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The Role of Malonate in the Bacterial Biosynthesis of 6-Methylsalicylic Acid

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Mycobacterium phlei has been shown by tracer techniques to utilize malonate in the biosynthesis of 6-methylsalicylic acid. The labeling pattern of 6-methylsalicylic acid obtained by chemical degradation is consistent with the acetate polymalonate biosynthetic pathway.

The first aromatic compound for which the polyacetate biosynthetic pathway was established was 6-methylsalicylic acid, 6-MSA (*1*). This acid had been isolated from *Penicillium griseofulvum* in 1931 during Raistrick's classical work (*2*), and, over the years, has come to be regarded as one of the prototype "secondary metabolites" of fungi (*3, 4*). In 1961, the original polyacetate biosynthetic hypothesis was extended to include a role for malonic acid (*5–7*) with acetate functioning as a "starter" unit in the synthesis of a polyketide chain. The biosynthesis of this acid has now been studied with purified enzyme extracts from *Penicillium patulum* (*8, 9*).

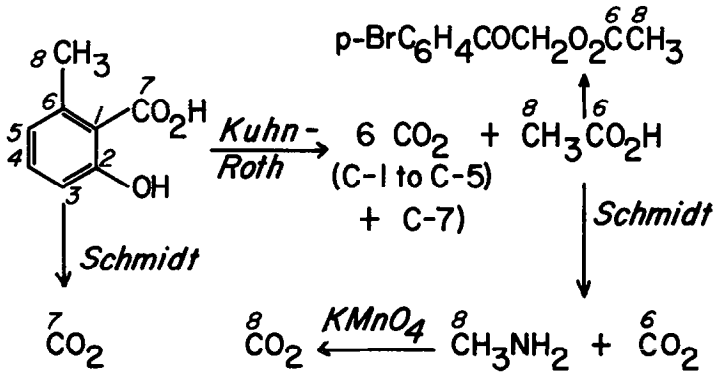
Although usually regarded as a typical fungal metabolite, a 6-MSA unit was found to occur in the structure of some of the mycobactins, iron-chelating compounds produced by various Mycobacteria, and free 6-MSA was isolated as a metabolite from *Mycobacterium phlei* (*10*). A further surprising observation was that 6-MSA is produced by chloroplasts isolated from dark grown barley leaves (*11*). It thus appears to have a wider distribution than previously assumed and possibly a wider biochemical significance.

In previous work, we examined 6-MSA biosynthesis in *M. phlei* (*12*). Neither labeled methionine nor shikimic acid were effectively utilized as precursors. Acetate was incorporated into the 6-MSA molecule, albeit not very efficiently (from 0.009 to 0.057% of added radioactivity); the labeling pattern found on chemical degradation was consistent with a polyacetate or acetate polymalonate pathway. It was, therefore, of interest to extend these studies of the bacterial biosynthesis of 6-MSA by examining the role of malonate, particularly in view of the rather poor utilization of acetate.

EXPERIMENTAL SECTION

General. All solvents were redistilled. Silicic acid for column chromatography was Unisil, Clarkson Chemical Co., mesh 200–325. The radioactive malonates were commercial preparations.

Growth of Mycobacterium phlei (*ATCC 10142*). This organism, obtained from the American Type Culture Collection, Rockville, MD, was maintained on nutrient agar slants at 37° and seed cultures (25 ml medium per 250-ml flask) were grown on medium of the following composition: L-asparagine, 7.66 g; K_2HPO_4, 1.4 g; sodium citrate, 7×10^{-1} g; $MgSO_4 \cdot 7H_2O$, 1.5 g; glycerol, 50 ml; and deionized water to make a final volume of 1 l. and a pH of 6.9. For the tracer experiments, 700-ml portions of this same medium were sterilized in 2.8-l Fernbach flasks, and a sterile solution of glucose (7.5 g) in water (25 ml) was added prior to inoculation. The tracers were dissolved in sterile water and were then distributed over eight such flasks. All growth conditions were those previously described (*12*).



Scheme I

For the isolation of 6-MSA, the filtered medium was acidified to pH 1 with 12 *N* HCl and continuously extracted with ether for 3 days. The crude extract (730 mg from the malonate-2-^{14}C tracer experiment or 900 mg from the malonate-1-^{14}C tracer experiment), obtained from removal of the ether *in vacuo*, was dissolved in benzene–ethyl acetate (1:1, v/v) and chromatographed on a column of silicic acid (60 × 2 cm) using benzene–ethyl acetate–acetic acid (80:20:1, v/v) and taking 10-ml fractions. Fractions 11–16 contained 6-methylsalicylic acid (34.7 mg from the malonate-2-^{14}C tracer experiment or 39 mg from the malonate-1-^{14}C tracer experiment) which was recrystallized from benzene–petroleum ether and further purified by sublimation (90°, 1 mm) to constant specific activity, mp 169–171°.

To locate radioactive atoms in the 6-MSA molecule, chemical degradations (Scheme 1) and radioactivity determinations were carried out as previously described (*12*). In the Kuhn–Roth oxidation, the liberated CO_2 was also collected as $BaCO_3$. The radioactivity in this sample was used as a measure of activity in C-1 + C-2 + C-3 + C-4 + C-5 + C-7.

RESULTS AND DISCUSSION

The utilization of added malonates by *M. phlei* is detailed in Table 1. There was considerable variation in the incorporation observed in the two experiments. The incorporation of malonate-2-^{14}C was 1.7%, a value which would be well thought of in a plant or fungal experiment; the incorporation with malonate-1,3-$^{14}C_2$, although much lower, was nevertheless about three times higher than the best acetate incorporation which we

observed previously. Furthermore, the dilution values tended to be lower than those observed in the acetate experiments. Thus, malonate can be regarded as a substantial precursor for 6-MSA.

TABLE 1

INCORPORATION OF ACTIVITY FROM LABELED MALONATES INTO 6-MSA BY *M. phlei*, ATCC 10142

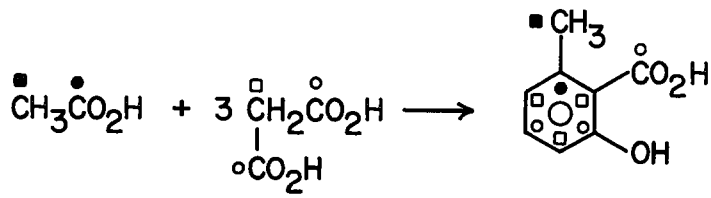
Labeled precursor[a]	6-Methylsalicylic acid		
	Specific activity dpm/mmole	Incorporation[b] %	Dilution[c]
Malonate-1,3-$^{14}C_2$	4.27×10^6	0.15	1.38×10^4
Malonate-2-^{14}C	4.5×10^7	1.7	1.25×10^3

[a] In each experiment, a total of 200 μCi was added; the specific activity of the precursor was 26.4 mCi/m*M* for malonate-1,3-$^{14}C_2$ and 23.7 mCi/m*M* for malonate-2-^{14}C.

[b] Incorporation is defined as the percentage of added activity recovered in isolated 6-MSA.

[c] Dilution is defined as specific activity of precursor/specific activity of product.

The results of the chemical degradations of the labeled 6-MSA samples are given in Table 2; individual values were obtained for C-6, C-7, and C-8. For an ideal acetate plus polymalonate condensation, the acetate "starter" unit, which is represented by C-8 and C-6 in 6-MSA would be devoid of radioactivity (see Scheme 2). In these experiments, malonate-2-^{14}C contributed 9% of the total activity to C-8, and malonate-1,3-$^{14}C_2$ contributed 10.8% of total activity to C-6. These values are considerably lower than that of 25% total activity which would be expected for labeling by way of acetate (four labeled positions per molecule).[1]



Scheme 2

For the ideal situation, the subsequent two carbon units extending the polyketide chain would be wholly derived from malonate; since three such units are required for the construction of 6-MSA, each labeled malonate would contribute three labeled carbon atoms which would each contain 33.3% of total activity. The only one of these atoms examined experimentally was C-7. As expected, malonate-2-^{14}C contributed at most, only 1% of total activity to this position; on the other hand, malonate-1,3-$^{14}C_2$ contributed 29.8% of total activity, a value approaching that required by theory.

[1] In the two acetate experiments previously reported the actual values were 23% for acetate-2-^{14}C-labeling of C-6 + C-8, and for the same combination, 24% for acetate-1-^{14}C. In a third experiment with acetate-2-^{14}C, with very low levels of activity, C-6 + C-8 accounted for 19% of total, but this value is subject to some error.

TABLE 2

CHEMICAL DEGRADATION OF LABELED 6-MSA SAMPLES

Precursor	6-Methylsalicylic acid[b]		C-1 + C-2 + C-3 + C-4 + C-5 + C-7		C-6[a]		C-7		C-8[a]	
	Specific activity dpm/mmole	% Total	Specific activity dpm/mmole	% Total	Specific activity dpm/mmole	% Total	Specific activity dpm/mmole	% Total	Specific activity dpm/mmole	% Total
Malonate-1,3-$^{14}C_2$	1.66×10^5	100	2.46×10^4	90	1.79×10^4	10.8	4.92×10^4	29.8	0	0
Malonate-2-^{14}C	4.9×10^5	100	4.4×10^5	90	4.5×10^3	0.9	4.5×10^3	0.9	4.4×10^4	9.0

[a] These values for C-6 and C-8 are those determined on the products of the Schmidt reaction. The combined value for C-6 + C-8 was also determined from the activity of the *p*-bromophenyl–acetate derivative; in the malonate-1,3-$^{14}C_2$ experiment, this sum was 2.12×10^4 dpm/mmole, and in the malonate-2-^{14}C experiment this sum was 4.5×10^4 dpm/mmole.

[b] The degradations were performed after 6-methylsalicylic acid had been diluted with 50–100 mg of cold material.

It is apparent from the finding of some activity in the "starter" unit, and a value lower than theoretical in the terminal unit, that some conversion of malonate to acetate had taken place.[2] This is a situation often observed in fungal studies. As a quantitative measure of the malonate to acetate conversion, Bu'Lock *et al.* (*13*) use the following ratio:

$$\frac{\text{Incorporation from malonate into acetate-derived } C_2 \text{ unit}}{\text{Incorporation from malonate into malonate-derived } C_2 \text{ unit}}.$$

This ratio varies from 0 (no malonate to acetate conversion) to 1.0 (nonspecific incorporation with all malonate decarboxylated to acetate). In the present experiments, this ratio can be evaluated from the malonate-1,3-$^{14}C_2$ experiment by taking the ratio as C-6/C-7 = 10.8/29.8 = 0.36. This value compares very well with that of 0.26 observed for 6-MSA synthesis in *Penicillium patulum* (*6*) and with that of 0.28 observed in tropolone biosynthesis where again a partial conversion of malonate to acetate must be postulated (*14*).

From the work reported here and earlier, it is clear that the bacterial synthesis of 6-MSA is a typical acetate plus polymalonate pathway; in terms of total incorporation of activity from the medium, malonate is preferred over acetate, perhaps for permeability reasons. Since good evidence has been presented for the acetate polymalonate condensation in barley chloroplasts this pathway is common to fungi, plants, and bacteria; in no case has any evidence been presented for alternate possibilities such as the methylation of a unit (*e.g.*, salicylate) derived from shikimate.[3] It is particularly striking that in *Mycobacterium fortuitum*, two mycobactins *F* and *H* are produced, *F* containing a salicylic acid unit and *H*, a 6-MSA unit (*18*). Both of the free acids are present in the culture fluid of this organism and, as shown recently by Hudson, are synthesized by totally independent pathways (*19*).

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[2] The presence of a malonyl–CoA decarboxylase enzyme has been reported in *Penicillium patulum* (*8*).

[3] Synthesis of salicylic acid by the shikimic acid pathway has been demonstrated by tracer experiments in *Mycobacterium smegmatis* and in cell-free extracts of the same organism (*15*, *16*). Furthermore, the uptake of this acid into mycobactin *S* occurs in growing cells of the same organism (*17*).

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