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 (Warndorf-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 Fc-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 brodimo-prim 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 triis (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 with 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, 751 82 Upspsala, 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) (3-10⁹ 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. 3×10⁹ 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 (10⁹-10¹⁰ organisms) were incubated at 34°C for 24 h in 50 mM HEPES with 5 mM NaCl, 1 mM MgSO₄, 0.2 mM Na₂HPO₄, 17 mM asparagine and 55 mM glucose; the pH was adjusted to 7.0 with 6 M KOH and 10 μM uracil, 10 μM thymidine and 10 μM cytosine and penicillin 50 u/ml were added (Khanolkar and Wheeler, 1983) followed by 1 μCi [G-³H] hypoxanthine (Amersham; 2-8 Ci/mmol) in a final volume of 300 μl. 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 μCi (U-¹⁴C) glucose (Amersham; 270 Ci/mol) added in a final volume of 200 μl 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 μl) 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 μM, and glucose, 18-5 μM.

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* (3×10⁹ 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 hycoxin 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 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 10⁶ (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 μl/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 μl of the
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 subsequent isolates and 39 800 SE 6000 dpm/10^10 *M. leprae*/24 h in all nine isolates.

It was decided to include 10^9–10^10 *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 24 h, 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 H_2O_2, 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>M. leprae Viability after treatment (%)</th>
<th>Relative activity</th>
<th>M. microti OV 254 Viability after treatment (%)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>γ-irradiation of suspension (2.5 MRad): hypoxanthine + incubation medium added immediately after irradiation</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>γ-irradiation of tissue (2.5 MRad): storage for 2 months at –70°C before M. leprae was harvested</td>
<td>0</td>
<td>3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H₂O₂ (23.5 mM), pH 7, 90 min</td>
<td>1†</td>
<td>24</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H₂O₂ (23.5 mM), pH 4.5, 90 min</td>
<td>...</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde 4%, 18 h</td>
<td>...</td>
<td>20</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Hycolin 1%, 18 h</td>
<td>...</td>
<td>11</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

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/10¹⁰ M. leprae/24 h and 59,200 dpm/10¹⁰ 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 μg/ml; for brodimoprim, 10 μg/ml and for SC-SE 60, 30 μg/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/10¹⁰ 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 μg/ml, 103; with SC-SE 60 100 μg/ml, 94; with Li 014 10 μg/ml, 55; and with Li 014 100 μg/ml, 46. With the exception of incubations with SC-SE 60 100 μg/ml these values were very similar to those in fig. 1. When penicillin and the pyrimidines were omitted from the incubation medium, relative incorporation values were as follows: with no agent, 100 (= 14,500 dpm/10¹⁰ 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...
Screening of anti-leprosy agents

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.

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

<table>
<thead>
<tr>
<th>Agent (b)</th>
<th>None</th>
<th>brodimoprim 1 μg/ml</th>
<th>brodimoprim 3 μg/ml</th>
<th>brodimoprim 30 μg/ml</th>
<th>SC-SE 60 10 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 (23)</td>
<td>107</td>
<td>80 (7)</td>
<td>57‡</td>
<td>85 (10)</td>
</tr>
<tr>
<td>1 ng/ml dapsone</td>
<td>86</td>
<td>70†</td>
<td>69†</td>
<td>53‡</td>
<td>95</td>
</tr>
<tr>
<td>3 ng/ml dapsone</td>
<td>84 (8)</td>
<td>67‡</td>
<td>50*‡</td>
<td>...</td>
<td>64‡</td>
</tr>
<tr>
<td>10 μg/ml SC-SE 60</td>
<td>85 (10)</td>
<td>...</td>
<td>80</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

* 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*

<table>
<thead>
<tr>
<th>Concentration of dapsone</th>
<th>Relative activity of anti-dihydrofolate reductase agents (below) with dapsone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>brodimoprim 3 µg/ml</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>73</td>
</tr>
<tr>
<td>30 ng/ml</td>
<td>62*</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>94</td>
</tr>
</tbody>
</table>

* 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.

Each figure represents the mean activity relative to activity with no additions (50 360 dpm/10^10 *M. leprae*/24 h). In column 1 (no anti-dihydrofolate reductase agents) each figure represents 4 determinations; all other figures represent 2-3 determinations.

(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 anomolous 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 ^14^CO₂ from [U-^14^C] glucose over 20 h was 6290 SE 506 dpm/10^10 *M. leprae* (17 determinations). Radioactivity taken up into the washed bacteria was 34 900 dpm/10^10 *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 ^14^CO₂ from [U-^14^C] glucose by *M. leprae*

<table>
<thead>
<tr>
<th>Hypoxanthine added</th>
<th>Relative activity of anti-leprosy agent (below) with hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dapsone 1 ng/ml</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>3·3 µM</td>
<td>97</td>
</tr>
<tr>
<td>80 µM</td>
<td>111</td>
</tr>
</tbody>
</table>

* Significant inhibition; p < 0.05

Each figure represents the mean activity relative to activity with no additions. Except for incubations with no additions (17 determinations; mean = 6290 dpm/10^10 *M. leprae*/24 h) each figure represents 3–4 determinations.

... = 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 $^{14}$CO$_2$ evolution from [H]$^4$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 [G-3H] 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 [G-3H] 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, 1979) 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.
REFERENCES


