Conversion of 6-Methylsalicylic Acid into Patulin by *Penicillium urticae*†

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ABSTRACT: *Penicillium urticae* (NRRL 2159A) produces the acetate-derived secondary metabolite patulin as an extracellular end product *via* the classical aromatic polyketide, 6-methylsalicylic acid. The existence of a preferred route for patulin biosynthesis in this fungus was investigated by a kinetic pulse-labeling study in which radiolabeled acetate and eight other pertinent secondary metabolites were fed to submerged fermentor cultures and the kinetics of the incorporation of radioactivity into subsequent metabolites examined. Fermentor growth characteristics and thin-layer chromatography R_F values, and ultraviolet absorption data for all pertinent secondary metabolites are given. A preferential accumulation of early pathway metabolites as opposed to patulin was noted in cultures grown at pH 4–5. The following metabolites were shown to be readily converted into patulin: acetate, 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde, and gentisaldehyde. Toluquinol and gentisyl alcohol were not incorporated, while patulin itself was not further converted to other secondary metabolites. Kinetic incorporation plots enabled pathway intermediates to be readily differentiated from end products and provided information on relative turnover rates which are in agreement with the pool sizes of the various metabolites. The sequence of incorporated metabolites mentioned above indicates the preferred route for patulin biosynthesis in *Penicillium urticae*.

Interest in the occurrence and biosynthesis of the antibiotic patulin began with the isolation of this compound from the culture medium of *Penicillium urticae* Bainier[1] (Birkinshaw *et al.*, 1943). Although the initial interest in patulin as an antibiotic diminished quickly because of its high toxicity to animals (Singh, 1967), it has been the biosynthesis of this fungal metabolite which has sustained the interest of many workers. The first postulated routes for patulin biosynthesis (Birkinshaw, 1953; Ehrensvärd, 1955; Tanenbaum and Bassett, 1959) were based upon structural comparisons of secondary metabolites produced by *Penicillium urticae* and *Penicillium griseofulvum*, and upon verification of the polyacetate origin and the precursor–product relationship of 6-methylsalicylic acid and patulin, by radiolabeling experiments. For further details, see Tanenbaum (1967), Corcoran and Darby (1970), and Turner (1971). Subsequent identification of *m*-hydroxybenzyl alcohol (Rebstock, 1964) and the detection of *m*-cresol, *m*-hydroxybenzaldehyde, *m*-hydroxybenzoic acid, and toluquinone (Bu'Lock *et al.*, 1965, 1969) as *P. urticae* metabolites provided the basis of a proposed matrix of reactions involving up to 16 metabolites, leading from 6-methylsalicylic acid to patulin, with no major route identified (Bu'Lock *et al.*, 1965). Finally, based on a demonstration that *P. urticae* could convert deuterio-labeled *m*-cresol into toluquinol, gentisyl alcohol, gentisaldehyde, and patulin, a major route for patulin biosynthesis *via* *m*-cresol, toluquinol, and gentisyl alcohol has been proposed (Scott and Yalpani, 1967).

Since from both a metabolic and enzymology point of view the patulin biosynthetic pathway is one of the more extensively studied secondary metabolite pathways and since it is therefore of value as a model system in examining both the *raison d'être* and the regulation of secondary metabolism, the question of whether a preferred route for patulin biosynthesis exists was examined. Using what could effectively be termed a pulse-chase radiolabeling technique, radiolabeled acetate and eight other key metabolites were fed to submerged fermentor cultures of *P. urticae* (NRRL 2159A) and in each case the kinetics of the incorporation of radioactivity into subsequent metabolites was examined. The principal sequence of reactions in the conversion of 6-methylsalicylic acid into patulin was thus determined.

Experimental Section

Culture Conditions. Penicillium urticase (NRRL 2159A) was the organism used in this study. Spores were stored in sterile soil at 28° to minimize strain variation. Vial slants of this organism were grown on Czapek–Dox agar (49 g of Czapek solution agar (Difco) plus 5 g of bacto agar (Difco) in 1 l. of distilled water) at 28° for a minimum of 7 days. A 3-ml detergent spore suspension prepared from one vial slant was used to inoculate a 2-l. erlenmeyer flask containing 250 ml of modified Czapek–Dox medium ($NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; $ZnSO_4 \cdot 7H_2O$, 0.0003 g; yeast extract, 3.0 g; glucose, 40.0 g; and distilled water to 1 l.). The flask was incubated at 28° on a reciprocating shaker (New Brunswick, Model R82) using a 3-cm stroke and 120 displacements per minute. After 24-hr growth, this shake flask was used to inoculate a 14-l. New Brunswick Microferm fermentor containing 6 l. of the modified Czapek–Dox medium. The culture was grown under the following standard growth conditions: temperature, 28°; aeration rate, 6 l./min; stirring rate, 500 rpm. Under these standard conditions the pH of the medium was not normally controlled. However it could be controlled if desired, by the automatic addition of 1 N NaOH and 1 N HCl using a Beck-

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[1] *Penicillium urticae* Bainier, *Penicillium patulum* Bainier, and *Penicillium flexuosum* Dale are synonyms, while *Penicillium griseofulvum* Dierckx is closely related (Smith, 1969; Raper *et al.*, 1949).

man combination electrode in conjunction with a Radiometer automatic titrator. Foaming was controlled by the automatic addition of small aliquots of Antifoam C (Dow Corning) as necessary.

Isolation of Metabolites. 6-METHYLSALICYLIC ACID, *m*-CRESOL, AND *m*-HYDROXYBENZYL ALCOHOL. An 18-hr fermentor culture in which the pH was held constant at 5.0 was filtered through Whatman No. 1 filter paper. The medium was adjusted to pH 2.0 with 6 N HCl and extracted with two equal volumes of ether. The combined ether extracts were extracted with an equal volume of sodium bicarbonate solution (5%, w/v) to separate the acidic and neutral compounds. The aqueous layer was then adjusted to pH 2.0 and was reextracted with two equal volumes of ether. This ether extract contained 6-methylsalicylic acid as the major constituent, whereas the original ether extract now contained *m*-cresol and *m*-hydroxybenzyl alcohol.

The two ether extracts were dried over anhydrous sodium sulfate and were reduced in volume by rotatory evaporation. The metabolites were isolated by preparative thin-layer chromatography (tlc) using 1 mm thick plates of neutral silica gel G, developed in chloroform–acetic acid (90:10, v/v). Further purification was by recrystallization (6-methylsalicylic acid from trichloroethylene, mp 174°; *m*-hydroxybenzyl alcohol from ethylene dichloride, mp 68°) except for *m*-cresol which was vacuum distilled, bp 203°.

PATULIN. A 50-hr standard fermentor culture was filtered through Whatman No. 1 filter paper. The medium was adjusted to pH 2.0 with 6 N HCl and extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, filtered, and the solvent removed by rotatory evaporation. The oily residue was extracted with 50 ml of boiling benzene. On cooling, crude patulin crystallized out. The crude product was further purified by recrystallization from hot benzene, mp 110°.

GENTISYL ALCOHOL. The residue, obtained after benzene extraction of the above-mentioned concentrated ethyl acetate extracts, was applied to a 2.5 × 50 cm column of silica gel G (Mallinckrodt-CC7). The column was eluted with chloroform–acetic acid (90:10, v/v) and 10-ml fractions were collected. The fractions were assayed by tlc and those fractions containing gentisyl alcohol were pooled. The solvent was removed by rotatory evaporation and the product was recrystallized from chloroform–methanol (95:5, v/v), mp 100°.

No attempt was made to isolate any of the other metabolites in quantity.

Identification of Metabolites. The various metabolites were identified by comparison of their physical properties to those of authentic samples. Melting points, using a Leitz microscopic hot stage apparatus, R_F data from both tlc and paper chromatography and ultraviolet (uv), infrared, and nuclear magnetic resonance (nmr) spectral characteristics were utilized. The tlc R_F data and uv spectral data are given in Table I. Any ambiguities which arose from the use of tlc were resolved by the paper chromatographic solvent systems designated, B, C, and F by Reio (1958, 1960).

Gentisyl alcohol was synthesized by sodium borohydride reduction of gentisaldehyde in aqueous solution with a 65% yield after ethyl acetate extraction and recrystallization. 6-Methylsalicylic acid was synthesized by the method of Eliel *et al.* (1953). Patulin was a gift from Dr. S. W. Tanenbaum. The remaining metabolites are available commercially (*m*-cresol, Fisher; *m*-hydroxybenzyl alcohol, Aldrich; toluquinol, Fisher; *m*-hydroxybenzaldehyde, Eastman; gentisaldehyde, Eastman; *m*-hydroxybenzoic acid, Eastman; gentisic acid, Eastman).

TABLE I: R_F and Ultraviolet Absorption Data for *P. urticae* Metabolites.

Metabolite	R_F[a] of Solvents A	B	λ_{max}, mμ (ε × 10^{-3}) 0.1 N HCl	0.1 N NaOH
6-Methylsalicylic acid	0.75	0.63	242 (3.5) 284 (1.3) 300 (1.3)	242 (6.5) 296 (3.0)
m-Cresol	0.71	0.71	292 (0.9)	240 (5.4) 290 (1.6)
Toluquinol	0.29	0.29	289 (2.8)	420 (0.8)
m-Hydroxybenzyl alcohol	0.17	0.25	274 (1.4)	240 (2.8) 294 (8.3)
m-Hydroxybenzaldehyde	0.64	0.46	256 (9.1) 315 (2.4)	266 (5.7) 360 (2.4)
m-Hydroxybenzoic acid	0.43	0.21	238 (6.9) 298 (2.3)	314 (2.6)
Gentisyl alcohol	0.04	0.08	293 (3.0)	
Gentisaldehyde	0.52	0.45	230 (16.1) 260 (7.9) 360 (3.3)	236 (10.8) 454 (4.1)
Gentisic acid	0.20	0.32	327	311 259
Patulin	0.32	0.20	277 (15.0)	295 (13.4)

[a] A 10-ml sample of filtered medium was acidified to pH 2.0 and extracted with two 10-ml portions of ethyl acetate. The volume of the combined extracts was reduced to 1 ml on a steam bath and a 5-μl aliquot was spotted onto a 20-cm, 250-μ thick, neutral silica gel plate which was then developed in solvents: (A) chloroform–acetic acid (9:1, v/v) or (B) ethyl acetate–petroleum ether (bp 60–70°)–acetic acid (60:90:2, v/v). After development the chromatogram was sprayed with either 10% (w/v) potassium permanganate in distilled water (yields white spots on pink) or diazotized *p*-nitroaniline (yields distinctive brown to orange spots on white).

Preparation of Radiolabeled Precursors. All radiolabeled precursors were recrystallized to constant specific activity and to an extent necessary to achieve chemical and radiochemical purity as determined by tlc followed by scanning in a Packard radiochromatoscanner. The specific activities and the amounts of the various precursors added to fermentor cultures are given in Table II.

[^{14}C]6-METHYLSALICYLIC ACID AND [^{14}C]*m*-HYDROXBENZYL ALCOHOL. Sodium [1-^{14}C]acetate (0.5 mCi; New England Nuclear) was added to a standard 18-hr fermentor culture in which the pH was held constant at 5.0. The culture was harvested 1.5 hr later and [^{14}C]6-methylsalicylic acid and [^{14}C]-*m*-hydroxybenzyl alcohol were isolated and purified as previously described.

[^{14}C]PATULIN. Sodium [1-^{14}C]acetate was added to a standard 17-hr fermentor culture in which the pH was not controlled. The culture was harvested 8 hr later and [^{14}C]patulin was isolated as previously described.

TRITIUM-LABELED PRESCURSORS. The tritium-labeled precursors were prepared by the method of Kirby and Ogunkoya (1965). The reaction times were varied from 2 to 24 hr depend-

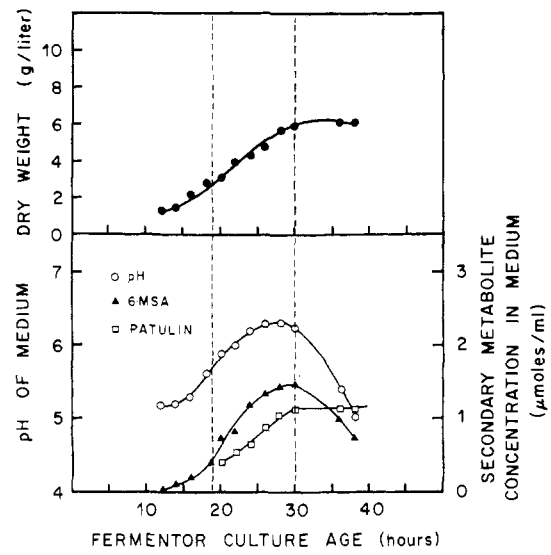

FIGURE 1: Growth characteristics of submerged fermentor cultures of *P. urticae* (NRRL 2159A). Growth conditions are described in the Experimental Section. Dry weight was determined by collecting 50-ml culture samples on preweighed filter papers, washing twice with distilled water, drying overnight at 110°, cooling, and weighing. The culture pH was determined either continuously using an electrode immersed in the culture, or intermittently by measuring the pH of a stirred sample before filtration. The 6-methylsalicylic acid concentration was determined by mixing 3 ml of ferric chloride (0.04 M $FeCl_3 \cdot 6H_2O$ in 0.02 M HCl) with 3 ml of filtered culture medium and 30 min later determining the optical density at 660 nm using a reagent blank prepared from sterile medium. A standard plot, prepared from synthetic 6-methylsalicylic acid in sterile medium was linear up to at least 1.6 μmoles/ml of medium. The patulin concentration was determined by an antibiotic bioassay in which a standard plot of growth rate for a *Bacillus subtilis* culture *vs.* mg of pure patulin was utilized.

ing upon the lability of the compound to be tritiated. Labile tritium was removed by extraction of the labeled precursor into 0.01 M NaOH. The basic solutions were then acidified and extracted twice with ether and the isolated compounds were purified as previously described. [G-^{3}H]Gentisyl alcohol was prepared by borohydride reduction of [G-^{3}H]gentisaldehyde as previously described.

Pulse-Labeling Experimental Procedure. KINETIC EXPERIMENTS. An aqueous solution of precursor was added at an appropriate time to a standard fermentor culture (the pH of the medium was held constant at 5.0 for the addition of sodium [1-^{14}C]acetate and [^{14}C]6-methylsalicylic acid, for all other experiments it was not controlled). Samples of the medium (50–100 ml) were withdrawn at appropriate time intervals after addition of the precursor. The samples were filtered and adjusted to pH 2 for the ^{14}C experiments and to pH 6 for the tritium experiments. Extraction with two equal volumes of ethyl acetate followed. The ethyl acetate extracts were reduced to 2.0 ml (rotatory evaporation) and 20-μl aliquots were spotted onto 5 × 20 cm neutral silica gel G tlc plates which were developed for 14 cm in solvent A (Table I). In the case of [^{14}C]6-methylsalicylic acid the ethyl acetate extracts were reduced to a minimum volume and the entire extract was applied to a tlc plate. In all cases two to three plates were run per sample.

The radiolabeled spots were located using a Packard radiochromatoscanner (Model 7201). These spots were scraped into glass scintillation vials containing 10 ml of a scintillation solution (naphthalene (Fisher), 100 g; 2,5-diphenyloxazole (Nuclear-Chicago), 7.0 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene (Nuclear-Chicago), 0.3 g; dioxane (Fisher) to 1000 ml; water (200 ml) was then added) developed by Snyder (1964) and counted in a Nuclear-Chicago scintillation counter. The efficiency of counting was determined by the channels ratio method.

NONKINETIC EXPERIMENT. [^{14}C]*m*-Hydroxybenzyl alcohol dissolved in 10 ml of distilled water was added to a 20-hr standard fermentor culture. After 10 hr the culture was harvested. The medium was filtered through Whatman No. 1 filter paper, and the pH adjusted to 2.0 and extracted with two equal volumes of ethyl acetate. Qualitative thin-layer plates were run as described for the kinetic experiments. The majority of the extract was used to isolate patulin which was recrystallized to constant specific activity.

TABLE II: Radiolabeled Precursors.

Precursor	Sp Act. (dpm/mg)	Quantity Added (mg)	Initial Concn in Fermentor (μmole/ml)	Incorp (%)[a] into Patulin
Sodium [1-^{14}C]acetate	1.54×10^9	0.72	0.0015	*b*
[^{14}C]6-Methylsalicylic acid	1.81×10^4	240	0.27	
[^{14}C]*m*-Hydroxybenzyl alcohol	1.61×10^4	70	0.093	12 (10)
[^{14}C]Patulin	4.4×10^4	100	0.108	
[G-^{3}H]*m*-Cresol	3.07×10^7	28.4	0.004	16.5 (4)
[G-^{3}H]*m*-Hydroxybenzyl alcohol	1.29×10^6	39.0	0.052	22 (6)
[G-^{3}H]*m*-Hydroxybenzaldehyde	2.84×10^6	18.0	0.024	41 (3)
[G-^{3}H]Gentisaldehyde	6.1×10^6	40.0	0.048	26 (2)
[G-^{3}H]Gentisyl alcohol	6.3×10^6	44.0	0.052	0
[G-^{3}H]Toluquinol	4.13×10^6	14.3	0.019	0

[a] Per cent Incorporation is defined as total activity in recovered product per total activity of administered precursor × 100. The incorporation time (hours) is given in parentheses. [b] Within a 4-hr incorporation time, significant incorporation into patulin was obtained only if the pH was allowed to change freely as opposed to being maintained at pH 5.

Results

The growth characteristics of fermentor cultures of *P. urticae* (NRRL 2159A), as shown in Figure 1, indicate a typical initial lag phase of 15 ± 6 hr, followed by a linear phase of ~15 hr and finally a stationary phase in which the cell mass remained constant at approximately 6–7-g/l. dry weight of cells. During the linear phase the pH and 6-methylsalicylic

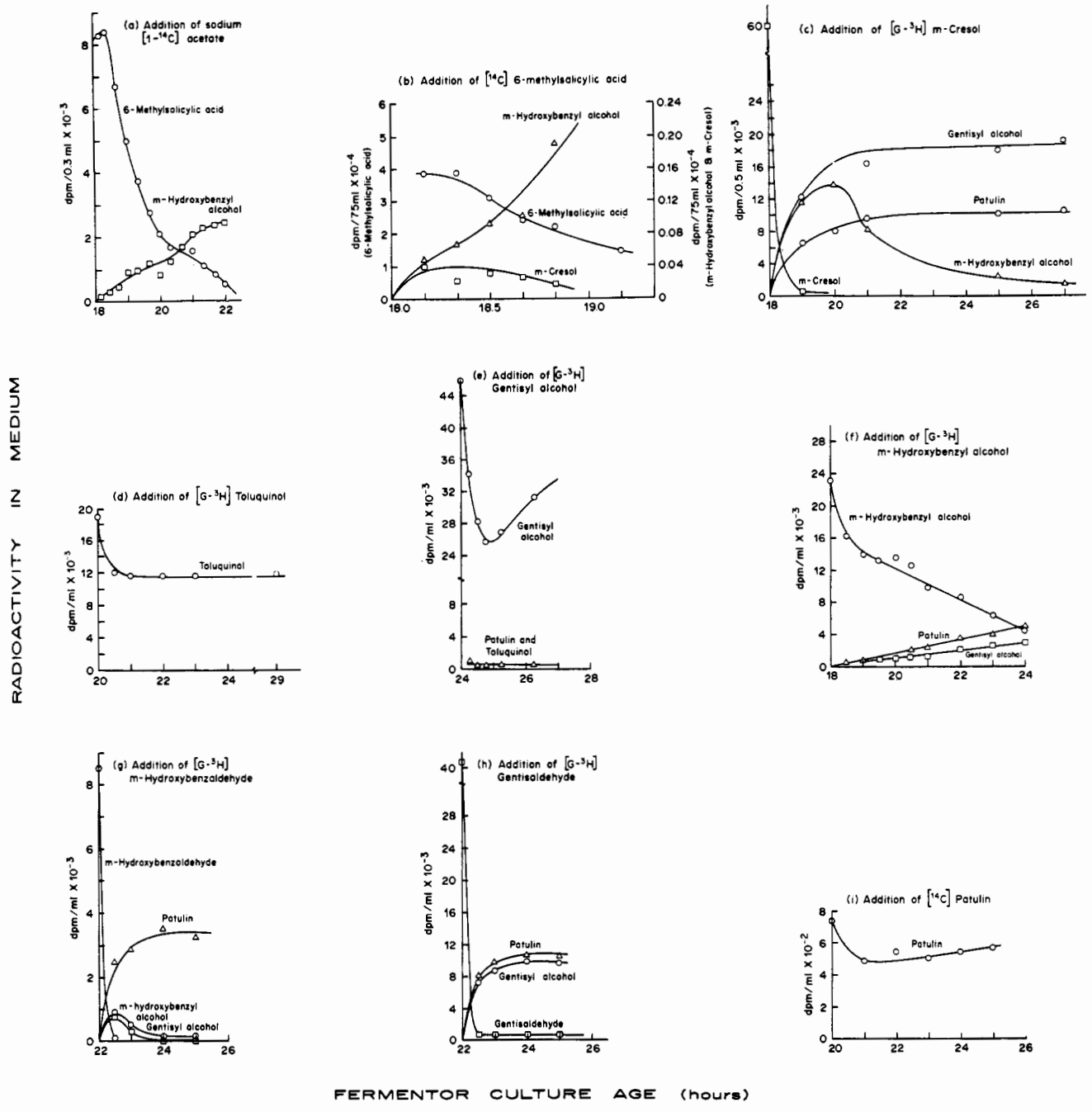

FIGURE 2: Kinetic analyses of pulse labelling experiments in which radiolabelled secondary metabolites were fed to fermentor cultures of *P. urticae*. Samples of medium were withdrawn at intervals to determine the progress of the radiolabel into other metabolites as described in the Experimental Section. The time course of each experiment is given in terms of the particular culture's age under the standard fermentor growth conditions indicated in Figure 1. The total radioactivity present in a particular metabolite per given volume of medium is expressed so that the actual number of dpm associated with a spot of metabolite on a tlc plate may be read from each plot. The per cent incorporation into metabolites at various times may also be calculated directly from these plots.

acid content of the medium rose and reached maximal values of 6.2–6.5 and ~1.5 μmoles per ml, respectively, at ~28 hr, while the patulin content reached a plateau value of ~1.2 μmoles/ml at ~30 hr. These fermentor cultures provided homogeneous mycelial cells and a means of reproducibly achieving identical conditions of temperature, aeration, and pH. Although variations in the lag phase occurred depending upon the inoculum used, the kinetic relationship between the various parameters were found to be consistently reproducible. Hence pH was the parameter of choice for rapid assessment of a culture's progress. All pulse labeling experiments were carried out during the 18- to 30-hr period of fermentor cultures in order to ensure maximal secondary metabolic activity.

Bearing in mind variations due to culture age and environmental conditions such as pH, *P. urticae* secondary metabolites may be *qualitatively* divided into three groups with respect to the relative amounts of each which accumulate. Thus 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, gentisyl alcohol, and patulin were isolated in sufficient amounts to allow their identification by comparison of their physical properties to those of authentic samples. Toluquinol

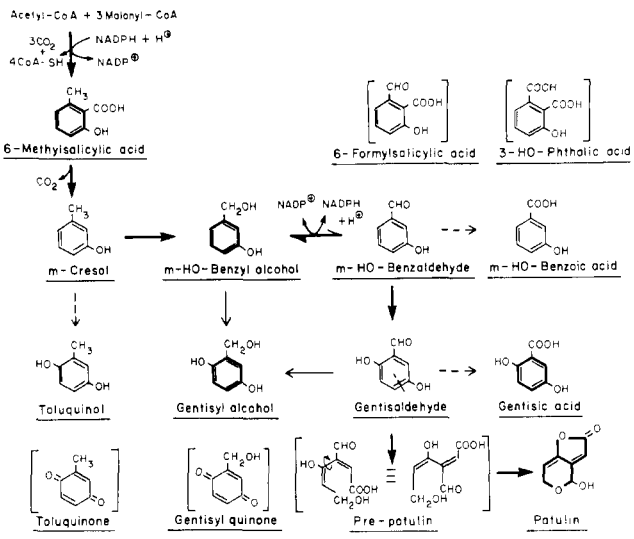

FIGURE 3: The major pathway for patulin biosynthesis in *P. urticae* (NRRL 2159A) is indicated by heavy arrows, with branch reactions indicated by light arrows and probable additional reactions by dashed arrows. The metabolites which usually accumulated to significant levels in the culture medium relative to the others, are drawn with heavy lines, while those generally present in trace amounts if at all, are enclosed in square brackets.

and *m*-hydroxybenzoic acid were identified solely by their chromatographic characteristics (Table I). Gentisic acid could have been readily isolated if desired but was not, while the two aldehydes *m*-hydroxybenzaldehyde and gentisaldehyde were always observed in such small amounts that they were never unambiguously identified. The effect of culture pH on the relative accumulation of various metabolites in the medium was noteworthy. Both qualitative tlc assays and quantitative [^{14}C]acetate incorporations into 6-methylsalicylic acid and patulin indicated that if the culture's pH was maintained at 4 or 5, the production of patulin decreased significantly while the levels of 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, and gentisyl alcohol increased.

The results of the pulse-labeling experiments are given in Figure 2 and Table II. As expected, [^{14}C]acetate was rapidly incorporated into 6-methylsalicylic acid (Figure 2a) and eventually patulin. [^{14}C]6-Methylsalicylic acid was also rapidly lost from the medium and in a short period of time the radiolabel appeared in *m*-hydroxybenzyl alcohol *via m*-cresol (Figure 2b). Similarly, [^{3}H]*m*-cresol was rapidly converted *via m*-hydroxybenzyl alcohol into gentisyl alcohol and patulin (Figure 2c). Separate longer term experiments with [^{3}H]toluquinol (Figure 2d) and [^{3}H]gentisyl alcohol (Figure 2e) indicated essentially no conversion of these metabolites to any other metabolites whatsoever. In a 10-hr nonkinetic experiment, [^{14}C]*m*-hydroxybenzyl alcohol was shown to be converted into gentisyl alcohol and patulin, while a short-term kinetic experiment indicated a *slow* conversion of [^{3}H]*m*-hydroxybenzyl alcohol into gentisyl alcohol and patulin (Figure 2f). [^{3}H]*m*-Hydroxybenzaldehyde was however rapidly incorporated into patulin and to a lesser extent into *m*-hydroxybenzyl alcohol and gentisyl alcohol (Figure 2g). In a further experiment [^{3}H]gentisaldehyde was rapidly converted to gentisyl alcohol as well as to patulin (Figure 2h). Finally [^{14}C]patulin did not appear to be converted into any of its precursors or to proceed to any new secondary metabolites (Figure 2i). Finally, two points concerning these results should be made. First, the extraction procedure for all of the tritium experiments was such that incorporation into the two acids, *m*-hydroxybenzoic acid and gentisic acid would not be detected. Second, since some of these pulse-labeling experiments were only carried out once, it is to be emphasized that it is the qualitative nature of the plots in Figure 2 rather that the absolute incorporations obtained which should be used in drawing conclusions from these results.

Discussion

From the results it may be concluded that in *P. urticae* a preferred route for patulin biosynthesis from 6-methylsalicylic acid does indeed exist, and that the pathway given in Figure 3 carries the predominant flux of acetate derived secondary metabolites in this fungus. Furthermore, examination of a *m*-hydroxybenzyl alcohol dehydrogenase from *P. urticae* (Forrester and Gaucher, 1972) has permitted the inclusion in Figure 3 of the cofactor requirement and equilibrium nature of the important dehydrogenase step of this pathway.

Although these conclusions appear straightforward, two important points must be taken into account when such a study is undertaken. Firstly, since the study utilized addition to and removal from extracellular metabolite pools, the cell permeability of all pertinent metabolites is of critical importance. In the majority of cases in which rapid uptake of added precursors followed by release of labeled products was obtained, permeability was obviously high. This is in agreement with the previously reported rapid uptake of 6-methylsalicylic acid by *P. urticae* in ~30–60 min (Bu'Lock *et al.*, 1966). However for the negative results obtained upon addition of labeled toluquinol, gentisyl alcohol, and patulin (Figure 2d,e,-i) the question of permeability had to be more closely examined. Hence examination of a centrifuged supernatant, prepared from sonicated, washed cells taken from the experiment in which [^{3}H]toluquinol was added (Figure 2d), indicated that greater than 10% of the added [^{3}H]toluquinol had been taken up by the cells. In addition all three relevant plots (Figure 2d,e,i) show an initial loss of radioactivity from the medium, followed, in the case of gentisyl alcohol and patulin additions, by a subsequent increase of radioactivity in the medium. This latter effect is undoubtedly due to the substantial synthesis and excretion of *nonradioactive* gentisyl alcohol and patulin.

A second point of concern in such a study is the addition of precursor to the extent of substantially increasing the normal pool size of this metabolite and hence perturbing the normal flux of the pathway. Bearing in mind the ~1-μmole/ml levels of the major metabolites such as 6-methylsalicylic acid and patulin, Table II shows that most precursor additions would not substantially increase pool sizes. However in the case of *m*-hydroxybenzaldehyde and gentisaldehyde the pool sizes probably were increased, since in both of these experiments (Figure 2g,h) incorporation into earlier metabolites on the pathway occurred.

Relative turnover rates of the various metabolites are indicated by the kinetic pulse labeling experiments plotted in Figure 2, while the accumulation of *P. urticae* metabolites noted in this study and previous studies provides information on the relative pool sizes of these metabolites. The agreement between these observations is excellent. Thus the intermediates undergoing obvious turnover, 6-methylsalicylic acid and *m*-hydroxybenzyl alcohol, exhibit the slowest turnovers in agreement with their large pool sizes while *m*-cresol, *m*-hydroxybenzaldehyde, and gentisaldehyde exhibit a very rapid turn-

over and small pool sizes. Similarly, the accumulating metabolites, gentisyl alcohol, patulin, and gentisic acid, are major end products and have large pool sizes while toluquinol and *m*-hydroxybenzoic acid are minor end products and have very small pool sizes. It is noteworthy here that, although neither incorporation into gentisic acid nor incorporation of gentisic acid was examined in this study, [^{14}C]acetate is incorporated into gentisic acid (Gatenbeck and Lönnroth, 1962) and [^{14}C]gentisic acid is not incorporated into patulin (Tanenbaum and Bassett, 1959).

The various incorporations into gentisyl alcohol merit further discussion. In the addition of [^{3}H]*m*-cresol (Figure 2c), gentisyl alcohol was a major product due to the hydroxylation of *m*-hydroxybenzyl alcohol, since toluquinol was not converted to gentisyl alcohol. The addition of [^{3}H]*m*-hydroxybenzaldehyde (Figure 2g) indicates some reversal of the pathway, to form gentisyl alcohol *via* *m*-hydroxybenzyl alcohol. Addition of [^{3}H]gentisaldehyde (Figure 2h) unexpectedly yielded substantial incorporation into gentisyl alcohol. Since no incorporation into *m*-hydroxybenzyl alcohol was observed and since aromatic hydroxylases are not usually reversible, it would appear that under abnormally high concentrations, gentisaldehyde can be directly reduced to gentisyl alcohol. Because added gentisyl alcohol was not metabolized this reduction must be essentially irreversible.

Reasons for the high per cent incorporations obtained in this study (Table II) compared to others are of interest. First, the metabolically isolated nature of this pathway allows little dilution of added precursors and secondly, the mycelial cells, of *P. urticae* appear to be exceedingly permeable to the precursors added. The previously reported almost exclusive incorporation of [^{14}C]acetate into 6-methylsalicylic acid and patulin (Tanenbaum and Bassett, 1959) bears this out. These incorporations represent minimum values since recovery of metabolites from the medium was not 100%. In addition all per cent incorporations were calculated without taking into account the possible loss of tritium, since it is not known whether the NIH shift[2] of protons (Guroff *et al.*, 1967) occurs in this case. Earlier results obtained when [^{2}H]*m*-cresol was incorporated into patulin (Scott and Yalpini, 1967) suggest that the NIH shift is not operating in this pathway. If this is so, loss of tritium in the hydroxylation of *m*-hydroxybenzyl alcohol and *m*-hydroxybenzaldehyde would result in a number of cases in larger per cent incorporations (*i.e.*, the value for *m*-hydroxybenzaldehyde in Table II would increase to 62%).

Finally in summary it is worthwhile to consider the principle factors which contributed to the elucidation of this pathway. To begin with the use of fermentor cultures meant that the time course of metabolic changes in *P. urticae* cultures were reproducible and the use of tritium-labeled precursors facilitated the preparation of a large number of precursors. However of most value was the use of *kinetic* pulse-labeling experiments which yielded far more information than the usual nonkinetic studies. Thus metabolites which are intermediates in the pathway could be readily differentiated from end products by the shape of the incorporation plots. These plots were also indicative of the relative turnover rates of the various metabolites and negative results obtained when end products were fed were not entirely worthless since they provided information on permeability and on the rate of synthesis of that end product. Lastly, the value of demonstrating and characterizing pertinent pathway enzymes is of unquestionable importance.

[2] NIH shift, an ortho shift of protons during aromatic hydroxylations.

Acknowledgments

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