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Reactive electrophilic metabolites of aromatic amine and amide carcinogens

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1. ABSTRACT

Aromatic and heterocyclic amines are a major class of chemical mutagens and carcinogens. The toxicity of these compounds is a consequence of their metabolic activation. The best-characterized enzymatic pathways for aromatic amine activation lead to the formation of reactive esters such as *N*-acetoxyarylamines, which are believed to be precursors of short-lived nitrenium ions. In the 1960s, nitrenium ions were invoked as likely intermediates in the formation of arylamine-derived DNA adducts. More recently, nitrenium ion chemistry has been studied by methods such as trapping with azide ion, laser flash photolysis, and the preparation of highly stabilized of examples (*e.g.*, dianisylnitrenium ion). In this review, we discuss the development of our understanding of nitrenium ion chemistry, with emphasis on their generation in biological systems and their reactions with critical targets such as DNA.

2. INTRODUCTION

In this review, we will focus on the formation and chemical reactivity of the electrophilic species that arise from the metabolism of heterocyclic and aromatic amines. These species – especially, nitrenium ions and related structures – were suggested to account for nucleic acid binding by arylamine carcinogens nearly 50 years ago, but recent advances have provided a clearer understanding of their properties and reactivities.

Exposure to aromatic amine carcinogens may have been an aspect of human life since our species began. The evolution of the large brain of Homo sapiens could have taken place, it has been argued, only because of the taming of fire and the development of cooking (1, 2). Chewing roasted food did not require the ape's large jaw muscles, and cooking made available a more abundant caloric supply, even while reducing the energetic costs of

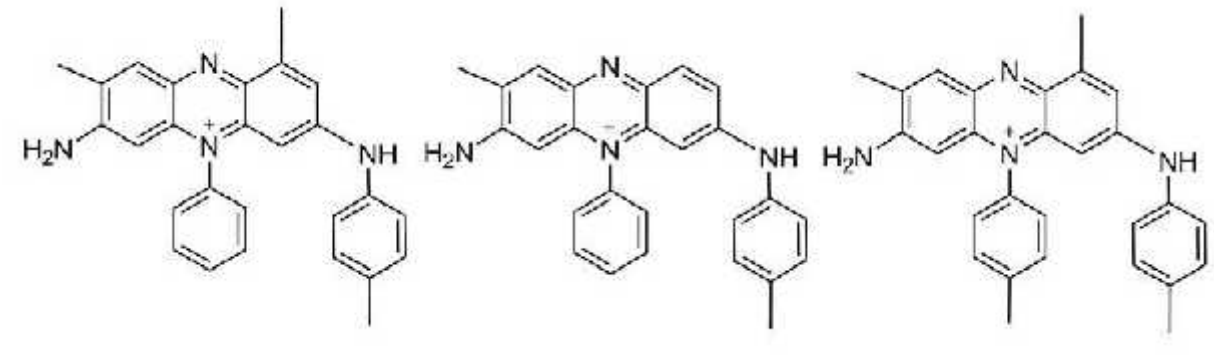

Figure 1. Major components of mauveine dye.

digestion. These changes made possible the massive expansion of the brain case. If this explanation is valid, dietary exposure to the heterocyclic amine carcinogens formed by roasting meat is as old as the human race. However, widespread exposure to the benzenoid arylamines did not take place until the rise of the chemical industry in the 19th Century.

3. EARLY SYNTHETIC CHEMISTRY OF AROMATIC AMINES

In 1856, William Henry Perkin synthesized mauveine, the first successful synthetic dye for silk and cotton, thereby launching the fashion revolution that reached its peak in the "Mauve Decade" of the 1890s. Mauveine was produced by the oxidation of aniline (in the form of an impure preparation that contained substantial amounts of toluidine isomers) with potassium dichromate. The history of Perkins' discovery and its influence on the rise of the chemical and pharmaceutical industries has been recounted in a popular book (3).

Less well-known than the role of mauveine in chemistry and fashion are the toxic consequences of its production and use. As early as the 1860s, industrial production of mauveine and other synthetic dyes was causing serious local groundwater contamination, and the leaching of synthetic dyes from clothing was being blamed for skin inflammations and rashes (3). The multiple constituents of mauveine, amino-substituted phenazines (the full structural characterization has been achieved only with the aid of modern instrumental analysis techniques (4); Figure 1) are polycyclic *N*-aryl compounds studded with "structural alerts" for mutagenicity and carcinogenicity. However, the hazards due to production of mauveine and other aniline dyes were soon eclipsed by those of the azo dyes, such as Congo red (Boettiger, 1883); *see review by Freeman in this series*. The occupational epidemic of arylamine-induced bladder cancer was recognized soon after the industrial production of polycyclic precursors of azo dyes (such as benzidine and 2-naphthylamine) began (*see review by Dietrich and Golka, and other chapters in this series*).

The structures of mauveine components reveal the tendency of oxidized anilines to react with additional monomer units, especially at the *para* position, to form extended *N*-aryl chains. Late in the 19th Century, Bamberger discovered the eponymous rearrangement of *N*-arylhydroxylamines to 4-aminophenols, another reaction that demonstrates the formation of an electrophilic *N*-aryl species that reacts with a nucleophile (in this case, water) at the *para* position (5). In 1951, consideration of the mechanism of the Bamberger rearrangement led Heller, Hughes, and Ingold to propose the formation of the nitrenium ion intermediate (see below); (6).

4. MONOCYCLIC AND POLYCYCLIC ARYLAMINES

Polycyclic arylamines (derivatives of fluorene, biphenyl, naphthalene, etc.) are generally more potent mutagens and carcinogens than are the monocyclic arylamines (aniline and derivatives). This greater potency may be due to their greater hydrophobicity (7, 8), although the correlation of hydrophobicity with other chemical properties, such as nitrenium ion stability, complicates interpretation of these trends (9). Nevertheless, aniline derivatives, although typically less toxic than polycyclic arylamines on a molar basis, are prevalent in the environment at much higher levels ((10) and references therein). Some monocyclic arylamines, including *ortho*-toluidine and 2,6-dimethylaniline, are established rodent carcinogens, and attention has recently been drawn to their potential significance as human environmental carcinogens (11).

5. METABOLIC N-HYDROXYLATION

The study of the biological oxidation of aromatic amines was initiated by the research of Kiese and colleagues on the induction of methemoglobinemia by aniline (12). Nitrosobenzene was detected in the blood of dogs treated with aniline (13); later work implicated *N*-phenylhydroxylamine as the metabolite that causes haemoglobin oxidation (14). At about the same time (1959), the relevance of aromatic amine N-oxidation in chemical carcinogenesis was recognized, in the laboratory of James and Elizabeth Miller, McArdle Laboratory for Cancer Research, University of Wisconsin. Male rats were fed a diet containing 2-acetylaminofluorene (AAF); urine

(1 L) collected from these animals was treated with beta-glucuronidase (to hydrolyze glucuronide conjugates), extracted, and then separated by chromatography on long columns of silica gel. These arduous efforts culminated in the isolation of about 10 mg of a new metabolite and previously unknown substance, the hydroxamic acid *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) (15), which proved to be a more active carcinogen than the parent compound (reviewed in (16)).

6. ENZYMATIC OXIDATION OF AROMATIC AMINES

Since the initial discoveries of arylamine *N*-hydroxylation, several distinct routes for the enzymatic oxidation of aromatic and heterocyclic amines have been identified, each of which has the potential to generate reactive intermediates of toxicological importance.

The AAF *N*-hydroxylation paradigm, in which hydroxylamines are recognized as activated metabolites of carcinogenic aromatic amines, was quickly extended to additional primary aromatic amines, such as 2-naphthylamine (17) and 4-aminostilbene. Uehleke demonstrated that rat liver microsomal preparations catalyzed the NADPH-dependent *N*-oxidation of arylamines, including 2-naphthylamine, 2-aminofluorene, 4-aminostilbene, and 4-aminobiphenyl (18). Cytochrome P450 was discovered in 1964 (19). In the following years, evidence for the role of the P450 enzyme system as a major catalyst of arylamine *N*-oxidation in the liver (20) was adduced, for example, on the basis of the reaction's sensitivity to P450 inhibitors such as piperonyl butoxide (21) and CO (22). More incisive analysis of the capacities of individual enzymes to catalyze arylamine N-oxidation required their purification from native sources (*e.g.*, (23)). The next step forward was the enabling technology of recombinant protein expression (24) and its combination with sensitive genetic systems for the detection of aryl-amine mutagenicity (25) and genotoxicity (26), which allowed researchers to study the catalytic properties of specific human enzymes of xenobiotic metabolism with respect to arylamine activation. Human cytochrome P450 1A2, an enzyme that is well expressed in the liver (27, 28), is considered to be the paragon of arylamine-activating P450 enzymes. Nevertheless, many other cytochrome P450 enzymes (*e.g.*, 1A1, 1B1, 3A4, etc.) display detectable activity with one or more aromatic or heterocyclic amines (26, 29-32). Furthermore, one must interpret cautiously the results of studies with recombinant proteins. Enzyme activity *in vitro* does not translate directly into *in vivo* significance, since it does not necessarily reflect biological factors governing metabolism in the living person or animal, such as organ-specific expression, level of expression, protein induction and turnover, etc.

In addition to cytochrome P450, flavin monooxygenase (FMO) (33, 34), EC 1.14.13.8, is capable of forming *N*-arylhydroxylamines. FMO is a family of non-P450 hepatic microsomal enzymes comprising five human forms, two of which are well expressed in the liver (35). The mechanisms of substrate oxidation catalyzed by P450 and FMO are very different; the activated state of P450 enzymes is an oxenoid heme iron-oxygen species (36) whereas that of FMO is an hydroperoxyflavin (37). FMO is much less studied than P450 as a catalyst of arylamine *N*-oxidation, but both 2-naphthylamine (38, 39) and 2-aminofluorene (40) have been shown to be *N*-hydroxylated by FMO.

Prostaglandin H synthase (PGHS) is the enzyme catalyzing the first two steps in prostaglandin biosynthesis from polyunsaturated fatty acids: its cyclooxygenase activity converts arachidonic acid to PGG_2 and its hydroperoxidase activity reduces the hydroperoxide PGG_2 to its corresponding alcohol, PGH_2 (41). Two forms of the enzyme are found in humans: PGHS-1, the "housekeeping" enzyme, and PGHS-2, the inducible form that drives inflammation and is the target of "COX-2 inhibitor" drugs, such as celecoxib and rofecoxib (42). Many xenobiotics and carcinogens, including aromatic amines, can undergo oxidative activation catalyzed by PGHS, in a cooxidation process whereby arachidonic acid and the carcinogen are both oxidized (43-49). Cooxidation of aromatic amines can generate free radicals (50, 51), azo dimers (44), and nitro compounds (52), presumably by mechanisms that do not involve hydroxylamine intermediates.

Aminophenol metabolites can be formed from aromatic amines by at least two distinct routes: ring-hydroxylation (e.g. catalysed by cytochrome P450; (40, 53, 54)) and the rearrangement of hydroxylamines (11, 32). Aminophenols may be readily oxidized to reactive quinoneimines. This bioactivation route is reviewed by Murata and Kawanishi, in another paper in this series, and will not be considered further here.

7. METABOLIC CONJUGATION OF HYDROXYARYLAMINES AND HYDROXYARYLAMIDES GENERATES REACTIVE AND MUTAGENIC METABOLITES

The concept that *N*-hydroxy metabolites can be further activated by conversion to electrophilic esters also arose in the laboratory of Miller and Miller. As they later recounted, "About the same time (1967) Poirier (in our laboratory), unable to synthesize *N*-hydroxy-MAB (*N*-methyl-4-aminoazobenzene), synthesized its benzoic acid ester … *N*-benzoyloxy-MAB proved to be an electrophilic reactant at neutrality … Analogous studies with *N*-acetoxy-AAF showed that it had similar, but stronger, electrophilic reactivity. Thus, *N*-acetoxy-AAF reacted at neutrality with … guanine residues in … polynucleotides; under appropriate conditions nearly all of the guanine residues in DNA or RNA could be modified. … Further studies emphasized the possible importance of the reactivity of the electrophilic esters of *N*-hydroxy-AAF …" (16).

Conjugation of O atoms of xenobiotics with endogenous electrophilic donors, such as UDP-glucuronic acid, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), or acetyl CoA, is an important theme in xenobiotic metabolism (55), and each of these routes was found to generate reactive electrophilic esters of *N*-hydroxy-AAF, at

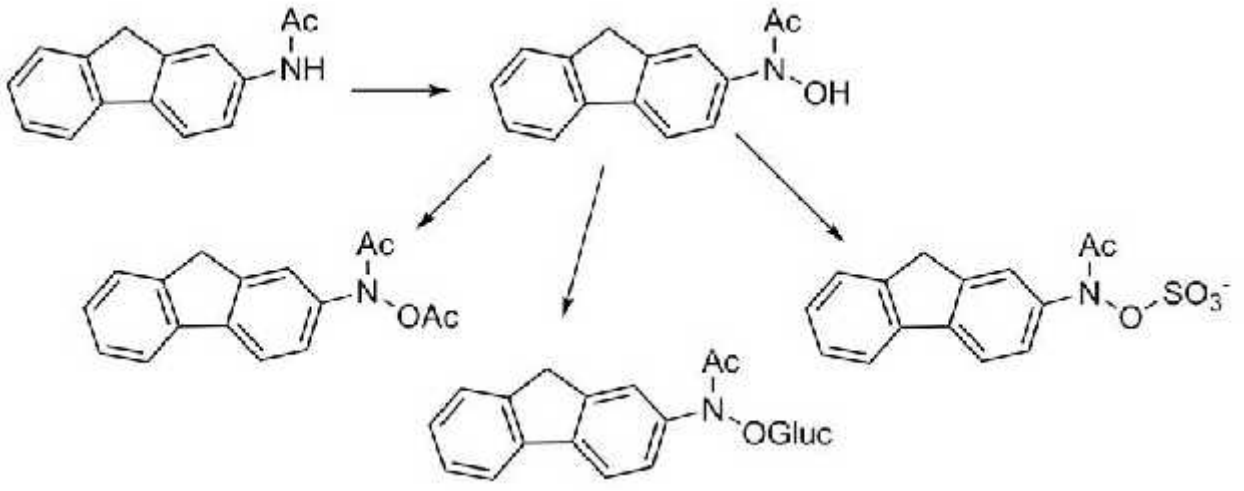

Figure 2. Electrophilic metabolites of N-hydroxy-2-acetylaminofluorene.

least *in vitro* (56-60); reviewed by Kadlubar and Beland (61); Figure 2. Further verification of the role of conjugation to form electrophilic esters was provided by studies in which the conjugating enzymes were either knocked out or overexpressed in (for example) bacterial cells. Thus, the mutagenicity of many aromatic amines (but not all; PhIP is an exception (62)) is greatly reduced in Ames test strains lacking acetyl CoA:arylamine *N*-acetyl-transferase (NAT) activity (63, 64) and is greatly increased in strains that overexpress bacterial (65) or human (66) NAT enzymes. Expressed human sulfotransferase 1A2 enhances the mutagenicity of *N*-hydroxy-AAF (67, 68). These pathways for conjugation of arylhydroxylamines and arylhydroxylamides are also discussed in the *contribution by Neumann in this series* and by Turesky and Le Marchand (69). *N*-Acetylation of arylamines can be a route for their detoxication and elimination, whereas *O*-acetylation of arylhydroxylamines is a mechanism of bioactivation. Consequently, the net effect on susceptibility to arylamine carcinogenesis - benign or malign - of the highly polymorphic human NAT enzymes is difficult to predict (70, 71).

8. DNA ADDUCTS OF AROMATIC AMINES: IDENTIFICATION OF GUANINE C-8 ADDUCTS AND POSTULATED ROLE OF NITRENIUM ION

Marroquin and Farber observed that when radioactively-labeled AAF was administered to rats, radioactivity became bound – presumably covalently – to nucleic acid (RNA) (72, 73). In a pivotal investigation, Kriek demonstrated that *N*-hydroxyaminofluorene (*N*-hydroxy-AF) reacts spontaneously with RNA or DNA *in vitro*, at a rate which is higher under mildly acidic conditions over the pH range 4-6 (74). He concluded that "the mechanism proposed by Heller, Hughes, and Ingold (1951) may be operative", and included a reaction scheme illustrating a nitrenium ion ("in which the nitrogen atom has only a sextet of valence electrons"), formed by loss of water from the protonated hydroxylamine. He noted that "If this ion is sufficiently resonance-stabilized, as might be the case if Ar = fluorenyl or biphenyl, it could react partly in an electrophilic substitution at C-8 of guanine, which is the most likely to be attacked by an electrophilic agent …" and he drew a "tentative" structure of a C-8 guanine adduct. The correctness of this assignment was confirmed by subsequent studies, including (Kriek, working in the laboratory of Miller and Miller, 1966) the chromatographic purification and identification of 8- (*N*-2-fluorenyl-acetamido)guanosine as the product of the reaction of *N*-acetoxy-2-AAF with guanosine (75). Kriek later observed that "This was one of the first examples … in which a carcinogen-DNA adduct was obtained by direct chemical synthesis. In the years that followed the same adducts were found *in vivo* in tissues of animals treated with AAF. Similar types of adducts were characterized for a number of other aromatic amines and other sites of reaction were identified, e.g. the exocyclic amino groups of guanine and adenine. In almost all instances, however, the major adduct found *in vivo* was an arylamine derivative in which the amino nitrogen was covalently bound to the C-8 of guanine …" (76) The structures of arylamine DNA adducts have been reviewed in detail by Beland and Kadlubar (77) and recently by Turesky and Le Marchand (69).

9. NITRENIUM IONS AS POSTULATED REACTION INTERMEDIATES; EVIDENCE FROM EARLY TRAPPING STUDIES

In 1951 Heller, Hughes, and Ingold showed that the rate of the Bamberger rearrangement of *N*-phenylhydroxylamine is proportional to the concentration of protonated hydroxylamine, reaching a constant value at high acid concentration (6). They also showed that the rate of the reaction is independent of (Cl^-) in aqueous HCl under conditions in which 4-chloroaniline and 2-chloroaniline are major reaction products along with the Bamberger rearrangement product, 4-aminophenol. The data are

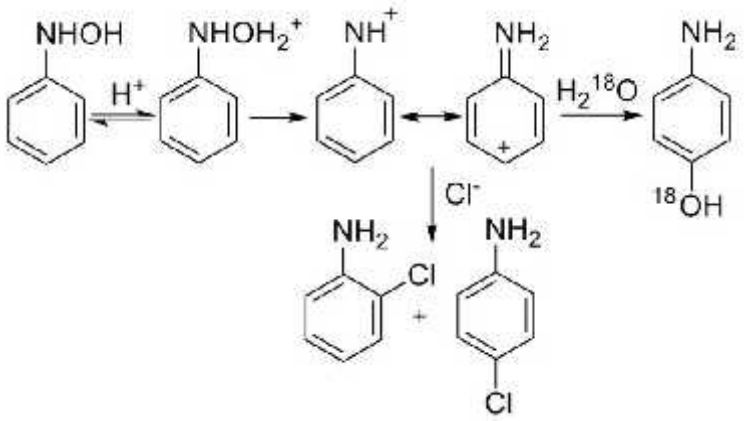

Figure 3. The nitrenium ion mechanism of the Bamberger rearrangement.

consistent with a two step mechanism involving formation of an "active electrophilic intermediate", which they formulated as a "carbonium ionic intermediate" (Figure 3), recognizing (without drawing it explicitly) the "mesomeric" nitrenium ion structure (Figure 3, the cation resonance structure to the left). The subsequent demonstration that the rearrangement occurs in ^{18}O-H_2O with incorporation of ^{18}O into 4-aminophenol, but with no detectable incorporation of ^{18}O into the hydroxylamine, provided the critical evidence for the modern formulation of the mechanism of the Bamberger rearrangement shown in Figure 3 (78, 79). Substituent effects, solvent deuterium isotope effects, and the lack of anchimeric assistance of an intramolecularly positioned nucleophile have provided additional support for the nitrenium ion mechanism of the Bamberger rearrangement (80-82).

Derivatives of *N*-arylhydroxylamines with better leaving groups than OH^- do not require acid conditions to react. Gassman and co-workers showed that the rates of solvolysis of ring-substituted *N*-*tert*-butyl-*N*-chloroanilines in EtOH correlate with the sigma$^+$ value of the substituent, with a rho$^+$ value of -6.4 (83, 84). The Hammett substituent constant, sigma$^+$, is used to correlate rates of reactions in aromatic substrates in which positive charge builds up at a site in resonance contact with the substituent; the substituent constant measures the electron donating (negative sigma) or withdrawing (positive sigma) ability of the substituent. The reaction sensitivity to substituents, rho$^+$, is the slope of a plot of the logarithm of the reaction rate constant vs. sigma$^+$. A negative rho$^+$ indicates a reaction in which electron-donating substituents increase the reaction rate, implying that positive charge builds up at the reaction site in the rate-limiting transition state. The large sensitivity to substituents, as measured by rho$^+$, indicates a transition state in which a full positive charge is developing at a reaction site directly adjacent to the aromatic ring. Solvolysis products in EtOH and MeOH, including rearranged 2-chloro-*N*-*tert*-butylanilines, are also consistent with reaction through short-lived nitrenium ion intermediates (84-86). Gassman and Granrud showed that rearrangement of *N*-methanesulfonatoöxyacetanilides in $CDCl_3$ correlated with sigma$^+$, with a rho$^+$ value of -9.2 (87). Novak and co-workers showed that the hydrolysis of *N*-sulfonatoöxy-acetanilides in aqueous solution was somewhat less sensitive to substituent effects, with rho$^+$ = -4.5, but products of reaction with water and non-solvent nucleophiles, as well as the lack of dependence of the hydrolysis rate on nucleophile concentration, were consistent with a nitrenium ion mechanism (88). The high yield of intramolecular rearrangement products generated in the presence of solvent and non-solvent nucleophiles suggested that a large proportion of the reaction occurs through an ion pair intermediate generated from the initial heterolysis of the N-Cl or N-O bond, and, therefore, that simple ring-substituted monocyclic *N*-arylnitrenium ions are short-lived species (≤ 1 ns) in aqueous or alcohol solvents.

Lifetimes of reactive cations in water can be determined indirectly by competition between an efficient non-solvent nucleophile such as azide, N_3^-, that reacts with the cation at the diffusion-controlled limit, and water (89-91). The "azide clock" method uses the change in the yields of azide- and solvent-derived products as a function of (N_3^-) to determine the ion's N_3^-/solvent selectivity, the ratio of the second-order rate constant for trapping of the ion by N_3^- and the pseudo-first-order rate constant for trapping of the ion by solvent: k_{az}/k_s. If k_{az} is diffusion limited at ca. 5×10^9 M^{-1} s^{-1}, $1/k_s$, the lifetime of the ion in water, can be estimated. If k_{az} is below the diffusion limit, the method provides a lower limit for the cation lifetime. In 1987, Fishbein and McClelland showed that N_3^- traps the 2,6-dimethylphenylnitrenium ion, generated by the Bamberger rearrangement of the corresponding hydroxylamine, with k_{az}/k_s of 7.5 M^{-1} (Figure 4) (92). If k_{az} is diffusion limited, the lifetime of the ion in H_2O is ca. 1.5 ns. The ion survives long enough to react inefficiently with non-solvent nucleophiles, but cannot be a selective species.

10. RELATIVELY STABLE ARYLNITRENIUM IONS

Flash photolysis of 4-azido-N,N-dialkylanilines in water generates unusually long-lived cationic N,N-dialkylquinonediimines, detected by UV absorption, that

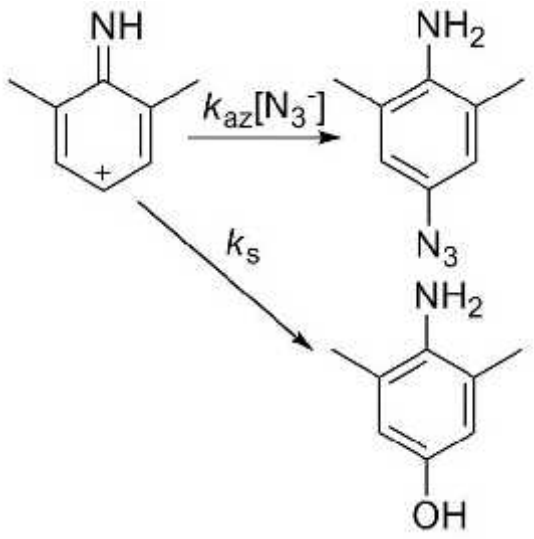

Figure 4. The "azide clock" applied to the 2,6-dimethylphenylnitrenium ion.

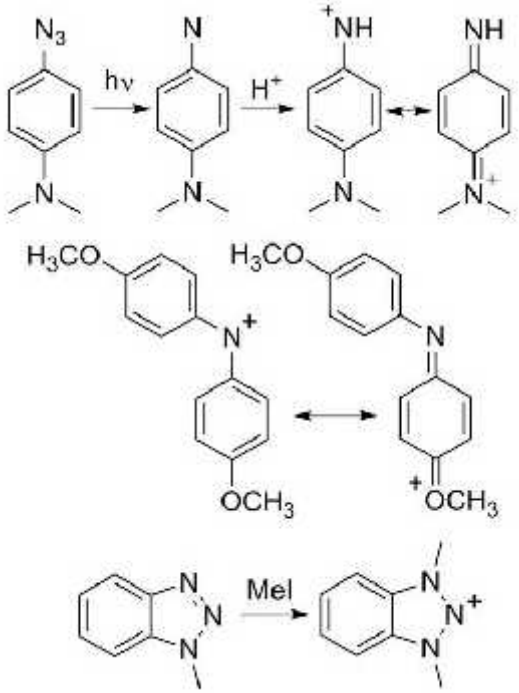

Figure 5. Stabilized nitrenium ions.

have a nitrenium resonance structure (Figure 5, top) (93). These species can also be produced by oxidation of the corresponding p-phenylenediamines. The N,N-dimethylcation shown in Figure 5 has a lifetime at neutral pH of 400 s at 25 °C, but it does decompose more rapidly under basic conditions (94). The dianisylnitrenium ion (Figure 5, middle) is generated in CH_3CN by two reversible one-electron oxidations of the corresponding amine (95, 96). Dianisylnitrenium ion can be detected by voltammetry or UV spectroscopy, and it has a lifetime of ca. 1 s in neutral CH_3CN. These cations are not typical of reactive arylnitrenium ions. They are highly stabilized by the strongly electron donating 4-*N*,*N*-dialkylamino or 4,4′-dimethoxy substituents, and do not react rapidly with nucleophiles. The *bis*-amino substituted nitrenium ion 1,3-dimethylbenzotriazolium (Figure 5, bottom) is generated by treatment of 1-methyl-benzotriazole with methyl iodide (97). This cation, and others of similar structure, are indefinitely stable, and their salts can be characterized by x-ray crystallography. Although these examples serve to illustrate that stable nitrenium species can be generated, they do not provide insight into the reactivity of transient nitrenium ions involved in carcinogenesis.

11. DIAMINES: PHENYLENEDIAMINE AND BENZIDINE

Benzidine (synthesized by the rearrangement of hydrazobenzene) and *para*-phenylenediamine (synthesized by reduction of 4-nitroaniline) are the prototypical aryldiamines, compounds possessing two amino substituents linked in a conjugated aromatic system. As mentioned earlier, and as discussed in several other articles in this series, benzidine and its derivatives are the starting materials for synthesis of a vast range of commercially important azo dyes (98). The relative ease of oxidation of aryldiamines to colored products was noticed immediately, and forms the basis of many analytical applications of these compounds, such as the routine use of diaminobenzidine as a peroxidase substrate in immunohistochemistry (99).

Wurster discovered the stable product of *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine (Wurster's blue) in 1879 (100). The oxidation of Wurster's blue was further studied by Michaelis (best-known for his fundamental work in enzyme kinetics), who noted that: "The univalent oxidation products of the aromatic *p*-diamines, or Wurster's salts, are free radicals which may polymerize in a sufficiently concentrated solution … it is impossible to obtain any pure solution of these radicals at all. They exist only in equilibrium with one molecular species at a lower level of oxidation, the diamine, and another at a higher level of oxidation, the diimine. Since the latter are very unstable compounds, liable to undergo irreversible changes, such a system may undergo irreversible changes, which are not directly due to any lability of the radical itself …" (101) Benzidine and its derivatives undergo corresponding chemical oxidations to free radical and diimine products (102-104). Peroxidase enzymes readily catalyze these oxidations (105) and the oxidation can also be performed electrochemically (*e.g.*, cyclic voltammetry (106)).

Holland and colleagues prepared 3,5,3',5'-tetramethylbenzidine (107) (TMB), a compound in which, as in Wurster's blue, the amino group is sterically protected by adjacent methyl substituents on the aromatic rings. The oxidation products of TMB are much less reactive than those of benzidine or dianisidine (3,3-dimethoxybenzidine (108)), and this relative stability made possible the direct detection of the electron paramagnetic resonance (EPR) spectrum of the TMB cation radical in a peroxidase

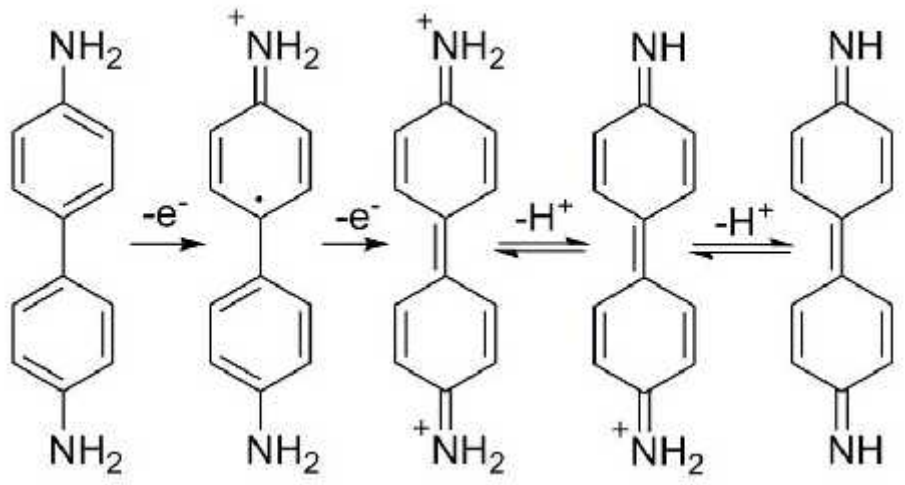

Figure 6. Benzidine oxidation to the benzidine cation radical and to the benzidine diimine dication, monocation, and neutral forms.

incubation, and measurement of the equilibrium between the radical and the charge-transfer complex of the diamine and diimine (109). Prostaglandin synthase also catalyzes the oxidation of TMB (110). The benzidine cation radical (Figure 6) is also detectable by EPR, although it is much less stable than in the case of TMB (111, 112). The monocation (Figure 6, diimine species with net charge +1) conjugate base of the benzidine diimine dication may be regarded as a stabilized arylnitrenium ion, as noted by McClelland *et al.* (113). It has an aqueous solution lifetime of ca. 100 s at neutral pH, but decomposes more rapidly under basic conditions (113) and its properties are very similar to those of the *N,N*-dialkylquinonediimines discussed above. Benzidine diimine (reacting predominately through its monocationic form that is dominant at neutral pH) is an electrophile that reacts to form covalent adducts with nucleophilic biological targets such as N-acetylcysteine (114), glutathione (115, 116), deoxyguanosine (117), and DNA (117-121).

Delocalization of positive charge over two N atoms is also seen in the case of the relatively stable nitrenium ion produced by oxidation of the antipsychotic drug clozapine (Figure 7) (122-125).

12. RAPID REACTION STUDIES AND MECHANISTIC/PRODUCT DISTRIBUTION STUDIES OF SHORT-LIVED NITRENIUM IONS; LIFETIMES; FLUORENE VS BIPHENYL SYSTEMS; EFFECT OF N-ACETYL GROUP ON STABILITY

Early studies on the Bamberger rearrangement and similar reactions of *N*-chloro, *N*-acetoxy, or *N*-sulfonatooxy derivatives had established that these reactions occurred through nitrenium ion intermediates, but the meagre available data on the lifetimes of monocyclic *N*-arylnitrenium ions suggested that these species had very short lifetimes in an aqueous environment that would not allow them to react selectively *in vivo*. As of the mid-1980s, the concept that nitrenium ions might be involved in the carcinogenicity of *N*-arylhydroxylamine derivatives was regarded with skepticism. Other possibilities had been demonstrated. The Boche and Novak groups had shown that aromatic amines can react with esters of hydroxylamines through an S_N2 mechanism, even in a polar solvent such as MeOH (126-128) and it was not at all clear that nitrenium ions played a biological role.

In 1993, the Novak group applied the azide clock methodology to the 4-biphenylyl-nitrenium ion and to the *N*-acetyl-4-biphenylylnitrenium ion, both of which were derived in aqueous solution from ester precursors (Figure 8) (129). The biphenylyl ions exhibit large k_{az}/k_s values: 2.9 $\times$ 10^3 M^{-1} and 1.0 $\times$ 10^3 M^{-1} for the NH and NAc ions, respectively. The π-conjugation provided by the distal phenyl ring has an unprecedentedly large effect on the kinetic lability of these ions that is not reproduced in similarly substituted benzyl cations, while the *N*-acetyl group has remarkably little effect. Assuming that the reaction with N_3^- is diffusion limited, the lifetimes of the 4-biphenylylnitrenium ion and *N*-acetyl-4-biphenylylnitrenium ion are ca. 0.6 micros and 0.2 micros, respectively. These relatively long lifetimes imply that *N*-arylnitrenium ions derived from metabolites of carcinogenic polycyclic arylamines and amides could act as very selective electrophiles in a biological environment.

Shortly afterwards, the Novak and McClelland groups demonstrated that the *N*-acetyl-4-biphenylyl- and *N*-acetyl-2-fluorenylnitrenium ions could be detected by fast UV spectroscopy in H_2O, following laser flash photolysis (lfp) of esters or *N*-chloro derivatives of the corresponding hydroxamic acids (130). The transients were identified as nitrenium ions by their second-order reactions with N_3^-, their insensitivity to O_2, and by the equivalence of k_{az}/k_s measured by direct observation and by the azide clock method. McClelland's group also demonstrated that the 4-biphenylyl- and 2-fluorenylnitrenium ions could be generated by *lfp of the corresponding azides (131). The lifetimes* $1/k_s$ *and* k_{az} *were measured directly for all four*

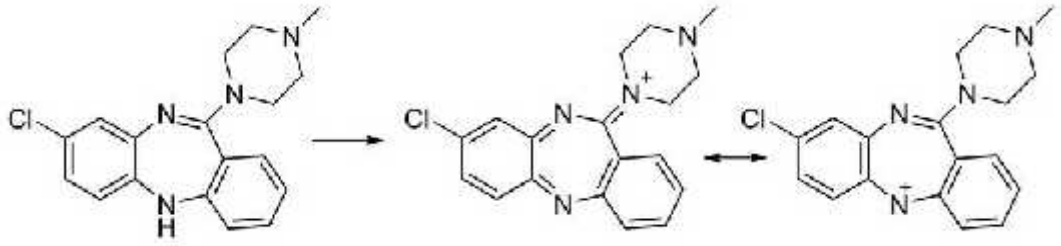

Figure 7. The nitrenium ion oxidation product of clozapine.

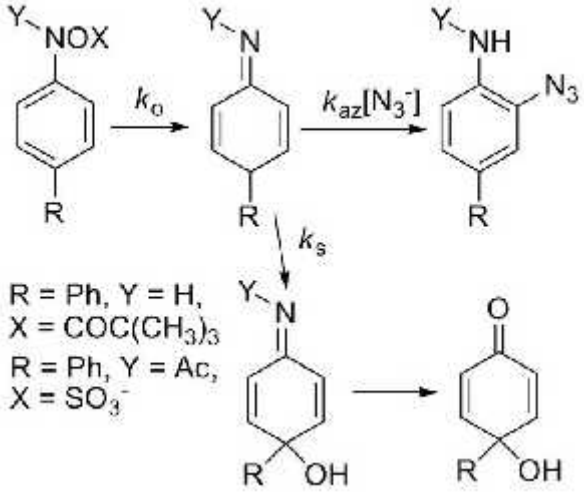

Figure 8. Reactions of the 4-biphenylylnitrenium ions with azide and water.

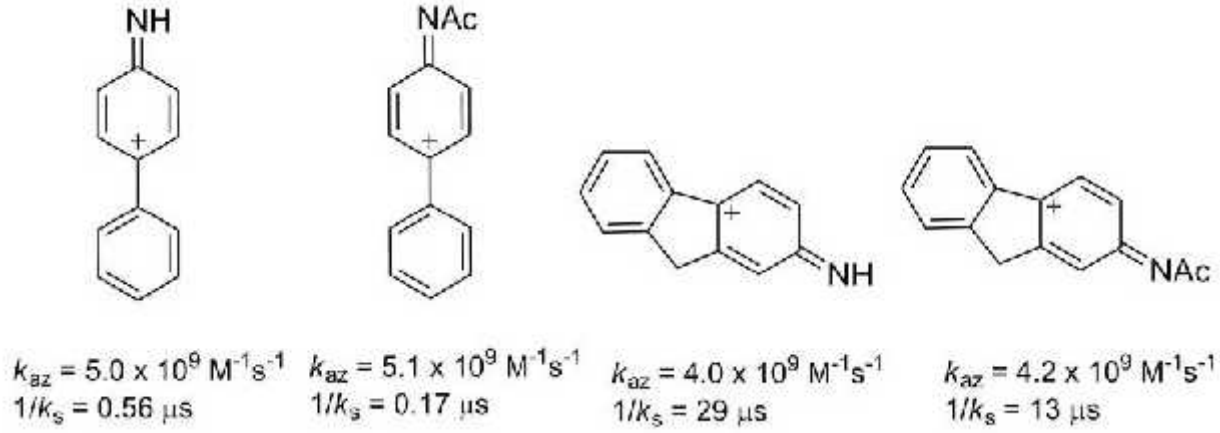

Figure 9. Aqueous solution lifetimes and k_{az} for 4-biphenylyl- and 2-fluorenylnitrenium ions.

cations (Figure 9). The results verified that the reactions of these ions with N_3^- is at or near the diffusion-controlled limit, and agreed with the lifetime estimates made by azide clock measurements. The lifetimes of the 2-fluorenyl ions, which are forced into planarity by the methylene bridge, are significantly longer than those of the 4-biphenylyl ions. A lfp study of the acid-base properties of the 4-biphenylyl- and 2-fluorenylnitrenium ions showed that they can be protonated in highly acidic solutions to generate dications, but these dications cannot be physiologically relevant (132).

The parent phenylnitrenium ion is too short-lived to be directly detected in aqueous solution by ns-lfp methods, but it has been estimated to have a lifetime in water of ca. 125-250 ps based on Br^- trapping data obtained during the Bamberger rearrangement of *N*-phenylhydroxylamine (133). A recent direct measurement

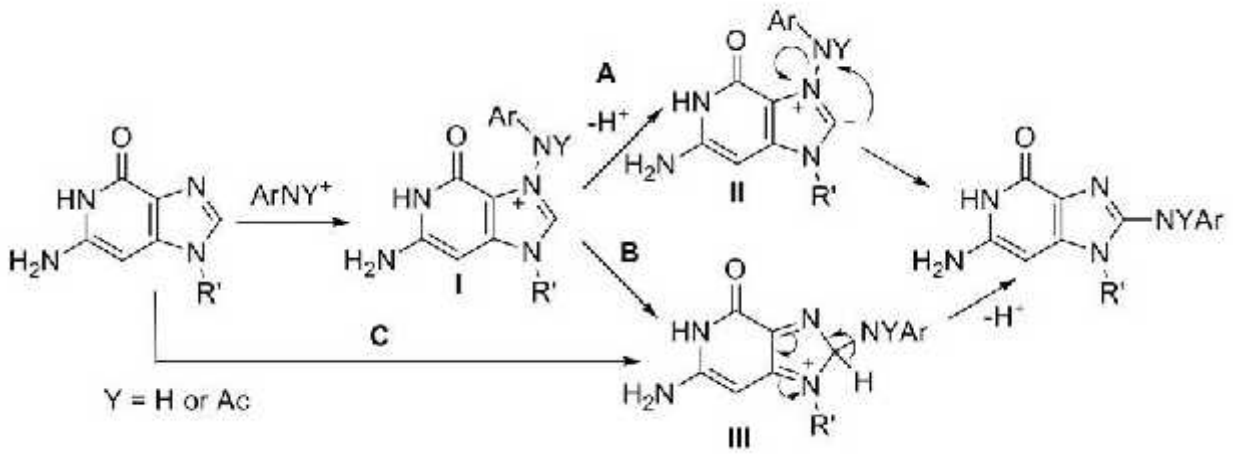

Figure 10. Mechanistic hypotheses for the formation of the C8 adduct.

of its lifetime (110 ps) by ultra-fast lfp of phenyl azide in formic acid is in reasonable agreement with that estimate (134). In general, it appears that the lifetimes of monocyclic *N*-arylnitrenium ions are too short for these species to be selective electrophiles in biological systems.

13. REACTIONS OF NITRENIUM IONS WITH dG; C-8 VS N-7/C-8 MECHANISMS OF dG ADDUCT FORMATION

Although the identity of the major C-8 adduct formed in the reaction of *N*-arylhydroxylamine derivatives with guanosine, 2′-deoxyguanosine, (dG), and the guanine moiety in DNA/RNA had been known since the mid-1960s, the detailed mechanism of this reaction was not understood until recently. The reaction was not established as involving a nitrenium ion until 1995, when Kennedy and Novak demonstrated that the rate of formation of the dG adduct is independent of dG concentration and equivalent to the rate of disappearance of the nitrenium ion precursor for the *N*-acetyl-4-biphenylylnitrenium and *N*-acetyl-2-fluorenylnitrenium ions (135). Trapping experiments with these two ions and, subsequently, with the 4-biphenylylnitrenium ion (136) provided k_{dG}/k_s values for each ion, and, since k_s was known, the magnitude of the second-order rate constant for the reaction with dG, k_{dG}, was obtained: 1.9×10^9 $M^{-1}s^{-1}$ for the 4-biphenylylnitrenium and *N*-acetyl-4-biphenylylnitrenium ions, and 6.2×10^8 $M^{-1}s^{-1}$ for the *N*-acetyl-2-fluorenylnitrenium ion. All three ions react efficiently with dG: at (dG) = 10 mM, the yield of the C-8 adduct exceeds 75%. The high selectivity is a direct consequence of the long aqueous solution lifetimes of the cations. McClelland and co-workers verified the magnitudes of $k_{d\text{-}G}$ for these and others ions via direct kinetic measurements following lfp generation of the ions (137, 138). Their results confirm that k_{dG} reaches an apparent diffusion-controlled limit of ca. 2.0×10^9 M^{-1} s^{-1} for many ions with $k_s \geq 10^6$ s^{-1}.

The intermediacy of nitrenium ions in the adduct-forming reactions of monocyclic *N*-arylhydroxylamine derivatives has not been tested. The *N*-acetoxy derivatives of 2-, 3-, and 4-tolylhydroxylamines, and 2,3-, 2,4-, 3,5-, and 2,6-dimethylphenylhydroxylamines react inefficiently with monomeric dG or with dG residues in DNA, and show a greater tendency to generate adducts with 2′-deoxyadenosine and 2′-deoxycytidine than do their 4-biphenylyl or 2-fluorenyl analogues (139-141). Whether these reactions involve the short-lived nitrenium ions that would be generated by these esters remains an open question.

The detailed mechanism for formation of the C-8 adduct has been of considerable interest. Mechanistic investigations have centered around the alternative mechanisms **A**, **B**, and **C** (Figure 10). Humphreys, Kadlubar, and Guengerich favored mechanism **A,** based on the reported isolation of an 8-methyl product analogous to **I** from the reaction of 8,9-dimethyl-guanine with *N*-acetoxy-2-aminofluorene and the isolation of the C-8 adduct from the reaction of 8-bromoguanosine with *N*-acetoxy-2-aminofluorene (142). It was reasoned that the 8-Br analogue of **I** would be reduced (by excess hydroxylamine present in the reaction mixture) to the intermediate **II**, which would then undergo a Stevens rearrangement to the C-8 adduct. Kennedy, Novak, and Kolb were not able to generate a product analogous to **I** from the reaction of the 4-biphenylyl- or *N*-acetyl-4-biphenylylnitrenium ions with 8-methylguanosine, but did detect metastable products that appeared to be the 8-methyl analogues of **II (**136). These metastable products were characterized by their decomposition products and by ^{1}H-NMR spectra taken during the reaction. Although products analogous to **I** were not detected, mechanism **B** was proposed on the basis of the reported observations of Humphreys, Kadlubar, and Guengerich, and the observation that the rate of formation of C-8 adducts for a number of purine nucleosides (adenosine, inosine, xanthosine, guanosine, dG) appeared to depend on the basicity of N-7.

McClelland's group observed the kinetics of the

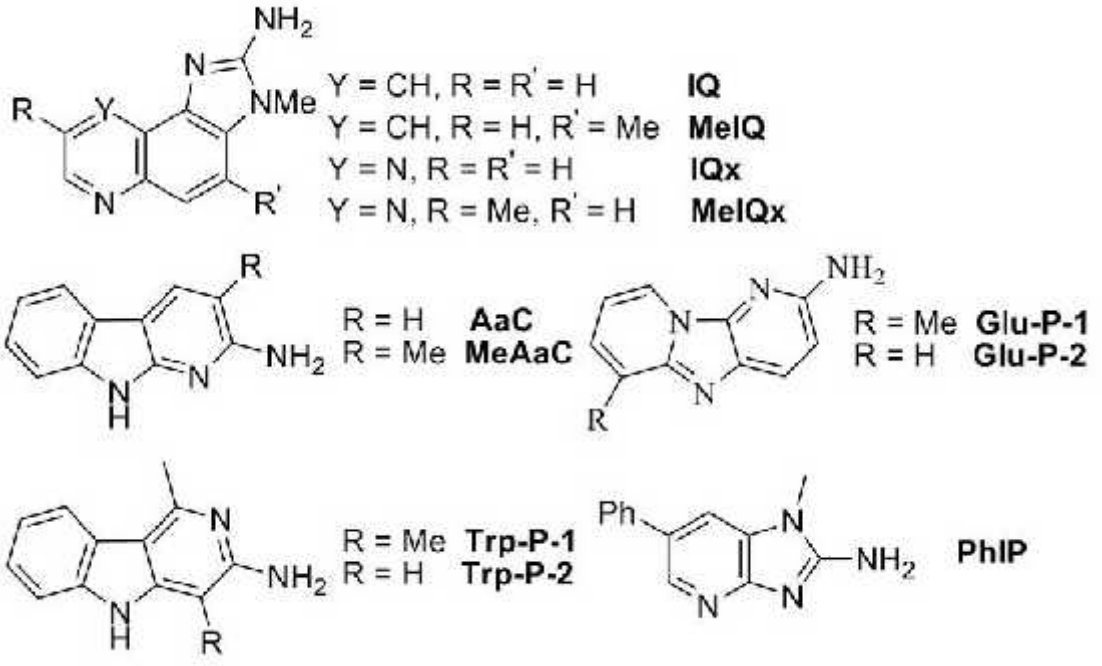

Figure 11. Selected carcinogenic heterocyclic arylamines (HCAs).

reaction of the 2-fluorenylnitrenium ion with dG following lfp to generate the cation (143). They identified the transient intermediate generated during the reaction as **III (**Ar = 2-fluorenyl, Y = H, R′ = 2′-deoxyribose) based on its absorption spectrum, which extends to 400 nm, indicating a highly conjugated species; a pK_a value of 3.9 that is consistent with deprotonation of **III**; and a rate constant for decomposition of the intermediate that is independent of Ar for Ar = 2-fluorenyl and 4-biphenylyl. The pH dependence of the kinetics of decomposition of the intermediate, and a large H/D kinetic isotope effect of ca. 6-7 for the decomposition of the intermediate derived from 8-deuterated-dG are also consistent with the identification. The rate of appearance of the intermediate was identical to the rate of disappearance of the nitrenium ion and no other intermediate was detected. The data are most readily interpreted in terms of mechanism **C**, which is analogous to an electrophilic aromatic substitution. The results cannot rule out initial attack by N-7, followed by rapid rearrangement to **III**, but the N-7 intermediate would have to be very short lived (< 5 ns) to escape detection.

Phillips and coworkers characterized the 2-fluorenylnitrenium ion in CH_3CN/H_2O mixtures by time-resolved resonance Raman (TR^3) spectroscopy (144), and they subsequently used the technique to monitor the reaction of the cation with guanosine (145, 146). They detected an intermediate that appeared to be **III** (Ar = 2-fluorenyl, Y = H, R′ = ribose).The TR^3 spectrum of the intermediate identified as **III** is consistent with its calculated (BPW91/cc-PVDZ) vibrational spectrum. Kinetics of formation of **III** are consistent with its direct formation from the nitrenium ion as concluded by McClelland. A recent DFT computational study employing the B3LYP method applied to 6-31G (d), 6-31G (d,p), 6-31+G (d,p), and cc-pVDZ50 basis sets provided additional evidence in favor of mechanism **C** (147). The weight of evidence now indicates that the reaction occurs via direct attack of C-8 on the N of the nitrenium ion, for the long-lived ions derived from polycyclic arylamine carcinogens.

14. HETEROCYCLIC ARYLAMINES

In 1939, Widmark demonstrated that extracts of fried horse meat caused cancers when applied to mouse skin (148). However, the identification of carcinogenic components of fried and broiled meats as heterocyclic arylamines (HCAs) took place in the 1970s and 1980s, mainly as a result of the investigations of Sugimura and coworkers (reviewed in (149-151)). More than 20 active HCAs have been identified in grilled meat; a selection is shown in Figure 11. In addition to their presence in cooked meats, HCAs have been detected in commercial food flavorings and sauces, beverages, and tobacco smoke (reviewed in (152)). Initial studies using the Ames (*Salmonella typhimurium*) mutagenicity assay showed that HCA mutagenicity required the presence of mammalian liver homogenates or purified cytochrome P450 preparations (153, 154). *N*-Hydroxylation is a necessary step in the metabolic activation of HCAs.

The further metabolic activation of HCAs and the generation of DNA adducts from their metabolites has been reviewed recently (69), and only a few highlights will be mentioned here. Acetyl-CoA enhances the binding of *N*-hydroxy-IQ to DNA in monkey and rat tissue, while PAPS

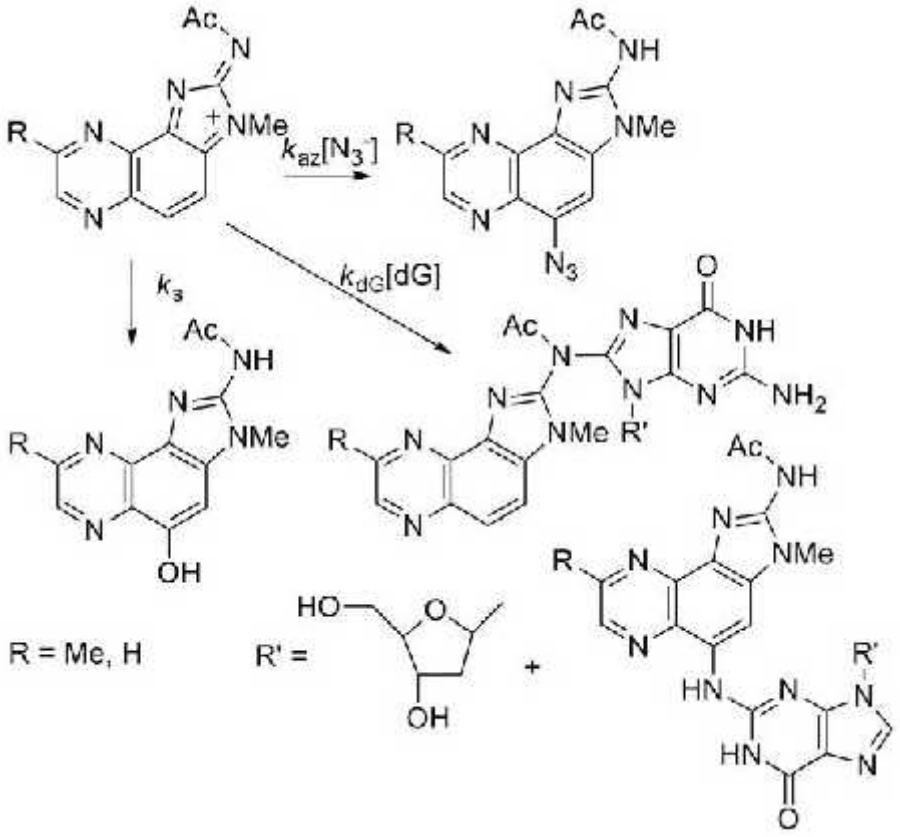

Figure 12. Reactions of the N-acetyl-IQx and N-acetyl-MeIQx nitrenium ions.

is ineffective at enhancing the binding of *N*-hydroxy-IQ to DNA (155). In contrast, PAPS increases the binding of *N*-hydroxy-PhIP to DNA, particularly in monkey tissues (155). This suggested that the major activation pathways for *N*-hydroxy-IQ and *N*-hydroxy-PhIP diverge, with acetyl CoA-dependent NAT responsible for activation of *N*-hydroxy-IQ and PAPS-dependent sulfotransferase (SULT) responsible for activation of *N*-hydroxy-PhIP. Subsequent studies using recombinant human NAT and SULT enzymes expressed in *S. typhimurium* confirmed that *N*-hydroxy-IQ is specifically activated by NAT and *N*-hydroxy-PhIP by SULT (67).

Adducts of HCAs with DNA and DNA bases have been extensively investigated since 1979 (69, 156). The major target in monomeric nucleosides, and in DNA *in vitro* and *in vivo*, is dG (69, 156). The major adducts are always C-8 adducts, but other structures have been identified in a few instances (69, 156). C-8 adducts of Glu-P-1 and Trp-P-2 were the first to be identified, in 1979 (157, 158). These adducts were isolated from DNA treated with Glu-P-1 or Trp-P-2 in the presence of rat hepatic microsomes (157, 158), from the reactions of the acetic acid esters of *N*-hydroxy-Glu-P-1 and *N*-hydroxy-Trp-P-2 with DNA (159, 160), and from hepatic DNA of rats fed the corresponding HCA (161).

The C-8 adducts of IQ, MeIQ, and MeIQx were synthesized by reaction of the *N*-acetoxy-HCA with dG or DNA (162-164) and were also isolated from *in vivo* experiments (163-167). Shortly after the C-8 adducts of IQ and MeIQx were discovered, minor N-2 adducts were isolated from the reaction of *N*-acetoxy-IQ and *N*-acetoxy-MeIQx with dG or DNA (168). These adducts, reminiscent of the N-2 adduct previously discovered for 2-AAF (169), accounted for 10-15% of the reaction with dG or DNA. The minor IQ adduct was discovered, along with the major C-8 adduct, in tissues of rats and monkeys that were administered single or multiple doses of IQ (170 -172).

The Novak group performed an extensive study of the chemistry of synthetic ester derivatives of a wide variety of *N*-hydroxy-HCAs (reviewed in 2004 (173)). At neutral pH, all of the ester derivatives investigated generated nitrenium ions that were trappable by N_3^-, with k_{az}/k_s values that are comparable to those previously measured for nitrenium ions derived from carcinogenic polycyclic aromatic amines. For example, the N-acetyl-IQx and N-acetyl-MeIQx nitrenium ions (Figure 12) were trapped by N_3^- with k_{az}/k_s values of 5.2×10^4 M^{-1} and 1.2×10^5 M^{-1}, respectively (174). Both ions were also trapped by dG to yield a mixture of C-8 and N-2 adducts in approximately an 80/20 ratio (174). This is a unique case, because the N-2 adducts of other nitrenium ions have not

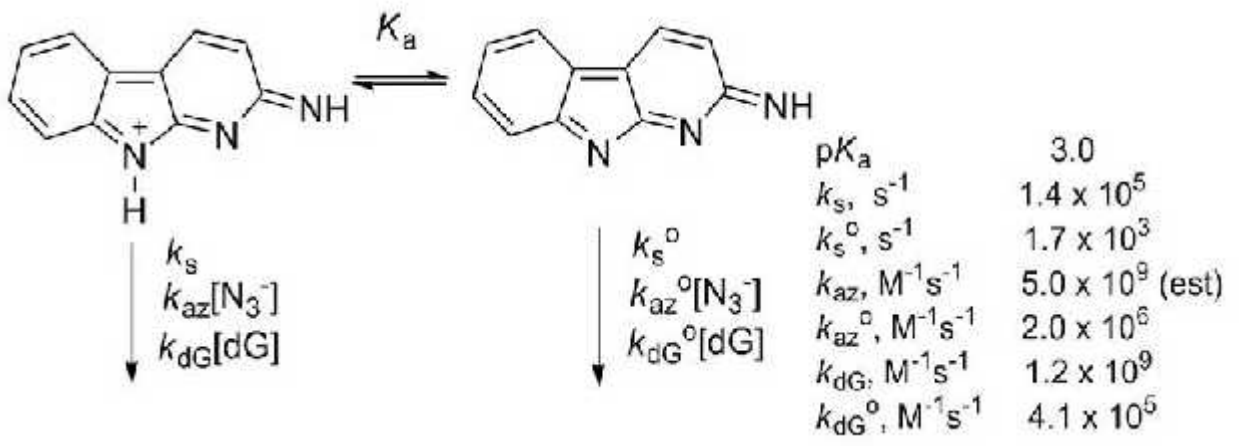

Figure 13. Acid-base properties of the AC nitrenium ion.

been generated from the reactions of the nitrenium ions with dG. Typically, these minor N-2 adducts are detected only in reactions with DNA (169)

Because of their heterocyclic structures, some HCA nitrenium ions have acid-base properties that are physiologically relevant. The AC nitrenium ion exhibits pH dependence of its azide/solvent and dG/solvent selectivity that can be traced to ionization of the 9-NH to form a neutral quinonoid (Figure 13) (175). The 9-NMe ion does not exhibit this pH dependence (175). It is interesting that both the cation and its neutral conjugate base are electrophiles that exhibit significant reactivity with both N_3^- and dG (175). Because the pK_a of the cation is 3.0, the neutral quinonoid is responsible for the observed reactions at physiological pH.

15. SUMMARY

Some 50 years after the discovery of the N-hydroxylation of AAF and the initial formulation of the nitrenium ion hypothesis, evidence from a wide variety of experiments has confirmed that N-arylnitrenium ions (or, occasionally, their neutral quinonoid conjugate bases) are responsible for the mutagenic and carcinogenic activity of polycyclic arylamines. Monocyclic arylamines are typically less potent carcinogens. The nitrenium ions derived from monocyclic arylamines have very short lifetimes, and it is less clear that they mediate the biological activity of these compounds. It is now recognized that the deoxyguanosine C-8 adducts of polycyclic arylamines are products of the reaction of nitrenium ions with DNA. The basis of the high regioselectivity of that reaction, and the role that DNA structure plays in determining it, are still not understood. Other types of arylamine-DNA adducts, while quantitatively minor, may nevertheless play significant biological roles. Investigation of the chemistry and biology of carcinogenic N-arylhydroxylamine derivatives has been a very productive area of research for the last fifty years and it promises to remain so for the foreseeable future.

16. ACKNOWLEDGMENTS

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