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 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 con-
centration 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-ethanesul-
phonic 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 treated with DNAase I(Sigma) 4 units/ml 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) (a)
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 x
10⁶ 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 x 10¹¹ 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 NaH₂PO₄, 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 ¹⁴C O₂ 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 6 M
KOH (Wheeler, 1983) and 1 µCi (U-¹⁴C) glucose (Amers-
ham; 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 concentra-
tions in the mixtures were: for hypoxanthine, 1-2 µM, and
glucose, 18-5 µM.

Suspensions of bacteria were usually added to incuba-
tions 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 x 10⁶ bacteria/ml) were made in PBS (pH 7.0) contain-
ing 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 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 me-
dium 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
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-diphenyl-loxazole 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 41 500 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 × 109 and 3·2 × 107 M. leprae. In incubations with 1·6 × 109 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 × 107 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

<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>...</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(\gamma)-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>(\gamma)-irradiation of tissue (2.5 MRad): storage for 2 months at (-70^\circ\text{C}) before (M.\ leprae) was harvested</td>
<td>0</td>
<td>3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>(H_2O_2) (23-5 mM), (pH) 7, 90 min</td>
<td>1†</td>
<td>24</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>(H_2O_2) (23-5 mM), (pH) 4-5, 90 min</td>
<td>...</td>
<td>...</td>
<td>8</td>
<td>9</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/10^11 \(M.\ leprae\)/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 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/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 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. 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...
Screening of Anti-leprosy Agents

171

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/10¹⁰ *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/10¹⁰ *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>brodimoprim None</th>
<th>brodimoprim 3 µg/ml</th>
<th>SC-SE 60 10 µg/ml</th>
<th>SC-SE 60 30 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>67</td>
<td>21*</td>
<td>80</td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>73</td>
<td>52</td>
<td>50</td>
<td>30 ng/ml</td>
</tr>
<tr>
<td>30 ng/ml</td>
<td>62*</td>
<td>15*</td>
<td>37*</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>8*</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

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.

* 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/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 14CO₂ from [U-14C] glucose by *M. leprae*

<table>
<thead>
<tr>
<th>Hyoxanthine added</th>
<th>dapsone None</th>
<th>dapsone 1 ng/ml</th>
<th>dapsone 50 ng/ml</th>
<th>dapsone 10 µg/ml</th>
<th>SC-SE 60 1 µg/ml</th>
<th>SC-SE 60 10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>110</td>
<td>57*</td>
<td>54*</td>
<td>73*</td>
<td>31*</td>
</tr>
<tr>
<td>3-3 µM</td>
<td>97</td>
<td>90</td>
<td>55*</td>
<td>55*</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>80 µM</td>
<td>111</td>
<td>78</td>
<td>55*</td>
<td>59*</td>
<td>105</td>
<td>34*</td>
</tr>
</tbody>
</table>

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.

* 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 $^{14}$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 [G-$^3$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 [G-$^3$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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