BIOLOGICAL OXIDATION OF NITROGEN IN ORGANIC COMPOUNDS AND DISPOSITION OF N-OXIDIZED PRODUCTS

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I. INTRODUCTION

The metabolic route of N-oxidation of a wide variety of nitrogenous compounds has received increasing attention during the past decade. Some of the reasons for this increased interest are the discovery of the natural occurrence of N-oxidized products in plant and animal tissues,[135,142,154] the use of certain N-oxy compounds in therapy,[199] and the observation that numerous drugs and other xenobiotics undergo transformation to the corresponding N-oxidized derivatives after administration to various species. N-Hydroxylation is a key reaction accounting for many of the toxic effects of aromatic amines: arylhydroxylamines act as catalysts in transferring oxygen to form methemoglobin;[429] certain N-hydroxy compounds are potent mutagens and carcinogens;[415] interactions in vivo of N-hydroxy derivatives of arylamines with proteins may yield specific antigens which lead to immunological reactions.[430]

The biotransformation of nitrogen containing molecules to N-oxidized products was discovered during the present century. Lintzel[153] detected conversion in vivo of trimethylamine to the corresponding N-oxide; this oxide had been found earlier in 1909 as a component of animal tissues.[431] In 1960, Baker and Chaykin[204] demonstrated that the enzyme system responsible for this metabolic reaction is located in the endoplasmic reticulum of living cells. The possible formation in vivo of hydroxylamines from aromatic amines was discussed by Heubner[432] in 1913. Shortly later Ellinger[433] claimed to have isolated small amounts of N-acetylphenylhydroxylamine from the blood of cats poisoned with acetanilide. Kiese[191] demonstrated formation of nitrosobenzene in the blood of dogs dosed with aniline. Liver microsomal conversion of imines to oximes was described by Parli et al.[68] More recently it has been recognized that heterocyclic compounds, such as benzodiazepines[174] or pyridines,[62,63,87] are also substrates for metabolic oxidation of the constituent nitrogen.

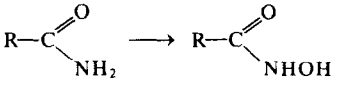
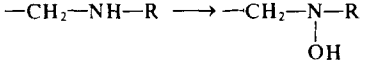
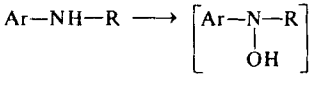
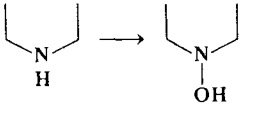
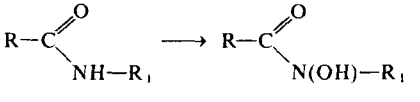
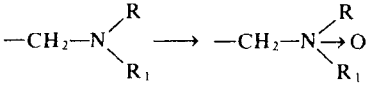
The biochemistry and pharmacology of hydroxylamines, hydroxamic acids, and N-oxides has been reviewed by Weisburger and Weisburger[434] and Bickel,[269] respectively. This review is intended to present the most important findings and to analyze the abundant data produced during the past decades.

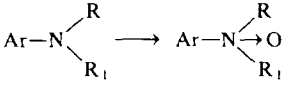
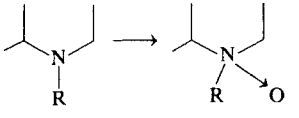
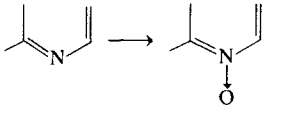
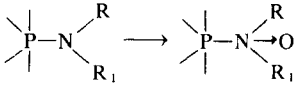
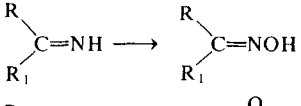
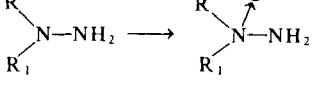
II. CHEMICAL ASPECTS OF N-OXIDATION

A. Types of Nitrogenous Compounds Undergoing N-Oxidation and Chemical Characteristics of the N-Oxidized Products

Table 1 summarizes a large variety of prototypes of nitrogen containing compounds

Table 1
TYPES OF NITROGENOUS COMPOUNDS UNDERGOING N-OXIDATION IN BIOLOGICAL SYSTEMS

Type of compound	Type of N-oxidation reaction	Model compound studied	Ref.
Aliphatic primary amine	$-CH_2-NH_2 \longrightarrow CH_2-NHOH$	Amphetamine	1, 2
		Mexiletine	3
		Phentermine	4—7
Aromatic primary amine	$Ar-NH_2 \longrightarrow Ar-NHOH$	Aniline and derivatives	8—24
		2-Naphthylamine	19, 22, 25
Primary amide		Urethane and related carbamates	26, 27
Aliphatic secondary amine		Amphetamine, N-monosubstituted	28—30
		Nortriptyline	31
		SKF 40652-A	32
Aromatic secondary amine		Aniline, N-monosubstituted	8—11, 18, 19, 22, 23
Heterocyclic secondary amine		Noratropine	36
		Norcodeine	31
		Norhyoscine	36
		Normorphine	31
		Phenmetrazine	31, 37
		2-Phenylindole	38
Secondary amide		Acetamidofluorene	39—47
		Acetamidostilbenes	48
		Acetanilides	49, 50
Aliphatic tertiary amine		Amitriptyline	51
		Chlorpromazine	52
		Dimethylamphetamine	53

Aromatic tertiary amine		Diphenhydramine	54
		Imipramine	55, 56
		Methadone	57
		Trimethylamine	58
		N,N-Dimethylaniline and analogues	8—14, 18, 19 22
Alicyclic tertiary amine		Fomocaine	59
		Nicotine	60
		Retrorsine	61
Hetero-aromatic tertiary amine		Pyridines	62—64
		Pyrimidines	65
		Quinoline	66
Tertiary amide		Octamethylpyrophosphoramide	67
Imine		2,4,6-Trimethylacetophenone imine	68
Hydrazine		1,1-Dimethylhydrazine	69

capable of being N-oxidized in biological systems: the metabolic N-oxidation of primary and secondary amines, amides and imines generally results in transformation of these compounds into the corresponding hydroxylamines, hydroxamic acids and oximes, respectively. Formation of a covalent bond between an oxygen atom and the nitrogen lone pair of electrons in tertiary amines, amides and N,N-disubstituted hydrazines yields N-oxides.

Aliphatic primary and secondary hydroxylamines have been found to be unstable compounds undergoing autoxidation; the rate of transformation to the corresponding nitroso compounds or nitrones increases as the pH of the solution is raised.[70,71] The primary oxidation products of N-hydroxyalkylamines with α-hydrogens isomerize to the oximes.[70,71] Oxidation is enhanced in the presence of trace heavy metal ions such as Cu^{2+} and Mn^{2+}, whereas other metal ions such as Fe^{2+}, Mg^{2+} or Ag^{+} have little effect on the rate of autoxidation.[70] Anions such as ClO_4^-, $SO_4^=$, Cl^- and Br^- greatly reduce the rate of autoxidation, halide ions being the most effective.[72] Furthermore, autoxidation is sensitive to small amounts of phosphate species in buffer systems.[71] There are also a few examples of stable aliphatic hydroxylamines such as N-hydroxyamphetamine,[1] N-methyl-N-hydroxyamphetamine,[30] N-hydroxy-β-methoxy-β-(3′-trifluoromethylphenyl) ethylamine,[32] N-ethyl-N-hydroxy-β-methoxy-β-(trifluoromethylphenyl)ethylamine,[32] or N-methyl-N-hydroxy-octylamine.[73]

The group of aromatic primary and secondary N-hydroxy compounds exhibits considerable differences with respect to stability of the N-oxidized products in aqueous solutions: whereas the N-hydroxy derivatives of 4-aminobiphenyl,[74] 4-aminobenzene-sulphonamide,[15] 4,4′-diaminodiphenylsulphone,[21] or 4-aminopropiophenone[75] have been found to be fairly stable in vitro, other hydroxylamines such as 4-chlorophenyl-hydroxylamine[76] or 2-naphthylhydroxylamine[77] have been shown to be labile substances. There are only a few reports in the literature on the occurrence of stable secondary arylhydroxylamines.[73,78,79] N-Hydroxynoratropine,[36] N-hydroxynorhyoscine,[36] and N-hydroxy-2-phenylindole[38] were reported to be stable heterocyclic secondary hydroxylamines.

N-Oxidation products of primary amides such as N-hydroxyurethane have been found to be stable at 22°C and pH 6.5 to 7.5; the compound decomposes in urine at pH 7.0.[26] The rate of decomposition of N-hydroxyurethane in alkaline solutions is increased by the presence of Ni^{2+}, Cu^{2+}, Mn^{2+} and decreased by Cr^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}, and Fe^{2+}.[27] Hydroxamic acids formed through N-oxidation of secondary amides usually are stable products.[39-50]

N-Oxides, formed through N-oxidation of aliphatic,[51-58] aromatic[8-14,18,19,22] and heterocyclic[59-66] tertiary amines, are salt-like, highly polar compounds of good stability under normal conditions. Because of resonance stabilization, heteroaromatic N-oxides have a more stable N-O-bond as compared with aliphatic N-oxides; they are less reducible and far less polar than the latter class of N-oxides.[80] In contrast to the thermolabile aliphatic and aromatic N-oxides, heteroaromatic N-oxides are considerably less sensitive to heat.[81]

Steric and stereochemical features of the parent amines have been recognized to govern transformation into the corresponding N-oxidation products. Table 2 shows that introduction of an ortho-, meta-, or parachloro group into the benzene moiety of aniline facilitates N-hydroxylation of the amine using rabbit liver microsomal preparations; similarly, introduction of an aliphatic residue into meta- or paraposition increases the rate of N-oxidation. N-Substitution of aniline has been found to cause a four- to five-fold increase in the rate of N-hydroxylation in rats;[84] within the class of aromatic secondary amines, the rate of N-hydroxylation appears to decrease as the chain length of the alkyl substituent on the nitrogen increases. With the exception of N,N-diethylaniline, only

Table 2
STRUCTURE-ACTIVITY RELATIONSHIP IN THE CONVERSION OF INDIVIDUAL NITROGENOUS COMPOUNDS TO THE CORRESPONDING N-OXIDATION PRODUCTS

Type of compound	Compound studied	Relative rate[a] of N-oxidation	Species	Ref.
Aromatic primary amine	Aniline	1.0	Rabbit	22
	2-Chloroaniline	2.1	Rabbit	22
	3-Chloroaniline	2.8	Rabbit	22
	4-Chloroaniline	3.8	Rabbit	22
	2,6-Chloroaniline	1.5	Rabbit	22
	2,4,6-Chloroaniline	1.8	Rabbit	22
	2-Methylaniline	0.9	Rabbit	22
	3-Methylaniline	1.9	Rabbit	22
	4-Methylaniline	2.9	Rabbit	22
	2,6-Methylaniline	0.6	Rabbit	22
	2,4,6-Methylaniline	0.2	Rabbit	22
	3-Aminopropiophenone	1.5	Dog	82
	4-Aminopropiophenone	4.0	Rabbit	83
	4-(2-Methoxy-ethoxy)-3-acetylaniline	4.7	Dog	82
Aromatic secondary amine	N-Methylaniline	1.0	Rat	84
	N-Ethylaniline	0.8	Rat	84
	N-Butylaniline	0.8	Rat	84
Aromatic tertiary amine	N-Ethyl-N-methylaniline	1.0	Guinea pig	85
	4-Chloro-N-ethyl-N-methyl-aniline	0.6	Guinea pig	85
	4-Methyl-N-ethyl-N-methyl-aniline	1.3	Guinea pig	85
	4-Phenyl-N-ethyl-N-methyl-aniline	0.2	Guinea pig	85
	N-Methyl-N-propylaniline	0.2	Rabbit	86
	N-Butyl-N-methylaniline	0.5	Rabbit	86
	N,N-Dimethylaniline	0.4	Rabbit	86
	N,N-Diethylaniline	0.4	Rabbit	86
Heteroaromatic tertiary amine	Pyridine	1.0	Rabbit	87
	3-Chloropyridine	0.9	Rabbit	87
	3-Methylpyridine	0.3	Rabbit	87
Aliphatic tertiary amine	Normethadone	1.0	Guinea pig	88
	(−)-Isomethadone	0.3	Guinea pig	88
	(−)-Methadone	0.1	Guinea pig	88
Primary amide	Methylcarbamate	1.0	Rat	26
	Ethylcarbamate	10.0	Rat	26
	Propylcarbamate	0.6	Rat	26
	Butylcarbamate	10.0	Rat	26
Secondary amide	2-Acetamidofluorene	1.0	Rabbit	89
	7-Fluoro-2-acetamido-fluorene	2.5	Rabbit	89
	3-Acetamidofluorene	0.02	Rat	47
	4-Acetamidostilbene	1.0	Hamster	48
	4'-Fluoro-4-acetamido-stilbene	0.03	Hamster	48
	4'-Chloro-4-acetamido-stilbene	0.04	Hamster	48
	4'-Bromo-4-acetamido-stilbene	0.2	Hamster	48
Hydrazine	Methylhydrazine	1.0	Pig	90
	1,1-Dimethylhydrazine	0.6	Pig	90
	1,2-Dimethylhydrazine	0.2	Pig	90

[a] Rates of N-oxidation are expressed relative to those for the reference compounds in each class of amines, which were taken as unity.

those N,N-dialkylanilines containing a N-methyl group form N-oxides on incubation with rabbit liver microsomal fractions;[86] there appear to be no strict dependence of rate of N-oxide formation on the chain length of the alternative N-alkyl residue. p-Substitution of N-ethyl-N-methylaniline with chlorine, methyl or phenyl residues partly inhibits, partly stimulates N-oxidation in guinea pigs.

N-Oxide formation from heteroaromatic tertiary amines such as pyridine is diminished, when the aromatic nucleus is 3-substituted with a chlorine or methyl residue.[87] Similarly, introduction of an α- or β-methyl substituent into the chain connecting the basic center with the aromatic system in a series of tertiary arylalkylamines gradually abolishes N-oxide formation from these compounds.[88,91]

Although formation in the rat of hydroxamic acids from a series of carbamates is markedly influenced by steric factors, the data presented in Table 2 do not allow recognition of a general principle governing N-oxidation of these primary amides. Substitution of 4-acetamidostilbene with a 4'-halogen produces a dramatic decrease in the rate of formation of hydroxamic acid in the hamster;[48] on the other hand, 7-fluoro-2-acetamidofluorene is N-hydroxylated more rapidly than 2-acetamidofluorene, the 3-isomer being almost not N-oxidized.[47,89]

Finally, monomethylhydrazine has been found to be N-oxidized more rapidly than N,N-dimethylhydrazine or 1,2-dimethylhydrazine.[90]

Stereoselective amine oxidation was observed with the R(−) and S(+) enantiomers of amphetamine: the level of N-hydroxyamphetamine formed from the S(+) enantiomer was half that formed from the R(−) form.[2] Similarly, (−)-N-benzylamphetamine was N-hydroxylated more rapidly than (+)-N-benzylamphetamine in the rabbit.[29] Further, in many species metabolism of (2'S)-(−)-nicotine leads to the preferential formation of the *cis*-diastereoisomer of nicotine-1'-N-oxide, whereas (2'R)-(+)-nicotine gives rise to more *trans*-diastereoisomer.[60]

B. Methods for the Detection of N-Oxidation Products

The detection of N-oxygenated metabolites is associated with many problems mainly arising from difficulties in managing relatively small amounts of labile products. Thus, by far the greater number of arylhydroxylamines have been quantified after conversion to the corresponding nitroso derivatives;[8-24,76,77,82-84] the amount of nitroso compound formed can be readily estimated by the method of Herr and Kiese.[92] Stable secondary hydroxylamines can be measured by their ability to reduce Fe^{3+}.[73] N-Hydroxycarbamates give colored pentacyanoferroate complexes in the presence of Mg^{2+} ions.[26] At acidic pH, N,N-dimethylaniline-N-oxide is quantitatively reduced by nitrous acid to N,N-dimethylaniline which readily forms the yellow p-nitroso derivative absorbing at 420 nm.[93] Other N-oxides can be determined by reacting the metabolites with SO_2 to yield an aldehyde and the secondary amine; the aldehyde formed is then measured by a standard colorimetric method.[94] N-Oxides formed from a series of pyrrolizidine alkaloids have been estimated by a modified Ehrlich reaction after conversion to pyrroles.[95] Clearly, all these methods, although highly sensitive, lack specificity.

Improved analytical methods for the quantification of N-oxidation products include chromatographic techniques taking into account the chemical peculiarities of the individual N-oxidized amines.

1. Paper Chromatography

Paper chromatography has been successfully employed in the separation of the N-oxidized metabolites formed from 4-chloroaniline,[96] 4-aminobiphenyl,[74] 2-naphthylamine,[25] carbamates,[26] 4-acetylaminobiphenyl,[74] 4-acetylaminostilbene,[97] 2-acetylaminofluorene,[39-44] 7-fluoro-2-acetylaminofluorene,[98] 2-acetylaminophenanthrene,[98] tri-

methylamine,[99] chlorpromazine,[52] imipramine,[55] N,N-dimethylaniline,[93] nicotine,[60,100] nictotinamide,[101] pyridylalanine,[102] and 2-phenyl-1,3-di(4-pyridyl)-2-propanol.[64] Detection of the separated N-oxy compounds is usually achieved by treatment after development of the chromatograms with specific chromogenic reagents or examination under UV light.[103] Identification of trace amounts of metabolites is often aided by the use of radio-labeled compounds.[39-44,55]

2. Thin-Layer Chromatography

Thin-layer chromatography has been found useful in the separation of N-oxidation products formed from aliphatic primary,[3] secondary,[28-30] and tertiary[51,57,104-108] amines; the same technique has been applied for the detection of hydroxylamines formed from aromatic primary amines.[16,21,77] Similarly, methods for the seperation by t.l.c. of a series of N,N-dialkylaniline-N-oxides have been described.[86,108,109] Furthermore, t.l.c. has enabled the identification of hydroxamic acids formed from primary[27] and secondary[44-50] amides. Alicyclic[36] and heteroaromatic[63,64,108,110] N-oxides, too, have been recently analyzed by t.l.c. NMR or mass spectrometry has been used to confirm the structures of the isolated metabolites[28-30,36,48,51,63,64,110]

3. Column Partition Chromatography

Column partition chromatography has been described for the separation of N-hydroxy-4-acetylaminobiphenyl[111] and N-hydroxy-2-acetylaminofluorene.[112,113]

4. Gas-Liquid Chromatographic Analysis

Gas-liquid chromatographic analysis of N-oxidation products has been hampered by the thermolability of many compounds under investigation. This made necessary the development of methods permitting conversion of these substances to heat-stable derivatives. Thus, trimethylsilylating or trifluoroacetylating agents stabilize aliphatic primary hydroxylamines to enable their analysis by g.l.c.[2-7,114]

The same procedure has been applied in the detection of labile secondary alkylhydroxylamines such as N-ethyl- and N-benzyl-N-hydroxyamphetamine[28,29] and N,N-dibenzylhydroxylamine.[33] Chemical conversion of hydroxylamines to the corresponding nitroso compounds by means of potassium ferricyanide has enabled quantification by g.l.c. of total N-oxidation products formed from 4-substituted anilines,[103] 2-naphthylamine,[115] and 4-aminobiphenyl.[115] Thermolabile hydroxamic acids formed from 4-acetamidobiphenyl[116] and 2-acetylaminofluorene,[117] have been subjected to g.l.c. after derivatization. Tertiary amine N-oxides have been chromatographed after reduction by $TiCl_3$ to the parent amines.[86,118-120]

Direct analysis by g.l.c. of more heat-stable products has been reported for the N-oxidized metabolites of certain secondary alkylamines,[30] imines,[68] and tertiary amines.[51,81,87] Usually, g.l.c. has been combined with mass spectrometry to confirm the structures of the separated metabolites.[2,4,28,51]

5. High-Pressure-Liquid Chromatography

High-pressure-liquid chromatography has been used rarely in the detection of N-oxygenated compounds. There are a few reports in the literature concerning the separation of dimethylnitrosamine,[129] azoxymethane,[129] azoxybenzene,[130] 4-chloronitrosobenzene,[121] N-hydroxy-2-acetamidofluorene,[122] fluorenone oxime,[123] N-hydroxy-4-aminobiphenyl,[437] and the N-oxides of some 3- and 4-substituted pyridines.[438]

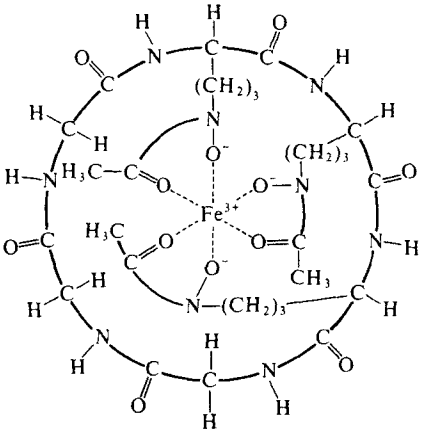
Nonchromatographic techniques for the identification of N-oxidation products include low- and high-voltage electrophoresis,[124] polarography,[131] and ESR spectrometry serving for the detection of metabolically formed nitroxide radicals.[125-128]

The problems associated with the identification and quantification of N-oxidized metabolites have been reviewed in detail by Patterson et al.[103]

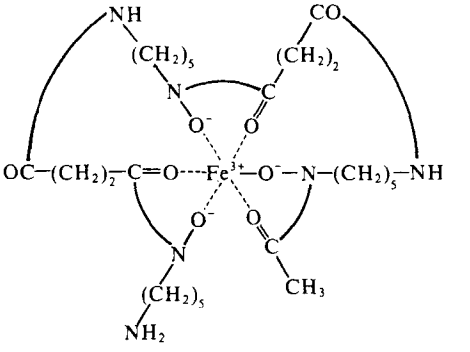
III. NATURAL OCCURRENCE OF N-OXIDIZED COMPOUNDS

A. The Occurrence of N-Oxidation Products in Microorganisms

A series of biologically active hydroxamic acids has been isolated from microorganisms, most of which contain three hydroxamic acid groups per molecule; the name *siderochrome* has been proposed for these substances. Ferrichrome (I) and its analogues are examples of this class of compounds. The substance was isolated in 1952



(I)



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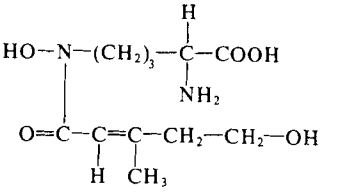
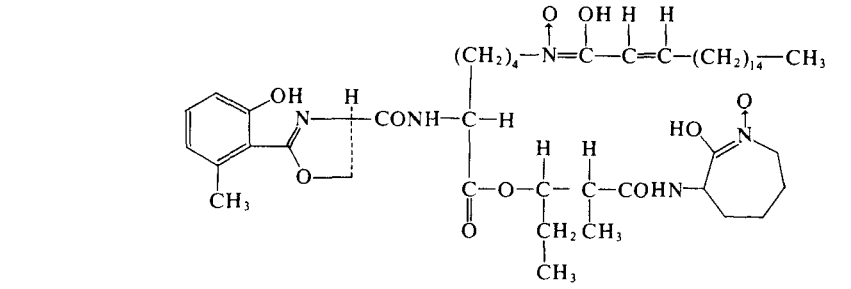


(III)

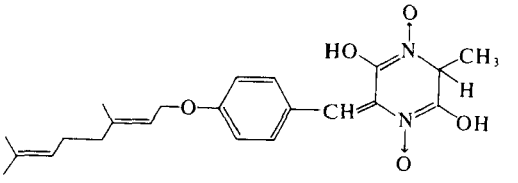
from the fungus *Ustilago sphaerogena.*[132] The significant structural feature of ferrichrome is the ability of all three hydroxamate ligands to group themselves octahedrally about the ferric ion. In contrast to the ferrichromes, ferrioxamine compounds such as ferrioxamine B (II) produced by a number of *Streptomyces* and *Nocardia* contain three hydroxamate groups inserted into a chain.[133] Fusarinine (III) constitutes the basic hydroxamate unit in fusarinine B, isolated from culture filtrates of *Fusarium roseum.*[134]

The biological importance of siderochromes is based on their ability to serve as growth factors for several microorganisms in that they are efficient and sometimes specific sources of heme iron; further, these substances serve as iron-transport agents.[135] An additional series of mono- and dihydroxamic acids have been isolated from different microorganisms: mycobactin P (IV) was purified from *Mycobacterium phlei* and is a potent growth factor for *Mycobacterium johnei.*[136] Mycelianamide (V), from *Penicillium griseofulvum,* has antibacterial properties and forms a red chelate with ferric ion.[135] Other antibiotics of hydroxamate structure are myxine (VI),[137] aspergillic acid (VII),[138] and pulcherriminic acid (VIII).[135] Hadacidin (IX) is produced by several *Penicillia;* its antitumor activity has been related to its antagonism to aspartic acid in adenylate

synthesis.[139] Iodinine (X), the pigment of *Chromobacterium iodinum,* exhibits a close structural relationship to myxine.[140] Finally, chloramphenicol (XI), a nitro compound with antibacterial activity, has been isolated from filtrates of *Streptomyces* species.[141]



(IV)



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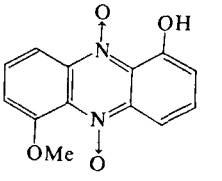

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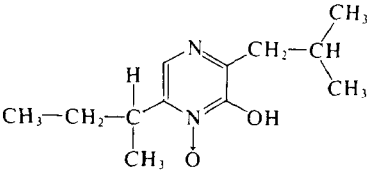

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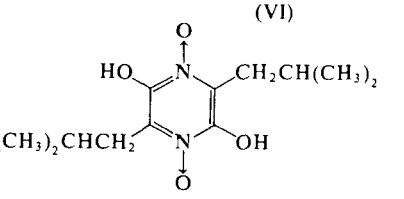

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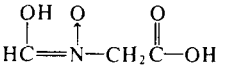

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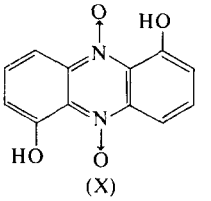

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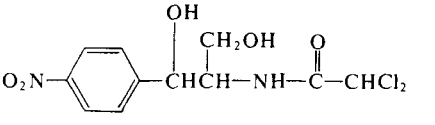


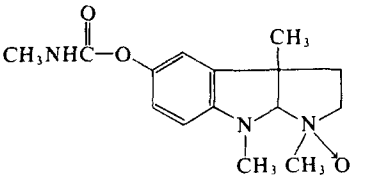
(XI)

B. The Occurrence of N-Oxidation Products in Plants

The topic has been extensively treated by Phillipson.[142] Interestingly, alkaloid N-oxides have been recognized to constitute the major part of natural N-oxidation products isolated from plant tissues. This class of compounds may be more widespread in the plant kingdom than is at present realized, since N-oxides occurring in minute amounts might have escaped detection. Further, alkaloid N-oxides tend to be more water soluble than the corresponding tertiary bases and hence are lost during normal isolation and separation procedures. On the other hand, formation of artifacts by autoxidation of a tertiary parent amine during the process of isolation cannot always be ruled out.

The first naturally occurring heterocyclic N-oxide reported was the *indole alkaloid* geneserine (XII) obtained from the Calabar bean *Physostigma venenosum.*[143] Other natural indole alkaloid N-oxides of importance are harmanine, reserpine N-oxide, and strychnine N-oxide.[142] The group of *pyrrolizidine alkaloid* N-oxides comprises some dozen of compounds such as senecionine (XIII) or indicine.[142] Severe hepatotoxic actions, observed after ingestion of the parent amines, are likely to be not related to transformation into the N-oxides[144] of the bases. Other groups of alkaloid N-oxides

include a few *quinolizidine alkaloid* N-oxides such as oxymatrine (XIV),[142] *piperidine alkaloid* N-oxides such as N-methylpiperidine N-oxide (XV),[142] and *nicotine* N-oxides. Nicotine-1′-oxide (XVI) has been observed as a constituent of fermented tobaccos.[145] There is, however, doubt as to whether nicotine N-oxide is a natural product or formed during tobacco fermentation. It has been proposed to be an intermediate in the formation of nornicotine from nicotine.[146]



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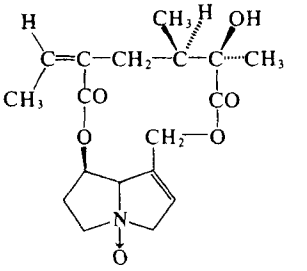

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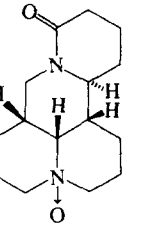

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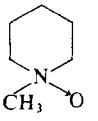

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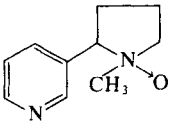


(XVI)

The role of alkaloid N-oxides in the plant is not understood. Although Wenkert[147] suggested that N-oxides formed in the plant might be intermediates in the pathway leading from amino acids to alkaloids, such concept lacks definite experimental proof.

β-Nitropropionic acid (hiptagenic acid) is another natural N-oxidation product occurring in the bark of the tree *Hiptage mandoblata;* the compound has been isolated from the hydrolysate of hiptagin, a crystalline toxic glycoside.[148]

C. The Occurrence of N-Oxidation Products in Animal Tissues

So far known, trimethylamine N-oxide represents the only example of a natural N-oxy compound occurring in animal tissues. The N-oxide was found to be abundant in marine fish such as herring,[149] teleosts,[150] and elasmobranchs,[150] but to be almost absent in freshwater species.[151] Further, trimethylamine N-oxide was found to occur in cephalopods,[150] crustaceans,[150] intervertebrates,[152] and marine mammals.[152] Small amounts of the N-oxide have also been detected in normal human urine; these are believed to be of dietary rather than endogenous origin.[153] For detailed information on the biochemistry of trimethylamine N-oxide the reader is referred to an article of Cheymol et al.[154]

There have been many speculations on the biological functions of trimethylamine N-oxide. It has been argued that the N-oxide might represent an end-product in nitrogen metabolism far less toxic than ammonia.[151] Further, the N-oxide has been proposed to play an important role in cellular redox processes.[155] In spite of all such concepts the biological importance of trimethylamine N-oxide remains obscure.

IV. N-OXIDATION OF AMINE COMPOUNDS AFTER IN VIVO ADMINISTRATION

Early attempts at demonstrating N-oxidation of aromatic amines in vivo were

unsuccessful. Thus, aniline is noticeably absent from Table 3 since evidence for its presence in urine as phenylhydroxylamine has not been shown.[189] The inability to detect phenylhydroxylamine in the urine of animals dosed with aniline mainly results from rapid reaction of phenylhydroxylamine with oxygen and hemoglobin to yield nitrosobenzene.[190] Hence, N-oxidation products of aromatic amines were first discovered in the blood.[96,191] Obviously, ring substitution of the aniline molecule favors urinary excretion of N-oxidized metabolites.[17,21,76,97,158-160] Cramer et al.[113] reported isolation of N-hydroxy-2-acetylaminofluorene from the urine of rats fed on a diet containing 2-acetylaminofluorene; the hydroxamic acid was present only in a conjugated form, later shown to be the O-glucosiduronic acid.[192] Other hydroxamic acids are excreted, too, as conjugates which are readily split by β-glucuronidase (EC 3.2.1.31).[26,113,158,185-187] N-Hydroxy metabolites of some arylamines have been found to be excreted in urine as conjugates which readily decompose in slightly acid solution.[76] Radomski et al.[193] obtained evidence of the presence of a glucuronic acid conjugate of N-hydroxy-4-aminobiphenyl in the urine of dogs, which was found to hydrolyze during incubation with β-glucuronidase or at pH 3.

Table 3 lists a large number of tertiary amines being converted to the corresponding N-oxides in vivo. These include drugs, such as hypnotics,[183] neuroleptics,[164-184] antidepressants,[51,163,165] tranquilizers,[174] analgesics,[57,181,182] antihistaminics,[166,178] and antihypertensives,[175,177,180] and other xenobiotics.[66,101,124,161,162,171-173] The discovery of transformation in vivo of drugs into N-oxides has stimulated interest in this class of N-oxidized products. Early reports of Polonovski et al.[194] suggesting morphine-N-oxide to have the same action as the parent compound, one fourth of its activity, but considerably lower toxicity did not stand the test of time.[195] Chlorpromazine-N-oxide is less potent than the parent amine or desmethylchlorpromazine, but more potent than chlorpromazine sulfoxide.[196] Imipramine-N-oxide has been found to exert less pronounced actions than imipramine with equal or decreased toxicity.[197] Similarly, chlorcyclizine-N-oxide[178] and guanethidine-N-oxide[180] exhibit lower activity than the unchanged drugs. On the other hand, diallylmelamine-N-oxide is 20 times more potent than the parent compound.[198] The lower toxicity of many N-oxides as compared with the parent amines has been attributed to the highly polar character of this type of N-oxidized metabolites facilitating renal excretion.[53] The observations with metabolically formed N-oxides have encouraged the search for synthetic N-oxides exerting biological activity; meanwhile, an increasing number of such products have been introduced into therapy.[199]

The amount of N-oxidation product excreted varies widely with the animal species,[76,89,171] strain,[200,201] and sex.[202] Several factors which affect the formation and excretion of N-oxidized metabolites have been described. Thus, after partial hepatectomy a seven-fold increase in urinary excretion of N-hydroxy-2-acetylaminofluorene has been observed in rats dosed with 2-acetylaminofluorene, while adrenalectomy decreased the excretion by 50%.[203] Rats continuously fed on a diet containing 2-acetylaminofluorene show increasing urinary excretion of N-hydroxy-2-aminofluorene.[113] Similarly, in rats pretreated with either 3-methyldimethylaminoazobenzene or ethionine the fraction of 2-acetylaminofluorene transformed into the N-hydroxy derivative is increased.[203] Pretreatment of dogs with phenobarbital stimulates urinary excretion of the N-oxidation products formed from p-phenetidine[17] and 2-naphthylamine.[77] Analogously, pretreatment of mice with phenobarbital increases the urinary level of pyridine-N-oxide after intraperitoneal administration of pyridine, whereas 3-methylcholanthrene has no appreciable effect.[171] The appearance of morphine-N-oxide in the urine of rats dosed with morphine has been claimed to be entirely dependent on the concomitant administration of tacrine or amiphenazole;[181] it has been concluded that these agents effect accumulation of the N-oxide by inhibition of its further metabolism.

Table 3
COMPOUNDS SHOWN TO BE EXCRETED IN THE URINE AS N-OXIDATION PRODUCTS

Type of compound	Compound administered	Species	Ref.
Aliphatic primary amine	Mexiletine	Man	3
	Phentermine	Rabbit	6
	Chlorphentermine	Man	156
		Guinea pig	156
		Rhesus monkey	156
	Cyclohexylamine	Rabbit	157
Aromatic primary amine	4-Chloroaniline	Rabbit	76
		Dog	76
	4-Ethylaniline	Rabbit	76
		Dog	76
	p-Phenetidine	Man	158
		Rat	17
	4-Aminopropiophenone	Guinea pig	76
	3-Aminopropiophenone	Rabbit	76
		Guinea pig	76
	4-Aminobiphenyl	Rhesus monkey	159
	Benzidine	Man	158
	Dichlorobenzidine	Man	158
	4,4'-Diaminodiphenylsulfone	Man	21
		Dog	21
	4-Aminoazobenzene	Rat	160
	4-Aminostilbene	Rat	97
	1-Naphthylamine	Man	158
	2-Naphthylamine	Dog	77
	2-Aminofluorene	Rabbit	76
Aliphatic tertiary amine	Trimethylamine	Chicken	124
	Diethylaminoethanol	Rat	161
	Dimethyloctylamine	Rat	162
	Amitriptyline	Dog	51
	Imipramine	Rat	163
	Chlorpromazine	Man	164
	Doxepin	Rat	165
		Dog	165
	Orphenadrin	Man	166
	Methadone	Rhesus monkey	57
	Clotexamide	Rat	168
	Benzydamine	Rat	169
Aromatic tertiary amine	Dimethylaniline	Rat	162
	4-Dimethylaminobiphenyl	Rat	170
	4-Dimethylaminostilbene	Rat	170
Heteroaromatic tertiary amine	Pyridine	Mouse	171
		Rat	171
		Hamster	171
		Guinea pig	171
		Rabbit	171
		Ferret	171
	3-Chloropyridine	Guinea pig	171
	3-Methylpyridine	Mouse	171
	3-Acetylpyridine	Rat	172
	Nicotinamide	Mouse	101
	Cotinine	Rhesus monkey	173
	Bromazepam	Dog	174
	2-Phenyl-1,3-di(4-pyridyl)-2-propanol	Dog	64
	N,N-Diallylmelamine	Dog	175
	Quinoline	Guinea pig	66

Table 3 (continued)
COMPOUNDS SHOWN TO BE EXCRETED IN THE URINE AS N-OXIDATION PRODUCTS

Type of compound	Compound administered	Species	Ref.
Alicyclic tertiary amine	Arecoline	Rat	176
	Cinnarizine	Rat	177
	Chlorcyclizine	Rat	178
	1-Diethylcarbamyl-4-methyl-piperazine	Rat	179
	Guanethidine	Rat	180
	Morphine	Rat	181
	3-(β-Morpholinoethoxy)-1H-indazole	Man	182
		Dog	182
		Rat	182
	Methaqualone	Man	183
	Nicotine	Man	119
	Perazine	Man	184
Primary amide	Urethane and related carbamates	Man	26
		Rabbit	26
		Rat	26
Secondary amide	Acetanilide	Man	158
	4-Chloroacetanilide	Hamster	185
	4-Cyclohexylacetanilide	Rabbit	186
	Phenacetin	Man	187
	3-Acetylaminobiphenyl	Rabbit	186
	4-Acetylaminobiphenyl	Rabbit	186
	4-Acetylaminoazobenzene	Hamster	188
		Mouse	188
	4-Acetylaminostilbene	Rat	97
	2-Acetylaminonaphthalene	Man	158
	2-Acetylaminofluorene	Rat	113
	7-Fluoro-2-acetylaminofluorene	Rat	98
	2-Acetylaminophenanthrene	Rabbit	186
	3-Acetylaminophenanthrene	Rabbit	186
Aliphatic imine	2,4,6-Trimethylacetophenone imine	Rat	68

In contrast to this report, Phillipson et al.[34] were unable to detect increased urinary excretion of morphine-N-oxide in rats simultaneously dosed with the tertiary alkaloid and tacrine. Simultaneous administration of SKF 525-A with either dimethylaniline or dimethyloctylamine to rats increases the urinary levels of the N-oxides formed from these amines.[162] This compound, however, does not influence excretion of the N-oxide metabolically formed from diallylmelamine.[175]

V. ENZYMOLOGY OF N-OXIDATION

A. Site of N-Oxidation and General Reaction Mechanism

The site of N-oxidation of amine compounds was discovered by Baker and Chaykin.[204] In experiments with hog liver homogenates and subcellular fractions, the microsomal fraction was detected to be abundant in N-oxygenating activity for trimethylamine; the reaction required oxygen and NADPH, NADH being a less efficient electron donor. Similar observations were made soon thereafter by Kiese and Uehleke,[84] who studied the N-oxidation of some aromatic amines by rat liver homogenates and microsomal subfractions. Meanwhile a large number of amines has been recognized to undergo conversion to the corresponding N-oxidation products in aerobic microsomal fractions

from various tissues fortified with NADPH. These include aliphatic primary,[1,5,7] secondary,[28,33] and tertiary[52,56] amines; aromatic primary,[9,10,12,14,15,17,20,21,23,24] secondary,[8-10] and tertiary[8-10,12-14,18,73,86,118,205,206] amines; heterocyclic secondary[36] and tertiary[61-63,207] amines; primary[27] and secondary[40,42,44,45,48,49] amides; hydrazines[69] and imines.[68]

By using ^{18}O Baker and Chaykin[58] demonstrated the oxygen atom inserted into the amine substrate during the N-oxidation reaction to be derived from molecular oxygen and not from water. Similar results were obtained when the incorporation of ^{18}O into p-thioanisidine,[23] (+)-amphetamine,[208] and 2,4,6-trimethylacetophenone imine[68] during N-oxidation of these compounds was investigated. From these findings the stoichiometry of microsomal N-oxidation reactions can be formulated according to

$$R-NH_2 + O_2 + NADPH + H^+ \longrightarrow R-NHOH + NADP^+ + H_2O \quad (1)$$

As can be seen, N-oxidation bears the character of a mixed-function oxidation reaction.[209]

As yet two microsomal mixed-function oxidase systems have been recognized to be involved in the N-oxidation of amine substrates; these will be dealt with below.

B. Cytochrome P-450-dependent N-Oxidation Reactions

In 1958, reports by Garfinkel[210] and Klingenberg[211] described the presence of a carbon monoxide binding pigment in mammalian hepatic microsomes. Evidence that this pigment is a typical b-type hemoprotein was presented by Omura and Sato.[212] Its name, cytochrome P-450, is derived from the unique wavelength of the absorption maximum in the Soret region in the difference spectrum produced by the CO-ferrocytochrome adduct. Cytochrome P-450 is ubiquitous in nature and is, presumably, an ancient enzyme in the evolutionary sense (Table 4). Induction,[213,214,215] immunological,[215,217] kinetic,[217-218] and binding[217,219] studies presented evidence for the existence of multiple forms of cytochrome P-450, some of which were purified to apparent homogeneity.[220]

Cytochromes P-450 purified after pretreatment of rats with either phenobarbital or 3-methylcholanthrene contain about 480 amino acid residues; notable differences are seen in the contents of lysine, glutamate, proline, glycine, alanine, valine, leucine, and tryptophan.[217] The heme moiety of the pigment is ferroprotoporphyrin IX.[221] Recent investigations indicate mercaptide sulfur ligation at the fifth ligand of heme in cytochrome P-450.[222] A molecular weight of about 50,000 daltons has been estimated for highly purified hemoprotein preparations by polyacrylamide gel electrophoresis in the presence of SDS.[223] The spectral properties include an extinction coefficient of 91 mM^{-1} cm^{-1} and peaks in the absolute oxidized spectrum at 390, 414 to 418, 530 to 535, 564 to 568, and 642 to 644 nm; the absolute reduced spectrum shows peaks at 410 to 414, and 540 to 542 nm in the absence of CO and 447 to 452 and 550 to 552 nm in its presence.[223]

In the molecular events occurring during mixed-function oxidation reactions, reducing equivalents are transferred from NADPH to cytochrome P-450 by a microsomal flavoprotein which functions as a NADPH-cytochrome P-450 reductase; this enzyme, most probably, is related to the flavoprotein known since 1950 as NADPH-cytochrome c reductase.[225] Evidence for the involvement of the reductase in mixed-function oxidation reactions comes from experiments with reconstituted systems containing highly purified cytochrome P-450 and detergent-solubilized NADPH-cytochrome P-450 reductase.[220] The detergent-solubilized flavoprotein has a molecular weight of about 78,000 daltons and contains two flavins (both FMN and FAD) as prosthetic groups;[226] the oxidized enzyme has absorption peaks at 380 and 455 nm and a

Table 4
OCCURRENCE OF CYTOCHROME P-450 IN THE MICROSOMES FROM SEVERAL ANIMAL SPECIES

Species	Organ	Cytochrome P-450 (ΔA/mg of protein)
Mouse	Liver	0.086
Rat	Liver	0.097
Rabbit	Liver	0.117
Pig	Liver	0.057
Sheep	Liver	0.071
Goat	Liver	0.070
Chicken	Liver	0.023
Bob White Quail	Liver	0.029
Japanese Quail	Liver	0.039
Turkey	Liver	0.031
Large Mouth Bass	Liver	0.022
Drosophila	Whole body	0.012
Eye gnats H. bishopi	Whole body	0.019
Eye gnats H. pallipes	Whole body	0.014
Eye gnats H. pusio	Whole body	0.015
Tobacco hornworm	Gut	0.012
Tobacco budworm	Gut	0.034
Housefly-CMSA	Abdomen	0.031
Housefly-Fc	Abdomen	0.034

From Kulkarni, A. P., Smith, E., and Hodgson, E., *Comp. Biochem. Physiol.*, 54B, 509, 1976. With permission.

shoulder at 476 nm.[226] Its *de novo* synthesis is stimulated by pretreatment of animals with phenobarbital.[227]

As illustrated in Figure 1, the sequence of reactions occurring during the cyclic function of cytochrome P-450 can be enumerated as follows:

1. The low spin ferric pigment reacts with a molecule of substrate resulting in the formation of an enzyme-substrate complex; due to modification of the electron density distribution surrounding the heme, addition of substrate produces a distinctive optical change in the difference spectrum.[228] Schenkman et al.[229] designated those difference spectra exhibiting absorption maxima around 385 to 390 nm and absorption minima at about 420 nm as type I and those exhibiting a characteristic peak around 420 to 435 nm and a trough between 390 to 410 nm type II.[229] Type II spectra are predominantly given by a series of basic amines, such as aniline, n-octylamine, phenylhydrazine, imidazole, pyridine, and nicotinamide.[230,231] N-Substituted anilines usually elicit type I spectral changes.[232] The type I spectral perturbation is associated with a change in the spin state of the ferric hemoprotein to its high spin form.[233]
2. The complex of substrate with ferricytochrome P-450 undergoes a one electron reduction to give the ferrocytochrome P-450-substrate complex;[234] this electron originates from NADPH and is transferred via NADPH-cytochrome P-450 reductase.[220] Type I compounds have been found to accelerate the NADPH-dependent reduction of ferric cytochrome P-450, whereas type II substrates usually decelerate it.[235]

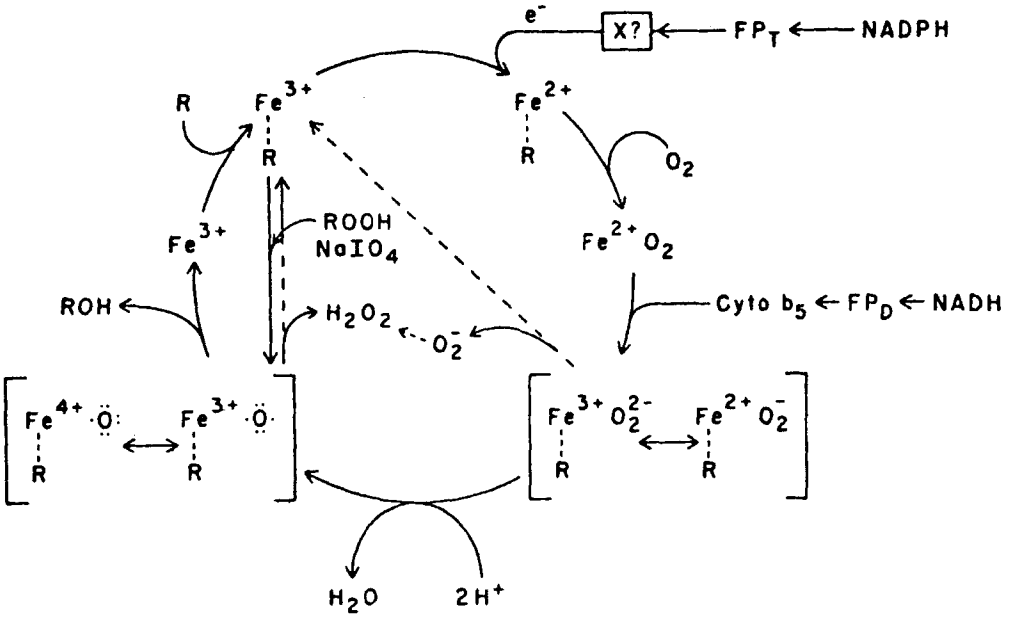


FIGURE 1. Mechanism of microsomal mixed-function oxidation reactions catalyzed by cytochrome P-450. Fe is the heme iron of the hemoprotein, R is the substrate and ROOH is an organic hydroperoxide.

3. In the presence of oxygen the reduced cytochrome P-450-substrate complex reacts rapidly to form a ternary complex of oxygen, substrate and hemoprotein, which is assumed to represent the initial step in oxygen activation by cytochrome P-450.[236]
4. Oxycytochrome P-450 is further reduced to a complex of oxygen, substrate, and ferric cytochrome P-450 which contains two electrons; the source of the electron required for the conversion of oxycytochrome P-450 to peroxycytochrome P-450 remains controversial.[237,238] Reduced cytochrome b_5, another microsomal hemoprotein, may function in this capacity.[239] A series of amine compounds, such as aniline, N,N-dimethylaniline, aminopyrine and ethylmorphine, were shown to decrease the steady-state reduced level of this pigment during NADPH-driven mixed-function oxidation.[238,239,240]
5. Peroxycytochrome P-450 is likely to lose one molecule of water resulting in the formation of an oxene complex of cytochrome P-450.[241] Studies on the peroxidatic activity of cytochrome P-450, using hydroperoxides to initiate substrate oxidation, indicate a close similarity of the proposed oxene complex to Complex I of other peroxidases.[242]
6. Cytochrome P-450 coupled with a molecule of substrate and active oxygen undergoes a transformation whereby oxygen reacts with the substrate to give oxidized product and ferric hemoprotein.

The oxygenating activity of the cytochrome P-450 system can be modified by various agents. Thus, pretreatment of animals with compounds such as barbiturates, aromatic hydrocarbons, steroids, insecticides, and a large number of drugs results in induction of different forms of cytochrome P-450 with differing drug metabolizing capacity.[243] On the other hand, pretreatment of animals with cobaltous chloride diminishes the heme content of hepatic microsomes and prevents induction of hemoproteins by phenobarbital.[244]

Other agents turned out to be potent inhibitors of the cytochrome P-450 enzyme system. Carbon monoxide competes with oxygen for ferrous cytochrome P-450 thus inhibiting many mixed-function oxidation reactions; the affinity of the oxidase for CO has been shown to depend on the rate of NADPH-driven electron flux.[245] The CO complex of reduced cytochrome P-450 can be dissociated by monochromatic light of the wavelength 450 nm, and this property has been used to identify the role of cytochrome P-450 in many mixed-function oxidation reactions.[246] Similarly, metyrapone produces a CO-like difference spectrum with reduced cytochrome P-450; this led to the suggestion that one of its interactions is with the oxygen binding site of the hemoprotein.[247] Noncompetitive inhibition occurs at low metyrapone concentrations and competitive inhibition at high concentrations of the base.[247]

Inhibition of mixed-function oxidation reactions by SKF 525-A and a series of methylenedioxyphenyl compounds such as piperonyl butoxide is likely to result from cytochrome P-450-mediated metabolism of these compounds: metabolic intermediates bind tightly to the oxidase, preventing its further participation in oxygenating reactions.[248] Imidazoles were detected to be even more potent inhibitors of the cytochrome P-450 system.[249] Other agents, such as mercurials[250,251] and detergents,[251] effect inhibition of mixed-function oxidations by converting cytochrome P-450 into the inactive P-420 form. Finally, treatment of animals with barbiturates containing an allyl side chain yields metabolites which cause breakdown of cytochrome P-450 heme.[252] For a detailed information on the biochemistry of cytochrome P-450 the reader is referred to a comprehensive treatise by Sato and Omura.[253]

The role of cytochrome P-450 in the N-oxidation of various amine compounds has been extensively studied. Among other techniques, modifiers of the mixed-function oxidase have been most successfully applied to probe the enzyme system. Thus, reports of Kampffmeyer and Kiese[254] suggested that cytochrome P-450 is not involved in the rabbit liver microsomal N-hydroxylation of aniline, since carbon monoxide apparently did not inhibit this reaction. A reappraisal of the effects of CO on this N-oxidation reaction[9] revealed that Kampffmeyer's observations were based on inadequate experimental conditions: inhibition by CO of rabbit liver microsomal N-hydroxylation of aniline can be only observed, when the concentration of microsomal protein in the assay media is considerably lower than 5 mg/mℓ (Figure 2). Under such conditions CO, at a pressure 50 times that of oxygen, inhibits N-hydroxylation of aniline by almost 50%. Surprisingly, calculations of the Warburg partition coefficient (K), which is an expression of the ratio of CO to O_2 required for 50% inhibition, disclosed that the CO-sensitivity of the aniline N-oxidase becomes markedly smaller and the O_2-sensitivity accordingly greater as the ratio of CO to O_2 in the atmosphere increases.[13] This has been interpreted to mean participation in this N-hydroxylation process of at least two forms of cytochrome P-450 with differing affinities for oxygen and carbon monoxide.[13] Similarly, in pork liver microsomal fractions CO transforms the system N-hydroxylating aniline to one with an approximately three-fold higher affinity for oxygen.[13] 4-Chloroaniline is another substrate investigated; at a ratio of CO to O_2 equal to 49, the rabbit liver microsomal N-hydroxylation of this compound is diminished by 44%.[22]

Further evidence for the involvement of cytochrome P-450 in the N-hydroxylation of anilines comes from experiments with inhibitors assumed to compete with aniline for the heme binding site of the hemoprotein. Indeed, metyrapone has been found to effect competitive inhibition of 4-chloroaniline N-hydroxylation in rabbit liver microsomal fractions.[20,107] Pyridine and n-octylamine diminish the rate of N-oxidation of aniline, 4-chloroaniline, and 4-toluidine in hepatic microsomal fractions from guinea-pigs and hamsters.[24] Moreover, SKF 525-A and piperonylbutoxide, known to block cytochrome P-450-catalyzed oxidations through tight binding of their metabolic intermediates to the

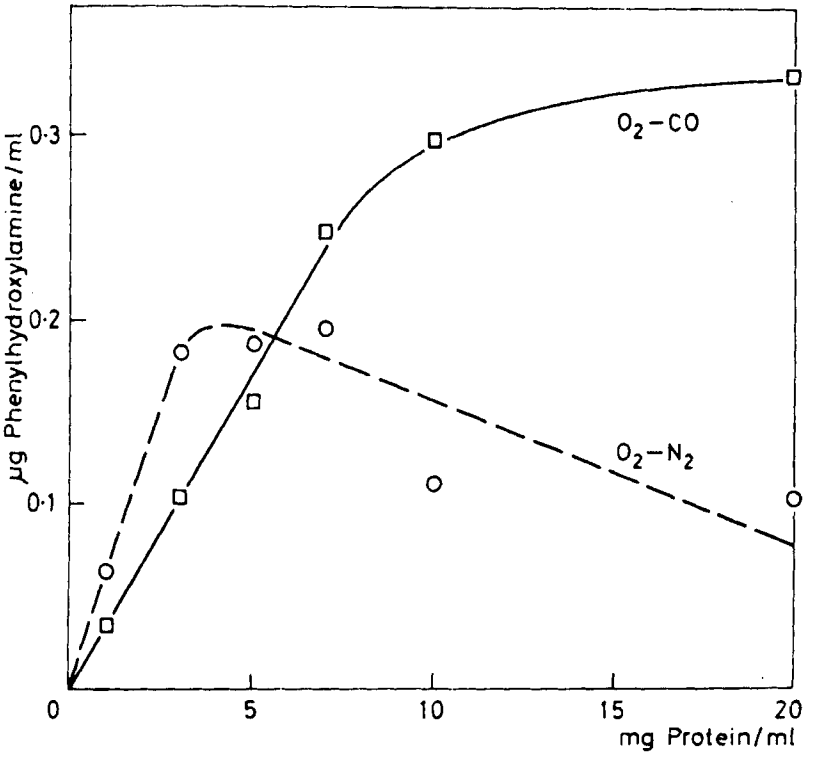


FIGURE 2. The influence of increasing concentrations of rabbit liver microsomal protein on inhibition by CO of N-hydroxylation of aniline. The symbols indicate: o, rate of N-hydroxylation under 13 torr oxygen and 650 torr nitrogen; □, rate of N-hydroxylation under 13 torr oxygen and 650 torr CO. (From Heinze, E., Hlavica, P., Kiese, M., and Lipowsky, G., *Biochem. Pharmacol.*, 19, 641, 1970. With permission.)

pigment, inhibit N-hydroxylation of 4-chloroaniline in rabbit liver microsomes.[22] The conversion of cytochrome P-450 into the P-420 form by mercurials or sodium deoxycholate results in a concomitant decrease in the rate of N-hydroxylation of aniline, 4-chloroaniline and p-phenetidine in rabbit liver microsomes.[8,18,22] Similar effects are observed after hemoprotein destruction by irradiation of microsomal suspensions with UV light.[18]

Pretreatment of rabbits with phenobarbital causes considerable stimulation of liver microsomal N-oxidation of aniline, 4-chloroaniline and 4-toluidine,[8,24] whereas pretreatment of the animals with 3-methylcholanthrene causes only minor effects.[24] Finally, the specific activity for 4-chloroaniline N-hydroxylation by liver microsomes of newborn rats closely parallels the increase of cytochrome P-450.[22]

The kinetic properties of the cytochrome P-450-dependent aniline N-oxidase have been studied in detail. Liver microsomal N-hydroxylation of aniline proceeds most rapidly at pH around 7.5.[8] With rabbit liver microsomes, the apparent Michaelis constant (K_m) for aniline has been determined to be 1 to 7 mM.[8,24] With $K_m = 0.2$ mM,4-chloroaniline exhibits higher affinity for cytochrome P-450.[22,24] These values differ considerably from that for the so-called optical dissociation constant (K_S) of aniline, which has been calculated to be 40 mM in rabbit liver microsomal fractions.[8] The apparent Michaelis constant for oxygen, as measured in terms of rabbit liver microsomal N-hydroxylation of aniline, was found to range from 5 to 6 μM.[8,12]

Studies on the influence of pH and temperature[10,14] on the apparent Michaelis

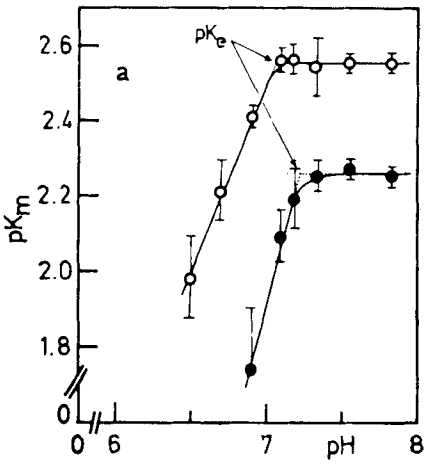


FIGURE 3. Effect of pH and temperature on the apparent K_m value for aniline. Measurements of N-hydroxylation of the arylamine were performed at 26°C (●) and 36°C (○). (From Hlavica, P. and Kehl, M., *Xenobiotica*, 6, 679, 1976. With permission.)

constant (K_m) for aniline and the maximal velocity of N-oxidation (V_{max}) of the amine in rabbit liver microsomes suggested the involvement in aniline activation of a free functional group in the enzyme with pK around 7 (Figure 3); the apparent heat of ionization of this group (ΔH_i) was calculated to be 26.8 kJ mol^{-1}.[14] Photo-oxidation experiments in the presence of rose bengal gave a pH titration curve closely resembling that of an amino acid with a single pK_a of about 6.0 (Figure 4).[14] Further, aniline N-oxidase was strongly inhibited by diethyl pyrocarbonate at pH 6.0; catalytic capacity was partially restored by treatment of the enzyme with neutral hydroxylamine.[14] These results are compatible with the assumption that imidazole might play a role in the catalytic conversion of aniline to phenylhydroxylamine.

The N-oxygenating capacity of cytochrome P-450 for anilines has been shown to vary with the species investigated. Hepatic microsomes from rabbits, cats, and guinea-pigs N-oxidize aniline at different rates.[255] With respect to N-hydroxylation of aniline, these laboratory animals also exhibit differing sensitivity toward several inhibitors of drug metabolism.[255] Similarly, the apparent K_m and V_{max} values for hepatic microsomal N-hydroxylation of aniline, 4-chloroaniline, and 4-toluidine demonstrate a large species difference.[24]

N-Hydroxylation of aniline occurs in a number of tissues other than liver.[9,22] Table 5 lists the rates of N-oxidation in microsomal fractions prepared from various organs of the pig. Most interestingly, microsomes from corpora lutea N-hydroxylate aniline to a greater extent than do hepatic microsomes although the concentration of cytochrome P-450 in corpus luteum microsomes is only 16% that in liver microsomes. Uehleke reported the maximal rate (V_{max}) of N-hydroxylation of 4-chloroaniline in rabbit lung microsomes to be twice that in liver microsomes;[22] the K_m values measured with both tissues were identical.

The involvement of cytochrome P-450 in the N-hydroxylation of aromatic primary amines has been confirmed by experiments with amine compounds other than aniline. In rabbit liver microsomal fractions, p-thioanisidine is N-hydroxylated to yield 4-methylmercapto phenylhydroxylamine;[23] a secondary, nonenzymic, reaction has been

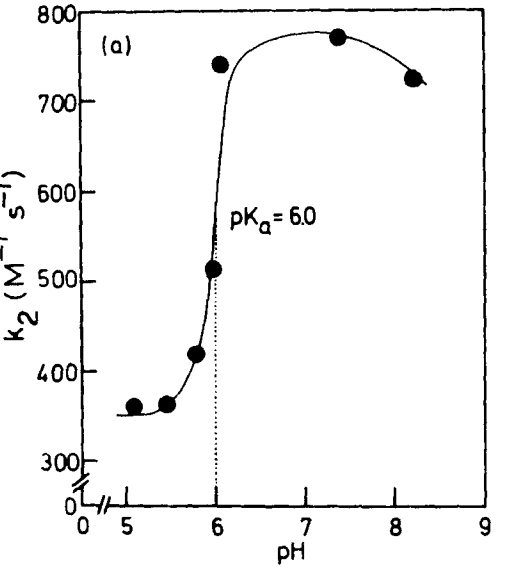


FIGURE 4. Effect of pH on the second-order rate constant for photoinactivation of the rabbit liver microsomal monooxygenase(s) catalyzing N-hydroxylation of aniline. Experiments were performed in the presence of 5 μM rose bengal. (From Hlavica, P. and Kehl, M., *Xenobiotica*, 6, 679, 1976. With permission.)

Table 5
N-HYDROXYLATION OF ANILINE IN MICROSOMAL FRACTIONS PREPARED FROM VARIOUS TISSUES OF THE PIG

Organ	Phenylhydroxylamine formed (μg/mℓ)	Cytochrome P-450 (nmol/mg of protein)
Liver	2.0	0.61
Corpus luteum	3.2	0.10
Ovary (stroma)	0.08	n.d.*
Ovary (young pig)	0	n.d.
Testicle	0.28	0.19
Retained testicle	0	0.12
Hermaphrodite testicle	0.63	0.22
Lung	0.43	n.d.
Kidney	0.07	0.30
Pancreas	0.03	n.d.
Spleen	0	n.d.
Bladder mucosa	0.15	n.d.
Thymus	0.04	n.d.
Thyroid	0.03	n.d.
Adrenal	0.04	0.3
Lymph node	0.03	n.d.
Brain	0	n.d.

* n.d. = Not detected.

From Heinze, E., Hlavica, P., Kiese, M., and Lipowsky, G., *Biochem. Pharmacol.*, 19, 641, 1970. With permission.

shown to transform the primary N-oxidation product to 4,4′-bismethylmercapto azobenzene. A 4:1 CO-O_2 mixture inhibits formation of the hydroxylamine by 30%, SKF 525-A being without effect.[23]

N-Hydroxylation of 4-aminobiphenyl by rabbit liver microsomes is considerably stimulated by pretreatment of the animals with phenobarbital.[16] Similarly, phenobarbital pretreatment of rabbits also increases the rate of N-hydroxylation of 4,4′-diaminodiphenylsulphone (dapsone) in hepatic microsomes.[21] Inhibition of the reaction by CO and metyrapone indicates that this process is a typical cytochrome P-450-dependent mono-oxygenation.[21] The enzymatically formed hydroxylamine is converted into 4-amino-4′-nitrodiphenylsulphone by nonenzymic oxidation in the presence of atmospheric oxygen.[256] Finally, administration of phenobarbital to rabbits enhances the rate of liver microsomal N-hydroxylation of 2-napthylamine.[257] Metyrapone effects competitive inhibition of the reaction.[107] The apparent Michaelis constant (K_m) for N-hydroxylation of the amine has been determined to be 0.3 to 0.5 mM;[257] this value is within the range of that for the optical dissociation constant (K_S).[257]

Cytochrome P-450 appears to participate, too, in the N-hydroxylation of aliphatic primary amines. In rat and rabbit liver microsomal fractions N-hydroxylation of phentermine is inhibited by CO, n-octylamine, SKF 525-A, and cyanide.[4,7] N-Oxidase activity is increased after phenobarbital but not after 3-methylcholanthrene administration to rats.[7] Evidence has been obtained for the involvement in this reaction of a two-enzyme system in rabbits,[4] whereas the kinetic data from rats are consistent with a single-enzyme system.[7] Storage at −70° C of rabbit liver microsomes greatly destroys the N-oxygenating activity;[4] under the same conditions rat liver microsomal activity is unchanged.[7] The relative order of N-hydroxylation of phentermine in three animal species examined is rabbit > guinea pig > rat.[4]

Spectral evidence for the participation of cytochrome P-450 in the N-oxidation of aliphatic and aromatic primary amines comes from the observation of formation of product-ferrocytochrome complexes absorbing between 448 and 455 nm. Thus addition of p-chloroaniline, sulfanilamide or dapsone to rat liver microsomal fractions in the presence of oxygen and NADPH results in the gradual formation of metabolite-ferrohemochromes absorbing at 448 to 452 nm (Figure 5).[258] Under comparable conditions amphetamine produces a 455 nm metabolic complex.[259,260] The preferred formation of such complexes from hydroxylamines[260,261,262] and the inhibition of complex formation from amphetamine but not from N-hydroxyamphetamine by the N-oxidase inhibitor dithiothreitol[260] suggests a key role of oxidation at the nitrogen in adduct formation. Experiments with compounds containing an oxidized nitrogen suggest a nitroso[263,264] or nitroxide[260] intermediate.

Pretreatment of animals with phenobarbital stimulates metabolic complex formation.[258,259] Carbon monoxide,[263] metyrapone,[258] SKF 525-A,[264] and sodium deoxycholate[263] inhibit formation of cytochrome P-450 product adducts. Complexes formed from aliphatic primary amines are stable in the presence of sodium dithionite, those formed from aromatic primary amines are destroyed by the reducing agent.[258]

Reports in the literature on the involvement of cytochrome P-450 in the N-oxidation of secondary amines are scarce. The formation of N,N-dibenzylhydroxylamine from dibenzylamine in rabbit liver microsomes has been studied by Beckett and Gibson.[33] The reaction is inhibited by modifiers of the cytochrome P-450 system such as CO, SKF 525-A, imidazole, and p-chloromercuribenzoate. A synergistic effect of NADPH and NADH has been observed suggesting the second electron to be supplied by cytochrome b_5. Pretreatment of rabbits with phenobarbital enhances the further metabolism of the hydroxylamine formed.

The stimulation of formation of N-hydroxy-2-phenylindole from 2-phenylindole in

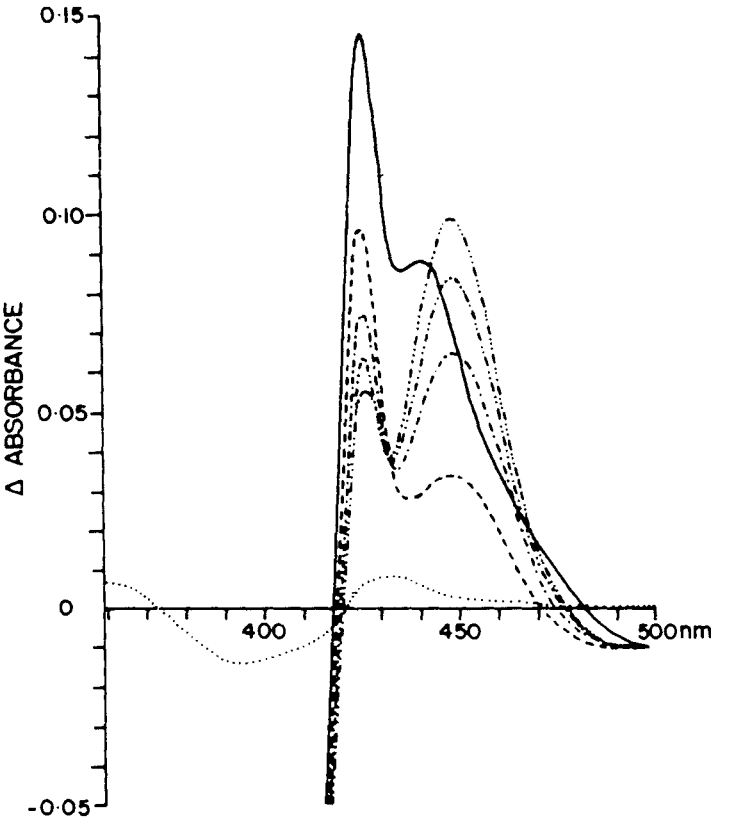


FIGURE 5. The interaction of p-chloroaniline with microsomal cytochrome P-450. The symbols indicate: (...), difference spectrum observed after the addition of the amine to oxidized hepatic microsomes from phenobarbital-treated rats; spectral change monitored 0.5 (— — —), 1.5 (— . —), 2.5 (— .. —), and 4.5 (— ... —) min after the addition of NADPH to the sample cuvette; (— ——), spectrum measured after the addition of sodium dithionite to the experimental cell. (From Franklin, M. R., *Chem.-Biol. Interact.*, 14, 337, 1976. With permission.)

hepatic microsomes from rabbits and guinea pigs after pretreatment of the animals with 3-methylcholanthrene suggests participation of the cytochrome P-448 system in the N-oxidation of the heterocyclic secondary amine.[38]

The metabolic route of N-hydroxylation of amides of yield hydroxamic acids has received increasing attention; this reaction is likely to be an essential activation step for the mutagenic and carcinogenic actions of some of these compounds. The formation of N-hydroxyurethane from urethane in vivo has been reported to be stimulated by pretreatment of mice with 3-methylcholanthrene; SKF 525-A inhibited N-hydroxylation at 24 hr after administration.[27] These findings hint at a cytochrome P-448-dependent mechanism involved in the N-oxidation of the primary amide. Similar results were obtained with hepatic microsomes from hamsters. Such preparations catalyze N-hydroxylation of 4-chloroacetanilide[49] and phenacetin.[50] Both reactions are inhibited by an atmosphere of CO-O_2 (9:1) or by the addition of piperonylbutoxide to the assay media. Pretreatment of the animals with 3-methylcholanthrene enhances the rate of N-oxidation of the secondary amides.[49,50] Liver microsomes from mice,[45] rats,[47] hamsters,[45] and guinea pigs[47] mediate N-hydroxylation of 2-acetamidofluorene. The

reaction is inhibited by carbon monoxide,[40,44,45,47] the Warburg partition coefficient (K) being 7.97 in control animals and 4.23 in hamsters pretreated with 3-methylcholanthrene.[40] Pretreatment of the animals with the polycyclic hydrocarbon stimulates N-hydroxylation,[40,44,47] treatment with cobaltous chloride[45,47] impairs N-oxidation of the acetamide. Similarly, purified immune-globulin against microsomal NADPH-cytochrome c(P-450) reductase partially inhibits liver microsomal N-hydroxylation of 2-acetylamino-fluorene.[45] The reaction requires phospholipid;[43] detergents impair N-oxidation of the amide.[42] Final evidence for the participation of cytochrome P-448 in the N-hydroxylation of 2-acetylaminofluorene was presented by experiments with a reconstituted system containing highly purified hemoprotein and NADPH-cytochrome c reductase.[39,41] Genetic factors controlling this N-oxidation process have been studied by Nebert[265] and Thorgeirsson et al.;[46] the responsiveness of different strains of animal species to induction of mono-oxygenase activity by polycyclic aromatic compounds has been claimed to be associated with the Ah^b allele.[265,266]

Imines appear to be another type of nitrogenous compounds N-oxidized by the cytochrome P-450 system. In rat liver microsomal fractions, oxime formation from 2,4,6-trimethylacetophenone imine is inhibited by CO, SKF 525-A, and DPEA; prior treatment of the rats with phenobarbital increases oxime formation.[68]

Cytochrome P-450 has been proposed to participate, too, in the N-hydroxylation of certain hydrazines. Using acetyl- and isopropylhydrazine, Nelson et al.[267] observed stimulation of covalent binding to rat liver microsomes of reactive metabolites by phenobarbital pretreatment of the animals; prior administration of cobaltous chloride diminished tissue binding. It was concluded that monoacyl- and monoalkyl hydrazines undergo N-hydroxylation by cytochrome P-450 with subsequent dehydration of the N-oxidized intermediates to yield the corresponding diazenes. Analogously, procarbazine may undergo conversion to the azo derivative.[69]

There is controversy as to the involvement of cytochrome P-450 in the N-oxide formation from tertiary-amine compounds. Among these substrates, N,N-dimethylaniline and analogues have been most extensively studied. Early observations suggested that cytochrome P-450 was not involved in the N-oxidation of the aromatic amine. This view was based on the fact that carbon monoxide rather stimulates N-oxide formation from N,N-dimethylaniline in rabbit and pig liver microsomal fractions.[8,13,206] This has been attributed to inhibition by CO of liver microsomal dimethylaniline N-oxide aldolase (EC 4.1.2.24) resulting in accumulation of the N-oxide.[206] Aldolase activity, however, has been shown to be negligible in rabbit liver microsomes.[8,268] Similarly, metyrapone increases the rate of rabbit liver microsomal N-oxidation of N,N-dialkylanilines at intermediate concentrations.[107,268] Other modifiers of drug metabolism, such as SKF 525-A or imidazole, do not appreciably affect N-oxidation of N-ethyl-N-methylaniline in rabbit liver microsomes.[268] Rabbit liver microsomal N-oxide formation from N,N-dimethylaniline exhibits lower sensitivity toward irradiation of the assay media with UV-light than N-hydroxylation of aromatic primary amines.[18] Similarly, N-oxidation of aliphatic tertiary amines appears to be insensitive toward classic inhibitors of the cytochrome P-450 system.[56,104] The subject has been comprehensively reviewed by Bickel.[269]

With intact rabbit liver microsomal fractions, Hlavica demonstrated a biphasic effect of carbon monoxide on the formation of N-oxide from N,N-dimethylaniline: plots of relative rate of N-oxidation versus oxygen concentration were not hyperbolic but sigmoidal,[12] the Hill interaction coefficient, n_H, being 2.1 (Figure 6). Carbon monoxide abolished the sigmoidal relationship between oxygen concentration and reaction rate,[12] increased the rate of N-oxidation at low oxygen pressures, and diminished K_m and V_{max} (Figure 7).[12,13] These findings suggested the involvement of different forms of

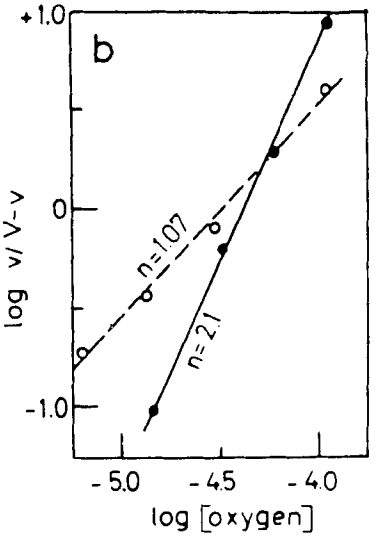


FIGURE 6. Binding of oxygen to rabbit liver microsomal mixed-function oxidase(s) mediating N-oxidation of N,N-dimethylaniline. Reactions were carried out either in the absence (●) or presence (○) of 0.6 mM CO. (From Hlavica, P., *Biochim. Biophys. Acta*, 273, 318, 1972. With permission.)

cytochrome P-450 in equilibrium in this N-oxidation process.[13] Metyrapone is presumed to stimulate N-oxidation of N,N-dialkylanilines[107,268] by displacing the equilbrium between two functionally different forms of the hemoprotein to the species with increased catalytic capacity for N-oxide formation;[281] such mechanism has been proposed to account, too, for the stimulation of other mixed-function oxidations by metyrapone.[270]

Studies on the influence of pH on the apparent Michaelis constant for oxygen and maximal rate of N-oxide formation from N,N-dimethylaniline presented evidence for the involvement in this reaction of a two-enzyme system: N-oxidation of the arylamine is associated with the formation of two enzyme-O_2 complexes, the pK_s of only one of them being affected by the addition of CO (Figure 8).[13] The existence of alternative metabolic routes of N-oxidation of N,N-dimethylaniline in rabbit liver microsomes was further confirmed by application of various modifiers of drug metabolism such as heat, 2-bromo-4′-nitroacetophenone, and menadione.[271,272]

Further evidence for the involvement of cytochrome P-450 in the N-oxidation of N,N-dialkylanilines comes from experiments with compounds effecting either inhibition of synthesis or destruction of the hemoprotein. Thus, pretreatment of rabbits with cobaltous chloride decreases the level of hepatic microsomal cytochrome P-450 by 70% and decelerates N-oxide formation from N,N-dimethylaniline by about 50%.[272] The decay in cytochrome P-450 produced by addition of bile salts to rabbit liver microsomal fractions closely follows the decrease in N-oxygenating activity for N-ethyl-N-methylaniline.[268] Similar observations were made, when the influence of "aging" of rabbit liver microsomal preparations on N-oxide formation was studied.[268] On the other hand, pretreatment of rabbits with pregnenolone-16α-carbonitrile, a selective inducer of cytochrome P-450 and NADPH-cytochrome c(P-450) reductase,[273] stimulates liver

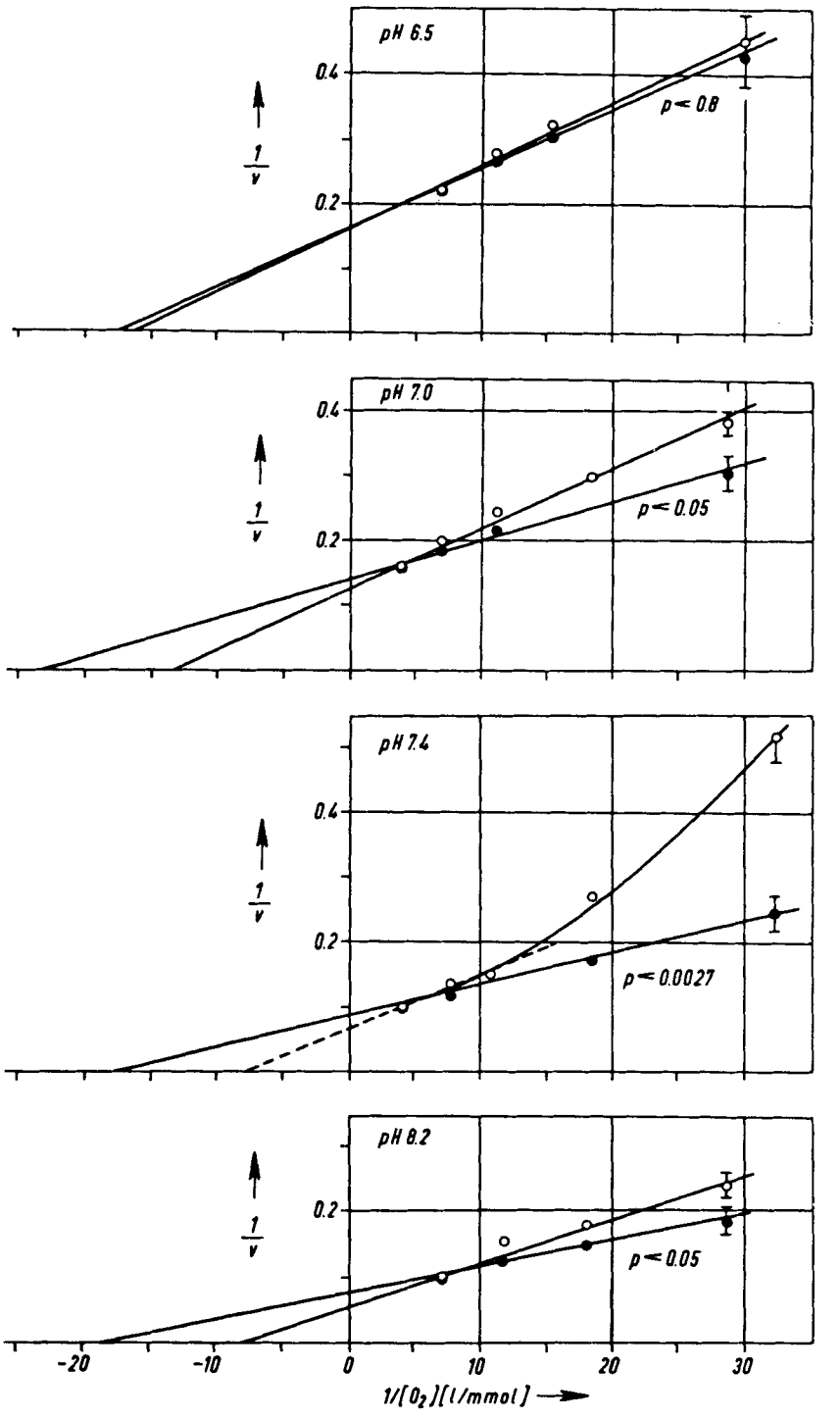


FIGURE 7. Rabbit liver microsomal N-oxide formation from N,N-dimethylaniline at various oxygen concentrations in the absence (○) or presence (●) of 0.49 mM CO. (From Hlavica, P. and Kehl, M., *Hoppe-Seyler's Z. Physiol. Chem.*, 355, 1508, 1974. With permission.)

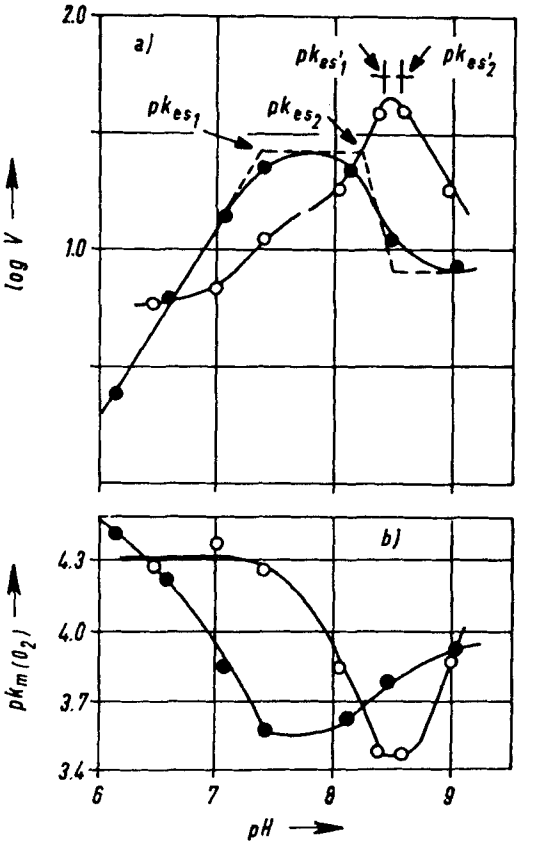


FIGURE 8. Effect of pH on the apparent Michaelis constant for oxygen and on maximum velocity of N-oxidation of N,N-dimethylaniline in the absence (●) or presence (○) of 0.49 mM CO. pK_{es1}, pK'_{es1}, pK_{es2}, and pK'_{es2} are the ionization constants of enzyme-oxygen complexes. (From Hlavica, P. and Kehl, M., *Hoppe-Seyler's Z. Physiol. Chem.*, 355, 1508, 1974. With permission.)

microsomal N-oxidation of N,N-dimethylaniline.[272] Pretreatment of laboratory animals with glucocorticoids, known to induce synthesis of cytochrome P-450,[274] produces similar effects.[275,276] Noteworthy, administration of phenobarbitone to rabbits results in an approximately two-fold stimulation of liver microsomal N-oxide formation from 4-chloro-N-ethyl-N-methylaniline.[277]

The concept of the involvement of cytochrome P-450 in the N-oxidation of N,N-dimethylaniline receives support by the observation of formation of 424- and 448-nm ferrohemochromes during the microsomal oxidative metabolism of the amine.[278,279] From the effects of temperature, pH, n-octylamine, extraction of the enzyme preparations with organic solvents, and pretreatment of rabbits with inducers of drug metabolism on both the formation of the spectral species and the enzymic C- and N-oxidation of N,N-dimethylaniline it was concluded that the 448 nm spectral change is the result of binding to cytochrome P-450 of a metabolite arising from N-oxidation of the arylamine (Figure 9).[279] Interestingly, tiaramide-N-oxide produces a pronounced spectral perturbation with reduced rat liver microsomal cytochrome P-450 with absorbance peak at 442 nm, which is antagonized by CO.[280]

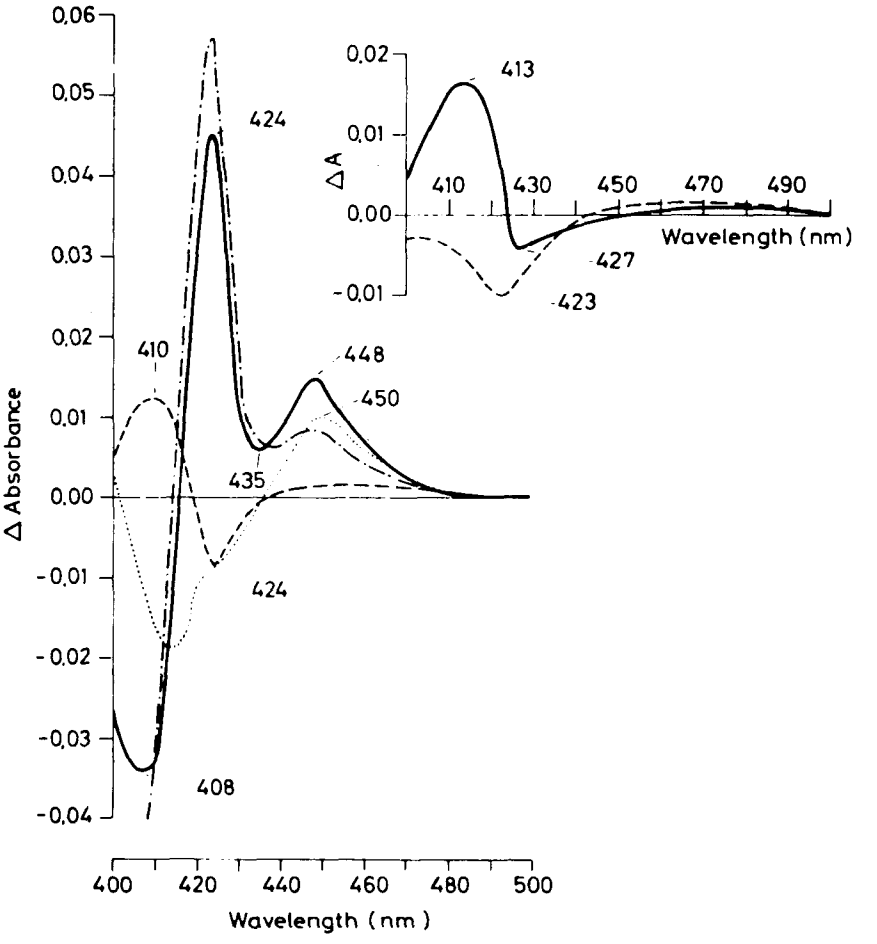


FIGURE 9. Difference spectrum produced during the NADPH-dependent oxidative metabolism of N,N-dimethylaniline by partially solubilized rabbit liver microsomal cytochrome P-450. The symbols indicate: spectral change recorded 0.5 (— — —), 15 (———), and 62 (. . .) min after substrate addition to the sample cell; (— . . —), spectrum generated in the presence of exogenous catalase. The inset of the figure shows difference spectra produced by N,N-dimethylaniline either under aerobic conditions in the absence of NADPH (———), or under anaerobic conditions in the presence of NADPH (— — —). (From Hlavica, P. and Aichinger, G., *Biochim. Biophys. Acta*, 544, 185, 1978. With permission.)

Ultimate proof of the ability of cytochrome P-450 to mediate N-oxidation of N,N-dimethylaniline was obtained by experiments with a reconstituted system containing highly purified cytochrome P-450 LM_4 from the rabbit, NADPH-cytochrome c(P-450) reductase, and lipid factor.[281] From the N-oxygenating capacity of this system and previous data,[272] the hemoprotein was roughly calculated to account for about 40% of the total amount of N,N-dimethylaniline-N-oxide formed in rabbit liver microsomes; as will be shown later, the remainder arises from catalysis by oxidase(s) other than cytochrome P-450. The inability to observe appreciable inhibition by modifiers of the cytochrome P-450 system of N-oxide formation from N,N-dialkylanilines in intact microsomal fractions[18,56,104,268] is thus easily understood: considering that the hemoprotein-dependent N-oxidation is likely to be only partially inhibited even by its specific inhibitors, total microsomal N-oxide formation is likely to be affected only to a minor extent by these agents.

The participation of cytochrome P-450 in the N-oxidation of other types of tertiary-amine compounds, such as heteroaromatic amines, has been confirmed by experiments with a series of 3-substituted pyridines. In hepatic microsomes from various animal species, N-oxidation of pyridines has been reported to be inhibited by CO, SKF 525-A, DPEA, n-octylamine, and other aliphatic and aromatic amines.[62,277] Any treatment of microsomes causing destruction of cytochrome P-450 also produces a fall in N-oxidation of the pyridines.[62] Pretreatment of animals with phenobarbitone, however, results in a 3- to 13-fold increase in the rate of N-oxide formation.[62] The reaction proceeds most rapidly at pH around 8.0.[87] The kinetic parameters, K_m and V_{max}, exhibit marked species and sex differences.[87] Further evidence for the ability of the cytochrome P-450 system to catalyze pyridine N-oxidation comes from spectral work: in hepatic microsomal fractions prepared from phenobarbital-pretreated rabbits the NADPH-driven oxidative metabolism of pyridine is associated with the gradual formation of a ferrocytochrome P-450-product adduct absorbing at 442 nm.[283] Analysis of the pyridine metabolites indicates that this spectral change results from binding to cytochrome P-450 of an intermediate arising from N-oxidation of the amine. Data obtained by means of NMR spectrometry suggest that the N-oxidized metabolite coordinates to the heme iron via the oxygen atom.[283]

The rat liver microsomal formation of two N-oxide metabolites from metyrapone, another pyridine derivative, is inhibited, too, by CO, SKF 525-A, and aniline; phenobarbital pretreatment of the animals stimulates formation of both N-oxides to different extents.[63] Moreover, the transformation of 2-phenyl-1,3-di(4-pyridyl)-2-propanol to a chiral N-oxide metabolite in rat liver 9000 × g supernatant is increased by prior administration to the animals of phenobarbital.[64]

Some involvement of cytochrome P-450 has been found, too, in the N-oxidation of retrorsine, an alicyclic tertiary-amine compound: the rat liver microsomal N-oxidation of this pyrrolizidine alkaloid is inhibited by CO, SKF 525-A, and mercurials; pretreatment of the animals with either phenobarbitone or DDT stimulates N-oxide formation.[61,282] The apparent K_m value for retrorsine has been determined to be 0.32 mM.[282]

Recently, Hallström et al[284] reported on the NADPH-dependent liver microsomal N-oxide formation from pargyline, a N,N-dialkylalkynylamine. Carbon monoxide exerted a pronounced inhibitory effect on N-oxide formation in hepatic microsomes from rats pretreated with phenobarbital. Such preparations catalyzed N-oxidation of the tertiary amine at a rate three times that of control microsomes. Interestingly, CO and phenobarbital pretreatment did not affect pargyline N-oxidation in rabbit liver microsomal fractions. Rabbit liver microsomes were about six times as active as rat liver microsomes in producing the N-oxide.

C. Mixed-Function Amine Oxidase-Dependent N-Oxidation Reactions

In 1964, Ziegler and co-workers[285] established evidence for a flavin-dependent route of N-oxidation of N,N-dialkylanilines in pork liver microsomal fractions. These observations led to partial purification from pork liver microsomes of a N-oxidase exhibiting spectral characteristics of a flavoprotein.[286] Ultimate proof of the flavoprotein nature of this oxidase, also called dimethylaniline monooxygenase (N-oxide forming; EC 1.14.13.8), was presented after successful purification of the enzyme to apparent homogeneity.[287] By means of immunological techniques, the flavoprotein amine oxidase was shown to be distinct from NADPH-cytochrome c reductase (EC 1.6.2.3), another microsomal flavoprotein.[288]

The prosthetic group of the mixed-function amine oxidase is flavin adenine dinucleotide; the enzyme contains 14 to 15 nmol of flavin per mg of protein.[287,289] The

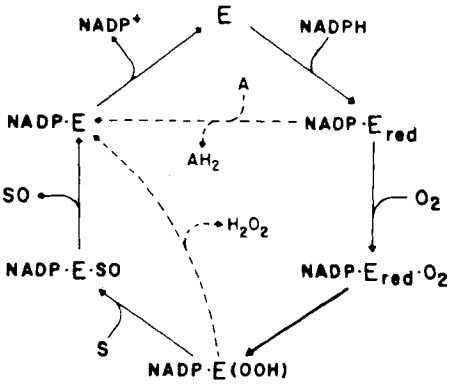


FIGURE 10. Possible intermediates in reactions catalyzed by the FAD-containing mixed-function amine oxidase. The abbreviations are: E, enzyme; S, substrate. The dashed lines indicate alternate reaction pathways, where A and AH_2 represent oxidized and reduced electron acceptor, respectively. (From Poulsen, L. L. and Zeigler, D. M., *J. Biol. Chem.*, 254, 6449, 1979.)

minimum molecular weight based on flavin is 64,000.[289] Sodium dodecyl sulfate-treated preparations of the purified porcine oxidase give a single protein band upon electrophoresis in polyacrylamide gels, and the rate of migration is near that of proteins of 60,000 molecular weight.[290] Similar observations were made with the purified rabbit liver oxidase.[281] However, the sedimentation rate of the native enzyme indicates that the enzymically active species exists as an octomer.[288] The oxidase does not contain iron, copper, or cytochromes.[287]

Anaerobically, the oxidized form of the amine oxidase exhibits absorbance maxima at 380 and 450 nm; both absorbance bands are completely destroyed after addition of NADPH to the enzyme solutions.[289] After reequilibration with oxygen, a new spectral species with absorption peak at 375 nm appears, which is believed to represent a peroxyflavin form of the oxidase.[289]

At saturating levels both NADPH and NADH support N-oxidation of amines equally well, but NADPH saturates the oxidase at less than one tenth the concentration of NADH.[290] With N,N-dimethylaniline as a substrate, the NADH- and oxygen-supported N-oxide formation proceeds most rapidly at pH 7.9, whereas maximum activity is observed at pH 8.5 in the NADPH-dependent reaction.[291] Kinetic studies are consistent with an ordered Ter-Bi mechanism with an irreversible step between the second and third substrate where NADPH is added first, followed by oxygen, and the oxidizable substrate is added last (Figure 10).[289]

Tertiary-amine N-oxidation catalyzed by the porcine oxidase is stimulated about two-fold by lipophylic primary alkylamines, such as n-octylamine or DPEA, and some guanidine derivatives.[291,292] These activators are not substrates for the oxidase, but appear to interact with a regulatory site that is distinct from the catalytic site of the enzyme.[292] The concentrations of the amines required to half-saturate the activator site are 0.25 mM for DPEA and 1.25 mM for n-octylamine.[292] There appear, however, to exist considerable species differences with respect to response of the oxidase to n-octylamine: the primary amine has little or only marginal effects on the N-oxygenating activities in rat and rabbit liver microsomes,[293] and the purified oxidase from rabbit liver is almost insensitive to 1 mM n-octylamine.[272]

Induction of the amine oxidase has been observed neither after pretreatment of experimental animals with phenobarbital[288] nor after administration of 3-methylcholanthrene.[78]

The oxidase is insensitive to carbon monoxide, SKF 525-A, cyanide, and azide, but is strongly inhibited by 1-(1-naphthyl)-2-thiourea.[287] The partially purified rabbit liver enzyme has been found to be sensitive to Hg^{2+}.[294] Nevertheless, an inhibitor that specifically blocks microsomal amine oxidase-dependent reactions without affecting cytochrome P-450-mediated oxidations has not been found.[290]

Owing to changes in the polypeptide backbone structure, the purified amine oxidase from pig liver shows irreversible inactivation when exposed to moderate temperatures between 30 to 40° C.[295] The relatively low temperature of this structural transition is possibly an artifact of the in vitro study; as an integral part of the hepatic microsomal system, the enzyme is membrane bound and may be stabilized by membrane lipids. Interestingly, there is a considerable increase in thermal stability of the oxidase when insolubilized by covalent attachment to nylon tubing, sepharose particles, or glass beads.[296] The purified rabbit liver enzyme appears to be more heat stable.[272]

The amine oxidase exhibits a broad substrate specificity for a large number of secondary and tertiary amine compounds.[286,287] With N,N-dimethylaniline, a substrate most frequently used to assay the enzyme, the concentration of amine required to half-saturate the porcine oxidase has been determined to be 2.9 to 28 μM.[292,297] With an apparent K_m of 89 to 340 μM, the partially and highly purified rabbit liver N-oxidase exhibits considerably lower affinity for this amine substrate.[272,294] The concentration of NADPH necessary for half-maximal activity in solutions at equilibrium with atmospheric oxygen is 5 μM.[290] This value does not change appreciably over a wide range in pH. The K_m for oxygen, as measured in terms of N-oxidation of N,N-dimethylaniline, has been estimated to be 45 nM at pH 7.4 and 37° C.[289] The affinity of the oxidase for O_2 increases with decrease in pH.[289] These findings are in accord with the kinetic data given in Figure 8.

Aromatic primary amines, such as aniline, are not substrates for the amine oxidase.[287,291] Exception to this rule is given by primary arylamines capable of forming imine tautomers. Thus, 2-naphthylamine is readily N-oxidized by the enzyme.[291,292] In pig liver microsomal fractions, a rapid autoxidation of the hydroxylamine formed is antagonized by NADPH-cytochrome c reductase.[25,291]

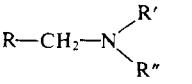
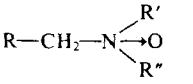
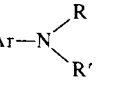
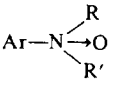
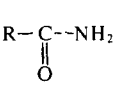
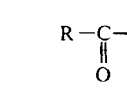
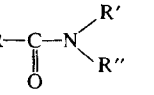
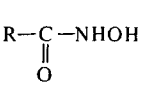
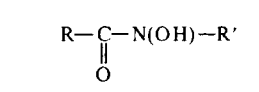
The flavoprotein oxidase also catalyses the formation of N-oxides from methylhydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, and procarbazine.[69,90] Methane is a decomposition product of methylhydrazine oxide.[90]

There is some evidence suggesting N-oxidation of N,N-disubstituted hydroxylamines by the purified mixed-function amine oxidase:[297] N-methyl-N-benzylhydroxylamine is likely to be transformed to the highly unstable hydroxylamine oxide, which dehydrates to a mixture of stable phenyl-N-methylnitrone and unstable benzylnitrone. The latter hydrolyzes rapidly to yield benzylhydroxylamine and formaldehyde. However, a recent report of Rauckman et al.[298] indicates that the N-oxidase does not directly attack the secondary hydroxylamines but, in the presence of NADPH and oxygen, releases an oxidizing agent, probably superoxide, that is responsible for hydroxylamine oxidation.

Most interestingly, the flavoprotein oxidase mediates S-oxidation of a series of sulphur-containing compounds, too.[290,293,299] The water-soluble aminothiol cysteamine, which is readily oxidized to the disulphide, is believed to be the physiological substrate.[290,300] With substrates containing both nitrogen and sulphur atoms, the flavoprotein preferentially catalyzes oxidation of the sulfur atom.[289] Hence, this enzyme would be more accurately termed an S-oxidase.

Recently, Patton et al.[167] reported on the isolation from hamster liver nuclei of a

Table 6
DIFFERENT TYPES OF ORGANIC NITROGEN COMPOUNDS AND THEIR METABOLIC N-OXIDATION PRODUCTS

	Primary (a)	Secondary (b)	Tertiary (c)
Group I (pK_a 8-11)	$R-CH_2-NH_2$	$R-CH_2-NH-R'$	
Product	$R-CH_2-NHOH$	$R-CH_2-N(OH)-R'$	
Group II (pK_a 1-7)	$Ar-NH_2$	$Ar-NH-R$	
Product	$Ar-NHOH$	$[Ar-N(OH)-R]$	
Group III (pK_a below 1)			
Product			

From Gorrod, J. W., *Chem. - Biol. Interact.*, 7, 289, 1973. With permission.

mixed-function amine oxidase that catalyzes the oxygenation of both nitrogen and sulfur compounds. The nuclear enzyme cross reacts with antibodies raised to the pig liver microsomal oxidase.

D. Differentiation of the Metabolic Routes of N-Oxidation

The enzymic mechanisms involved in the oxidation of nitrogen in organic compounds have been reviewed by Gorrod,[301] who attempted differentiation by a consideration of species difference, age, pH, optima, induction, inhibition, and stereochemical effects. From the data collected, a simple three category classification for nitrogenous substrates was established, which is given in Table 6. As can be seen, Group I includes basic aliphatic amines (pK_a 8 to 11), Group II aromatic amines containing an electron-withdrawing substituent (pK_a 1 to 7), and Group III comprises acetamides with pK_a below one. Group I compounds were proposed to undergo N-oxidation by the mixed-function amine oxidase, whereas Group III substrates should be N-oxidized by the cytochrome P-450-dependent system. Group II compounds were suggested to act as substrates for both systems, a lower pK_a favoring N-oxidation by cytochrome P-450 and a higher pK_a favoring oxidation by the flavoprotein oxidase. However, there are many exceptions from this concept and amine substrate specificities for these two microsomal monooxygenases based on pK_a are only a very approximate guide. Thus, N-hydroxylation of phentermine (Group Ia) is obviously mediated by the cytochrome P-450 system[4,7] and not by the mixed-function amine oxidase.[292] Similarly, N,N-dibenzylamine (Group Ib) appears to be predominantly N-hydroxylated by cytochrome P-450.[33] Pargyline (Group Ic) is likely to be a substrate for cytochrome P-450 in the rat, but not in

the rabbit.[284] 2-Naphthylamine (Group IIa)[25,107,257] and N,N-dimethylaniline (Group IIc) are metabolized by both microsomal N-oxidases.[12,13,272,279,281,287,289,291,292] Pyridine, a heterocyclic tertiary amine with pK_a equal to that of N,N-dimethylaniline, is not N-oxidized by the flavoprotein enzyme,[290] but is converted into the corresponding N-oxide by the cytochrome P-450 system.[62,277,283] Substitution of the pyridine ring with an electron withdrawing or electron donating group, which can induce a dramatic change in pK_a of the molecule,[277] is not associated with a change in enzymology.[62,277] Finally, procarbazine, a hydrazine derivative, has been proposed to be N-oxidized by cytochrome P-450 in rat hepatic microsomes;[69] this compound is metabolized, too, by the pig liver microsomal flavoprotein oxidase.[90] These findings clearly indicate that additional factors, such as nucleophilicity[290,302] or steric features,[290] may govern interaction of the amine substrates with the microsomal N-oxidases.

Obviously, certain amines can act as substrates for more than one enzyme. In these cases the enzymology of N-oxidation will depend on the catalytic capacities of the individual oxidases involved and the molar ratio of the enzymes present. Both variables are likely to exhibit a marked species difference. Thus, the flavoprotein monooxygenase appears to be abundant in pig liver, where it accounts for 3 to 4% of the total microsomal protein.[289,290] On the other hand, the specific content in pig liver microsomes of cytochrome P-450 is only one third that in rabbit liver microsomes,[272] the latter being relatively poor in flavoprotein amine oxidase. The porcine flavoprotein enzyme shows high affinity for N,N-dimethylaniline,[292,297] whereas the corresponding rabbit liver oxidase exhibits considerably lower N-oxygenating capacity for this substrate.[272,294] It is, therefore, conceivable that cytochrome P-450-dependent N-oxidation of N,N-dimethylaniline is insignificant in the pig, but is of importance in the rabbit.[272]

E. Peroxidase-Dependent N-Oxidation Reactions

Böttcher and Kiese[303] reported on the formation of small amounts of nitrosobenzene from aniline in the presence of catalase (EC 1.11.1.6) and hydrogen peroxide. The concentration of nitrosobenzene was higher in incubation mixtures containing horseradish peroxidase (EC 1.11.1.7) and H_2O_2. N-Hydroxylation was stimulated by temperature. With 4-chloroaniline, Corbett et al.[304] failed to demonstrate horseradish peroxidase- and H_2O_2-dependent N-oxidation of the amine. Transformation of 4-chloroaniline to the corresponding nitroso compound has been found to be mediated by chloroperoxidase (EC 1.11.1.10).[304,389] This enzyme is of special interest, since it exhibits characteristics similar to those of cytochrome P-450.[305] The N-hydroxylation reaction proceeds most rapidly at pH 4.4 with an apparent K_m of 0.81 mM and catalytic-centre activity of 312. The initial rate of the reaction is strongly affected by the presence of halide ions. The peroxidase also catalyses H_2O_2-dependent oxidation of N-methyl-4-chloroaniline to give 4-chloronitrosobenzene as a major product.[121]

Kadlubar et al.,[306] by studying the hydroperoxide-supported oxidation of amines in hepatic microsomal fractions, failed to detect significant N-oxide formation from N,N-dimethylaniline.

F. N-Oxidation in Plants and Microorganisms

There are only few data in the literature concerning the enzymology of N-oxidation of amine substrates in plants and microorganisms. Thus, Møller and Conn[307] reported on the NADPH- and oxygen-dependent formation of N-hydroxytyrosine from L-tyrosine during the biosynthesis of the cyanogenic glucoside dhurrin in microsomal fractions from etiolated *Sorghum* seedlings. The reaction was not affected by carbon monoxide, SKF 525-A or cyanide, but was strongly inhibited by cytochrome c, flavins, and quinones.

Kawai et al.[308] observed transformation of p-aminobenzoate, p-aminobenzaldehyde, and p-aminophenylacetate to the corresponding nitro compounds in *Streptomyces thioluteus*. Experiments with ^{18}O demonstrated that both oxygen atoms in the nitro group of p-nitrobenzoate were derived from atmospheric oxygen. The reaction proceeded most rapidly at pH around 7.5 and was sensitive to inhibitors such as cyanide, azide, mercurials, and hydroxyquinoline. N,N-dimethylaminobenzoate was oxidized to the corresponding N-oxide.

Strains of *Fusarium oxysporum* produce 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene when grown on 4-chloroaniline.[309] In *Pseudomonas aminovorans,* trimethylamine is converted into the N-oxide by a NADPH- and oxygen-dependent tertiary-amine mixed-function oxidase.[310] The enzyme is insensitive to carbon monoxide, SKF 525-A, or cyanide, but is inhibited by excess amine substrate.

VI. FURTHER FATE OF N-OXIDIZED PRODUCTS

A. Reduction of N-Oxidized Compounds

A large variety of nitrobenzenes have been found to undergo reduction to the corresponding primary amines via the nitroso and hydroxylamine intermediates both in vivo[311-314] and in vitro.[315-319] Attempts to characterize mammalian nitro reductases have been very confusing, since evidence indicates the existence of several reductase systems in the cytosol and the microsomal fraction of liver cells. It is, therefore, meaningless to carry out substrate, cofactor and inhibition specificity studies on mixture of the systems, as has been the case in a number of studies based on whole tissue homogenates[316] or postmitochondrial fractions.[319]

Among the nitro reductases in the soluble fraction of liver, xanthine oxidase (EC 1.2.3.2) has been proposed to be active in the NADH-dependent reduction of a series of nitro compounds, such as niridazole,[320] nitrofurans,[321] and trinitrotoluene,[317] Maximal NADH-supported niridazole reductase activity is observed at pH 7; the apparent K_m is 0.13 mM.[320] The reaction is blocked by potent inhibitors of xanthine oxidase.

Fouts and Brodie[322] reported on the presence in both the soluble fraction and the microsomes of the liver cell of an enzyme system which utilized NADPH and NADH and effected reduction of p-nitrobenzoate and a number of other aromatic nitro compounds. The cytosol enzyme was shown to include a flavoprotein with low prosthetic group specificity, since activity was restored by the addition of FAD, FMN, or riboflavin to preparations previously inactivated by acid treatment. The reaction was highly sensitive to oxygen, which might, in part, result from autoxidation of the phenylhydroxylamine intermediate to yield the nitroso derivative. The cytosol reductase was inhibited by SKF 525-A,[322] cyanide, and boiling.[323] Pretreatment of rats with either phenobarbital or DDT did not result in increased capacity for reduction of the soluble enzyme.[323] Kato et al.,[324] by reinvestigating the NADPH- and NADH-linked system(s) effecting conversion of p-nitrobenzoate, concluded that both reductases were separate enzymes because of differences in their intracellular localization. The NADH-dependent nitro reductase activity was located in the cytosol and catalyzed the reduction of p-nitrobenzoic acid to p-hydroxylamino-benzoate; the NADPH-supported microsomal activity converted p-hydroxylamine-benzoate to p-aminobenzoate. The latter activity was stimulated by FAD or FMN, inhibited by O_2, and induced by pretreatment of rats with phenobarbital.

Experiments by Gillette et al.[325] demonstrated that in mice, rats, and rabbits NADPH-driven formation of the primary amine from p-nitrobenzoate occurred almost extensively in the microsomal fraction of liver. The reaction was blocked by CO and pretreat-

ment of the animals with CCl_4, but stimulated by prior administration of phenobarbital. These findings suggested the involvement of cytochrome P-450 in the reductive process. This view was supported by the observation that compounds known to either accelerate or slow the rate of reduction of cytochrome P-450 exerted an analogous effect on the microsomal NADPH-dependent reduction of p-nitrobenzoate.[326] Although cytochrome P-450 can mediate reduction of p-nitrobenzoate under artificial anaerobiosis, it appears to be a rather inefficient nitro reductase in living animals, since, owing to its high affinity for O_2 ($K_m < 0.1\ \mu M$), only a minor portion is present in the reduced form in the liver cell.

Another microsomal enzyme capable of catalyzing reduction of nitro compounds is NADPH-cytochrome c reductase (EC 1.6.2.3). The flavoprotein has been proposed by Feller et al.[327] to mediate reduction of niridazole to give hydroxyaminothiamidazol. The reaction is insensitive to CO, but is stimulated by pretreatment of rats with phenobarbital. Under air the hydroxylamine is readily reoxidized to niridazole. By using metronidazole, Perez-Reyes et al.[328] succeeded to demonstrate that rat hepatic microsomal fractions supplemented with NADPH reduce the nitro compound to the corresponding nitro anion radical; under aerobic conditions, O_2 reacts with the radical to produce superoxide and to regenerate metronidazole. Under anaerobic conditions, the radical is further reduced. Similar results were obtained during studies on the reductive metabolism of nitrofurazone in rat hepatic microsomes.[329] Interestingly, nitro reduction of the strong carcinogen 4-nitroquinoline-N-oxide is, in part, O_2-insensitive.[330] Recently, Köchli et al.[440] detected NADH-supported reduction of dinitrobenzene in rat brain mitochondria.

Mammalian cytosolic and microsomal reductases also catalyze metabolism of nitroso compounds. Kuwada et al.[435] reported NADH-dependent reduction by porcine liver alcohol dehydrogenase (EC 1.1.1.1) of p-nitrosophenol to yield p-aminophenol. Horie and Ogura[436] described NADPH-dependent reduction of p-nitrosophenol by partially purified reductase from porcine heart cytosol. Furthermore, nitrosobenzene is readily transformed to phenylhydroxylamine by NADPH-supported catalysis of methemoglobin reductase.[331] This enzyme, which is normally operative in the red cell to compensate for the slow, continuous chemical oxidation of hemoglobin by molecular oxygen, has been purified by Huennekens et al.[332] The reductase is a hemoprotein with an apparent molecular weight of 185,000. Finally, Gillette et al.[325] observed anaerobic formation of aniline from nitrosobenzene in mouse liver microsomes fortified with NADPH; the reaction was strongly inhibited by CO.

Hydroxylamines have been demonstrated to act as substrates for the reductase systems, too. Poirier and Weisburger[319] found conversion of 2-naphthylhydroxylamine into 2-naphthylamine in the cytosol of mouse and rat liver cells in the presence of NADPH and nitrogen. Similarly, under anaerobic conditions phenylhydroxylamine is reduced to the parent amine in mouse liver microsomes supplemented with NADPH the reaction is severely blocked by CO.[325] Beckett and Bélanger[6] detected phentermine in the urine of rabbits dosed with N-hydroxyphentermine. The reduction of N-hydroxyphentermine was further studied in liver microsomes isolated from rats, guinea-pigs, and rabbits.[333] The reduction requires the presence of NADPH and is inhibited by O_2 and CO. In the rat, the rate of reduction is increased by phenobarbital pretreatment. Kinetic analysis of the reductase activity in rat liver microsomes suggests that the reduction of the hydroxylamine is mediated by at least two enzyme systems. Kadlubar et al.[334] described a hepatic microsomal, cyanide-insensitive, reduced pyridine nucleotide-dependent hydroxylamine reductase activity that is independent of oxygen concentration and catalyzes the reduction of a number of primary and secondary hydroxylamines to the parent amines. At the pH optimum of 6.3, NADH is the preferred cofactor.

There is also evidence for nonenzymic reduction of nitro compounds in mammalian tissues. Juchau et al.[335] observed only slight loss of reductase activity in boiled placental microsomes fortified with NADPH and FMN; a model system containing NADPH, FMN, and reduced glutathione in phosphate buffer was active in reducing p-nitrobenzoate. Similarly, nitrosobenzene has been reported to be nonenzymically reduced to phenylhydroxylamine by NADH or NADPH in aqueous solutions.[336] In vitro, under anaerobic conditions nitrosobenzene rapidly reacts with reduced glutathione to yield phenylhydroxylamine.[130] It seems unlikely, however, that these mechanisms have any functional role in living animals, since relatively high concentrations of reduced nucleotides, flavins, and glutathione are required to effect reduction of the N-oxidized compounds.

Owing to the anaerobic conditions that exist in the gut, it is possible that bacterial flora play an important role in the conversion nitro compounds to aromatic amines in animals receiving nitro compounds orally. Thus, simultaneous oral administration of p-nitrobenzoic acid and tetracycline to rats results in strongly reduced urinary excretion of p-aminobenzoate as compared with animals not treated with the antibiotic.[337] Intestinal contents of rats show several-fold higher reductase activity for p-nitrobenzoate than liver and intestinal wall, respectively.[337]

Saz and Slie[338,339] provided evidence for the ability of *Escherichia coli* to reduce the nitro groups of p-nitrobenzoate, chloramphenicol, and other aromatic nitro compounds. The bacterial reductase is a NAD-dependent system which requires a sulphydryl donor, such as cysteine or glutathione, and Mn^{++}. The enzyme is blocked by azide and low concentrations of chlortetracycline, which form chelates with Mn^{++}. *E. coli* has been detected to contain two different nitrofurazone-reducing activities distinguishable primarily by their sensitivity to oxygen.[329] Partial purification of nitro reductase activity has been achieved from *Neurospora crassa*[340] and *Aspergillus niger*.[341] m-Dinitrobenzene is the most effective electron acceptor in both systems. Both NADH and NADPH serve as electron donors. Cysteine stimulates enzyme activity, metal chelators are inhibitory. Highly purified nitro reductase has been obtained from *Nocardia V*.[342] The enzyme converts p-dinitrobenzene into p-nitroaniline. Zucker and Nason[343] solubilized a hydroxy amine reductase from *Neurospora crassa* that is specific for hydroxylamine. NADPH is the preferred source of electrons. Reduction of p-nitrobenzoate has been further observed in enteric bacteria, such as *Aerobacter aerogenes, Proteus vulgaris, Salmonella typhimurium,* and *Streptococcus faecalis*.[337] In each case, activity was strongly inhibited by the presence of oxygen.

N-Oxides are reduced, too, to the corresponding tertiary parent amines in animal tissues. The reduction of trimethylamine-N-oxide by mammalian liver preparations was described as early as 1927.[344] Nevertheless, conflicting results and the fact that a significant amount of nonenzymic reduction appears to occur have made it difficult to characterize the enzymology of N-oxide reduction. Thus, reduction of trimethylamine-N-oxide is heat-stable and can be brought about also by Fe^{++}, cysteine, or reduced glutathione.[344] Similarly ferrous ions catalyze reduction of chlorpromazine-N-oxide.[345] In rat and rabbit liver microsomal fractions, dimethylaminoazobenzene-N-oxide is reduced to the tertiary amine even in the absence of NADPH.[346] According to Bickel and associates,[104,347] reduction of imipramine-N-oxide in rat hepatic preparations does not require the presence of reduced pyridine nucleotides; the reaction is sensitive to O_2, but is not inhibited by CO or SKF 525-A. These findings are at variance with reports of other workers,[348-350] who established evidence for a cofactor requirement of reduction of imipramine-N-oxide in rat hepatic microsomes. It appears that in the rat hepatocyte most of the activity for the reduction of substrates such as N,N-dimethylaniline-N-oxide, imipramine-N-oxide, or tiaramide-N-oxide is located in the microsomal fraction, the activity in other subcellular fractions being low or

negligible.[348] The microsomal NADPH-supported N-oxide reductase activity is inhibited by an atmosphere of carbon monoxide; the concentration of CO required for 50% inhibition of imipramine-N-oxide reduction is 1.0 μM.[348] Reductase activity is also blocked by the presence of O_2, with 50% inhibition occurring at an oxygen concentration of 2 to 3 μM.[348] The apparent K_m values for reduction of the N-oxides of imipramine, tiaramide, and N,N-dimethylaniline are on the order of 0.1 mM.[348] N-Oxide reduction is markedly inhibited by nitro, nitroso, and azo compounds as well as hydrazide, n-octylamine, and DPEA.[348] Pretreatment of rats with phenobarbital stimulates reductase activity.[348] These findings hint at a possible role of cytochrome P-450 in N-oxide reduction. This view receives support from experiments with levopropoxyphene-N-oxide: the anaerobic, NADPH-dependent reduction of the N-oxide in rat liver 15,000 x g supernatant is inhibited by CO, cyanide, and dipyridyl.[351] Flavins (FMN, FAD, riboflavin) effect a pronounced stimulation of rat liver microsomal tertiary-amine oxide reduction.[348,352] Kato et al.[350] believe that the flavin- or viologen-induced enhancement of NADPH-dependent N-oxide reduction consists of the reduction of these electron acceptors by NADPH-cytochrome c(P-450) reductase, which, in their turn, reduce cytochrome P-450.

During studies on the rat liver microsomal reduction of tiaramide-N-oxide, a combination of NADPH and NADH was found to stimulate reduction to a greater extent than NADPH alone. This finding prompted a closer investigation of the NADH-supported reduction of a number of N-oxides in rat liver microsomal fractions.[349] The reaction was found to be sensitive to CO and was strongly inhibited by antibodies to NADH-cytochrome b_5 reductase (EC 1.6.2.2) and cytochrome b_5. At an oxygen concentration of 4 μM, 50% inhibition of imipramine-N-oxide reduction was observed. The NADH-dependent amine oxide reduction was markedly less sensitive to modifiers of the cytochrome P-450 system as compared with the NADPH-supported activity. These results would suggest that reducing equivalents for N-oxide reduction are transferred from NADH to cytochrome P-450 mainly via NADH-cytochrome b_5 reductase and cytochrome b_5 in the microsomal membranes. Powis and Wincentsen[354] feel that the rat liver microsomal NADH- and NADPH-dependent reduction of indicine-N-oxide involves different pathways: the NADH-supported reaction is inhibited 48% by 0.5 mM KCN and 45% by 0.8 M acetone, while the NADPH-supported reduction is inhibited only 3% by KCN and stimulated 28% by acetone. Phenobarbital pretreatment produces a selective increase in the maximal rate of the NADPH-dependent N-oxide reduction.

Ultimate proof of the ability of cytochrome P-450 to mediate N-oxide reduction comes from experiments with a reconstituted system containing highly purified rabbit liver microsomal cytochrome P-450 and NADPH-cytochrome c(P-450) reductase.[355] Such system catalyzes the anaerobic reduction of tiaramide-N-oxide by NADPH at a rate comparable to that in intact rabbit liver microsomes. The reaction is blocked by O_2 and CO. When reduced methyl viologen is used as electron donor, N-oxide reduction proceeds at higher rate in the absence of NADPH-cytochrome c(P-450) reductase.

Dajani et al.[352] studied the hepatic and extrahepatic reduction of nicotine-1′-N-oxide in various tissues of the rat. Most tissues reduced the N-oxide in the following order: liver > small intestine > kidney > heart > lung. The authors found substantial reductive activity in both the soluble and the microsomal fraction of rat liver and intestine. the reductases were nonspecific with regard to their requirement for NADH and HADPH. Air or boiling abolished the reducing activity in all fractions. There was, however, a marked difference in the sensitivities of the individual fractions toward various modifiers of drug metabolism. It was concluded that, in addition to cytochrome P-450, part of the reductase activity might be linked to a NADPH-dependent flavoprotein enzymes, such as NADPH-cytochrome c reductase or xanthine oxidase.

However, it has to be mentioned that solubilized rat liver NADPH-cytochrome c reductase shows no N-oxide reductase activity by itself.[348] Murray and Chaykin[356] succeeded in solubilizing an enzyme from hog liver that reduces nicotinamide-N-oxide to nicotinamide. The reductase is a metalloflavoprotein and shows dependence on NADH and some other cytoplasmic cofactor(s). In addition, nicotinamide-N-oxide is reduced by xanthine oxidase from liver and milk; the reaction is inhibited by cyanide and oxygen.[357] Xanthine oxidase also reduces a series of purine N-oxides to the corresponding parent amines.[358] This metabolic step appears to be not related to the oncogenic actions exerted by some of the purine N-oxides, but probably should be considered to represent a detoxification mechanism.[359]

Moreover, N-oxide reduction has been detected to occur in the mitochondrial inner membrane of rat liver.[360] NADPH is the preferred cofactor. The reductase activity is markedly inhibited under aerobic conditions. Isocitrate and α-ketoglutarate stimulate N-oxide reduction. The NADPH-dependent reaction is not inhibited by metyrapone, n-octylamine, or DPEA, but is partially suppressed by CO.

The ability of heme-complexed Fe^{++} to effect N-oxide reduction is not surprising. Thus, the N-oxides of N,N-dimethylaniline, N,N-dimethylaminoazobenzene or imipramine are readily reduced to the corresponding parent amines in erythrocytes or in aerobic solutions of hemoglobin.[109,347,361] Methemoglobin is formed during this process as a result of transformation of aromatic N-oxides to aminophenols, which, in the presence of O_2, oxidize hemoglobin.[361] The biological importance of extrahepatic N-oxide reduction, particularly in the blood, has been pointed out by Bickel[269] who demonstrated that N-oxide reduction in vivo is decreased by only 5 to 10% in hepatectomized rats.

Finally, N-oxide reductase activity has been detected in microorganisms. Resting cells of *E. coli* reduce nicotinic acid N-oxide to nicotinic acid.[362] Pyridine-N-oxide is reduced to pyridine in fermenting sucrose solutions by using baker's yeast.[363] 4-Picoline-N-oxide is not reduced under the same conditions.

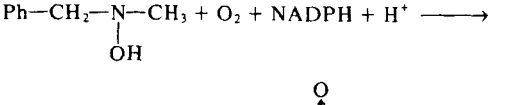
B. Oxidation of N-Oxidized Compounds

Aerobic incubation of N-methyl-,[30,364] N-ethyl-,[28] N-propyl-,[365] or N-benzylamphetamine[29] with rat and rabbit liver preparations fortified with NADPH results in formation of the corresponding secondary hydroxylamines along with significant amounts of nitrone analogues. The latter are believed to represent intermediates in the liver microsomal conversion of the hydroxylamines to ketoximes, another type of metabolites regularly formed from amphetamines.[30,364] When N-hydroxy-N-methylamphetamine is used as the substrate, considerably more nitrone is formed in the presence of rat liver 12,000 x g supernatant fortified with NADPH than with supernatant or cofactor alone;[364] on the other hand, appreciable quantities of nitrone are obtained, when N-hydroxy-N-propylamphetamine is incubated with mixtures containing supernatant but no cofactor, or boiled supernatant with NADPH.[365] These findings clearly indicate that both enzymic and nonenzymic[70] mechanisms are operative in the oxidative transformation of the hydroxylamines to the nitrones.

Aerobic incubation of phentermine[5,366] with rabbit and guinea pig liver preparations supplemented with cofactor or oral administration of the arylalkylamine to man[367] results in production of the corresponding primary hydroxylamine along with appreciable amounts of nitroso and nitro derivatives. More nitroso analogue is formed in the liver microsomal fraction than in the 9,000 x g supernatant of the same liver under identical conditions.[5] Unexpectedly, the nitroso compound appears to be not formed through enzymic or nonenzymic oxidation of N-hydroxyphentermine, since the hydroxylamine is not further oxidized when incubated with rabbit liver homogenate in the presence of NADPH and O_2.[5,366] The nitro compound is presumed to derive from the

nitroso analogue by autoxidation at alkaline pH.[366] Alternatively, formation of the nitro derivative from N-hydroxyphentermine has been proposed to involve the catalytic production of H_2O_2 by the cytochrome P-450 system.[114] Thus, the oxidation of N-hydroxyphentermine to 2-methyl-2-nitro-1-phenylpropane in washed rat liver microsomal fractions is increased by phenobarbital treatment and decreased by CO and DPEA. Oxidation is further severely blocked by hemoglobin and catalase. These findings have been interpreted to mean consumption by the hemoproteins of H_2O_2 generated during the NADPH-supported mixed-function oxidation followed by impairment of the H_2O_2-dependent oxidation of N-hydroxyphentermine. This view is supported by the stimulatory effect of azide on the formation of the nitro analogue. Nitro compounds have been further found to arise from oxidation of aralkyl oximes.[368,369] The latter are probably formed through dehydrogenation of N-hydroxyarylalkylamines.[30,364] Incubation in the presence of NADPH and O_2 of 1-phenyl-2-propanone oxime with 9,000 x g liver supernatant fraction from a number of species yields 2-nitro-1-phenylpropane.[368,369] Nitro formation is almost absent in assay media containing no cofactor or heat-denaturated supernatant.[369] The nitro compound, therefore, must arise largely from enzymic metabolism of the oxime, the reaction sequence probably being N-oxidation followed by isomerization.[369]

The formation of nitrone intermediates reported above is probably catalyzed by the mixed-function amine oxidase (EC 1.14.13.8). The enzyme has been demonstrated to mediate formation of an unstable oxide from N-methyl-N-benzylhydroxylamine which preferentially dehydrates to the stable phenyl-N-methylnitrone.[297] The reaction sequence is given by Equations 2 and 3.



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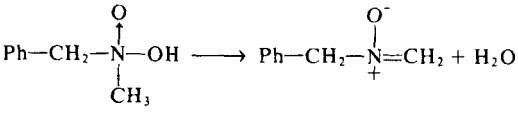
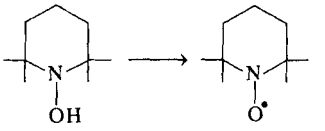


(3)

The enzyme also catalyzes oxidation of N-hydroxy-2,2,6,6-tetramethylpiperidine and some other alicyclic secondary hydroxylamines to yield stable nitroxide radicals (Equation 4).[126,370]



(4)

NADPH-dependent hepatic microsomal formation of nitroxide radicals has been furthermore shown to occur during oxidative activation of the hydroxylamines of a series of amine compounds, such as 2-aminofluorene, 2-naphthylamine, 4-aminostilbene, or carcinogenic aminoazo dyes.[127,371] It has been postulated that tumor formation may be initiated by such reactive species.[371,439]

Bartsch and Hecker[372] detected a nitroxyl free radical of N-hydroxyacetamidofluorene in a horseradish peroxidase-H_2O_2 system and predicted a free radical dismutation type

mechanism to account for the nitrosofluorene and N-acetoxy-2-acetamidofluorene products observed. Similar results were obtained with a hematin-cumene hydroperoxide system;[373] the dismutation rate constant of the nitroxyl free radical of N-hydroxyacetamidofluorene was calculated to be $2.7 \times 10^5\ M^{-1}s^{-1}$. The nitroxide is most probably generated via the spin trapping of a cumene hydroperoxyl radical.[374]

In the presence of oxygen, hemoglobin rapidly oxidizes phenylhydroxylamine to nitrosobenzene.[375] Reactions involved in this oxidation process have been formulated tentatively as follows:

$$HbFe^{2+}O_2 + Ph-NHOH \longrightarrow HbFe^{2+}OOH^- + Ph-\overset{+}{N}-OH \quad (5)$$

$$HbFe^{2+}OOH^- + Ph-NHOH \longrightarrow HbFe^{3+} + 2OH^- + Ph-\dot{N}-OH \quad (6)$$

$$HbFe^{2+}OOH^- + Ph-\dot{N}-OH \longrightarrow HbFe^{3+} + OH^- + O^{2-} + Ph-\overset{+}{N}-OH \quad (7)$$

$$Ph-\overset{+}{N}-OH + OH^- \longrightarrow Ph-N=O + H_2O \quad (8)$$

$$Ph-\overset{+}{N}-OH + 1/2\ O^{2-} \longrightarrow Ph-N=O + H_2O \quad (9)$$

N-Alkylphenylhydroxylamines, such as N-benzylphenylhydroxylamine, react with oxyhemoglobin to yield the corresponding nitrones.[376]

C. N-Dealkylation of N-Oxidized Compounds

Machinist et al.[377] described the properties of a pork liver microsomal dealkylase, which catalyzes the N-dealkylation of a number of closely related N,N-dialkylarylamine N-oxides (dimethylaniline-N-oxide aldolase; EC 4.1.2.24). The reaction proceeds according to

$$Ar-\overset{O}{\overset{\uparrow}{N}}-(CH_3)_2 \longrightarrow Ar-NH-CH_3 + CH_2=O \quad (10)$$

and has been proposed to include a N-C transoxygenation step;[378,379] it does not require the presence of reduced pyridine nucleotides or O_2. The enzyme is strongly inhibited by SKF 525-A and pyridine. N-Dealkylase activity is also blocked by CO in NADH- or NADPH-reduced microsomal fractions. These findings suggest the enzyme to be identical with cytochrome P-450. This view is in accord with the ability of barbiturates to induce hepatic dealkylase activity.[380] In mammals, the enzyme is most concentrated in liver microsomes and appears to be abundant in the pig.[380]

N-Oxides have been proposed to be obligatory intermediates in oxidative N-dealkylation.[93] This view was mainly based on the observation that in rat and pig liver microsomes the rate of demethylation of N,N-dimethylaniline-N-oxide is several times higher than that of overall demethylation of N,N-dimethylaniline.[378] However, there is a body of evidence against such concept:

1. The high apparent K_m value (80 mM) for N,N-dimethylaniline-N-oxide[377] appears to be prohibitive for the demethylation of N-oxide formed at relatively low concentrations in incubation mixtures containing N,N-dimethylaniline.

2. In the steady state, the rate of reduction of imipramine-N-oxide is several times higher than that of N-oxide demethylation.[104]
3. Dithiothreitol, a selective N-oxidase inhibitor, strongly blocks guinea pig liver microsomal N-oxide formation from normethadone without affecting the rate of overall demethylation of the tertiary amine.[88]
4. Pretreatment of rats with 2-aminofluorene stimulates liver microsomal N-oxidation of N,N-dimethylaniline without affecting demethylation of the amine.[275]
5. In the course of poisoning of rats with thioacetamide, hepatic microsomal N,N-dimethylaniline demethylation recovers while demethylation of N,N-dimethylaniline-N-oxide remains depressed.[381]
6. Separation of the rat hepatic endoplasmic reticulum into smooth and rough fractions results in partial separation of the activities for N-demethylation and N-oxidation of N,N-dimethylaniline.[382]
7. The patterns of differentiation of N-oxidation and N-demethylation of N,N-dimethylaniline are different in developing rat liver.[73]
8. Various animal species readily N-dealkylate tertiary-amine compounds in spite of negligible concentrations of N-oxide dealkylase in their tissues.[8,104,268,383]
9. Certain tertiary amines, such as N-methylcarbazole, which cannot form N-oxides, readily undergo N-dealkylation in fortified liver microsomal fractions.[35] These findings hint at a N-oxide-independent pathway of N-dealkylation of tertiary amines.

From incubates of ferrihemoglobin, or ferricytochrome c, with N,N-dimethylaniline-N-oxide, Kiese et al.[361] isolated large amounts of N-methylaniline. Similarly, N,N-dimethylaminoazobenzene-N-oxide undergoes rapid demethylation to N-methylaminoazobenzene in aerobic solutions of hemoglobin.[109,384] The azo dye amine-N-oxide is also demethylated in aqueous solutions in the presence of Fe^{++}-ions.[109] The mechanism of N-demethylation in these systems remains to be elucidated.

Certain strains of bacteria have been recognized to mediate nonoxidative demethylation of trimethylamine-N-oxide to dimethylamine. The system from *Pseudomonas aminovorans*[385] shows pH optimum at 6.0; the apparent K_m for trimethylamine-N-oxide is 2 mM. The N-dealkylase differs from the corresponding mammalian enzyme in that it is strongly blocked by cyanide and mercurials, but is insensitive to CO, SKF 525-A, and pyridine. A similar enzyme has been purified from *Bacillus* PM 6 that, in addition, catalyzes N-dealkylation of chlorpromazine and propoxyphene N-oxide.[386]

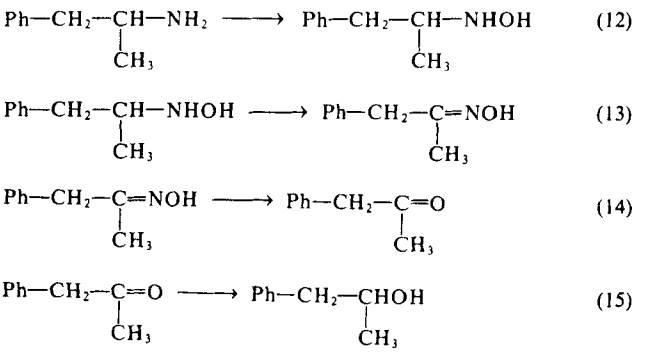
N-Methyl-N-alkylhydroxylamines can undergo N-dealkylation by the mixed-function amine oxidase system (EC 1.14.13.8). The reaction proceeds via the formation of nitrones as given in Equations 2 and 3; the latter hydrolyze to yield the primary hydroxylamines and formaldehyde:[297]

$$\mathrm{Ph{-}CH_2{-}\overset{+}{N}(O^-){=}CH_2 + H_2O \longrightarrow Ph{-}CH_2{-}NHOH + CH_2{=}O} \qquad (11)$$

D. Deamination of N-Oxidized Compounds

In 1955, Axelrod[387] demonstrated that amphetamine is metabolized by rabbit liver microsomes to phenylacetone and ammonia. The enzyme system catalyzing this reaction is a typical mixed-function oxidase in that it requires oxygen and NADPH. Later experiments revealed the formation of significant amounts of phenylacetone oxime along with N-hydroxyamphetamine and phenylpropanol in rabbit liver 9,000 x g supernatant fractions containing amphetamine and cofactor.[2,388] These findings suggested that the oxime might be an intermediate in the oxidative deamination of the

primary arylalkylamine.[388] The proposed reaction sequence is given in Equations 12 to 15:



(12)

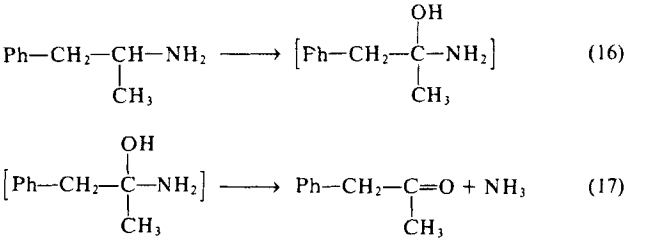
(13)

(14)

(15)

However, there is some doubt whether phenylacetone oxime is a true metabolite of amphetamine, since N-hydroxyarylalkylamines have been shown to readily decompose to the corresponding oximes (Equation 13).[70,71] The inability of boiled rabbit liver preparations to form significant amounts of oxime from R(−) N-hydroxyamphetamine and the stereoselectivity of this process suggest that at least part of the oxime arises from metabolic transformation.[2]

There is further controversy as to the importance of N-oxidation in deamination of amphetamines (Equation 12). Although Caldwell[156] detected a close relationship between N-oxidation and deamination in vivo of amphetamines, experiments in vitro with ^{18}O are ambiguous: when rabbit liver microsomal fractions containing amphetamine and NADPH are incubated in an atmosphere of ^{18}O, the carbonyl group of phenylacetone formed contains either ^{18}O or ^{16}O.[390] Thus, only part of the ketone formed results from hydrolytic mechanisms. The other portion arises from direct C-hydroxylation of the amine as originally proposed by Brodie et al. (Equations 16 and 17).[391]



(16)

(17)

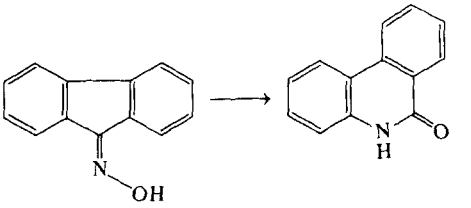
E. Rearrangement of N-Oxidized Compounds

Miller and Miller[392] demonstrated that in rats dosed with N-hydroxy-2-acetylaminofluorene, urinary excretion of 1-hydroxy-2-acetylaminofluorene was consistently higher than after administration of 2-acetylaminofluorene. It was concluded that the N-hydroxy derivative may rearrange in vivo to form the ortho-aminophenol, possibly in a manner analogous to the rearrangement of arylhydroxylamines to phenolic amines in acid.[393] Similarly, rats treated with N-hydroxy-4-acetylaminostilbene excrete more of the 3-hydroxy derivative than after injection of 4-acetylaminostilbene.[97]

N-Hydroxyacetanilide, N-hydroxy-4-acetamidobiphenyl, N-hydroxy-2-acetamidonaphthalene, and N-hydroxy-2-acetamidofluorene are isomerized to the corresponding o-hydroxy derivatives by the soluble fraction of rat or rabbit liver.[74] The enzyme-catalyzed isomerization of N-hydroxy-2-acetamidofluorene to the o-amidophenol has been studied in detail by Gutmann and Erickson.[394,395] Rearrangement is dependent on the

synergistic action of two components. One of these is inducible by 3-methylcholanthrene and is associated with the microsomal fraction of rat liver; the other, nondialyzable, component is localized in the soluble fraction of rat liver. The process of isomerization proceeds optimally at a pH near seven and does not require the presence of NADPH. The reaction appears to be restricted to arylhydroxamic acids in which the nitrogen is linked to an aromatic system capable of extended conjugation. Experiments with N-hydroxy-2-acetamidofluorene labeled with ^{18}O in the hydroxyl group indicate that the hydroxyl group is transferred in toto from the nitrogen to the carbon atom of the aromatic moiety of the molecule.

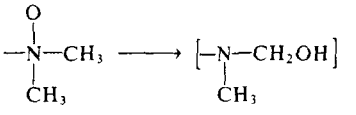
An interesting oxime-amide rearrangement has been recently reported by Sternson and Hincal:[123] rat liver homogenates convert fluorenone oxime to phenanthridinone (Equation 18). The reaction is catalyzed by enzyme(s) located in the mitochondrial and microsomal fraction



(18)

of rat liver. The enzyme(s) do not require O_2 but transformation is accelerated by NADPH. Rearrangement is insensitive to CO, but is stimulated by pretreatment of rats with phenobarbital.

Studies on tertiary-amine N-oxide rearrangements have established that a number of N-oxides undergoes enzymic and ferric ion-catalyzed isomerization to carbinolamines (Equation 19);[378,379] the latter decompose to yield secondary amines and formaldehyde (Equation 20). The overall reaction is a model for oxidative N-dealkylation (see above). By using trimethylamine-N-oxide, Craig et al.[396] proposed the involvement in this process of a free radical mechanism.



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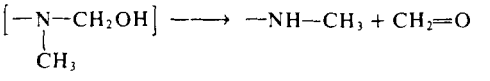


(20)

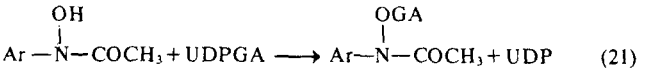
With N,N-dimethylaniline-N-oxide, an additional pathway of isomerization was detected resulting in aminophenol formation. Thus, Bamberger and Leyden[397] identified 2- and 4-dimethylaminophenol among the products of thermolysis of the N-oxide. These compounds were also isolated from incubates of N,N-dimethylaniline-N-oxide with either ferricytochrome c or ferrihemoglobin.[361] Analogously, Terayama[384] found 3-hydroxy-4-dimethylaminoazobenzene in incubation mixtures of 4-dimethylaminoazobenzene-N-oxide with hematin.

Coccia and Westerfeld[345] demonstrated a novel type of intramolecular N-oxide rearrangement: anaerobic solutions of Fe^{++}-ions convert chlorpromazine-N-oxide to chlorpromazine sulphoxide.

F. Metabolic Conjugation of N-Oxidized Compounds

The initial activation reaction of aromatic amines and amides was first discovered by Cramer et al.[113] who detected a new metabolite in the urine of rats continuously fed on a diet containing N-2-acetylaminofluorene. This metabolite was identified as the

glucuronide of N-hydroxy-2-acetylaminofluorene. Meanwhile, a large number of N-hydroxy compounds have been found to be converted to the corresponding glucosiduronic acids.[21,26,158,185–187,193] The reaction proceeds via catalysis of microsomal UDP glucuronyltransferase (EC 2.4.1.17) and requires uridine diphosphate glucuronic acid (UDPGA).[398]



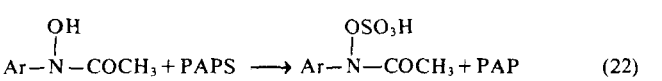
$$\mathrm{Ar{-}\overset{\overset{\mathrm{OH}}{|}}{N}{-}COCH_3 + UDPGA \longrightarrow Ar{-}\overset{\overset{\mathrm{OGA}}{|}}{N}{-}COCH_3 + UDP} \qquad (21)$$

The glucosiduronic acids of N-hydroxy derivatives of arylamines are readily split by mammalian or bacterial β-glucuronidase (EC 3.2.1.31).[399,400]

Glucuronide conjugates have been considered to be rather inert chemically and are generally regarded as end products in the metabolism of foreign compounds. However, the glucuronide of N-hydroxy-2-acetylaminofluorene has been found to have unusual reactivity with certain nucleophiles. At neutral pH, the glucuronide reacts with methionine and guanosine to yield 1- and 3-methylmercapto-N-2-acetylaminofluorene as well as a mixture of N-(guanosin-8-yl)-N-2-acetylaminofluorene and N-(guanosin-8yl)-aminofluorene;[398,401] reaction is facilitated in more alkaline medium. These findings suggest a possible role of the glucuronide conjugate in the mechanism of binding of the carcinogen to nucleic acids associated with mutagenic and oncogenic effects.[398]

Although there is no direct evidence for the formation in vivo of sulfate esters of N-hydroxy compounds, this type of conjugate appears to represent biologically active material.[402] If produced in vivo, such conjugates would exist only transiently because of their instability and high reactivity.[398] Indirect evidence that the sulfate ester of N-hydroxy-2-acetylaminofluorene might be an ultimate carcinogen comes from experiments with acetanilide:[402] the compound inhibits hepatocarcinogenicity of N-hydroxy-2-acetylaminofluorene by diminishing the availability of sulfur. Injection of sulfate ions in rats given N-hydroxy-2-acetylaminofluorene increases the toxicity of the carcinogen.[403] On the other hand, studies with carcinogenic N-oxidized purines failed to show correlation between sulfate conjugation and tumor-producing capacity.[404] Furthermore, the sebaceous gland of the external auditory canal (Zymbal's gland) and the mammary gland of rats lack N-hydroxy-2-acetylaminofluorene sulfotransferase activity although these tissues are highly susceptible to the carcinogen.[405]

The formation of sulfate conjugates of N-hydroxy compounds has been demonstrated in vitro in fortified soluble fractions of rat liver.[406] Synthesis involves the transfer of sulfate from adenosine-3′-phospho-5′-phosphosulphate (PAPS) to a N-hydroxy-N-arylacetamide catalyzed by one or more sulphotransferases (Equation 22). The esterifi-



$$\mathrm{Ar{-}\overset{\overset{\mathrm{OH}}{|}}{N}{-}COCH_3 + PAPS \longrightarrow Ar{-}\overset{\overset{\mathrm{OSO_3H}}{|}}{N}{-}COCH_3 + PAP} \qquad (22)$$

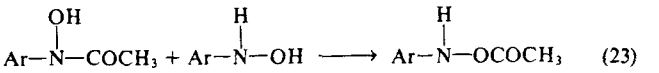
cation in rats is strongly affected by sex hormones.[407] N-Hydroxy-2-acetylaminofluorene sulphotransferase appears to be different from liver sulphotransferases forming phenolic sulphates.[398] Andrews et al.[408] described the formation of a dimer of 2-acetylaminofluorene during sulphation of N-hydroxy-2-acetylaminofluorene by rat liver soluble fraction.

N-Acetoxy derivatives of N-hydroxy compounds represent another type of highly reactive species.

Nonenzymic formation of o-acetyl derivatives of a number of N-hydroxy-N-arylacetamides proceeds via acylation by acetyl coenzyme A, carbamoyl phosphate, or acetyl phosphate.[409,410] N-Hydroxy-2-acetylaminofluorene is acetylated to a much greater extent than 2-acetylaminofluorene or 2-aminofluorene, the pH optimum being

10.[409] Carbamoyl phosphate is a more powerful acetyl donor with the N-hydroxy derivatives of 2-acetylaminonaphthalene, 2-acetylaminophenanthrene, and 4-acetylaminostilbene; reactions proceed most rapidly at pH 4.5.[410]

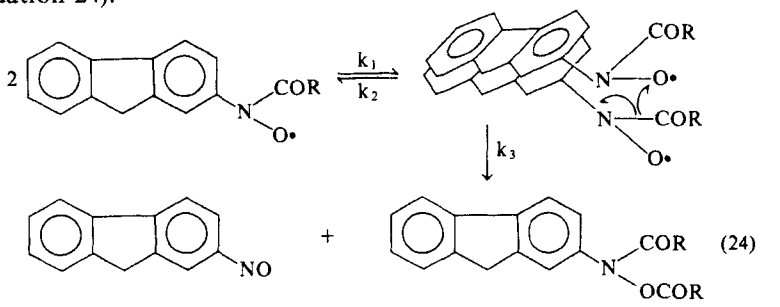
Bartsch et al.[411,412] detected enzymic formation of N-acetoxy arylamines in the cytosol of liver and other tissues of the rat by transacetylation of N-hydroxy-N-arylacetamides



(23)

(Equation 23). Considerable difference was observed between hepatic and mammary transacetylase with respect to stability, pH optimum and substrate specificity.

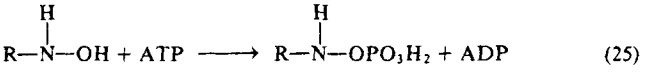
Finally, dismutation of a nitroxide free radical formed from N-hydroxy-2-acetylaminofluorene yields N-acetoxy-2-fluorenylacetamide and nitroso compound (Equation 24).[372,413]



(24)

The N-acetoxy derivatives formed along either of these pathways are too reactive to permit isolation, and their formation, therefore, can be assessed only through the reaction products with nucleophiles such as methionine or guanosine.[409,414,415]

Hydroxylamine kinase activity of pyruvate kinase (EC 2.7.1.40) has been detected by Cottam et al.[416] The reaction, proceeding according to Equation 25, exhibits an absolute requirement for bicarbonate, a nucleoside triphosphate, and a divalent metal (Mn^{++} or



(25)

Zn^{++}). N-Methyl- and N,N-dimethylhydroxylamine also undergo phosphorylation. The apparent K_m value for hydroxylamine has been determined to be 24 mM. Similarly, N-hydroxy-2-acetylaminofluorene is transformed to the phosphate ester in the presence of ATP and Mg^{++}.[417]

O-Methylation of N-hydroxy-2-acetylaminofluorene and other related N-hydroxy derivatives is performed by a soluble rat liver fraction in the presence of cysteine and S-adenosylmethionine as a methyl donor.[418] The enzyme system is different from catechol O-methyltransferase (EC 2.1.1.6).

In alkaline media, nitrosobenzene is likely to undergo nonenzymic transformation to a short-lived glutathione conjugate which rearranges to glutathionesulphinanilide in the presence of H^+-ions.[130]

G. Acylation and Deacylation of N-Oxidized Compounds

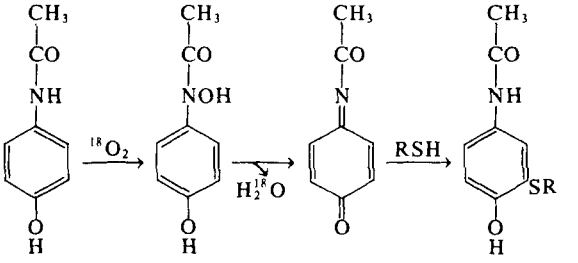
Administration of N-hydroxy-2-aminofluorene to rats results in substantial urinary excretion of N-hydroxy-2-fluorenylacetamide due to in vivo N-acetylation of the hydroxylamine.[419] The enzyme system responsible for N-acetylation has been recognized by Lotlikar and Luha[420] to reside in the cytosol of the livers of various species; it requires the presence of acetyl coenzyme A.

The reverse reaction, N-deacylation of N-hydroxyacetamides, has been noted in a number of species. There appear to exist two types of deacylases located both in the cytosol[421] and the microsomal fraction[422] of different tissues. Microsomal deacetylase activity for N-hydroxy-2-acetylaminofluorene is most concentrated in the livers of guinea pigs and hamsters; the guinea pig enzyme is blocked by SKF 525-A and fluoride. Järvinen et al.[423] succeeded in purifying two deacetylases from guinea pig liver microsomal fractions mediating hydrolysis of N-hydroxy-2-acetylaminofluorene. Enzyme I (molecular weight 200,000) hydrolyzes the hydroxamic acid 265 times faster than 2-acetylaminofluorene, whereas enzyme II (molecular weight 41,000) hydrolyzes 2-acetylaminofluorene 1.4 times faster than the corresponding hydroxamic acid. Both enzymes exhibit the character of esterases. N-Hydroxy-2-acetylaminofluorene is most readily split at pH 6.5 by enzyme I, the apparent K_m value being 0.44 mM. The enzyme is strongly inhibited by diethyl p-nitrophenylphosphate, fluoride, and mercuric chloride.

The simultaneous occurrence of N-acylation and N-deacylation of N-hydroxy compounds in the cytosol of living cells appears to regulate the concentration of free hydroxylamine available for binding to macromolecular receptors.[417]

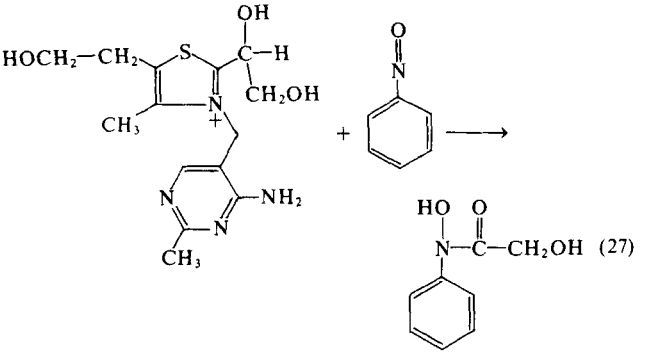
H. Miscellaneous Reactions of N-Oxidized Compounds

A further example serves to illustrate that dehydration of N-hydroxy compounds is a metabolic route contributing to the formation of active arylating species which can react with nucleophiles. By using paracetamol, Hinson et al.[424] did not observe incorporation into the molecule of atmospheric ^{18}O during incubation in the presence of glutathione with fortified hamster liver microsomal fraction. This has been interpreted to mean loss of heavy label through dehydration of the N-hydroxy intermediate to yield the corresponding acetimidoquinone which then reacts with glutathione (Equation 26).



(26)

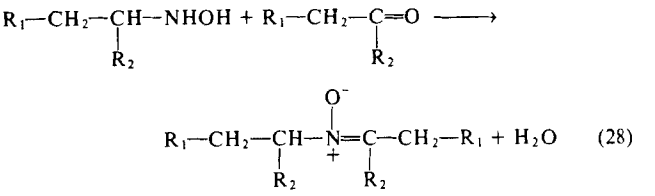
A novel metabolic pathway to hydroxamate production has been detected by Corbett and co-workers.[425,426] It results from the action of thiamine-dependent enzymes, such as pyruvate decarboxylase (EC 4.1.1.1) or transketolase (EC 2.2.1.1), on arylnitroso compounds. With α-hydroxyethylthiamine and nitrosobenzene the transketolase-catalyzed reaction proceeds as depicted in Equation 27:



(27)

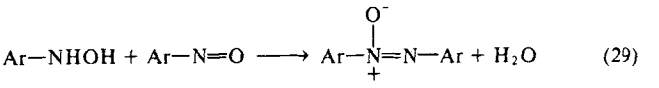
The reaction has been also shown to occur in the soluble fraction of rat liver.

Reaction of primary hydroxylamines with aldehydes or ketones furnishes nitrones.[427] The simultaneous formation of carbonyl- and N-hydroxy derivatives has been demonstrated during oxidative deamination of amphetamines.[2,387,388] Under simulated biological conditions, Beckett et al.[428] showed condensation of the metabolically formed N-hydroxy derivatives of amphetamine, mexiletine, and norfenfluramine with their products of deamination to give the corresponding nitrones. The reaction can be formulated as follows:



(28)

Arylhydroxylamines couple with nitroso arenes to form azoxy compounds (Equation 29); this reaction may occur during partial oxidation of metabolically formed arylhydroxylamines to the nitroso derivatives. Thus, an azoxybenzene is the main



(29)

product resulting from rabbit liver microsomal N-oxidation of p-thioanisidine.[23] Similarly, Irving[89] reported formation of azoxyfluorene in incubation mixtures containing N-hydroxy-2-acetylaminofluorene and fortified rabbit liver microsomal fraction. 4,4′-Dichloroazoxybenzene was identified in incubates of 4-chlorophenylhydroxylamine with chloroperoxidase (EC 1.11.1.10) and H_2O_2.[304] 4,4′-Dichloroazoxybenzene is also produced during N-oxidation of 4-chloroaniline by certain types of soil fungi.[309]

VII. CONCLUDING REMARKS

Research on the oxidation of nitrogen in organic compounds has been stimulated owing to the development of highly sensitive techniques permitting identification and quantification of N-oxidized products in biological material.

Naturally occurring hydroxamates have been recognized to serve as growth factors and iron-transport agents in microorganisms. However, the role of naturally occurring N-oxides in plant and animal tissues is less well understood.

A large number of nitrogenous compounds undergo N-oxidation after in vivo administration to various animal species. The endoplasmic reticulum of the cell has been detected to be the site of metabolic transformation. Two microsomal N-oxidase systems have been recognized to be involved in the N-oxidation of amine substrates: the cytochrome P-450-dependent electron transport chain (EC 1.14.14.1), and the mixed-function amine oxidase (EC 1.14.13.8). The former predominantly N-oxidizes primary amines, amides, and imines; it further appears to be of some importance in the N-oxidation of certain types of tertiary amine compounds. The latter system excels in N-oxidation of numerous secondary and tertiary amines although the natural role of this enzyme appears to be participation in S-oxidation reactions. Differentiation of both metabolic pathways has been attempted by considering some physicochemical properties of amine substrates, such as basicity or nucleophilicity. However, there are no clear-cut data indicating predominance of either of these factors in the interaction of amines with the individual N-oxidases.

Many N-oxidized compounds undergo further transformation through various reactions, such as reduction, oxidation, dealkylation, deamination, rearrangement, or conjugation. Some of the secondary metabolites represent stable end-products, others, such as nitroxides or certain conjugates of hydroxamic acids, are highly active species reacting with macromolecular cell constituents. Their function as ultimate carcinogens awaits further elucidation.

ABBREVIATIONS USED

DDT:	1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane
DPEA:	2,4-dichloro-6-phenylphenoxyethylamine
FAD:	flavin adenine dinucleotide
FMN:	flavin mononucleotide
NADH:	nicotinamide-adenine dinucleotide, reduced
NADPH:	nicotinamide-adenine dinucleotide phosphate, reduced
PAPS:	adenosine-3′-phospho-5′-phosphosulphate
SKF 525-A:	2-diethylaminoethyl-2,2-diphenylvalerate
UDPGA:	uridine diphosphate glucuronic acid

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