1,2-Naphthalene Oxide as an Intermediate in the Microsomal Hydroxylation of Naphthalene*

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ABSTRACT: The microsomal metabolism of naphthalene has been reinvestigated and the pathways leading to naphthol, *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, and the conjugate, *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione, have been clarified. In the microsomal system synthetic 1,2-naphthalene oxide was found to be converted into all major naphthalene metabolites. In the presence of microsomes 1,2-naphthalene oxide rearranges nonenzymatically almost exclusively to 1-naphthol, the preponderant phenolic oxidation product from naphthalene. Racemic 1,2-naphthalene oxide is hydrated enzymatically to an optically active diol identical with the diol from naphthalene with respect to absolute stereochemistry and the source of the oxygen atom at the 2 position. Soluble liver enzymes catalyze the addition of glutathione to 1,2-naphthalene oxide to form a conjugate identical with that obtained on oxidation of naphthalene. The formation of 1,2-naphthalene oxide from naphthalene in the microsomal system was demonstrated both by radiotracer trapping technique and by isolation.

When increasing amounts of glutathione were added to the microsomal system, the conjugate increased commensurate with a decrease in naphthol and dihydrodiol. When a competitive substrate for the epoxide hydrase is added, the yield of naphthol goes up at the expense of the diol. These two observations strongly suggest that the oxide is an obligatory intermediate for all naphthalene metabolites. The relevance of these findings to naphthalene metabolism in particular and phenol formation in general is discussed.

One of the most important biological reactions of aromatic substrates is oxidation to phenolic compounds. This reaction can proceed with the incorporation of half of the obligatory oxygen molecule into water and the other half into the phenolic product. Because of this, the enzymes have been termed "mixed-function oxidases" (Mason, 1957) or "monooxygenases" (Hayaishi, 1964), and include such specific enzymes as phenylalanine hydroxylase, tryrosine hydroxylase, and tryptophan hydroxylase, as well as the nonspecific "drug-metabolizing" oxygenases of liver microsomes. The microsomal enzymes are ideally suited for investigations on the mechanism of aromatic hydroxylation because of the broad range of possible substrates. Although these enzymes appear to catalyze a direct hydroxylation of the aromatic ring, our investigations on the intramolecular migration of ring substituents during hydroxylation of aromatic substrates (the "NIH shift") (Guroff *et al.*, 1967; Daly *et al.*, 1968) led us to believe that arene oxides[1] might be involved as intermediates in the enzymatic formation of phenols. The arene oxides, a class of compounds which have only recently become synthetically available, readily isomerize to phenols (Vogel and Günther, 1967); all of the migrations which have been shown to occur during the NIH shift are mechanistically compatible with observed rearrangements of arene oxides to phenols (Jerina *et al.*, 1968a).

Investigations on the possible role of arene oxides as intermediates in biological oxidation of aromatic compounds have been conducted in three stages: (i) In order to qualify as intermediates, arene oxides should be converted enzymatically or nonenzymatically into all the products formed by biological systems from the corresponding aromatic substrate. Indeed, benzene oxide is converted *in vitro* into phenol, (−)-*trans*-1,2-dihydro-1,2-dihydroxybenzene, catechol, and the conjugate *S*-(1,2-dihydro-2-hydroxyphenyl)glutathione (Jerina *et al.*, 1968b) all of which are known metabolites of benzene *in vivo*. (ii) On rearrangement to a phenol, arene oxides must display the special feature of enzymatic phenol formation, *i.e.*, the NIH shift. Rearrangement of 3,4-toluene-4-^{2}H oxide to 4-hydroxytoluene-3-^{2}H was found to occur with deuterium retentions as high as 85% (Jerina *et al.*, 1968a). (iii) Finally, the actual formation of an arene oxide in a biological system had to be established. This paper demonstrates that 1,2-naphthalene oxide is formed from naphthalene in the presence of the liver microsomal hydroxylating system, and presents evidence for the oxide being the initial product of oxygenation and the intermediate leading to other oxidative metabolites.

Material and Methods

Materials. GSH,[2] NADP, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, Gibbs reagent, and other reagents employed are commercially available. Naphthalene-1-

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[1] The term arene oxide refers to the compounds resulting from the epoxidation of one of the double bonds of an aromatic nucleus. Through valence bond isomerization, such compounds can exist either as the oxide with the three-membered oxirane ring fused to the aromatic skeleton, or can undergo ring expansion to the seven-membered oxygen heterocycle, oxepin.

[2] The following standard abbreviations are used throughout: *N*-2,6-trichlorobenzoquinonimine (Gibbs reagent), and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene (dihydrodiol).

TABLE I: Effect of a Competitive Substrate for Epoxide Hydrase on Naphthalene Metabolism.[a]

Substrate (50 μmoles)	μmoles of Product	
	α-Naphthol	*trans*-1,2-Dihydro-1,2-dihydroxynaphthalene
Naphthalene	0.04	0.76
Naphthalene + styrene oxide	0.54	0.07

[a] Incubations with 10 ml of rabbit liver microsomal preparation and 2 ml of 0.5 M Tris buffer (pH 9) for 15 min at 37° as previously described (Jerina *et al.*, 1970). Products isolated by thin-layer chromatography and assayed with Gibbs reagent (Booth and Boyland, 1958).

^{14}C (2 mCi/mmole) was obtained from Nuclear-Chicago and diluted to 0.05 mCi/mmole before use, oxygen-18-enriched water (60% ^{18}O) from Isotopes, Inc., and fluorescent silica gel thin-layer chromatography plates from Brinkmann and from Analtech. The levels of ^{18}O in isotopic incorporation experiments were measured directly on water and the dihydrodiol with an LKB 9000 mass spectrometer was operated at 70 eV.

The synthesis of racemic 1,2-naphthalene oxide has been described (Vogel and Klärner, 1968).

Racemic dihydrodiol was prepared according to Booth *et al.* (1950).

S-(1-Naphthyl)cysteine, *S*-(2-naphthyl)cysteine, and the *N*-acetyl derivative of each compound were prepared according to described procedures and were found to have melting points comparable with those reported in the literature (West and Mathura, 1954). *N*-Acetyl-*S*-(1- and 2-naphthyl)cysteine were individually converted into their methyl esters by treatment with excess ethereal diazomethane. The esters were homogeneous after preparative thin-layer chromatography (R_F 0.35 each) in benzene–chloroform–ethyl acetate (1:1:1).

Preparation of Subcellular Fraction. Rat liver microsomes were obtained from animals which had been given intraperitoneal injections of phenobarbital (40 mg/kg) for 4 days in order to induce high levels of drug-metabolizing enzymes. Microsomes were prepared according to an earlier procedure (Mitoma *et al.*, 1956) with the following modifications: the first centrifugation was performed at 20,000*g*, and the pellets from a second centrifugation at 1000,000*g* were stored at −18° after washing once with 1.15% KCl. Before use, the pellets were suspended in phosphate buffer (pH 7.4, 0.05 M).

Rat liver soluble fraction containing glutathione-conjugating enzymes (Booth *et al.*, 1960a) was prepared according to the method of Booth *et al.* (1961).

Rabbit liver microsomes were prepared as previously described by (Jerina *et al.*, 1970).

Incubation Procedures and Assays. A. NAPHTHOL AND DIHYDRODIOL FROM NAPHTHALENE. The standard naphthalene incubation contained 2.0 ml of pyrophosphate buffer (0.1 M, pH 8.0), 1.2 μmoles of NADPH, 25 μmoles of glucose 6-phosphate, 2.4 units of glucose 6-phosphate dehydrogenase, 50 μmoles of nicotinamide, 25 μmoles of magnesium choride, 0.3 ml of microsomal suspension (from 250 mg of rat liver), and 4 μmoles of naphthalene (in 0.1 ml of ethylene glycol monoethyl ether); final volume 3.5 ml. The suspension was agitated under air for 15 min at 37°. Production of naphthol and dihydrodiol was estimated colorimetrically with Gibbs reagent (Booth and Boyland, 1958). Under these conditions, 0.08–0.16 μmole of naphthol and 0.2–0.3 μmole of dihydrodiol were formed per flask, dependent upon the microsomal preparation.

B. CONJUGATION OF GLUTATHIONE WITH NAPHTHALENE-^{14}C. The standard naphthalene incubation mixture (A) was used with naphthalene-^{14}C as substrate and with the additions of 0.5–1.0 ml of soluble rat liver fraction and 0.5 ml of 0.06 M GSH solution. Reactions were run at 30 or 37° for 30 min and terminated by addition of acetic acid. The products from the incubation were adsorbed on charcoal and eluted with methanol–benzene–aqueous ammonia as previously described (Booth *et al.*, 1961). Concentrated elutions were applied to Whatman No. 3MM paper and chromatographed in 1-butanol–acetic acid–water (12:3:5). The conjugate, which was made visible on the paper (R_F 0.39) by ultraviolet light or ninhydrin, was eluted with 50% ethanol for further study. Recoveries were low and variable. The isolated GSH conjugate was assayed radiometrically.

C. CONJUGATION OF GLUTATHIONE WITH 1,2-NAPHTHALENE OXIDE. Reaction mixtures containing 0.5 ml of soluble rat liver fraction, 0.5 ml of 0.06 M GSH solution (30 μmoles), 3.5 ml of pyrophosphate buffer (0.1 M, pH 8.0), and 2 μmoles of 1,-2-naphthalene oxide (in 20 μl of ethanol) were incubated at 30 and 37° for 30 min and the conjugate was isolated as in part B above. Under these conditions 54% of the reaction was enzymatic. Nonenzymatic formation of conjugate was shown in the same way except that the soluble liver fraction was omitted. When the concentration of GSH was lowered to 10 μmoles/reaction mixture and the amount of soluble liver fraction doubled, nonenzymatic reaction was reduced to about 23% of the enzyme-catalyzed rate. In this second experiment, the total yield of conjugate was decreased about 30%. The conjugate was assayed as described (Booth *et al.*, 1961).

D. DIHYDRODIOL-^{18}O FROM 1,2-NAPHTHALENE OXIDE. The microsomal pellet obtained from 10 ml of rabbit liver homogenate was resuspended in isotonic KCl (2.0 ml, 60% ^{18}O) and 0.1 ml of Tris buffer (pH 9.0, 1 M) together with 3 mg of racemic 1,2-naphthalene oxide (in 50 μl of ethanol) were added. The mixture was incubated under nitrogen at 37° for 30 min and extracted with 5 ml of ethyl acetate. The amount of ^{18}O in the water of the final incubation mixture was measured on an aliquot of the aqueous phase. The ethyl acetate was dried (Na_2SO_4), and concentrated, and the dihydrodiol was isolated by thin-layer chromatography. The yield was estimated at 0.9 mg by ultraviolet spectroscopy.

E. EFFECT OF INHIBITION OF EPOXIDE HYDRASE. Naphthalene was converted into dihydrodiol as described previously (Jerina *et al.*, 1970) with or without styrene oxide as a cosubstrate for epoxide hydrase (details shown in Table I).

F. 1,2-NAPHTHALENE-^{14}C OXIDE FROM NAPHTHALENE-^{14}C. The standard naphthalene incubation mixture (A) but with naphthalene-^{14}C was incubated at 30° for 3 min and the reaction was stopped by extraction with 1 volume of chilled ethyl acetate containing 0.5% triethylamine. These extracts were stored at −18°. The combined extracts from 75 incubations

were dried (Na_2SO_4), and concentrated, first *in vacuo* (<30°) and then under N_2, to 0.1 ml for countercurrent distribution.

Countercurrent Distribution. The distribution coefficient (K) and distribution curve for naphthalene and its products were determined by the use of 2.5 ml of methanol–water–ethyl acetate (20:4:1) as the lower phase and 5 ml of heptane as the upper phase. The distribution of these compounds, with the exception of the dihydrodiol which remains almost entirely in tubes 0 and 1, is shown in Figure 1.

Naphthalene. K = 9.8, determined by running a countercurrent distribution on naphthalene-^{14}C and counting 0.5-ml aliquots of each phase in 10 ml of Bray's solution.

Dihydrodiol. K = 0.02, determined by ultraviolet spectroscopy ($OD_{270\ m\mu}$).

1-Naphthol. K = 0.12, determined by colorimetric assay. For the lower phase, an aliquot was made up to 1.5 ml in methanol, and 0.1 ml each of fresh Gibbs reagent (0.1% in EtOH) and borate buffer (0.5 M, pH 9.2) was added. After 3 min the $OD_{605\ m\mu}$ was measured and compared with a standard curve. For the upper phase, 4 ml of heptane along with 0.2 ml of Gibbs reagent and 1.5 ml of 10% Na_2CO_3 were agitated and the $OD_{605\ m\mu}$ was measured for the aqueous phase.

1,2-Naphthalene Oxide. (a) Synthetic: K = 0.9–1.0, determined spectroscopically by $OD_{270\ m\mu}$ on each phase; (b) enzymatically formed: aliquots from the lower phase were assayed with Gibbs reagent before and after acid treatment to convert the oxide into naphthol. In the assay 1 ml of methanol phase was mixed with 0.2 ml of methanol or 0.1 N HCl in methanol, 0.1 ml of Gibbs reagent, and 0.1 ml of borate buffer (pH 9.2), followed by reading the $OD_{605\ m\mu}$ after 3 min. Since naphthalene oxide does not give a color with Gibbs reagent, the oxide concentration was calculated from the difference between the two readings. The tubes containing the oxide from upper phase were combined and saved for thin-layer chromatography.

Gas Chromatography. Mixtures of 1- and 2-naphthol were easily separated as their trimethylsilyl ethers by the use of 3% SE-30 on Gas Chrom Z (100–120 mesh) columns (6 ft × 0.25 in.) operated at 135°. For preparation of the derivatives, anhydrous extracts containing the phenols were concentrated to dryness in small tubes and 0.1–0.2 ml of silylating reagent (5 ml of pyridine, 1 ml of trimethylchlorosilane, and 1 ml of hexamethyldisilazane) was added. The resulting solution was chromatographed directly. Ratios of isomers were estimated from peak areas.

Thin-Layer Chromatography. It was possible to separate naphthalene (R_F 0.57), dihydrodiol (R_F 0.09), 1-naphthol (R_F 0.32), and 1,2-naphthalene oxide (R_F 0.53) by thin-layer chromatography on Brinkmann analytical silica gel GF plates. In order to prevent extensive rearrangement of naphthalene oxide before and during chromatography, the areas of the plate, which were to be spotted, were pretreated with triethylamine. The solvent (benzene–chloroform–ethyl acetate, 1:1:1) contained 5% triethylamine. In this system, 2-naphthol moved somewhat more slowly than 1-naphthol, but did not separate completely.

Results

Enzymatic Formation of 1,2-Naphthalene Oxide. 1,2-Naphthalene oxide readily isomerizes to naphthol. The rate of rearrangement is enhanced in polar solvents, such as water.

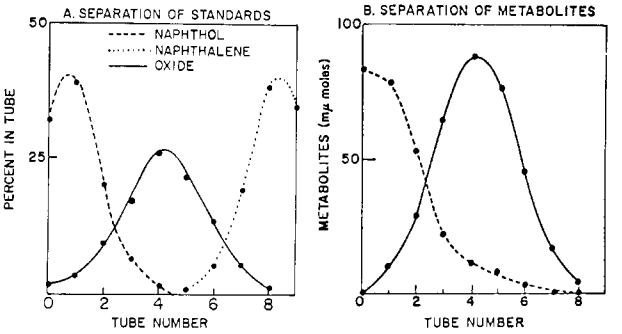


FIGURE 1: Identification of 1,2-naphthalene oxide as an *in vitro* metabolite of naphthalene. (A) Countercurrent distribution of 1-naphthol, 1,2-naphthalene oxide (oxide), and naphthalene; ten tubes, lower phase 2.5 ml of methanol–water–ethyl acetate (20:4:1) and upper phase of 5 ml of *n*-heptane. Diol with K = 0.02 in this system is found (>97%) in tubes 0 and 1, and thus is not indicated in the figure. (B) Countercurrent distribution of naphthalene metabolites. Aliquots from each tube were assayed colorimetrically with Gibbs reagent before and after treatment with dilute hydrochloric acid. The broken line represents naphthol present in the lower phase before acid treatment, while the solid line indicates the increments in naphthol appearing in the lower phase in each tube as a result of the acid treatment which rearranges naphthalene oxide. Naphthalene and diol were not estimated and do not interfere with the Gibbs assay under these conditions. Since the lower phase contains 88% of the naphthol and 50% of the naphthalene oxide, the total naphthol assayed was 0.27 μmole and total naphthalene oxide was 0.66 μmole. Extraction of α-naphthol from microsomes under the basic conditions employed in this experiment is, however, very inefficient.

Heat or acid lead to rapid or explosive isomerization. Stock solutions of the oxide in anhydrous ethanol were kept at −20° for several months without appreciable decomposition. For storage of the crystalline solid, temperatures below −20° are recommended. Because of its instability, the first experiments were directed toward determining the lifetime of naphthalene oxide in the standard incubation medium. The stability of the oxide did not appear to vary in the pH range 7–9. When the temperature was lowered and the incubation time shortened, the recovery of oxide increased, but the extent of naphthalene metabolism decreased. In an attempt to reach the best possible compromise, the standard naphthalene incubation was run for 3 min at 30°. The half-life of 1,2-naphthalene oxide at this temperature was less than 3 min.

Naphthalene-^{14}C was incubated with microsomal preparations under the above conditions along with nonradioactive carrier naphthalene oxide with the aim of trapping a radioactive pool of naphthalene oxide. Experiments were run with carrier added either at the beginning or at the end of the reaction. Incubations with heat-denatured microsomes served as controls. The ethyl acetate extracts were subjected to thin-layer chromatography, and the band corresponding to naphthalene oxide, now completely free of all naphthol as judged by rechromatography, was eluted with methanol. Due to the poor separation of naphthalene and naphthalene oxide, it was not possible to count the eluted fraction directly. The eluted carrier oxide was then rearranged to napthol by adding a few drops of dilute HCl. Rechromatography after neutralization with triethylamine gave a newly formed naphthol band which was removed and found to be radioactive by liquid scintillation spectrometry. No radioactivity was found in the newly formed naphthol fractions from the heat-denatured control

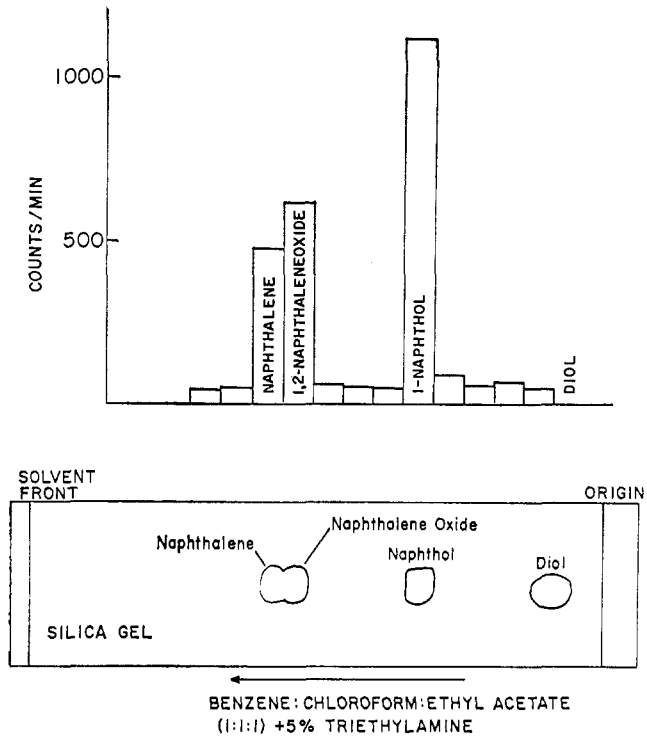


FIGURE 2: Identification of 1,2-naphthalene-^{14}C oxide as an *in vitro* metabolite of naphthalene-^{14}C. The lower portion of the diagram presents a typical separation of naphthalene, 1,2-naphthalene oxide, 1-naphthol, and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene. The upper portion presents the thin-layer chromatography of naphthalene-^{14}C metabolites obtained after the following treatment: (1) purification of the materials from the upper phases of tubes 3–5 of countercurrent distribution tubes shown in Figure 1 by thin-layer chromatography to obtain a fraction containing only naphthalene and naphthalene oxide, (2) partial acid-catalyzed rearrangement of the naphthalene oxide in this fraction to naphthol, and (3) rechromatography on thin-layer chromatography to show the presence of newly formed naphthol. The last step is necessary since bands due to naphthalene and naphthalene oxide are so close together in this system. Residual triethylamine from the thin-layer chromatography scrapings would often be responsible for incomplete acidification. In some experiments, this would lead to incomplete isomerization to naphthol and recoverable naphthalene oxide as shown here.

incubations. Only slightly more radioactivity was observed when the carrier oxide was added at the beginning rather than at the end of the incubation because of the small amounts of carrier which were recoverable. Based on the amounts of 1-naphthol and dihydrodiol formed from naphthalene under these incubation conditions, only a small fraction of the oxidized products from naphthalene-^{14}C was trapped and identified as radioactive 1,2-naphthalene oxide.

Following the above studies, attempts were made to isolate the oxide without carrier by carrying out large-scale incubations with naphthalene-^{14}C. The combined extracts (see incubation procedure F) of 75 incubation flasks were concentrated and subjected to countercurrent distribution (Figure 1).

Only naphthol and naphthalene oxide were estimated by the methods described in the legend. In this experiment, approximately 0.66 μmole of 1,2-naphthalene oxide was isolated. This is, however, a minimal value, since there were large losses when the oxide was carried through the incubation and extraction procedure. For comparison, the total naphthol and naphthalene oxide which would be formed under these conditions is 2.3 μmoles. The upper phases of tubes 3–6 from the countercurrent distribution were pooled, concentrated to a small volume, and resolved by thin-layer chromatography. The 1,2-naphthalene oxide and naphthalene bands together with a small amount of naphthol were visible on the plate under ultraviolet light. When a narrow strip of the plate was sprayed with Gibbs reagent, the oxide and naphthol bands gave positive reactions.[3] The oxide band was eluted with methanol containing a trace of triethylamine. An aliquot of this eluate was rechromatographed, and scrapings from every area along the length of the second plate were counted showing the sample to be essentially free of radioactive naphthol (<75 cpm in the naphthol region and >1000 cpm in the naphthalene–naphthalene oxide region). Another aliquot of eluate from the first plate was partially acidified to rearrange most of the oxide to naphthol and rechromatographed; this plate showed a radioactive (1100 cpm), Gibbs-positive band corresponding to naphthol (Figure 2).

In Vitro Dihydrodiol Formation. With rabbit liver microsomes, dihydrodiol was formed both with naphthalene and with naphthalene oxide as substrates. Dihydrodiol obtained by hydration of the oxide had the *trans* configuration on the basis of the nuclear magnetic resonance spectrum. Thus the same relative stereochemistry obtains for the diol from naphthalene (Holtzman *et al.*, 1967) as well as from naphthalene oxide.

As far as the absolute stereochemistry of diol obtained from either naphthalene or naphthalene oxide is concerned, in both cases mixtures are obtained in which there is an excess of the (−)-enantiomer. *trans*-2-Dihydro-1,2-dihydroxynaphthalene has been resolved (Booth *et al.*, 1950) and the (−)-isomer ($[\alpha]_D^{25}$ −167; *c* 1.0, ethanol) assigned the (1R, 2R) absolute configuration (Miura *et al.*, 1968). Based on the observed rotations ($[\alpha]_D^{25}$ −54° and $[\alpha]_D^{25}$ −47°, respectively), both naphthalene and racemic 1,2-naphthalene oxide lead to diol composed of a similar mixture of the two enantiomers. Considering the error associated with measuring optical activity on small biological samples, the two sources of diol have very nearly the same amount of optical activity.

Naphthalene oxide was incubated with microsomes in ^{18}O-enriched water. At the end of the incubation an aliquot of the water was found to contain 43% ^{18}O, the isolated dihydrodiol contained 45% ^{18}O. Since the cleavage patter of dihydrodiol in the mass spectrometer does not allow a decision as to the location of ^{18}O, it was dehydrated to 1- (95%) and 2- (5%) naphthol by treatment with 1 N HCl on the steam bath for 10 min. The resulting phenols were silylated, separated by gas chromatography, and simultaneously examined by mass spectrometry using the gas chromatographic inlet of an LKB 9000 mass spectrometer (*cf.* Holtzman *et al.*, 1967). The 1-naphthol contained less than 1% ^{18}O while the 2-naphthol contained 39% ^{18}O; *i.e.*, the oxide was selectively hydrated at the 2 position. Nonenzymatic hydration of this oxide has never been observed (Vogel and Klärner, 1968).

Incubation of naphthalene with microsomes generally leads

[3] The formation of a Gibbs-positive color from 1,2-naphthalene oxide is usually not immediate and requires varying amounts of time (5 min to 1 hr). The color results from reaction of the Gibbs reagent with naphthol formed by rearrangement of the oxide on the plate.

TABLE II: Chemical Isomerization of 1,2-Naphthalene Oxide to 2-Naphthol.[a]

Isomerization Method	% 2-Naphthol
Acetic acid, 1 min	7
Methanol solvent with acetic acid (trace)	10
Crystalline solid at room temperature, 24 hr	5
pH 7.0 buffer	2
pH 7.0 buffer, 10% acetamide	8
Methylene chloride saturated with acetamide	12

[a] The major product is 1-naphthol. Separation and quantitation by gas chromatography as described in the text.

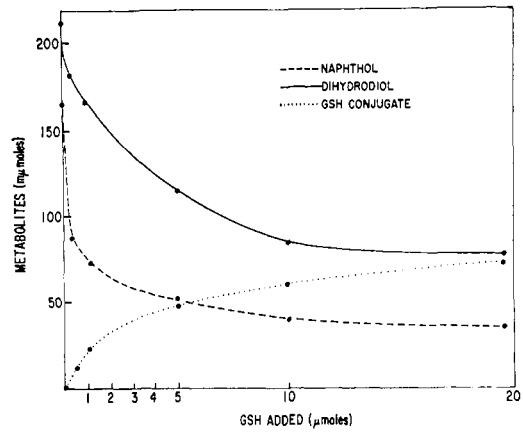


FIGURE 3: Effect of GSH on the products formed from naphthalene with liver microsomes. Recoveries of the GSH conjugate from the incubations were low (<30%).

to more dihydrodiol than naphthol. Presumably, the dihydrodiol results from an enzymatically catalyzed hydration and the naphthol from rearrangement of the oxide. Evidence for this interpretation was obtained by carrying out microsomal oxidation of naphthalene in the presence of styrene oxide, a substrate (Jerina *et al.*, 1968b) and competitive inhibitor of the epoxide hydrase. This experiment (Table I) produced far more naphthol than dihydrodiol, suggestive of the oxide as the common intermediate for both metabolites of naphthalene.

In Vitro Formation of Naphthol. Since microsomal oxidation of naphthalene produces both 1- and 2-naphthol, it was important to determine whether naphthalene oxide could yield both products in the same ratio that is observed for naphthalene metabolism *in vitro*. The *in vitro* ratio was found to be somewhat variable, but the amount of 1-naphthol always greatly predominated. Incubations with liver microsomes from phenobarbital-induced rats provided 12–15% 2-naphthol, while normal rabbit liver microsomes formed only 1–3% of this isomer. When 1,2-naphthalene oxide was allowed to rearrange to naphthols in the presence of each of the microsomal preparations, less than 3% of 2-naphthol was formed. For purposes of comparison, naphthalene oxide was allowed to rearrange to naphthols in several nonprotein environments. The amount of 2-naphthol varied from 2 to 10% (Table II) thus demonstrating that changes in the environment can affect the proportion of each isomer in a manner not readily predictable. The important point to be made from these studies is that 2-naphthol is formed in small and variable amounts during isomerization of naphthalene oxide under any of the conditions studied. This same situation obtains when the naphthols are formed enzymatically from naphthalene. Due to the great instability of naphthalene oxide it has not been possible to determine whether there are enzymes present in microsomes which are capable of catalyzing the isomerization of naphthalene oxide to naphthol.

Enzymatic Formation of Mercapturic Acid Precursor. Incubation of naphthalene with rat liver microsomes in the presence of liver supernatant and GSH yields a GSH conjugate along with the naphthol and dihydrodiol normally observed in the absence of GSH (Booth *et al.*, 1961). On careful examination it was shown that the amount of the GSH conjugate increased at the expense of naphthol and dihydrodiol, when increasing amounts of GSH were added to the incubation (Figure 3). The same GSH conjugate was formed both by addition of GSH to 1,2-naphthalene oxide under enzymatic and nonenzymatic conditions. *N*-Acetylcysteine did not add to the oxide under these conditions indicative of the unusual ability of GSH to serve as a nucleophile.

The GSH conjugate, *S*-(1,2-dihydro-2-hydroxynaphthyl)-glutathione, formed enzymatically from naphthalene, provides another test for naphthalene oxide as the microsomal intermediate from naphthalene. Reaction of GSH with naphthalene oxide could, in principle, occur either at the 1 or 2 position producing either or both of the isomers. Experiments were designed to show that the same isomer of the mercapturic acid precursor is formed with either naphthalene or the oxide as substrates. The GSH conjugate obtained either enzymatically or nonenzymatically from naphthalene oxide, or enzymatically from naphthalene, displayed a λ_{max}^{MEOH} 260 mμ and λ_{min}^{MEOH} 238 mμ. In all cases treatment with warm, dilute HCl for a few minutes resulted in aromatization to a *S*-naphthylglutathione together with a small amount of naphthol. In each instance the naphthol was isolated by extraction into ether and identified as 1-naphthol (>95%) by gas chromatography. The remaining aqueous solution was concentrated to dryness and *S*-naphthylglutathione was purified by paper chromatography in 1-butanol–pyridine–water (1:1:1). It was not possible to separate *S*-(1-naphthyl)cysteine and *S*-(2-naphthyl)cysteine by paper chromatography in acidic, basis, or neutral solvents, nor could either of the *S*-naphthylcysteines by eluted from an automated amino acid analyzer under conditions suitable for *S*-phenylcysteine (Jerina *et al.*, 1968b). Attempts to oxidize the thio ether linkage to a sulfone with performic acid (Moore, 1954) to provide compounds that would be amenable to ion-exchange chromatography led to destruction of the amino acids. The two isomers could, however, be distinguished by their ultraviolet spectra, a method which easily identified the *S*-naphthylglutathione obtained by acid dehydration of the GSH conjugate. The ultraviolet spectra of the *S*-naphthylcysteine-*N*-acetyl methyl esters are shown in Figure 4. The presence or absence of the *N*-acetyl and the methyl group does not markedly effect the ultraviolet spectra. The *S*-naphthylglutathione obtained from acid treatment of the GSH con-

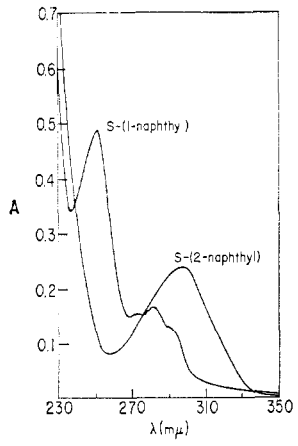


FIGURE 4: The ultraviolet spectra of *N*-acetyl-*S*-(1-naphthyl)-cysteine methyl ester and *N*-acetyl-*S*-(2-naphthyl)cysteine methyl ester in methanol.

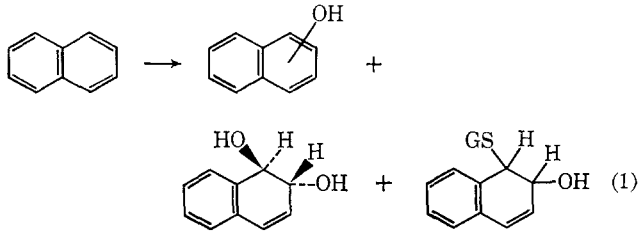
jugate was eluted from paper chromatograms with 50% ethanol and concentrated to dryness *in vacuo* several times, after addition of dilute ammonia to free the sample of pyridine. The ultraviolet spectrum of this *S*-naphthylglutathione was identical with that of synthetic *N*-acetyl-*S*-(1-naphthyl)cysteine methyl ester. The anomalous formation of 1-naphthol during the acid treatment will be commented on in the Discussion.

Further evidence for the nature of the GSH conjugate and its identity with the products from each of the three sources was obtained by Raney nickel desulfurization as previously described (Boyland *et al.*, 1961). This treatment of the conjugate led in each case to 2-naphthol (>95%) which was identified by gas chromatography.

This evidence requires that both naphthalene and naphthalene oxide form the same mercapturic acid precursor.

Discussion

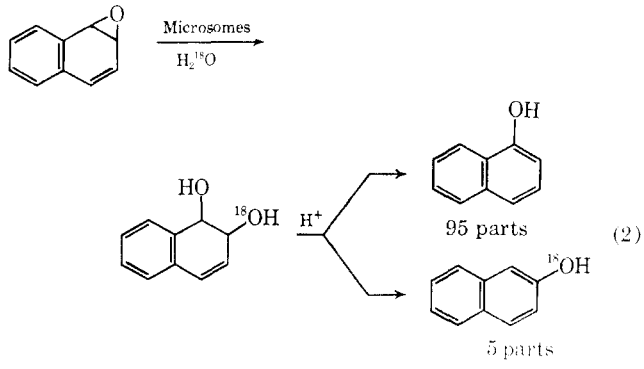
A key requirement for establishing arene oxides as intermediates in the enzymatic oxidation of aromatic substrates to phenols and other metabolites is, of course, the isolation of such an oxide from a metabolic system. The microsomal oxidation of naphthalene seemed ideally suited for this, since naphthalene is converted into a dihydrodiol (1,2-dihydro-1,2-dihydroxynaphthalene) and a GSH conjugate (*S*-(1,2-dihydro-2-hydroxynaphthyl)glutathione) as well as phenol (principally 1-naphthol) when incubated with liver homogenates (Booth *et al.*, 1960a) (eq 1). Formation of a dihydrodiol or GSH con-



(1)

jugate (mercapturic acid precursor) has never been demonstrated for a monocyclic arene in a microsomal system (Jerina *et al.*, 1968b). It was not possible to demonstrate the presence of benzene-^{14}C oxide during microsomal oxidation of benzene-^{14}C by the addition of reisolation of carrier benzene oxide. This failure may have been due in part to the low recovery of added benzene oxide as a maleic anhydride adduct and in part to the fact that benzene itself is a poor substrate for microsomal oxygenases. Since the enzymes responsible for dihydrodiol formation and GSH conjugation are, at least partially, located in the soluble liver fraction, a highly reactive, oxidized form of naphthalene appears to be produced by microsomes and released into solution for further reaction. 1,2-Naphthalene oxide has now been isolated and identified as this intermediate.

Racemic 1,2-naphthalene oxide was found to be hydrated by microsomal epoxide hydrase to form (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene of 28–35% optical purity (Jerina *et al.*, 1970). The dihydrodiol obtained as a microsomal metabolite of naphthalene had the same absolute stereochemistry and approximately the same optical purity. Formation of dihydrodiol from naphthalene by microsomes has been shown to involve incorporation of oxygen from air into the 1 position (Holtzman *et al.*, 1967). It follows that the oxygen in the 2 position must be derived from water. Hydration of 1,2-naphthalene oxide in ^{18}O water led to incorporation of the water at the 2 position (eq 2). Thus dihydrodiol forma-



(2)

tion from naphthalene and from racemic 1,2-naphthalene oxide is identical with regard to the source of oxygen at the 2 position and the absolute stereochemistry of the diol. Based on the stereochemical course of dihydrodiol formation, arene oxides were implicated as intermediates in the metabolism of a number of aromatic substrates (Jerina *et al.*, 1970). In addition, the enzymatic formation of 1,2-naphthalene oxide was suggested to be another example of asymmetric synthesis (<10% optical purity).

The ratio of 1-naphthol to 2-naphthol formed during the isomerization of 1,2-naphthalene oxide to phenols is another criterion which determines whether 1,2-naphthalene oxide is the intermediate in the microsomal metabolism of naphthalene. In general, isomerizations of 1,2-naphthalene oxide with acid, acetamide, etc. (Table II), produce mainly 1-naphthol. This can be readily understood on the basis of resonance contributions to the cationoid intermediate leading to 1-naphthol which do not interfere with the resonance of the fully aromatic ring. By the same argument, dehydration of the dihydrodiol must lead predominantly to 1-naphthol. Small amounts (2–12%) of 2-naphthol were also formed during isomerization of the oxide under various conditions.

The earlier literature on microsomal phenol formation from

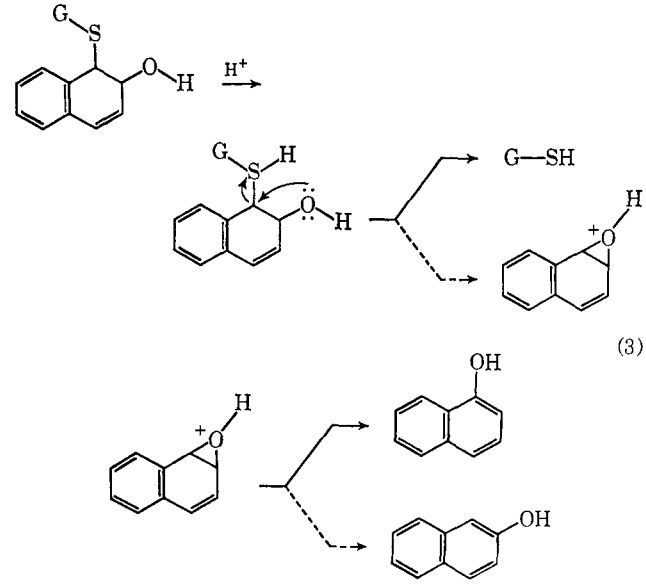
naphthalene suggested that 1-naphthol was the sole product (Mitoma *et al.*, 1956; Booth and Boyland, 1958). However, more recent studies (Boyland *et al.*, 1964; Ullrich and Staudinger, 1966) with improved techniques have shown that 2-naphthol is also a product, although a minor one. The various incubation conditions employed in the current investigation led to mixtures containing 1–15% 2-naphthol. The ratio of naphthols obtained from naphthalene or from the oxide are, therefore, in close agreement. Furthermore, it is no longer necessary to postulate a second enzyme system to explain 2-naphthol formation.

Conjugation with GSH to form a mercapturic acid precursor was utilized in these studies to furnish additional proof for 1,2-naphthalene oxide as an obligatory intermediate in the microsomal oxidation of naphthalene. When the metabolite was obtained *in vitro* from naphthalene, the structure was assigned as *S*-(1,2-dihydro-2-hydroxynaphthyl)glutathione (Booth *et al.*, 1960b).

Similarly, the urinary metabolite, in which the glutathione was degraded to an *N*-acetylcysteine, was also thought to have the sulfur attached to the 1 position of the naphthalene ring (Boyland and Sims, 1958). In both cases, minor amounts of the isomers with sulfur in the 2 position might not have been detected. These earlier assignments were also confused by reports that both naphthols are formed on treatment of GSH conjugates with dilute acid. The instability of these compounds and the difficulty of their separation made the earlier structural assignments a formidable task. Formation of a glutathione conjugate has now been studied under the following three conditions: (i) chemical addition of GSH to 1,2-naphthalene oxide in pH 8.0 buffer, (ii) enzymatic addition of GSH to the oxide by the conjugases of liver supernatant, and (iii) enzymatic formation of the conjugate from naphthalene in the complete *in vitro* system. The products were identical in all three cases. The structure was established as *S*-(1,2-dihydro-2-hydroxynaphthyl)glutathione by chemical degradations similar to those employed in earlier studies. Desulfurization with Raney nickel produced 2-naphthol as the sole phenol and hydrolysis with acid gave *S*-(1-naphthyl)glutathione as determined spectroscopically. The acid treatment also provided a small amount of naphthols which consisted mainly (>90%) of the 1 isomer. This remarkable migration of oxygen[4] may proceed *via* the protonated oxide (eq 3). It is noteworthy that such a migration does not occur during dehydration of the dihydrodiol.

Thus, all the *in vitro* metabolites of naphthalene can originate from 1,2-naphthalene oxide. The oxide itself has been shown to be a metabolite of naphthalene both by the use of naphthalene-^{14}C and carrier naphthalene oxide and by direct isolation. The metabolite was identified as 1,2-naphthalene oxide by countercurrent mobility, R_F on thin-layer chromatography, and facile isomerization to naphthol.

It then remained to establish that 1,2-naphthalene oxide was *the obligatory intermediate* in the formation of all the *in vitro* naphthalene metabolites. Evidence for this was obtained by



demonstrating that naphthol and dihydrodiol formation from naphthalene are greatly diminished, while conjugation with glutathione increases, when increasing amounts of GSH are added to the incubations. That is, the reactive 1,2-naphthalene oxide is removed from the system as the glutathione conjugate. Similarly, the addition of styrene oxide, a competitive substrate for the epoxide hydrase, to microsomal incubation mixtures containing naphthalene causes a marked decrease in dihydrodiol formation accompanied by a stoichiometric increase in naphthol formation. From a kinetic standpoint, 1,2-naphthalene oxide qualifies as the initial intermediate in the oxidation of naphthalene, since its rate of conversion into all other metabolites greatly exceeds the rates of product formation with naphthalene in the whole system, *i.e.*, naphthalene oxide formation is the rate-limiting step in naphthalene metabolism.

These experiments indicate that 1,2-naphthalene oxide is *the intermediate* responsible for all of the metabolites formed during oxidation of naphthalene by isolated liver microsomes. The possibility that some other oxide of naphthalene might be involved is negligible, since most of the other structurally similar oxides of naphthalene have been synthesized (Vogel and Klärner, 1968; Sims, 1965) and shown either to rearrange rapidly to stable oxepins or to be far too stable compared with the newly identified naphthalene metabolite. Other postulated oxidation intermediates of naphthalene have been suggested, but fail to explain all the pathways of metabolism.

A naphthalene metabolite which has not been discussed is 1,2-dihydro-1-naphthol glucuronide, the so-called acid-labile precursor of naphthalene (Boyland and Solomon, 1955). The metabolite may have arisen *via* microbial metabolism in the intestinal tract, since in subsequent experiments it was not detected in the urine of animals receiving naphthalene (E. Boyland, personal communication).

As a class of metabolites, dihydrodiols have been known since 1935 when Boyland and Levi (1935) isolated 1,2-dihydro-1,2-dihydroxyanthracene from the urine of rats treated with anthracene. Since then, numerous compounds including benzene (Sato *et al.*, 1963), halobenzenes (Jerina *et al.*, 1967b; Smith *et al.*, 1950), naphthalene (Young, 1947), phenanthrene (Young, 1947; Boyland and Wolf, 1950), and several other polycyclic aromatic hydrocarbons (Boyland and Sims,

[4] An alternate and equally acceptable explanation for the 1-naphthol formation is that the samples of conjugate contained small amounts (undetectable by ultraviolet spectroscopy) of the isomeric metabolite with sulfur attachment at the 2 position. Since the carbonium ion at C-2 is favored, this metabolite could produce the small amount of 1-naphthol observed as its principal aromatization product.

1964a,b) have been observed to form dihydrodiols *in vivo*. In all instances, these diols have been shown or suggested to have *trans* configuration thus leading Boyland to suggest in 1950 that they might arise *via* the enzymatic addition of water to the corresponding arene oxide, compounds which at the time had not been synthesized. The addition of thiols was also suggested as a plausible metabolic route. These predictions have now been confirmed in the case of naphthalene in which the dihydrodiol arises *via* an arene oxide intermediate.

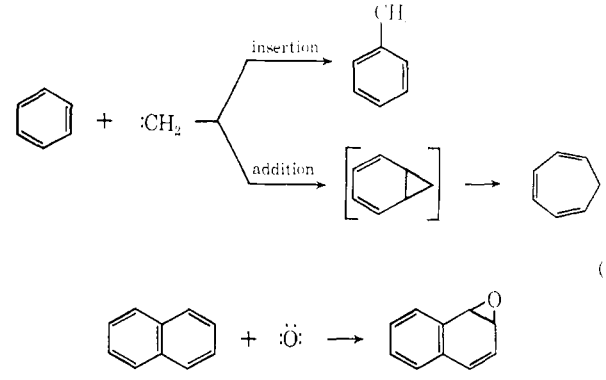
In deciding whether arene oxides are intermediates in the formation of other dihydrodiols in mammalian and bacterial systems (Gibson, 1968) there are several criteria which may be applied. First, the dihydrodiol would be expected to have a *trans* configuration relative to the two hydroxyl groups, since *cis*-glycols are not the typical products of nonenzymatic epoxide openings and since enzymatic *cis* opening of epoxides has not been observed. Second, the diol should contain one oxygen atom from air and the other from water. Finally, phenol formation should be observed as a side reaction, in view of the characteristic lability of arene oxides. The *cis*-dihydrodiols obtained from benzene (Gibson *et al.*, 1968a) and 4-chlorotoluene (Gibson *et al.*, 1968b) with *Pseudomonas putida* probably do not arise *via* the corresponding arene oxides, since the enzyme appears to be a dioxygenase (D. Gibson, personal communication).

The present investigation represents the first direct demonstration of an arene oxide intermediate in the metabolic formation of a phenol from an aromatic substrate. This observation opens up several interesting areas of speculation. The most important of these is whether arene oxides are ubiquitous intermediates on the metabolic route to phenols. That is, do all the mixed-function oxidases, including such specific enzymes as phenylalanine and tyrosine hydroxylase, form phenols *via* the arene oxide pathway; or is arene oxide formation limited to the microsomal metabolism of naphthalene and perhaps certain other aromatic substrates. If the oxide pathway is ubiquitous, the enzymes involved should be renamed aryl oxygenases, since they would no longer be truly aryl hydroxylases.

Other areas of speculation concern the long-range toxic effects of aromatic drugs mediated *via* arene oxide metabolites and the possible role of arene oxides in the carcinogenic activity of various polycyclic aromatic hydrocarbons. Preliminary studies on relatively unreactive arene oxides showed them to be of very low carcinogenic activity compared with the parent hydrocarbons (Miller and Miller, 1967; Boyland and Sims, 1967; Sims, 1967). The more reactive arene oxides would, however, if generated *in situ*, have excellent properties for binding to proteins and nucleic acids. The recent demonstration that covalent reaction of carcinogens with nucleic acids and proteins requires prior metabolism by the microsomal system (Gelboin, 1969) or chemical activation (Umans *et al.*, 1969) is in line with these suggestions.

Formation of 1,2-naphthalene oxide also provides a significant clue to the nature of the active oxygen species associated with mixed-function oxidases. The most likely mechanistic model would be one in which an oxygen atom transfer process or "oxenoid mechanism" involving an oxygen atom with six electrons around it ("oxene") is the reactive oxidant.

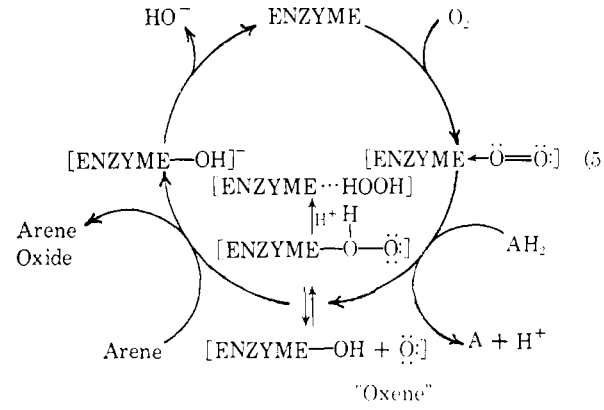
The use of the term "oxene" for the oxygen atom derives from the fact that the oxygen atom is isoelectronic with two well-known reactive intermediates in organic chemistry: carbene and nitrene. These species are known to undergo insertion and addition reactions which are analogous to the hydroxylation and epoxidation reactions proposed for oxygen atom. Carbene adds to benzene to form norcaradiene[5] just as oxygen atom might add to naphthalene to form 1,2-naphthalene oxide (eq 4). By analogy to carbene, the postulated



(4)

oxygen atom ("oxene") could explain all the oxidation processes (aliphatic and aromatic hydroxylation, epoxidation, S and N oxidation, etc.) manifested by the microsomal mixed function oxidases. The suggestion of an oxenoid mechanism has been made in the past (Ullrich and Staudinger, 1966; Hamilton, 1964). *However, it has not been recognized until now that oxygen atoms can add to arenes to form arene oxides which isomerize to phenols.*

In delineating all the reasonable paths by which molecular oxygen might be activated by the mixed-function oxygenases, Mason (1957) considered an enzyme-bound neutral oxygen atom as the oxidant. Hamilton (1964) later elaborated on this possibility and described a mechanism essentially the same as that which we propose (eq 5). The most important



(5)

feature of this postulated mechanism is that it generates an oxidant (coordinated oxygen atom, oxene) which would be capable of displaying reactions characteristic of mixed function oxidases, including the possible generation of peroxide (Gillette *et al.*, 1957; Storm and Kaufman, 1968). The mechanism does not, however, intend to depict an actual

[5] For a discussion of the reaction, see Kirmse (1964) and Streith and Cassal (1967).

sequence of events, which will, of course, be quite different with different enzyme systems. For energetic reasons, it does seem likely that an oxygen atom is transferred to the substrate rather than being liberated into the medium. Reduction of enzyme or of a bound cofactor may occur before oxygen is bound. The substrate may be bound at some other point in the sequence, for example, prior to reduction of the enzyme or the enzyme–oxygen complex. The four-electron reduction of oxygen and two-electron oxidation may actually occur in a concerted reaction. Finally, reduction of half of the oxygen molecule to water might occur before oxidation of substrate.

Chemical hydroxylating systems which operate *via* an oxygen atom transfer mechanism should then be good models for the mixed-function oxidases. The only known model system (Jerina *et al.*, 1967a) which displays the NIH shift, peroxytrifluoroacetic acid, functions *via* an oxygen atom transfer reaction (Henbest, 1965). Hamilton has devised a model hydroxylating system (Hamilton *et al.*, 1966) based on the suggested mechanism (eq 5) which has been found not to display the NIH shift (Jerina *et al.*, 1967a). This hydrogen peroxide system with catalytic amounts of ferric ion and catechol may hydroxylate by insertion or perhaps by radical process (Norman *et al.*, 1965) rather than addition; thus circumventing the necessary arene oxide intermediate.

The studies presented here seem to rule out the cyclic peroxides which have been suggested as intermediates *via* singlet oxygen (Soloway, 1966; Baldwin *et al.*, 1968). However, such a species could account for the incorporation of two oxygen atoms into the aromatic nucleus as is the case with the dioxygenases. A search for arene oxides in the metabolism of aromatic substrates other than naphthalene and studies on oxygen atom transfer reactions are currently underway.

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The Transient Inactivation of Trypsin by Mild Acetylation with *N*-Acetylimidazole*

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ABSTRACT: Two species of acetylated trypsin could be detected during the progress of acetylation of bovine trypsin with *N*-acetylimidazole. At an early stage Ser^{183} is O acetylated and the enzyme inactivated. This derivative can be isolated at low pH but rapidly deacylates and reactivates at neutral pH. At a later stage a second acetyl group is introduced (possibly on a histidyl residue) which appears to greatly retard the deacetylation of Ser^{183}. These two acetyl groups, which interfere with the catalytic mechanism, are exclusively removed by dilute imidazole, thus distinguishing them from acetyl groups simultaneously introduced onto ϵ-amino groups of lysyl residues and phenolic groups of tyrosyl residues.

It has been well established that trypsin is not readily inactivated by acetylation of lysyl residues with acetic anhydride. Extensive acetylation yielded largely active trypsin preparations (*e.g.*, Labouesse and Gervais, 1967) in spite of the acetylation of 85–100% of the ϵ-amino groups. In contrast to these studies where the site of the acetylation was lysine, acetylation of up to ten tyrosyl residues (in 3.2 M guanidine hydrochloride with acetylimidazole) led to complete inactivation (Riordan *et al.*, 1965b). Milder acetylation with the same reagent in the absence of denaturant modified only 6.7 tyrosyl residues and left the enzymatic activity intact. Riordan *et al.* (1965b) concluded from these studies that three "buried" and chemically unavailable tyrosines were involved in maintaining the active center of the enzyme in a configuration required for catalytic activity.

Attempts to acetylate the α-amino group of trypsin have shown that it is chemically unavailable for acetylation, whereas Scrimger and Hofmann (1967) have demonstrated that its deamination by nitrous acid is paralleled by a loss of activity.

The present study was initiated as an attempt to identify in the amino acid sequence the tyrosine residues which are exposed in the native enzyme; however, the goal was redefined when it was discovered that very mild treatment with *N*-acetylimidazole led to a transient inactivation of the enzyme as a labile acetylated intermediate. Since previous attempts in several laboratories have failed to demonstrate a monoacyltrypsin intermediate in the course of the tryptic catalysis of various acyl-x substrates, the nature of this derivative was examined to identify the site(s) of acetylation and its relation to the functional active center.

Independent studies of the acetylation of trypsin in other laboratories have demonstrated an *activation* of tryptic activity against certain esters (Trenholm *et al.*, 1966, 1969; Labouesse and Gervais, 1967; Chevallier *et al.*, 1968). This activation (obtained under different experimental conditions, see Table I) is reported to be the result of tyrosyl acetylation. Thus, the modification of trypsin with acetylating agents under various conditions has yielded products ranging from completely inactive to superactive. The present work is directed toward both a study of the effects of limited acetylation, which appears to interfere with the catalytic action, and an identification of the loci of acetylation. The conditions of acetylation are milder than others cited in Table I and employ both low temperature and a less reactive acetylating agent. Under these conditions transient intermediates could be observed and isolated.

Materials and Methods

Acetylimidazole (mp 101°) was either synthesized by the method of Boyer (1952) or obtained from K and K Laboratories. Frequent recrystallizations from dry benzene or isopropenyl acetate were carried out on material stored *in vacuo* over P_2O_5 or NaOH. [Acetyl-^{14}C]*N*-acetylimidazole, from New England Nuclear Corp. (specific activity 0.23 μCi/μmole), was diluted approximately tenfold with cold reagent and recrystallized from dry benzene. The specific radioactivity was determined for this dried material.

DNS-amino acids,[1] TES, HEPES, and imidazole were

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[1] Abbreviations used are: AcIm, *N*-acetylimidazole; *N*-Ac-L-TyrEt, α-*N*-acetyl-L-tyrosine ethyl ester; Bz-L-ArgEt, α-*N*-benzoyl-L-arginine ethyl ester; Bz-Arg-NA, α-*N*-benozyl-DL-arginine-*p*-nitroanilide; DIP,