BBA 65959

# 6-METHYLSALICYLIC ACID DECARBOXYLASE FROM *PENICILLIUM PATULUM*

ROBLEY J. LIGHT
*Department of Chemistry, Florida State University, Tallahassee, Fla. 32306 (U.S.A.)*

(Received May 12th, 1969)

## SUMMARY

1. A crude extract catalyzing the decarboxylation of 6-methylsalicylic acid has been isolated from *Penicillium patulum*.

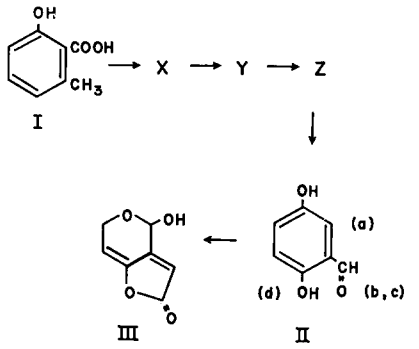
2. Culture conditions which lead to the formation of decarboxylase activity are described.

3. No other activity metabolizing 6-methylsalicylic acid can be detected *in vitro* with extracts containing the decarboxylase.

4. The decarboxylase has a broad pH optimum between 7 and 8, and the $K_m$ for 6-methylsalicylic acid is 7 $\mu$M. Decarboxylase activity is eluted from a Bio-Gel A5m column at a position corresponding to a Stokes radius of about 30 Å.

## INTRODUCTION

6-Methylsalicylic acid (I) is a polyketide secondary metabolite produced under certain culture conditions by *Penicillium patulum* and several related organisms[1-3]. It is synthesized from acetyl-CoA and malonyl-CoA by an enzyme complex presumably similar in some respects to the fatty acid synthase complex[4,5]. 6-Methylsalicylic acid is metabolized in the same organism to a number of other phenolic products[1,6]. Ring opening and rearrangement of one of these, presumably gentisaldehyde (II), leads to patulin (III)[7]. The conversion of 6-methylsalicylic acid to gentisaldehyde involves a minimum of four steps: (a) decarboxylation; (b) transformation of the methyl group to hydroxymethyl; (c) oxidation of this hydroxymethyl to an aldehyde; and (d) hydroxylation of the aromatic ring (see Scheme 1). It is not clear in what order these reactions take place, and the specificity of some of the enzymes involved may be broad enough that several pathways are possible. The detection of *m*-cresol in continuous culture[1] and the conversion of deuterated *m*-cresol to patulin[8] in good yield suggest that the first step in at least one of the pathways is the decarboxylation of 6-methylsalicylic acid. This paper reports the isolation of a 6-methylsalicylic acid decarboxylase from *P. patulum* and culture conditions under which the decarboxylase activity is found.




Scheme 1

## EXPERIMENTAL PROCEDURE

### *Culturing methods*

Isolate RL-17 (early 6-methylsalicylic acid strain) of *P. patulum* NRRL 2159A (ref. 2) was employed in this study. Cultures were maintained on malt agar slants as previously described[2]. Growth of mycelium for isolation of 6-methylsalicylic acid decarboxylase was carried out in aerated fermentor cultures as described for 6-methylsalicylic acid synthase isolation[5] except that the procedure was modified slightly when the cultures began producing large quantities of acid. In a typical transfer culture in Czapek–Dox medium, acid production became as high as 3–5 mequiv/l per h, the pH of the Czapek–Dox medium remained at 4, and 6-methylsalicylic acid production was markedly decreased. Thin-layer chromatography of the medium from one culture after 10 h showed the presence of material with the same $R_F$ as gluconic acid in quantities sufficient to account for the acid production. When sucrose was substituted for glucose, and the Czapek–Dox medium initially titrated to pH 6.5, acid production was decreased to about 0.5 mequiv/l per h. Consequently sucrose was substituted for glucose in both the germinating medium[5] and the Czapek–Dox medium[2] and the following procedure was followed. Spores were washed from an agar slant into 150 ml of germinating medium contained in a 500-ml erlenmeyer flask and incubated on a rotary shaker at 25° for 24 h. The contents of this flask were added to 4 l of germinating medium contained in a 14-l fermentor jar (New Brunswick model MF-114) and the culture grown for 16 h at 26° with aeration (4 l/min) and agitation (500 rev./min). Antifoam C (Dow Corning) was added automatically to control foaming. Mycelium was harvested by filtration and washed with Czapek–Dox medium. A portion (120 g wet wt.) of the mycelium was added to a second fermentor assembly containing 6 l of Czapek–Dox medium and cycloheximide at a concentration of 0.3 μg/ml. The culture was maintained at 26° under aeration (4 l/min), agitation (500 rev./min), and continuous antifoam addition. Samples were harvested at various times by filtration, washed with 0.02 M phosphate buffer (pH 7.3), suspended in the same buffer and lyophilized.

### *Extraction and assay of 6-methylsalicylic acid decarboxylase*

6-Methylsalicylic acid decarboxylase activity could be released from the my-

celium by several procedures. Large-scale extraction was carried out with a colloid mill. The preparation used for most of the experiments described in this paper employed mycelium which has been grown in Czapek–Dox as described above for 16.5 h, harvested (140 g wet wt.), and suspended in 400 ml of cold 0.1 M phosphate buffer containing 1 mM EDTA and 0.02 M ascorbate. The suspension was mixed with 800 g damp washed glass beads (0.2 mm), and run through an Eppenbach Micro Mill (Model MV-6-3, Gifford-Wood) for 45 sec at a gap setting of 32. The mill was washed out with an additional 400 ml of buffer, and the material was centrifuged for 60 min at 13 000 rev./min (27 000 $\times g$). The supernatant contained 5.5 mg/ml of protein (biuret[9]) and 19.1 units/ml 6-methylsalicylic acid decarboxylase activity. The crude extract was moderately stable frozen, retaining about 30% of its activity after 24 days. The presence of ascorbate seemed to improve stability, but its effect was not studied systematically. Sonication was employed for small scale isolation and for routine determination of activity levels. Lyophilized mycelium (0.5 g) was suspended in 50 ml of cold 0.02 M phosphate buffer (pH 7.3) and the suspension was sonicated for 10 min at the top setting of a Branson model S-110 Sonifier. Cooling was maintained with a Rosett cooling cell (Branson Instruments) in an ice-salt bath, and sonication was interrupted at 2-min intervals to allow the temperature to return below 3°. The mixture was centrifuged for 20 min at 18 000 rev./min (39 000 $\times g$), and the supernatant assayed for activity.

Extracts for 6-methylsalicylic acid synthase activity were prepared by grinding lyophilized mycelium with sand[5] and the assay of 6-methylsalicylic acid synthase has also been described elsewhere[2]. In some cases these same extracts were used for 6-methylsalicylic acid decarboxylase activity assays.

$(NH_4)_2SO_4$ fractionation precipitated 6-methylsalicylic acid decarboxylase activity between 25 and 50% satn., but in a number of attempts recovery of activity amounted to only 2–10% of the total activity in the crude extract. Assay of this fraction in the presence of 1 mM $Zn^{2+}$ did not restore the lost activity, nor have other attempts to improve the recovery or restore the lost activity been successful so far. The preliminary characterizations of the enzyme described in this paper were carried out on either the crude extract or this $(NH_4)_2SO_4$ fraction, and it was felt that more extensive characterization should wait until conditions for further purification in higher yield could be worked out. For preparation of the $(NH_4)_2SO_4$ fraction employed here, 700 ml of cold crude extract was slowly brought to 25% satn. by addition of 100.8 g of $(NH_4)_2SO_4$, and the pellet was collected by centrifugation and discarded. Further $(NH_4)_2SO_4$ (118.3 g) was added slowly to the supernatant, stirring was continued for one hour, and the pellet from this fraction (AS-2) was dissolved in 140 ml of 0.02 M phosphate buffer (pH 7.2) containing 1 mM EDTA. The solution was dialyzed overnight against the same buffer, centrifuged, and stored in frozen aliquots. All steps were carried out at 4° in the cold room. Insoluble material produced upon thawing was centrifuged before assays were run. The frozen and thawed AS-2 fraction contained 5.8 mg/ml protein (biuret[9]), and 0.9 unit/ml 6-methylsalicylic acid decarboxylase activity, and this fraction was somewhat more stable frozen than the crude extract, retaining about 60% of its activity after two months.

6-Methylsalicylic acid decarboxylase activity was determined by measuring the release of radioactive $CO_2$ from biosynthetically labeled 6-methyl[$^{14}$C]salicylic acid. One unit of activity is defined as the quantity of enzyme which will catalyze the

decarboxylation of 1 nmole of 6-methylsalicylic acid per min under the conditions of the standard assay (usually in 0.02 M phosphate, pH 7.2). Incubations were carried out for 60 min at room temperature in either the side arm or main compartment of a Warburg flask, with sufficient $H_2SO_4$ contained in the other compartment to acidify the reaction mixture. Hyamine hydroxide (0.3 ml of a 1 M solution in methanol) was placed in the center well. Incubations contained the specified quantity of extract, 10–20 nmoles of 6-methyl[$^{14}$C]salicylic acid, and buffer to a final volume of either 0.12 ml (side arm) or 2.0 ml (main compartment). At the end of the incubation period the side arm and main compartment contents were mixed, the flasks were kept an additional 2 h to allow complete absorption of the $CO_2$ by the hyamine solution, and the contents of the center well were washed into a scintillation bottle with 15 ml of scintillation fluid (4 g of 2, 5-diphenyloxazole, 0.05 g of 1, 4-bis-[2-(5-phenyloxazolyl)]-benzene, 1 l of toluene). Radioactivity was assayed in a Tri-Carb model 3214 liquid scintillation counter at about 70% efficiency.

A larger scale incubation was carried out to identify the other product of the decarboxylase. The incubation contained 2.0 ml of AS-2 fraction and 55 nmoles of 6-methyl[$^{14}$C]salicylic acid. After incubation for 90 min, acidification, and equilibration for 2 h, the center well contained 33 nmoles of $CO_2$. The acidified reaction mixture was extracted twice with ether. The ether extracts were concentrated under a stream of nitrogen to approx. 30 ml, and a 10-ml aliquot was assayed for *m*-cresol by gas–liquid chromatography on a 6 ft × 0.25 inch glass column packed with 5% ethyleneglycol adipate on 100–120 mesh gas chrom Q. The column was operated at 170° with an argon flow of 40 ml/min and an argon ionization detector. The concentrated extract was found to contain approx. 10 nmoles of *m*-cresol. Considering the potential losses on evaporation and inaccuracy in measuring the concentrated extract volume, this 30% recovery is not bad and indicates that *m*-cresol is at least a principle product in the reaction. In other experiments in which reaction mixtures were assayed by thin-layer chromatography, no non-volatile products were observed.

*Preparation of 6-methyl[$^{14}$C]salicylic acid*

Mycelium from a germinating culture was transferred to a 125-ml erlenmeyer fask containing 50 ml of Czapek–Dox medium and 0.3 μg/ml of cycloheximide. After shaking for one h, 125 μC of sodium [1-$^{14}$C]acetate (New England Nuclear) was added and the shaking was continued for 4 h. The culture medium was filtered, acidified, and extracted 4 times with ether. The ether was dried over $Na_2SO_4$ and evaporated and the residue was suspended in warm benzene and applied to a 10 g silicic acid column prepared in benzene. The column was eluted with 200 ml of benzene and 200 ml of 10% ether in hexane, and fractions were assayed for 6-methylsalicylic acid by thin-layer chromatography[10]. The 6-methylsalicylic acid containing fractions were combined and evaporated and the residue (9.0 mg) was recrystallized from 0.5 ml of chloroform producing 3.0 mg of 6-methylsalicylic acid with a specific activity of $8.2 \cdot 10^5$ counts/min per μmole. Previous degradations[10–12] of biosynthetic 6-methylsalicylic acid have shown that 25% of the radioactivity is in the carboxyl group. A second batch of 6-methyl[$^{14}$C]salicylic acid was prepared in a similar fashion and had a specific activity of $1.16 \cdot 10^6$ counts/min per μmole.

*Other materials*

Adsorbosil-1 (Applied Science Laboratories) was used for thin-layer chromatography. SiliCAR CC-4, 100–200 mesh (Mallinckrodt Chemical Co.) was used for silicic acid column chromatography. Cycloheximide was obtained from Upjohn under the registered trademark Actidione. Scintillator materials and hyamine hydroxide were obtained from Packard. Tricine buffer, *N*-tris-(hydroxymethyl)glycine, was obtained from Calbiochem. Bio-Gel A5m was obtained from Bio-Rad Laboratories. Blue dextran was obtained from Pharmacia. Ethyleneglycoladipate and gas chrom Q were obtained from Applied Science Laboratories.

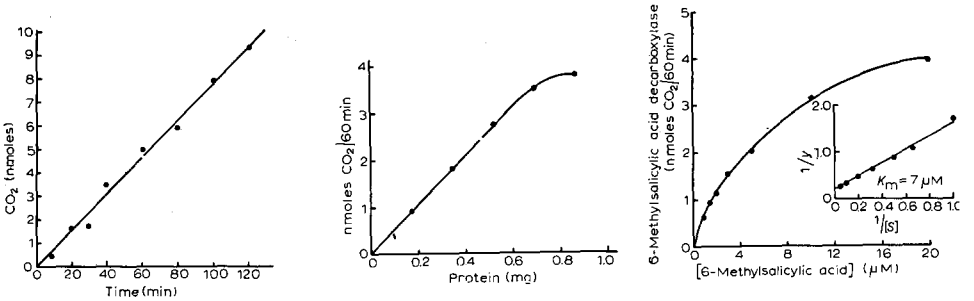



Fig. 1. Time course of 6-methylsalicylic acid decarboxylase assay. Incubations contained 15 nmoles of 6-methyl[$^{14}$C]salicylic acid and 0.1 ml of fraction AS-2 (0.58 mg protein) in a final volume of 0.81 ml. The assay was carried out at room temperature as described in EXPERIMENTAL PROCEDURE.

Fig. 2. Dependence of 6-methylsalicylic acid decarboxylase activity on enzyme concentration. Incubations contained 15 nmoles of 6-methyl[$^{14}$C]salicylic acid and the indicated quantity of fraction AS-2 in a final volume of 0.18 ml, and proceeded for 60 min at room temperature as described in EXPERIMENTAL PROCEDURE.

Fig. 3. Effect of 6-methylsalicylic acid concentration on 6-methylsalicylic acid decarboxylase activity. Incubations contained 0.15 ml of fraction AS-2 (0.87 mg protein) and the indicated concentration of 6-methyl[$^{14}$C]salicylic acid in a final volume of 2.0 ml. The incubation proceeded for 60 min at room temperature as described in EXPERIMENTAL PROCEDURE. Units of $v$ and $[S]$ in the reciprocal plot are the same as shown for the direct plot.

## RESULTS

Fig. 1 shows the time course of the decarboxylase reaction and Fig. 2 shows that the activity is proportional to enzyme concentration. From the dependence of the activity on 6-methylsalicylic acid concentration shown in Fig. 3, the $K_m$ is calculated to be about 7 $\mu$M. The decarboxylase is active over a fairly broad pH range (Fig. 4) with a broad optimum between pH 7 and 8.

The enzyme is unstable below pH 5 and is unstable to heating at 60° for 5 min. Activity in the AS-2 fraction is not inhibited by 0.001 M acetylacetone and only slightly inhibited (20%) by 0.01 M $NaNO_3$.

Gel-filtration of the crude extract on Bio-Gel A5m is shown in Fig. 5. Correlation of elution position with Stoke's radius for markers on the same column is shown in Fig. 6. This correlation gives a value of about 30 Å for the Stoke's radius of 6-methylsalicylic acid decarboxylase. Several other methods of correlation are possible, but

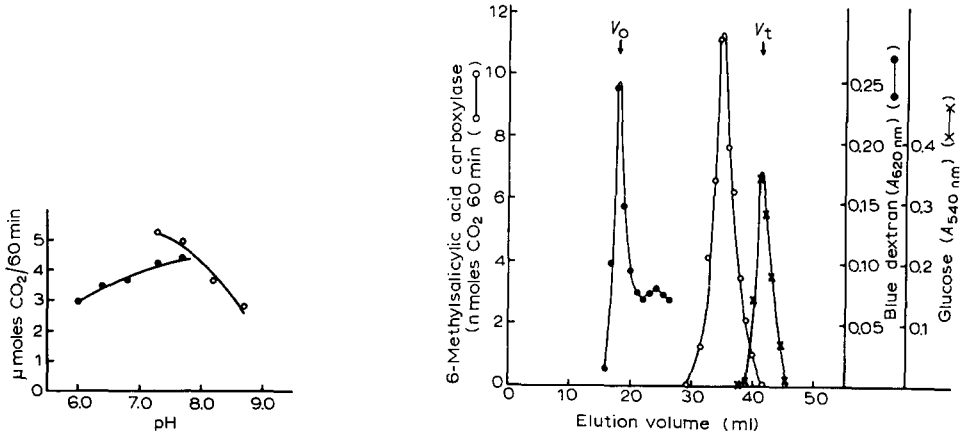



Fig. 4. Effect of pH on 6-methylsalicylic acid decarboxylase activity. Incubation contained 20 nmoles of 6-methyl[$^{14}$C]salicylic acid, 0.15 ml of fraction AS-2 (0.87 mg protein) and either 185 $\mu$moles of phosphate buffer (●) or 185 $\mu$moles of Tricine buffer (○) at the indicated pH. Incubations proceeded for 60 min at room temperature as described in EXPERIMENTAL PROCEDURE.

Fig. 5. Gel-filtration of 6-methylsalicylic acid decarboxylase on Bio-Gel A5m (6% agarose). The column (1.5 cm diameter) was packed in buffer containing 0.2 M phosphate (pH 7.2), 1 M NaCl, $10^{-3}$ M EDTA, and $10^{-4}$ M dithiothreitol to a height of about 28 cm. It was charged with 0.75 ml of 6 weeks frozen crude extract and eluted with the same buffer at a flow rate of 7 ml/h. Fractions of 1.05 ml were collected. The assay mixture contained 15 nmoles of 6-methyl[$^{14}$C]salicylic acid and 0.8 ml of the column fraction, and incubations were carried out for 120 min at room temperature as described in EXPERIMENTAL PROCEDURE. The void volume was determined by elution of blue dextran (●) and the total volume was determined by elution of glucose (×) which was assayed by the reduction of 3,5-dinitrosalicylic acid[13].

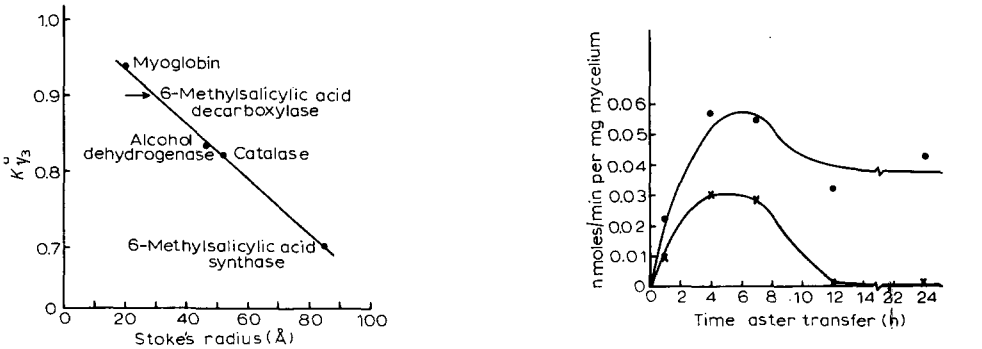



Fig. 6. Correlation of Stoke's radius with elution volume by gel-filtration according to the method of PORATH[14]. Here $K_d$ is calculated as $(V_e - V_0)/(V_t - V_0)$ where $V_e$ is the elution volume of the component in question, $V_0$ is the elution volume of blue dextran, and $V_t$ is the elution volume of glucose rather than the total bed volume. Markers were eluted from the column under conditions similar to those described in Fig. 5.

Fig. 7. Time course of appearance of 6-methylsalicylic acid decarboxylase (●) and 6-methylsalicylic acid synthase (×) activity in fermentor transfer culture. Mycelium was grown in germinating medium and transferred to Czapek–Dox medium containing 0.3 $\mu$g/ml cycloheximide as described in EXPERIMENTAL PROCEDURE. Aliquots were harvested and lyophilized at the appropriate times. 6-Methylsalicylic acid decarboxylase activity was assayed in sonicated extracts as described in EXPERIMENTAL PROCEDURE and 6-methylsalicylic acid synthase activity was assayed in extracts prepared by grinding with sand as described previously[5]. For comparison, both activities are expressed on a mg dry wt. of mycelium basis and represent either nmoles of 6-methylsalicylic acid formed from [$^{14}$C]malonyl-CoA (×), or nmoles of $CO_2$ liberated (●).

they all give similar values for the Stoke's radius[15]. Assuming that this method gives a correct estimation of Stoke's radius, then the molecular weight will still depend upon the partial specific volume and frictional ratio. The molecular weight can be estimated at about 70 000 assuming values of 0.725 $cm^3$ for the partial specific volume and 1.1 for the frictional ratio, or at about 54 000 assuming values of 0.725 $cm^3$ and 1.2, respectively, for these quantities.

6-Methylsalicylic acid decarboxylase activity is not present in mycelium grown in a rich germinating medium under conditions in which 6-methylsalicylic acid synthase activity is not present. Upon transfer of this mycelium to a fermentor culture of Czapek medium containing 0.3 $\mu$g/ml cycloheximide, a condition which leads to the production of 6-methylsalicylic acid synthase[3,5], decarboxylase activity appears at about the same time as the synthase activity. Fig. 7 shows the results from one typical experiment. The two activities are compared on a mg mycelium basis. Since different methods of extraction were required to give optimum release of activity (sonication for the decarboxylase and grinding in sand for the synthase), the comparison can only be approximate. It does illustrate that the activities are of the same order of magnitude.

In such fermentor experiments the concentration of 6-methylsalicylic acid did not build up in the medium to the extent previously found in similar transfers to shake culture[2,3]. The culture medium gave a greenish-brown color with $FeCl_3$, indicating the presence of other phenolic compounds in addition to 6-methylsalicylic acid which gives a deep purple color. Other compounds were evident in the fermentor culture medium on silicic acid thin-layer chromatography, including a substance migrating with the same $R_F$ as *m*-hydroxybenzyl alcohol ($R_F$ = 0.18; developing solvent, benzene:acetic acid, 85:15). Media from shake cultures harvested in the first 5–10 h after transfer gave a deep purple color with $FeCl_3$ and showed 6-methylsalicylic acid as the principle spot on thin-layer chromatography. This would indicate that activity metabolizing 6-methylsalicylic acid does not appear as rapidly in shake culture as the fermentor culture.

It should also be noted from Fig. 7 that the decarboxylase activity remains present up to 24 h even though the synthase activity has decayed. Addition of 20 $\mu$g/ml cycloheximide to a similar culture 4 h after transfer did not cause the decarboxylase activity to decay. Since this concentration of cycloheximide is sufficient to block protein synthesis[3], these observations indicate that the decarboxylase is not turning over rapidly.

## DISCUSSION

BU'LOCK *et al.*[1] have demonstrated at least three periods in the growth of *Penicillium urticae* which differ with respect to 6-methylsalicylic acid metabolism. Young cultures do not produce 6-methylsalicylic acid, and 6-methylsalicylic acid added to the medium is not metabolized. During a transition period occurring between 24 and 36 h of growth, 6-methylsalicylic acid production begins but this 6-methylsalicylic acid is not degraded. A short time after this transition, activity metabolizing 6-methylsalicylic acid appears, and 6-methylsalicylic acid concentration in the medium drops to a lower steady-state level. Thus the appearance of the metabolizing enzymes can be separated in time from the appearance of the 6-methylsalicylic acid synthase activity. Our observations *in vitro* are consistent with these observations *in vivo* even though

our culturing conditions are somewhat different. Extracts of mycelium grown in germinating medium contain neither 6-methylsalicylic acid synthase activity nor 6-methylsalicylic acid decarboxylase activity. After transfer to Czapek–Dox medium under conditions which stimulate 6-methylsalicylic acid synthase production, 6-methylsalicylic acid decarboxylase activity appears at about the same time in fermentor culture but not in shake culture.

As already pointed out (Scheme 1) most of the metabolic products of 6-methyl salicylic acid lie along the pathway to patulin[1,6]. The specificity of the enzymes, and hence the order of the steps, is not clear. The appearance of 6-methylsalicylic acid decarboxylase under the conditions reported here, and the failure to observe any non-volatile products of 6-methylsalicylic acid *in vitro* with crude extracts which contain 6-methylsalicylic adid decarboxylase, are both consistent with *m*-cresol being the initial metabolic product. These observations are also consistent with results obtained *in vivo* in which *m*-cresol was detected in the culture medium[1] and was converted to patulin in good yield[8].

REBSTOCK[16] has identified *m*-hydroxybenzyl alcohol in a different strain of *P. urticae*, and G. M. GAUCHER (personal communication) has demonstrated the conversion of radioactive *m*-hydroxybenzyl alcohol to patulin *in vivo*. Thus hydroxylation of the methyl group also appears to be an early step on the way to patulin. Whether decarboxylation and methyl hydroxylation must occur sequentially cannot be decided until the enzyme specificities are determined. The identification of 6-formylsalicylic acid[6], 3-hydroxyphthalic acid[6], and toluquinol[8] in culture media certainly require another sequence of reactions, although it has not been demonstrated whether any of these compounds lie on the pathway to patulin. Deuterated *m*-cresol was incorporated into toluquinol[8].

Studies on phenomena related to or responsible for polyketide biosynthesis must take into account the position of the metabolite in relationship to potential diversifying reactions. It is possible that a given set of conditions which stimulate one enzyme in a sequence may have an adverse effect on other enzymes. Consequently the effect of culture conditions upon the overall pattern of polyketide production can be quite complex. Stimulation of the synthesis of 6-methylsalicylic acid, the first polyketide product in this series, appears to be somehow related to a partial inhibition of protein synthesis[3]. On the other hand, BU'LOCK *et al.*[1] have suggested that the enzymes metabolizing 6-methylsalicylic acid may be induced sequentially by newly appearing metabolites. Such a pattern would be similar to the sequential induction of enzymes for mandelic acid degradation in Pseudomonas[17,18]. Optimum conditions for 6-methylsalicylic acid formation therefore do not necessarily represent optimum conditions for the production of *m*-cresol, gentisaldehyde, or patulin. The concentration of any metabolite is increased not only by an increase in the enzyme synthesizing it but also by a decrease or lack of the enzyme metabolizing it. While these factors should be obvious, some studies reporting effects on the concentration of a secondary metabolite have sometimes lost sight of them. An adequate evaluation of such phenomena will only be possible when each of the enzymes involved has been identified and characterized and can be studied individually.

Decarboxylation represents a common modification reaction of polyketide secondary metabolites, and one of the few modification reactions which have been demonstrated *in vitro*. Decarboxylases from several other systems have been isolated

and studied. These include a 2,3-dihydroxybenzoic acid decarboxylase from *Aspergillus niger*[19], stipitatonic acid decarboxylase from *Penicillium stipitatum*[20], and orsellinic acid decarboxylase from *Gliocadium roseum*[21]. BENTLEY[22] has reviewed additional cases in which decarboxylation must be involved.

ACKNOWLEDGMENTS

This research was supported by Grants GB-5754 and GB-8325 from the National Science Foundation and by a Public Health Service Career Development Award (No. K3-AM-11 762) from the Institute of Arthritis and Metabolic Diseases.

The technical assistance of Mrs. M. Clark is gratefully acknowledged. The author is indebted to Mr. R. Bryant for the gel-filtration experiment with 6-methylsalicylic acid decarboxylase and for calibration of the Bio-Gel A5m column.

REFERENCES

1 J. D. BU'LOCK, D. HAMILTON, M. A. HULME, A. J. POWELL, H. M. SMALLEY, D. SHEPHERD AND G. N. SMITH, *Can. J. Microbiol.*, 11 (1965) 765.
2 R. J. LIGHT, *J. Biol. Chem.*, 242 (1967) 1880.
3 R. J. LIGHT, *Arch. Biochem. Biophys.*, 122 (1967) 494.
4 F. LYNEN, *Federation Proc.*, 20 (1961) 941.
5 R. J. LIGHT AND L. P. HAGER, *Arch. Biochem. Biophys.*, 125 (1968) 326.
6 S. W. TANENBAUM AND E. W. BASSETT, *Biochim. Biophys. Acta*, 28 (1958) 21.
7 S. W. TANENBAUM AND E. W. BASSETT, *J. Biol. Chem.*, 234 (1959) 1861.
8 A. I. SCOTT AND M. YALPANI, *Chem. Commun.*, (1967) 945.
9 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 450.
10 R. J. LIGHT, T. M. HARRIS AND C. M. HARRIS, *Biochemistry*, 5 (1966) 4037.
11 A. J. BIRCH, R. A. MASSY-WESTROPP AND C. J. MOYE, *Australian J. Chem.*, 8 (1955) 539.
12 R. J. LIGHT, *Arch. Biochem. Biophys.*, 112 (1965) 163.
13 P. BERNFELD, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 149.
14 J. PORATH, *Pure Appl. Chem.*, 6 (1963) 233.
15 L. M. SIEGEL AND K. J. MONTY, *Biochim. Biophys. Acta*, 112 (1966) 346.
16 M. C. REBSTOCK, *Arch. Biochem. Biophys.*, 104 (1964) 156.
17 J. MANDELSTAM AND G. A. JACOBY, *Biochem. J.*, 94 (1965) 569.
18 I. L. STEVENSON AND J. MANDELSTAM, *Biochem. J.*, 96 (1965) 354.
19 G. TERUI, T. ENATSU AND H. TOKAKU, *Technol. Rept. Osaka Univ.*, 2 (1952) 283.
20 R. BENTLEY AND C. P. THIESSEN, *J. Biol. Chem.*, 238 (1963) 3811.
21 G. PETTERSSON, *Acta Chem. Scand.*, 19 (1965) 2013.
22 R. BENTLEY, in Z. VANĚK AND Z. HOŠŤÁLEK, *Biogenesis of Antibiotic Substances*, Academic Press, New York, 1965, p. 241.