Effect of Phenobarbital Treatment on the Stereochemistry of the *in Vitro* Metabolism of Ethylbenzene[†]

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The metabolism of ethylbenzene has been studied using isolated subcellular hepatic fractions to ascertain the relationship of enzyme induction to the decreased stereoselectivity of metabolism reported in intact animals. It was observed that both ethylbenzene and 1-phenylethanol were oxidized by microsomal enzymes and that phenobarbital treatment increased the rate of metabolism. Acetophenone, the oxidation product of 1-phenylethanol, was reduced by a soluble reductase which was not altered by phenobarbital treatment. Kinetic studies revealed that phenobarbital treatment produced only quantitative changes in the metabolism of ethylbenzene. No great differences in the metabolism of (*R*)-(+)-, (*S*)-(−)-, or (*RS*)-(±)-1-phenylethanol were observed when microsomal fractions were employed. However, more (*S*)-(−)-1-phenylethanol disappeared from incubation mixtures than (*R*)-(+)-1-phenylethanol when 9000g supernatant fractions were used as the enzyme source. (*R*)-(+)-1-Phenylethanol accumulated during the course of the incubation when 9000g supernatant fractions were employed; no change in enantiomeric composition was noted when microsomal fractions served as the source of the enzyme. Acetophenone was stereospecifically reduced to (*S*)-(−)-1-phenylethanol by a soluble reductase. These results suggest that quantitative alterations in the pathway, ethylbenzene → 1-phenylethanol → acetophenone → 1-phenylethanol, as well as the selective destruction of (*S*)-(−)-1-phenylethanol could account for the changes in optical purity observed after phenobarbital treatment.

Although it is well known that many factors influence microsomal drug metabolism, most investigators have been concerned with those factors which alter the hepatic drug metabolizing enzymes.[1] While it is important to study these factors, it is equally important to consider physical and chemical properties of substrates employed in drug metabolism. For example, the role of lipid solubility in microsomal oxidations has been discussed by McMahon[2] and Hansch.[3] Another physical factor, stereochemical configuration, has received relatively little attention in drug metabolism although the stereochemical factors involved in biological activity are well known.[4]

Stereochemically, an enzyme can attack two basic types of substrates: (a) enantiomers and (b) optically inactive compounds which become optically active as a result of biotransformation. A number of investigators have shown that enantiomers of sympathomimetic amines,[5,6] hexobarbital,[7,8] narcotic analgesics,[9,10] and local anesthetics[11] are metabolized at different rates. On the other hand, McMahon and Sullivan observed that the benzylic hydroxylation of ethylbenzene to optically active 1-phenylethanol is a stereoselective reaction.[12,13] Although earlier workers[14] recognized the stereochemistry involved in the reactions, McMahon and Sullivan demonstrated that the formation of 1-phenylethanol proceeds with a high degree of stereoselectivity.[12] In addition, they observed that rats treated with phenobarbital and other compounds hydroxylated ethylbenzene with a decreased stereoselectivity both *in vivo* and *in vitro*.[12] They suggested that a qualitatively altered microsomal enzyme could account for this reduced stereoselective microsomal hydroxylation. While it has been established that treatment with polycyclic hydrocarbons alters the microsomal enzymes qualitatively as well as quantitatively,[15-17] most evidence suggests that phenobarbital treatment produces only quantitative alterations.[18] Thus, a quantitatively altered metabolic pathway could account for the observed changes in optical activity of 1-phenylethanol seen after phenobarbital treatment. Evidence to support this contention is presented herein.

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Results

Characterization of *in Vitro* Reactions. The metabolic pathway forming (*RS*)-(±)-1-phenylethanol was studied to characterize the enzymes involved. The hydroxylation of ethylbenzene and the oxidation of (*RS*)-(±)-1-phenylethanol were catalyzed by enzymes located in the microsomal fraction of the liver and acetophenone was reduced by the soluble enzyme fraction. Cofactor requirements are shown in Table I. Ethylbenzene was hydroxylated by a TPNH-dependent mixed function oxidase. Carbon monoxide inhibited the hydroxylation of ethylbenzene 69.5% when a gas mixture in which $CO/O_2 = 2$ and an ethylbenzene concentration of 1 m*M* was employed. (*RS*)-(±)-1-Phenylethanol was oxidized to acetophenone by both TPNH- and DPN-dependent microsomal enzymes. Preliminary studies indicate that the TPNH-dependent enzyme is inhibited by carbon monoxide; no inhibition by carbon monoxide was observed in the DPN-dependent pathway. Furthermore, phenobarbital

Table I. Cofactor Requirements for the *in Vitro* Metabolism of Ethylbenzene[a]

$$\text{ethylbenzene} \xrightarrow{\text{rn 1}} (RS)\text{-}(\pm)\text{-1-phenylethanol} \underset{\text{rn 3}}{\overset{\text{rn 2}}{\rightleftarrows}} \text{acetophenone}$$

	Reaction		
Cofactor	1[b]	2[b]	3[c]
TPNH	0.80 ± 0.01 (4)	1.71 ± 0.01 (4)	0.71 ± 0.02 (4)
TPN	ND[d]	ND	ND
DPNH	ND	ND	ND
DPN	ND	0.20 ± 0.03 (3)	ND

[a]Incubation mixtures were complete as described in the Experimental Section except for pyridine nucleotides (1 m*M*) which were added as shown above; the substrate concentrations were 5 m*M*. Reaction velocities are expressed as nmol of product/mg of protein/min ± S.E.M. The number of experiments is in parentheses. [b]Enzyme source, microsomal fraction. [c]Enzyme source, soluble fraction. [d]ND = no reaction detected.

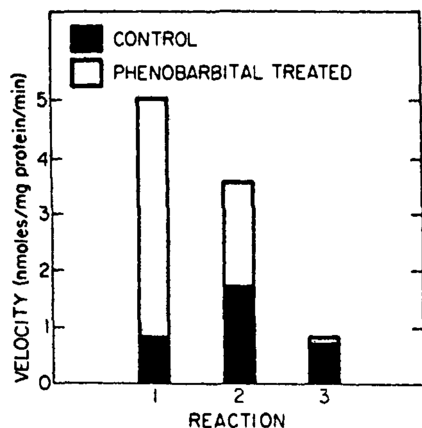

Figure 1. Effect of phenobarbital treatment of rates of ethylbenzene metabolism. Reaction 1: hydroxylation of ethylbenzene to 1-phenylethanol (enzyme source, microsomal fraction). Reaction 2: oxidation of 1-phenylethanol to acetophenone (enzyme source, microsomal fraction). Reaction 3: reduction of acetophenone to 1-phenylethanol (enzyme source, soluble fraction). Incubation mixtures were prepared and assayed as described in the Experimental Section. Rats received phenobarbital sodium (40 mg/kg) once daily for 5 days.

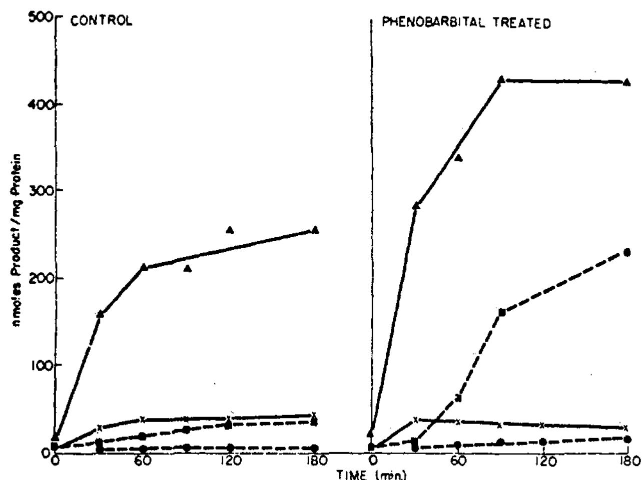

Figure 2. Formation of 1-phenylethanol and acetophenone from ethylbenzene in microsomal and 9000g supernatant fractions derived from control and phenobarbital-treated rats: (–X–) 1-phenylethanol–9000g supernatant fraction; (–▲–) 1-phenylethanol–microsomal fraction; (- - ● - -) acetophenone-9000g supernatant fraction; (- - ■ - -) acetophenone–microsomal fraction.

treatment did not increase the rate of the DPN-dependent reaction. The reduction of acetophenone was catalyzed by a soluble enzyme requiring TPNH as cofactor and was not inhibited by carbon monoxide.

Effect of Phenobarbital Treatment on Ethylbenzene Metabolism *in Vitro*. The effect of phenobarbital treatment on ethylbenzene metabolism *in vitro* is shown in Figure 1. The hydroxylation of ethylbenzene by hepatic microsomes to 1-phenylethanol increased in activity from a control value of 0.8 to 5.0 nmol/mg of protein/min in phenobarbital treated rats. Similarly, the oxidation of (*RS*)-(±)-1-phenylethanol to acetophenone increased from a control value of 1.7 to 3.5 nmol/mg of protein/min following phenobarbital treatment. The reduction of acetophenone was not altered by treatment with phenobarbital. These results show that the oxidation of both ethylbenzene and 1-phenylethanol is increased by phenobarbital treatment.

The effect of phenobarbital treatment on the time course

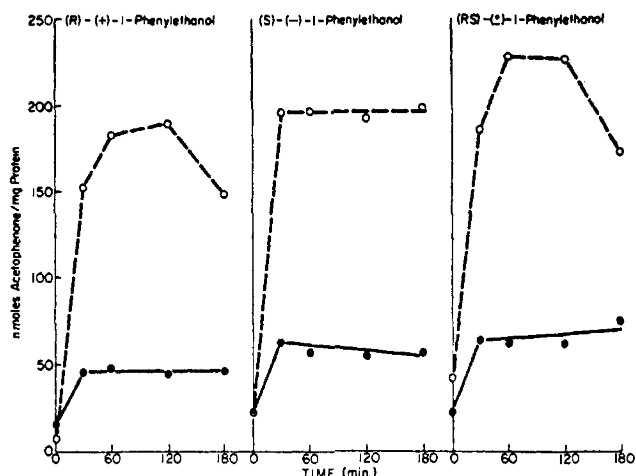

Figure 3. Metabolism of (*R*)-(+), (*S*)-(–), and (*RS*)-(±)-1-phenylethanol to acetophenone by hepatic microsomal fractions: (- - -) phenobarbital-treated; (—) control.

of conversion of ethylbenzene to 1-phenylethanol and acetophenone by both microsomal fractions and 9000g supernatant fractions was also studied. The results are shown in Figure 2. It can be seen that the conversion of ethylbenzene to 1-phenylethanol by hepatic microsomal fractions is increased considerably by phenobarbital treatment; in contrast, 1-phenylethanol formation by 9000g supernatant fractions was comparable in both induced and noninduced animals. Acetophenone formation was more rapid in microsomal fractions derived from phenobarbital-treated animals as compared to controls. Relatively little difference in acetophenone formation was noted between induced and noninduced animals when 9000g supernatant fractions were employed. The accumulation of acetophenone when microsomal fractions from phenobarbital-treated animals are employed is attributable to both increased oxidation of 1-phenylethanol and the absence of the soluble reductase.

Effect of Phenobarbital Treatment on Kinetics and Inhibition of Ethylbenzene Hydroxylation. The kinetic data obtained for the *in vitro* conversion of ethylbenzene to 1-phenylethanol are summarized in Table II. It can be seen that phenobarbital treatment does not alter the Michaelis constant (K_m) [$p > 0.5$] while the maximal velocity (V_{max}) was increased significantly ($p < 0.05$). SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) was a competitive inhibitor of the microsomal hydroxylation of ethylbenzene; phenobarbital treatment did not alter the inhibition constant (K_i) of the reaction. These are the expected results if phenobarbital treatment produces only quantitative changes.

Metabolism of (*R*)-(+)-, (*S*)-(–)-, and (*RS*)-(±)-1-Phenylethanol by Hepatic Microsomal Fractions and by 9000g Supernatant Fractions. The conversion of both enantiomers as well as the racemic modification of 1-phenylethanol to acetophenone by microsomal fractions has also been examined as shown in Figure 3. It can be seen that although the conversion of 1-phenylethanol to acetophenone is markedly increased following phenobarbital treatment, the separate enantiomers do not show great differences.

The disappearance of (*R*)-(+)-, (*S*)-(–)-, and (*RS*)-(±)-1-phenylethanol and the simultaneous formation of acetophenone by 9000g supernatant fractions are shown in Figure 4. In control animals, 33.3% of (*R*)-(+)-1-phenylethanol and 59.2% of (*S*)-(–)-1-phenylethanol disappeared after 180 min. In phenobarbital-treated animals the corresponding values

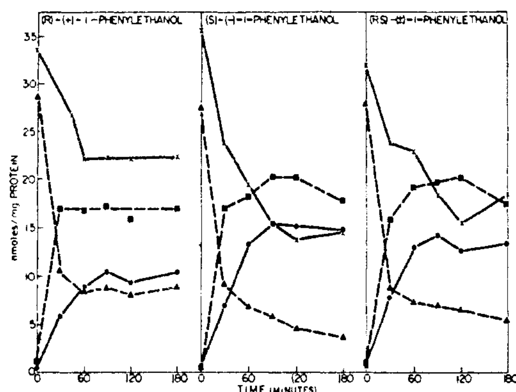

Figure 4. Disappearance of 1-phenylethanol and simultaneous formation of acetophenone in 9000g supernatant fractions derived from control and phenobarbital-treated rats: (–X–) 1-phenylethanol, control; (–●–) acetophenone, control; (- -▲- -) 1-phenylethanol, phenobarbital-treated; (- -■- -) acetophenone, phenobarbital-treated.

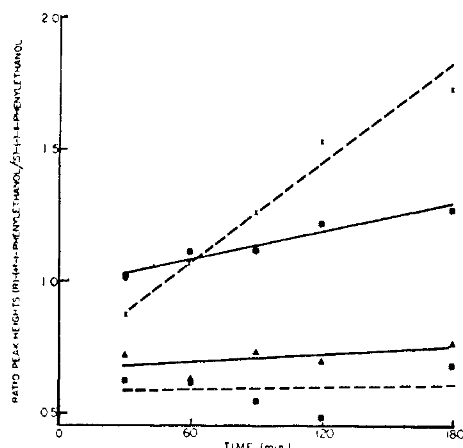

Figure 5. Enantiomeric composition of 1-phenylethanol formed enzymically from ethylbenzene: (–●–) 9000g supernatant fraction, control; (- -X- -) 9000g supernatant fraction, phenobarbital-treated; (–▲–) microsomal fraction, control; (- -■- -) microsomal fraction, phenobarbital-treated.

for (*R*)-(+)- and (*S*)-(–)-1-phenylethanol were 69.5 and 86.9%, respectively.

Effect of Phenobarbital Treatment on the Stereochemistry of Metabolism of Ethylbenzene to 1-Phenylethanol by Microsomal Fractions. The availability of a sensitive gas chromatographic method[19] for the determination of absolute optical purity of 1-phenylethanol allows assessment of the stereochemical course of the benzylic hydroxylation of ethylbenzene. The results of these experiments are summarized in Figure 5. It can be seen that the enantiomeric composition of enzymically formed 1-phenylethanol remains constant when microsomal fractions from normal and phenobarbital-treated rats are employed. In contrast, when 9000g supernatant fractions were employed, (*R*)-(+)-1-phenylethanol accumulated during the course of the experiment; this effect is even more pronounced when enzyme preparations derived from phenobarbital-treated rats are employed.

Reduction of Acetophenone by 105,000g Supernatant Fractions. The enzyme catalyzing the reduction of acetophenone to 1-phenylethanol was located in the soluble fraction of the hepatocyte and required TPNH as cofactor (Table I). Using the gas chromatographic method,[19] it was observed that only optically pure (*S*)-(–)-1-phenylethanol was formed when acetophenone was reduced enzymically. Phenobarbital treatment was without effect on the stereochemical course of acetophenone reduction.

Discussion

McMahon and Sullivan[12] observed that treatment of rats with phenobarbital results in a decreased stereoselectivity of the *in vivo* hydroxylation of ethylbenzene. Normal rats excreted optically active 1-phenylethanol as 90.0% (*R*)-(+)-1-phenylethanol. They also observed that phenobarbital pretreatment resulted in a decreased stereoselective hydroxylation of ethylbenzene from a control value of 80.0 to 66.6% (*R*)-(+)-1-phenylethanol when they used the 15,000g supernatant of hepatic homogenates containing both microsomal and soluble fractions. These findings have been confirmed by Maylin and Anders.[20] On the basis of these results McMahon and Sullivan[12] suggested that a qualitative change in the structure of the endoplasmic reticulum could account for the reduced stereoselectivity observed both *in vivo* and *in vitro*.

Alternatively, a quantitatively altered metabolic pathway rather than a qualitatively changed endoplasmic reticulum could account for the apparent changes seen in the stereoselective hydroxylation of ethylbenzene following phenobarbital treatment. The data in Figure 1 show that the steps in the metabolic pathway of ethylbenzene to 1-phenylethanol to acetophenone to 1-phenylethanol were altered quantitatively by phenobarbital treatment. The increased rates for both the hydroxylation of ethylbenzene and the oxidation of 1-phenylethanol provide an increased amount of acetophenone for the soluble reductase, which serves to stereospecifically convert acetophenone to (*S*)-(–)-1-phenylethanol. This should contribute to the overall reduction in optical purity seen after phenobarbital treatment and is consistent with a quantitatively altered metabolic pathway.

An alternative way by which phenobarbital treatment could alter the optical purity of 1-phenylethanol would be to selectively increase the degradation of one of the enantiomers. The data in Figure 3 show that phenobarbital treatment produces quantitative changes in the microsomal conversion of 1-phenylethanol to acetophenone but the enantiomers show no great differences. However, when 9000g supernatant fractions were employed (Figure 4), more (*S*)-(–)-1-phenylethanol was metabolized than the (*R*)-(+) enantiomer and the effect was enhanced by phenobarbital treatment. It can also be seen from Figure 4 that the amount of 1-phenylethanol metabolized is in excess of the acetophenone formed, suggesting additional pathways. In this connection it should be noted that McMahon and Sullivan reported that while (*S*)-(–)-1-phenylethanol was metabolized to (*S*)-(–)-mandelic acid *via* a pathway involving acetophenone as an intermediate, (*R*)-(+)-1-phenylethanol was excreted as the glucuronide.[21]

The results in Figure 2 show that the time course of ethylbenzene hydroxylation and 1-phenylethanol oxidation differs qualitatively when microsomal or 9000g supernatant fractions are employed. Neither 1-phenylethanol nor acetophenone accumulates in the reaction mixture when 9000g supernatant fractions from either control or phenobarbital-treated rats are employed. In contrast, acetophenone accumulation is marked when microsomal fractions from phenobarbital animals are used. This is not unexpected since the reductase metabolizing acetophenone is absent.

If the effect of phenobarbital is to produce quantitative changes in the metabolism of ethylbenzene, it may be pre-

Table II. Effect of Phenobarbital Treatment on the Kinetics and Inhibition of Ethylbenzene Hydroxylation[a]

Enzyme	K_m, $M \times 10^3$	V_{max}	K_i, $M \times 10^4$, SKF 525-A
Normal enzyme	5.5 ± 1.2 (3)[b]	1.5 ± 1.2 (3)[c]	0.42 ± 0.13 (3)[b]
Phenobarbital induced	5.4 ± 3.2 (3)[b]	9.5 ± 2.8 (3)[c]	0.44 ± 0.03 (3)[b]

[a]Rats were treated for 5 days with 0.9% NaCl or phenobarbital sodium (40 mg/kg). Rat liver microsomal fractions were prepared as described in the Experimental Section. The maximal velocity is expressed as nmol of 1-phenylethanol formed/mg of protein/min. The inhibitor was SKF 525-A (concentration = 0.5 and 1.0 m*M*). The values are shown as the mean ± S.E.M.; the numbers in parentheses refer to the number of determinations. [b]Not significantly different ($p > 0.5$). [c]Significantly different ($p < 0.05$).

dicted that the enantiomeric composition of the 1-phenylethanol produced should change during the course of incubation. The data in Figure 5 show that this is the case only when the soluble fraction is present; phenobarbital treatment further enhances this change. It should be noted that (*R*)-(+)-1-phenylethanol appears to accumulate during the course of the incubation. However, in the intact animal a greater amount of (*S*)-(−)-1-phenylethanol is excreted following phenobarbital treatment. The basis for this discrepancy between the *in vitro* and *in vivo* situations is not clear.

The kinetic data obtained (Table II) support the contention that the change in the stereochemistry of ethylbenzene metabolism following phenobarbital treatment is the result of a quantitative change. This is also in agreement with the results reported by Rubin, *et al.*,[22] Netter and Seidel,[23] and Anders and Mannering[24] showing that phenobarbital treatment does not alter the K_m for the microsomal metabolism of several substrates. No change in either the K_m or K_i after phenobarbital treatment suggests that the reduced stereoselectivity is due to a quantitative change. It should be pointed out, however, that phenobarbital treatment has been shown to qualitatively alter the kinetics of the metabolism of aniline, a type II substrate.[25] Lack of qualitative change in the metabolism of type I substrates after phenobarbital treatment was also shown by the work of Ullrich,[18] who demonstrated equal turnover numbers for the microsomal metabolism of cyclohexane in both control and phenobarbital-induced rats. Unpublished results from this laboratory show that ethylbenzene is a type I substrate.

Finally, preliminary experiments have been carried out to characterize the enzymic reactions involved. The cofactor requirements, subcellular location, and inhibition studies indicate that the benzylic hydroxylation of ethylbenzene is catalyzed by a microsomal mixed function oxidase. The oxidation of 1-phenylethanol to acetophenone is catalyzed by both a microsomal TPNH-dependent oxidase and a microsomal dehydrogenase, the former predominating (Table I). Inhibition by carbon monoxide, requirement of TPNH as a cofactor, induction by phenobarbital treatment, and microsomal location suggest a mixed function oxidase in the oxidation at 1-phenylethanol. Alternatively, a peroxidative pathway may be involved.[26,27] The soluble enzyme catalyzing the reduction of acetophenone to 1-phenylethanol with TPNH as a cofactor is similar to other reductases which have been described.[28]

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured on a Bendix 969 polarimeter equipped with a 0.2-dm cell. The ir of all compounds was consistent with the structures.

Animals. Long Evans strain male rats (Blue Spruce Farms, Inc., Altamont, N. Y.) weighing 100–150 g were employed as experimental animals. A commercial ration and tap water were given *ad libitum.* For the induction experiments, phenobarbital sodium was dissolved in 0.9% sodium chloride solution and was administered intraperitoneally (40 mg/kg) once daily for 5 days. Rats were sacrificed 24 hr after the last dose.

Chemicals. Unless otherwise stated, chemicals were obtained from commercial sources and used without further purification. All water was distilled over EDTA and glassware used in enzyme studies was acid-washed. Ethylbenzene, 99.9%, was purchased from Chemical Samples Co.

Preparation of (*R*)-(+)- and (*S*)-(−)-1-Phenylethanol. (−)-1-Phenylethylene glycol was prepared essentially as described by Bakshi and Turner.[29] (−)-Mandelic acid, 15.2 g (Aldrich Chemical Co.), mp 132–134°, $[\alpha]^{24}$D −160° (*c* 1, MeOH), was reduced with 5.4 g of lithium aluminum hydride in the usual manner. The yield of (−)-1-phenylethylene glycol was 10.2 g; this was recrystallized from ether-petroleum ether: mp 59–62°; $[\alpha]^{23}$D −44.7° (*c* 0.76, MeOH). Bakshi and Turner[29] obtained a product of mp 66–67°, $[\alpha]^{24}$D −39.9 ± 0.3° (*c* 6.562, EtOH). (−)-1-Phenylethylene **glycol 2-tosylate** was prepared by reacting 6.84 g of (−)-1-phenylethylene glycol in 20 ml of pyridine, cooled to −10°, with 9.49 g of *p*-toluenesulfonyl chloride. The yield of tosylate was 14.2 g; this was recrystallized from ether-petroleum ether: mp 69–71°; $[\alpha]^{25}$D −33.5° (*c* 1, MeOH). (*S*)-(−)-1-Phenylethanol was prepared by reducing 8.36 g of (−)-1-phenylethylene glycol 2-tosylate with 1.7 g of lithium aluminum hydride in the usual manner to give 3.2 g of (*S*)-(−)-1-phenylethanol. The product distilled at 80° (5 mm), $[\alpha]^{25}$D −45.6° (*c* 1.48, MeOH). The reported rotation of this compound is $[\alpha]^{20}$D −43.76° (neat).[30] (+)-1-Phenylethylene **glycol** was prepared by reduction of 24.75 g of (+)-mandelic acid (Aldrich Chemical Co.), mp 130–133°, $[\alpha]^{24}$D +160° (*c* 1, MeOH), with 9.4 g of lithium aluminum hydride to give 20.0 g of (+)-1-phenylethylene glycol. The product was recrystallized from ether-petroleum ether: mp 59–65°; $[\alpha]^{24}$D +43.2° (*c* 1.02, MeOH). (+)-1-Phenylethylene **glycol 2-tosylate** was synthesized by reacting 12.9 g of (+)-1-phenylethylene-glycol in 40 ml of pyridine, cooled to −10°, with 19.0 g of *p*-toluenesulfonyl chloride. The yield of tosylate was 27.4 g; this was recrystallized from ether-petroleum ether: mp 69–72°; $[\alpha]^{26}$D +33.6° (*c* 1.1, MeOH). (*R*)-(+)-1-Phenylethanol was prepared by reduction of 21.3 g of 1-phenylethylene glycol 2-tosylate with 4.16 g of lithium aluminum hydride to give 8.85 g of (*R*)-(+)-1-phenylethanol. The product distilled at 80° (5 mm), $[\alpha]^{25}$D +45.9° (*c* 3.32, MeOH). The reported rotation of this compound is $[\alpha]^{19}$D +42.88° (neat).[30]

Enzyme Assay. Microsomal fractions and 9000g supernatant fractions were isolated by differential centrifugation of homogenates prepared in 0.25 *M* sucrose solution. The microsomal fractions were washed twice by resuspending and recentrifuging. If not used immediately, the enzyme was stored frozen at −10° for up to 3 weeks with no apparent loss of activity.

For the enzyme assays, the usual reaction mixture contained TPN (8.0 μmol), DL-isocitrate (80 μmol), isocitrate dehydrogenase (8.0 enzyme units), phosphate buffer, pH 7.4 (2.5 mmol), magnesium chloride (375 μmol), substrate, inhibitor (when used), 5 ml of enzyme solution containing either 50 mg of microsomal or soluble enzyme protein or 250 mg of protein derived from the 9000g supernatant fraction and water to a final volume of 25 ml. The substrate concentrations varied in different experiments. When product appearance and stereochemistry were measured, the substrate concentration was 10 m*M* in order to ensure saturation of the enzyme. When the disappearance of 1-phenylethanol was studied, the substrate concentration was lowered to 0.4 m*M* to facilitate the detection of small changes in the substrate concentration. When ethylbenzene served as the substrate the amounts of TPN, DL-isocitrate, and isocitrate dehydrogenase added were increased threefold to provide adequate amounts of cofactors for the entire series of reactions. The reaction mixtures were incubated with shaking at 37° in an air atmosphere. Unless otherwise stated, the incubation time was 30 min. In some cases the reactions were stopped by rapid cooling in an ice bath; the 1-phenylethanol and acetophenone contents of the mixture were determined by injecting an aliquot of the reaction mixture directly into the gas chromatograph. Alternatively, the reaction was stopped by adding 10 ml of benzene to each flask. After shaking and centrifuging, the benzene layer was separated and dried over magnesium sulfate, and an aliquot was injected into the gas chromatograph. Using the latter procedure, 90% of the metabolite (1-phenylethanol and acetophenone) was recovered. 1-Phenylethanol

and acetophenone were chromatographed on either a 6 ft × 6 mm glass column packed with 10% poly(ethylene glycol) (Carbowax 4000) on 80–100 mesh Gas Chrom Q and operated at 100° on a Barber-Colman 5000 gas chromatograph or a 6 ft × 1.8 mm stainless steel column packed with 10% poly(propylene glycol) (Ucon 50-HB-2000) on 80–100 mesh, silylated Gas Chrom S held at 100°, on a Varian Aerograph 1200 gas chromatograph. Both instruments were equipped with flame ionization detectors.

The enantiomeric composition of the enzymically formed 1-phenylethanol was measured gas chromatographically following diastereomer formation as previously described by Anders and Cooper.[19] For these estimations, each benzene extract was transferred to an evaporator tube (Kontes K-288250) and 20 μl of pyridine and 3 mg of 3-β-acetoxy-Δ^5-etienic acid chloride were added. The mixture was maintained at 90° in a Kontes tube heater for 30 min; the temperature was then increased to 130° to concentrate the reaction mixture to about 0.1 ml. The residue (5–10 μl) was injected directly into the gas chromatographic column.

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Conformation of Histamine Derivatives. 1. Application of Molecular Orbital Calculations and Nuclear Magnetic Resonance Spectroscopy

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The conformational energies of the histamine dication and of the two tautomeric forms of its monocation are calculated using EHT and CNDO molecular orbital procedures. The relative populations of trans/gauche conformers predicted by the EHT method agree well with nmr-derived aqueous solution data, but the CNDO predictions do not agree. The applicability of EHT calculations is further indicated by comparing predicted with observed (nmr-derived) rotamer populations for the series 2-, 3-, 4-, and *N*-methyl-, *N*,*N*-dimethyl-, and *N*,*N*,*N*-trimethylhistamine dications. The nmr data indicate that the mole fraction of trans rotamers is somewhat greater for the histamine dication (0.54) than for the monocation (0.45) and that monomethylation has but little additional influence. Further methylation at the amino group of histamine increases the trans rotamer mole fraction from 0.57 (for $-N^+H_2Me$) to 0.72 (for $-N^+HMe_2$) to 0.92 (for $-N^+Me_3$).

Conformational analysis of histamine in relation to biological activity is of contemporary interest[1,2] and there have been several recent studies using quantum mechanical (molecular orbital) techniques.[3-6] Following Ash and Schild's suggestion[7] that the pharmacological actions of histamine could be mediated by at least two types of histamine receptor in tissues, Kier proposed[3] that the drug-receptor interactions involved two distinct histamine conformations and adduced evidence from extended Hückel theory (EHT) that the lone gaseous histamine molecule has two preferred minimum energy conformations. Subsequent studies indicated[8] that this might also be the case for histamine in aqueous solution.

Others have taken a different view of the molecular orbital calculation procedure and made different predictions.[4,5] In any case, there is no *a priori* reason why a minimum energy conformation should be the biologically active one and, although the proposition is attractive, there is as yet no corroborative evidence that relates the dual activity of histamine to its conformation. The problem is further complicated by the fact that histamine exists as an equilibrium mixture of different tautomeric and ionic species. Although Kier selected the species that is probably the most prevalent under physiological conditions, there are others which should be considered.

For these reasons it seems necessary to examine the prob-

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