Biosynthesis of the Antibiotic 2,5-Dihydrophenylalanine by *Streptomyces arenae*†

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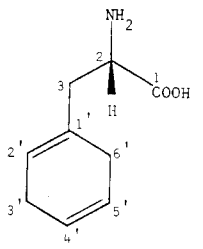
ABSTRACT: The biosynthesis of L-2,5-dihydrophenylalanine (DHPA) in *Streptomyces arenae* strain Tü 109 was studied in tracer experiments with [U-^{14}C]- and [1,6-^{14}C]shikimic acid followed by chemical degradation of the labeled product. The results indicate that shikimic acid (II) provides only the ring carbons of DHPA, that the side chain of DHPA is attached at the carbon derived from C-1 of II, and that in the transformation of II into DHPA the asymmetry of the ring of II is preserved, with C-6 of II giving rise to C-6′ of DHPA. Both generally ^{14}C-labeled chorismate and prephenate, but not L-[3-^{14}C]serine, are incorporated into DHPA. By preparing and feeding 5,6-dihydro[4-^{3}H]prephenate it was shown that this compound is not an intermediate in the biosynthesis of DHPA. A reaction sequence is proposed for the conversion of prephenate to DHPA, involving an allylic rearrangement, followed by 1,4 reduction of the resulting conjugated diene and a combined decarboxylation/dehydration.

A rather ubiquitous constituent of *Actinomycetes* is L-2,5-dihydrophenylalanine (I), which has been isolated from



I: 3-(1',4'-cyclohexadienyl)alanine (dihydrophenylalanine, DHPA)

a variety of *Streptomyces* species by several groups (Yamashita et al., 1970; Scannell et al., 1970; Fickenscher et al., 1971). This antibiotic had actually been prepared synthetically before it was recognized as a natural product and some of its biological properties had been studied (Snow et al., 1968; Shoulders et al., 1968; Genghof, 1970). DHPA[1] is an antagonist of phenylalanine both in the rat (Snow et al., 1968) and in microorganisms (Yamashita et al., 1970; Scannell et al., 1970; Fickenscher et al., 1971; Snow et al., 1968; Shoulders et al., 1968; Genghof, 1970), a finding which is plausible in light of the demonstrated planar conformation of the 1,4-cyclohexadiene ring (Shoulders et al., 1968). In addition, one group reported activity against sarcoma-180 in the mouse (Scannell et al., 1970). The antibacterial activity in *Escherichia coli* is the result of false feedback inhibition of prephenate dehydratase and the phenylalanine-sensitive DAHP synthetase by DHPA (Fickenscher & Zähner, 1971). The amino acid is incorporated into the cellular protein in phenylalanine-requiring mutants of *E. coli* and in sarcoma-180 (Pine, 1975). The compound is also an inhibitor of mammalian tryptophan hydroxylase (Okabayashi et al., 1977).

Scannell et al. (1970) carried out two experiments on the biosynthesis of DHPA. Lack of incorporation of phenylalanine (<0.02%) but good incorporation of [U-^{14}C]shikimic acid (2.5%) indicated that the biosynthesis does not involve reduction of phenylalanine but presumably a new variant of the shikimic acid pathway.

In this paper we report evidence which shows that the biosynthetic pathway to DHPA proceeds via chorismic and prephenic acid and which defines to some extent the mode of incorporation of prephenic acid.

Experimental Procedure

All experimental details are given in the supplementary section of the microfilm edition of the journal.

Results and Discussion

Feeding experiments on the biosynthesis of DHPA were carried out using 100-mL shake cultures of *Streptomyces arenae* strain Tü 109, which were grown as described by Fikenscher et al. (1971). Labeled precursors were added 24 h after inoculation, the cultures were harvested 48 h later, and DHPA was isolated by ion-exchange chromatography and purified by recrystallization from water–ethanol. In an initial series of experiments we confirmed the results of Scannell et al. (1970) by demonstrating that L-[U-^{14}C]phenylalanine is not incorporated into DHPA (data not shown), whereas D-(−)-[U-^{14}C]shikimic acid and DL-[1,6-^{14}C]shikimic acid[2] are efficiently incorporated (Table I). Thus, DHPA is indeed derived from the shikimate pathway but not via the aromatic amino acids.

Considering various possible modes of conversion of shikimic acid (II) into DHPA, one can envision a condensation of the shikimate carboxyl carbon with a two-carbon unit, e.g., derived from acetate or malonate, to provide the three-carbon side-chain of DHPA. In this case, C-3 of DHPA should contain one-seventh of the radioactivity incorporated from D-[U-^{14}C]shikimic acid. To test for this possibility, DHPA from a feeding experiment with [U-^{14}C]shikimate was degraded by aromatization to phenylalanine and oxidation to benzoic acid (0.0445 μCi/mmol), which was then subjected to the Schmidt reaction to give barium carbonate from C-3 (0.2% of specific radioactivity of benzoic acid) and aniline, isolated as acetan-

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[1] Abbreviation used: DHPA, L-2,5-dihydrophenylalanine.

[2] The systemic numbering of shikimic acid according to the IUPAC rules is used throughout this paper.

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TABLE I: Incorporation of Labeled Shikimic Acid and Serine into DHPA.

radioact. precursor[a]	sp act. of start. material (μCi/mmol)	sp act. of product (μCi/mmol)	sp incorp rate (%)
D-[U-^{14}C]shikimic acid (2.19 mmol)	3.48	0.071	2.04
D-[U-^{14}C]shikimic acid (2.16 mmol)	5.54	0.146	2.64
L-[3-^{14}C]serine (1.0 mmol)	11.70	6.73×10^{-3}	0.058
DL-[1,6-^{14}C]shikimic acid (2.24 mmol)	4.97	0.185	3.72

[a] The amount of precursor indicated was added to four 100-mL cultures. [b] DL-[1,6-^{14}C]Shikimic acid (13.9 mCi/mmol) was diluted with nonlabeled D-shikimic acid; the figures in the table refer only to the D-shikimic acid in the mixture.

ilide, representing carbons 1′–6′ (cf. Figure 1) (87.6% of specific radioactivity of benzoic acid). The results clearly show that C-3 is not labeled; the slightly low specific radioactivity of the acetanilide is probably a reflection of the fact that the amount isolated was very small and the material could therefore not be purified rigorously. The results nevertheless clearly rule out a $C_7 + C_2$ pathway.

Another possibility is the condensation of a six-carbon moiety with serine or a derivative of serine, as for example in the formation of tryptophan or in the biosynthesis of the aromatic plant amino acid orcylalanine (Hadwiger et al., 1965). However, as shown in Table I, serine is not significantly incorporated, ruling out such a $C_6 + C_3$ pathway.

By process of elimination, these results point to a mode of formation of DHPA involving a rearrangement of chorismic acid (III) or a derivative thereof, in which the side chain of DHPA is derived from the enopyruvyl side chain of III. Two such processes are known. The one involved in the formation of the normal aromatic amino acids leads by attachment of the side chain at C-1 of the ring to prephenic acid, which is then aromatized (cf. Haslam, 1974). The other proceeds via isochorismic acid (Young et al., 1969) and a hypothetical isoprephenic acid and leads to the unusual plant amino acids *m*-carboxyphenylalanine and *m*-carboxytyrosine (Larsen et al., 1975) with attachment of the side chain at the original C-3 of shikimate. Applied to the biosynthesis of DHPA these two processes would lead to the labeling patterns indicated in Figure 2. A distinction between these two possibilities was made in two ways. One was to determine whether deuterium from [3-^{2}H]shikimic acid was incorporated into DHPA. Shikimic acid was oxidized to 3-dehydroshikimate, which was then reduced with $NaBD_4$ to give a mixture of [3-^{2}H]shikimic and epi[3-^{2}H]shikimic acid. This mixture was fed to a 100-mL culture and the isolated DHPA was analyzed by mass spectrometry and found to contain 13.8% of one deuterium. [U-^{14}C]Shikimic acid fed with the deuterated material was also efficiently incorporated, although the exact specific incorporation rate could not be calculated. Since the hydrogen from C-3 of II is expected to be retained in the "normal" and lost in the "isochorismate" pathway, this result favors operation of the normal chorismate/prephenate pathway in the biosynthesis.

More conclusive evidence in support of this view was obtained by degradation of DHPA biosynthesized from DL-[1,6-^{14}C]shikimic acid to determine the location of the ^{14}C in

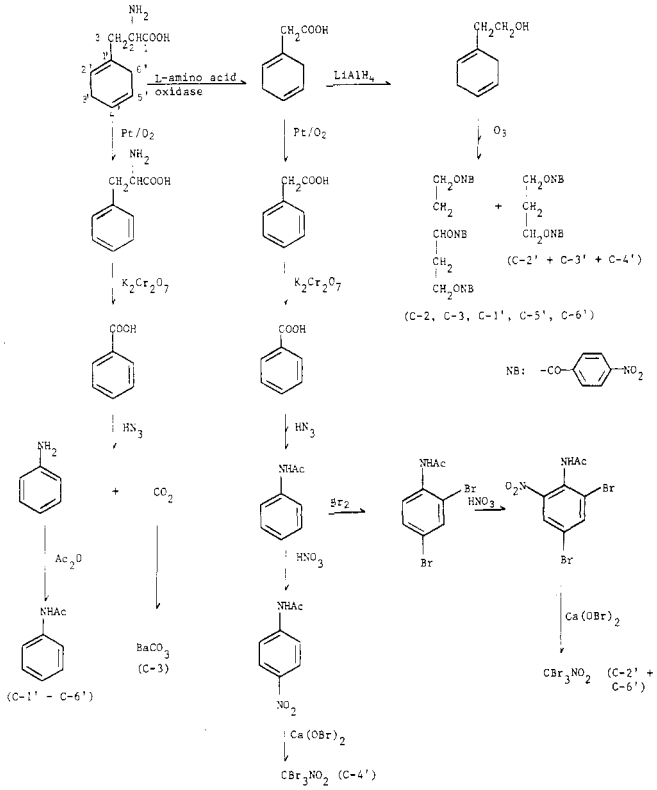

FIGURE 1: Degradations of DHPA to determine the ^{14}C distribution.

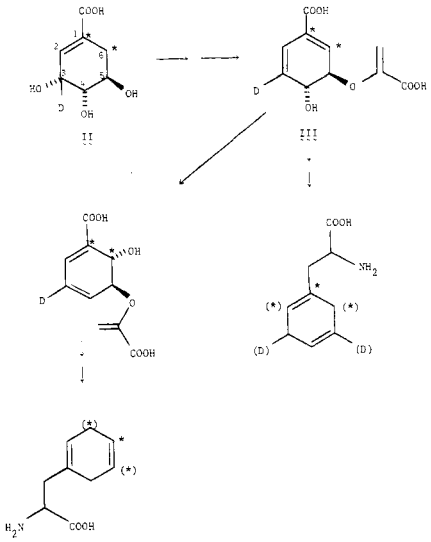

FIGURE 2: Two possible modes of rearrangement of chorismate leading to DHPA.

the ring. DHPA was converted into acetanilide as shown in Figure 1, which was then derivatized to allow isolation of either C-4′ or C-2′ + C-6′ as bromopicrin as described by Baddiley et al. (1950). The results are summarized in Table II. The bromopicrin from C-2′ + C-6′ had 23% of the specific radioactivity of the starting material, indicating that about half of the radioactivity of the DHPA resides at C-2′ and/or C-6′. The bromopicrin from C-4′, on the other hand, was essentially inactive. These results agree with the expectations for the "normal" pathway involving attachment of the side chain at C-1. If the isochorismate route were operating, the bromopicrin from C-4′ should have had half the specific radioactivity of the DHPA and that from C-2′ + C-6′ should have been inactive.

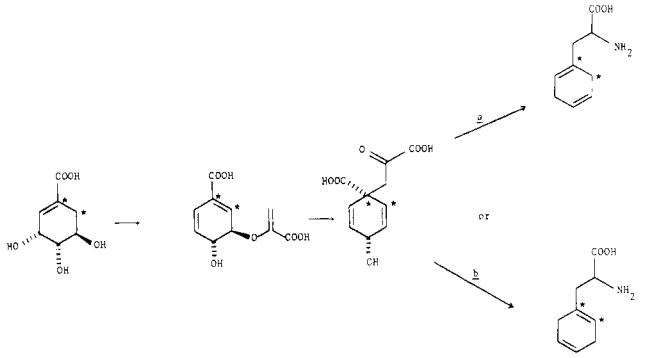

FIGURE 3: Two stereochemical alternatives for the conversion of prephenate into DHPA.

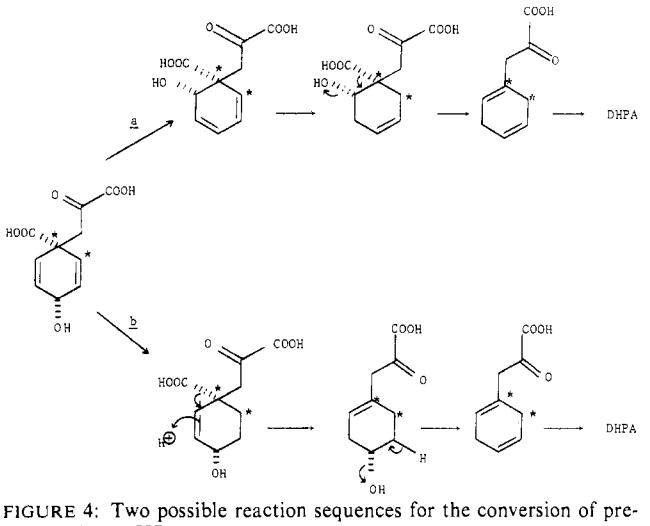

FIGURE 4: Two possible reaction sequences for the conversion of prephenate into DHPA.

TABLE II: Degradation of DHPA from Feeding Experiments with DL-[1,6-^{14}C]Shikimic Acid to Bromopicrin.

compound	sp act. (μCi/mmol)	% of sp radioact.[c]
DHPA	0.050	100
dihydrophenylacetic acid	0.057	
	0.0265[a]	114
acetanilide	0.025	
	0.017[b]	108
p-nitroacetanilide	0.0148	94
2,4-dibromoacetanilide	0.0156	99
bromopicrin (C-4′)	0.00085	5
bromopicrin (C-2′ + C-6′)	0.00358	23

[a] Combined products from two experiments diluted with carrier. [b] After dilution with carrier. [c] Corrected for dilutions.

TABLE III: Incorporation of Chorismate and Prephenate into DHPA.

radioact. precursor[a]	sp act. of start. material (μCi/mmol)	sp act. of product (μCi/mmol)	sp incorp rate (%)
DL-[1,6-^{14}C]shikimic acid[b]	16.41	0.064	0.39
[G-^{14}C]chorismic acid	7.98	0.116	1.45
sodium [G-^{14}C]prephenate	7.17	0.029	0.40

[a] In each experiment 0.37 mmol of precursor was fed to four 100-mL cultures; all 12 cultures were inoculated with the same inoculum. [b] DL-[1,6-^{14}C]Shikimic acid (13.9 mCi/mmol) was diluted with carrier D-shikimic acid; the data in the table refer to the D-shikimic acid only.

The conclusion that the biosynthesis of DHPA proceeds via chorismate and prephenate was further substantiated by demonstrating incorporation of these compounds. Both chorismate and prephenate were prepared in ^{14}C-labeled form by biosynthesis from [U-^{14}C]glucose using appropriately blocked bacterial mutants. Both compounds and shikimic acid for comparison were fed to cultures of *S. arenae* under identical conditions. To minimize decomposition, the precursors were added in several portions during the course of the fermentation. As the data in Table III indicate, chorismate is a substantially better precursor than shikimate. Prephenate is also significantly incorporated, although not any better than shikimate. The relatively poorer incorporation compared to chorismate may well reflect the instability of sodium prephenate under the incubation conditions.

The finding that prephenic acid is a likely intermediate in the biosynthesis of DHPA raises an interesting stereochemical question. Both in the starting material, shikimate, and in the product, DHPA, the two "halves" of the six-membered ring, assuming the ring is bisected along the C-1/C-4 axis, are not identical, whereas prephenate is a symmetrical compound. However, in the latter the two "halves" of the ring are in an enantiotropic relationship and should therefore be distinguished by an enzyme. Since the mode of incorporation of shikimate into prephenate is known (Figure 3), one can thus ask which side of the prephenate ring gives rise to C-2′ and C-3′ of DHPA and which gives rise to C-5′ and C-6′. To decide this question, DHPA biosynthesized from DL-[1,6-^{14}C]shikimic acid was degraded by a reaction sequence which preserves the asymmetry of the cyclohexadiene ring. Ozonolysis of DHPA and several of its derivatives and of dihydrophenylacetic acid gave complex mixtures of products. However, reduction of dihydrophenylacetic acid (0.063 μCi/mmol) to the corresponding alcohol followed by ozonolysis and decomposition of the ozonide with $NaBH_4$ gave cleanly a mixture of 1,3-propanediol and 1,3,5-pentanetriol, which were separated and isolated as their *p*-nitrobenzoyl derivatives (Figure 1). Analysis of the two *p*-nitrobenzoyl derivatives showed that the propanediol is inactive (0.6% of specific radioactivity of dihydrophenylacetic acid), whereas the pentanetriol contains essentially all the radioactivity (93.7% of specific radioactivity of dihydrophenylacetic acid). Together with the earlier degradation data, these results prove that one half of the label incorporated into DHPA from [1,6-^{14}C]shikimate resides at C-6′, indicating that the pathway a of Figure 3 is operating.

The conversion of prephenic acid into DHPA requires a reduction as well as elimination steps which must not ultimately lead to a conjugated diene system. We envisioned two plausible reaction sequences by which this could be accomplished. These are shown in Figure 4. Pathway a involves 1,4 reduction of a conjugated diene system followed by a decarboxylation/dehydration, whereas pathway b involves initial reduction of one double bond of prephenate followed by decarboxylation and dehydration as separate steps. Pathway a seems chemically more plausible, although the postulated 1,4 reduction of a diene is rather unprecedented in biological systems. The availability of both isomers of dihydroprephenic acid from the total synthesis in the laboratory of Danishefsky (Danishefsky and Hirama, 1977; Danishefsky, S., personal communication) enabled us to examine pathway b experimentally. A mixture of the racemic cis and trans isomers of

TABLE IV: Comparison of the Incorporation of Dihydroprephenate, Prephenate, and Shikimate into DHPA.

radioact. precursor[a]	sp act. of start. material (μCi/mmol)	sp act. of product (μCi/mmol)	radioact. of start. material (μCi)	radioact. of product (μCi)	sp incorp rate (%)	incorp rate (%)
DL-[1,6-^{14}C]shikimic acid[b]	≫1000		10.7	1.76×10^{-1}		1.64
[4-^{3}H]dihydroprephenic acid dimethyl ester[b]	≫1000		29.5	4.19×10^{-3}		0.01
[4-^{3}H]dihydroprephenic acid[b]	≫1000		19.8	8.59×10^{-3}		0.04
DL-[1,6-^{14}C]shikimic acid, 0.38 mmol[b,c]	31.42	1.04×10^{-1}			0.33	
sodium [G-^{14}C]prephenate, 0.38 mmol	6.29	2.59×10^{-2}			0.41	

[a] All cultures were inoculated from the same inoculum; one 100-mL culture each was used in the first three experiments; four each were used in the last two experiments. [b] The data in the table are calculated for the "natural" isomer only; it is assumed that only one enantiomer and, in the case of VI and VII, only one cis-trans isomer is biologically active. [c] DL-[1,6-^{14}C]Shikimic acid (13.9 mCi/mmol) was diluted with nonlabeled D-shikimic acid.

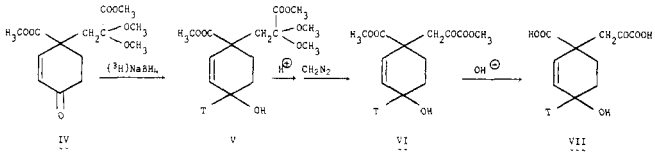

FIGURE 5: Synthesis of [4-^{3}H]dihydroprephenic acid.

dihydroprephenic acid tritiated at C-4 was prepared by reduction of the enone IV (Figure 5), kindly provided by Professor Danishefsky, with tritiated $NaBH_4$. Removal of the ketal function and reesterification gave the dimethyl ester of dihydroprephenate as an approximately 1:1 mixture of the cis and trans isomers. An aliquot of the mixture was hydrolyzed to the free acid. Both the free acid VII and the ester VI were fed to cultures of *S. arenae* in order to compare their incorporation with that of prephenate. Since VI and VII had a high specific radioactivity and no carrier material was available, whereas prephenate had a low specific activity, a direct comparison by feeding equimolar quantities was not possible. Instead, VI and VII were fed together with DL-[1,6-^{14}C]shikimic acid of high specific activity under comparable conditions and the incorporation rate for each compound was determined. In parallel cultures we compared the specific incorporation rate of the same shikimic acid with that of prephenate fed at high concentrations. The results (Table IV) clearly indicate good incorporation of shikimate and prephenate but no significant incorporation of dihydroprephenate. Since neither the free acid nor the ester is incorporated, it seems unlikely that lack of cellular uptake of the precursor is the reason for the negative result. Thus, these results render pathway b of Figure 4 highly improbable.

In summary, the data presented indicate that DHPA is formed from shikimic acid via chorismic acid and prephenic acid by a reaction sequence in which the asymmetry of the ring of shikimic acid is preserved, C-6 of shikimate giving rise to C-6′ of DHPA. For the conversion of prephenate to DHPA, a reaction sequence involving an allyl rearrangement, the 1,4 reduction of a conjugated diene and a combined decarboxylation/dehydration (pathway a, Figure 4), is a likely possibility.

Acknowledgments

We are greatly indebted to Professor H. Zähner, Tübingen, for providing *S. arenae* Tü 109, to Professors R. Somerville and H. Zalkin, Lafayette, for bacterial mutants, and particularly to Professor S. Danishefsky, Pittsburgh, for providing enone IV as well as unpublished procedures for its conversion into dihydroprephenate.

Supplementary Material Available

All experimental details of this work are given in the supplementary material (18 pages). Ordering information is given on any current masthead page.

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Effect of Hydroxyl Radical Scavengers on Microsomal Oxidation of Alcohols and on Associated Microsomal Reactions†

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ABSTRACT: The possibility that oxidation of alcohols by liver microsomes represents a system potentially dependent upon the interaction of the alcohols with hydroxyl radicals was evaluated. Mannitol, benzoate, and dimethyl sulfoxide, three compounds that react rapidly with hydroxyl radicals, inhibited microsomal oxidation of ethanol. Whereas only slight inhibition was observed in the absence of a catalase inhibitor, azide, all three scavengers strikingly decreased microsomal oxidation of ethanol in the presence of azide. The inhibition was competitive with respect to ethanol. These agents had no effect on xanthine oxidase-mediated (H_2O_2-mediated) oxidation of ethanol, nor did they inhibit microsomal electron transport (NADH- or NADPH-cytochrome *c* reduction, NADH- or NADPH-dependent oxygen uptake) or the metabolism of aminopyrine or aniline. The oxidation of l-butanol, which is not an effective substrate for the peroxidatic activity of catalase, was inhibited by dimethyl sulfoxide even in the absence of azide. Whereas microsomal oxidation of ethanol was inhibited 15–25% by azide, butanol oxidation was doubled in the presence of azide. The addition of H_2O_2 in the presence of azide resulted in a stimulation of microsomal oxidation of ethanol and butanol in short term experiments. These results are consistent with a mechanism of microsomal oxidation of alcohols which involves the interaction of the alcohols with hydroxyl radicals that are generated from the microsomal electron transfer pathway. Differences in the metabolism of ethanol and l-butanol may reflect the fact that, whereas ethanol can be oxidized by both a catalase-dependent pathway and a hydroxyl radical pathway, butanol is oxidized only by the latter.

The oxidation of ethanol to acetaldehyde by isolated rat liver microsomes requires NADPH and is similar in many regards to the oxidation of drugs by the microsomal mixed function oxidase system (Lieber & DeCarli, 1968, 1970, 1972). The presence of catalase in isolated microsomes provides for an alternate route for the production of acetaldehyde, namely, the peroxidatic activity of catalase (Keilin & Hartree, 1945; Tephley et al., 1961; Thurman et al., 1972; Thurman, 1973). The H_2O_2 required for peroxidatic activity arises from the oxidation of NADPH by NADPH oxidase (Gillette et al., 1957; Thurman, 1973). Other experiments, however, have shown that the catalase pathway cannot account for all of the NADPH-dependent, ethanol-oxidizing activity of isolated microsomes (Lieber, 1975; Teschke et al., 1974, 1976). Recent experiments have strongly implicated a role for a cytochrome P-450 dependent pathway in the oxidation of ethanol by microsomes (Joly et al., 1977; Ohnishi & Lieber, 1977; Teschke et al., 1977).

Alcohols are well known scavengers of hydroxyl radicals (Anbar & Neta, 1967; Dorfman & Adams, 1973). Recent reports have suggested that hydroxyl radicals are generated by the NADPH-dependent microsomal electron transfer system (Fong et al., 1973; Lai & Piette, 1977). It therefore seemed possible that the microsomal oxidation of ethanol to acetaldehyde could represent, at least in part, the hydroxyl radical scavenging activity of ethanol. In a preliminary communication (Cederbaum et al., 1977), we reported that four scavengers of hydroxyl radicals, namely, dimethyl sulfoxide (Me_2SO),[1] benzoate, mannitol, and thiourea, inhibited the catalase-independent pathway for the oxidation of ethanol by rat liver microsomes. Urea, a compound that reacted poorly with hydroxyl radicals, did not inhibit the oxidation of ethanol and served as a negative control. In the latter studies, azide was added to the system in order to inhibit the oxidation of ethanol by microsomal catalase. In the present study, the effects of hydroxyl radical scavengers are contrasted in the presence and absence of azide. Additional experiments in which H_2O_2 was added directly and experiments with l-butanol as substrate help to clarify the roles of H_2O_2 and of hydroxyl radicals in the microsomal alcohol-oxidizing system.

Methods

Liver microsomes were prepared from male Sprague-Dawley rats as previously described (Cederbaum et al., 1976), washed once, and suspended in 125 mM KCl. Protein was determined by the method of Lowry et al. (1951).

Microsomal oxidation of ethanol or l-butanol was assayed as previously described (Cederbaum et al., 1977), with the use of flasks containing 0.6 mL of 15 mM semicarbazide in the center well. In most experiments, the final concentration of ethanol or butanol was 51 mM. The reaction was initiated with the NADPH-generating system and was terminated after 15 min at 37 °C by the addition of trichloroacetic acid (final

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[1] Abbreviation used: Me_2SO, dimethyl sulfoxide; SEM, standard error of the mean.

0006-2960/78/0417-3058$01.00/0