Deuterium Isotope Effects during Formation of Phenols by Hepatic Monoxygenases. Evidence for an Alternative to the Arene Oxide Pathway†

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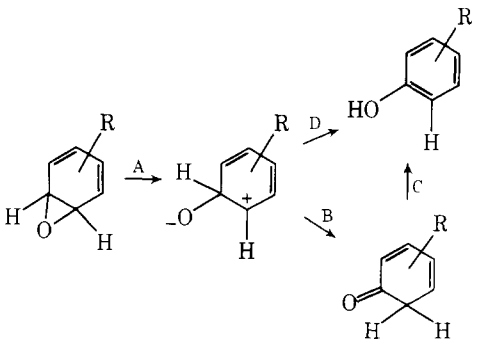
ABSTRACT: The in vivo and in vitro metabolisms of normal and deuterated aromatic substrates have been investigated in rats. Significant isotope effects (k_H/k_D = 1.3–1.75) were associated with in vivo formation of meta-hydroxylated metabolites from 1:1 mixtures of normal and perdeuterio-(aryl ring) nitrobenzene, methyl phenyl sulfide, and methyl phenyl sulfone. Since isotope effects of this magnitude are incompatible with arene oxides as intermediates in the formation of phenols, the results provide evidence that multiple pathways are responsible for the formation of phenols in mammals. Significant isotope effects were not associated with the formation of the other phenolic isomers of nitrobenzene, methyl phenyl sulfone, or methyl phenyl sulfide or with the formation of phenolic products from anisole, bromobenzene, chlorobenzene, fluorobenzene, benzonitrile, naphthalene, zoxazolamine, acetanilide, biphenyl, diphenylhydantoin, benzene, *o*- and *p*-xylene, toluene, and mesitylene. Significant isotope effects might not be observable with the latter substrates if the kinetic parameters for oxidation of substrate change or if the arene oxide pathway greatly predominates. Furthermore, extensive in vivo metabolism of any substrate would make isotope effects unobservable by the procedure employed, namely the analysis of isotope content in metabolites formed from 1:1 mixtures of normal and deuterated substrates.

Oxidative metabolism of the aromatic nucleus by monoxygenases has been established to proceed via initial formation of arene oxides which (i) spontaneously isomerize to phenolic products, (ii) are enzymatically hydrated to *trans*-dihydrodiols, (iii) react with glutathione to form conjugates, or (iv) are covalently bound to tissue constituents (cf. review by Jerina and Daly, 1974). While several different mechanisms of isomerization have been demonstrated for arene oxides (Kasperek and Bruice, 1972; Yagi et al., 1972; Kasperek et al., 1972a,b; Bruice et al., 1973),the initial and rate-limiting step under physiological conditions is invariably the heterolytic cleavage of a carbon–oxygen bond to form a carbonium ion (step A). The carbonium ion either



isomerizes to the keto tautomer of the phenol (step B) or forms the phenol directly (step D). Formation and subsequent enolization of the keto tautomer is generally the principal pathway to the phenol (step C). The migration and enolization steps (step B and C) account for the NIH shift of isotopic hydrogen and other substituents which have been observed following the hydroxylation of aromatic substrates by monoxygenases (Daly et al., 1972). Comparison of rates of isomerization to phenols between suitably deuterated arene oxides and their normal hydrogen congeners has shown this reaction sequence to proceed wihout detectable isotope effects (Kasperek et al., 1972a). Furthermore, formation of an arene oxide from deuterated aromatic substrates should proceed without a significant isotope effect since carbon–hydrogen bonds are not broken in the oxidation. The general lack of isotope effects in formation of phenols from aromatic substrates (Guroff and Daly, 1967; Daly and Jerina, 1969; Tanabe et al., 1967; Perel et al., 1967) is, therefore, compatible with the formation of arene oxide intermediates.

The report of a significant isotope effect for hydroxylation[1] of zoxazolamine in vitro (Tanabe et al., 1970) is incompatible with the intermediacy of an arene oxide and suggests alternate pathways for formation of phenols. Such pathways might be similar to the pathways involved in the metabolic formation of alcohols from alkanes which do show primary isotope effects. Although the results with zoxazolamine could not be reproduced in the present study, two other examples of isotope effects for the hydroxylation of aromatic substrates in vivo have been detected.

Experimental Section

Materials. Completely ring deuterated aromatic substrates with the exception of zoxazolamine, methyl phenyl sulfide, and methyl phenyl sulfone were obtained from Isomet. 2-Amino-5-chlorobenzoxazole-*4,6-d*$_2$ (dideuteriozoxazolamine) was synthesized from 2-amino-5-chlorophenol as described (Tanabe et al., 1970). Ring-deuterated methyl

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[1] The term "hydroxylation" in the present paper is used for simplicity to describe the overall process involved in the formation of phenols. While most of these reactions proceed via arene oxides, mechanistic significance should not be attached to this term as it is used in this paper.

phenyl sulfide was synthesized from benzene-d_6 by the following routine sequence: bromination (Ricker and Zienek, 1965), Grignard formation, reaction of the Grignard reagent with sulfur, and alkylation of the resultant thiophenol with methyl iodide (cf. Cymerman-Craig and Loder, 1963). Hydrogen peroxide in acetic acid oxidized the sulfide to the sulfone.

Metabolic Studies. Animals were induced either with 0.2% sodium phenobarbital in their drinking water for 4 days or by intraperitoneal injection of 3-methylcholanthrene (40 mg/kg in 1 ml of cottonseed oil/day) for 2 days and were then used on day 5 or 3, respectively. Female Sprague-Dawley rats weighing 250–300 g were used, except where indicated otherwise.

In vitro studies on 1:1 mixtures of normal and deuterated acetanilide, anisole, and naphthalene were carried out essentially as described (Jerina et al., 1968). The in vitro metabolism of 1:1 mixtures of normal and dideuteriozoxaxolamine was conducted at pH 8.2 for 30 min with resuspended microsomal pellets under incubation conditions previously described for a variety of other substrates (Daly et al., 1968) or according to the procedure of Tanabe et al. (1970).

In vivo metabolism was conducted as follows. Benzonitrile, bromobenzene, chlorobenzene, fluorobenzene, diphenylhydantoin, methyl phenyl sulfide, methyl phenyl sulfone, and nitrobenzene (160 mg per kg per injection) and benzenesulfonanilide, naphthalene, acetanilide, anisole, *p*-xylene, *o*-xylene, mesitylene, and benzene (100 mg per kg per injection) were administered intraperitoneally as aqueous emulsions (1 ml/injection) with 0.1% triethanolamine oleate as the emulsifying agent. Biphenyl (200 mg per kg per injection) and zoxazolamine (100 mg per kg per injection) were administered in propylene glycol solutions (1 ml/injection). All compounds were administered as 1:1 mixtures of normal and deuterated substrates. Each rat received two injections 24 hr apart, and two rats were used in each experiment. Urine was collected in an ice-cooled flask for the 48 hr subsequent to the first injection. After adjustment to pH 5 with acetate buffer, the urine was then incubated for 18 hr at 37° with 50,000 units/100 ml of urine of a β-glucuronidase preparation with sulfatase activity (Sigma Chemical Company).

Products from both in vitro and in vivo studies were extracted into ether which was dried ($MgSO_4$) and concentrated in vacuo. Phenolic products of the following substrates were isolated by thin-layer chromatography on silica gel GF plates developed with the solvent systems indicated in the parentheses: diphenylhydantoin ($CHCl_3$–CH_3OH–HOAc, 90:10:1); acetanilide (benzene–CH_3OH–HOAc, 90:16:8); anisole, benzenesulfonanilide, and naphthalene ($CHCl_3$–benzene–EtOAc, 6:3:1); methyl phenyl sulfide and sulfone (benzene–Et_2O, 70:30); nitrobenzene, benzene, *o*- and *p*-xylenes, and mesitylene (benzene–EtOAc, 95:5); zoxazolamine ($CHCl_3$–CH_3OH, 90:10); and biphenyl (benzene–2-propanol–aqueous NH_3, 90:9:1). The *m*- and *p*-hydroxybiphenyl were further purified by chromatography on Avicel plates developed with water. Halophenols were methylated prior to separation and mass spectral analysis (see below). Cyanophenols were separated with a Du Pont 830 high-pressure liquid chromatograph (0.5-m Varian MicroPak analytical column with 4% MeOH in hexane as mobile phase at 700 psi and flow rate of 0.9 ml/min, injection solvent MeOH). Relative proportions of metabolites were determined either by gas or high-pressure liquid chromatography or were estimated by size of thin-layer chromatographic bands.

Chemical Studies. The preparation of the diazomethane and the irradiation of a 1:1 mixture of normal and deuterated benzene solution containing diazomethane were conducted as described (Lemmon and Strohmeier, 1959). Reaction of the above benzene mixture with diazomethane in the presence of cuprous chloride was conducted as described (Muller and Fricke, 1963). The normal benzene employed for these reactions was distilled twice before use to remove residual toluene. The toluene and cycloheptatriene produced in the above reactions were analyzed for deuterium content as described below after removal of excess benzene by fractional distillation.

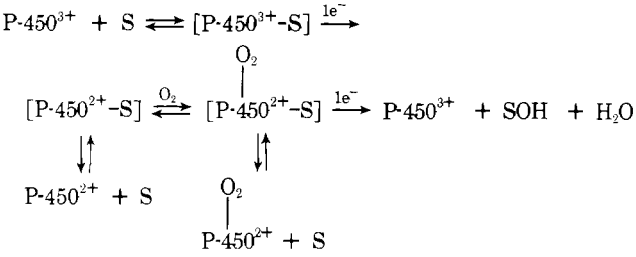
Mass Spectral Analysis. The deuterium content of the substrates and their hydroxylation products were determined by mass spectrometry using the direct probe or the liquid inlet system on a Hitachi RMU-6E mass spectrometer operated at 70 eV. For compounds which showed significant M-1 ions at this potential, the spectrometer was operated at the threshold for the appearance of the desired molecular ion to eliminate this fragmentation. The mole fraction of deuterium in the substituted positions was greater than 0.98 in all substrates except nitrobenzene (0.97), anisole (0.95), zoxazolamine (0.94) and biphenyl (0.87). Bromo-, chloro-, and fluorophenols in the ethereal extracts from urine were methylated with dimethyl sulfate in alkaline solution prior to separation of isomers and determination of deuterium content on a LKB 9000 gas chromatograph–mass spectrometer at 20 eV or a Finnegan 1015 chemical ionization mass spectrometer using Carbowax columns as previously described (Jerina et al., 1968). Deuterium contents of the toluene and cycloheptatriene formed by reaction of diazomethane with benzene were determined on the LKB spectrometer with a 15% OV-17 column operated at 60°.

Accurate determination of the isotopic composition of mixtures of deuterated and normal compounds by mass spectral analysis was found to require several precautions. In instances where loss of hydrogen or fragmentation is extensive, the compound must be analyzed at its threshold voltage to maximize the molecular ion. For combined gas chromatographic–mass spectral analysis, multiple determinations must be made through the peak since normal and deuterated compounds need not have identical retention times. For example, when a mixture of normal and perdeuterio- (aryl ring) 4-bromophenol was analyzed by direct inlet probe, the ratio of hydrogen to deuterium species was found to be 1.12:1.00. After methylation and combination gas chromatography–mass spectrometry, the 4-bromoanisole had a ratio of 0.98:1.00 early in the gas chromatographic peak 1.11:1.00 at the maximum, and 1.48:1.00 late in the peak. In all cases, spectra at the maximum of chromatographic peaks were found to be representative of the sample. A more complete discussion of these and other considerations is available (Biemann, 1962). Peak heights were measured and results calculated from data taken on 5–10 individual traces.

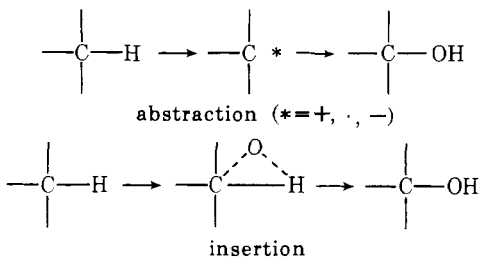
Results and Discussion

Nonpolar foreign substances are generally metabolized in mammals by hepatic cytochrome P-450 monoxygenases. Whether or not significant primary isotope effects with selectively deuterated substrates can be detected for oxidations at carbon will be dependent on the mechanism of ac-

tion for this class of enzymes. The generally accepted mechanism (Estabrook, 1971) for cytochrome P-450 catalyzed oxidations is shown below; oxidized enzyme [P-450^{3+}] binds substrate (S) to yield [P-450^{3+}-S] followed by a one-electron reduction to [P-450^{2+}-S]. Molecular oxygen then binds to form [P-(O_2)450^{2+}-S]. Further reduction by one electron regenerates oxidized enzyme and releases the product (SOH) and water without detectable intermediates. Either of the reduction steps, depending on availability of reducing equivalents and the nature of the substrate, can be rate limiting for the overall sequence (Gigon et al. 1969; Schenkman and Cinti, 1970; Diehl et al. 1970). Since deuteration of the substrate will not significantly affect the rate of either reduction step, a change *in overall reaction rate* will only be observable when an oxidation of substrate involving a carbon–hydrogen bond is rate limiting. In addition, regardless of which step is rate limiting the only situation in which a primary isotope effect can be detected by available methods for analysis of product composition is when the reduced enzyme–substrate–oxygen complex equilibrates substrate with the medium more rapidly than oxidation of substrate occurs. Under such conditions an isotope effect for the oxidation is detectable even when substrate oxidation is not rate limiting for the overall sequence. Thus, a partially deuterated substrate could cause a change in product composition or show an isotope depletion in product even though the deuterated and normal substrate might not be distinguishable kinetically. In the case of a partially deuterated substrate, a high percentage of metabolism would mask an isotope effect measured by isotope content of the product.

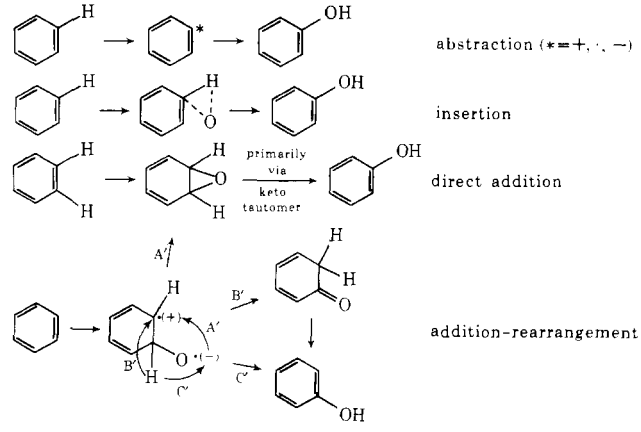


Detailed mechanisms which describe the nature of substrate oxidation by P-450 and related enzymes are as yet unavailable. Such details are important to predicting whether or not carbon–hydrogen bond breaking and hence isotope effects will be associated with the rate-limiting step of oxidation at aliphatic and aromatic positions. In oxidation at saturated carbon either the substrate must be activated by loss of hydride, hydrogen atom, or a proton to form a readily oxidized carbonium ion, radical, or carbanion (abstraction), or transfer of the equivalent of singlet oxygen atom to the substrate must occur (insertion). For



oxidations of aromatic hydrocarbons to phenols, abstraction or insertion reactions analogous with oxidation at saturated carbon must again be considered. In addition, transfer of a singlet oxygen atom to a formal aromatic double bond would produce an arene oxide in one step (*direct addition*), while transfer of a triplet oxygen atom or related species would form an intermediate which could generate a phenol by any of the three steps A′, B′, or C′ (addition–rearrangement). Nearly all these mechanisms must be considered as possible for these enzyme-catalyzed hydroxylations.

Enzymatic hydroxylation at saturated carbon often shows a primary isotope effect (see below) and invariably proceeds with a high degree of retention of configuration (cf. review by Daly, 1971). Both of these results are compatible with an insertion mechanism involving transfer of a singlet oxygen atom. The basis of such "oxenoid" reactions have been discussed elsewhere (Jerina et al., 1970; Jerina, 1973; Hamilton, 1974). Abstraction pathways, while generally less likely for energetic reasons, are equally compatible with primary isotope effects. Imposition of rigid geometry on the intermediate by the enzyme could result in a high degree of retention of configuration. In addition, radical reactions are known which do proceed with a marked degree of retention of configuration (Baldwin et al., 1970).



Formation of phenols from aromatic hydrocarbons is now widely accepted to occur by the direct addition pathway (Jerina and Daly, 1974), since isotope effects are generally not observed, intermediate arene oxides have been isolated, and the NIH shift occurs. The abstraction pathway for phenol formation can probably be excluded for energetic reasons, although it would be expected to show primary isotope effects. Alternatives A′ and B′ of the addition–rearrangement pathway would be expected to show kinetic and other properties similar to the direct addition pathway. Thus, only insertion and alternative C′ of addition–rearrangement are likely pathways to explain the presence of a primary isotope effect during hydroxylation of an aromatic ring. Notably, the NIH shift cannot occur by either of these mechanisms. Further consideration of alternative C′ requires that the depicted intermediate must be reversible with substrate in order to detect an isotope effect (cf. discussion of SE2 and SE3 mechanisms, Zollinger, 1964). Such reversibility may not be realistic on energetic grounds. Thus, the most attractive explanation for an isotope effect observed during phenol formation is an insertion pathway.

Biological half-life in vivo, duration of pharmacological activity, rate of product formation in vitro, and effects of heavy isotopes on product composition in vivo and in vitro have been employed as measures of isotope effects during metabolism of various substrates by hepatic monoxygenases. In vivo approaches are valid only when deuterium or tritium is substituted at the principal site of metabolism and when oxidation is the primary mode of deactivation.

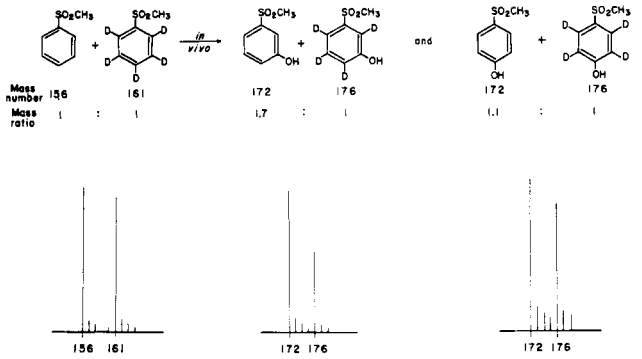


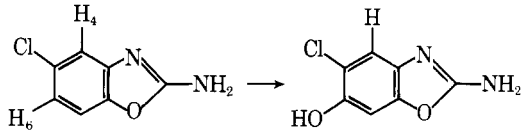
FIGURE 1: Typical mass spectra for methyl phenyl sulfone and phenolic metabolites. For calculation of isotope effects see Biemann (1962).

Significant in vivo isotope effects ($k_H/k_D > 1.2$) on pharmacological activity or biological half-life have been reported for compounds where aliphatic hydroxylation represents a major pathway of metabolism as in the 3-hydroxylation of *N*-demethyldiazepam (Marcucci et al., 1973), the 3′-hydroxylation of 5-*n*-butyl-5-ethylbarbituric acid (Tanabe et al., 1969) and pentobarbital (Mark et al., 1971), the benzylic hydroxylation of ethylbenzene (McMahon et al., 1969), the ω, ω-1, and ω-2 hydroxylation of the propyl groups in propyl *p*-nitrophenyl ether (Mitoma et al., 1971), and the 7-α-hydroxylation of taurodeoxycholic acid (Bjorklem, 1971). No isotope effects were observed during microsomal aliphatic hydroxylation of cyclohexane (Ullrich, 1968), in certain steroid hydroxylations (Bjorklem, 1971), or during benzylic hydroxylation of tolbutamide (Tagg et al., 1967). The biological half-life of tolbutamide (Lemieux et al. 1961; Kimbrough, 1972) was not affected by deuterium substitution at the principal site of oxidative metabolism, the aryl methyl group. Significant in vitro isotope effects ($k_H/k_D > 1.2$) have been reported for the oxidative dealkylation of morphine (Elison et al., 1961, 1963), *N*-isopropylamphetamine (Henderson et al., 1974), norcodeine (Thompson and Holtzman, 1970) and 4-nitroanisole (Mitoma et al., 1967). In the case of dimethylnitrosamine, where oxidative metabolism yields a proximate carcinogen, deuterium substitution significantly decreases the carcinogenicity (Keefer et al., 1973).

Previous studies had not shown isotope effects for the in vitro formation of phenols from phenylalanine with phenylalanine hydroxylase (Guroff and Daly, 1967) or from acetanilide (Tanabe et al., 1967) and anisole (Daly and Jerina, 1969) with hepatic microsomal preparations. The pharmacological activity of phenobarbital was unaffected by deuterium substitution at the 4 position of the aromatic ring (Perel et al., 1967). Only in the case of in vitro conversion of zoxazolamine to a phenolic metabolite has a significant isotope effect ($k_H/k_D = 1.47$) been reported for an aryl "hydroxylation" (Tanabe et al., 1970). In vivo pharmacological activity of zoxazolamine was, however, not significantly altered with the dideuterated drug.

Investigation of isotope effects during formation of phenols has been extended by the present study in which the deuterium content of in vivo and in vitro metabolites from 1:1 mixtures of a variety of normal and ring deuterated aromatic hydrocarbons have been examined. The principle of the mass spectrometric analysis employed is based on measurement of deuterium in product phenols as illustrated in Figure 1 for hydroxylation of methyl phenyl sulfone at the meta and para positions. Data generated in this way are

Table I: Deuterium Isotope Effect (k_H/k_D) during 6-Hydroxylation of Zoxazolamine.



	k_H/k_D
In vivo 1:1 mixture of zoxazolamine and zoxazolamine-*4,6-d*$_2$, based on mass spectrum of product.	
Control rats	1.06
3-Methylcholanthrene-induced rats	1.20
In vitro 1:1 mixutre of zoxazolamine and zoxazolamine-*4,6-d*$_2$, based on mass spectrum of product	1.06, 0.95[a]
Kinetic analysis of rate of substrate disappearance in separate experiments with zoxazolamine and zoxazolamine-*4,6-d*$_2$	0.92[a,b]

[a] Preparation and incubation according to Tanabe et al. (1970). [b] Analysis of substrate by high-pressure liquid chromatography with a 1-m ETH column eluted with 2% MeOH–hexane at 460 psi and a flow rate of 1.0 ml/min, and CH_2Cl_2 as injection solvent. Tanabe et al. (1970) report an isotope effect of 1.47 ± 0.07 using a spectral assay of substrate disappearance. This method, while satisfactory for many applications, did not prove sufficiently precise for in vitro kinetic studies in our hands.

presented in Tables I–IV for 17 aromatic substrates. With certain exceptions, which will be considered as examples of primary isotope effects, the values of k_H/k_D fall in the range of 0.88–1.22. For the entire study, standard deviations ranged from 0.02 to 0.11 (av ± 0.07), where multiple metabolism studies had been conducted on given substrates. Examination of all the data in the range of 0.88–1.22 shows the vast majority of the results are significantly greater than 1.00. Whether this represents a significant primary isotope effect or is the consequence of a systematic artifact cannot be said with certainty. Values of k_H/k_D less than 0.70 and greater than 1.30 have been designated as significant primary isotope effects with the intention of excluding any systematic errors and secondary isotope effects.

The finding that zoxazolamine displayed a significant primary isotope effect on hydroxylation of the aromatic ring at the 6 position (Tanabe et al., 1970) was subjected to careful scrutiny (Table I). When 1:1 mixtures of the drug and its cogener deuterated at the position of hydroxylation were examined in vivo with normal animals and with animals induced with 3-methylcholanthrene or in vitro with liver microsomes from noninduced and benzo[*a*]pyrene-induced animals, significant isotope effects associated with the production of 6-hydroxyzoxazolamine were not detected. Furthermore, *rate* of substrate disappearance for the normal and deuterated drug with microsomes showed no significant isotope effect with a sensitive and accurate assay by high-pressure liquid chromatography. The variance between the present results and the initial study (Tanabe et al., 1970) may reflect changes in the rate-limiting step for different preparations of microsomes or may simply be due to insensitivity of the initial spectrophotometric assay. The "NIH shift" of deuterium for 6-hydroxylation of zoxazolamine-*4,6-d*$_2$ was estimated at less than 10%. Prediction of a retention value for such an activated ring is difficult (cf. Daly et al., 1972).

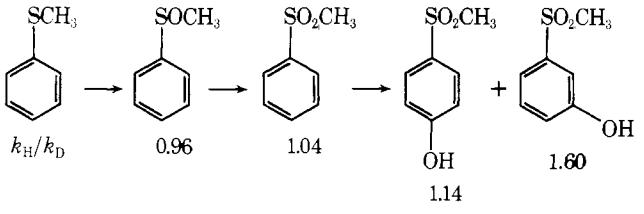
Nitrobenzene and methyl phenyl sulfone (Table II) were the only substrates for which significant primary isotope ef-

Table II: In Vivo Metabolism of 1:1 Mixtures of Normal and Deuterated Aromatic Substrates: Deuterium Isotope Effect (k_H/k_D) on Formation of Phenolic Products.[a]

Substrate	No. of Deuteriums in Substrate	Position of Hydroxylation and k_H/k_D of Phenolic Products				Other Phenolic Products	k_H/k_D
		Ortho	Meta	Para	Catechols		
Nitrobenzene[b]	5	1.18 ± 0.06 (4)	1.40 ± 0.07 (7)	1.04 ± 0.04 (7)		4-Hydroxy-acetanilide	0.98 ± 0.04 (5)
Methyl phenyl sulfone	5 (ring)		1.75	1.13			
Bromobenzene[c]	5	1.09 ± 0.11 (7)	1.22 ± 0.06 (9)	1.17 ± 0.07 (11)	1.08 ± 0.06 (9)		
Chlorobenzene	5	1.11 ± 0.09 (3)	1.17 ± 0.11 (3)	1.12 ± 0.02 (3)			
Fluorobenzene	5	0.95	1.03	0.94			
Benzonitrile	5	1.18, 1.17	1.19, 0.98	1.08, 1.14	1.00		
Benzene-sulfonanilide	5 (aniline ring)			0.94			
Acetanilide	5 (ring)			1.09			
Anisole	5 (ring)	1.00		1.06		Phenol	1.04
Biphenyl[d]	10	1.11 ± 0.10 (3)	1.08, 1.10	0.94 ± 0.03 (3)	1.07 ± 0.03 (3)	4,4′-Dihydroxy-biphenyl	1.00 ± 0.04 (3)
						3,4-Hydroxy-4-methoxy-biphenyl	0.88, 0.95
Diphenyl-hydantoin	10 (phenyl rings)			0.97			

[a] The k_H/k_D values are means and standard deviation with the number of experiments in parentheses or are results of single experiments. [b] Similar results were obtained in normal, 3-methylcholanthrene-induced, and phenobarbital-induced Sprague-Dawley rats, and in germ-free rats. In experiments with germ-free rats, urines from the first and second 24-hr period after administration of the compound were analyzed separately and afforded similar results. [c] Similar results with normal, 3-methylcholanthrene-induced, and phenobarbital-induced Sprague-Dawley rats and in germ-free and hormone-assay rats. Analysis of the urines collected during the first and second 24-hr period after administration of the compound to germ-free rats gave similar results. [d] Similar results from normal, 3-methylcholanthrene-induced, and phenobarbital-induced Sprague-Dawley rats. Small amounts of a trihydroxylated metabolite of unknown structure were detected when methylcholanthrene-induced rats were employed.

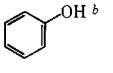
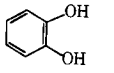
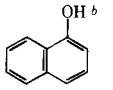
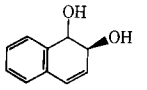
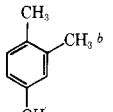
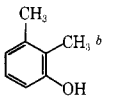
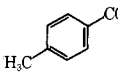
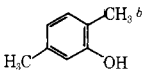
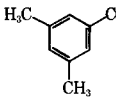
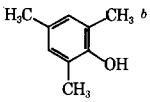
fects were observed in vivo (Tables II and III). Hydroxylation at the meta position of these substrates gave values of k_H/k_D = 1.40 and 1.75, respectively. Hydroxylation at both meta and para positions are major metabolic routes for these substrates. Methyl phenyl sulfide is probably converted in vivo to hydroxyphenyl methyl sulfones as shown (cf. McBain and Menn, 1969) and, therefore, displays isotope effects similar to those observed when the sulfone was studied. Several possible explanations of the isotope results have



been considered. Factors such as K_m, solubility, and membrane transport could well be different for normal and completely deuterated substrates. Other authors have argued (cf. Elison et al., 1961; Thompson and Holtzman, 1970, 1973; Mitoma et al., 1967) that changes in these factors due to deuteration could not produce isotope effects of the magnitude observed here. Results of the present study support these views: urine from animals dosed with nitrobenzene was collected on consecutive days (Table II, footnote *a*), and no change in isotope effects were detected; the para-hydroxylated product from methyl phenyl sulfone as well as ortho- and para-hydroxylated products from nitrobenzene show no significant isotope effect; the isotope effect is not altered when deuterium is present only at the two equivalent meta positions in nitrobenzene or methyl phenyl sulfone (results not reported).

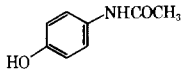
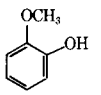
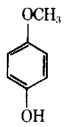
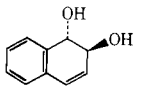
Pathways leading from substrate to metabolite in vivo are, however, difficult to delineate. Hence, it is possible that phenols may not be primary products, but result from further metabolism of a primary metabolite such as an arene oxide. For example, the meta-hydroxylated metabolites derived from nitrobenzene and the sulfone might have originated from dihydrodiols which were dehydrogenated to catechols (cf. Ayengar et al., 1959) and finally dehydroxylated to meta-substituted phenols by gut flora (cf. Scheline, 1973). Dehydrogenation of diols to catechols is accompanied by a significant isotope effect as can be seen from the *increased* deuterium content of the dihydrodiol of naphthalene (Tables III and IV). In vitro rate studies have shown that deuterium-containing naphthalene dihydrodiol is indeed converted to 1,2-dihydroxynaphthalene at a slower rate than the hydrogen-diol (Oesch, Jerina, and Daly, unpublished results). Thus, partial conversion of dihydrodiols to catechols will result in increased deuterium content of the diols and decreased deuterium content of the catechols. The catechols obtained from benzene and substituted benzenes (Tables II and III) do not show this effect, since conversion of diol to catechols is nearly complete for these substrates (Sato et al., 1963; Jerina et al., 1967) and only traces of diols are present in the urines. Catechols derived from nitrobenzene and methyl phenyl sulfone might lead to a meta-hydroxylated metabolite via dehydroxylation by gut microorganisms. These meta-hydroxylated metabolites would presumably have been enriched in deuterium due to isotope effects at the dehydrogenation stage. This possibility was eliminated: germ-free animals produce major amounts of *m*-nitrophenol and only minor amounts of catechols as do normal animals; administration of 4-nitrocatechol to normal rats results in excretion of the free and conjugated catechol with no detectable *m*-nitrophenol. Spontaneous or enzyme-catalyzed dehydration of dihydrodiols to meta-substituted phenols as an origin of the isotope effect appears unlikely, since there is no precedent for this reac-

Table III: In Vivo Metabolism of 1:1 Mixtures of Normal and Deuterated Aromatic Substrates.[a]

Substrate	No. of Deuteriums in Substrate	Products	
Benzene	6	 1.08, 1.12	 1.03
Naphthalene	8	 1.13	 0.53
o-Xylene	4 (ring)	 0.89	 0.96
	4 (methyl)		
p-Xylene	4 (ring)	 1.00	 1.09, 1.00
Mesitylene	12	 1.55	 0.55

[a] Deuterium isotope effects (k_H/k_D) are presented below the structures of the products. [b] Minor or trace metabolite. [c] Major metabolite.

Table IV: In Vitro Metabolism of 1:1 Mixtures of Normal and Deuterated Aromatic Substrates.[a]

Substrate	No. of Deuteriums in Substrate	Products		
Acetanilide	5 (ring)	 1.03		
Anisole	5 (ring)	 0.96	 1.03	 1.09
Naphthalene	8	 0.67, 0.60		

[a] Deuterium isotope effects (k_H/k_D) are presented below the structures of the products.

tion in vivo (Posner et al., 1961; Boyland and Sims, 1962). In addition, such a reaction would not on mechanistic considerations be expected to show an isotope effect. Neither the sulfone nor nitrobenzene are metabolized to a sufficient extent in vitro for the present type of isotope experiments. Results with substrates that were examined in vitro (Table IV) were not significantly different from the in vivo results (Tables II and III).

Since large deuterium isotope effects were only observed when hydroxylation occurred at the position which is meta to the benzene ring substituent, metabolism of halobenzenes (Jerina et al., 1967; Daly et al., 1968), benzonitrile (Smith and Williams, 1950), and biphenyl (Raig and Ammon, 1972) was examined as hydroxylation was either known or suspected to occur at the meta position in these compounds.

Metabolism of the halobenzenes presents a particularly intriguing case since the two expected arene oxides from chlorobenzene (cf. Kaubisch et al., 1972) have been synthesized and neither isomerizes to *m*-chlorophenol (Selander, Jerina, and Berchthold, unpublished results). Chlorobenzene is converted to *p*-, *m*- and *o*-chlorophenol in the ratio of 5:3:2 in Sprague-Dawley rats. No significant isotope effects were observed (Table II). Metabolism of bromobenzene was examined in more detail. In vivo metabolism was examined in normal, phenobarbital-induced, and methylcholanthrene-induced Sprague-Dawley rats, germ-free rats, and Hormone-assay rats. The ratio of phenols produced was approximately 6:5:1 (para:meta:ortho) in all cases, with 20–50% of the total metabolites represented by catechols and methylated catechols. Statistically significant isotope effects were not observed for any of these products (Table II). Complete or very extensive depletion of the serum level of the deuterated and normal substrate mixtures employed in this study, on a single pass through the liver, would preclude the observation of an isotope effect by the present method of product analysis.

Normal Sprague-Dawley rats produce *p*-, *m*- and *o*-hydroxybenzonitriles and 3,4-dihydroxybenzonitrile (ratio 15:2:2:1) from benzonitrile without significant isotope effects (Table II). Metabolism of biphenyl in phenobarbital- and methylcholanthrene-induced rats results mainly in para hydroxylation with minor amounts of meta and ortho hydroxylation. Significant isotope effects were not detected (Table II). Of the several other compounds examined (Tables II and III), only the metabolism of mesitylene requires further comment. Production of mesitol is accompanied by an inverse isotope effect of $k_H/k_D = 0.55$. This unusual result is easily explained by a metabolism-linked increase in deuterium content of the substrate in vivo. The principal mode of metabolism is aliphatic oxidation to produce 3,5-dimethylbenzoic acid with $k_H/k_D = 1.55$.

In toto, the in vivo results reported in this paper allow no steric or electronic predictions as to when a significant primary isotope effect will be detected. The general tendency toward values >1.00 is suggestive that many of the hydroxylations may be accompanied by minor amounts of pathways which display isotope effects. The observed isotope effects associated with meta hydroxylation of nitrobenzene and methyl phenyl sulfone would actually be reduced if, in addition to pathway influenced by isotopic hydrogen, an arene oxide pathway plays a significant role in producing these metabolites.

Radical and electrophilic substitution reactions of the aromatic ring are known to display isotope effects as high as 2.6 and 6.6, respectively (Zollinger, 1964). Insertion reactions into the aryl carbon–hydrogen bond have not been studied. Insertion of carbenes into aliphatic carbon–hydrogen bonds is, however, known to proceed with an isotope effect of 1.6–1.8 (Goldstein and Baum, 1963; Simons and Rabinovitch, 1963; Franzen and Edens, 1969). The reaction of diazomethane with a 1:1 mixture of normal and deuterated benzene to produce cycloheptatriene via norcaradiene (addition of carbene) and toluene (insertion of carbene) have been examined in the present study as models for the enzymatic oxygen atom transfer processes. Photolytic decomposition of diazomethane in benzene produced toluene and cy-

cloheptatriene in the ratio of 1:4 with k_H/k_D of 1.09 ± 0.07, $n = 3$ and 1.10 ± 0.07, $n = 3$, respectively. Absence of an isotope effect for production of toluene suggests the reaction proceeds via addition and rearrangement rather than insertion. Alternatively, the transition state for insertion could precede carbon–hydrogen bond breaking. Only cycloheptatriene was produced in significant amounts when the diazomethane was decomposed with cuprous chloride ($k_H/k_D = 1.02 \pm 0.06$, $n = 3$). The carbenoid reagent generated in this way is noted for its preference toward addition reactions (House and Blankley, 1968). Thus, neither of the above carbene reactions have provided a chemical analogy for the direct oxygen-insertion reaction into an aromatic carbon–hydrogen bond, which is the preferred mechanism to explain the isotope effects detected in the present metabolic studies.

Arene oxides of aryl sulfones or nitrobenzene are presently unavailable. While it is anticipated that such arene oxides will not display primary isotope effects on isomerization, it is conceivable that such highly stable arene oxides could isomerize by presently unknown mechanisms. If an isotope effect is operative for these compounds, the phenols produced would be enriched in hydrogen (apparent isotope effect), while other pathways, such as dihydrodiol and catechol production, would be enriched in deuterium. Sufficient catechols could not be isolated from the aryl sulfone or nitrobenzene to test this hypothesis. The only remaining alternatives to an isotope effect on isomerization of certain arene oxides are that cytochrome P-450 operates by a different mechanism with selected substrates or that a separate enzyme which does not function by arene oxide pathway is present. Should the latter possibility prove true, there are profound implications on the area of drug metabolism: aromatic compounds would still be metabolized but the cytotoxic (Brodie et al., 1971) and carcinogenic (Grover et al., 1971, Huberman et al., 1971) pathway via arene oxides would be avoided.

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Stable, Nonreducible Cross-Links of Mature Collagen†

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ABSTRACT: During in vivo maturation, and also during in vitro incubation with physiological buffers, native collagen fibers display a progressive increase in tensile strength and insolubility. Paralleling these physiologically important changes is a progressive loss of the reducible cross-links which initially join the triple-chained subunits of collagen fibers. Although there is evidence suggesting that the reducible cross-links are gradually transformed into more stable, nonreducible cross-links during maturation, the nature of the transformation process and the structure of the stable "mature" cross-links has remained a mystery. In order to test the possibility that cross-link transformation involves addition of a nucleophilic amino acid residue to the reducible cross-links, histidine, arginine, glutamate, aspartate, lysine, and hydroxylysine residues were chemically modified, and the effect of each modification procedure on the in vitro transformation of reducible cross-links was ascertained. The results of these experiments indicated that destruction of histidine, arginine, glutamate, and aspartate residues has no measurable effect on the rate and extent of reducible cross-link transformation in hard tissue collagens. In contrast, modification of lysine and hydroxylysine residues with a wide variety of specific reagents completely blocks the transformation of reducible cross-links. Removal of the reversible blocking groups from lysine and hydroxylysine residues then allows the transformation to proceed normally. These results indicate that collagen maturation involves nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible cross-links, yielding derivatives which are not only more stable but also capable of cross-linking more collagen molecules than their reducible precursors.

Collagen, the most abundant protein of higher vertebrates, is unusual in several respects. Not only do its large, triple-chained subunits undergo spontaneous self-assembly to form fibrils, but in addition, these fibrils exhibit a unique maturation process which gradually transforms them into more stable aggregates (Fowler and Bailey, 1972). Furthermore, this process appears to occur more quickly in vitro and in the collagen fibers of healing wounds than in normal, developing tissues. Collagen, which is formed extracellularly by proteolytic cleavage of a soluble precursor, procollagen (Bellamy and Bornstein, 1971; Layman et al., 1971), contains reactive aldehyde residues which are produced extracellularly by oxidative deamination of a few lysine and/or hydroxylysine residues (Piez, 1968). When the collagen molecules aggregate to form native fibrils, aldehyde residues on one molecule can then react with lysine or hydroxylysine residues on another molecule to form aldimine cross-links (Bailey and Peach, 1968; Tanzer et al., 1970; Mecha-

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