TWENTYFIVE YEARS OF RESEARCH ON TUBERCULOSIS AT THE INDIAN INSTITUTE OF SCIENCE, BANGALORE*

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1982 is the centenary year of the discovery by the German microbiologist Dr. Robert Koch of *Mycobacterium tuberculosis*, the rootcause of tuberculosis. For this discovery made on 24 March 1882, Dr Koch was awarded the Nobel Prize and the German Government set up a special institute to help him pursue further research on the tubercle bacillus. Some 60 years later in 1945, Dr Selman Waksman discovered streptomycin, the first drug which was found to be specific against this organism, and he also received the Nobel Prize.

A hundred years after Koch's discovery of the cause of the disease, tuberculosis remains the single most important infectious disease in terms of numbers of deaths and economic losses. The World Health Organization estimates that there are 7 million new cases every year, adding to a tragic total of atleast 20 million deaths annually. The disease continues to be one of our biggest health problems. In India about 8 million people suffer from active pulmonary tuberculosis, of which 25% are infectious cases. Nearly 2 million fresh cases are reported every year, thus making India the country with the largest number of tuberculosis patients. The problem is complicated by the fact that a high incidence of tuberculosis in India is resistant to currently available drugs. Therefore there is a crying need for newer antitubercular drugs.

Almost all western countries and Japan have eradicated tuberculosis and are no longer interested in research on this organism. Basic research on the biochemistry and molecular biology of the organism, especially the basis of drug resistance, is urgently needed. It can come only from the poorer countries of Asia and Africa, where tuberculosis continues to be a major problem.

The genus Mycobacterium belongs to the order Actinomycetales, a group of fungus-like bacteria and includes in its family, in addition to *Mycobacterium tuberculosis* (*M. tuberculosis*), *M. leprae*, the causative organism of leprosy, *M. smegmatis*, a non-pathogenic, saprophytic organism, etc. Their DNA has a very high GC content, *M, tuberculosis* having 66-68% GC and *M. smegmatis* 70–80% GC(compare it to the DNA of the more common bacterium *Escherichia coli* with 50% GC and *Clostridium perfringens* with 30% GC.) They are also the slowest growing bacteria in culture though it has been possible to speed up the growth of *M. tuberculosis* by a factor from coconut water.[1,2].

When work on *M. tuberculosis* $H_{37}Rv$ and other mycobacteria was started in 1957, the aims of the programme were threefold:

(1) To study the enzyme systems of the virulent tubercle bacilli and compare them with those reported in the human host, so that any differences between them could be exploited for possible chemotherapeutic attack against tuberculosis;
(2) To compare the enzyme systems of the drug-sensitive and drug-resistant strains of the tubercle bacilli and to study the genetics of resistance to drugs, like isoniazid and streptomycin, in order to combat the high incidence of drug-resistance in India;
(3) To compare the enzyme systems and genetic make-up of the virulent and avirulent tubercle bacilli to find out the possible cause of virulence.

The work carried out by our group at the Indian Institute of Science has been published in a review[3] as well as in a recent book[4] dealing with the metabolism and genetics of mycobacteria in general. A summary of the research carried out

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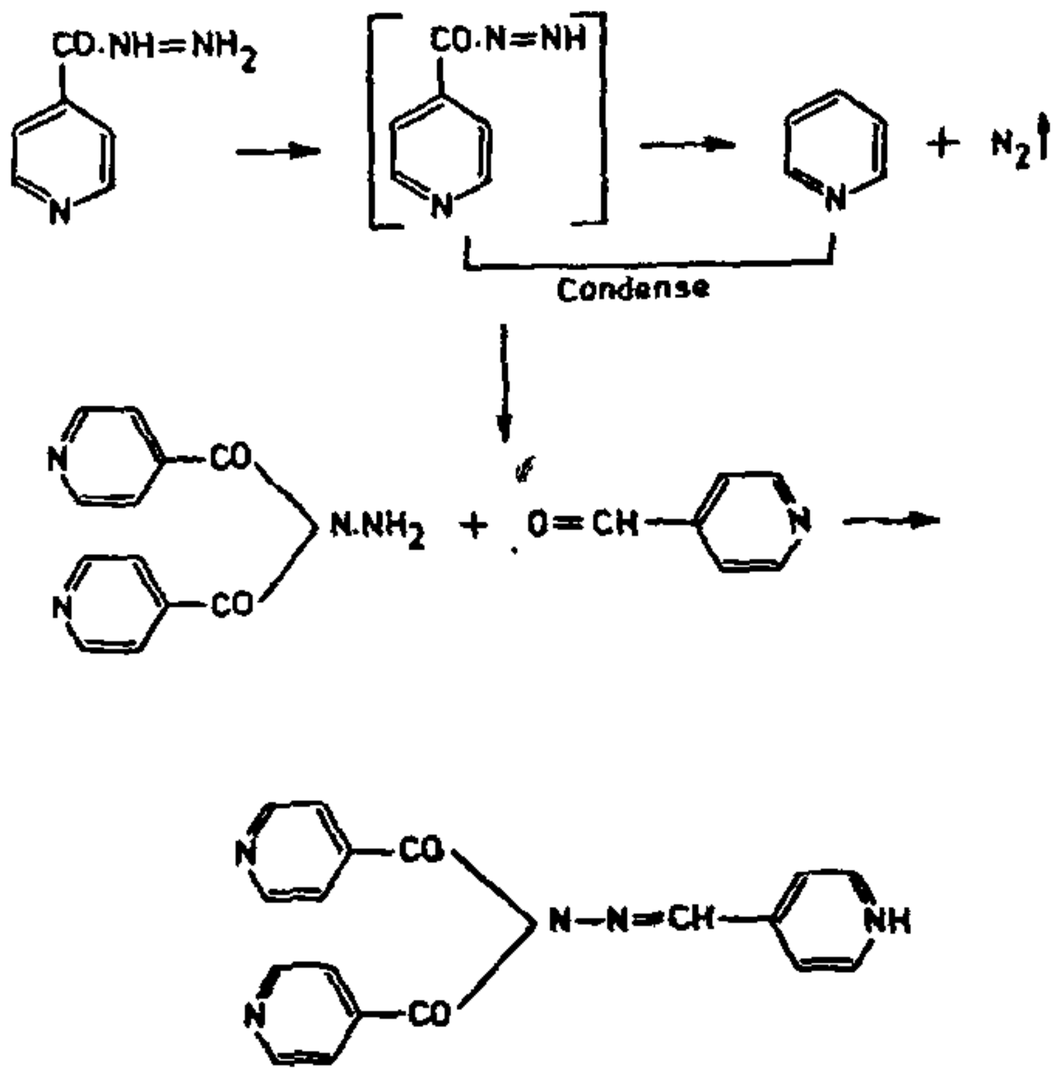
during the last 25 years (1957–1982) is given in what follows.

1. Studies on the metabolic response of tubercle bacilli to oxygen showed that contrary to conventional beliefs that tubercle bacilli are strict aerobes, the virulent strains of tubercle bacilli possess a functional glycolytic system[5]. Of utmost interest was the observation of Ramakrishnan *et al*[6] who studied the relative extents to which two strains $H_{37}Rv$ and $H_{37}R_a$ dissimilated glucose by the glycolytic and oxidative pathways. It was found that the virulent strain dissimilated glucose by the glycolytic pathway to a greater extent than the avirulent strain. This metabolic property of the virulent strain might account for the fact that it can multiply and survive in the host more successfully than the avirulent strain, even in increasingly anaerobic conditions of the developing lesions.

2. *M. tuberculosis* $H_{37}Rv$ was also found[7] to have all the enzymes of the tricarboxylic acid cycle and the glyoxylate bypass. It has been reported[4] that the avirulent strain does not possess the enzymes of the glyoxylate bypass. This is one of the few qualitative biochemical differences found between the virulent and avirulent strains of the tubercle bacillus.

3. To understand the role played by the nicotinamide adenine nucleotides in the oxidative metabolism of *M. tuberculosis* described above, a study of these coenzymes was undertaken. The organism was found to possess a nicotinamide adenine dinucleotide glycohydrolase (NADase) and also its protein inhibitor, which under normal conditions, prevents the NADase being active[8]. These two proteins have been purified to homogeneity[9]. After our work was published, Bekierkunst[10] reported that the antitubercular drug isoniazid acts by binding to the inhibitor and thus allowing the NADase to break down the NAD necessary for the viability of the organism. However, the isolation of INH resistant mutants by Sriprakash and Ramakrishnan[11] showed that in some of the resistant strains the inhibitor was still sensitive to INH and hence the theory postulated by Bekierkunst may not be correct. It was also shown that during the mutation from INH sensitivity to INH resistance three proteins—catalase, peroxidase and Y enzyme—were altered at the same time[12]. During purification of these proteins from the crude extract of the bacillus, the same fold purification was found for all of them, suggesting that mutation affects one protein with three different activities. In the sensitive strains INH is converted by this protein in the presence of NAD to a toxic compound which inhibits the growth of *M. tuberculosis*. Though this compound has not been purified and identified, the molecular weight of the enzymatic product has been determined with the help of Sephadex gels and a tentative formula assigned to it (figure 1). We hope to synthesize this compound chemically and determine whether INH resistant strains of the bacilli are inhibited by it.



4. We have also carried out experiments on the nucleic acid metabolism and its regulation in tubercle bacilli as a part of the overall studies on the molecular biology of this organism. Initially, the biosynthesis of nucleic acid purines was examined. The precursors for the 8 atoms of the purine ring have earlier been worked out in *E. coli*. When this aspect was studied in *M. tuberculosis*[13], carbons 2 and 8 of the purine ring

in this organism came from formate and not methionine. On the other hand, labelled methionine was incorporated into the nucleic acids of the organism, suggesting that methylated bases were present in the nucleic acids of tubercle bacilli. This aspect will be discussed in detail later.

5. The protein synthesis in *M. tuberculosis*, especially with reference to the mode of action of the antitubercular drug streptomycin has also been studied. It was found[14,15] that protein synthesis in this organism is inhibited not only by streptomycin but by all broad-spectrum antibiotics, though they have no effect on the growth of the organism. It was also shown that if the permeability barrier to these antibiotics is removed by a detergent like sodium dodecyl sulphate, they inhibit the growth of tubercle bacillus. The same holds good in streptomycin-resistant strains, where streptomycin is able to inhibit the growth of these mutants if the permeability barrier is removed.

It was during this stage that we had the good fortune to discuss the whole problem of drug resistance and other facets of work on tubercle bacilli with Jacques Monod when he visited India in 1961. Monod had just then published his classical paper on the *lac* operon and he suggested that only the application of the then emerging science of molecular biology to the problem would lead to its solution. Some experience in this science was gained by the present author working for 2 years in Yale University on the isoleucine-valine operon of *E. coli*. On returning to Bangalore, work on the molecular biology of tubercle bacillus was started.

In order to work on the molecular biology of *M. tuberculosis*, an inducible or repressible system in this organism was required, as well as a genetic system like conjugation, transduction or transformation to transfer genes from one mutant strain of the organism to another. Since none of these was available in this organism, we set out first of all to find such systems.

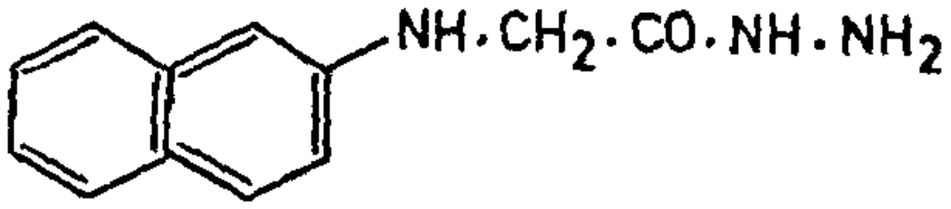
6. Since *M. tuberculosis* uses L-asparagine as a preferred source of nitrogen in its growth medium, we first concentrated our attention on L-asparaginase in the organism. Metabolic differences were found between the avirulent and virulent strains of tubercle bacilli in the metabolism of L-asparagine by the two strains[16,17]. The avirulent strain was found to possess two L-asparaginases, one of which is inducible, while the virulent strain possesses only the constitutive L-asparaginase. The inducible L-asparaginase was found to have inhibitory activity against Ehrlich ascites tumors. Since the DNA of *M. tuberculosis* is highly GC rich, it would be of interest to sequence the promoter of the inducible L-asparaginase in this organism to see whether it is still AT rich as in other organisms or not.

7. The virulent strain had all the enzymes of the isoleucine-valine biosynthesis. Excess of valine in the medium inhibited the growth of the organism, which was reversed by the addition of isoleucine. This phenomenon, brought about by valine repressing the enzymes of the common isoleucine-valine pathway, has earlier been reported by Umbarger in *E. coli* K_{12}.

One of the interesting side-products of this study[18-20] is that tubercle bacilli require vitamin C specifically for an essential enzymatic step in the biosynthesis of these branched amino acids. This is the first time that vitamin C has been shown to act as a coenzyme, and this finding has simplified the bioassay of this vitamin and its analogues, for which previously only tedious animal experimentation, using scorbutic guinea pigs, was available.

8. Since the phenomena of induction and repression in bacteria have been shown to act at the level of transcription, the biochemical aspect of transcription was studied in *M. tuberculosis*. Investigations on the RNA metabolism of the bacillus have shown[21,22] that the RNA polymerase of this organism has properties different from that of *E. coli*. It is 1000 times more sensitive to the antitubercular drug rifampicin than that of *E. coli*. The reason for this was shown by incubating both enzymes with labelled rifampicin and trying to leach out the label with cold rifam-

picin. The label is leached out from the mycobacterial enzyme much more slowly than the *E. coli* enzyme. These experiments were carried out with the mycobacterial enzyme purified to homogeneity. Thus a drug like rifampicin which acts at low concentrations on the RNA polymerase of *M. tuberculosis* not only does not affect the human host, whose enzyme is different from



prokaryctic RNA polymerase, but also the normal flora of the human intestine, like *E. coli*, at these low concentrations. Based on this finding a new potent antitubercular drug, N-naphthyl glycine hydrazine (figure 2) has been developed[23] This drug, which inhibits RNA polymerase of *M. tuberculosis* at low concentrations in turn inhibits the growth of the bacilli *in vitro* at 1 ug/ml. Dr. P. R. Mahadevan, in a personal communication to the author has reported that the compound also showed anti-*Mycobacterium leprae* activity when it was tested using labelled dihydroxyphenylalanine (DOPA). The drug is now undergoing trials against experimental tuberculosis in guinea pigs at the Patel Chest Institute, Delhi.

9. It has also been shown[24] that the slow growth of *M. tuberculosis* is reflected in the slow rate of its macromolecular synthesis. For instance, by using labelled (C^{14}) bases during the growth of the organism and pulse labelling the 3' end of the growth end of the RNA chain by ^{3}H labelled bases, it has been shown that the rate of chain elongation of RNA in this organism is 4 nucleotides/sec as against 40 nucleotides/sec in *E. coli*. Studies on the DNA polymerase[25] and elongation of DNA chain in this organism using rifampicin to stop initiation of new chains but not their elongation[26] have shown that the DNA chain elongation rate in *M. tuberculosis* is approximately one-twentieth that of *E. coli*.

10. Metabolic differences between the virulent and avirulent strains of *M. tuberculosis* have also been found in the methylation of the bases in their DNA[27]. 5-Methyl cytosine has been found only in the virulent strain and in *M. smegmatis* lysogenized by a phage. One of the possible conclusions from this work is that the virulence of the tubercle bacillus may be coded by a plasmid. Electron microscopic studies support the presence of such a plasmid in the virulent strain of *M. tuberculosis*, and no such plasmid can be demonstrated in the avirulent strain.

11. The analysis of methylated bases in the transfer RNA of the organism revealed[28] that it contains 1-methyl adenine and lacks ribothymidine. In this respect it differs from the tRNA of a prokaryote like *E. coli* and resembles that of eukaryotes. The initiator tRNA of *M. smegmatis* has been sequenced to find out the disposition of these methylated bases[29]. The structure of this tRNA also appears to be unique in that it occupies a place midway between prokaryotes and eukaryotes, as shown by the sequences at positions 1,54,57 and 72 of the tRNA.

12. In addition, a significant breakthrough in understanding the genetics of mycobacteria and their drug resistance was achieved by the isolation of a transducing mycobacteriophage for the first time[30,31]. Amber mutants of this bacteriophage have been isolated[32] using genetic engineering techniques. Studies are under way to map the genes responsible for drug resistance in mycobacteria.

Thus, the orginal objectives with which our group started work on *M. tuberculosis* have been largely attained by 1982. Basic knowledge of the metabolism of this important organism obtained through these studies, should prove invaluable for an effective control of tuberculosis.

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ANNOUNCEMENT

Dr. B. C. ROY NATIONAL AWARD

Dr. B. C. Roy National Award has been given to medical practioners for their contribution to medical science. The award is of the value of Rs. 5000 and a medal. The following is the list of awardees for this year.

Eminent medical teacher category: Dr. T. S. Chelvakumaran (Manipal), Dr. B. S. Sadasivudu (Hyderabad) Dr. R. M. L. Mehrotra (Lucknow), Dr. B. L. Agarwal (Allahabad), Dr. M. P. Vaidya (Varanasi) and Dr. K. D. Sharma (Bombay).

Development of specialities category: Dr.T. Desiraju (Bangalore), Dr. K. B. Sharma (M.A. Medical College, New Delhi).

Dr. M. Viswanathan (Madras), Dr. P. B. Desai (Bombay) and Dr. P. K. Kakar (M.A. Medical College, New Delhi).

Socio-medical relief category: Dr. J. G. Kannappan (Madurai), Dr. Arun Sen (Calcutta) and Dr. Sivanand Adhvaryoo (Rajkot). Oration: Prof. N. Rangabashyam (Madras).