GENERAL INTRODUCTION
MYCOBACILLIN

In the light of prevailing fungus infection in the eastern part of India, an extensive programme to screen out successful antifungal antibiotic producing strains from Indian soils, vegetables and fruits was undertaken in late 1950's in this laboratory, which resulted in the isolation of 178 antifungal organisms. A strain of *Bacillus subtilis* was tipped amongst these isolates as the most active organism. This strain produced a homogeneous cyclic peptide antibiotic, subsequently named 'Mycobacillin'. The molecule which was found to be essentially homogeneous contains 13 residues of 7 different amino acids whose sequence was also determined (Fig. 10) (114-116). It was found that all the constituent glutamic acids of the molecule and 4 out of 5 residues of aspartic acid were of D-configuration, the rest of the amino acids being of L-variety (117). Molecular weight as calculated from its diffusion rate through a porous membrane is 1775 (which comes to 1527 when calculated from the molecular weights of constituent amino acids). It was found to be active specifically against fungi, including plant and skin pathogens, but inactive against actinomycetes and bacteria.

**Media and condition**

Of the different complex media tested those enriched with peptone or casein hydrolysate gave sufficiently high yields of the antibiotic. Supplementation with glucose also enhanced antibiotic yield which indicated that the formation of mycobacillin was an energy linked process accelerated by the
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FIG. 10
supply of glucose. Another interesting observation was that enrichment of medium with beef-extract also stimulates mycobacillin production. It provided beside the utilisable nitrogenous compounds, minerals for the synthesis. Potassium and magnesium were required in fair amounts. In addition to these, trace elements like Fe^{++}, Mn^{++} and Cu^{++} stimulated the synthesis in concentration as low as 0.5, 1.25 and 0.05 parts respectively per million (118, 119).

The role played by individual amino acids on mycobacillin synthesis was studied by their incorporation in the synthetic medium either singly or in combination of two with glutamic acid as the common constituent. It was observed that amino acids, glutamic and aspartic, gave good yields of mycobacillin in synthetic medium. The other amino acids necessary for the growth of the organism and its antibiotic formation might be synthesized from either of these two amino acids. The other amino acids hardly proved useful unless they were used in combination with glutamic acid (120).

Relationship between antibiotic production and basic cellular metabolism

Mycobacillin fermentation might be divided into three distinct phases on the basis of changes that took place in the composition of fermentation broth containing casein hydrolysate, glucose and beef-extract under stationary condition. The first phase was characterised by low mycobacillin
production, rapid cell synthesis, glucose and amino nitrogen consumption, the second by enhanced rate of mycobacillin production but slow cell synthesis and glucose utilisation although amino acid consumption still continues and the third by a slight decay of mycobacillin already formed. Initial fall in pH during first phase despite continuous formation of ammonia might be due to the formation of organic acids which were intermediates in glucose utilisation. In the second phase extending up to the 5th day cell synthesis markedly decreased but disappearance of amino acids and total nitrogen from the broth still continued. In the last phase of fermentation commencing on the 5th day, fewer changes in the composition of the broth occurred. This was perhaps due to aging effect when metabolic activity was slowed down. This phase was, however, marked by a slight decrease in concentration of mycobacillin due to its degradation and also by a slight increase in amino and ammonium nitrogen of the broth due to autolysis of producer cells (120, 121).

There was no correlation observed between the uptake of amino acids from the broth and the formation of antibiotic. Studies on the disappearance of free amino acids from cells and mycobacillin synthesis indicated that there might possibly be some relation between the two. During the period (72-96 hours) of rapid mycobacillin synthesis, free amino acids of cell pool like glutamic acid, aspartic acid, alanine, serine, tyrosine, lysine and valine disappeared of which the first six were constituents of the antibiotic molecule (Thesis of S.K. Majumdar...
Mycobacillin was found to be a broad spectrum antibiotic being active against skin pathogens, plant pathogens and also saprophytic fungi. Regarding its action on a sensitive strain of *Candida albicans* it was observed that it enhanced 'lag period' but did not affect energy yielding processes like respiration and glycolysis (122). Though mycobacillin was found to affect slightly the oxidation of its constituent amino acids, protein synthesis as measured in terms of incorporation of labelled leucine, serine or glycine into TCA precipitable fraction, remained unaltered in its presence. None of the constituent amino acids of mycobacillin could antagonise the growth inhibiting property of the antibiotic. It brought about agglutination of cells of the sensitive strain of *C. albicans*, changed its Gram-character and caused release of UV absorbing materials from the cells (123, 124) (Thesis of N.D. Banerjee for degree of Doctor of Philosophy of the University of Calcutta, 1969).

Now, detailed kinetic studies on mycobacillin sensitive reactions indicated that visible agglutination lags behind fall in viability which was also not quantitatively related to release. Thus none of the above reactions alone could be considered responsible for the antifungal action of the compound. In order that the primary site of action of the drug might be
isolated, it was considered desirable to select the most sensitive microbe as the test organism for studying the mode of action of the drug. Mycobacillin being specifically active against *Aspergillus niger* whose inhibitory dose is 20 μg/ml as against 50 μg/ml for *C. albicans*, the problem was therefore reinvestigated with reference to *A. niger* a test organism.

It was found that the antibiotic also specifically affected 'lag period' of *A. niger* and also had no marked inhibitory effect on energy yielding processes at growth inhibitory concentration. It also caused release of 260 and 280 mμ absorbing materials from vegetative growth of *A. niger*. The releasing action was considerably reduced in presence of osmotic stabilizer. Mycobacillin also agglutinated spores of *A. niger* at 37°C but not at 4°C and the agglutinated spores were all viable (125).

It was found that sterols e.g. cholesterol, ergosterol, ergocalciferol and also lipid like lecithin could antagonise growth inhibitory property as well as the leakage action of the antibiotic. Lipid like compounds were isolated from the sensitive organism *A. niger* and fractionated into neutral and phospholipid components which were found to contain mainly cholesterol and lecithin respectively. Both the lipid fractions antagonised the growth inhibiting property of mycobacillin. But the antibiotic did not interfere with their biosynthesis (126, 127).

The spectral behaviour of mycobacillin in UV range in presence of cholesterol or cholesterol and digitonin suggested
that antagonism might owe its origin to a chemical reaction between the antibiotic and the antagonist. Sterol mycobacillin interaction had further been supported by monolayer properties of sterols and mycobacillin either alone or in a binary mixture with the antibiotic as the common ingredient (128) (Thesis of Miss A. Halder for the degree of Doctor of Philosophy of the University of Calcutta, 1971).

Antifungal action of di and tri acetyl derivatives of mycobacillin was not antagonised by cholesterol or lecithin which antagonised the action of its heptamethyl derivative. Considering the reactive groups of mycobacillin, the antagonism might involve the tyrosine hydroxyl groups of the antibiotic and the 3-hydroxyl group of cholesterol or the oleic acid components of lecithin having unsaturation at the 9:10 position with cis-configuration (129).

It has been observed that mycobacillin sensitive A. niger normally possesses transport mechanism for the release of a few selective metabolites such as lysine, proline, ATP, P_i, Na^+, K^+ and Ca^{2+}. The presence of mycobacillin enhanced the release of these metabolites. Thus, it appears that mycobacillin reacts with the transport system, causing selective release. The mycobacillin sensitive sites on the cell all differ in the degree not only of their mycobacillin sensitivity but also of their efficiency in causing release. For example, some metabolites like ATP is more released than others against a given mycobacillin concentration and also for the maximum
release, the concentration of the antibiotic required appeared to differ from one metabolites to another. Release by preloaded cells further identified the process to be a concentration dependent physical diffusion process. It has also been observed that once the release was initiated, it attained a constant value conditioned by the dynamic equilibrium between the intra- and extra-cellular concentrations of any one of the releasable constituent. Inherently, the *A. niger* cells possess transport mechanism for the uptake of many metabolites including those for which the cells were found to be competent to cause release irrespective of the presence of mycobacillin. Mycobacillin appears to enhance the uptake process at low concentration (up to 20 µg/ml) only of those metabolites for which the antibiotic possessed releasing action. Probably the same transport mechanism might be operating both for release and uptake. The presence of a higher concentration of the antibiotic appears to have an effect on the uptake process (communicated).

Studies on the site of binding of mycobacillin on protoplast, the nature of binding, interaction and the membrane structure in the region of mycobacillin sensitive site revealed by change of turbidity of protoplast suspension even in presence of the osmotic stabilizer by mycobacillin which did not cause any change of turbidity of whole cell suspension indicating that membrane may be the possible site of action of the antibiotic. The pattern of kinetics of turbidity change, first
increase, then decrease, and again increase indicates that mycobacillin differed entirely from other polyene antibiotics and that there might be more than one type of non-identical mycobacillin binding sites on the protoplast, causing efflux of more than one type of some specific cell constituents. That mycobacillin possesses at least two types of binding sites on the protoplast was further confirmed by Scatchard plot of the data on binding studies.

The site and nature of mycobacillin binding reaction was then studied by fluorescence technique using intact protoplast. Since the quantum yield of ANS was not altered by mycobacillin the decrease in fluorescence during protoplast ANS interaction in its presence might be caused by the antibiotic affecting this interaction. As the interaction of mycobacillin and ANS with the protoplast was found to be reversible, the data emerging from equilibrium binding studies were analysed which showed that ANS possessed two types (A type and B type) of binding on the protoplast and mycobacillin competed with ANS only for the A type of binding and the binding of B type was not affected. That these studies further showed that mycobacillin and ANS possessed competitive non-identical binding sites on protoplast membrane, mycobacillin being structurally different may be taken as an indication that membrane is the site of its action.

The nature of binding of mycobacillin was studied by noting the temperature dependence of the binding process and it was
found that reaction between mycobacillin and protoplast was favoured at higher temperature, in other words, structural disorderlines favoured the reaction. Therefore the binding reaction between mycobacillin and protoplast might be physicochemical in nature (communicated).

**Biosynthesis**

Studies on biosynthesis of mycobacillin was undertaken in 1961 when a well-known hypothesis to account for the biosynthesis of protein had already been formulated and that of small peptides like glutathione by non-template mechanism was just been confirmed (130). But little information was available at that time regarding the biosynthesis of peptide antibiotics except for the fact that RNA template was not involved in biosynthesis of gramicidin S (9). In context of this work it was observed that chloramphenicol, a well established inhibitor of protein synthesis, inhibited both growth and mycobacillin formation and so its effect could not be interpreted. But when nucleic acid base antagonists were added at the 66th hour of growth in stationary culture, growth, RNA and DNA synthesis stopped, whereas mycobacillin production continued as usual indicating that RNA of high molecular weight is not involved in the formation of the antibiotic.

A streptomycin dependent variant was isolated from the antibiotic producer strain by single step mutation process. Streptomycin inhibited the growth of the mutant in concentrations
exceeding an optimal level. It was also observed that when this mutant was grown in the presence of streptomycin but subsequently deprived of it, mycobacillin production remained quite normal, but protein synthesis was seriously impaired. This non-interference of streptomycin deprivation therefore, was taken as a further evidence in support of the previous conclusion that ribosome did not take part in mycobacillin biosynthesis (131).

Studies on biosynthesis of mycobacillin indicated that RNA-template is not involved and the synthesis occurs in stepwise fashion by linear addition of amino acids. The cell pool of the producer organism contained four nucleotide linked peptides whose amino acid composition was found to be similar to that of mycobacillin (132). An active cell-free system by lysozyme lysis of producing strain was developed. The incorporation of radioactive amino acids into mycobacillin by the system was observed to be energy dependent. The non-participation of RNA-template in the biosynthesis of mycobacillin was further confirmed when the cell-free system was incubated in the presence of exogenous ribonuclease which did not affect to any extent, the incorporation of amino acids into mycobacillin molecule (Thesis submitted by A.B. Banerjee for the degree of Doctor of Science of the University of Calcutta, 1966).

Before further investigations on biosynthesis of mycobacillin, chemical characterisation of the molecule e.g., D-, L-sequence of amino acids and side chain linkage was carried out (133).
The non-participation of ribosomal mechanism in the synthesis of the molecule so characterised was further confirmed in cell-free system by showing its insensitivity to protein synthesis inhibitors and by localising the enzyme system in the soluble supernatant of the producer organism (134). During the studies on the mode of activation of mycobacillin amino acids, it was observed that mixture of mycobacillin amino acids catalyse a ATP-[^32P]P\textsubscript{i} exchange in cell-free system when tested individually, only (L)-proline amongst all these amino acids catalysed such an exchange. This exchange was found to be increasingly stimulated by the sequential addition of other non proline mycobacillin amino acids as they are arranged in the molecule (135). By amino acid deprivation technique, it has also been possible to isolate from the mycobacillin synthesizing cell-free system containing 2 to 6 mycobacillin amino acids a series of peptide starting from L-proline. Those peptides were found to be di-, tri-, hexa-, octa- and undecapeptide containing respectively 2,3,4,5,6, different mycobacillin amino acids. Studies on C- and N-terminal and stereoisomeric configuration of constituent amino acids of these peptides suggested that they might be considered as growing mycobacillin peptide chain starting from L-proline along C\rightleftharpoons N and interrupted at various points for absence of requisite amino acids (136, 137). The peptides isolated from the cell-free system by amino acid deprivation technique and supposed to be mycobacillin intermediates were not however incorporated into mycobacillin in presence of complementary amino acids (138),
which raises an important question as to their role as intermediates as well as to the structural organisation of the enzyme to function as a protein template in place of genetic code either as a multisite single enzyme or a multiple enzyme complex, functionally linked in an unknown way.

**Structure-function relationship**

As mentioned earlier, mycobacillin is a unique molecule containing ten free reactive moieties viz., seven carboxyl, two phenolic and one aliphatic hydroxyl groups and possessing both antifungal and haemolytic activities and was inactivated to the extent of 90% in presence of serum (139). Di- and triacetyl derivatives of mycobacillin have been prepared. Acetylation of the antibiotic at two tyrosine hydroxyl groups adversely affected the antifungal activity. The minimum inhibitory concentration was 35–40 μg/ml whereas for mycobacillin it is only 15–20 μg/ml. Interestingly the derivatives were not at all inactivated in presence of serum, if incorporated to the extent of 50% (v/v) in the medium whereas mycobacillin was under identical condition inactivated by serum by more than 90%. Triacetyl mycobacillin had almost the same biological activity as the diacetyl derivative and its serum inactivation was also nil (140).

Esterification of the carboxyl groups of mycobacillin with different alcohols decreased its antifungal activity but protected it partially from inactivation by serum (141). Alkyl or aryl radicals had no specific effect on its antifungal
activity or its susceptibility to inactivation by serum.
Acetylation of tyrosine hydroxyl groups of a water-soluble ethyl ester or esterification of triacetyl mycobacillin considerably lowered the antifungal activity of the antibiotic (paper published by P.C. Banerjee and Prof. S.K. Bose, 1972 and 1973).

Significance of mycobacillin production in relation to the spore forming metabolism of the producer B. subtilis B₃

Earlier studies dealing with the effect of mycobacillin on the life process of its producer organism showed that it extended the normal lag period of growth of its producer B. subtilis B₃ and the non-producer mutants. Mycobacillin sensitive reactions like release of UV absorbing materials, change of Gram-character and agglutination of whole cells observed with a sensitive strain of C. albicans were also exhibited by producer B. subtilis B₃. Exponentially growing cells were more susceptible to the antibiotic than the cells at late log or post log phase. Protoplast made from insensitive late log phase cells showed considerable response with regard to the above mentioned reactions when treated with mycobacillin (13).

As in the case of other peptide antibiotics produced by the genus Bacilli, mycobacillin synthesis was found to be parallel to the spore forming metabolism. The antibiotic was elaborated during endotropic sporulation of B. subtilis B₃ in distilled water plus CaCl₂ (0.2 mg/ml) while no antibiotic was formed during germination of a highly dense spore suspension in complex growth medium (14, 142).
The close association between sporulation and mycobacillin synthesis has been demonstrated by inhibition of both the processes by common inhibitors like glucose (in excess), ethyl malonate, sodium bisulphite, β-phenethyl alcohol, acriflavin, α-picolinic acid, fluoro acetic acid and m-tyrosine. This close association was further supported by the findings that oligosporogenous mutants produced lesser amounts of mycobacillin than the parent whereas the asporogenous mutants completely lacked the ability to form the antibiotic (14). When asporogenous mutants were tried for transformation by wild DNA of the parent, 3 out of 8 asporogenous mutants showed transformation as screened for spore formation. All the transformants were also found to be antibiotic producers.

One adenine requiring auxotrophic mutant of *B. subtilis* B3 producing normal level of mycobacillin was further mutated by UV irradiation which gave three mycobacillin producer and three strictly non-producer mutants. The non-producer mutants did not produce the antibiotic even intracellularly. All the adenine auxotrophs including those three mycobacillin-negative mutants were spore formers. Spores of mycobacillin producer mutants were more heat-resistant than those of non-producer ones. Spore of mycobacillin producer mutants had greater ion-exchange properties, they germinated better in some germination media and were also more susceptible to germination inhibitors when compared to those of non-producer mutants. Mycobacillin neither helped nor interfered with germination of spores of the two types. Sporogenous mycobacillin non-producer mutants showed less
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(one third to one half) exoprotease ability than sporogenous producer mutants did. Asporogenous mycobacillin non-producer mutants possessed much less exoprotease than both sporogenous producer and non producer mutants (143, 144).

When induced by UV irradiation, two out of eight asporogenous non-producer mutants gave five My+ revertants from Sp⁻My⁻ mutants screened as My+ retained Sp⁺ character. Some of the revertants regained wild-type level of different spore-related parameters while some other revertants, instead of having wild-type levels of different spore-related parameters, showed gradation in them (145).

Sporulation is considered a favourable biological process for studies on differentiation in unicellular system. It is well established that sporulation genes are sensitive to glucose and other catabolites. Majumdar et al. (146) have isolated some glucose resistant mutants which are able to sporulate in presence of glucose and other carbon sources. It has been also observed that mycobacillin and dipicolinic acid synthesis are subjected to catabolite repression (147). Some spore specific and spore associated enzymes are also subjected to catabolite repression in parent strain B. subtilis B₃₄ which are indifferent in the glucose resistant mutants. Derepression of sporulation and synthesis of dipicolinic acid and mycobacillin by cyclic GMP under conditions of glucose repression has been observed (2, 3). Derepression of spore specific and spore associated enzymes by cyclic GMP has also been observed under condition of glucose repression (148).
VERSICOLIN

Lack of clinical usefulness of mycobacillin initiated a further screening of microorganisms particularly effective against dermatophytes like Trichophyton rubrum which causes 90% of skin infections in the eastern India. This screening resulted in the isolation of an antagonist strain of Aspergillus versicolor. A new antifungal antibiotic was elaborated by this strain which was subsequently named versicolin. Homogeneity of versicolin was tested using various technique involving paper chromatography-cum-bioautography and thin layer chromatography which showed that the preparation contained only one component (149, 150).

A tentative structure was suggested (151) on the basis of physico-chemical analysis (Fig. 11A)*. It was found to be mainly active against pathogenic fungi and had practically no activity against bacteria. The action of versicolin was fungicidal.

Media and condition

It was found that A. versicolor could produce the antibiotic only in a complex medium containing 4% glucose and 1% peptone. The optimum condition for production by A. versicolor in a

*Another structure had also been suggested for versicolin by Prof. R.W. Rickards as indicated in Fig. 11B (R.W. Rickards; J. Antibiotics, Vol. XXIV, 715, 1971). However, it has not yet been possible to produce confirmatory evidence in support of either structure for versicolin.
**FIG. 11A Versicolin, according to Dhar & Bose**

**FIG. 11B Versicolin, according to Rickards**
glucose peptone medium were determined. Those were as follows: pH 3.0, temperature 25°C, period of incubation 7 to 9 days under stationary condition.

Attempt at developing a synthetic medium for versicolin production showed that A. versicolor failed to produce versicolin in a purely synthetic medium containing an inorganic nitrogen source, although its growth was fairly normal. If the synthetic medium was supplemented with any of the amino acids viz., L-asparagine, L-aspartic acid and valine, fairly good yields of versicolin were obtained. Of these three amino acids L-asparagine seemed to be the best. In synthetic medium inorganic nitrogen had no influence on the yield of versicolin. Glucose was the best carbohydrate source for versicolin production in L-asparagine supplemented synthetic medium. The antibiotic production was also dependent on the presence of phosphate, sodium, potassium and magnesium in suitable concentrations. In addition to these, trace elements like Fe$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$ when in combination gave the highest yield of 80 μg/ml. Cobalt was, however, inhibitory to antibiotic synthesis in all combinations (152).

Relationship between antibiotic production and basic cellular metabolism:

Studies on biochemical changes during versicolin fermentation showed that there was no lag between growth and antibiotic production. Versicolin synthesis took place during the log phase of cellular growth. pH rapidly rised and continued even after
optimum antibiotic titre had been reached. Sugar was rapidly utilised during antibiotic synthesis. It was also observed that antibiotic synthesis was accompanied by rapid depletion of medium nitrogen which appeared mostly as ammonia in the medium and thereby increased its pH. Cell nitrogen did not show, however, an appreciable increase during the process (153) (Thesis submitted by A.K. Dhar for the degree of Doctor of Science of the University of Calcutta, 1969).

**Evaluation as an antifungal drug**

The first task for evaluation was to develop a simple method for the preparation of the antibiotic. The isolation and purification of versicolin from culture filtrate of *A. versicolor* was therefore effected by a modified procedure based on repeated fractional column chromatography (154). The efficiency of recovery by the process was 60% as against 40% by the earlier method (154). Studies had been made on the structure and function relationship of versicolin. It was found that trisubstituted acyl-derivatives had no activity against *T. rubrum* whereas monosubstituted acyl-derivative and O-alkyl derivatives were less active than the parent compound. All the acyl- and O-alkyl derivatives were inactivated to a greater extent in presence of serum. It had no haemolytic activity like other antibiotics. It was however inactive against fungi causing systemic infection.

Studies on the toxicity of versicolin by different routes indicated that the LD$_{50}$ value (mg/kg body weight) of the
antibiotic against mice were 33, 61, 80 and 330 respectively when administered intravenously, intraperitoneally, subcutaneously and orally.

Studies on the distribution of versicolin indicated that the antibiotic was well distributed in different organs. Blood could maintain twenty times the fungicidal concentration even for four hours after administration of a single maximum tolerable dose whatever might be the routes of administration. Versicolin was excreted in the active form mostly in urine but only slightly in faeces. Versicolin was effective against experimental infection by *T. rubrum* in guinea pigs even at a low dose of 2.5 mg/kg body weight when administered orally. The lower dose took a month or so for complete cure whereas, higher doses only 15 days, though both the doses were administered successively for 15 days. The antibiotic had no subacute toxicity at these curable doses as revealed by growth rate, blood picture, weight of different organs and their microscopic and histological examination (155).

**Evaluation of mycobacillin and versicolin as agricultural fungicides:**

In search for finding newer uses, the two antifungal antibiotics isolated in our laboratory had been further evaluated as agricultural fungicides. Antimicrobial spectrum and phytoxicity of these two antibiotics had been studied recently. Both the antibiotics were active against some common rice and jute pathogens. It was found that mycobacillin was specifically
active against a rice pathogen *Pyricularia oryzae* at concentration of 10 µg/ml. Versicolin was specifically active against *Colletotrichum gloeosporioides*, a jute pathogen at a concentration of 2-5 µg/ml. The activity of both the antibiotics against a very common rice pathogen *Helminthosporium oryzae* and a common jute pathogen *Macrophomina phaseolina* were very low e.g. 50 µg/ml (156).

Germination studies showed that mycobacillin had no toxic effect against germination of seeds even for a long exposure period of two days. It had no effect against the growth of germinated seeds measured in terms of root and shoot length at a concentration of 500 ppm both for short and long exposure. While studying the phytotoxicity of mycobacillin against rice and jute seeds it was curiously observed that mycobacillin not only stimulated germination but also enhanced the growth of root or shoot of germinated seeds. Versicolin had no phytotoxicity against the germination of seeds and growth of seedlings at a concentration of 500 ppm for 2 hours exposure, but detectable phytotoxicity was observed for both the processes at the same dose for 24 hours exposure or longer (156).

The effect of paddy soils on mycobacillin and versicolin was investigated. Soil inactivated mycobacillin as determined by spectral analysis and microbiological assay. Soil could inactive mycobacillin only at or above the threshold concentration (125-130 µg/10 mg of soil), the excess being unreacted. Soil was without any effect on versicolin (157) (Thesis of J. Nandy)
Aspergillus versicolor (N^) was an active regenerate from a partially degenerated culture of A. versicolor once producing versicolin (158). That active isolate also degenerate completely during repeated subculture.

The inactive mutant of A. versicolor (N^) was then mutagenised with a chemical mutagen, HNO₂ and from the mutant population so obtained were isolated eighteen active strains by developing a rapid screening technique (159). These were designated as A. versicolor (N₅)₁⁻(N₅)₁₈. Studies on the phenotypic identity of the producer revertant derivatives showed all the eighteen isolates to be different. However, none of the derivatives resembled the phenotype of the parental mutant (160).

A new antibiotic was subsequently obtained by the fermentation of an arbitrarily chosen strain (N₅)₁₇ in Sabouraud broth. Physicochemical and biological properties of the antibiotic were then studied and it was designated as mycoversilin (161, 162). The following studies have yet been done on the new antibiotic.

**Media and condition**

Experiments for the development of a medium with a complex nitrogen source indicated that peptone was the best nitrogen source for mycoversilin synthesis. The optimum condition for
antibiotic production in a glucose peptone medium with respect to pH, temperature and incubation period were as follows: pH 3.5, temperature 28°C and period of incubation 8 to 9 days under stationary condition. Kinetics of antibiotic production and growth in glucose peptone medium showed that they were very much associated showing the absence of any marked lag between them. Basal synthetic medium with inorganic nitrogen sources viz., NaNO₃, (NH₄)₂SO₄, NH₄NO₃ etc. either alone or in combination did not support mycoversilin synthesis, although its growth was unaffected. Basal synthetic medium containing NaNO₃ and any one of the amino acids viz., L-aspartic acid, L-valine, L-proline and D-methionine gave fairly good yield of mycoversilin and of these L-aspartic acid was the best amino acid for mycoversilin production. The synthetic basal medium with 1% L-aspartic acid gave much higher yields of the antibiotic than that was obtained in a complex medium containing 4% glucose and 1% peptone. Of the different carbohydrate sources viz., glucose, sucrose, lactose, starch etc. tested for the production of the antibiotic in the L-aspartic acid supplemented synthetic medium, glucose seemed to be the best source. Metal ions like Fe²⁺, Ni²⁺ when added singly to the trace element free synthetic basal medium showed an optimum with respect to cell growth. The metal ions viz., Fe²⁺, Mn²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ when added singly neither stimulated nor inhibited mycoversilin production with the exception of Co²⁺ which stimulated the production at a concentration of 0.5 μg/ml.
The antibiotic production was slightly enhanced by all the combinations of two ions (taking Fe\(^{2+}\) as a common ingredient) except that containing copper. Fe\(^{2+}\) and Co\(^{2+}\) gave the best yield. The combination of the three ions viz., Fe\(^{2+}\), Mn\(^{2+}\) and Co\(^{2+}\) gave better yield of the antibiotic. The combination of four metal ions viz. Fe\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\) gave the best yield of the antibiotic (communicated).

**Relationship between antibiotic production and basic cellular metabolism**

Studies on relationship between mycoversilin synthesis and basic cellular metabolism showed that growth and antibiotic productions appeared to be very much closely associated and that there was no lag between growth and antibiotic synthesis. The pH of the medium decreased slightly when antibiotic production reached the peak value. Glucose was more rapidly utilised from the medium than amino nitrogen during growth and antibiotic production (communicated).

**Isolation and chemistry**

The antibiotic was isolated from the fermented broth by solvent extraction followed by chromatography on acid alumina and silica gel columns. The purified crystalline product was shown to be homogenous by thin layer chromatography and paper chromatography. It was soluble in polar organic solvents and in water but insoluble in non polar solvent. It was found to be optically inactive. From the elemental and mass spectral analysis,
mycoversilin was found to have the molecular formula $\text{C}_{18}\text{H}_{16}\text{O}_8$.
The UV spectrum showed the presence of phenolic moiety and IR spectrum also confirmed the presence of phenolic moiety. The presence of either function was also shown by IR spectrum.
Considering the 18 carbon atoms, functional groups (-OH, -C=O-C, polynuclear aromatic system and unsaturation), the distribution of hydrogen atoms as arrived at by proton signals of the $^1\text{H}NMR$ spectrum and the environment of carbon atoms as indicated by $^{13}\text{C}NMR$ spectrum of mycoversilin, the antibiotic might be imagined as having the structures I and II (Figs. 12A and 12B) agreed with the molecular formula, colour reactions and most of the physicochemical properties. But since mycoversilin formed an acetonide which did not show any colour test with FeCl$_3$
mycoversilin has the structure I (163).

**Mode of action**

Mycoversilin, a narrow spectrum antibiotic, was found to be active specifically against dermatophytes. The MIC against the most active dermatophyte, *T. rubrum* is 15 µg/ml. Regarding its action on the sensitive organism it was shown to affect both the lag period and maximum growth supportable at 15–20 µg/ml concentrations. The antibiotic is fungistatic or fungicidal depending on its concentration. It has no inhibitory effect on mycelial growth, membrane permeability, respiration and *in vivo* [$^{14}\text{C}$]uracil incorporation in RNA at any concentrations tested. At higher concentrations it affected spore germination and *in vivo* [$^{14}\text{C}$]thymine incorporation into DNA. At MIC level it
MYCOVERSILIN

FIG. 12

A

B
specifically inhibited sporulation and \textit{in vivo} \textsuperscript{14}C-leucine incorporation into protein. The \textit{in vivo} inhibitory action of mycoversilin on protein synthesis was further confirmed by its action \textit{in vitro} in inhibiting polyU directed cell-free protein synthesis. In localizing the exact site of action on \textit{in vitro} protein synthesis it was observed that the antibiotic inhibited not the amino acyl-AMP formation \textit{ATP-\textsuperscript{32P}}P\textsubscript{i} exchange reaction but the amino acyl-tRNA formation. However, the action of mycoversilin which specifically inhibits sporulation remained to be explained (164).
GENESIS AND SCOPE OF THE PRESENT INVESTIGATION
Mycobacillin, elaborated by a strain of *Bacillus subtilis* B₃, is a cyclic trideca antifungal peptide. The chemistry of this peptide including amino acid composition, dL sequence and side chain linkages have all been worked out. A cell-free system including partial purification of the enzyme by \((\text{NH}_4)_2\text{SO}_4\) fractionation had been developed for the synthesis of the antibiotic. In course of study it was observed that mycobacillin synthesizing activity starts at the late exponential phase whereas mycobacillin synthesis at the early stationary phase which continues for about six days when most of the vegetative cells have been converted into spores. Hence, the distribution of mycobacillin synthesizing activity (under standardised condition) in vegetative cells or in spores and also its localisation into subcellular particle seems to be an interesting query. It was also observed that the synthesizing enzyme system under condition of amino acid deprivation synthesizes series of intermediate peptides whose amino acid composition and C- and N-terminal residues agree with the equivalent segments of mycobacillin. However, this enzyme system could not recognise the preformed intermediate peptides and incorporate them into mycobacillin. It is therefore equally interesting to study the substrate recognition capability of the enzyme if localized in the subcellular particle. The other relevant properties of the localized enzyme in comparison to those of the non-localized or soluble enzyme will also be studied to follow the mechanism of biosynthesis of mycobacillin. In light of this discussion
the following lines of work may be pursued:

**/SCHEME OF WORK/**

1) STANDARDISATION OF THE CONDITIONS FOR MYCOBACILLIN SYNTHETASE ACTIVITY


ii) Effect of isotopic dilution on $^{14}$C-amino acid incorporation into mycobacillin.

iii) Effect of different protein concentrations on the rate of enzymatic activity.

iv) Effect of different pH and buffer concentrations for optimum synthesis.

v) Effect of ATP/Mg$^{++}$ ratio on enzyme activity.

vi) Effect of sulphydryl reagents on enzyme activity.

vii) Effect of energy sources on the incorporation of $^{14}$C amino acid into mycobacillin.

viii) Inhibition of antibiotic synthesis by adenosine diphosphate and monophosphate.

ix) Effect of metal ions.

x) Effect of pantothenic acid on mycobacillin synthetase activity.
II] TRANSLOCATION OF MYCOBACILLIN SYNTHETASE INTO MEMBRANE

i) Kinetics of cellular growth, antibiotic production and synthetase activity in subcellular fractions.

ii) Distribution of synthetase activity under conditions of inhibition of protein synthesis.

iii) Localisation of the 10000 g particulate synthetase activity of stationary-phase cells.

iv) Characterisation of particulate mycobacillin synthetase system.
   a) Effect of lysozyme treatment.
   b) Presence of succinate dehydrogenase activity and
c) Effect of detergents.

v) Site of membrane binding of particulate mycobacillin synthetase activity.

III] ROLE OF MEMBRANE-BOUND ENZYME IN SUBSTRATE RECOGNITION FOR SYNTHESIS

i) Synthesis of peptides of different chain length under amino acid deprivation condition by membrane-bound enzyme.
ii) Incorporation of free or enzyme-bound intermediate peptides of different chain length into mycobacillin by membrane-bound enzyme.

iii) Activation energy of membrane-bound mycobacillin synthetase.

iv) Temperature dependence of $K_m$ of membrane-bound mycobacillin synthetase.

IV] ROLE OF SUBSTRATE-BOUND ENZYME IN SUBSTRATE RECOGNITION FOR SYNTHESIS

i) Incorporation of enzyme-bound preformed intermediate peptides of different chain length into mycobacillin by soluble cytosol enzyme.

ii) Incorporation of free penta and nona peptides into mycobacillin by soluble mycobacillin synthetase.

iii) Activation energy of soluble enzyme.

iv) Temperature dependence of $K_m$ of soluble mycobacillin synthetase.

V] CHARACTERISATION OF THREE-FRACTION MYCOBACILLIN SYNTHETASE

i) Absence of aspartic, alanine and glutamic acid racemase activity in mycobacillin synthetase.
ii) Effect of L or D forms of constituent amino acids on ATP/P\textsubscript{i} exchange.

iii) Role of different L or D forms of constituent amino acids on \textsuperscript{14}C amino acid incorporation into antibiotic.

iv) Inhibitory effect of optical isomers on ATP/P\textsubscript{i} exchange.

v) Absence of pantothenic acid in different enzyme fractions of mycobacillin synthetase.

vi) Requirement of ATP for each of the peptide bonds of mycobacillin.