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SHORT COMMUNICATION

THE BIOSYNTHESIS OF 6-METHYLSALICYLIC ACID AND SALICYLIC ACID BY *MYCOBACTERIUA4 FORTUITUM*

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Abstract-The biosynthesis of 6-methylsalicylic acid and salicylic acid by *M. fortuitum* has been investigated using sodium acetate-2-^{14}C.6-Methylsalicylic acid was observed to be 18 times more radioactive than salicylic acid, thus, strongly suggesting that the biosynthesis of the two acids proceeds via totally differing routes. Methyl 6-methylsalicylate, methyl salicylate and o-methoxyphenol were also obtained from the organism.

INTRODUCTION

PREVIOUS investigations on the biosynthesis of 6-methylsalicylic acid and salicylic acid by *Mycobacteria* have shown 6-methylsalicylic acid to be of polyketide origin[1] (*Mycobacterium phlei*) and salicylic acid to be derived from shikimic acid[2,3] (*Mycobacterium smegmatis*). Both metabolites, in addition to occurring extracellularly, are also produced intracellularly and are incorporated into the growth promoting agents, the mycobactins.[4] Recently, Snow and White[5] found that *M. fortuitum* (NCTC 8573) produced two mycobactins differing only in the aromatic component. These mycobactins (designated F and H) were identical in every respect except that F contained a salicylic acid unit and H a 6-methylsalicylic acid unit. Since the two acids are not normally co-metabolites this organism offered a unique opportunity to study the biogenesis of these structurally similar aromatic compounds.

RESULTS AND DISCUSSION

M. fortuitum was grown for 10 days in shake culture at 34". The filtered and acidified culture medium was extracted with ether and the extract examined (TLC) for the presence of aromatic constituents. This resulted in the isolation of the following compounds: 6-methylsalicylic acid, salicylic acid, methyl salicylate, methyl 6-methylsalicylate, *o*-methoxyphenol. 6-Methylsalicylic acid and salicylic acid were separated by preparative TLC and found to be present in the ratio 1:3. Examination of the naturally occurring methyl esters by gas chromatography showed the ratio of methyl 6-methylsalicylate to methyl salicylate to be of the order 50:1.

Isolation and rigorous purification of the two acids biosynthesized in the presence of

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[4] *G.* N O *Bacteriol. Rev. 34, 99 (1970).*
[5] *G.* A. SNOW and A. J. WHITE, *Biochem. J.* **115**, 1031 *(1969).*

sodium acetate-2-^{14}C showed 6-methylsalicylic acid to have a specific activity 18 times greater than that of salicylic acid (Table 1). This provides good evidence that the two acids are not biosynthesized by related pathways. It is highly likely that as established in M. *phlei*[1] 6-methylsalicylic acid is polyketide derived while as in *M. smegmatis*[2,3] salicylic acid arises via the intermediacy of shikimic acid.*

TABLE 1. INCORPORATION OF SODIUM ACETATE-2-^{14}C INTO METABOLITES OF *M. fortuitum*

Metabolite	*Specific* activity (dis/min/μmole)	* Dilution value
6-Methylsalicylic acid	1727	5744
Salicylic acid	93	106,666
Methyl 6-methylsalicylate (counted as acid)	8649	1147

* Dilution value is defined as specific activity of precursor/specific activity of product.

The incorporation of radioactivity into methyl 6-methylsalicylate was also measured. This ester, containing a trace of methyl salicylate, was hydrolysed and the resulting 6-methylsalicylic acid purified by TLC. Surprisingly, this ester-derived acid was over 5 times more radioactive than the unesterified acid occurring naturally. Although several explanations of this result are possible further work is required to clarify this finding.

The co-occurrence of o-methoxyphenol with the two acids and their esters is of some interest. Although, unfortunately no measure was obtained of the incorporation of acetate-2-^{14}C into this metabolite it seems likely that this compound is of shikimate origin. A feasible mode of biogenesis of this material is via the intermediacy of 2,3-dihydro-2,3-dihydroxybenzoic acid. The latter compound has been shown to arise from isochorismic acid, a metabolite recently implicated in salicylic acid biogenesis.[6] However, further work will be required to clarify the biosynthetic origin of this metabolite and its possible relationship to salicylic acid.

EXPERIMENTAL

Gas chromatography was performed on an F & M 402 gas'chromatograph operating at 100° with a 6 ft glass column packed with 3 % OV-17 on silanized Gas-Chrom Q, 60-80 mesh. Preparative TLC was carred out on commercially prepared silica gel F-254 plates (Brinkmann Instruments) of 0·25 mm thickness. Radioactivity determinations were made in a liquid scintillator[7] using a Packard Tri-Carb Model 3310 scintillation counter.

Growth of Organism

M. fortuitum (NCTC 8573) was grown in shake culture on a New Brunswick Gyrotory shaker operating at 140 oscillations/min. The culture medium (essentially similar to that designated 'II' by White and Snow)[8] had the following composition—L-asparagine, 5 g; KH_2PO_4, 1 g; Na_2HPO_4, 2 g; $MgSO_4$–$7H_2O$, 0·2 g; glycerol, 30 ml; H_2O, 1 l. The organism was first grown in this medium in seed culture for 1 day at 34". The seed cultures were then transferred to Fernback flasks containing 700 ml of medium plus 7 g of glucose in 25 ml H_2O.

* In a preliminary experiment the acids have been biosynthesized in the presence of low levels of shikimic acid-G-^{14}C and the specific activity of salicylic acid observed to be double that of 6-methylsalicylic acid.

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[7] G. A. BRAY, *Anal Biochem, 1, 279 (1960).*
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Feeding of Sodium Acetate-2-^{14}C

150 μc of acetate-2-W (specific activity 4·47 mc/mM) were evenly distributed between 3 flasks of the organism grown for 120 hr. A further 150 μc were added after 168 hr. Growth was then continued for a further 72 hr (i.e. total growth period = 240 hr).

Isolation of Metabolites

The filtered growth medium (2 1.) was acidified (HCl) and continuously extracted with Et_2O for 12 hr. Removal of the ether afforded an oil (54 mg) which was columned on silicic acid with benzene–HOAc (99 : 1) to give the following: 6-methylsalicylic acid-salicylic acid (6 mg), o-methoxyphenol (5 mg), methyl 6-methylsalicylate-methyl, salicylate (2 mg). The mixture of the two acids was separated by preparative TLC in $CHCl_3$–HOAc (98: 2), 3 developments of the plate being required. After separation each acid was further purified by TLC to give finally salicylic acid (3 mg) and 6-methylsalicylic acid (1 mg). Salicylic acid was recrystallized several times; no further purification was effected with 6-methylsalicylic acid.

The naturally occurring esters of the acids, after examination by gas chromatography, were hydrolysed in 5% KOH solution at 100" for 1 hr. The resulting mixture (predominantly 6-methylsalicylic acid) was separated as described to give 6-methylsalicylic acid (1 mg, 8649 dis/min/μmole) and a minute trace of salicylic acid. To establish the absence of radioactive impurities in the 6-methylsalicylic acid inactive acid (5 mg) was added and the activity of the material redetermined (1108 dis/min/μmole). The material was then recrystallized twice without loss of activity (1182 dis/min/μmole).

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