m-Hydroxybenzyl Alcohol Dehydrogenase from *Penicillium urticae*†

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ABSTRACT: The enzyme, *m*-hydroxybenzyl alcohol (NADP) dehydrogenase, a key enzyme in the conversion of 6-methylsalicylic acid to patulin in *Penicillium urticae* (NRRL 2159A), was partially purified and characterized and its kinetics of occurrence in fermentor cultures was compared to a variety of other parameters. This intracellular, soluble dehydrogenase was found to have a high substrate specificity, a pH optimum of 7.6, an approximate molecular weight of 120,000, and a K'_{app} (pH 7.6, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 30°) of 0.18. The enzyme exhibited a single band on disc gel electrophoresis and was inhibited by iodoacetate and diethyl pyrocarbonate. The calculated *in vivo* activity of the dehydrogenase was shown to be more than sufficient to account for the average maximum patulin biosynthetic rate of *P. urticae* cultures. An examination of a number of other fungi suggests that the occurrence of this enzyme is restricted to closely related fungi which possess all or part of the patulin biosynthetic pathway. Of the pathway end products the only possible feedback inhibitor was gentisaldehyde. The enzyme does not appear to be constitutive, its synthesis is inhibited by *p*-fluorophenylalanine, it is not induced by early pathway metabolites in the presence of fresh medium, and a dibutyryl derivative of cyclic adenosine monophosphate was found to promote an *in vivo* increase in activity.

Although well established by radiotracer experiments, the biosynthesis of acetate-derived secondary metabolites has received little confirmation at the enzymatic level. As indicated in recent reviews (Light, 1970; Corcoran and Darby, 1970) enzymes uniquely associated with such metabolic pathways have rarely been detected, let alone isolated and characterized. Despite the substantial effort which has been expended in examining 6-methylsalicylic acid and patulin biosynthesis in cell-free extracts from *Penicillium urticae*, progress in characterizing enzymes uniquely associated with secondary metabolism in this fungus has been slow.

A *P. urticae* cell-free extract capable of converting glucose, acetyl-CoA,[1] or 6-methylsalicylic acid into patulin was first reported by Bassett and Tanenbaum (1960). Investigation of 6-methylsalicylic acid synthase as well as a few other polyketide synthases followed (Light, 1970; Corcoran and Darby, 1970) and the former enzyme has now been purified 100-fold and shown to be a multienzyme complex very similar to the fatty acid synthase complex which also occurs in *P. urticae* (Dimroth *et al.*, 1970).

Finally, of the enzymes required for the conversion of 6-methylsalicylic acid to patulin, only 6-methylsalicylic acid decarboxylase has been detected in cell-free extracts of *P. urticae* (Light, 1969). Little characterization of this enzyme was possible because of a lack of stability.

Since the detection and characterization of individual pathway enzymes provides the only unambiguous proof for the existence and regulation of a metabolic pathway, the detection and characterization of an enzyme on the major route from 6-methylsalicylic acid to patulin was the object of this investigation. Upon having determined the major patulin pathway, an examination of the various steps suggested that the probable conversion of *m*-hydroxybenzyl alcohol to *m*-hydroxybenzaldehyde by an alcohol dehydrogenase might provide the convenient marker enzyme desired. This was indeed the case and the enzyme, *m*-hydroxybenzyl alcohol: NADP-oxidoreductase (EC 1.1.1-) which catalyzes reaction 1, was detected in crude cell-free preparations and subsequently characterized. The enzyme's occurrence, substrate specificity, and equilibrium constant provide both additional support for the previously determined route (Forrester and Gaucher, 1972) and additional insight into possible regulation of the pathway.

m-hydroxybenzyl alcohol + $NADP^+$ ⇌ *m*-hydroxybenzaldehyde + NADPH + H^+ (1)

Methods

Preparation of Cell-Free Extracts. Cells of *P. urticae* were harvested after an appropriate period of growth in a 14-l. fermentor under standard conditions as previously described (Forrester and Gaucher, 1972). These cells were suction filtered through Whatman No. 1 filter paper, washed twice with distilled water, lyophilized, and stored dry at −15°. Lyophilized cells (2 g) were suspended in 60 ml of 10 mM TES buffer (Sigma) at pH 7.6 which contained 1 mM dithiothreitol (Sigma) and 1 mM $MgCl_2$. The cell slurry was sonicated in a 100-ml rosette cell immersed in an ice bath for 7 min at the maximum setting of a Branson sonicator (Model 5125). Cellular debris was removed by centrifugation at 30,000*g* (Sorvall SS-34 rotor) for 30 min. This supernatant is the crude cell-free extract referred to below. If fresh cells were used they were treated as above except for the use of 10 g of wet cells/60 ml of sonicating buffer.

m-Hydroxybenzyl Alcohol Dehydrogenase Assay. An ap-

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[1] Abbreviations used are: CoA, coenzyme A; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; cAMP, 3′,5′-cyclic adenosine monophosphate; Bu_2cAMP, 6-*N*,2′-*O*-dibutyryl-3′,5′-cyclic adenosine monophosphate; *m*HBADH, *m*-hydroxybenzyl alcohol dehydrogenase; G-6-PDH, glucose 6-phosphate dehydrogenase; *m*HBAlc *m*-hydroxybenzyl alcohol.

propriate amount of enzyme solution (usually 0.05–0.20 ml) was added to 0.5 μmole of NADPH (Sigma) in 10 mM TES (pH 7.6) and 1 mM $MgCl_2$, previously equilibrated to 30°. The reaction was started by adding 0.4 μmole of *m*-hydroxybenzaldehyde to yield a total volume of 3.0 ml in a quartz cuvet in the 30° thermostated cell compartment of a Beckman DBG spectrophotometer. The reaction was monitored by the decrease in optical density at 340 nm using a Beckman 10-in recorder. One unit of enzyme activity was equal to 1 nmole of product/min. Since both NADPH ($\epsilon_{340}^{pH\ 7.6}$ 6.22 × 10^3) and *m*-hydroxybenzaldehyde ($\epsilon_{340}^{pH\ 7.6}$ 1.6 × 10^3) absorb at 340 nm the sum of their extinction coefficients at 340 nm was used in converting ΔOD/min into enzyme units. The optical density change per minute was in all cases linear with increasing enzyme concentration and a typical assay using 0.1 ml of crude extract yielded a ΔOD/min of 0.2–0.3.

Glucose-6-Phosphate Dehydrogenase Assay. An appropriate amount of enzyme solution (usually 0.1–0.2 ml) was added to 1 μmole of $NADP^+$ (Sigma) in 10 mM TES (pH7.6) and 1 mM $MgCl_2$, previously equilibrated to 30°. The reaction was started by the addition of 20 μmoles of glucose 6-phosphate (Sigma) to yield a total volume of 3.0 ml in a quartz cuvet, in the thermostated cell compartment of a Beckman DBG spectrophotometer. The reaction was monitored by the increase in optical density at 340 nm. One unit of enzyme activity was equal to 1 nmole of NADPH ($\epsilon_{340}^{pH\ 7.6}$ 6.22 × 10^3) per min.

Protease Assay. The crude supernatant (1 ml) was added to 1 ml of 2% casein in 0.1 M phosphate buffer (pH 7.2). This solution was incubated at 37° for 30 min and the reaction terminated by the addition of 3 ml of 5% trichloroacetic acid. This mixture was gravity filtered through Whatman No. 1 filter paper and 1 ml of the filtrate was added to 5 ml of 0.4 M NaOH. Phenol reagent (1 ml of 1 N) (Fisher) was added and the optical density of 690 nm was determined after 30 min of color development at room temperature. Blanks were run concurrently with the assays as described above except that the trichloroacetic acid was added prior to the incubation period. One unit at enzyme activity was defined as the liberation of products equivalent to 1 μmole of tyrosine/min as determined by a standard plot prepared from L-tyrosine.

Protein Assay. Both the Folin–Lowry method (Lowry *et al.*, 1951) and the 280:260 nm ratio method were used to determine the protein concentration in the crude extract. Bovine serum albumin (Sigma) was used as a standard in these assays.

Partial Purification. All procedures were carried out at 0–5° and all centrifugations were performed in a Sorvall RC-2B centrifuge using the SS-34 rotor. The crude extract was brought to 2% (w/v) in streptomycin sulfate (Sigma), and after 10 min was centrifuged at 10,000*g* for 10 min. Solid ammonium sulfate (Mann Ultra Pure) was added to the resulting supernatant until 40% saturation was reached. After centrifugation at 10,000*g* for 10 min the resulting precipitate was discarded and the supernatant was brought to 65% saturation in ammonium sulfate. The precipitate obtained by centrifugation at 10,000*g* for 10 min was dissolved in a minimum volume of 10 mM TES (pH 7.6) which was 1 mM in dithiothreitol and $MgCl_2$. This ammonium sulfate fraction was applied to a Pharmacia K25/100 column packed with Sephadex G-200 (fine) which had been previously equilibrated with 10 mM TES (pH 7.6) which was 1 mM in dithiothreitol and $MgCl_2$ and 0.02 mM in $NADP^+$. Elution was carried out using the above equilibration buffer and 5-ml fractions were collected. Fractions were assayed for *m*-hydroxybenzyl alcohol dehydrogenase activity and protein concentration. Active fractions were pooled.

Molecular Weight. A Pharmacia K25/100 column of Sephadex G-200 (fine) was calibrated with 5-mg samples of apoferritin, γ-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen, and myoglobin (Mann). Using the same column, the partition coefficient, K_{av}, for *m*-hydroxybenzyl alcohol dehydrogenase was determined and the molecular weight estimated from a standard plot of K_{av} *vs.* molecular weight.

Equilibrium Constant. To 1.0 μmole of NADPH and 2.0 μmoles of *m*-hydroxybenzaldehyde in 10 mM TES (pH 7.6, 1mM $MgCl_2$) was added 0.5 ml of enzyme solution to yield a total volume of 5.0 ml. This was incubated at 30° for 30 min, at which time, equilibrium having been reached, the reaction was quenched by immersion in a steam bath for 5 min. The equilibrium amount of $NADP^+$ was then assayed for by a modification of an established method (Klingenberg, 1965). A 0.5-ml aliquot of the equilibrium mixture was added to 10 mM TES (pH 8.0) containing 1 mM $MgCl_2$, 7 mM glucose 6-phosphate, and 300 units of glucose-6-phosphate dehydrogenase (Sigma) to yield a total volume of 3.0 ml. The amount of $NADP^+$ added was calculated from the change in optical density at 340 nm. As an independent check of the reaction's major direction, the relative initial rate for the reaction of *m*-hydroxybenzyl alcohol and $NADP^+$ was determined and compared to that for *m*-hydroxybenzaldehyde and NADPH. Care was taken to ensure that the initial concentrations of substrates and enzyme were identical in each case.

Modulation of Cellular Increases in m-Hydroxybenzyl Alcohol Dehydrogenase. To obtain cells with a low *m*-hydroxybenzyl alcohol dehydrogenase specific activity, cells from a 16-hr standard fermentor culture were harvested by suction filtration and washed twice with distilled water. Then, 0.25-g cells were added to 500-ml erlenmeyer flasks containing 100 ml of standard medium which was 1 mM in one of the following: 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, or *m*-hydroxybenzaldehyde. After 1-hr incubation on a reciprocal shaker at 28°, the cells were assayed for *m*-hydroxybenzyl alcohol dehydrogenase activity in the usual manner. In a similar experiment, details of which are given in Table V, the effect of *p*-fluorophenylalanine, cAMP, and Bu_2cAMP was examined.

Results

The specific activity of *m*-hydroxybenzyl alcohol dehydrogenase and a typical primary metabolic enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in fermentor culture cells of *P. urticae* at various culture ages is compared to other culture parameters in Figure 1. The occurrence with culture age of these two dehydrogenases differ markedly and indicate that while glucose-6-phosphate dehydrogenase appears to be constitutive the *m*-hydroxybenzyl alcohol dehydrogenase is probably not. The double maximum for the *m*-hydroxybenzyl alcohol profile was found to be reproducible. Intracellular protease content rises steadily once the secondary metabolism phase is established.

Because of stability problems the characterization of *m*-hydroxybenzyl alcohol dehydrogenase was limited to meaningful experiments which could be performed on a crude extract. Thus, while lyophilized *P. urticae* cells were found to retain approximately 75% of their *m*-hydroxybenzyl alcohol dehydrogenase activity after 2 months when stored dry at −15°, the cell-free extract prepared from these cells retained only

TABLE I: Partial Purification of *m*-Hydroxybenzyl Alcohol Dehydrogenase.

Purification Step	Vol (ml)	Total Units	Protein Concn (mg/ml)	Sp Act. (units/mg of protein)	Yield (%)	-fold/ Purifcn
Crude supernatant	275	115,500	4.2	100	100	
Streptomycin sulfate precipitation	275	115,500			100	
Ammonium sulfate fractionation	12	70,000	13	455	61	4.55
Sephadex G-200	42	17,000	0.55	655	15	6.55

about 11% activity after 24 hr at 3°. Previously reported means of stabilizing dehydrogenases (George *et al.*, 1969; Katagiri *et al.*, 1967) were generally unsuccessful. Thus additions to the crude extract of 1% bovine serum albumin, 1% gelatin, and 10% acetone were negative while 10% ethanol, 10% glycerol, and 1 mM dithiothreitol yielded slight stability and 10% dimethyl sulfoxide yielded approximately 27% activity in 24 hr at 3°. Inspite of the enzyme's instability a preliminary attempt to purify the enzyme is given in Table I.

High-speed centrifugation of the cell-free extract (180,000*g* for 30 min in an International B-60 A-321 rotor) and resonication of lyophilized cells with and without detergent indicated no particulate *m*-hydroxybenzyl alcohol dehydrogenase activity whatsoever. In addition no detectable activity was found in the culture medium. The dehydrogenase is thus a soluble intracellular enzyme. A partition coefficient (K_{BV}) of 0.32 for *m*-hydroxybenzyl alcohol dehydrogenase indicated an approximate molecular weight of 120,000.

Even though a crude enzyme preparation was used, the substrate specificity study given in Table II clearly indicates a dehydrogenase (EC 1.1.1) which is NADPH and *m*-hydroxy- or *m*-methoxybenzaldehyde specific. It is also noteworthy that gentisaldehyde is not a substrate. Under the assay conditions used ethanol and benzyl alcohol dehydrogenase (EC 1.1.1) activities were present but no *m*-hydroxybenzaldehyde dehy-

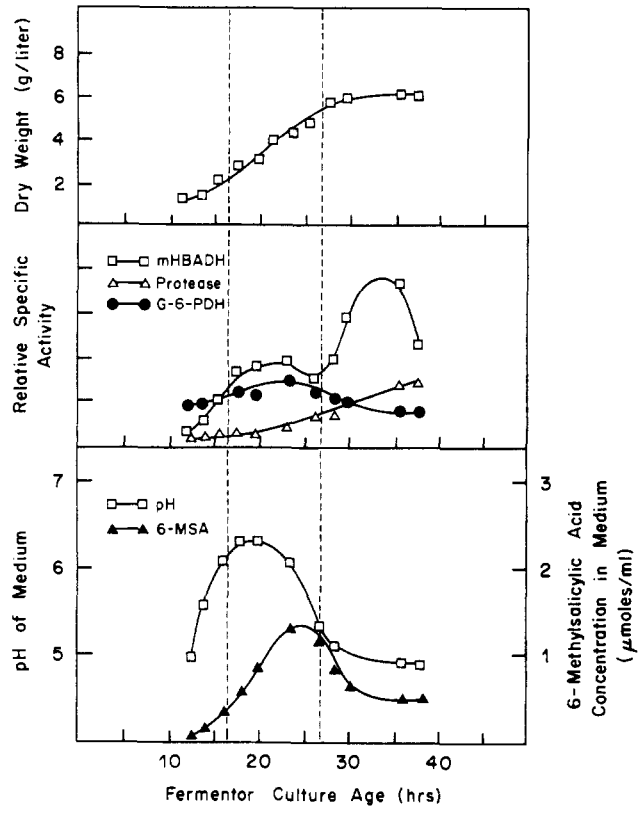

FIGURE 1: Kinetics of *m*-hydroxybenzyl alcohol dehydrogenase occurrence in fermentor cultures of *P. urticae* (2159A). 200-ml samples were suction filtered, and the cells washed twice with distilled water. Lyophilized cells (500 mg) were sonicated in 40 ml of sonicating buffer. The resulting slurry was centrifuged at 30,000*g* for 30 min and the specific activities (enzyme units/mg of protein) of the supernatant determined. The dry weight of the culture, pH of the medium, and 6-methylsalicylic acid concentration in the medium were monitored as previously described (Forrester and Gaucher, 1972).

TABLE II: Substrate Specificity of Unpurified *m*-Hydroxybenzyl Alcohol Dehydrogenase.

	Rel Substrate Act. (%)[a]	Rel Inhibitor Act. (%)[b]
m-Hydroxybenzaldehyde[c]	100	
m-Methoxybenzaldehyde[d]	100	0
Acetaldehyde[e]	29	16
Benzaldehyde	20	0
p-Hydroxybenzaldehyde	10	37[f]
o-Hydroxybenzaldehyde	0	0
Acetophenone	0	0
m-Hydroxyacetophenone	0	0
p-Hydroxyacetophenone	0	50[f]
p-Hydroxyoctaphenone	0	70[f]
Gentisaldehyde[g]	0	36

[a] In each case the cofactor NADPH and standard assay conditions of pH 7.6 and 30° were used. [b] Per cent decrease in *m*-hydroxybenzyl alcohol dehydrogenase activity upon addition of an equimolar amount of various substrate analogs. [c] Substitution of NADH for NADPH yielded a relative activity of ~12%, while substitution of *m*-hydroxybenzyl alcohol and $NADP^+$ for substrate and cofactor respectively yielded a relative activity of ~18%. Furthermore substitution of $NADP^+$ or NAD^+ for NADPH yielded no activity. [d] Substitution of *m*-methoxybenzyl alcohol and $NADP^+$ for substrate and cofactor, respectively, yielded a relative activity of ~9%. [e] Substitution of NADH for NADPH yielded a relative activity of 35%. [f] Concentration of added compound equal to only one-tenth the substrate concentration since these compounds absorb strongly at 340 nm. [g] Substitution of NADH for NADPH yielded a relative activity of ~12%.

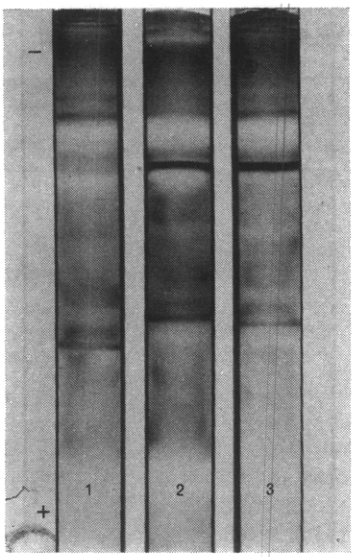

FIGURE 2: Polyacrylamide disc gel electrophoresis of *m*-hydroxybenzyl alcohol dehydrogenase in crude extracts of *P. urticae*. Using the basic method of Ornstein and Davis (1964), the gels were incubated in the dark at 25° for ~30 min in 10 mM TES (pH 8.5) which contained 1 mM $MgCl_2$, 20 mM substrate, 0.5 mM cofactor, 0.1% nitroblue tetrazolium chloride, and 0.02% phenazine methosulfate. The reaction was quenched with 7% acetic acid and the purple dehydrogenase bands faded slowly. Using the same enzyme extract in each case, gel 1 was stained in the presence of $NADP^+$ alone while gel 2 was stained in the presence of $NADP^+$ and *m*-hydroxybenzyl alcohol and gel 3 in the presence of $NADP^+$ and *m*-methoxybenzyl alcohol. Gels stained in the presence of $NADP^+$ and ethanol were virtually identical with gel 1.

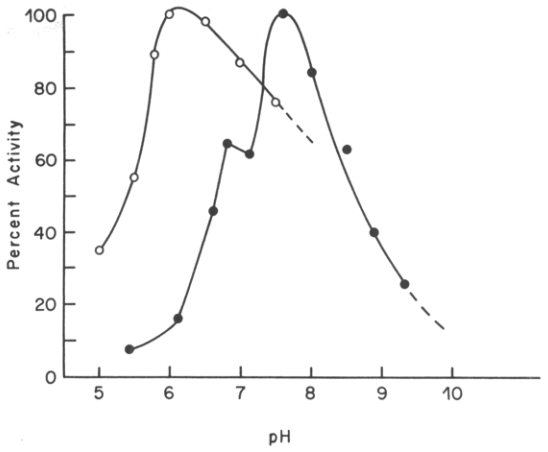

FIGURE 3: pH–activity profiles for ethanol (NADP) dehydrogenase (○) and *m*-hydroxybenzyl alcohol (NADP) dehydrogenase (●) activity in crude extracts of *P. urticae*. Equal aliquots of enzyme and substrate were assayed at various pH's in 0.1 M TES and 0.01 M citrate, respectively. The profile for *m*-hydroxybenzyl alcohol dehydrogenase was reproducible in three separate experiments.

drogenase (EC 1.2.1) activity was detected. Finally, *p*-hydroxybenzaldehyde, -acetophenone, and -octaphenone appear to be potent inhibitors while acetaldehyde and gentisaldehyde inhibit to some extent. As indicated in Figure 2, polyacrylamide disc gel electrophoresis indicated one band of *m*-hydroxy-(*m*-methoxy)benzyl alcohol dehydrogenase activity which was distinguishable from any ethanol (NADP) dehydrogenase activity.

As shown in Figure 3, the pH–activity profiles for these two enzymes differ. The ethanol dehydrogenase exhibits a pH optimum of 6.2 while the *m*-hydroxybenzyl alcohol dehydrogenase exhibits a pH optimum of 7.6 with a reproducible shoulder at pH 6.8. At their respective pH optima, the ethanol dehydrogenase activity (units per milliliter) was generally 70% of the *m*-hydroxybenzyl alcohol dehydrogenase activity. No change in the pH profile of *m*-hydroxybenzyl alcohol dehydrogenase was obtained upon comparison of fresh *vs.* lyophilized cells or upon comparison of "early" (16–26 hr) *vs.* "late" (26–47 hr) harvested fermentor culture cells.

Substantial protection against the inhibition of *m*-hydroxybenzyl alcohol dehydrogenase with iodoacetate and diethyl pyrocarbonate is obtained by prior incubation of the enzyme with the cofactor NADPH ($NADP^+$) while no protection is afforded by the substrate, *m*-hydroxybenzaldehyde (Table III). Diethyl pyrocarbonate has been shown (Ovádi and Keleti, 1970) to react specifically with histidine residues at pH 6.0. Thus cofactor binding appears to protect essential active site cysteine and histidine residues.

Although no attempt was made to determine accurate K_m values, initial rate *vs.* substrate concentration plots were typically hyperbolic indicating classical Michaelis–Menten kinetics. The equilibrium constant and standard free-energy change for the reaction (1) were calculated as: K'_{app} = [aldehyde][NADPH]/[alcohol][$NADP^+$] = 0.18 (pH 7.6, TES, 30°) and $\Delta G^{0\prime}$ = +1.04 kcal/mole (pH 7.6, TES, 30°), respectively. The reaction clearly favors reduction of the aldehyde to alcohol, since in addition to the equilibrium constant the reverse rate relative to the forward rate was found to be approximately 6:1. Finally under standard assay conditions (*i.e.*, pH 7.6, 30°) the average *in vitro* *m*-hydroxybenzyl alcohol dehydrogenase activity per gram of dry weight cells was calculated to be approximately 2000 μmoles of *m*-hydroxybenzyl alcohol/

TABLE III: Inhibition Study of Unpurified *m*-Hydroxybenzyl Alcohol Dehydrogenase.

Preassay Treatment[a]	Rel Act. (%)
Control	100
+Iodoacetic acid	46
+NADPH, then iodoacetic acid	97
+$NADP^+$, then iodoacetic acid	89
+*m*-Hydroxybenzaldehyde, then iodoacetic acid	45
+Diethyl pyrocarbonate	19
+$NADP^+$, then diethyl pyrocarbonate	65
+*m*-Hydroxybenzaldehyde, then diethyl pyrocarbonate	19

[a] 10-ml portions of crude supernatant were incubated at 0° with either 1 μmole of *m*-hydroxybenzaldehyde, 1 μmole of $NADP^+$, or 1 μmole of NADPH. After 5-min equilibration, 10 μmoles of iodoacetic acid (BDH, recrystallized before use) was added to each sample. After a further incubation at 0° of 2 hr the activity of each sample was determined by assaying 0.1-ml aliquots. Similarly, 10-ml portions of crude supernatant, adjusted to pH 6.0 with 1 N HCl, were incubated at 0° with 1 μmole of $NADP^+$ or with 1 μmole of *m*-hydroxybenzaldehyde. After 5-min equilibration, 10 μmoles of diethylpyrocarbonate (Eastman) was added. After a further 3 min at 0° the activity of each sample was determined by assaying 0.1-ml aliquots.

TABLE IV: Occurrence of *m*-Hydroxybenzyl Alcohol Dehydrogenase in Various Fungi.[a]

Fungus	Enzyme	Secondary Metabolites			
		6-MSA[i]	*m*-Cresol	*m*HBAlc	Patulin
Penicillium urticae (NRRL 2159A)	+	+	+	+	+
Penicillium patulum (NRRL 2329)[b]	+	+	+	+	Trace?
Penicillium patulum (CMI 59511)[c]	+	+	+	+	Trace?
Penicillium griseofulvum (NRRL 2300)[d]	−	+	−	Trace?	−
Penicillium expansum (NRRL 973)[e]	−	−	−	−	−
Aspergillus terreus (NRRL 255)[f]	−	−	−	Trace?	−
Polystictus versicolor (PRL 1736)[g]	Trace[h]	−	−	−	−

[a] Shake cultures, grown as previously described, were harvested in midstationary phase. In each case crude cell-free extracts of the cells were assayed for mHBADH activity and the culture media were qualitatively examined for phenols by tlc of ethyl acetate extracts as also previously described (Forrester and Gaucher, 1972). [b] Reported to produce gentisyl alcohol (C. W. Hesseltine, NRRL, personal communication, 1963). [c] Presumably a griseofulvum producer from Glaxo Labs, 1955. [d] Reported to produce 6-MSA (Anslow and Raistrick, 1931) and griseofulvum (Oxford *et al.*, 1939). [e] Reported to produce small amounts of patulin (Anslow *et al.*, 1943). [f] Reported to produce 6-MSA (Curtis *et al.*, 1964) and patulin (Kent and Heatley, 1945). [g] Reported to produce an extracellular aromatic alcohol oxidase (Farmer *et al.*, 1960), and an intracellular mHBAlc dehydrogenase (Hurst, 1963). [h] No activity found in the culture medium. [i] 6-MSA = 6-methylsalicylic acid.

hr per g dry weight of cells for aldehyde → alcohol or approximately 330 μmoles of *m*-hydroxybenzaldehyde/hr per g dry weight of cells for alcohol → aldehyde. Given that the dehydrogenase activity at pH 6.3 is approximately 25% that at pH 7.6 (Figure 3), a pH correction may be made to yield an average *in vitro* *m*-hydroxybenzaldehyde biosynthetic capacity for *P. urticae* cells of approximately 82 μmoles/hr per g dry weight of cells at pH 6.3. This is to be compared to the average maximum patulin biosynthesis rate of *P. urticae* cells during the linear growth phase of a standard fermentor culture which was determined to be approximately 20 μmoles/hr per g dry weight of cells at 28° and an average pH of 6.3 (Forrester and Gaucher, 1972).

A preliminary examination of a few fungi reported to produce patulin, its precursors, or a *m*-hydroxybenzyl alcohol dehydrogenase (Table IV) indicates a correlation between the occurrence of patulin or its precursors in the culture medium and the occurrence of *m*-hydroxybenzyl alcohol dehydrogenase activity in the cells.

A very preliminary check on some of the more obvious types of regulation which might operate *via* *m*-hydroxybenzyl alcohol dehydrogenase was made. First in analogy to other biosynthetic pathways the role of the patulin pathway's end products as modifiers of *m*-hydroxybenzyl alcohol dehydrogenase activity was examined. Patulin, gentisic acid, *p*-hydroxy-, or *m*-hydroxybenzoic acids all at 0.13 mM concentrations had no effect on the *in vitro* activity of the enzyme. As indicated in Table II only gentisaldehyde inhibited to any extent.

Secondly, the ability of early intermediates of the patulin pathway to induce *m*-hydroxybenzyl alcohol dehydrogenase activity in fermentor grown *P. urticae* cells with very little initial dehydrogenase activity was examined. No increase in cellular *m*-hydroxybenzyl alcohol dehydrogenase activity was obtained in the presence of 1 mM 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, and *m*-hydroxybenzaldehyde. Finally, the effect of *p*-fluorophenylalanine and cAMP as well as its dibutyryl derivative (Bu_2cAMP) on the normal cellular increase in *m*-hydroxybenzyl alcohol dehydrogenase activity was examined. As indicated in Table V, *p*-fluorophenylalanine completely inhibited the normal increase in dehydrogenase activity of a standard 18-hr fermentor culture while such a culture when transferred to fresh medium devoid of glucose and yeast extract and containing Bu_2cAMP exhibited a substantial increase in activity. cAMP itself did not yield such an increase.

TABLE V: Inhibition and Stimulation of *m*-Hydroxybenzyl Alcohol Dehydrogenase Synthesis.

Medium Modification	Rel Increase in Enzymatic Act. (%)[a]
Control[b]	25
+*p*-Fluorophenylalanine (2.2 mM)[b]	0[d]
+cAMP (1.2 mM)[c]	26
+Bu_2cAMP (0.8 mM)[c]	43

[a] For a 2-hr shake flask incubation of 2 g of wet cells harvested from a 16-hr standard fermentor culture, washed, and resuspended in 50 ml of medium. [b] 16-hr standard fermentor culture medium was used. [c] Freshly prepared medium in which glucose and yeast extract were omitted was used. [d] No inhibition of dehydrogenase activity was obtained upon addition of *p*-fluorophenylalanine to cell-free extracts.

Discussion

The preceding results describe a soluble, intracellular, NADP-specific, alcohol dehydrogenase whose substrate specificity indicates it is an *m*-hydroxybenzyl alcohol dehydrogenase and probably not an ethanol dehydrogenase of low specificity. The demonstrated oxidation of alcohol to aldehyde indicates that according to Enzyme Commission rules the enzyme is not a reductase. The occurrence of a single enzymatically active band on polyacrylamide disc gel electrophore-

sis indicates the absence of *m*-hydroxybenzyl alcohol dehydrogenase isozymes. The enzyme's pH optimum of 7.6, molecular weight of 120,000, and K'_{app} (pH 7.6, TES, 30°) of 0.18 are all typical of known alcohol dehydrogenases (Barman, 1969). Although the forward reaction (1) is somewhat more favored than usual, the reverse reaction is clearly favored. The inactivation of the dehydrogenase by the sulfhydryl reagent, iodoacetate, and the histidine reagent, diethyl pyrocarbonate, and the protection from such inactivations by NADPH are also in agreement with what has been found for other dehydrogenases (DiSabato and Kaplan, 1963; Auricchio and Bruni, 1969; Ovádi and Keleti, 1970). The presence of a shoulder at pH 6.8 in the dehydrogenase's pH profile (Figure 3) raises the possibility of a mixture of two slightly different *m*-hydroxybenzyl alcohol dehydrogenases. This might result from (1) modification of the enzyme by aging or lyophilization, (2) synthesis of different enzymes at early and late stages in fermentor cultures, (3) the occurrence of both soluble and particulate enzymes, and (4) the occurrence of isozymes. Since the examination of each possibility proved negative, further attempts at explaining the occurrence of this shoulder must await purification of the enzyme. It is of interest to note here that 6-methylsalicylic acid synthase (Dimroth *et al.*, 1970) and 6-methylsalicylic acid decarboxylase (Light, 1969) also have pH optima of 7.6.

Literature reports of enzymes which are similar to *m*-hydroxybenzyl alcohol dehydrogenase are few. In investigations of the catabolism of soil lignins and humic acid by *Polystictus versicolor*, the ability of this fungus to interconvert hydroxy- and methoxy-substituted benzoic acids and their corresponding aldehydes and alcohols was correlated with the presence of a soluble, intracellular *m*-hydroxybenzaldehyde reductase (Hurst, 1963) and an extracellular *p*-methoxybenzyl alcohol oxidase capable of also oxidizing *m*-methoxybenzyl alcohol (Farmer *et al.*, 1960). The detection of a benzyl alcohol (NAD(P)) dehydrogenase in *Mycobacterium tuberculosis* has been reported (Sloane and Rao, 1963) and a catabolic, intracellular benzyl alcohol (NAD) dehydrogenase has been purified from *Pseudomonas putida* grown on toluene as the sole carbon source (Suhara *et al.*, 1969). Although sufficient comparative data on these enzymes is not available, it would appear that the dehydrogenase reported here is substantially different from these other enzymes.

Although the dehydrogenase described in this report is of interest in itself, it is its relationship to the patulin biosynthetic pathway of *P. urticae* which is of principal interest. Thus the characteristics of this enzyme support and explain a number of observations made during the elucidation of the major route for patulin biosynthesis (Forrester and Gaucher, 1972). The fact that the equilibrium constant for the *m*-hydroxybenzyl alcohol dehydrogenase reaction does not favor the forward reaction, particularly at pH's below 7.6, explains the pulse-labeling experiments which indicated that *m*-hydroxybenzyl alcohol has a considerably slower turnover rate than the other pathway metabolites. The substantial pH dependence of this reaction, due largely to the $NADP^+$ requirement, also explains the accumulation of early pathway metabolites in *P. urticae* cultures grown at acidic pH's. In addition, although no explanation can be provided for the occurrence of a "late" peak of dehydrogenase activity in fementor cultures (Figure 1), it is probable that the low pH of cultures at this time nullifies this increase in dehydrogenase and hence explains the absence of increased pathway activity. Finally, this work provides additional support for the presently proposed major route of patulin biosynthesis. It is accepted (Scrutton and Utter, 1968) that the *in vitro* determined maximum catalytic activity of a pathway's pacemaker enzyme(s) provides an estimate of the maximum intracellular catalytic capacity of the pathway itself and hence should be greater or equal to the observed *in vivo* metabolic flux of that pathway. Assuming a pacemaker role for *m*-hydroxybenzyl alcohol dehydrogenase and an average intracellular pH of approximately 6.3, the *m*-hydroxybenzaldehyde biosynthetic capacity of *P. urticae* fermentor cultures (82 μmoles/hr per g of dry weight cells) and hence the maximum patulin biosynthetic capacity is calculated to be approximately four times the observed average maximum patulin biosynthetic rate (20 μmoles/hr per g of dry weight cells). Although the potential errors in calculating maximum *in vivo* catalytic capacities from *in vitro* assays are many (Scrutton and Utter, 1968), the significance of this exercise is simply to provide some assurance that the observed levels of *m*-hydroxybenzyl alcohol dehydrogenase are sufficient to warrant its suggested role as an enzyme catalyzing what is probably the rate determining step of the major patulin biosynthetic pathway.

Since the major pathway utilized in patulin biosynthesis is predominately oxidative in nature, the question of whether some of the pathway's steps utilize enzymes whose function is primarily catabolic rather than biosynthetic is of interest. The microbial degradation of aromatic compounds by bacteria has been extensively studied (Gibson, 1968; Evans, 1969) while for the fungi much less is known (Henderson, 1963). In a recent relevant study (Hopper and Chapman, 1971; Hopper *et al.*, 1971) *m*-cresol and xylenols were shown to be catabolized by a *Pseudomonas* by an NAD-dependent oxidation of a methyl to a carboxyl group, probably *via* hydroxymethyl and aldehyde groups, followed by hydroxylation para to the first ring hydroxyl to yield gentisates which then underwent C_1–C_2 ring cleavage. While the patulin pathway is similar in terms of methyl group oxidation, ring hydroxylation to a gentisyl compound, and oxidative ring cleavage, the biosynthesis of patulin requires oxidation of the methyl group to the aldehyde only, utilization of $NADP^+$ and a unique C_4–C_5 ring cleavage. These differences plus the association of *m*-hydroxybenzyl alcohol dehydrogenase with those fungi possessing all or part of the patulin pathway (Table IV) support the unique role of this enzyme in *P. urticae* secondary metabolism. However further investigation of this point is necessary.

Finally the regulation of patulin biosynthesis is of interest. Fine or rapid control over the patulin pathway might be expected *via* classical feedback inhibition. Examination of patulin pathway end products as inhibitors of *m*-hydroxybenzyl alcohol dehydrogenase indicated that only gentisaldehyde might be construed as a feedback inhibitor. With respect to other pathway enzymes only the product inhibition of 6-methylsalicylic acid synthase has been reported (Arihood and Light, 1966; Bu'Lock *et al.*, 1966).

Coarse or slower control *via* induction or repression of enzyme synthesis has been more extensively investigated, although in most cases the conclusions are inferred rather than proven. Experiments in which the progress of *P. urticae* fermentor cultures have been arrested by continuous culture techniques (Bu'Lock *et al.*, 1965, 1969), disrupted by transfer from rich germinating medium to poorer Czapek–Dox medium (Light, 1967a, 1969) or partially inhibited by addition of cycloheximide or fluorophenylalanine (Light, 1967b, 1969; Bu'Lock *et al.*, 1969) all indicate the coappearance of 6-methylsalicylic acid synthase and decarboxylase in a nonconstitutive manner. Although less conclusive, these same experiments also indicate the probability that the enzymes convert-

ing *m*-cresol to gentisaldehyde are coordinately induced, and that induction of the ring-cleavage oxygenase which produces patulin, follows. These observations are supported by our proposed major route for patulin biosynthesis (Forrester and Gaucher, 1972) and by this report in which *m*-hydroxybenzyl alcohol dehydrogenase does not appear to be constitutive (Figure 1) and fluorophenylalanine is shown to indeed inhibit synthesis of the dehydrogenase and not the dehydrogenase itself (Table V).

Seemingly contradictory are our results which indicate that neither 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, nor *m*-hydroxybenzaldehyde promote an increase in the *m*-hydroxybenzyl alcohol dehydrogenase content of *P. urticae* cells resuspended in fresh medium. However supplementation and continuous culture experiments (Bu'Lock *et al.*, 1965) as well as resuspension experiments (Bu'Lock *et al.*, 1969) indicate that fresh, complete Czapek–Dox medium in the presence of added or accumulated 6-methylsalicylic acid inhibits the conversion of 6-methylsalicylic acid to subsequent metabolites. Thus our results confirm these findings. In addition both 6-methylsalicylic acid synthase and decarboxylase are absent when *P. urticae* is grown in a rich germinating medium (Light, 1969).

It would thus appear that patulin biosynthesis in *P. urticae* may be controlled *via* counterbalanced systems of induction and catabolite repression. Although 6-methylsalicylic acid may well function as an inducer, the possible identity of the repressor is completely unknown. The knowledge that cAMP regulates the synthesis of a number of bacterial enzymes which are subject to control by catabolite repression (De Crombrugglie *et al.*, 1969) prompted a preliminary examination of the effect of cAMP on *m*-hydroxybenzyl alcohol dehydrogenase synthesis. As indicated in Table V, the dibutyryl derivative of cAMP (Bu_2cAMP) promoted a significant increase in dehydrogenase activity. The effectiveness of the dibutyryl derivative as opposed to cAMP itself may reflect the greater metabolic stability and membrane penetrability of this derivative (Henion *et al.*, 1967). The known morphogenetic effects of cAMP (Jost and Rickenberg, 1971) prompt the speculation that cAMP may also play a role in the sporulation of fungi and hence provide an explanation of the recognized correlation between secondary metabolism and sporulation (Bu'Lock, 1965).

In conclusion it would appear that further experiments at the enzyme level will prove fruitful in providing further insight into the complexities of fungal regulation as it pertains to secondary metabolism.

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