Chapter 2

Review of Literature

I. Actinomycetes

A. History

Von Langenbeck (1845), who was the first to observe the occurrence of the fungus infection on a patient; but it was James Israel (1878) who published the observations of Von Langenbeck and described an actinomycete as a causative agent of human disease.

Initial work on the actinomycetes was done by Ferdinand Cohn in 1872-5, and C.O. Harz in 1878.[4] Cohn named an organism he found in the tear duct of a human eye *Streptotrix foersteri*. It was soon followed by a description by Harz of another organism, named *Actinomyces bovis*, found in a 'lumpy jaw' of a cow. These findings include organisms causing saprophytes,occuring in soil, dust, water basins and in other natural substrates.[4,5]

Cohn's work on actinomycetes and on the matter of bacteria in general was completely neglected by nearly all botanists following him.

Simultaneously with the work of Bollinger and Harz in 1878, Israel was, in 1878, studying pathological material from pyaemia and suppuration in the neck of man, and he observed granules, which contained mycelium, similar to those described by Bollinger in cattle and with in the past had been diagnosed as scrofulosis or osteosarcoma, Israel

named this parasitic infection 'actinomycosis'.

Bostroem published in 1890 the important paper on 'Investigation of Actinomycosis in Man'. He declared that *Actinomyces* was a fungus, which provoked inflammation in the tissues of animals.[5]

This work was followed by that of numerous other investigators, notably Bergey (1907), who was the first to isolate *Actinomyces* from a normal human mouth. Waksman and Curtis (1916) and Chirurco (1921) identified Bergey's theory with that of Klinger and Colebrook, namely that the actinomycetes have a destructive tendency through symbiosis with aerobic schizomycetes.[5]

The actinomycetes have always been a strange group of organisms to the bacterial taxonomists. The number of investigators studying this group of microbes has always been small and for the part these organisms have been neglected by medical bacteriologists, physiologists and biochemists. Such affairs were to be expected when one recalls that until recently not even their taxonomic positions were certain.[6] Bacteriologists considered them as bacteria and mycologists generally considered them as fungi. The taxonomy position of the actinomycetes has been one of the most debatable questions in microbiology, bacteria or fungi.

The size (width of thallas) and staining properties of the actinomycetes have usually placed them with the bacteria but their branching and manner of sporulation have related to the fungi. Avery and Blank (1954)[7] included that "from the chemical point of view *Actinomycetales* have nothing in common with the true fungi, but rather

with the bacteria." From the study on the cell-wall composition, chitin is absent from the cell substance of actinomycetes as well as from bacterial cells, but is present in fungus mycelium and spores.[8] Neither actinomycetes nor bacteria have been shown to contain true nuclei, they both lack nuclear membranes and mitochondria.[9] Actinomycetes are usually sensitive to antibiotics that are active upon bacteria, they are usually resistant to those antibiotics like the polyenes, that are active upon fungi but not upon bacteria.[10]

This era is now over and the actinomycetes are generally accepted as bacteria.[11,12]

B. Distribution of actinomycetes

Actinomycetes are among the most widely distributed group of microorganisms in nature. Members of the actinomycetes which live a saprophytic existence can be found with greater or less frequency in most ecological setting, soil having the greatest population density. Numerically they are less dominant than the other bacteria and more prominent than the fungi. The numbers vary a great deal but it is not uncommon in fertile soils from many different parts the world.[13] Very few natural substrates are entirely free from them. The ability of actinomycetes to survive for a long time is indicated from the slime of a mammoth's nose.

Actinomycetes are found in all soils throughout the world. They are usually found in the surface layers where oxygen is present. They occur in greatest numbers in the top few inchs of the soil and decrease with depth. They are found in aqueous habitats both in freah water and marine habitats. Erikson isolated from lake mud and in one

instance from lake water.[16] Umbreit and Mc Coy found that 10 to 20 percent of the total microbiolgical population found in the water of the lakes of the northern highland region of Wisconsin comprised species of the genus *Micromonospora*.[17] There is a natural actinomycetes flora in marine littoral and in bottom mud.[18,19] Other types are favoured by particular oxygen environments;*Actinomyces israeli*, for sample, is anaerobic, whilst a number of other species are micro-aerophilic.

C. Characterization of actinomycetes

Bacteria that tend to form branching filaments which in some families develop into a mycelium. The filaments may be extremely short or well developed. The diameter varies from 0.5-2.0 μm, generally less than 1.0 μm. Filaments are not always observed because in certain families the filaments tend to fragment and can be seen only in some cultural stages of development or in host tissue. Fragmentation of filaments lead to the formation of coccoid,elongate or diphteroid elements.

Spores may be produced singly on the hypha, as a pair or as chains of various numbers of spores. If sufficient numbers of spores are present the chains can be straight, looped or spiral. Such chains arise singly from the hyphae or in a verticillate manner.

Gram-positive, though this reaction may vary with the age of the culture. Some are acid-alcohol-fast and some members are weakly acid-fast.

D. Classification of actinomycetes

Many systems of classifying the actinomycetes have been suggested. These were based upon their activities in a natural environment such as pathogenic and nonpathogenic forms, upon their cultural characteristics such as pigmentation and gelation liquefaction, or upon their morphology, especially the manner of sporulation.[4]

According to Bergey's manaul of determinative bacteriology, actinomycetes in place in the order *Actinomycetales* with eight families.[6]

This literature review only dealed with the well recognized genera of the actinomycetes as *Actinomyces*, *Streptomyces*, *Nocardia*, *Micromonospora*, *Microbispora*,*Actinoplanes* and *Streptosporangium*. The great detail was in genus *Streptomyces* that members of it were mostly found to be the antibiotic-producing organisms.

The genus *Actinomyces*

The genus *Actinomyces* is very sparse development of erect hyphae in growth produced in an atmosphere of reduced oxygen tension. These hyphae are occasionally septate, but no definite spores are formed; aerial mycelium heavier than vegetative mycelium, 1 μm or even more in diameter. Arthrospores about 2 μm long. The substrate mycelium is initially unicullular, and the branches may external into long filaments, causing the colony to adhere to the medium, or may give rise more or less quickly to irregular segments and characteristic angular branching. The colonies exhibit a considerable degree of polymorphism, but no stable variants have been established. Liquid media are usually clear.[20]

This genus comprises gram-positive, irregularly staining, non-spore forming, non-motile anaerobic or micro-aerophilic organisms.[12]

They are mostly pathogenic in nature. The pathogenic form are non-acid fast, non-proteolytic, and non diastatic. These have been isolated from granules in the pus of morbid tissues of a human and animal disease known as actinomycosis. They produce no filterable stages and show no serological reactions with other genera. There were also recorded observations concerning the occurrence in various natural substrates of non-pathogenic, mesophilic, anaerobic actinomycetes that could with full justifications be included in this genus. Although few of these have been sufficiently studied, one such species is included. The saprophytic forms may be proteolytic, actively fermentative and may possess marked reducing properties. The natural relationship of this genus to the other genera of the actinomycetes, based primarily upon morphological and cytological studies, has recently been examined.[21]

The genus *Streptomyces*

Morphology

Streptomyces is the generic name given by Waksman and Henrici in 1943[22] to the aerobic sporulating species which belong to the family of *Streptomycetaceae*. The substrate mycelium of a *Streptomyces* does not segment spontaneously into bacillary or coccoid forms.

It produces leathery or tough-textured growth,remaining nonseptate and coherant even in old cultures. The aerial mycelium is usually thicker than the substrate mycelium. While the morphology of the substrate mycelium is usually undifferentiated. The arrial mycelium, under fixed conditions of culture, shows sufficient differentiation. This is one of the most important criteria for classification in the genus *Streptomyces*.[10] Several aspects relating to the aerial mycelium may

be considered :

a) Gross macroscopic appearance The relative abandance structure (cottony, velvety, powdery), formation of rings or concentric zones, and pigmentation of the aerial mycelium are important diagnostic criteria.

b) Microscopic properties The microscopic structure of the aerial mycelium gives a clear picture of the morphology and reproductive structures of the organism. The hyphae may be long or short, with extensive or little branching. The branching may be simple or complex, monopodial or synpodial, brown-shaped or verticillate. The fruiting bodies or sporophores are short or long, occurring singly, inclusters or as verticils; they are straight, wavy or spiral-forming. The spirals or coils are either long and open or short and compact. Spiral formation may take place on one medium and not on others.

Pridham et al. (1958)[23] proposed four sections : straight to flaxous (Rectus - Flexibilis), hooks, loops or open spirals (Retinaculum-Apertum), tight spirals or coils (Spira) and verticillate (Biverticillus). Verticillate strains were placed in the genus *Streptoverticillium* by some workers. Spore chain morphology has nevertheless proved to be a consistent and reliable character for the majority of strains included in both the present study and the International Streptomyces Project (I.S.P.).

c) Spores The spores, also called conidia, produced from, or in, certain hyphae of the aerial mycelium, or the "sporogenous hyphae", may be oblong, oval or spherical. Krassilnikov (1949)[25] attached great importance of this character, as determined by the light microscope,

as a diagnostic feature.

Kriss et al. (1945)[26] were the first to use the electron microscope for study of spores of *Streptomyces*. Flaig et al. (1952) found that some strains had smooth surfaces while others had spiny surface. They later detected spores with hairy and warty surfaces; the nature of the nitrogen source influenced the appearance of the spore surface,organic nitrogen favouring spine formation.[10]

Kuster (1955) classified *Streptomyces* spores into two groups: (a) those producing a smooth surface and (b) those having a rough surface. Each of these groups was divided into three subgroups, based on shape of the spores. Thus there are spores with smooth surfaces, with spines, with hairs or with warty protuberance and spores that are globose,long-ovoid and cylindrical.[10]

Streptomyces species produce a slender,coenocytic hyphae. The diameter of the hyphae is usually 0.5-2.0 µm. The aerial mycelium at maturity forms chains of three to many spores 0.5-2.0 µm in diameter, gram positive, aerobes. On isolation colonies are small (1-10 nm diameter)discrete and lichenoid, leathery or butyrous; initially relatively smooth surfaced but later develop a welf of aerial mycelium that may appear granular, powdery, velvety or floccose. Spores or conidia are formed in spiral spore-bearing hyphae or sporophores which arise from the aerial mycelium either monopodially or in the form of tufts or verticils.[10,11]

Cultural and biochemical characters

1. Formation of pigments

The formation of soluble and insoluble pigments of the substrate and aerial mycelium in organic and synthetic media play a

major role in characterizing species. Unfortunately, color characteristics vary greatly with age of the culture, composition of the medium, temperature of incubation and nature of the inoculum.

With the synthetic media, it comes to be recognized that different organisms are able to produce a great variety of pigments, some are water soluble. The presence of oxygen is essential for pigment, both soluble and insoluble. The formation of deep brown to black pigments in organic media containing proteins and protein derivatives, notably the amino acid tyrosine, is an important species characteristic. Since about one third of all species of *Streptomyces* now recognized are melanin-positive.[27,28]

Lechmann and Sano (1908) first suggested the expression "tyrosine reaction". They used for their studies a tyrosine-containing medium, melanin being known to be an oxidation product of tyrosine. Shinobu (1958)[29] attached great importance to the "tyrosine reaction" in the species characteristic of *Streptomyces*. This ability to produce pigment, led many of the earlier investigators to designate certain species as "chromogenic".

The formation of yellow, red, blue, green, and other soluble pigments is also highly characteristic of the species growing on synthetic media. There is considerable variation in the intensity of these pigments, depending upon the strain of organism. In view of the fact that color standards are not always available, it is suggested a series of color designation which are simple and convenient. Pigment formation is considered as a constant specific property, although the nature of the pigments varies with the composition of the medium.[30]

The color of the aerial mycelium is not considered as constant and is greatly influenced by the composition of the medium.

2. Utilization of carbon sources

The ability to utilize as sources of carbon and energy various organic compounds, such as carbohydrates, alcohol, salts of organic acid, fats and amino compounds, can be offered a criteria of certain diagnostic value. These studies date from the early work of Waksman in 1919.[27] The use of liquid substrate was later found to give misleading results. Numerous studies indicate that solid substrates and different basal media were later used.[31,32]

Hata et al. (1953)[33] found a correlation between the groups and types of organisms established on the basis of carbon utilization and their antistreptomytic and antibacterial spectra.

The best carbon source for characterizing *Streptomyces* species were found to be raffinose, L-xylose, D-fructose, L-arabinose and D-mannitol. Gordon and Mihm (1959)[34] considered as a species characteristic the utilization of acetate, malate, propionate, pyruvate and succinate. None of the utilization of the actinomycetes produce gas. Some are able to form acid from certain carbon sources.[35]

3. Utilization of nitrogen compounds and others

The ability to use different nitrogen compounds for their growth may offer criteria of certain diagnostic value. Certain chemical changes such as proteolytic activity, nitrate reduction and ammonia formation. Shinobu (1958)[29] considered the utilization of urea, creatinine and certain amino acids, as of some importance in species.

characterization.

The use of hydrogen sulfide production as a taxonomic implementation in the differentiation of *Streptomyces* species has been suggested by Pridham (1948).[31] When employed in conjunction with other physiological, cultural and morphological criteria, hydrogen sulfide production was said to give promise as an aid in the systematics of the genus *Streptomyces*.

Among the proteolytic activities of diagnostic value in separating genera, liquefaction of gelatin, hydrolysis of casein and peptonization of milk are very important. Some species show strong activity and other show less or no liquefaction. This property, as well as milk peptonization, when combined with the ability to produce brown to black pigments could provide significant criteria for species characterization. Reports of inability to liquefy gelatin of certain species may often be questioned.[27,36] Repeated tests with different inocula might have shown different results.

The reduction of nitrate to nitrite has been universally used among the criteria for species differentiation. In view, however, of the influence of nutritional factors upon this reaction, and its quantitative rather than qualitative nature.

4. Ecology

The natural substrate of an organism, especially diseased plants or animals, and composts of stable manures and plant resisued at high temperature, is of some systemic significance. Various attempts have been made of utilize the ecological characteristic of the actino-

mycetes as a basis of classification. Thus, the following rather broadly defined ecological categories have been proposed at various time to classify actinomycetes: animals parasites, plant parasites, soil inhibitants, water inhibitants, mesophilic forms, thermophilic forms, inhibitants of acidic (pH 3 to 6.5) substrated and inhibitants of neutral to alkaline substrated (pH 6.5 and above). The temperature at which an organism is grown greatly affects the nature and amount of growth, the nature and extent of sporulation, and the degree of formation of soluble pigments. The optimum temperature for the growth of most species of *Streptomyces* is between 25° and 30°C. Only a few of these organism are thermophilic. The optimum reaction for the growth of actinomycetes is pH 6.8 to 7.5. When these organisms are grown on complex organic media and on many synthetic media, the reaction usually becomes alkaline

5. Production of specific chemical compounds

These characteristics include the production of vitamins, enzymes and antibiotics. Streptomyces have been found to produce these highly valuable chemical substances, especially antibiotics. The ability of these organisms to produce various kind of antibiotics, in some strains produce more than one antibiotic, can provide a potential diagnostic aid. It should be emphasized, however, that the characteristic of producing a particular antibiotic is a strain-characteristic rather than a species characteristic. The fact that a large proportion of all the culture of *Streptomyces* isolated from natural substrates show some degree of inhibition of growth of other microorganisms, when tested on suitable media, suggested the ability to form antibiotics.

Some strains of *Streptomyces griseus* reportedly produce one or more different kinds of antibiotics, including cyclohexamide, albomycins, chromomycin and costreptomycin.[37]

Krassilnikov[38,39] tended to overwhelmphasize the importance of antibiotics formation in species characterization of actinomycetes. Many species and even individual cultures are able to form a variety of different antibiotics; on the other hand, the same antibiotic may be produced by different organisms.

6. Antibiotic susceptibility

William (1967)[40] showed the correlation of taxonomic information on *Streptomyces* and their antibiotic susceptibility patterns. However, the significance of this has not been firmly established yet.

7. Actinophage sensitivity

Phage typing of actinomycetes might be of some help in identifying unknown isolates. Actinophages vary greatly in their ranges. Most actinophages which were tested against a large number of organisms proved to be polyvalent.[41] Some phages have been found to be specific, causing the lysis of strains of only a few species or of only certain strains of only a few species or of only certain strains of one species. The separation of streptomycin-producing strains from grisein-producers and other members of the former *S. griseus*, which is now regarded as a species group rather than a single species.[42] Some streptomycin-producing cultures have been found, however, that are resistant to these specific phages.[43,44]

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The genus *Nocardia*

The genus *Nocardia* represents a group of aerobic actinomycetes which includes both pathogens and saprophytes. Numerous culture of nocardia have been isolated from human and animal infection. The colonies produces by nocardias are either smooth or rough and much folded. The first pathogenic aerobic actinomycete was describe by Nocard and later was named *Nocardia farcinica* by Treuisan. Many species of *Nocardia* do not form any aerial mycelium. Nocardias multiply by concentration and segmentation of the protoplasm within a filamentous cell, followed by dissolution of the cell membrane. The fragmented portions of the mycelium usually develop in to fresh mycelium under favorable conditions. The colonies of nocardias never form on aerial mycelium but there are culture whose colonies are covered with a thin coating of short aerial hyphae which break up into cylindrical oidiospores.[45] The genus *Nocardia* has slender filaments or rods, frequently swollen and branched, shorter rods and coccoid forms are found in older cultures.

The genus *Micromonospora*

The genus *Micromonospora* is well developed, branched, septate mycelium averaging 0.5 μm in diameter. Spores borne single, sessile or short long sporophores which often occur in branched clusters. Sporophore development monopodial or in some cases sympodial. Aerial mycelium absent or in some culture appearing irregularly as a restricted white or grayist bloom, gram-positive, not acid fast.[12] *Micromonospora* species are aerobic or anarobic and mesophilic. They grow readily at 25-40°C. Thermal death point of the mycelium is 70°C, in 2 to 5 minutes; spores resist 80°C for 1 to 5 minutes.[10]

The genus *Microbispora*

Spores in characteristic longitudinal pairs are formed as aerial mycelium. Spore (1.2-1.8 µm diameter) may be sessile or on short sporophores; paried spores may be closely arranged along the aerial hyphae, giving the appearance of a catkin or borne at longer intervals. In most species aerial mycelium is pink.[12]

The genus *Actinoplanes*

Occur on sterilized leaves in water, forming a very inconspicuous mycelium which branches throughout the leaf tissue. The external hyphae are scattered or in tufts on the leaf surface and form a fringe around the edge of the leaf. Aerial mycelium is lacking or sparingly formed; usually pinkish to reddish. Spores in coil, nearly straight chains, or irregularly arranged, in sporangia, motile by a tuft of polar flagella 2-6 µm in lengh. Sporangial wall evanescent or persistent. *Actinoplanes* is aerobic, gram-positive and acid fast. This genus also occurs saprophytic in soils and in fresh water.

The genus *Streptosporangium*

Sporangia spherical to ovoid, 7-18 µm(usually 7-20 µm). Sporangiospores arranged in a single coil within the sporangium,spherical to ovoid, 1.0-1.3 µm by 1.5-3.5 µm, non-motile.[12] Occurs on sterized leaves of Paspalum grass in water, forming an inconspicuous which overgrows the leaves. The aerial mycelium is white to pinkish on the leaves.[7]

II The search for antibiotics

A. History of antibiotics

The word"antibiosis" was used to describe a type of association in which one living creature was destroying another in order to sustain its own life. Waksman (1947)[46] published the following definition of the word : "Antibiotic is a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganism." Benedict and Langlykke modified this definition to comprise substances which act upon certain organisms at least in very dilute solutions.[47] Umezawa (1956)[48] suggested the inclusion among antibiotics not only of substances of microbial origin but also those produced by higher forms of life; their action should not be limited to only microbes, but should also include tumors. Abraham and Newton[49] descrihed the word: "Antibiotics are natural compounds derived from organisms (Mainly on microorganisms) which themselves or after chemical modification are able at low concentration to inhibit or kill other microorganisms and/or abnormal cells in higher animals".

In the second part of the last century the study of antibiotics began to take place as part of scientific medicine. Emmerich and Low reported that experimental anthrax could be cured by the local application "pyocyanase", an enzyme produced in the culture medium in which *Pseudomonas pyocyanae* had been grown. Fleming discovered penicillin in 1929 during an investigation of the destruction of bacteria by leucocytes. An agar plate on which staphylococci were growing became contaminated by an air-borne mould and around the area of con-

tamination the growth of the staphylococci was inhibited.[50]

The development of antibacterial chemotherapy was first achieved with dye prontosil, a drug discovered by Domagh in 1935. Prontosil, a red dye, is a complex molecule containing a number of groups known to promote fastness to wool and Domagh had hoped that these groups would promote affinity for bacteria.

As in so much of the history of chemotherapy, success depended more on good fortune, as Dubos rightly described, and on extensive testing than on a correct preconception. Dubos[51] successfully isolated in 1938 a group of active substances known as tyrothricin, a product of *Bacillus brevis* thus opening the field of antibacterial antibiotics. Tyrothricin was later separated into two active principles, gramicidin and tyrocidine.

In 1940-1 Waksman and Woodruff[52] reported the isolation of actinomycin, the first actinomycete-produced antibiotic to be obtained in a crystalline form. Waksman and Schatz (1945)[53] isolated streptomycin which was active against Gram-negative and Gram-positive bacteria and mycobacteria.

In 1954 Johnson, Anker and Melency isolated a Gram-positive sporulating bacillus of the *B. subtilis* group from a compound fracture of the tibia; by growing this organism in a suitable medium. They produced an antibacterial substance to which was given the name bacitracin.[54]

Duggar (1948)[55], working in the Lederle Laboratories, announced the discovery of chlortetracycline. This was the first of

the really true broad-spectrum antibiotics and in 1960 Finlay from the Pfizer Laboratory discovered oxytetracycline which we know as Terramycin.[56] Since then, many antibiotics produced by actinomycetes have already found extensive application in the treatment of various human and animal diseases.

B. Antibiotic screening

Primary screening

Screening may be defined as the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest from among a large microbial population. Screening must in a few step allow the discarding of many valueless microorganisms, while at the same time allowing the easy detection of the small percentage of useful microorganisms that are present in the population. With numerous variations of each, there are two methods of screening for antibiotics. One involves the direct screening of cultures growing on agar and the other the determination of activity in liquid media.[57] Waksman and Starkey[58] used the plate method for counting has observed that some of the colonies of actinomycetes on the plate are surrounded by clear zones free from the growth of bacteria and fungi. By far the most successful method in the search for antibiotics has consisted in testing the antagonistic properties of large numbers of microorganisms. The method comprises briefly the following steps.[59]

The first, the substrate to be studied is plated out on media which permit the growth of actinomycetes. The simplest screening technique for antibiotic producers is the 'crowded-plate' procedure. The crowded-plate technique has only limited application, since usually

we are interested in finding a microorganism producing antibiotic activity against specific microorganisms and not against the unknown microorganisms that were by chance on the plate in the vicinity of an antibiotic-producing microorganism.

Second, each actinomycete culture is inoculated in petri dishes containing agar media considered favorable for the production of antibiotics; the inoculation is usually made as a broad streak as that incubation yields a ribbon of growth of even width. Antibiotic screening is improved, therefore, by the incorporation into the procedure of a "test organism" that is,an organism used as an indicator for the presence of specific antibiotic activity.

Third, after growth of the actinomycete, at what is considered a favorable temperature (25-30°C) for a favorable length of time (3 to 7 days) test organisms against which antagonists are sought, are streaked at right angle to the actinomycetic ribbon.

Final, after incubation of the test organisms under optimal conditions for their growth, the antagonistic potentialities of the actinomycetes are estimated by the width of the inhibition zone.

There is no ideal medium,which permits the plating out of a natural substrate with the resulting growth of all the actinomycetes present in the substrate and which inhibits the growth of all other microorganisms. Porter et al (1960)[60] advocate the use of an arginine-glycerol agar. The addition of selective inhibitors permits reduction of the number of fungi or true bacteria and helps in the isolation of actinomycetes in pure cultures. Corks and Chase[61] have used with success the antifungal antibiotic cycloheximide to eliminate fungal

growth. Lawrence (1956)[62] reduced the number of contaminating bacteria and fungi by pretreating the sample to be plated out for 10 minutes with a 1:140 dilution of phenol.

Another method consists in increasing the number of actinomycetes present in a soil sample before plating out. Tsao et al (1960) dried soil samples and then incubated them, buffered with calcium carbonate, in a moist atmosphere. This resulted in an increase in the percentage of viable actinomycetes in these soil samples.

The media used for the actual cross-streak test must be favorable for growth of both the actinomycete and the test organisms. The ideal medium for such tests should also be free from chemicals that might inhibit the action of the antibiotics produced by the actinomycetes.

Demonstration that an antagonist can produce a diffusible substance effective upon the test organisms chosen in a given screening program must be followed by demonstration that this substance can also be produced in liquid media.[59] This is a prime importance, since antibiotics must be obtained in liquid media for large-scale production.

Secondary screening

Primary screening allows the detection and isolation of microorganisms that possess potentially interesting industrial aplications. This screening is usually followed by a secondary screening to further test the capabilities of and gain information about these organisms. Primary screening determines which microorganism are able to produce a compound without providing much idea of the production or yield potential for the organisms.

Secondary screening is conducted on agar plates, in flasks or small fermentors containing liquid media. The use of agar plates, although not as sensitive as liquid culture, is of advantage for initial secondary screening, because more information is obtained with the expenditure of a similar amount of affort. Liquid culture provides a much better picture of the nutritional, physical and production responses of an organism to actual fermentation production conditions. Routien and Finlay (1952)[63] screened thousands of soil samples obtained from widely scattered geographical areas. Certain organisms producing streptomycin, streptothricin, chloramphenicol, actinomycin and xanthomycin-like antibiotics were extremely common. Tetracycline-producing cultures were isolated only a few times.

Secondary screening can be qualitative or quantitative in its approach. The qualitative tell as the spectrum or range of microorganisms which is sensitive to antibiotic. The quantitative approach tell us the yields of antibiotic. Secondary screening should reveal whether there are pH, aeration or other critical requirements associated with particular microorganisms,both for the growth of the organism and for the formation of antibiotic. Secondary screening should show whether certain medium constituents are missing or to its ability to accumulate fermentation products.

The preceding discussion emphasizes the fact that secondary screening can provide a broad range of information which helps in deciding which of various microbial isolates possess possible usefulness as an industrial organism.

C. The study of antibiotic by thin layer chromatography

Over the years many investigators have devised numerous procedures for classification and identification of antibiotics by use of chromatographic techniques. In earlier years, these various chromatographic systems were quite usable because of the relatively small number of antibiotics compared to the present. With the thousands of antibiotics currently known, systematic chromatographic classification of this large number of compounds is extremely difficult. In 1959 Miyazaki et al.[64] described a method of grouping antibiotics according to their salting out chromatograms. The antibiotics were examined by means of ascending paper chromatography. Paris and Theallet were able to separate 23 antibiotics which were described in the French Pharmacopia into seven groups utilizing paper and TLC as well as electrophoresis. Blinov and co-workers in 1969 were able to separate over 300 antibacterial preparations into five groups according to a chromatographic scheme.

Probably the greatest single influence in the systematic analysis of antibiotics was that of Betina[65] who, in 1964, attempted to establish a systematic chromatographic separation of 62 known antibiotics. These were distributed into five classes and further into 14 sub-classes according to their Rf values in four principle solvent systems. Betina further analyzed antibiotics by means of "pH chromatography." By the use of this chromatographic method the ionic character of unknown antibiotics and also the general possibilities of their isolation can be determined.

The behavior of 16 antibiotics were examined by Dobrecky and co-workers[66] by means of thin layer chromatography using Silica

Gel G, Aluminum Oxide G and Cellulose MN 300, A number of solvents were used. Antibiotics zones were detected by spraying with ninhydrin, sulfuric acid or by UV. An extension of the classification of antibiotics by thin layer chromatography was proposed by Azalos and co-workers[67]. They used 84 antibiotics in this study. They stated that the method would not identify an individual antibiotic in a crude mixture.

Sephadex has been used in thin layer chromatography for the identification of antibiotics by Zuidweg et al.[68] With this medium a buffer solution was used instead of organic solvents. Thin layer chromatography using Kieselgel G. (Merck) was utilized by Schmitt and Mathis for separation of 42 antibiotics. Utilizing three selective solvent systems, they were able to distribute these antibiotics into four groups.

Thin layer chromatography was utilized by Ikekawa et al[69] for resolving approximately 50 antibiotics utilizing seven different solvent systems. The method used Silica Gel G. TLC plates and detection of the spot with 10% potassium permanganate and 0.2% bromophenol blue solution or by color reactions characteristic to the particular antibiotic under test. Of particular interest are several solvent systems for separation of certain groups of antibiotics. They separated the antibiotics into five groups. The groups were as follow : (1) macrolide antibiotics, (2) water-soluble basic antibiotics, (3) peptide antibiotics, (4) polyene antibiotics, (5) nucleoside antibiotics. The solvent system selected by the thin layer chromatography can be applied to column chromatography for preparative separations.

Using the solvent system for the water-soluble antibiotics on cellulose powder thin layer plates gave excellent separations

according to Ito et al.[70]

Silica Gel G thin layer plates were utilized by Ochab and Borowiecka for separation of the macrolide group, which was difficult to identify on paper. Cephalosporin C and its semi-synthetic derivatives have been separated and identified by thin layer chromatography using Silica Gel G layers by Buri[71]. This layer chromatography of compounds with chelating ability, particularly a variety of tetracyclines and their derivatives,was carried out by Nishimoto and co-worker.[72] Silica gel thin layer plates pretreated with disodium-EDTA were utilized with excellent results.

D. Bioautographic detection of antibiotics in preparation chromatogram

Numerous methods are used for the detection of antimicrobial agents on chromatograms and these are devided into several categories, chemical detection by use of suitable reagents, the use of ultraviolet light and bioautographic detection of biologically active components. Paper chromatograms are placed in contact with the agar. In analytical techniques where paper chromatograms are used to determine unknown quantities of antibiotics in sample for assay. Generally, alternate standards and unknowns are plated on a plate, such as described by Wagman et al.[78]

Bioautography of thin layer chromatograms is used routinely for the detection of antimicrobial substances, but is somewhat more difficult to handle because of the inflexibility of a glass backed plate which does not always permit the layer to conform to the agar. This lack of contact batween the entire surface and the agar can result in

poorly defined or missing spots. To avoid the adherence of the adsorbent to the agar surface a numbers of methods have been used.

The most common technique is that described by Meyers and Smith[74] who inserted a sheet of filter paper between the plate and the agar surface. As a modification of the Meyers and Smith method, Meyers and Erikson[75] have altered the technique by incorporating 0.1% potassium nitrate into bath the basal and seed agar layers and have found that good growth of the organism occurred under the glass plate. The theory of this technique is that an organism grow under these conditions if given a compound capable of replacing oxygen as an oxidant in terminal respiration.

An interesting method used to plate thin layer chromatograms is described by Narasimhachari and Ramachandran[76] who used the method of taking a micro thin layer of the developed, dry thin layer chromatogram by pressing a transparant cellulose adhesive tape. The tape is then carefully removed from the plate and gently tapped on the nonadhesive side to remove any loose adsorbent meterial. It is then stretched on a nutrient agar plate freshly seeded with a suitable test organism. Another interesting technique was devised by Homans and Fuchs[77] who found that it was possible to directly spray a thin layer chromatogram with a spore suspension of a fungus contained in a glucose minimal medium and which gave most reliable results.

Thin layer chromatograms have the disadvantage of adherence of the absorbent to the agar surface when carrying out bioautographic methods. This problem can be avoided as shown by Wagman and Bailey[78]

by the use of a silicic acid-glass fiber sheet. A useful method for increasing the detection sensitivity of paper strips during bioautography is to leave them in contact with the agar surface throughout the incubation period.