UPTAKE OF SALICYLIC ACID INTO MYCOBACTIN S BY GROWING CELLS OF *MYCOBACTERIUM SMEGMATIS*

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1. Introduction

Mycobactins are a unique series of compounds which chelate very strongly with iron and other metals and are produced only by the mycobacteria [1]. The structure of mycobactin S, produced by *Mycobacterium smegmatis*, is shown in fig. 1.

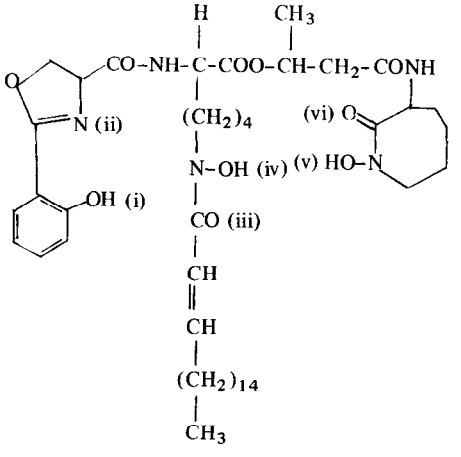


Fig. 1. *Mycobactin* S, after White and Snow [ref. 2]. The atoms which coordinate with iron are numbered (i) to (vi).

One of the features of all mycobactin molecules is the aromatic moiety which is either salicylic acid or 6-methylsalicylic acid [1, 2]. Either one of these acids, depending upon which one is found in the mycobactin molecule, also appears in the free form in the medium after growth of mycobacteria [3, 4]. The formations of the free aromatic acid and mycobactin have both been reported to be stimulated by a deficiency of iron in the growth medium [2, 3]. The extracellular salicylic acid and the salicylate moiety of mycobactin are formed from shikimic acid [5, 6], but hitherto it has not been shown whether the extracellular compound can penetrate into the cell or if it can participate in mycobactin biosynthesis. This report describes experiments which demonstrate that both these events do occur in growing cells of *M. smegmatis*.

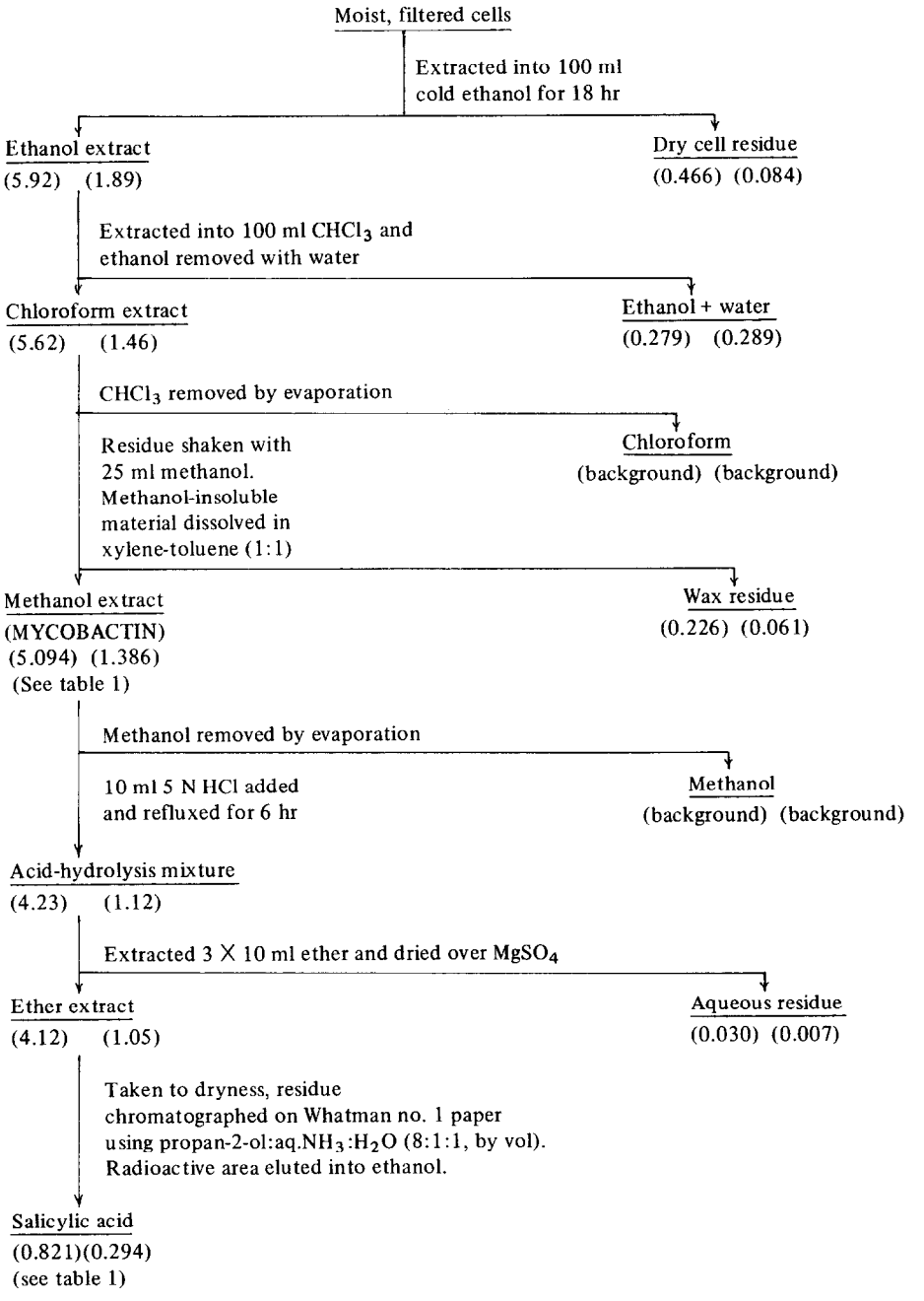
2. Materials and methods

The strain of *M. smegmatis* used was the same as that of all previous work [3]. The medium was glycerol 20 ml, L-asparagine 5 g, KH_2PO_4 5 g and $MgSO_4 . 7H_2O$ 0.4 g with NaOH added to pH 6.8 and distilled water to make 1 litre. Metal ions were removed by first autoclaving the medium with 0.5% alumina at pH 7.5, and readjusting the pH with 2 M H_2SO_4 [3, 7, 8]. Medium, just prior to inoculation, was supplemented with Zn^{2+} 0.46 μg/ml, and, for iron-sufficient medium, Fe^{2+} 2.0 μg/ml; for iron-deficient medium, iron was omitted. All glassware was cleaned as previously described [3]. Medium (100 ml) was held in 250 ml conical flasks and was inoculated with 0.4 ml of a suspension of *M. smegmatis* formed by shaking in iron-deficient medium a small amount of pellicle taken from a 4 day culture grown on iron-sufficient medium. ^{14}C-carboxy-Salicylic acid (40 μg, sp. act. 3.43×10^7 dpm/μmole) was added to each flask at the time of inoculation. Cultures were grown under stationary conditions for 6 days to give maximum formation of mycobactin [8].

Cultures were harvested by filtration and the moist cells extracted with ethanol to remove mycobactin [9]. The subsequent procedure is given in scheme 1 and is

Scheme 1

Procedure for the isolation of mycobactin and its hydrolysis to salicylic acid. Total radioactivities ($\times 10^{-6}$ dpm) of the various fractions are given in parentheses: the figure on the left-hand side refers to material from iron-deficient cultures and that on the right-hand for iron-sufficient cultures. Total ^{14}C-carboxy-salicylic acid added was 79.52×10^6 dpm and 19.88×10^6 dpm for iron-deficient and iron-sufficient cultures respectively.



based on the work of White and Snow [2, 9]. Radioactivity in the dry cells after mycobactin extraction was determined by digesting a weighed amount (about 250 mg) in 15 ml M NaOH at 37° for 24 hr in a stoppered tube. To this solution, 22.5 ml M KH_2PO_4 was added and samples taken for counting. Mycobactin was determined as its ferric complex after solution in methanol using $A^{1\%}_{1cm}$ = 42.8 at 450 nm [2]. Salicylic acid was determined spectrophotometrically using $A_{302nm} - A_{350nm}$ and the amount calculated from a molar extinction coefficient of 3680. All radioactivity was measured by scintillation with a Beckman counter, model LS 233, with scintillation fluid as described by Bray [10].

Paper chromatograms were scanned using a windowless scintillation detector (model CS-03, Panax Equipment Ltd, Redhill, Surrey).

^{14}C-carboxy-Salicylic acid was obtained from the Radiochemical Centre, Amersham, Bucks.

3. Results and discussion

The incorporation of ^{14}C-carboxy-salicylic acid into iron-deficient and iron-sufficient growing cells of *M. smegmatis* after 6 days was determined and the radioactivities which were recovered in the various fractions are given in scheme 1 and table 1. The chromatograms of material from mycobactin hydrolysis were scanned before the elution of salicylic acid and no compound having more than 1% of the radioactivity of salicylic acid was detected with either sample.

The results show that with both iron-deficient and iron-sufficient cultures salicyclic acid is effectively taken up from the medium into the cell. In both cases about 95% of the incorporated material was recovered in the ethanol-soluble fraction. Of the radioactivity in the mycobactin no significant amount of radioactivity was recovered from any fraction following acid hydrolysis except for that found in the salicylic acid moiety. It is concluded, therefore, that the salicylic acid molecule is incorporated intact into the mycobactin molecule. The considerable dilution of the specific activity of the added salicylic acid to its eventual specific activity in the salicylate moiety is an obvious indication that *de novo* synthesis of salicylic acid was still continuing, it having previously been reported that mycobactin production was not arrested by as much as 20 μg of salicylate/ml of medium [11].

Table 1
Incorporation of ^{14}C-carboxy-salicylic acid into mycobactin S during growth of *M. smegmatis* for 6 days on iron-deficient and iron-sufficient medium.

	Medium used	
	Iron deficient	Iron sufficient
Cell dry wt.*/100 ml medium	153 mg	568 mg
Mycobactin extracted (μg/mg cell dry wt.)	1.90	2.22
Sp. act. of mycobactin** (dpm/μmole)	1.72×10^6	0.45×10^6
Incorporation of total ^{14}C-salicylate added into mycobactin (see scheme 1)	6.4%	7.0%
Sp. act. of salicylate from mycobactin hydrolysis (dpm/μmole)	1.24×10^6	0.40×10^6
Retention of activity in salicylate from mycobactin	72%	89%

Eight cultures from iron-deficient medium and two from iron-sufficient medium were used. Procedure for isolation of mycobactin and its subsequent hydrolysis given in scheme 1. Sp. act. of salicylate added 34.3×10^6 dpm/μmole.

* Cell dry wt. determined after mycobactin extraction.
** Mycobactin S, although a mixture of similar molecules [2], assumed to have mean M.W. of 827.

Although the uptake of salicylic acid by growing cells of *M. smegmatis* is now established the reason for this is still not clear. It is possible that its chelating properties with iron offer a mechanism by which iron is transported into the cell [11] in a manner similar to that reported to occur in other organisms with 2,3-dihydroxybenzoic acid, or its conjugate with serine [12, 13]. (The formation of the analogous compound, *N*-salicyloylserine, does not appear to occur in extracts of *M. smegmatis*, C.Ratledge, unpublished work). Nevertheless the possibility cannot be excluded that this uptake of salicylic acid may only be an exchange with intracellular salicylic acid which is functioning just as a precursor of mycobactin. The function of mycobactin itself is still completely unknown.

Acknowledgement

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