# A Novel Pathway to the Ultimate Mutagens of Aromatic Amino and Nitro Compounds

## by Dieter Wild*

Photolysis of arylazides in aqueous media was recently found to generate presumed nitrenium ions, species which are generally considered as the ultimate mutagens/carcinogens derived from arylamines and nitroarenes. The primary photolysis products of arylazides, the arylnitrenes, can possibly react as electrophiles themselves, or they can be protonated and thus form the electrophilic nitrenium ions. Numerous arylazides and aryldiazides can be photoactivated to short-lived mutagens detectable in *Salmonella typhimurium* TA98. Structure-activity comparisons between arylazides and the matching arylamines and nitroarenes show correlations; e.g., phenyl azide and methyl-substituted phenyl azides are not mutagenic or only weakly mutagenic like aniline, nitrobenzene, and their methyl homologues, whereas 4-azidodiphenyl, 2-azidofluorene, 1-azidopyrene, azido-IQ, and azido-isoIQ are increasingly mutagenic in that order, like the matching amino and nitro compounds. It is hypothesized on the basis of these data that the nitrene/nitrenium ion is the reactive intermediate common to the three mutagenic pathways and that the reaction of the nitrene/nitrenium ion with DNA is rate limiting for the overall mutagenic process in Salmonella. The photochemical generation from arylazides of the reactive species, the nitrene/nitrenium ions, opens new perspectives for the understanding of the genotoxic activity of arylamines and nitroarenes in general and, specifically, of the food mutagens/carcinogens of the IQ type.

## Introduction

DNA can be damaged by numerous endogenous or exogenous chemicals; the nature of the primary DNA lesions and the chemical processes involved are manifold, but most of them can be classified as oxidative reactions, hydrolytic reactions (e.g., base deamination, depurination), or electrophilic substitution reactions. The last can be triggered by electrophiles; therefore, the occurrence of xenobiotic electrophiles and metabolic formation of electrophiles and their reactions with nucleic acids have been studied intensively. Two main classes of electrophiles are those with a positive charge at a carbon atom (carbenium ion) and those with a positive charge at a nitrogen atom (nitrenium ion). Carbenium ions are produced by various alkylating agents; nitrenium ions are produced metabolically from aromatic amino and nitro compounds.

Experimental studies on DNA lesions and on their further processing within cells (repair or conversion into mutations) frequently require the preparation of DNA and nucleotides containing specific lesions, DNA adducts, at high frequency. We have recently found that arylazides offer a new, simple, and efficient pathway to electrophilic, DNA-binding and mutagenic species that are indistinguishable from the electrophilic species (nitrenium ions) produced from aromatic amino and nitro compounds (1,2). The following report extends and sum-

marizes our data on the mutagenic potential of photoactivated arylazides, the electrophilic species involved, and the role of this species in the genotoxic potency of aromatic amino and nitro compounds.

## Materials and Methods

### Arylazides

The arylazides were synthesized from the arylamines by diazotization and coupling of the diazonium salts with sodium azide. They were purified by column chromatography on $SiO_2$ and were characterized with respect to their purity and identity by thin layer chromatography, UV-spectra, and thermospray mass spectra. We have previously reported on phenyl azide, 4-azidodiphenyl, 6-azidochrysene, 2-azidofluorene, 1-azidonaphthalene, 2-azidonaphthalene, 1-azidopyrene, azido-IQ, and azido-MeIQx (1,2). Published syntheses were followed for the preparation of 2,6-dimethylphenyl azide (3), 2,4,6-trimethylphenyl azide (4), 4,4'-diazidodiphenylmethane (5), and 4,4'-diazidodiphenyl (5). 2,4,5-Trimethylphenyl azide was prepared like the 2,4,6-trisubstituted compound. The amines were from the following sources: 2,6-dimethylaniline, 2,4,5-trimethylaniline, and 2,4,6-trimethylaniline from Aldrich-Chemie (Steinheim, FRG), benzidine from Merck (Darmstadt, FRG), and 4,4'-diaminodiphenylmethane from Fluka (Neu-Ulm, FRG). All arylazides were handled exclusively under yellow light instead of daylight.

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## Mutagenesis by Arylazides in Salmonella

*Salmonella typhimurium* TA98 cells and the azides were mixed with top agar and poured onto Vogel-Bonner minimal medium (6); the plates were immediately irradiated with near ultraviolet light (NUV, 365–366 nm) from an Osram black light bulb (HQV 125 W); this plate irradiation technique has been described in detail elsewhere (2).

## Results

The new azide, 2,4,5-trimethylphenyl azide (1-azido-2,4,5-trimethylbenzene) was purified by column chromatography with *n*-hexane on $SiO_2$; it was a liquid at room temperature and crystallized in the refrigerator. The thermospray mass spectrum of a solution in ammonium acetate/methanol presented the masses 134, 136, 166 (base peak), and 194, attributable to ArN + $H^+$, $ArNH_2$ + $H^+$, ArNHOH + $H^+$, $ArNHOCH_3$ + $H^+$ and $ArNHOCOCH_3$ + $H^+$. This spectrum is analogous to that obtained previously under comparable conditions with azido-IQ (1). The sample was pure according to thin layer chromatography on $SiO_2$ (*n*-hexane): $R_f$ = 0.65; in UV light the spot turned yellow-brown.

Figure 1 presents a set of mutagenicity data obtained with 4,4'-diazidodiphenyl, the diazide analogous to the diamine benzidine. Mutagenic activity was absent in the absence of NUV and increased with the NUV irradiation up to a maximal value (Fig. 1A), which corresponds to practically complete photolysis of the diazide; these conditions were not bactericidal. From this time-effect relationship a $t_{50}$ value of 5.5 sec was derived; this is the NUV-irradiation period required for the half-maximal mutagenic effect of this compound under the experimental conditions. The dose-effect relationship (Fig. 1B) was obtained using 20 sec of NUV irradiation; the initial linear slope of the relationship (1400 revertants/µg or 330 revertants/nmole) is a measure of the specific mutagenic activity of this diazide.

It is important to note that the mixture of azides and

bacteria in the top agar layer was irradiated immediately after pouring the plate; the azides apparently penetrate the cells very rapidly. An extended pre-equilibration did not increase the mutagenic effect in most cases but quenched it due to decomposition of the azide, as has been shown previously in the case of 2-azidofluorene (2).

The mutagenic activities of all azides and diazides were assayed with *Salmonella typhimurium* TA98 in the same way as for 4,4'-diazidodiphenyl; the findings are compiled in Table 1. The simple phenylazides were either not mutagenic or only weakly mutagenic, even at high concentrations. Azides with more aromatic rings were increasingly mutagenic: 4,4'-diazidodiphenylmethane containing two unconjugated aromatic rings and thus resembling phenylazide was nevertheless several-fold more mutagenic than the 2,4,5- and 2,4,6-trimethyl-substituted phenylazides. Increasing activity was found in the order 1-azidonaphthalene < 2-azidonaphthalene < 4-azidodiphenyl < 4,4'-diazidodiphenyl < 2-azidofluorene < 6-azidochrysene < 1-azidopyrene; the most active were the heterocyclic azides azido-IQ, azido-methyl-IQ, and azido-isoIQ (Fig. 2) (8).

## Discussion

The reactive, electrophilic, and mutagenic species formed by arylazide photolysis is either an arylnitrene or an arylnitrenium ion formed from the nitrene by protonation. Arylhydroxylamines or arylnitroso compounds which could theoretically be formed are very likely not the major agents. This conclusion is based on the very short lifetime of the mutagenic species, on the high mutagenic effect of photoactivated azides in a hydroxylamine-resistant Salmonella strain, TA98/1,8-$DNP_6$, and on structure-activity comparisons of the mutagenic azido-, nitro-, and amino-imidazoarenes (2,7,8). Therefore, the mutagenic activities of photoactivated
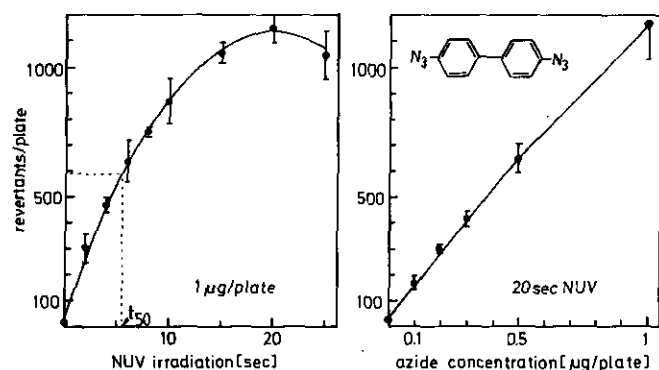


FIGURE 1. Mutagenic activity of 4,4'-diazidodiphenyl and NUV light in *Salmonella typhimurium* TA98. (A) Activity as a function of the NUV light irradiation period; (B) activity as a function of the azide concentration.

Table 1. Mutagenic activity of arylazides and aryldiazides in *Salmonella typhimurium* TA98 upon photoactivation by near ultraviolet light.

| Arylazide | Mutagenic activity, revertants/nmole | | $t_{50}$, min |
|---|---|---|---|
| Phenyl azide[a] | < 0.04 | | — |
| 2,6-Dimethylphenyl azide | < 0.04 | | — |
| 2,4,5-Trimethylphenyl azide | 0.21 ± | 0.05 | 1.3 |
| 2,4,6-Trimethylphenyl azide | 0.55 ± | 0.11 | 1.1 |
| 4,4'-Diazidodiphenylmethane | 3.3 ± | 0.4 | 0.3 |
| 1-Azidonaphthalene[a] | 2.4 ± | 0.2 | 0.25 |
| 2-Azidonaphthalene[a] | 16.1 ± | 0.5 | 0.43 |
| 4-Azidodiphenyl[a] | 20.3 ± | 1.8 | 1.0 |
| 4,4'-Diazidodiphenyl | 330 ± | 40 | 0.1 |
| 2-Azidofluorene[a] | 695 ± | 22 | 1.0 |
| 6-Azidochrysene[a] | 936 ± | 61 | 0.17 |
| 1-Azidopyrene[a] | 3470 ± | 165 | 0.01 |
| Azido-IQ[b] | 13400 ± | 670 | 0.5 |
| Azido-methyl-IQ[c] | 39200 ± | 2930 | 1.2 |
| Azido-isoIQ[c] | 44000 ± | 2420 | 0.5 |

[a] Data from Wild et al. (2).
[b] Data from Wild and Dirr (1).
[c] Data from Wild and Dirr (8).

arylazides reported here are a direct manifestation of the electrophilic reactivities of the nitrenes/nitrenium ions that are governed by the structure of their aromatic rings.

Nitrenium ions are, on the other hand, also formed metabolically, as the ultimate mutagens and carcinogens of aromatic amines and aromatic nitro compounds (9). Thus, we suggest that the ultimate species from aromatic azides, amino, and nitro compounds are identical and that therefore their reactivity is the same without regard to their origin. The mutagenic activities of azido, amino, and nitro compounds should be interrelated. We have indeed presented evidence for an azido-nitro-amino mutagen interrelationship in a series of eight heterocyclic compounds related to the food mutagen/carcinogen IQ (8); the data obtained with two of these amino compounds (IQ and isoIQ) and their nitro and azido analogues are shown in Figure 2. In addition, Figure 2 shows similar comparisons of 4-substituted diphenyls, 2-substituted fluorenes, and 1-substituted pyrenes with azido, nitro, and amino substituents.

The azido-nitro-amino trios (Fig. 2) reveal similarities as well as differences. Although some differences within trios are considerable, there is a basic within-trio sim-ilarity. In other words, variations of the ring structures affect the mutagenic activities of the azido, nitro, and amino compounds in a similar way. This finding confirms our hypothesis that one common kind of intermediate, the nitrene/nitrenium ion, is formed from the three series of compounds and that this is the main determinant of the mutagenic activity in Salmonella (8). Differences are also obvious in the activity profiles of the trios: in the diphenyl, fluorene, and pyrene trios, the azide is the most active, whereas in the IQ and isoIQ trios, the azide is the least active. Such profile differences can be due to the different polarities of the compounds and their rate of penetration through the Salmonella cell wall and membrane: the hydrocarbon-derived azides are more lipophilic than their nitro and amino analogues and can most rapidly penetrate, whereas the heterocyclic compounds are all relatively polar due to the hetero atoms. This could result in a relative disadvantage for the heterocyclic azides (note that the plate irradiation technique used requires fast penetration of the azides).

Other pharmacokinetic properties of azido, nitro, and amino compounds and of their metabolites possibly also contribute to the differences within trios; for instance, the balance between activation and inactivation of ar-
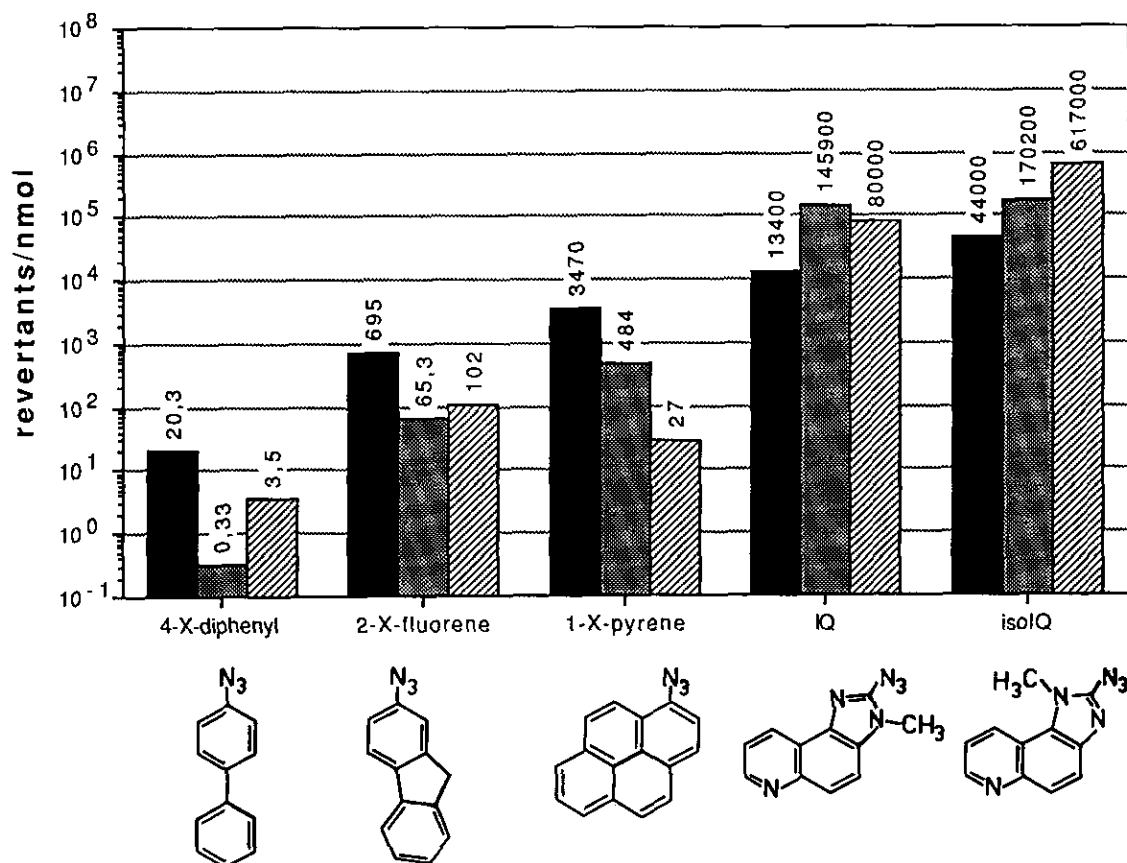


FIGURE 2. Specific mutagenic activities of azido-, nitro-, and amino analog (trios) in Salmonella typhimurium TA98; solid columns, X = azido; dotted columns, X = nitro; hatched columns, X = amino. The chemical structures of the azides are shown in the lower part of the figure. Sources of values for the compounds are: 4-nitrodiphenyl (12); 4-aminodiphenyl (13); 2-nitrofluorene (7); 2-aminofluorene (D. Wild, unpublished); 1-nitropyrene (14); and 1-aminopyrene (13).

ylamines by S9 mix may vary from compound to compound. Furthermore, it cannot be excluded that an enzyme that activates amino or nitro compounds in general still discriminates against certain specific substrates; there is some evidence for this in the case of the nitroreduction of nitrobenzopyrenes by Salmonella (10).

The mutagenic activities shown in Table 1 span more than five orders of magnitude; as mentioned above, this can be considered an expression of corresponding differences in the electrophilic character of the ultimate species. The two properties probably have their roots in the delocalization into the aromatic ring of the positive charge of the nitrenium ion (or of the electron deficiency of the nitrene), which is supported by characteristics of the aromatic ring structure to a greater or lesser extent.

The heterocyclic imidazoaromatic ring of IQ and related heterocyclic compounds (with a methyl group next to the azido group) supports the mutagenic and electrophilic activity most efficiently. We have recently concluded that the extremely high potency of these compounds is due to extremely efficient delocalization mediated by an imidazole-nitrenium and an imidazole-ammonium ion resonance structure (with the positive charge at an imidazole nitrogen). Further, the role of the imidazole-methyl group is to stabilize the ammonium resonance structure against deprotonation. In addition, carbenium ion resonance structures can contribute to the total delocalization (8).

The carbocyclic nitrenium ions can delocalize their positive charge only to carbon ring atoms and thus form only carbenium resonance structures with the charge in positions ortho and para to the nitrogen substituent and in vinylogous ring positions. The charge delocalization thus achieved appears to be less than in the heterocyclic compounds and the mutagenic activities are therefore lower. Parameters relevant for the extent of this mode of delocalization are the number of conjugated or condensed aromatic rings, the position of the nitrenium substituent on the aromatic ring system (compare 1- and 2-azidonaphthalene), and substitution by methyl and by amino groups. The presence of an amino group in addition to the nitrenium group is a likely cause of the 16 times higher activity of 4,4'-diazidodiphenyl compared with 4-azidodiphenyl (Table 1). The photolysis of this diazide very likely proceeds via the 4-amino-4'-azidodiphenyl (formed from 4-nitreno-4'-azidodiphenyl by hydrogen abstraction) to the aminodiphenylnitrenium ion. The amino group of this ultimate intermediate possibly increases the delocalization by way of an ammonium resonance structure.

## Conclusions and Perspectives

Photolysis of arylazides conveniently allows the nonenzymatic, single-step formation of electrophilic species that are probably identical with the arylnitrenium ions formed metabolically from amino- and nitroaromatic compounds. The presumed nitrenium ions formed are obviously not N-acetylated and contain the aromatic ring of the parent compound, without modification by microsomal oxidation.

The mutagenicity of arylnitrenium ions in Salmonella is probably correlated with their electrophilicity. It should be noted in this context that DNA binding and mutagenic processing of DNA adducts can be influenced by the DNA sequence and conformation in the neighborhood of an adduct (11). Such site specificity does not necessarily weaken the overall correlation between electrophilicity and mutagenicity of the reactive species. This question needs further study.

In Salmonella the electrophilic reactivity of arylnitrenium ions appears to be rate limiting for mutagenesis by arylamines, nitroarenes, and arylazides. In another cellular system metabolic formation of nitrenium ions may be slower and become rate limiting. Even then, however, the order of mutagenic effectiveness in a series of arylamines or nitroarenes can still reflect the order of electrophilicity of their nitrenium ions. Experiments in this direction with special emphasis on IQ-related food mutagens/carcinogens are currently under way.

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