 ml of de-ionised water and pH 7 after sterilization media were designated as medium K, M, C, N & F respectively.

(g) To study the effect of atmospheric CO_2 and N_2 two sets of experiments were performed.

i) Flasks plugged with rubber-buck and centre-wall containing concentrated KOH were used for the experiment.

ii) Experiment was performed in an atmosphere of oxygen by placing the inoculated flasks into McIntosh & Filder's jar and flushing it with O_2. In both the above experiments aim being to remove the CO_2 present in the culture flask.

EXPERIMENTAL & RESULTS

This chapter of this thesis is divided, for convenience, into Sections: I, II, III & IV.

Section I, deals with the investigations carried out to study (1) the decomposition of streptomycin in the soil, in laboratory conditions; and (2) streptomycin decomposition by mixed population of soil (obtained by extraction of soil repeatedly treated with streptomycin) in laboratory media.

In the Section II, isolation, identification, and characterization of streptomycin decomposing organism from the above mixed population, are described.

Section III, includes investigations carried out to study the effect of environmental factors on the growth characteristics of an isolate number "38" namely Cellulomonas fimi.

Last section (IV) deals with the effect of such environmental factors (mentioned in Section III) on the pattern of streptomycin decomposition, by the organism.

Definition:

Induction period - is the period during (the incubation of the culture) which no decomposition of streptomycin took place.

EXPERIMENTAL AND RESULTS

SECTION I

Experiments with soil

The Decomposition of Streptomycin in the Soil

I. (a) The examination of Soil treated with Streptomycin at 1000µg/g.

To study the fate and effect of streptomycin in the soil, the first step was to introduce the antibiotic in the soil and study its stability, and the course and cause of disappearance from the soil.

Soil both, sterile and non-sterile, were treated with 1,000µg/g as described in the method. Changes in the recoverable streptomycin as estimated (as described in material:method section) together with the corresponding change in the number of micro-organisms as determined by plating parallel samples on Thronton's Agar (Thronton 1922) and soil extract agar (Jacobs and Dadd 1959), were studied.

TABLE 9

The recovery of Streptomycin from soil treated with 1000ug of Streptomycin/g.

Period of incubation Days. 1hr.	Streptomycin recovered in µg/g from Soils. Non-sterile	Sterile.
0 hr.	310	460
5 hrs.	310	460
1 day	310	460
5 days	240	460
10 "	100	460
20 "	8	460
30 "	0	438

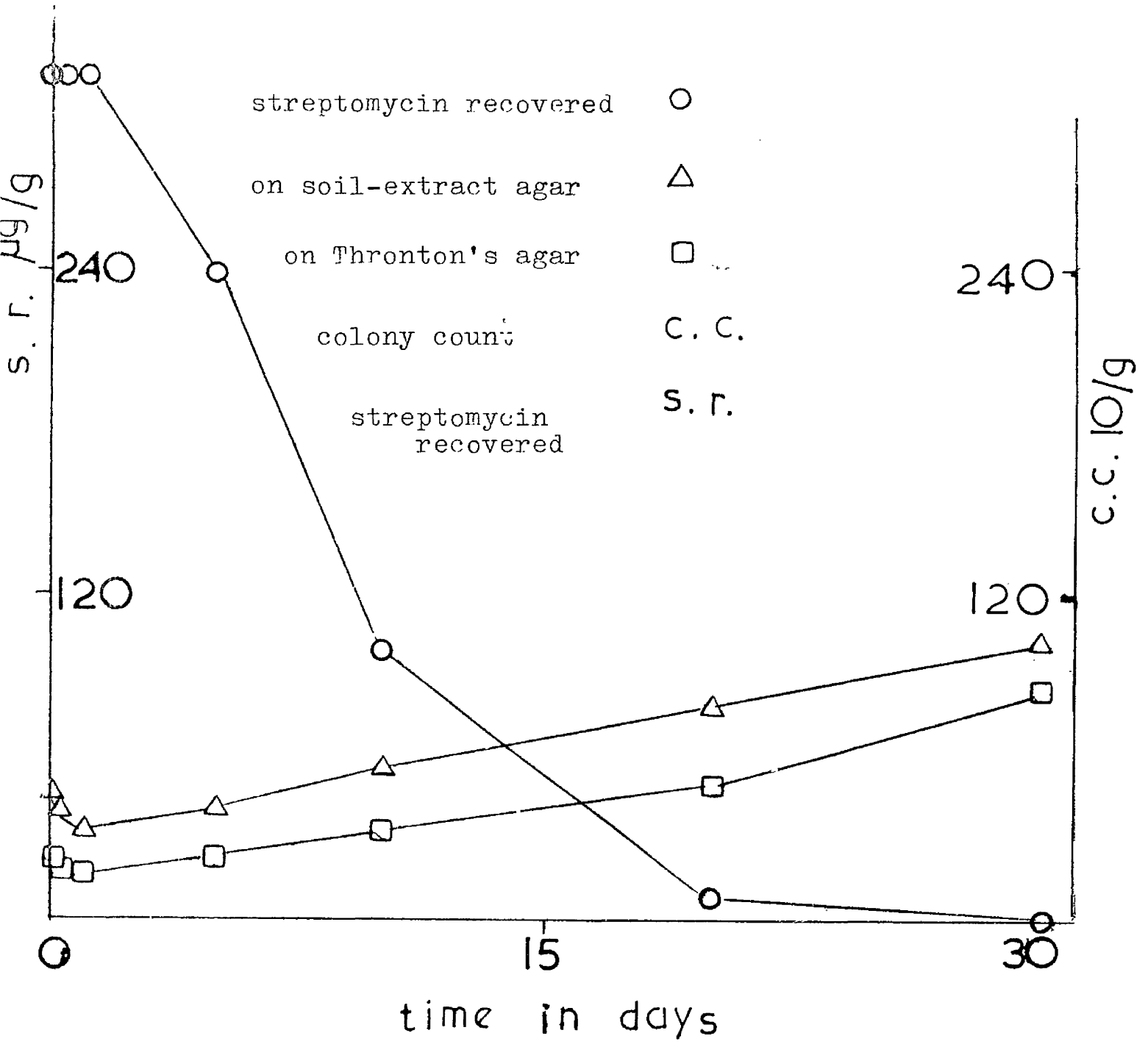


Fig. 3:- The relationship between the recovery of streptomycin from treated soil, and the microbial count on soil-extract agar and on Thronton's agar.

TABLE 10

The changes in the number of Organisms developing on Thronton's agar and Soil extract agar during incubation of Soil treated with 1,000µg of Streptomycin/g.

Period of incubation		Member of Organisms millions/g of Soil on Thronton's agar		Soil-extract agar.	
days	hr.	Treated	Control	Treated	Control
	0 hr.	22.8	22.8	45.2	45.2
	5 hrs.	18.5	24	39.0	45.8
1	day	17.5	26.5	32.1	48.1
5	days	22.7	25.3	41.0	47.9
10	"	32.2	26.2	55.2	50.5
20	"	50.5	24.5	78.0	49.2
30	"	84.2	23.8	102.0	48.5

The results show (see Table 9 - Fig. 3) that about 46% of the added Streptomycin was recoverable from the sterile soil immediately after its addition. This remained constant throughout the experimental period. As compared to this, there was only 31% of initial recovery from the non-sterile soil which did not change for a further 24 hours. After 24 hours, the amount of extractable streptomycin decreased, first at an accelerated rate and slowing down later. More than 50% of the recoverable antibiotic disappeared within 10 days and by the end of 30 days no recoverable streptomycin was detected. The

"immediate recovery" which did not exceed more than 46% from either type of soils showed that the antibiotic was strongly absorbed by soil colloids. Difference in the behaviour of the sterile and non-sterile treated soil suggests that micro-organisms present in the non-sterile soil might be responsible for the streptomycin disappearance from that soil.

The results of the parallel experiments of bacterial count show (Table 10) that the number in the control soil fluctuated but did not very greatly from the initial level throughout the experimental period, where as in the treated non-sterile soil (Table 10-Fig.3) there was decrease in the number in the first 24 hours (presumably the antibiotic exerted a lethal effect), which recovered within 5 days and continued to rise steadily thereafter until after 30 days when it was almost double the control (on soil-extract agar). Thus bacterial multiplication began when the streptomycin disappearance started.

It was interesting to note that in the treated non-sterile soil an amine like odour became noticeable after 7 days which became more and more pronounced later on; it was strongest between 12-17 days, i.e. during the period of most rapid streptomycin disappearance. Later it became weaker and could not be detected after 22 days.

If we combine all the above results it becomes apparent that in the non-sterile treated soil, streptomycin content started decreasing after the 5th day and consequently the

bacterial multiplication began which in turn gave the amine-like odour. In the last stage, by the end of 20 days period very little streptomycin was recovered from the soil, bacterial count was more than double the initial count, and odour was also faint. Finally after 30 days, all the streptomycin disappeared from the soil, and the amine-like odour had gone also, and bacterial multiplication continued unchecked (probably utilising decomposed products of streptomycin).

(b) The disappearance of streptomycin from the Soil treated with successive doses of 1, 000 µg of Streptomycin/g.

From the above experiments two points were evident, (i) Biological decomposition of streptomycin was proceeding in the Soil; and (ii) streptomycin has inhibitory action as well as stimulating action on certain type of organisms. The latter action seems to be mainly on streptomycin-resistant or decomposing organisms. To facilitate the isolation of the later type of organisms, repeated treatments of non-sterile soil with 1,000 µg of streptomycin/g of soil, was carried out to enrich the soil in this type of organisms. Since it seemed probable that the amine-like odour was associated in some way with the biological decomposition of streptomycin, successive doses of antibiotic were added as soon as the odour had disappeared.

The results show (Table 11) that successive doses of streptomycin disappeared more tapidly than did the previous doses which allowed addition of fresh streptomycin at shorter intervals with each dose. By the end of 4th treatment the soil became very active when successive doses of streptomycin disappeared within 4-5 days. It was found that each time the amine-like odour was associated with streptomycin disappearance. The results also indicates the evidence of biological decomposition of streptomycin. At this stage, it was believed that the soil would be quite rich in streptomycin-decomposing organisms so the first step in isolation procedure was begun by inoculating "soil extracts" into synthetic medium containing streptomycin as the sole source of carbon and nitrogen; this step has been discussed separately in the next experiments.

TABLE 11

The disappearance of Streptomycin from the Soil treated with successive doses (1,000 µg/g).

Treatment number	Time required for total disappearance of Streptomycin (in days).
I	22.
II	12.
III	7.
IV	5.
V	4-5

II. The decomposition of Streptomycin by mixed bacterial population in laboratory media.

The earlier workers (Sinha 1950; and Gladialy 1961) found that 1000 µg of streptomycin/1 ml of synthetic medium was too high a concentration to be decomposed by micro-organisms in the synthetic medium, though in fact the same dose level concentrations was decomposed in the soil from which the "soil extracts" were used to inoculate the synthetic medium.

The experiments were performed to verify this finding. The following two experiments (a) and (b) were carried out simultaneously.

(a) Preliminary experiments at 1000 µg of streptomycin/ml.

As mentioned earlier samples were withdrawn from the above 'active soil' at different intervals of time as shown in the Table 12 . Five grammes of soil was taken in 1 oz crew cap bottle each time, and to that 10 ml of sterile distilled water was added, mixed well, and allowed to settle. 1 ml supernatant of the soil solutions were then added to 50 ml of synthetic medium containing 1000 µg of streptomycin/ml and incubated along with uninoculated control flasks at 25°C. Repeated biological and occassional chemical estimations of streptomycin contents in the inoculated synthetic medium were carried out, in usual manner.

There was no disappearance of streptomycin from any of the cultures even after 4 months. When the samples from the culture flasks were plated on nutrient agar medium containing either of 1000, 2000 or 5000 µg of streptomycin/ml numerous colonies appeared. This result showed that the micro-organisms present in the culture flasks were highly resistant to streptomycin but were unable to decompose high concentration of streptomycin such as 1000 µg/ml. These results also raise the possibility that the disappearance of streptomycin in the original soil culture may be due to the presence of micro organisms which are unable to reproduce on the test media.

TABLE 12

The scheme of sampling soil repeatedly supplemented with 1000 µg of streptomycin/g, to obtain enrichment cultures

Label	Time in days from start	Number	Time of collection of samples
1	0	1/1	One day after first treatment
	15	1/15	15 days after first treatment
2	23	2/1	One day after second treatment
	33	2/10	10 days after second treatment
3	38	3/1	One day after third treatment
4	46	4/1	One day after forth treatment
5	53	5/1	One day after fifth treatment

Number of the treatment/days after that treatment has been applied before the sample was taken.

(b) Experiments with low concentration of streptomycin

It was thought that if the conditions in the synthetic medium flasks were made closer to those in soil by using some colloidal absorbent which will absorb most of the streptomycin and leave behind small residue for micro-organisms to act upon, it might be helpful, so, as suggested by Ghadially (1961) when 10 ug of bentonite/ml was added to the synthetic medium flasks containing (1000 µg/ml) streptomycin, most of the streptomycin was absorbed by bentonite and small residue of 20 ug of streptomycin/ml was left free in the medium. It was thought that this could be a good level to use in further work. So experiments were carried out with this level.

In this study parallel experiments were performed with two sets of media.

(1) 'BSM' medium - synthetic medium containing 1000 µg of streptomycin/ml + bentonite.

(2) "SSM' medium - Synthetic medium containing 20 µg of streptomycin/ml and no bentonite.

In both media the amount of free streptomycin in the synthetic medium thus finally becomes approximately the same (20 µg/ml).

(i) 'BSM' medium

Forty eight ml portions of synthetic medium were taken into 150 ml flasks and to each flask 500 µg of bentonite

was added. The flasks were autoclaved at 15 lbs pressure for 20 minutes. The sterile streptomycin solution was then added to bring the dose level to 1000 µg of streptomycin/ml, and the volume in each flask was made up 50 ml with sterile synthetic medium.

(ii) 'SSM' medium

Forty eight ml portions of sterile synthetic medium were added to sterile flasks and sterile streptomycin solution was added to bring the dose level to 20 µg of streptomycin/ml in the final volume of 50 ml of medium in each flask which was made good by the addition of sterile synthetic medium.

Procedure.

Fifty ml portions of both media were inoculated with 1 ml of supernatant of soil extract obtained from streptomycin treated soil as used and described in experiment (a) (See scheme Table 12). There were 7 samples in all. The inoculated media, together with uninoculated media which served as control, were incubated at 25°C.

Biological and chemical estimations of free streptomycin present in the culture media were carried out at certain intervals. The results were compared and corrected according to that of the control medium which served as 'reference concentration'.

TABLE 13

The changes in the free streptomycin content of a synthetic medium containing bentonite (BSM), after inoculation with various mixed cultures.

Incubation period in days	Free Streptomycin content (µg/ml) in cultures inoculated with various soil samples.						
	1/1	1/15	2/1	2/10	3/1	4/1	5/1
0-37	20	20	20	20	20	20	20
38	16	16	16	20	10	10	10
39	8	8	8	16	3	2	2
40	2	2	2	8	0	0	0
41	0	0	0	2	0	0	0
42	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0

Control - no Decomposition.

TABLE 14

The changes in the streptomycin content in the synthetic medium (SSM) after inoculation with various mixed cultures.

Incubation period in days	Streptomycin content (µg/ml) in cultures inoculated with various soil samples.						
	1/1	1/15	2/1	2/10	3/1	4/1	5/1
2-26	20	20	20	20	20	20	20
27	20	20	20	20	20	20	15
28	12	12	12	20	10	10	15
29	4	4	4	15	2	2	0
30	0	0	0	5	0	0	0
31	0	0	0	0	0	0	0

Control - no Decomposition.

As shown in table 13 the free streptomycin content in 'SSM' medium remained constant for 37 days; it then started decreasing and none was detectable after 40-42 days. Though the free streptomycin content did not change in the medium over as long a period as 37 days, the peculiar odour associated with biological decomposition was noticed in all the flasks on the 7th day.

In the case of 'SSM' medium (See table 14), the streptomycin content remained unchanged upto 27 days induction period and the whole amount was decomposed thereafter within 3 to 4 days time. The odour was noticed on the 28th day when streptomycin disappearance began in the medium. It is important to note here that the number of streptomycin treatments given to the soil from which the cultures are derived did not markedly affect the results.

(c) <u>The effect of successive transfers of culture on streptomycin decomposition.</u>

When the decomposition of streptomycin in the above experiment was complete, transfers of 1.0ml from all the cultures were made into fresh media. The media were incubated together with the uninoculated controls and the disappearance of streptomycin from the medium was followed in the usual way by biological assay. The procedure was repeated twice more.

The remaining culture in each 'SSM' medium flask was

centrifuged, the supernatant decanted, and the bentonite residue extracted with acid methanol.

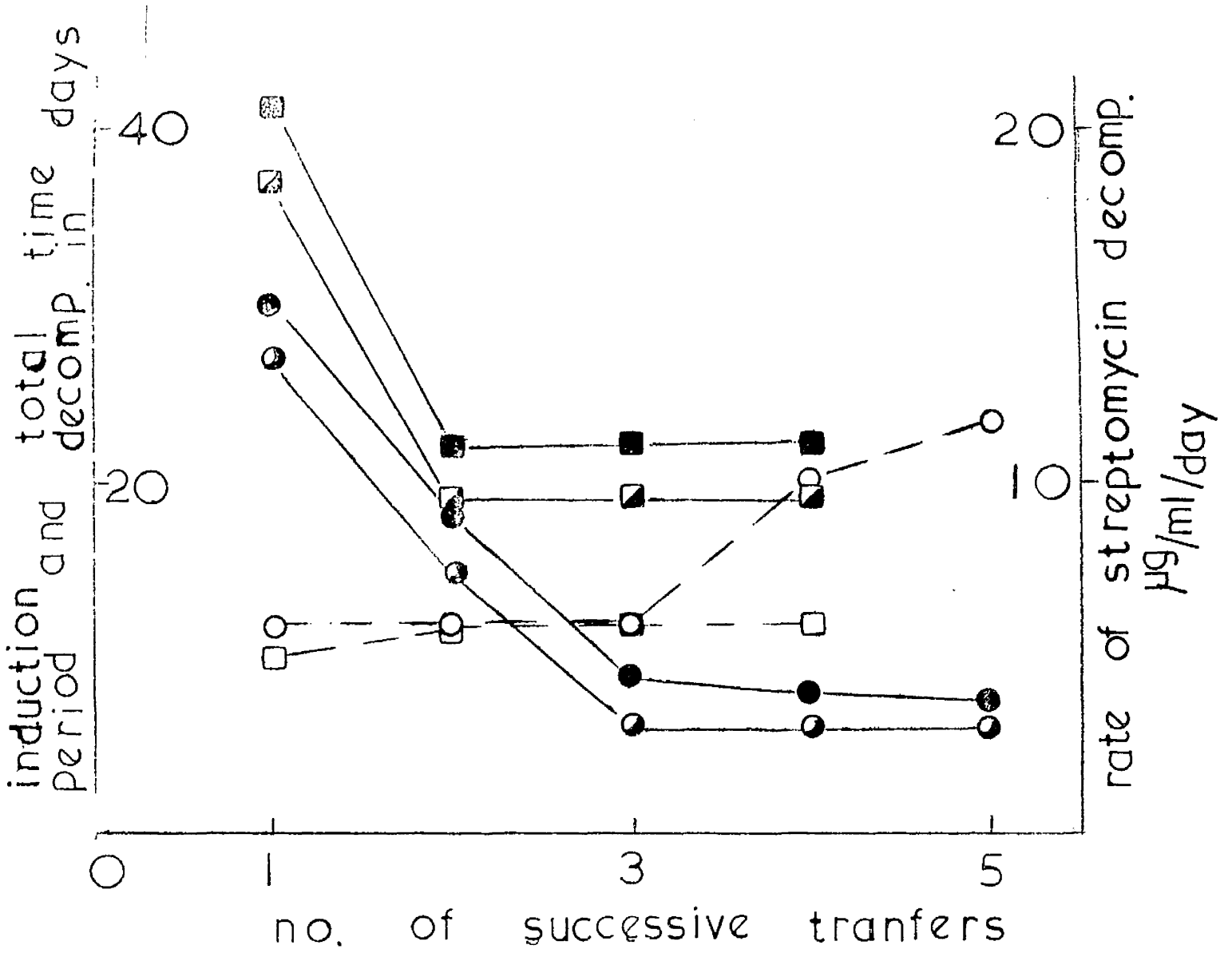
The results of sub-culturing show that (Tables 15 & 16 - Fig.4) rapid decomposition of streptomycin occurred in both media. In sub-cultures from 'BSM' medium free streptomycin remained constant for 19 days and all of it was decomposed in 22 days. The odour was noticed as in the inital culture on the 7th day. In 'SSM' medium there was an induction period of 17 days in which initial streptomycin content did not decrease, and between 18-21 days the streptomycin decomposition was complete. The odour was apparent on the 17th day.

Acid methanol extract of bentonite also did not show the presence of any streptomycin.

Also successive sub-culturing in the 'BSM' medium did not change the decomposition pattern (Table 15. Fig.4).

The interpretation of the results obtained in the experiments b & c could be that in the BSM medium streptomycin decomposition began in each culture after a short induction period of 6-7 days but the release of streptomycin from the bentonite maintained the concentration of free streptomycin in the medium.

Therefore the total decomposition of streptomycin took place in 45 days as compared to 30 days in 'SSM' medium. The amine-like odour associated with biological decomposition



	GSM medium	BSM medium
Rate of Decomposition	○	□
Induction period	◒	◪
Total decomposition time	●	■

Fig 4: The effect of successive transfers on streptomycin decomposition in BSM and GSM medium.

TABLE 15

The disappearance of free streptomycin in bentonite-synthetic medium (BSM) in successive transfers.

Sample number	Time for complete decomposition of Streptomycin (in days).			
	Primary Culture	Transfer number 1.	2.	3.
1/1	40	22	22	22
1/15	40	22	22	22
2/1	40	22	22	22
2/10	40	22	22	22
3/1	39	21	21	21
4/1	39	21	21	21
5/1	39	21	21	21

Control - no Decomposition

TABLE 16

The change in the streptomycin content in streptomycin-synthetic medium in successive transfers.

Sample number	Time for complete decomposition of Streptomycin (in days).			
	Primary Culture	Transfer number 1.	2.	3.
1/1	30	18	9	8
1/15	30	18	9	8
2/1	30	18	9	7
2/10	31	18	9	8
3/1	30	17	7	6-7
4/1	30	17	7	6-7
5/1	29	17	7	6-7

Control - no Decomposition

was noticed on the 7th day in 'BSM' medium which also suggest the proposed mechanism. Furthermore, no streptomycin was detected in the extract of bentonite which shows that all the streptomycin originally added (1000 µg/ml) had been decomposed.

To shorten the duration of experiments and as the presence of bentonite did not help in shortening the induction period or increasing the rate of streptomycin decomposition, further studies were carried out only in the synthetic medium containing streptomycin only, and no bentonite.

(d) The effect of successive transfers of culture in media with increasing concentration of streptomycin on the decomposition of streptomycin.

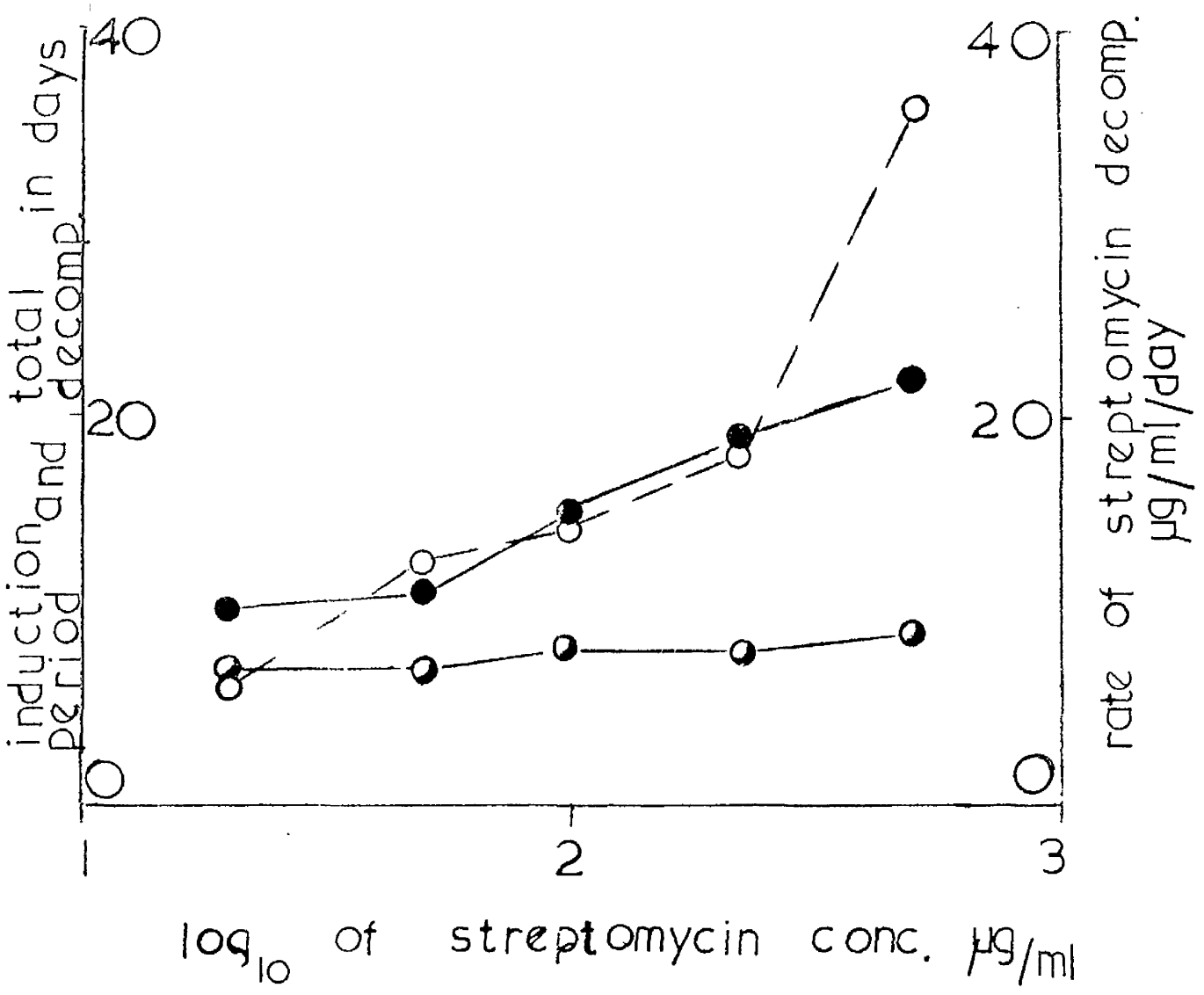
In the above experiment by serial transfers of the culture to the synthetic medium with the same dose level i.e. 20 µg/ml (SSM medium) shortening of the induction period was obtained. Experiments were therefore performed with synthetic medium containing different concentrations of streptomycin and their effect on the decomposition was studied.

For this study, duplicate flasks containing 49 ml of sterile synthetic medium with 20 µg of streptomycin/ml (SSM medium) were inoculated with 1.0 ml of each of 7 samples, taken from the first transfer from the above

experiment (see Table 16). Inoculated media were incubated along with uninoculated (control) media, and the course of streptomycin decomposition was followed by means of biological assays. As soon as the total amount of streptomycin had disappeared from the medium, transfers of 1 ml samples were made from each of the 7 pairs of flasks into flasks containing 49 ml of synthetic medium with 50 µg of streptomycin-ml and the course of streptomycin decomposition was followed. The process was repeated, the successive initial concentrations being 100, 200 and 500 µg of streptomycin/ml.

Parallel experiments were performed with flasks containing 49 ml of synthetic medium with 100 µg of streptomycin/ml and inoculated (1 ml) with the original mixed cultures (Scheme - Table 12) directly extracted from the soil. The culture media were examined in the usual manner together with uninoculated control media, during incubation for streptomycin decomposition.

From the results (Table 17 Fig.5) it can be seen that the concentration of streptomycin did affect the pattern of streptomycin decomposition. While the induction period remained almost the same in each case, the subsequent rate of streptomycin decomposition increased with increase in streptomycin content in the medium. The results show that there was gradual adaptation of the organism to streptomycin decomposition.



○ Rate of decomposition

◑ Induction period

● Total decomposition time

Fig 5: The effect of streptomycin concentration on the course of its decomposition in subsequent transfer by mixed culture.

In the synthetic medium containing 50, 100 µg of streptomycin/ml and inoculated directly with the original mixed culture (Soil extracted), streptomycin decomposition did not take place. This also suggested that ability to decompose higher concentration of streptomycin was developed by gradual adaptation of the micro-organisms involved.

TABLE 17

The effect of streptomycin concentration on the course of decomposition in subsequent transfers.

Sample number	Induction period (I) and time for total decomposition of streptomycin (in days) at streptomycin concentration (µg/ml).									
	20		50		100		200		500	
	I	T	I	T	I	T	I	T	I	T
1/1	7	10	7	11	8	15	8	19	8	22
1/15	7	10	7	11	8	15	8	19	8	22
2/1	7	10	7	11	8	15	8	19	8	22
2/10	7	10	7	11	8	15	8	19	8	22
3/1	7	10	7	11	8	15	8	19	8	22
4/1	7	10	7	11	8	15	8	19	8	22
5/1	7	10	7	11	8	15	8	19	8	22

Control - no Decomposition

SECTION II

Isolation, Identification and Characterization of Streptomycin decomposing organisms.

The Isolation of Streptomycin-decomposition Organisms.

Samples from all the mixed cultures which displayed maximal activity of streptomycin decomposition upon successive addition of the antibiotic were plated out on nutrient agar containing 1000 µg of streptomycin/ml and the plates were incubated at 25°C for a month. The colonies which developed were isolated and the cultures were further purified by streaking on nutrient agar containing streptomycin. The isolates were maintained on nutrient agar slants containing 1000 µg of streptomycin/ml, sixty two colonies were thus isolated.

These cultures were then inoculated in duplicate into a synthetic medium containing 20 µg of streptomycin/ml and incubated. By repeated biological and chemical assays their ability to decompose streptomycin was studied. Twelve cultures, out of 62 showed complete decomposition of streptomycin in the synthetic medium, the time for total decomposition ranging from 31 to 45 days. There were periods of no measurable streptomycin decomposition of 27 to 41 days (induction period), the phase of streptomycin destruction lasting in each case four days, (See table). The remaining cultures were inactive. The 12 cultures were

once again tested for purity by repeated streaking on nutrient agar as above.

From these 12 isolates an active culture, number "38" was selected for further studies. Also the detailed characterization of the isolate was carried out.
The reason for selecting this culture were:
i) The bacterium was one of the most active ones, decomposing streptomycin (20 µg/ml) in the synthetic medium in 31 days, with an induction period of 27 days. Also (ii) the colonies of this organism were noticed in greater number in culture samples isolated from the mixed cultures (scheme Table 12). So it was thought that the organism plays an active role in streptomycin decomposition in the mixed culture, and therefore, probably also in the soil under natural conditions.

Identification and Characterization of Streptomycin-decomposing isolate No. 38.

(a) Morphology and Staining reactions:

Cells from nutrient broth and nutrient agar cultures were straight to angular or slightly curved pleomorphic rods, usually banded or beaded with metachromatic granules and occasionally clubbed, branched or coccoid cells depending upon the age and conditions of the sub-cultures. Gram-variable, sometimes young and sometimes old cells lose the stain easily resulting in cells with irregular stained segments,

with granules invariably gram-positive. The size of cells ranged from 0.4 - 0.5 u wide by 1.2 - 2.5 u long. The pleomorphism was more pronounced on solid media.

Cells were non-sporing, non-capsulated and non-acid fast. Culture obtained from potassium-tellurite medium showed marked granulation. When grown on peptone-agar containing carbohydrates, the storage of fatty material was observed by Burdon's (1946) technique. Cells were feebly motile as examined by hanging-drop preparation, whereas flagella staining technique failed to show the presence of any flagella.

(b) Growth Characters:

In Liquid media.

In nutrient broth and yeast-extract broth (YE broth) there was a steadily increasing turbidity with a slow accumulation of sticky cream-coloured sediment. In nutrient broth with different dilutions (to have different concentration of lab-lemco), the growth was visible and luxurient within 24 hours at dilutions 1:1, 1:2, 1:3, and within 48-72 hours at dilutions 1:4 and 1:5.

In synthetic medium the organism grew in such a way as not to give the measurable turbidity in the medium. The growth, in the synthetic medium was therefore measured (studied) by plate-count technique.

On solid media.

White, smooth, glistening, opaque, with entire margin, and butyrous growth was obtained on nutrient agar within 20-24 hours. On nutrient agar diluted to have different concentration of Lab-lemco, growth was obtained within 20-24 hours with dilutions 1:1, 1:2, 1:3 and in 48-72 hours with dilutions 1:4 and 1:5.

On synthetic medium-agar growth obtained was similar to that obtained on nutrient agar.

When the culture was streaked on MacConkey's agar white colonies were obtained, the colonies revealed the morphology, staining and biochemical reactions, similar to those obtained with the parent culture. This suggest that the organism can grow in the presence of bile-salt. Also, blue coloured colonies obtained on Eosin-methylene-blue agar (E.M.B.agar) show the capability of organism to grow in the presence of bile-salt. On Bismuth sulphite agar (Wilson and Blair's) medium, brown growth was obtained which suggest that organism can grow in the presence of bismuth sulphite. No haemolysis was observed on blood (horse) agar plates. Starch was hydrolysed in 24 hours at temperatures 25° and 30°C whereas it was hydrolysed in 48 hours at 15°C. Brown to black colonies were obtained on potassium-tellurite medium, bulture obtained from this medium showed marked granulation. On potato slants cream

coloured growth appeared within 2-3 days.

(c) Physiological characteristics:

Aeration:-

Surface growth was obtained when culture was added to melted nutrient agar (cooled to 45°c) and incubated. Also growth studied in thioglycolate medium showed pellicle formation. Furthermore growth was obtained in inoculated nutrient broth and nutrient agar media when incubated anaerobically in the presence of atmosphere of H_2. These results suggest that the organism is aerobic, facultatively anaerobic.

Temperature:-

The organism can grow at a wide range of temperature ranging from 10°C to 37°C as observed by growth in nutrient broth and nutrient agar inoculated and incubated at temperatures 10°, 15°, 25°, 30°, 33.5°, 37° and 55°C. Luxurient growth was obtained at temperatures 25°, 30° and 33.5°C, within 12-24 hours, whereas at temperatures 10° and 15°C, less growth was observed in 36-48 hours. At 37°C feeble growth was obtained in 24 hours, and no growth was obtained at 55°C. This shows that the optimum temperature for the growth of organism is between 25°C and 33.5°C.

pH value of the medium:-

Better growth was obtained at pH 7 as compared to that

of the growth obtained at pH 6 and 8 which was similar. No growth appeared at pH 3, 4 or 5.

(d) Biochemical Reactions:

Acid was produced in the peptone water containing: Arabinose (0.5%), rhamnose (0.25%), xylose (0.5%), glucose (1.0%), fructose (1.0%), mannose (0.5%), sucrose (1.0%), maltose (1.0%), melibiose (0.25%), raffinose (0.25%), dextrin (1.0%), glycerol (1.0%), salicin (1.0%) and -methyl glucoside (0.5%), whereas no acid was produced form mannitol (1.0%) and dulcitol (1.0%).

M.R and V.P tests were negative. Whereas Indole was formed. Nitrate was reduced to nitrite in 72 hours in peptone water containing 1% of KNo_3; scant production of ammonia was observed. Cultures obtained from nutrient agar and nutrient broth were catalase positive. Urea was hydrolysed as observed by purple pink colour of the Christensen's medium, and also there was growth in Koser's Uric acid medium. Citrate was utilized as observed by growth in Koser's Citrate agar. H_2S was not formed. Starch and gelatin were hydrolysed at all the three temperatures used: 15°, 25° and $37^{\circ}C$, though the process was very slow at $15^{\circ}C$. No haemolysis was observed on the blood agar plates. No growth was obtained on the Dorset's egg medium, whereas hydrolysis of serum slopes was observed.

Cellulose test:-

i) Filter paper strip method:

Filter paper strip in 0.5% peptone broth was weakened (to pulpy mass) so that on slight agitation, of the culture tube, fibres started seperating from the strip.

This shows that the organism can attack cellulose.

ii) C.M.C. method:

Further proof of the ability of organism to decompose cellulose was obtained by its ability to decompose synthetic form of cellulose-Carboxy-methyl-cellulose (C.M.C.). Cell-free filtrate of the peptone culture was able to hydrolyse 10% of the C.M.C. within one hour incubation at 37°C, as observed by calorimetric estimation of reducing sugar (see details of these two methods in the Method section).

From all the above results it seems that the organism-isolate No.38 is a member of Genus Cellulomonas namely Cellulomonas fimi. This genus Cellulomonas is in turn a member of Family Corynebacteriaceae.

The detailed discussion about the identification of the organism as Cellolomonas fimi is given in the Discussion Chapter.

TABLE 18

Biochemical fermentations and other growth Characterization of Isolate "38".

Test & Media	Biochemical fermentation and growth characterization by bacterium at temperatures.		
	15°C	25°C	37°C
1) Peptone water containing carbohydrates			
i) Monosaccharides:			
Arabinose (0.5%)	+	+	+
Rhamnose (0.25%)	+	+	+
Xylose (0.5%)	+	+	+
Glucose (1.0%)	+	+	+
Fructose (1.0%)	+	+	+
Galactose	+	+	+
Mannose	+	+	+
ii) Disaccharides:			
Sucrose (1.0%)	+	+	+
Lactose (1.0%)	-	-	-
Maltose (1.0%)	+	+	+
Treholse (0.25%)	-	-	-
Melibiose (0.25%)	±	+	+
Cellibose (0.5%)	-	-	-
iii) Polysaccharides:			
Raffinose (0.5%)	±	+	+
Inulin (1.0%)	-	-	-
Dextrin	-	+	+
Glycogen (0.25%)	-	-	-
iv) Alcohols			
Glycerol	-	+	+
Erythritol	-	-	-
Adinitol	-	-	-

Table 18 - contd.

Test & Media	15°C	25°C	37°C
Mannitol	-	-	-
Sorbitol	-	-	-
Dulcitol	-	-	-
v) Glucosides:			
Salicin (1.0%)	-	+	+
Aesculin (0.25%)	-	-	-
-methyl glucoside(0.5%)	+	+	+
2) Nitrate Broth	+	+	+
3) Methyl Red Test	-ve	-ve	-ve
4) Voges-Proskauer test.	-ve	-ve	-ve
5) Indole Production.	+ve	+ve	+ve
6) Urea hydrolysis test.			
i) Christen's medium	Alkali Prod.	Alkali Prod.	Alkali Prod.
ii) Koser's Uric Acid.	Growth	growth	growth
7) Koser's citrate medium	growth	growth	growth
8) H_2S Production:			
i) Tryptone Iron agar	-ve	-ve	-ve
ii) Pb-acetate agar	-ve	-ve	-ve
9) Litmus milk	-ve	-ve	-ve
10) Dorset's egg slops	no growth	no growth	no growth
11) Serum hydrolysis	+ve	+ve	+ve
12) Gelatin tequefaction			
13) Starch hydrolysis			
14) Rotation to O_2			
i) N. agar(culture added to melted cool agar)	surface growth	surface growth	surface growth
ii) Thioglycollate medium	Pellicle growth	Pellicle growth	Pellicle growth
15) MacConkey's agar	White Colonies	White Colonies	White Colonies

Table 18 - contd.

Test & Media	15°C	25°C	37°C
16) E.M.B. Medium	Blue Colonies	Blue Colonies	Blue Colonies
17) Wilson & Blair's medium	Brown Colonies	Brown Colonies	Brown Colonies (feeble growth)
18) Blood agar plates.	No haemolysis	No haemolysis	No haemolysis

\+ - Acid production - - No acid & gas production.

\+ Acid and gas production.

+ve - Positive test.

-ve - Negative test.

SECTION III

The Effect of environmental factors on the growth of cellulomonas.

I. Growth characteristics in a Synthetic-medium containing 20 ug of Streptomycin/ml.

Because most of the studies regarding streptomycin decomposition were carried out in a synthetic medium containing streptomycin as sole source of carbon and nitrogen for growth and energy (SSM medium) it was necessary, first, to study the growth characteristics of the bacterium in that

medium. The following experiment was therefore performed to study growth of the organism, in the SSM medium.

Fifty ml portions of the synthetic medium containing 20 µg of streptomycin/ml (SSM medium) were inoculated with a suspension of washed cells from 48 hour culture as described under Methods. The culture media were well shaken, 1-ml samples withdrawn from each flask by means of a sterile pipette to give zero time data, and the remainder was incubated at 25°C. Serial tenfold dilutions of the samples were used for plate counts at zero time and at suitable intervals. Enough samples were withdrawn in order to form a complete growth curve.

The growth of the organism in SSM medium is shown in Table 19 in Fig.6. It can be seen that there was no lag phase in this medium; organisms began multiplying first at a higher rate of growth which slowed down later on and maximum growth being obtained after 12 hours. There was no significant increase in the Viable Counts of the organisms thereafter.

As streptomycin is the sole source of carbon and nitrogen in the SSM medium, and there was sixty fold increase in the Viable counts of the organism, it was assumed <u>at this stage</u> that the organisms were growing at the expense of streptomycin. But as will be shown later there was no decrease in the concentration of streptomycin from the medium at that time.

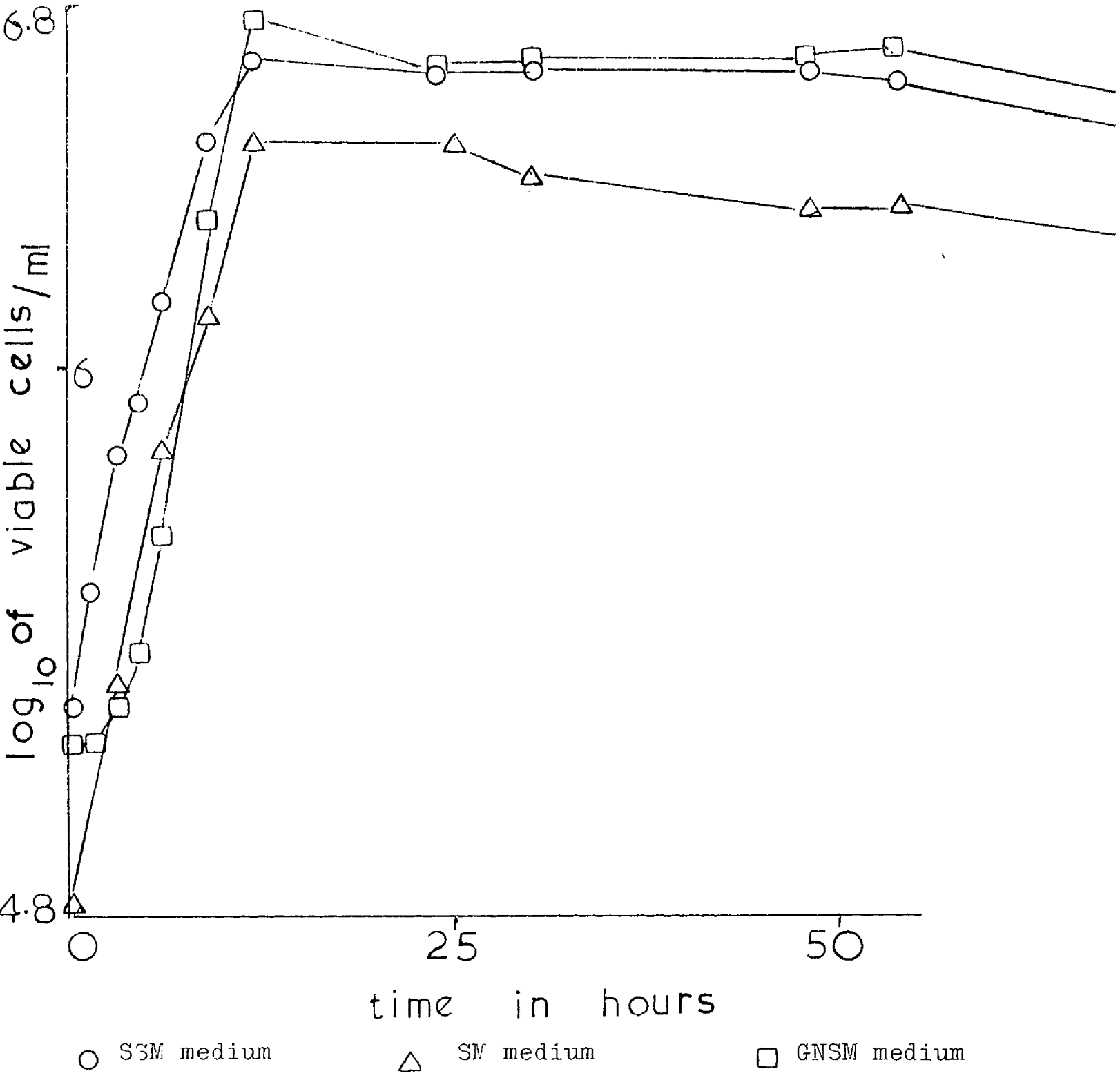


Fig 6: Growth of the organism in synthetic medium(SM); containing streptomycin(SSM); and containing glucose+ammonium sulphate (GNSM).

TABLE 19

Viable Count of Cellulomonas (strain No.38) in the Synthetic media.

Incubation period in Hours.	Log_{10} of Viable cells/ml of medium	
	Streptomycin medium (SSM medium)	Glucose medium (GNSM medium)
0	5.2543	5.1654
1½	5.5109	5.168
3	5.80	5.250
4½	5.92	5.372
6	6.135	5.63
9	6.490	6.32
12	6.67	6.76
24	6.65	6.65
36	6.65	6.71
48	6.655	6.67
54	6.62	6.70
72	6.50	6.57
96	6.49	6.51

II Effect of Sources of Carbon and nitrogen on growth.

(a) Supplementing different carbon and nitrogen sources in the medium.

It has been shown by various workers that the nature and concentrations of sources of carbon and nitrogen in the medium, play great role on the growth and on the activity of micro-organisms. (Feniksova 1957, Shu & Blackwood 1951, Tikhomirova 1954, Steiner 1960, Asia et al 1951, Ono &

Kanbayashi 1954, Roy 1955, Smirnova 1916, 1917 etc.).

The experiment was therefore conducted to find the capability of bacterium to utilize sources of carbon and nitrogen, other than streptomycin. For this purpose 50 ml portions of SSM medium were inoculated as in the previous experiment. Another variant was tested in which the medium GNSM was inoculated in a similar way with approximately the same size of inoculum. (Former medium contains streptomycin as sole source of carbon and nitrogen; the latter contains glucose and ammonium sulphate as sources of carbon and nitrogen respectively). During incubation at 25°C, 1 ml samples were withdrawn from the culture flasks at a suitable interval together with zero time, and growth was estimated in the usual manner by plate counts.

The results with regard to formation of viable cells are shown in Table 19 and Fig.6, which show that there was an initial lag phase of 2 hours in GNSM medium as compared to no lag phase in the control - SSM - medium. The organism then began to multiply first at a lower rate of growth up to 6 hours, followed by a higher rate of growth, and the maximum viable cell content was obtained after 12 hours. The rate of growth in the exponential phase, was higher in GNSM medium as compared to that of SSM medium. The maximum cell content was almost the same in both the media and was obtained in the same interval of time. There was no decrease in viable cell numbers thereafter.

The results show that different sources of carbon and nitrogen did not affect to a great extent the growth characteristic of the organism in the synthetic medium.

(b) Concentration of Sources of Carbon and Nitrogen in the medium

Concentrations of sources of carbon and nitrogen, and even together different concentrations of ingredients of the substrate, influence the growth of micro-organisms.

To study this phenomenon, a set of experiments with three different concentrations of streptomycin, glucose and ammonium sulphate in synthetic media was performed.

Forty eight ml portions of basal-salts synthetic medium (SM medium) were pipetted into 12 sterile 150 ml flasks and divided into two sets. To each of two flasks in the first set sterile streptomycin solution was added so as to give concentrations of 20, 100 and 400 µg/ml of the medium respectively in a final volume of 50 ml. Similarly duplicate flasks in the other set were supplemented with glucose so as to give 20, 100 and 400 µg/ml of medium, and the ammonium and sulphate concentrations were adjusted to 5, 25, 100 µg/ml respectively. Media were inoculated and were incubated at 25°C.

The viable counts at various stages of the culture development are shown in Table 20 and Fig.7, from which it can be seen that the maximum yields of cells obtained in

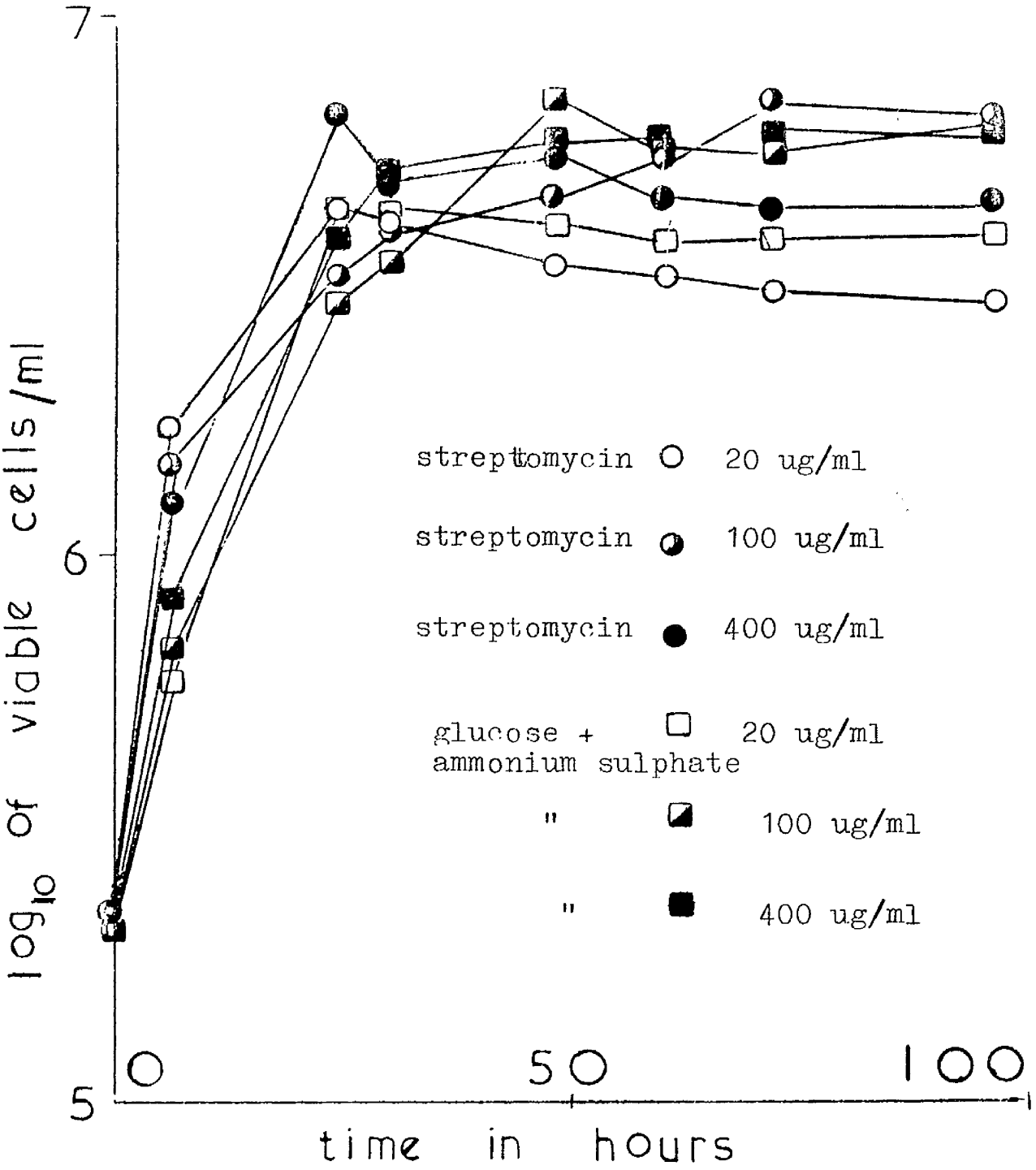


Fig 7: growth of organism in synthetic medium containing higher concentrations of carbon & nitrogen sources.

the synthetic medium with higher concentrations of streptomycin (100 or 400 µg/ml) was slightly higher than with the low concentrations of streptomycin (20 µg/ml) in the same medium. There was no noticeable influence on growth by a supplimentation on the substrate with various concentrations of glucose and ammonium sulphate.

TABLE 20

Growth of Isolate 38 in synthetic-media containing various concentrations of streptomycin, glucose and ammonium sulphate.

Incubation period in hours.	Log_{10} of Viable cells/ml in Synthetic medium contg. Streptomycin			Glucose & Ammonium Sulph.		
	20µg/ml	100µg/ml	400µg/ml	20µg/ml 5µg/ml	100µg/ml 25µg/ml	400µg/ml 100µg/ml
0	5.34	5.34	5.34	5.32	5.32	5.32
6	6.24	6.168	6.10	5.78	5.83	5.92
24	6.65	6.53	6.82	6.65	6.48	6.59
30	6.62	6.60	6.70	6.63	6.55	6.71
48	6.55	6.67	6.75	6.62	6.85	6.73
60	6.53	6.74	6.67	6.59	6.74	6.76
72	6.50	6.85	6.67	6.60	6.76	6.79
96	6.48	6.83	6.66	6.61	6.83	6.81
102	6.48	6.83	6.65	6.58	6.81	6.80

(c) Additional carbon and nitrogen sources in the medium.

As for different concentrations of sources of carbon and nitrogen, additional sources of different carbon and nitrogen also affect the microbial growth. To study this effect experiments were performed with synthetic-medium to which

additional sources of carbon and nitrogen were added. They were as follows:

Medium 'SSM' - To basal-Salts-Synthetic medium (SM medium) Streptomycin (20 µg/ml) was added as sole source of carbon and nitrogen.

Medium SSM_1 - To 'SSM' medium, glucose (20 µg/ml) was added as additional carbon source.

Medium SSM_2 - 'SSM' medium containing glucose (20µg/ml) and ammonium Sulphate (5µg/ml) as additional sources of carbon and nitrogen respectively.

Medium 'GNSM' - Similarly to medium 'GNSM' streptomycin (20 µg/ml) was added.

Flasks containing 50 ml portions of SSM, SSM_1, SSM_2, GNSM, were inoculated with the same sizes of inocula from respective media and flasks were incubated at 25°C. Development of culture in media during incubation was estimated by plate counts in usual way.

As can be seen from the results obtained (Table 21 and Fig.8) it is clear that the addition of sources of carbon and nitrogen to the medium did not affect the growth. Total growth obtained in SSM_1 and SSM_2 media was similar to the growth in control 'SSM' in 'GNSM' media.

From the results obtained in the above three sets of experiments, it can be seen that different sources of carbon and nitrogen (Exp.(i)), different concentrations of these

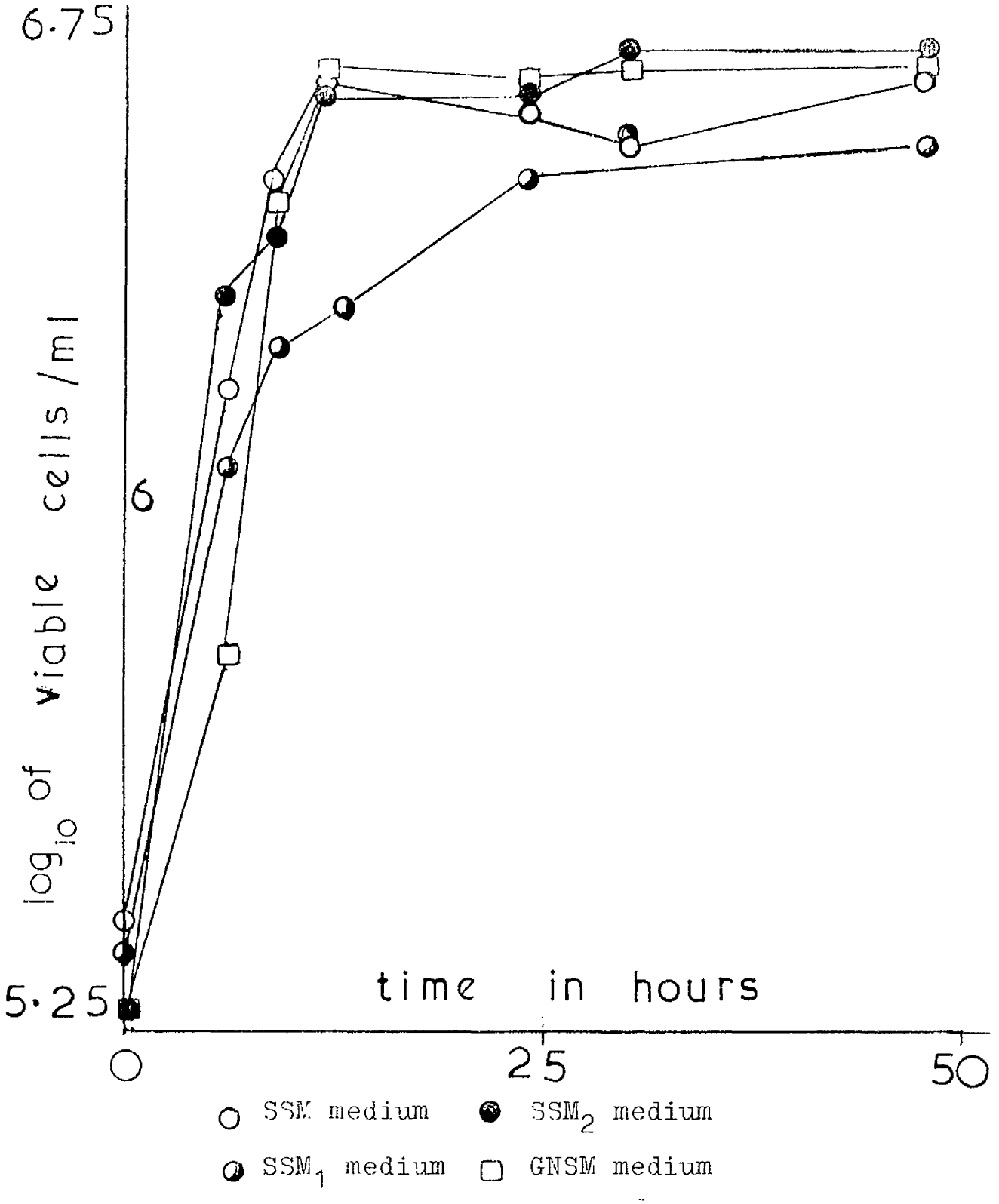


Fig. 8: Growth of organism in SSM and Gnsm media with additional carbon & nitrogen sources.

TABLE 21

Growth of organism in 'SSM' and 'GNSM media with additional Carbon and nitrogen sources.

Incubation period in	Log_{10} of Viable Cells/ml in media.			
	SSM	SSM Glucose (20µg/ml)	SSM Gluc.(20µg/ml), Ammon.Sulph. (5µg/ml)	
Hours	SSM	SSM_1	SSM_2	GNSM
0	5.336	5.307	5.233	5.233
6	6.168	6.05	6.313	5.78
9	6.48	6.22	6.30	6.45
12	6.63	6.293	6.62	6.65
24	6.579	6.496	6.64	6.63
30	6.54	6.552	6.657	6.65
48	6.637	6.541	6.693	6.63
72	6.255	6.170	6.48	6.25

(exp.(ii), and additional sources of carbon and nitrogen (exp.(iii) in the basal-salts synthetic medium, did not affect the growth of organisms, and the maximum growth obtained remained almost the same in all the media.. It was thought to be useful to consider another factor, which is known to effect the growth and the maximum yield of the micro-organisms in certain cases, i.e. size of inoculum.

The experiment is described below.

III Effect of Size of inoculum on the growth of organisms.

It has been shown by various workers that the size of inoculum is one of the major factors to be considered in any kind of microbial study. It affects the growth rate and maximum yield of cell material in Aperfillus (McIntosh, A.F. and Meyrath, J, 1963, 1964; Meyrath, J. 1962, 1963, 1964; and Meyrath J. and McIntosh A.F. 1963), Clavicaps (Taber, W.A. 1957), Mucor (Bartnicki-Gureias and Nickerson, W.J. 1962), Hirsutell (MacLeod, D.M. 1959, 1959a) and Saccharomyces (Wiken, T; Som, H; and Sulzer F. 1953). This factor also affects the carbon metabolism (Meyrath J, 1963; Steele et al, 1954), nitrogen metabolism (McIntosh A.F. and Meyrath J. 1963) and the respiratory activity (Meyrath, J and McIntosh, A.F. 1963) of micro-organisms. It also affects the fermentations, such as alcoholic fermentation in saccharomycetes (Pfenning, N, & Wiken, T 1960) and alkaloid production in clavialps (Taber W.S. & Vining, L.C. 1958). The production of enzymes, such as Amalyse, is also influenced (Meyrath, J. 1965). Few examples are available in case of bacteria which show the effect of size of inoculum on the growth and activity of bacterium (Parker R.F. 1946; Hinshelwood C.N. 1946); Nakamura M.L. and B.L. Pitsch, 1961, and Allen W.L. and J. McVergh, 1963).

To study this phenomenon a set of experiments was

performed with three different sizes of inocula.

50 ml quantities of 'SSM' medium and 'GNSM' medium were inoculated with "large inoculum", (i.e. 1.5×10^7 cells) per 50 ml of substrate, medium inoculum (i.e. 4×10^3 cells per 50 ml) or 'small inoculum' (i.e. 6×10^1 cells per 50 ml). All the inoculated culture flasks together with uninoculated control medium flasks, were incubated at 25°C on a mechanical shaker. Growth was estimated by plate counts in usual manner.

Table 22 and Fig. 9 show that the size of inoculum did affect the growth of the organisms to a certain extent. With large inoculum there was no lag phase and maximum growth was obtained in 10 hours interval. In the case of the medium inoculum, however there was a lag period of 2-3 hours in the beginning which was followed by a higher rate of growth and maximum growth was obtained within 24-48 hours. But with the small inoculum measurable growth was only obtained 10 hours after the inoculation after which a higher rate of growth occurred which attained maximum after 53-58 hours time interval. However, in all the cases, maximum growth obtained was similar to that obtained in previous experiments. Thus size of inoculum did not affect the maximum yield.

As mentioned earlier, up to now, in all the above experiments inspite of increase in cell number under different

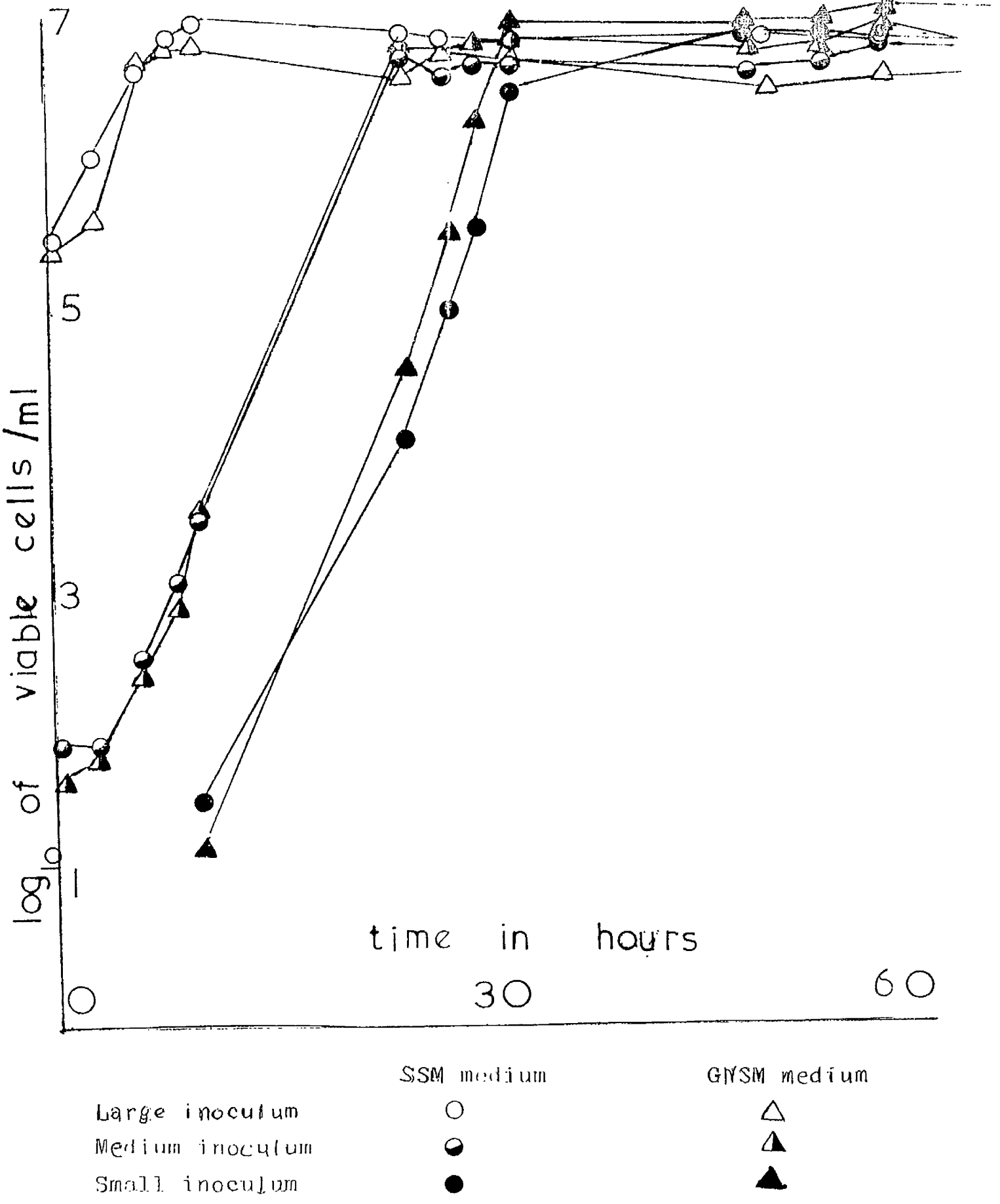


Fig. 9:- Effect of size of inoculum on the growth of organism.

TABLE 22

Growth of organism in 'SSM' and 'GNSM' medium with different sizes of inocula.

Incubation Period in	Log_{10} of Viable cells/ml Streptomycin substrate			Log_{10} of Viable cell/ml Glucose Substrate		
Hrs. Mins.	Large Inoc.	Medium Inoculum	Small Inoculum	Large Inoc.	Medium Inoculum	Small Inoculum
0	5.42262	1.9490		5.44191	1.6983	
3	6.01493	1.9396		5.57020	1.8354	
6	6.62008	2.6719		6.62127	2.4133	
8-30	6.81508	3.0834		6.78189	2.87142	
10	6.90694	3.50133	1.5422	6.76276	3.35349	1.2031
24-20	6.88649	6.70078	4.08934	6.5376	6.75679	4.51030
27-5	6.83570	6.59176	4.93600	6.71236	6.74833	5.48040
29-15	-	6.61119	5.595	-	6.76465	6.26318
32	6.82456	6.634	6.48806	6.62051	6.79865	6.87093
48-5	-	6.58070	6.85636	-	6.71977	6.83251
49-35	6.83410	-	-	6.46979	-	-
53-5	-	6.61508	6.84813	-	6.79118	6.93591
57-30	6.86308	6.70753	6.75587	6.50910	6.88927	6.99674
72	6.76663	6.70140	6.72962	6.52673	6.58095	6.93543
73-30						
77	-	6.76938	6.65839	-	6.67182	6.95595
99-10	6.75587	6.55204	6.78733	6.60958	6.67062	6.92428

conditions, there was no decrease in the streptomycin concentration from the medium. The following experiments were therefore performed to obtain more data in this direction.

IV Growth of organisms in a Synthetic medium to which no sources of carbon and nitrogen have been added.

Since in the previous experiments inspite of using higher concentrations of carbon and nitrogen sources or with additional carbon and nitrogen sources in 'SSM' and 'GNSM' medium, the results obtained were almost similar to each other. Maximum growth obtained did not exceed $5 \times 10^6 - 10^7$ cells/ml of medium. Experiments were therefore performed with the synthetic medium (SM medium) which does not contain added sources of carbon or nitrogen.

Flasks containing 50 ml quantities of synthetic medium (SM medium) were inoculated with washed cell suspension of 48 hours growth in 'SSM' medium. For comparison flasks containing 50 ml volumes of 'SSM', GNSM medium were inoculated in similar way with culture from respective medium. Samples for zero time data were withdrawn from each culture media and the remaining ones incubated. At suitable time intervals parallel samples from all the three culture media were withdrawn and growth estimated.

The results of viable counts show (Table 23. Fig.6) that the growth obtained in synthetic medium (SM medium) was similar to growth in streptomycin-synthetic-medium (SSM) except that in the former the peak of viable count was slightly lower than the later.

TABLE 23

Growth of Organisms in Synthetic-Medium (no added sources of Carbon and Nitrogen).

Incubation period in hours.	Log_{10} of viable cells/ml of medium 'SM' medium
0	4.8112
3	5.30
6	5.81
9	6.1
12	6.49
24	6.49
30	6.44
48	6.336
54	6.35
96	6.20

V <u>The effect of serial sub-culturing in synthetic medium on growth</u>

In the above experiment synthetic medium (SM medium), in

which hundred fold growth was obtained, was inoculated with washed cell suspension of 48 hours old growth in streptomycin-synthetic-medium (SSM medium). It was thought that the impurities which could have been present in the 'SSM' medium may have been transferred to synthetic medium with the inoculum. To study this phenomenon serial sub-culturing in the 'SM' medium after every 48 hours was carried out and growth was studied in subsequent sub-cultures by plate counts. Seven such sub-cultures were done.

The results obtained show that (Table 24) the same amount of growth was obtained in each sub-culturing.

The question then arises as to what is the possible source or sources of nutrient for the organism to multiply in synthetic medium, because a hundredfold growth was obtained in this medium. There are two possibilities.
(1) From the internal source i.e. present in the medium in the form of impurities (2) from the external source such as atmospheric carbon and nitrogen. Some experiments were performed in the hope of elucidating this problem.

V Sources of energy supply in the Synthetic medium

Internal Sources.

The impurities in synthetic medium (SM) could have come either from de-ionised water with which 'SM' medium was prepared or with ingredients (chemicals) composing 'SM' medium.

TABLE 24

The effect of Serial Sub-culturing in Synthetic Medium on growth of organism.

Sub Culture number	Log_{10} of viable cells/ml of synthetic medium	
	Inoculum	After 24 hours.
I	4.80	6.44
II	4.788	6.37
III	4.75	6.29
IV	4.81	6.27
V	4.91	6.17
VI	4.72	6.21
VII	4.80	6.20

In this study three sets of experiments (i,ii,iii) were performed as shown below.

(i) Experiment with 'SM' medium prepared in double glass distilled water

For this study experiments were performed in synthetic-medium (SM) prepared with double glass distilled water, against synthetic-medium (SM) prepared with de-ionised water.

The rest of the medium composition being the same for both media.

Parallel experiments were performed with both types of 'SM' media (mentioned above) in the usual manner by

inoculating 50 ml portions of media, incubating at 25°C and studying the growth by plate counts.

The results obtained show that the same growth was obtained in both media.

(ii) Experiment with 'SM' medium deficient in either of the salts constituents.

The other possibility through which impurities could have been incorporated in the medium was with the chemicals used for the preparation of synthetic-medium (SM medium). However as mentioned in the method sections all chemicals used were of 'Analar grade' except K_2HPO_4 (B.D.H. Laboratory reagent).

In this set of experiments 'SM' medium used in each case was deficient in either of its salts constituents. They were as follows:

(1) SM_1 Medium: 'SM' medium without K_2HPO_4

(2) Medium 'SM_2: 'SM' medium without $MgSO_4$ $7H_2O$

(3) Medium SM_3: 'SM' medium without $CaCl_2$

(4) Medium SM_4: 'SM' medium without Nacl.

(5) Medium SM_5: SM medium without $FeCl_3$

Fifty ml volumes of the media SM_1, SM_2, SM_3, SM_4, SM_5 and SM (as a control) were inoculated with the same size of inoculum of washed cell suspension of 48 hours grown culture

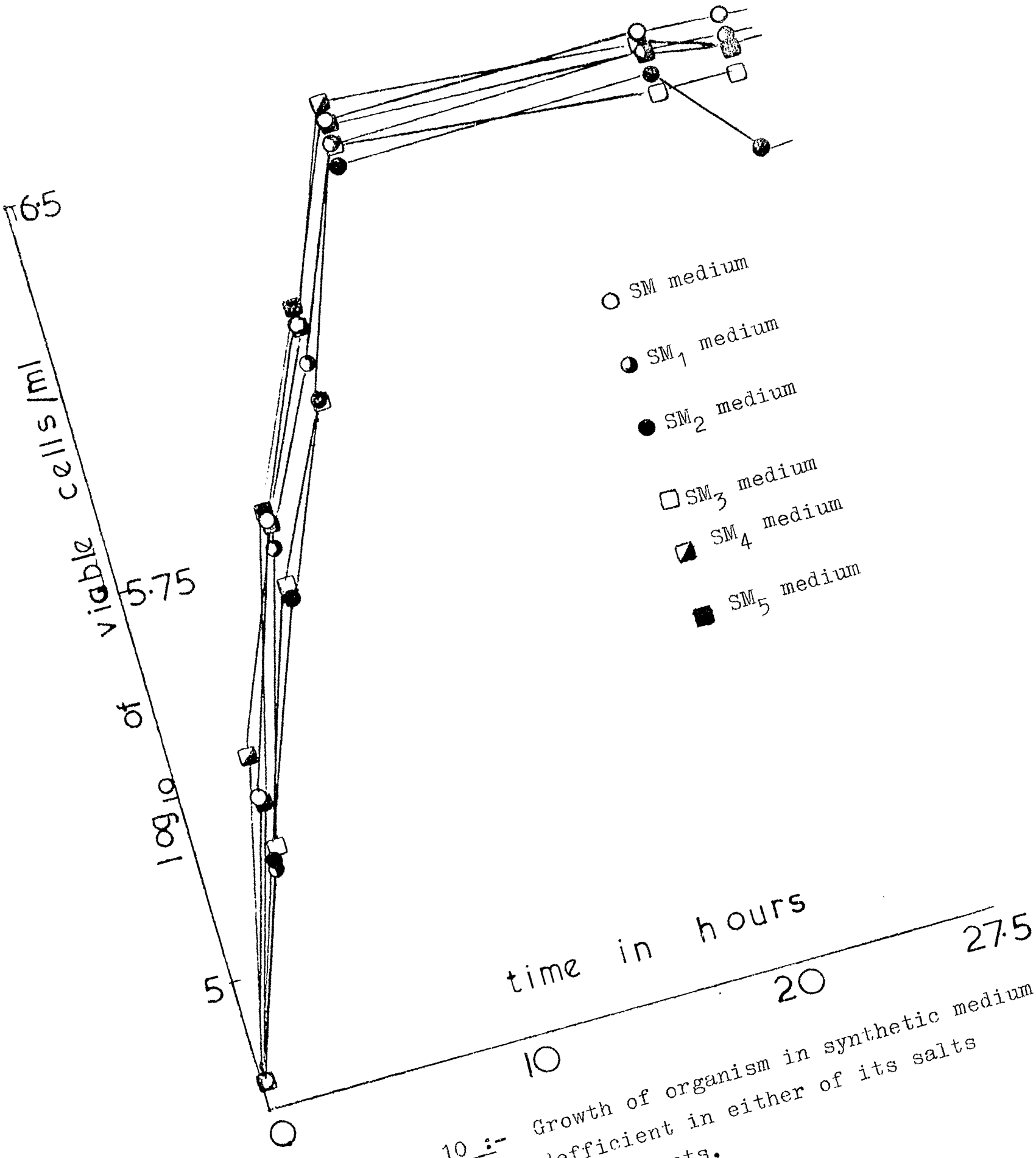


Fig. 10 :- Growth of organism in synthetic medium defficient in either of its salts constituents.

in 'SM' medium. During incubation 1 ml aliquot from each flask, including samples for zero time data, were withdrawn at suitable intervals of time and growth was estimated by plate counts.

The results obtained show (Table 25, Fig.10) that once again the same growth was obtained in all the media used in this set of experiments except in the medium SM_2 and SM_3 where maximum growth obtained was slightly less.

TABLE 25

Growth of Organism in Synthetic-Medium deficient in either of its salts constituents.

Incubation	Log_{10} of Viable cells/ml in media.					
in hours	Sm Control	SM_1	SM_2	SM_3	SM_4	SM_5
0	4.805	4.805	4.805	4.805	4.805	4.805
3	5.316	5.175	5.18	5.20	5.38	5.30
6	5.813	5.70	5.65	5.67	5.80	5.80
9	6.196	6.072	6.0	6.0	6.13	6.15
12	6.492	6.45	6.39	6.43	6.52	6.48
24	6.499	6.47	6.41	6.37	6.45	6.46
27	6.44	6.45	6.21	6.37	6.41	6.41
48	6.336	6.42	6.22	6.35	6.37	6.38

(iii). In the above experiment growth was obtained in each 'SM' medium deficient in one of its salt constituents.

Further experiments were therefore performed, this time in the media prepared with individual ingredients of 'SM' medium the pH of all media being adjusted to 7.2. The media were as follows:

Medium K: It was prepared by dissolving 1 gm of K_2HPO_4 in 1000 ml of de-ionised water.

Medium M: Contained $MgSO_4 . 7H_2O$ - 0.2 g and 1000 ml of de-ionised water.

Medium C: Contained $CaCl_2$ - 0.1 g and de-ionised water 1000 ml.

Medium N: Contained Nacl - 0.1 g and de-ionised water 1000 ml.

Medium F: Contained $FeCl_3$ - 0.002 g and de-ionised water 1000 ml.

The pH in all the media was 7 after sterilization.

50 ml portions of the media K,M,C,N,F and SM (control) were inoculated as in the previous experiment with the same size of inoculum, culture flasks incubated and growth studied in the usual manner by plate counts.

Results obtained were as shown in Table 26 & Fig.11. It can be seen that in media K,N and F organisms could survive but did not multiply, whereas in medium 'M' and medium C there was growth, although not to the same extent as in the synthetic-medium (SM). The results suggest that

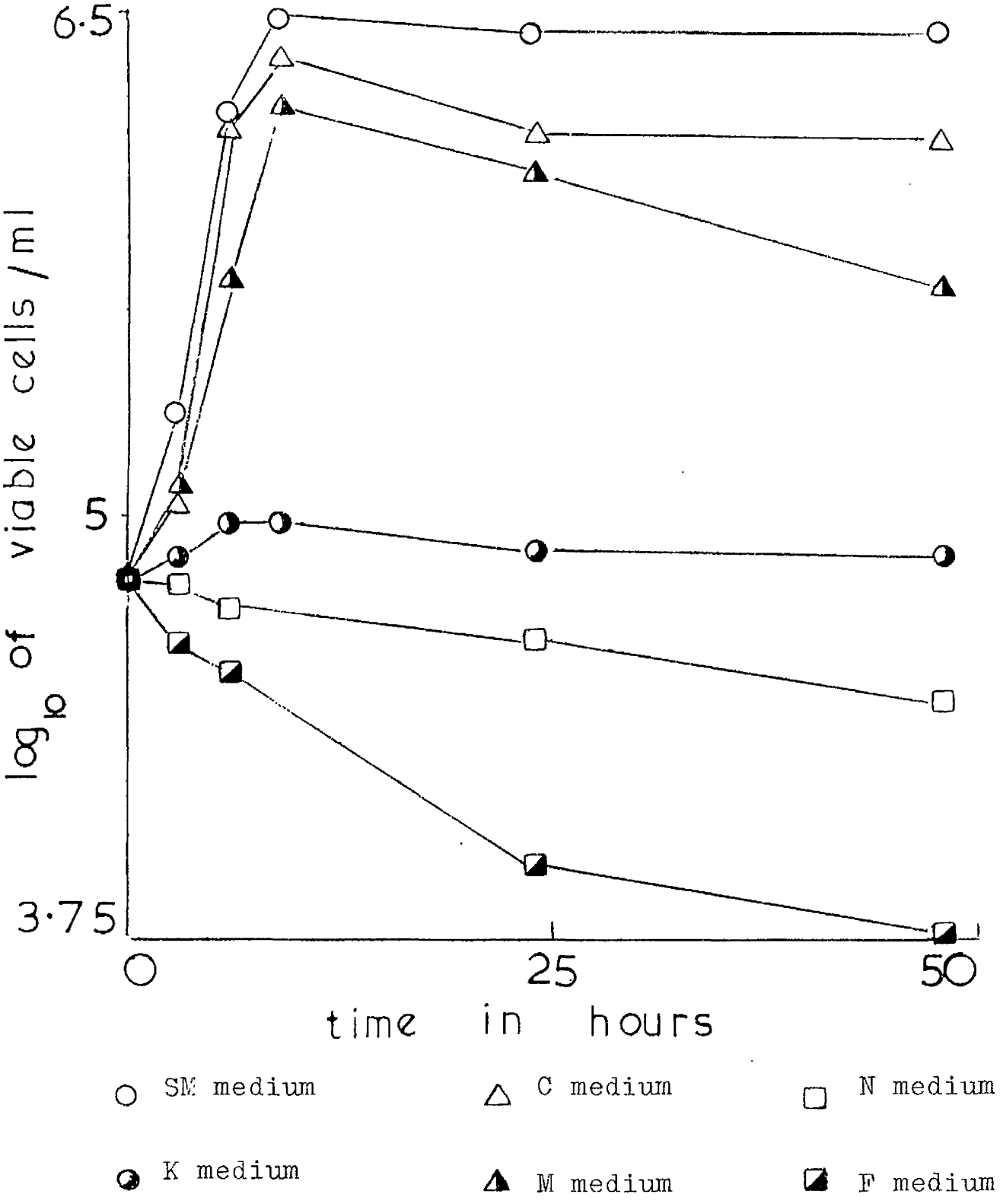


Fig. 11:- Growth of organism in salts media.

growth obtained in the complete synthetic-medium may have been supported, at least in part, by the salts - $mgSO_4 . 7H_2O$ and $CaCl_2$.

TABLE 26

Growth of Organisms in SM, K,M,C,N & F media.

Incubation period in hours	Log_{10} of viable cells/ml of medium.					
	Sm Control	K	M	C	N	F
0	4.81	4.81	4.81	4.81	4.81	4.81
3	5.31	4.88	5.05	5.07	4.7	4.62
6	6.2	4.95	5.70	6.15	4.73	4.538
9	6.49	4.91	6.21	6.35	-	-
24	6.44	4.90	6.03	6.135	4.64	3.97
48	6.44	4.89	5.665	6.12	4.45	3.73
72	6.42	4.91	5.31	6.08	4.39	3.73

(b) External Sources

As suggested earlier, another possible source of supply of energy sources for the organism growing in the synthetic medium (SM' medium) could be atmospheric carbon dioxide (and possibly nitrogen too), i.e. organism may be utilizing atmospheric CO_2 (and N_2) as energy sources. The following two sets of experiments were performed to study the mechanism.

Experiment (i)

In this set of experiment flasks with central-well were used and cotton-wool plugs were replaced by rubber-bunks.

Fifty ml. portions of sterile 'SM' medium were pipetted in above mentioned sterile flasks. In the central-well 0.3 ml of 20% KOH was placed in order to absorb carbon dioxide (CO_2) present in the flask. This portions were then inoculated with different sizes of inocula (5×10^3, 5×10^4 and 1×10^5 cells/ml) and 1 ml samples were withdrawn for zero time data. The cotton-plugs were replaced by rubber-bunks and inoculated portions of medium were incubated. During incubation development of the culture in the medium was measured in usual way by plate count technique.

As can be seen from the results obtained (Table 27) there was great increase in the viable cells in all the cultures within 24 hours which remained in the equilibrium state thereafter. The maximum yield of viable cells obtained with all the different sizes of inocula was about 10^6 cells/ml, which is lower than that normally obtained in 'SM' medium when incubated aerobically. The results show that the growth in the 'SM' medium (when incubated aerobically) was not supported by atmospheric CO_2 as growth was obtained in the conditions where CO_2 from the culture flask was removed. There is a suggestion that though it is assumed that KOH placed in the central-well of the flask would absorb all the CO_2 present in the flask, it is not certain that it would also absorb CO_2 dissolved (diffused)

in the medium as well and which might have been utilized by the organism.

TABLE 27

Growth of organism in the Synthetic medium from which CO_2 has been eliminated.

Incubation period in days/hours.	Log 10 of viable cells/ml with inocula.		
	$5x10^3$ cells/ml	$5x10^4$ cells/ml	$1x10^5$ cells/ml.
0 hours	3.5211	4.5380	5.1139
1 day.	6.0241	6.1139	6.1502
2 days	6.0240	6.0211	6.0472
3 days	5.8779	5.9586	6.021

Experiment (ii)

In this set of experiments 50 ml portions of sterile synthetic medium contained in sterile flasks (plugged with cotton-wool) were inoculated in usual way. The culture flasks were then placed in a McIntosh and Fildes's jar in which air was then replaced by oxygen (O_2). For this purpose the jar was evacuated by means of a pressure pump and then it was filled with oxygen. At certain time interval samples were withdrawn to study the growth in the medium and remainder was incubated in the atmosphere of O_2 as mentioned above.

From the results obtained (Table 28) it seems that

organism was unable to multiply in this conditions and after certain time interval there was no measurable growth in the medium. The results are discussed in Disdussion chapter.

TABLE 28

Growth of organism in the synthetic medium in the atmosphere of O_2

Incubation period in days/hours.	Log_{10} of Viable cells/ml.
0 hours	4.7877
1 day	3.0472
2 days	1.2402
3 days	No measurable growth.

SECTION IV

Decomposition of Streptomycin by Cellulomonas

As described in the previous section, the effect of environmental factors on the growth of cellulomonas sp. was studied in detail. Further studies were carried out to find the effect of some of those factors on streptomycin decomposition by the organisms. In this section, work has been more concentrated to the study of streptomycin decomposition. However, from time to time, as thought useful, growth was also studied.

Streptomycin decomposition studies were conducted in a basal-salts synthetic medium (see detail in method Section F) to which streptomycin was added as the sole source of carbon and nitrogen for supply of energy and cellular building material. The synthetic medium was used because it's a simple medium and its exact composition is known. It is therefore easier to study the specific nutritional requirements of a particular micro-organism in this type of medium, whereas in the case of a complex medium the results of such studies are often influenced by the nutrients composing the medium. In all the following studies the same synthetic medium - 'SM' medium - was used to which streptomycin and/or other energy sources were added which are mentioned specifically.

I Decomposition of Streptomycin in a Synthetic medium containing 20 µg of Streptomycin/ml.

Forty nine ml portions of sterile synthetic medium (SM) were pipetted into 150 ml acid-washed and sterile flasks. To each flask 1 ml of sterile streptomycin solution (of concentration 1000 µg/ml) was added so as to give concentration of 20 µg/ml in final volume of 50 ml of medium. Flasks thus containing streptomycin-synthetic-medium (SSM) were inoculated with a washed cell suspension of 48 hours old growth in 'SSM' medium. 1 ml aliquot from each flask was withdrawn for zero time data and the remainder incubated for further studies. Parallel uninoculated flasks of 'SSM' medium were incubated as control. During incubation 1 ml aliquots were withdrawn at regular intervals (every 48 hours) and the course of streptomycin decomposition and growth were estimated in the usual manner.

Parallel samples were also withdrawn from uninoculated control medium and were used as the reference concentration of streptomycin in the assay technique.

The results obtained are given in Table 29 and shown graphically in Fig.12. It can be seen that the total amount of streptomycin was decomposed in 22 days with an induction period of 18 days in which no decomposition took place. Streptomycin decomposition was associated with the amine-like odour as in the case of streptomycin decomposition

by mixed populations.

Parallel results of plate counts show that there was an increase in viable cells in the first 24-48 hours. But thereafter, the total viable cells, remained more or less the same; the total amount of streptomycin was decomposed without even a further increase in cell number. The results show that the streptomycin decomposition does not support further growth of the organism; in other words products of streptomycin decomposition do not support growth of the organism.

TABLE 29

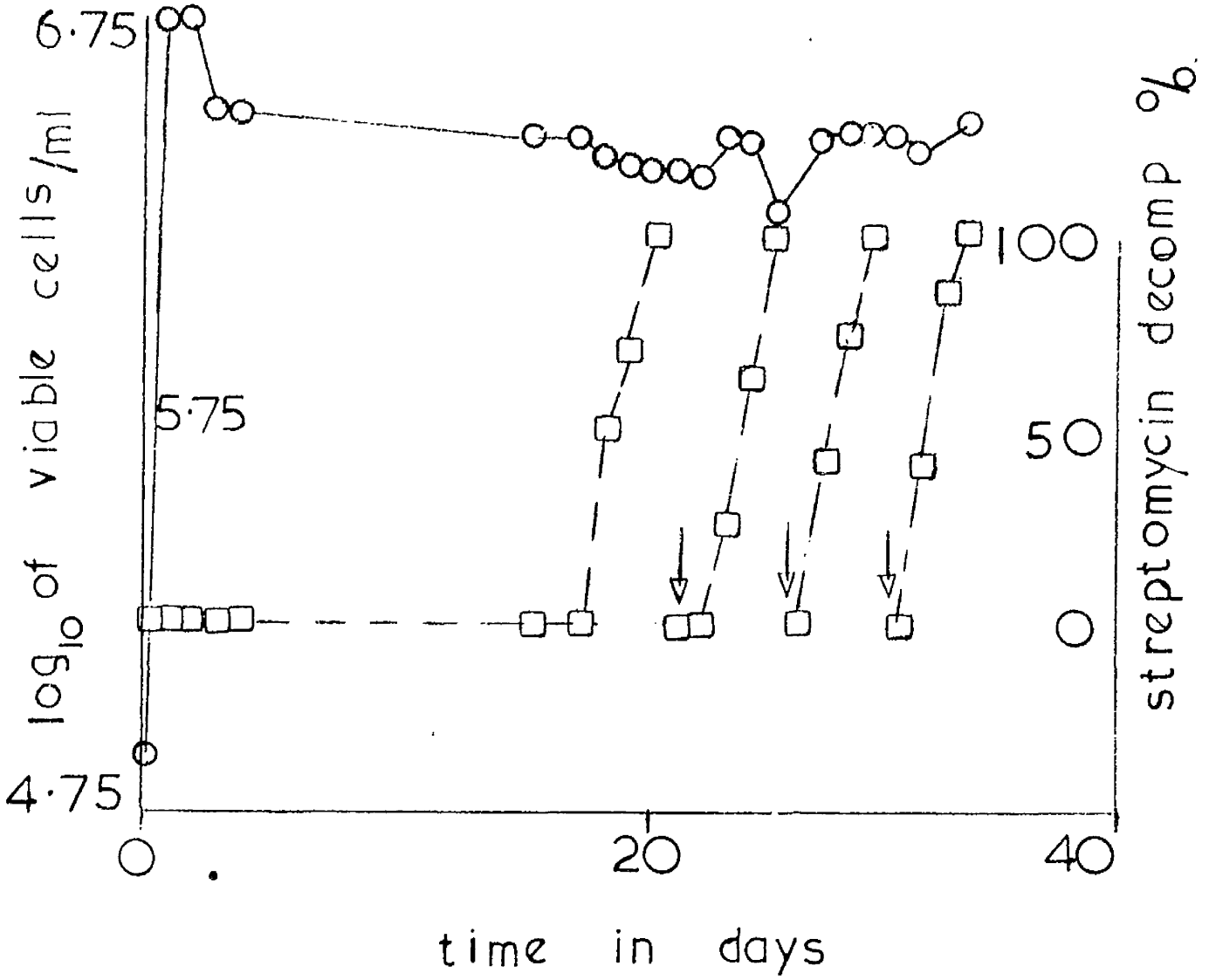
Streptomycin decomposition and corresponding growth of organisms in synthetic-medium containing 20 µg of streptomycin/ml (SSM).

Incubation period in days-hours	Log_{10} of viable cells/ml	Streptomycin degraded (%)
0 hr.	5.51	0%
1 day	6.82176	0%
2 days	6.81614	0%
3 days	6.61	0%
4-17 days	6.534	0%
18 days	6.57	0%
19 days	6.496	50%
20 days	6.44	80%
21 days	6.420	100%
22 days	6.45	-

II Effect of successive additions of streptomycin on the course of its disappearance.

In this experiment, 50 ml portions of sterile 'SSM' medium were prepared and inoculated as mentioned above, samples were taken for the assay and plate counts to give zero time data, and cultures were incubated together with uninoculated control medium. During the course of incubation streptomycin decomposition and growth were estimated in the usual manner. As soon as the total amount of streptomycin had disappeared, more of sterile antibiotic solution was added to the culture medium to restore the original concentration (20 µg/ml). Cultures were re-incubated and the course of streptomycin decomposition was followed and corresponding estimates of growth were made. The procedure was repeated twice more.

From the results (Table 30, Fig.12) it can be seen than streptomycin in successive additions disappeared rapidly. The second addition of streptomycin disappeared with a shorter induction period, and successive additions, disappeared immediately without any induction period. The results of plate counts show that upon further additions of streptomycin to the culture medium there was no marked increase in the total viable cells. The results suggest, as mentioned earlier, that streptomycin decomposition does not result in an increase in viable cells.



○ Viable cells/ml

□ streptomycin decomposed (%)

↓ addition of antibiotic solution

Fig. 12:- Relationship between growth of organism and streptomycin decomposed in SSM medium.

TABLE 30

Decomposition of Streptomycin added to the substrate on three successive additions of 20 µg/ml.

Time in Days.	Log_{10} of Viable cells/ml	Streptomycin Degraded (%)
0 hour.	4.8574	0 %
1 day.	6.82176	0 %
2 days	6.8161	0 %
3 days	6.53403	0 %
19 days	6.4962	50 %
20 "	6.2293	80 %
21 "	6.420	100%
22 "	6.421	-
	More antibiotic solution added (20µg/ml)	
22 days	6.4217	0 %
23 "	6.4038	0 %
24 "	6.5198	25 %
25 "	6.50	63 %
26 "	6.3052	100%
	More antibiotic solution added (20µg/ml)	
27 "	-	0 %
28 "	6.50	40 %
29 "	6.52	75 %
30 "	6.53	100%
	More antibiotic solution added (20µg/ml)	
31 days	6.51	0 %
32 "	6.47	40 %
33 "	-	85 %
34 "	6.55	100%

From the foregoing results it seems that the rapid decomposition of streptomycin in the successive additions could have been due to an adaptation phenomenon. When first inoculated into the 'SSM' medium the culture took 18 days for its adaptation and thus presumably the same time for the induction of the streptomycin-decomposing enzyme in that medium. But once the culture was adapted to the medium and induction of the specific enzyme has begun, it continued to be formed and therefore streptomycin in the successive additions was attacked immediately without any induction period. From the studies so far it is not known whether the streptomycin decomposing enzyme is intracellular or extracellular. The following experiment was therefore performed to study this phenomenon.

III Streptomycin Decomposition by Cell-free Filtrates.

Portions of sterile 'SSM' medium were inoculated with different amounts (1,2,3,4 or 5 ml) of cell-free filtrates and the final volume was made up to 50 ml. Cell-free filtrates were obtained by filtering through Seitz's filter the culture medium in which the first batch of streptomycin has just been decomposed. The inoculated media were incubated together with uninoculated control medium. During incubation an amount of streptomycin present in the medium was estimated in usual manner.

The results of assay technique show that there was no

decrease in the streptomycin content, in any medium inoculated with cell-free filtrates.

IV Effect of Serial transfers of culture under optimal conditions on Streptomycin decomposition.

From the previous results it is clear that once the streptomycin decomposing enzyme became available, it continued, and the streptomycin in successive additions was decomposed immediately without any induction period. Also the results of the above experiment show that the streptomycin decomposing enzyme is an intracellular enzyme. Further experiments were therefore performed to study whether the ability to decompose streptomycin without induction period can be retained by the culture when transferred into fresh 'SSM' medium; in other words to study whether the acquired ability to decompose streptomycin can be transferred from parent culture to its progeny.

Fifty ml portions of sterile 'SSM' medium were inoculated with the washed cell suspension of the culture which had just completed the decomposition of the first batch (20µg/ml) of streptomycin; the size of inoculum being the same as in the initial transfer (Table 30). The inoculated media were incubated together with uninoculated control medium. During incubation the course of streptomycin decomposition and growth of the culture was studied in the usual way. Similar transfers

were made twice more into fresh 'SSM' medium, each time after the culture had decomposed all the streptomycin in the previous medium, and the course of streptomycin decomposition was followed in the subsequent cultures.

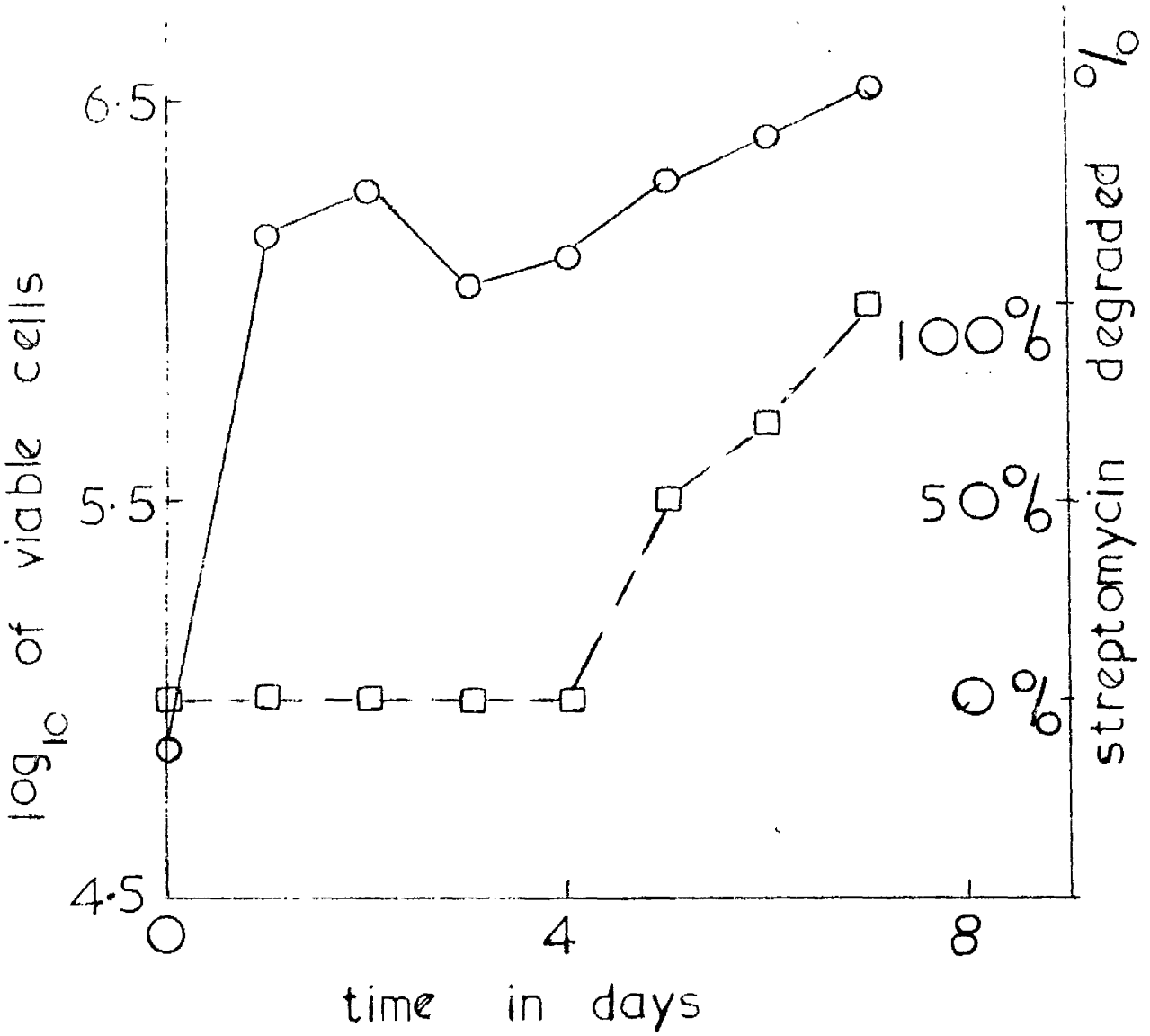
As shown in Table 31 and Fig.13, the decomposition of streptomycin in the sub-cultures occurred within 7 days with as short an induction period as 4 days. Also the peculiar amine-like odour which is usually associated with streptomycin decomposition was noticed each time the active phase of streptomycin decomposition began. The results of plate counts show that the maximum yield of viable cells was obtained in 7 days. The results suggest delay in growth as compared to the growth in the initial culture (Table 29,30), where same maximum yield of viable cells was obtained in 1 day (24 hours).

The above results show that the ability to decompose streptomycin without (or with a shorter) induction period can be retained and can be transferred to its progeny, by the organism. The results are discussed, in detail, in the Discussion section.

V <u>The Effect of Size of Inoculum on Streptomycin decomposition.</u>

It is well established that size of inoculum plays an important part in microbial growth.

Also from the previous experiments two things are clear: (i) Each time the same maximum yield ($5 \times 10^6 - 10^7$) of viable cells is obtained (Tables 19,20,21,22,23,25,29,30,31 etc)



○ $\log_{10}$ of viable cells/ml

□ streptomycin degraded %

Fig 13:- Effect of serial subculturing (after decomposition) on the course of decomposition.

TABLE 30

Effect of Serial transfers on Streptomycin Decomposition.

Time in days	Log_{10} of cell number/ml.	Streptomycin Degraded (%)
0 hours.	4.85	0 %
1 day.	6.10694	0 %
2 days.	6.12086	0 %
3 "	6.04934	0 %
4 "	6.10223	0-5%
5 "	6.31856	50%
6 "	6.42121	70%
7 "	6.50473	100%

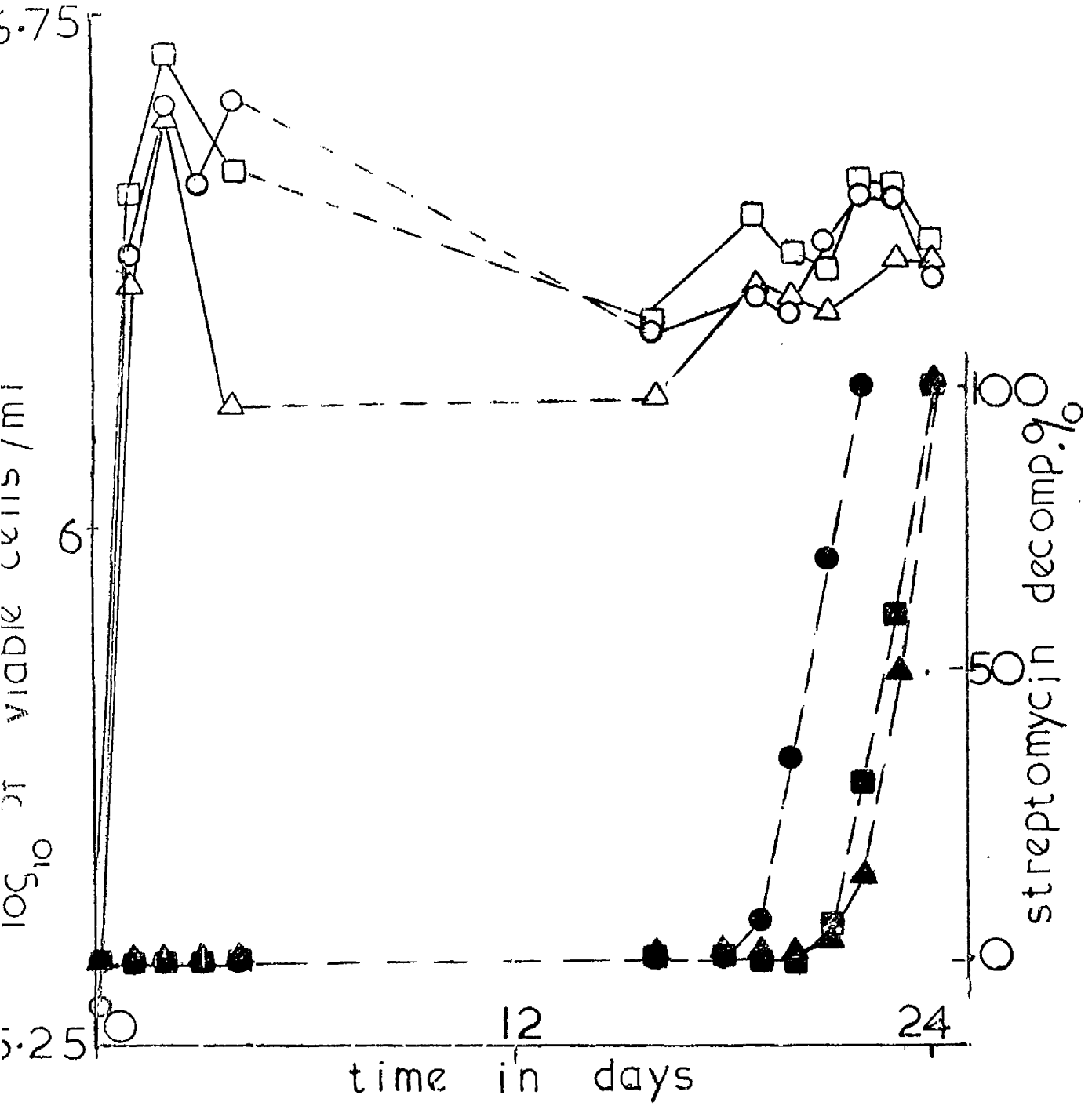
In the case of initial culture (Table 29,30) maximum yield was obtained within 24 hours whereas in the case of subsequent cultures (Table 31) it was delayed and was obtained within 7 days time interval. Moreover, in the case of experiments performed with different sizes of inocula the results show (Table 23) that once again almost the same maximum yield was obtained in each case.

(ii) Though the same maximum yield of viable cells was obtained with different sizes of inocula, the time required to obtain that maximum yield was different with different sizes of inocula.

It is therefore natural to enquire as to what could be the effect of different sizes of inocula, and specifically the effect of inoculum size equal to that of maximum yield of viable cells, on the streptomycin decomposition pattern. The following set of experiments was performed to study this phenomena.

Fifty ml portions of sterile 'SSM' medium contained in sterile flasks were inoculated with different sizes of inocula. Inoculum approximately equal to the maximum yield which for convenience could be described as "Largest inoculum" was of 5.5×10^8 viable cells/50 ml of medium, "Large inoculum of 1.5×10^7 cells/50 ml medium (as in the previous experiment see page 118), "Medium inoculum" of 4×10^3 cells/50 ml and "Small Inoculum" 6×10^1 cells/50 ml of medium. Inoculated media were incubated together with uninoculated control medium and course of streptomycin decomposition and growth of the culture were followed in usual manner.

The results show (Table 33, Fig.14) that the 'Largest', 'Large' and 'medium' inoculum there was an induction period as usual, of 18 days and total amount of streptomycin was decomposed in 3 days thereafter. But in the case "Small" inoculum decomposition of streptomycin was considerably delayed. It occured only at the end of 50 days with an induction period of 46 days.



$\log_{10}$ of viable cells/ml

○

△

□

streptomycin decomposed %

●

▲

■

Fig 14:- The effect of additional carbon and nitrogen sources on the growth of the organism and on the course of streptomycin decomposition.

TABLE 35

Effect of Size of inoculum on Streptomycin Decomposition.

Time in days/hrs.	Log_{10} of viable cells/ml and streptomycin degraded (%) with different sizes of inocula.							
	Largest Inoculum Viable cell/ml	Strepto degraded %	Large Inocul. Viable cell/ml	Strep. Degd %	Medium Inoc. Viable cell/ml	Strep Degd %	Small Inoc Viable cell/ml	Str Dgd %
0 - 0hr.	6.8630	0%	5.4226	0%	1.9490	0%		
0 - 3 "	6.8750	"	6.0149	"	1.9396	"		
0 - 6 "	6.8621	"	6.62008	"	2.6719	"		
0 - 9 "	6.8245	"	6.81508	"	3.0834	"		
0 -10 "	6.85502	"	6.90694	"	3.50133	"	1.5422	0%
1 - 0day	6.8682	"	6.88649	"	6.70078	"	4.08934	"
1 - 6 "	6.8750	"	6.8357	"	6.59176	"	4.9360	"
2 - 0 "	6.8542	"	6.8340	"	6.5807	"	6.8563	"
3 - 0 "	6.8409	"	6.7666	"	6.70140	"	6.7296	"
4 -16 "	6.8395	"	6.75587	"	6.55207	"	6.7873	"
19 "	6.8025	50%	6.7650	40%	6.57	35%	6.76	0%
20 "	6.82	80%	6.76	75%	6.56	75%	6.75	"
21 "	6.83	100%	6.75	100%	6.57	100%	6.73	"
22 "	6.832	-	6.75	-	6.56	-	6.73	"
46 "	-	-	-	-			6.74	"
47 "							6.73	5%
48 "							6.737	25%
49 "							6.73	65%
50 "							6.71	100%

The results of plate counts show (Table 33, Fig.14) that with the 'largest inoculum' there was not much increase in the total viable cells. It could be expected as the initial inoculum was almost the same size as of the maximum yield usually obtained in the synthetic medium. In the case of "Large inoculum" and "medium inoculum" the maximum yield of viable cells was obtained in 10 hours and 24 hours respectively. Where as "Small inoculum" required 72 hours to reach the same maximum yield of cells. However, in each case the maximum yield of viable cells was almost the same. In all cultures after the maximum peak was reached, there was no further change in the maximum yield until and after the total amount of streptomycin was decomposed.

VI <u>The effect of Additional Carbon and Nitrogen sources in the Medium on Streptomycin Decomposition.</u>

As can be seen from the previous experiments the earlier decomposition of streptomycin was obtained when antibiotic was added to culture media as soon as previously, amount of antibiotic was decomposed. The successive doses of antibiotics were decomposed within 3-4 days without (or with 1-2 days) induction period. Furthermore when the inoculum was taken from the culture which had just decomposed streptomycin, the subsequent culture was able to decompose streptomycin earlier in 7 days, with shorter induction period of 4 days. But in this case though the earlier decomposition of

antibiotic was obtained the maximum yield of viable cell in 'SSM' medium was obtained in 7 days whereas the same yield was usually obtained in 1 day in the initial culture. It seems here as if there may be some competition between the enzyme(s) responsible for the synthesis of cell building material and the induction of streptomycin decomposing enzyme(s). The use of larger inocula in the initial culture did not help to obtain earlier decomposition of streptomycin. The following experiment was performed to see whether the additional sources of carbon and nitrogen would help the synthesis of above mentioned enzymes.

In this experiment three types of media were used, (see also method and materials).

1) 'SSM' medium - Synthetic medium containing streptomycin.

2) SSM_1 medium - Synthetic medium containing streptomycin + glucose.

3) SSM_2 medium - Synthetic medium containing streptomycin + glucose + ammonium sulphate.

50 ml portions of media were inoculated with 48 hours old culture and were incubated together with uninoculated control media. Course of streptomycin decomposition, and development of the culture, in the medium were followed in usual manner.

The results obtained (Table 34, Fig.14) show that it took 21-24 days to affect the total decomposition of streptomycin

with induction period of 18-21 days respectively. The maximum yield of viable cells obtained was also the normal one.

TABLE 34

The effect of additional carbon and nitrogen sources on the course of Streptomycin decomposition

Incubation period in	Log_{10} of viable cells/ml & streptomycin decomposed in the synthetic medium containing					
	Streptomycin (SSM)		Streptomycin + glucose (SSM_1)		Streptomycin + glucose + Ammon.Sulph. (SSM_2)	
	viable cell/ml	strept. Deg.%	viable cell/ml	strept. Deg.%	viable cell/ml	strept. Deg.%
0 hours.	5.305	%	5.3079	%	5.2335	%
1 day	6.39002	%	6.3579	%	6.485	"
2 days	6.63745	"	6.6062	"	6.6937	"
3 "	6.502	"	-			"
4 "	6.62552	"	6.1708		6.5263	"
4-16 days	6.2788	"	6.193	"	6.297	"
19 days	6.348	7%	6.35		6.457	"
20 "	6.32	35%	6.325		6.40	
21 "	6.42	70%	6.32	3%	6.375	5%
22 "	6.48	100%	-	15%	6.50	30%
23 "	6.48	--	6.39	50%	6.49	60%
24 "	6.37	--	6.39	100%	6.42	100%

DISCUSSION AND CONCLUSION

Antibiotics when added to the normal soil may undergo various physical, chemical and biological changes depending upon the nature of the antibiotic and the type of soil in question. These inactivation phenomena include absorption by soil colloids, possible inactivation either by reacting with substances already present in the soil or due to the instrinstic instability of the antibiotic molecule. Even if the above phenomena are absent in a particular case there is every possibility of microbial attack and hence inactivation of the antibiotic under discussion. Basic antibiotics such as streptomycin and tyrothricin when added to the normal soil are strongly adsorbed by soil colloids (Nandi, 1948, Sinha 1950, Gottlieb and Siminoff 1951; Fremer and Starkey 1951; Shirodkar, 1953 and Ghadially 1961) and also they are decomposed biologically (Nandi 1948, Sinha 1950; Gottlieb and Siminoff 1951). But it is not known whether or not they undergo chemical inactivation also.

In the present work, from the sterile-soil, the results of recoverable streptomycin show that the amount recovered immediately after its addition to the soil was 46%; and this amount did not change during the long incubation period of 30 days (see Table 5). The fate of unrecoverable material however is obscure. It is not known whether the remainder was too firmly bound to be extracted with the

acid-methanol technique or that it was decomposed soon after its addition due to the chemical degradation. Extensive work by Sinha (1950) showed that the same amount of streptomycin was lost each time a dose of antibiotic was added to a given soil sample. As for the possible occurrance of the substances reacting with streptomycin in the soil, it could be expected that these should have been exhausted with the first batch of antibiotic added. However, it is possible that some catalytic conditions other than enzymic are involved here. The results suggest that the physiochemical absorption of the antibiotic might be the cause of this specific loss of antibiotic. Experimental results show (Table 5) that the initial recovery from non-sterile treated soil was 36% in the present case. It was presumed here also that the remainder was firmly adsorbed by the soil colloids. Microbial degradation seems to be very less probable since the interval of time between addition of the antibiotic and its extraction from the soil was very short (2 hours). Results of initial recovery show that the non-sterile soil seemed to bind more antibiotic than the sterile soil, and this suggests that the autoclaving process might have altered the adsorptive capacity of soil colloids whereby the penetration of the solvent used (acid methanol) was facilitated. Thus, streptomycin, when added to the normal soil is strongly absorbed.

Now, let us first consider the fate and effect of the comparatively small amount of streptomycin which was left free in the soil. The decrease in the recoverable streptomycin from non-sterile soil, which completely disappeared between 20-25 days; as compared to the constant recovery of streptomycin from sterile soil at that time (Table 9) suggests that micro-organisms were mainly responsible for the disappearance of streptomycin from non-sterile treated soil. The more rapid disappearance of successive additions of antibiotic from the non-sterile soil (Table 7) further supports the phenomenon that microbial degradation of streptomycin was proceeding in the soil. From the results of parallel experiments (See Table 9,10) of recoverable streptomycin from the non-sterile treated soil and corresponding bacterial numbers as revealed by plate counts, it is clear that:- (1) streptomycin exerts a limited lethal action on the soil microflora; (2) After an initial depression, an increase in bacterial number began usually when the amount of recoverable streptomycin began decreasing. This increase in bacterial number could either be due to the multiplication of organisms which can metabolize streptomycin, or due to the streptomycin-resistant organisms (survivors) utilizing the products of streptomycin-killed organisms as nutrients; (3) Since this increase in bacterial cell continued even after the total

amount of recoverable streptomycin disappeared it could be ascribed to the multiplication of organisms utilising decomposed products of antibiotic. These results of recoverable streptomycin from non-sterile treated soil and correspondingly its effect on organisms, strongly suggest that streptomycin undergoes microbial degradation when added to the normal soil. It is interesting to note that an amine-like odour, appeared in the non-sterile treated soil at the time when recoverable streptomycin began decreasing and correspondingly the total bacterial cells began increasing; this odour disappeared after the total decomposition of streptomycin. It is assumed that this amine-like odour, is associated with biological decomposition of streptomycin.

The second point to be considered is the fate of adsorbed antibiotic considering that the majority of added streptomycin was firmly adsorbed by soil colloids, the question arises as to whether such substances can have any effect on soil fertility. Gottlieb and Siminoff (1951) stated that basic antibiotics such as streptomycin could exert no effect in soil. However, the work of Nandi (1948) with tyrothricin (another basic antibiotic) and that of Sinha (1950) and Shrodkar (1953) with streptomycin showed that absorbed antibiotics were subjected to the biological degradation. Pramer and Starkey (1951) also demonstrated the decomposition of absorbed streptomycin. Moreover in the present work

(described on page 140) experiments performed with mixed culture, in bentonite-streptomycin-synthetic-medium (BSM) (see Table 13,15) showed that decomposition of adsorbed antibiotic was preceeding in the apparent induction period of 18 days, as evident by the presence of amine-like odour at that time. Since the adsorbed antibiotics are accessible to the soil micro-flora, it can be assumed that they may also be able to exert their characteristic inhibitory effects when present in soil.

Studies with mixed bacterial populations obtained from the soil in which successive doses of antibiotic were rapidly decomposed showed that streptomycin decomposition by soil micro-organisms can be demonstrated in the synthetic medium, provided the initial concentrations of antibiotic is low. Since in the preliminary experiments performed in synthetic medium containing 1000 µg of streptomycin/ml of medium streptomycin decomposition did not take place in the experimental period (100 days). When the conditions in the synthetic medium containing 1000 µg of streptomycin/ml were made closer to those in soil by addition of colloidial absorbed such as bentonite (BSM medium) all except 20 µg of streptomycin/ml was absorbed; and decomposition of streptomycin occurred. In subsequent cultures, made after decomposition, in fresh 'BSM' medium, all the free streptomycin disappeared in 21 days with an induction period

of 18 days in which no decrease in free streptomycin content was observed. The amine-like odour was noticed in this medium (BSM) on the 7th day and disappeared after 21 days though the free streptomycin content did not change till the long induction period of 18 days had elapsed. It is possible that decomposition of streptomycin (free) was proceeding during the induction period but due to equilibrium phenomena concentration of free streptomycin was maintained in the medium by release of bound material from the soil colloids. However, later on as can be seen from the experiments performed in 'BSM' and 'SSM' medium (Table 13,14,15,16) it seems more probable that during the induction period the decomposition of absorbed antibiotic was proceeding and which in turn gave amine-like odour on 7th day.

When experiments were performed in synthetic medium containing (low concentration) 20 µg of streptomycin/ml (SSM medium) all the antibiotic was decomposed in 21 days with an induction period of 18 days in which no decomposition took place. In subsequent cultures made after decomposition in fresh SSM medium all antibiotic was decomposed rapidly and finally an active culture was obtained which decomposed all the added antibiotic in 7 days with an induction period of 4 days. Each time the amine-like odour was noticeable once the phase of streptomycin decomposition had begun; the odour was unnoticeable in the induction period. When subsequent transfers after decomposition were made into the

synthetic medium containing higher concentration of streptomycin it was possible to obtain cultures which could decompose 800 µg of streptomycin/ml of medium, but parallel experiments performed with the initial culture (soil extract) showed that the culture was unable to decompose 50, 100 µg of streptomycin/ml in synthetic medium. All these results suggest the mechanism of adaptation. The initial cultures (soil extracts Table 12) were unable to decompose higher concentration (1000 µg/ml) of streptomycin and even as low as 50 µg/ml, but subsequent sub-cultures made after decomposition (i.e. adapted culture) could decompose as high as 500 ug of streptomycin/ml; (Table 17) this may not have been the limit, but higher concentrations were not tried. Successive additions of streptomycin at dose levels of 20 µg/ml were rapidly decomposed with shorter induction (Table 29) which also supports the proposed mechanism.

Further studies regarding streptomycin decomposition and growth kinetics were performed with a Cellulomonas sp, a pure culture isolated from an actively streptomycin decomposing mixed culture. Since this isolate actively decomposed streptomycin in streptomycin-synthetic-medium it was presumed to be an organism associated with streptomycin decomposition in soil. When the organism was inoculated into synthetic medium containing 20 µg of streptomycin/ml ("SSM' medium) it first decomposed

streptomycin in 21 days with a lag period of 18 days in which no decomposition took place. When experiments were repeated with larger inocula, once again similar results were obtained in which decomposition of total amount of streptomycin occurred in 21 days with an induction period of 18 days (Table 33). Though with very small inocula (10 cells/ml) streptomycin decomposition was delayed; total amount of streptomycin was decomposed in 50 days with an induction period of 46 days in which no decomposition occurred. The results suggest that after an initial number of viable cells the inoculum size did not have much bearing on the pattern of streptomycin decomposition. When experiments were performed with 'SSM' medium containing additional carbon and nitrogen sources (Table 34), the same pattern of streptomycin decomposition was observed. Thus, in the case of an initial culture, inspite of the use of larger inocula and additional carbon and nitrogen sources, the pattern of streptomycin decomposition remains the same.

When subsequent cultures were made after decomposition into fresh (see Table 30) streptomycin-synthetic-medium (SSM) all the added antibiotic was decomposed in 7 days with a shorter induction period of 4 days in which no decomposition took place; sizes of inocula being the same in initial culture (mentioned earlier) and in subsequent cultures.

Another way in which quicker decomposition of streptomycin could be obtained was by successive additions of streptomycin at the same dose level (20 µg/ml) to the culture medium immediately after the previous dose had disappeared (Table 29); immediate decomposition of the streptomycin in the successive additions occurred without any induction period except for the first two additions where the induction period was 1-2 days, and total decomposition occurred in 3 days thereafter. The enhanced activity of the culture observed in the case of successive additions of antibiotic to the culture flask and in subsequent transfers, does not seem to be the result of mutation or/and selection as for the sample isolated from the 'active culture' revealed only one type of colony and behaved exactly like the parent culture. When first inoculated into 'SSM' medium the culture took like the parent culture, 21 days to decompose the total amount of streptomycin with an induction period of 18 days in which no decomposition took place.

The results of pure culture studies further support the phenomenon of adaptation. The possible explanation for the phenomenon of 18 days induction period in the initial culture could be due to the maintenance of the culture on solid medium (its true with streptomycin) it lost its adaptability and therefore took longer (18 days) when

transferred to streptomycin-synthetic-medium (liquid medium) for the adaptation and synthesis of streptomycin decomposing enzyme in the new medium. Once the culture was adapted and was able to decompose streptomycin, immediate decomposition of successive additions of antibiotic took place. Similarly, if subsequent cultures were done in the same medium, after decomposition, all streptomycin in subsequent transfers was decomposed in 7 days, with a shorter induction period of 4 days.

When the organism was grown in synthetic medium without streptomycin and sub-cultured after every 48 hours in the same medium 6-7 times, the sub-culture was unable to decompose streptomycin when transferred into synthetic-medium containing 20 ug of streptomycin/ml (SSM). These results suggest that the streptomycin decomposing enzyme is probably an induced enzyme. Another explanation for the 18 days induction period could be that the culture requires 18 days when first transferred into 'SSM' medium - a new environment - for induction of the enzyme synthesis. Once the enzyme became available or synthesis began, it continued to be synthesized and therefore successive additions of antibiotic doses into the culture flasks were decomposed immediately in 3 days without any lag period; supported by the quickly available nutrients - the decomposed products of the antibiotic. Similarly subsequent cultures made in fresh 'SSM'

medium all the antibiotic was decomposed in 7 days with a shorter induction period of 4 days; this induction period could be the time required for a general adjustment in the fresh medium or for the formation of streptomycin decomposing enzyme(s) (presuming that the enzyme forming system is unstable).

There is some indication of a general adjustment taking place in this culture since the maximum cell number is reached only after 7 days, where as in previous cultures with inocula not derived from a streptomycin decomposing phase this maximum has been reached after 1 day. Since streptomycin decomposition starts only after 4 days, the possible explanation could be that before this time there was not sufficient cell material to form the required amount of streptomycin decomposing enzyme. However, after 4 days already 1/3rd of the maximum cell number has been reached, and this should have given a noticeable streptomycin decomposition. Therefore, the conclusion seems inevitable that there was a delay in the induction process of the streptomycin decomposing enzyme despite the fact that the inoculum had been taken from a streptomycin decomposing phase.

The reason for this delay is not easy to understand but it is possible that, because of the formation of new cell material, the induction of the streptomycin decomposing

enzyme has been subjected to a competition by the formation of other enzymes important in the synthesis of cell material. While the formation of the streptomycin-decomposing enzyme had suffered, it is also possible that the eventual formation of this enzyme(s) has improved the formation of the other enzymes. This could then in turn explain the delay in growth of the culture.

The results of the parallel experiments, on streptomycin decomposition and growth of the organism in 'SSM' medium show (Table 28,29), that there was no decrease in the streptomycin content for 18 days while there was a hundred fold increase in the total viable cells. The results suggest that the organism remains indifferent to the streptomycin and multiplying at the expense of energy sources other than streptomycin.

From the observations mentioned above, there was no decrease in the streptomycin content during the incubation period, and the streptomycin decomposing enzyme being intracellular, two more explanations based on cell-permeability can be put forth for the particular pattern of streptomycin decomposition.

Information concerning the uptake of streptomycin by bacteria is conflicting, Anand, Davis and Armitage (1960) reported an initial uptake of streptomycin by resistant cells of Escherichia coli which was completed a minute

after addition of an antibiotic and was equal at both 0^{o} and $37^{o}C$. On the other hand, Szybalski and Shizuyoshi (1959) observed that there was little if any, streptomycin uptake by cells of a streptomycin-resistant strain of E. Coli growing at $37^{o}C$. Klein and Framer (1962) reported that uptake of streptomycin by an antibiotic - utilizing Pseudomonad was negligible at $5^{o}C$ as compared to the uptake of streptomycin at $28^{o}C$ in 3 minutes. Moreover, Anand, Davis and Armitage (1960) showed that the initial uptake of streptomycin was on the surface of the cell or at the cell-wall only and which later affected the cell permeability. It seems that organisms which resist streptomycin may not either absorb it or if absorbed, it may not reach the vital site to create its harmful effect. In the present work, as the organisms under discussion was highly resistant to streptomycin, two possibilities can be considered. It might have been the case that in the lag period the organism either did not absorb the antibiotic or did to a very small extent, which was not detected by the assay technique used, or another possibility is that the cells were permeable to streptomycin but streptomycin was not utilized; in either case no loss in streptomycin content in the medium would be detected. According to the former possibility the organism remains indifferent to streptomycin and multiply at the expense of impurities in the medium, slight absorption of

streptomycin will help in the induction of specific enzyme and enzyme will be synthesized, but decomposition of the antibiotic will not take place as cells were impermeable to streptomycin. After a certain time interval (induction) the cell-permeability would have altered, either due to the exhaustion of nutrients in the medium by which cells will starve, or due to ageing of cells, or more probably due to the constant presence of streptomycin in the medium cell-wall will be affected. Streptomycin may then enter the cell and could react with specific enzyme present in the cell, and thus be decomposed or inactivated. This phenomenon would also explain the quicker decomposition of streptomycin in the successive additions to the culture medium. This phenomenon could possibly be studied by tracer techniques using labelled streptomycin.

According to the latter possibility the cells were permeable to streptomycin and multiply and synthesis streptomycin decomposing enzyme(s) at the expense of energy sources other than streptomycin; lag period of 18 days, being either time required for synthesis of enzyme or if the enzyme was present (or synthesized) from the very beginning then some unknown factor was preventing the specific reaction to take place, possibly a barrier "the active centre" of the enzyme-molecule. Once the specific enzyme was synthesized and/or the barrier was broken it came in contact with

streptomycin and decomposition of antibiotic resulted.

Considering all the above possibilities it seems that the streptomycin-decomposing enzyme is produced through recessive gene or genes and requires a constant presence of its substrate since no decomposition takes place if the antibiotic was not present from the very beginning. Moreover, it is possible that the streptomycin molecule remains unaffected so long as the complete mole of streptomycin-decomposing enzyme is formed. In the light of above hypothesis different lag periods are in fact the time required to form a complete molecule of the enzyme under different environmental conditions. Also, whatever may be the source or sources for the growth of organism in streptomycin-synthetic-medium, they are also required to provide enough food and energy for the induced enzyme synthesis; the phenomenon calls for comments.

Light might be shed on the above mentioned hypothesis if experiments are performed with inocula taken at different time intervals during the lag period of 18 days. Also experiments could be performed by inoculating streptomycin-synthetic-medium (SSM) with cell free sonicates obtained by disrupting washed cells suspension; samples being taken at suitable time intervals during the lag period of 18 days.

Further studies with pure culture isolate were performed in connection growth kinetics. Growth curve

obtained in synthetic-medium containing 20 µg of streptomycin/ml as the sole source of carbon and nitrogen for growth and energy (SSM medium), showed that the organism began multiplying immediately after inoculation, first at a higher rate of growth which slowed down later on and maximum growth was obtained within 24 hours (Table 19). The growth remained almost the same thereafter. Against, this, when glucose (20 µg/ml) and ammonium-sulphate (5µg/ml) were used instead of streptomycin as sources of carbon and nitrogen in the synthetic-medium (GNSM medium), results (Table 19) showed that there was an initial lag phase of 2 hours in this medium, followed by higher rate of growth and maximum growth obtained was same as in the previous medium (SSM) and requiring same time. When growth was studied in the synthetic-medium containing higher concentrations of streptomycin (100 µg/ml, 400 µg/ml), or higher concentrations of glucose (100 µg/ml, 400 µg/ml) and correspondingly ammonium-sulphate (5 µg/ml, 100 µg/ml), or with additional sources of carbon and nitrogen (Table 21) once again growth to some extent was obtained (Table 20 & 21) each time. Also experiments performed with different sizes of inocula showed the growth to be of the same extent (Table 33). As mentioned earlier, no decrease in streptomycin content in streptomycin-synthetic-medium (SSM) was observed during the growth (to hundredfold) of organism.

All the above results suggest that the organism was multiplying in the synthetic-medium (SM) at the expense of energy sources, other than that the streptomycin, glucose and ammonium-sulphate, even though synthetic-medium in each case contained either streptomycin or glucose ammonium sulphate as sole energy source or sources. Experiments were therefore performed in synthetic-medium which did not contain carbon and nitrogen source or sources, results obtained (Table 17) were similar to those obtained in previous experiments i.e. in 'SSM' and 'GNSM' medium and which further supports the proposed mechanism that organisms were indifferent to added energy sources in synthetic-medium.

The question then arises as to what is the energy source or sources for organisms multiplying to hundredfold in synthetic-medium which does not contain any energy source. There could be two possibilities, either the impurities present in the synthetic-medium served as energy sources for growth or may be that the organism was able to utilize atmospheric carbon and/or nitrogen as its energy sources. Impurities in the synthetic-medium may either have been incorporated with the de-ionised water used for the preparation of medium and/or may have come from the ingredients composing the medium. Experiments performed with synthetic-medium prepared with double-glass distilled water gave similar results as compared to those obtained with

synthetic-medium prepared with de-ionised water. The result show that impurity in de-ionised water was not responsible for the growth obtained in synthetic-medium. Experiments performed with synthetic-medium deficient in either of its salt constituents did not give different results (Table 25). But, when experiments were performed with media prepared with individual ingredients - of synthetic medium - such as medium K,M,N and F (See experiments on page 126), results obtained showed (Table 26) that medium 'M' ($MgSO_4 . 7H_2O$) and medium 'C' ($CaCl_2$) supported the growth of organisms to some extent. The results suggest that growth obtained in synthetic-medium may have been supported, at least in part, by the salts - $MgSO_4 \ 7H_2O$ and $CaCl_2$, i.e. impurities present in these (ANALAR) salts. Suggestion requires further work in which media K, M, C, N, F could be purified such as treatment with ion-exchange.

As mentioned earlier, another possible source for energy could be atmospheric carbon and nitrogen. Experiments performed with flask plugged with rubber-bunk and containing concentrated KOH in the central-well gave results similar to other (above) experiments (Table 27). In this experiment though it was assumed that KOH placed in the centre-well of the flask used, would absorb all the carbon dioxide present in the flask (i.e. present in the atmosphere

and in the medium), but it is not certain that KOH would also be able to absorb carbon-dioxide dissolved in the medium, and, which might have been utilized by organisms.

Another set of experiments in this connection was performed in the atmosphere of oxygen. In these experiments inoculated synthetic-medium were placed in a McIntosh and Filde's Jar in which air was then replaced by oxygen. Results obtained in this set of experiments showed that organism was unable to multiply in the conditions mentioned and after certain time intervals there was no measurable viable growth (Table 28). One of the possible explanations for the result obtained in this experiment could be that due to replacing air with oxygen in the jar it is possible that along with H_2, N_2 was also removed (from the jar) as it being lighter than O_2, but all CO_2 might not have been removed as it is heavier than O_2. But CO_2 and O_2 in the jar were not able to support the growth possibly because O_2 may have formed inert compounds with CO_2 and therefore the organism was unable to utilise CO_2 for growth. As in this experiment to replace air by oxygen in jar, technique used was first to evacuate the jar by means of a pressure pump and then it was filled with oxygen, it is possible that it was not able to remove all CO_2 present in the flask. Experiments should be performed in which repeated evacuating and filling of jar with O_2 should be done to remove CO_2 completely from the flask and

jar.

From the foregoing results it seems possible that either impurities present with salts compositing the synthetic-medium or atmospheric CO_2 and N_2 (also) or both mechanisms simultaneously supported the growth in synthetic-medium which did not contain any source or sources for growth and energy; the suggestion warrents further study. Also, impurities present in the medium and/or, atmospheric carbon and nitrogen in this case may be required to supply sufficient energy for growth and for induce enzyme synthesis.

The system followed for the classification of the isolate "38" was as given in the Bergey's Manual of Determinative Bacteriology, 7th edition 1957.

According to its morphological characteristics and specially marked pleomorphism, arrangement of cells, and granulation, the organism seems to be a member of family Corynebacteriaceae which belongs to Order Eubacteriales and class Schizimycetes.

The division of family Corynebacteriaceae into genera prilarily depends upon its habitat and as this organism is isolated from the soil, it belongs to second part of the family Corynebacteriace which includes genera Microbacterium, Cellulonae, and Arthrobacter. The first part of this family mainly consists of organism primarily pathogenic on anilams and plants.

As the bacterium is an active decomposer of cellulose it is placed in the genus Cellulomonas. This genus consists of 10 species, the separation of which depends upon biochemical fermentation and motility. As this organism produces nitrites and nitrate, ferments sugars xylose and arabinose besides other sugars, and is motile, it is probably Cellulomonas fimi. This organism-isolate "38"- Cellulomonas fimi seems to be of interest as it is an active reducer of nitrate to notrite. Moreover as mentioned above it is an active decomposer of cellulose. Sinha (1950) also mentioned that there was marked increase in the denitrifying bacteria and in cellulose decomposer, in the soil treated with streptomycin.

The studies regarding environmental effects on growth and on the ability to decompose streptomycin (discussed earlier in detail), of the organism, show that the organism can grow in a synthetic medium which does not contain any source(s) of carbon and nitrogen for growth and energy. This suggest that organism has capacity to synthesis cell building material and even active enzymes which can decompose antibiotic like streptomycin, from very minute quantities of energy sources such as present as impurities in the ANALAR salts composing the synthetic medium (SM medium). Also it is possible that organism may be utilising atmospheric carbon and nitrogen for these activities. The suggestion requires further work. The organism is also able to grow at a wide range of temperature ranging from 10°C to 37°C.

SUMMARY

Investigations concerning the decomposition of streptomycin were carried out according to the following lines. For convenience the procedures were divided into four (I,II,III,IV) sections:

I) Standard curves of biological assays of streptomycin in water, and in soil extracts of sterile and non-sterile soil were constructed to determine the recovery of streptomycin from treated soils and, from laboratory media.

Soil, sterile and non-sterile, was treated with 1000µg of streptomycin/g and its stability and course of decomposition was followed. The "initial recovery" of streptomycin from sterile treated soil was 460 µg/g and that from non-sterile treated soil was 310 µg/g of soil. This shows that antibiotic is strongly adsorbed by soil colloids. The extraction of streptomycin from treated soil was done with solvent acid-methanol. The amount of streptomycin recovered from sterile treated soil remainad constant during the incubation whereas it constantly decreased and none was recovered after 30 days from non-sterile treated soil. An amine like odour was noticeable during the course of streptomycin disappearance from soil. There was an increase in the bacterial population of soil at the time of streptomycin disappearance. The evidence of disappearance of streptomycin from non-sterile treated soil as compared

to constant recovery from sterile treated soil, and increase in the bacterial population at the time of streptomycin disappearance from treated soil, suggest that micro-organisms were mainly responsible for the disappearance of streptomycin from treated soil.

Soil was repeatedly treated with 1000 µg of streptomycin/g. The successive additions of streptomycin, made in the soil, after the previous dose has disappeared were decomposed rapidly in shorter time; after fourth edition, dose of (1000 µg/g) streptomycin, was decomposed in 4-5 days. "Soil extracts" from the above active soil were then inoculated into a laboratory synthetic medium (SM) containing streptomycin as the sole source of carbon and nitrogen, to study degradation of streptomycin in the laboratory media and also to facilitate the isolation of streptomycin decomposing organisms. Preliminary experiments at concentration 1000 µg of streptomycin/ml of the synthetic medium were performed but it was found to be too high to be decomposed by the mixed population (Soil extracts) of the soil. Further experiments were performed with lower concentration of streptomycin. To make conditions closer to those in soil a colloidial absorbent namely bentonite was added to the synthetic medium containing 1000 µg of streptomycin/ml (BSM medium). It was found that all streptomycin except 20 µg/ml was absorbed by the bentonite, and this small amount of free streptomycin

was decomposed by the mixed culture. Parrel experiments were performed with synthetic medium containing 1000 µg of streptomycin/ml and bentonite, and synthetic medium containing only 20 µg of streptomycin/ml. In both the media streptomycin, by mixed culture, was decomposed in the same interval of time. No antibiotic was recovered when bentonite was extracted with acid-methanol after the apparent total decomposition. This shows that decomposition of adsorbed antibiotic had also taken place. Successive additions of streptomycin at the same dose level, and at higher concentrations, were made in these media and course of its decomposition was studied.

Section II

From the above mixed cultures, isolation of streptomycin decomposing organisms was carried out. Culture samples were isolated on nutrient agar containing streptomycin and isolated colonies, after streaking on the medium for purity, were inoculated in the synthetic medium containing 20 µg of streptomycin/ml (SSM medium), to study its ability to decompose streptomycin. Twelve cultures out of 62 isolates were found to be able to decompose streptomycin in SSM medium. Out of these cultures an active isolate no."38" was used in further studies. This isolate "38" was characterised in detailed and was classified according to the system followed in Bergey's Manual of Determinative Bacteriology, 7th edition

1957. This organism proved to be a member of family Corynebacteriaceae namely Cellulomonas fimi. The organism was found to be an active reducer of nitrite to nitrate and was also able to attack cellulose such as filter-paper strip in peptone water and semi-synthetic cellulose such as Carboxy-methyl-cellulose. Detailed studies regarding effect of environmental factors on the growth and on the ability to decompose streptomycin were performed with this organism - Cellulomonas fimi.

Section III

Effect of environmental factors on the growth kinetics of the bacterium were studied. This investigations included experiments such as, effect of different carbon and nitrogen sources, concentration of such carbon and nitrogen sources in the medium, additional carbon and nitrogen sources in the medium, effect of source and size of inoculum, and effect of serial subculturing, on the growth of organism were performed.

In all the above experiments maximum growth obtained, as estimated by plate counts, was between 5×10^6-10^7 cells/ml irrespective of concentration or presence of additional carbon nitrogen sources in the medium. Also as shown in the Section IV there was no decrease in the streptomycin content in the induction period of 18 days whereas there was hundredfold increase in the viable cells/ml within

24 hours. This shows that organism was utilizing energy sources other than that of streptomycin, glucose, and ammonium sulphate, incorporated in the medium as the sole source of energy sources. In the synthetic which does not contain any carbon and nitrogen source (SM medium), once again growth to the same extent was obtained. Further experiments were performed to study the supply of energy sources in the synthetic medium. Experiments performed with SM medium prepared in double-glass-distilled water did not give any different results than that obtained with SM medium prepared in de-ionised water. But when experiments were performed with SM medium defficient in either of its salts constituents, and media composed of individual salts of SM medium i.e. media K, M, N, C, & F, it was found that impurities present in the ANALAR salts such as $MgSo_4$ and $CaCl_2$ were, at least, if not entirely, responsible for supporting growth in the SM medium. It may be possible that organism was able to utilize atmospheric carbon dioxide and nitrogen even, as its energy sources. Experiments were performed in this direction.

Section IV

Degradation of streptomycin by the organism in synthetic medium was studied. This investigation included experiments such as; decomposition of streptomycin in SSM medium; effect of successive additions of streptomycin to the culture medium after the previous dose had been decomposed, and its effect on

the pattern of streptomycin decomposition. Also experiments were performed in which subculturing of the culture was done under optimal conditions and its effect on streptomycin was studied. Effect of, size of inoculum, and additional carbon and nitrogen sources in the medium, on the streptomycin decomposition was studied.

REFERENCES

Aaslestad H.G., and Larson A.D.	(1964)	J. Bact, 88 1296-1303.
Abram, D.	(1957)	Ph.D. Thesis, Univ. of London.
Afanasief, M.M., and Sharp, E.L.	(1958)	Plant Disease Reptr. 42, 1071-73.
Anand, N, David B.D. and Armitager A.K.	(1960)	Nature, 185, 22-24.
Anderson, A.L.	(1957)	Phytopathology, 47, 515.
Anderson, H.W. and Gottlieb D.	(1952)	Econ. Botany, 6, 294-308.
Anderson, H.W. and Nienow T.	(1947)	Phytopathology, 37, 1.
Andus, L.J.	(1950)	Nature, Lond; 166, 356.
Anslow, W.K, Raistrick, H. Smith G.	(1943)	J. Chem. Soc. Ind. 62, 236-238.
Ark, P.A.	(1953)	Plant Disease Reptr. 37, 404-6
" "	(1955)	Plant Disease Reptr. 39, 926-28.
" " and Alcorn S.M.	(1956)	Plant Disease Reptr. 40, 85-92.
" " and Thompson J.P.	(1957)	Plant Disease, 41.
" "	(1960)	Plant Disease Reptr. 44, 197-99.
Asia et al.	(1951)	J. Agr. Chem. Soc. Japan, 25, 352-57.

Babayan, A.A, Karapetyan K.A, & Sarksyan, M.A.	(1958)	Agrobiologiya, 5, 101-4.
Balanchandran et al.	(1960)	Madras Agr. J., 47, 29-30.
Baron, A.L.	(1950a)	Handbook of Antibiotics, Reinhold Publ. Corpor, N.Y.
" "	(1950b)	Ibid. P.85.
Bartnicki-Gt ar ei, S, & Nickerson, W.J.	(1962)	J. Bacteriol. 84, 841-58.
Betto, E. & Bonde, P.	(1960)	Agr. Ital, 60, 125-47.
Bonde, R. & Johnson, B.	(1958)	Plant Disease Reptr. 42, 781-84.
Boyd, A.E.W., & Paton A.M.	(1958)	Plant Pathol., 7, 88-91.
Boxer, G.E., Jelinek, V.C., and Leghorn P.M.	(1947)	J. Biol. Chem. 169, 153-65.
Brian, P.W.	(1946)	Trans. Brit. Mycol. Soc. 29, 211.
" "	(1951)	Bot. Rev. 17, 357.
" "	(1957)	Microbiol. Ecology seventh Sympos. Soc. Gen. Microbiol.
" " Curtis, P.J. & H.G. Hemming.	(1946)	Trans. Brit. Mycol. Soc. 29, 173.
Brian P.W., and Hemming H.G.	(1945)	Ann. Appl. Biol. 32, 214-20.
Brian P.W, Hemming H.G. and J.C. McGowan	(1945)	Nature, Lond., 155, 637.
Brian, P.W. et al	(1951)	Nature, Lond, 167, 347.

Brown, J.G., and Boyle A.M.	(1944)	A.M. Science, 100, 528.
Brown, J.G. and Heep, D.M.	(1946)	Science, 104, 208.
Burdon, K.L.	(1946)	J. Bact, 52, 665-678.
Buxton, E.W, Khalifa, O. &, V. Ward.	(1965)	Ann. Appl. Biol. 1965, 55, 83-88.
Byrde, R.J.W.	(1959)	Plant Pathol., 8, 90-93.
Callow, K.K, and Hart P.D.	(1946)	Nature, 157, 334-335.
Chandler, W.A.	(1958)	Plant Disease Reptr, 42, 652-55.
Claus, D, and Walker, N.	(1964)	J. Gen. Micro., 36, 107-22.
Cole, J.E.	(1959)	Plant Lisease Rept, 43, 81.
Conover, R.A.	(1954)	Plant Disease Reptr, 38, 405-9.
Cordon, T.C. and Haenseler, C.M.	(1939)	Soil Sci., 47, 207-215.
Cox, R.S.	(1955)	Plant Disease Reptr. 39, 484-86.
Crosse, J.E, and Bennett, M.	(1957)	Plant Pathol., 6, 31-35.
Crosse et al.	(1960)	Ann. Appl. Biol., 48, 270-78
Crowdy, S.H, and Pramer, D.	(1955)	Chem. & Ind. (London) 7, 160-162.
Curtis, P.J, and J.F. Grove	(1947)	Nature, Lond., 160, 574.
Davey, C.B. & Papavize G.C.	(1959)	Agron. J. 51, 493.

Davey, C.B. & Papavizas G.C.	(1960)	Phytophthology, 50, 522.
Deep, I.W.	(1958)	Plant Disease Reptr, 42, 476-78.
Deep, I.W. and Bartlett, A.	(1961)	Plant Disease Reptr, 45, 628-31.
Dekker, J.	(1963)	Ann. Rev. of Microbiology, 17, 243-63.
Dubos, R.J.	(1939)	J. Exp. Med. 70, 1-17.
Ducheesne, E.	(1897)	Thesis, Lyon.
Dunegan, J.C, J.R. Kienholz, R.A. Wilson & W.T. Morris.	(1954)	Plant Dis. Reptr., 38, 666-669.
Dye, E.N, and Dye M.H.	(1954)	New Zealand J. Sci, Technal., A.36, 21-26.
Eaton, E.O., and King C.J.	(1934)	J. Agri. Res., 42, 1109-1113.
Endos, S.	(1931-33)	Bull. Miyazaki Coll. Agr. For., 4, & 5.
Erhlich, J., Anderson L.E, Coffey, G.L.& Gottlieb, D.	(1952)	Antibiot. Chemother. 2, 595.
Evans, W.C, Smith, B.S.W, Lingtead, R.P and Elvidge J.A	(1951)	Nature, Lond; 168, 772.
Erikson, D.	(1941)	J. Bact. 41, 277.
Fellows, H.	(1929)	Phytopathology, 19, 103.

Ⓦ Quoted in Microbial Antagonism and Antibiotic substances, S.A. Waksman, 1947.

Fenikseva, R.V.	(1957)	Proceeding Int. Symp. Enz.Chem. Tokyo & Kyoto, 482-85.
Fleming, A.	(1929)	Brit. J. exp. Path. 10, 226-236.
Florey et al.	(1949)	Antibiotics. Vds. I & II, Oxford, Univ. Press.
Folin, O, and Ciocalteau, V.C	(1927)	J. Biol. Chem. 73, 627.
Ford, J.H, Klomparens W, and Hamner, C.L.	(1958)	Plant Disease Reptr. 42, 680-95.
Forsyth, W.G.E, Hayward A.C, & Robert, J.B.	(1958)	Nature, Lond., 182, 800.
Frank et al.	(1959)	Antibiot. Ann., 1958-59, 898-902.
Fred, E.B. & Waksman, S.A.	(1928)	Laboratory Manual of Gen. Microbiology, New York, M C Graw-hill
Frost, W.D.	(1904)	J. Infect. Dis., 1, 599.
Garrett, S.D.	(1939)	Waltham, Massachusetts, Chronica, Botanica.
" "	(1944)	Root Disease Fungi, 1st ed. Waltham, Mass. U.S.A.
Gause, G.F.	(1946)	Science, 104, 289-290
Ghadially, N.	(1961)	Ph. D. Thesis, Univ. of London.
Ghillini, C.A. & Alghisip	(1960)	Notis. Mal. Piante, Italy, 52, 15-22.
Gilliver, K.	(1946)	Ann. Bot., 10, 271-282.

Goodman, J.J., & Henry A.W.	(1947)	Science, 105, 320.
Goodman, R.N.	(1953)	Am. Fruit Grower, 73, 7, 16, 17.
" "	(1954)	Proc. Am. Soc. Hort. Sci. 64, 186-90.
" "	(1954)	Plant Disease Reptr. 38, 874-72.
" "	(1959)	Antibiotics, Their Chemistry & Nonmedical uses, 322-448 (Goldberg H.S. Ed; Van Nostrand Co., Princeton, N.J. 1959.
Gottlieb, D. & Siminoff, P.	(1950)	Phytopathology (abs) 50, 11.
" "	(1951)	Phytopathology 41.
Gottlieb, D. and his Colleague	(1951)	Phytopathol., 41, 393.
" and Siminoff, P.	(1952)	Phytopathology, 42, 91-97.
" " and Martin, M.M.	(1952)	Phytopathol., 42, 493-96.
Gregory, K.F, Allen, O.N., Riker, A.J. & Peterson W.H.	(1951)	Bact. Proce. Vol, for 1951, P.18.
Gregory, K.F. Allen. O.N., Riker, A.J. & Peterson W.H.	(1952)	Am. J. Botany, 39, 405-415.
Gregory, K.F. Allen. O.N., Riker, A.J. & Peterson W.H.	(1952b)	Phytopathology 42, 613-22.
Greig - Smith.	(1912-15)	Proc. Linn. Soc. N.S. Wales, 36, 679, 40, 631, 42, 162.
Griffiths, D.J. & Peregrine W.T.H.	(1960)	Plant Pathol., 9, 11-14.

Grossbard, E.	(1948)	Plant disease IV. The control of plant disease by microbial antagonism Rept. Exptl.Research Sta. Cheshunt, pp. 29-39.
Grossbard, E.	(1948b)	Rep. Exp. Res. Stat. Cheshunt, 34, 37.
"	(1952)	J. Gen. Microbiol, 6. 295-310.
"	(1952b)	Ph. D. Thesis, Univ. of London.
Grover, R.K.	(1960)	Dissertation Abstr. 21, 1323.
Halliwell, G.	(1961)	Biochem. Journ. 79, Part I, 135-92
Heggestad, H.E., and E.E.Clayton	(1954)	Plant Dis. Reptr., 38, 661-65.
Hessayon, D.G.	(1951)	Nature, 168, 998.
Hildreth, A.A. & P.M. West.	(1941)	Canad. J. Res. C., 19, 183.
Hitier, H, and Izard, C.	(1960)	Biol. Abstr., 35, 11, No.30515.
Hutchinson, H.P. & Thayson, A.C	(1918)	J. Agric. Sci., 9, 43-62.
Jacobs, S.E. & Dadd, A.H.	(1959)	Ann. Appl. Biol. 47, 666-672.
Jeffreys.	(1952)	Journ. Gen. Microb. 7, 295-312.
Kalyan Sundaram, R.	(1955)	Current Sci., 24, No.9, 310.

Katz, E., and P. Pienta.	(1957)	Science, 126, 402-3
Keil, H.L., & Wilson G.A.	(1962)	Plant Dis. Reptr., 46, 397-400.
Khalifa and Ward.	(1965)	Ann. App. Biol. 55, 83-88.
Klein, D., and Premer D.	(1962)	J. Bact., 83, 309-313.
Klinkowski, M.	(1961)	Proc. Int. Congr., Botany Conger. Montreal 1958, 393-96.
Ktisiewicz, J.M., & Pound G.S.	(1961)	Phytopathol., 51, 495-500.
Klos, E.J.	(1958)	Mich. State Univ. Agr. Expt. Sta. Quart. Bull. 40, 653-58.
Kock, P.C.	(1958)	Mededel. Landbouw, Logeschool Opzoekingsskat, Stoat Gent, 23, 805-8.
Koperoff, N. & Beerman, P.	(1922)	Science, 126, 402-3.
Koreniyako et al.	(1955)	Microbiologia, 25, No.5.
Korenyako A.T. & Koveshnikov A.D.	(1960)	Doklady Akad. Nauk SSSR, 25, 746-52.
Krasilnikov, N.A.	(1954)	Dokl. An. SSSR, 94 No.5.
"	(1954)	E.R. Acad. Sci, U.R.SS. 94, 957.
Kriger, W.S.	(1959)	African J. Agr. Sci, 2, 195-205.
"	(1961)	Phytopathol, 7, 42, 362-74.

Lal, A.	(1939)	Ann. Appl. Biol., 26, 247-261.
Leach, L.D., and Davey A.E	(1942)	J. Agri. Res. 64, 1-18.
Lichstein, H.C. & Van de Sand V.F.	(1945)	J. Infect. Dis., 76, 47-51
"	(1946)	J. Bact. 52, 145-46.
Linford, M.B.	(1942)	Soil Sci., 53, 93-103.
Logsdon, C.E.	(1961)	Am. Potato. J., 38, 1-5.
Lochhead, A.G. & Thexton, R.H.	(1947)	Can. J. Research C., 25, 20-26.
" " & Landerkin G.B.	(1949)	Plant & Soil I, 271-76.
Luepschen, N.S.	(1960)	Cornell Univ., Agr. Expt. Sta. Mem. No.375.
MacLeod, D.M.	(1959)	Can. J. Bot. 37, 695-714.
"	(1959a)	Can. J. Bot. 37, 819-834.
Maier, C.R.	(1960)	Phytopathol., 50, 351-56.
Martin, N. & Gottlieb D.	(1952)	Phytopatho., 42, 294.
" "	(1955)	Phytopatho., 45, 407-408.
McFadden, L.A.	(1961)	Phytopatho., 51, 663-68.
McCowan, J.C.	(1946)	Trans. Brit. Mycol. Soc. 29, 188.

McIntosh A.F. and Meyrath J. (1963) J. Gen. Microbiol., 33, 57-62.

" " (1964) Arch. Microbiol., 48, 368-380.

McNew, G.L., (1955) Proc. Intern. Conf. Antibiot., Agr. 1955, No.397, 189-91 (Natl. Acad. Sci. 1956).

Meyrath, J. (1962) Experientia, 18, 41-42.

" (1963) Antonie Van Leeuwenhoek, J. Microbiol. Serol. 29, 57-78.

" (1964) Experientia, 20, 257-258.

" and McIntosh A.F. (1963) J. Gen. Microbiol., 33, 47-56.

Millard, W.A. (1923) Ann. Appl. Biol. 10, 70.

" and C.B. Taylor (1927) Ann. Appl. Biol. 14, 202-216.

Miller, P.W. (1959) Plant Disease Reptr., 43, 401-2.

Mirchink, T.G., and Greshnykh K.P. (1961) Microbiologiya, 30, 1045-49.

Monib, M. (1953) Ph. D. Thesis. Univ. of London.

Moss, V.D. (1957) Plant Dis. Reptr., 41, 709-14.

" (1958) Plant Dis. Reptr., 42, 703-6.

Moss, V.D.	(1961)	Forest Sci., 7, 380-96.
Moss et al.	(1960)	J. Forestry, 58, 691-95.
Müller, K.O., Mackay, J.H.E, and J.L. Friend	(1954)	Nature, 174, 878-79.
Nimmer, S.	(1950)	Ph. D. Thesis, Univ. of London.
Nandi, P.N.	(1948)	Ph. D. Thesis, Univ. of London.
Napier et al.	(1954)	Ann. Appl. Biol. 44, 145-51.
Nalti, J.J.	(1959)	Plant Disease Reptr., 43, 735-40.
Nauta et al	(1946)	Rec. Trav. Chim. Pays-Bas, 65, 865.
Neilson and Jones, E.	(1941)	J. Agric. Sci., 31, 379.
Newman, A.S. and Norman A.G.	(1943)	Soil Sci, 55, 377-391.
Nikitina, Mme, E.T.	(1958)	Trud. Inst. Microbiol. Virol; Acad. Sci. Kazaki S.S.R. 2,24-41 Crev.Appl.Mycol., 38, 29-30, 1959.
Nissen, V.T.	(1954)	Nature Lond; 174, 226-227.
Novogrudsky, D.M.	(1936)	Adv. Mod. Biol. (U.S.S.R) 5, 509-536 Bull. Acad. Sci., (U.S.S.R), 1, 277-293.
Nultsch, W.	(1958)	Zentr. Bakterid, Parasitenk; Abt.II, 111, 387-404.

Ono, H, and A. Kanbayashi.	(1954)	J. Ferment. Assoc. (Japan) 12, 49-52.
Palmer et al.	(1959)	Plant Dis. Reptr., 43, 494-95.
Palmer J.G. and Henneberry T.J	(1960)	Am. Hort. Mag. 39, 108-10.
Park, J.J. and Johnson M.J.	(194a)	J. Bio. Chem. 181, 149.
Parker, K.G.	(1961)	N.Y. State, Hort. Soc. Proc, 106, 171-777.
Perlman. D.	(1952)	Bact. Proc., Boston, Massachusetes 58.
Pfenning, N. and Wiken T.	(1960)	Zentr, Bakteriol. Parasitent, Abt.II 113, 491-510.
Pramer, D.	(1953)	Ann. Appl. Biol. 40, 617-622.
Pramer, D. and Starkey R.L.	(1950a)	Bacteriol. Proc., Baltimore, Maryland, P.18-19.
" "	(1950b)	Proc.VII Intern. Botanical. Congr; Stockholm, Sweden, P.256-57.
Pramer, D. and Starkey R.L.	(1951)	Science, 113, 127.
Pramer D. et al.	(1956)	Phytophtho., 46, 341-42.
Quastel, J.H. and Scholefield P.G.	(1951)	Bact. Rev, 15, 1.
Raistrick et al	(1941)	Chem. and Ind. 60, 828-830.

Rangaswami, G.,	(1957)	Indian Phytopathol., 10, 8-10.
" and Ethiraj	(1962)	Phytopathol., 52, 989-92.
" & Rao, Rama Rao, and A.R. Lakshmanan, A.R.	(1959)	Phytopathol., 49, 224-29.
Rangaswami, G. and P. Vidyasekaran.	(1963)	Phytopathol., 53, 995.
Rayner, M.C.	(1934)	Forestry, 8, 96.
"	(1936)	Forestry, 10, 1.
"	(1939)	Forestry, 13, 19.
"	(1941)	Forestry, 15, 1.
Rhodes, A.	(1962)	In Antibiotics in Agr. 101-22 (Woodbine M. Ed, Butterworths London, 1962).
Robinson et al	(1954)	Phytopathol., 44, 646-50.
Rodriguez et al	(1957)	Plant Dis. Reptr., 41, 560-63.
Roy, D.K.	(1955)	Science & Culture (India) 20, 565-66.
Sabet, K.A.	(1956)	Ann. Appl. Biol., 44, 152-66.
Sakakibara, E.	(1951)	Acta. Schol. Med. Univ. Kioto, 29, (quoted from Brit. Abst. 1952, A, III, 1938).
Sanford, G.B.	(1926)	Phytopathol., 16, 525-47.

Sanford, G.B.	(1959)	In C.S. Holton, et al (ed.) Plant Pathology Problem and Progress; 1908-1958 Univ. Wisconsin Press Madison
Sanford, G.B. and Broadfoot W.C.	(1931)	Sci., Agri. 11, 512-528.
Schenk J.R. and Spielman M.A	(1945)	J. Am. Chem. Soc., 67, 2276.
Shaw, L., Lucas, G.B. and Wilson J.H.	(1959)	Plant Disease Reptr., 43, 753-54.
Shiradkar, S.	(1953)	Ph. D. Thesis, Univ. of London.
Shu, P., and A.C. Blackwood	(1951)	Canad. J. Bot., 29, 113-124.
Siminoff, P., and Gottlieb D.	(1951)	Phytopathol., 41, 420-30.
Sinha, P.	(1950)	Ph. D. Thesis, Univ. of London.
Singh, P., R.S. Vasudeva and B.S. Bajaj.	(1965)	The Annals of App. Biol. 55, 89-97.
Slag C.N. and Fellows, H.	(1947)	J. Agr. Res. 75, 279.
Smith, W.J.	(1949)	J. Colo. Wyo. Acad. Sci., 4, 49.
Smith et al	(1948)	J. Bact., 55, 425-47.
Smith N.A. and Guyer G.	(1961)	Mich. State Univ. Expt. Sta. Quart. Bull. 44,-226-29.
Smith G.N. and Worrel C.S.	(1950)	Arch. Biochem, 28, 232-41.
Snirnova	(1956)	Microbiologija 25, 423-28.

Sowell, G.	(1959)	Plant. Dis. Reptr., 43, 193-97.
Sprague, R.	(1958)	Plant Disease Reptr., 42, 1208-9.
Stall, R.E.	(1959)	Plant Dis. Reptr., 43, 725-28.
" and Thayer P.L.	(1962)	Plant Dis. Reptr., 46, 389-92.
Staněk, M., and Pre slička M	(1960)	Rost linna Výroba, Pragua, 33, 1191-1204.
Staněk et al	(1959)	Z. Rostlinna Výroba, Prague, 22, 941-50.
Staněk, M, and Wasserbauer R.	(1960)	Rostlinna Výroba, Prague, 33, 1147-62.
Starkey, R.L. and D. Pramer	(1953)	Proc. VI, Intern. Congr. Microbiol. Rome, Italy, 6, 344-45.
Steel et al	(1954)	Can. J. Microbiol. 1, 150-57.
Steiner, K..	(1966)	Thesis, Eidgen. Techn. Hochschule, Zurich.
Stevenson, I.L.	(1956)	J. Gen. Microbiol. 14, 440-48.
"	(1956)	J. Gen. Microbiol. 15, 372-80.
" and A. Lockhead	(1953)	Canad. J. Bot. 31, No. 23.
Stoddard E.M.	(1957)	Plant Disease Reptr., 41, 536.
Subramanian, C.V.	(1946)	J. Indian Bot. Soc. 25, 89.

Synder, W.C. Schroth, M.N and Christen, T.	(1959)	Phytopathology, 49, 755.
Szybalski, W.	(1952)	Bact. Proc., 36.
" and Shizuyoshi M.	(1959)	Biochem. Biophys. Research Commun. 1, 249-52.
Szirmai, I., and Vörös J.	(1960)	Kisérlet,Közlemények Budapest, 2, 25-37.
Taber, W.A.	(1957)	Can. J. Microbiol. 3, 803-812.
" and Vining L.C.	(1958)	Can. J. Microbiol. 4, 611-626.
Tanner, F.W. and Beesch S.C.,	(1958)	Advanc. Enzymol., 20, 383-406.
Thayner, P.L and Stall R.E.	(1961)	Phytopathol, 51, 568-71.
Thompson, H.S.	(1961)	Can. J. Plant. Sci., 41, 227-30.
Thronton H.G.	(1922)	Ann. Appl. Biol. 2, 241-74.
Tikhomirova, A.S.	(1954)	Microbiologija (engl. Trans) 28, 40-46.
Tim. E.C.	(1932)	Phytopathol. 22, 27.
Valaskova, E.	(1958)	Cesk Mikrobiol., 3, 325-35.
Valaskova E.	(1961)	Tagungsberichte Nr.33. Deut. Akad. Landwirtschaftswissenschaften, Berlin. 217-32.

Waksman S.A.		(1927)	Principles of Soil Microbiology. Waverley Press U.S.A
"		(1944)	Proc. Soc. Exp. Biol. and Med. 55, 66.
"		(1948)	Antibiotics Biol. Rev. 23, 452.
"		(1949)	Ann. Appl. Biol., 36, 257.
"	Bugie E.S. and Spencer E.L.	(1943)	J. Bact. 45, 233-248
"	Horning E.S. and Spencer E.L.	(1943)	J. Bact. 45, 233-248
"	and Lechevalier	(1949)	Science, 109, 305.
"	and Woodruff H.B.	(1942)	Soil Science 53, 233-239.
Webb, F.W.		(1958)	Agr. (London) 64, 493-97.
Weindling, R.		(1932)	Phytopathol. 22, 837-45.
"		(1934)	Phytopathol. 24, 1153-79.
"		(1945)	Acta Path. Microbiol. Scand., 22, 379-91.
"		(1946)	Soil Sci, 61, 23-30.
"	and Emerson O.H	(1936)	Phytopathol, 26, 1068-70.
Wiken, T, Somm, H, & Sulzer F		(1953)	Arch. Mikrobiol. 20, 201-220.

Wilkins, W.H.	(1949)	Ann. Appl. Biol., 36, 257.
Winfree et al	(1958)	Phytopathol. 48, 311-16.
Winogradisky, S.N.	(1949)	Microbiologie du sol; problems et methods masson et cie, paws.
Winter, G.	(1940)	Ztschr. Pflanzenk., 1, 113-34.
Winter and Willeke	(1951)	Natu Wissenschaften 38, 437.
Wood, R.K.S.	(1951)	Ann. Appl. Biol. 38, 203-30.
Wood, R.K.S. and M. Treit	(1955)	Botan. Rev. 21, 441-492
Woodthrope T.J. and Ireland D M.	(1947)	J. Gen. Microbiol. 1, 344-352.
Wright, J.M.	(1954)	Ann. Appl. Biol. 41, 280-289.
"	(1955)	Ann. Appl. Biol. 43, 288-296.
"	(1956)	Ann. Appl. Biol. 44, No.3
" and J.F. Grove	(1957)	Ann. Appl. Biol. 45, 36.
Zaumeyer, W.J.	(1955)	Agr. Food Chem., 3, 112-16.
"	(1956)	Phyto., 46, 32.
"	(1958)	Ann. Rev. of Micro. 12, 415-40.
Zaumeyer W.J. Thomas H.R. and Mitchell J.W. Fisher H.H.	(1953)	Phytopathology, 43, 407
Zentmyer, G.A.	(1964)	Phytopathology, 53,1383.