Migration of Substituents during Hydroxylation of Aromatic Substrates (NIH Shift). Oxidations with Peroxytrifluoroacetic Acid*

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ABSTRACT: Migration and retention of deuterium during microsomal and peroxytrifluoroacetic acid oxidations have been measured for a series of selectively deuterated aromatic substrates. Nonenzymatic and enzymatic retention values were comparable except for highly reactive aromatic rings, such as anisoles and anilides. When peroxytrifluoroacetic acid was hydrolyzed in [^{18}O]H_2O, [^{18}O]oxygen was not incorporated into the hydrogen peroxide, making it unlikely that OH^+ is generated under these conditions. An oxygen-transfer mechanism is proposed for the hydroxylation of aromatic rings by peroxytrifluoroacetic acid which involves either the direct formation of cationoid intermediates or their generation from an (protonated) arene oxide precursor. Hydroxylation of chloro- or bromobenzene by peroxytrifluoroacetic acid leads to di- and trihalophenols in addition to the expected monohalophenols. No evidence for intramolecular migration of halogen was obtained during these studies.

The intramolecular migration and retention of aromatic ring substituents such as deuterium, tritium, halogens, and alkyl groups, which occur during enzymatic hydroxylation by mixed-function oxidases, provide a unique new criterion for evaluating chemical model systems capable of introducing the hydroxyl moiety into aromatic rings. Such migrations, commonly referred to as the NIH shift (Daly *et al.*, 1968; Jerina *et al.*, 1970b), are so characteristic of the enzyme reactions that oxidants which do not produce them may no longer be considered meaningful models for mixed-function oxidases. A preliminary examination of various oxidizing systems revealed that peroxytrifluoroacetic acid was a reagent capable of causing migrations similar to those observed during enzymatic hydroxylations (Jerina *et al.*, 1967b). Model hydroxylating systems are of interest in elucidating the mechanism of the far more complex enzymatic reactions. It therefore became meaningful to compare the magnitude of the NIH shift obtained in nonenzymatic (peroxytrifluoroacetic acid) and microsomal hydroxylations. In addition, experiments were conducted to clarify the mechanism of the peroxytrifluoroacetic acid reactions.

Experimental Section

Labeled Compounds. Specifically deuterated compounds were prepared by catalytic hydrogenolysis of the appropriately chlorinated or brominated compound with 5% palladium on charcoal and deuterium gas in tetrahydrofuran solvent. Excess triethylamine was added to neutralize the acid generated during the reaction. In a typical experiment, a 1 M solution of the halo compound in tetrahydrofuran containing a 10% excess of triethylamine, 5% [^{2}H]H_2O, and 5% palladium on charcoal (10% of the weight of the halo compound) was agitated under deuterium gas (99.8% $^2H_2$) for 5–10 hr. All products were either recrystallized or distilled. The melting point or boiling point of the labeled compounds was nearly identical with those of the normal hydrogen analogs. This procedure usually provided products with greater than 90% incorporation of isotope, exclusively located (>98%) in the desired position on the ring. Phenolic compounds, however, gave poor incorporation and considerable (10–20%) randomization of label.

Other methods of incorporation were also explored. Deuterolysis of isobenzoxazole derivatives of phenols (Musliner and Gates, 1966) proceeded with excellent incorporation and negligible randomization. Hydrolysis of arylmagnesium halides (Jerina *et al.*, 1967a) or aryllithium compounds (Jerina *et al.*, 1967b) with [^{2}H]H_2O was also satisfactory but often led to low incorporation of deuterium, unless *extreme* care was taken to avoid traces of water prior to hydrolysis. Raney nickel catalyzed tritiolysis or deuterolysis of the tosyl derivatives of phenols proceeded with considerable randomization of label (Jerina *et al.*, 1967b). Labeled compounds, method of synthesis, and deuterium content are given in Table I.

Specificity of Labeling. All compounds employed for labeling were judged to be free of isomeric impurities (<0.5%) by gas–liquid chromatography or thin-layer chromatography prior to introduction of deuterium. Random incorporation was determined by bromination or by hydroxylation with Fentons reagent at the desired position of label. Both reactions proceed by direct substitution without significant migration and retention of isotope (Jerina *et al.*, 1967b). Deuterium contents were determined by comparison of the molecular ion for each derivative with that of the same compound containing only the natural abundance of deuterium. Multiple determinations were made in each case. The extent of random deuterium label is given in Table I.

Hydroxylations with Peroxytrifluoroacetic Acid. Reactions were run in either heterogeneous or homogeneous mixtures. The heterogeneous system consisted of 1.0 ml each of $CHCl_3$, 90% H_2O_2, and trifluoroacetic anhydride and 100 mg of aromatic substrate. The homogeneous system contained 20 mmoles of peroxytrifluoroacetic acid and 100 mg of substrate in 10 ml of CH_2Cl_2. Solutions of peroxytrifluoroacetic acid

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were readily prepared by the cautious addition of 4.1 ml (0.15 mole) of 90% H_2O_2 to 26 ml (0.18 mole) of trifluoroacetic acid anhydride in 100 ml of CH_2Cl_2 at 0° with stirring until the mixture became homogeneous (Emmons and Pagano, 1955). The resulting solution was stable for several days at 0°. Reactions were run for 15 hr at 5° unless otherwise noted. Products were isolated by slowly pouring the reaction mixture into an excess of cold $NaHCO_3$ solution which was then extracted several times with CH_2Cl_2 or ethyl acetate. The combined organic extracts were dried (Na_2SO_4) and concentrated. The various components were separated by thin-layer chromatography, gas–liquid chromatography, or paper chromatography as previously described (Daly *et al.*, 1968b). Special care was taken to ensure that all hydroxylated isomers were completely separated before determination of deuterium content. Conversions of 0.1–2% proved adequate for the measurement of deuterium retentions. For the halobenzenes, the hydroxylated products were methylated with dimethyl sulfate in aqueous sodium hydroxide prior to gas chromatography (Hamilton *et al.*, 1966). Methylation of unlabeled phenols in $[^2H]H_2O$–$[^2H]NaOH$ demonstrated that the reaction proceeds without exchange of aromatic protons. Deuterium retentions in phenolic products were measured on a LKB 9000 combination mass spectrometer–gas chromatograph or on a Hitachi RMU6E mass spectrometer. The results are presented in Table II, together with the retentions obtained with the same substrate during microsomal hydroxylations (Daly *et al.*, 1968b).

Decomposition of Peroxytrifluoroacetic Acid in $[^{18}O]H_2O$. The position of bond cleavage during the hydrolysis of peroxytrifluoroacetic acid into H_2O_2 and trifluoroacetic acid was determined in a two compartment reaction vessel with a volume of 50 ml. One arm of the flask contained 0.60 ml of $[^{18}O]H_2O_2$ (20.0% ^{18}O), while the other arm held 2.0 ml of 0.33 N cerric (IV) sulfate in 1 N H_2SO_4 in normal water. Both solutions were prepared free of oxygen by boiling and subsequent cooling under nitrogen. Then 0.12 mmole of peroxytrifluoroacetic acid in 0.10 ml of CH_2Cl_2 was added to the $[^{18}O]H_2O$. The vessel was closed and evacuated. The peroxy acid solution was warmed to 40° for 10 min. Alternate thawing of the solutions and evacuation after freezing (−78°) removed traces of atmospheric oxygen. When the contents of the two compartments were mixed, there was a vigorous evolution of oxygen. Determination of ratio of mass 32 to mass 34 on the evolved oxygen gas provided a measure of incorporation of the labeled $[^{18}O]H_2O$ into the hydrogen peroxide. The oxygen gas contained 0.38% ^{16}O–^{18}O which represents an incorporation of less than 2% of water. Thus, peroxytrifluoroacetic acid in analogy with peroxyacetic and peroxyformic acids (Bunton *et al.*, 1956) undergoes exclusive acyl-oxygen cleavage. The mass 32/34 ratio in the sample was determined on a Consolidated Engineering Corp. mass spectrometer, Model 21-401.

Results and Discussion

Comparison of Hydroxylations by Peroxytrifluoroacetic Acid and Microsomes. Hydroxylations of aromatic substrates either enzymatically or with peroxytrifluoroacetic acid proceed with migration and retention of substituents (the NIH shift). In this respect, peroxytrifluoroacetic acid provides a meaningful mechanistic model for mixed-function oxidases. Typical electrophilic aromatic substitution reactions, such as halogenation or nitration and hydroxylation of aromatic rings by hydroxyl radical (Fenton's reagent), however, *do not* cause migration of substituents (Jerina *et al.*, 1967b). In addition to peroxytrifluoroacetic acid, other chemical oxidants (pyridine *N*-oxide, *hν*) have recently been found to cause the NIH shift (Jerina *et al.*, 1970a). Deuterium retentions observed during hydroxylation of a number of compounds by liver microsomes and the peroxy acid system are compared in Tables II and III.

The retentions observed with peroxytrifluoroacetic acid and liver microsomes are quite similar for many substrates, such as nitro-, cyano-, and methylbenzenes and the biphenyls (Table II). For the halobenzenes, retentions are uniformly higher with peroxytrifluoroacetic acid as oxidant, whereas with highly activated rings, such as methoxy- and phenoxybenzenes (Table II) and the various anilides (Table III), the retentions with peroxytrifluoroacetic acid are much lower. Migration and retention of deuterium are not unique to oxidations by peroxytrifluoroacetic acid, but are also observed during hydroxylation of aromatic substrates with peroxyacetic and *m*-chloroperoxybenzoic acids. The reten-

TABLE I: Synthesis of Labeled Compounds.[a]

Substrate	Deuterium Inc	Random Deuterium
[4-^{2}H]Aniline	0.90	0.00[b]
[4-^{2}H]Acetanilide	1.00	0.00[b]
N-([4-^{2}H]Phenyl)benzamide	0.89	0.00[c]
N-([4-^{2}H]Phenyl)formamide	0.89	0.00[c]
N-([4-^{2}H]Phenyl)trifluoroacetamide	0.87	0.00[c]
N-([4-^{2}H]Phenyl)benzenesulfonamide	0.71	0.00[b]
N-Methyl-*N*-([4-^{2}H]phenyl)-benzenesulfonamide	0.71	0.00[d]
[5-^{2}H]Salicyclic acid	0.38	0.05[b]
[4-^{2}H]Anisole	1.00	0.00[b]
[4-^{2}H]Diphenyl ether	1.00	
[4-^{2}H]Biphenyl	1.00	
[4-^{2}H]Toluene	0.84	
[4-^{2}H]Fluorobenzene	0.98	0.00[e]
[4-^{2}H]Chlorobenzene	1.00[f]	<0.01[e]
[4-^{2}H]Bromobenzene	1.00[g]	<0.02[e]
[4-^{2}H]Benzoic acid	0.83	
[4-^{2}H]Benzonitrile	1.00	<0.02[e]
[4-^{2}H]Nitrobenzene	0.92	0.00[c]

[a] The table indicates the average number of deuterium atoms per molecule after making appropriate corrections for the natural abundance of heavier isotopes. The general method of synthesis consisted of deuterolysis of the corresponding chloro or bromo precursor as described in the Experimental Section. [b] Based on remaining deuterium in excess of natural abundance following bromination or chlorination at the indicated ring position. [c] Prepared from the preceding aniline sample. [d] Prepared by methylation of the preceding sulfonamide. [e] As indicated in footnote *b* except that substitution conducted with Fenton's reagent as described to produce minimal retentions (Jefcoate *et al.*, 1969). [f] Prepared by neutralization of 4-chlorophenylmagnesium bromide with 99.8% $[^2H]H_2O$. [g] As in footnote *f* using the 4-bromo analog.

TABLE II: Comparison of Deuterium Retentions Found with Peroxytrifluoroacetic Acid and Liver Microsomes.[a]

$RC_6H_4{}^2H$, R =	Microsomes	Peroxytrifluoroacetic Acid
NO_2	40	49
CN	41	47
$CONH_2$	42	
COOH		52
Br	40	76
F	47	73
Cl	54	72
$NCH_3SO_2C_6H_5$	53	6
CH_3	54	68
OC_6H_5	55	12
OCH_3	60	8
C_6H_5-4′-F	61	58
C_6H_5	64	59

[a] The values in the table are percentages of deuterium retained following hydroxylation at the position of the label. Separation of oxidation products and microsomal data are described elsewhere (Daly *et al.*, 1968a).

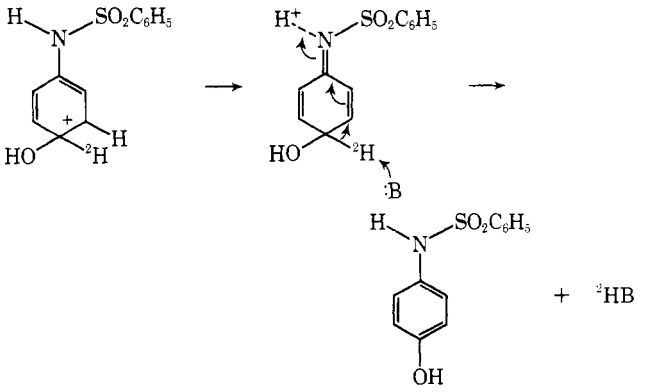
TABLE III: Comparison of Deuterium Retentions for Substrates with an Ionizable Hydrogen on a Heteroatom Adjacent to the Aromatic Ring.[a]

RC_6H_4-4-^2H, R =	Microsomes	Peroxytrifluoroacetic Acid	$\sim pK_a$ of Parent Acid
OH (salicyclic acid)	0	0	
$NHSO_2C_6H_5$	1	3	0.7
NH_2	6	3	
NHCOH	19	6	3.8
$NHCOC_6H_5$	21	7	4.2
$NHCOCH_3$	(25–55)	8	4.8
$NHCOCF_3$	(12, 34)[b]	24	0.2

[a] The values in the table are percentages of deuterium retained following hydroxylation at the position of the label. Separation of oxidation products and microsomal data are described elsewhere (Daly *et al.*, 1968a). [b] These values were obtained with rabbit or rat microsomes at pH 8.0, respectively, and represent a correction of an earlier value of 0% reported for hydroxylation with rabbit microsomes at pH 8.0. Conversions with rabbit microsomes are quite low and a contaminant exhibiting a peak at *m/e* 219 cochromatographed with the *N*-trifluoroacetyl-4-hydroxyaniline (*m/e* 219), so that mass spectral determination of deuterium content was difficult.

tions, 21 and 23%, respectively, for para hydroxylation of [4-^{2}H]anisole with these peroxy acids are higher than the retention observed with peroxytrifluoroacetic acid.[1]

The data obtained with microsomes and peroxytrifluoroacetic acid for substrates containing an ionizable hydrogen on a heteroatom attached to the aromatic ring are presented in Table III. As discussed in earlier papers (Daly *et al.*, 1968b; Jerina *et al.*, 1968), such substrates show low retentions with microsomes because the ionized substituent as an electron donor provides for resonance stabilization without migration. When deuterated benzenesulfonanilide was hydroxylated by microsomes, deuterium retention was negligible (Daly *et al.*, 1968b). In *N*-methylbenzenesulfonanilide where

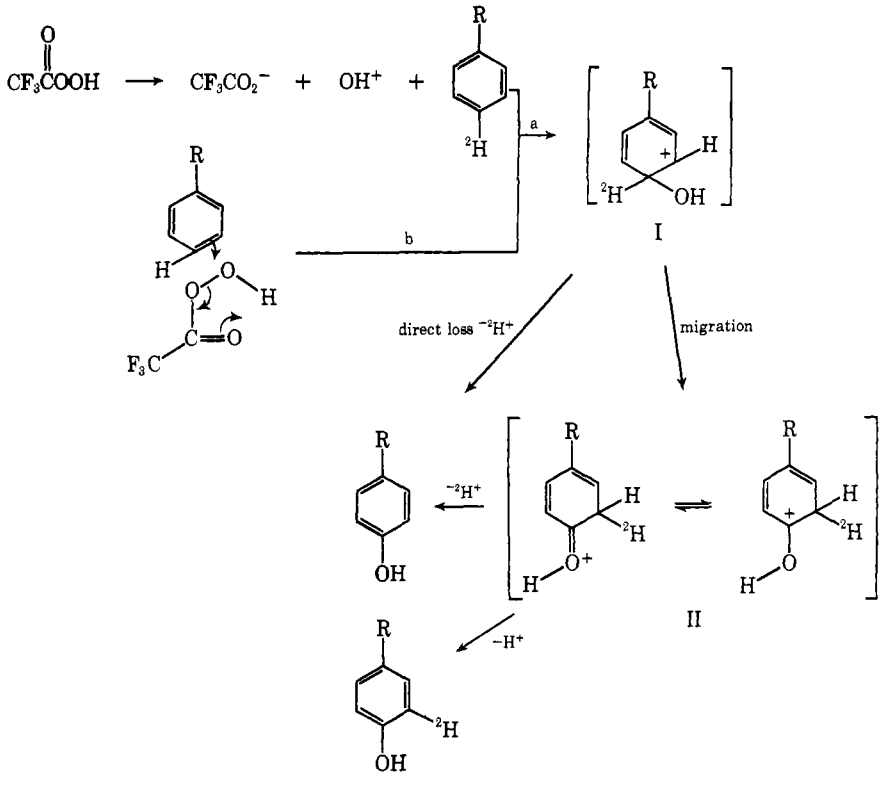


ionization is blocked, the deuterium retention increased from 1 to 53% for microsomal hydroxylation. The corresponding values for hydroxylation by peroxytrifluoroacetic acid were 3 and 6%. The failure to increase retention in the latter case may be due to the high acidity of the peroxytrifluoroacetic acid medium. Both enzymatic and nonenzymatic systems show a general decrease in retention as the acidity, *i.e.*, ionizability, of the anilide NH increases. The pK_a for the acid moieties are given in Table III as a guide to the relative pK_a's of the anilides. The effect is far more pronounced for microsomal hydroxylations. Trifluoroacetylaniline provides an exception to this generalization with both peroxytrifluoroacetic acid and microsomal oxidations, perhaps related to the fact that in this substrate the aromatic ring is strongly deactivated toward hydroxylation by either peroxytrifluoroacetic acid or microsomal preparations.

Despite quantitative differences in deuterium retention observed with peroxytrifluoroacetic acid and enzymatic oxidation (Table II), chemical hydroxylation with peroxytrifluoroacetic acid is a useful model for comparison with the enzyme system, since unlike other model systems, such as the Fenton reaction (Norman and Lindsay-Smith, 1965) and the systems of Udenfriend (Udenfriend *et al.*, 1954), Hamilton (Hamilton and Friedman, 1963), and others (Ullrich and Staudinger, 1969a,b), the peroxytrifluoroacetic acid system *does elicit* the NIH shift. For both microsomes and peroxytrifluoroacetic acid, the extent of oxidation of aromatic substrates and the ratios of isomeric phenols obtained indicate attack by a weak and selective electrophile. With peroxytrifluoroacetic acid, the phenol formed from an equal mixture of C_6H_6 and $[^2H]C_6H_5$ consists within 1% of equal amounts of C_6H_5OH and $[^2H]C_6H_5OH$. Thus, hydroxylations of aromatic rings by either enzymes (Tanabe *et al.*, 1967; Guroff and Daly, 1967; Perel *et al.*, 1967; Daly and Jerina, 1970) or by peroxytrifluoroacetic acid (Ullrich and Staudinger, 1969b) do not exhibit a significant primary isotope effect.

[1] The authors thank Dr. D. R. Boyd for these unpublished reports.

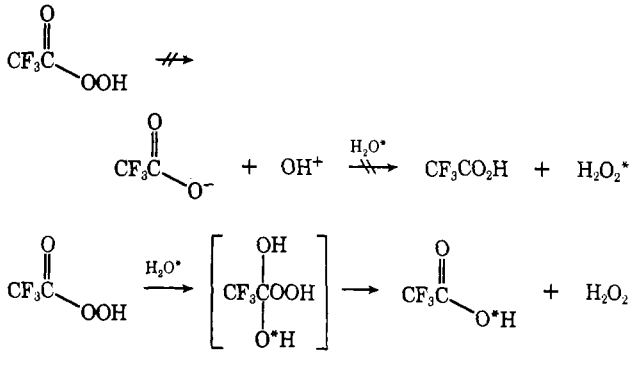
SCHEME I



The single exception occurs during microsomal hydroxylation of zoxazolamine (Tanabe *et al.*, 1970).

Mechanism of Oxidation by Peroxy Acids. The simplest mechanisms for the peroxy acid oxidation involve, at least in a formal sense, either generation (reaction a) or direct transfer of OH^+ (reaction b) to form the cationoid intermediate I by an electrophilic substitution reaction. Direct loss or migration of deuterium can then occur. The driving force for the migration pathway would be stabilization of the positive charge in the intermediate II (Scheme I).

Heterolytic cleavage of the peroxide bond of peroxytrifluoroacetic acid, such as would be involved in the intermediate formation of OH^+ in the above mechanism, should also occur in water. Peroxytrifluoroacetic acid, unlike the other peroxy acids such as peroxyformic, -benzoic, or -acetic acid, is extremely reactive and quite unstable in water, where it rapidly hydrolyzes to trifluoroacetic acid and H_2O_2 (Emmons



and Pagano, 1955). Whether this hydrolysis occurs *via* heterolytic cleavage of the peroxide bond with intermediate formation of OH^+ which further reacts with water to form hydrogen peroxide has now been tested with the aid of [^{18}O]-H_2O. The results indicate that less than 2% of the H_2O_2 formed contains an oxygen atom derived from [^{18}O]H_2O. Therefore, hydrolysis of peroxytrifluoroacetic acid, as previously reported for peroxyacetic and formic acid (Bunton *et al.*, 1956), occurs by attack of water at the carbonyl carbon rather than by heterolysis of the peroxide bond to form OH^+. In view of this evidence and arguments by others (Norman and Taylor, 1965; Davies, 1961), assumption of free HO^+ as the active oxidizing species becomes tenuous and need not be considered further. The direct transfer of OH^+ within a donor–acceptor complex (reaction b) with generation of the postulated cationoid intermediate I at the present time still serves as an acceptable and reasonable mechanism.

If such a mechanism is valid for either enzymatic or peroxy acid hydroxylation, the magnitude of deuterium retention should be a function of the R substituent on the ring. Substituents which readily donate electrons to the ring system should stabilize the cationoid intermediate I, and thus favor low retention of deuterium. The results of the peroxy acid hydroxylations offer better support for this assumption than the data on microsomal reactions. Thus, [4^2-H]anisole gave a predictably low (8%) retention with peroxytrifluoroacetic acid while a surprisingly high (60%) retention was found with microsomes (Table II). In general, the retention values for oxidation by peroxytrifluoroacetic acid or by microsomes *do not correlate* with the Hammett σ^+ constants for the various substituents studied (Figure 1). The simple assumption of cationoid intermediates thus fails to explain the quantitative aspects of deuterium retention.

Oxidation by the peroxy acid could also lead to a (protonated) arene oxide, a reaction for which there is ample analogy in the epoxidation of olefins by peroxy acids. Generation

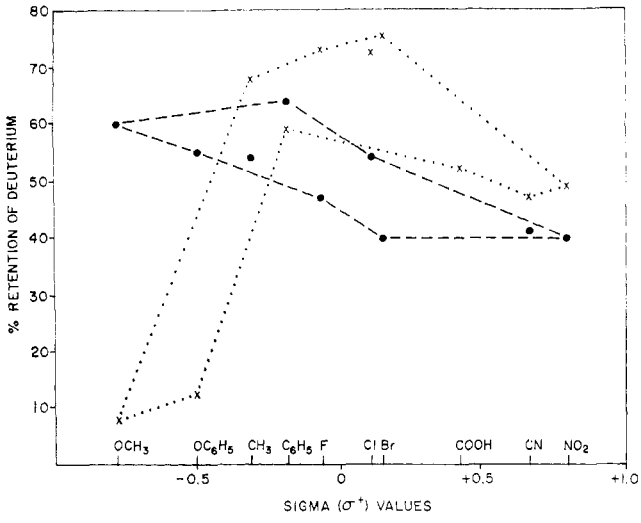
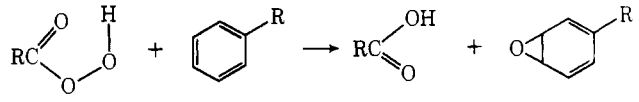
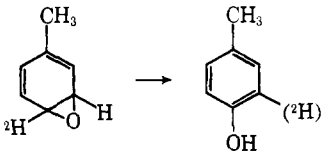


FIGURE 1: Hammett plot of σ^+ values (Palin, 1961) against per cent migration and retention of deuterium after hydroxylation of aromatic substrates by peroxytrifluoroacetic acid (×) or microsomes (●).

of an arene oxide with peroxytrifluoroacetic acid would merely extend the reaction to epoxidation of an aromatic double bond. This suggestion becomes more tenable with the demonstration of the direct enzymatic (Jerina *et al.*, 1968a,

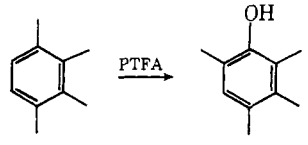


1970c) and chemical (Jerina *et al.*, 1970a) epoxidation of an aromatic double bond. Arene oxide intermediates are compatible with the results obtained with peroxytrifluoroacetic acid. The arene oxide should be formed at the region of highest electron density and should rearrange to produce the phenol (Jerina *et al.*, 1968a,b; Kaubisch *et al.*, unpublished results) expected as a result of attack by an electrophilic reagent, in agreement with the isomer distribution obtained with peroxytrifluoroacetic acid. No primary isotope effect would be expected for the formation of an arene oxide, as is the case for hydroxylations by peroxytrifluoroacetic acid. Deuterated arene oxides are known to rearrange to phenols with migration and retention of deuterium (Jerina *et al.*, 1968b; D. R. Boyd, D. M. Jerina, and J. Daly, in preparation, 1970), in agreement with migrations noted for peroxytrifluoroacetic acid oxidations.

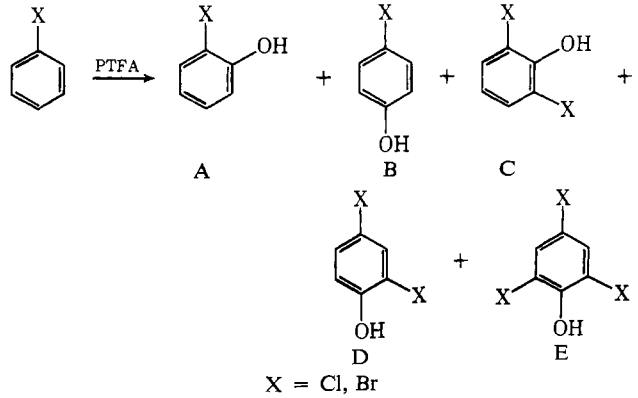


An arene oxide is formed enzymatically from naphthalene (Jerina *et al.*, 1968a, 1970c), suggestive of arene oxides as intermediates in many enzymatic hydroxylations. If both oxidations by enzymes and peroxytrifluoroacetic acid proceed through intermediate arene oxides, the deuterium retentions seen with these systems should be the same. The data presented in Tables II and III show this is not the case. The nature of the isomerization of arene oxides to phenols might be responsible for this discrepancy. The deuterium retained during isomerization is strongly dependent on environment. Thus, with [4-^{2}H]toluene 3,4-oxide, retention of deuterium in the product, *p*-cresol, varies from ~40 to 85% under different conditions (Jerina *et al.*, 1968b). Retentions are lower under acidic conditions. [4-^{2}H]Anisole was hydroxylated with peroxytrifluoroacetic acid in the presence of 1,3,5-trinitrobenzene in the hope that the charge-transfer complex would be less capable of stabilizing the positive charge and, therefore, show higher retention. Only a small increase in retention to 10% was observed with the complexed species. Further investigation of the isomerization of deuterated arene oxides should define more exactly the parameters affecting the extent of migration and retention. The correspondence between the deuterium retentions obtained in the oxidations by microsomes and peroxytrifluoroacetic acid is least satisfactory for compounds containing highly activated rings, such as anisole, anilides, and diphenyl ether. It is, therefore, possible that peroxytrifluoroacetic acid proceeds by a mechanism different from the enzymatic oxidations in the case of such reactive compounds. Unfortunately, oxidations with peroxytrifluoroacetic acid are not carried out under conditions where it is likely that acid-labile intermediate arene oxides could be isolated.

Migrations of Other Substituents. Before the suggestion of peroxytrifluoroacetic acid as a mechanistic model for mixed-function oxidases (Guroff *et al.*, 1967), migration of a methyl group had been noted with this reagent during the conversion of prehnitene to 2,3,4,6-tetramethylphenol (Buehler and Hart, 1963). Migrations of halogen substituents



during hydroxylations with peroxytrifluoroacetic acid have now been reported (Ullrich *et al.*, 1968; Foulkes, 1969). Our investigation did not concentrate on such migrations, but during hydroxylation of chloro- and bromobenzene, a surprising variety of di- and trihalogenated phenols was observed. Such products presumably arise *via* halogenation of the primary products, the 2- or 4-halophenols. The origin of the halogenating species is unknown. Perhaps the overall

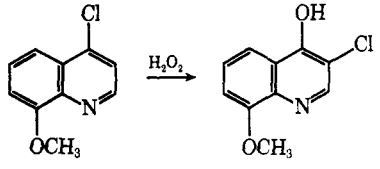


X = Cl, Br

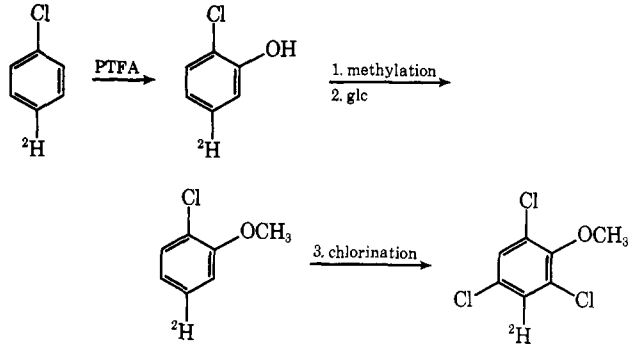
% of Product

X = Cl	A	B	C	D	E
1 hr	52	24	0	24	0
16 hr	6	4	4	86	1

reaction is similar to that seen in the formation of 3-chloro-4-hydroxy-8-methoxyquinoline from 4-chloro-8-methoxyquinoline, in which an *apparent* halogen migration occurs. The mechanism suggested for the latter reaction required hydrolysis of the starting material to form a phenol and chloride ion which, after oxidation to hypochlorite, chlorinated the phenol (Fujita and Price, 1968).



In the present investigation, chloro- and bromobenzene produced polyhalogenated products, while fluorobenzene did not. That *o*-chlorophenol arose entirely by attack at C-2, in contrast to the alternative of attack at C-1 and migration of halogen, was demonstrated by isolation of *o*-chlorophenol in the form of its *O*-methyl ether by preparative gas–liquid chromatography, followed by chlorination with Cl_2. All of the deuterium was *retained* after chlorination to [5-^{2}H]-2,4,6-trichloroanisole, *i.e.*, no chlorine migration had occurred. The source and nature of the halogenating species in the polyhalogenation accompanying oxidation by peroxytrifluoroacetic acid are presently under study. In view of such dualistic pathways, it is necessary to differentiate clearly



between intermolecular halogenations and the intramolecular migration of halogen (the NIH shift).

Conclusions

The migration and retention of deuterium during oxidative formation of phenols with peroxy acids may be rationalized in terms of an oxygen-transfer mechanism which leads to the formation of cationoid species either directly or *via* intermediate arene oxides. Similar mechanisms and formation of arene oxides also pertain in hydroxylation of aromatic substrates with other model systems (Jerina *et al.*, 1970a) and with enzymes (Jerina *et al.*, 1970c). At the present time, the extent of retention of deuterium is only qualitatively explained by such mechanisms. However, if arene oxides are the initial products formed during oxidation with peroxytrifluoroacetic acid, the retentions will be strongly dependent on both environment and structure of the arene oxide. Further studies on the chemical and enzymatic formation of arene oxides and their role in the metabolism of aromatic substrates are in progress.

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Isolation of Highly Purified γ-Glutamylcysteine Synthetase from Rat Kidney*

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ABSTRACT: The presence in mammalian kidney of substantial amounts of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase, enzymes which catalyze the degradation of glutathione and γ-glutamylamino acids, suggested that kidney might also possess catalytic activity capable of synthesizing γ-glutamylamino acids such as γ-glutamylcysteine. However, the determination of γ-glutamylcysteine synthetase in crude tissue preparations which contain enzymes that catalyze the degradation of γ-glutamylamino acids presents a difficult analytical problem. A reliable procedure for the determination of γ-glutamylcysteine synthetase activity in crude tissue preparations was developed and is described here. In the new method, the tissue preparation is incubated with [^{14}C]L-glutamate, adenosine 5′-triphosphate (ATP), magnesium ions, L-α-aminobutyrate, and an excess of purified brain γ-glutamyl cyclotransferase; under these conditions, the [^{14}C]glutamyl moiety of the [^{14}C]γ-glutamylamino acid formed is quantitatively converted into [^{14}C]pyrrolidonecarboxylate, which is separated from other ^{14}C compounds and determined. Application of this procedure indicates that a number of rat tissues contain γ-glutamylcysteine synthetase activity and that the most active tissue is kidney (specific activity, about 10 units (micromoles per hour) per milligram of protein). The enzyme has been purified in about 40% yield from rat kidney leading to an apparently homogeneous (by analytical ultracentrifugation ($s_{20,w}$ = 5.6 S) and acrylamide gel electrophoresis) protein exhibiting a specific activity of 540 units/mg. The present findings indicate that γ-glutamylcysteine synthetase constitutes 2–3% of the protein in the soluble fraction of rat kidney homogenates. The rat kidney preparation described here is about 100 times more active than previously reported preparations of γ-glutamylcysteine synthetase (from hog liver and bovine lens). The pH optimum and the apparent K_m values for the substrates have been determined. The purified rat kidney enzyme loses activity progressively when stored at 0° and may be reactivated by exposure to dithiothreitol. The enzyme is markedly inhibited by *p*-mercuribenzoate, *p*-mercuribenzenesulfonate, and iodoacetamide. The enzyme is active when α-methylglutamate and β-methylglutamate are substituted for glutamate; it interacts with D-glutamate as indicated by the formation of inorganic phosphate at about 2% of the rate observed with L-glutamate. L-α-Aminobutyrate can be replaced by L-cysteine and several other amino acids; replacement with hydroxylamine leads to formation of an hydroxamic acid. The possibility is suggested that γ-glutamylamino acids are involved in amino acid transport.

Earlier work in this laboratory on enzymes that catalyze reactions involving γ-glutamylamino acids led to the isolation of highly purified preparations of γ-glutamyl transpeptidase (Orlowski and Meister, 1963, 1965) and γ-glutamyl cyclotransferase (Orlowski *et al.*, 1969). Since substantial amounts of these enzymes are present in mammalian kidney (Orlowski, 1963; Orlowski and Meister, 1970a,b), it became of interest to us to determine whether the kidney also possesses catalytic activity capable of synthesizing γ-glutamylamino acids. The synthesis of γ-glutamylcysteine, the first of the two steps involved in the enzymatic synthesis of glutathione, was initially studied by Bloch and his collaborators (Bloch, 1949; Johnston and Bloch, 1949, 1951) in pigeon liver preparations. In this work, liver preparations from a number of animals were examined and found to be rather poor sources of the enzyme; no activity was detected in extracts of acetone dried rabbit kidney. Later, Mandeles and Bloch (1955) and Strumeyer (1959) succeeded in obtaining a partially purified preparation of the enzyme from hog liver. A purified preparation of γ-glutamylcysteine synthetase has also been obtained from bovine lens (Rathbun, 1967). γ-Glutamylcysteine synthetase catalyzes the following reaction: L-glutamate + L-cysteine + ATP → L-γ-glutamyl-L-cysteine + ADP + P_i. When L-cysteine is replaced by L-α-aminobutyric acid, L-γ-glutamyl-L-α-aminobutyrate is formed at about the same rate as found for the synthesis of γ-glutamylcysteine. With relatively purified enzyme preparations the reactions may be followed quantitatively by determination of the inorganic phosphate released. However, this method is not applicable to determination of the enzyme in crude tissue extracts because of the presence of other systems that catalyze the dephosphorylation of ATP. For this reason, an alternative procedure was devised by Mandeles and Bloch (1955) in which the incorporation of [^{14}C]glycine or of [^{14}C]glutamate into glutathione was determined in the presence of an excess of glutathione synthetase, the enzyme that catalyzes the

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