structure was confirmed by elemental analysis and molecular weight (271; osmometry in $CHCl_3$).

Oxide **3** was dehydrohalogenated to 3,4-toluene-4-^{2}H oxide (**4**) with potassium *t*-butoxide in THF or ether at −20° for 15 min. Water and ether were added. The ether layer was separated and dried over $MgSO_4$. All operations were conducted at 0° in glassware which had previously been washed with 10% NaOH to prevent acid-catalyzed isomerization to cresol. In order to obtain an nmr spectrum (Figure 1), the ether solution was concentrated to a small volume *in vacuo* at 0° after addition of an equal volume of CCl_4. The uv spectrum of the bright yellow oxide–oxepin mixture [$\lambda_{max}^{85\%\ methanol}$ 267 mμ (ε 2200); $\lambda_{max}^{cyclohexane}$ 273 mμ with tailing to longer λ (ε 1910)][7] resembles benzene oxide, suggestive of the presence of both valence isomers in solution. 3,4-Toluene oxide is much more unstable than benzene oxide. It could not be distilled and had a half-life (CCl_4 solution) of ~24 hr at room temperature in *base-treated* glassware. The structure of oxide **4** was further confirmed by formation of the Diels–Alder adduct with maleic anhydride in ether at 0° (mp 141–142°; 50% yield based on **3**; *m/e* 207). By comparison of the mass spectra of the Diels–Alder adducts of **4** and **5** (calcd *m/e* 206.0579; found 206.0577), the isotopically labeled oxide had neither lost nor randomized the deuterium initally present.

The isomerization of oxide **4** was studied under a variety of conditions. According to tlc, glpc, and the nmr spectra (Figure 1), **4** isomerizes almost exclusively to *p*-cresol. Such a strong orienting influence of a methyl substituent is noteworthy. During isomerization deuterium migrates from the 4 position of oxide **4** to the 3 position of the product (4-hydroxytoluene), as is clearly shown by the nmr of this material (Figure 1). The deuterium retention values in Table I can be compared with values of 56 or 68%

Table I[a]

Isomerization method	% deuterium[b] retention
0.1 *N* HCl	37
Room temperature in CCl_4	58
Liver microsomes[c] at pH 8.0	70
10% aqueous acetamide	75[d]

[a] The isomerization products obtained in the three aqueous systems were isolated by extraction into ether and concentration to a small volume for glpc. Deuterium retentions were measured on an LKB 9000 mass spectrometer–gas chromatograph at 70 eV using a column (6 ft × 1/8 in. o.d.) of Bentone 34–tricresyl phosphate–80–100 mesh Gas Chrom CL (1:1:1) operated at 125°. [b] Values are corrected to represent amount of initial 4-^{2}H retained in the product *p*-cresol. [c] This is a protein-catalyzed nonenzymatic rearrangement which occurs both with active and boiled microsomes. [d] Mass spectrometric deuterium analysis for hydroxytoluene and toluene is difficult because of the large M − 1 peaks which in the 3,5-dinitrobenzoates of the three *p*-cresols become negligible. In this way it was determined that 4-hydroxytoluene 3,5-dinitrobenzoate obtained by rearrangement of the oxide in acetamide solution retained 85% deuterium.

when toluene-4-^{2}H is hydroxylated either with rabbit liver microsomes or with peroxytrifluoroacetic acid, respectively.[8] Migration and retention of deuterium during rearrangement of the oxide **4** is similar to that obtained during enzymatic hydroxylation of toluene-4-^{2}H. *Arene oxides are thus possible metabolic intermediates in the formation of phenols since they undergo spontaneous or catalyzed "NIH shifts" to an extent comparable to enzymatic hydroxylation.*

Due to the great instability of 3,4-toluene oxide, it has not been possible to demonstrate its role as a substrate for the enzymes which add water or glutathione to benzene oxide.[1] Although at present no direct evidence is available for the existence of a monocyclic arene oxide in a biological system, our preliminary observations suggest that 1,2-naphthalene oxide is an intermediate in the metabolism of naphthalene.[9]

(7) The oxide stock solution (CCl_4) was washed with base to remove cresol immediately prior to running the spectra. Extinction coefficients were estimated from the *p*-cresol which spontaneously formed in the cell after 24 hr.

(8) D. M. Jerina, J. W. Daly, and B. Witkop, *Arch. Biochem. Biophys.*, in press.

(9) D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend, *J. Am. Chem. Soc.*, **90**, 6525 (1968).

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The Role of Arene Oxide–Oxepin Systems in the Metabolism of Aromatic Substrates. III. Formation of 1,2-Naphthalene Oxide from Naphthalene by Liver Microsomes[1]

Sir:

The possibility that arene oxides function as intermediates during the oxidative metabolism of aromatic substrates to phenols, catechols, dihydrodiols, and premercapturic acids has been investigated in three stages. First, benzene oxide was shown to serve as an *in vitro* precursor for phenol, catechol, (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene, and S-(1,2-dihydro-2-hydroxyphenyl)glutathione.[2] Then the nonenzymatic rearrangement of 3,4-toluene-4-^{2}H oxide to 4-hydroxytoluene was demonstrated to occur with up to 85% deuterium retention,[1] making the isomerization of arene oxides to phenols compatible with the "NIH shift."[3,4] In this communication *we describe the first isolation and identification of an arene oxide from a biological system.*

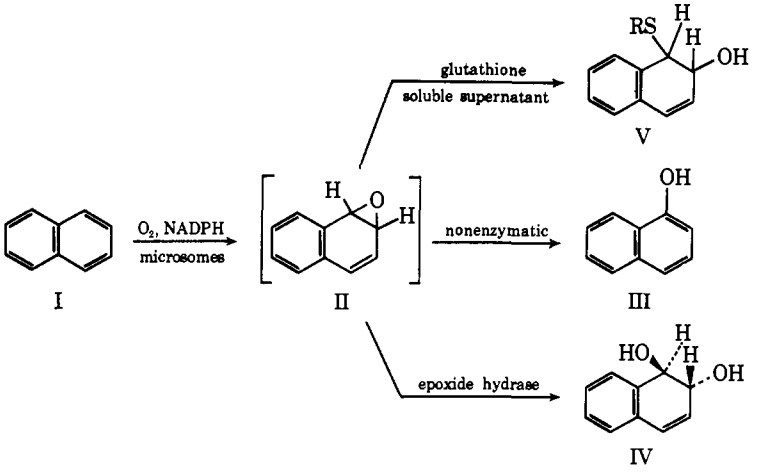
Attempts were made to demonstrate the *in vitro* formation of benzene oxide in an earlier study,[2] but the results were inconclusive, since benzene is a poor substrate for the aromatic hydroxylating system in liver microsomes. Naphthalene (I), on the other hand, is readily metabolized by rat liver microsomes to 1-naphthol (III) and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene (IV). If the liver supernatant fraction and glutathione are included in the incubation, the principal product becomes a glutathione conjugate which has been assigned the structure S-(1,2-dihydro-

(1) Paper II: D. M. Jerina, J. W. Daly, and B. Witkop, *J. Am. Chem. Soc.*, **90**, 6523 (1968).

(2) D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend, *Arch. Biochem. Biophys.*, **128**, 176 (1968).

(3) G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, S. Udenfriend, and B. Witkop, *Science*, **157**, 1524 (1967).

(4) J. W. Daly, G. Guroff, D. M. Jerina, S. Udenfriend, and B. Witkop, Advances in Chemistry Series, American Chemical Society, Washington, D. C., in press.



2-hydroxynaphthyl)glutathione (V).[5] In the absence of microsomes, however, the glutathione-conjugating enzyme in the supernatant is incapable of forming V from naphthalene. Furthermore, the metabolic products, III, IV, or V, are not interconvertible. These results suggest an oxidized, reactive naphthalene intermediate which requires for its formation intact microsomes. The following describes the isolation of such an oxidized naphthalene species and its identification as 1,2-naphthalene oxide (II).[6]

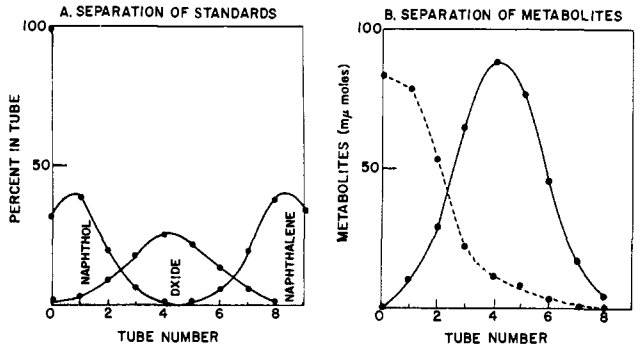


Figure 1. (A) Countercurrent distribution of 1-naphthol, 1,2-naphthalene oxide (oxide), and naphthalene; ten tubes, lower phase 2.5 ml of methanol–water–ethyl acetate (20:4:1) and upper phase 5 ml of *n*-heptane. Diol IV with $K = 0.02$ in this system is found (>97%) in tubes 0 and 1. (B) Countercurrent distribution of naphthalene metabolites. The ethyl acetate extract from the incubation of naphthalene with liver microsomes at 30° for 3 min was dried over Na_2SO_4, concentrated *in vacuo*, and partitioned as above. Aliquots from each tube were assayed colorimetrically with Gibbs reagent before and after treatment with dilute hydrochloric acid. The broken line represents preformed naphthol, while the solid line indicates the increments in naphthol appearing in each tube as a result of the acid treatment which rearranges naphthalene oxide. Naphthalene and diol IV were not estimated and do not interfere with the Gibbs assay under these conditions.

In Vitro **Formation of 1,2-Naphthalene Oxide.** Naphthalene-^{14}C and cold racemic 1,2-naphthalene oxide as carrier were incubated (pH 8.0 pyrophosphate, 30°, 3 min) with rat liver microsomes obtained from animals pretreated with phenobarbital. The reaction mixture was extracted with ethyl acetate. The extract was concentrated and purified by tlc on silica gel GF with benzene–ethyl acetate–chloroform (1:1:1) containing 5% triethylamine. This solvent system completely separates diol (R_f 0.10) from 1-naphthol (R_f 0.26) but only partially separates 1,2-naphthalene oxide (R_f 0.51) from naphthalene (R_f 0.57). The naphthalene oxide band (free of all naphthol) was eluted from the plate and rearranged with dilute acid to 1-naphthol. Rechromatography in the same system cleanly separates the newly formed naphthol. In this way 0.1–0.2% of the oxidized products from naphthalene-^{14}C were trapped and identified as radioactive 1,2-naphthalene oxide.

By running a large incubation in the absence of carrier it was possible to demonstrate directly the formation of 1,2-naphthalene oxide. The products from the incubation were partially separated by countercurrent distribution. The naphthalene oxide peak (Figure 1) was located by differential colorimetric assay before and after acid treatment of the fractions. The product was further identified by tlc before and after acid treatment. Approximately 5% of the oxidized metabolites could be accounted for as 1,2-naphthalene oxide. This is, however, a minimal value since there are large losses when the oxide is carried through the incubation and extraction procedure. The possibility that this oxide is optically active is under study.

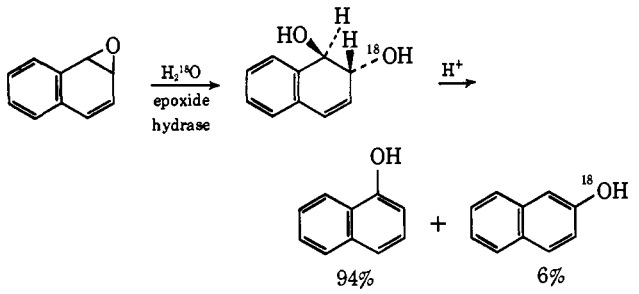
In Vitro **Metabolism of Racemic 1,2-Naphthalene Oxide.** Rabbit liver microsomes (pH 8.0 Tris, 30 min at 37°, oxidative cofactors absent) contain an enzyme which hydrates 1,2-naphthalene oxide to (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene (IV, 35–40% yield) which has the same absolute stereochemistry as the diol obtained directly on incubation of naphthalene with the microsomal hydroxylating system. The diol formed directly from naphthalene has oxygen from air only in the 1 position.[7] Incubation of 1,2-naphthalene oxide in $H_2{}^{18}O$ leads to diol which contains ^{18}O only in the 2 position. Thus, diol formation from the (racemic) oxide is both stereochemically and topologically identical with diol formation from naphthalene. The absolute stereochemistry of this diol is under study.

Rat liver supernatant (pH 8.0 pyrophosphate, 30 min at 37°) contains an enzyme which catalyzes the opening of 1,2-naphthalene oxide by glutathione to a product which on paper chromatography is indis-

(5) J. Booth, E. Boyland, and P. Sims, *Biochem. J.*, **74**, 117 (1960).

(6) For synthesis of 1,2-naphthalene oxide, see E. Vogel and F. G. Klärner, *Angew. Chem.*, **80**, 402 (1968). We are greatly obligated to Professor E. Vogel for an advance copy of this manuscript.

(7) J. Holtzman, J. R. Gillette, and G. W. Milne, *J. Am. Chem. Soc.*, **89**, 6341 (1967). Oxygen-18 in the diol IV was analyzed by this procedure.



tinguishable from the conjugate obtained directly from naphthalene with microsomes, supernatant, and glutathione. The opening of the oxide II by glutathione also occurs nonenzymatically, but at a much slower rate. During these incubations the oxide also undergoes spontaneous isomerization to 1-naphthol. Likewise, the phenol formed from naphthalene by microsomes is also 1-naphthol.

When the glutathione-conjugating system and increasing amounts of glutathione are added to liver microsomal perparations, III and IV decrease as more and more of the oxide is trapped as V. This experiment suggests that 1,2-naphthalene oxide is the *obligatory intermediate* in the enzymatic hydroxylation of naphthalene. That arene oxides are in general intermediates on the pathway of the hydroxylation of aromatic substrates becomes now an attractive assumption.

The current view on microsomal mixed-function oxygenation favors an oxygen atom transfer reaction[8] rather than endoperoxide intermediates.[9,10] Complete biochemical details will be published elsewhere.

(8) *Cf.* V. Ullrich and H. J. Staudinger in "Biological and Chemical Aspects of Oxygenases," K. Bloch and O. Hayaishi, Ed., Maruzen Co., Ltd., Tokyo, 1966, p 235; *Physiol. Chem.*, **349**, 85 (1968).
(9) A. H. Soloway, *J. Theoret. Biol.*, **13**, 100 (1966).
(10) J. E. Baldwin, H. H. Basson, and H. Krauss, *Chem. Commun.*, 984 (1968).

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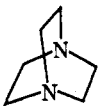
Quenching of Singlet Oxygen by Tertiary Aliphatic Amines. Effect of DABCO

Sir:

While studying the reactions of amines with singlet oxygen ($^1O_2$) generated by an electrodeless radiofrequency discharge,[1] we observed that tertiary aliphatic amines not only yield no reaction products, but act as inhibitors of the oxidation of known reactive acceptors of singlet oxygen. Thus, no reaction product could be detected[2] after treating 2 mmol of 1,4-diazabicyclo[2.2.2]octane (DABCO, **1**) in 25 ml of bromobenzene with $^1O_2$ during 2 hr at 0°. Yet when a small amount of DABCO was added to a solution of 1,3-diphenylisobenzofuran (**2**), the complete oxidation[3] of **2** took much longer than in the absence of DABCO. The oxidation of dibenzyl sulfide and rubrene was also inhibited (see Table Ia). This inhibitory property is not limited to that tertiary aliphatic amine. All those tried, ethyldiisopropylamine, N,N′-tetramethylethylenediamine, and N-allylpiperidine, were also found to be apparently unreactive toward $^1O_2$, yet to retard the oxidation of **2**.



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It therefore seemed interesting to find out if an actual quenching of singlet oxygen took place. Indeed, reactions involving $^1O_2$ generated by entirely different techniques were found to be retarded or suppressed by DABCO. In its presence, not only was the rate of rubrene photooxidation[4] reduced (Table Ib), but a correspondingly smaller volume of oxygen was absorbed by the solution. If the quenching of the rubrene photooxidation had been due to the competitive faster oxidation of the quencher, the rate of oxygen intake would have been unchanged or increased by DABCO. Thus the DABCO is not oxidized. Chemically generated singlet oxygen in aqueous solution is also quenched by DABCO. 2,5-Dimethylfuran, one of Foote's most efficient acceptors,[5] was chosen for this test (Table Ic).

Quenching of $^1O_2$ by DABCO must be very efficient since it affects the rate of oxidation of **2**, the most reactive substrate known,[6] when **2** and DABCO are present at concentrations of the same order. A gas-phase experiment supports this conclusion. A small amount of DABCO was sublimed into a stream of oxygen rich in $^1O_2$ (at a total pressure of 6 torr), while the intensity of the emission band at 635 mμ[7] which is proportional to the square of the concentration of $O_2(^1\Delta_g)$[8] was being monitored downstream. At an estimated flow rate of $\sim 4.5 \times 10^{-3}$ mol/min, the gradual introduction of $< 3 \times 10^{-4}$ mol of DABCO into the gas stream resulted in >100-fold decrease in the intensity of the 635-mμ band, lasting 15 min, suggesting that each molecule of DABCO is able to deactivate several molecules of $O_2(^1\Delta_g)$.[9] This nonreactive quenching of $O_2(^1\Delta_g)$ is remarkable in view of its known stability;[10a] for comparison, naphthalene, water, pyridine, and bromobenzene, introduced in the oxygen stream in the same manner as DABCO, caused no or negligible quenching of $O_2(^1\Delta_g)$ (the 635-mμ band).

(1) E. J. Corey and W. C. Taylor, *J. Am. Chem. Soc.*, **86**, 3881 (1964). In the present experiments, a mixture of 10% oxygen in helium, at a total pressure of 50 torr, passed through the discharge tube, then bubbled through the reacting solution in a vessel with a reflux condenser at Dry Ice temperature.
(2) By nmr, ir, and tlc.
(3) Indicated by the discoloration of 2.
(4) At 540 mμ DABCO does not interfere with light absorption by the rubrene. Besides, DABCO had no measurable quenching effect on the fluorescence of rubrene, even at a concentration of 4×10^{-2} *M*, greater than in the oxidation experiments. (No effort was made to remove oxygen in the fluorescence measurements, because of the obvious presence of oxygen in the oxidation experiments.)
(5) C. S. Foote, M. T. Wuesthoff, S. Wexler, I. G. Burstain, R. Denny, G. O. Schenck, and K. H. Schulte-Elte, *Tetrahedron*, **23**, 2583 (1967).
(6) T. Wilson, *J. Am. Chem. Soc.*, **88**, 2898 (1966).
(7) Through a Baird-Atomic interference filter, Standard Visible Type B-3, with peak wavelength at 6350 Å, by means of an EMI 9558B photomultiplier tube.
(8) L. W. Bader and E. A. Ogryzlo, *Discussions Faraday Soc.*, **37**, 46 (1964); S. H. Whitlow and F. D. Findlay, *Can. J. Chem.*, **45**, 2087 (1967).
(9) Assuming that about 10% of the oxygen is in the $^1\Delta_g$ stage. See L. Elias, E. A. Ogryzlo, and H. I. Schiff, *ibid.*, **37**, 1680 (1959).