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Biosynthesis of Patulin.[1] Dehydrogenase and Dioxygenase Enzymes of Penicillium Patulum

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Two aromatic dehydrogenases catalyzing the reversible conversions of gentisyl alcohol and *m*-hydroxybenzyl alcohol to their corresponding aldehydes have been partially purified. These partially purified dehydrogenases were shown to require NADPH. In the case of the gentisyl alcohol–gentisaldehyde interconversion, a 46-fold purification was achieved using POLYCLAR AT and DEAE-cellulose chromatography.

A cell-free system capable of converting gentisaldehyde to patulin was prepared with a pH optimum of 8.0. The system was dependent on O_2 and NADPH, was stimulated by ATP and inhibited by the Fe^{2+} chelators, α, α-dipyridyl and *o*-phenanthroline. These results suggest a dioxygenase mechanism for patulin synthesis from gentisaldehyde.

INTRODUCTION

Fermentations of the mold *Penicillium patulum*, like many other antibiotic producing fungi, have two distinct physiological phases, with a sharp transition between them. These phases correspond to the "logarithmic growth" and "stationary" phases of bacterial cultures and have been called "trophophase" and "idiophase" respectively (*4*, *5*).

In *P. patulum* the idiophase is characterized by the production of phenolic secondary metabolites, derived *via* the acetate-polymalonate pathway, and the conversion of some of these metabolites to the antibiotic patulin.

The first of the phenolic secondary metabolites involved in patulin biosynthesis is 6-methylsalicylic acid (6-MSA). 6-MSA is synthesized from acetyl-CoA and malonyl-CoA, thus forming the biosynthetic link between "primary" and "secondary" metabolites in this organism. 6-MSA was the first intermediate in patulin biosynthesis to be demonstrated by radioactive tracer experiments (*6*, *7*). Using this fact and structural comparisons of known metabolites isolated from *P. patulum*, several postulated biosynthetic schemes have been proposed (*4–7*).

Experimental evidence for the biosynthetic sequence leading from 6-MSA to patulin and the biosynthetic relationships of the phenolic secondary metabolites of *P. patulum* has been obtained by Scott et al. (*1–3*) by using various ^2H, ^{14}C and $^{14}C/^3H$ labeled intermediates in replacement cultures of the organism. Scheme 1 presents a summary of these results.

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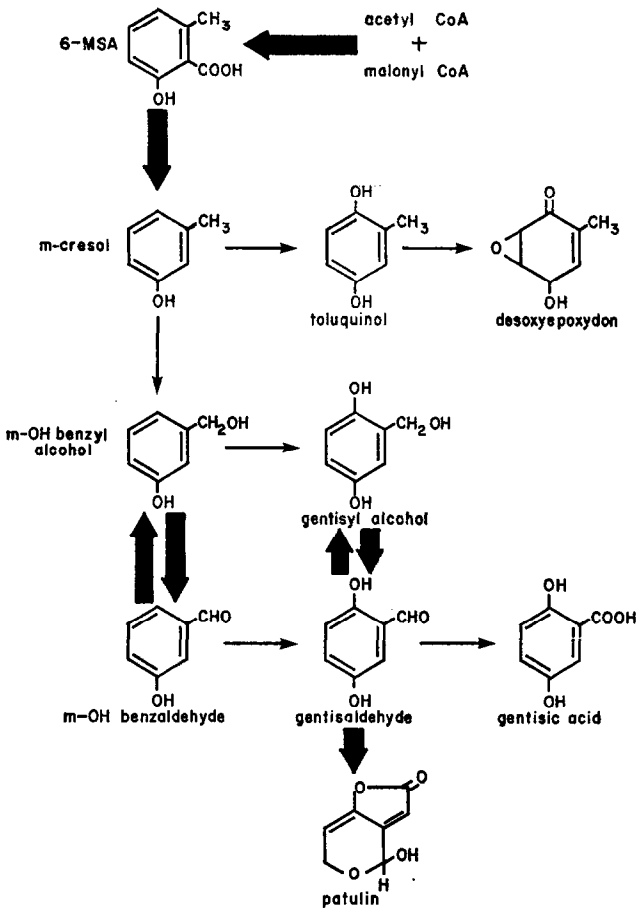


SCHEME I. The Pathway of Patulin Biosynthesis. (Darker arrows represent biosynthetic conversions which have been demonstrated enzymatically by this study or previous studies.)

Recently Gaucher has used a kinetic pulse-labeling technique with radio-labeled acetate and other metabolites to determine the preferred route of patulin biosynthesis (*8*). His results are in agreement with Scott et al. (*1–3*) except in the case of gentisyl alcohol which was not incorporated into patulin in Gaucher's studies (*8*), but which has been shown to be a precursor of patulin by Scott et al. (*1*).

There has been very little success in demonstrating at the enzyme level the individual biosynthetic transformations depicted in Scheme I. 6-MSA decarboxylase has been demonstrated in *P. patulum*, but little characterization of the enzyme was possible because of its instability (see note added in proof) (*10*). A crude cell-free system capable of reversible, NADPH dependent, *m*-hydroxybenzyl alcohol dehydrogenase activity has been detected (*9*). An earlier cell-free system capable of converting glucose, acetyl-CoA, or 6-MSA to patulin has been reported, but none of the later intermediates were shown to be substrates (*11*).

This study describes the partial purification and catalytic properties of an NADPH dependent aromatic dehydrogenase(s) from *P. patulin* capable of the reversible conver-

sion of gentisyl alcohol to gentisaldehyde, and *m*-hydroxybenzyl alcohol to *m*-hydroxybenzaldehyde. Also, the conversion of gentisaldehyde to patulin in a cell-free system is demonstrated, and the requirements for enzymatic activity are discussed in terms of the proposed mechanism of patulin formation from gentisaldehyde.

EXPERIMENTAL

Materials

Experimental materials were obtained from the following sources: ammonium sulphate (enzyme grade) from Mann Research Laboratories; bovine serum albumin from Sigma; EM-reagent pre-coated thin layer plates from Brinkman Instruments Inc.; NADH, NADPH, FAD, FMN from P–L Biochemicals; liquid scintillation counting was done using Bray's solution (*12*) in a Packard Tri Carb liquid scintillation counter; DEAE Cellulose DE23 from Whatman; Polyclar AT from GAF Corp., New York, New York. Patulin was isolated from the culture broth of *P. patulum* as described previously (*1*, *20*).

Protein Determination

Protein was determined by the Biuret method of Gornall, Bardwill and David (*13*) using bovine serum albumin as an internal standard, or by the method of Warburg and Christian (*14*).

Synthesis of [Ring Labeled -^{14}C] Gentisaldehyde

30.8 mg of [U-^{14}C] aniline-HCl containing 1.00 mCi was purchased from New England Nuclear and diluted to 500 mg with cold material. [U-^{14}C] Hydroquinone was prepared from the aniline–HCl by oxidation to benzoquinone with H_2SO_4 and $Na_2Cr_2O_7$ in the cold followed by reduction of the quinone with SO_2 gas (*15*). [^{14}C]gentisaldehyde was prepared from the hydroquinone by a Riemer–Tieman reaction (*16*), and purified on a silica gel column eluted with $CHCl_3$. The [^{14}C]gentisaldehyde (80 mg) was crystallized to constant activity from benzene (mp 99–100°C). The uv spectra and thin layer chromatography in benzene:dioxane:acetic acid 90:25:4 and $CHCl_3$:acetic acid 9:1 on silica plates showed it to be pure. Specific activity 0.088 mCi/m*M*. [1-^{14}C]*m*-Hydroxybenzaldehyde (specific activity 6.35×10^{-3} mCi/m*M*) was a gift of Dr. Lolita Zamir.

Synthesis of [^3H]Gentisaldehyde

[^{3}H]-Hydroquinone was prepared by the method of A. I. Scott et al. (*1*) using ^3H_2O for exchange instead of ^2H_2O. 160 mg Hydroquinone in 1.0 ml of ^3H_2O (100 mCi New England Nuclear) and a small piece of Na metal were sealed in an evacuated tube and heated in an oven at 130°C for 3 days. The tube was cooled and opened, the solution extracted with ether, and evaporated after the addition of 150 mg of hydroquinone. [^{3}H]-Gentisaldehyde was synthesized from [^{3}H]-hydroquinone by the method of Riemer–Tieman (*16*). 300 mg of [^{3}H]-hydroquinone was dissolved in a hot solution of 0.7 g NaOH in 1.1 ml H_2O under N_2. 0.9 ml of $CHCl_3$ was added and the reaction mixture refluxed for 1–1/4 hr. The reaction mixture was cooled, extracted with ether

and the ether dried and evaporated. The residue was titrated with $CHCl_3$ and chromatographed on a 36 g silica gel column eluted with $CHCl_3$. The [^{3}H]-gentisaldehyde was eluted as the second yellow band. The [^{3}H]-gentisaldehyde was crystallized 3 times from benzene. Yield: 28 mg. Specific activity: 2.31×10^9 dpm/m*M*.

Preparation of Polyclar AT

Polyclar AT (polyvinyl pyrrolidone) was prepared according to the method of Loomis (*17*). The powder was boiled for 10 min in 10% HCl and washed with distilled water until free of Cl^-. Then it was equilibrated with 0.05 *M* K-phosphate buffer pH 7.5, and the excess buffer decanted. The Polyclar AT was used as a very thick slurry.

Microbiological Methods

(*I*) *For Isolation of Aromatic Dehydrogenase(s)*

Penicillium patulum (NRRL2159A) was grown on Czapek-Dox medium containing 40 g dextrose, 3 g $NaNO_3$, 1g K_2HPO_4, 0.5 g $MgSO_4$–$7H_2O$, 0.5 g KCl, 0.02 g $FeSO_4$, 0.1 mg $ZnSO_4$ per liter water.

The spores from 1 subculture grown on Czapek–Dox agar slopes were transferred to two 250 ml conical flasks containing 100 ml of medium and grown at 180 rpm, 26–28°C on a rotary shaker for 7–10 days.

(*II*) *For Preparation of Patulin Synthesizing Cell Free System*

One slope of *P. fatulum* (NRRL 2159A) subculture was transferred to 1 liter of Czapek-Dox medium (prepared as described in the aromatic dehydrogenase experimental) in a 5 liter Pivotsky bottle and grown in still culture for 6–10 days at 26°C. Patulin production was measured by taking the uv spectra of an acidified sample of the growth media. A single peak with λ_{max} 276 nm (ε 13,350) indicated patulin production.

Assays of Enzyme Activity

(*I*) *Aromatic Dehydrogenase(s)*

Thin layer chromatography of radioactive products. The incubation mixture contained in a volume of 1.1 ml: 100 μM K-phosphate pH 7.5; 1.0 μM NADPH, 0.2 μM [U-^{14}C] gentisaldehyde (0.088 mCi/m*M*) and 0.5–2.0 mg enzyme. The incubations were stopped by the addition of 10 drops of acetone. 0.05 mg of gentisyl alcohol was added and the tubes extracted with 2×5 ml of ether. The ether extracts were evaporated by a stream of N_2 and chromatographed on silica gel thin layer plates (Brinkman F254, 20×20 cm and 0.2 mm thick) developed by benzene:dioxane:acetic acid (90:25:4). The gentisyl alcohol was detected by uv light, recovered, and assayed for radioactivity in Bray's solution (*12*). When [1-^{14}C]*m*-hydroxybenzaldehyde was used a substrate, carrier *m*-hydroxybenzyl alcohol was added. R_f values: gentisaldehyde 0.60, gentisyl alcohol 0.23, *m*-hydroxybenzaldehyde 0.69, *m*-hydroxybenzyl alcohol 0.45.

Optical estimation. The cuvette held in 1.1 ml: 100 μM K-phosphate pH 7.5, 0.25 μM NADPH, 0.5 μM gentisaldehyde or *m*-hydroxybenzaldehyde and enzyme. $d = 1.0$ cm, $T = 25$°C. After a stable baseline was achieved (about 1–2 min) the incubation was started by the addition of 0.5 μM gentisaldehyde (or *m*-hydroxybenzaldehyde) and the decrease in O.D. at 340 nm was followed.

Since both gentisaldehyde and *m*-hydroxybenzaldehyde absorb at 340 nm $\varepsilon_{340}^{pH\,7.5}$ 2.7×10^3 and $\varepsilon_{340}^{pH\,7.5}$ 1.6×10^3, respectively, the sum of the extinction coefficients was used in calculating the amount of NADPH (ε_{340} 6.22×10^3) oxidation from the change in optical density.

(II) Enzyme Assay of Patulin Synthesis

Thin layer chromatography of radioactive patulin. The incubation mixture contained in a volume of 1.0 ml: 0.2 μM [^{14}C]-gentisaldehyde (0.088 mCi/mM) or [^{3}H]-gentisaldehyde (1.04 mCi/mM) 1.0 μM NADPH, 1.0 μM ATP–Mg^{2+}, 1.0 μM patulin, 1.0–2.5 mg of protein in the enzyme extract, and H_2O. The incubations were started by the addition of enzyme and continued for 30 min at 28°C. The incubations were stopped by the addition of 3 drops of 60% perchloric acid, 0.25 mg of carrier patulin was added and the mixture extracted with 3 × 5 ml of ether (approximately 75% recovery of patulin). The ether extracts were combined, dried with Na_2SO_4, evaporated under a stream of N_2, and chromatographed on silica gel thin layer plates (Brinkman F254, 20 × 20 cm, 0.2 mm thick) developed with benzene: dioxane: acetic acid (90:25:4). The patulin was detected by uv light, recovered, and assayed for radioactivity in Bray's solution (*12*). R_f values: gentisaldehyde 0.60, patulin 0.38.

RESULTS

Purification Procedure for Aromatic Dehydrogenase(s)

All procedures were carried out at 4°C.

Crude extract. 8 day old mycelium was filtered, washed with 0.25 M NaCl solution, dried between filter papers and weighed. 30 g of cells were suspended in 100 ml of 0.05 M K-phosphate buffer, pH 7.6 containing $1 \times 10^{-3} M$ DTT and passed through a French press at 20,000–10,000 psi.

Polyclar AT treatment. To the French press extract 30 g (wet wt) of the Polyclar AT slurry was added and the suspension stirred for 10 min. The suspension was then centrifuged at 10,000*g* for 15 min and then at 100,000*g* for 1 hr. The supernatant was saved.

40–55% ammonium sulphate fractionation. Solid ammonium sulphate was added to the 100,000*g* Polyclar treated crude extract to 40% saturation. The extract was spun 45,000*g* for 10 min and the pellet discarded. Solid ammonium sulphate was again added to 55% saturation, and the centrifugation carried out as above. The resulting pellet was dissolved in 8.0 ml of 0.05M K-phosphate buffer pH 7.6 containing $1 \times 10^{-3} M$ DTT.

Dialysis. The 40–55% ammonium sulphate pellet solution was dialyzed for 3 hr against one change of buffer in the above K-phosphate buffer.

DEAE cellulose column. The DEAE column (2 × 15 cm) was equilibrated with 0.05M K-phosphate buffer pH 7.6 with 1×10^{-3} DTT. The dialyzed enzyme was placed on the column and eluted with a linear gradient 0.05M → 0.50M K-phosphate pH 7.6 with $1 \times 10^{-3} M$ DTT. 1.0 ml fractions were collected and the protein was monitored continuously by a Uvicord II analyzer. The flow rate was 60 ml/hr. The enzyme assay used to follow the purification was the optical estimation using gentisaldehyde and NADPH as the substrates.

Enzyme Purification

The French press method of preparing the crude extract was found to yield higher aromatic dehydrogenase activity than the following methods: grinding in a mortar with sand, grinding in a homogenizer with glass beads or using a Ten Brock homogenizer.

The Polyclar AT treatment not only resulted in a 2-fold purification of the enzyme, but also gave a 1.5 fold increase in total activity as shown in Table 1. The optimum ratio of Polyclar AT slurry to mycelium was shown by the results given in Table 2 to be 1/1 (wt/wt). The Polyclar AT also removed materials that were interfering with the Biuret protein assay.

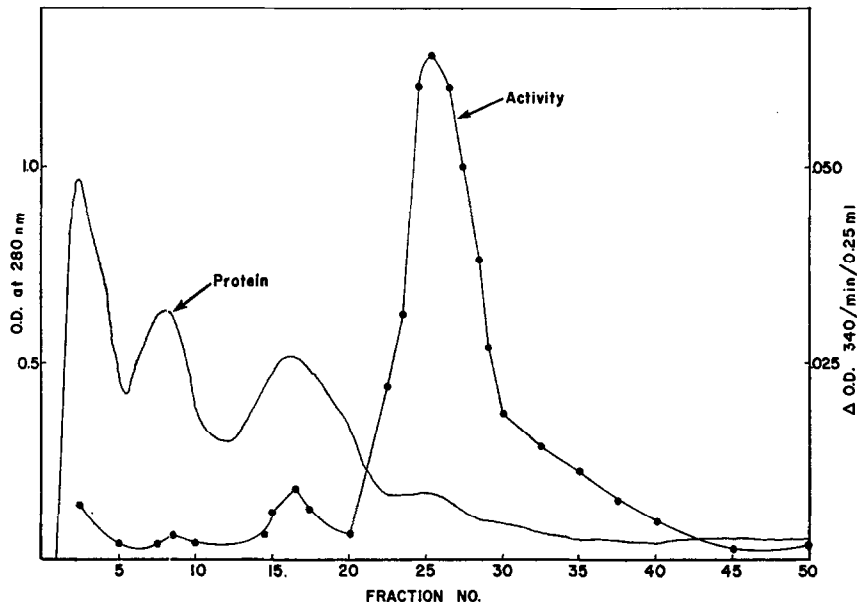


FIG. 1. DEAE Cellulose Chromatography of Dehydrogenases. The procedure is described in the text.

The results of the enzyme fractionation by DEAE cellulose chromatography are shown in Fig. 1. The enzyme activity was eluted in the fourth protein band at approximately 0.25*M* K-phosphate concentration.

The summary of the purification is given in Table 1. The enzyme was purified 46.4 fold with a total yield of 25%. The purified enzyme retained 50% of its activity after 48 hr.

Crystallization of gentisyl alcohol (tribenzoyl derivative) to constant radioactivity preparation of the enzyme. 10 day old *P. patulum* mycelium was homogenized with a Ten Brock homogenizer in 45 ml of 0.05*M* K-phosphate, $1 \times 10^{-3}M$ dithiothreitol buffer, pH 6.5. This homogenate was centrifuged 15 min at 10,000*g* and 1 hr at 105,000*g* producing a clear yellow solution used as the enzyme source.

Incubation and isolation of gentisyl alcohol. 10 tubes each containing 1.0 ml of enzyme (4.0 mg), 0.2 μM [^{14}C]gentisaldehyde, and 1.0 μM NADPH in a total volume of 1.1 ml

TABLE 1

SUMMARY OF ENZYME PURIFICATION[a]

Procedure	Protein mg/ml	Total Protein mg	Specific Activity mU/mg	Total Activity mU	Rel. Yield %	Purification-fold
Crude 100,000*g* (not treated with Polyclar before centrifugation)	3.8	328	3.48	1140	100	—
Polyclar AT Treated Crude 100,000*g*	2.9	236	7.16	1690	148	2.0
40–55% $(NH_4)_2SO_4$	6.75	54	12.7	686	60	3.7
Dialysis	6.5	54	13.5	729	64	3.9
DEAE Cellulose column fractions #24–29	0.16	2.8	161	451	25	46.4

[a] One milliunit (mU) of enzyme activity = 1n*M* NADPH oxidized/min. Protein was estimated by the Biuret (*13*) method or the method of Warburg and Christian (*14*) for the column fractions.

TABLE 2

TESTING POLYCLAR AT CONCENTRATIONS[a]

wt of Polyclar AT slurry / wt of mycelium	mg protein	spec. act. (mU/mg)	total activity (mU)
0	39.0	3.64	142
0.5	35.0	4.06	142
1.0	29.4	7.28	214
1.5	18.2	6.38	116

[a] The crude French press extract was prepared from 16 g of mycelium and divided into 4 equal volumes. Assuming that 4 g of mycelium was used for each portion of crude extract, the appropriate amount of Polyclar AT slurry was added and the 10 min stirring followed by centrifugation carried out as described in the enzyme preparation. The assay used was the optical method with gentisaldehyde as substrate.

were incubated at 28°C for 1 hr. Just after addition of 10 drops of cold acetone, 0.025 mg carrier gentisyl alcohol was added to each tube. The tubes were extracted and chromatographed as described in the TLC assay method. The gentisyl alcohol band was recovered and eluted by stirring with 200 ml ether for 4 hr. Total recovery 7500 dpm. 20 mg additional carrier gentisyl alcohol was added to the ether, and the ether was dried, evaporated on a rotary evaporator and placed on a vacuum line overnight.

Preparation of the tribenzoyl derivative of gentisyl alcohol. Since a small quantity of gentisyl alcohol is hard to crystallize, the more crystalline tribenzoyl derivative was prepared (*18*).

The gentisyl alcohol residue was dissolved in 0.5 ml pyridine and cooled in an ice bath. 0.5 ml benzoyl chloride was added and the reaction allowed to stand for 3 hr at 0°C. Water was added forming a yellow oily ppt., which was repeatedly washed with water until it became a solid. It was recrystallized 2 times from methanol before being counted (Table 3).

TABLE 3

CRYSTALLIZATION OF GENTISYL ALCOHOL TO CONSTANT ACTIVITY

No. of crystallizations of tribenzoyl derivative	wt of tribenzoyl sample (mg)	dpm in sample	background dpm	dpm/mg in gentisyl alcohol
3	1.587	179	30	309
4	1.344	157	30	314
5	2.032	228	30	321
6	2.405	263	30	319
7	2.205	243	30	319

Formula used for calculation of dpm/mg in gentisyl alcohol (G.Alc):

$$\frac{\text{MW tribenzoyl G. Alc}}{\text{MW G. Alc.}} \times \frac{\text{dpm above bkg. in sample}}{\text{wt sample}} = \text{dpm/mg G. Alc.}$$

7500 dpm eluted from gentisyl alcohol band
22.5 mg carrier added ∴ expected 333 dpm/mg G. Alc.

Crystallization of meta-hydroxybenzyl alcohol to constant activity preparation of enzyme. 8 day old *P. patulum* cells weighing 13.5 g were disrupted in 50 ml of K-phosphate $1 \times 10^{-3} M$ DTT buffer, pH 7.5, by passing through a French press at 20,000 psi. The 105,000*g* 1 hr supernatant was used as the enzyme source.

Incubation and isolation of meta-hydroxybenzyl alcohol. 10 incubations containing in 1.1 ml volume, 1 ml (4.2 mg) of enzyme solution, 1.0 μM NADPH were used for the isolation of *m*-hydroxybenzyl alcohol. The incubations were at 30°C for 30 min. The incubations were terminated, extracted, and chromatographed as described in the TLC assay method. The *m*-hydroxybenzyl alcohol band was recovered, eluted with ether, 20 mg carrier *m*-hydroxybenzyl alcohol added, evaporated under vacuum and crystallized to constant activity from 1,2-dichloroethane (Table 4).

TABLE 4

CRYSTALLIZATION OF META-HYDROXYBENZYL ALCOHOL TO CONSTANT ACTIVITY[a]

Crystallization Number	Wt Sample in mg	dpm/mg in *m*-hydroxybenzyl alcohol
2	1.472	2412
3	1.039	2261
4	2.744	2304
5	1.211	2286

[a] 53,200 dpm eluted from *m*-hydroxybenzyl alcohol band.
22.5 mg carrier *m*-hydroxybenzyl alcohol added ∴ expect 2360 dpm/mg.

pH Dependence of the Dehydrogenase Activity

Figure 2 shows the pH dependence of the dehydrogenase activity. The pH maximum is about pH 7.5 with the enzyme activity falling off faster on the acidic side than on the basic side of the pH maximum. Experiments done using *m*-hydroxybenzaldehyde as substrate yielded an identical curve.

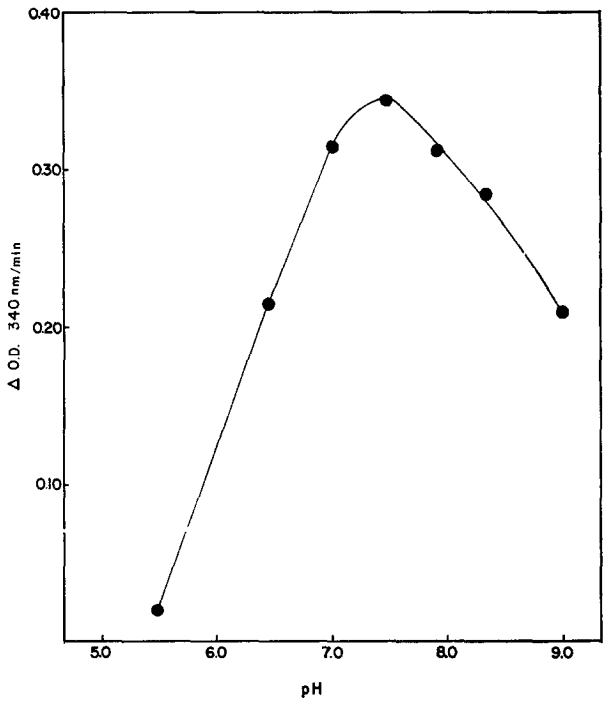


FIG. 2. pH Dependence of Dehydrogenase activity. The pH of the incubation mixture was adjusted with 0.1*M* HCl or 0.1*M* NaOH. The assays were performed by the optical method.

Substrate Specificity of the Partially Purified Dehydrogenase(s)

Table 5 shows the reaction rates of the purified enzyme(s) with various combinations of substrates and cofactors. The rate of *m*-hydroxybenzaldehyde reduction is 7 times that of gentisaldehyde reduction. Substitution of NADH for NADPH resulted in a 63% reduction in rate of the gentisaldehyde to gentisyl alcohol conversion, and a 68% reduction in the *m*-hydroxybenzaldehyde to *m*-hydroxybenzyl alcohol conversion. The rate of the alcohol → aldehyde is 10.7 times slower in the gentisyl alcohol → aldehyde case, and 8.1 times slower in the *m*-hydroxybenzyl alcohol → aldehyde conversion. The dehydrogenase(s) appear(s) to prefer NADPH and be reversible favoring the nonphysiological conversion (aldehyde → alcohol).

Preparation of a Patulin Synthesizing Cell Free System

All procedures were carried out at 4°C. The mycelial pad was filtered, washed with cold 0.25 *M* NaCl, and dried between filter papers. The mycelium was mixed with a 4-fold w/v excess of buffer (0.1*M* Tris pH 8.0, $2 \times 10^{-3}M$ EDTA, $1 \times 10^{-3}M$ DTT,

TABLE 5

REACTION RATES OF VARIOUS SUBSTRATES AND THE PURIFIED DEHYDROGENASE ENZYME(S)[a]

Substrate	Cofactor	Relative rate in mU
Gentisaldehyde	NADPH	11
Gentisaldehyde	NADH	4
m-hydroxybenzaldehyde	NADPH	75
m-hydroxybenzaldehyde	NADH	24
Gentisyl Alcohol	$NADP^+$	1
m-hydroxybenzyl alcohol	$NADP^+$	9

[a] Incubation was done with the DEAE cellulose purified enzyme (0.072 mg) following the optical assay procedure with 0.5 μM substrates and 0.25 μM cofactors in each case. One milliunit of activity = conversion of 1nM substrate/min.

3 mg/ml BSA, in 15% glycerine) and macerated for 30 sec (speed 6) in a Sorvall omnimixer. The macerated mycelium was then passed through a French press at 15,000 psi and the extract centrifuged first at 10,000*g* for 15 min, then at 100,000*g* for 60 min. The 100,000*g* supernatant was used as the enzyme source.

Crystallization of Patulin to Constant Radioactivity

The enzyme was prepared as described above except the 10,000*g* supernatant was used as enzyme source. Twenty incubations each containing 1.0 ml:0.5 ml enzyme extract, 0.2 μM [^{14}C]-gentisaldehyde, 1.0 μM NADPH, 1.0 μM patulin, were incubated for 1 hr at 28°C. The incubations were stopped, extracted and chromatographed as described in the enzyme assay procedure. The patulin bands from the TLC were combined and stirred overnight with 250 ml of ether containing 20 mg of carrier patulin. The ether extract contained 44,000 dpm. The ether was dried, filtered and evaporated. An additional 20 mg of patulin was added and the patulin crystallized from ether. The results are shown in Table 6.

TABLE 6

CRYSTALLIZATION OF PATULIN TO CONSTANT RADIOACTIVITY

Crystallization No.	Weight of sample (mg)	dpm/mg patulin
1	1.189	674
2	1.038	660
3	0.475	657
4	1.350	663

Intracellular Location of Enzyme Activity

The intracellular location of the enzyme(s) that synthesize patulin was examined by assaying the supernatants of the French press extract after centrifugation at various

TABLE 7

INTRACELLULAR LOCATION OF PATULIN SYNTHESIZING ENZYME(S)[a]

Fraction	mg of protein in assay	Substrates	n*M* Patulin in 30 min
1,000*g* supernatant	10.0	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) —	14.4
1,000*g* supernatant	10.0	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) ATP 1.0 μM	26.9
18,000*g* supernatant	6.8	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) —	16.7
18,000*g* supernatant	6.8	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) ATP 1.0 μM	24.3
100,000*g* supernatant	2.5	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) —	14.2
100,000*g* supernatant	2.5	^{14}C Gentisaldehyde (0.2 μM) NADPH (1.0 μM) ATP 1.0 μM	22.3

[a] Each assay contained in a total volume of 1.0 ml:0.5 ml of one of the above supernates, the indicated substrates and cofactors, and 1.0 μM of patulin. The incubations and assay of radioactivity were performed as described in the experimental section.

times and speeds. The supernatants assayed were: the 1000*g*, 5 min supernatant which should contain the microsomal, mitochondrial and soluble cellular fractions; the 18,000*g*, 15 min supernatant which should contain the microsomal and soluble fractions; and the 100,000*g*, 60 min supernatant which should contain only the soluble fraction (*19*). The results of the assays are shown in Table 7. Since equal volumes of the enzyme extract had the same activity even after the highest speed centrifugation, the patulin synthesizing enzyme(s) are most likely located in the soluble portion of the cell rather than in any particulate fraction. The 2-fold stimulation of patulin synthesis by ATP was also constant in each supernatant tested, indicating that this stimulation is due to some soluble rather than particulate component(s).

Time Dependence of Patulin Synthesis

Figure 3 shows the amount of [^{14}C]-patulin recovered from incubations of the cell free extract stopped at various times. The formation of [^{14}C]-patulin is linear for about 30 min, reaches a maximum amount at about 60 min, and thereafter the amount of [^{14}C]-patulin recovered decreases.

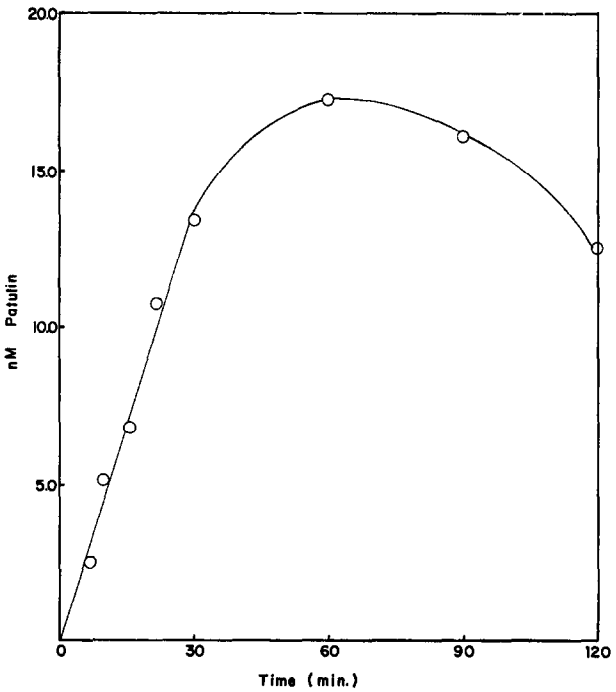


FIG. 3. Time Dependence of Patulin Synthesis. The enzyme assays were performed as described in the experimental procedure using 2.5 mg of protein (100,000*g* supernatant) except the time was varied and ATP was not added.

Protein Dependence of Patulin Synthesis

Figure 4 shows the effect of protein concentration on patulin synthesis. The formation of patulin was linear up to 3.0 mg/ml under conditions described in the assay procedure.

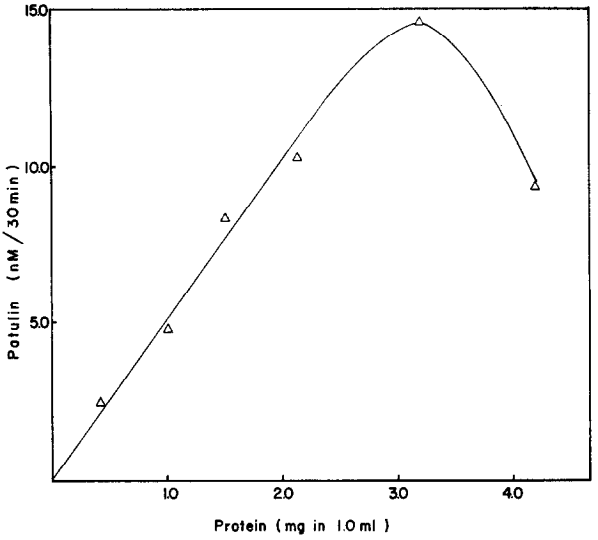


FIG. 4. Protein Dependence of Patulin Synthesis. Assays were performed as described in the text varying the protein concentration over the range shown. ATP was not added to the incubations.

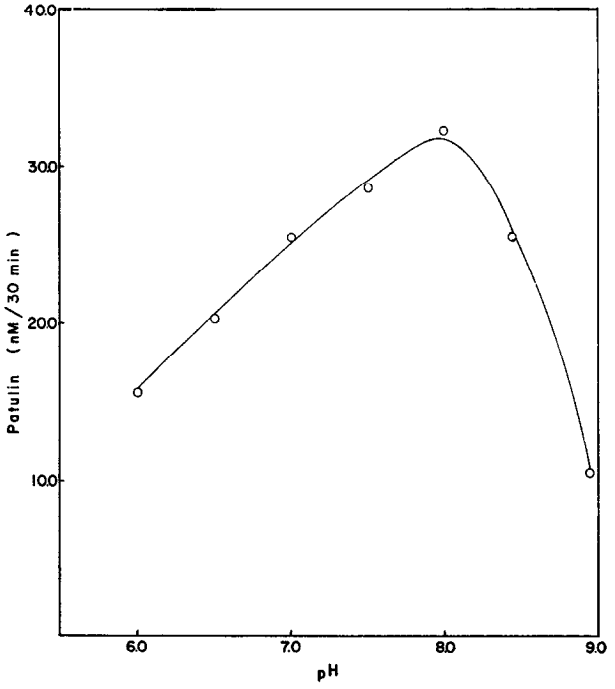


FIG. 5. pH Dependence of Patulin Synthesis. The pH of the incubation mixture was adjusted with 0.1*M* HCl or 0.1*M* NaOH. The reaction was started by the addition of substrates (0.2 μM ^{14}C gentisaldehyde, 1.0 μM NADPH, 1.0 μM ATP) and assayed as described in the experimental methods.

At higher protein concentrations there was a decrease in rate. The nature of this effect has not been investigated but all studies were carried out with levels of protein concentration which were in the linear part of the curve.

pH Dependence of Patulin Synthesis

The pH dependence of patulin synthesis is shown in Fig. 5. The maximum is near pH 8.0 with the rate of synthesis falling off faster on the basic side of the maximum than on the acidic side of the maximum.

Stability of Patulin at Various pH's

Figure 6 shows the stability of patulin in Tris buffers of various pH's. Patulin undergoes decomposition in basic solution, the more basic the solution the faster the rate of

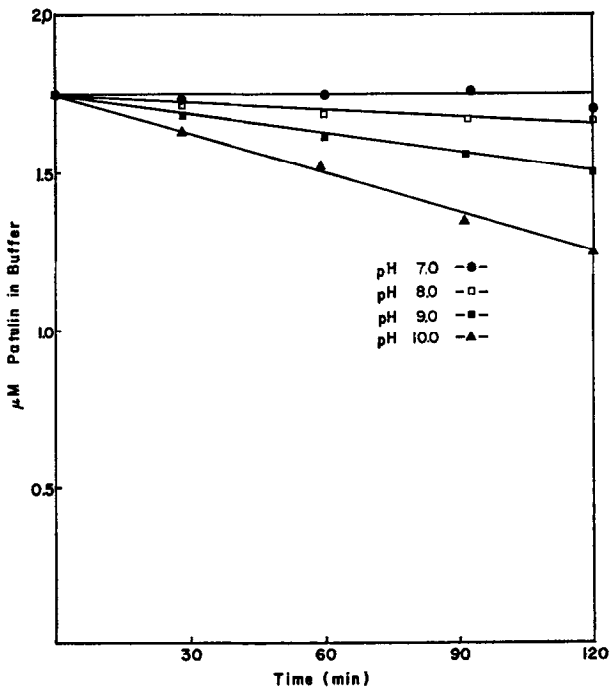


FIG. 6. Stability of Patulin at Various pH's. 0.025 ml of a 10 mg/ml solution of patulin was added to 1.0 ml of 0.1*N* tris–HCl buffer of different pH's and incubated at 28°C. 0.10 ml of the buffer–patulin solution was assayed at various times by placing it in 2.9 ml of 0.1*N* HCl in a uv cell. The O.D. at 276 nm was measured and the amount of patulin remaining in the buffer was calculated using 13,350 as the molar extinction coefficient of patulin at 276 nm.

decay. Patulin in strong base (1*N* NaOH) shows a spectral shift from 276 nm to 286 nm. Immediate reacidification of the basic solution results in approximately a 60% reduction in absorption at 276 nm (corrected for dilution) and the appearance of a peak at 232 nm. The nature of the basic decomposition product was not investigated.

Requirements for the Cell-Free Synthesis of Patulin

Table 8 shows the results of omitting various components of the patulin synthesizing cell-free system. The omission of NADPH or O_2 results in a drastic reduction of patulin synthesis. ATP addition results in a two-fold stimulation of patulin synthesis over the complete system without ATP. The omission of carrier patulin results in about 40% reduction in the amount of [^{3}H]-patulin recovered after the 30 min incubation. The Fe^{2+} chelating agents α,α-dipyridyl and *o*-phenanthroline at $10^{-3}M$ concentration had a very strong inhibitory effect on the cell-free system.

TABLE 8

REQUIREMENTS FOR THE CELL FREE SYNTHESIS OF PATULIN[a]

Incubation	n*M* Patulin/30 min	% of Complete System
complete	32.0	100
–ATP	16.3	51
—NADPH	0.8	3
–Patulin	18.5	58
$-O_2$	6.0	19
Complete + α,α-dipyridyl ($10^{-3}M$)	5.7	18
Complete + *o*-phenanthroline ($10^{-3}M$)	0.3	1

[a] The complete system contained in a final volume of 1.0 ml; 0.2 μ*M* [^{3}H]-gentisaldehyde, 1.0 μ*M* NADPH, 1.0 μ*M* Mg^{2+}–ATP, 1.0 μ*M* patulin, and 3 mg protein. The incubations were done at 28°C for 30 min and were assayed as described in the text.

The incubations minus O_2 were done in Thunberg tubes which were evacuated and filled with N_2 three times before the incubations were started by mixing the substrates and enzyme compartments.

The inhibition studies with α,α-dipyridyl and *o*-phenanthroline were done by preincubating inhibitor with enzyme, patulin, and ATP 10 min at 28°C and starting the incubation by the addition of [^{3}H]-gentisaldehyde and NADPH. The final concentration of α,α-dipyridyl and *o*-phenanthroline was $10^{-3}M$.

Testing Various Cofactors

Table 9 shows the effect of various cofactors in different combinations on the ability of the cell-free system to synthesize patulin. ATP gives a 2-fold stimulation over NADPH alone. The cell-free system is most active using NADPH, but shows appreciable activity with $NADP^+$ or NADH giving 66% and 82%, respectively, of the activity with NADPH. FAD and FMN show almost no activity alone and when used with NADPH gives no stimulation of activity.

Effect of Metal Ions

Table 10 shows the results of adding various metal ions to the cell-free system. Mn^{2+} and Mg^{2+} result in a slight stimulation of activity, whereas Fe^{2+} has a definite inhibitory effect.

Meta-hydroxybenzaldehyde as Substrate

This cell-free system was incapable of making patulin using [^{14}C]-metahydroxybenzaldehyde as substrate. Radioactivity in the patulin band in the TLC assay system

TABLE 9

TESTING VARIOUS COFACTORS[a]

Cofactors in Incubation	n*M* Patulin/30 min	% of Control
NADPH, ATP	20.8	100
NADPH	10.2	49
$NADP^+$, ATP	14.0	67
$NADP^+$	6.8	33
NADPH, FMN	7.4	36
NADPH, FAD	7.4	36
FMN	0.9	4
FAD	0.9	4
NADH	8.4	40

[a] Incubations contained in a final volume of 1.0 ml; 0.2 μM [^{3}H]-gentisaldehyde, 1.0 μM patulin, 2.1 mg protein and 1.0 μM of the various cofactors as given in the table. Incubations were assayed as described in the text.

TABLE 10

EFFECT OF METAL IONS[a]

Incubation	n*M* Patulin/30 min	% of Control
Control	13.8	100
+ Fe^{2+}	8.5	61
+ Mn^{2+}	14.7	107
+ Mg^{2+}	14.9	108
+ Fe^{2+} + Mg^{2+}	9.1	66

[a] Incubations contained in a final volume of 1.0 ml; 0.2 μM [^{14}C]-gentisaldehyde, 1.0 μM NADPH, 1.0 μM patulin, 2.5 mg protein, and $10^{-3} M$ final concentration of metal ions. Incubations were assayed as described in the text.

did not remain after crystallization of the eluted patulin. The radioactivity was due to streaking of the [^{14}C]-*m*-hydroxybenzyl alcohol band which is formed from [^{14}C]-*m*-hydroxybenzaldehyde in this cell-free system. (Patulin R_f, 0.38; *m*-hydroxybenzyl alcohol R_f, 0.45).

Inducibility of Patulin Synthesizing Enzyme(s)

The level of activity in the patulin synthesizing cell-free system could not be raised by growing the cells with any of the following precursors of patulin added to the growth media: 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde, gentisyl alcohol, or gentisaldehyde. Concentrations of precursors ranging from $10^{-6} M$ to $10^{-2} M$, and times of exposure ranging from one to 14 days were used with no positive increase in enzymic activity.

DISCUSSION

This study describes the partial purification, and catalytic properties of enzymes catalyzing three of the biosynthetic reactions involved in patulin formation in *Penicillium patulum*. The enzymes and biosynthetic reactions examined were an aromatic dehydrogenase (or dehydrogenases) capable of the reversible interconversion of *m*-hydroxybenzyl alcohol to *m*-hydroxybenzaldehyde, and gentisyl alcohol to gentisaldehyde to patulin.

The purification of the dehydrogenase(s) was largely due to the ability of Polyclar AT to stabilize the crude extract, enabling further fractionations to be carried out. As shown in Table 2 Polyclar AT treatment not only resulted in a purification of the crude extract, but also in an increase in total activity. This indicates the removal of some inhibitory materials by the Polyclar AT, which is a commercially available form of insoluble crosslinked polyvinyl pyrrolidone (*21*). Loomis applied the use of Polyclar AT to the isolation of plant enzymes since the presence of plant phenolic compounds often hinders the isolation of active enzymes from plant tissues (*17*) (*22*).

Because of the large amount of phenolic compounds present, isolation of enzymes from the fungus *Penicillium patulum* is beset with many of the same problems encountered in the isolation of plant enzymes. Therefore, Polyclar AT was used to aid in the isolation of active enzymes from *P. patulum* extracts.

The partially purified enzyme(s) eluted from the DEAE cellulose column was capable of catalyzing the redox equilibrium between gentisaldehyde and gentisyl alcohol and between *m*-hydroxybenzaldehyde and *m*-hydroxybenzyl alcohol. The gentisaldehyde → alcohol conversion was shown by the isolation of [^{14}C] gentisyl alcohol formed enzymatically from [^{14}C] gentisaldehyde, and the crystallization to constant radioactivity of the crystalline tribenzoyl derivative of the [^{14}C] gentisyl alcohol (Table 3). The *m*-hydroxybenzaldehyde → alcohol conversion was likewise shown by the isolation and crystallization constant radioactivity of [^{14}C] *m*-hydroxybenzyl alcohol formed enzymatically from [^{14}C] *m*-hydroxybenzaldehyde (Table 4). The reverse (alcohol → aldehyde) conversion was shown by the ability of both gentisyl alcohol and *m*-hydroxybenzyl alcohol to reduce $NADPH^+$ (Table 5) in the presence of the enzyme(s).

The dehydrogenase(s) prefers $NADPH^-$ rather than NADH as shown by the 3-fold increase in rate with gentisaldehyde or *m*-hydroxy-benzaldehyde as substrate. The aldehyde to alcohol conversion is favored in the enzymatic reaction, the rate being 10.7 times faster in the *m*-hydroxy-benzaldehyde → alcohol conversion, and 8.1 times faster in the gentisaldehyde → alcohol conversion over their corresponding reverse reactions. Considered in terms of Scheme 1, the above result means that the nonphysiological reaction is favored *in vitro*. Since the aldehydes are closer in the biosynthetic pathway than their corresponding alcohols to the end metabolite patulin, the *in vivo* implied direction is from alcohol to aldehyde to patulin. However, in the gentisaldehyde to gentisyl alcohol case, the *in vitro* favored reaction has been shown to occur *in vivo* (1), indicating that this enzyme is reversible under the correct conditions even in the living organism.

The partially purified aromatic dehydrogenase reported here is similar in some respects to the *m*-hydroxybenzyl alcohol dehydrogenase reported by Forrester and

Gaucher (*9*). Both dehydrogenases have a pH maximum at 7.6, and are precipitated in approximately the same ammonium sulphate fraction. Both are NADPH dependent, the enzyme(s) reported here being 30% and Gaucher's enzyme 12% as active with NADH and *m*-hydroxybenzaldehyde as substrates. Finally, both dehydrogenases are reversible with the aldehyde to alcohol conversion favored.

However, there is a major difference between the two dehydrogenase preparations in their ability to use gentisaldehyde as a substrate. The preparation reported here catalyzes the reversible conversion of gentisaldehyde to gentisyl alcohol, where as Gaucher's dehydrogenase was inhibited by this substrate. The results presented above demonstrate the *in vitro* reversible enzymatic conversion of gentisyl alcohol and gentisaldehyde, which previously had been shown to occur *in vivo* in replacement cultures of *P. patulum* (*1*).

Although the dehydrogenase activity emerged as a single peak from the DEAE cellulose column, there is no conclusive evidence to determine if the activity is due to a single enzyme with low substrate specificity, or is due to two dehydrogenases, one specific for *m*-hydroxybenzaldehyde and one specific for gentisaldehyde, which co-purify. However, since Gaucher's crude extract, prepared from the same organism used in this study, contained only *m*-hydroxybenzaldehyde activity, the latter possibility seems the most attractive.

The fact that the partially purified dehydrogenases convert *m*-hydroxybenzyl alcohol to *m*-hydroxybenzaldehyde nine times faster than gentisyl alcohol to gentisaldehyde suggests that the preferred pathway of patulin biosynthesis is *m*-hydroxybenzyl alcohol → *m*-hydroxybenzaldehyde → gentisaldehyde → patulin. This pathway is also suggested by short term kinetic feeding studies on whole cells (*8*).

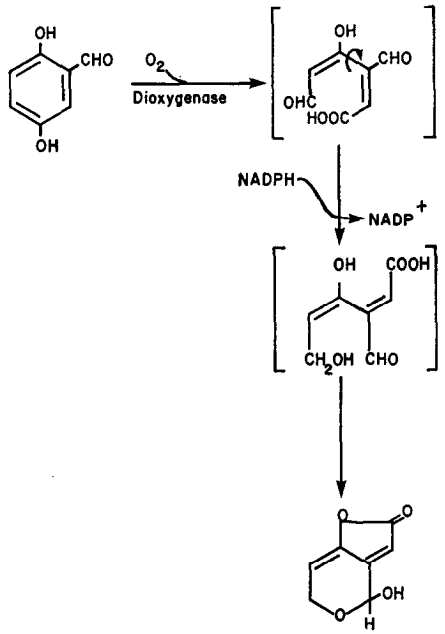
On the other hand, the gentisyl alcohol to gentisaldehyde conversion rate in the crude extract is 0.35 n*M*/min/mg of crude extract protein (from Table 1; assuming the alcohol to aldehyde rate is 10.7 times slower than the reverse rate as in Table 5) and is the same order of magnitude as the gentisaldehyde to patulin conversion rate of 0.47 n*M*/min/mg of crude extract protein (Table 7). The extrapolation of *in vitro* enzyme rates to *in vivo* metabolic flux is beset with many pitfalls such as separation of subunits, protease activity, and contact with denaturing agents. But if the intracellular rates of conversion of gentisyl alcohol to gentisaldehyde and gentisaldehyde to patulin are similar to the rates in crude extracts, then the gentisyl dehydrogenase activity is high enough to account for patulin biosynthesis. Therefore, it is quite possible that hitherto undetected mono-oxygenases converting *m*-hydroxybenzyl alcohol to gentisyl alcohol, and *m*-hydroxybenzaldehyde to gentisaldehyde, rather than the dehydrogenase activities, could determine the preferred *in vivo* pathway.

The isolation of a cell-free system capable of patulin synthesis proved to be very difficult due to the extreme liability of the enzymes involved. The problem was compounded by the instability of the product (patulin) above pH 7.0, making it necessary to add carrier to trap the enzymatically formed radioactive product. Even using the most successful method, one out of five preparations was still inactive for no apparent reason.

The instability of patulin at pH 8.0 probably accounts for the decrease in amount of patulin isolated from the enzymatic reaction at times greater than 60 min (Fig. 3). This product instability could also account for the rapid decrease in activity on the alkaline side of the pH maximum (Fig. 4).

The proposed mechanism of patulin biosynthesis from gentisaldehyde involving an oxidative cleavage of gentisaldehyde followed by a molecular rearrangement to form patulin, has been widely accepted without any experimental proof. An expanded version of this mechanism supported by the experimental results of this study is presented in Scheme 2 with the proposed intermediates shown in brackets.

The first proposed step in the reaction involves the cleavage of gentisaldehyde at the 4,5 position by a dioxygenase. Dioxygenases catalyze the incorporation of both atoms



SCHEME II. The Proposed Mechanism of Patulin Biosynthesis from Gentisaldehyde. [Brackets indicate proposed intermediates.]

of molecular oxygen into a molecule of substrate (*23–25*). A major reaction catalyzed by dioxygenases is the cleavage of an aromatic carbon-carbon bond (*26–31*).

Typical dioxygenases which have been obtained in crystalline form usually contain either Fe^{2+} or Fe^{3+} as the sole cofactor (*25*). It is assumed that the iron plays an active role in the catalytic process by the activation of oxygen as well as the substrate. Fe^{2+} containing enzymes are inhibited by the Fe^{2+} chelating agents α,α-dipyridyl and *o*-phenanthroline, although the mechanism of this inhibition is not entirely clear (*32–34*).

The proposal (Scheme 2) that the first step of patulin formation involves a dioxygenase-mediated cleavage of gentisaldehyde at the 4,5 position is supported by the following experimental evidence presented in this study. 1) The reaction is dependent on molecular oxygen (Table 8) as in all dioxygenase catalyzed reactions. 2) The enzyme activity is located in the soluble portion of the cell (Table 7), implying a dioxygenase rather than a mono-oxygenase activity. Mono-oxygenase in eukaryotic organisms are most often found in the particulate fraction (*33*) where as dioxygenases are soluble enzymes. 3) The enzymatic activity is inhibited by the Fe^{2+} chelating agents, α,α-

dipyridyl and *o*-phenanthroline which suggest the involvement of ferrous iron as a cofactor as is the case with many dioxygenases (*25*). However, added Fe^{2+} does not stimulate the activity suggesting that Fe^{2+} is tightly bound.

The complete inhibition of patulin formation by the cell-free system in the absence of NADPH is evidence for the reduction step included in the proposed mechanism. Earlier evidence supporting the reduction step was the finding that optically active patulin could be isolated from replacement cultures in which deuterated *m*-cresol, *m*-hydroxybenzyl alcohol, or gentisaldehyde served as the precursor to [2,5 ^{2}H]patulin (*1*). Since C_5 of patulin is prochiral, the presence of ^{2}H at this position, which is stereospecifically reduced from aldehyde to alcohol by a reductase in the proposed mechanism, allows the isolation of a single diastereomer. The reductase activity of the cell-free system prefers NADPH—rather than NADH—as a cofactor (Table 9). The rather high rate of activity with $NADP^+$ is probably due to the ability of the crude system to generate NADPH.

This study of the cell-free system has not clarified the order or mechanism of the lactonization and hemiacetal formation involved in the final steps of patulin formation. The two-fold stimulation of activity by ATP (Tables 7–9) may be related to these later steps, but also it may be just a general nonspecific energy requirement of some component(s) of the cell-free system. Any mechanism that is proposed concerning the hemiacetal formation must assume *either* that the final hemiacetal closure is non-specific *or* that racemization occurs rather easily to explain the fact that patulin is isolated as a racemate from culture broths.

Another experimental observation that requires more study at the enzyme level is the lack of incorporation of the side chain protons of *m*-hydroxybenzyl alcohol, gentisyl alcohol, and gentisaldehyde into the hemiacetal proton at C_1 of patulin (1). The role of patulin lactone in this process, as well as the nature of the open chain intermediates are other details of the mechanism of patulin biosynthesis that need elucidation at the enzyme level.

This cell-free study does allow one to distinguish between the mechanism presented here and another proposed mechanism in which *m*-hydroxybenzaldehyde is the immediate precursor of patulin (1). *m*-Hydroxybenzaldehyde was *not* converted to patulin with this cell-free system not under a variety of conditions modifying this system. Therefore, *m*-hydroxybenzaldehyde is most likely incorporated into patulin *via* gentisaldehyde and not directly.

The above result emphasizes the fact that a type of biosynthetic conversion involved in the secondary metabolism of *P. patulum*, which has not been shown at the enzyme level, is the mono-oxygenase or hydroxylase catalyzed reaction. Scheme 1 implies that mono-oxygenases must catalyze the conversions of *m*-cresol to *m*-hydroxybenzyl alcohol, *m*-hydroxybenzyl alcohol to gentisyl alcohol, and *m*-hydroxybenzaldehyde to gentisaldehyde, but so far none of these conversions have been shown in cell-free systems.

One final result that is worthy of discussion, although negative, concerns the inducibility of the patulin cell-free system. If the *in vitro* assay is a reliable indication of *in vivo* metabolic activity (and admittedly the interpretation of synthetase assays in cell extracts are subject to unsuspected complications) then the lack of any increase in patulin synthesizing activity of extracts of cells grown under various conditions of

exposure to patulin precursors indicates the non-inducibility of these enzymes by precursors. This observation is complemented by the results of Forrester and Gaucher (*9*), who could *not* demonstrate the promotion of *m*-hydroxybenzyl alcohol dehydrogenase activity by the addition of 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol or *m*-hydroxybenzaldehyde, and by Bu'Lock et al. (*5*) who found the formation of the later enzymes involved in patulin biosynthesis could be halted solely by exposing the mycelium to better nutritional conditions, even though the metabolites already formed were not removed.

Thus, it appears that the control of secondary metabolism in *P. patulum* is more complex than once thought (*4*), and a variety of interacting factors such as substrate induction, catabolite repression and nutrient levels may be involved.

Note added in proof. 6-MSA decarboxylase has recently received detailed study by F. Lynen et al. (private communication).

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