

STUDIES ON DRUGS FOR KANSEN'S DISEASE

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Studies on Drugs for Hansen's Disease

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286

286

CONTENTS

Page No.

Acknowledgements	...	...	
Abbreviations	...	...	
CHAPTER I : <u>INTRODUCTION</u>		...	
1. Introduction	...	...	1
1.1. Incidence of Leprosy	...	...	3
1.2. Leprosy, the disease	...	...	3
1.3. Treatment	...	...	6
1.3.1. Introduction	...	...	6
1.3.2.1. Historical	...	...	9
1.3.2.2. Substituted Sulfones	...	...	10
1.3.2.3. DDS : Active nucleus	...	...	14
1.3.3. Drugs other than sulfones	...	...	25
1.3.3.1. Introduction	...	...	25
1.3.3.2. Rifampicin	...	...	32
1.3.3.3. Clofazimine	...	...	36
1.3.3.4. Ethionamide / Prothionamide	...	...	40
1.3.3.5. Thiacetazone	...	...	43
1.3.3.6. Thiourea derivatives	...	...	44
1.3.3.7. Sulfonamides	...	...	45
1.3.4. Multi-drug therapy	...	...	46
1.4. The problem	...	...	48

1.4.1.	Reasons (hypothesis) for the problems	50
1.5.	Search for New drugs	52
1.5.1.	A new approach	53
1.6.	Discussion	53

CHAPTER II : CHEMISTRY

2.	Introduction	59
2.1.	Use of active transport system	60
2.1.1.	The target	62
2.1.2.	Choice of the drug	66
2.1.3.	Choice of the carrier	68
2.1.4.	Method of conjugation	75
2.2.	Materials and methods	82
2.2.1.	Materials	82
2.2.2.	Methods	83
2.2.2.1.	Synthesis of compounds	83
2.2.2.2.	Purification of compounds	85
2.2.2.3.	Characterisation of compounds	85
2.3.	Results and discussion	87

CHAPTER III : PHARMACOLOGY

3.	Introduction	91
3.1.	<u>In-vitro</u> cultivation of <u>M. leprae</u> .. (Review of literature)	94
3.1.1.	Use of tissue culture systems	94
3.1.2.	Use of bacteriological media	97
3.1.3.	Use of various extracts	99
3.1.4.	Chemotherapeutic studies in leprosy <u>in-vitro</u>	101

3.1.5.	ICRC bacilli	...	...	103
3.1.6.	Materials and methods	...	...	108
3.1.6.1.	Materials	...	...	108
3.1.6.2.	Methods	...	...	110
3.1.6.2(a)	Experiments	...	...	112
3.2.	Experimental animal models	...	...	113
3.2.1.	Attempts to transmit human leprosy to various animals	...	...	115
3.2.2.	The mouse foot-pad technique	...	...	119
3.2.3.	Development of new animal models..			126
3.2.4.	Chemotherapeutic studies in leprosy : <u>in-vivo</u>	...	...	128
3.2.5.	<u>Mycobacterium leprae</u>	...	...	130
3.2.6.	Materials and methods	...	...	131
3.2.6.1.	Materials	...	...	131
3.2.6.2.	Methods	...	...	133
3.2.6.2(a)	Experiments	...	...	137
3.3.	Results and discussion	...	...	139

CHAPTER IV : DISCUSSION AND CONCLUSION

4.	Discussion and conclusion	...	...	146
4.1.	Summary	...	...	154
REFERENCES				156
APPENDIX - 1				156
SYNOPSIS				1 - x11
STATEMENT				

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ADITYA MOON

ABBREVIATIONS

AFB	- Acid fast bacilli
B 663	- Clofazimine
CNS	- Central Nervous System
DDS	- Diaminodiphenyl sulfone
DDC	- Diethyl dithio carbamate
EHL	- Erythema leprosum nodosum
LL	- Lepromatous leprosy
BL	- Borderline lepromatous leprosy
BT	- Borderline tuberculoid leprosy
TT	- Tuberculoid leprosy
T/900r	- Thymectomised and irradiated
RFU	- Ready for use
HAS	- Human albumin serum
PPD	- Purified protein derivative
INH	- Isoniazide
PAS	- Para amino salicylic acid

CHAPTER : I

INTRODUCTION

1. INTRODUCTION

Over the past several millenia, the human race has fallen heir to thousands of recognisable diseases. Few have assumed such special place in history, in literature, or in popular consciousness as bubonic plague (the Black Death), syphilis, tuberculosis, leprosy and small-pox, which have taken on a mythology and a metaphorical significance quite beyond their simple clinical explanation.

Among these few special diseases, none has stirred more imagination or greater dread than leprosy. Leprosy is an ancient affliction; physical evidence for it exists in skeletons of Egyptian mummies from the fifth century A.D. and reference is rife since the dawn of civilisation (Griffin, 1981). In India, Africa and China, the disease has been prevalent since ancient times, and with the passage of time it was introduced into various other countries of the world.

It may be said, that earliest authentic reference to the disease is found in the ancient literature of India.

In ancient medical writings like that of Charaka, Sushruta and Vag-Bhata of our country the disease is known as Yat-Rakta or Yat-Shonita and Kushta. Reference to Kushta is also made in much earlier Indian literature eg, Manu Smriti and Atharva Ved, which clearly bring out the difference between Shvitra (i.e., Leucoderma) and Kushta which outwardly resemble (Dharmendra, 1978). Reference to this disease are found in Egyptian Papyri written thousand of years before the birth of Christ as well as in the writings of ancient China (Dharmendra, 1978).

Although leprosy is considered primarily a tropical disease, it was a major problem throughout Europe and the British Isles in the middle ages and medical historians remain baffled by its mysterious decline. Western Europe was largely leprosy free by the 17th century, although small pockets of the disease remained through the 19th century, most notably in Norway. It was here that Norwegian physician Armauer Hansen first discovered the causal organism, Mycobacterium leprae, in 1873. This landmark event relabled leprosy, Hansen's disease and the discovery of this specific bacillus, ruined many of the cherished hypothesis including popular and religious beliefs of punishment by Gods.

1.1. INCIDENCE OF LEPROSY

On a worldwide basis, leprosy today is estimated to affect 12 - 15 million persons (WHO, 1977; Bechelli and Martinez Dominguez, 1968). (It is likely that no more than 25% of those afflicted with leprosy are now receiving treatment.) It is quite disturbing that out of these almost one-fifth i.e., around 4 million cases occur in India (Dharmendra, 1978 a). The prevalence rate in India is 0.5 per 1000. The states of India where leprosy is highly prevalent are; Tamil Nadu, Andhra Pradesh, Orissa, W. Bengal, Maharashtra, Bihar, Uttar Pradesh and Madhya Pradesh. These eight states between them account for 95% of the total leprosy in India (Dharmendra, 1978 a).

1.2. LEPROSY, THE DISEASE

Hansen's disease, or leprosy, is a chronic infectious disease, caused by Mycobacterium leprae. This organism is rod like in appearance, and displays acid-fast staining qualities. In many respect it closely resembles tubercle (TB) bacilli.

Leprosy chiefly affects skin and peripheral nerves. In some forms of the disease, M. leprae can be found in large numbers in the nasal mucus membrane and in muscle tissues, liver, spleen, lymph nodes, testes and blood vessel walls. If untreated, nerve involvement

may lead to loss of the sense of pain, muscle strength and circulation resulting in the development of deformities.

Leprosy is found two to three times more frequently in males than females (Sengal, 1979). The disease occurs in children and adults. It is believed to have a variable incubation period, ranging from 2 -20 years. The mode of transmission has not definitely been established, but leprosy is probably most often contacted by breathing dust or droplets laden with the M. leprae bacilli. Other modes of transmission may also be possible (Moss, 1974).

Leprosy assumes several distinct clinical forms. These differences are related solely to the patient's resistance to the disease. The "indeterminate" form of leprosy is characterized by one or more vague hypopigmented spots on the skin. The course of indeterminate leprosy is variable. It may remain quiescent, recover spontaneously or progress insidiously. Its further progress depends upon the immunological responses of the body. It may either form into lepromatous, tuberculoid type or an unstable group of leprosy.

* Tuberculoid leprosy : Patients with fairly high resistance will develop tuberculoid leprosy, initially marked by the presence of a few well-defined hypopigmented skin patches or lesions. A partial or complete loss of feeling is associated with these lesions. The patches enlarge slowly

and leprosy bacilli are usually difficult to find. Hard nodules or tubercles also develop in the skin, thus the term tuberculoid leprosy.

If untreated, nerve involvement may lead to loss of the sense of pain, muscle strength and circulation. These effects are most commonly seen in the forearm and lower leg and can lead to development of foot deformities and the so called 'claw-hand'.

* Lepromatous leprosy : At the other extreme, subjects showing little or no resistance to M. leprae will develop the lepromatous form of the disease. Without treatment, M. leprae bacilli multiply almost unchecked and may be found in many body organs in addition to the skin.

The skin of the face typically thickens and the individual develops a leonine (lion-like) appearance. Soft nodules appear on the ears, nose and about the face. The nose is usually teeming with bacilli, which can result in eventual destruction of the nasal septum and palate.

* Borderline leprosy : (Dimorphous leprosy) This classification includes patients with intermediate grades of resistance to the infection. Lesions may resemble either the tuberculoid or lepromatous forms, depending on whether the individual shows greater or lesser resistance. In the midrange of resistance, the lesions will appear as mixed.

An important consideration in treating borderline cases is the fact that host resistance is unstable. Without treatment, the disease may drift towards the lepromatous pole. By the same token, under treatment, the resistance can recover and upgrade towards the tuberculoid end of the spectrum.

1.3. TREATMENT

1.3.1. INTRODUCTION

Leprosy is one of the most dreaded diseases and almost all branches of traditional medicine including psychotherapy, therapeutic occultism and herbal medicine have been employed in its treatment. In addition, social ostracism was practiced. In orthodox medicine, plant extracts played an important role in the treatment of leprosy. Chaulmoogra oil, obtained from plants of Hydnocarpus spp., dominated the treatment until the advent of sulfones.

Great advances have been made in the treatment of leprosy during the last three decades. New and potent drugs have been introduced and search is continuing for more effective and fast acting drugs. Various methods of reconstructive surgery have been applied for the treatment of deformities in leprosy and the importance of physiotherapy and occupational therapy in the prevention and management of early deformities has been recognised.

Drugs used in the treatment of leprosy patients may be divided into two categories.

- (i) the drugs against the causative organism of the disease - routine drugs.
- (ii) the drugs for the treatment of special symptoms and complications - specially indicated drugs.

Only the first category is reviewed here, the second category does not merit extensive review in the present context.

In the first category has been the age-long treatment with hydnocarpus oil and its preparations, which till about three decades ago were the mainstay in the treatment of leprosy. These drugs have now generally been discarded in favour of the new chemotherapeutic drugs which have brought about a revolution in the field of leprosy treatment. The first chemotherapeutic drugs to be introduced were the sulfone drugs, and in one form or the other, they still continue to be the main drugs for the treatment of the disease.

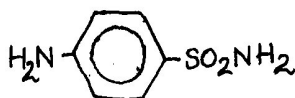
Although other drugs have proved of value as alternative methods of treatment, or as adjuncts to sulfones, generally they have not been found to surpass them; their main use is in sulfone - intolerant or sulfone resistant patients. However, drugs like rifampicin, clofazimine and ethionamide have been found to be very

actively bactericidal for the leprosy bacilli and clofazimine is useful in reactional states of leprosy as well. Moreover, the modern trend in chemotherapy of leprosy is treatment with a combination of drugs i.e., multiple drug therapy, as in the treatment of tuberculosis and other mycobacterial diseases.

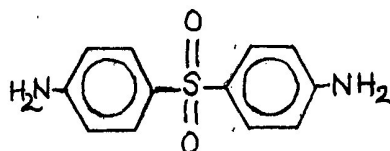
Individual drugs are discussed in the following pages. Since sulfones form the main class of drugs used in the treatment of leprosy, they are reviewed under one head, all other drugs are reviewed under 'Drugs other than sulfones'.

1.3.2. SULFONE DRUGS

The sulfones, as a class, are derivatives of 4,4'-diaminodiphenylsulfone (Dapsone; DDS). The parent compound, dapsone, is allied to the sulphonamides. These compounds are inexpensive and highly stable.



Sulphanilamide
(A sulphonamide)



Dapsone
(4,4'-diaminodiphenyl
sulfone)

1.3.2.1. HISTORICAL

Dapsone (4,4' - diaminodiphenylsulfone; DDS) is the parent compound of the sulfone drugs. Although synthesised in 1908 (Fromm and Wittmann, 1908), it was first tested only in the aftermath of the sulphonamide explosion. As a standard antibacterial agent it was disappointing (Buttle *et al.*, 1937) because it was 25 times as toxic as sulfanilamide. In 1938, Feinstein and co-workers concluded their experimental work with DDS in streptococcal, pneumococcal, meningococcal and staphylococcal infections in mice with the statement : "Its toxicity, in our opinion, precludes its use in human beings." Also in the same year, Bauer and Rosenthal found that the therapeutic index of DDS (maximum tolerated dose / minimum effective dose) against streptococci in mice was 6 as compared to 3.3 for sulphanilamide. Yet they found it desirable to obtain derivatives with lower toxicity without a corresponding decrease in therapeutic action.

In 1939, DDS was proved to have antituberculosis activity (Rist *et al.*, 1939; 1940; Feldman *et al.*, 1944) which in turn led to the discovery of its action against Mycobacterium leprae (Hilesworth *et al.*, 1949; Lowe, 1950). First however, some of the substituted sulfones were proved to be active against leprosy (Faget *et al.*, 1943; 1945; 1946 a). It was only in 1947 that dapsone injections were employed for leprosy (Cochrane *et al.*, 1949) and later oral treatment with one-fifth the earlier dose was introduced

(Lowe and Smith, 1949; Lowe, 1950). Both the trials were very successful and as a result the use of dapsone has been established all over the world.

1.3.2.2. SUBSTITUTED SULFONES

The derivatives of DDS may be divided into (i) 'Simple derivatives, and (ii) 'Nuclear substituted' derivatives. In the simple derivatives, one H of the NH_2 radical is replaced by various side chains; and in the nuclear derivatives, the side chain replaces an H at the ortho - or meta - position in the benzene ring.

(i a) Disubstituted simple derivatives : These include the most commonly known and widely used preparations e.g. promin, diasone and sulphetone. A much recent disubstituted derivative is 4,4' - diacetyldiamino diphenylsulfone (DADDS : Acedapsone) a repository preparation of sulfone.

(i b) Monosubstituted simple derivatives : These have been prepared and used to a limited extent only e.g., sulfone cilag, hydroxyethylsulfone and succisulfone.

(ii) Nuclear derivatives : These have been used to a very limited extent. They include promacstin, promisole and propiosulfone. A drug which is closely related to DDS is diamino-diphenylsulphoxide. This has been found as effective as DDS but it is too toxic for prolonged use.

Despite the study and development of a large variety of sulfones, dapsone remains the most useful clinically.

DADDS (Acetarsone)

The first reports of the clinical use of a sulfone in man dealt with a disubstituted sulfone DADDS (p-diacetylaminodiphenyl sulfone) in the treatment of gonorrhea (Heitz Boyer *et al.*, 1937; Palazzoli and Bovet, 1937). It was also successfully tried in experimental malaria (Coggeshall *et al.*, 1941) but no favourable effect could be obtained in experimental tuberculosis (Smith *et al.*, 1942). It was only after the establishment of the foot-pad method by Shepard (Shepard, 1960) that the antileprotic activity of the compound was established (Shepard, 1967; Shepard *et al.*, 1968). Considering the long term findings reported by Russel *et al.* (1975), it can be said that as far as the therapeutic effects are concerned, there is no difference between oral DDS and parenteral DADDS, except that DADDS provides a means of long interval intermittent treatment, as it has to be administered once every two months.

Still investigational in most countries, DADDS is used primarily as part of the combined therapy in the treatment of lepromatous leprosy, but may be used alone in treating non-lepromatous cases not without the danger of drug resistance.

first clinically used in leprosy by Muir (1944). Numerous other favourable reports followed and the drug attained wide usage after it was made available commercially in 1946 (Doull, 1963). Diasone acts the same way as DDS, but is less completely absorbed, has a lower therapeutic ratio, more frequent side effects, and is more expensive.

Sulphetrone (tetra sodium 2,4' - di [3 - phenyl - 1,3 - disulfopropylamino] - diphenyl sulfone) was first used by Wharton in 1947. Sulphetrone proved to be very well tolerated in effective dosage and came into extensive use (Doull, 1963; Bushby, 1964). This drug has been recently discarded after prolonged use in favour of the parent compound dapsone.

Other substituted sulfones

Early experiments were done with 4,4' - dinitrodiphenyl sulfone (Buttle *et al.*, 1937; Fournneau *et al.*, 1937), disodium formaldehydesulphoxylate diaminodiphenyl sulfone (Bauer and Rosenthal, 1938), a benzylidene derivative of DDS (Buttle *et al.*, 1938) and many more compounds derived from DDS (Buttle *et al.*, 1938). Often a promising activity was found against streptococcal or pneumococcal infections, though none of these derivatives was as effective as the parent sulfone, DDS, itself. Likewise, many substituted sulfones were tried out for leprosy. Among them were promacatin (or Internal antiseptic 307)

(Faget *et al.*, 1943) and promizole (Faget *et al.*, 1946) but none of these were used very extensively (Bushby, 1964). Till this date hundreds of mono and disubstituted derivatives have been synthesised in search for compounds with lower toxicity and greater solubility (Buttle *et al.*, 1938; Levy 1978; Colwell *et al.*, 1974). Most of these substituted compounds act the same way as DDS, are less completely absorbed, have a lower therapeutic ratio, more frequent side effects and are more expensive.

1.3.2.3. ACTIVE NUCLEUS : DDS

Faget (1947), while comparing promin, diasone and promizole, notes his impression that "the progress of improvement is very similar with each of the three drugs. The diaminediphenyl sulfone parent radical, common to all, appears to be active principal." It seems that only around 1948 was it fully realised that the proprietary sulfones were both active and toxic through the DDS nucleus (Floch and Destombers, 1949; Lowe, 1950 and 1950 a), although there had been many early suggestions in that direction (Buttle *et al.*, 1938; Johnson, 1940; Hinshaw and Feldman, 1941).

It now looks doubtful whether much useful purpose has been served by the production of various DDS derivatives, since evidence is now available that most of these complex sulfones are active by virtue of their being broken down in the body to the parent substance and/or because of

containing DDS as a contaminant in the derivatives (Francis and Spinks, 1950; Love, 1952; Gelber *et al.*, 1974).

Summarising the brief history of dapsone, Cochrane (1964) states : "DDS should have been the first sulphone shown to possess activity against mycobacteria, yet was not widely advocated for the treatment of leprosy, till its derivatives had been given for almost ten years, makes an interesting object lesson in the difficulties that can arise in the application of form of treatment, for which there is no experimental basis."

In as far back as 1935, Muir could state in an editorial of Leprosy Review : "Now that the rules of its use have been standardised, DDS has been accepted universally as the treatment of choice" and till this date the statement holds good. Since DDS is the most economical and convenient sulfone, it remains the drug of choice in the treatment of leprosy (Love and Smith, 1949; Love, 1950; Gómez Orbaneja, 1953).

DDS : Antileprotic activity

The exceptional sensitivity of *M. leprae* to dapsone is indicated by its MIC of only 0.003 µg/ml in rats (Levy and Peters, 1975; Peters *et al.*, 1975). At concentrations near to its MIC, dapsone is essentially bacteriostatic, but at concentrations in excess of 100 times this value it is

weakly bactericidal (Fieldsteel and Levy, 1976; Levy, 1976; Colston *et al.*, 1978). The *in-vivo* activity of dapsone in patients with leprosy has also been studied using the technique of foot-pad inoculation (Shepard *et al.*, 1968) and its estimated sensitivity is between 1 and 10 ng/ml for organisms recovered from untreated patients (Shepard *et al.*, 1969; Galber *et al.*, 1971). A dose of 100 mg dapsone results in peak concentrations that exceed the MIC by a factor of about 500 - fold and maintains inhibitory levels for about 10 days (Ellard *et al.*, 1975; Glasko *et al.*, 1968; Galber and Rees, 1975; Peters *et al.*, 1972; 1974; 1975 a; 1976; 1977). Several studies have shown that 39% or more of leprosy outpatients are grossly irregular in self-administering their dapsone treatment (Low and Pearson, 1974; Ellard *et al.*, 1975; Huikeshoven *et al.*, 1976; Balakrishnan, 1977).

Viable dapsone sensitive leprosy bacilli ("persisters") can be recovered in upto 50% of lepromatous patients after as many as ten years of continuous dapsone monotherapy (Waters *et al.*, 1974). Estimates of the prevalence of dapsone resistant strains of *M. leprae* among lepromatous patients have ranged from 3% to 20% (Levy *et al.*, 1977; Pearson *et al.*, 1975; 1977; Peters *et al.*, 1976). In the worst situation new dapsone resistant cases were occurring at a rate of about 3% per annum of those at risk (Pearson *et al.*, 1977 a) Dapsone resistance is a stable

characteristic of Malarexan (Jacobson and Hastings, 1976; Waters, 1977) and resistant strains are clearly infectious for man since dapsone resistant strains of Malarexan have been isolated from previously untreated patients (Jacobson and Hastings, 1978; Pearson et al., 1977 a).

DDS : Mode of action.

It is interesting to see how the ideas about the mode of action of DDS has changed over the years. The chemotherapeutic action was thought to be through the antagonism of p-aminobenzoic acid (PABA) in folic acid biosynthesis (Steenken and Heise, 1943; Brownlee et al., 1948; Denovick et al., 1962; Browne, 1967). Throughout the years most authors (Haylor, 1968; Haylor and Hanks, 1961; Pattyn and Van Ermenegen, 1968; Morrison, 1971; Colwell et al., 1973; Panitch and Levy, 1978; Seydel et al., 1980) have considered this at least as one important part of the essentially bacteriostatic action of DDS on microorganisms.

In 1952 however, Rath de Souza and DeSouza Lima drew attention to the degeneration of the histiocytic (Virchow) cells, caused by DDS. This process appeared to begin before the disintegration of Malarexan present in these cells. Their hypothesis therefore was that the sulfones act principally on the Virchow cells, in some way altering

its metabolism and making its cytoplasm unsuitable for life of Malariae.

In 1956 and repeatedly thereafter Bergel (1956; 1967) interpreted the mechanism of action of the sulfones against leprosy on the basis of an antioxidant activity, protecting the lipoproteic membrane of lysosomes and thus increasing the defensive capacity of the organism. The concept that DDS may be acting on, or through, lysosomal enzymes was further investigated by Prabhakaran and Bapat in 1966, who showed an elevation of acid phosphatase in mouse peritoneal macrophage by DDS, and by Palekar and Nagar (1967). A few years later Morrison (1971) hypothesized that "DDS interaction with lysosomal membranes results in permeability changes and the subsequent concentration of DDS within the organelle.... Thus through discharge of lysosomal contents into the phagosome a localized concentration gradient of DDS is available to exert anti-folate effects upon phagocytosed mycobacteria." Tautsumi and co-workers explained in 1978 some experimental results in terms of a "tidal wave action of DDS", first stabilizing lysosomal membranes, and in the later stage accelerating the NAD - NADH system inside the macrophage.

Also today the specifically low MIC of DDS for Malariae continues to puzzle the researchers, inducing them to consider alternative modes of action. Recently, Panitch and Levy (1978) showed that Malariae accumulates DDS against a concentration gradient, explaining the prolonged

bacteriostatic action of DDS. The activity was quantitatively antagonised by PABA, but "the 100 - fold greater MIC for Makansasi than for Malanras suggests that the actions of the drug on the two organisms may differ qualitatively as well as quantitatively." Extensive biochemical studies by Seydel et al., (1980) support this idea. They outline several hypotheses, two of which are of particular interest. The first is the powerful effect of the inhibition of folate metabolism by DDS that would occur if folate metabolism is the rate determining step for the extremely slow generation rate of Malanras. The second is the possible inhibitory effect of DDS, also at a second stage in the pathway of folic acid biosynthesis.

Perhaps the most recent hypothesis for the mechanism of action of DDS against Malanras comes from Prabhakaran and colleagues (1980). They found that DDS inhibits orthodiphenoloxidase, "the only enzyme proven to be present in the leprosy organisms." They suppose that the sulfur atoms of DDS bind the copper of the enzyme and thus inactivate it, if only the drug is able to penetrate the bacterium.

Thus the mechanism of leprostatic action of dapsone remains obscure.

DPS - Metabolism

Work on metabolism of DPS in man and animals has been done by several workers (Smith, 1949; Dharmendra *et al.*, 1950; Ross, 1950; Simpson and Molesworth, 1950; Bushby and Weiswood, 1955; Chatterji and Poddar, 1957; Dhople and Nagar, 1963; Bushby, 1967; Gita Dutta *et al.*, 1973). The metabolism of dapsone is discussed here, especially with reference to oral administration, since Dharmendra *et al.* (1950) found the blood concentrations of the drug were comparable when the various doses were given at varying intervals by either route and it is the only route used today.

a) Absorption

Dapsone is quickly and nearly completely absorbed from the gastrointestinal tract (Love, 1952) and undergoes enterohepatic circulation (Glasko *et al.*, 1968). Peak concentrations in plasma are reached between 2 - 4 hours, and is detectable for several hours. Its half life is around 21 hours (Alexander *et al.*, 1970).

b) Distribution

Dapsone is distributed throughout the total body water and is present in all tissues. It tends to be retained in skin and muscle and a higher concentration is found in liver, kidney (Simpson and Molesworth, 1950)

and leprosy lesions (Chatterji and Poddar, 1957) than plasma. In blood, about 50 - 80% of the drug is bound to plasma proteins (Graham, 1975; Riley and Levy, 1973; Gita Dutta *et al.*, 1973). The sulfones are retained in the circulation for a long time because of intestinal reabsorption from the bile.

c) Excretion

Dapsone is acetylated in the liver and the degree of acetylation is genetically determined, but the host's acetylator status has no effect on its elimination (Gelber *et al.*, 1971). Besides DDS in small amounts, less than 5% is excreted unchanged. (Bushby and Wolwood, 1955; 1956), the drug is present as an acid labile mono-N-glucuronide and mono-N-sulfamate in addition to an unknown number of unidentified metabolites (Shepard, 1969; Gita Dutta *et al.*, 1973; Alexander *et al.*, 1970). Another important metabolite of DDS found in urine is mono - acetylated DDS (MADDS) (International Leprosy Congress, Bergen, 1973 b) thought to be an active metabolite (Gita Dutta *et al.*, 1973).

DDS - Untoward effects

Sulfones are potentially very toxic (Buttle *et al.*, 1937; Cochrane *et al.*, 1949) but with doses under 300 mg daily, toxic reactions to dapsone are uncommon (Lowe, 1950).

The adverse reactions to dapsone have been extensively reviewed in a recent editorial in Lancet (Lancet, 1981). Most of the reactions are dose related and independent of the underlying disease. Some, however are idiosyncrasies and some arise only in particular disease setting.

Haemolysis occurs in most of the patients treated with dapsone, but upto a dose of 100 mg a day, it is mostly subclinical. Higher doses might produce haemolytic anaemia (De Gwin, 1967; Dharmendra and Chatterji, 1963; Powell *et al.*, 1967) usually of the Heing body haemolytic type (Cream and Scott, 1970). This anaemia is due to the decrease of the life span of RBC (Desforges *et al.*, 1959) and continues till the bone marrow is able to meet the increased demand. There is an increased risk in those with glucose-6-phosphate dehydrogenase deficiency (De-Gwin *et al.*, 1966). One common unwanted effect is cyanosis due to methaemoglobinaemia and sulphaemoglobinaemia (Alexander *et al.*, 1970).

Anorexia, nausea and vomiting may follow the oral administration of sulfones. Isolated instances of headache, nervousness, insomnia, blurred vision, paresthesia, drug fever, haematuria, pruritus and a variety of skin rashes have been reported. Similarly, reversible peripheral neuropathy (thought to be due to axonal degeneration) and

psychosis are rare complications (Rapoport and Guss, 1972). An infectious mononucleosis - like syndrome, which may be fatal occurs occasionally (Leiker, 1986).

The association of DDS with Lepra reactions is debatable (Huikeshoven, 1981). In 1973 Helmy and co-workers reported that DDS is at least no direct cause of EHL. Also in recent studies in India, it was concluded that EHL was not related to DDS therapy (Dutta, 1979). Clinical manifestations of EHL include fever, malaise, exfoliative dermatitis, jaundice with hepatic necrosis, lymphadenopathy, methaemoglobinaemia and anaemia (De-Gowin, 1967).

Although a large number of untoward effects have been described above, the sulfones may be given for many years in doses adequate for successful therapy of leprosy if proper precautions are observed. Treatment should be initiated with a small dose and quantity then increased gradually. Patients must be under consistent supervision for reactions, especially those related to exacerbation of the disease, which may be very severe and demand cessation of treatment as well as the institution of specific measures to reduce the threat to life.

DDS - Limitations

The sulfone drug, dapsone has no doubt marked a great advance in the treatment of leprosy, all the same it

suffers from certain limitations.

a) Treatment has to be continued for long periods

With DDS, the treatment has to continue for several years - specially in the case of lepromatous cases - before the disease subsides clinically and bacteriologically. In lepromatous cases, it takes five or more years for the bacilli to disappear from the skin, and in some cases the bacilli may persist even for ten years or more of treatment. Moreover, the bacilli may "persist" in bone marrow, nerves, muscles etc... after they have disappeared from the skin. Further, the treatment has to be continued even after the complete subsidence of the disease, for varying periods.

b) Set backs during treatment

There are some patients who improve upto a certain extent and then the improvement stops, followed by recrudescence of the disease even with continued treatment. This may be due to the emergence of 'sulfone resistant' strains of *M. leprae*. The question of 'dapsone resistance' will be discussed at length further in this chapter.

c) Untoward effects

The toxic manifestations of DDS, which have already been discussed, constitute another limitation in the use of DDS, for treatment of leprosy in some cases.

Secondly, a very small percentage of patients may be sensitive to the sulfone drugs, and alternative drugs have to be used. However, the really efficient alternative drugs are highly expensive, and may not be available for many cases till their cost is considerably reduced.

1.3.3. DRUGS OTHER THAN SULFONES

1.3.3.1. INTRODUCTION

The introduction of the sulfone drugs has no doubt marked a great advance in the treatment of leprosy, and they continue to be the sheet anchor, and the first line of treatment in leprosy. However, because of certain limitations, which will be referred to later in this chapter, the search has continued for more effective and fast acting drugs against leprosy. Also, it is a great advantage to have alternative drugs available, so that they may be used in cases which do not tolerate or benefit by the sulfones or in the sulfone resistant cases.

The earlier method of selecting drugs was based on clinical and bacteriological findings in groups of lepromatous cases. The findings of Rees and Valentine (1962), and Shepard and McKee (1975) regarding the change in staining characters of the leprosy bacilli under effective

treatment provided a rapid method of ascertaining progress under treatment by following the changes in Morphological Index (MI) i.e., by a fall in the proportion of solidly staining bacilli, was a breakthrough in that era.

With the introduction of mouse-footpad method by Shepard, experimental chemotherapy came to be established and rationality for the use of any drug in leprosy was put on a more precise ground before extensive clinical trials. More about experimental chemotherapy would be discussed in Chapter III. Results on experimental chemotherapy have been reported by several workers. Here a reference to the survey of the results by Shepard (1971), and a review of the matter by Ellard (1975; 1980) would put the drugs in leprosy treatment in right perspective.

Shepard (1971) made a survey of the drugs studied by the mouse foot-pad technique by himself and by others. The survey brings out the fact that apart from DDS and DADDS, the most active drugs are clofazimine, rifampicin, ethionamide, and long acting sulphonamides. Drugs like isoniazid, thiacetazone, thiambutosine, streptomycin and PAS are less effective; and drugs like pyrazinamide, ethambutal and etisul are inactive.

In the reviews by Ellard (1975; 1980), he has

compared the pharmacological and bacteriological aspects of the treatment of leprosy with various drugs in a tabular form, the condensed version of which is reproduced here (Table 1). It is possible to compare likely potencies of the drug in clinical treatment by considering the MIC against *M. leprae*, their bactericidal activity in mouse foot-pad and pharmacological studies in man. The important parameters are.....

- a) the ratio of peak serum or tissue levels achieved with well tolerated doses in man to the MIC against *M. leprae*, which is approximate indication of therapeutic margin of the drug.
- b) for bactericidal drugs, the likely rapidity and extent of bacterial killing at the concentrations attainable in man, and
- c) for purely bacteriostatic drugs, the duration after a single dose during which body levels exceed the MIC, since this gives an approximate indication of the regularity with which successive doses need to be taken if treatment failure caused by multiplication of leprosy bacilli between doses is to be prevented.

From the information given in the table, Ellard concludes that amongst the bacteriostatic drugs, dapsone

Table : 1

Minimum inhibitory concentrations against *M. leprae* (MICs), peak serum concentrations, duration of coverage and bactericidal activity of antileprosy drugs.

Drug	Human dosage (mg)	%dose absorbed	MIC (ug/ml)	Ratio peak ^a serum conc. to MIC	Duration which ^b serum conc. exceed MIC (Days)	Bactericidal ^c activity
Dapsone	100	>90	0.003	500	10	+
Sulphadimethoxine ^e	1500	>90	(20)	7	4	-
Sulphadoxine ^e	1500	>90	(35)	4	14	-
Acedapsone	225	100	0.003 ^g	15	200	-
Rifampicin	600	>90	0.3	30	1	+++
Clofazimine ^d	300	< 50	-	-	-	-
Thiambutosine ^f	1500	10	0.05	1	? < 1	-
Thiocarlide	2000	2	-	-	? < 2	-
Thiacetazone	150	>90	0.2	8	2	-
Ethionamide	375	-	0.05	60	1	++
Prothionamide	375	-	0.05	40	1	++

a : Ratio of peak serum concentrations in man after a single dose of MIC determined in the mouse.

b : Serum concentrations in man after a single dose.

c : Acedapsone is active against *M. leprae* by conversion to dapsone; the figures for MIC and peak serum concentration refer to the values for dapsone.

d : Because of uneven tissue distribution, estimate of MIC is not possible.

e : Cross-resistant with dapsone.

f : Manufacture discontinued.

g : Purely bacteriostatic (-) : relative degrees of bactericidal activity (+, ++, +++).

surpasses all other drugs, that aedapone (DADDS) is the long acting preparation of choice and that rifampicin is the only bactericidal drug against the leprosy bacillus. Regarding other bacteriostatic drugs, the long acting sulphonamides, much less adequate levels of antileprosy activity are attainable in contrast to those of DDS and DADDS, and there is no justification available for their continued use in the treatment of leprosy. Clinical and bacteriological evidence suggests that the potency of clofazimine is similar to that of aedapone. Regarding thiambutosine, thiocarlide and thiacetazone, it is possible that bacteriostatic concentrations of these drugs are not maintained for more than two days after giving single doses and thus are the weakest.

Ethionamide and prothionamide would be expected to show bactericidal activity intermediate between that displayed by rifampicin and DDS, but this has yet to be established in controlled clinical trials.

Reference to the drugs, other than sulfones can be made under two heads : (i) The earlier drugs used before the precise methods of assessment were available; and (ii) the newer drugs used after the introduction of such precise methods i.e., after the advent of experimental therapy.

(1) The earlier drugs

Search for alternatives to sulfones, were mostly on the basis of their antitubercular activity and some promising results were reported by several workers.

Of the earlier drugs used as alternatives to sulfones in the treatment of leprosy, a mention may be made of the following :

- 1) Thiacetazone
- 2) Isoniazide
- 3) Thiourea derivatives; the most popularly used one is thiambutosine. Another preparation which has been used to a limited extent only is thiocarlide.
- 4) Phenazine dye compounds; clofazimine (B 669 Gaigy, Lamprene) and B 1912.
- 5) Long acting sulfonamides; sulphamethoxypyrazine, sulphamethoxydiazine and sulphorthomidine.
- 6) Antibiotics used in the treatment of tuberculosis: Cycloserine, rifamycin, streptomycin, oxytetracycline, kanamycin, doxycycline, aureomycin etc...
- 7) Other drugs in the treatment of tuberculosis; para amino salicylic acid (PAS), pyrazinamide, ethionamide, prothionamide and thiol compounds especially etisul (etip).

8) Miscellaneous; Methimazole - Tapazole Lilly, a thyroid depressant drug and vadrine - an oxidiazoline compound.

(11) Drugs after experimental chemotherapy

Judged by the mouse foot-pad method, of the earlier drugs, only clofazimine has been found to be very effective. Long acting sulphonamides, although active, have been found to have MIC close to toxic blood levels in man. Of the new drugs, two have been found to be very effective; these are rifampicin and ethionamide, the latter producing frequent gastro-intestinal distress in man. (International Leprosy Congress, Bergen, 1973 a).

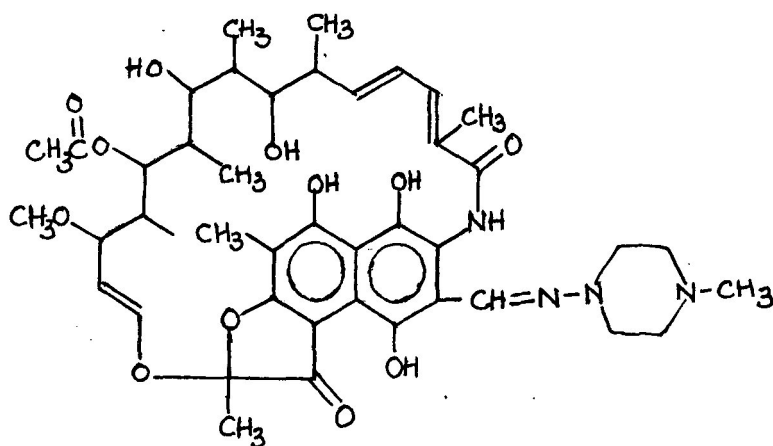
At the International Leprosy Congress, Bergen (1973 b), the Committee on Therapy recommended clofazimine and rifampicin. It further stated "Thiambutosine, streptomycin and thiasemicarbazone (thiacetazone) are less effective bacteriostatic drugs, and are therefore used less frequently. Long acting sulphonamides are reported to have given good results by some leprologists, but because of its toxic effects, the Committee has stressed the need of caution in mass leprosy campaigns."

Thus in addition to DDS and DADDs, the two drugs that claim serious attention are clofazimine and rifampicin,

used alone or in combination with other drugs. Ethionamide is also a more promising drug for the years to come. It has a high bactericidal activity - only surpassed by rifampicin, but more clinical data is awaited for it to be used on a mass scale. In case of multi-drug therapy, however, drugs like thiambutosine, thiacetazone, isoniazide etc... can play an important role.

1.3.3.2. RIFAMPICIN (Rifadin, Rifampin)

Rifampicin is a semi-synthetic derivative of Rifamycin - an antibiotic obtained from Streptomyces mediterraneus.



Rifampicin

Rifampicin inhibits the growth of most gram positive bacteria as well as many gram negative organisms (Atlas and

Turck, 1968; Kunin *et al.*, 1969). Rifampicin is bactericidal for *Mycobacterium tuberculosis* *in-vitro* and *in-vivo* (Verbist and Gyselen, 1968; Lorian and Finland, 1969) and *M. leprae* infections in mice and clinical trials in man (Rees *et al.*, 1970; Shepard, 1971; Shepard *et al.*, 1972 a; 1972 b; 1974; Pattyn *et al.*, 1974; Holmes and Hilson, 1972; Hilson *et al.*, 1971). Rifampicin inhibits DNA - dependent RNA polymerase of mycobacteria and other microorganisms, leading to suppression of initiation of chain formation (but not chain elongation) in RNA synthesis (Konne *et al.*, 1973) leading to bactericidal activity.

Over the years, rifampicin is used to a limited extent in leprosy because of its high cost and limited availability. This drug is indicated (Int. Lepr. Congr., Bergen, 1973 b) when it is important to render patients non infectious as rapidly as possible, in combination therapy (with DDS) for prevention of resistance (Peters *et al.*, 1972), in treatment of cases already resistant to other drugs (e.g., DDS) (Rees *et al.*, 1976) and concurrent presence of leprosy and tuberculosis (Languillon, 1973). Although this drug is so far the most potent against *M. leprae*, results of the long term treatment with the drug are not very promising (Rees, 1975; Leiker, 1975; Galber, 1976).

Various aspects of metabolism of rifampicin have been studied (Cohn, 1969; Furusaki, 1970; Janne and Beggs,

1975; Radner 1973; Dickinson *et al.*, 1974). The oral administration of rifampicin produces a peak plasma concentration in 2 to 4 hours. Aminosalicyclic acid delays its absorption (Radner, 1973). Following absorption from the gastrointestinal tract, it is quickly excreted through the biliary route, thus requiring frequent administration (Keberle *et al.*, 1968; Dickinson *et al.*, 1974). The half life of rifampicin varies from 1.5 to 5 hours.

Rifampicin is distributed throughout the body and is present in effective concentrations in many organs and body fluids including cerebrospinal fluid. (Sippel *et al.*, 1974). The drug imparts an orange-red colour to the urine, feces, saliva, sputum, tears and sweat; patients should be so warned. Rifampicin also penetrates into phagocytic cells and kills microorganisms that survive in the intracellular environment.

Rifampicin is fully active against dapsone-resistant strains (Rees *et al.*, 1970; 1975) but it interferes with the dapsone metabolism and reduces the levels of dapsone in the body (Gelber *et al.*, 1975; Gelber and Rees, 1975; Peters *et al.*, 1978). For this reason (as also its high cost) its use should not be prolonged. There are presently no clinical data to support the superiority of combination

therapy with rifampicin and dapsone over that with dapsone alone. Every species of bacterium, including *M. leprae* against which rifampicin was employed has developed strains resistant to the antibiotic (Stottmeier, 1976; Jacobson and Hastings, 1976). The problem of bacterial 'persistence' is also noticed during treatment with this drug (Rees, 1975; Gelber, 1976). Drug-sensitive persisters have been isolated after combined treatment with dapsone plus rifampicin (Gelber *et al.*, 1977).

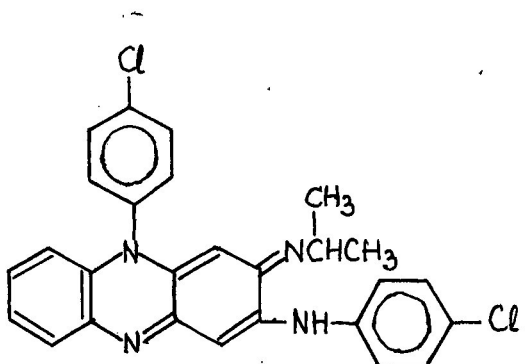
Rifampicin on the whole is well tolerated, although the occurrence of pruritus, headache, gastralgia, gastric disturbances of moderate degree, photosensitisation have been reported. Intermittant higher doses of rifampicin has been associated with frequent and more adverse reactions (Aquinas *et al.*, 1972) including flu-like syndrome and thrombocytopenia (Pujet *et al.*, 1974). The most notable problem is the development of jaundice (Scheuer *et al.*, 1974) especially during liver damage and hepatorenal syndrome (Flynn *et al.*, 1974). The more serious side effects such as thrombocytopenia, transient leukopenia, purpura, haemolysis hemoglobinuria, hematuria, renal insufficiency and acute renal failure are fortunately rare. An extensive review of adverse reactions to rifampicin appeared recently (Girling and Hitzge, 1980).

In tuberculosis, potentially serious side effects

were caused (Aquinas *et al.*, 1972) by the formation of antibodies to rifampicin and the possibility of similar effects in leprosy cannot be ruled out. The incidence of EHL has generally been slightly higher with rifampicin therapy (Pattyn *et al.*, 1975) although not statistically significant.

1.3.3.3. CLOFAZIMINE (B. 663. Geigy; Lamprene)

Clofazimine (B. 663) is a riminophenazine derivative, one of the entirely new series of antimycobacterial drugs, which has showed antituberculosis activity both *in-vitro* as well as in experimental animals (Barry *et al.*, 1967) but not in infections in man (Browne *et al.*, 1981).



Clofazimine

B-1912, a new analogue of B 663 (Barry, 1973) is cheaper and has the same activity as B 663 against a wide range of mycobacteria (Shepard *et al.*, 1972). Unfortunately

it has now been shown regularly to produce abortion in mice (Morrison, 1979) and will not be manufactured.

Clofazimine has a two fold activity - antibacterial and anti-inflammatory. Although the multiplication of M. leprae is prevented by clofazimine (Shepard and Chang, 1964) at 0.0001% (Shepard, 1969 a), its MIC cannot be estimated because of the marked accumulation of the drug by reticulo-endothelial cells (Conalty et al., 1971) and for the same reason, its mode of action (bacteriostatic or bactericidal) cannot be decided, although Hilson and Banerjee (1974), report a slow bactericidal activity. The mode of action of clofazimine may be by inhibition of the template function of DNA by binding to it (Morrison and Marley, 1976). Clofazimine was first reported to be active in LL patients by Browne and Hogerzeil (1962 a; 1962 b) and since then various reports, including a recent extensive trial (Browne et al., 1981) have confirmed the results. The therapeutic response obtained with daily doses of 200-300 mg. is similar to that achieved with dapsone (Pettit and Rees, 1966; Pettit, Rees and Ridley, 1967; Levy, Shepard and Fasal, 1972). Intermittent clofazimine treatment is highly successful in mice (Shepard et al., 1971), but results in man have been disappointing (U.S. Leprosy Panel/Leonard Wood Memorial, 1972).

Together with anti-leprotic activity, this drug

also possesses a strong anti-inflammatory activity (Browne, 1965; 1966). It aids the control of erythema nodosum leprosum (Hastings and Trautman, 1968; Karat *et al.*, 1970; Helmy, Pearson and Waters, 1972) and has been reported to have beneficial effects in the treatment of reversal (upgrading) reactions and neuritis in borderline (dimorphic) forms of leprosy. (Pfaltzgraff, 1972; Schultz, 1971).

The pharmacology of clofazimine in both mouse and man appears complex (Banerjee *et al.*, 1974; Levy, 1974). On oral administration 20% of the crystalline substance and about 50% of the micronised substance is absorbed, but the exact mode of absorption is not clear. The drug slowly passes into the blood stream and an average blood level of 1.5 $\mu\text{g/ml}$ is seen with a 300 mg. daily dose. After absorption, the drug passes into the various organs and is stored in the reticulo-endothelial cells and macrophages in these organs. Mansfield (1974), Desikan and Balakrishnan, 1976 and Balakrishnan *et al.* (1976), found clofazimine in all organs except brain. The sites where the drug accumulates, apart from the skin, are subcutaneous fat, adrenals, heart, liver, lungs, lymph nodes, small intestines, kidneys, pancreas and spleen. Excretion of this drug is very slow and only small amounts are excreted unchanged partly through the kidney and partly through the sebaceous and sweat glands.

Various publications give a detailed account of side effects and toxic manifestations of this drug (Hastings *et al.*, 1976; Jopling, 1976; Ramu and Iyer, 1976; Plock and Leiker, 1976) which are mostly due to the accumulation of the drug in the skin, mucous membranes and internal organs. A major side effect is the reddish-brown or blackish pigmentation of the skin, which may be very distressing to light skinned individual (Levy and Randall, 1970; Browne, 1965 a). Conjunctiva also shares the pigmentation of the skin, and there may be red colouration of urine, stools, sputum and sweat. Many type of gastro-intestinal manifestations are also seen. The usual ones are dyspepsia, nausea, vomiting, loss of appetite, loss of weight, epigastric pain, colicky pain in abdomen, constipation and diarrhoea. Occasionally these symptoms may be severe and clofazimine may have to be discontinued (Atkinson *et al.*, 1967; Schuls, 1971; Karat, 1975; Bergeyck *et al.*, 1980). Eosino-philiic enteritis has also been described as an adverse reaction of this drug (Mason *et al.*, 1977).

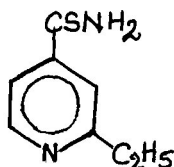
Because of its high cost, its slower action than dapsone and its various side effects, it cannot be used as a basic drug, but its combination of anti-inflammatory action and anti-leprosy activity places it in a unique position amongst the drugs available for treatment of leprosy. The main indications for the use of clofazimine are :

- a) Patients intolerant to dapsone. Clofazimine offers a second line of treatment as its activity is found comparable to dapsone.
- b) Reactive states in leprosy.
- c) Dapsone resistant cases, as confirmed by several workers (Levy et al., 1972; Taylor and Alexander, 1976).
- d) Neuritis;

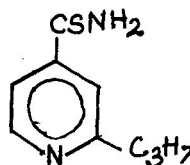
Unfortunately, there is now some evidence regarding the possibility of the development of clofazimine resistant strains of M. leprae (Browne and Hogerzeil, 1982 or Theophilus, 1978). Persisters can also be isolated after many years of continuous clofazimine treatment (Waters, 1977).

1.3.3.4. ETHIONAMIDE / PROTHIONAMIDE

Ethionamide (α -ethyl thioisonicotinamide) and more recent prothionamide (α -propyl thioisonicotinamide) are both congeners of thioisonicotinamide



Ethionamide



Prothionamide

Colston *et al.* (1978) have shown the high bactericidal activity of both the compounds, only surpassed by rifampicin. Prothionamide, a compound similar to ethionamide, was found to be as active as ethionamide against *M. leprae* (Colston *et al.*, 1978) and better tolerated in man (Fox *et al.*, 1969; British Tuberculosis Association, 1968). The antileprotic activity of ethionamide in mice has been studied by Shepard (1969 a, b; 1976) whereas prothionamide has been studied by Colston *et al.* (1978 a) and Pattyn (1978) and their MICs, are about 0.05 µg/ml. These drugs probably act by inhibiting the incorporation of sulfur containing aminoacids (Tsukamura *et al.*, 1964).

Both ethionamide and prothionamide have been used to a very limited extent in leprosy. They are mostly used along with other drugs, for if used alone resistance develops in about six months (Bragina, 1968). Strains of *M. leprae* exhibit cross-resistance between ethionamide, prothionamide, thiacetazone and thiambutosine (Pattyn and Colston, 1978), but are not cross-resistant with dapsone or rifampicin. (Rees, 1967, a). Although reports of clinical trials with ethionamide are available (Flouch *et al.*, 1966; Bragina, 1968) those of prothionamide are still awaited.

On oral administration, these drugs reach a peak serum concentration in about 3 hours. The drugs have a

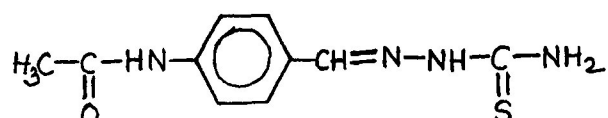
short half-life (Gray *et al.*, 1962). Ethionamide is rapidly and widely distributed; the concentrations in the blood and various organs are approximately equal. Significant concentrations are present in cerebrospinal fluid (Hughes *et al.*, 1962). Less than 1% of the drug is excreted in active form in the urine along with various other metabolites.

The most common reactions to ethionamide are anorexia, nausea, vomiting. Severe postural hypotension, mental depression, drowsiness and asthenia are common. Other reactions of nervous system include olfactory disturbances, blurred vision, diplopia, dizziness, restlessness and tremors. Severe allergic skin reactions, purpura, stomatitis, gynecomastia, impotence, menorrhagia, acne and alopecia and acute rheumatic symptoms have been noted. Hepatitis has been reported in 5% of the cases (Mandell and Sande, 1980). Prothionamide also has similar toxic effects, but probably less frequently than ethionamide.

Both prothionamide and ethionamide form the second line of drugs in the treatment of leprosy. They have to be used in conjunction with other drugs in order to restrict the possibility of drug resistant strains developing.

1.3.3.5. THIACETAZONE (TB-1; Amithiazone, Thiosemicarbazone)

Of the series of thiosemicarbazones synthesised (Domagk *et al.*, 1948), thiacetazone (4-acetylamino benzaldehyde thiosemicarbazone) was the most effective as tuberculostatic agent.



Thiacetazone

Ryrie (1950) first showed the chemotherapeutic activity of this drug in leprosy and later studied extensively by Lowe (1952 a, 1953, 1954, 1954 a) and other workers. It was considered to be a good alternative in cases intolerant to DDS, and had some additional beneficial effect on neurological symptoms. It also appears to exert a greater effect on the tuberculoid than on lepromatous forms of the disease. The Committee on Therapy of International Leprosy Congress, Tokyo (1958) reported "the relapses are rare in patients who have been treated with alternate courses of DDS and thiosemicarbazone."

In experimental leprosy (Colston and Hilson, 1976), the MIC of TB-1 is found to be 0.3 µg/ml to 1.0 µg/ml and Rees (1968) has shown that DDS resistant strains are

sensitive to thiacetazone (thiambutosine and clofazimine).

Thiacetazone is well absorbed from the gastrointestinal tract and large amounts are excreted in the urine (Robson and Sullivan, 1963).

The most common untoward effects of thiacetazone are anorexia, nausea and vomiting. The drug may depress bone marrow function, resulting in anaemia. Leukopenia and agranulocytosis occur. Skin rashes and albuminuria may also occur. The high incidence of jaundice in patients suggest that it is nephrotoxic.

Generally, thiacetazone can be substituted for sulfones, when the latter, for some reason cannot be administered.

1.3.3.6. THIOUREA DERIVATIVES

Thiambutosine (Ciba 1906; p-butoxy-p' - dimethyl aminodiphenyl-thiourea), thiocarlide (Isorexyl; p,p' - diisobutoxy-diphenyl-thiourea), a number of other diphenylthioureas have been shown to prevent the multiplication of M. leprae in mouse. (Colston and Hilson, 1976; Rees, 1967; Shepard, 1971; Hilson, Banerjee and Holmes, 1971).

Various clinical trials (Doull *et al.*, 1961; Davey, 1968; Davey and Currie, 1966; Griffiths, 1966) show that these drugs are not as effective as DDS, nor are they free from side effects. Some DDS sensitive cases are sensitive to these drugs (thiambutosine) and combined DDS and DPT treatment does not produce better results than those produced by either drug alone. Drug resistance develops after 2-4 years (Davey, 1968).

These drugs have been found to be bacteriostatic (Shepard, 1971) and cross resistance is shown by these drugs (Rees, 1967 a; 1967). Rees (1968) has shown that DDS resistant strains are sensitive to these drugs and vice-versa.

On oral administration both thiambutosine and thioacarlide are poorly absorbed (10% and 6% respectively) (Ellard, 1976; Ellard and Maylor, 1961).

Although thioacarlide had not been popularly adopted, as against thiambutosine which had been widely used, both have given way to the newer drugs like clofazimine, etc.

1.3.3.7. LONG-ACTING SULFONAMIDES

Several groups of long acting sulfonamides have

been tried in the treatment of leprosy. The most commonly used ones being :

- a) Sulphamethoxypyrazine (Sulphamethoxypyridazine)
- b) Sulphadimethoxine (Sulphamethoxydiazine)
- c) Sulphorthomidine (Sulphermethoxine, Sulphadoxine)

Languillon (1969; 1971) reported on satisfactory chemotherapy with various long acting sulfonamides including the above mentioned ones. Opronolla (1971) concluded that although the long-acting sulfonamides were effective, none was superior to DDS. This was confirmed in mouse-foot-pad model but the minimum inhibitory concentrations of these compounds appear close to toxic blood levels. (Ellard *et al.*, 1970). These compounds are inactive against dapsone-resistant strains (Adams and Waters, 1966; Rees, 1968). Besides, since these compounds are considerably more expensive than dapsone, their continued use in the treatment of leprosy remains unjustified.

1.3.4. MULTI-DRUG THERAPY

As in the case of tuberculosis and other mycobacterial diseases, there is a great need for use of multi-drug therapy in leprosy for the treatment of multi-bacillary (LL and BL) cases. This need hardly arises in paucibacillary non-lepromatous cases, and this kind of therapy is therefore not indicated in non-lepromatous cases.

Although dapsons is very effective in the treatment of leprosy, because of the limitations, discussed before, it cannot be called on ideal drug and recourse has to be made to multi-drug therapy.

There are three main reasons for the use of multi-drug therapy in multi-bacillary leprosy patients. These are :

- (i) Prevention of development of drug resistance during mono therapy;
- (ii) To successfully deal with the question of persistence of viable bacilli still sensitive to the drug, after treatment with the drug for long periods.

The drug resistance and persistence of the bacilli are the two factors responsible for relapses of the disease in varying percentage of cases after discontinuation of treatment.

- (iii) A third advantage of multi-drug therapy is that clinical improvement and bacteriological clearance may be quicker with the combination of drugs than with monotherapy even with potent anti-leprosy drugs like dapsons and rifampicin. However, this is disputed.

The extensive literature on this subject is beyond the scope of this thesis, suffice to say that the multi-drug therapy, besides being highly effective, should also be cheap enough for mass treatment of large number of patients of the developing countries. Naturally, dapsone has to be one of the constituents in such therapy together with a bactericidal drug like rifampicin or ethionamide.

1.4. THE PROBLEM

Before setting out for the search of new drugs one should necessarily analyse the problems with the existing drugs. With this rational it was felt necessary to highlight the problems that exist with the present battery of drugs for leprosy, before the look-out for new drugs. The two major problems noticed with the anti-leprosy drugs are : the problem of "Drug resistance" and the problem of "Persistent ^aMalan₂" which have been described before in this chapter as responsible for treatment failures and relapse in leprosy (Levy, 1976 a). A good discussion of these problems is now available (Freerksen, 1975; 1978 a; World. Hlth. Org., 1982).

Drug Resistance

As has been mentioned before in this chapter, Malan₂ has been shown to develop resistance to almost all the drugs used in the treatment of leprosy. All the

primary drugs including dapsone (Pettit *et al.*, 1968), rifampicin (Jacobson and Hastings, 1976) and clofazimine (Browne and Hogerweil, 1982 c) have developed resistant strains. The problem of resistance is on the increase and no time should be lost to combat this problem or these drugs will soon be obsolete. Whereas some drugs like dapsone take years to develop resistance, other drugs like thiambutosine, develop resistance within a matter of months.

Amongst all the drugs, dapsone takes the longest time to develop resistance and that too is at a dose like for example... if *M. leprae* are resistant against dapsone at a dosage of 0.1 mg/kg body weight, they are still sensitive to 1 mg/kg.

PERSISTENT *M. leprae*

The problem of bacterial persistence in leprosy has been reviewed in a recent article (Toman, 1981). Bacterial persistence is a phenomenon commonly defined as the capability of the microorganisms to survive in the host despite adequate microbial treatment and this phenomenon in leprosy has been rather recently observed in small number of patients under prolonged chemotherapy (Waters *et al.*, 1974; Russell *et al.*, 1976; Rees *et al.*, 1976; Pattyn *et al.*, 1976). The fact that viable, drug susceptible *M. leprae* could be found after years of

treatment with dapsons, clofazimine, rifampicin, and thiambutosine has been taken as evidence that none of these drugs is capable of eradicating the viable organisms from the body of patients with multibacillary leprosy.

Persisters cannot be directly identified in a bacterial population; their presence may only be inferred. Persistence is probably induced by factors unfavourable to multiplication or those that inhibit bacterial metabolism such as aging and crowded bacterial populations, old necrotic lesions, sparsely vascularised sites with low oxygen tension (pO_2) and intracellular locations within macrophages where pH is acid. Persisters are cells that are capable of adapting to such adverse condition by reducing their metabolism to minimum. They may remain in this state for some time; a proportion of them dies but some cells may regain normal metabolism and multiply as do normal organisms.

1.4.1. REASONS

Reasons for drug resistance could be many. Many a time, the resistance may be due to the failure of the patient to take adequate dose of an effective drug regularly (Browne, 1980), but the mechanism of resistance development may be

varied (Franklin and Snow, 1975). Although this mechanism of resistance development is understood for some anti-leprosy drugs, e.g., microbial resistance to rifampicin is due to an alteration of the target of this drug - DNA - dependent RNA polymerase (di Mauro *et al.*, 1969), that for other drugs like dapsone is still not clear. Instances of organisms developing resistance by putting up a permeability barrier are known (Franklin and Snow, 1975). It is now also known that DDS does not penetrate M. leprae to any appreciable extent (Prabhakaran *et al.*, 1981). Could this then be the reason for dapsone resistance?

The problem of 'bacterial persistence' could also mean that the antileprosy drugs do not penetrate deep into nerves which harbour the M. leprae (Kahn and Scott, 1974) or they are not taken in by the organism (Waters *et al.*, 1978). In sheep and dogs fed with dapsone or rifampicin, the drugs can be traced in extracts of exsanguinated sciatic nerve (Allen *et al.*, 1975) and DDS is also shown to readily penetrate the nerves in man (Peters *et al.*, 1978 a), but in a recent study (Boddington and Stols, 1981), the authors state, that there seems to be a blood-nerve barrier in peripheral (sciatic) nerves in mice with advanced leprosy neuropathy. This barrier prevents markers given intravenously from penetrating deep into the endoneurium. Although in mice with very severe leprosy neuropathy this

barrier is defective, allowing leakage of protein markers deep into the endoneurium and into the bacillated macrophages located there, the markers were not seen in schwann cells loaded with bacilli. On the basis of the above results the authors suggest, that since intact blood-nerve-barriers can prevent penetration of proteins, anti-leprosy drugs which combine with plasma proteins might also be prevented from entering the endoneurium, unless the drug protein complex is broken down. Secondly, extrapolation to man of experiments in mice suggests that even in patients with a leaking barrier, the schwann cell will escape penetration by foreign substances thus leaving *M. leprae* within schwann cells unaffected by therapy. It may be mentioned here that the preferential sites of lodging of *M. leprae* are nerves and schwann cells of unmyelinated fibres besides smooth (dartos) muscle and striated muscle.

These could be some of the reasons for the problems with the present drugs as described before.

1.5. SEARCH FOR NEW DRUGS

Although the newer drugs are found to be active against leprosy, none has been found to be better than sulfones, discovered almost 30 years ago. The treatment of the disease is still far from satisfactory and combined therapy has to be resorted to. Search continues, therefore,

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barrier is defective, allowing leakage of protein markers deep into the endoneurium and into the bacillated macrophages located there, the markers were not seen in schwann cells loaded with bacilli. On the basis of the above results the authors suggest, that since intact blood-nerve-barriers can prevent penetration of proteins, anti-leprosy drugs which combine with plasma proteins might also be prevented from entering the endoneurium, unless the drug protein complex is broken down. Secondly, extrapolation to man of experiments in mice suggests that even in patients with a leaking barrier, the schwann cell will escape penetration by foreign substances thus leaving *M. leprae* within schwann cells unaffected by therapy. It may be mentioned here that the preferential sites of lodging of *M. leprae* are nerves and schwann cells of unmyelinated fibres besides smooth (dartos) muscle and striated muscle.

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for more effective and fast acting drugs.

In the search for new drugs there are basically two approaches.

- a) Chemical modification of existing drugs.
- b) Synthesis of totally new types of drugs.

Various chemical modifications of the existing anti-leprotic drugs have been attempted, but with little success. The only notable drug synthesised on this principal which has proved to be of some use is the DADDS, a repository preparation of dapsone (Shepard, 1967; Shepard *et al.*, 1968). Even this drug is not better than DDS, except that DADDS provides a means of long interval intermittent treatment (Russel *et al.*, 1975).

B 1912, a synthetic new analogue of B 663 (clofazimine) is also reported. This compound is easier to synthesise and therefore cheaper to make (Barry, 1973). Work is also underway on more structural analogs of clofazimine (Levy, 1981; Lepr. Rev., 1981), chaulmoogric (Levy, 1975) and long acting rifampicin analogs (Pattyn and Saerens, 1977). Of these the rifampicin analogs appear the most promising for further investigation.

Inspite of the great amount of work being done on this line, a success in the near future is doubtful since

it is difficult to come across a drug better or equivalent in activity to DDS and of lower cost to be used for mass treatment.

The synthesis of new structures is a much more difficult approach, especially when quick methods of screening their activity are lacking; as in the case of leprosy. The only ~~in-vivo~~ method (foot-pad method) available takes months to give results. This could be a frustrating experience for both the chemist and the pharmacologist. Since the introduction of sulfones in the treatment of leprosy was based on its antitubercular activity, in the search for better drugs attention has been directed mostly to the drugs found effective in tuberculosis. Now, one has to look for new leads.... One could screen potent non-steroidal anti-inflammatory drugs for antileprotic activity. The rationale being that quite a few effective drugs in use against leprosy possess anti-inflammatory activity e.g. dapsone and clofazimine (Gidheh *et al.*, 1979). Tilorone hydrochloride, a non-steroidal anti-inflammatory agent (Ford *et al.*, 1978) is an interesting example of one such drug shown to possess antileprotic activity (Levy *et al.*, 1978). All the same, it would be wrong to make any generalisation, as effective anti-leprosy drugs could come from other classes as well e.g., dihydrofolate reductase inhibitors

(Galber and Levy, 1976), antithyroid compounds (Levy and Anandan, 1978; Levy and Ullmann, 1975), interferon inducers (Levy et al., 1978). Thus, if the search for new antileprotic drugs should not be a work of Sisyphus, one has to have a rational basis of designing new drugs.

The designing of new drugs could have been easier, if the biochemistry of the disease process or the metabolic pattern of the causative organism was known. Agents could be designed which could specifically correct a particular altered process. Similarly, in the case of chemotherapy, agents could be designed or known effective blockers could be tapped which could specifically interfere with some essential metabolic step of the parasite only, without affecting the host (Nitya Anand, 1973). Unfortunately, our knowledge about biochemical processes, at the molecular level in leprosy, is still not developed to the extent that drugs could be designed with sufficient precision. Only a few studies have been conducted to elucidate different biochemical path-ways present in the organism (Prabhakaran, 1967; Prabhakaran and Braganca, 1962). One of the reasons for the lack of studies in this field is the inability to cultivate *M. leprae* in an artificial medium.

Thus the search for new drugs, resembles the hunt for the needle in a hay-stack. However, one may be the

happy man who strikes oil in his back garden. It is understandable, therefore, that one should look for another approach.

1.5.1. A NEW APPROACH

As has been mentioned earlier, DDS is the main weapon in the fight against M. leprae infection, in many countries, including India, with high incidence of the disease.

Now, fears have been expressed that the usefulness of this weapon might be lost forever. As stated earlier, strains of M. leprae resistant to DDS have emerged in different parts of the world, and is on an increase (Browne, 1980). Besides, there is the problem of "persisters" which account for relapse in many cases of leprosy (Toman, 1981).

The above mentioned problems could well be the result of inavailability of the drug at the site of lodging of M. leprae or the failure of the drug to permeate the cell membrane of the organism. The later hypothesis is very recently proved to be true by Prabhakaran et al. (1981), who have shown that DDS does not penetrate M. leprae to any appreciable extent.

This is not an unusual feature. Many therapeutic

agents are ineffective because of the failure of the active molecule to permeate the cell membranes and cases of bacteria are known which develop resistance to drugs by forming a permeability barrier (Franklin and Snow, 1975).

Attempts to overcome these problem include modification with lipophilic groups, attachment to macromolecules and entrapment in the liposomes. Another approach employs the active transport systems to bring potentially cytotoxic drugs into the cells. The advantage of this approach is that it can result in a 100- to 1000-fold concentration of the toxic agent inside the cell.

An approach to the development of new drugs for leprosy, or improving the efficacy of the existing ones, that exploits normal metabolic processes as a means of selectively delivering toxic agents into the bacterial cell is proposed in this thesis.

Amino-acids coupled to the DDS molecule to appear as a peptide act as discriminating drug carriers selectively permeating the target cell, *M. leprae*. Once inside the cell, normal metabolism could either result in the release of the drug which is toxic to the cell or the drug-amino-acid complex itself could interfere with the normal metabolism of the cell. Making an antibacterial

agent to permeate its target organisms should enhance its effectiveness (Gregoriadis, 1979).

1.6. DISCUSSION

It is concluded, that the treatment of leprosy, is far from satisfactory and there is a need for new drugs with better activity. Even DDS, which till this date had been the sheet anchor in leprosy treatment is facing limitations of "resistance" and "persistance" which may be due to poor penetration of DDS in the causative organism, M. leprae.

Subsequent chapters present the work done as an attempt to overcome the problems.

CHAPTER : II

CHEMISTRY

2. INTRODUCTION

A major obstacle to the successful use of drugs in medicine is their failure to perform in the living animal as well as they do in-vitro. Between the site of administration of a drug and its site of action stand molecules, cells, membranes and organs which must be bypassed or traversed. Most drugs, however, are toxic to normal tissues, have limited or no access to the target, or are prematurely inactivated or excreted. Anti-leprosy drugs are no exception. Many therapeutic agents are ineffective because of the failure of the active molecule to permeate the cell membrane or reach its target. Attempts to overcome this problem include modification with lipophilic groups, or by the use of carrier systems (Gregoriadis, 1977; 1979; deDuve et al., 1974; Trout, 1978). A variation of the latter approach employs the active transport systems to potentially deliver cytotoxic drugs into the cell. The advantage of this approach is that it can result in a 100- to 1000- fold concentration of the toxic agent inside the cell.

In the recent years there is a growing optimism that problems or difficulties with a drug can be circumvented by the use of carrier systems that will protect the environment from the drug they carry (and vice-versa) and

at the same time guide them to where they are needed or facilitate their release there. In a typical targeting sequel the drug-carrier unit would preserve its integrity, avoid interception by normal cells, penetrate interposing membranes, and selectively recognise and associate with the target. This should then facilitate both the release of the drug from its carrier and disposal of the carrier (Gregoriadis, 1977).

In the following pages an attempt to synthesise such drug-carrier units is described with a view to circumvent the problems faced with the drug in question, as mentioned in the previous chapter.

2.1. USE OF ACTIVE TRANSPORT SYSTEM

In the past some of the molecular biologists had hoped that they would be able to synthesise new useful compounds according to their needs. But the idea, that drug cannot only enter the cell in passive diffusion but they should be taken up by an active transport system or facilitated diffusion although may be too revolutionary, is still true (Table : 2).

Table 1.2

<u>Drugs (antibiotics)</u>	<u>"Misused" Transport System</u>
Sideromycins :	sideramin transport system
albomycin (grisein)	
ferrimycin	
A 22765	
succinimycin	
tripeptides :	oligopeptide transport system
phosphinothricin- ala-ala	
L-(N ⁵ -phosphono) - methionine- β -sulfoximyl- ala-ala.	
plumbamycin B	
dipeptides :	dipeptide transport system
bacillysin	
D-cycloserin	D-alanine transport system
phosphonomycin	transport system for glycer- ophosphate + system for glucose-6-phosphate
showdomycin	cytidine transport system
streptomycin	PEP-sugar-phosphotransferase system, part specific for N-acetylglucosamine
neojirimycin	PEP-sugar-phosphotransferase system, part specific for glucose
streptomycin	polyamine transport system

(Z^aehner, 1980)

The bacterial cell does not possess special transport systems for drugs. The drugs are appropriating some of the existing systems for substrate transport. In some cases it has been possible to identify the substrate transport system which is taken over by a certain group of drugs. Thus if we know of a substrate transport system for a particular substrate, it should be possible to design new drugs, or to modify a known inhibitor in such a way that the inhibitor can enter a larger spectrum of bacteria or accumulate in their cells, which leads to a higher activity (Zachner, 1980).

A new drug designed by this hypothesis would then have two parts:

- i) The drug, and
- ii) The carrier

Successful targeting depends on the drug-carrier unit, the target, and the biological milieu by which the two are separated (Gregoriadis, 1981).

2.1.1. THE TARGET

Obviously, the target for any action in leprosy is the causative organism Mycobacterium leprae. Before the designing of any drug-carrier unit, the target has to be well defined or the whole exercise is fruitless. It is

not just enough to define M. leprae as the target, it is equally important to find an essential part or an essential metabolic activity, which when blocked would result in the death of the organism. This then would be the target. Rational chemotherapeutic agent that acts directly on an essential metabolic activity in M. leprae would provide the most effective answer to the problems of leprosy (Prabhakaran, Harris and Kirchheimer, 1979).

Few biochemical studies have been done on M. leprae probably because of the inability to cultivate the organism in an artificial medium to get a sufficient quantity of organisms required for such studies.

The presence of mycolic acids in M. leprae was first demonstrated by Etenadi and Convit (1974). Like other actinomycetales, M. leprae has in its wall mycolic acids, arabinogalactan and peptidoglycan (Draper, 1976). The mycolic acids resemble two of the three found in M. tuberculosis, and differ from those found in M. avium, M. leprae and M. mageritensis and other cultivable acid fast bacilli (Young, 1980). The peptidoglycan contains diaminopimelic acid, D-alanine, glutamic acid, glucosamine, muramic acid and substantial amount of glycine. The simultaneous occurrence of glycine and mesodiaminopimelic acid in bacteria is rare and does not occur in other mycobacteria analyzed so far. At the

present time this is an important differential characteristic of Maleprae.

Likewise, knowledge of metabolic processes in Maleprae remains limited. Among enzyme activities reported to occur are dopa oxidase (Prabhakaran, 1967; Prabhakaran and Kirchheimer 1968), glutamate decarboxylase (Prabhakaran and Braganca, 1962), β -glucuronidase and N -acetyl- β -glucosaminidase (Matsuo and Skinsnes, 1974). In each case controversy persists about the origin of the activity, whether it is bacterial or host-derived. More investigation is needed.

The organism is reported to take up, and to incorporate into insoluble products, radiolabeled thymidine and DOPA (Talwar *et al.*, 1974; Ambrose *et al.*, 1974). An unusual form of the enzyme *o*-diphenoloxidase (*o*-diphenol : oxygen [EC 1.10.3.1]) has been identified as occurring in Maleprae (Prabhakaran and Kirchheimer, 1966; Prabhakaran *et al.*, 1975). Although phenolase, phenol oxidase, phenolase complex and dopa oxidase or tyrosinase occur in a variety of organisms, including vertebrates, invertebrates, higher plants, and fungi, the only report on the possible occurrence of the enzyme in bacteria relates to the oxidation of tyrosine to pigmented products by a small vibrio Microspira tyrosinatica (Larway and Evans, 1962). Several species of mycobacteria tested,

including *Malepraecurium*, *Malebacteriosis* and some so-called *Maleprae* cultures showed no phenolase activity. Therefore oxidation of DOPA has been proposed as an identification test for the leprosy bacilli (Kirchheimer and Prabhakaran 1968, Prabhakaran, 1976). Extensive work on the o-diphenoloxidase enzyme of *Maleprae* has been done by Prabhakaran and his co-workers. They conclude that it is a true - orthodiphenol oxidase and that other copper proteins and catalase or peroxidase are not involved in the enzyme activity observed (Prabhakaran, Harris and Kirchheimer, 1972), that it is an intracellular enzyme firmly attached to particulate elements in the bacterial cell (Prabhakaran *et al.*, 1973; Prabhakaran, 1967 a). The phenolase activity is important in the survival and proliferation of *Maleprae*. Although inhibitors of tyrosinase from mammalian and plant sources like penicillamine and mimosine did not inhibit the enzyme in *Maleprae*, either intact or disrupted or suppress multiplication of bacilli in mice, other inhibitors like diethyldithiocarbamate (DDC) which penetrate the bacilli and inhibits the enzyme are bactericidal, as proved by foot-pad technique (Prabhakaran, Harris, and Kirchheimer, 1972). Some other compounds partially inhibited the enzyme (Prabhakaran, Harris and Kirchheimer, 1969). DDC is a potent copper chelator and phenolase is known to be a copper enzyme. Thus, o-diphenoloxidase activity of *Maleprae* is probably essential for its multiplication.

However, the exact role of this enzyme is not clear (Prabhakaran, 1973). It is suggested that o-diphenoloxidase might serve as an alternative respiratory pathway. This would be important in organisms in which the cytochrome and flavoprotein systems are not fully active, as is probably the case in the leprosy bacillus (Prabhakaran, 1967).

Thus, o-diphenoloxidase, a constitutive enzyme in *M. leprae* appears to be the target and specific inhibitors of the enzyme would be of immense therapeutic value.

2.1.2. CHOICE OF THE DRUG

Based on the description of the target enzyme o-diphenoloxidase, DDC (diethyldithiocarbamate) appeared to be the drug of choice. The 100% inhibition of the phenolase enzyme (Prabhakaran, Harris and Kirchheimer, 1972) by DDC and bactericidal effect on multiplication of bacilli in foot-pad strongly support its choice as an anti-leprosy drug at least in cases where the leprosy bacilli are resistant to conventional forms of treatment. However, several problems regarding use of DDC in patient remains to be worked out - for DDC is unstable under acid conditions and has to be buffered before administration, moreover, it might produce copper depletion.

Other metal chelators also do not give good results although o-diphenoloxidase is known to be a copper protein (Makino *et al.*, 1974). Besides DDC, cyanide is a potent inhibitor of metallo enzymes and penicillamine is effective copper chelator, used in the treatment of hepatolenticular degeneration in man. Although, these metal chelators produced total inhibition of mammalian and plant tyrosinases, they show little effect on the o-diphenoloxidase activity of the bacilli (Prabhakaran, Harris and Kirchheimer, 1975).

Secondly, reducing agents are known to be inhibitors of tyrosinase (Lerner and Fitzpatrick, 1950). Reducing agents like reduced glutathione, ascorbic acid, and cysteine although completely inhibit plant and mammalian tyrosinase, show little activity on phenoloxidase of Malepnae even with disrupted bacilli (to exclude permeability barrier of the bacterial cell membrane), suggesting that the active site of the enzyme is not readily accessible to the inhibitors, unlike in plant and mammalian tyrosinases. Probably, the configuration of the enzyme protein in Malepnae is such that these inhibitors are unable to penetrate the enzyme molecule and bind its copper moiety (Prabhakaran, Harris and Kirchheimer, 1975). The activity of DDC, the only compound which completely suppressed the o-diphenoloxidase of Malepnae, is explained by the presence of the two non-polar ethyl groups in the DDC molecule which more or less shadow the sulfurs which

constitute the polar region (Prabhakaran, Harris, and Kirchheimer, 1975 a).

Of the anti-leprosy drugs in use today none is known to act by the inhibition of the enzyme o-diphenoloxidase in *M. leprae* except DDS. DDS is shown to inhibit o-diphenoloxidase, and this has recently been hypothesised as the mechanism of action of DDS (Prabhakaran et al., 1981). They suppose that the S atoms of DDS bind with the copper of the enzyme and thus inactivate it, if only the drug is able to penetrate the bacterium. Presently DDS causes only about 10% inhibition of the o-diphenoloxidase activity in intact *M. leprae* (Prabhakaran, Harris and Kirchheimer, 1981).

Thus dapsone, a clinically used drug was selected for attachment to the carrier. The exceptional sensitivity of *M. leprae* to inhibition by dapsone, as indicated by its MIC of only 0.003 µg/ml (Peters et al., 1975) which is much lower than those of other anti-leprosy drugs, speaks for the selection. Besides, DDS is a small molecule, and the cheapest drug available.

2.1.3. CHOICE OF THE CARRIER

Since little can be done to influence the target and surroundings, the carrier must be chosen or designed

appropriately. To that end advantage can be taken of the plethora of naturally occurring targeting devices, which, as well as functioning in the living animal, can teach us how to build our own carriers. Many macromolecules, cellular and synthetic carrier systems (Table : 3) have been tested in animals and some already in man.

The carriers described (Table : 3) have been used to achieve different purposes, but for designing of antibacterial agents, the carriers most exploited are the peptides and aminoacids. Proteins cannot be utilised because of their antigenic nature. Peptides and amino-acids are ideally suited as carrier species because of chain length, side chain and other functional group specificities. Furthermore, since the structural requirements for transfer differ for bacteria and mammalian cells, drug / drug-carrier conjugates can in principle be designed so as to be selective (Gregoriadis, 1979; Becker and Haider, 1979; Payne, 1972, 1977; Gilvarg, 1972). The molecular basis of cellular permeation of peptides and amino-acids have been studied in bacteria (Becker and Haider, 1979; Payne and Gilvarg, 1978; Sussman and Gilvarg, 1970).

The simplest carrier seems to be some amino-acid if the warheads (drugs) are also amino-acids (unusual type). The dipeptide compounds formed would be transported by the dipeptide and tripeptide systems and split in the cell

Table 1.2

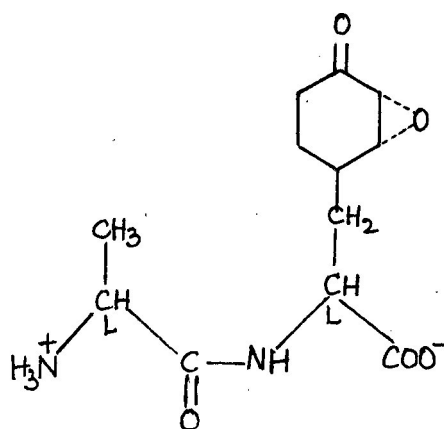
DRUG CARRIERS OF POTENTIAL USE IN MEDICINE

<u>Carrier</u>	<u>Target or mode of action</u>
Macromolecular :	
Antibodies and F(ab') ₂	Various cells (e.g. malignant).
Proteins, enzymes, hormones	
Asialoglycoproteins (galactose)	Hepatic parenchymal, sinusoidal cells.
Asialoglycophorin	Kupffer, endothelial cells.
Glycoproteins (mannose, glucose, N-acetyl-glucosamine)	Mononuclear phagocytes, spleen and bone marrow cells.
α -L-iduronidase (mannose-6-phosphate)	Fibroblasts.
β -hexosaminidase A (various sugars)	Brain synaptosomes.
β -D-glucuronidase (mannose-6-phosphate)	Fibroblasts.
Neoglycoproteins(fucose)	Alveolar macrophages.
Asialo-LDL	LDL-receptor-deficient fibroblasts.
Polylactose-LDL	Hepatic parenchymal cells.
Melanotropin	Melanoma cells.
Other tropic peptide hormones	Various cells.
Concanavalin A	Various cells (e.g., fibroblasts, Ehrlich ascites).
Other macromolecules :	
Monophosphopentamannose	Fibroblasts.
Polylysine	Drug resistant Chinese hamster ovary cells.

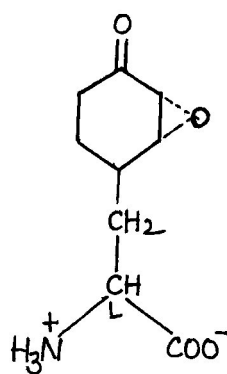
DNA	Phagocytic cells.
Dextrans	Increased half lives of drugs.
Polyethyleneglycol	Increased half lives of drugs.
Cellular :	
Mixed leucocytes, neutrophils	Inflamed areas.
Platelets	Macrophages.
Asialoerythrocytes (galactose, glucose)	Kupffer cells.
Erythrocytes	Kupffer and splenic cells, other cells by fusion, as scavengers of unwanted molecules, as depots for drug release, as suppliers of naturally contained agents.
Islets	As suppliers of insulin.
Fibroblasts	As suppliers of enzymes.
Pseudoviruses	Various cells.
Synthetic :	
Magnetic polymers, various types	Areas subjected to electromagnetic field.
Acrylic microspheres	Phagocytic cells.
Albumin microspheres	Phagocytic cells, lung cells.
Polyalkylcyanoacrylate nanocapsules	Phagocytic cells, as depots for drug release.
Poly(ϵ-caprolactone polylactic acid and polyglycolic acid.	As depots for drug release.
Liposomes	Phagocytic, hepatic parenchymal cells, other cells (with targeted liposomes) as depots for drug release (liposomes sensitive to HDL, temperature and pH) As vaccines.

Sugars in parenthesis denote molecules on the carrier which bind to cell receptors. LDL, low density lipoproteins; HDL, high density lipoproteins.

by its own peptidase into the normal amino-acid, which was only one part of the carrier and the unusual amino acid which is the active war head. The existence of antibiotic bacillysin and anticapsin (Kenig and Abraham, 1976) show that the idea of combining a war-head with a carrier is very likely more than a dream.

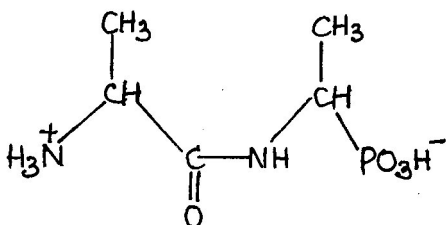


Bacillysin



Anticapsin

The dipeptide antibiotic bacillysin enters the cells of sensitive organisms through the peptide transport system (Kenig *et al.*, 1976) and these carriers have been exploited in the development of the antibacterial agent alaphosphin (Allen *et al.*, 1978).

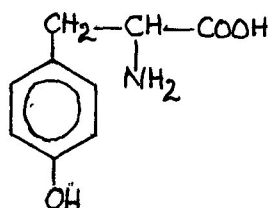


Alaphosphin

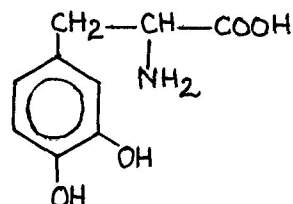
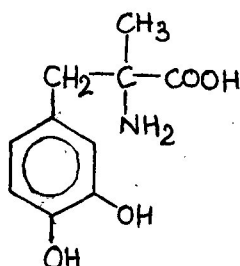
Various attempts have been made to carry normally impermeant molecules into various microorganisms (Steinfeld and Haider, 1979; Fickel and Gilvarg, 1973; Kenig and Abraham, 1976; Haider et al., 1974; Becker et al., 1973; Ames et al., 1973). Although most of these attempts have not been successful for various reasons, they do provide an insight to the mechanism of action and the problems arising in such a study. Whereas, various structural requirements for some organisms are well defined, for others they have to be yet studied. The requirements for permeases in E. coli have been well defined; these include the nature of any substitution in the amino or carboxyl termini (Payne, 1974; Payne and Gilvarg, 1968), the stereospecificity of the residues (Levine and Simmonds, 1962), the peptide bond (Payne, 1972, 1972 a), the chain length (Payne and Gilvarg, 1968 a) and the nature of the amino-acid residues.

Unfortunately, not much is known about the requirements of M. leprae as regards substrate specificity and requirements for permease. Investigations of the metabolism of M. leprae (Prabhakaran, 1967) have revealed a few of the metabolic characteristics of this microorganism. As stated earlier, o-diphenoloxidase, is the only specific enzyme proven to be present in the leprosy bacilli (Prabhakaran and Kirchheimer, 1968). This enzyme is shown to have very high affinity for and selectively binds DOPA (3,4 - dihydroxy - phenylalanine) as substrate

(Prabhakaran *et al.*, 1976; Prabhakaran, 1977). It is for this reason that DOPA was selected as a carrier amino-acid. For the present study, L-DOPA, DL-DOPA - [since mammalian tyrosinase oxidises L-DOPA at a much faster rate than D-DOPA (Brown *et al.*, 1959)] were used as carriers. Since DOPA is an unstable compound and can be oxidised readily by many substances (Lerner, 1953), methyl-DOPA, a more stable derivative was also used. Besides, Tyrosine which is a precursor of DOPA in body (Lerner, 1953) was also tried.



Tyrosine

DOPA
(3,4 - dihydroxy
phenylalanine)

methyl-DOPA

Dapsone-amino acid conjugates were synthesised with

a view that because of the structural resemblance with DOPA, these compounds would be actively transported into the cell of the bacilli where it could be acted upon by the enzymes of the cell to break and release free dapsons, which will bind competitively with o-diphenoloxidase of the bacilli or alternatively the complex itself could be active - resulting in the death of the organism because of higher concentration of the drug in the cells.

2.1.4. METHOD OF CONJUGATION

No purpose is served by the proper selection of the drug and the carrier unless they are bound or conjugated in such a way that the carrier carries the drug to the target. Conjugation methods are essentially the chemical procedures employed to link the drug to the carrier via covalent bonds. The selection of a particular method is determined by the physico-chemical characteristics of the drug, the carrier and the ingenuity of the investigator. There are innumerable methods of conjugation of two compounds but clues to some feasible methods can be had from the extensive literature especially on the preparation of drug protein conjugates for immunological studies and radioimmuno-assays (Erlanger, 1973; Kennedy *et al.*, 1976; Landon and Moffat, 1976; Miya *et al.*, 1976).

In order to successfully link a drug to a carrier, the active group or functional groups available on both the

drug and the carrier molecule have to be identified and once the functional group/s of a drug has been identified a suitable method can be selected (Table : 4).

As regards the drug of choice, dapsone, it is known that there are two functional groups available for reaction. These are sulfone group and the two free terminal aromatic amino groups. Other active sites for substitution are hydrogen atoms on the benzene rings of the DDS molecule. On review of the SAR of dapsone and its derivatives (Lewis and Shepard, 1970), it appears that the sulfone group is essential for activity, and any addition on the benzene nucleus in the structure results in decrease of activity. These therefore cannot be used. The terminal aromatic amino groups of DDS although important for activity can be utilised using a suitable conjugation method if it can result in free amino groups in the bacterial cell on hydrolysis.

As far as carrier is concerned, active groups for various amino acids have been described (Table : 5).

Table 1.4METHODS OF COVALENTLY LINKING A DRUG TO A CARRIER

Functional group on drug	Method of conjugation
Carboxyl	1) Use of isobutyl chloroformate to form a mixed anhydride, which will react directly with amino groups to form peptide bonds. 2) Use of carbodiimides ($R-N=C=N-R'$, where R and R' are aryl or alkyl groups) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or 1-cyclohexyl-3-[2-morpholinyl-(4) ethyl] carbodiimide can be used in aqueous solutions and dicyclohexylcarbodiimide in organic solvents to form peptide bonds. 3) Use of N-hydroxysuccinimide, in presence of dicyclohexylcarbodiimide to form the N-hydroxy-succinimide ester, which may be more active than the free carboxyl group.
Aromatic amino	Direct diazotization and coupling to the active positions of tyrosine, tryptophan and histidine residues.
Aliphatic amino	1) Use of carbodiimides (see above) 2) Use of glutaraldehyde to link amino acids 3) Use of 2-xylylene diisocyanate or toluene-2,4-diisocyanate to link amino groups. 4) Use of 4-nitrobenzoyl chloride to form 4-nitrobenzoylamide, which can then be reduced to the 4-aminobenzoyl derivative and linked by diazotisation as for an aromatic amino group.
Nitro	Reduction to a primary amino group and then as above.
Hydroxyl	1) Addition of equimolar proportions of anhydrides of dicarboxylic acids (e.g.,

succinic) to the drug gives half esters (e.g., hemisuccinate) and the free carboxyl group can then be linked as above.

- 2) Introduction of a carboxyl group using ω -halo-substituted carboxylic acids.
- 3) Formation of a chlorocarbonate with phosgene and use of the Schotten - Baumann reaction to link it directly to amine groups on the carrier molecule.
- 4) Use of oxidising agents to convert the alcohol into a carboxylic acid.

Carbonyl

- 1) Formation of the O-(carboxymethyl) oxime using O-(carboxymethyl) hydroxylamine and reaction of the free carboxyl group as above.
- 2) Introduction of a carboxyl group using 4-hydrazinebenzoic acid.
- 3) Schiff base formation directly with primary amino groups on the carrier molecule.

Active hydrogen

- 1) Direct conjugation using the Mannich reaction.
- 2) Reaction with diazotised 4-aminobenzoic acid to give a derivative with a free carboxyl group.

based on (Landon and Moffat, 1976)

Table 1.5**ACTIVE GROUPS ON AMINO ACIDS FOR CONJUGATION**

ε - Amino group of lysine.

Phenolic group of tyrosine.

Sulphydryl group of cysteine.

Carboxyl group of aspartic or glutamic acid.

Terminal amino and carboxyl groups.

Active positions on rings of tyrosine, tryptophan and histidine which can be attached to molecules by diazonium salts.

Imidazole ring of histidine.

Hydroxyl group of serine.

} Less reactive than the above

(Landon and Moffat, 1976)

Of the amino-acids decided to be used as carriers, the active groups available are free amino and carboxylic groups on the side chains. Although the importance of these groups for permease activity in *Malassezia* have not yet been studied, substrate specificity of tyrosinase from other sources are known (Lerner *et al.*, 1961), and one has to recourse to the data of the experiments with tyrosinase from other sources, till such time that concrete data on o-diphenoloxidase of *Malassezia* is available. From one such study of the substrate specificity of the mammalian tyrosinase, it was concluded that an α -amino group is essential for tyrosinase action, although the carboxyl group is relatively less important. Although the ethyl ester of tyrosine is shown to have similar activity as tyrosine, substitution of carboxyl group by methyl group or hydrogen results in a marked loss of activity as substrate (Lerner *et al.*, 1961). Thus the most feasible method of conjugation using the carboxyl group of the carrier amino acid and the aromatic amino groups of dapsons appears to be the formation of a peptide bonds.

Unfortunately, in a recent attempt at designing of anticandidal drugs by forming 5-fluorocytosine - peptide conjugates using the peptide bonds (amide bond between α -amino group of the fluorinated pyrimidine and carboxyl function of the peptide) (Steinfeld *et al.*, 1979) the workers were unsuccessful. They conclude that "the type of linkage used may be susceptible to nonspecific

hydrolysis." They have therefore turned to synthesising compounds using other linkages to get compounds stable to such nonenzymatic hydrolysis.

Besides nonenzymatic hydrolysis of peptide linkages, This linkage is also prone to series of proteolytic enzymes, peptidases in the body and can be easily broken down before the conjugate reaches the target (Appel, 1974). For the above mentioned reasons, the α -amino group, as well as the carboxyl group cannot be exploited for the synthesis of drug-amino-acid conjugates. The high specificity of the tyrosinase in general and diphenoloxidase of Malepran in particular, for the phenolic hydroxyl group preclude its use for the reaction. Thus the only active positions available on the amino-acid molecule are the active sites on the benzene rings of tyrosine and phenylalanine moiety. These can be used without much changing the nature of the molecule.

DIS (dapsone) molecule can thus be linked to active sites on the rings of tyrosine, DOPA and m-DOPA through the azo-(-N=N-) linkage. This can be achieved by the standard diazotisation and coupling procedure (Vogel, 1971).

The azo linkage is a stable linkage and azo analogs have been synthesised in the past and have been shown to be

active against mycobacterial infections (Lewis and Shepard, 1970).

The azo linkages can be reduced by azo-reductase. Azo reductase are microsomal enzymes which occur in tissues. They require NADPH and are stimulated by flavins. In most mammals azo-reductase activity is rather low but is high in microorganisms (Creasey, 1979). On reduction, the amino group is set free, thus in the present case, on the reduction of the azo-group dapsone would be set free, - this is a much required feature.

Thus the said diazotization and coupling procedure has been utilised for coupling the drug dapsone to amino acids.

2.2. MATERIALS AND METHODS

2.2.1. MATERIALS

1. Chemicals

Dapsone (4,4' -diaminodiphenyl sulfone) Bengal Chemical

L-Tyrosine : (E. Merck)

L-DOPA : (Loba-Chemie)

DL-DOPA : (BDH)

methyl-DOPA :

All other routine chemicals used were of A.R. Grade.

2. GLASSWARE

All glassware used were from Borosil, Corning.

2.2.2. METHODS

2.2.2.1. SYNTHESIS OF COMPOUNDS

a) Diazotization of Dapsone (Vogel, 1971)

A thin paste is made of 0.01 Mbl. of finely powdered dapsone* and 30 ml of water in a 50 ml beaker. To this 2.5 gm (2.1 ml) of concentrated hydrochloric acid is added and warmed until dapsone dissolves. This solution is cooled to 10°C with ice and stirred mechanically. 2.5 gm (2.1 ml) of concentrated hydrochloric acid are further added to the cooled solution.

A solution of 1.5 gm (0.022 Mbl.) of sodium nitrite in 3 ml of water is added to the above solution as rapidly as possible, taking care that the temperature does not rise above 15°C. Care should also be taken to see that excess of sodium nitrite solution is not added. Excess of nitrous acid formed in the reaction mixture are detected by immediate blue colouration of starch-iodide paper as also the decolourisation of bromine water and potassium permanganate solution.

The solution assumes an orange-red colouration as diazotization of dapsone takes place and is maintained below

5°C using an ice-salt mixture.

b) Coupling of diazonium salt with amino acid

0.02 M of the amino acid (Tyrosine, L-DOPA, DL-DOPA, methyl DOPA)* (a slight excess was used) was dissolved in a minimum amount of 10 M (40%) sodium hydroxide solution. The beaker containing solution was surrounded by an ice-salt bath contained in a large crystallising dish with a flat bottom; this permits magnetic stirring of the contents of the beaker. The solution was maintained between 0-4°C.

The solution of diazonium salt (a), kept at 0-4°C was added dropwise with rapid stirring to the cold amino-acid solution. The pH was maintained between 9 - 9.5 with further additions of dilute sodium hydroxide/hydrochloric acid as necessary. When all the solution had been added, the reaction was allowed to continue with slow stirring for at least one hour, maintaining pH 9 - 9.5.

After reaction, the solution acquired a deep bright colour (red to violet) and the compound was precipitated out by lowering the pH to 1.5 - 2.0. The solid brightly

* Molecular Wt. :	DDS	: 243.3
	L-Tyrosine:	181.2
	DOPA	: 197.19
	m-DOPA	: 211.21

coloured precipitate was filtered through fluted Whatman filter paper using suction.

2.2.2.2. PURIFICATION OF COMPOUNDS

The precipitate collected was washed extensively with cold dilute hydrochloric acid to get rid of any excess dapsone and then with chilled water to remove the acid.

The compound was then solublised by adjusting the pH to 9 - 9.5 using ammonia solution and reprecipitated by acidifying it with hydrochloric acid to pH 1.5 - 2 and filtering the precipitate. This procedure was repeated three times to get rid of any impurities. The compound was checked for impurities of dapsone by thin layer chromatography (TLC).

The compound free from dapsone contamination was finally recrystallised from large excess of water and dried in a vacuum desiccator over anhydrous calcium chloride and stored in a desiccator till further use.

2.2.2.3. CHARACTERISATION OF COMPOUNDS

As stated above, the compounds were checked for impurities of dapsone by TLC using silica gel as an

adsorbent and comparing it with authentic sample of dapsone.
The solvent system used was :

Ethyl acetate

Water

TLC of the compounds was also performed using various solvent systems described for dyes (Schweppe, 1969) especially the following, to understand the purity of the synthesised compounds. Silica gel was used as adsorbant for all TLC work.

a) n-Butanol	4 ml		
Ethanol	1.5 ml		
Pyridine	2.0 ml		
20% Ammonia	2.0 ml		
Water	1.5 ml		
b) n-Butanol	6 ml		
Acetic acid	1.5 ml		
Water	1.5 ml		
Acetone	0.01 ml		
c) n-Butanol	4 ml	1 1 1	Top layer taken as the system
Acetic acid	1 ml		
Water	8 ml		
d) Hexane	6.5 ml		
Benzene	2.0 ml		
Pyridine	1.5 ml		

Carbon, hydrogen and nitrogen estimations of all the compounds was done. Sulfur was not estimated.

Absorption spectra of the four compounds synthesised was taken on a Perkin-Elmer, PE-402, UV-VIS spectrophotometer

using 1 cm cell to confirm the coupling of dapsone to amino acids. A solution of compounds in 0.1 N sodium hydroxide was used for the spectra analysis.

2.3. RESULTS AND DISCUSSION

Dapsone was coupled to L-tyrosine, L-DOPA, DL-DOPA and methyl DOPA by diazotisation of dapsone and coupling to the amino acids. The conjugates, so formed have been coded as ADTy, AD-Dp, AD₂Dp and ADmDp respectively.

The conjugates formed were intensely coloured (bright red to reddish violet) a characteristic of azo dyes formed due to azo linkage. The coupling was further confirmed by the absorption spectra of the conjugates which were found similar to those obtained by Pauly for such compounds, prepared by similar procedure (Pauly, 1915).

All the four conjugates viz., ADTy, AD-Dp, AD₂Dp and ADmDp were found free from impurities of dapsone, as determined by TLC by comparison to authentic sample of dapsone.

The compounds synthesised showed lack of purity by TLC in various solvent systems. The compounds showed two to four bands with colours ranging from yellow to brown to dark red. No attempt was made to separate the components of the mixture in quantities suitable for further

characterisation. Impurities have been detected in similar compounds synthesised in the past (Tabachnick and Sobotka, 1959). These workers point out that it is not possible to prepare compounds of sufficient purity when the reaction was carried out with the amino acid itself instead of their *N*-acyl derivative. For reasons described before in this chapter, the *N*-blocked amino acid could not be used for this study.

In the absence of *N*-substituted derivative of the amino-acid, it is most likely that the diazonium ion reacts with the free amino group of the amino acids. Howard and Wild (1957) have shown that amino group of α -amino acids react with aromatic diazonium ions by loss of nitrogen. This fact is substantiated by the C.H.N. analysis of the compounds as shown below.

Table 1.6C, H, N & ANALYSIS OF THE COMPOUNDS

Compound		Formula	% C	% H	% N
ADTy	Calculated	a $C_{30}H_{28}N_6O_8S_1$	56.96	4.43	13.29
		b $C_{30}H_{26}N_4O_8S_1$	59.60	4.30	9.27
	Found		57.32	4.35	10.56
AD-Dp	Calculated	a $C_{30}H_{28}N_6O_{10}S_1$	54.21	4.21	12.65
		b $C_{30}H_{26}N_4O_{10}S_1$	56.78	4.10	8.83
	Found		54.06	4.28	9.64
AD Dp	Calculated	a $C_{30}H_{28}N_6O_{10}S_1$	54.21	4.21	12.65
		b $C_{30}H_{26}N_4O_{10}S_1$	56.78	4.10	8.83
	Found		54.84	4.19	9.04
ADnDp	Calculated	a $C_{32}H_{32}N_6O_{10}S_1$	55.49	4.62	12.13
		b $C_{32}H_{30}N_4O_{10}S_1$	58.06	4.53	8.45
	Found		55.98	4.54	8.85

A : Formula 'a' represents expected compound where the diazonium ion couples with the amino acids on the benzene nucleus.

B : Formula 'b' represents expected compound if the diazonium ion reacts totally with the free α -amino group of the amino acids with loss of nitrogen.

In agreement with the above assumption, the nitrogen content of the derivatives is found to be low (refer table : 6) but not low enough to prove that all the diazonium ions react with free α -amino group of the amino acid with loss of nitrogen as indicated by N content of Formula 'b'. Since no further separation of the compounds of the mixture was attempted the molecular weights of the compounds have been calculated based on formulas 'a' of the various derivatives for testing of their activity in in-vitro and in-vivo systems.

CHAPTER : III

PHARMACOLOGY

3. INTRODUCTION

The leprosy bacillus was among the first micro-organism ever described to be associated with human disease. Despite the early discovery, about more than a hundred years ago, leprosy remains an enigma. Though it is accepted that Mycobacterium leprae is the causative agent of leprosy, it has posed a number of questions in attempts to satisfy Koch's postulates (1884), for establishing the identity of the organisms. For over 80 years the problems of cultivation of M. leprae in-vitro and transmission of leprosy to animals were not resolved. During the last few decades, however, newer techniques of tissue culture, biochemistry, histopathology, electron microscopy, as well as supply of inbred animals have greatly contributed to the elucidation of these problems.

In the field of cultivation of M. leprae in-vitro, tissue culture methods have provided considerable data on the behaviour of fresh M. leprae in culture. Early study of cultures of lepromatous nodule explants have shown that the bacilli retain their morphology; they are easily phagocytosed by cells, but the number does increase, the apparent increase being due to release of bacilli from cells to medium. The studies on cultivation of M. leprae - rat leprosy bacilli, have achieved some

success, and provided both methods and criteria for growth and its quantitative estimations. Biochemical studies on respiration of Malacrasmarius and inhibitory activity of serum against these bacilli, pointed out various pitfalls in the management of cultivation of Malacras. By adapting tissue culture methods and employing bacterial and tissue extract supplements, limited success has been achieved during the last 30 years. The bacilli grown in-vitro do resemble in morphology, bacteriological characters, generation time and exhibit low virulence pathogenicity to animals. At present these studies have merely opened up experimental models for exploration of growth requirements of Maleppae and its physiological characteristics.

Transmission of Malacras to animals has been successfully achieved in recent years, especially, the transmission to inbred strains of mice has met with good success. The first attempts during the 20 years were directed towards selection of strain of mice, choice of site for inoculation and evolution of quantitative methods for evaluation of results on multiplication of the bacilli in-vivo. Among these, the foot-pad technique introduced by Shepard (1960) was found to give reproducible results and was widely adapted for studies on various aspects of

pathogenesis of leprosy. Though this technique showed only a limited multiplication of *M. leprae* in mice (Rees, 1964), later variation by Rees (1965), of complete destruction of immune mechanisms by thymectomy and total body irradiation, yielded continuous growth of bacilli as well as dissemination all over the body. Considerable data on pathogenesis of *M. leprae* and their viability in various tissues have shown that among the various tissues examined histopathologically, nerves and muscle tissue are the most favoured sites of lodging of *M. leprae*. Electron microscopic studies have also confirmed these data and has yielded much information on host parasite relationship of the bacilli with the phagocytic cells.

The foot-pad technique has been adapted for chemotherapeutic investigation, both for examining new drugs and sensitivity of *M. leprae* to standard drugs, as an aid to prognosis in the treatment of leprosy and lately to study and understand the problem of drug resistance.

In this chapter, after an initial discussion of the various available methods to test the efficacy of the anti-leprosy drugs, details of the experiments done, to evaluate the efficacy of the synthesised compounds are presented.

3.1. IN-VITRO CULTIVATION OF M. LEPRAE

Review of literature

Countless efforts have been spent on isolation and cultivation of M. leprae in-vitro since its discovery. Since the first reported apparent success in growing M. leprae in-vitro in 1880, to date, vigorous attempts are made to cultivate this highly fastidious organism. These efforts include diverse approaches viz., use of cell substrates, bacteriological media, addition of some extracts or exotic growth intermediates (Prabhakaran, 1975).

Many of these efforts report, that during cultivation of M. leprae, emergence of a saprophyte or chromogenic acid-fast bacilli usually occur. The materials and methods deployed by these investigators have been described in extensive reviews by McKinley (1939), Yoshie (1973), Prabhakaran (1975) and Pattyn (1977).

3.1.1. USE OF TISSUE CULTURE SYSTEMS

Cells of simian or human origin

Shepard (1958) made a survey of the growth of mycobacteria in human and monkey tissue cells. Baylet *et al.* (1960) reported failure in the multiplication of

Malepree using monkey kidney cells. A survey of the suitability of some cell lines of human origin was made by Morris and Nakamura (1959) without any evidence of multiplication. Garbutt (1965) has claimed success in cultures of human diploid cells, but this has not been confirmed.

Primary cultures of human peripheral tissues

DeSouza Aramjo (1956) tried tissue culture from a histiocytic tumour taken from a patient with lepromatous leprosy and observed acid-fast bacilli until the 7th passage. Delville (1963) described erratic and limited multiplication in cultures of human amniotic cells. Tissue cultures from lepromatous tissue was also tried by Yoshie (1963), while Jadin *et al.* (1967) claimed to have obtained multiplication of Malepree in cells derived from lymph nodes and human skin, after which the bacilli multiplied in the cell-free medium.

Cultures of human or murine nervous tissue

This was tried frequently, in view of the well-known affinity of the leprosy bacillus for nervous tissue. Banadive *et al.* (1953) tried cultures of human foetal spinal ganglion (and skeletal muscle) cells in plasma clot. The organisms were readily phagocytosed and found to be

healthy - solidly stained - inside the cells for 8 to 10 days.

Human macrophages

Samuel *et al.* (1973) cultivated human macrophages *in-vitro* and infected them with *M. leprae*. In 50% of the experiments, a 2 to 9 fold increase in the number of acid fast bacilli was observed within 12 to 3 months of cultivation, both at 32°C and 37°C. This limited multiplication could be suppressed, using anti-leprosy drugs, thereby establishing conclusively that multiplying organisms were *M. leprae*, in the macrophage *in-vitro*, irrespective of the origin of the latter i.e., a healthy individual or a LL or a TT case.

Various other cell cultures of human and animal origin have also been tried for multiplication of *M. leprae* and extensive studies with *M. leprae* *in-vitro* have also been undertaken (Rees and Garbutt, 1962) which has given a lot of information on the behaviour of these mycobacteria inside tissue cells.

In an extensive objective study on survival of *M. leprae in-vitro*, Lumsden (1964) observed that among the various tissue substrates, schwann cells have the highest phagocytic activity against *M. leprae* with 76% cells showing parasitization. The intracellular bacilli had

little or no adverse effect on the cell. These organisms retained their morphology and staining properties for a long time. The other cell types, viz., HeLa, HEP 2, human amnion cells, sympathetic ganglia, schwannoma cells and nerve tissue from a variety of animals did not support the growth of Maleprae.

3.1.2. USE OF BACTERIOLOGICAL MEDIA

Various bacteriological media and cell free synthetic media have been tried out for the cultivation of Maleprae with some success. Freire (1956) used slide culture method for growing Maleprae, the medium consisted 1% agar and 20% LL. serum and observed new globi formation, but Ghosh and Mukherjee (1982) in an attempt to repeat the work of Freire could not find any evidence of proliferation of the organisms or formation of new globi.

DeSouza, Araujo (1943-1951 a) published a series of reports on the cultivation of Maleprae. He isolated sixty unclassified strains of acid-fast bacilli on L-J medium directly from leprosy material and from insects harbouring the clothes of the patients.

Makayama (1968) observed colonial growth of Maleprae, but most of them turned out to be passenger organisms, except a group showing remarkable pleomorphism.

Murohashi and Yoshida (1971; 1972; 1974; 1976; 1976 a; 1978) formulated a series of new semi-synthetic soft agar media of M-Y-series which consisted of glucose, glycerol, yeast RNA in the medium. The colony-like bacterial masses were observed after 20 weeks of incubation at 37°C. These masses consisted of acid-fast organisms, which persisted in the medium even after one year of incubation. The increase in the size and the number of bacteria was low. Subsequent subculturing resulted in the increase in the number of acid-fast bacilli. The intradermal injection of a Dharmendra type of antigen, prepared from these bacteria, elicited the reactions comparable to 'lepromin'. Drug sensitivity tests of the cultivated organisms have also been undertaken using liquid media (Murohashi and Yoshida, 1976 b, 1978 a).

Skinsness et al. (1975) claimed that LA-3 medium enriched with hyaluronic acid and fresh yeast extract, yielded an in-vitro culture of M. leprae in 6 weeks of incubation at 37°C. The inoculum consisted of bacteria isolated from trypsin and alkali treated lepromas. These bacilli, as reported by Skinsness, have a phase in their life cycle in which non-acid-fast protoplast - like bodies or short rods could be observed. At the end of sixth week, non-acid-fast material could be traced, but with predominance of acid-fast rods. On LA-3P- the solid

medium - macroscopic, orange-yellow colonies consisting of acid-fast rods, were detected. The subculturing of bacilli also presented a similar growth pattern.

Similar organisms have also been isolated by Chatterjee (1976) and extensively studied (Chatterjee, 1980). Both isolates of Chatterjee and Skinasness have a non-acid fast cultivable bacilli.

3.1.3. USE OF VARIOUS EXTRACTS

Various exotic extracts, extracts of mycobacteria etc. have been used in an attempt to cultivate the fastidious organism M. leprae.

Jackson (1951) used glycerol broth enriched with lung extract and isolated a pleomorphic organism from the blood of leprosy patients. The growth occurred during 5-18 days and could be subcultured.

Nishimura and Honda (1957) attempted to grow M. leprae in a medium with minced chick embryo tissue, dextrose, brain broth, egg and heat-killed M. leprae, but failed to obtain any growth.

Oltzki and Godinger (1967) added extracts of atypical mycobacteria to the Eagle's medium and observed

growth within 4-6 weeks. The subculturing was also successful.

Hirsal and Treo (1968) inoculated Malerbas in Dacry Hart Medium enriched with the filtrates of atypical mycobacteria, nocordia, solution of ferrioxamin B. The results were negative. The symbiosis on L-J medium with the same atypical mycobacteria also yielded negative results.

Conditioned medium & ICRC bacilli

In 1968, Bapat et al., isolated mycobacteria from human leprosy nodule in cultures of fibroblast cells (cell strain SPG) derived from dorsal root ganglion of human foetus. They studied the behaviour of leprosy bacilli and their affinity towards the ganglion elements. They were able to subculture the bacilli present in the supernatant of the fibroblast monolayer culture. The bacilli were maintained on tissue culture conditioned fluid and later adapted to bacteriological media containing serum or egg. The organisms were strongly acid fast, non-chromogenic and slow growing (Bapat et al., 1961). These in-vitro cultivated organisms were designated as 'ICRC' bacilli.

This work was repeated during the period 1968-72

and five more acid-fast strains of the ICRC bacilli were cultivated and extensively studied. Detailed information regarding this bacillus is included separately in this chapter.

To summarise, the attempts to grow M. leprae ~~in-vitro~~ by employing cell culture systems (schwann cells, human fibroblasts, macrophages etc...) have had limited success. Bacteriological media and cell free synthetic media supplemented with tissue and mycobacterial extracts, growth intermediates, have shown to be possessing growth supporting properties for M. leprae. However, no further systematic study on various aspects like nutritional requirements have been undertaken in most of the cases and it is also surprising that quantitative method was rarely applied to estimate the growth. The strain ICRC bacillus, which is slow growing and non-chromogenic is one model organism which has been studied extensively and can thus prove to be useful for various studies.

3.1.4. CHEMOTHERAPEUTIC STUDIES IN LEPROSY : IN-VITRO

The discovery of new drugs for the treatment of bacterial infection has mostly depended upon ~~in-vitro~~ screening methods for sensitivity of the causative organisms to new compounds. This system is not fully applicable for introducing new drugs in leprosy, since the ~~in-vitro~~

culture of the pathogen is not available; neither the induction of progressive lesions in laboratory animals was possible till 1960. Till then the chemotherapy in leprosy was either empirically devised or tested with Malepraeurium or the antituberculosis drugs were used on the assumption that Maleprae and Matuberculosis have very close physiological relationship.

With the introduction of the foot-pad technique (Shepard 1960, Rees, 1964) the arbitrarily chosen drugs were tested for the first time against Maleprae (Shepard and Chang, 1962; 1964, Shepard, 1967 a, 1971) and were thus put on a more solid footing. The results by the foot-pad technique were parallel to those for human patients, and the technique soon came to be established as the final experiment in-vivo before large scale clinical trials. However, the in-vivo method is time consuming and expensive procedure for screening the activity of new drugs.

The lack of culture of Maleprae has put a severe restriction on in-vitro screening of compounds against leprosy. Yet Mayer (1964), in his extensive review strongly advocated the use of mycobacterial strain closely resembling Maleprae. Such in-vitro method is rapid and

will yield some information prior to extensive in-vivo tests.

The sensitivity of few of the cultivated M. leprae has been tested against standard antileprosy drugs (Samuel et al., 1973; Chatterjee, 1980) but quantitative methods were rarely applied to estimate the effect. Whelan et al. (1985) found no satisfactory pattern in the sensitivity of 12 strains of mycobacteria to standard anti-leprosy drugs. Khanolkar (1972) used ICRC bacilli as a model strain and estimated the activity of 50 newly synthesised compounds by turbidimetric method in-vitro. Murohashi and Yoshida (1974; 1976 b; 1976 c) also used the in-vitro cultivated M. leprae isolates, and estimated the effect of DDS, rifampicin, streptomycin and INH by counting the number of microcolonies in the smears of the respective cultures after 20 to 40 weeks of incubation. More recently, Modak (1977) has tested the activity of known anti-leprosy drugs against ICRC bacilli. In light of these data, ICRC bacilli was used for in-vitro testing of the compounds synthesised before confirmation on the mouse foot-pad model.

3.1.5. THE ICRC BACILLI

Bapat et al. (1968) reported in-vitro isolation and cultivation of an acid-fast mycobacterium from five human

lepomatous leprosy cases. The cultures were maintained on 'conditional fluid', obtained from stock SPG cell strains (Bapat *et al.*, 1968; 1961). This acid-fast bacillus was designated as the ICRG Bacillus.

During the period 1968-72, five more acid-fast strains were isolated and cultivated in-vitro from untreated lepomatous leprosy nodules, in the similar fashion (Khanolkar, 1972). To-date, all the strains are being maintained in 'conditional fluid' in Cancer Research Institute, Bombay.

ICRG Bacilli : Bacteriology

It is a strongly acid-fast bacilli, 1.5 to 5 μ in length and gram-positive. They can be grown in bacteriological media formulated for mycobacteria, e.g., L-J medium, showing tiny, smooth, light creamy colonies. There is no pigment formation on exposure to light or on storage. These bacteria, being fastidious, can be cultivated only on media enriched with human serum and/or egg yolk. These organisms are weakly catalase positive. This acid-fast bacillus can retain its viability at 4°, 25°, and 37°C for 5 months (Bapat *et al.*, 1968; 1961; Ganguli, 1963).

ICRC Bacilli & Pathogenicity

The pathogenicity of these in-vitro cultivated ICRC bacilli, has been tested in mice as well as in golden hamsters. The intraperitoneal injection of ICRC bacilli in mice resulted in early and late histiocytic granulomata in visceral organs, showing progressive lesions in the liver, spleen, pancreas and lymphnodes. The cellular reaction during the progress of the pathological lesion was similar to that observed in man. In addition, the mice developed limb deformities such as foot-drop, resorption of the digits, ultimately resulting in the loss of limbs. Thus, it develops a disease with symptoms parallel to human lepromatous leprosy (Ranadive and Bapat, 1962).

Pathogenicity of the ICRC bacilli in the foot-pad of mice is also shown to be identical to that of M. leprae. The organisms show limited multiplication in normal mice while a continuous one in T/900r mice. The muscle tissue involvement is common for both normal and T/900r. mice. The spread of AFB to dermal nerves and liver granuloma and foot-drop was observed only in T/900r mice (Bapat and Madak, 1978).

Intratesticular inoculation of ICRC bacilli in mice and hamsters, resulted in a moderate spread of

infection within six months; to testes, liver and other visceral organs producing histiocytic granuloma (Ranadive, 1963; Ranadive and Bapat, 1966). In hamsters, the tropic changes of testicular architecture were observed.

ICRC bacilli : Antigenicity

Immunological investigations carried out using Ouchterlony gel diffusion method and haemagglutination technique demonstrated that, both ICRC and Maladras show a cross reaction with HAS (Rao et al., 1964). But in-vitro studies on the macrophage migration inhibition, using antigens prepared from the ICRC bacilli, viz. - ICRC - in, demonstrated that the ICRC bacilli show cross reactivity only with lepromin (prepared in a similar method) and particularly with H 37 Rv but not with HAS (Gangal and Khanolkar, 1974). In an in-vivo comparative study of the antigens in mice, sensitised with Maladras, ICRC bacilli, BCG, and human serum, a typical delayed type of hypersensitivity reaction occurred to the corresponding antigens (Khanolkar, 1972). These were measured quantitatively. The pattern of reaction of Maladras and ICRC bacilli was identical but it differed from PPD and human serum, i.e., ICRC bacilli share antigens with Maladras but not with PPD or human serum.

Bhide and Haik (1975) while studying the mode of action of thalidomide in lepra reaction, observed that ICRC

Bacillary antigen forms a specific complex with serum from leprosy patients and could induce histamine release. The reaction was specific to leprosy sera and was prevented by thalidomide, as in lepra reaction (Sheskin and Sager, 1971).

In another comparative study using antigens prepared from ICRC bacilli culture and fresh M. leprae from human lepromata, it was shown that the reaction of leprosy patients to these two antigens was more or less similar. The data provided evidence that ICRC bacilli are immunologically identical to M. leprae (Bapat et al., 1977).

ICRC bacilli I Chemotherapeutic studies

Khanolkar (1972) showed the inhibitory effect of thiacetazone on ICRC bacilli which was later confirmed by Modak (1977). On the extension of the series, the inhibitory activity of DDS was demonstrated when it was dissolved in hydrochloric acid or ethanol and diluted as such in physiological solution without precipitation. Partial inhibition of ICRC bacilli was observed when DDS was used as a suspension in distilled water (Modak, 1977).

Thiacetazone, a water soluble compound was inhibitory to ICRC bacilli at 80 µg/ml. Rifampicin was

also bactericidal at 10 $\mu\text{g/ml}$. Probably, both these drugs have a similar mode of action - inhibition of RNA synthesis (Modak, 1977).

In the spectrum of antibiotics, the ICRC bacilli were found to be resistant to penicillin, streptomycin and kanamycin, while gentamycin had bacteriostatic action. Similarly, the antituberculosis drugs INH and PAS too, have been shown to have bacteriostatic activity. On removal of drug influence, the ICRC bacilli showed growth when transferred to fresh medium (Modak, 1977).

All the studies on ICRC demonstrate that physiologically, the ICRC bacilli may possess sensitivity similar to that of M. leprae and differ from other mycobacteria including M. tuberculosis. Thus, the ICRC bacilli provides a model strain for in-vitro chemotherapeutic primary screening, and also for the study of mechanism of action of the drugs.

3.1.6. MATERIALS AND METHODS

3.1.6.1. MATERIALS

(a) ICRC bacilli (Bapat et al., 1958; 1961; Khanolkar, 1972)

ICRC bacilli are acid fast bacilli grown in-vitro from M. leprae, isolated from human lepromatous nodules.

In the current studies ICRC strain Case-49 bacilli was extensively used for investigations. The seed inoculum was obtained through courtesy of Dr. C.V. Bapat, Cancer Research Institute.

(b) Medium

The medium used for maintenance and the in-vitro studies with ICRC bacilli was a modified Dubos medium (Dubos and Davis, 1946) as described in Appendix 1

The media prepared was sterilised by autoclaving at 121°C and 15 lbs. pressure for 20 minutes. The medium was supplemented with sterile 10% HAS, penicillin (100 u/ml) and streptomycin (100 µg/ml). For the present study the 'ready for use' (RFU) media was provided by Dr. C.V. Bapat, Cancer Research Institute.

(c) Drugs/Biochemicals/Compounds

Compounds coded as R-4651, R-4754, R-3652 and R-3647 are representative of series of antituberculosis compounds synthesised at the Chemotherapy Department, Haffkine Institute and have shown promising antitubercular activity. Biochemicals like L-Tyrosine, L-DOPA, DL-DOPA and methyl-DOPA were of the same origin as those described in the previous chapter.

Compounds coded as AD₁Ey, AD-Dp, AD₂Dp and AD₃Dp are the compounds synthesized as described in Chapter II.

(A) Glassware

Culture bottles (Vasir Glass Works), test tubes and other glassware (Borosil, Corning) were used. For turbidimetric assay, Bausch and Lomb 1/2" Spectronic-20 cuvettes were used and for enumeration of the bacilli, specially prepared ring slides (1 cm. diameter circles) were used.

3.1.6.2. METHODS

i) Maintenance of the ICRC bacilli (Modak, 1977)

The stock cultures of the ICRC bacilli were serially maintained in Dubos Medium (RFU). These were regularly subcultured at every 3 to 4 weeks. At the time of subculturing, smears were prepared, stained and the morphology of the bacteria was recorded.

ii) In-vitro experiments on ICRC bacilli

a) Preparation of solution of the test compound

Stock solution of the various compounds to be tested was prepared freshly each time and sterilised by autoclaving

at 121°C and 15 lb. pressure for 15 minutes. No special attempt was made to dissolve the compounds. The concentrations at which the various compounds were screened are described further in this chapter.

b) Turbidimetric assay (Bapat et al., 1961; Modak, 1977)

For turbidimetric assay, the cultures were set up in standard Bausch and Lomb, Spectronic-20, 1" cuvettes. 4 ml of medium (RFU. Dubos with 10% HAS) was added to each tube. 1ml of the test solution was added to the first tube, followed by 1:5 serial dilutions in subsequent tubes. Usually the final concentration of the compounds was 400, 80 and 16 µg/ml. Two week old culture of the ICRC bacilli from Dubos medium was used as inoculum. The inoculum was so adjusted that the original turbidity reading would be around 90% transmission against a blank (Corresponding to an approximate count of 1×10^9 AFB/ml). The tubes were closed with rubber stoppers. A tube containing plain medium served as negative control and one with only inoculum and without test solution served as positive control, or growth control. The total amount of fluid in each tube was 5 ml.

Before measuring the turbidity on day zero, the cultures were refrigerated for 72 hours. The cultures were then incubated at 37°C for 12 days. The organisms settle

at the bottom of the tubes to form a pellet, leaving the supernatant clear (or the tubes are spun at 1500 rpm. for 15 minutes). Thus, each tube served as its own control for 100% transmission setting, before shaking. The reading of both supernatant and the suspension (after shaking thoroughly) were taken on the Spectrophotometer fitted with a red filter (660 mμ). Turbidity was estimated on day-0 followed by reading at 3 days interval upto 12 days. In this system, for turbidimetric measurements, the values were only comparative and not absolute as per manual, because of increased optical path. Each experiment was repeated at least three times and the readings were recorded graphically as % transmission versus time in days.

This method was followed for studying the effect of various compounds on the growth of ICRC bacilli.

3.1.6.2 (a) EXPERIMENTS: GROUP I

a) Standardisation of turbidimetric assay and correlation of in-vitro and in-vivo techniques

This experiment was conducted to establish the reliability of the in-vitro turbidimetric assay using ICRC bacilli as the test organism for screening of compounds for antileprosy activity as also to establish the correlation with the standard foot-pad technique.

Compounds R-4651, R-4754, R-3652 and R-3647, found to have antituberculosis activity were tested at concentration of 400, 80 and 16 $\mu\text{g/ml}$ each. The results are summarised in Table : 7 and represented graphically (Figs 1-4).

b) Effect of amino-acids on growth of ICRC bacilli

L-Tyrosine, L-DOPA, DL-DOPA and methyl-DOPA were tested for their effects on ICRC bacilli at concentrations of 400 and 80 $\mu\text{g/ml}$. The results are summarised in Table : 7 and represented graphically (Figs 5-8).

c) Effect of the DDS-amino acid conjugates on the growth of ICRC bacilli

The four DDS-amino acid conjugates synthesised viz., ADTy, AD-Dp, ADdDp and ADmDp were tested at concentrations of 400, 80 and 16 $\mu\text{g/ml}$ DDS equivalents of the conjugates. DDS was also tested at the above three concentrations. The results are summarised in Table : 7 and represented graphically (Figs 9-13).

3.2. EXPERIMENTAL ANIMAL MODELS

Ever since the discovery of the leprosy bacillus, attempts are being made to transmit the infection to laboratory animals.

Table 17

EFFECT OF DRUGS ON THE GROWTH OF ICRC BACILLI

Sr. No.	Drug/ compound	Concentration tested $\mu\text{g/ml}$	Inhibition rating	Approximate MIC $\mu\text{g/ml}$
1.	DDG	400	+++	400
		80	++	
		16	+	
2.	L-Tyrosine	80	=	
		16	=	
3.	L-DOPA	80	=	
		16	=	
4.	methyl-DOPA	80	=	
		16	=	
5.	R-3647	400	+	400
		80	=	
		16	=	
6.	R-3682	400	+	400
		80	=	
		16	=	
7.	R-4651	400	++	400
		80	=	
		16	=	
8.	R-4754	400	=	
		80	=	
		16	=	
9.	ADTy	400	++	400
		80	+	
		16	=	
10.	AD-Dp	400	+++	80
		80	+++	
		16	++	
11.	AD ₂ Dp	400	+++	16
		80	+++	
		16	+++	
12.	AD ₂ Dp	400	+++	400
		80	++	
		16	++	

Key : +++ Complete inhibition
= No inhibition

++ $\frac{1}{2}$ Partial inhibition
+ 0

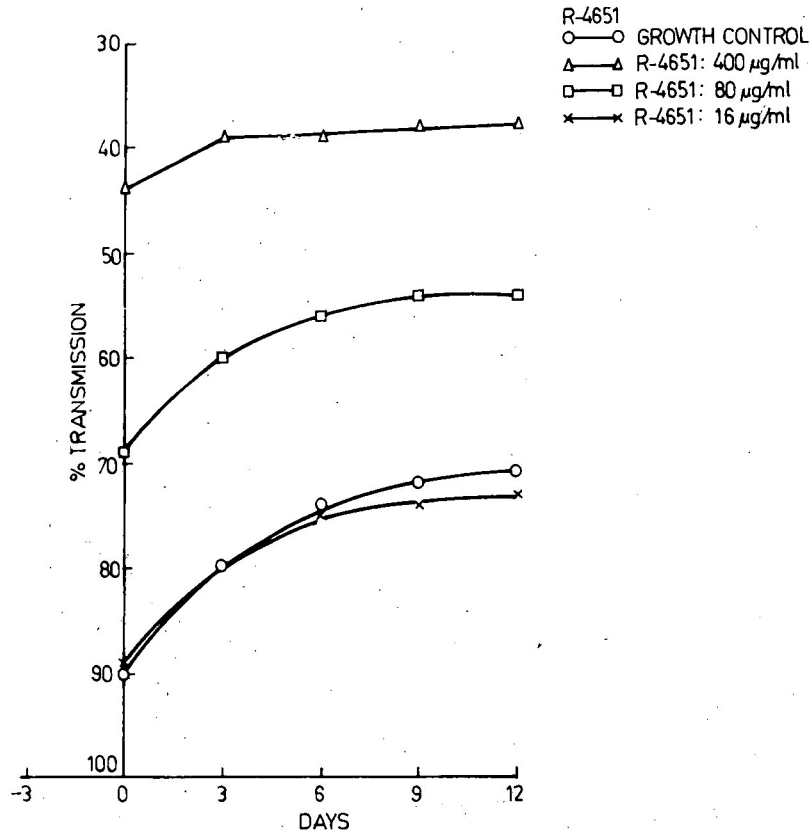


Fig.1 : Effect of R-4651 on the growth of ICRC bacilli

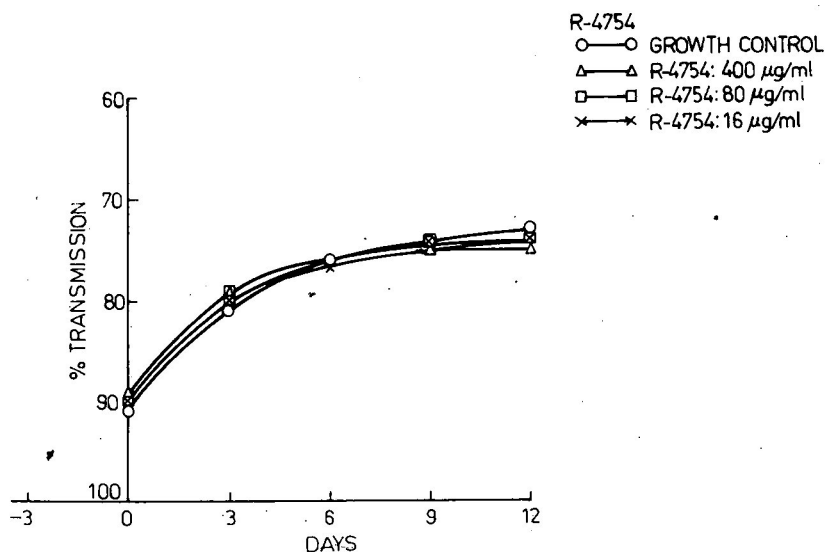


Fig.2 : Effect of R-4754 on the growth of ICRC bacilli

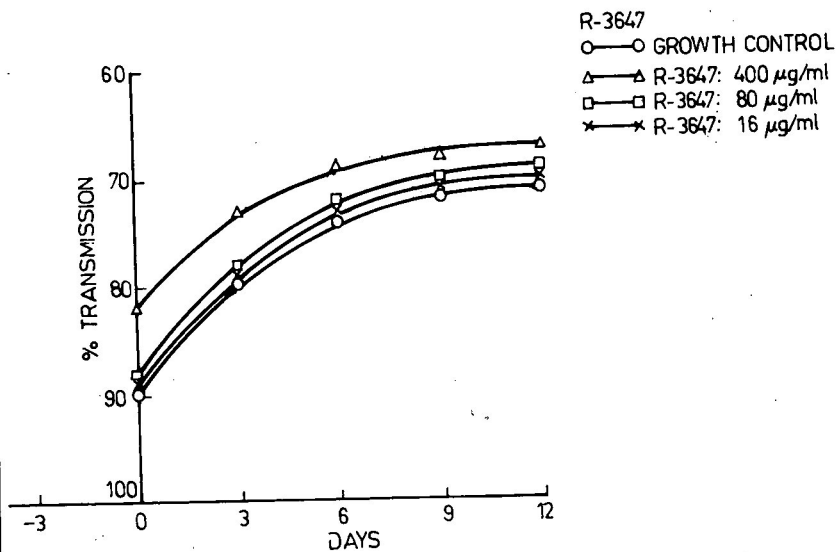


Fig.4 : Effect of R-3647 on the growth of ICNC bacilli

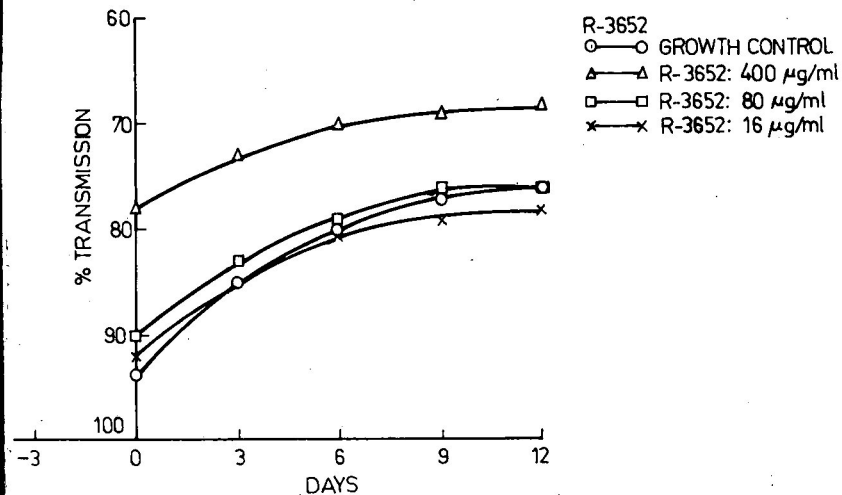


Fig.3 : Effect of R-3652 on the growth of ICNC bacilli

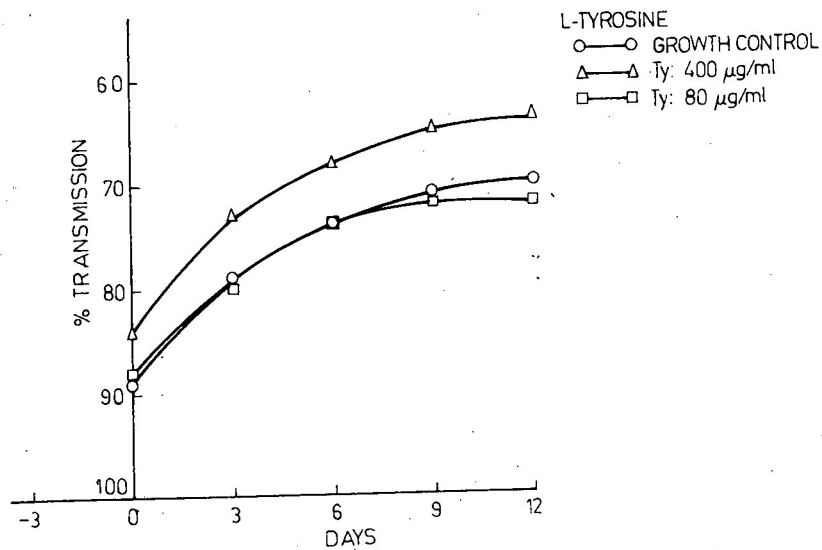


Fig.5 : Effect of L-tyrosine on the growth of XERC bacilli

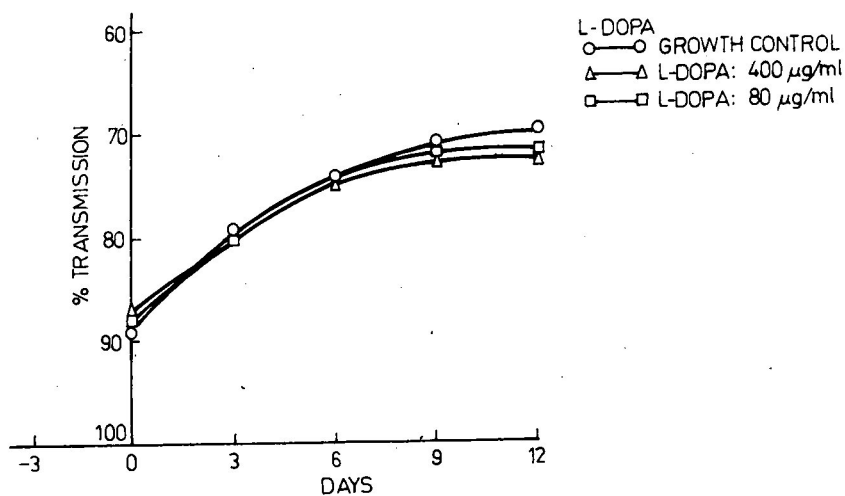


Fig. 6. : Effect of L-DOPA on the growth of XERC bacilli

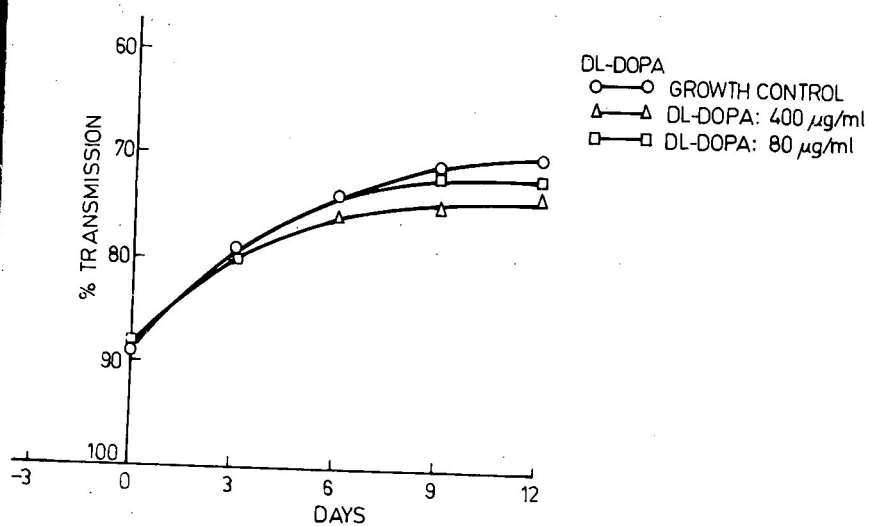


Fig.7 : Effect of DL-DOPA on the growth of ICRC bacilli

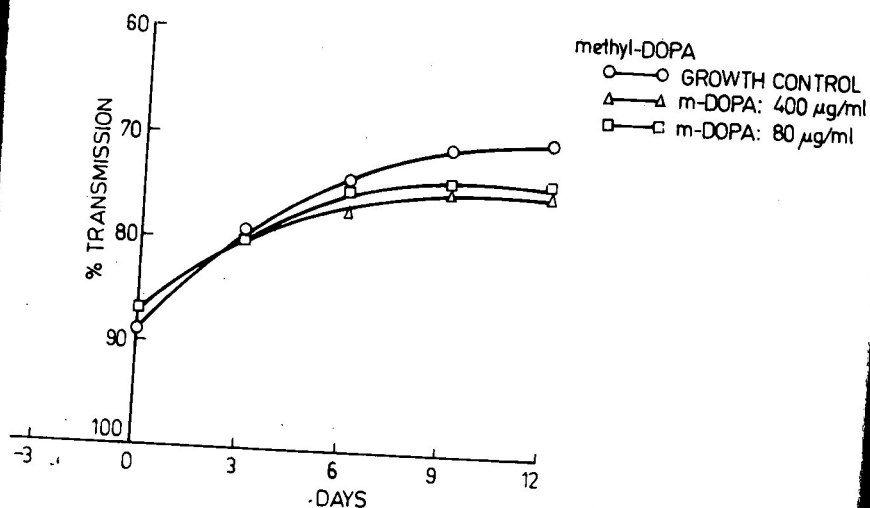


Fig.8 : Effect of methyl-DOPA on the growth of ICRC bacilli

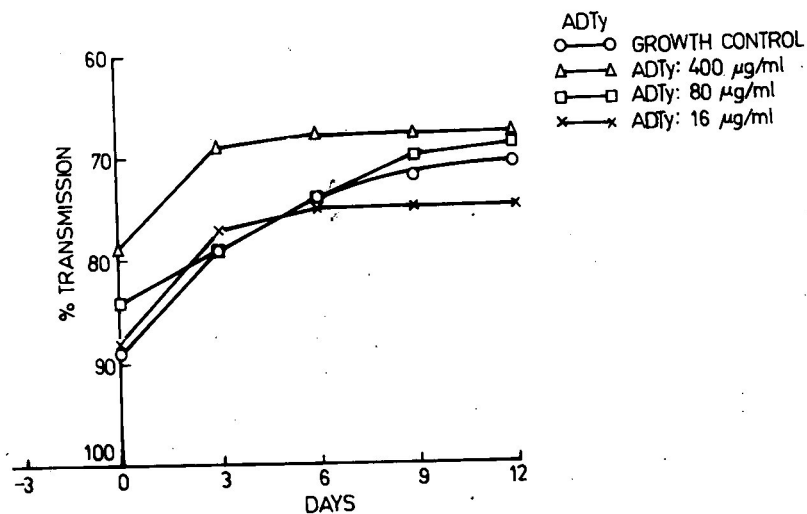


Fig.9 : Effect of ADTy on the growth of XERC bacilli

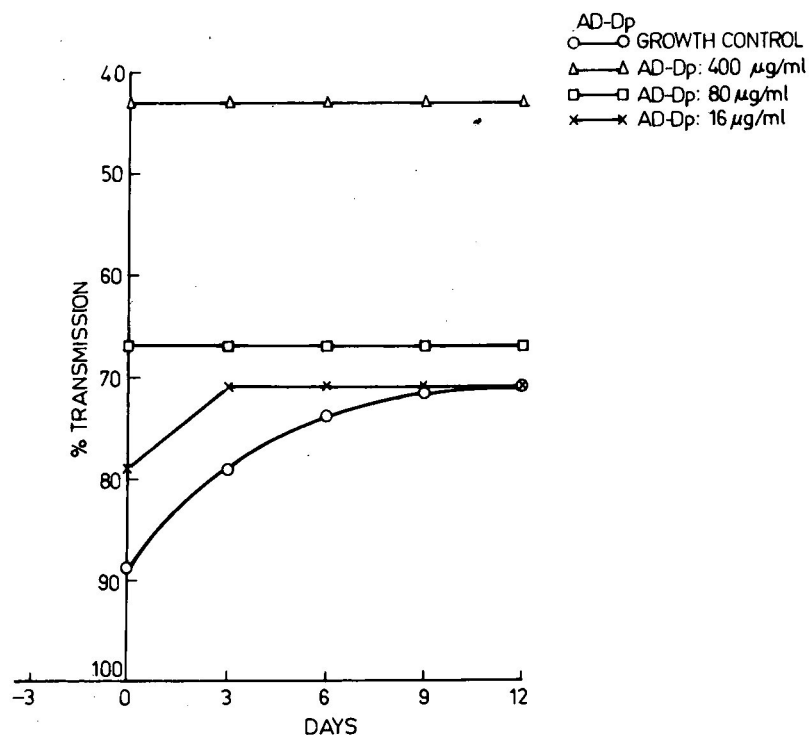


Fig.10 : Effect of AD-Dp on the growth of XERC bacilli

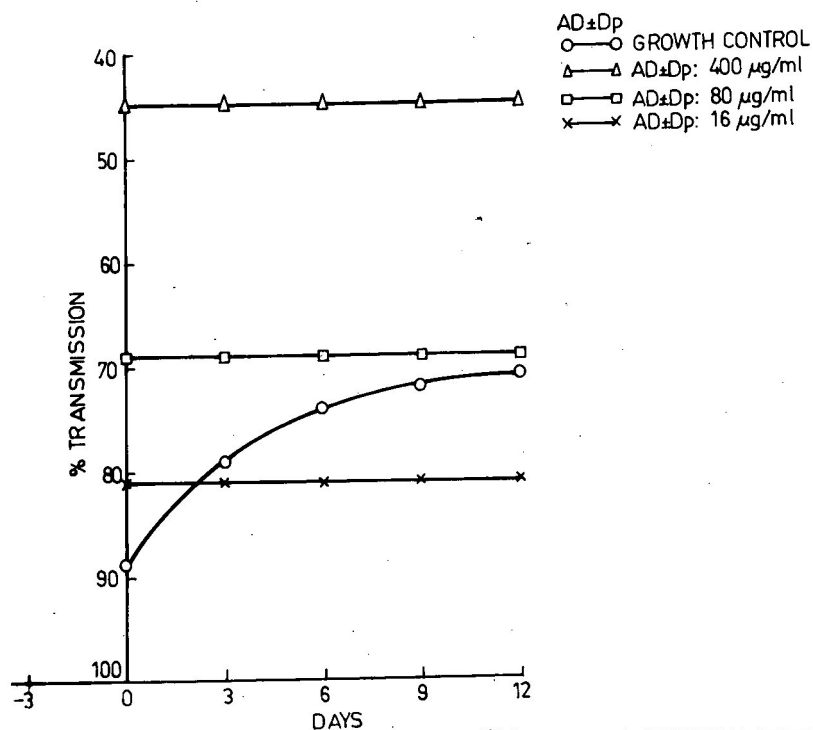


Fig. 11 : Effect of AD±Dp on the growth of IHC bacilli

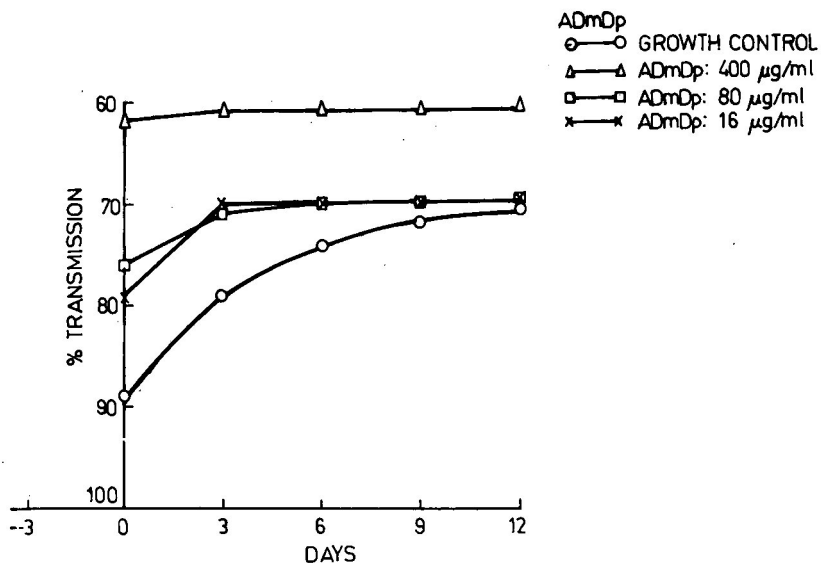


Fig. 12 : Effect of ADmDp on the growth of IHC bacilli

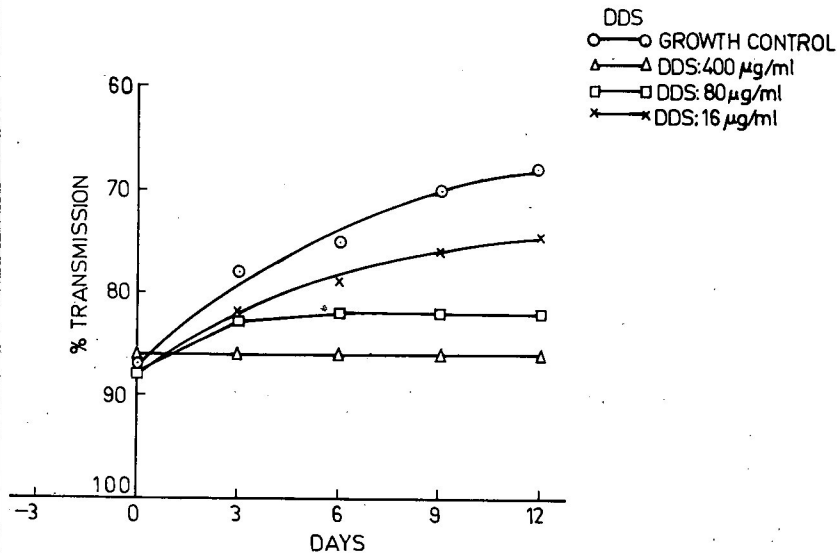


Fig. 13 : Effect of DDS on the growth of ICRC bacilli

One of the first attempts, that met with limited success, was by Adler (1937) who showed a heavy infection within 6 weeks after implantation of a lepromatous nodule in a splenectomised syrian hamster. Since then, several animal models have been tried for transmission of human leprosy. This data is presented as follows :

- a) Attempts of transmission of human leprosy to various animals.
- b) Evolution of the mouse foot-pad technique and its contribution to experimental leprosy.
- c) Development of new animal models viz - Armadillo, etc...

3.2.1. ATTEMPT TO TRANSMIT HUMAN LEPROSY TO VARIOUS ANIMALS

A lot of work on animal transmission was carried out during the period 1939-1960 involving various animal species. For all these attempts, leproma piece or ear lobe nodules served as source of M. leprae, but no quantitation about the number of organisms injected was carried out.

Primate : Collier (1940) injected M. leprae in the skin of the back of monkey to observe the nasal smears after 8 months. Cochrane et al. (1945) infected splenectomised monkeys. The subcutaneous and intraperitoneal injections resulted only in erythematous rash without any progressive lesions. Lui (1955)

gave intramuscular, intraperitoneal injections of M. leprae to Taiwan monkeys and also implanted skin-nodules. Seven out of 21 animals showed clinical signs of leprosy but there was no dissemination of the infection.

Gander (1958) infected chimpanzee intravenously or intraperitoneally and observed nodules and depigmented patches that regressed subsequently. McFadsean and Ridley (1961) used normal and X-irradiated monkey as animal model. The intravenous and intradermal injection produced lesions indicating progressive infection, but no multiplication was observed.

Golden Hamster was another animal model that was used extensively for the disease transmission. Dharmendra and Lowe (1944) reported that splenectomy of syrian hamster did not produce lesions. While Burnet (1943) could not succeed in transmitting the disease, Kelkar and Ranadive (1958) succeeded in producing granuloma in the subcutaneous tissue and with 50% of the golden hamsters showing limited multiplication of AFB when the animals were treated with cortisone and given a whole body irradiation with a dose of 150 r. to impair the immune response. Binford (1958) studied the transmission to the golden hamster extensively. Normal, cortisone treated and irradiated animals were used. The injection of M. leprae

intestis and ear lobe, induced histiocytic granuloma after 21 weeks, that could be successfully passaged to new animals. Niven and Waters (1963) and Waters and Niven (1965), in attempting to repeat Binford's work observed granuloma containing AFB and nerve involvement in ear lobe but no lesions in testis. This infection was passaged upto two passages resulting in the limited multiplication of Malapras.

Mouse was the animal model which assisted in transmission of Malapras, in almost every attempt. Chatterjee (1958, 1958a) selected a hybrid black mouse and injected it subcutaneously, intraperitoneally and intracerebrally. A heavy and generalised infection was observed that was serially passaged upto third passage. But these results could not be confirmed by other laboratories immediately. Sato (1962) failed to produce progressive lesions in F_2 hybrid mice, but pin-headed lepromas were observed in mesentery, serosa of liver and spleen. Janssens and Pattyn (1963) infected foot-pad of mice and passaged the infection till four passages. But Kirchheimer (1964) failed to transmit the infection via foot-pad to mice.

Vitamin E. deficient diet was the treatment for rats, that led to globi formation in the testis (Bergel, 1969). Wilkinson (1962) failed in inducing multiplication

in white rat on intratesticular injections. The white Wister rat, model of Hilson (1965) demonstrated limited multiplication of M. leprae, yielding 10^7 AFB. In the lesions, the macrophages harboured the bacilli.

Many miscellaneous animals like fowl (Jagai and Hayashi, 1945), rainbow perch (Chausinand and Bessel, 1962), Chick embryo (Habil *et al.*, 1964), turtle-Anolis lizard -(Fite *et al.*, 1964), guinea pigs (Kirchheimer, 1964), goldfish (Pattyn and Van Loo, 1974), to name a few, were tried for transmission, but obtained only negative data. The salient features of these experiments have been summarised as follows :

- i) Most of the experiments were only of exploratory nature without any quantitative parameter for determination either multiplication of organisms or histopathogenesis.
- ii) Despite the aim to find a suitable animal model with above ideas in mind, none of the workers waited long enough for the lesions to develop.
- iii) Inconsistency cropped in; due to indifference to quantitative parameters.
- (iv) A few apparently successful attempts were neither followed up nor applied for any specific enquiry, e.g., chemotherapy or histopathogenesis.

3.2.2. EVOLUTION OF THE MOUSE FOOT-PAD TECHNIQUE AND ITS CONTRIBUTION TO EXPERIMENTAL LEPROSY

The mile-stone in the experimental human leprosy was introduction of foot-pad technique by Dr. C.C. Shepard (1960) which was a systematic approach towards transmission of M. leprae to mice.

Two other mycobacteria viz, M. ulcerans and M. balnei causing human skin infection were found to multiply in the foot-pad of mice which is a relatively cooler tissue. Therefore, foot-pad was chosen as a site for injection. The injection of 10^3 to 10^4 organisms in the foot-pad yields 10^6 to 10^7 organisms per foot-pad, within 8 to 10 months. The growth reaches its peak at this stage, and thereafter, the number decreases but the bacilli do not disappear completely from the foot-pad. Thus, the M. leprae multiply to a limited extent, locally in the foot-pad (Shepard, 1960). Histologically, the infected foot-pad showed microscopic granuloma without necrosis. Mouse to mouse passage had no effect on the growth pattern of M. leprae (Shepard, 1962).

The identity of this organism, multiplying in the foot-pad, as M. leprae, was confirmed by testing the lepromin preparation of these bacilli in leprosy patients, which evoked a negative reaction in LL cases (95%) and a positive

response in TT cases (63%) (Shepard, 1963).

In 1963, at the Rio de Janeiro Congress, 'the limited multiplication of M. leprae in the mouse foot-pad' was proposed as a specific character of M. leprae.

In a comparative study of M. coli and M. leprae by electron microscopy and staining properties, it was observed that, the solidly stained organisms are uniformly electron dense, whereas irregularly stained organisms are irregularly electrondense. For M. coli, the colony count decreased with the increase in the number of irregularly stained organisms (Rees and Weddell, 1968). For M. leprae, in the absence of any other criterion for viability, it was generally accepted that the solidly stained organisms are viable, while beaded or irregularly stained organisms are non-viable (Shepard, 1965, 1971).

This limited multiplication of M. leprae in the foot-pad of a mouse was confirmed by Rees in 1964.

The technique was immediately applied to studies on (i) Chemotherapy, (ii) Pathogenesis and (iii) Immunology. These studies with mouse foot-pad system have revealed a number of unknown features about leprosy.

(1) In-vivo chemotherapeutic studies

One of the problems in leprosy, that needed attention was the assay for new drugs. Therefore, Shepard diverted his studies towards the experimental chemotherapy using mouse foot-pad technique and studied the effects of different drugs on the multiplication of *M. leprae* (Shepard and Chang, 1964, Rees, 1969).

The minimum inhibitory concentration of DDS required was found to be 1 to 10 ng/ml of serum level (Shepard, 1969; Galber *et al.*, 1971). This explains the effectiveness of DDS in the treatment of leprosy.

The presence of DDS resistant organisms in the LL patients not responding to the treatment was demonstrated for the first time using the mouse foot-pad model (Pettit *et al.*, 1966). The Kinetic method - which mimics the actual therapeutic treatment - made possible the study of the effect of the anti-leprosy drugs on an established infection in mice. This revealed that only DDS, related sulfones releasing DDS, clofazimine and rifampicin are bactericidal, while the rest of the drugs are only bacteriostatic (Shepard, 1967 a, 1971). The rapid bactericidal activity of rifampicin was demonstrated by simultaneous clinical and experimental trials (Rees *et al.*, 1970).

The mouse foot-pad technique fully justifies its role in experimental chemotherapy, which was used to study the effect of new drugs having a broad spectrum of activity especially against mycobacteria. Many newer drugs and derivatives of older drugs have been tested against M. leprae using this technique before large scale clinical trials.

The effect of combination of drugs on the multiplication of M. leprae, in the foot-pad shows that clofazimine, ethionamide, dapsone and rifampicin on combination, potentiate each others activity (Shepard, 1976). Also studies with resistant organisms show that rifampicin is fully active against dapsone resistant strains (Rees, et al., 1970).

(11) Pathogenesis in leprosy

The injection of 10^3 to 10^4 organisms in the foot-pad of a normal mouse yields 10^6 organisms after 6-8 months (Shepard, 1960; Rees, 1964). Microscopic granuloma was observed histologically without necrosis or foot-drop of the inoculated foot-pad. The systematic histological study of the foot-pad demonstrated that M. leprae involves the striated muscle fibre initially and throughout the period of bacterial multiplication (Palmer et al., 1965). Other mycobacteria studied in comparison including

M. leprae could not show muscle invasion; revealing that "Unequivocally none of the other species of mycobacteria i.e., M. chelonae, M. fortuitum, and M. goodii, M. marinum, M. tuberculosis, B.C.G. had a comparable predilection for muscle fibres" (Rees and Weddell, 1970; Rees, 1969). This observation drew the attention towards the importance of muscle biopsy in human patients. A series of muscle biopsies were then examined (from LL to TT cases). The acid-fast M. leprae were always observed in the striated muscle fibres, non striated muscle fibres of the breast and scrotum (Rees, 1971).

The spread of infection also occurs in normal mice inoculated intravenously and is confined to the same sites of predilection (Weddell et al., 1971; Rees and Weddell, 1968).

Still, the infection of foot-pad remained localised and did not yield a very high number. Therefore, an attempt was made to drastically suppress the immunological capacity of the animal and then study the pattern of infection. The animal was thymectomised and irradiated (whole body irradiation - 900r) (Rees, 1965). The animals so treated, yielded 1000 times more organisms than in normal i.e., 10^6 per foot-pad. These organisms, on injecting back into normal mice showed a peak growth of 10^6 within

6 months, remaining steady upto 12 to 15 months again (Rees et al., 1967; Shepard, 1968).

The phenomenon of foot-drop was observed in T/900r mice injected locally or intravenously with 10^4 Maladras after 20 months - involving peripheral nerves. Five percent of such animals develop functional abnormalities of one or both hind limbs, progressively worsening as the animal grows older (Rees and Weddell, 1968).

Thus, the normal animal replicated 'BT' type of human infection; the T 900/r, animals were always detected in the endothelial cells of the blood capillaries, indicating the spread of the infection by bacillaemia (Rees, 1969; Rees and Weddell, 1970).

(iii) Experimental Leprosy & Role of Immunity

A limited multiplication of Maladras in the foot-pad of normal mouse confined for the first 6 months, resembled borderline leprosy. The prolonged incubation time, suggested a weak pathogenicity (Rees, 1970).

The T/900r mice totally suppressed for their cell mediated immune (CMI) responses, showed a spread of infection and the pattern of infection resembled BL form

in human patients (Rees, 1969). The replacement of immunocompetent lymphoid cells in mice with an established infection changed the pattern of the disease from LL to BB or BL type (Rees and Weddall, 1970). These findings demonstrate the role of CMI in leprosy. Also, it is the immune competence of the individual patient that may be playing a very important role in determining the type of disease (Rees, 1970 a; 1971).

Studies like the one by Navalkar et al. (1974) have demonstrated that foot-pad model can be used to study progression of immune status along with the disease state.

More recently, studies in vaccination of mice against M. leprae infection, has proved the feasibility of a vaccine against leprosy which will be a major breakthrough if achieved (Shepard, 1965 a; 1966; 1976 a).

The important contributions of the mouse foot-pad to experimental human leprosy are summarised as follows (Rees, 1973; 1969).

- i. For the first time, it was possible to grow and enumerate M. leprae in an animal model with consistency and regularity.
- ii. The normal and T/900r mice helped in understanding the pathogenesis of leprosy, revealing the importance of

muscle biopsy, study of the viability of the organisms, from the biopsied tissue.

iii. The mouse foot-pad technique, allowed to screen different anti-leprosy drugs before its actual use in therapy. It was possible with the help of mouse foot-pad technique to demonstrate the presence of drug resistant organisms in the patients not responding to DDS. Now, it has also made easy to monitor clinical efficacy of the drugs.

iv. It is the immune competence of the individual that determines the type of disease in human, was also understood from the foot-pad infection of normal and T/900 r. groups and the replacement of immune competent lymphoid tissue in T/900 r animals.

v. It plays now a major role in the search for vaccine against leprosy.

3.2.3. DEVELOPMENT OF NEW ANIMAL MODELS

In spite of the success of mouse foot-pad technique, the search for an animal model for getting higher yields of bacilli continued with vigour. The spectacular discovery of this search for a new model, was the infection of nine-banded armadillo (*Dasypus novaeboracensis*, Linn). The low

body temperature, a possible weak immune response and the longer life span of this primitive mammal attracted the attention and may have contributed to the success of a model system for leprosy (Storrs, 1971).

On infection with MalanKas, the armadillo developed a massive infection of lepromatoid type of leprosy within 14 months (Kirchheimer and Storrs, 1971). The inoculated skin sites showed massive nodular lesions with intracellular organisms and typical lepra cells were observed. Histologically, it showed a severe spread of infection to skin, liver, spleen and lymph nodes. The infection differed from 'LL' type in leprotic pneumonitis, leprotic meningitis (i.e., involvement of CNS), a severe wide spread bone marrow infection and oesophageal involvement (Kirchheimer et al., 1972, Binford et al., 1975). The acid-fast bacteria isolated from skin and lymph node of a armadillo showed multiplication in the mouse foot-pad after 6 months, and also a dopaoxidase positive reaction (Kirchheimer et al., 1972; Storrs, 1973). Peripheral nerve involvement due to MalanKas infection was also observed (Issan and Binford, 1973). The pyridine extraction test and the immune response to the antigen prepared from these bacilli presented results identical to MalanKas (Convit and Pinardi, 1974). Comparative studies on lepromin with armadillo bacterial antigen in leprosy patients, have shown that both antigens give similar weak reactions in

LL type while corresponding strong reactions in TT patients (Meyers et al., 1975; Miller et al., 1975).

These observations confirmed that armadillo develops lepromatoid type of leprosy with M. leprae infection. However, the frequency of 'takes' is low - 40%. Yet, this high yielding model - giving 100 gms. of infected tissues per animal is indeed of a great advantage and this material is being extensively used for various investigations in leprosy and for biochemical studies (Storrs et al., 1974).

Still, there is need in leprosy research for experimental animals which would present fewer problems than do the armadillo and the mouse. Work on many old models is being revived e.g., chimpanzee (Binford, 1968; Donham and Leininger, 1977), neonatally thymectomized Lewis rats (Fieldsteel and Levy, 1976 a) and newer animals are being tried. Kirchheimer (1978) has reviewed the recent advances in experimental transmission of leprosy to animals where reference is made to the European hedge-hog, the slender loris (India), the Korean Chipmunk and the most recent white handed gibbon (Waters et al., 1980), in all of which some preliminary work has shown promise.

3.2.4. CHEMOTHERAPEUTIC STUDIES IN LEPROSY : IN-VIVO

The foot-pad infection, in spite of its limited

nature, has been used successfully for testing drugs for antileprosy activity. Thus, it has been shown that when Maladras is injected into the foot-pads of mice treated with known antileprosy drugs the organisms fail to multiply. Therefore, the mouse foot-pad infection provides, for the first time, a specific test for screening new antileprosy drugs and the search is no longer restricted to drugs that are known to be active against M. tuberculosis (Rees, 1969). This technique is used not only for screening drugs but has also been specifically applied to determine, for the first time, the minimal inhibitory concentration (MIC) of drugs like DDS, in-vitro against strains of Maladras from previously untreated patients (Shepard, 1967 b; Rees, 1967; 1967 a). That Maladras in the mouse is exquisitely sensitive to DDS is an unexpected finding because all other species of mycobacteria are relatively insensitive to sulfones. Extrapolation of the findings from mouse to man, suggests that a daily dose of 1 mg of DDS would be effective in man (Rees, 1969). This lends support for using lower doses of DDS in man, to reduce the incidence of reactions without the fear of diminishing the therapeutic efficacy of the drug. Unfortunately, such low doses cannot be used because of the possibility of the organisms developing resistance, which has once again been proved by the same technique (Pettit et al., 1966; Rees, 1967 a). The possibility of drug resistance, must therefore be weighed

against the advantages to be gained by a significant diminution in the incidence of serious reactions. Similar, but less extensive studies have demonstrated the emergence of resistant M. leprae to many other drugs as well.

The foot-pad infection technique provides an experimental model for investigating the prophylactic effects of various vaccines (Shepard, 1965 a; 1966; 1976a).

Thus foot-pad infection fully justifies its role in the experimental chemotherapy in leprosy.

3.2.5. MYCOBACTERIUM LEPRAE

Mycobacterium leprae belongs to Genus - Mycobacterium, Family - Mycobacteriaceae, Class - Schizomycetes, Order - Actinomycetales.

The organisms are straight or slightly curved rods, 1.5 to 8 μ in length and 0.2 to 0.5 μ in diameter. The ends are rounded or blunt but may be pointed also. The organisms are acid-fast, Gram-positive; with Ziehl Neelsen's stain, it stains more deeply than M. tuberculosis. It may also show beaded forms. They are non-motile and non-sporing. In smears, it appears in glomeration termed "Globi" in the form of tightly packed bundles of cigars;

a large number of free acid-fast bacilli are also seen. The 'globi' are characteristic of leprosy organisms as seen in smears or sections of lepromatous nodules. It has been suggested that 'globi' are colonies of bacilli growing within an intracellular membrane.

Electron microscopic studies showed uniform electron dense areas along the cell wall of the living bacilli; whereas only pits were seen in the cell wall of dead bacilli. The colonies of bacilli are found to be surrounded by a peripheral 'halo' zone (Brieger et al., 1959; Hishiura, 1960; Hanks, 1961).

3.2.6. MATERIALS AND METHODS

3.2.6.1. MATERIALS

a) Mycobacterium Leprae

Bacteriologically positive nodules (3+) were excised from the ear lobes of the LL patients, attending the O.P.D. of the Aeworth leprosy Hospital, Bombay. The nodules were collected in sterile B33 containing penicillin (100 u/ml) and streptomycin (100 µg/ml) and brought to the laboratory in few hours and used as a source of fresh M. Leprae. The reference data of the case from which M. Leprae was used for the current studies is as follows :

Name : Sayanna Balanna
Reg. No. : 1252/80
Date : 4.9.80

b) mice

3 months old mice (average weight 18-20 gm) of the inbred strain CBA were used for foot-pad inoculations. these animals were maintained in an air-conditioned animal house and were fed on pellet food (Hindustan Lever).

c) Drugs/Chemicals/Compounds

The drugs and compounds tested for anti-leprotic activity by the 'foot-pad' technique are as described previously in this chapter.

Trypsin (2000 units/gm) was from Merck. Other routinely used chemicals were of A.R. grade with no preferences regarding the manufacturer.

d) Glassware

Test tubes, petri dishes etc. (Borosil, Corning) were used. For enumeration of the bacilli, specially prepared ring slides (1 cm dia circles) were used.

3.2.6.2. METHODS

(a) Purification of *M. leprae* from biopsies

The nodule obtained from a human patient was cut into small pieces with the help of sharp scissors and homogenized using 0.25% w/v trypsin solution. The bacilli were separated from the tissue by using Mickle shaker. The suspension was allowed to stand for some time, so that the tissue fragments settle down. The supernatant was cautiously decanted into a centrifuge tube and spun slowly to settle down tissue particles. The supernatant was decanted once again and spun at 3000 xg to settle down the bacteria. After discarding the supernatant the pellet was suspended in 2.0 ml of sterile Normal Saline and stored in a refrigerator till further use. The whole procedure was carried out in aseptic condition.

(b) Standard smear preparation

The method is based on the Breed's technique for counting bacteria in milk (Topley and Wilson, 1964). The principle of this method is as follows :

A known or standard amount of bacterial suspension is spread on a standard area and stained. The average number of bacteria or organisms per unit area or per oil immersion field is counted. From this number, the total number of organisms smeared on the standard area can be

estimated. This gives a good estimation of the number of organisms per ml in the bacterial suspension. The method described by Hart and Rees (1960) was used in the present study.

Procedure

The smears were prepared on a marked slide with four circles of 1 cm. diameter. A platinum loop of standard wire gauge which was previously calibrated was used. It held 0.0002 ml per drop of the suspension. A loopful of the bacterial suspension was spread on the marked area and allowed to dry and later the smears were fixed on steam (100°C) for 2 minutes.

(c) Staining of the smears

The fixed smears were flooded with freshly filtered Ziehl Neelson's Carbol Fuchsin solution. The stain was heated until it started steaming gently. Heating was continued for five minutes intermittently, taking care that the smear is always flooded with the stain. The slides were later allowed to stand for 15 minutes. The smears were washed under tap water, decolourised with acid-alcohol mixture (3% hydrochloric acid in 95% ethanol) and again washed with water. The smears were counter-stained with 1% Methylene blue for 2 minutes, and air dried.

(d) Preparation for enumeration of the bacilli in standard smears (Shepard and McRae, 1968)

Counting was carried out under a particular oil immersion lens (100x) and eyepiece (10x) throughout the experiment. Twenty five fields along the diameter of each smear were counted. Total of 100 fields from four standard smears prepared, were scored. The average number of organisms ($\bar{n}$), per field was determined. The area of the field of oil immersion lens (A) and the area of the standard smear (A_s) was determined. Hence the average number of organisms per standard smear = $\bar{n} \times A$ was calculated. This represented the number of organisms in 0.0002 ml of original bacterial suspension and thus the number of organisms per ml (N) of the original suspension was estimated. If the original suspension was diluted before preparation of standard smears, the dilution factor was taken into consideration for determining the number of organisms per ml or per foot-pad.

(e) Method of injection of the bacilli in the foot-pad

The dose and route of administration was as described by Shepard (1960; 1962). The original bacterial suspension or inoculum was diluted with sterile Normal Saline, so as to contain approximately 2.5×10^5 AFB/ml of the suspension. From this final inoculum, 0.03 ml (corresponding to 7.5×10^3

AFB) was injected into the right hind foot-pad of each mice superficially. This volume ballooned upon injection and then spread longitudinally throughout the foot-pad.

(f) Harvesting the bacilli from foot-pad : (Shepard, 1960, 1962; Rees, 1964)

Harvests were performed by cutting off the foot-pad with scissors and forceps just below the hair line. The tissue was then minced ^othoroughly with sharp scissors, placed in the cup of a Mickle disintegrator with about twenty, 2-3 mm. glass beads and 1 to 2 ml of 0.25% w/v freshly prepared trypsin solution. Fifteen minutes vibration in Mickle disintegrator with minimum amplitude was used.

The fluid from the Mickle cup was transferred to a tube. Clumps of tissue were allowed to settle for 10 minutes, and the supernate was carefully removed with bulb and pipette. This was used for preparation of standard smears. The volume of trypsin solution used was recorded at each operation and taken into account in the final calculation of the number of acid-fast bacilli harvested per foot.

Infectious precautions were observed throughout the procedure.

3.2.6.2. (a) EXPERIMENTS : GROUP : II

About 400 normal CBA mice were injected with 7.5×10^6 AFB per foot-pad (right hind foot pad). The inoculated animals were kept in airconditioned room throughout the experiment. These animals were divided equally into 15 groups, including the one which served as control. All the animals were provided food and water ad libitum during the period of experiment.

The chemotherapeutic activity of the drugs was studied according to Shepard's kinetic method of testing of drugs (Shepard, 1967 a). The groups of inoculated animals were fed with different doses of drugs to be tested, as follows :

Table 1 B

Drug tested	Concentration of drug in the feed w/v.
Dapsone, DDS.	0.01% 0.001%
R-4651	0.01%
R-4754	0.01%
R-3652	0.01%
R-3647	0.01%
ADT [*]	0.01% 0.001%
AD-Dp [*]	0.01% 0.001%
AD+Dp [*]	0.01% 0.001%
AdmDp [*]	0.01% 0.001%
Control	-Nil-

* concentrations expressed in terms of
Dapsone content of the molecule.

The animals were fed with the drug mixed feed (powdered Hindustan Lever pellet food) from the 21st day (3rd month) of the injection of the bacilli and continued upto 180th day (6th month) post inoculation.

From the 6th month onwards and every month thereafter counting of the bacilli was done from the infected foot-pads.

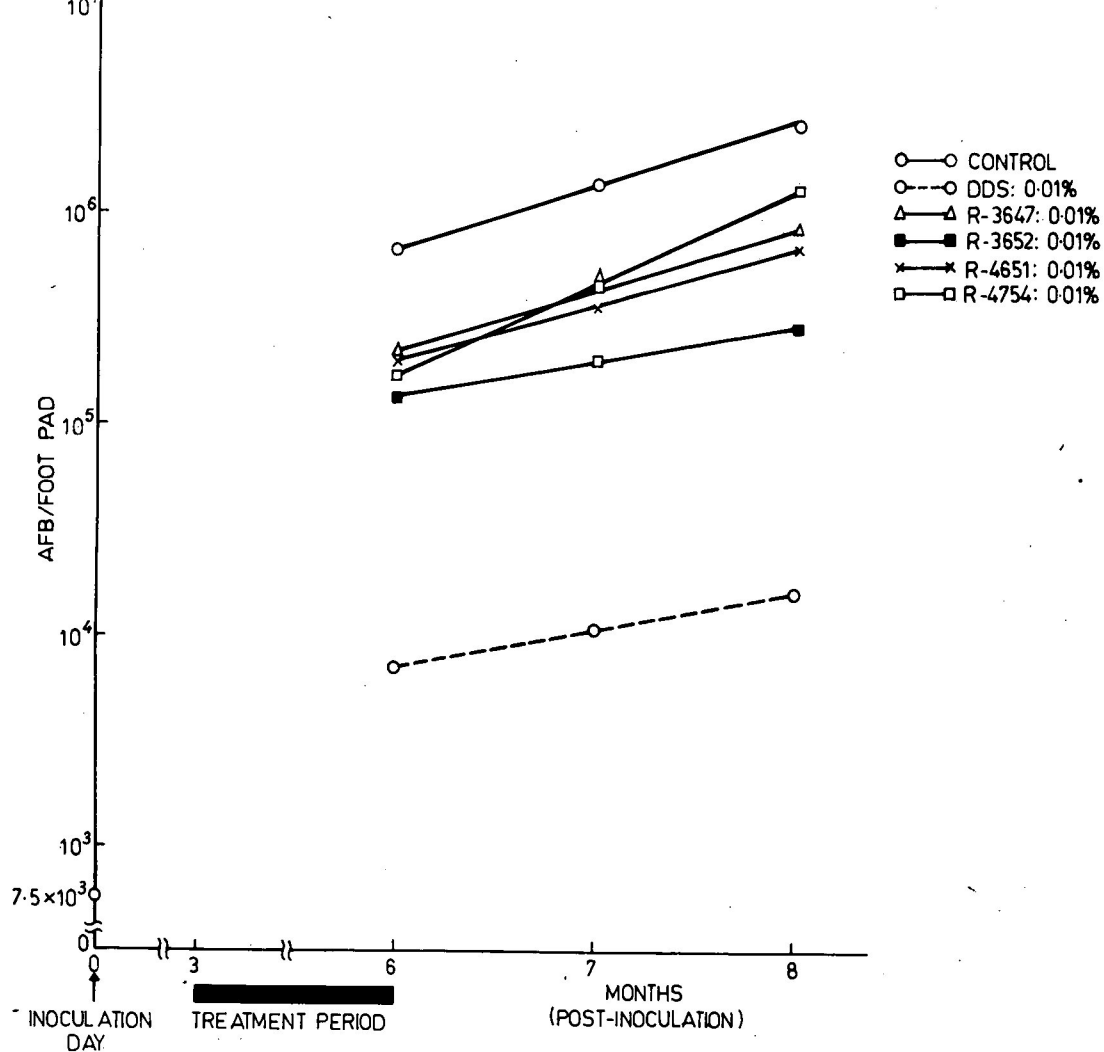
A batch of 2 to 4 animals was sacrificed each month from each group under treatment as also the control group and bacilli were harvested and the counting done. The maximum period of observation for the experiment was 10 months after injection. During the course of the study, a few animals died accidentally.

Growth curves were plotted with bacillary count/foot pad on y-axis and time in months on the x-axis (Figs: 14 & 15).

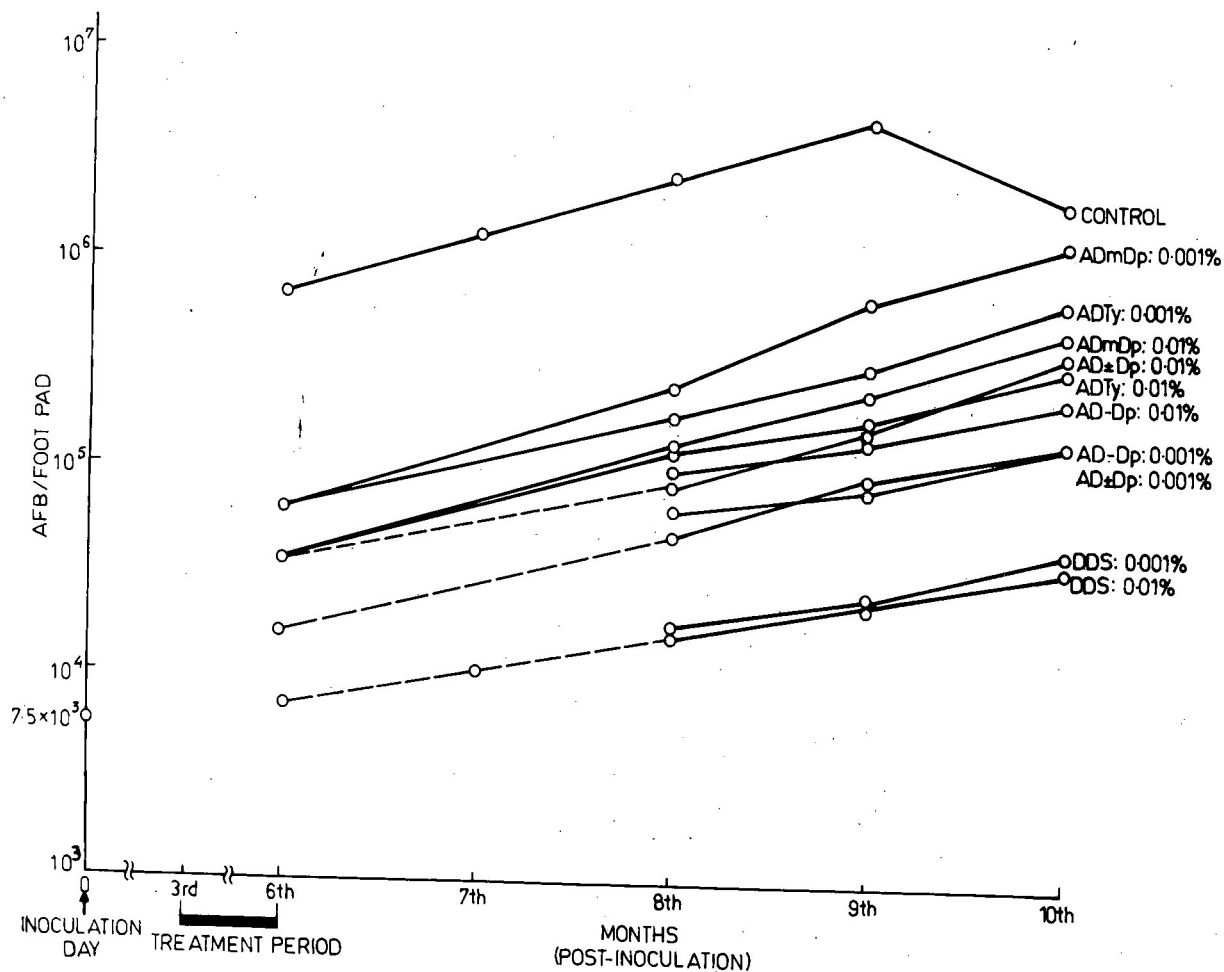
3.3. RESULTS AND DISCUSSION

The present series of experiments were primarily designed to study the anti-leprotic activity of the synthesised DDS-amino acid conjugates, viz., ADTy, AD-Dp, AD+Dp, and ADmDp, as compared to that of the parent sulfone DDS.

Mayer (1964) has strongly advocated the in-vitro screening of newly synthesised anti-leprosy drugs, since it is rapid and requires a small quantity of the drug. In comparison with other assay systems used in the past, the turbidity assay system using ICRC bacilli has several advantages. The results of the extensive studies by Khanolkar (1972) and Modak (1977) show that the requirements of in-vitro drug screening are fulfilled by this slow growing organism - ICRC bacilli.



**Fig. 14 : Antileprotic activity of compounds :
Foot-pad method**



**Fig. 16 : Antileprotic activity of compounds :
Foot-pad method**

Studies by Modak (1977) demonstrate that the sensitivity profile of the ICRC bacilli to standard anti-leprosy drugs closely resembles that of M. leprae and differs from other mycobacterial strains. DDS was found to be bactericidal to the ICRC bacilli at 100 $\mu\text{g/ml}$; rifampicin and thiacetazone were bactericidal at 10 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$ concentrations respectively. INH, PAS could only inhibit the cultures while streptomycin and kanamycin had no effect at all. On comparison with in-vivo data on drug sensitivity of M. leprae by foot-pad model (Shepard, 1971), the above data on drug sensitivity of ICRC bacilli is very impressive and thus strongly suggests that it can serve as an excellent model for primary in-vitro screening work.

To test the suitability of turbidimetric assay system using ICRC bacilli, Khanolkar (1972) subjected a series of 50 compounds for antileprotic activity testing. These compounds had shown activity against M. tuberculosis (H₃₇R_v). Although 18 of these compounds were found to be active in the in-vitro system against ICRC bacilli, the much needed confirmation by the in-vivo system was not done, to determine the validity of the model for in-vitro chemotherapeutic screening programmes.

The first of the series of experiments conducted was thus to determine the validity of the model for

in-vitro chemotherapeutic screening. With a view to establish a correlation between the in-vitro turbidimetric assay and in-vivo foot-pad technique, four compounds (coded as : R-4651, R-4754, R-3652 and R-3647) were tested by both systems. Incidentally, the compounds were obtained from the same source as those used by Khanolkar (1972) and showed activity against M. tuberculosis ($H_{37}R_v$).

Of the four compounds, compounds R-3647 and R-4754 had almost no inhibitory effect on the ICRC bacilli (Ref. Figs: 2 & 4) at the concentrations tested. Compounds R-4651 and R-3652 showed some activity at 400 $\mu\text{g/ml}$ concentration in the in-vitro system. This lack of activity the compounds was also reflected in the in-vivo testing by mouse foot-pad technique (Ref. Fig: 14), where the bacterial counts for all these drugs was between 10^5 and 10^6 AFB/foot-pad between 6th and 8th months. Of the four, compounds R-4651 and R-3652 were found to be more active, in both the in-vitro and in-vivo systems.

A good correlation was observed between the in-vitro and in-vivo techniques and the in-vitro technique was found to be an indicator of the activity of the drug in-vivo.

In another set of experiments, the effect of amino-acids L-tyrosine, L-DOPA, DL-DOPA and methyl-DOPA used in

the synthesis of the compounds, and which are shown to be acted upon by Melanrag activity were studied. The results show (Ref. Figs. 5 to 8) that tyrosine, L-DOPA and DL-DOPA and methyl-DOPA had very little effect on the growth of the ICRC bacilli at the concentrations tested.

The results for tyrosine and DOPA were consistent with the results of Modak (1977), who had tested these amino acids for their suitability in the culture media for growth of ICRC bacilli.

DOPA oxidase enzyme has been observed to be specific only to Melanrag. It is claimed that DOPA provides alternate energy source (Prabhakaran, 1967 b) yet, it will be difficult to even observe the role of DOPA in in-vitro growth of Melanrag because of auto-oxidation of DOPA in solution. These results also prove that any contamination of the respective amino-acids in the compounds will not interfere with the activity of the synthesised compounds.

The effects of the synthesised DDS-amino acid conjugates viz., ADTy, AD-Dp, AD₂Dp, AD_mDp as compared to DDS on the ICRC bacilli was studied, before extensive in-vivo studies by the foot-pad technique.

DDS (Fig:13) showed a complete inhibition of the

growth at 400 $\mu\text{g/ml}$ concentration with a minimum inhibitory concentration around 100 $\mu\text{g/ml}$. These results confirm the results of Modak (1977).

In comparison, ADTy (Fig: 9) showed no complete inhibition even at the highest concentration tested and was thus inactive in the in-vitro studies against ICRC bacilli.

Compound ADmDp (Fig: 12) demonstrated an activity profile similar to DDS, with nearly complete inhibition of the ICRC bacilli at 400 $\mu\text{g/ml}$ (DDS equivalent) concentration.

The two DOPA conjugates viz., AD-Dp and AD₂Dp have shown better inhibitory activity than DDS in the in-vitro system against the ICRC bacilli (Figs: 10 & 11). AD-Dp and AD₂Dp showed complete inhibition of the growth of the ICRC bacilli at low concentration of 16 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$ (DDS equivalents) respectively. It appears that the minimum inhibitory concentrations of these compounds is around 20 $\mu\text{g/ml}$ (DDS equivalents) much below that of DDS (100 $\mu\text{g/ml}$). These results are very impressive.

With a view to confirm the results of in-vitro testing, the above mentioned four DDS-amino acid conjugates and DDS were subjected to in-vivo testing by the foot-pad technique at two concentrations each.

The growth of Maleprrae in foot-pad for the control group was found to be good with 8.2×10^5 AFB/foot-pad at the 6th month, reaching a peak at 9th month (6.5×10^6 AFB/foot-pad) and a marginal decrease in the count at the end of 10th month post inoculation (Ref. Fig. 15).

In normal mice, Maleprrae multiply locally to yield 10^6 to 10^7 organisms per foot-pad, within 6-8 months. The growth curve reaches its peak - a plateau - at this stage and by 12 months the number of organisms in the foot-pad declines. This pattern of growth curve has been shown to be a characteristic of Maleprrae alone and a similar growth pattern which has been observed in the present experiment.

No AFB were detected at the 6th month harvesting of foot-pads from DES, AD-Dp and AD₂Dp groups, indicating, the high inhibitory activity of these compounds. A very low bacillary count was however observed for the compounds AD₁Y and AD₂Dp (less than 10^5 AFB/foot-pad), thus necessitating the extension of the experiment period. Subsequent harvesting and counting was done at the end of 8th, 9th and 10th months.

The DDS-amino-acid conjugates ADmDp, ADTy, AD-Dp and AD+Dp were all found to be effective by the mouse foot-pad technique (Fig. 15) with a bacterial counts between 10^4 and 10^6 AFB/foot-pad for 8th to 10th month readings. The corresponding readings for DDS were between 10^4 and 10^5 for the same months.

Of the four DDS conjugates, ADmDp and ADTy showed dose dependant inhibition of the growth of the bacteria in the foot-pad, while lower concentrations of AD-Dp and AD+Dp showed better activity.

CHAPTER : IV

DISCUSSION AND CONCLUSION

4. DISCUSSION AND CONCLUSION

Leprosy, is one of the oldest and the most dreaded disease afflicting mankind. Although great advances have been made in the treatment of this disease during the last three decades, the treatment is far from satisfactory. One of the first chemotherapeutic agents - dapsone - still continues to be the main drug in the treatment of leprosy, because of its low cost, high efficacy and lesser side effects.

In the past few years, the emergence of drug resistant strains of M. leprae is becoming a serious problem in the treatment and control of leprosy. Sulfone resistance, which has now been known for several years, is on the increase. Besides, the problem of 'persistance' of the bacilli even after years of treatment with drugs is another important aspect to reckon with in the fight against the disease.

To add to the dismay, is our little knowledge of Mycobacterium leprae, the causative organism now known for almost over a century. M. leprae remains the most obstinate microorganism known, resisting all attempts of culturing in an artificial medium. Besides, transmission of the disease to animal models is also a limited and

wherever possible, is not an exact replicate of the human form. Maleprae does not fulfil Koch's postulates and has been a great hindrance in research work in Leprosy.

It is only in the last decade that with the establishment of the 9 banded armadillo as a model for the study of leprosy and other biomedical research (Kirchheimer and Storrs, 1971; Storrs, 1971) that a good supply of Maleprae is available to the researchers. The result is the extensive immunological and biochemical studies being reported.

One of the most significant discovery reported in the recent years is the presence of the characteristic enzyme o-diphenoloxidase in Maleprae by Prabhakaran and his coworkers (Prabhakaran and Kirchheimer, 1966; Prabhakaran et al., 1975) and the uptake of DOPA by Maleprae as a substrate for its energy requirements (Prabhakaran, 1973).

A recent finding by the same group of workers has revealed that the drug dapsone does not penetrate Maleprae in-vitro to any appreciable extent (Prabhakaran et al., 1980) and consequently does not fully inhibit the enzyme o-diphenoloxidase, necessary for the survival of the bacilli.

Could the development of the permeability barrier be the reason for the problems like 'dapsons resistance' and 'bacterial persistence'? It was based on this hypothesis that compounds ADTy, AD-Dp, AD₂Dp and ADmDp were synthesised so that, because of the substrate specificity of M. leprae, the compounds could be actively taken up, resulting in the accumulation of the drug in the bacterial cell with higher inhibition of the enzyme. O-diphenoloxidase is a copper protein. The S atom of DDS probably binds the copper and inactivates the enzyme. As such, mutations involving the enzyme protein would not impair the effectiveness of the drug. Rifampicin, which is bactericidal to the organism exerts its antibacterial action by binding the enzyme RNA polymerase. When one amino acid in the enzyme protein is substituted due ^{to} a single step mutation, the antibiotic is no longer effective. If M. leprae becomes resistant to DDS by developing permeability barrier for the drug, dapsons combined with amino acids - the normal substrate - or other similar compounds, would not only be more effective against leprosy than dapsons alone, but would also be active against drug resistant strains of M. leprae.

The need to test the activity of the synthesised DDS conjugates on an in-vitro culture system, especially for such a permeability problem study like the present one, was obvious. A confirmed M. leprae cultivation method

in-vitro is not available till this date. Mayer (1964) has strongly advocated the use of bacterial strain closely resembling Malaprag. The slow growing ICRC bacilli fulfilled most of the requirements of an in-vitro test strain with sensitivity profiles similar to Malaprag. Besides, the turbidity assay system had several advantages and could be easily adopted. Thus, ICRC bacilli were used as the test organisms for in-vitro studies.

Testing of the inhibitory activity of compounds - R-4651, R-4754, R-3652 and R-3647 on ICRC bacilli by in-vitro turbidimetric assay and against Malaprag by mouse foot-pad technique revealed striking correlation between the results of the two techniques. This further strengthened the reasons in favour of the use of ICRC bacilli for in-vitro testing before confirmation of the activity by the foot-pad technique. This experiment was an extension of the earlier experiment by Khanolkar (1972), who screened a series of 50 synthetic compounds by the in-vitro turbidimetric assay using ICRC bacilli, but the activity was not confirmed by the foot-pad method.

None of the amino acids tested viz., Tyrosine, L-DOPA, DL-DOPA and methyl DOPA had any significant effect on the growth of ICRC bacilli. Dopa oxidase enzyme has been observed to be specific only to Malaprag. It is claimed that DOPA provides alternative energy source (Prabhakaran,

1967 b), yet it will be difficult to even observe the role of DOPA in in-vitro growth of Malariae because of autooxidation of DOPA in solution. Tyrosine - the precursor of DOPA - also showed no effect on the growth. These results were consistent with those obtained by Modak (1977).

In the final set of experiments four compounds ADTy, AD-Dp, AD₂Dp and ADmDp were tested for their activity by the in-vitro system using the ICRC bacilli. The results of the growth inhibitory activity of the compounds synthesised as compared to DDS against ICRC are briefly summarised as follows which lead to some striking conclusions.

In-vitro testing : Decreasing order of potency.



The results clearly indicate that better penetration of DDS and consequently better activity can be achieved by coupling DDS to a suitable carrier molecule e.g., an aminoacid like DOPA and thus proves the correctness of the hypothesis, on which the present work is based.

Another important feature which strikes out is the importance of the phenolic groups in the drug molecule / conjugated compound. A review of the Structure Activity

Relationship (SAR) of dapsone derivatives substantiates this fact. Of the dapsone derivatives synthesised in the past, the ones with free terminal phenolic groups were surprisingly more active than other compounds (Colwell *et al.*, 1974). It is also known that phenolic groups increase lipid solubility of the compounds, a factor which plays prime role in the penetration of a drug into a bacterial cell, like M. leprae which has a thick waxy coat. The higher activity of DOPA and its isomers (with two phenolic groups) as against tyrosine (with one phenolic group) conjugates can only be explained on this basis.

A recent study by Prabhakaran, Harris and Kirchheimer (1980) proves that the hypothesis on which the present work was carried out is true. These workers have shown that DDS in suspension or solution showed very little inhibitory effect on the oxidation of DOPA by intact M. leprae. In the disrupted bacilli, the inhibition was greater, but it was still less than 30%. However, when mixed with polylysine, DDS produces 100% inhibition of the enzyme o-diphenoloxidase in both intact and disrupted bacilli.

These results demonstrate that polylysine enables dapsone not only to pass through the bacterial cell membrane, but also to penetrate the enzyme molecule readily. It has been shown that this peptide can interact

with lipids. Polylysine can pass through the lipid bilayers of the cell membrane easily. This observation is consistent with the earlier data of the same workers (Prabhakaran, Harris and Kirchheimer, 1972) on the complete inhibition of o-diphenoloxidase of M. leprae by DDC (diethyl dithio carbamate), which contains lipid soluble ethyl groups. DDC has been found to have a potent bactericidal effect on the leprosy bacilli. However, it is unstable under acid conditions and cannot be clinically used.

Unfortunately, studies by Prabhakaran et al. (1980) are not complete. These workers have assayed the inhibition of the enzyme, o-diphenoloxidase by the inhibitory effect of DDS and DDS + polylysine on the oxidation of DOPA by M. leprae. No direct estimation of the enzyme or inhibitory activity (bacteriostatic or bactericidal) on the organism has been shown.

Secondly, in their said study DDS has not been linked by a covalent bond to the peptide polylysine, which is most important if the two molecules have to move together in-vivo. The hydrogen bond between DDS and polylysine only works in an in-vitro system.

The work reported in this thesis is definitely a step further. The DDS-carrier unit is covalently linked

in the present study and tested directly on the growth of the ICRC bacilli in the in-vitro system. Further, the conjugates have also been tested by the mouse foot-pad method. All the DDS-amino acid conjugates viz., ADmDp, ADTy, AD+Dp and AD-Dp were found to be effective in-vivo with bacterial counts between 10^4 and 10^6 AFB/foot-pad for 8 to 10 month readings, but were not as effective as DDS. The reasons for this failure in-vivo could be many and one is at a loss to define all the parameters that could play a part. Few are presented below :

- a) The inherent difference between the in-vitro and in-vivo systems.
- b) The availability of the drug at the site of infection i.e., foot-pad.
- c) The breakdown of the drug conjugates in-vivo. Azo linkages are reported to be broken down by the intestinal flora and other enzymes (Azo-reductase____) in the body (Watabe et al., 1980).

The results of in-vivo testing show that L-DOPA and DL-DOPA conjugates have similar activity. This is consistent with the finding of Prabhakaran (1973) that o-diphenoloxidase of Melancon acts on both the D & L isomers with same specificity.

In conclusion, it can be said, that this unique drug-carrier delivery system has been tried out for the first time in the fight against *M. leprae* infection to overcome some of the problems faced with the presently available drugs for the treatment of leprosy. It is to be frankly admitted, that the work presented in this thesis, is of a preliminary nature and although the investigation path has been defined, a lot of work still has to be done to achieve the goal.

Further studies have to be done, using different vehicles, different linkages, different concentrations of conjugates as also determine the effect of drug carrier units on the growth of drug resistant *M. leprae*, to assess the toxicity of new conjugates and to explore newer techniques. All this would depend on the ingenuity of the investigator, advances in research in interdisciplinary sciences in the years to come, but it can be said with confidence that research on these lines would definitely lead to significant results.

Summary

DDB amino-acid conjugates were synthesised with a view to overcoming the problems of bacterial resistance to the presently available battery of drugs against leprosy

and of 'bacterial persistence.' Two of the conjugates synthesised showed better activity than DDS in the in-vitro system. Unfortunately, these drugs were less active than DDS when tested against M. leprae by the foot-pad method. But it can be asserted that this drug delivery system can be utilized for providing more effective drugs against the most dreaded disease afflicting mankind, LEPROSY.

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Appendix 1.1

Formula : (Difco Manual, 9th Edition, 1971)

The stock solutions A, B, C and D are derived from Difco.

Solution A (10 X)

KH_2PO_4	10.0 gms.
$\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$...	63.0 gms.
Asparagine	10.0 gms.
Tryptone	12 gms.
Na Citate ^{iv}	12.5 gms.
D/W	1000 ml.

Solution B (100 X)

$\text{Fe(II)Amn. Citrate}$...	50 mg.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	100 mg.
CaCl_2	5 gms.
ZnSO_4	1 mg.
CuSO_4	1 mg.
D/W	100 ml.

Solution C :

Tween 80 (100%)..	0.5 ml.
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Solution D :

Glycerol	25 ml.
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The solution B substitutes yeast extract and solution F are experimental modification of the Dubos medium.

Solution E : (Paul, 1970)

Vitamine mixture (100 X) ..	10ml.
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Solutions F :

(a) Na Glutamate	10 mg(D/W)
(b) Na Pyruvate	10 mg(D/W)

Final medium was prepared as follows :

Solution A	100 ml.
Solution B	10 ml.
Solution C	0.5 ml.
Solution D	25 ml.
Solution E	10 ml.
Solution F	1 ml. each of (a) & (b).
0.1% Phenol Red	4 ml.
pH	7.2 to 7.4
D/W to make	1000 ml.

SYNOPSIS

INTRODUCTION

Hansen's disease continues to be a major health problem in the world, with over 20 million people suffering from the disease today. The most widely used drug in the treatment of this disease is Dapsone (DDS; 4,4'-diaminodiphenyl sulfone). The dramatic actions of DDS in Hansen's disease were first demonstrated by Cochrane ¹ et al. Prior to this no effective chemotherapeutic agent against Mycobacterium leprae, the causative organism was available. DDS is inexpensive and for this reason, it is the main weapon in the fight against M. leprae infection in many countries, including India, with high incidence of the disease.

Now, fears have been expressed that the usefulness of this weapon might be lost forever. Strains of M. leprae resistant to DDS have emerged in different parts of the world. ² As the treatment of Hansen's disease is prolonged, irregular and insufficient intake of the drug could result in secondary sulfone resistance. Few cases of primary drug resistance are also reported. ³ Besides, even after years of treatment with DDS, viable bacilli persist in the tissues of lepromatous cases. ⁴

The above mentioned problems could well be the result of inavailability of the drug at the site of lodging of Malaria or the failure of the drug to permeate the cell membrane of the organism. The later hypothesis is very recently proved to be true by Prabhakaran et al.⁵ whose studies show that the drug does not penetrate intact Malaria in-vitro.

Many therapeutic agents are ineffective because of the failure of the active molecule to permeate the cell membranes. Attempts to overcome this problem include modification with lipophilic groups, attachment to macromolecules and entrapment in the liposomes. Another approach employs the active transport systems to bring potentially cytotoxic drugs into the cells. The advantage of this approach is that it can result in a 100- to 1000- fold concentration of the toxic agent inside the cell.

The molecular basis of cellular permeation of peptides and amino-acids have been studied in bacteria.^{6, 7} Peptides and amino-acids are ideally suited as carrier species, because of chain length, side chain and other functional group specificities. Furthermore, since the structural requirements for transfer differ for bacteria and mammalian cells, drugs/drug conjugates can in principle be designed so as to be selective.

DDS has been reported to inhibit folic acid synthesis in other bacteria⁸ but the mechanism of action of DDS in Hansen's disease remains unknown. Now, it is also known that the drug does not penetrate *M. leprae* so an approach to the development of new drugs for Hansen's disease or improving the efficacy of the existing ones, that exploits normal metabolic processes as a means of selectively delivering toxic agents into the cell, is proposed. Amino-acids coupled to the DDS molecule to appear as a peptide acts as a discriminating drug carrier selectively permeating the target cell, *M. leprae*. Once inside the cell, normal metabolism could either result in the release of the drug which is toxic to the cell or the drug amino-acid complex itself could interfere with the normal metabolism of the cell. Making an antibacterial agent to permeate its target organisms should enhance its effectiveness.

α -Diphenoloxidase, a copper containing enzyme, is the only specific enzyme proven to be present in the Hansen's bacilli.⁹ This enzyme is shown to have very high affinity for and selectively binds DOPA (3,4-dihydroxy-phenylalanine)¹⁰ as substrate. For this reason DDS conjugates with L-DOPA (AD-Dp), DL-DOPA (AD+Dp), L-tyrosine, a precursor of DOPA (ADTy) and methyl DOPA, a more stable derivative of DOPA (ADmDp) were synthesised.

The DDS-amino-acid conjugates were synthesised with a view that because of structural resemblance with DOPA, these compounds would be actively transported into the cell of the bacilli where it could be acted upon by the enzymes of the cell to break and release free DDS or alternatively the complex itself could be active as the S atom of DDS molecule is free to bind with Cu of the enzyme o-diphenol-oxidase, resulting in the death of the organism, as it has been shown that Cu chelating compounds, which penetrate the cell are bactericidal in nature, for ex. diethyldithio-¹¹carbamate.

COUPLING OF DDS TO AMINO-ACIDS

DDS was diazotized by sodium nitrite and hydrochloric acid. The diazonium salt was then added to alkaline solution of amino-acid maintained in an ice-salt bath between 0-5°C. pH maintained between 9 and 9.5 throughout the addition. Bright red conjugates were obtained. After one hour of standing with intermittent slow stirring, the conjugates were precipitated by bringing the pH to 1.5-2.

The solution was filtered and washed extensively with cold dil. hydrochloric acid to get rid of any excess DDS and then with chilled water to remove acid. The compounds were reprecipitated from water by pH changes

and finally recrystallised from large excess of water. The conjugates were checked for impurities of free DDS by TLC, dried under vacuum and stored in a desiccator.

From UV absorption data¹² it was concluded that the coupling of amino-acid to DDS had occurred.

IN-VITRO STUDIES

For ~~in-vitro~~¹³ activity of the conjugates synthesised, ICRC Bacilli¹³ was used as a test organism. Stock culture C-49 : ICRC Bacilli was obtained from Cancer Research Institute and was maintained in modified Dubos medium. These were regularly subcultured at every three to four weeks. At the time of subculturing smears were prepared, stained and morphology of the bacteria was recorded.

Turbidimetric assay

For turbidimetric assay standard Bosch and Lomb spectronic-20 cuvettes were used. Two week old culture of the ICRC Bacilli from Dubos medium was used as inoculum. The inoculum was adjusted to 90% Transmission against a blank. A tube containing plain medium served as negative control and one with only inoculum and without test solution served as positive control or growth control. The amount

of fluid in each tube was 6 ml.

Before measuring the turbidity on day zero, the cultures were refrigerated for 72 hours. The cultures were then incubated at 37°C for 12 days. Readings at 660 mμ were taken on every third day and graphs of % transmission vs. time in days plotted.

The organisms inoculated, settle down at the bottom of the tubes, leaving the supernatant clear or the tubes are spun at 1500 rpm for 15 minutes. Thus, each tube served its own control for 100% Transmission, before shaking. In this system, for turbidimetric measurements, the values were only comparative and not absolute.

This system was used for studying the effects of drugs on the growth of ICRC Bacilli.

Effect of amino-acids on growth of ICRC Bacilli

L-tyrosine, L-DOPA, DL-DOPA and methyl-DOPA were tested for their effects on ICRC Bacilli at concentrations of 400 μg and 80 μg/ml.

Effects of the DDS-amino-acid conjugates on the growth of ICRC Bacilli

The four DDS-amino-acid conjugates viz., ADTy,

AD₂Dp, AD-Dp, and ADaDp were tested at concentrations 400, 80 and 16 µg/ml DMS equivalents of the conjugates.

DMS was also tested at the above concentrations.

Correlation of in-vitro and in-vivo techniques

With a view to establish a correlation between the 'in-vitro' turbidimetric assay and 'in-vivo' Shepard's¹⁴ mouse foot-pad technique, four newly synthesised compounds (coded as; R-4651, R-4754, R-3652 and R-3647) which showed promising anti-tuberculosis activity were obtained from Chemotherapy Department, Haffkine Institute. These compounds were tested at concentrations of 400, 80 and 16 µg/ml in the in-vitro system.

IN-VIVO TESTING

The chemotherapeutic activity of the drugs was studied according to Shepard's method of kinetic testing of¹⁵ drugs. Malanrae were isolated from human biopsy, collected from untreated lepromatous leprosy patient at the Achworth Leprosy Hospital, Bombay.

The counting of the bacilli was done according to Shepard and McRae¹⁶ and suitably diluted to give approximately 2.5×10^5 AFB/ml suspension.

400 mice of CBA strain were injected with 7500 bacilli in 0.03 ml of suspension, each in the right hind foot-pad and the inoculated animals were kept in airconditioned room throughout the experiment.

The animals were fed with drug mixed feed (Hindustan Lever) from the 91st day (i.e., 3rd month) of the injection of the bacilli and continued upto 180th day (i.e., 6th month) post inoculation.

The drugs R-4651, R-4754, R-3652 and R-3647 were tested at a single dose of 0.01% of feed whereas other drugs were tested at 0.01% and 0.001% DDS or DDS equivalents. The control group of animals received normal diet.

From the 6th month onwards and every month thereafter counting of the bacilli was done from the infected foot-pads. A batch of two to four animals were sacrificed each month from each group and foot-pads pooled for reading.

The procedure for harvests was similar to that adopted by Shepard¹⁷ and average number of acid-fast bacilli (AFB) was estimated per foot-pad.

Growth curves were plotted against time with AFB/foot-pad on Y-axis and time in months on X-axis.

RESULTS

The growth of *Maleptra* in foot-pad for the control was found to be good, with close to 10^6 AFB/foot-pad at 6th month, reaching a peak at 9th month (around 10^7 AFB/footpad).

The amino-acid tested had no effect on the growth of ICRC Bacilli in the in-vitro system. This was consistent with the results of Modak¹⁸ who had tested these amino-acids for their suitability in the culture media for ICRC Bacilli.

The DDS-amino-acid conjugates ADaDp, ADTy, AD+Dp foot-pad technique with bacterial counts between 10^4 and 10^6 AFB/foot-pad for 8th to 10th month readings. The readings for DDS were between 10^4 and 10^5 for the same months. Of the four DDS conjugates, the first two showed dose dependant inhibition of the growth of the bacteria in the foot-pad. The in-vitro turbidimetric assay showed some correlation with the in-vivo results.

The four drugs R-4651, R-4754, R-3652 and R-3647 were not found to be effective in Shepard's technique. The bacterial counts for all these drugs was between 10^5 and 10^6 AFB/foot-pad between 6th and 9th month.. This lack of activity was also reflected in the in-vitro testing using ICRC Bacilli. Thus a good correlation was observed between the in-vitro and in-vivo techniques and the in-vitro technique was found to be an indicator of the activity

of the drug in-vivo.

SUMMARY

DDS was conjugated to amino-acids L-tyrosine, L-DOPA, DL-DOPA and methyl DOPA. The conjugates were tested for activity by in-vitro and in-vivo techniques and were found to be effective.

Four anti-tuberculosis drugs were also tested for their activity against ICRC Bacilli in-vitro and Malsprae in-vivo. A good correlation was found in the results obtained by the two techniques.

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STATEMENT UNDER UNIVERSITY RULE

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As required by the University Rule No. O.412 and O.413, I state that the work presented in this thesis is original in nature and to the best of my knowledge has not been submitted for any degree of this or any other University.

Whenever reference has been made to the work of others, it has been clearly indicated along with the source of information in the "References".

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Signature of the Candidate

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