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Arene Oxides and the NIH Shift: The Metabolism, Toxicity and Carcinogenicity of Aromatic Compounds

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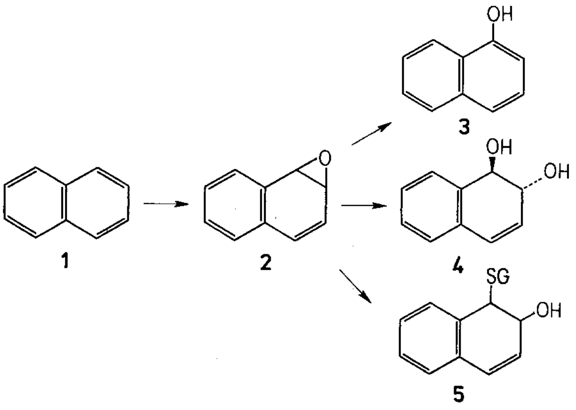
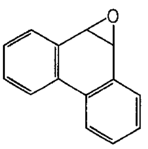
Arene oxides: formation and metabolism in biological systems.

In 1968[1] naphthalene-1,2-oxide **2** was identified as the obligatory intermediate in the hepatic metabolism of naphthalene **1**. This isolation ended years of speculation on the possible role of such arene oxides in the metabolism of aromatic compounds [cf. ref.[2]]. Hepatic monooxygenases convert naphthalene to naphthalene-1,2-oxide, which then either spontaneously isomerizes to 1-naphthol **3**, undergoes enzymatic hydration to the *trans*-dihydrodiol **4**, or conjugates both spontaneously and by enzymic catalysis with glutathione to form **5**. The spontaneous isomerization **2** to **3** is accompanied by migration of deuterium from the 1- to the 2-position[3]. This phenomenon, the 'NIH Shift', has now been observed concomitant with the isomerization of many arene oxides to phenols[3–6].



Recently, the conversion of polycyclic hydrocarbons to oxides, such as phenanthrene 9,10-oxide **6**, benz[a]anthracene 5,6-oxide **7**, and dibenz[a,h]anthracene 5,6-oxide **8** has been reported[7,8].



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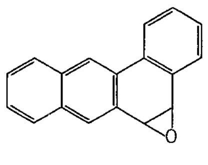

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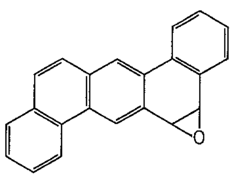

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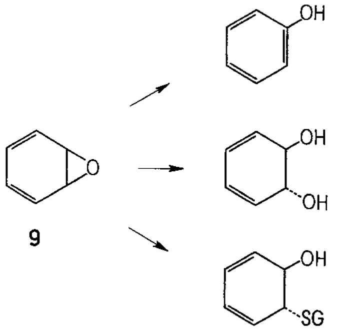
The nature and distribution of metabolites which are formed from an intermediate arene oxide depend on a number of factors: 1. intrinsic stability of the oxide with regard to isomerization to phenols, 2. susceptibility to enzymatic hydration to *trans*-dihydrodiols as catalyzed by microsomal epoxide hydrase, 3. susceptibility to conjugation with glutathione as catalyzed, in part, by a soluble hepatic glutathione S-epoxide transferase and 4. the affinity to nucleophilic groups of macromolecular tissue components. As an example, benzene oxide **9** isomerizes spontaneously to phenol and, enzymatically, is converted to a *trans*-dihydrodiol and a glutathione conjugate[9].



The interrelationship of hepatic monooxygenase and epoxide hydrase activity has recently been examined. Both enzymes are localized in microsomal fractions and both are inducible in rodents by pretreatment of ani-

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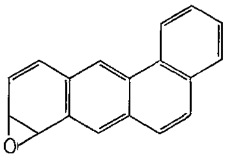
mals with 3-methylcholanthrene or phenobarbital[10]. The extent and mechanism of induction of total levels of these two enzymes are, however, not comparable and are under separate genetic control[11]. Soluble preparations of the mono-oxygenases, cytochrome P-450 or P-448, contain high levels of epoxide hydrase activity[12], suggestive of a close association of mono-oxygenase and epoxide hydrase in liver membranes. Reconstituted cytochrome P-450 or P-448 systems containing NADPH, cytochrome P-450 reductase and a lipid fraction convert naphthalene to **2**, **3** and **4**[12]. Total conversion of naphthalene to metabolites with cytochromes P-450 and P-448 is comparable, but the ratios of naphthol **3**, which represents rearranged oxide, and the *trans*-dyhydrodiol **4** are markedly different. This difference is expressed by a ratio of $\sim$3:1 with cytochrome P-450 and 1:1 with P-448. This is in spite of the fact that the P-450 preparation contains significantly higher total levels of hydrase activity, which would be expected to favor dihydrodiol formation. These results suggest that hepatic mono-oxygenases are associated with epoxide hydrases in complexes of varying structures and stabilities. Radioactive naphthalene oxide, generated from radioactive naphthalene, should not completely equilibrate with an added pool of carrier naphthalene oxide, if such coupled mono-oxygenase-epoxide hydrase systems do catalyze the overall conversion of naphthalene to dihydrodiol; i.e., the specific activity of dihydrodiol should be greater than that of naphthol. This is exactly what has been observed with liver homogenates[13], and strengthens the argument for the existence of a coupled mono-oxygenase-epoxide hydrase system. The relevancy of such coupled systems to the metabolism of other mono-, bi- and polycyclic hydrocarbons is under investigation. Varying degrees of association of the two systems for different substrates could lead to virtually, complete conversion of an arene oxide to a dihydrodiol on the one hand, and to phenols and glutathione conjugates in the opposite extreme case.



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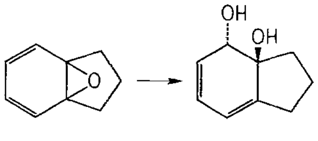
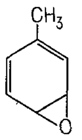


11 **12**



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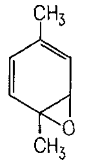

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Microsomal epoxide hydrases convert naphthalene 1,2-oxide **2**[1,14], benzene oxide **9**,[9,14] phenanthrene 9,10-oxide **6**,[15,16] benz[a]anthracene 5,6-oxide **7**[16], benz[a]anthracene 8;9-oxide **10**[17], and indan-8,9-oxide **11**[18] to *trans*-dihydrodiols. The enzymatic hydration of **11** to form **12** is unique in that it occurs by 1,6- rather than 1,2-addition of water. The enzymatic hydration of toluene-3,4-oxide **13** or of *p*-xylene-3,4-oxide **14** could not be demonstrated in microsomal preparations, probably because **13** isomerizes rapidly to *p*-cresol[4], while **14** is not a substrate for the hydrase because of the sterically hindered trisubstituted oxirane ring[19].

Epoxide hydrase has been solubilized and partially purified from guinea-pig liver microsomes[20] with the help of an assay based on the hydration of styrene-^{3}H-oxide[10]. The activity of the 'purified' enzyme was increased for naphthalene 1,2-oxide, phenanthrene 9,10-oxide and styrene oxide to the same degree, approximately 30-fold[15]. Activity of the purified enzyme for benzene oxide was increased only 4-fold[15]. Cross inhibition studies with various epoxides[19] also suggest that 'benzene oxide hydrase' differs from the other relatively non-specific enzyme(s) of guinea-pig liver microsomes.

Arene oxides are readily opened by nucleophiles, especially those containing sulfur[21,22]. Addition of the thiol group of glutathione to arene oxides leads to glutathione conjugates and the cases of **2**, **9** and **10**, enzyme catalysis has been demonstrated[1,9,17]. In vivo such glutathione conjugates are metabolized to premercapturic acids by loss of the glycine and glutamic acid residues followed by N-acetylation of the S-substituted cysteine. The resultant premercapturic acids are readily dehydrated to mercapturic acids by treatment with acid. From soluble hepatic proteins a glutathione S-epoxide transferase which is highly active toward many simple epoxides, but fails to act on benzene and naphthalene oxide, has now been purified[23]. Formation of glutathione conjugates during microsomal metabolism of estradiol has been discussed in terms of quinone and arene oxide intermediates[24].

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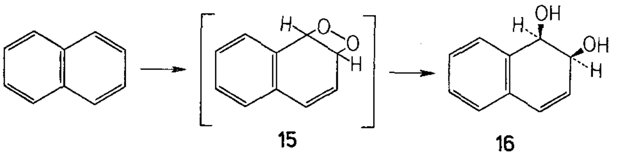
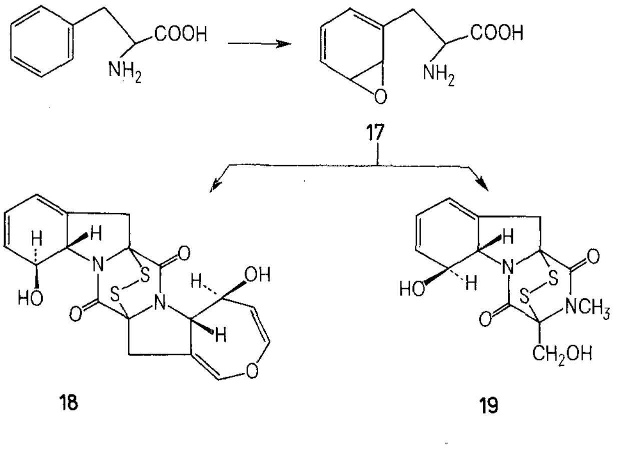
Aromatic compounds are metabolized in higher organisms to phenols, *trans*-dihydrodiols, glutathione conjugates, and (pre)mercapturic acids via intermediate arene oxides. The dihydrodiols are conjugated with glucuronic acid or are dehydrogenated to catechols by a soluble hepatic dehydrogenase[25]. Catechols may then be methylated to guaiacol derivatives by the action of catechol-*O*-methyltransferase. Except for phenols, occurrence of any of these compounds as urinary metabolites provides evidence for the in vivo formation of the corresponding arene oxide. A few examples of such metabolites are given in Table I.

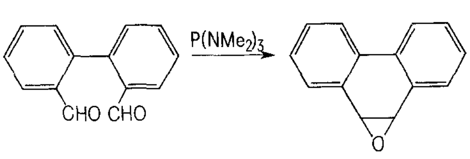
In lower organisms, *cis*- rather than *trans*-dihydrodiols are formed by the action of dioxygenases[49], as in the formation of a *cis*-dihydrodiol **16** from naphthalene[50]. Cyclic peroxides, such as **15**, have been postulated as possible intermediates. The NIH shift does, however, occur during arene metabolism with certain lower organisms and plants, suggestive of intermediate arene oxides. The biosynthesis of fungal metabolites, such as aranotin **18** and gliotoxin **19**[51–53], appears to involve an arene oxide intermediate, presumably a 2,3-oxide of phenylalanine **17**. Aranotin **18** represents an example of a metabolite derived from the oxepin form of an arene oxide.



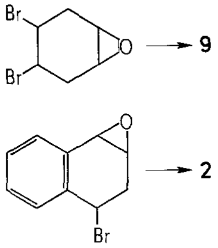


Stability of arene oxides-rearrangements to phenols and the mechanism of the NIH shift.

Arene oxides, aromatic compounds in which one of the formal double bonds has been epoxidized, have first been approached by NEWMAN and BLUM[54] who prepared oxides of phenanthrene and benz[a]-anthracene. The route employed Mark's reagent, *tris*-dimethyl-aminophosphine, to close the oxirane ring from the appropriate aromatic *bis*-aldehyde precursor:



Subsequently, VOGEL et al.[55] developed the dehydrohalogenation reaction of halo-epoxides. Variations on this theme are the synthesis of benzene oxide **9** and napthalene-1,2-oxide **2**. A direct transfer of oxygen from photo-excited pyridine-N-oxide to an aromatic



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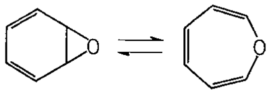
acceptor as in the formation of **2**[56] is of greater mechanistic than preparative interest.

Two reactions dominate the chemistry of arene oxides: the valence bond tautomerism to oxepins, and the facile rearrangement to phenols. Benzene oxide[55], for example, is in equilibrium with the seven-membered oxepin **20**. Both extremes of the oxide-oxepin equilibrium can be observed: Napthalene 1,2-oxide exists solely as the oxide[57,58], since the oxepin **21** could only exist with loss of aromaticity in the benzene ring. By contrast, attempts to synthesize naphthalene 2,3-oxide **22** have produced only 3-benzoxepin **23**[59]. Irreversible oxepin tautomers, such as **23**, are quite stable and do not rearrange to phenols. As yet, only arene oxides which exist partly or entirely in the oxide form have been established as metabolic intermediates.



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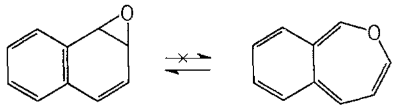

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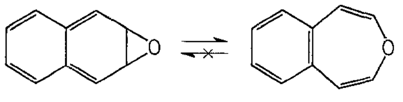


22 **23**

Recently, considerable insight has been gained into the mechanism by which arene oxides rearrange to phenols. This mechanism is compatible with the intramolecular migration and retention of substituents observed during enzymatic aryl hydroxylation, *the NIH shift*[60–62]. Kinetics for the isomerization of arene oxides

Table I. Mammalian metabolism of aromatic compounds to dihydrodiols and mercapturic acids: Evidence for the intermediacy of arene oxides[a]

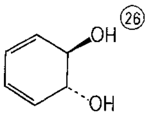
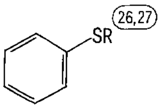
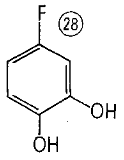
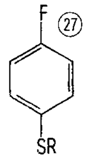
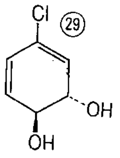
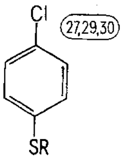
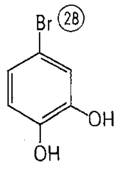
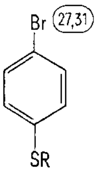
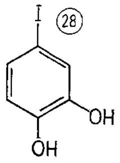
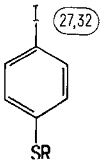
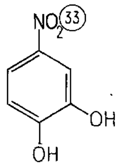
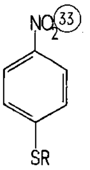
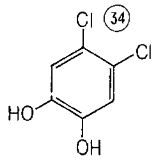
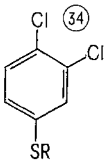
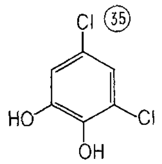
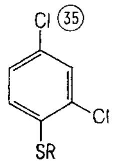
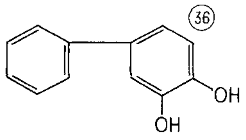
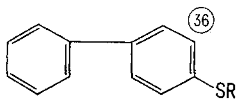
Compound	Metabolite $R = CH_2\text{-}\underset{\substack{\vert \\ NHCOCH_3}}{CH}COOH$	
Benezene		
Fluorobenzene		
Chlorobenzene		
Bromobenzene		
Iodobenzene		
Nitrobenzene		
1-2-Dichlorobenzene		
1,3-Dichlorobenzene		
Biphenyl		

Table I continued on p. 1133

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[56] D. M. JERINA, D. R. BOYD and J. W. DALY, Tetrahedron Lett. *1970*, 457.

[57] E. VOGEL and R. G. KLÄRNER, Angew. Chem. ,Int. Edition *7*, 374 (1968).

[58] D. R. BOYD, D. M. JERINA and J. W. DALY, J. org. Chem. *35*, 3170 (1970).

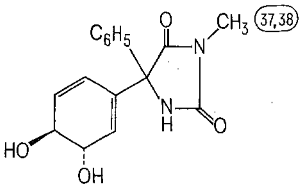
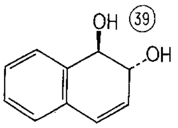
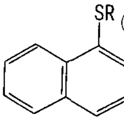
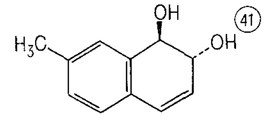
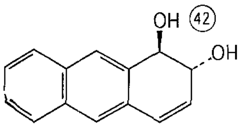
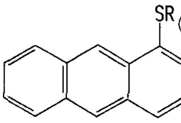
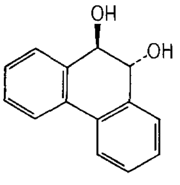
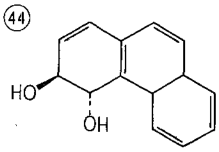
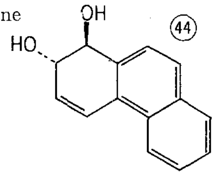
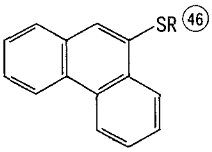
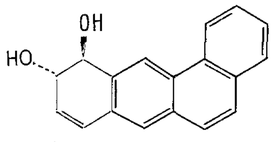
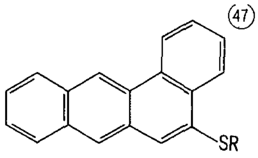
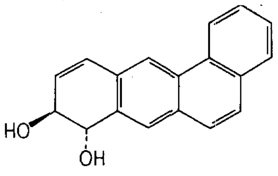
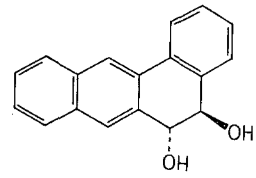
[59] A. M. JEFFREY and D. M. JERINA, J. Am. chem. Soc. *94*, 4048 (1972) and references therein.

[60] G. GUROFF, D. JERINA, J. RENSON, S. UDENFRIEND and B. WITKOP, Science *157*, 1524 (1967).

[61] J. W. DALY, G. GUROFF, D. M. JERINA, S. UDENFRIEND and B. WITKOP, in *Advances in Chemistry, Oxidation of Organic Compounds III* (Ed. R. F. GOULD, American Chemical Society, Washington, D. C. 1968), vol. 77, p. 279.

[62] D. M. JERINA, J. W. DALY and B. WITKOP, in *Biogenic Amines and Physiological Membranes in Drug Therapy, Part B.* (Eds. J. H. BIEL and L. G. ABOOD; Marcel Dekker, Inc., New York 1971), p. 413.

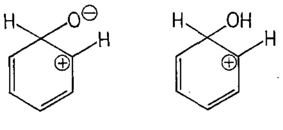
Table I (continued). Mammalian metabolism of aromatic compounds to dihydrodiols and mercapturic acids: Evidence for the intermediacy of arene oxides[a].

Compound	Metabolite $R = CH_2\text{-}\underset{\textstyle NHRCH_3}{\underset{\vert}{CH}}COOH$
Diphenylhydantoin	 (37,38)
Naphthalene	 ㊴  ㊵
2-Methylnaphthalene	 ㊶
Anthracene	 ㊷  ㊸
Phenanthrene	 ㊹  ㊺  ㊹  ㊻
Benz(a)anthracene[b]	 ㊼  ㊼  

[a] The table is not intended as a complete survey of the literature on dihydrodiol and (pre)mercapturic acid formation. Instead, selected citations to dihydrodiol and mercapturic acid formation are given. In certain compounds, where dihydrodiol was not reported, the formation of catechol has been cited.

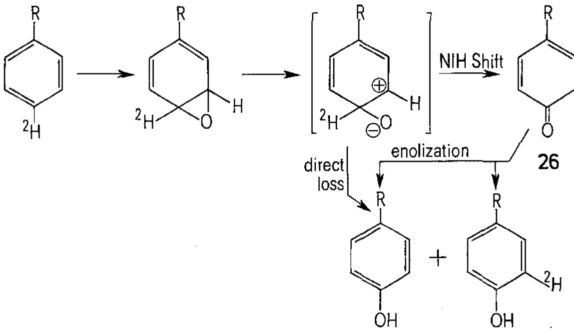
[b] Microsomal conversion of a series of polycyclic hydrocarbons to phenols and dihydrodiols has been recently investigated[48]. Earlier literature is cited.

to phenols generally indicate two types of reactions[63–66]: a spontaneous rearrangement in the neutral to basic region, and an acid-catalyzed rearrangement at low pH. The rate-determining step in the region of spontaneous isomerization is the opening to the zwitterion **24**, while in acid the protonated form **25** of this species



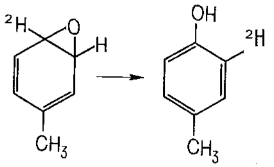
24 **25**

is predominant[64]. A complete mechanism for rearrangement of the arene oxide to the phenol must be compatible with the various retentions observed during the NIH shift over a wide pH-range. The intermediate keto tautomer **26** of the phenol satisfies these requirements. The mechanism shown is for the spontaneous reaction which predominates at physiological pH. The keto-tautomer **26** requires tritium retentions higher than



those of deuterium, since enolization of **26** to the phenol is known from analogies to show a substantial isotope effect which might not be markedly affected by the nature of the group R. Since retentions are, however, strongly dependent on R, an alternate path leading to direct loss of heavy hydrogen is also proposed. Discussion of these points will be resumed later (p. 1135).

This scheme, retention of heavy isotope during isomerization of an arene oxide, was confirmed when 1-^{2}H-4-methylbenzene oxide **27** was observed to rearrange



27 **28**

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[63] G. J. Kasperek and T. C. Bruice, J. Am. chem. Soc. *94*, 198 (1972).

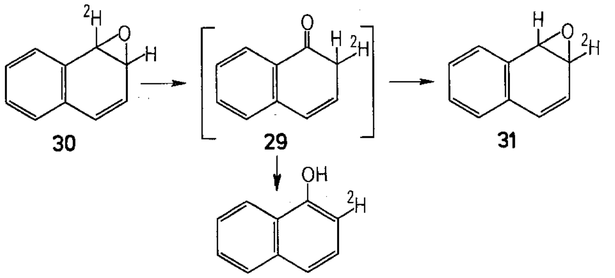
[64] G. J. Kasperek, T. C. Bruice, H. Yagi and D. M. Jerina, J. chem. Soc. D., Chem. Commun. *784*, (1972).

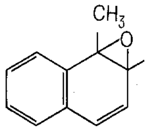
[65] H. Yagi, D. M. Jerina, G. J. Kasperek and T. C. Bruice, Proc. natn. Acad. Sci., USA *69*, 1985 (1972).

[66] G. J. Kasperek, T. C. Bruice, H. Yagi, N. Kaubisch and D. M. Jerina, J. Am. chem. Soc., in press (1972).

to 4-methylphenol **28** with up to 85% retention of deuterium depending on conditions[4]. Rearrangement of the oxide **27** or hydroxylation of toluene-4-^{2}H at the same pH lead to comparable retentions.

Evidence for the intermediacy of a keto-tautomer was provided in the metabolism of naphthalene, where either 1-^{2}H- or 2-^{2}H-naphthalene lead to 1-naphthol with 70% deuterium retained[3]. This requires a common intermediate, presumably the keto-tautomer **29**, whose formation predominates over the pathway of direct loss. Both 1-^{2}H- and 2-^{2}H-naphthalene-1,2-oxide (**30** and **31**) rearrange to 1-naphthol with comparable retention, a result again explicable in terms of the common intermediate keto-tautomer, a species which may be observed directly when generated photochemically from naphthalene oxide at –198°. This unstable intermediate isomerizes spontaneously when warmed to –78°[67]. Rearrangement of 1,2-dimethylnaphthalene 1, 2-oxide **32** leads to the stable methyl substituted ketone **33**[5,6].





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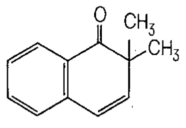
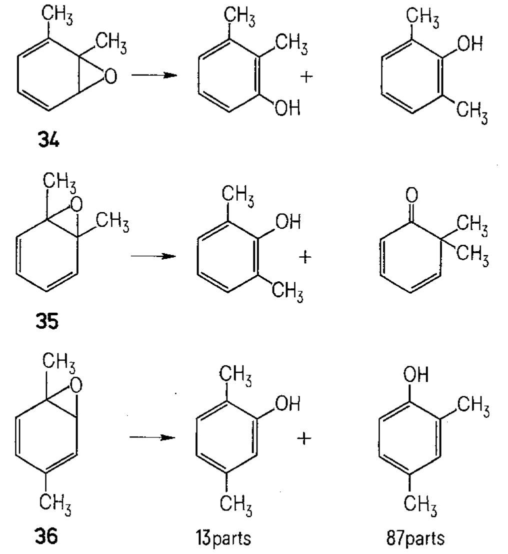
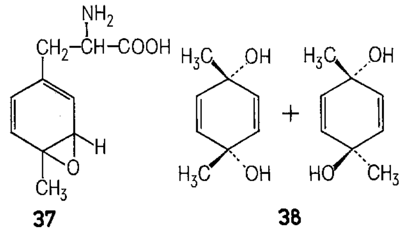


33

The 3 monomethyl-, the 9 dimethyl-, and the symmetrical trimethylbenzene oxides have all been synthesized and their rearrangement products compared with the phenolic metabolites obtained from the corresponding alkyl benzenes by incubation with liver microsomes[5,6]. In all cases, one or more of the arene oxides gave a matching distribution of phenolic metabolites. However, isolation of the alkylarene oxides in vitro was unfeasible because of their instability. The relative stability of arene oxides is a function of the number and type of substituents, e.g., electron-donating substituents decrease, electron-withdrawing substituents increase the stability of arene oxides[64].

Of the methyl-substituted benzene oxides, those with alkyl substitution on the oxirane ring, proved relatively stable. However, when the microsomal metabolites were compared with the phenols obtained on rearrangement of these oxides, it seemed apparent that the microsomal system does not epoxidize aromatic double bonds substituted by one or two methyl groups, presumably because of steric hinderance[5,6]. Such methyl-substituted oxiranes (**34**, **35** and **36**) rearrange to phenols with and without methyl migrations. Whereas the NIH shift of methyl groups has not been observed with the non-specific microsomal system (cf. ref.[68]) the highly specific enzyme, phenylalanine hydroxylase, oxidizes 4-methylphenylalanine to 3-methyltyrosine (11 parts) and 4-methyl-3-hydroxyphenylalanine (1 part)[69] with methyl migration presumably via the methyl-substituted oxide **37**. The spontaneous non-enzymatic rearrangement of 1,4-dimethylbenzene oxide **36**[6,65,66] provides a pertinent model for this reaction: The ratios of methyl-migrated and nonmigrated phenols obtained from **36** at physiological pH, or from 4-methylphenylalanine during enzymatic oxidation, are comparable. The ratio of xylenols from **36** is strongly dependent on pH. Acid-catalyzed isomerization leads to nearly equal amounts of both compounds, while spontaneous isomerization forms preponderantly the methyl-migration product, 2,4-dimethylphenol. Under acid conditions the arene oxide **36** is in equilibrium with the 1,4-dihydrodiols **38**[65].





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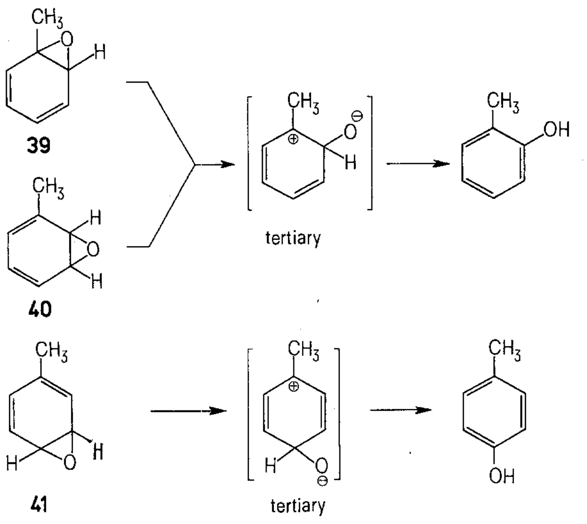
[67] D. JERINA, B. WITKOP, C. MCINTOSH and O. CHAPMAN, in preparation.

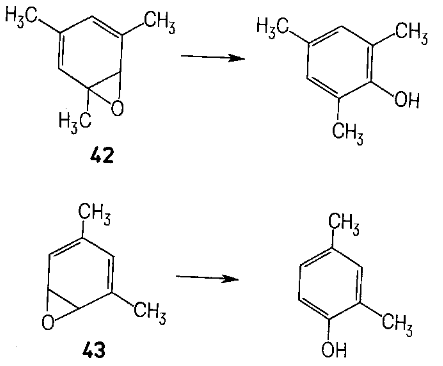
[68] J. DALY, Biochem. Pharmac. *19*, 2979 (1970).

[69] J. DALY and G. GUROFF, Arch. Biochem. Biophys. *125*, 136 (1968).

Further study of the rearranged phenols from the methyl-substituted benzene oxides[5, 6] has led to a simple rule for predicting the direction of opening with unsymmetrical oxides: the set of cationoid canonical contributors to the transition state which contains more tertiary than secondary ions will be favored. Thus with the three toluene oxides **39**, **40**, and **41**, only *o*- and *p*-



cresol are formed. Likewise, in the microsomal metabolism of toluene only *o*- and *p*-cresol but no *m*-cresol are observed. Other examples of such directed openings are the rearrangement of 1,3,5-trimethylbenzene oxide **42** to 2,4,6-trimethylphenol and 3,5-dimethylbenzene oxide **43** to 2,4-dimethylphenol. As predicted by the

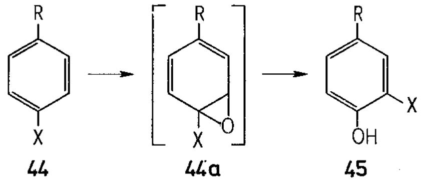


'rule' of cationoid canonical contributions, methyl does not migrate in the first case, and the oxirane ring opens selectively in the second. The 'rule' rationalizes the preponderant formation of 1- rather than 2-naphthol from naphthalene 1,2-oxide **2**, since the allylic carbonium ion should be more stable than the alternative benzylic ion.

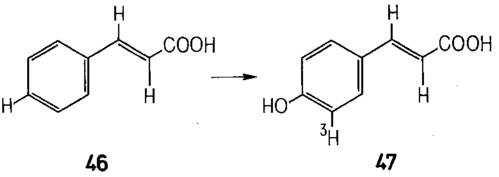
All examples of the NIH shift involving migration of isotopic hydrogen, methyl and probably of halogen are explicable in terms of isomerization of an intermediate arene oxide. Conversely, the NIH shift now provides a priori evidence for the intermediacy of an arene oxide.

The NIH shift: Migration of deuterium and tritium.

This section attempts to review and tabulate all of the known examples of the NIH shift represented in a general form by **44** → **45**, as brought about by mono-



oxygenases. The source of the enzyme has little effect on the migration of deuterium or tritium, which depends solely on the nature of the substrate, i.e., the chemical properties of the intermediate arene oxide (**44a**). Rare exceptions to this generalization are the acylanilides to be discussed below (p. 1140). Hydroxylation of phenylalanine-4-^{3}H to *p*-tyrosine (Table II) occurs with > 90% retention of tritium regardless of whether phenylalanine hydroxylase from *Pseudomonas*[70, 71], *Penicillium*[72] or liver[70, 71] or whether tyrosine hydroxylase from adrenal glands[73] is used. Cinnamic acid-4-^{3}H **46** is converted to 4-hydroxycinnamic acid-3-^{3}H **47** in vivo and in vitro in different plants[75, 76] and plant preparations[77, 78] to an extent of 85–90%.



A proposed keto-intermediate **26** which tautomerizes to the phenol with significant isotope effects requires that tritium be retained to a greater extent than deuterium, unless the nature of isotopic hydrogen changes the ratio between the two major pathways of isomerization of arene oxides: direct loss and formation of the keto-tautomer.

Indeed, tritium usually migrates and is retained to a higher degree than deuterium during formation of the same phenolic metabolite. Hydroxylations of anisole,

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[70] G. Guroff and J. Daly, Arch. Biochem. Biophys. *122*, 212 (1967).

[71] G. Guroff, C. A. Reidsnyder and J. Daly, Biochem. biophys. Res. Commun. *24*, 720 (1966).

[72] L. Nover and M. Luckner, FEBS Letters *3*, 292 (1969).

[73] J. Daly, J. Levitt, G. Guroff and S. Udenfriend, Arch. Biochem. Biophys. *126*, 593 (1968).

[74] G. Guroff, M. Levitt, J. Daly and S. Udenfriend, Biochem. Res. Commun. *25*, 253 (1966).

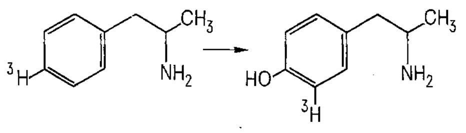
[75] D. Reed, J. Vimmerstedt, D. M. Jerina and J. W. Daly, Arch. Biochem. Biophys., submitted.

[76] M. H. Zenk, Z. Planzenphysiol. *57*, 477 (1967).

[77] N. Amrhein and M. H. Zenk, Phytochemistry *8*, 107 (1969).

[78] D. W. Russell, E. E. Conn, A. Sutter and H. Grisebach, Biochim. biophys. Acta *170*, 210 (1968).

aniline and phenylalanine are typical examples (Table II). Amphetamine-4-^{3}H **48** is oxidized to **49** both in vivo and in vitro by rodent enzymes with 80–90% retention of tritium[79], a value significantly higher than the deuterium retention of 54% observed with the analogous toluene-4-^{2}H (compound **54**, Table III). By

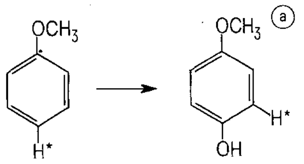
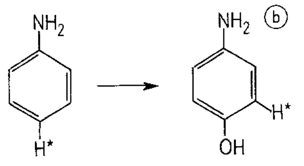
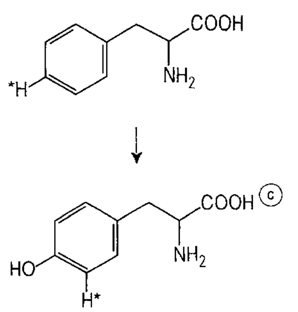


48 **49**

contrast, labeled benzenesulfonaide, **68**, and salicyclic acid, **78**, lost either deuterium (Table III) or tritium[79,80] completely during hydroxylation. Acetanilide sometimes, but not always, retains tritium to a greater extent than deuterium (Table IV).

In certain unsymmetrical compounds, isotopic hydrogen can selectively migrate and be retained in one of the two adjacent positions. This was first noted during the hydroxylation of 5-^{3}H-tryptophan **79** with trytophan hydroxylase[86]. Migration occurred selectively to

Table II. Comparison of migration and retention of deuterium and tritium

Metabolic pathway	% Migration and retention of	
	deuterium	tritium
	60	79
	6	12
	70	94

[a] Either in vitro with hepatic microsomal preparations or in vivo metabolism in rodents[75,77]. [b] In vitro with hepatic microsomal preparations from rodents[77,78]. [c] In vitro with phenylalanine hydroxylase[70,71,74].

Table III. Phenol formation from substituted benzene with hepatic monooxygenases: migration and retention of deuterium[a]

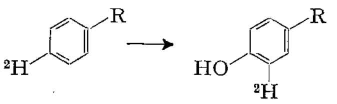
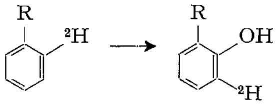
Para-Hydroxylation	% Migration and retention
	
R = **50** $-OCH_3$	60[b]
51 $-OC_6H_5$	65
52 $-C_6H_5$	64
53 $-p-C_6H_4F$	63[c]
54 $-CH_3$	54
55 -F	47
56 -Cl	54[d]
57 -Br	40
58 $-\overset{O}{\overset{\|}{C}}NH_2$	42[e]
59 -CN	41[e]
60 $-NO_2$	40[e]
61 $-NCH_3SO_2C_6H_5$	53
62 $-NH_2$	6
63 $-NH\overset{O}{\overset{\|}{C}}CH_3$	30
64 $-NH\overset{O}{\overset{\|}{C}}NH_2$	26
65 $-NH\overset{O}{\overset{\|}{C}}C_6H_5$	21
66 $-NH\overset{O}{\overset{\|}{C}}H$	19
R = **67** $NH\overset{O}{\overset{\|}{C}}CF_3$	12[f]
68 $-NHSO_2C_6H_5$	1
Ortho-hydroxylation	
	
R = **69** $-OCH_3$	60
70 $-CH_3$	43
71 $-NH\overset{O}{\overset{\|}{C}}CH_3$	59

Table III continued p. 1137

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[79] J. DALY, G. GUROFF, S. UDENFRIEND and B. WITKOP, Arch. Biochem. Biophys. *133*, 318 (1967).

[80] J. W. DALY, Analyt. Biochem. *33*, 286 (1970).

[81] J. DALY, D. JERINA and B. WITKOP, Arch. Biochem. Biophys. *128*, 517 (1968).

[82] J. DALY and D. JERINA, Arch. Biochem. Biophys. *134*, 266 (1969).

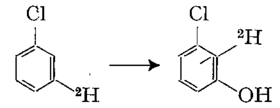
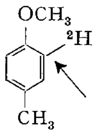
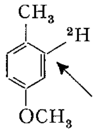
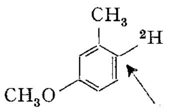
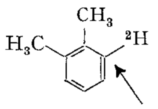
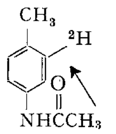
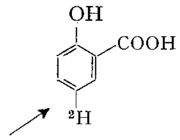
[83] D. JERINA, G. GUROFF and J. DALY, Arch. Biochem. Biophys. *124*, 612 (1968).

[84] J. DALY, D. JERINA, J. FARNSWORTH and G. GUROFF, Arch. Biochem. Biophys. *131*, 238 (1969).

[85] D. M. JERINA, J. W. DALY and B. WITKOP, Biochemistry *10*, 366 (1971).

[86] J. RENSON, J. DALY, H. WEISSBACH, B. WITKOP and S. UDENFRIEND, Biochem. biophys. Res. Commun. *25*, 504 (1966).

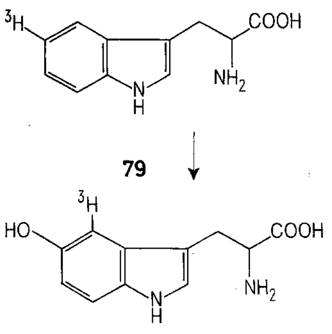
Table III (continued). Phenol formation from substituted benzene with hepatic monooxygenases: migration and retention of deuterium[a].

Para-Hydroxylation	% Migration and retention
Meta-hydroxylation	
R = **72** 	24
Di- and tri-substituted benzenes[g]	
R = **73** 	53
R = **74** 	41
R = **75** 	34
R = **76** 	35
R = **77** 	38
R = **78** 	0

[a] Results obtained in vitro with guinea-pig or rabbit microsomal preparations at pH 8 unless otherwise noted. Values for compounds **60**–**68** and **78** from reference[81] unless otherwise noted. Values for compounds **69**–**77** from reference[82]. [b] Reference[81] and[83]. [c] Reference[84]. [d] Identical value obtained in vivo with guinea-pig. [e] Value obtained in vivo with guinea-pig. [f] Reference[86]. [g] Arrow indicates position of phenolic group.

the 4-position of the 5-hydroxyindole **80**. Other examples of selective migration are observed in the metabolism of steroids **81** and **82** in rabbits[87]. Migration of isotopic hydrogen to alternate positions implies the intermediacy of two arene oxides, as for example, the 2,3 and 3,4-oxides of **81**, while selective migration implies the intermediacy of only one oxide substituted on the oxirane ring with isotopic hydrogen, as for example, the 4,5-oxide of **79** and the 2,3-oxide of **82**.

In the simplest case deuterium or tritium would be expected to migrate quantitatively during isomerization of an intermediate arene oxide to the keto-tautomer and to show an isotope effect of 3–10 in the isomerization of the ketone to the phenol. Such a simplified mechanism requires that the enzyme does not assist in abstracting isotopic hydrogen from an adjacent

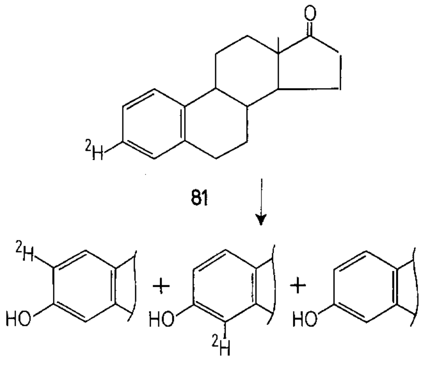


79

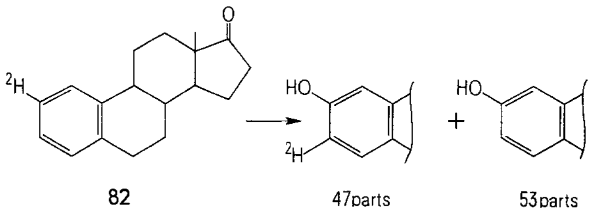
>85% retention

80

position and that retention of isotopic hydrogen be the same, regardless of whether it is initially present at the ultimate position of the phenolic hydroxyl group or in the adjacent position. This simplified mechanism adequately rationalizes the NIH shift in the formation of 1-naphthol **83**, tyrosine **84**, and 5-hydroxytryptophan **85**, but fails to explain many other compounds, such as 4-hydroxyacetanilide **86**, 4-chlorophenol **87**, 4-hydroxyanisole **88**, and others where isotopic hydrogen is preferentially retained when it is initially in the adjacent



81



82

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[87] T. Nambara, M. Numazawa and S. Akiyama, Chem. Pharma. Bull., Tokyo *17*, 2394 (1969).

[88] S. Udenfriend, P. Zaltzman-Nirenberg, J. Daly, G. Guroff, C. Chidsey and B. Witkop, Arch. Biochem. Biophys. *120*, 413 (1967).

cent position. The retentions for compounds **83–90** have been corrected where necessary for the presence of isotopic hydrogen at only one of two equivalent positions. In a single case, the biosynthesis of norpluviine **91** from cinnamic acid, the converse is true, and tritium, adjacent to the position of hydroxylation, is selectively lost[89].

A number of other aromatic substrates lose some deuterium from a position adjacent to that of the incoming phenolic hydroxyl group (**92 abc**, **93**, **94**, **95**). The possibility of selective migration, or lack of data preclude a valid comparison of the results with those that pertain during hydroxylation of the same substrate labeled with isotopic hydrogen at the point of oxygenation. The loss of tritium during in vivo conversion of geissoschizine **96** to vindoline **97** in a plant[91] is not expected.

The keto forms **99** and **102** of the phenols **98** and **101** should exhibit a significant isotope effect in the isomerization to the phenols **100** and **103**. More tritium should be retained in **100** than in **103**, since in the latter case, the tritium was originally flanked by deuterium. This expectation was verified with acetanilide **104** and anisole **105** both in vitro and in vivo in rodents, and with cinnamic acid **106** in vitro with a plant enzyme[75].

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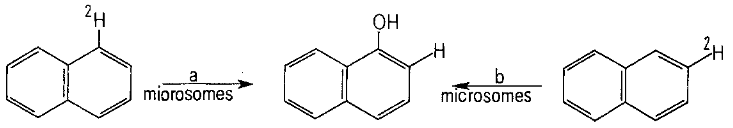
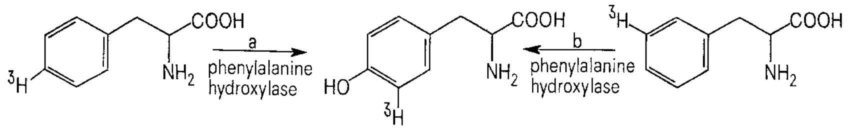
[89] W. R. BOWMAN, I. T. BRUCE and G. W. KIRBY, J. chem. Soc. D, *1969*, 1075.

[90] J. DALY and D. JERINA, unpublished results.

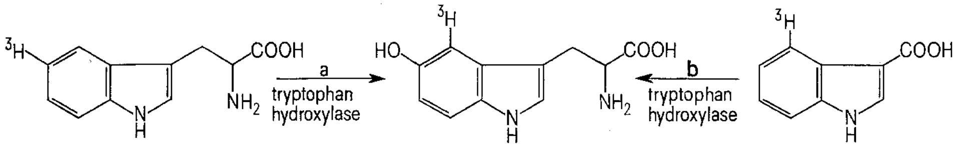
[91] A. R. BATTERSBY and E. E. HALL, Chem. Commun. *1969*, 793.



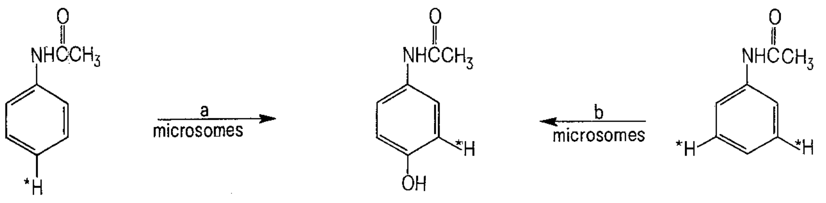
83
a) 68% ^{2}H ③
b) 68% ^{2}H



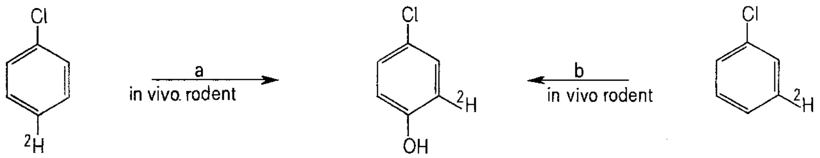
84
a) 94% ^{3}H (70)
b) 95-96% ^{3}H



85
a) 85% ^{3}H (86)
b) 85% ^{3}H

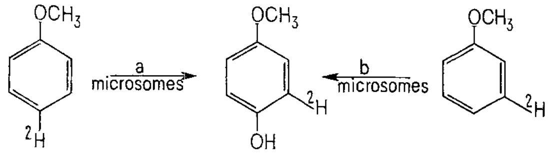


86
a) 30% ^{2}H, 45% ^{3}H (84,88)
b) 98% ^{2}H, 98%-101% ^{3}H

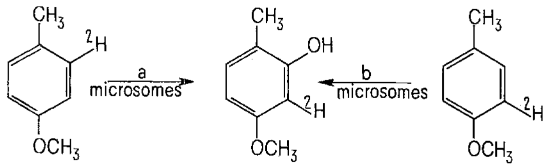


87
a) 54% ^{2}H (81,82)
b) 96% ^{2}H

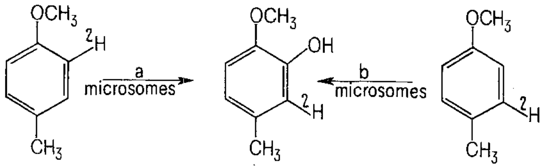
However, the multistep biosynthesis of capsaicin **129** (p. 1142) and norpluviine **91**, proceeds with identical tritium retentions (~50%) regardless of whether cinnamic acid-4-^{3}H or cinnamic acid-4-^{3}H,3,5-^{2}H serve as precursors[89]. Further investigation will be needed to clarify these apparent inconsistencies.



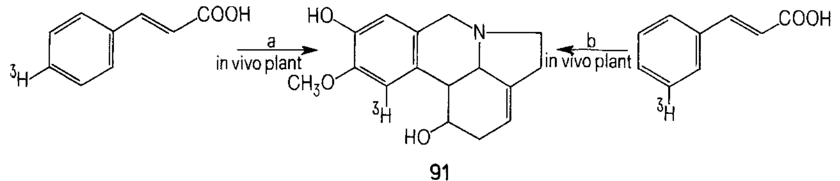
88
a) 60% ^{2}H
b) 86% ^{2}H (75,81)



89
a) 41% ^{2}H
b) 95% ^{2}H (82)

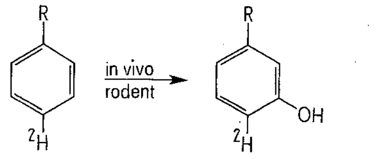


90
a) 53% ^{2}H
b) 92% ^{2}H (82)

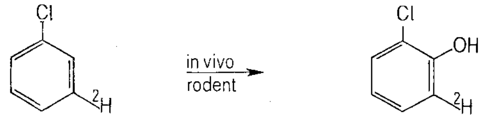


91
a) 51% ^{3}H
b) 28% ^{3}H (89)

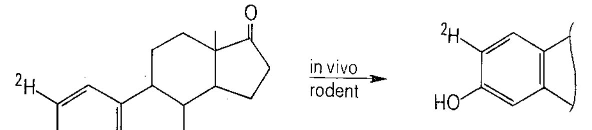
Extensive studies on the migration and retention of deuterium or tritium indicate that the magnitude of the retention is greatly influenced by the nature and position of other ring substituents. Table III presents data obtained in mammalian systems with a variety of deuterated substrates. Similar results have been obtained with anisole-4-^{2}H, **50**, and toluene-4-^{2}H, **54**, in various fungi[92, 93].



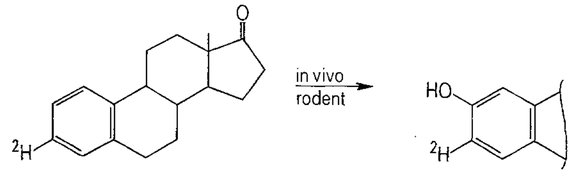
92a, R=Cl, 84% ^{2}H (78)
92b, R=F, 87% ^{2}H (90)
92c, R=CONH$_2$, 78% ^{2}H (90)



93, >93% ^{2}H (82)



94, 81% ^{2}H (87)



95, 81% ^{2}H (87)

Deuterated benzenes containing an un-ionizable substituent *para* to the deuterium (Table III, compounds **50–61**) show relatively high migration and retention of deuterium (40–64%), while those substrates (Table III, compounds **62–68** and **78**) with ionizable substituents *para* to the deuterium retain only 0–30% of deuterium. These results may be rationalized in terms of an alternate stabilization of an intermediate cation **107** (p. 1140) by ionization of the substituent to yield **108**. Such a pathway would compete with the migration of isotopic hydrogen that yields **109**. Regardless of the mechanism for the increase in the direct loss pathway, the observations have provided an empirical rule that enzymatic 'hydroxylation' *ortho*- or *para*- to a phenolic group or other highly activating groups, such as –NH$_2$ in compound **62** or –NHSO$_2$C$_6$H$_5$ in compound **68** of Table III, will result in virtually complete loss of isotopic hydrogen from the 'hydroxylated' position.

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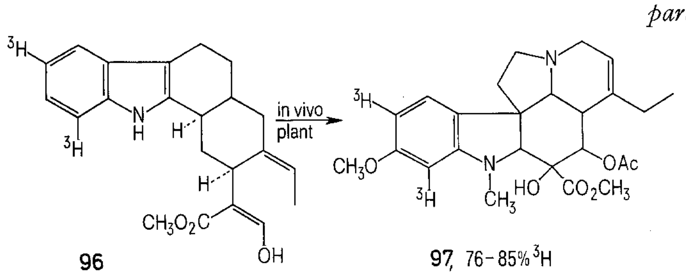
[92] B. J. Auret, D. R. Boyd, P. M. Robinson, C. G. Watson, J. W. Daly and D. M. Jerina, J. chem. Soc. D., Chem. Commun. *1971*, 1585.

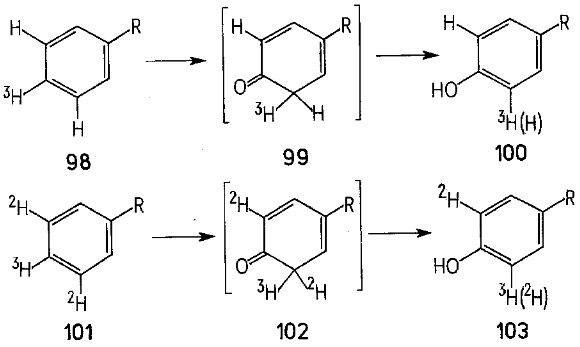
[93] J. P. Ferris, M. Fasco, F. Stylianopoulou, D. M. Jerina, J. W. Daly and A. M. Jeffrey, Biochemistry, submitted.

With acetanilide-4-^{2}H, **63**, an unusual situation exists with respect to the NIH shift. Retention values are dependent on the pH of the microsomal incubation medium, on the presence or absence of acetone in the medium, on the animal source of the microsomes and on pretreatment of animals with phenobarbital or polycyclic hydrocarbons[79, 81, 84, 88, 94]. Retentions varying from 26–58% (Table IV), have been interpreted as caused by the effects of pH and microenvironment on alternate pathways leading from **107** to **108** and **109** (X = NAcyl)[81, 84]. Likewise, retention values for closely related acylanilines, such as **63**–**67** (Table III), should also depend on conditions. Retentions for benzanilide, **65**, vary with pH[84] and for trifluoroacetanilide, **67**, with species[85]. Retentions with other aromatic substrates which do not contain an ionizable substitutent, such as compounds **50**, **52**, **53** and **56** of Table III, were not influenced by the above factors[84]. Surprisingly, hydroxylation *ortho* to the acetamido function (compound **71**, Table III) led to *ortho*-hydroxyacetanilide which retained 60% deuterium independent of species[82].



96

97, 76–85% ^{3}H



98 **99** **100**

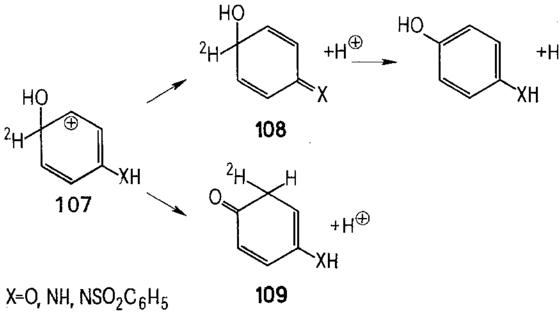
101 **102** **103**

Substrate	% Retention ^{3}H in 100	103
104, R = $\mathrm{NH}\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}\mathrm{CH_3}$, in vivo, rodent	40	26
in vitro, rodent microsomes	62	40
105, R = OCH_3, in vivo, in vitro, rodent	77–80	61–64
106, R = trans-CH-CHCOOH, in vitro, plant	90–92	67–68

Ordinarily migrations and retentions of deuterium during *ortho*- or *para*-hydroxylation are comparable as exemplified by the hydroxylations of **50** and **69** or **54** and **70** (Table III), and by hydroxylation of tritiated cinnamic acids. Thus, cinnamic acid-4-^{3}H, **46**, is *para*-hydroxylated in plants with retention of 84–90%[75–78] compared with 92% retention in *ortho*-hydroxylation of cinnamic acid-2,6-H_3, **110** to **111**, in vivo in a plant[95].

The same plant hydroxylates benzoic acid-2,6-^{3}H, **112** to **113**, with a tritium migration and retention of only 15%. The low tritium retention in the salicylic acid **113** obtained from the metabolism of **110** in this plant, is proof for the sequence in vivo **110** → **112** → **113** rather than for an alternative pathway involving initial hydroxylation of **110**. Retention values for *para*-hydroxylation of benzoic acid-4-^{3}H have as yet not been reported.

Meta-hydroxylation of mono-substituted benzenes results in a lower migration and retention of deuterium as in compounds **56** and **72** (Table III), or in the in vivo conversion of phenylalanine-3-^{3}H **114** to the mold metabolite cyclopenin **115**[72], which retains only 35% tritium as compared with >90% in the conversion of phenylalanine-4-^{3}H to tyrosine (Table II).

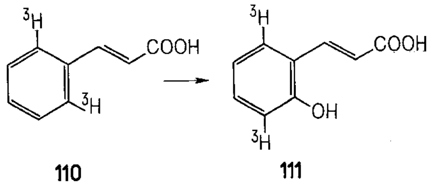


107 **108** **109**

X=O, NH, $NSO_2C_6H_5$

As mentioned above, *ortho*- and *para*-hydroxylations of phenolic substrates occur with complete loss of isotopic hydrogen from that position. A few additional examples, **116**, **117**, and **118ab**, illustrate the generality of this observation.

Recognition of the NIH shift and its mechanism, and quantitation as a function of substrate structure has



110 **111**

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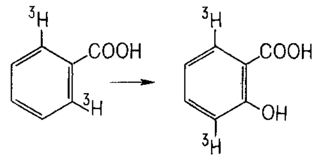
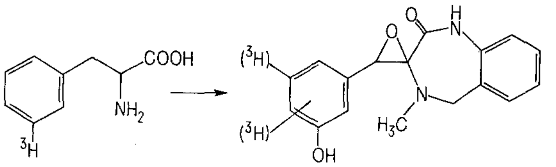
[94] M. Tanabe, D. Yasuda, J. Tagg and C. Mitoma, Biochem. Pharmac. *16*, 2230 (1967).

[95] B. E. Ellis and N. Amrhein, Phytochemistry *10*, 3069 (1971).

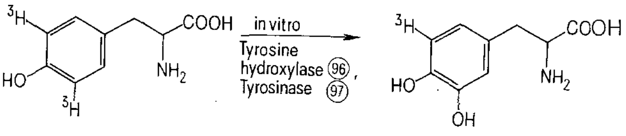
removed many uncertainties from biosynthetic studies with tritium or deuterium ring-labeled aromatic precursors. Indeed, the retention of isotopic hydrogen has been used, in conjunction with basic knowledge of the NIH shift, to elucidate in vivo biosynthetic pathways (as for example with **110** → **112**). Similarly, the mold metabolism of **119** to patulin **120** containing only 2 deuteriums[100] was predictable, since initial hydroxylation *para* to a phenolic hydroxyl should proceed with complete loss of one deuterium.



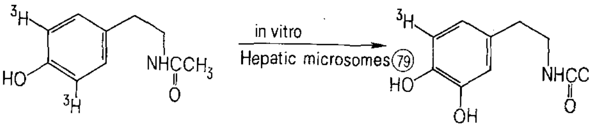
112 **113**



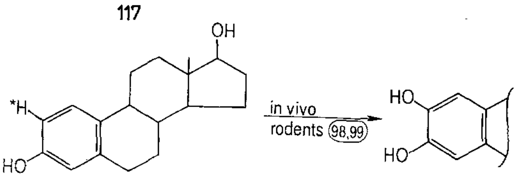
114 **115**



116

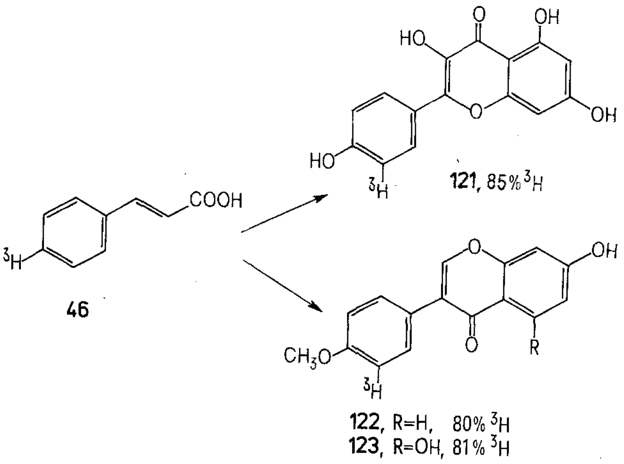


117



118a $^*H = {}^2H$
118b $^*H = {}^3H$

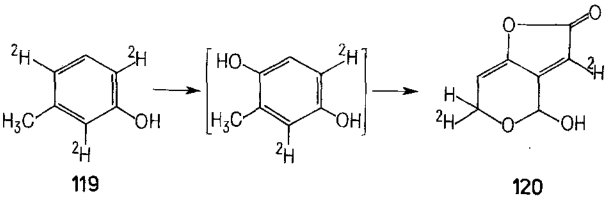
In the multistep biosynthetic pathways from cinnamic acid-4-^{3}H **46** to kaempferol **121**, formonetin **122**, and biochanin A **123** in plants[101], the initial hydroxylation of **46**, as expected, is accompanied by a tritium migration and retention of 80–85% (see **46** → **47**, p. 1135). In the conversion in plants of **46** to dihydroxylated products including quercetin **124**, rutin **125**, cyanidin **126**, leucocyanidin **127** and chlorogenin **128**[77,101] the first hydroxylation must have proceeded with a tritium migration of 60%, while the second hydroxylation, *ortho* to the new phenolic group, entails, as expected, the complete loss of hydrogen from that position; i.e., the apparent percent migration and retention of tritium has been halved by the second hydroxylation.



46

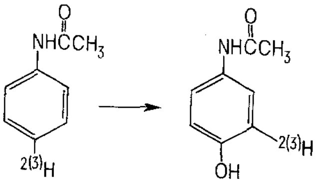
121, 85% ^{3}H

122, R=H, 80% ^{3}H
123, R=OH, 81% ^{3}H



119 **120**

Table IV. Migration and retention of deuterium and tritium during hydroxylation of acetanilide-4-^{2}(^{3})H under various conditions[79,84]

	% Migration and retention of	
	Deuterium	Tritium
Microsomal preparations		
Rabbit, pH 8	31 ± 1	45
Rabbit, pH 8, 0.45 *M* acetone	38	—
Rabbit, pH 9	24	—
Rat, pH 8	44 ± 1	45 ± 3
Rat, induced phenobarbital, pH 8	50 ± 1	63 ± 3
Rat, induced 3-methylcholanthrene, pH 8	34 ± 1	24 ± 3
In vivo		
Rat	34	38
Rat, induced phenobarbital	40	—
Rat, induced 3-methylcholanthrene	30	

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[96] T. Nagatsu, M. Levitt and S. Udenfriend, Analyt. Biochem. *9*, 122 (1964).

[97] S. H. Pomerantz, Biochem. biophys. Res. Commun. *16*, 188 (1964).

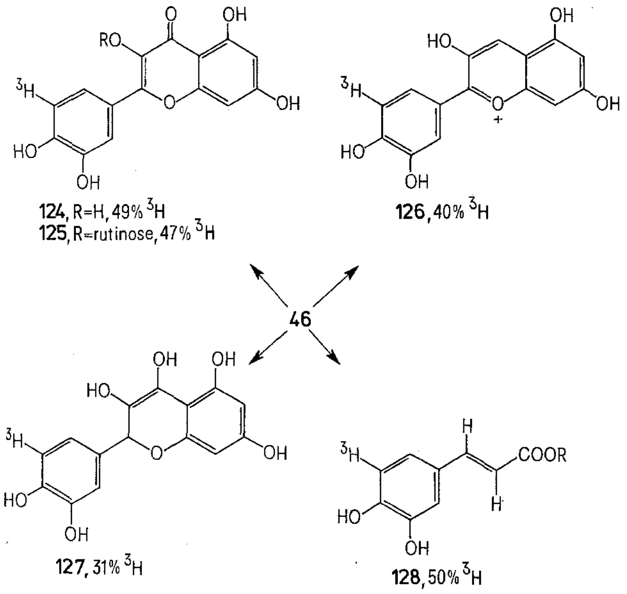
[98] T. Nambara, S. Akiyama and S. Honma, Chem. pharm. Bull., Tokyo *19*, 1727 (1971).

[99] J. Fishman, H. Guzit and L. Hellman, Biochemistry *9*, 1593 (1970).

[100] A. I. Scott and M. Yalpani, Chem. Commun. *1967*, 945.

[101] A. Sutter and H. Grisebach, Phytochemistry *8*, 101 (1969).

In plants cinnamic acid **46** is converted in vivo to capsaicin **129** and norpluviine **91** with final retentions of 50%[89]. The pathways involved in the biosynthesis of **129** and **91** are unique, since the tritium retentions still are about 50%, even with the use of cinnamic acid-3,5-^{2}H-4-^{3}H in place of cinnamic acid-4-^{3}H. Tritium is apparently lost selectively from cinnamic acid -3-^{3}H during biosynthesis of **91**[89].



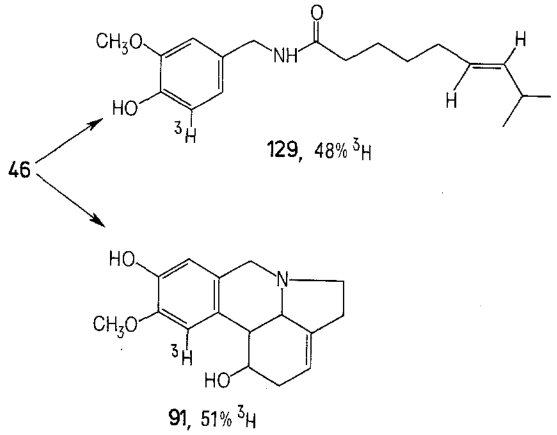
124, R=H, 49% ^{3}H
125, R=rutinose, 47% ^{3}H

126, 40% ^{3}H

127, 31% ^{3}H

128, 50% ^{3}H

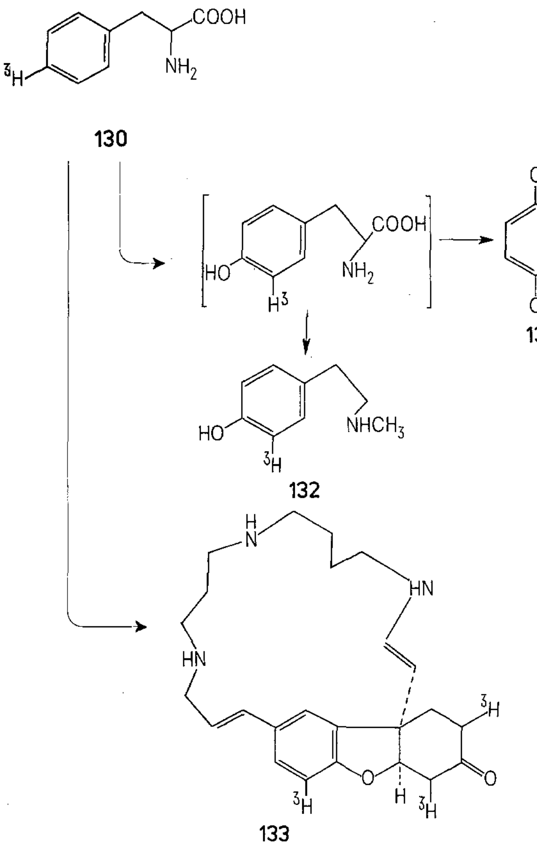
The oxidation of phenylalanine-4-^{3}H **130** in a plant to hydroquinone-3-^{3}H **131** with 87% retention of tritium[102] is not unexpected. The multi-step conversion of phenylalanine-4-^{3}H-β-^{14}C **130** in barley yields N-methyltyramine **132** with 88% retention of tritium[103]. Presumably, the reaction sequence involves initial formation of tyrosine, followed by decarboxylation and N-methylation. The biosynthesis to lunarine **133** from **130** in the plant *Lunaria blennis* occurs with a final tritium retention of 43%[104]. The migration and retention of tritium expected during 4-hydroxylation of an intermediate cinnamic acid-4-^{3}H should have led to a final retention of 68% in **133**. However, in **133**, the tritium is labilized by the keto group and may undergo partial exchange during isolation.



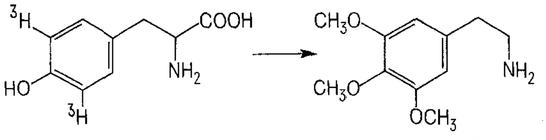
129, 48% ^{3}H

91, 51% ^{3}H

The lack of retention of tritium in mescaline **135**, derived from tyrosine-3,5-^{3}H **134**, is proof that 4-methoxyphenethylamine and 4-methoxy-3-hydroxyphenethylamine are not on the biosynthetic pathway, since such tritiated intermediates should on further hydroxylation yield mescaline containing tritium in the 2-position[105].



Selective migration of tritium may be involved in the plant biosynthesis of acronidine **137** and skimmianine **138**, from the anthranilic acid prescursor **136**[106], containing varying amounts of tritium. The existence



134

135, 0% ^{3}H

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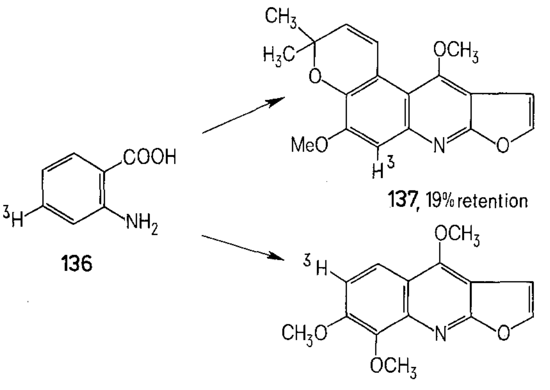
of an alternate minor pathway which involves initial hydroxylation of **136**, *para* to the amine group, followed by complete loss of tritium during formation of **138** can, however, not be excluded.



136

137, 19% retention

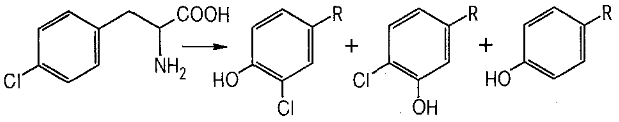
138, 10% retention

Enzyme assays.

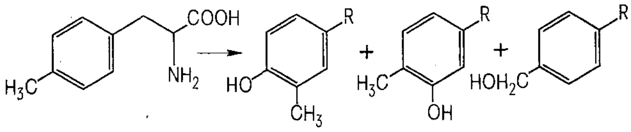
Knowledge gained from studies of the NIH shift in deuterated and tritiated aromatic substrates aided in the development of assays for the major mono-oxygenases. After phenylalanine-4-^{3}H is converted to tyrosine-3-^{3}H with phenylalanine hydroxylase[70], tritium may be selectively and quantitatively liberated from the product as water by iodination with N-iodosuccinimide. The tritiated water is then readily separated and assayed by scintillation spectrometry[107]. Tryptophan-5-^{3}H **79** is oxidized by tryptophan hydroxylase to 5-hydroxytryptophan-4-^{3}H **80**[86] from which the tritium is readily released into water by brief treatment with acid[108]. This method provides a simple and sensitive assay for tryptophan hydroxylase[109]. For a rapid assay of microsomal 'aryl hydroxylase' activity, the substrate of choice is benzene-sulfonanilide-4'-^{3}H (**68**, Table II) which on microsomal hydroxylation quantitatively generates tritiated water in the medium[110]. Tyrosine hydroxylase[96] and tyrosinase[97] are assayed by measuring formation of tritiated water during enzymatic conversion of tyrosine-3,5-^{3}H **116** to dopa. When estradiol-2-^{3}H, **118b**, is hydroxylated, tritiated water is released stoichiometrically and this reaction has been used to follow in vivo metabolism[99].

The NIH shift: Migration of halo- and alkyl-substituents.

Phenylalanine hydroxylase accepts a variety of 4-substituted phenylalanines as substrates and converts them to 3-substituted tyrosines with migration of chloro[111], bromo[111] and methyl substituents[69]. 4-Fluoro-[111] or 4-iodo-substituents[112] are, however, replaced by hydroxyl to yield tyrosine as the sole product. Such replacement of substituents also occurs to a minor extent with 4-chlorophenylalanine **139**, and 4-bromophenylalanine, but not with 4-methylphenylalanine, **140**. In the latter case, benzylic hydroxylation becomes a major metabolic route. No other examples of *simple alkyl migration* during enzymatic aryl hydroxylation have been reported. The nonspecific hepatic microsomal 'aryl hydroxylases' instead effect benzylic hydroxylation of alkyl substituents[5,6,68,113]. Phenylalanine hydroxylase does not metabolize 4-methoxyphenylalanine[114]. Tyrosine-4-^{18}O is converted to dopa-4-^{18}O by tyrosine hydroxylase without any evidence for migration of the oxygen from position 4 to 3[73].

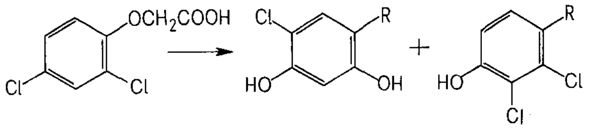


139 17parts 1part 2parts

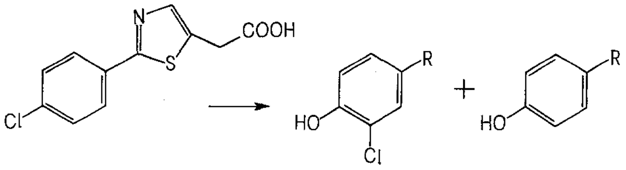


140 11parts 1part 15parts

Halogen migrations are elicited by the action of nonspecific mono-oxygenases from animals, plants and fungi. In plants[115] and fungi[116] 2,4-dichloro-phenoxy-



141 **142** 10parts **143** 1part



144 **145** 1part **146** 2-3parts

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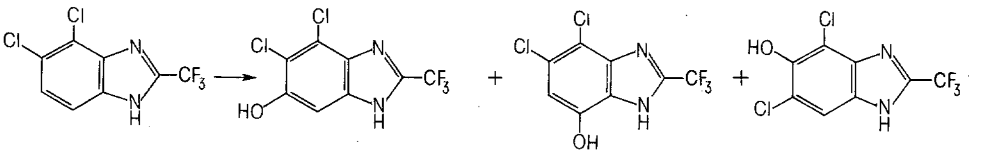
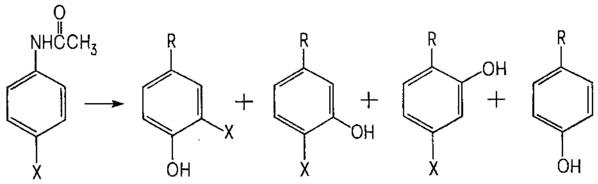


147 major minor **148** minor

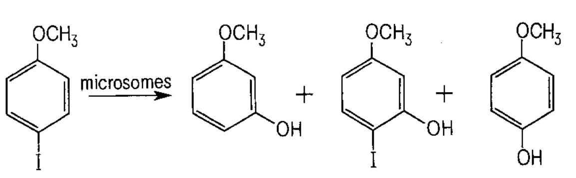
acetic acid **141** undergoes selective migration of the halogen to yield **142** as the major product.

In mammals the antiinflammatory agent **144** is metabolized to a mixture of products in which the chloro-substituent either migrates (**145**) or is replaced (**146**) by hydroxyl[117,118]. The in vivo and in vitro metabolism of the insecticidal agent **147** in rodents yields a small amount of a phenolic product **148** in which halogen has migrated[119].

Microsomal mono-oxygenases metabolize 4-chloro- and 4-bromoacetanilides, **149 ab**, to a variety of phenolic products among which a *minor* metabolite is the migration product, 3-halo-4-hydroxyacetanilide[68,120]. 4-Chloroacetanilide yields only 4-hydroxyacetanilide with rabbit liver microsomal fractions[113]. With 4-fluoroacetanilide, reminiscent of 4-fluorophenylalanine[111], the 4-hydroxy product is formed with loss rather than migration of the fluorine[113,120]. Surprisingly, 4-iodoanisole **150** yields 3-hydroxyanisole with loss of the iodo-substituent from the position adjacent to oxidative attack[68] in the same way as 3-fluoroaniline is degraded to aniline and fluoride[121]. The mechanism of loss of halogen from positions either adjacent to or at the position of the phenolic hydroxyl and its relationship to the intermediacy of arene oxides is at present unknown. Arene oxides with a halogen-substituted oxirane ring have as yet not been synthesized.

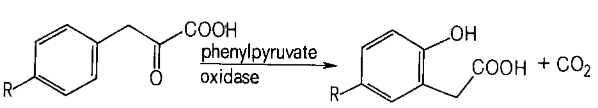


149ab				
a)X=Cl (104,11),	8parts,	18parts,	47parts,	27parts
b)X=Br (111),	4parts,	21parts,	50parts,	25parts

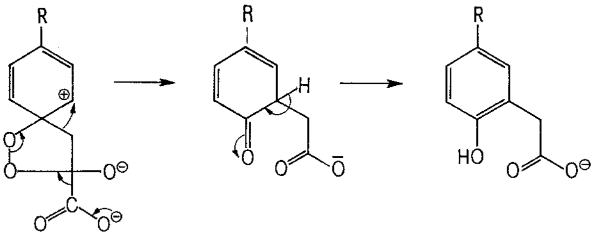


150 2parts 1part 2parts

Although the migration of the side chain in the breakdown of phenylpyruvates (**150abc**) to phenylacetic acids might be considered an example of the NIH shift, a variety of considerations indicate that an entirely different mechanism is involved. This reaction actually provides an intramolecular example of a new class of oxidative enzymes which utilize a keto acid as the reducing agent for oxygen to effect aryl or alkyl oxygenations. Examples of this class of enzymes are thymine hydroxylase[125], proline hydroxylase of collagen[126], butyrobetaine hydroxylase[127] and phenylpyruvic acid oxidase[128]. Mechanisms[128–130] proposed for the conversion of phenylpyruvates to phenylacetic acids invoke the intermediacy of a cyclic peroxide, **151**, which decarboxylates with concomitant migration of the side chain and regeneration of the aromatic nucleus.



150a R=^{3}H (122)
150b R=F (123)
150c R=OH (124)



151

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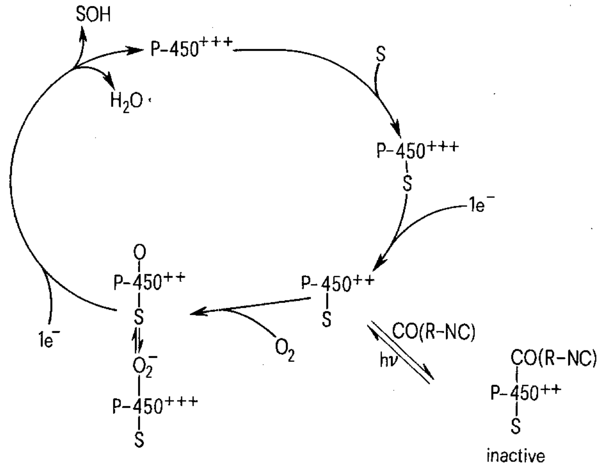
Nonenzymatic oxidations accompanied by the NIH shift and a mechanism for mono-oxygenase oxidations.

There are a great number of mono-oxygenases or mixed-function oxidases which catalyze the incorporation of molecular oxygen into aromatic and aliphatic substrates. These enzymes share a common stoichiometry; i.e., two reducing equivalents are consumed for each mole of substrate and molecular oxygen with the production of oxygenated substrate, water, and the oxidized form of the reducing agent:

$$\text{Substrate} + 2e^- + 2H^+ + O_2 \rightarrow \text{Substrate-OH} + H_2O$$

The present discussion centers on the enzymes found in the endoplasmic reticulum of hepatic cells, microsomal P-450, since the system has been well studied. Many of the other mono-oxygenases may function by similar mechanisms.

The microsomal hemoprotein cytochrome P-450 is looked upon as the terminal oxidase for oxygen activation and substrate oxidation in the electron transport chain in liver. Intensive investigation from several laboratories has lead to a working hypothesis for the action of this enzyme system[131]. In essence, the oxidized form of the hemoprotein (P-450^{+++}) binds substrate(s) and undergoes a one electron reduction. The reduced enzyme-substrate complex binds molecular oxygen. At this point superoxide, the radical anion of the oxygen molecule, has been implicated in the scheme[132]. Addition of the second electron leads, without detectable intermediates, to oxidized substrate (SOH) and water. Little is known about the key step during which substrate is oxidized.



The observation that the NIH shift is mediated by arene oxides allows considerable insight into the mechanism of oxygen activation and substrate oxidation catalyzed by mono-oxygenases. From a chemical standpoint, the formation of arene oxides would be termed an oxygen atom transfer reaction, with the reactive oxidant consisting of a neutral oxygen atom with 6 valence electrons. Such a species, equivalent to '*oxene*' in analogy to the reactions of the well known isoelectronic species *carbene* and *nitrene*, should add to alkenes and aromatics to form epoxides and arene oxides and should also insert itself between carbon-hydrogen bonds to form alcohols (or phenols). Addition reactions might be expected to be favored for oxene in the singlet state and insertion reactions in the triplet spin state. Mechanisms for the enzymatic generation of oxene have been discussed (ref. 1 and 62 and references therein).

The concept of an 'oxeneoid mechanism' agrees with the wide range of oxidations catalyzed by microsomal P-450. Oxene should be a weak and thus selective electrophile in its attack on carbon-hydrogen and carbon-carbon double bonds. In the carbon-hydrogen bond case, this accounts for the preponderance of dealkylation at heteroatoms, for benzylic and allylic oxidations, and for oxidations at tertiary positions. All of these reactions occur by insertion at electron rich carbon hydrogen bonds. Insertion reactions would be expected to take place with an isotope effect and this is indeed the case with a number of substrates[133]. Addition reactions of oxene to isolated or conjugated double bonds would form epoxides or arene oxides as observed with hepatic mono-oxygenases[133]. The product distribution of phenols from various aromatic substrates would reflect not only the electrophilic character of oxene, but also directed openings of intermediate arene oxides and specificity of the mono-oxygenase. Addition of oxene to form an intermediate arene oxide should occur without a primary isotope effect. Isomerization of arene oxides to phenols also occurs without a primary isotope effect[65]. Thus, the rate of enzymatic phenol formation should be uneffected in tritiated or deuterated aromatic substrates. This is generally the case[70,82,134,135]. Hydroxylation of deuterated zoxazoleamine is the only reported exception[136]. *Meta*-hydroxylation of nitrobenzene in vivo also occurs with an isotopic effect (K_H/K_D) of approximately 1.5[90]. These exceptions indicate that phenols are formed enzymatically in certain instances by insertion, rather than by addition.

Since carbon monoxide and ethyl isocyanide are isoelectronic with oxene it is not suprising that they inhibit P-450. The actual enzyme-generated oxidant

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[131] R. W. ESTABROOK, in *Handbook of Experimental Phyrmacology* (Eds. B. B. BRODIE and J. R. GILLETTE; Springer-Verlag, New York 1971), vol. 28, part 2, p. 264.

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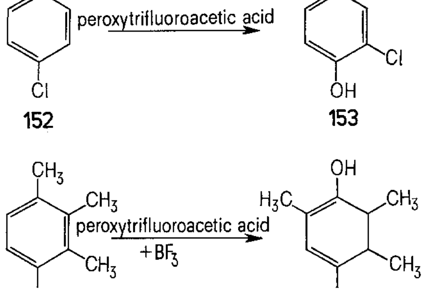
is probably an oxo-iron species (Fe=O) which shows little radical character when it reacts with aromatic substrates by addition and substantial radical character when it attacks saturated positions by insertion. This mechanistic dualism is worthy of note.

Over the past 20 years, since UDENFRIEND et al.[137–139] described a chemical system which utilized molecular oxygen and was capable of hydroxylating aromatic rings, a variety of model systems have been investigated. Criteria, such as relative reactivity of saturated positions, relative reactivity of substituted benzenes and their *o-m-p*-product ratios, as well as the range of different types of oxidation served to evaluate the resemblance of these models to enzyme systems. The advent of the NIH shift provided a new and highly sophisticated criterion by which to judge such models[62]. When phenols are formed without the NIH shift, the model system has no relevance to the enzyme-catalyzed reaction and probably hydroxylates by a radical mechanism. Systems, such as those described by FENTON (Fe^{++}, H_2O_2)[140], UDENFRIEND (Fe^{++}, O_2 and an organic reducing agent, such as ascorbate[137,138] reduced pteridines[141,142] or thiosalicylate[143]) and by HAMILTON (Fe^{+++}, H_2O_2, catalytic amount of catechol[144]) do not exhibit the NIH shift[62,145]. The model system, Fe^{++}, O_2 and thiosalicylate does form dihydrodiols from naphthalene as reported[143], but the mechanism is radical in nature and does not involve the intermediate formation of naphthalene-1,2-oxide[146]. Both *cis*- and *trans*-dihydrodiols are formed from naphthalene. Hydroxylation of deuterated acetanilide or anisole with this system did not occur with a concomitant NIH shift[90].

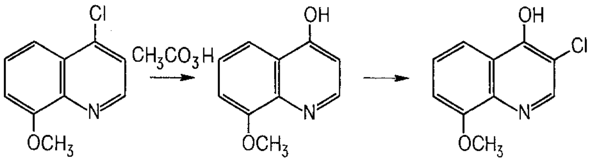
Phenols are formed from labeled anisole and acetanilide with horseradish peroxidase by a radical mechanism since the NIH shift does not occur[147]. Oxidation of phenylacetic acid-4-^{3}H to 2-hydroxyphenylacetic acid-4^3-H with plant peroxidases proceeded without migration of the side chain[148].

Peroxyacids hydroxylate a variety of deuterium- and tritium-labeled aromatic substrates with concomitant NIH shift[85,145]. Retention in general was substantially lower than in the corresponding enzymatic hydroxylations, possibly due to extensive direct loss of label during isomerization of the intermediate arene oxide in the strongly acidic medium. Migrations of halo- and alkyl-substituents have also been observed during peracid oxidation of suitable aromatic substrates[117,120,149], e.g., of **152** to **153**[120], **154** to **155** and **156**[149]. Halogen substituents may undergo an NIH shift which appears to be intramolecular but in reality is simulated by a multi-step pathway as shown for the conversion of **157** to **159** with peroxyacetic acid[150]. Initial hydrolysis yields chloride ion and **158**, which is then chlorinated to yield **159** (cf., multiple halogenations reported in ref.[85]).



152 **153**

154 **155** **156**



157 **158** **159**

Photo-activated N-oxides hydroxylate deuterated anisole, acetanilide, toluene, chlorobenzene and bromobenzene[56], with NIH shifts comparable to those of enzymatic 'hydroxylation' of the corresponding aromatic substrates. The results obtained with different photo-activated systems are, however, variable and somewhat difficult to interpret, since the intermediate arene oxides are isomerized to phenols by light[56] and as yet little is known of what effect this will have on the ultimate magnitude of migration and retention of isotopic hydrogen. However, in the case of naphthalene, the *photo*-isomerization of the intermediate naphthalene oxide appeared to be negligible under the conditions employed for the reaction[56]. Nonetheless, equal amounts of naphthalene 1,2-oxide and 1-naphthol were isolated as products from photo-activated pyridine-N-oxide

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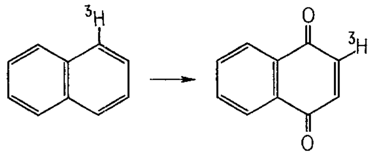
and naphthalene[56]. Presumably, a portion of this 1-naphthol was formed by an insertion mechanism rather than by isomerization of the intermediate oxide.

Perhaps model systems closest to mono-oxygenases are those which involve oxy-transition metal complexes and, in addition, show the NIH shift. A Sn^{++}, O_2 system[151,152] converts deuterated anisole, acetanilide and chlorobenzene to phenols with a significant NIH shift[62]. When 2,6-xylenol-4-^3H was oxidatively polymerized with a Cu^{++}, O_2 system, tritium migrated and was retained[153]. Similarly, chromyl acetate effects a substantial migration and retention of tritium during oxidation of **160** to **161**[154].

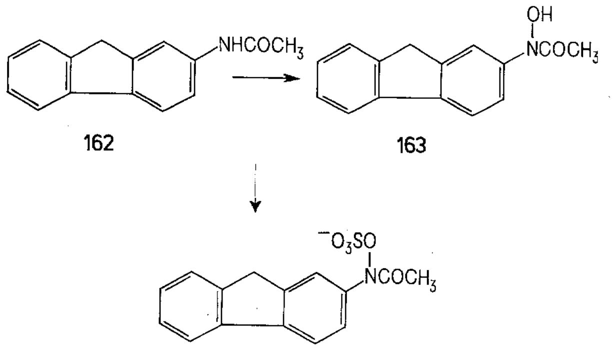


160 **161**

A combination of $Mo(CO)_6$ and *tert*-butylhydroperoxide hydroxylate deuterated anisole with an NIH shift comparable to the enzymatic process[56]. There are still no effective model systems resembling the oxo-iron species apparently involved in enzymatic reactions.

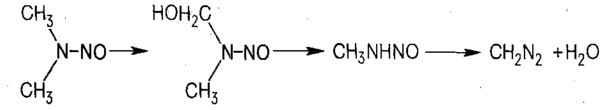
Carcinogenicity and toxicity of arene oxides.

Some xenobiotic compounds are converted in vivo to toxic or carcinogenic metabolites[155,156]. Thus, 2-acetylaminofluorene **162** is hydroxylated to the N-acetylhydroxamic acid **163** and then to the active carcinogen **164** by esterification with sulfate or acetate[157,158]. Such esters reach with nucleophilic tissue constituents. The toxic effects of dialkyl nitrosamines **165** have also been linked to the metabolic formation of a reactive electrophile, in this case a diazoalkane **166**[159], which reacts with hepatic proteins[160].



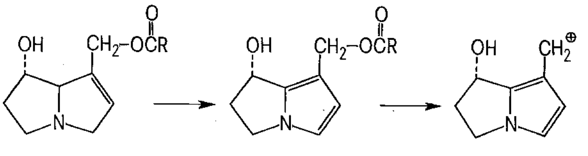
162 **163**

164

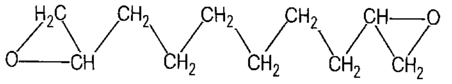


165 **166**

The hepatotoxicity of pyrrolizidine alkaloids **167** has been ascribed to the metabolic formation of an alkylating agent such as the carbonium ion species **169** derived from dehydroheliotrine **168**[161] or of a toxic epoxide[162]. Certain diepoxides, such as **170**, are carcinogenic presumably due to their alkylating ability[163]. The destruction of the heme moiety of cytochrome P-450 seen during metabolism of the allylic substrates **171** and **172**[164,165] may be linked to the fact that both compounds are metabolized to epoxides[166–168]. An alternate metabolic pathway may be allylic hydroxylation, reverse aldolization and liberation of acrolein, a highly reactive aldehyde capable of undergoing additions and condensations.



167 **168** **169**



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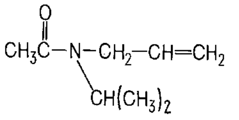

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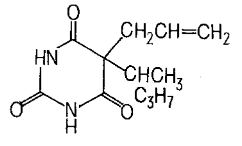

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The carcinogenicity of polycyclic compounds, such as 7,12-dimethyl-benzanthracene, is due to metabolic conversion to an active species[169], for which carbonium ions generated at the benzylic positions[169], radical cations[170] and arene oxides[1] have been suggested. With benzpyrene only the epoxide possibility need to be considered. Initial in vivo studies with polycyclic arene oxides were inconclusive[163,171,172] perhaps because such reactive compounds must be generated in situ.

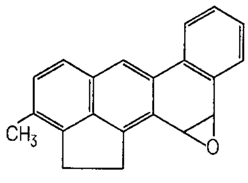
Thus, in a wide variety of compounds the metabolic generation of an electrophilic species with alkylating properties appears responsible for toxicity or carcinogenicity.

Arene oxides are opened by a variety of nucleophiles, such as azide, water, methanol and mercaptides[1,9,21,65]. Accordingly, arene oxides as reactive alkylating agents might be responsible for the metabolism-linked hepatotoxicity of halobenzenes[173], the bone marrow toxicity of benzene[174], the cytotoxicity of benzpyrene[175], lesions resulting from long-term use of aromatic drugs and carcino-genicity of polycyclic hydrocarbons. These aspects are now under active investigation in several laboratories.

Bromobenzene **173** is converted by hepatic monooxygenases to a reactive intermediate, presumably the arene oxide **174**[173]. Reaction of **174** with glutathione yields **177**, while reaction with tissue constituents evokes necrotic lesions. The metabolism-linked reaction of radioactive bromobenzene with tissue constituents provides protein fractions in which radioactivity is associated with sulfur-containing amino acids[176].

The toxicity of bromobenzene is enhanced by pretreatment of animals with phenobarbital[173], an agent which increases the hepatic levels of the monooxygenases responsible for formation of the intermediate arene oxide and is lowered by SKF-525 a[177], a potent inhibitor of these enzymes. Surprisingly, 3-methylcholanthrene, another inducer of mono-oxygenases, had the opposite effect of phenobarbital: the toxicity of bromobenzene decreased[178,179]. 3-Methylcholanthrene may induce alternate metabolic pathways for bromobenzene, such as formation of 2-bromophenol via 2-bromobenzene oxide, or else the dihydrodiol **176** might now be formed by an enzyme complex in which mono-oxygenase and epoxide hydrase are coupled. Such a concerted reaction would convert the arene **173** to the relatively nontoxic diol **176** without significant release of **174** into the medium. Evidence for such coupled mono-oxygenase-hydrase systems, inducible by 3-methylcholanthrene, has recently been adduced[12,13].

The evidence is growing that arene oxides are the active carcinogens formed from polycyclic aromatic hydrocarbons[180–182]. The 5,6-oxide of benz(a)anthracene **7**, the 5,6-oxide of dibenz(a, h)anthracene **8** and the 11,12-oxide of 3-methylcholanthrene **178** are highly active in inducing malignant transformations in rodent cells[180] in contrast to the comparatively inactive *cis*-dihydrodiols, phenols and parent hydrocarbons. A non-K-region epoxide, such as the relatively less stable 8,9-epoxide of benz(a)anthracene **10** is much



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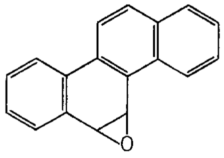

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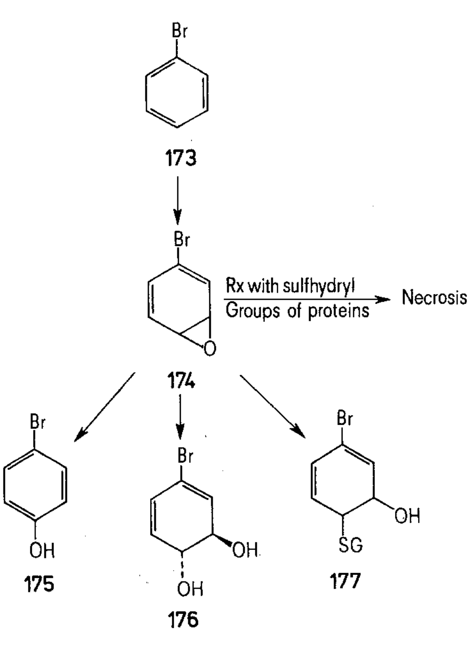

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less active than the corresponding K-region expoxide **7** in this test system[182]. K-region epoxides of noncarcinogenic hydrocarbons such as phenanthrene 9,10-oxide **6** and chrysene-5,6-oxide **179** were completely inactive[182]. Polycyclic arene oxides are active alkylating agents that bind to, and react with DNA, RNA and proteins[183–186]. Certain polycyclic arene oxides are potent mutagens in strains of *Salmonella*[187], in bacteriophages[188] and in a clone of chinese hamster cells[189], possibly because of interaction with DNA.

Arene oxides are important as metabolic intermediates capable of initiating tissue necrosis and carcinogenesis. Correlation of stability and reactivity with the structure of arene oxides will provide a sound basis for predictions of cytotoxicity. For example, alkylarene oxides which have low stability and rapidly rearrange to phenols, should not be cytotoxic or give diols[5,6]. In addition, phenols may be formed by oxygenases either directly or via intermediate arene oxides and this dualism may depend on the structure of the aromatic substrate. Compounds which form phenols directly by an insertion reaction would be expected to be nontoxic. The cytotoxic activity of intermediate arene oxides could probably be counteracted and offset by enhancing the rate of detoxification of such oxides, either through increases in levels of gluthatione or epoxide-hydrase. The present review indicates how efforts initiated to elucidate an unprecedented phenomenon, 'the NIH shift', have now opened up active research in the multidisciplinary area of formation, metabolism, toxicity, carcinogenicity and chemical reactivity of the novel labile metabolites known as arene oxides.

Zusammenfassung.

Nach der Isolierung des ersten Arenoxyds als labiler Zwischenverbindung im oxydativen Abbau von Naphthalin mit Lebermikrosomen besteht kein Zweifel mehr, dass im Stoffwechsel aromatischer Substrate ganz allgemein Arenoxyde obligatorische Primärprodukte sind, von denen sich durch enzymatische und nichtenzymatische Additionen und Umlagerungen zwangsläufig Dihydrodiole und (Prä-)Merkaptursäuren ableiten. Die Mono-Oxygenasen des Tier- und Pflanzenreichs einschliesslich der Pilze und Bakterien führen die Hydroxylgruppe unter Wanderung des ursprünglichen Substituenten ein, eine Umlagerung, die als *NIH-Verschiebung* bekannt wurde und als Beweis für das intermediäre Auftreten von Arenoxyden gültig ist. Die kurz- und langfristige Toxizität aromatischer Arzneimittel sowie die krebserregende Wirkung polyzyklischer aromatischer Kohlenwasserstoffe, wie Modellversuche andeuten, lässt sich von der Öffnung von Arenoxyden durch nukleophile Gruppen im Gewebe und kovalente Bindung an Biopolymere erklären.

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SPECIALIA

Compounds of Papilionid Caterpillars (*Baronia brevicornis* S.)

In recent years the chemical study of insects has induced a big interest in that field, specially with the discovery of the metamorphosis hormones which play a vital role within the postembrionic development of the insect.

The defense mechanism of the caterpillars of butterflies of the family Papilionidae have been investigated recently[1], finding that the principal products everted by the defensive gland, the osmeterium, are two aliphatic acids, isobutyric and 2-methyl butyric acids, which are known to play the role of larvae protectors, especially from ants[2].

Since *Baronia brevicornis* Salv. is a singular specimen of the Mexican fauna and its philogenetic characters have been studied[3], therefore it was interesting to study the larvae from the chemical point of view. We wish to report now on some of the products found in the bodies of the caterpillars of *Baronia brevicornis* S.

The caterpillars were collected in km 23 of the road from Jojutla to Cuautla, Estado de Morelos, México, being in the 5th stage of its living cycle; in average, the weight per larvae was 350 mg.

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