Utilization of Shikimic Acid for the Formation of Mycobactin S and Salicylic Acid by *Mycobacterium smegmatis**

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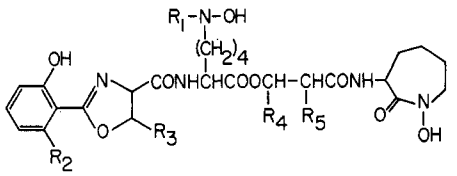
ABSTRACT: The rates of production of extracellular salicylic acid, and intracellular mycobactin S which contains bound salicylic acid, have been studied in shake cultures of *Mycobacterium smegmatis*. The salicylic acid production in the culture medium is maximal after 24-hr growth (3.6 mg/g dry weight of cells) while the maximal production of mycobactin S (28.1 mg/g dry weight of cells) occurred 24 hr later. When shikimate-*G*-^{14}C was added to cultures at zero time, extracellular salicylic acid isolated at 48 hr had a higher specific activity than did the mycobactin S. Chemical degradation of the labeled extracellular salicylic acid, and the labeled salicylic acid obtained by hydrolysis of myobactin S, indicated that in both cases all seven carbon atoms of shikimate had been incorporated intact into the salicylic acid. Essentially all of the activity of the mycobactin S was accounted for by the salicylic acid component.

Many mycobacteria produce a series of compounds known as the mycobactins (Snow and White, 1969). These intracellular metabolites, formed in response to conditions of iron deprivation, appear to function in *Mycobacterium johnei* as growth promoters (Snow and White, 1969). All of the mycobactins so far isolated can be divided into two structural types by reference to the aromatic acid which they contain. Those such as mycobactin P (I) contain 6-methylsalicylic acid, while those such as mycobactin S (II) contain salicylic acid as the aromatic component. Both of these aromatic compounds have also been detected as the nonconjugated acids in culture media of mycobacteria; here again their production is stimulated by iron deficiency (Ratledge and Winder, 1962; Hudson and Bentley, 1969).



I $R_1 = CH_3(CH_2)_n CH{=}CHCO$, $n = 10, 12, 14, 16$; $R_2 = CH_3$, $R_3 = H$, $R_4 = C_2H_5$, $R_5 = CH_3$

II R_1 as in I, $R_2 = R_3 = R_5 = H$, $R_4 = CH_3$

The biosynthetic origin of these aromatic acids in bacteria is an intriguing question which has received little attention. *A priori*, salicylic acid would be regarded as a shikimate derived metabolite, and some preliminary evidence substantiating this biosynthetic route has been obtained (Ratledge, 1969). Other possibilities would be catabolism of phenylalanine to a C_6–C_1 moiety such as benzoic acid, followed by hydroxylation, or derivation from 6-methylsalicylic acid by removal of the methyl group (Snow, 1965). 6-Methylsalicylic acid, a well-known fungal metabolite, is, in these organisms, the classical example of an aromatic compound produced by the polyketide pathway (Dimroth *et al.*, 1970; Light, 1970). While this pathway may operate in mycobacteria another route, namely, C methylation of salicylic acid or a related compound, was worth considering.

In view of these uncertainties, detailed studies of the biosynthesis of these two aromatic acids have been undertaken. The work described in this paper is concerned with (a) the relative rates of production of extracellular salicylic acid and of the salicylic acid component of mycobactin S by *Mycobacterium smegmatis*, and (b) a study of the biosynthesis of both extracellular and myocobactin-incorporated salicylic acids from generally labeled shikimic acid (shikimate-*G*-^{14}C). Work on the biosynthesis of 6-methylsalicylic acid in *Mycobacterium phlei* is described in the following paper (Hudson *et al.*, 1970).

Experimental Section

General. All solvents were redistilled. Silicic acid for column chromatography was Clarkson Chemical Co.'s "Unisil," mesh 100–200. Thin-layer chromatography was carried out on Merck precoated silica gel F-254 plates. All radioactive materials were obtained from commercial sources. Samples were assayed for radioactivity using a Packard Tri-Carb Model 3310 scintillation counter. Organic ^{14}C-labeled intermediates were counted in a liquid scintillator (Bray, 1960). The activity of barium carbonate samples was obtained by suspension in a thixotropic gel prepared by adding Cab-O-Sil (700 mg) to 18 ml of the following solution: 2,5-diphenyloxazole (15.2 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.19 g), and toluene (3.8 l.).

Growth of Organism. The organism employed, *M. smegmatis* (NCIB 8548), was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. It was maintained on nutrient agar slopes at 37°. For experimental cultures, the organism was grown in shake culture in medium essentially similar to that previously reported (White and Snow, 1968). However, this medium was supplemented with zinc since this

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SCHEME I

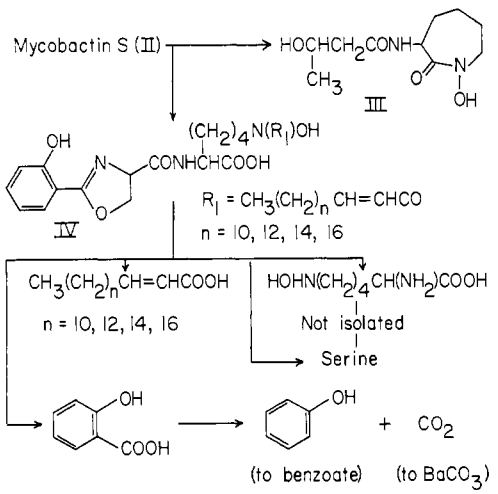


TABLE I: Time Course of Salicylic Acid and Mycobactin S Production by *M. smegmatis* NCIB 8548.

		Salicylic Acid		Mycobactin S	
Growth Period (Days)	Dry Wt of Cells (g/Flask)	Mg/g Dry Wt of Cells	Mg/ Flask	Mg/g Dry Wt of Cells	Mg/Flask
1	0.62	3.60	2.23	4.16	2.58
2	1.62	2.46	3.99	28.10	45.52
3	2.00	2.00	4.00	*a*	
4	2.25	0.18	0.41	23.50	52.88
5	2.62	Not detectable		14.86	38.93

[a] Not recorded.

metal has been reported to stimulate mycobactin S production in surface cultures by an unspecified strain of *M. smegmatis* (Ratledge, 1968). The exact medium composition was: L-asparagine, 5 g; KH_2PO_4, 1 g; Na_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 0.001 g; glycerol, 10 ml; and deionized water to make a final volume of 1 l. and a pH of 6.8. Seed inocula were prepared by growth for 24 hr at 37° as shake cultures in 250-ml erlenmeyer flasks containing 25 ml of the above medium. These seed cultures were then used to inoculate 2.8-l. fernbach flasks containing 700 ml of the above medium now supplemented with 35 g of glucose in 50 ml of water, and growth was continued in shake culture at 37° normally for a period of 2 days.

Isolation of Metabolites. The cells were filtered from the growth medium, dried *in vacuo*, and stirred with aqueous ethanol (70%) for 2 days. The mixture was filtered, saturated ethanolic ferric chloride solution was added to the filtrate (2 ml/100 ml of filtrate), and the resulting deep red solution was extracted with chloroform (White and Snow, 1969). Removal of the chloroform afforded a deep red oil which was chromatographed on a column of silicic acid (22 × 2 cm) employing as solvent system chloroform–methanol (96:4, v/v) and collecting 8-ml fractions. Ferric mycobactin S was obtained in fractions 8 and 9; thin-layer chromatography on silica gel F plates with chloroform–methanol (96:4, v/v) showed the material to be homogeneous. Ferric mycobactin S was converted to mycobactin S which was obtained as a white powder, mp 167–168° (lit. mp 163° (White and Snow, 1969)). For purification of radioactive mycobactin S the material was reprecipitated several times from chloroform with ether until constant specific activity had been reached.

Salicylic acid was isolated from the filtered growth medium by continuous ether extraction at pH 1 for 12 hr. Removal of the ether afforded an oil which was chromatographed on a silicic acid column (20 × 1 cm) with benzene–acetic acid (99:1, v/v) to give crystalline salicylic acid, mp 157°. Recrystallization from benzene–petroleum ether raised the melting point to 158–159°; recrystallization was continued in the tracer experiments until constant specific activity had been reached.

Degradation of Salicylic Acid (Scheme I). Salicylic acid (50 mg) was decarboxylated with copper chromite catalyst in quinoline (Bentley, 1963), the evolved carbon dioxide being collected as barium carbonate (70 mg). The remaining reaction mixture was filtered, dissolved in ether, and washed several times with HCl (2 N) and water. Removal of the ether gave an oil which was chromatographed on a column of silicic acid (24 × 1 cm). Elution with benzene–acetic acid (99:1, v/v) afforded phenol (24 mg) as an oily solid. This was converted to phenol benzoate by reaction with benzoyl chloride–sodium hydroxide. Purification of this derivative by recrystallization from aqueous ethanol gave needles, mp 68–69°.

Degradation of Mycobactin S (Scheme I). Mycobactin S (110 mg) was hydrolyzed with 1 N NaOH (White and Snow, 1969) to give cobactin S (III, 32 mg) and mycobactic acid S (IV, 70 mg). Cobactin S was purified by recrystallization from acetone to give needles, mp 122–123° (lit. mp 123° (White and Snow, 1969)). Mycobactic acid S was not further purified but was hydrolyzed with acid as previously described. The acid mixture was then extracted with ether, removal of the ether affording a colorless oily solid. This was chromatographed on a column of silicic acid (24 × 1 cm) with benzene–acetic acid (99:1, v/v) to give fatty acids (20 mg) and salicylic acid (14 mg). The salicylic acid was further purified by recrystallization from benzene–petroleum ether (bp 30–60°) as previously described. Serine and 2-amino-6-hydroxyaminohexanoic acid, also produced in the acid hydrolysis of mycobactic acid S, were not isolated.

Results

Time Sequence of Metabolite Production. A group of 20 fernbach flasks was inoculated uniformly with *M. smegmatis*. Four flasks were removed at daily intervals, and from the combined cells and culture medium were isolated, respectively, mycobactin S and salicylic acid. Table I shows the yields of these two metabolites over a 5-day period. The yields recorded in this table are based on the actual weight of ferric mycobactin S and salicylic acid isolated and purified once by chromatography on silicic acid. In terms of the amounts of metabolites produced per gram dry weight of cells, the salicylic acid production was a maximum after 1 day, while the mycobactin S production was maximal after 2 days. In terms of total metabolites produced per flask, salicylic acid showed a

TABLE II: Incorporation of Shikimate-*G*-^{14}C into Salicylic Acid and Mycobactin S.

Compd Isolated	Incorp (%)	Sp Act. (dpm/μmole)	Dilution Value[a]
Salicylic acid	0.138	709	5,824
Mycobactin S	0.112	322[b]	12,800

[a] Dilution value is defined as specific activity of precursor/specific activity of product. [b] In assessing activity incorporated into mycobactin S, calculations were originally based on a molecular weight of 855 (Hudson and Bentley, 1970). However, analysis of the distribution of fatty acids in the mycobactin showed that a more accurate value would be 827. Hence the values quoted herein are based on the latter value.

maximum which varied little over days 2 and 3, whereas for mycobactin S, a plateau was maintained from about day 2 to day 4. Using either measure of production, the salicylic acid maximum was attained some 24 hr before the maximum of mycobactin S.

Incorporation of Shikimate-G-^{14}C into Salicylic Acid and Mycobactin S. Details of the incorporation of shikimate-*G*-^{14}C into salicylic acid and mycobactin S are shown in Table II. In order to obtain maximum yields of each metabolite the tracer (20 μCi, specific activity 4.1269×10^6 dpm/μmole) was equally distributed between two fernbach flasks of the organism at the time of inoculation and growth allowed to continue for a further 48-hr period. Isolation of salicylic acid (12 mg) revealed 0.138% of the initial radioactivity to have been incorporated into this metabolite. Mycobactin S (128 mg) contained 0.112% of the total radioactivity fed as shikimic acid.

Degradation of Mycobactin S (Table III). Mycobactin S was degraded initially to mycobactic acid S and cobactin S. The cobactin S contained only 2.2% of the total activity present in mycobactin S. Further degradation of mycobactic acid S to salicylic acid and fatty acids revealed the latter to be completely inactive and salicylic acid to contain 98.4% of the activity originally present in the mycobactin.

Degradation of Extracellular Salicylic Acid and Salicylic Acid Obtained from Mycobactin S. The salicylic acid from each source was diluted with inactive material and separately decarboxylated. The results, shown in Table IV, revealed the same distribution pattern in each case. The phenol benzoate contained 84–85% of the original radioactivity of the salicylic acids and the barium carbonate, 14–15%.

TABLE III: Degradation of Mycobactin S.

Compd Counted	Sp Act. (dpm/μmole)	Act. (%)
Mycobactin S	322	100
Cobactin S	7	2.2
Salicylic acid	317	98.4
Fatty acids	0	0

TABLE IV: Degradation of Salicylic Acid.

Compd Counted	Sp Act. (dpm/μmole)	Act. (%)
Salicylic acid (extracellular)	50	100
Phenol benzoate	42	84
Barium carbonate	7	14
Salicyclic acid (from mycobactin S)	75	100
Phenol benzoate	64	85
Barium carbonate	11	15

Discussion

The incorporation of radioactivity from shikimate-*G*-^{14}C into both mycobactin S and salicylic acid represents the first evidence for participation of shikimic acid in mycobactin biosynthesis and provides definitive evidence that shikimic acid is a precursor of extracellular salicylic acid in *M. smegmatis*. In the mycobactin S, radioactivity was incorporated almost entirely into the salicylic acid component. A very low level of activity was present in the cobactin S component and in the nonsalicylate portion of mycobactic acid S.

The chemical degradation of the labeled salicylic acid samples indicated a utilization of all seven carbon atoms of shikimic acid in the biosynthetic process. The atoms of the benzene ring contained six-sevenths of the total salicylic acid radioactivity. *Anal.* Calcd, 85.7. Found, 84–85. The carboxyl group contained one-seventh of the total radioactivity. *Anal.* Calcd, 14.3. Found, 14–15.

Both the levels of activity incorporated into the two metabolites and their dilution values are encouraging in view of the presence of large amounts of glucose in the medium. The difference in dilution value obtained for salicylic acid and mycobactin S (5824 and 12,800, respectively) is taken as a reflection of the different times of synthesis of each metabolite. This deduction is fully in accord with the growth study results (Table I) which showed the maximum production of salicylic acid to precede that of mycobactin S by about 24 hr. These results also suggest that excess salicylic acid, not required for mycobactin synthesis, is removed by excretion into the medium. Thus when mycobactin S synthesis is minimal (1-day's growth) considerable quantities of extracellular salicylic acid are formed.

Ratledge (1969) had earlier provided some preliminary evidence for the incorporation of shikimic acid into extracellular salicylic acid in an unspecified strain of *M. smegmatis*. In this work, washed cell suspensions of the organism were incubated with various ^{14}C compounds leading to very small amounts of salicylic acid (0.04 mg). Consequently, no attempt was made to locate the distributions of activities by chemical degradation of the molecule. Since mycobacteria are capable of metabolizing shikimic acid *via* a pathway which allows incor-

poration of radioactivity into other metabolic pools, *e.g.*, acetate (Campbell *et al.*, 1967), resort to detailed chemical degradation is required to provide rigorous proof of the biosynthetic origin of salicylic acid.

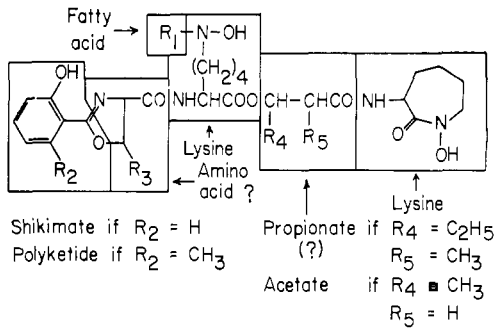
The demonstration of the common origin of the aromatic moiety of mycobactin S and the extracellular salicylic acid strongly suggests a common origin for the extracellular 6-methylsalicylic acid and the aromatic unit of mycobactin P in *M. phlei*. Since extracellular 6-methylsalicylic acid biosynthesis in *M. phlei* most likely proceeds by a polyketide route (Hudson *et al.*, 1970), the 6-methylsalicylic acid unit of mycobactin P is also presumed to have a polyketide origin. Thus the mycobacteria possess widely different pathways for producing two aromatic moieties which differ only in a single methyl group. A similar situation has been noted in the biosynthesis of some benzoquinones by the beetle, *Eleodes longicollis* (Meinwald *et al.*, 1966). In the defensive secretion of this beetle, *p*-benzoquinone is produced largely from utilization of the aromatic ring of tyrosine or phenylalanine, while methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone originate by acetate plus polymalonate and propionate plus polymalonate condensations, respectively.

Other bacterial iron sequestering agents containing aromatic acids are enterobactin (Pollack and Nielands, 1970) and the conjugates of 2,3-dihydroxybenzoic acid with glycine (Ito and Nielands, 1958), serine (Brot *et al.*, 1966), and lysine (Corbin and Bulen, 1969). The 2,3-dihydroxybenzoic acid in these agents presumably arises from shikimic acid, as has been documented for the free acid in *Aerobacter aerogenes* and *Escherichia coli* (Young and Gibson, 1969).[1] Isochorismic acid is an intermediate to 2,3-dihydroxybenzoic acid, and has been proposed as an intermediate beyond shikimic acid in salicylic acid biosynthesis (Young *et al.*, 1969). This suggestion is reinforced now that shikimic acid has been unequivocally implicated in salicylic acid synthesis in mycobacteria.

The essential components for the synthesis of a complete mycobactin molecule are most likely those shown in Scheme II. Since the incorporation of lysine into the hydroxylamine unit of mycobactin P (I) was shown by Allen *et al.* (1970), the only major area of uncertainty remaining concerns the origin of the groups R_4, R_5, and the carbon atoms attached to them. Snow (1965) has suggested that for mycobactins where $R_4 = CH_3$ and $R_5 = H$ the β-hydroxybutyric acid moiety would be formed by condensation of two acetate units. Similarly, for mycobactins where $R_4 = CH_3CH_2$ and $R_5 = CH_3$ the required 3-hydroxy-2-methylvaleric acid fragment has been postulated to arise *via* condensation of two propionic acid units (Asselineau, 1962). The recent isolation of mycobactins in which $R_4 = CH_3(CH_2)_n$ (where n is 14–17) and $R_5 = CH_3$ is suggestive of a similar condensation between a long-chain fatty acid and propionic acid (Snow and White, 1969).

[1] In some fungi, however, this acid is derived from tryptophan (Gröger *et al.*, 1965).

SCHEME II



References

Allen, M., Birch, A. J., and Jones, A. R. (1970), *Aust. J. Chem. 23*, 427.

Asselineau, J. (1962), Les Lipides Bactériens, Paris, Hermann, p 118.

Bentley, R. (1963), *J. Biol. Chem. 238*, 1889.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brot, N., Goodwin, J., and Fales, H. (1966), *Biochem. Biophys. Res. Commun. 25*, 454.

Campbell, I. M., Coscia, C. J., Kelsey, M., and Bentley, R. (1967), *Biochem. Biophys. Res. Commun. 28*, 25.

Corbin, J. L., and Bulen, W. A. (1969), *Biochemistry 8*, 757.

Dimroth, P., Walter, H., and Lynen, F. (1970), *Eur. J. Biochem. 13*, 98.

Gröger, D., Erge, D., and Floss, H.-G. (1965), *Z. Naturforsch. 20 B*, 856.

Hudson, A. T., and Bentley, R. (1969), *Chem. Commun.*, 830.

Hudson, A. T., and Bentley, R. (1970), *Tetrahedron Lett.* 2077.

Hudson, A. T., Campbell, I. M., and Bentley, R. (1970), *Biochemistry 9*, 3988.

Ito, T., and Neilands, J. B. (1958), *J. Amer. Chem. Soc. 80*, 4645.

Light, R. J. (1970), *J. Agr. Food Chem. 18*, 260.

Meinwald, J., Koch, K. F., Rogers, J. E., and Eisner, T. (1966), *J. Amer. Chem. Soc. 88*, 1590.

Pollack, J. R., and Neilands, J. B. (1970), *Biochem. Biophys. Res. Commun. 38*, 989.

Ratledge, C. (1968), *Biochem. J. 110*, 23P.

Ratledge, C. (1969), *Biochim. Biophys. Acta 192*, 148.

Ratledge, C., and Winder, F. G. (1962), *Biochem. J. 84*, 501.

Snow, G. A. (1965), *Biochem. J. 97*, 166.

Snow, G. A., and White, A. J. (1969), *Biochem. J. 115*, 1031.

White, A. J., and Snow, G. A. (1968), *Biochem. J. 108*, 593.

White, A. J., and Snow, G. A. (1969), *Biochem. J. 111*, 785.

Young, I. G., and Gibson, F. (1969), *Biochim. Biophys. Acta 177*, 348.

Young, I. G., Gibson, F., and Macdonald, C. G. (1969), *Biochim. Biophys. Acta 192*, 62.