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Metabolism in *Mycobacterium leprae:* possible targets for drug action

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Introduction

Most of this paper deals with attempting to identify actual and possible targets for existing and future antileprosy agents. However, the application of some of this research into devising a suitable method for drug screening which uses [^{3}H]-hypoxanthine, i.e. radioisotopically labelled, will also be mentioned.

I want to emphasize biosynthetic pathways in *Mycobacterium leprae* because experience tells us that it is in these pathways that targets for antibacterial agents usually occur. This presents an immediate problem when considering leprosy bacilli since little work has been done on such pathways. Most of the work done on intermediary metabolism has been done on catabolic pathways, where a major finding is that *M. leprae* organisms can use a variety of carbon sources to release energy, by pathways including oxidative pathways like most other mycobacteria.[1]

A general scheme for many—but not all—biosynthetic pathways is shown in Figure 1. Examples of some pathways that fit the scheme in Figure 1 are shown in Table 1 and it is suggested that in such pathways, development of new agents should be directed against the synthesis of characteristic molecules from intermediates. This is because the intermediates in these pathways may be acquired directly from the environment by mycobacteria and in that case the synthesis of intermediates by mycobacteria themselves would be stopped. This is a useful thing for the bacteria to do since all biosynthetic pathways require a great deal of energy, and bacteria can save some energy by not synthesizing intermediates unnecessarily.

Although most mycobacteria can synthesize the intermediates shown in Table 1, when they have to, the detailed biochemistry of the pathways has rarely been worked out. Instead, the evidence for synthesis of intermediates is usually that mycobacteria—such as tubercle bacilli, *M. avium*—can grow in culture media

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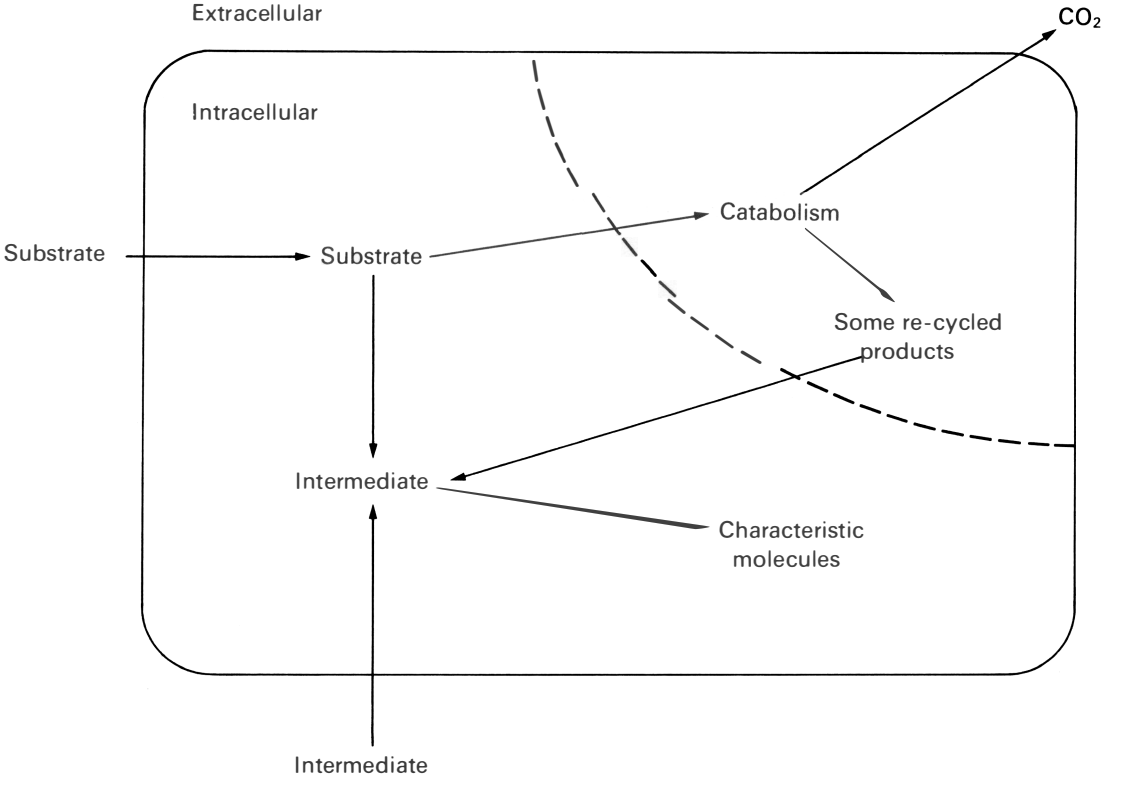

Figure 1. General scheme for biosynthesis of characteristic molecules. See Table 1 for examples of some important pathways. Only the area to the left of the broken line is discussed in this paper.

without a supply of intermediates. It therefore follows that in the case of *M. leprae*—which may have access to intermediates from the host—we do not yet know whether or not some intermediates can be synthesized at all.

There now follows a detailed discussion of four biochemical functions which involve characteristic molecules. Figure 1 illustrates the third and fourth examples but not the first two.

Biosynthesis of tetrahydrofolate

It is the first part of biosynthesis of tetrahydrofolate (Figure 2)—the production of dihydropteroate—that is only found in bacteria. Both the host and bacteria can convert folate to tetrahydrofolate. The well-known sulphonamide class of drugs act against the part of the pathway which is characteristically bacterial while benzylpyrimidines act selectively against bacterial, rather than mammalian

Table 1. Biosynthesis of some important characteristic molecules.

Extracellular substrate*	Intracellular substrate†	Intracellular intermediate†	Extracellular intermediate‡	Characteristic molecules synthesized
Simple carbon & nitrogen sources (e.g. carbohydrates, pyruvate, NH_3)	mainly tetroses pentoses and keto acids plus NH_3	amino acids	amino acids	Proteins
Simple carbon & nitrogen sources	pentoses glutamine aspartate C_1 units§	purine nucleotides	purine bases and nucleosides¶	Nucleic acids
Simple carbon & nitrogen sources	glutamine CO_2 aspartate pentoses C_1 units§	pyrimidine nucleotides	pyrimidine bases**	Nucleic acids
Carbon sources (e.g. acetate pyruvate glucose glycerol)	acetyl-CoA malonyl-CoA	fatty-acyl-CoAs	fatty acids	Complex lipids, e.g. mycolates, phenolic glycolipid

* Molecules obtained from the environment.

† Molecules which can participate in biosynthetic pathways (see Figure 1). Additionally *all* biosynthetic pathways require a great deal of energy which the bacteria must generate.

‡ Molecules obtained from the environment; all these examples have been shown to be scavenged by *M. leprae* organisms isolated from host tissue.[1]

§ Serine is an efficient source of C_1 units in the mycobacteria[19,20] and tetrahydrofolate is required for the use of C_1 units in these biosynthetic pathways.

¶ Purine nucleotides are not taken up directly by *M. leprae*[20,21].

** It is not known whether pyrimidine nucleosides or nucleotides can be taken up by *M. leprae*.

dihydrofolate reductase (see Figure 2). DDS[2] and a number of benzylpyrimidines (see contribution of J K Seydel in this supplement) have their primary sites of action in *M. leprae* against these two respective activities.

Uptake of iron

All living organisms require iron for their survival and growth. However, many bacteria growing in host tissue exist in an environment which is limiting for

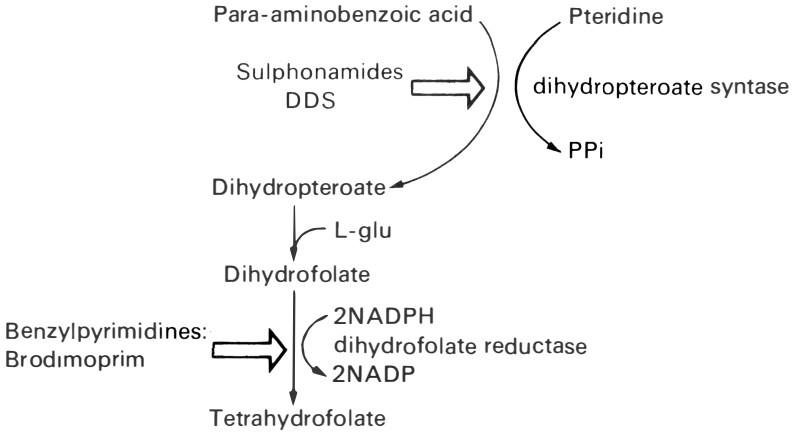

Figure 2. Latter stages of tetrahydrofolate synthesis.

available iron. Thus bacteria have evolved ways of scavenging iron using chelating molecules with a very high affinity for iron. Mycobacteria are no exception, producing iron chelators known as exochelins and mycobactins when grown in conditions with limited free iron.[3]

Exochelins are molecules which can chelate iron in the environment and present it to mycobacteria so that they take up the iron. It is not yet known whether *M. leprae* elaborates its own exochelins but when incubated with a panel of exochelins from nine different sources—from four strains of armadillo derived mycobacteria (ADMs: almost always isolated from armadillos infected with *M. leprae*[4]) *M. bovis* BCG, *M. vaccae*, *M. smegmatis*, *M. avium* and *M. neoaurum*—iron bound to the exochelins from one of the ADM strains and *M. neoaurum*[5] was taken up by *M. leprae*.

Since exochelins are used exclusively by mycobacteria for acquisition of iron, there appear to be some intriguing chemotherapeutic possibilities. Scandium and Indium can be chelated to bacterial siderophores and the resultant complexes were bacteriostatic to *E. coli*[6] and protected mice from infection with Klebsiella organisms.[7] Thus a preparation of exochelins might be used similarly to deliver toxic metals specifically to mycobacteria and as such might be of use in inhibiting growth of, or killing mycobacteria, including *M. leprae*.

Lipid biosynthesis

A most important metabolic activity in mycobacteria growing in simple culture media is the *de novo* fatty acid syntase, a multi-enzyme complex which has acetyl-CoA (C_2, i.e two carbons) as its initial substrate and adds further C_2 units, derived from malonyl-CoA, to it (see Table 1). The products are fatty acids, mainly C_{16} to

C_{32}.[8] However this complex is probably not a suitable target for potential antimycobacterial agents because its activity is repressed or inhibited in mycobacteria when fatty acids can be obtained from the environment. Such abolition of syntase activity has been shown in *M. convolutum* grown on alkenes[9] and *M. lepraemurium* grown on Ogawa medium, a lipid-rich medium.[10] Also, when cell-free extracts of *M. phlei* grown in the absence of lipids were assayed for *de novo* syntase with and without fatty acyl-CoAs, the syntase activity was strongly inhibited by fatty acyl-CoAs. I have recently shown that the uptake and incorporation into lipids of acetate into *M. microti* is strongly inhibited in *M. microti* grown in the presence of fatty acid or in mice (unpublished data) suggesting that in host tissue *M. microti* adapts to an environment where fatty acids are available for metabolic purposes.

Thus it may be necessary to search for targets in the biosynthesis pathways for characteristic, complex lipids for which fatty acyl-CoAs (derived from fatty acids such as C_{18} obtained from the environment) can be the starting substrates. Substrates and products of many pathways of lipid synthesis are likely to be restricted to mycobacteria, so any agents having a primary effect on these pathways would be very selective in acting against the bacteria, and not host enzymes. The likely biosynthetic pathways for α-mycolic acid (Figure 3) and phenolic glycolipid (Figure 4) are shown as examples of such pathways (see Minnikin (1982)[11] for review and further references). Many of the enzymes themselves have not been demonstrated. For instance, in α-mycolate biosynthesis (see Figure 3 for identification of letters) steps (C) and (D) are deduced from identification of intermediates in intact bacteria while (A) and (B) have been demonstrated in enzyme assays necessarily using cell-free extracts. Steps (E) and (F) are conjectural. Condensation reactions have been shown only using up to C_{10} acids in cell-free extracts of mycobacteria when two C_{10} acids condensed to give C_{20} acid.[12] But it would be so favourable in terms of energy conservation for the mycobacteria and in terms of the enzymes required to use awkward (long and very hydrophobic) substrates to carry out condensation reactions possibly using fatty acids scavenged from the host that it is likely that step (F) and probably even (E) are condensation reactions. However, it appears that *M. tuberculosis* can add C_2 units to fatty acids up to C_{54} acids if necessary;[13] so step (E) might be attained by elongation rather than condensation.

In the synthesis of phenolic glycolipid—or even characteristic molecules like phthiocerol, an intermediate (G) which has no counterpart in the host must be synthesized before an unusual addition of C_3 units (probably as propionyl-CoA: step H) occurs. Phthiocerol is probably converted to phenol phthiocerol dimycocerosate by step (M) shown with [^{14}C]-tyrosine followed by step (N). The incorporation of label from tyrosine suggests a role for step (L), although *p*-hydroxybenzoate is synthesized—only in bacteria—by the chorismic acid pathway (K). Step (N) requires fatty acids such as mycocerosate in *M. leprae* and tuberculostearate in *M. kansasii*, both synthesized (O) using enzymes which are

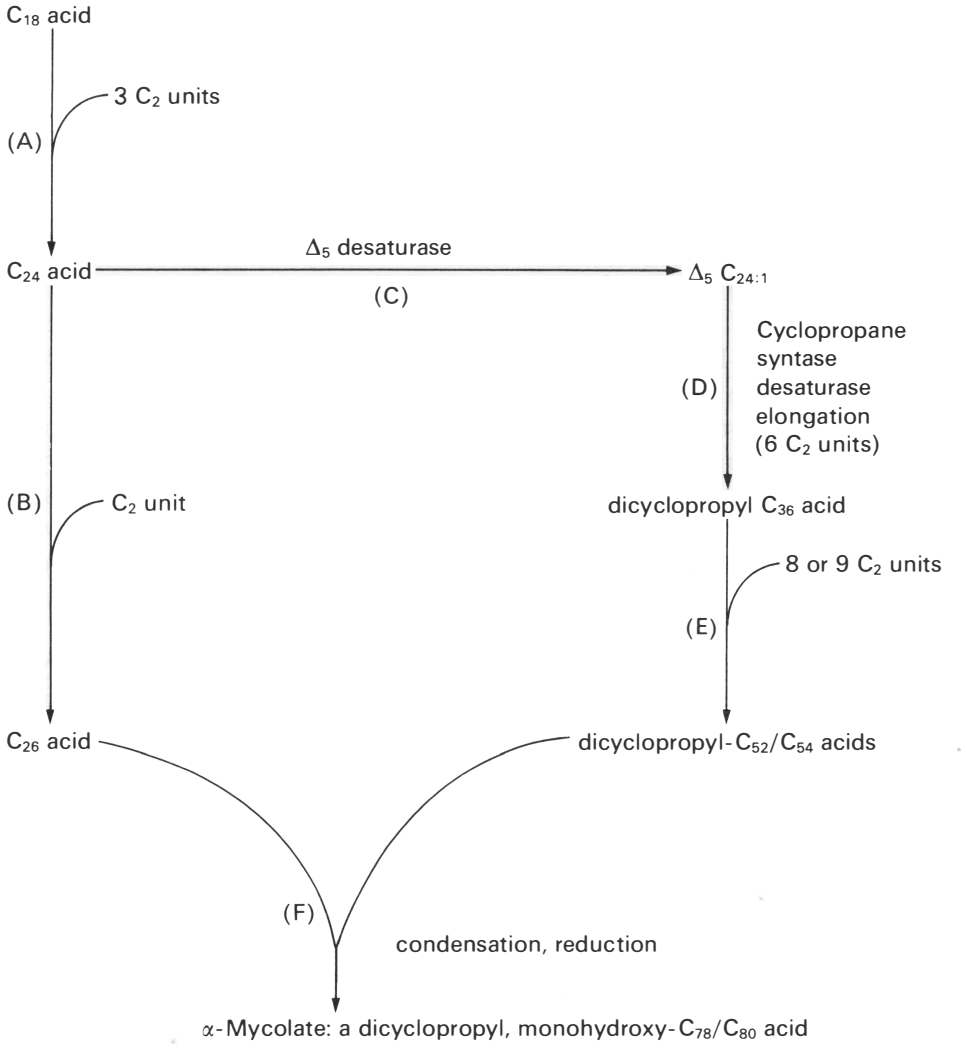

Figure 3. Synthesis of α-mycolate in *Mycobacterium tuberculosis*.

characteristically mycobacterial (for mycocerosate syntase[14]). Thus, in the synthesis of phenolic glycolipid from phthiocerol, there are four steps (K,M,N,O) which have both substrates and products synthesized only in bacteria—and some found only in mycobacteria. The final glycosylation step (P) also involves unusual sugars in many mycobacteria. Perhaps somewhere in these steps lies a target for a novel antimycobacterial agent!

What is probably needed now is a detailed study of the biosynthetic pathways of characteristic lipids of mycobacteria. The pathways seem promising for containing target enzymes for antimycobacterial agents because the substrates

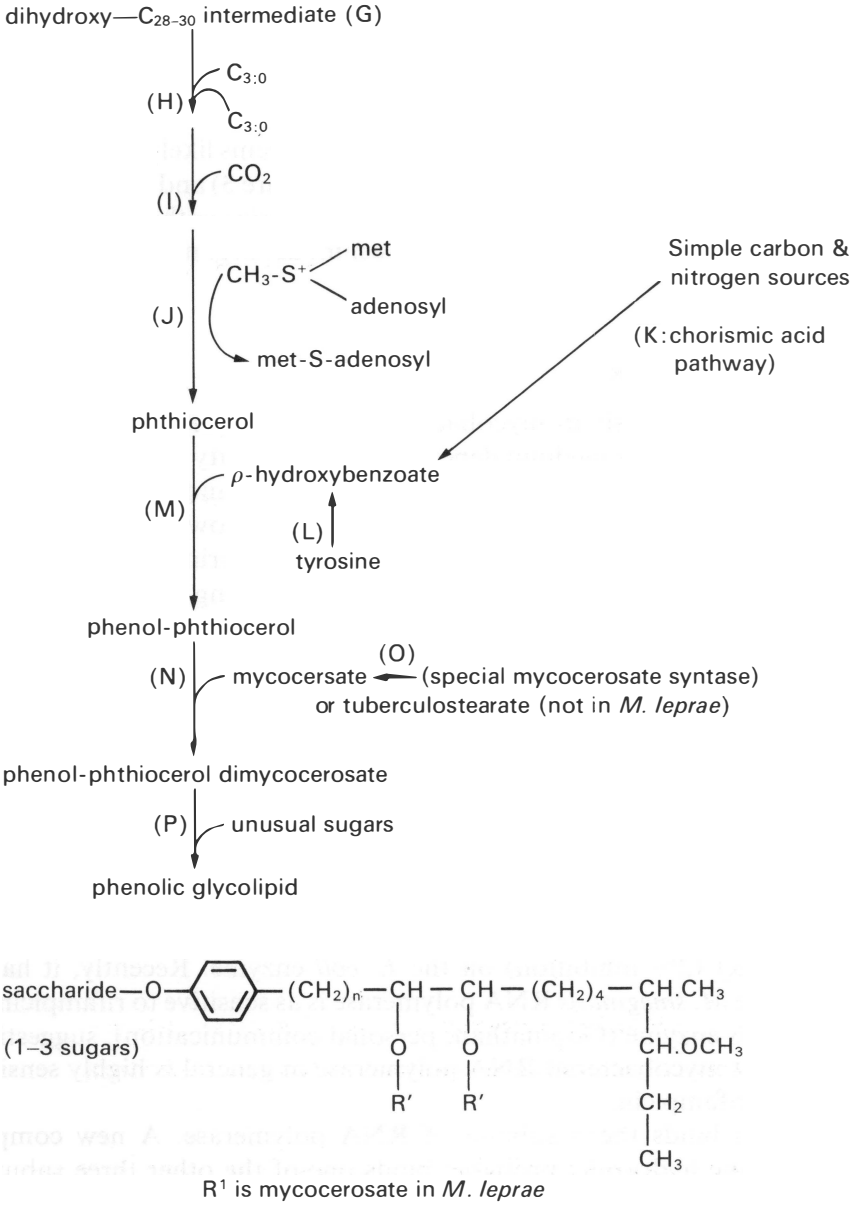

Figure 4. Synthesis of phenolic glycolipids.

and intermediates (Figures 3 and 4) are so different from any in mammalian systems, where fatty acids greater than C_{24} acids are not found. Thus agents acting directly on enzymes with characteristically mycobacterial substrates might be expected to have little effect on metabolism in the host. It would be most desirable to demonstrate individual enzymes, using cell-free extracts of mycobac-

teria. Only in this way, the effect of any agents could be tested on single enzymes. When agents inhibit an activity in whole bacteria (e.g. mycolate synthesis from, say, palmitic acid-C_{16}) it is always possible that they are having only a secondary, or indirect effect on the activity that is being measured. These suggestions may prove very difficult to follow up in *M. leprae*, but it seems likely that the inevitably complex biosynthetic pathways for α-mycolate (Figure 3) and phenolic glycolipid (Figure 4) will be conserved in whichever mycobacteria synthesize those complex lipids and thus, at least initially, easily grown model organisms could be used in place of *M. leprae*.

Nucleic acid biosynthesis

Nucleic acid biosynthesis in mycobacteria growing in relatively simple media such as Modified Dubos medium depends upon the ability first to use the carbon and nitrogen sources to synthesize nucleotides *de novo* and then the assembly of nucleotides into the nucleic acids, DNA and RNA. However, mycobacteria—including *M. leprae*[15]—can scavenge the purine and pyrimidine bases from the environment and convert them to nucleotides, thus saving a great deal of energy (see Table 1). Indeed, it is not known whether *M. leprae* can synthesize nucleotides *de novo* at all.

Thus, again, the best strategy seems to be to develop antimycobacterial drugs against the synthesis of characteristic molecules—the nucleic acids—from nucleotides. The substrates (nucleotides) for nucleic acid synthesis are common to both mammalian or bacterial systems so agents are needed which selectively inhibit the bacterial nucleic acid synthesis. Such agents are in use, and a notable example is rifampicin. Rifampicin is selectively active against its target—RNA polymerase—in mycobacteria amongst bacteria in general. Thus 10^{-8} rifampicin completely inhibits the *M. tuberculosis* enzyme[16] while having barely any detectable effect (2% inhibition) on the *E. coli* enzyme. Recently, it has been shown that the *M. smegmatis* RNA polymerase is as sensitive to rifampicin as the *M. tuberculosis* enzyme (Gopinathan, personal communication), suggesting the possibility that mycobacterial RNA polymerase in general is highly sensitive to inhibition by rifampicin.

Rifampicin binds the β subunit of RNA polymerase. A new compound, naphthyl-glycine hydrazide, probably binds one of the other three subunits. It inhibits the growth of *M. tuberculosis* and rifampicin resistant mutants are sensitive to it.[17] Because it acts on a different part of the same enzymes as rifampicin, there is the intriguing possibility that naphthyl-glycine hydrazide and rifampicin might act synergistically against mycobacteria.

Hypoxanthine incorporation for drug screening

During the work on purine metabolism in *M. leprae*, it became evident that the

Table 2. Incorporation of selected purines & pyrimidines into *M. leprae*.

Substrate	μM	Incorporation pmol/10^{10} *M. leprae*/24h
Hypoxanthine	1·2	8
	3·3	21
	17	74
	80	280
Adenosine	17	120
	80	465
Thymidine	0·07	0·06
	17	6
	80	19

Incorporation is into material insoluble in ice-cold 5% (w/v) trichloroacetic acid.

Substrate (in pmol) incorporated refers to pmol substrate supplied extracellularly and in calculary incorporation no account was taken of any possible dilution supplied substrate in intracellular pools of metabolites. For incubation conditions, see Khanolkar & Wheeler (1983).[15]

incorporation of radioactive hypoxanthine into acid insoluble material in *M. leprae* organisms[15] was far more rapid than radioactive thymidine incorporation (see Table 2). Adenosine incorporation was slightly more rapid than hypoxanthine, but also more variable. Thus the effect of a number of antileprosy agents on hypoxanthine incorporation was tested. Rifampicin and deoxyfructoserotonin inhibited the activity strongly.[15] Then the lowest concentration needed significantly (statistically: using Wilcoxon Rank-sum test) to inhibit hypoxanthine incorporation was determined for a number of other agents.[22] The concentrations were as follows: for DDS 10 ng/ml, for clofazimine 100 ng/ml, and for brodimoprim 30 μg/ml. These concentrations may be considered as MIC values for hypoxanthine incorporation in intact *M. leprae*. In DDS-resistant *M. leprae*, hypoxanthine incorporation was not inhibited by DDS (at up to 100 ng/ml) alone but with only 3 μg brodimoprim/ml (0·1 times MIC), almost complete inhibition of hypoxanthine incorporation was observed. A similar, synergistic effect of brodimoprim with DDS even in DDS-resistant mycobacteria is reported elsewhere in this book, and was shown previously.[18]

Since the variety of agents which inhibit hypoxanthine incorporation in *M. leprae* precludes them all having a primary effect on hypoxanthine incorporation,

it is likely that hypoxanthine incorporation is inhibited as an indirect effect—possibly, overall impaired metabolic competence of the intact *M. leprae* organisms incubated with the agents. If this is so then measuring hypoxanthine incorporation looks a very good way of screening novel, potentially antileprosy agents with a wide range of primary effects.

There seems little advantage in using substrates radiolabelled with extremely high specific activity in incubations with intact *M. leprae* since at the low concentrations of substrate which result (see Table 2) incorporation is almost proportional to the extracellular concentration of substrate, and adding unlabelled substrate or adding the same amount of radioactivity but using a substrate of relatively low specific activity has little effect on the amount of *radioactivity* incorporated.

Discussion and summary

I have emphasized the development of antileprosy drugs to inhibit synthetic pathways for characteristic bacterial molecules although at present the target enzyme dihydrofolate reductase has been shown to be a good target for possible new antileprosy agents.[18] However where intermediates are synthesized first (as shown in Figure 1 and Table 1) then characteristic molecules are made for the intermediates, it must be the second step which is sought to be inhibited. This is because, while intermediates (like nucleotides and fatty acyl-CoAs) need not be synthesized by mycobacteria all mycobacteria including *M. leprae* must synthesize their own characteristic molecules. Since products and often substrates in the synthesis of characteristic molecules are often only found in bacteria—or sometimes only in mycobacteria—such pathways ought to be able to be inhibited by agents acting highly selectively against mycobacteria. Finally, since many characteristic molecules of mycobacteria such as phthiocerol, phthiocerol based phenolic glycolipids, mycolates, and nucleic acids occur not only in *M. leprae* but also other mycobacteria, a search for new agents based on their synthesis might reveal agents which kill many pathogenic mycobacteria, not only *M. leprae*.

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