Measurement of hypoxanthine incorporation in purified suspensions of *Mycobacterium leprae*: a suitable method to screen for anti-leprosy agents *in vitro*

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Summary. The rate of incorporation of hypoxanthine was measured in suspensions of *Mycobacterium leprae*, with and without added anti-leprosy agents. Dapsone, clofazamine and brodimoprim, as well as other benzylpyrimidines, inhibited hypoxanthine incorporation, and their minimum inhibitory concentrations for incorporation with intact *M. leprae* were near the minimum inhibitory concentrations at which the agents have antibacterial effects. At sub-inhibitory concentrations for hypoxanthine incorporation, some combinations of benzylpyrimidines and dapsone were inhibitory, suggesting that synergic effects of anti-leprosy agents might also be detected by the inhibition of hypoxanthine incorporation. Thus, demonstration of inhibition of hypoxanthine incorporation in *M. leprae* could be a rapid method for screening anti-leprosy agents and especially for preliminary testing of new, potential anti-leprosy agents. The rate of hypoxanthine incorporation was generally lower in suspensions of *M. leprae* with lower viability, but it was not proportional to viability so the technique would not be suitable for accurate determination of viability.

Introduction

Multi-drug therapy, usually with three anti-leprosy drugs, is now recommended for the treatment of leprosy (World Health Organization, 1985). However, there are problems with all the drugs in use. Drug resistance is well known with dapsone and has been reported recently with clofazamine (Warndorff-van Diepen, 1982) and rifampicin (Guelpa-Lauras *et al.*, 1984). Also, during treatment with thioamides in combination with other anti-leprosy agents, serious toxic effects were observed in up to 15% of patients (Cartel *et al.*, 1983; Pattyn *et al.*, 1984). Clearly, there is an urgent need for new chemotherapeutic agents active against leprosy bacilli.

A major difficulty in testing potential anti-leprosy agents is the absence of a rapid, reliable method for drug screening. *M. leprae* can be grown reliably in the foot-pads of mice (Shepard, 1960; Colston *et al.*, 1978) but it takes 6–12 months to assess the growth of the bacteria. Several alternatives to the mouse foot-pad have been suggested for screening anti-leprosy drugs and for detecting drug-resistant *M. leprae*. These include observations of the effects of live *M. leprae* on cholesterol metabolism (Nair and Mahadevan, 1984) and F_c-receptors of macrophages infected with leprosy bacilli (Mankar *et al.*,

1984), and fluorescence of *M. leprae* organisms incubated with fluorescein diacetate (Kvach *et al.*, 1984; Mankar *et al.*, 1984). Other studies have used radio-isotopically labelled substrates to investigate the incorporation by *M. leprae* of dihydroxyphenylalanine (DOPA) (Ambrose *et al.*, 1978), thymidine (Nath *et al.*, 1982; Mittal *et al.*, 1983), and hypoxanthine (Khanolkar and Wheeler, 1983). All of these effects and activities were inhibited by anti-leprosy agents when tested against intact *M. leprae*.

In this paper, hypoxanthine incorporation in *M. leprae* is examined in detail to ascertain whether it is (a) suitable for estimating the viability of suspensions of *M. leprae* by killing the bacteria in ways which would be expected to leave enzymes or even whole metabolic pathways active for some time and (b) suitable for screening anti-leprosy drugs. Anti-leprosy agents were tested alone, and in combination, to search for possible synergic effects.

Materials and methods

Anti-leprosy chemotherapeutic agents

Dapsone (4,4-diaminodiphenylsulphone) (Sigma) 2 mg/ml in 1M HCl was diluted in distilled water. The benzylpyrimidines, SC-SE 60 and Li 014, were gifts from Professor J. K. Seydel, Forschungsinstitut Borstel, Par-

kallee 1-40, 2061 Borstel, West Germany, and brodimoprim was a gift from both Professor J. K. Seydel and Dr P. Jenner, National Institute for Medical Research, Mill Hill, London NW7 1AA. Clofazamine was a gift from Dr S. O'Sullivan, Medical Research Council of Ireland, Dublin. Except for dapsone, agents were dissolved in dimethylsulphoxide (Analar) at 100-500 times the concentration needed in incubations: dimethylsulphoxide 1% v/v itself did not affect hypoxanthine incorporation in *M. leprae*.

Suspensions of *M. leprae*

M. leprae was isolated from armadillo spleen or liver as described previously (Wheeler, 1983). Briefly, tissues were homogenised in 'homogenisation medium' [0.2 M-tris (hydroxymethylaminomethane) base in 14 mM NaCl with 1 mM MgSO₄]. Homogenisation medium was used at the rate of 4 ml/g (wet weight) of infected tissue, resulting in a homogenate at pH 8.7. Bacteria were collected as a pellet by centrifugation at 8000 *g* for 10 min. The pellet was washed (all washings were done by resuspension and centrifugation) once in buffer [30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) adjusted to pH 7.2 with NaOH, 136 mM NaCl, 1 mM MgSO₄ and Tween 80 0.1% w/v], and treated with DNAase I (Sigma) 4 units/ml in Tween-80 0.1% w/v with 3 mM HEPES and 1 mM MgSO₄, pH 7.2, used at rate of 4 ml/g (wet weight) original tissue, for 1 h at 20° C. The suspension was filtered through a sieve (0.5 mm stainless steel mesh) to remove large pieces of debris, the pellet was collected (8000 *g*, 10 min) and the bacteria were resuspended in Tween 80 0.1% w/v and separated from tissue debris in a Percoll (Pharmacia, S-751 82 Uppsala, Sweden) 30% v/v gradient. The bacteria were then washed three times in Tween 80 0.1% w/v in 5 mM MES (2-[*N*-morpholino] ethanesulphonic acid) adjusted to pH 6.8 with 6 M NaOH (buffered Tween 80) and resuspended in buffered Tween 80 at three times the density required in incubations. All manipulations were done at 4° C unless otherwise stated. Further details about the isolation of *M. leprae* from infected tissues can be obtained from the report to the WHO (World Health Organization, 1980).

Suspensions of *M. microti*

M. microti strain OV 254 (obtained from the National Institute for Medical Research, London NW7 1AA) (3×10^9 organisms) were inoculated into 250 ml flasks each containing 100 ml of modified Dubos Medium (pH 7). They were grown statically at 37° C for 14 days by which time $c. 3 \times 10^{11}$ organisms were present in each flask. Organisms were centrifuged, washed twice with buffered Tween 80 and resuspended in buffered Tween 80 at three times the density required in incubations.

Incubation with radio-labelled substrates and anti-leprosy agents

For hypoxanthine incorporation studies bacteria (10^9 - 10^{10} organisms) were incubated at 34° C for 24 h in

50 mM HEPES with 5 mM NaCl, 1 mM MgSO₄, 0.2 mM NaH₂PO₄, 17 mM asparagine and 55 mM glucose; the pH was adjusted to 7.0 with 6 M KOH and 10 μ M uracil, 10 μ M thymidine and 10 μ M cytosine and penicillin 50 u/ml were added (Khanolkar and Wheeler, 1983) followed by 1 μ Ci [*G*-³H] hypoxanthine (Amersham; 2.8 Ci/mmol) in a final volume of 300 μ l. For ¹⁴CO₂ evolution studies bacteria were incubated at 34° C for 20 h in 50 mM HEPES with 1 mM MgSO₄, 5 mM K₂HPO₄ and penicillin 50 U/ml; the pH was adjusted to 7.0 with 6M KOH (Wheeler, 1983) and 1 μ Ci (U-¹⁴C) glucose (Amersham; 270 Ci/mol) added in a final volume of 200 μ l in a Warburg flask. The above concentrations are those in incubations; incubation mixtures were made up at 1.5 times those concentrations and filter-sterilised. Then 1 vol of bacterial suspension was added to 2 vol of incubation mixture to start incubations. CO₂ was absorbed during incubation in 1 M NaOH (150 μ l) in the centre well. These are essentially incubation mixtures used in previous studies (Khanolkar and Wheeler, 1983; Wheeler, 1983), except that the radiochemicals were used with no non-radioactive carrier present, so the concentrations in the mixtures were: for hypoxanthine, 1.2 μ M, and glucose, 18.5 μ M.

Suspensions of bacteria were usually added to incubations immediately after harvesting as described above. However, some suspensions were either treated or stored as follows: (a) suspensions of *M. leprae* and *M. microti* (3×10^9 bacteria/ml) were made in PBS (pH 7.0) containing Tween 80 0.05% v/v and treated at 25° C with 23.5 mM H₂O₂ for 90 min (Sharp *et al.*, 1985), or formaldehyde 4% v/v for 18 h, or hycolin 1% v/v for 18 h. During treatment with H₂O₂, the concentration of H₂O₂ was monitored; after 45 min, it had fallen from 23.5 mM, to 21.6 mM so further H₂O₂ was added to restore the concentration to 23.5 mM. After the above treatments, the bacteria were centrifuged and washed three times in buffered Tween 80 before being added to the incubation medium. (b) *M. leprae*, either as a suspension or before isolation (i.e., in infected armadillo tissue) was subjected to a 2.5 MRad dose of γ -irradiation from the ⁶⁰Co source at the National Institute for Medical Research, London NW7 1AA. This dose was calculated to leave 1 surviving bacterium in 10⁶⁰ (P. Draper and R. J. W. Rees, personal communication). (c) Suspensions of *M. leprae* at three times the density used in incubations were stored at 4° C for up to 27 days in either buffered Tween 80, 5 mM MES adjusted to pH 6.8 with 6 M NaOH, or modified Dubos Medium at pH 6. After storage, the bacteria were washed twice with buffered Tween 80, suspended in a small volume of buffered Tween 80, and added to incubation medium at the rate of 100 μ l/incubation.

Anti-leprosy agents were added, when required, to the incubation media, and *M. leprae* added afterwards. In general, leprosy bacilli were added to incubation media to start incubations. However, when they were pre-incubated with anti-leprosy agents, the incubation medium was complete except for [*G*-³H] hypoxanthine, which was added after the pre-incubation period (24 h at 34° C) to start incubations.

At the end of the incubation period, 5 μ l of the

suspension was spread on to nutrient agar plates to check for contamination. Results from any contaminated incubations were discarded. Incorporation of [G-³H] hypoxanthine was measured by collecting the remaining bacteria from each incubation on a Whatman GFC filter (Whatman Ltd, Maidstone, Kent), then washing each filter twice with 10 ml of buffered Tween 80 and three times with 10 ml of ice-cold trichloroacetic acid 5% v/v. Uptake of [U-¹⁴C] glucose was measured by collecting bacteria as above and washing through the filters by vacuum. Filters were dried under a lamp, placed in scintillation vials and scintillation fluid was added so that radioactivity was determined on the filters. After experiments with [U-¹⁴C] glucose, the NaOH, with absorbed ¹⁴CO₂, was also added to scintillation vials and scintillation fluid added. Bray's Scintillation Fluid (2, 5-diphenylloxazole 4 g, naphthalene 60 g, methanol 100 ml, ethanediol 20 ml, made up to 1 l with dioxan) was used but most water-tolerating scintillation fluids that render glass-fibre filters transparent could be used. Radioactivity was determined by scintillation counting. In presenting the results, background incorporation of hypoxanthine in heat-killed (100° C for 15 min) bacteria was subtracted from incorporation in bacteria which were not heat-killed—except where the terms 'live' or 'heat-killed' are specified in the results. Storage at 4° C, treatments and incubation with anti-leprosy agents did not affect background incorporation.

Tests of statistical significance and minimum inhibitory concentrations (MICs)

Significance tests were done by the Wilcoxon (Rank-Sum) test (Colquhoun, 1971) on relative activity values for individual incubations, where the mean relative activity in incubations of freshly isolated bacteria without anti-leprosy agents was 100.

Minimum inhibitory concentrations (MICs) of anti-leprosy agents are defined for the purpose of this paper as 'the concentration of agent required to give significant ($p < 0.05$) inhibition of hypoxanthine incorporation and above which significant ($p < 0.05$) inhibition of hypoxanthine incorporation is always observed'.

Results

*Hypoxanthine incorporation by live and killed *M. leprae**

The mean rate of incorporation of [G-³H] hypoxanthine into material insoluble in cold trichloroacetic acid with four isolates of *M. leprae* from infected tissues (three frozen, one fresh) was 41500 SE 3800 dpm/10¹⁰ *M. leprae*/24 h. There was no marked increase in hypoxanthine incorporation by *M. leprae* isolated from fresh tissue and it was not significantly lower in isolates from the same tissues stored at -70° C for up to 11 months. The mean rate of incorporation was 38 400 SE 9200 dpm/10¹⁰ *M. leprae*/24 h in five

subsequent isolates and 39 800 SE 6000 dpm/10¹⁰ *M. leprae*/24 h in all nine isolates.

It was decided to include 10⁹–10¹⁰ *M. leprae* organisms in each incubation after a comparison of incubations with 1.6×10^9 and 3.2×10^7 *M. leprae*. In incubations with 1.6×10^9 organisms for 24h, radioactivity incorporated in all incubations with heat-killed bacteria (712 dpm: 6 determinations) was lower than in all incubations with live bacteria (6051 dpm: 14 determinations). In incubations with 3.2×10^7 organisms for 7 days, radioactivity incorporated by the live bacteria (1161 dpm: 20 determinations) was still significantly higher ($p < 0.05$) than by heat-killed bacteria (716 dpm: 9 determinations). However, in many individual incubations with heat-killed bacteria, the radioactivity incorporated was higher than in some individual incubations with live bacteria. Thus the rank-sum of the incubations with heat-killed bacteria was 82 (minimum possible rank-sum for $n = 9$ is 45).

When suspensions of *M. leprae* were treated by incubation in H₂O₂, hycolin, or formaldehyde or by γ -irradiation to kill >99% of the organisms, hypoxanthine incorporation was significantly inhibited ($p < 0.05$) by all treatments, which abolished 67–97% of the activity depending on the treatment (table I). Similar effects were observed with *M. microti* OV 254 grown on modified Dubos Medium at pH 7, showing that hypoxanthine incorporation reflected the viability of suspensions of at least one other mycobacterium grown in culture medium (table I).

*Effect on hypoxanthine incorporation of storage of *M. leprae* at 4° C*

When freshly isolated purified leprosy bacilli were stored at 4° C as a suspension in Tween 80 0.05% buffered with 5 mM MES at pH 7, their viability, as judged in the mouse foot-pad, decreased exponentially with time (A. C. R. E. Lowe, personal communication). However, their hypoxanthine-incorporating activity increased for the first 15 days of storage, then fell. Only after 27 days was the hypoxanthine-incorporating activity significantly below the activity of freshly isolated bacteria (fig. 1). When Tween 80 was omitted, the effect of storage on hypoxanthine incorporation in *M. leprae* was similar to when Tween 80 was present (fig. 1) showing that it was not Tween 80 that was responsible for the initial increase in the ability of *M. leprae* to incorporate hypoxanthine.

The ATP content of *M. leprae* falls rapidly during storage in buffered Tween 80 at 4° C (Nam Lee and Colston, 1985) but considerably less rapidly in

Table I. Effect on hypoxanthine incorporation of killing without destroying enzymes

Treatment	<i>M. leprae</i>		<i>M. microti</i> OV 254	
	Viability after treatment (%)	Relative* activity	Viability after treatment (%)	Relative* activity
None	...	100	100	100
γ -irradiation of suspension (2.5 MRad): hypoxanthine + incubation medium added immediately after irradiation	0	33	0	0
γ -irradiation of tissue (2.5 MRad): storage for 2 months at -70°C before <i>M. leprae</i> was harvested	0	3	...	...
H_2O_2 (23.5 mM), pH 7, 90 min	1†	24	...	...
H_2O_2 (23.5 mM), pH 4.5, 90 min	...	...	8	9
Formaldehyde 4%, 18 h	...	20	<0.01	...
Hycolin 1%, 18 h	...	11	<0.01	...

Viability determinations for *M. microti* were done by plating on to Sauton's medium.

*Each figure represents the mean activity relative to the activity (in one experiment) when no treatments were done; 100 represents 26 400 dpm/ 10^{10} *M. leprae*/24 h and 59 200 dpm/ 10^{10} *M. microti*/24 h. Each figure represents 3–5 determinations.

† from Sharp *et al.*, 1985.

... = Not done

modified Dubos Medium adjusted to pH 6 and without serum albumin (H. S. Heine, personal communication). As the ATP content might reflect the content of intracellular nucleotides in general and such changes might affect hypoxanthine uptake and incorporation, the effect of storing *M. leprae* in modified Dubos Medium, pH 6 at 4°C was investigated. In this medium, the ability of the bacteria to incorporate hypoxanthine decreased continuously throughout the period of storage (fig. 1).

Effect of anti-leprosy agents on hypoxanthine incorporation by *M. leprae*

The anti-leprosy agents dapsone, brodimoprim and clofazamine significantly inhibited hypoxanthine incorporation in *M. leprae* as did the experimental agents SC-SE 60 and Li 014 (fig. 2). However, dapsone was the only agent which produced nearly complete inhibition of hypoxanthine incorporation. MICs for hypoxanthine incorporation were, for dapsone, 10 ng/ml; for clofazamine, 100 ng/ml; for Li 014, 3 $\mu\text{g}/\text{ml}$; for brodimoprim, 10 $\mu\text{g}/\text{ml}$ and for SC-SE 60, 30 $\mu\text{g}/\text{ml}$ (deduced from data in fig. 2).

When *M. leprae* was pre-incubated with anti-leprosy agents in the incubation medium before hypoxanthine was added, relative incorporation values were as follows: with no agent, 100 (= 76 400 dpm/ 10^{10} *M. leprae*/24 h); with DDS 3 ng/ml, 109; with DDS 10 ng/ml, 63; with DDS 30 ng/ml, 41; with SC-SE 60 10 $\mu\text{g}/\text{ml}$, 103; with SC-SE 60 100 $\mu\text{g}/\text{ml}$, 94; with Li 014 10 $\mu\text{g}/\text{ml}$, 55; and with Li 014 100 $\mu\text{g}/\text{ml}$, 46. With the exception

Fig. 1. Effect of storage at 4°C on hypoxanthine incorporation in *M. leprae*: ○—● storage in 5 mM-MES, pH 6.8 + Tween 80 0.05%; ▲—▲ storage in 5 mM-MES, pH 6.8; □—■ storage in modified Dubos Medium, pH 6—no serum albumin but Tween 80 0.05% was included. Significant difference ($p < 0.05$) of incorporation in stored *M. leprae* from incorporation in freshly isolated *M. leprae* is shown by filled symbols. Each symbol represents the mean activity relative to incubations with freshly isolated *M. leprae*. 100 represents 39 800 dpm/ 10^{10} *M. leprae*/24 h.

of incubations with SC-SE 60 100 $\mu\text{g}/\text{ml}$ these values were very similar to those in fig. 2. When penicillin and the pyrimidines were omitted from the incubation medium, relative incorporation values were as follows: with no agent, 100 (= 14 500 dpm/ 10^{10} *M. leprae*/24 h); with DDS 1 ng/ml, 81; with DDS 3 ng/ml, 66; with DDS 10 ng/ml, 41; with DDS 30 ng/ml, 41; with DDS

Fig. 2. Effect of anti-leprosy agents on hypoxanthine incorporation in dapsone-sensitive *M. leprae*: ●—● dapsone; ○—○ clofazimine (B663); ▲—▲ Li-014; △—△ brodimoprim; ■—■ SC-SE 60. Significant inhibition (taken as $p < 0.05$ but in fact p always < 0.01) of hypoxanthine incorporation occurred at concentrations of agents shown by symbols connected by unbroken lines. Each symbol represents the mean activity relative to activity with no additions, and represents 3 or 4 determinations except at the MIC, where 6–10 determinations were done; 100 represents 39 800 dpm/ 10^{10} *M. leprae*/24 h.

100 ng/ml, 21; and with Li 014 100 µg/ml, 50. These relative values were similar to those shown in fig. 2, for which the complete incubation medium was used. Thus penicillin and either dapsone or Li 014 were not acting synergically, and penicillin can be used in the incubation medium.

Combinations of dapsone and agents acting on dihydrofolate reductase acted synergically by inhibiting hypoxanthine incorporation in *M. leprae* significantly at concentrations which, when used alone, did not have a significant effect on hypoxanthine incorporation (table II). In four out of five of these combinations, the combined effect was greater than the sum of the inhibition caused by each agent alone. However, if a more stringent test for synergic action—whether the inhibition by any combination of agents significantly inhibits the activity of hypoxanthine incorporation in the presence of each agent alone—was applied, synergy was observed only between dapsone 3 ng/ml and brodimoprim 3 µg/ml (table II).

Synergy between dapsone and brodimoprim was more easily detected in dapsone-resistant *M. leprae* which were harvested from Armadillo 603. Armadillo 603 was inoculated with *M. leprae* from two patients in Madras. The bacteria were resistant to dapsone in the plasma of mice at up to 1 µg/ml (Rees, personal communication). 603 was part of the NIMR bank of infected armadillos.

In dapsone-resistant organisms, hypoxanthine incorporation was not significantly inhibited by dapsone 100 ng/ml (table III) although curiously dapsone 30 ng/ml did cause significant inhibition. However, if hypoxanthine incorporation in control incubations (i.e., without dapsone) was compared with activity in (a) any concentration of dapsone or

Table II. Effect of combinations of anti-leprosy agents on hypoxanthine-incorporation in dapsone-sensitive *M. leprae*

Agent (b)	Relative activity of agents (a) (below) with agents (b)				
	None	brodimoprim 1 µg/ml	brodimoprim 3 µg/ml	brodimoprim 30 µg/ml	SC-SE 60 10 µg/ml
None	100 (23)	107	80 (7)	57†	85 (10)
1 ng/ml dapsone	86	70†	69†	53‡	95
3 ng/ml dapsone	84 (8)	67‡	50*‡	...	64‡
10 µg/ml SC-SE 60	85 (10)	...	80	...	...

* Significant inhibition ($p < 0.05$) of hypoxanthine incorporation by combinations of agents, when compared with inhibition by agent (a) or (b) alone.

† Inhibition of hypoxanthine incorporation by combination of agents was significant (†: $p < 0.05$, or ‡: $p < 0.01$) compared with hypoxanthine incorporation in the absence of any anti-leprosy agents.

Each figure represents the mean activity relative to activity with no additions (39 800 dpm/ 10^{10} *M. leprae*/24 h), and represents 3–6 determinations except where the number of determinations is shown in brackets.

... = Not done.

Table III. Effect of combinations of dapsone and agents acting on dihydrofolate reductase on hypoxanthine incorporation in dapsone-resistant *M. leprae*

Concentration of dapsone	Relative activity of anti-dihydrofolate reductase agents (below) with dapsone				
	None	brodimoprim 3 µg/ml	brodimoprim 30 µg/ml	SC-SE 60 10 µg/ml	SC-SE 60 30 µg/ml
0	100	67	21*	80	48*
10 ng/ml	73	52	...	50	...
30 ng/ml	62*	15*	...	57	...
100 ng/ml	94	8*	...	37*	...

Each figure represents the mean activity relative to activity with no additions (50 360 dpm/10¹⁰ *M. leprae*/24 h). In column 1 (no anti-dihydrofolate reductase agents) each figure represents 4 determinations; all other figures represent 2–3 determinations.

* Significant inhibition; $p < 0.05$. The effect on hypoxanthine incorporation of each anti-dihydrofolate reductase agent was compared with hypoxanthine incorporation in all incubations without anti-dihydrofolate inhibitors.

... = Not done.

(b) dapsone 30–100 ng/ml (by pooling the results used in table III) no significant inhibition of activity by dapsone could be shown. This suggests that the inhibition with dapsone 30 ng/ml was an anomalous result and perhaps it could be considered to be the one 'false positive' in these results. However, when brodimoprim or SC-SE 60 at concentrations which did not by themselves inhibit hypoxanthine incorporation were added to suspensions of dapsone-resistant *M. leprae* together with dapsone 0–100 ng/ml, inhibition of hypoxanthine incorporation was clearly observed. Indeed, with a combination of brodimoprim 3 µg/ml and dapsone 30 or 100 ng/ml, complete inhibition of hypoxanthine incorporation was nearly achieved (table III), an effect otherwise only observed with dapsone-sensitive *M. leprae* plus dapsone 100 ng/ml.

Effect of anti-leprosy agents and hypoxanthine on evolution of CO₂ from glucose

The mean rate of evolution of ¹⁴CO₂ from [U-¹⁴C] glucose over 20 h was 6290 SE 506 dpm/10¹⁰

M. leprae (17 determinations). Radioactivity taken up into the washed bacteria was 34 900 dpm/10¹⁰ *M. leprae* after incubation for 20 h.

SC-SE 60 and dapsone significantly inhibited evolution of CO₂ from glucose (table IV) at similar concentrations to those at which they inhibited hypoxanthine incorporation (fig. 2). These observations were exploited to screen for any possible effect of hypoxanthine on *M. leprae* by using CO₂ evolution from glucose as an 'alternative drug-screening' method. Hypoxanthine on its own had no effect and did not potentiate the effect of SC-SE 60 or dapsone (table IV). In these experiments hypoxanthine was used at 3.3 µM (the concentration in incubations when 1 µCi of hypoxanthine at 1 Ci/mmol was used in studies of hypoxanthine incorporation) or 80 µM, at which concentration it potentiates the effect of trimethoprim (another benzylpyrimidine) on *E. coli* (Bruce *et al.*, 1984). Similar effects of anti-leprosy agents and hypoxanthine were observed on uptake of glucose (results not presented).

Table IV. Effect of anti-leprosy agents and hypoxanthine on production of ¹⁴CO₂ from [U-¹⁴C] glucose by *M. leprae*

Hypoxanthine added	Relative activity of anti-leprosy agent (below) with hypoxanthine					
	None	dapsone 1 ng/ml	dapsone 50 ng/ml	dapsone 50 ng/ml	SC-SE 60 10 µg/ml	SC-SE 60 100 µg/ml
None	100	110	57*	54*	73*	31*
3.3 µM	97	90	...	55*	111	...
80 µM	111	78	55*	59*	105	34*

Each figure represents the mean activity relative to activity with no additions. Except for incubations with no additions (17 determinations; mean = 6290 dpm/10¹⁰ *M. leprae*/24 h) each figure represents 3–4 determinations.

* Significant inhibition; $p < 0.05$

... = Not done.

Discussion

During studies on purine metabolism in *M. leprae*, it was shown that three anti-leprosy agents inhibited the incorporation of hypoxanthine (Khanolkar and Wheeler, 1983). Those observations are extended here by showing statistically significant inhibition of hypoxanthine incorporation by five further anti-leprosy agents which have different primary effects. These included four agents with targets in folate metabolism: dapsone, which inhibits dihydropteroate synthetase (Kulkarni and Seydel, 1983) and three benzylpyrimidines, which inhibit dihydrofolate reductase (Seydel *et al.*, 1983; Seydel and Kraus, personal communication). The fifth agent, clofazamine, probably works by intercalating nucleic acid molecules and consequently blocking transcription. Thus only one agent, clofazamine, has a direct effect on the pathway for hypoxanthine incorporation—uptake, conversion to nucleotides, and assembly of nucleotides into nucleic acids—which has been shown to exist in *M. leprae* (Khanolkar and Wheeler, 1983). The established agents, dapsone (Ellard, 1974) and clofazamine (Shepard, 1960) significantly inhibited hypoxanthine incorporation at concentrations at which they exert their pharmacological effects. The benzylpyrimidines, which are experimental agents, significantly inhibited hypoxanthine incorporation at or near concentrations that inhibited dihydrofolate reductase in extracts of *M. leprae* (Seydel and Kraus; personal communication) or in killed suspensions of *M. lufu* (Seydel *et al.*, 1983), the latter being the organism of choice as a model for folate metabolism in *M. leprae* (Kulkarni and Seydel, 1983; Seydel *et al.*, 1983). In these experiments, maximum inhibition of hypoxanthine incorporation was only 50–60% (table II) for agents other than dapsone. This degree of inhibition may not be exceeded as many of the agents came out of solution at concentrations higher than those tested. An important observation was that dapsone did not inhibit hypoxanthine incorporation in dapsone-resistant *M. leprae*, showing that when an agent had no chemotherapeutic effect it did not affect hypoxanthine incorporation either. It appeared possible to detect synergy between dapsone and inhibitors of dihydrofolate reductase, although the synergy was only obvious when high concentrations of dapsone were used against dapsone-resistant *M. leprae*. Synergy has been shown with these agents in similar combinations against *M. lufu*, including dapsone-resistant strains (Seydel *et al.*, 1983; Seydel and Kraus, personal communication).

Two agents in the incubation mixtures routinely

used could possibly have potentiated the effect of some of anti-leprosy agents tested. In particular, hypoxanthine itself potentiates the effect of a benzylpyrimidine, trimethoprim, in *E. coli* (Bruce *et al.*, 1984) so a similar effect might have been observed in *M. leprae*. However, no potentiating effects of either hypoxanthine or penicillin were observed in these studies. To seek any effect of hypoxanthine on *M. leprae* an alternative screening method, relying on $^{14}\text{CO}_2$ evolution from [^{14}C] glucose was devised. This activity, which was inhibited by dapsone and SC-SE 60, may itself be suitable for screening anti-leprosy agents, although it is hypoxanthine incorporation which, as a result of these findings, is recommended as a method for screening.

Hypoxanthine incorporation in suspensions of *M. leprae* was not directly related to the viability of the bacteria as judged by infectivity for the mouse foot-pad, but, generally, suspensions with a greater proportion of viable cells had higher activities of hypoxanthine incorporation. However, during storage of leprosy bacilli at either 4° C or 34° C (i.e., the latter in experiments requiring pre-incubation for 24 h) hypoxanthine incorporation increased initially before falling. Concomitantly, an exponential decrease in viability occurred. Thus the initial increase in [$\text{G-}^3\text{H}$] hypoxanthine incorporation may have been a result of intracellular nucleotides becoming depleted during storage so that a greater proportion of the purine nucleotide pool was derived from [$\text{G-}^3\text{H}$] hypoxanthine in stored *M. leprae* than in fresh *M. leprae*. This suggestion is supported by the observation that the initial increase in hypoxanthine incorporation on storage occurred when *M. leprae* organisms were kept in a medium in which ATP (i.e., the one nucleotide that has been measured in *M. leprae*) content was rapidly depleted. During an earlier study of thymidine incorporation into *M. leprae*, more radioactivity was incorporated into leprosy bacilli kept for 9 days in an alleged culture medium (M-Y medium; Murohashi and Yoshida, 1975) than into freshly isolated leprosy bacilli suspended in the same medium (Ambrose *et al.*, 1978). At that time the higher incorporation at 9 days was explained by suggesting that 'M-Y' medium, might be promoting nucleic acid synthesis or even growth. Subsequently, 'M-Y' medium was dismissed as a culture medium for *M. leprae* (Nakamura *et al.*, 1982). Thus the observations of Ambrose *et al.* (1978) may also reflect depletion of nucleotides during storage in 'M-Y' medium. These results show how important it is to test a potential method for determining viability with organisms which have been killed

without destroying the activity being measured. For an activity to be related to viability, it must be shown to decrease rapidly as the organism loses its metabolic competence after killing.

Thus, the usefulness of hypoxanthine incorporation seems to be greatest as a method for preliminary screening of new potential anti-leprosy agents. Those agents that inhibit hypoxanthine incorporation could then be tested in experimental animals and, eventually, patients. Clear-cut results were obtained easily and quickly with $\sim 10^9$ *M. leprae*/incubation, and at this rate a heavily infected armadillo liver could supply enough bacteria for 1000 incubations. More replicates would have to be done with $\sim 10^7$ *M. leprae*/incubation in 6 day incubations, but with only about 50% inhibition by

some agents, clear cut results would be more difficult to obtain. Whether or not the method is suitable for evaluating drug resistance in biopsies from patients needs further evaluation, although dapsone resistance could easily be detected. Perhaps the greatest advantage in measuring hypoxanthine incorporation in *M. leprae* is that it is rapid relative to thymidine incorporation (Khanolkar and Wheeler, 1983) and can readily be measured by a fairly simple technique with *M. leprae* suspended in an incubation medium.

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REFERENCES

- Ambrose E J, Khanolkar S R, Chulawalla R G 1978 A rapid test for bacillary resistance to dapsone. *Leprosy in India* **50**: 131–143.
- Bruce I, Hardy J, Stacey K A 1984 Potentiation by purines of the growth inhibitory effects of sulphonamides on *Escherichia coli* K12 and the location of the gene which mediates this effect. *Journal of General Microbiology* **130**: 2489–2495.
- Cartel J L, Millan J, Guelpa-Lauras C C, Grosset J H 1983 Hepatitis in leprosy patients treated by a daily combination of dapsone, rifampin, and a thioamide. *International Journal of Leprosy* **51**: 461–465.
- Colquhoun D 1971 Numerical and rank measurements. Lectures on Biostatistics, Clarendon Press, Oxford: pp 137–151.
- Colston M J, Hilston G R F, Banerjee D K 1978 The "proportional bactericidal test": a method for assessing bactericidal activity of drugs against *Mycobacterium leprae* in mice. *Leprosy Review* **49**: 7–15.
- Ellard G A 1974 Growing points in leprosy research. 4. Recent advances in the chemotherapy of leprosy. *Leprosy Review* **45**: 31–40.
- Guelpa-Lauras C C, Grosset J H, Constant-Desportes M, Brucker G 1984 Nine cases of rifampin-resistant leprosy. *International Journal of Leprosy* **52**: 101–102.
- Khanolkar S R, Wheeler P R 1983 Purine metabolism in *Mycobacterium leprae* grown in armadillo liver. *FEMS Microbiology Letters* **20**: 273–278.
- Kulkarni V M, Seydel J K 1983 Inhibitory activity and mode of action of diaminodiphenylsulfone in cell-free folate synthesizing systems prepared from *Mycobacterium lufu* and *Mycobacterium leprae*: a comparison. *Chemotherapy* **29**: 58–67.
- Kvach J T, Munguia G, Strand S H, 1984 Staining tissue derived *Mycobacterium leprae* with fluorescein diacetate and ethidium bromide. *International Journal of Leprosy* **52**: 176–182.
- Mankar M V, Jagannathan R, Mahadevan P R 1984 In vitro drug screening system using membrane alteration in macrophages by *Mycobacterium leprae*. *Journal of Biosciences* **6**: 709–716.
- Mittal A, Sathish M, Seshadri P S, Nath I 1983 Rapid, radiolabeled-microculture method that uses macrophages for *in vitro* evaluation of *Mycobacterium leprae* viability and drug susceptibility. *Journal of Clinical Microbiology* **17**: 704–707.
- Murohashi T, Yoshida K 1975 Attempts to culture *Mycobacterium leprae* in culture media. *Acta Leprologica* **58**: 5–21.
- Nair I, Mahadevan P R 1984 An *in vitro* test using cholesterol metabolism of macrophages to determine drug sensitivity and resistance of *Mycobacterium leprae*. *Journal of Biosciences* **6**: 221–231.
- Nakamura M *et al.* 1982 Failure to validate the growth of *Mycobacterium leprae* on M-Y 14b agar medium. *International Journal of Leprosy* **50**: 480–487.
- Nam Lee Y, Colston M J 1985 Measurement of ATP generation and decay in *Mycobacterium leprae* *in vitro*. *Journal of General Microbiology* **131**: 3331–3337.
- Nath I, Prasad H K, Sathish M, Sreevatsa, Desikan K V, Seshadri P S, Iyer C G S 1982 Rapid radiolabeled macrophage culture method for detection of dapsone-resistant *Mycobacterium leprae*. *Antimicrobial Agents and Chemotherapy* **21**: 26–32.
- Pattyn S R *et al.* 1984 Hepatotoxicity of the combination of rifampin-ethionamide in the treatment of multibacillary leprosy. *International Journal of Leprosy* **52**: 1–6.
- Seydel J K, Wempe E G, Rosenfeld M 1983 Bacterial growth kinetics of *Escherichia coli* and mycobacteria in the presence of brodimoprim and metioprim alone and in combination with sulfamerazine and dapsone. *Chemotherapy* **29**: 249–261.
- Sharp A K, Colston M J, Banerjee D K 1985 Susceptibility of *Mycobacterium leprae* to the bactericidal activity of mouse peritoneal macrophages and to hydrogen peroxide. *Journal of Medical Microbiology* **19**: 77–84.
- Shepard C C 1960 The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. *Journal of Experimental Medicine* **112**: 445–453.
- Warndorff-van Diepen T 1982 Clofazimine-resistant leprosy—a case report. *International Journal of Leprosy* **50**: 139–142.
- Wheeler P R 1983 Catabolic pathways for glucose, glycerol and 6-phosphogluconate in *Mycobacterium leprae* grown in armadillo tissues. *Journal of General Microbiology* **129**: 1481–1495.
- World Health Organization 1980 UNDP/World Bank/WHO Special programme for research and training in tropical diseases. Report of the fifth meeting of the scientific working group on the immunology of leprosy (IMMLEP). TDR/IMMLEP-SWG(5)/80-3, Annex 4 World Health Organization, Geneva p. 23.
- World Health Organization 1985 UNDP/World Bank/WHO Special programme for research and training in tropical diseases. Tropical disease research, seventh programme report (1 January 1983–31 December 1984). Leprosy, Chapter 8. World Health Organization, Geneva.