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# THE RELATIONSHIP OF THE CARCINOGENIC/MUTAGENIC POTENTIAL OF ARYLAMINES TO THEIR SINGLET-TRIPLET NITRENIUM ION ENERGIES

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SUMMARY

Utilizing intermediate neglect of differential overlap (INDO) and ab initio methodology, trends in the energy differences between the singlet and triplet states for mono- and polycyclic aryl nitrenium ions have been estimated. Calculations reveal an empirical correlation between the energy separation of the singlet and triplet states of the nitrenium ion and the ability of the parent amine to behave as a carcinogen or mutagen. Non-carcinogenic/non-mutagenic arylamines were characterized by nitrenium ions whose singlet states were much less stable than the triplet. Carcinogenic/mutagenic amines were characterized by nitrenium ions whose singlet states were of similar or greater stability than the triplet. By examination of the charge density at key ring atoms of the singlet and triplet species, a rational approach to the stabilization of one species relative to the other has emerged and forms the basis for prediction of genotoxicity in closely related structures. The application of this empirical correlation to the prediction of the carcinogenic/mutagenic potential of arylamines is discussed.

## INTRODUCTION

A crucial advance in elucidating the mechanisms by which chemicals cause cancer, has been the recognition that numerous types of molecules are metabolically activated in vivo to chemical intermediates of pronounced electrophilic reactivity which initiate the cellular events leading to carcinogenesis. Among the structural classes which have been so identified, arylamines occupy a prominent place, as already several members are known to be carcinogens for man [1].

Abbreviations: 2-AAF, 2-acetylaminofluorene; INDO, intermediate neglect of differential overlap; 3-PAPS, 3'-phosphoadenosine-5'-phosphosulfate; UHF, unrestricted Hartree-Fock.

Early studies with arylamines indicated that, in animals, tumors almost invariably appeared in organs distant from the site of administration, suggesting the intervention of metabolites in the tumor genesis [2,3]. This notion was further delineated by the Millers [4] who found that a metabolite of *N*,*N*-dimethyl-4-aminoazobenzene was covalently bound to the hepatic proteins of rats fed this compound. In 1960, Cramer et al. [5] reported that metabolic activation of the carcinogen 2-acetylaminofluorene (2-AAF) afforded *N*-hydroxy-2-AAF as a major urinary excretion product. Subsequent work indicated both that *N*-hydroxy-2-AAF was a more potent carcinogen in the rat than was 2-AAF [6] and that further metabolic transformation of the hydroxamic acid was likely, since *N*-hydroxy-2-AAF itself was not chemically reactive in vitro [7]. The final activation step, at least for liver tumor formation in the male rat, was found to involve conversion of *N*-hydroxy-2-AAF to its sulfate ester by the cytosol-soluble enzyme 3'-phosphoadenosine-5'-phosphosulfate (3-PAPS) [8,9]. In addition to the sulfate ester pathway, recent work has indicated that at least three other enzyme pathways exist for conversion of *N*-hydroxy-2-AAF to its ultimate carcinogenic species [10].

Despite the uncertainty as to the complete sequence of intermediates and paths of biosynthesis, it is generally accepted that metabolic activation of arylamines involves initial N-oxidation and leads subsequently to an electrophilic species which can covalently interact with cellular macromolecules [11]. This ultimate carcinogenic species, as shown in Fig. 1 for 2-aminofluorene, is the aryl nitrenium ion. Aryl nitrenium ions have been postulated as the metabolically derived ultimate carcinogenic form of numerous arylamines [12—15] and as such have provided an intellectually satisfying mechanistic concept.

Although the aryl nitrenium ion has gained impressive popularity in the role described, little is factually known about its control of cellular chemical events. Scribner and Naimy suggested that the adduct-forming abilities of *N*-acetoxy-*N*-arylacetamides could be related to their ability to form radical cations [16], i.e., triplet nitrenium ions and also that their adenine-guanine specificity could be rationalized on the basis of frontier orbital coefficients [17]. These workers suggested that the less likely the precursor *N*-acetoxy-*N*-arylacetamide was to generate a radical cation, the better carcinogen it would be [16]. More recently, Loew et al. [18], utilizing quantum mechanical methods, studied the relation between the electrophilic properties of the aryl nitrenium ion and both the pattern of metabolites and mutagenic potential

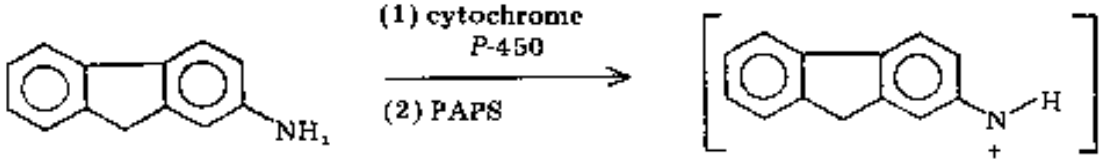



Fig. 1. Conversion of 2-aminofluorene to its nitrenium ion.

associated with the parent molecule. Despite these efforts, nothing of any predictive value is yet known about the relationship of the electronic nature of the arylnitrenium ion species to that ion's potential to act as a mutagen or carcinogen.

Aryl nitrenium ions have been the subject of intensive chemical and physical-chemical investigations [19—21]. Of crucial significance is the observation by Gassman and Cryberg [22] who showed that a dialkyl nitrenium ion could exist discretely in either the singlet or the triplet spin state and that the two species could display different chemical reactivity. These authors suggested that the singlet, which would necessarily be the initial intermediate derived from heterolytic cleavage in the nitrenium ion precursor, underwent typically ionic type reactions, whereas the triplet, derived from the singlet by spin inversion, behaved chemically as a free radical.

This paper reports the utilization of INDO and ab initio methodology to calculate trends in the energy difference between the lowest singlet and triplet states for the nitrenium ions derived from a variety of carcinogenic and non-carcinogenic arylamines. Our working hypothesis was that the ability of a particular nitrenium ion to act biologically as an ultimate mutagen or carcinogen might be related to its inherent potential to react predominantly either as a singlet or as a triplet, depending on the relative energies of the two spin states. Our data indicates that this is indeed the case and, for the first time, we can outline principles for the prediction of the genotoxic potential of the parent amine, based purely on a consideration of the electronic structure of the corresponding nitrenium ion. We have sought not merely to prepare a list of compounds which broadly support the notions described above, but have tried to treat groups of compounds, members of which possess similar chemical structure but different oncogenic potential.

## MATERIALS AND METHODS

Calculations of total energies and charge distributions were made with the standard INDO molecular orbital method [23]. The observed trends were checked by repeating key calculations with ab initio computations using Gaussian 70 [24] and the STO-3G basis set. Standard values for bondlengths and angles were employed in constructing the molecules with the aid of the Merck Molecular Modeling System [25]. In the case of the ab initio calculations, the frequently encountered convergence problems for both the singlet and triplet nitrenium ions usually could be overcome using strong damping in the SCF procedure.

For the open shell calculations, the unrestricted Hartree-Fock (UHF) formalism was employed. In the ab initio calculations differing amounts of higher spin states become mixed into the triplet, depending on whether a monocyclic or polycyclic aryl nitrenium ion is treated. Thus, a naphthyl or a biphenyl nitrenium ion calculation can be reliably compared with others of the same parent ring system, but not with the phenyl system. This problem did not arise in the INDO computations.

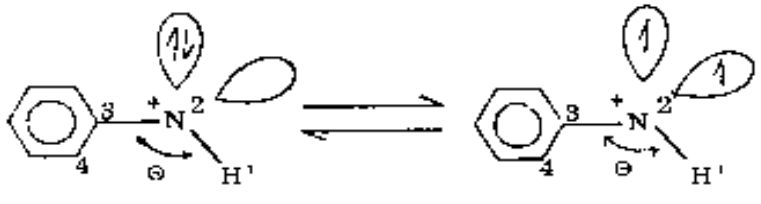
## RESULTS AND DISCUSSION

Singlet triplet energy differences are notoriously difficult to obtain accurately [26], either by experiment or from theory, as indicated by the continuing controversy over $CH_2$, which is isoelectronic with the simplest nitrenium ion $^+NH_2$. However, in the present study, we are not interested in the actual magnitudes of the singlet-triplet separation, but only in the trends and changes in the singlet-triplet gaps. Furthermore, the nitrenium ions examined herein are all closely related; the electron-deficient nitrogen atom is directly attached to an aromatic hydrocarbon system and the structural variations are primarily in substituents remote from the site of the nitrogen atom. With such stringent restrictions, even a semi-empirical method such as INDO should be able to reveal the major trends. Where possible, we have checked the predictions of the INDO calculations by ab initio methods.

The energy surfaces of the singlet and triplet states of the phenyl nitrenium ion were explored as a function of the geometry about the nitrenium ion center. It was found that the total energy of each spin state varied both with the angle $\Theta$ and the dihedral angle described by atoms 1234 (Fig. 2). The data in Fig. 3 indicate that from INDO the singlet and triplet states of the phenyl nitrenium ion are of lowest energy when $\Theta$ is 120° and 160°, respectively. The data in Fig. 4 indicate that the singlet ($\Theta$ = 120°) and triplet ($\Theta$ = 180°) are most stable when the dihedral angle about the nitrenium ion center is 0°. Similar calculations for numerous other aryl nitrenium ions indicate that these geometries are usually characteristic of the lowest energy states, although as shown for the triplet in Fig. 3, the energy differences are quite small as $\Theta$ varies from 160° to 180°.

Table I shows the energy differences between the singlet and triplet states for the nitrenium ions of nine arylamines as given by INDO calculations. A representative number of ab initio calculations is also included. Each of these amines has been found to be non-carcinogenic in animals and/or non-mutagenic in the Ames test. In each case, INDO found the triplet to be of lower energy than the singlet. The difference in energy between the two states, i.e., $E_s - E_t$, varied from 20.7 for *N*-phenyl-1-aminonaphthalene to 11.8 for aniline.

Table II shows the singlet-triplet energy differences, as given by the INDO and ab initio methods, for the nitrenium ions derived from eighteen carcinogenic and/or mutagenic arylamines. This list comprises monocyclic, bicyclic




singlet triplet

Fig. 2. Singlet and triplet spin states of the phenyl nitrenium ion.

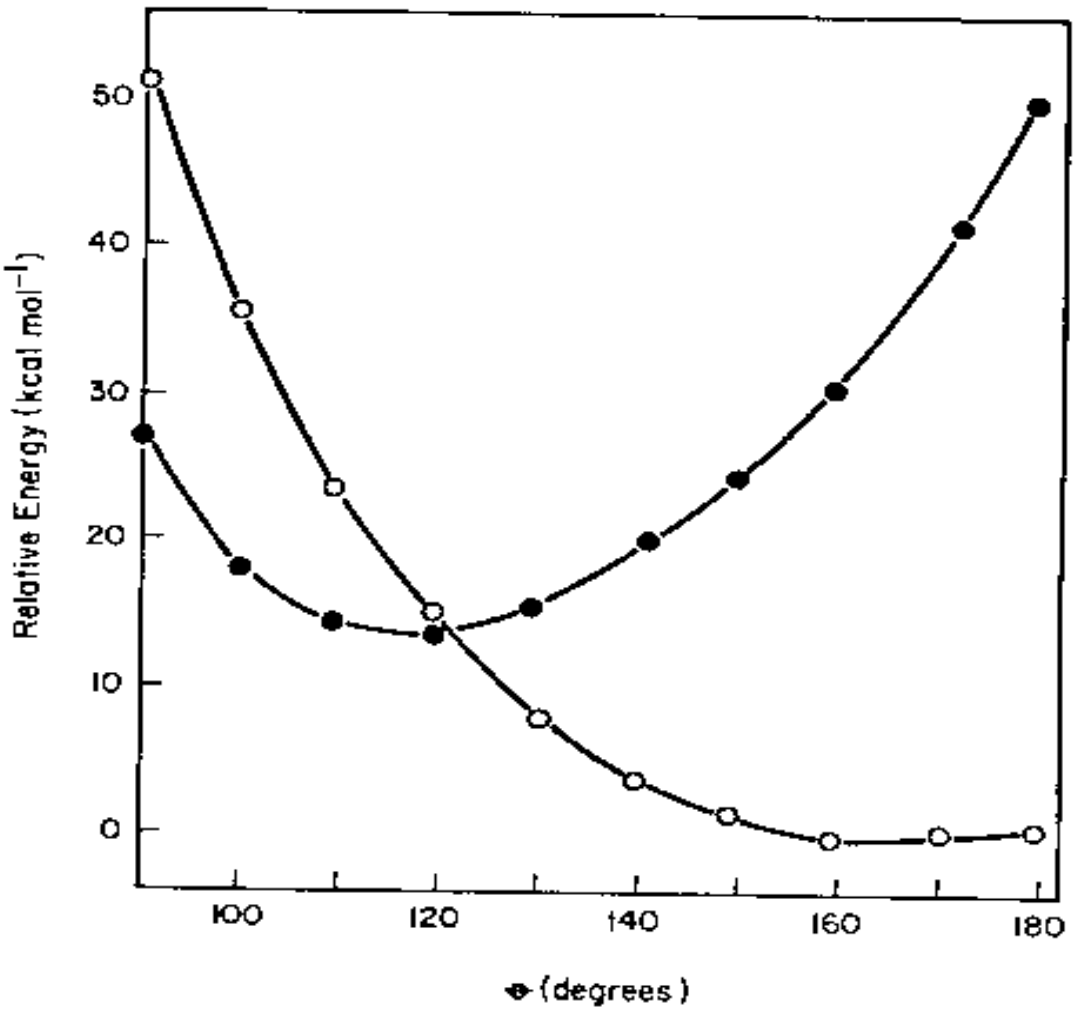



Fig. 3. Relative energy of the singlet (•) and triplet (○) states of the phenyl nitrenium ion as a function of Φ, as given by INDO methodology.

and polycyclic amines and includes both classical carcinogens as well as others specifically chosen for study as structural analogs of compounds in Table I. For the nitrenium ions studied, the $E_s - E_t$ values from INDO varied from 9.1 for 1-aminofluorene to −3.2 for 2,4-dimethoxyaniline. The negative sign in the latter case is significant only in relation to the other values shown; it does not mean that the singlet is experimentally more stable than the triplet, since INDO is not reliable for prediction of the actual magnitude of the singlet-triplet separation.

For structurally similar amines, the ab initio calculations confirm the trends found in the INDO computations, in that $E_s - E_t$ is consistently larger for non-carcinogenic/non-mutagenic amines than for carcinogenic/mutagenic amines. In particular, monocyclic arylamines exhibit $E_s - E_t > 30$ kcal mol$^{-1}$ for non-carcinogenic/non-mutagenic amines and $E_s - E_t < 30$ for the carcino-

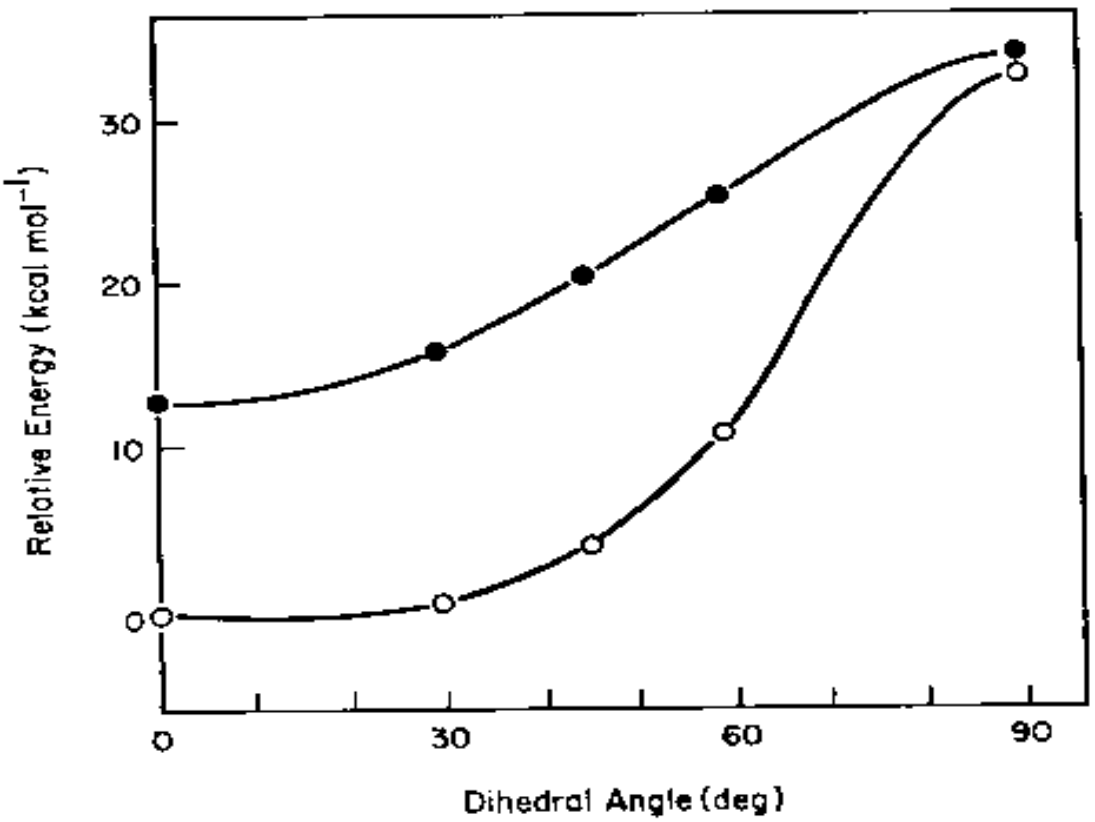



Fig. 4. Relative energy of the phenyl nitrenium ion for the singlet (•) at $\Theta = 120°$ and triplet (○) at $\Theta = 180°$ as a function of the dihedral angle, as given by INDO methodology.

TABLE I

ENERGY DIFFERENCES OF SINGLET ($E_s$) AND TRIPLET ($E_t$) NITRENIUM IONS DERIVED FROM NON-MUTAGENIC/NON-CARCINOGENIC ARYLAMINES

| Compound | $E_s - E_t$ (kcal mol$^{-1}$) | | Carcinogen Ref. No. | Mutagen Ref. No. |
|---|---|---|---|---|
| | INDO | ab initio | | |
| *N*-Phenyl-1-Aminonaphthalene | 20.7 | | | −27 |
| *N*-Ethylaniline[a] | 20.5 | | 28 | |
| 3,5-Dimethoxyaniline | 15.2 | | | −29 |
| 3-Methoxyaniline | 13.6 | | | 29 |
| 3,5-Dimethylaniline | 13.5 | 31.5 | | 29,33 |
| *p*-Aminobenzoic acid | 12.9 | | −30 | |
| 3-Methylaniline | 12.6 | 31.8 | −31,32[b] | 33 |
| 3-Aminobiphenyl | 12.2 | 49.2 | | −29 |
| Aniline | 11.8 | 32.1 | −34, +35 | 36 |

[a] Hydroxylamine was tested for carcinogenicity.
[b] Questionable activity.

TABLE II

ENERGY DIFFERENCES OF SINGLET ($E_s$) AND TRIPLET ($E_t$) NITRENIUM IONS DERIVED FROM MUTAGENIC/CARCINOGENIC ARYLAMINES

| Compound | $E_s$-$E_t$ (kcal mol$^{-1}$) | | Carcinogen Ref. No. | Mutagen Ref. No. |
|---|---|---|---|---|
| | INDO | ab initio | | |
| 2,5-Dimethylaniline | 8.85 | | +32 | +33 |
| 2-Methylaniline | 8.66 | 28.2 | +32 | −33,36 |
| 3,4-Dimethylaniline | 8.47 | 27.1 | +37 | +33 |
| 2-Aminoanthracene | 8.08 | | +38,39 | +40 |
| 2-Aminonaphthalene | 7.91 | 43.2 | +41 | +36 |
| 2-Aminophenanthrene | 6.59 | | +42 | |
| 2-Aminobiphenyl | 6.09 | | | +36 |
| 2,4,5-Trimethylaniline | 5.88 | | +31 | +33 |
| 4-Aminobiphenyl | 4.77 | 40.4 | +43 | +36 |
| 2,4-Dimethylaniline | 4.64 | 26.6 | +32 | +33,44 |
| 1-Aminonaphthalene | 4.20 | 35.1 | ?45, +46[a] | +36 |
| 2-Methoxyaniline | 3.42 | 20.3 | +47 | +29 |
| 2-Aminofluorene | 3.39 | | +48 | +36 |
| 4-Amino-*t*-stilbene | 2.63 | | +49 | +36 |
| 4,4'-Diaminobiphenyl | 2.44 | | +50 | +36 |
| 6-Aminochrysene | 0.06 | | +51 | +36 |
| 1-Aminopyrene | 2.94 | | | +36 |
| 2,4-Dimethoxyaniline | −3.2 | | −52 | +29 |

[a]Hydroxylamine was tested for carcinogenicity.

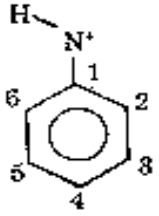
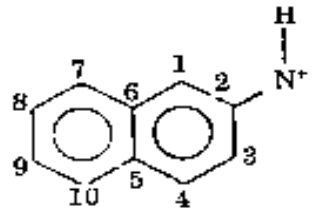
genic/mutagenic amines. Because of difficulties in the ab initio calculations (see Materials and Methods) the naphthyl and biphenyl amines cannot be fit into the same rule.

Taken together, the data in Tables I and II indicate that non-carcinogenic/non-mutagenic arylamines are characterized by nitrenium ions which from INDO methodology have $E_s—E_t > 10$ kcal mol$^{-1}$, i.e., the triplet is the more stable species. Carcinogenic/mutagenic arylamines are characterized by nitrenium ions exhibiting values of $E_s—E_t < 10$ kcal mol$^{-1}$. That is to say, arylamines whose nitrenium ions display large differences in $E_s$- and $E_t$-values, with the triplet the more stable, were non-carcinogenic/non-mutagenic, while those amines giving rise to nitrenium ions wherein the singlet and triplet states were of similar energy, or the singlet was of lower energy were carcinogenic/mutagenic.

The empirical correlation between $E_s—E_t$ values and arylamine genotoxicity is potentially a valuable predictive tool. We have already used it as such with good success throughout this study, particularly in identifying non-mutagenic arylamines. Of special importance is the ability of this technique to predict correctly the genotoxic potential of structural isomers within a given ring system. This involves both knowledge of the $E_s—E_t$ correlation and a comparison of charge density at key positions in the singlet and triplet species. For example, the INDO charge density data in Table III

TABLE III

CHARGE DENSITIES (INDO) FOR THE PHENYL NITRENIUM ION AND THE 2-NAPHTHYL NITRENIUM ION

| Nitrenium ion | Atom | Singlet | Triplet | $\Delta_{t-s}$ |
|---|---|---|---|---|
|  | 1 | +0.11 | +0.16 | +0.05 |
| | 2 | +0.12 | +0.06 | −0.06 |
| | 3 | +0.01 | +0.04 | +0.03 |
| | 4 | +0.17 | +0.10 | −0.07 |
| | 5 | +0.01 | +0.04 | +0.03 |
| | 6 | +0.12 | +0.06 | −0.06 |
|  | 1 | +0.16 | −0.06 | −0.10 |
| | 2 | +0.12 | +0.17 | +0.05 |
| | 3 | +0.02 | −0.03 | −0.05 |
| | 4 | +0.02 | +0.06 | +0.03 |
| | 5 | +0.11 | +0.06 | −0.05 |
| | 6 | +0.11 | +0.04 | +0.03 |
| | 7 | +0.08 | −0.04 | −0.04 |
| | 8 | +0.02 | +0.03 | +0.01 |
| | 9 | +0.11 | +0.07 | −0.04 |
| | 10 | 0.0006 | +0.02 | +0.02 |

indicates that for the phenyl nitrenium ion, the triplet has a greater positive charge at C-3 and C-5 than does the singlet. On the other hand, the singlet has a greater positive charge at C-2, C-4 and C-6 than does the triplet, as is expected from resonance theory. This analysis would indicate that substitution of cation-stabilizing groups at C-3 and/or C-5 would tend to preferentially stabilize the triplet relative to the singlet, while similar substitution at C-2, C-4 and C-6 should preferentially stabilize the singlet. In the case of the 2-naphthyl nitrenium ion similar analysis would indicate that stabilization of the triplet relative to the singlet would result from substitution of cation-stabilizing groups at C-4 and C-10, while similar substitution at C-1, C-3, C-7 and C-9 would favor stabilization of the singlet. By applying these concepts we predicted correctly, as outlined in Table IV, that: (a) 2-methylaniline was carcinogenic while 3-methylaniline was not; (b) 2,4-, 2,5- and 3,4-dimethylaniline were mutagenic/carcinogenic while 3,5-dimethylaniline was not; (c) 2- and 4-aminobiphenyl were mutagenic while 3-aminobiphenyl was not; (d) 2-methoxyaniline and 2,4-dimethoxyaniline were mutagenic while 3-methoxyaniline and 3,5-dimethoxyaniline were not.

One interpretation of this empirical correlation is that the calculated energy differences roughly reflect the propensity of the initially-formed singlet species (Fig. 2) to react as a singlet or to spin invert to the triplet. When, as with non-carcinogenic amines, the triplet is much more stable, the nitrenium ion will display reactivity predominantly via the triplet species. However, for carcinogenic amines, the singlet displays stability more comparable to that of the triplet and the nitrenium ion will, therefore, possess a relatively greater opportunity to react as a singlet. The implication here is

TABLE IV

COMPARISON OF GENOTOXIC POTENTIAL OF ISOMERIC ARYLAMINES

| | Mutagenic/Carcinogenic | Non-mutagenic/Non-carcinogenic |
|---|---|---|
| (A) | 2-Methylaniline | 3-Methylaniline |
| (B) | 2,4-Dimethylaniline<br>3,4-Dimethylaniline<br>2,5-Dimethylaniline | 3,5-Dimethylaniline |
| (C) | 2-Aminobiphenyl<br>4-Aminobiphenyl | 3-Aminobiphenyl |
| (D) | 2-Methoxyaniline<br>2,4-Dimethoxyaniline | 3-Methoxyaniline<br>3,5-Dimethoxyaniline |

that enhanced opportunity for reaction as a singlet, as compared to the triplet, is important for initiation of the mutagenic/carcinogenic process.

In studying the mechanistic basis of arylamine genotoxicity, it is interesting to consider the stability of the singlet nitrenium ion as a determinant of precursor, i.e., hydroxylamine ester, reactivity. It is attractive to reason that the more stable the nitrenium ion, relative to its precursor, the more reactive and possibly genotoxic that precursor might be. However, efforts by Scribner et al. [13] and Loew et al. [18] to quantitatively correlate hydroxylamine ester reactivity and nitrenium ion stability with carcinogenicity and mutagenicity have not been successful. This lack of quantitative correlation is not surprising in view of the complexity of the chemical and biological processes for metabolic activation and cellular transformation. Our data in this regard indicate that the reactivity of the hydroxylamine ester, as given by the stability of the singlet nitrenium ion vs. that of the parent amine, does not correlate with the observed genotoxic potential. Specifically, INDO calculations show that for two non-genotoxic amines, *N*-ethylaniline and *N*-phenyl-1-aminonaphthalene, the energy separation between the parent amine and the corresponding nitrenium ion is less than that for many of the mutagenic/carcinogen amines listed. Therefore, the reactivity of the hydroxylamine esters for these two non-genotoxic amines should be greater than that of many genotoxic arylamines. Calculations further reveal that other non-mutagenic amines should give rise to hydroxylamine esters which are less reactive than those esters of any genotoxic amine. It, therefore, does not seem possible at this time to correlate arylamine genotoxicity with the reactivity of the nitrenium ion precursor.

Although the present method holds great promise as a tool for prediction of the genotoxic potential of arylamines, several limitations are apparent. Our approach assumes that: (a) metabolic activation to the nitrenium ion stage occurs, (b) biologically significant concentrations of ion or precursor do penetrate to crucial cellular targets and (c) that subsequent reaction of

nitrenium ions constitutes an important genotoxic pathway. Important exceptions are possible. For example, hindrance to enzymic N-hydroxylation, as has been suggested for 1-aminonaphthalene and 2-aminobiphenyl [53], or any other activation step, as well as enhanced metabolism at some other functionality in the molecule could diminish or eliminate the biological import of the nitrenium ion pathway. It is also possible that other mechanisms, for example, epoxidation in the case of the topical carcinogen 3-methyl-2-aminonaphthalene [54] could be important in determining tumorigenicity.

The present method is in design and methodology an effort to delineate the inherent genotoxic potential of the aryl nitrenium ion on the basis of electronic characteristics. The extent to which this measure of inherent activity has already correlated with observed biological activity is unprecedented. We expect these concepts to prove valuable in the recognition and structural modification of genotoxic arylamines.

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