APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3366–3370 Vol. 62, No. 9
0099-2240/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Comparison of the Efficacies of Chloromethane, Methionine, and *S*-Adenosylmethionine as Methyl Precursors in the Biosynthesis of Veratryl Alcohol and Related Compounds in *Phanerochaete chrysosporium*

DAVID B. HARPER,[1,2]* W. C. McROBERTS,[2] AND J. T. KENNEDY[2]

Microbial Biochemistry Section, Department of Food Science, The Queen's University of Belfast,[1] and Food Science Division, Department of Agriculture for Northern Ireland,[2] Belfast BT9 5PX, United Kingdom

Received 16 January 1996/Accepted 27 June 1996

The effect on veratryl alcohol production of supplementing cultures of the lignin-degrading fungus *Phanerochaete chrysosporium* with different *methyl*-$^2H_3$-labelled methyl precursors has been investigated. Both chloromethane (CH_3Cl) and L-methionine caused earlier initiation of veratryl alcohol biosynthesis, but *S*-adenosyl-L-methionine (SAM) retarded the formation of the compound. A high level of C^2H_3 incorporation into both the 3- and 4-*O*-methyl groups of veratryl alcohol occurred when either L-[*methyl*-$^2H_3$]methionine or C^2H_3Cl was present, but no significant labelling was detected when *S*-adenosyl-L-[*methyl*-$^2H_3$]methionine was added. Incorporation of C^2H_3 from C^2H_3Cl was strongly antagonized by the presence of unlabelled L-methionine; conversely, incorporation of C^2H_3 from L-[*methyl*-$^2H_3$]methionine was reduced by CH_3Cl. These results suggest that L-methionine is converted either directly or via an intermediate to CH_3Cl, which is utilized as a methyl donor in veratryl alcohol biosynthesis. SAM is not an intermediate in the conversion of L-methionine to CH_3Cl. In an attempt to identify the substrates for O methylation in the metabolic transformation of benzoic acid to veratryl alcohol, the relative activities of the SAM- and CH_3Cl-dependent methylating systems on several possible intermediates were compared in whole mycelia by using isotopic techniques. 4-Hydroxybenzoic acid was a much better substrate for the CH_3Cl-dependent methylation system than for the SAM-dependent system. The CH_3Cl-dependent system also had significantly increased activities toward both isovanillic acid and vanillyl alcohol compared with the SAM-dependent system. On the basis of these results, it is proposed that the conversion of benzoic acid to veratryl alcohol involves *para* hydroxylation, methylation of 4-hydroxybenzoic acid, *meta* hydroxylation of 4-methoxybenzoic acid to form isovanillic acid, and methylation of isovanillic acid to yield veratric acid.

Veratryl alcohol (3,4-dimethoxybenzyl alcohol) is a secondary metabolite produced by many species of white rot fungi (8, 10, 11). It is believed to play a key role in lignin degradation by such fungi by stabilizing lignin peroxidase (15) and enhancing the ability of the enzyme to oxidize other compounds which in the absence of veratryl alcohol would be poor substrates for the enzyme (1, 4, 7, 12, 13). The biosynthetic pathway for veratryl alcohol in *Phanerochaete chrysosporium* was investigated by Shimada et al. (14), who concluded from ^{14}C-trapping experiments that the compound was formed from phenylalanine via cinnamic acid, 3,4-dimethoxycinnamyl alcohol, and veratryl glycerol. However, more recent work by Jensen et al. (9), involving ^{14}C pulse-labelling and isotope-trapping experiments, has demonstrated that only the first step in the pathway proposed by Shimada et al. (14) can be regarded as correct. Cinnamate is not converted to 3,4-dimethoxycinnamyl alcohol but is cleaved to either benzoate or benzaldehyde, which then undergoes hydroxylation, methylation, and reduction to yield veratryl alcohol. It is not known whether the *meta* or *para* position of the aromatic ring is the site of the initial hydroxylation, nor is the sequence in which subsequent methylation and hydroxylation occur clear.

Harper et al. (6) employed C^2H_3-labelled substrates to investigate the origin of the 3- and 4-*O*-methyl groups of veratryl alcohol and showed that CH_3Cl was as effective a precursor as L-methionine, thus implicating CH_3Cl as a methyl donor in the biosynthesis of veratryl alcohol. Further evidence that CH_3Cl was involved in veratryl alcohol biosynthesis in *P. chrysosporium* was afforded by a study of the effect of supplementation of fungal culture media with CH_3Cl (5). In the presence of 0.6 mM CH_3Cl, not only was veratryl alcohol biosynthesis induced earlier in the growth cycle, but peak concentrations of the idiolyte were significantly increased. Later work by Coulter et al. (2) on the methylation of the synthetic substrate acetovanillone (4-hydroxy-3-methoxyacetophenone) by whole mycelia of *P. chrysosporium* provided compelling evidence for the existence of two biochemically distinct systems for O methylation of this substrate, one utilizing *S*-adenosylmethionine (SAM) as a methyl donor and the other utilizing CH_3Cl as a methyl donor. The enzyme using SAM was induced early in the growth cycle and attained maximum activity at the beginning of the exponential phase. This SAM-dependent *O*-methyltransferase, which was purified from cell extracts and characterized by Coulter et al. (3), was strongly *para* specific, attacking a range of 2,4-disubstituted phenols. The activity of the enzyme in vivo could be almost completely inhibited by *S*-adenosyl-L-homocysteine (SAH). By contrast, the CH_3Cl-utilizing methylation system was absent in early growth but attained maximal activity in the middle of the growth phase. Unlike the SAM-dependent enzyme, CH_3Cl-dependent methylating activity was not inhibited by SAH in whole mycelia, and even complete suppression

* Corresponding author. Mailing address: Microbial Biochemistry Section, Department of Food Science, The Queen's University of Belfast, Newforge Lane, Belfast, BT9 5PX, United Kingdom. Phone: 44-1232-255343. Fax: 44-1232-669551.

of the SAM-dependent system by SAH did not appear to be associated with a significant change in CH_3Cl-dependent activity. This finding appears to eliminate the possibility that CH_3Cl is converted to SAM prior to use in methylation. However, all attempts to detect CH_3Cl-dependent *O*-methyltransferase activity in cell extracts have to date been unsuccessful, indicating that the enzyme is either highly labile or membrane bound.

Although the findings described above establish quite unequivocally that both SAM and CH_3Cl can act as methyl donors in the methylation of the synthetic substrate acetovanillone, in *P. chrysosporium* the relative efficacies of SAM and CH_3Cl as precursors of the 3- and 4-*O*-methyl groups of veratryl alcohol have yet to be determined. Accordingly, in the present study we have used C^2H_3-labelled compounds to compare the extents to which the methyl groups of CH_3Cl, L-methionine, and SAM are used in the biosynthesis of veratryl alcohol by cultures of *P. chrysosporium* supplemented with these compounds early in the growth cycle, prior to the normal initiation of veratryl alcohol biosynthesis. Additionally, in an attempt to identify the intermediates in the metabolic conversion of benzoic acid to veratryl alcohol, we have assayed, using isotopic techniques, the relative activities of the CH_3Cl- and SAM-dependent methylating systems with a number of possible hydroxylated intermediates as substrates.

MATERIALS AND METHODS

Organism and maintenance medium. *P. chrysosporium* Burds INA-12 (CNCM I-398), a strain previously utilized in investigations by Harper and coworkers (2, 3, 5, 6), was employed in the study. Fungal cultures were maintained on 4% (wt/vol) malt extract agar.

Chemicals. C^2H_3Cl (99 atom% ^2H), L-[*methyl*-$^2H_3$]methionine (99.4 atom% ^2H), and [*N, N, N*-trimethyl-$^2H_3$]choline chloride (99.2 atom% ^2H) were purchased from MSD Isotopes, Montreal, Canada. *S*-Adenosyl-L-[*methyl*-$^2H_3$]methionine (99 atom% ^2H) (C^2H_3-SAM) was acquired as the *p*-toluenesulfonate salt from C/D/N Isotopes, Montreal, Canada. L-Methionine, *S*-adenosyl-L-methionine *p*-toluenesulfonate salt, and SAH were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide reagent was purchased from Pierce Chemical Co., Rockford, Ill. CH_3Cl, acetovanillone, and all substituted benzoic acids, benzaldehydes, and benzyl alcohols were obtained from Aldrich Chemical Co., Gillingham, Dorset, United Kingdom.

Culture media and culture conditions. *P. chrysosporium* was grown as described by Coulter et al. (2) without agitation at 37°C in 100-ml and 2-liter conical flasks containing 10 and 200 ml, respectively, of medium.

For experiments with isolated mycelia in which CH_3Cl- and SAM-dependent activities with different substrates were assessed, mycelia were harvested after 72 h from cultures grown in 2-liter flasks. Superficial moisture was removed by pressing the mycelia between pieces of filter paper, and the mycelia were used directly in an assay of methylating activity. The experiment was repeated with different batches of 72-h mycelia on three separate occasions, and similar results were obtained in each case. (The results presented in Table 3 are those from one such batch.)

For experiments which required supplementation of the growth medium with labelled precursors at a particular stage of growth, the following procedure was adopted. The organism was grown in 100-ml flasks, the polytetrafluoroethylene-coated rubber stopper of each of which was fitted with two stoppered injection ports, one of which consisted of a capillary tube extending to within 2 mm of the base of the flask. After growth for 28, 41, or 54 h, the medium was supplemented with various labelled and unlabelled methyl donors, in a volume not exceeding 1.5 ml, through the capillary injection port. CH_3Cl, L-methionine, and SAM and their labelled analogs were added so as to give a final concentration in the medium of 0.5 mM, while SAH was added to give a final concentration of 1 mM. Medium of 41-h cultures was also supplemented with labelled choline chloride (0.5 mM) in some instances. Each treatment was replicated five times. After a further 16 h of incubation, the cultures were harvested, veratryl alcohol in the supernatant was assayed, and the labelling pattern in the 3- and 4-*O*-methyl groups was determined.

Determination of veratryl alcohol and percent C^2H_3 incorporation. Veratryl alcohol in the culture medium was extracted with chloroform and quantified by gas chromatography-mass spectrometry, and the labelling pattern was determined as described by Harper et al. (6). As it was not possible on the basis of the mass spectrometry fragmentation pattern to distinguish between veratryl alcohol monosubstituted with C^2H_3 in position 3 and that monosubstituted with C^2H_3 in position 4, veratryl alcohol with a single C^2H_3 substituent is referred to simply as monolabelled. The compound giving an ion of *m/e* 168 (substitution OCH_3, OCH_3) is referred to as unlabelled compound, that giving an ion of *m/e* 171 (substitution OCH_3, OC^2H_3) is referred to as monolabelled, and that giving an ion of *m/e* 174 (substitution OC^2H_3, OC^2H_3) is referred to as dilabelled.

TABLE 1. Veratryl alcohol production 16 h after addition of various precursors to cultures of *P. chrysosporium* of different ages

Precursor(s) added[a]	Veratryl alcohol present (nmol flask^{-1}) (mean ± SD) with addition of precursor at the following age of culture (h):		
	28	41	54
H_2O	0	133 ± 86	237 ± 174
CH_3Cl	1 ± 1	575 ± 545	872 ± 109
L-Methionine	39 ± 13	235 ± 130	349 ± 284
SAM	0	0	130 ± 122
SAH	0	141 ± 211	376 ± 160
CH_3Cl + SAH	2 ± 3	745 ± 546	726 ± 233
L-Methionine + SAH	41 ± 40	252 ± 58	619 ± 213
SAM + SAH	0	0	410 ± 236
CH_3Cl + L-methionine	126 ± 56	556 ± 312	864 ± 367
CH_3Cl + SAM	0	0	166 ± 235
L-Methionine + SAM	0	21 ± 37	194 ± 203

[a] CH_3Cl, L-methionine, and SAM were each added to cultures as described in Materials and Methods to give a final concentration in the medium of 0.5 mM, while SAH was added so as to give a final concentration of 1 mM.

Assay of CH_3Cl- and SAM-dependent methylation activities in whole mycelia with different substrates. In the assay of CH_3Cl-dependent methylating activity, mycelia (0.1 g) harvested from 72-h cultures as described above were incubated for 3 h at 37°C in triplicate 40-ml screw cap septum vials fitted with polytetrafluoroethylene-lined silicone discs. The vials contained, in a total volume of 2 ml, 50 mM 2,2-dimethylsuccinate buffer (pH 5.5), 1 mM C^2H_3Cl, 1 mM SAH, and 0.5 mM substrate. The C^2H_3Cl concentration referred to is that after equilibration of the gaseous and aqueous phases. Control incubations in which mycelia were incubated under the above-described conditions in the absence of the substrate were also performed. Conditions for assay of SAM-dependent methylating activity were similar to those described above except that the vials contained, in a total volume of 2 ml, 50 mM phosphate buffer (pH 7.75), 1 mM C^2H_3-SAM, and 0.5 mM substrate. After 3 h of incubation, the assay mixtures were quickly frozen at −15°C and stored at this temperature until extraction and quantitation of the methylated products were done.

Extraction and determination of methylated products other than veratryl alcohol. Extraction and determination of methoxylated benzaldehydes, benzyl alcohols, and 3,4-dimethoxyacetophenone were conducted as described by Coulter et al. (3) with gas chromatography-mass spectrometry. After adjustment of the incubation mixture to pH 2, methoxylated benzoic acids were extracted by the method described above for veratryl alcohol. *m*-Methoxybenzoic acid was added as the internal standard when veratric acid, 3,4,5-trimethoxybenzoic acid, or *p*-methoxybenzoic acid was present, and *p*-methoxybenzoic acid was employed as the internal standard when *m*-methoxybenzoic acid was present. Quantitation of methoxylated benzoic acids was performed after derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide reagent. The chloroform extract (100 μl) was evaporated to dryness under nitrogen, and the residue was reconstituted in *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide reagent (100 μl). This derivatized extract was analyzed by gas chromatography-mass spectrometry under conditions described by Coulter et al. (3). With all methylated compounds, ion currents at *m/e* values corresponding to the molecular ion (M) and M + 3 were monitored. The percentage of *O*-methyl compound labelled with C^2H_3 was determined as the ratio of ion currents *m/e*(M + 3)/[*m/e* (M) + *m/e* (M + 3)] at the retention time of the compound.

RESULTS AND DISCUSSION

Effect on veratryl alcohol production of supplementation of *P. chrysosporium* cultures with different methyl donors. Previous work by Harper et al. (5) indicated that veratryl alcohol biosynthesis by *P. chrysosporium* Burds INA-12 on normal unsupplemented medium does not occur to a significant extent before 55 to 60 h of growth. Therefore, in order to achieve maximum incorporation from the labelled methyl donors used to supplement the medium in this investigation, the additions were made at culture ages of 28, 41, and 54 h so as to minimize the dilution of label in veratryl alcohol by unlabelled compound synthesized prior to addition of precursor. Table 1

TABLE 2. Incorporation of C^2H_3 into veratryl alcohol 16 h after addition of various C^2H_3-labelled precursors to cultures of *P. chrysosporium* of different ages

Labelled precursor(s) added[a]	% C^2H_3 substitution in positions 3 and 4 of veratryl alcohol (mean ± SD) with addition of precursor at the following age of culture (h):								
	28			41			54		
	Di-CH_3	$CH_3C^2H_3$	Di-C^2H_3	Di-CH_3	$CH_3C^2H_3$	Di-C^2H_3	Di-CH_3	$CH_3C^2H_3$	Di-C^2H_3
H_2O	—[b]	—	—	100	0	0	100	0	0
C^2H_3Cl	0	13 ± 22	87 ± 22	5 ± 1	34 ± 1	61 ± 1	30 ± 7	22 ± 2	48 ± 5
L-[*methyl*-$^2H_3$]methionine	0	13 ± 1	87 ± 1	4 ± 2	13 ± 3	82 ± 5	46 ± 12	14 ± 3	40 ± 14
C^2H_3-SAM	—	—	—	—	—	—	99 ± 1	1 ± 1	0
SAH	—	—	—	100	0	0	100	0	0
C^2H_3Cl + SAH	0	35 ± 2	65 ± 2	6 ± 1	34 ± 2	60 ± 2	37 ± 4	22 ± 2	41 ± 2
L-[*methyl*-$^2H_3$]methionine + SAH	0	10 ± 7	90 ± 7	3 ± 1	12 ± 2	85 ± 3	60 ± 9	11 ± 2	29 ± 9
C^2H_3-SAM + SAH	—	—	—	—	—	—	100	0	0
L-Methionine + C^2H_3Cl	49 ± 4	43 ± 6	8 ± 5	40 ± 13	43 ± 4	17 ± 10	50 ± 7	33 ± 5	17 ± 8
L-[*methyl*-$^2H_3$]methionine + CH_3Cl	12 ± 2	42 ± 2	46 ± 3	24 ± 5	47 ± 1	29 ± 5	59 ± 6	29 ± 4	12 ± 3
L-[*methyl*-$^2H_3$]methionine + C^2H_3Cl	0	8 ± 1	92 ± 1	1 ± 1	6 ± 1	93 ± 1	26 ± 16	7 ± 2	67 ± 14
C^2H_3-SAM + CH_3Cl	—	—	—	—	—	—	98 ± 1	2 ± 1	0
C^2H_3-SAM + L-[*methyl*-$^2H_3$]methionine	—	—	—	2 ± 4	7 ± 9	91 ± 12	54 ± 13	11 ± 3	35 ± 11
SAM + C^2H_3Cl	—	—	—	—	—	—	71 ± 2	10 ± 1	19 ± 2

[a] All compounds except SAH were added to cultures as described in Materials and Methods to give a final concentration in the medium of 0.5 mM. SAH was added to give a final concentration of 1 mM.

[b] —, no veratryl alcohol formed.

shows the amounts of veratryl alcohol synthesized 16 h after the addition of various methyl donors at the different culture ages.

In cultures to which chloromethane or L-methionine was added, veratryl alcohol biosynthesis was initiated at an earlier stage of growth than in control cultures to which water alone was added. Moreover, the chloromethane-supplemented culture at 41 and 54 h showed a severalfold enhancement in the concentration of veratryl alcohol relative to that not only in the unsupplemented culture but also in the L-methionine-supplemented culture. When chloromethane and L-methionine were jointly added to cultures, their individual effects were not additive and veratryl alcohol production did not appear to be significantly different from that observed with chloromethane alone. The earlier induction of veratryl alcohol biosynthesis in the presence of chloromethane is consistent with the findings of Harper et al. (5), who showed that *P. chrysosporium* cultured in the presence of 0.6 mM chloromethane produced veratryl alcohol up to 36 h earlier.

The addition of SAM markedly delayed veratryl alcohol production, with the metabolite becoming detectable only in cultures supplemented at 54 h. Coaddition of chloromethane did not appear to remove the inhibitory effect of SAM, but L-methionine did afford partial relief. Since SAH can act as a powerful inhibitor of many SAM-dependent metabolic processes, the effect of this compound on veratryl alcohol production was examined. SAH did not appear to influence veratryl alcohol production either alone or in the presence of chloromethane, but there was evidence of some stimulation in cultures supplemented at 54 h with SAH and L-methionine and possibly also with SAH and SAM.

Effect of different C^2H_3-labelled methyl donors on incorporation of C^2H_3 into veratryl alcohol by *P. chrysosporium* cultures. Table 2 shows the percent C^2H_3 substitution in the 3 and 4 positions of veratryl alcohol produced by cultures supplemented at several stages of growth with different C^2H_3-labelled methyl donors both separately and in combination. The level of incorporation of C^2H_3 from both C^2H_3Cl and L-[*methyl*-$^2H_3$]methionine was very high when the methyl donors were added to 28-h cultures; 87% of veratryl alcohol was doubly labelled, and no unlabelled compound was present. Supplementation at later stages of growth led to progressively lower levels of incorporation from both methyl precursors, but even those at 54 h yielded veratryl alcohol that was more than 50% labelled. In contrast, no appreciable labelling was detectable in cultures supplied with C^2H_3-SAM. Although not recorded in Table 2, labelling was also undetectable in veratryl alcohol produced by cultures supplemented at 41 h with [*N*, *N*, *N-tri-methyl*-$^2H_3$]choline chloride.

The presence of SAH had little effect on the pattern of incorporation from the various methyl precursors other than slightly reducing that from C^2H_3Cl. This effect may arise from an increased availability of cellular methionine as a result of the known inhibitory effects of SAH on SAM utilization within the cell. Indeed, the sensitivity of incorporation of C^2H_3 from C^2H_3Cl to increased concentrations of methionine is illustrated by the results obtained upon supplementation of the medium with a combination of C^2H_3Cl and unlabelled methionine. Under these circumstances, the percentage of doubly labelled veratryl alcohol at 28 h fell from 87 to 8% and the percentage of unlabelled veratryl alcohol rose from 0 to 49%. This finding implies that incorporation of C^2H_3 from C^2H_3Cl is readily antagonized by L-methionine. The extent of the reverse effect, i.e., the antagonism of incorporation of C^2H_3 from L-[*methyl*-$^2H_3$]methionine by unlabelled chloromethane, can also be gauged from Table 1. While it is clear that the presence of unlabelled CH_3Cl greatly reduces incorporation of C^2H_3 from labelled methionine into veratryl alcohol, the effect after supplementation at 28 h is not nearly as dramatic as that produced by the opposite combination referred to above. Thus, the level of doubly labelled veratryl alcohol falls only from 87 to 46% and that of unlabelled compound rises merely from 0 to 12%. Unsurprisingly, when a combination of L-[*methyl*-$^2H_3$]methionine and C^2H_3Cl is employed to supplement the medium, C^2H_3 incorporation into veratryl alcohol rises to very high levels, with over 90% of the label being detected in both 28- and 41-h cultures.

The most plausible explanation of the results described above is that L-methionine is converted in *P. chrysosporium* either directly or, more probably, via an intermediate to

TABLE 3. Comparison of the activities of CH_3Cl- and SAM-dependent methylating systems with different substrates in whole mycelia of *P. chrysosporium*

Substrate	C^2H_3Cl as methyl donor[a]				C^2H_3-SAM as methyl donor[b]			
	Total methylated product formed (nmol $flask^{-1}$) (mean ± SD)	% C^2H_3 label in product (mean ± SD)	Total labelled product formed (nmol $flask^{-1}$)	Relative activity[c]	Total methylated product formed (nmol $flask^{-1}$) (mean ± SD)	% C^2H_3 label in product (mean ± SD)	Total labelled product formed (nmol $flask^{-1}$)	Relative activity
Vanillic acid	136 ± 11	59 ± 2	80	100	428 ± 49	89 ± 1	381	100
Isovanillic acid	68 ± 3	70 ± 1	48	60	179 ± 11	92 ± 1	165	43
Syringic acid	69 ± 9	54 ± 2	37	46	381 ± 46	88 ± 1	335	88
4-Hydroxybenzoic acid	33 ± 3	73 ± 1	24	30	20 ± 5	82 ± 2	16	4
3-Hydroxybenzoic acid	9 ± 7	57 ± 1	5	6	26 ± 4	91 ± 1	24	6
Acetovanillone	128 ± 26	71 ± 2	91	114	395 ± 4	87 ± 2	344	90
Vanillyl alcohol	56 ± 11	64 ± 1	36	45	116 ± 11	84 ± 2	97	25
Isovanillyl alcohol	<2		<2	<2	<2		<2	<1
4-Hydroxybenzyl alcohol	<2		<2	<2	<2		<2	<1
3-Hydroxybenzyl alcohol	<2		<2	<2	<2		<2	<1
4-Hydroxybenzaldehyde	(i) 7 ± 1, (ii) 15 ± 4[d]	(i) 63 ± 1, (ii) 60 ± 1	13	16	(i) 13 ± 1, (ii) 25 ± 6	(i) 77 ± 3, (ii) 73 ± 3	28	7
3-Hydroxybenzaldehyde	<2		<2	<2	<2		<2	<1

[a] The assay was conducted as described in Materials and Methods with 0.5 mM substrate, 1 mM C^2H_3Cl, and 1 mM SAH in 50 mM 2,2-dimethylsuccinate buffer (pH 5.5).
[b] The assay was conducted as described in Materials and Methods with 0.5 mM substrate and 1 mM SAM in 50 mM Tris buffer (pH 7.75).
[c] The activity with vanillic acid was assigned a value of 100.
[d] The product was composed of 4-methoxybenzaldehyde (i) and its reduction product, 4-methoxybenzyl alcohol (ii).

CH_3Cl, which is then utilized as the methyl donor in biosynthesis of veratryl alcohol. Despite the isolation of a SAM-dependent 4-*O*-methyltransferase from *P. chrysosporium* (3) and the demonstration by Coulter et al. (2) that whole mycelia of the fungus readily take up and utilize SAM in the methylation of acetovanillone, there is no evidence from the labelling studies described in this paper of a role for SAM in direct methylation of veratryl alcohol precursors or for any involvement of choline in methyl transfer. Moreover, it seems highly unlikely that SAM acts as a metabolic intermediate in the conversion of L-methionine to CH_3Cl. The failure of a supplement of C^2H_3-SAM and CH_3Cl to introduce significant labelling into veratryl alcohol establishes the complete absence of methyl exchange between the two compounds. Although there is a slight decrease in labelling when unlabelled SAM in combination with C^2H_3Cl is added to the medium, this effect is explicable, as with that of SAH, in terms of an increased availability of endogenous methionine for CH_3Cl biosynthesis, arising in this case from the repression of SAM biosynthesis within the cell by high levels of exogenous SAM.

Activities of CH_3Cl-and SAM-dependent methylating systems on different substrates in isolated mycelia. In order to distinguish as clearly as possible between CH_3Cl- and SAM-dependent methylations of different substrates by isolated mycelia of *P. chrysosporium*, it was necessary to develop specific assays for quantifying methylation by each methyl donor. Coulter et al. (2) have shown that the SAM-dependent methylation system is present in mycelia from an early stage of growth, has a pH optimum of 7.5 to 8.0, and can be completely suppressed by 1 mM SAH. By contrast, the CH_3Cl-dependent system is absent in early growth but reaches maximum activity in the middle of the growth phase at about 72 h. The pH optimum of the system is 5.5 to 6.0, and its activity is not inhibited by SAH. Therefore, the assay of SAM-dependent methylation was conducted in 50 mM Tris buffer (pH 7.75) with 1 mM C^2H_3-SAM as the methyl donor, while the assay of CH_3Cl-dependent methylation was performed in 50 mM 2,2-dimethylsuccinate buffer (pH 5.5) with C^2H_3Cl as the methyl donor in the presence of 1 mM SAH. The total methylated product formed with each substrate from each methyl donor after 3 h of incubation with mycelia isolated from 72-h cultures was measured, and the percentage of C^2H_3 label incorporated was determined. The results of this experiment are recorded in Table 3. The total labelled product formed in each case is also indicated, and the methylating activity relative to that with vanillic acid is calculated. Control experiments in which mycelia were incubated in the absence of a substrate were performed to confirm that no significant endogenous formation of methylated product occurred. In addition, the possibility of oxidation or reduction of the substrate under the assay conditions used was investigated. No significant reduction of any of the benzoic acids used as substrates to either the corresponding benzaldehydes or benzyl alcohols was detected during the course of the incubation. Similarly, no oxidation of the benzyl alcohols to the corresponding benzaldehydes or benzoic acids was found. However, a number of benzaldehydes, e.g., vanillin, isovanillin, and syringaldehyde, underwent rapid reduction to the extent that up to 75% conversion to the corresponding alcohol had occurred after 3 h of incubation with mycelia. Such reduction was not confined to substrate aldehydes but also occurred in the methylated products derived from them. Consequently, the results obtained with aldehydes as substrates were difficult to interpret and of limited value. Hence, in Table 3, results for only two aldehyde substrates of particular interest in the context of veratryl alcohol biosynthesis are shown. No results for dihydroxylated substrates such as 3,4-dihydroxybenzoic acid are presented in Table 3, since the mixture of products obtained and the difficulties associated with interpreting the labelling pattern of a dimethoxylated product made an accurate assessment of the relative activities of the methylating systems on the substrates impossible. With 3,4-dihydroxybenzoic acid as the substrate, the total product formed (sum of mono- and dimethoxylated compounds) with C^2H_3Cl as the

methyl donor was 21 ± 3 nmol $flask^{-1}$ and that with C^2H_3-SAM as the methyl donor was 222 ± 24 nmol $flask^{-1}$.

It is clear from Table 3 that in general the amount of labelled product synthesized from C^2H_3Cl is considerably less than that formed from C^2H_3-SAM. With vanillic acid as the substrate, the CH_3Cl-dependent system displayed only 21% of the activity of the SAM-dependent system. The pattern of activity with CH_3Cl as the methyl donor differed in several respects from that with SAM as the methyl donor. Thus, the CH_3Cl-dependent system appears to have significantly increased activities relative to vanillic acid on both isovanillic acid and vanillyl alcohol compared with the SAM-dependent system, while activity on syringic acid was substantially less. However, the major divergence in relative activity occurred with respect to the substrate 4-hydroxybenzoic acid, which was a much better substrate for CH_3Cl-dependent methylation than for SAM-dependent methylation (sevenfold when the rate with vanillic acid is used for normalization). In contrast, 3-hydroxybenzoic acid was a poor substrate for both systems, and 3- and 4-hydroxybenzyl alcohols were not methylated by either system. When 4-hydroxybenzaldehyde was employed as a substrate, the corresponding methoxybenzaldehyde and methoxybenzyl alcohol were formed, as with the other aldehydes as explained above. However, since the 4-hydroxybenzyl alcohol was not itself capable of being methylated by either system, reduction must have occurred subsequent to methylation, and therefore, a reasonably accurate assessment of the relative activities of the two systems with 4-hydroxybenzaldehyde as the substrate can be obtained by summing the amounts of labelled 4-methoxybenzaldehyde and 4-methoxybenzyl alcohol. On this basis, 4-hydroxybenzaldehyde, like 4-hydroxybenzoic acid, appears to be a rather poor substrate for the SAM-dependent system. However, the aldehyde was more readily utilized by the CH_3Cl-dependent system, although the rate of methylation was still only half of that of the 4-hydroxybenzoic acid.

These findings have important implications with regard to the route of biosynthesis of veratryl alcohol in *P. chrysosporium* in the light of results in Table 2, which demonstrate quite conclusively that the CH_3Cl-dependent methylation system alone is involved in veratryl alcohol biosynthesis and that SAM has no role in the formation of the compound. Jensen et al. (9) have proposed that in the biosynthesis of veratryl alcohol, cinnamate is cleaved to either benzoate, benzaldehyde, or a closely related derivative. One of these metabolites then serves as a substrate for hydroxylation and methylation to give veratryl alcohol. The relatively efficient methylation of 4-hydroxybenzoic acid by the CH_3Cl-dependent system suggests that *p*-hydroxylation of benzoic acid is the first stage in conversion of the latter compound to veratryl alcohol. The resulting *p*-methoxybenzoic acid may then be hydroxylated in the *meta* position to form isovanillic acid, which is also readily methylated by the CH_3Cl-dependent system. The final stage would be the reduction of the resulting veratric acid to veratryl alcohol. It could be argued that 4-hydroxybenzaldehyde is a sufficiently good substrate for CH_3Cl-dependent methylation for a route involving hydroxylation of benzaldehyde to be feasible. Inconsistent with such a possibility is the observation that 4-methoxybenzaldehyde is, as is clear from Table 3, very rapidly reduced in whole mycelia to 4-methoxybenzyl alcohol. Hydroxylation of the latter compound in position 3 would give isovanillyl alcohol, which is not a substrate for the CH_3Cl-dependent system. It therefore seems unlikely that the route to veratryl alcohol involves hydroxylation of benzaldehyde. Another conceivable biosynthetic route is via 3,4-dihydroxybenzoic acid. Although for the technical reasons outlined above it was not feasible to gauge the relative activity of this compound as a substrate, the overall production of methylated product upon incubation of 3,4-dihydroxybenzoic acid with C^2H_3Cl did not suggest that such a pathway was likely to be of major importance.

Even bearing in mind that the CH_3Cl-dependent methylation system probably comprises more than one enzyme, its relative lack of specificity is difficult to reconcile with a sole function in metabolism of methylating 4-hydroxybenzoic and isovanillic acids in the biosynthesis of veratryl alcohol. One possibility is that CH_3Cl participates in the methylation of phenolic compounds in other metabolic pathways. Alternatively, the relaxed substrate specificity of the CH_3Cl-dependent methylation system may not detract from its effectiveness if its role is confined to catalyzing a tightly channelled series of reactions, possibly on a membrane surface segregated from other metabolic activities and substrates within the cell.

REFERENCES

1. **Akamatsu, Y., D. B. Ma, T. Higuchi, and M. Shimada.** 1990. A novel enzymatic decarboxylation of oxalic acid by the lignin peroxidase system of the white rot fungus *Phanerochaete chrysosporium*. FEBS Lett. **269:** 261–263.
2. **Coulter, C., J. T. G. Hamilton, and D. B. Harper.** 1993. Evidence for the existence of independent chloromethane- and *S*-adenosylmethionine-utilizing systems for methylation in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**1461–1466.
3. **Coulter, C., J. T. Kennedy, W. C. McRoberts, and D. B. Harper.** 1993. Purification and properties of an *S*-adenosylmethionine:2,4-disubstituted phenol *O*-methyltransferase from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**706–711.
4. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. E. Fiechter.** 1986. Oxidation of benzo(a)pyrene by extracellular ligninase of *Phanerochaete chrysosporium*: veratryl alcohol and stability of ligninase. J. Biol. Chem. **261:**6900–6903.
5. **Harper, D. B., J. A. Buswell, and J. T. Kennedy.** 1991. Effect of chloromethane on veratryl alcohol and lignin peroxidase production by the fungus *Phanerochaete chrysosporium*. J. Gen. Microbiol. **137:**2867–2872.
6. **Harper, D. B., J. A. Buswell, J. T. Kennedy, and J. T. G. Hamilton.** 1990. Chloromethane, methyl donor in veratryl alcohol biosynthesis in *Phanerochaete chrysosporium* and other lignin-degrading fungi. Appl. Environ. Microbiol. **56:**3450–3457.
7. **Harvey, P. J., H. E. Schoemaker, and J. M. Palmer.** 1986. Veratryl alcohol as a mediator and the role of radical cations in lignin degradation by *Phanerochaete chrysosporium*. FEBS Lett. **195:**242–246.
8. **Hattakka, A., A. Kantelinen, A. Tervila-Wilo, and L. Viikari.** 1987. Production of ligninases by *Phlebia radiata* in agitated cultures, p. 185–189. *In* E. Odier (ed.), Lignin enzymic and microbial degradation. INRA Publications, Paris.
9. **Jensen, K. A., Jr., K. M. C. Evans, T. K. Kirk, and K. E. Hammel.** 1994. Biosynthetic pathway for veratryl alcohol in the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **60:**709–714.
10. **Kawai, S., T. Umegawa, and T. Higuchi.** 1986. *De novo* synthesis of veratryl alcohol by *Coriolus versicolor*. Wood Res. **73:**18–21.
11. **Lundquist, K., and T. K. Kirk.** 1978. *De novo* synthesis and decomposition of veratryl alcohol by a lignin degrading basidiomycete. Phytochemistry **17:** 1676.
12. **Paszczynski, A., and R. L. Crawford.** 1991. Degradation of azo compounds by ligninase from *Phanerochaete chrysosporium*: involvement of veratryl alcohol. Biochem. Biophys. Res. Commun. **178:**1056–1063.
13. **Popp, J. L., B. Kalyanaraman, and T. K. Kirk.** 1990. Lignin peroxidase oxidation of Mn^{2+} in the presence of veratryl alcohol, malonic or oxalic acid, and oxygen. Biochemistry **29:**10475–10480.
14. **Shimada, M., F. Nakatsubo, T. K. Kirk, and T. Higuchi.** 1981. Biosynthesis of the secondary metabolite veratryl alcohol in relation to lignin degradation in *Phanerochaete chrysosporium*. Arch. Microbiol. **129:**321–324.
15. **Wariishi, H., and M. H. Gold.** 1989. Lignin peroxidase compound III. Formation, inactivation and conversion to native enzyme. FEBS Lett. **243:**165–168.