TABLE III: Metabolism of Vitamin K_1 and Reversal of Warfarin Inhibition.[a]

Injected	Prothrombin % of Normal: Zero	2 hr	5 hr	Oxide:K_1 in Liver at 2 hr
200 μg of [^{3}H]K_1 + 4 mg of warfarin	12	53	34	0.92 ± 0.19 (3)
25 μg of [^{3}H]K_1	12	18	28	1.4 ± 0.12 (4)

[a] Rats injected intraperitoneally with 0.4 mg of sodium warfarin 24 hr previously were injected with the indicated amounts of [^{3}H]K_1. One group was also given an additional 4 mg of warfarin as shown. The prothrombin concentrations are the average for five animals. Duplicate thin layers were run for determination of the oxide:K_1 ratio for each liver. The number of livers analyzed is shown in parentheses.

which the K_1–oxide interconversion is considered may reveal differences. If our hypothesis concerning the mechanism of action of warfarin is correct, resistance to the anticoagulant might occur by a mutation which renders the conversion of phylloquinone oxide to vitamin K_1 no longer sensitive to warfarin inhibition.

Bell and Matschiner (1972) found that vitamin K_1 and phylloquinone oxide were competitive antagonists while the relationship between the vitamin and coumarin anticoagulants is not a simple competitive one (Lowenthal and MacFarlane, 1964; Lowenthal and Birnbaum, 1969). We proposed that warfarin is unable to counteract large doses of vitamin K_1 because the oxide:K_1 ratio does not reach an inhibitory level. The experimental results are consistent with this idea (Table III).

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Rearrangement of [1-^{2}H]- and [2-^{2}H]Naphthalene 1,2-Oxides to 1-Naphthol. Mechanism of the NIH Shift†

D. R. Boyd,‡ J. W. Daly, and D. M. Jerina*

ABSTRACT: Synthetic [1-^{2}H]- and [2-^{2}H]naphthalene 1,2-oxides rearrange predominately to 1-naphthol, which retains 60–85% of the original deuterium. The magnitude of deuterium retention is dependent on the pH of rearrangement. Under neutral or basic conditions, both deuterated oxides give 1-naphthol with a deuterium retention of approximately 80%. Thus, a common intermediate, on the pathway from the deuterated naphthalene oxides to 1-naphthol, is indicated. This intermediate is probably the keto tautomer of 1-naphthol, enolization of which to 1-naphthol in neutral and basic pH regions must then be accompanied by an isotope effect (k_H/k_D) of 4.0. Under acidic conditions, [1-^{2}H]naphthalene 1,2-oxide forms 1-naphthol with a lower retention of deuterium than is the case with [2-^{2}H]naphthalene 1,2-oxide. Thus, acid-catalyzed isomerization of naphthalene 1,2-oxide is mechanistically distinct from the spontaneous isomerization observed in the neutral and basic range. Microsomal hydroxylation of *either* [1-^{2}H]- or [2-^{2}H]naphthalene at pH 9 produces 1-naphthol with approximately 65% retention of deuterium providing additional evidence for naphthalene 1,2-oxide as an intermediate in the metabolism of naphthalene. Hydroxylation of the deuterated naphthalenes with a peracid or a photolytic model system affords deuterium retentions similar to those observed with microsomes.

The NIH shift, an intramolecular migration of aryl ring substituents, has been established as a characteristic of monoxygenase-catalyzed oxidation of aromatic compounds to form phenols (Daly *et al.*, 1968a; Guroff *et al.*, 1967). Despite numerous investigations, the mechanism of this intramolecular migration is still not known with certainty. However,

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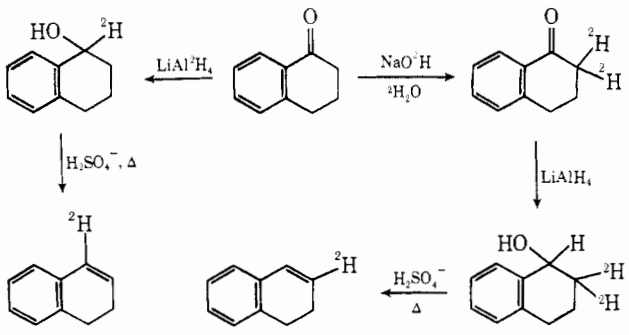
SCHEME I



the facile rearrangement of arene oxides to the isomeric phenols does occur with the requisite migration and retention of deuterium or alkyl substituents (Jerina *et al.*, 1968a, 1971a; Kaubisch *et al.*, 1972[1]). Such arene oxide intermediates are compatible not only with phenol formation and NIH shift, but also with the metabolism of aromatic compounds to dihydrodiols and glutathione conjugates (Jerina *et al.*, 1968b,c, 1970a). The enzymatic formation of an arene oxide from naphthalene (Jerina *et al.*, 1968c, 1970a) and from dibenz[*a,h*]anthracene (Selkirk *et al.*, 1971) has been demonstrated. Arene oxide intermediates have also been implicated in chemically induced carcinogenesis (Grover *et al.*, 1971) and tissue necrosis (Brodie *et al.*, 1971). As yet, however, an arene oxide has been firmly established as the obligatory intermediate in the conversion of an aromatic substrate to a phenol in only one case, the conversion of naphthalene to 1-naphthol (Jerina *et al.*, 1968b, 1970a). Therefore, the occurrence and mechanism of the NIH shift have been examined both during microsomal "hydroxylation" of [1-^{2}H]- and [2-^{2}H]naphthalene and during isomerization of the intermediate [1-^{2}H]- and [2-^{2}H]naphthalene 1,2-oxides. The results are compatible with the intermediacy of [1-^{2}H]- or [2-^{2}H]naphthalene 1,2-oxides which rearrange *via* deuterium or hydrogen migration, respectively, to a common intermediate, the keto form of 1-naphthol, which then enolizes to 1-naphthol with a preferential loss of hydrogen rather than deuterium.

Experimental Section

Mass Spectrometric Analysis. The deuterium content of the synthesized deuterionaphthalenes and deuterionaphthalene dihydrodiols was measured on a Hitachi RMU-7 spectrometer using the solids inlet probe. Naphthalene dihydrodiol samples were run at 70 eV while naphthalene samples were run at 15 eV to eliminate M − 1 and M − 2 peaks in these spectra. The naphthols and 1-bromonaphthalene were examined for deuterium content with an LKB combined gas chromatograph–mass spectrometer operated at 70 eV. Analysis of naphthols was done on the trimethylsilyl ethers as previously described (Jerina *et al.*, 1970a) while 1-bromonaphthalene was introduced through a 6 ft column of OV-1 (3% on gas chrom Q) operated at 180°. Calculation of deuterium content in all samples was done by direct comparison of the group of peaks at the molecular ion for the labeled and unlabeled species run under identical conditions.

Hydroxylation by Liver Microsomes. Enzymatic hydroxylations were carried out for 15 min at pH 9 with a crude microsomal preparation (9000*g* supernatant) prepared from livers of phenobarbital-induced Sprague–Dawley rats (Daly *et al.*, 1969) as described (Daly and Jerina, 1969). Substrate and products were extracted into ethyl acetate and isolated by thin-layer chromatography (Jerina *et al.*, 1970a). The trimethylsilyl derivatives of the naphthols were assayed for deuterium content by mass spectrometry.

Enzymatic Hydrations to trans-Dihydrodiols. The deuterated naphthalene oxides were converted to dihydrodiols with microsomes obtained from rabbit liver homogenates as described (Jerina *et al.*, 1970a). The proton magnetic resonance spectra of these dihydrodiols confirmed the assignment of line positions and coupling constants in the unlabeled *trans*-diol (Jerina *et al.*, 1971b) in that the appropriate absorptions and couplings were now absent. Both the [1-^{2}H]- and [2-^{2}H]-oxides gave dihydrodiol containing >0.99 atom of deuterium.

Preparation of [1-^{2}H]- and [2-^{2}H]Naphthalene. Both 1-bromo- and 2-bromonaphthalene were obtained pure and free of each other by several recrystallizations of their complexes with picric acid from ethanol saturated with picric acid. Regeneration from the complex and simultaneous separation from picric acid were achieved by passing solutions of the complexes over columns of deactivated alumina. Reductive deuterolysis as previously described for benzene derivatives (Jerina *et al.*, 1971c) provided the desired [1-^{2}H]- and [2-^{2}H]naphthalene with greater than 98% incorporation of one deuterium atom.

Specificity of labeling in the samples was determined by chemical reaction. A solution of [1-^{2}H]naphthalene (100 mg in 5 ml of CCl_4) was brought to reflux and bromine (140 mg, 1 ml of CCl_4) was added over a 2-hr period. After continued reflux for an additional 2 hr, the sample was analyzed for deuterium content. Only 1-bromonaphthalene is formed in this reaction. Deuterium content for this material was 0.75 deuterium atom/molecule, indicating 100% of the deuterium as localized at C-1, since there are four equivalent 1 positions in naphthalene. Hydroxylation of [2-^{2}H]naphthalene by Fentons reagent (Breslow and Lukens, 1960) gave a mixture of naphthols. Deuterium analysis of the trimethylsilyl ethers showed the 2-naphthol to contain 0.77 atom of deuterium suggesting 92% of the deuterium localized at C-2. Fentons reagent and bromination do not cause migration of deuterium (Jerina *et al.*, 1967b).

Preparation of Deuterated Naphthalene 1,2-Oxides. Synthesis of the appropriate deuterium-labeled naphthalene oxides followed directly from the previously reported synthesis of naphthalene 1,2-oxide (Vogel and Klärner, 1968). Specific introduction of label was achieved at the dihydronaphthalene stage (Scheme I). The deuterated olefins were then converted to the naphthalene oxides by epoxidation with *m*-chloroperoxybenzoic acid (Boyd *et al.*, 1970), followed by bromination and dehydrohalogenation (Vogel and Klärner, 1968). Deuterium content of the labeled naphthalene oxides (>99% monodeuterio) was not determined directly since rearrangement to naphthol and subsequent loss of label *via* exchange can occur in the ion source of the spectrometer. Thus, the measurement was made on the corresponding dihydrodiols prepared enzymatically from these oxides (see enzymatic hydration results).

(*A*) *[1-^{2}H]Naphthalene 1,2-Oxide.* A solution of 1-tetralol (43 g) in ether (100 ml) was slowly added to a stirred mixture of [$^2H_4$]$LiAlH_4$ (4.5 g) in ether (100 ml), and the resulting suspension stirred at reflux for 4 hr. Hydrolysis with 0.1 N HCl followed by the usual work-up and distillation provided

[1] N. Kaubisch, J. W. Daly, and D. M. Jerina, submitted for publication.

39 g (89% yield) of 1-[1-^{2}H]tetralol (bp 122–126° (8 mm)). All of this alcohol along with potassium hydrogen sulfate (18 g) and a trace of hydroquinone (100 mg) were heated under vacuum (40 mm) with stirring at 110–120° for 1.5 hr. The desired 1,2-[4-^{2}H]dihydronaphthalene distilled from the reaction mixture during the course of the dehydration. Redistillation provided 26 g (77% yield) of the pure olefin (bp 97° (28 mm)) which was then converted to [1-^{2}H]naphthalene 1,2-oxide. The proton magnetic resonance spectrum of the product differs from that of the unlabeled oxide (Vogel and Klärner, 1968) in that the doublet ($J_{1,2}$ = 3.7 Hz) at δ 4.30 for the C-1 hydrogen is now missing and a doublet of doublets of doublets ($J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 3.7 Hz, and $J_{2,4}$ = 1.8 Hz) at δ 3.93 for the C-2 hydrogen has collapsed to a doublet of doublets with coupling to the two vinyl hydrogens.

(*B*) *[2-^{2}H]Naphthalene 1,2-Oxide.* Label was introduced in the 2 position by an initial exchange reaction on 1-tetralone to produce 1-[2,2-$^2H_2$]tetralone which was converted to naphthalene oxide as above with the exception that $LiAlH_4$ instead of $[^2H_4]LiAlH_4$ was used in the reduction. The exchange reaction was conducted by mixing the ketone (14.6 g) with $[^2H_2]H_2O$ (60 g) and adding pyridine (20 ml) until the solution became homogeneous. Potassium *tert*-butoxide (0.5 g) was added and the mixture kept at room temperature for 15 min. Saturation with NaCl and extraction into ether permitted recovery of the ketone. The exchange was conducted twice more on the recovered ketone (>95% recovery) to ensure complete incorporation of deuterium. The proton magnetic resonance spectrum of the [2-^{2}H]naphthalene 1,2-oxide synthesized from the above ketone was consistent in that the signal for the C-2 hydrogen at δ 3.93 is now missing, while the signal for the C-1 hydrogen becomes a singlet and the vinyl hydrogens appear as a pair of doublets.

Results and Discussion

Intermediacy of Arene Oxides in Enzymatic Formation of Phenols. Recent investigations on the pathways for formation of phenols with monoxygenases suggest the intermediacy of labile arene oxides (Jerina *et al.*, 1968c, 1970a). Such intermediates are quite compatible with present knowledge of monoxygenase-catalyzed oxidation of aromatic rings; (i) arene oxides are unstable and rapidly rearrange to the phenols observed as metabolic products with the requisite migration of substituents (Jerina *et al.*, 1968a, 1971a; Kaubisch *et al.*, 1972[1]), (ii) formation of an arene oxide should not involve a primary isotope effect and indeed enzymatic formation of phenols is only rarely associated with a primary isotope effect (*cf.* Jerina *et al.*, 1971c), and (iii) the magnitude of migration and retention of a substituent during isomerization of an arene oxide would depend on the nature of other substituents on the aromatic ring and not on the enzyme involved in its formation; this is usually the case for the NIH shift as delineated with various aromatic substrates and with different monoxygenases (*cf.*, Daly *et al.*, 1968a,b). It is also known that, with a few exceptions (Bowman *et al.*, 1969), when deuterium or tritium is present flanking the position which is to be hydroxylated, little of this isotope is lost during oxidation to phenols by monoxygenases (Guroff *et al.*, 1967), and that the magnitude of tritum migration and retention is usually reduced by the presence of deuterium rather than hydrogen in the flanking positions (Reed *et al.*, 1972[2]). Such observations would be compatible with isomerization of an arene oxide to a species such as the keto form of the phenol containing both migrated and flanking isotopic species of hydrogen on the same carbon atom. Enolization of this species to the phenol would retain or lose different proportions of isotopic species of hydrogen dependent on the pertinent isotope effects. In order to investigate the mechanism of the NIH shift, the role of arene oxides, and the mechanism of isomerization of arene oxides, it became necessary to study these phenomena in an aromatic compound, naphthalene, for which the corresponding arene oxide, naphthalene 1,2-oxide, had been shown to be the obligatory intermediate in the formation of the phenolic metabolite, 1-naphthol (Jerina *et al.*, 1968c, 1970a).

Isomerization of Deuterionaphthalene Oxides. The isomerization of [1-^{2}H]- and [2-^{2}H]naphthalene 1,2-oxides to 1-naphthol was studied under a variety of conditions (Table I). The most striking feature of the isomerization of these two deuterated arene oxides is that under neutral or basic conditions, the same retention of deuterium in 1-naphthol pertains. Thus, at pH 7 and 8.5, 80–81% of the deuterium is retained in the 1-naphthol from either oxide. During rearrangement in the presence of liver microsomes at pH 8, a slightly lower retention of 72–75% pertains with both oxides. These results suggest formation of a common intermediate during isomerization of either oxide. In all likelihood, this intermediate is the keto form of 1-naphthol which arises by migration of either deuterium or hydrogen from the 1 to the 2 position (Scheme II). Retention of approximately 80% of the deuterium in the 1-naphthol requires an isotope effect (k_H/k_D) of approximately 4 for the enolization of the keto form of 1-naphthol. Differing isotope effects may well pertain with other keto–phenol tautomers. The keto tautomer of 1-naphthol has now been generated photochemically from naph-

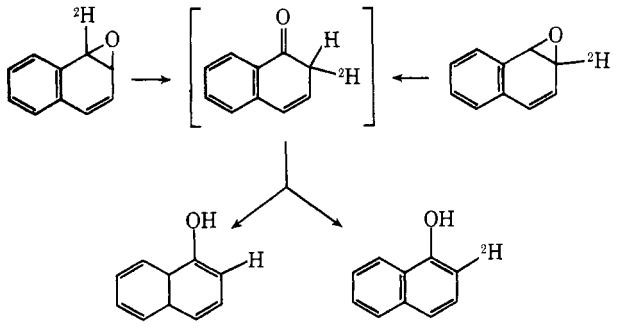
TABLE I: Deuterium Retention after Isomerization of [1-^{2}H]- and [2-^{2}H]Naphthalene Oxide to 1-Naphthol. The Oxides Contained 1.00 Deuterium Atom and Retentions Are Accurate to ± 2%.[a]

Isomerization Conditions	% Deuterium Remaining in 1-Naphthol after Isomerization	
	1-Deuterio	2-Deuterio
Liver microsomes (pH 9.0 Tris buffer)	75	72
Acetic acid	70	83
pH 3[b]	59	85
pH 4	58	85
pH 5.5	71	84
pH 7.0	80	81
pH 8.5	81	80

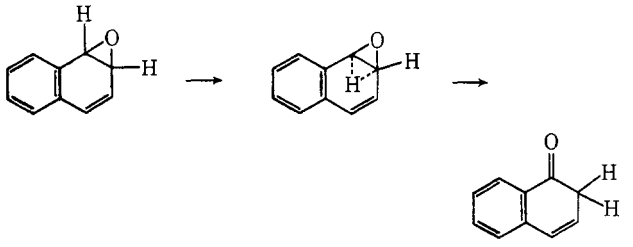
[a] Ring proton exchange of 1-naphthol does not occur with the media under these conditions. [b] The isomerizations at given pH values were done in standard buffer solutions for pH meters and mixtures thereof except the pH 3 medium which was the pH 4 buffer adjusted with concentrated HCl.

[2] D. Reed, D. M. Jerina, and J. W. Daly, manuscript in preparation.

SCHEME II



SCHEME III



thalene oxide at −196° (Jerina *et al.*, 1972[3]). Its thermal stability precludes isolation at room temperature.

Isomerization of the two oxides under acidic conditions produces 1-naphthol with differing deuterium retentions. Thus, in pH 3 and 4 aqueous buffer, only 58–59% of the deuterium migrates and is retained in the 1-naphthol formed from [1-^{2}H]naphthalene 1,2-oxide, while 85% is retained in 1-naphthol formed from [2-^{2}H]naphthalene 1,2-oxide. These observations clearly indicate that different mechanisms are operative at high and low pH. Similar changes in product distribution with pH have been observed during rearrangement of methyl-substituted arene oxides (Jerina *et al.*, 1971a; Kaubisch *et al.*, 1972[1]).

Interpretation of these results is greatly facilitated by consideration of elegant kinetic data on the rearrangement of arene oxides (Kasperek and Bruice, 1972). Isomerization of naphthalene oxide and benzene oxide proceeds by an acid-catalyzed mechanism below pH 6 and by a spontaneous, pH-independent mechanism at higher pH values. The isomerization of naphthalene oxide above pH 6 was suggested to proceed *via* a concerted migration of hydride to the unprotonated keto form of naphthol (Scheme III). The kinetic results and the present data are thus complementary with regard to keto tautomer as the common intermediate. For both oxides, complete migration of the hydrogen isotope from C-1 to C-2 occurs to form the common intermediate. This complete migration of the C-1 substituent as the first step in the neutral and basic range may be peculiar to naphthalene 1,2-oxide.

Considering *only* the acid-catalyzed region, the kinetic data of Kasperek and Bruice (1972) were interpreted to reflect concerted (path a) or stepwise (path b–d) rearrangement of the protonated oxide to the protonated keto form of the phenol (Scheme IV). Such a mechanism, with the alternate pathways whereby isotopic hydrogen could either migrate or be lost from the C-1 position, is compatible with the deuterium retentions observed with the two naphthalene oxides at low pH. Thus, if the isotope effect (k_H/k_D) of 4 for enolization of the keto form is independent of pH and if the stepwise pathway (path b) leads principally to loss (path c) rather than migration (path d) of isotopic hydrogen, a 3:1 fractionation between the concerted (path a) and stepwise (path b) rearrangement of protonated oxide should lead to 60% retention of deuterium in 1-naphthol from the [1-^{2}H]-naphthalene oxide and 85% in the 1-naphthol from the 2-^{2}H isomer. These calculated values are in agreement with observed values of 58–59% and 85%, respectively (Table I). However, the possibility that the isotope effect for enolization (k_H/k_D) is dependent on pH or that fractionation between loss (path b,c) and migration (path a and b–d) pathways depends on the species of isotopic hydrogen originally present at C-1 cannot be excluded. It does appear likely that the stepwise pathway of isomerization of the protonated naphthalene oxide, which involves a cationoid species, will lead to aromatization preferentially by loss of isotopic hydrogen (path c) rather than *via* migration of isotopic hydrogen. Experimental support for the latter statement has been obtained in the acid-catalyzed dehydration of the 3,4-dihydrodiol of 4-deuteriochlorobenzene (Jerina *et al.*, 1967a). The dehydration led to only a small migration and retention of deuterium (25%) (Scheme V), whereas oxidation of [4-^{2}H]-chlorobenzene by microsomes at pH 8 leads to a 60% retention (Daly *et al.*, 1968b). A similar examination of the deuterium retained on dehydration of 1,2-[1-^{2}H]- and 1,2-[2-^{2}H]dihydroxy-1,2-dihydronaphthalene could thus be most informative. The appropriate diols were prepared from the labeled oxides by the action of the microsomal epoxide hydrase (see Experimental Section). Unfortunately, 1-naphthol exchanges ring hydrogens with the medium rather easily, and conditions could not be found which caused dehydration but did not exchange 1-naphthol.

Migration and Retention of Deuterium During 1-Naphthol Formation from Naphthalene. [1-^{2}H]Naphthalene and [2-^{2}H]-naphthalene were prepared from the corresponding bromo compounds and converted to 1-naphthol with hepatic microsomes. With either labeled substrate, the product retained approximately 64% of the deuterium (Table II). An experi-

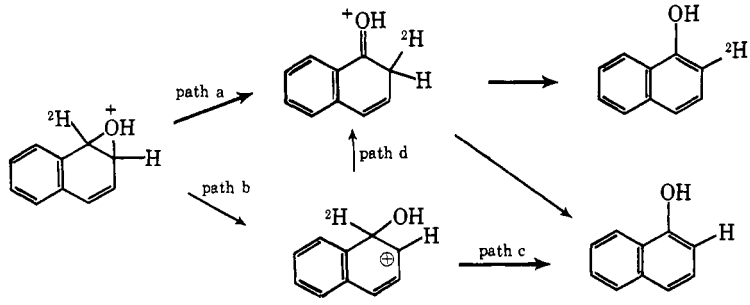
TABLE II: Hydroxylation of [1-^{2}H]- and [2-^{2}H]Naphthalene with Liver Microsomes and Two Chemical Model Systems. The Retentions Are Accurate to ±8%.[a]

Oxidant	% Retention in 1-Naphthol from [1-^{2}H]-Naphthalene	% Retention in 1-Naphthol from [2-^{2}H]-Naphthalene
Microsomes[b]	64	64
Pyridine *N*-oxide, *hν*[c]	68	64
m-Chloroperoxybenzoic acid[d]	60	68

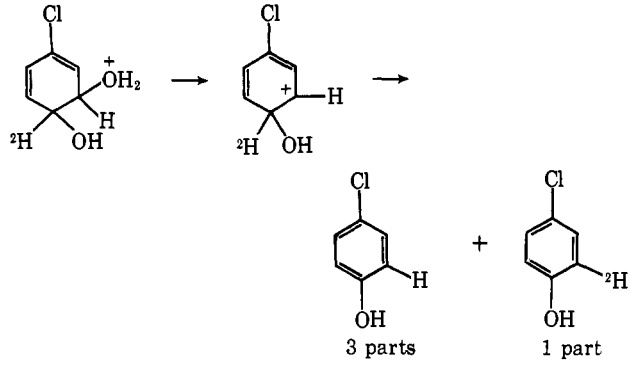
[a] Ring proton exchange of 1-naphthol with the media does not occur under these conditions. [b] See Experimental Section for conditions. [c] See Jerina *et al.* (1970b) for conditions. [d] See Jerina *et al.* (1971c) for conditions.

[3] D. M. Jerina, B. Witkop, C. McIntosh, and O. Chapman, manuscript in preparation.

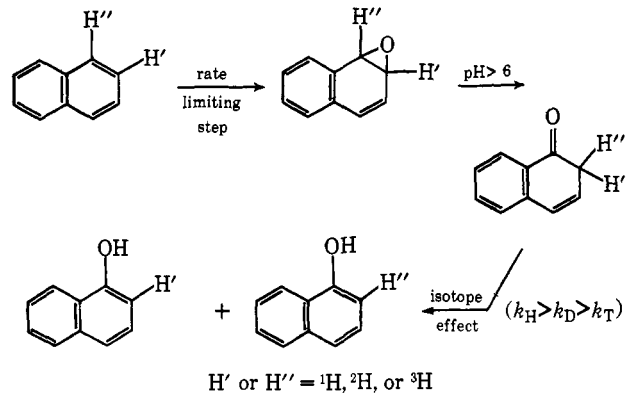
SCHEME IV



SCHEME V



SCHEME VI



H′ or H″ = ^{1}H, ^{2}H, or ^{3}H

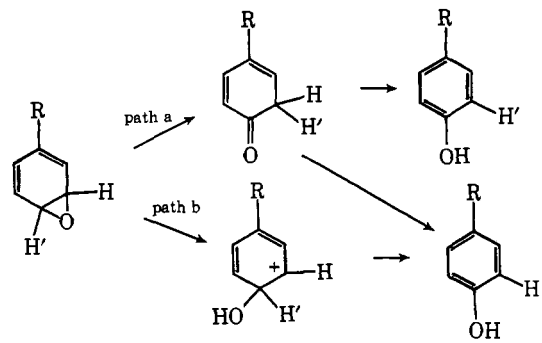
mental error of ±8% pertains with this experiment since 4 equivalent 1 or 2 positions are present in substrates and only one is occupied by deuterium (deuterium contents were measured to ±1–2%). In view of this, the results of isomerization of the deuterionaphthalene oxides under biological conditions and the results of microsomal metabolism of deuterionaphthalenes are not statistically different. However, the lower values for oxidation *vs.* isomerization of preformed oxide are suggestive that formation of 1-naphthol from naphthalene may proceed to a small extent *via* an insertion rather than an arene oxide mechanism. The biological formation of 1-naphthol from a 50:50 mixture of naphthalene and perdeuterionaphthalene did not show the isotope effect that would be expected for an insertion reaction (Daly and Jerina, 1972[4]), but such experiments might not have detected the existence of a minor pathway.

Similar retentions of deuterium were also observed during oxidation of the deuterionaphthalenes to 1-naphthol with two chemical model systems (Table II). Both of these systems (Jerina *et al.*, 1971c, 1970b), in analogy to the liver microsomal system (Jerina *et al.*, 1970a), appear to be mediated by the intermediate formation of naphthalene oxide.

Mechanism of the NIH Shift. The present results suggest that metabolism of naphthalene to 1-naphthol proceeds by the following mechanism: (i) oxidative formation of naphthalene oxide in the rate-limiting step, (ii) concerted isomerization of naphthalene oxide at physiological pH to the keto form of naphthol, (iii) enolization of the keto compound to 1-naphthol with an isotope effect (k_H/k_D) of approximately 4 (Scheme VI).

In considering a general mechanism for the NIH shift, it is, however, apparent that more complex mechanisms with alternate pathways are probably operative with other substrates. Thus, the simple mechanism of Scheme VI is valid only when a substrate, such as naphthalene, gives the same retention of isotope whether the isotopic hydrogen is present initially at the position of phenol formation or in a flanking position. This is not the case in the metabolism of certain monocyclic aromatic substrates, such as chlorobenzene (Daly and Jerina, 1969). Chlorobenzene is an excellent example, since the 3,4-oxide is strongly implicated in its metabolism to phenols and other products (Jerina *et al.*, 1967a; Brodie *et al.*, 1971). Approximately 54% of deuterium migrates from the 4 position and is retained during formation of 4-chlorophenol, while 93% of deuterium is retained when originally present in the flanking (3) position. Such results suggest that, not only a concerted mechanism (Scheme VII), but also a direct loss mechanism is relevant during isomerization of many arene oxides even under neutral or physiological conditions. If fractionation between path a and path b is not dependent on the nature of H′ (Scheme VII), then the

SCHEME VII



[4] J. W. Daly and D. M. Jerina, manuscript in preparation.

deuterium retentions observed during formation of 4-chlorophenol from [4-^{2}H]chlorobenzene and from [3-^{2}H]chlorobenzene (Daly and Jerina, 1969) require an isotope effect (k_H/k_D) for enolization of 8–9. The significance of such mechanisms to the formation of phenols with substrates other than naphthalene is under investigation. The course of rearrangements of alkyl-substituted arene oxides and their relationship to the metabolism of the parent arenes are the subject of a subsequent paper (Kaubisch *et al.*, 1972[1]).

Acknowledgment

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