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Arene Oxides as Intermediates in the Oxidative Metabolism of Aromatic Compounds. Isomerization of Methyl-Substituted Arene Oxides†

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ABSTRACT: Arene oxides are strongly implicated as key intermediates in monoxygenase-catalyzed metabolism of aromatic compounds. To provide further information on their metabolic significance, the arene oxides of toluene, of the three xylenes, of mesitylene, and of 2-methyl- and 1,2-dimethylnaphthalene 1,2-oxide were synthesized, and their phenolic rearrangement products were compared to the phenols obtained by hepatic metabolism of the parent hydrocarbons. The range of phenolic products obtained from microsomal metabolism of the alkyl-substituted aromatic substrates was compatible with the intermediacy of certain extremely labile arene oxides. Formation of arene oxides with alkyl substituents on the oxirane ring did not appear to be a significant pathway for hepatic metabolism except with mesitylene. Migrations of methyl groups as well as apparent migrations of oxygen occurred during rearrangement of methyl-substituted arene oxides. Formation of 2,4-dimethylphenol from 1,4-dimethylbenzene oxide provides a chemical model for the conversion of 4-methylphenylalanine to 3-methyltyrosine by phenylalanine hydroxylase. Phenolic isomers formed on rearrangement of the arene oxides were qualitatively predictable by a simple carbonium ion theory. The *ratios and nature* of isomerization products, however, varied with reaction conditions, indicating that multiple mechanistic pathways are operative in these rearrangements.

The arene oxide, naphthalene 1,2-oxide, has been established as the obligatory intermediate in the metabolic formation of naphthol, a dihydrodiol and a glutathione conjugate from the bicyclic hydrocarbon, naphthalene (Jerina *et al.*, 1968c, 1970a). In addition, an arene oxide was recently reported as a metabolite of the polycyclic hydrocarbon, dibenzanthracene (Selkirk *et al.*, 1971). Arene oxides derived from monocyclic hydrocarbons have yet to be isolated from a biological system (*cf.* Jerina *et al.*, 1968b). However, a variety of evidence does *suggest* arene oxides as key intermediates in the hepatic metabolism of monocyclic hydrocarbons. For example, benzene and a variety of other monocyclic aromatic compounds are converted to dihydrodiols and premercapturic acids (Sato *et al.*, 1963; Jerina *et al.*, 1967; Smith *et al.*, 1950; Chang *et al.*, 1970, etc.), presumably *via* intermediate arene oxides. In addition, formation of phenols from both monocyclic (Daly *et al.*, 1968b) and bicyclic (Boyd *et al.*, 1972) aromatic hydrocarbons occurs with varying degrees of retention of the ring substituent originally present at the position to which the hydroxyl group is introduced. This migration and retention of substituent, known as the NIH shift (Guroff *et al.*, 1967; Daly *et al.*, 1968a; Jerina *et al.*, 1971b), is *incompatible* with the direct formation on phenols by an insertion reaction, but is compatible with the migration of substituents which occur during isomerization of deuterated arene oxides to phenols (Jerina *et al.*, 1968a; Boyd *et al.*, 1972). The present investigation has attempted to determine whether the intermediacy of arene oxides is compatible with the observed metabolism of various alkylated aromatic hydrocarbons and whether such arene oxides would have the requisite stability for isolation from the metabolic system.

Experimental Section

General. All compounds synthesized were routinely checked for structure and purity by proton magnetic resonance (Varian HA-100) and mass spectrometry (Hitachi RMU-7). In many instances, combustion data were also obtained. The general synthetic schemes are shown in Table I and the proton magnetic resonance spectra of the olefins are presented in Table II.

Preparation of Dihydrobenzenes and Dihydronaphthalenes. Birch reductions of methyl-substituted benzoic acids and

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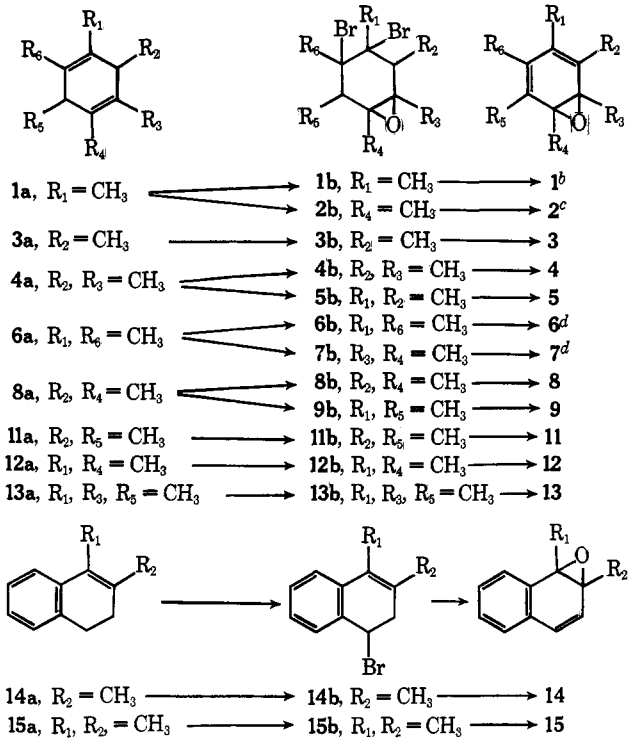
TABLE I: General Schemes for Synthesis of Alkyl-Substituted Arene Oxides.[a]



[a] R = H unless otherwise specified. [b] Prepared as described (Jerina *et al.*, 1968a). [c] Prepared as described (Günther *et al.*, 1967). [d] Prepared as described (Vogel and Günther, 1967).

TABLE II: Proton Magnetic Resonance Spectra of Cycloalkenes (δ Values at 60 MHz in $CDCl_3$, s = Singlet, d = Doublet, m = Multiplet).

3a, CH_3 (3 H, 1.06, d, 7 Hz), CH and CH_2 (3 H, 2.43–2.83, m), vinyl (4 H, 5.4–5.85, m)

4a, CH_3 (3 H, 1.08, d, 7 Hz), CH_3 (3 H, 1.65–1.8, m), CH and CH_2 (3 H, 2.4–2.8, m), vinyl (1 H, 5.3–5.5 m), vinyl (2 H, 5.55–5.75, m)

8a, CH_3 (3 H, 1.04, d, 7 Hz), CH_3 (3 H, 1.6–1.75, m), CH and CH_2 (3 H, 2.4–2.7, m), vinyl (1 H, 5.2–5.46, m), vinyl (2 H, 5.58–5.7, m)

11a, CH_3 (6 H, 1.06, d, 7 Hz), CH (2 H, 2.5–2.95, m), vinyl (4, H, 5.5–5.7, m), methyls cis

12a, CH_3 (6 H, 1.57–1.70, m), CH_2 (4 H, 2.4–2.6, m), vinyl (2 H, 5.25–5.45, m)

13a, CH_3 (3 H, 0.98, d, 7 Hz), CH_3 (6 H, 1.55–1.75, m), CH and CH_2 (3 H, 2.3–2.9, m), vinyl (2 H, 5.2–5.45, m)

14a, CH_3 (3 H, 1.83, broad), CH_2 (4 H, 2.05–2.95, m), vinyl (1 H, 6.18, broad), aromatic (4 H, 6.9–7.3, m)

15a, CH_3 (3 H, 1.87, s), CH_3 (3 H, 2.00, s), CH_2 (4 H, 2.05–2.95, m), aromatic (4 H, 6.9–7.4, m)

methyl-substituted benzenes were conducted as described for the reduction of 3,5-dimethylbenzoic acid (Chapman and Fitton, 1963).

1,4-DIHYDROTOLUENE (**3a**). The carboxyl group of 1,4-dihydrobenzoic acid (Kuehne and Lambert, 1963) was reduced to a hydroxymethyl group with an excess of $LiAlH_4$ in ether (80% yield, bp 56–57° (2 mm)), followed by esterification with an excess of toluenesulfonyl chloride in pyridine (86% yield, an oil), and displacement of the tosyl group with hydride by an excess of $LiAlH_4$ in ether to afford **3a** (75% yield, bp 47° (70 mm)) essentially as described (Chapman and Fitton, 1963). Conditions for removal of the tosyl group consisted of dropwise addition during 2 hr of 66 g (0.25 mole) of the tosylate in 100 ml of ether to a stirred slurry of 15 g (0.4 mole) of $LiAlH_4$ in 70 ml of ether. The reduction was run under dry N_2 and stirred for 24 hr to ensure complete reaction. Unreacted hydride was decomposed by slow, cautious addition of the reaction mixture to a stirred suspension of ice in 1 N HCl. After separation from the aqueous phase, the ether was dried ($MgSO_4$) and concentrated to a small volume by a slow distillation at atmospheric pressure to prevent loss of diene. A trace of hydroquinone was added and the diene **3a** was purified by fractionation through a 6-cm Vigreaux column *in vacuo*.

1,2-DIMETHYL-1,4-DIHYDROBENZENE (**4a**). Birch reduction of *o*-toluic acid provided 1,4-dihydro-2-methylbenzoic acid (76% yield, mp 73°, crystallized from ether–petroleum ether) which was reduced with $LiAlH_4$ to 1,4-dihydro-2-methylbenzyl alcohol (92% yield, bp 60° (1.8 mm)). Reaction of this alcohol with tosyl chloride was quantitative. The tosylate was then reduced with $LiAlH_4$ as in the preparation of **3a** to provide **4a** (75% yield, bp 62° (71 mm)).

1,3-DIMETHYL-1,4-DIHYDROBENZENE (**8a**). Birch reduction of *m*-toluic acid proceeded to only 85% completion. Pure product was obtained by crystallization from ether–petroleum ether (75% yield, mp 86°). Reduction to the alcohol with $LiAlH_4$ (90% yield, bp 59° (1.8 mm)), tosylation (93% yield, waxy semisolid), and further reduction with $LiAlH_4$ as in the preparation of **3a** provided **8a** (75% yield, bp 62° (71 mm)).

1,4-DIMETHYL-1,4-DIHYDROBENZENE (**11a**). Synthesis of **11a** was achieved by a modification of a procedure for the preparation of 1,4-dihydrobenzene (Norris, 1968). Diels–Alder reaction of 4.93 g (60 mmoles) of *trans*-hexadiene-2,4 with 6.4 g (60 mmoles) of *trans*-β-chloroacrylic acid (sealed tube, 87 hr, 100°) gave a 33% yield (3.7 g) of 2-chloro-3,6-dimethylcyclohex-4-enoic acid (mp 106°, crystallized from hexane). The mixture of 15 mmoles of the sodium salt of the acid and 15 mmoles of NaI dissolved in 30 ml of freshly distilled hexamethyl phosphoramidate was heated for 4–5 hr at 70–75° under 0.8-mm vacuum while collecting the distillate in a trap at −78°. The contents of the trap were diluted with pentane. After washing with water and drying with Na_2SO_4, the pentane solution was fractionally distilled to provide 1 g (61% yield, bp 133° (760)) of **11a**.

2,5-DIMETHYL-1,4-DIHYDROBENZENE (**12a**). Birch reduction of *p*-xylene (Aldrich Chemical Co.) yielded **12a** containing 30% of unreduced *p*-xylene.

1,3,5-TRIMETHYL-1,4-DIHYDROBENZENE (**13a**). Birch reduction of mesitylene to **13a** proceeded to 30% completion. The material was used as such in the preparation of **13** since, after the initial bromination, the mesitylene was readily removed by distillation.

3-METHYL-1,2-DIHYDRONAPHTHALENE (**14a**). 1-Tetralone was converted to 2-hydroxymethylene-1-tetralone and reduced to 2-methyl-1-tetralone. The procedure was similar to that used by Ireland and Marshall (1962) to prepare α-methyl

cyclic ketones. The overall yield of 2-methyltetralone-1 was 43% [bp 98° (8 mm); lit. (Stille and Wu, 1965) bp 98° (3mm)]. The ketone was reduced with $NaBH_4$ in ethanol, and the crude alcohol was dehydrated with dry $KHSO_4$ at 100° and 40 mm for 30 min. The pressure was lowered to 6 mm, and the desired hydrocarbon **14a** distilled at 87–88° (83% overall yield from ketone).

3,4-DIMETHYL-1,2-DIHYDRONAPHTHALENE (**15a**). 2-Methyl-1-tetralone was allowed to react with methyl Grignard reagent in tetrahydroduran. The crude alcohol was dehydrated as above to provide **15a** (67% overall yield, bp 93° (7 mm)).

Preparation of Bromo Epoxide Precursors to Arene Oxides. Generation of the appropriate arene oxide precursors from the 1,4-dihydrobenzenes took advantage, in many cases, of the greater reactivity of the trisubstituted double bonds (Jerina *et al.*, 1968a). Thus, both **4b** and **5b** were synthesized from **4a** and both **8b** and **9b** were prepared from **8a**. In the cases of **5b** and **9b**, bromination of the more reactive bond was the first reaction, followed by epoxidation of the remaining double bond while in the cases of **4b** and **8b**, the first reaction was epoxidation. In the cases of **2a**, **11a**, **12a**, and **13a**, the same products will be produced regardless of the order of epoxidation and bromination. Bromination was routinely the first reaction in these cases.

When bromination was the first reaction, the diene was dissolved in CCl_4 (10% solution) and cooled to −30 to −40° A 5% solution of bromine in CCl_4 was then added dropwise in the dark. Addition of bromine was terminated when the bromine color of the reaction mixture persisted. This reaction procedure proved quite satisfactory as reaction at the second double bond is much slower than the first. Fractional distillation readily separated small amounts of unreacted diene and tetrabromo compounds from the desired dibromocyclohexenes. All of the dibromocyclohexenes having a bromine atom at a tertiary position proved very unstable and were epoxidized promptly. All epoxidations of the dibromocyclohexenes were performed with peroxytrifluoroacetic acid as previously described (Jerina *et al.*, 1968b). The reactions were monitored by gas–liquid chromatography (glc), and sufficient oxidant was added to drive the reactions to completion. Overall yields from diene exceeded 60%.

When epoxidation was the first reaction, slightly less than 1 equiv of *m*-chloroperoxybenzoic acid in CH_2Cl_2 was added dropwise to a stirred solution of the diene in CH_2Cl_2 at 0°. The resulting epoxycyclohexenes were purified by fractional distillation to remove diene and diepoxide. Bromination of the remaining double bond was done at −78° in $CHCl_3$ in the dark. Overall yields from diene exceeded 60%.

The crude dibromo epoxides had satisfactory mass and proton magnetic resonance (pmr) spectra and were used as such for preparation of arene oxides, since further purification by thin-layer chromatography (tlc) or column chromotography had little or no effect on the isomerization studies described later. In general, these dibromo epoxide precursors were quite close to analytical purity. However, most of them exist as noncrystalline oils due to the presence of several stereoisomers (*cf.* Jerina *et al.*, 1968a). For example, **9b** could be formed as a mixture of four isomers, neglecting optical isomers and assuming that the two bromine atoms enter *trans*. The pmr spectrum at 100 MHz is strongly suggestive of this. Thus far, these mixtures of isomers have been isolated free from each other in only one case, that of **12b** where only two isomers are formed. An analytical pure sample of **12b** (crystals from cold pentene, mp 40–55°), which contained equal amounts of each isomer as judged by pmr of the methyl signals, stood at room temperature for 2 years. Remarkably, one of the isomers rearranged to the other providing a stereoisomerically and analytically pure sample (pmr (given in δ values) in $CDCl_3$, methyl on oxirane 1.36 and methyl on carbon bearing bromine 1.93, mp 64°).

A question more important to this study than stereoisomerism or analytical purity is the positional purity of substituents when more than one arene oxide can be generated from a given dihydrobenzene (*e.g.*, **8b** and **9b** from **8a**; *i.e.*, how much cross contamination of **8b** is in **9b** and *vice versa*?). Contamination of this sort would lead to erroneous isomerization results. The nmr spectra of the precursors are quite helpful in deciding this, and *cross contamination can be eliminated to a level of 2–4%* by the position of methyl groups in these series of compounds.

Generation of the precursors for the two naphthalene oxides, **14** and **15**, from the corresponding dihydronaphthalenes, **14a** and **15a**, followed the original synthesis of naphthalene 1,2-oxide (Vogel and Klärner, 1968): epoxidation of the double bond, followed by *N*-bromosuccinimide halogenation at the benzylic methylene. Epoxidation reactions were run at 0° in CH_2Cl_2 with *m*-chlorperoxybenzoic acid. The presence of anhydrous Na_2CO_3 in the reaction mixture improved the yield by reducing rearrangement to ketone (Boyd *et al.*, 1970). The intermediate methyl-substituted epoxides are far more unstable than tetralin 1,2-epoxide, and the bromination reactions were not always successful. Thus, 1-methyltetralin 1,2-epoxide was prepared (*cf.* preparation of 1,2-dimethyltetralin 1,2-epoxide) but could not be successfully brominated.

Preparation of Arene Oxides. Naphthalene oxides were generated as previously described for naphthalene 1,2-oxide (Vogel and Klärner, 1968) by dehydrohalogenation with diazabicyclononaene. Methyl-substituted benzene oxides were prepared as follows. To a stirred solution of 1.8 g (16 mmoles) of potassium *tert*-butoxide in 10 ml of tetrahydrofuran at −78° was added 4 mmoles of precursor in 5 ml of pentane. Addition of the precursor was done over a 5-min period, and stirring was continued an additional 5 min before adding five volumes of ice water. The pentane layer was separated from the aqueous phase, dried over Na_2CO_3, and used in isomerization studies. The basic aqueous phase was extracted three times with its volume of ether to remove neutral components, acidified, and extracted again with ether to isolate phenols formed during generation of the arene oxide.

The 2-methyl- and 1,2-dimethylnaphthalene 1,2-oxides were crystalline solids from pentane. Methyl-substituted benzene oxides were relatively stable when one methyl group was on the oxirane ring. Proton magnetic resonance spectra could in some cases be obtained after concentrating the pentane solutions with added CCl_4. The complete spectral and physical properties of these arene oxides are being determined and will be reported elsewhere.

Isomerization of Arene Oxides. One ml of pentane containing the arene oxide (5–15 mg) was added to 5 ml of pH meter standard buffer at the designated pH. The mixture was shaken for 5 min and the pentane phase was evaporated *in vacuo* at 20°. After storage for 15 hr at room temperature, the pH was adjusted to 12 and the solutions were extracted three times with 5 ml of fresh ether to remove neutral contaminants. The resulting solutions were adjusted to pH 1, saturated with salt, and extracted again with ether as above. The combined ether extracts were dried (Na_2SO_4), concentrated to 0.5 ml, and analyzed as indicated in the section on chromatography. Isomerizations were also carried out in pentane solution with a trace of trifluoroacetic acid. Rearrangements of

TABLE III: Metabolism of Alkylbenzenes with Rat Liver Microsomes.[a]

Substrate	Products	Conversion	R_F	Gibbs Reagent
Benzene	Phenol	Minor	0.54	Blue
Toluene	4-Hydroxytoluene (**16**)	Minor	0.56	Faint blue
	2-Hydroxytoluene (**17**)	Major	0.70	Purple
Ethylbenzene	4-Hydroxyethylbenzene	Major	0.61	Faint blue
	2-Hydroxyethylbenzene	Major	0.77	Purple
Isopropylbenzene	4-Hydroxyisopropylbenzene	Major	0.58	Faint blue
	2-Hydroxyisopropylbenzene	Trace	0.76	Purple
tert-Butylbenzene	4-Hydroxy-*tert*-butylbenzene	Minor	0.59	None
o-Xylene	2,3-Dimethylphenol[b] (**20**)	Major	0.62	Purple
m-Xylene	2,4-Dimethylphenol (**19**)	Major	0.55	Faint purple
	2,6-Dimethylphenol (**22**)	Trace	0.68	Purple
p-Xylene	2,5-Dimethylphenol (**18**)	Major	0.54	Purple
Mesitylene	2,4,6-Trimethylphenol (**24**)	Major	0.68	Purple
1,2,3-Trimethylbenzene	2,3,4-Trimethylphenol (**25**)	Minor	0.68	Faint orange
1,2,4-Trimethylbenzene	2,4,5-Trimethylphenol (**27**)	Trace	0.62	Faint grey

[a] For incubation conditions, isolation, and estimation of products, see Experimental Section. Major product >1 μmole, minor product 0.1–1 μmole, trace product <0.1 μmole. [b] Also the exclusive phenolic product with guinea pig liver microsomes.

the naphthalene oxides were done in $CHCl_3$ solution with a trace of acetic or trifluoroacetic acid and the products isolated by preparative thin-layer chromatography on silica gel.

Enzyme Preparations. Livers of male Sprague–Dawley rats (300 g) that had been pretreated with 3-methylcholanthrene (1 injection daily of 40 mg/kg in cottonseed oil for 2 days, sacrificed on day 3) were removed and homogenized with three volumes of isotonic KCl, followed by centrifugation at 10,000*g* for 20 min to remove cell debris and mitochondria. The supernatant is referred as the microsomal preparation. Microsomal preparations were also obtained in a similar manner from livers of untreated guinea pigs.

Microsomal Metabolism of Alkylbenzenes. Incubations were carried out at 37° for 15 min with 10 ml of microsomal preparation, 3.5 ml of Tris buffer (pH 9.0, 0.5 M), 15 μmoles of ATP, 10 μmoles of NADP, 50 μmoles of glucose 6-phosphate, 10 units of glucose-6-phosphate dehydrogenase, 2 mg of Tween 80 and 50 μmoles of substrate. The latter was added in 0.1 ml of acetone to a final volume of 15 ml. After incubation, the mixture was extracted with an equal volume of ethyl acetate. The extract was dried (Na_2SO_4) and concentrated *in vacuo*, and the phenolic products were analyzed by tlc (silica gel GF plates, with benzene–ethyl acetate, 95:5) and in some cases by glc (see below). Phenolic products were detected and estimated with Gibbs and/or Folins reagent as compared to known amounts of authentic compounds (Table III). Side-chain hydroxylation to form benzylic alcohols and acids was a major metabolism with all the alkylbenzenes except *tert*-butylbenzene. These alcohols or acids exhibit low R_F s in the solvent system used for tlc.

Microsomal Metabolism of Alkylnaphthalenes. Incubations and isolations were carried out as for the alkylbenzenes. Chromatography was on silica gel GF plates with chloroform–benzene–ethyl acetate (1:1:1).

Metabolism of 1-methylnaphthalene with guinea pig microsomes afforded 1-naphthoic acid (molecular ion, *m/e* 172) as a major product. In addition, a methylnaphthol (molecular ion, *m/e* 158) was detected as a minor product. R_F and color reaction with Gibbs reagent indicated that it was not 1-methyl-2-naphthol or 2-methyl-1-naphthol. A methylnaphthalene dihydrodiol (molecular ion, *m/e* 176) was also isolated as a minor product. Dehydration of the latter with acid did not yield either 1-methyl-2-naphthol or 2-methyl-1-naphthol.

Metabolism of 2-methylnaphthalene afforded 2-naphthoic acid (molecular ion, *m/e* 172) as the major product. A methylnaphthalene dihydrodiol (molecular ion, *m/e* 176) was formed as a minor product. Dehydration of the latter with acid gave neither 1-methyl-2-naphthol or 2-methyl-1-naphthol.

Metabolism of 1,2-dimethylnaphthalene afforded methylnaphthoic acid(s) (molecular ion, *m/e* 186) as major products. A dimethylnaphthol (molecular ion, *m/e* 172) and a dimethylnaphthalene dihydrodiol (molecular ion, *m/e* 190) were obtained as minor products.

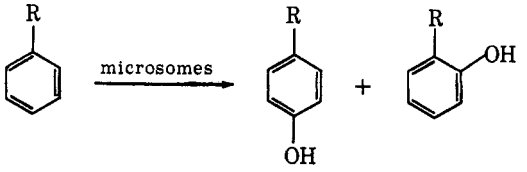
Chromatographic Separation and Identification of Phenols. In all instances, combinations of several procedures were used before identity of a given phenol was considered proven. The three cresols are readily separated by glc using 3% tricresyl phosphate on 100–120 mesh Gas Chrom Q as the stationary phase (6-ft column, 135°). The six dimethylphenols could not be simultaneously separated on any of the columns used. On the above column, operated at 130° or with 15% SE-30 on 100–120 mesh Gas Chrom Q (6-ft column 130°), the 2,4- and 2,5-dimethylphenols do not separate well. However, silylation (0.2 ml of Regisil added to 0.5 ml of ether containing phenols) of the phenol mixtures permits the separation of these two isomers using 3% OV-17 on 100–120 Gas Chrom Q (6-ft column, programmed from 100° at 2°/min after a 6-min post-injection interval) or 3% diisodecyl phthalate on 100–120 mesh Gas Chrom Q (6-ft column, 150°). The 2,4- and 3,5-dimethylphenols, after silylation, cochromatograph on the OV-17 column. Ambiquities arising from cochromatography were readily resolved by the use of two columns. Additional confirmation was obtained by tlc on silica gel followed by color reactions with Gibbs reagent or Folins reagent (Table III) and by combined mass spectrometry–gas chromatography on selected samples. The trimethylphenols were separated by tlc (Table III) and identified by color reaction with Gibbs reagent.

Rearrangement products and metabolites of the naphthalene derivatives were separated by tlc. Both 1-hydroxy-2-methyl- and 2-hydroxy-1-methylnaphthalene were used as reference compounds. Identity of the rearrangement product from 2-methylnaphthalene 1,2-oxide was established as 1-hydroxy-2-methylnaphthalene by comparison of pmr and infrared spectra (ir) to an authentic sample. Rearrangement of 1,2-dimethylnaphthalene 1,2-oxide with trifluoroacetic acid gave a ketone and an ester. The ketone (C=O, 1670 cm^{-1}; pmr, two CH_3 as a singlet at 1.24 and two vinyl hydrogen doublets at 6.00 and 6.42) was assigned as 2,2-dimethyl-1(2*H*)-naphthalenone based on comparison of spectral data for related ketones (Hart and Murray, 1970). The ester (C=O, 1780 cm^{-1}) was clearly a dimethylnaphthol trifluoroacetate with all the substituents on the same ring as judged from the pmr and mass spectra. The most likely structure is 1-trifluoroacetoxy-2,3-dimethylnaphthalene which might arise *via* a dienone–phenol rearrangement of the ketone (Marvell and Geiszler, 1952).

Results and Discussion

Nearly all of the arene oxides of benzene with mono- and dimethyl substituents as well as mesitylene oxide and the 2-methyl- and 1,2-dimethylnaphthalene 1,2-oxides have now been synthesized and their isomerization has been studied. The products obtained on isomerization have been compared to the metabolites formed from the parent hydrocarbons with the drug metabolizing system of liver microsomes. At present, most of these arene oxides have been obtained only as dilute pentane solutions. Their isolation and complete characterization as pure compounds are in progress, but will require considerable additional effort in view of their often remarkable instability. Thus, **5**, **9**, **11**, and **13** are so unstable that their pmr spectra have not as yet been determined.

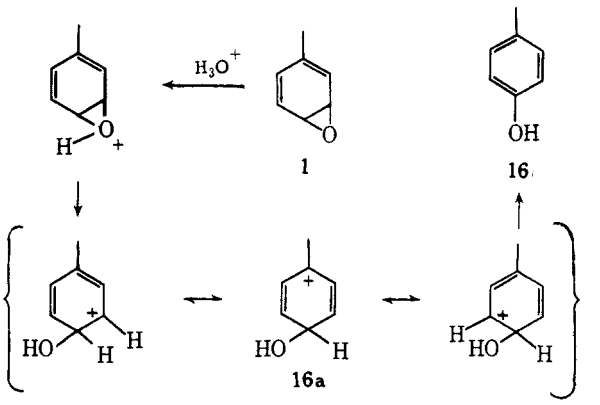
Toluene and Monoalkylbenzenes. Toluene and monoalkylbenzenes are metabolized with rat liver microsomes to mixtures of ortho- and para-hydroxylated products (Table III). The relative amounts of **16** and **17** formed from toluene vary,



16 **17**

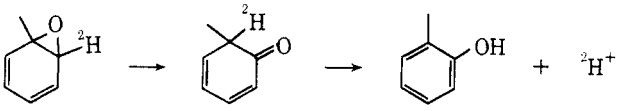
with the biological system being 2:1 with rat microsomes from 3-methylcholanthrene-induced animals (Table III) and 1:1 with rabbit microsomes (Daly *et al.*, 1968b). Toluene forms predominantly **16** *in vivo* in rats (Bakke and Scheline, 1970). "Hydroxylation" of toluene at either the ortho or para position is accompanied by an NIH shift of deuterium (Daly *et al.*, 1968b; Daly and Jerina, 1969) suggesting in each case the intermediacy of an arene oxide. Indeed, the extent of migration and retention of deuterium is similar when toluene-4-^2H is "hydroxylated" and when the corresponding toluene-4-^2H oxide **1** isomerizes (Jerina *et al.*, 1968a).

The toluene oxide **1** rearranges exclusively to **16** (Jerina *et al.*, 1968a). The pathway leading to **16** has the slight energetic advantage of additional stabilization of a cationoid intermediate *via* hyperconjugation with the methyl group (**16a**). This small, but apparently significant, amount of added stabilization seems a dominant factor in controlling the opening of all of the arene oxides studied here. The intermediacy of **1** is thus compatible with microsomal formation of **16** from



16a

toluene. The remaining two toluene oxides, **2** and **3**, rearrange exclusively to **17**. Stabilization by hyperconjugation with

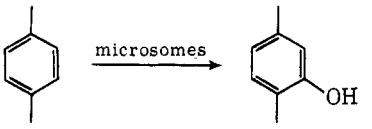


2 **17**

methyl again causes selective opening. Either of these oxides could be the intermediate leading to metabolic formation of **17**, but results obtained with *p*-xylene and *o*-xylene (see below) suggest that formation of arene oxide with a substituted oxirane ring as in **2** is not generally significant in the microsomal metabolism of alkylbenzenes. The marked effect of the size of the alkyl substituent on the extent of ortho hydroxylation (Table III) also indicates that steric factors will strongly militate against formation of an oxide such as **2**. Kinetic studies (Kasperak and Bruice, 1972) and isotopic studies (Boyd *et al.*, 1972) suggest that spontaneous rearrangement of arene oxides to the keto form of the phenol may be a concerted process not requiring acid catalysis under physiological conditions. The arene oxides **2**, with deuterium in the 2 position, would, therefore, be expected to isomerize as shown to **17** with complete loss of deuterium, a result incompatible with the NIH shift of deuterium observed during formation of 2-hydroxytoluene from [2-^2H]toluene (Daly and Jerina, 1969). In fungi, intermediacy of arene oxides with substituents on the oxirane ring has been proposed to account for the low NIH shift of deuterium observed in ortho-hydroxylated metabolites (Auret *et al.*, 1971).

The present evidence indicates that the very unstable arene oxides **1** and **3** are likely intermediates in hepatic, ring metabolism of toluene. The oxide **2**, however, appears to be the only oxide in this series with sufficient stability to have been demonstrated in a biological system.

1,4-Dimethylbenzene (p-Xylene). *In vitro* metabolism of *p*-xylene leads to **18** as the exclusive phenolic product. In addition, large amounts of *p*-toluic acid are produced by oxidation of a methyl group (*cf.* the para effect of substituents in the



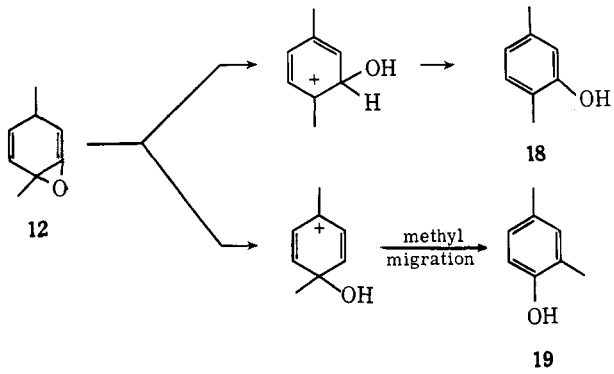
18

anisole and acetanilide series, Daly, 1970). *In vivo* in rats, *p*-xylene is also metabolized to **18** (Bakke and Scheline, 1970).

Absence of the methyl-migrated product **19** from the metabolites eliminates arene oxide **12** as an intermediate, since **12** always rearranges to a mixture of both **18** and **19** (see below). Thus, **11** must be the metabolic intermediate if an arene oxide is involved. Exclusive formation of **11** might be expected if steric factors militate against formation of oxides substituted on the oxirane ring as in **12**. Unfortunately, **11** is so unstable that it spontaneously rearranges to **18** at 0° in aprotic media, and thus probably could never be demonstrated in a biological system.

The xylene oxide **12**, with methyl substitution on the oxirane ring, is quite stable and could be distilled as a yellow-orange oil. Rearrangement of **12** occurs in methanol at room temperature to a mixture of **18** and **19**. The methyl migration product **19** predominates. The ratio of methyl migration to direct opening on isomerization was found to be a function of pH (Figure 1) with maximal migration (87% under neutral and basic conditions.) High migration of deuterium under basic conditions has been reported during isomerization of [4-^{2}H]-toluene 3,4-oxide (Jerina *et al.*, 1968a) and [1,2-^{2}H]naphthalene 1,2-oxide (Boyd *et al.*, 1972). Clearly multiple mechanistic pathways are operative in these isomerizations. This fact has also emerged from a kinetic study of the rearrangement of benzene and naphthalene 1,2-oxide (Kasperek and Bruice, 1972). Kinetic study of the isomerization of **12** is in progress (Kasperek *et al.*, 1972). The xylene oxide **12** and the methyl migration observed during its isomerization to phenols provide a simple chemical model for the formation of 3-methyltyrosine and 3-hydroxy-4-methylphenylalanine from 4-methylphenylalanine with phenylalanine hydroxylase (Daly and Guroff, 1968). Under physiological conditions (pH 8), the ratio (9:1) of methyl migration *vs.* direct opening of **12** is similar to the ratio (11:1) of 4-hydroxy-3-methylphenylalanine to 3-hydroxy-4-methylphenylalanine obtained on enzymatic oxidation of 4-methylphenylalanine.

Substituted phenylalanines with ^{2}H, ^{3}H, Cl, Br, and methyl in the 4 position have been shown to undergo the NIH shift during the hydroxylation with phenylalanine hydroxylase (Guroff *et al.*, 1966a–c; Daly and Guroff, 1968). In view of the possibility that these reactions proceed *via* an arene oxide at the 3,4 position, experiments were aimed at the synthesis of the parent compound, phenylalanine 3,4-oxide. The route was patterned after the synthesis of benzene oxide (Vogel *et al.*, 1965). Synthesis of the immediate precursor, the dibromoepoxide with the amino acid residue blocked as the *N*-trifluoroacetyl *O*-methyl ester, was readily accomplished. Although this compound underwent dehydrohalogenation, acid treatment of the crude mixture to remove the amino acid blocking groups and isomerize the presumed intermediate arene oxide to the phenol failed to produce even trace amounts of tyrosines. The actual nature of the dehydrohalogenation products was not determined.



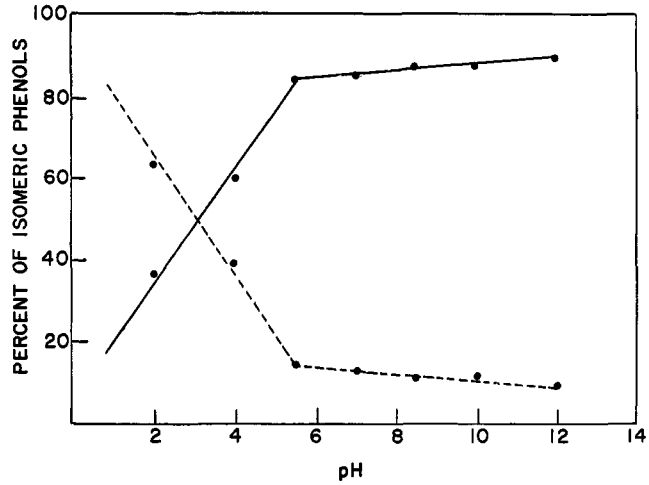
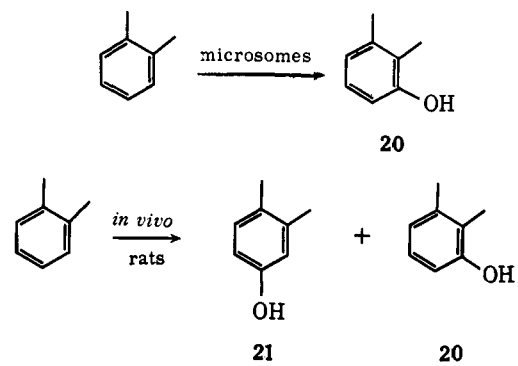


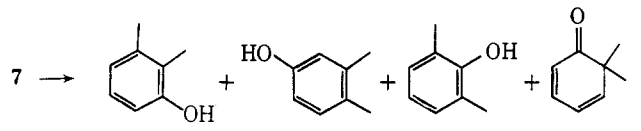
FIGURE 1: Isomerization of 1,4-dimethylbenzene 1,2-oxide (**12**) to 2,4-dimethylphenol (**19**, ●–●) and 2,5-dimethylphenol (**18**, ●---●) as a function of pH. Isomerizations carried out as described in the Experimental Section.

1,2-Dimethylbenzene (*o-Xylene*). *o*-Xylene is oxidized by rat or guinea pig liver microsomes exclusively to **20** (Table III). This hydroxylation is accompanied by an NIH shift of deuterium (Daly and Jerina, 1969). However, in the intact rat, **21** is the predominant metabolite along with only a trace of



20 (Bakke and Scheline, 1970; Daly and Jerina, 1972[1]). Possibly more than one arene oxide is formed *in vivo* and a specific enzyme activity responsible for the formation of the arene oxide that isomerizes to **21** is lost on preparation of microsomes. The absence of **22** as a phenolic metabolite of *o*-xylene excludes the arene oxides **4** and **7** as significant metabolites, since both give substantial amounts of **22** on isomerization. Thus, with *o*-xylene as with *p*-xylene, arene oxides with substituted oxirane rings are not formed during microsomal metabolism. Rearrangement of **7** in acid yields the phenols **20**, **21**, **22**, and the dienone **23** (Schubart, 1967). The same phenols are formed from **4** with the ratios dependent on the conditions of isomerization. Direct opening of **4** yields **20**, while methyl migration yields **22**. Formation of **21** would appear to result either from addition and elimination of water or *via* a migration of oxygen.

[1] In preparation.



	20	21	22	23
1 N $HClO_4$	2%	48%	21%	27%

4 → 20 + 21 + 22

F_3CCOOH	57%	6%	37%
pH 4	52	30	18
pH 7	33	14	53
pH 10	34	12	54

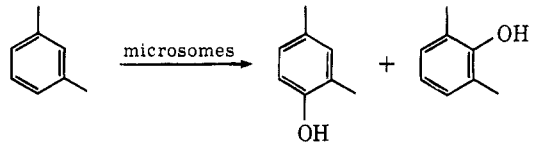
The two other arene oxides of *o*-xylene, **5** and **6**, which do not have methyl substitution on the oxirane ring, are quite unstable. Spontaneous rearrangement of **6** was reported to occur in pentane at room temperature to give **21** (Schubart, 1967). Rearrangement of **5** under a variety of conditions always gave

5 → 20 + 21

F_3CCOOH	33%	65%
pH 4	11	89
pH 7	20	78
pH 10	23	75

mixtures in which opening of the oxirane ring had occurred in both directions to provide both **20** and **21** as predicted by the hyperconjugation theory. Intermediacy of both **5** and **6** is, therefore, compatible with the *in vivo* metabolism of *o*-xylene. The exclusive formation of **20** *in vitro* remains a paradox. However, when the dehydrohalogenation reaction which generates **5** is allowed to proceed for 30 min rather than 5 min, **20** is nearly the only phenol found in the base-soluble fraction (see experimental). Attempts are presently under way to prepare pure **5** and ascertain the factors controlling its isomerization.

1,3-Dimethylbenzene. m-Xylene is converted to **19** and **22**



19 **22**

in ratio of greater than 10:1 by rat liver microsomes (Table III). *In vivo* in rats, the major phenolic metabolite is also **19** (Bakke and Scheline, 1970).

Of the three possible arene oxides, **8**, **9**, and **10**, only **10** (see structure of Table I, R_1, R_3 = CH_3) remains to be synthesized. The oxide **8** gave only **22** under a variety of conditions as predicted by the hyperconjugation theory. The oxide **10** would be predicted to afford exclusively **19** on isomerization. However, neither **8** nor **10** seems likely metabolic intermediates in that both have methyl substitution on the oxirane ring. The extremely unstable arene oxide **9** rearranges in aqueous media to both of the metabolic phenols (**19** and **22**), although never in the 10:1 ratio observed for

9 → 19 + 22

F_3CCOOH	57%	22%
pH 4	56%	44%
pH 7	58%	42%
pH 10	62%	38%

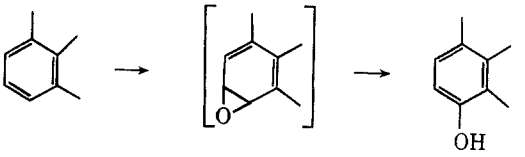
microsomal metabolism. The origin of the apparent oxygen migration to form **22** from **9** is unknown. A firm prediction regarding which arene oxide(s) might be involved in *m*-xylene metabolism is not as yet possible. The proportions of phenols found in the base-soluble fraction from the dehydrohalogenation reaction which generates **9** (see Experimental Section) corresponds closely to the proportions of phenolic metabolites obtained from the parent hydrocarbon. Efforts to prepare and study pure samples of **9** are in progress.

1,3,5-Trimethylbenzene (*Mesitylene*). The sole phenolic product obtained from mesitylene is **24** both *in vitro* (Table

24

IV) and *in vivo* (Bakke and Scheline, 1970) and **24** is also the only product obtained from the very unstable arene oxide **13**. Metabolism of mesitylene may thus proceed *via* formation of an arene oxide with a substituted oxirane ring. This is, however, the only possible arene oxide that can be formed from mesitylene. When possible, formation of unsubstituted oxiranes appears the preferred metabolic pathway (*cf. o*- and *p*-xylene).

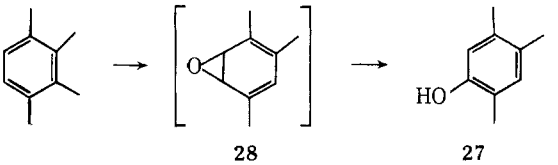
1,2,3-Trimethylbenzene. The only detectable phenolic product from 1,2,3-trimethylbenzene was **25**. Only one unsubstituted oxirane can be formed from 1,2,3-trimethylbenzene and this oxide **26** would be predicted, on the basis of hyper-



26 **25**

conjugation, to isomerize almost exclusively to **25**. Synthesis of the arene oxide was not attempted.

1,2,4-Trimethylbenzene. Only one phenolic product, **27**, is detected from 1,2,4-trimethylbenzene; this phenol would be expected as the predominate product from the only possible unsubstituted oxirane intermediate (**28**). Synthesis of the



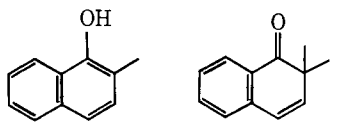
28 **27**

arene oxide was not attempted. The same phenolic metabolite, **27**, was produced *in vivo* in rats (Bakke and Scheline, 1970).

Methylnaphthalenes. Since naphthalene oxide is sufficiently stable to be demonstrated in an *in vitro* system (Jerina *et al.*, 1970a) and since alkyl substitution on the oxirane ring generally stabilizes arene oxides, the synthesis of several methyl-substituted naphthalene oxides was attempted following the procedure for the original synthesis of naphthalene 1,2-oxide (Vogel and Klärner, 1968). 2-Methylnaphthalene 1,2-oxide (**14**) and 1,2-dimethylnaphthalene 1,2-oxide (**15**) proved to be crystalline compounds more stable than naphthalene oxide.

The attempted synthesis of 1-methylnaphthalene 1,2-oxide was unsuccessful due to the instability of intermediate 2-methyltetralin 1,2-epoxide.

Rearrangement of **14** with acetic acid proceeds exclusively to **29**; *i.e.*, no migration occurs. This directed opening is analogous to the preferential isomerization of naphthalene 1,2-oxide to 1-naphthol where the greater stability of the allylic carbonium ion directs the isomerization.



29 **30**

The oxide **15** is sufficiently stable so that acids weaker than trifluoroacetic do not catalyze its rearrangement at room temperature. Rearrangement forms only one (**30**) of the two possible ketones. Despite interaction of the cis-eclipsed methyl groups in **15**, no evidence was found for valence bond tautomerism to the oxepin structure (*cf.* Boyd *et al.*, 1970). The relatively high stability of **15** makes it quite suitable for identification as a metabolite. Unfortunately, electronic or steric factors appear to prevent microsomal oxidation of 1,2-dimethylnaphthalene at the 1,2 bond. Neither the oxide nor the isomeric ketone **30** could be detected after incubation of 1,2-dimethylnaphthalene with microsomes. The principal pathway of microsomal metabolism of 1- and 2-methylnaphthalene and of 1,2-dimethylnaphthalene led instead to naphthoic acids and methylnaphthoic acids, respectively. Naphthols and dihydrodiols were formed as products *via* metabolism at ring positions other than the 1,2-double bond. In the case of 1,2-dimethylnaphthalene these products could not have arisen *via* the arene oxide **15** since isomerization of **15** does not yield a dimethylnaphthol and **15** would not be a substrate for the microsomal epoxide hydrase that forms dihydrodiols (Oesch *et al.*, 1971). A 7,8-dihydrodiol has been reported as an *in vivo* metabolite of 2-methylnaphthalene (Grimes and Young, 1956).

Conclusions

The present study has delineated a number of factors which influence the stability and reactivity of alkylated arene oxides and has demonstrated that, in every case, certain of these oxides are compatible with the observed enzymic formation of phenols from the parent hydrocarbon. The stability of arene oxides appears to be markedly increased by alkyl substitution of the oxirane ring and markedly decreased by alkyl substitution in other ring positions. Since microsomal metabolism of aromatic hydrocarbons does not usually appear to proceed *via* formation of arene oxides with alkyl-substituted oxirane rings, this suggests that microsomal metabolism of the alkyl-substituted aromatic compounds will generate only very unstable arene oxides. An exception to this generalization is mesitylene, where the only possible arene oxide contains a substituted oxirane ring. This arene oxide (**13**) is, however, very unstable due to the two alkyl substituents in other positions on the dihydroaromatic ring. The literature on metabolism of benzene derivatives contains no mention of dihydrodiol formation from alkylated benzenes. Perhaps this is to be expected since alkyl groups on the oxirane ring virtually prevent the action of epoxide hydrase (Oesch *et al.*, 1971), while alkyl groups in other positions sufficiently destabilize the arene oxide so that rapid isomerization to phenols prevents enzymatic hydration (Jerina *et al.*, 1968a).

The selectivity and variability of isomerization of the arene oxides have indicated: (i) that the relative stability of intermediate cationoid species plays a role in product formation and (ii) that multiple mechanisms for isomerization pertain. Prediction of phenolic products can be based on the anticipated stability of intermediate carbonium ions, but such predictions must be made with caution; *e.g.*, a firm prediction of the direction of opening (migration *vs.* nonmigration) of an arene oxide postulated as an intermediate (Hamilton, 1971) in the conversion of phenylpyruvates to homogentisate (Schepartz and Gurin, 1949; Kindl, 1969; Taniguchi *et al.*, 1964) is not as yet possible. The mechanism(s) for apparent oxygen migrations with **4** and **9** are under investigation.

Instability of arene oxides of the alkylbenzenes toward rearrangement may preclude implication in tissue necrosis as suggested for halogen-substituted arene oxides (Brodie *et al.*, 1971). Further studies on substituted arene oxides are in progress and concern their enzymatic formation, metabolism, possible reaction with macromolecular constituents of tissue, stability, and chemical properties.

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Reaction of *N*-Bromosuccinimide with Reduced Ring Systems. Apparent Protection of Glutamate Dehydrogenase by Reduced Nicotinamide–Adenine Dinucleotide†

Murray R. Summers

ABSTRACT: In response to the use of reduced molecules to protect a protein from oxidation by *N*-bromosuccinimide, this reagent can be shown to react stoichiometrically with NADH, dihydrofolic acid, and 3,5-dimethyldihydropteridine. In each case, spectra characteristic of the oxidized molecule are generated. The reaction of *N*-bromosuccinimide with NADH is shown to occur even in the presence of a competing protein molecule (glutamate dehydrogenase). In this system, the NADH is oxidized preferentially to the protein; only when all NADH is reacted, does the *N*-bromosuccinimide begin to modify protein molecules, as determined by decreasing enzyme activity. This reaction of *N*-bromosuccinimide should be given consideration when one is "protecting" a protein from modification by adding reduced cofactors.

Dihydrofolate reductase has been shown to be irreversibly inhibited by oxidation with *N*-bromosuccinimide (Freisheim and Huennekens, 1969). The same investigators found that this inhibition was markedly delayed when the *N*-bromosuccinimide reaction was carried out in the presence of NADPH or dihydrofolic acid, both of which molecules are substrates for the enzyme. While evidence of this nature is suggestive of a modification in the active site, one must be certain that the modifying reagent does not react with the substrates themselves. This control was never done in the dihydrofolate reductase study. Experiments reported in this communication show that as expected *a priori* (Filler, 1963), a rapid reaction does occur between *N*-bromosuccinimide and reduced molecules, even in the presence of a competing protein molecule. The extent of protection, if any, by these reduced substrates is therefore difficult to judge, when molecules such as these are used to protect proteins from oxidation by reactive chemicals.

Experimental Section

Materials. *N*-Bromosuccinimide was purchased from Eastman Chemical Co. and was recrystallized once from H_2O before use. Both glutamate dehydrogenase (Type II) and NADH (Grade III) were obtained from Sigma Chemical Co. and were used with no further purification. The H_2folate and

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