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N-Nitrosamines

In N-Nitrosamines; Anselme, J.;
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N-Nitrosamines

Jean-Pierre Anselme, EDITOR
University of Massachusetts at Boston

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FOREWORD

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that in order to save time the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable since symposia may embrace both types of presentation.

PREFACE

Over the past twenty-odd years, *N*-nitrosamines, previously compounds of moderate academic interest, have assumed a prominent place in our daily lives. In Jekyllesque fashion, *N*-nitrosamines have revealed themselves as carcinogens in animals and possibly as ubiquitous guests of man's environment. Even the average public is already fully aware of the term "nitrosamine," thanks to the extensive coverage accorded the topic in the news media. The progress made since the "modern era" began in the mid-fifties has made it apparent that a great deal of the basic organic chemistry of *N*-nitrosamines remains unexplored. Equally evident is the fact that a sound knowledge of the chemistry of these compounds is essential if chemists and biologists are to unravel the mechanism of biological action. The important discoveries of the magnetic non-equivalence and of the base-lability of the α-hydrogens of nitrosamines have not only opened new vistas on the chemistry of this class of compounds but also have suggested the α-position as a possible trigger of the carcinogenic process (α-hydroxylation). The major concern over the existence of unrecognized sources of nitrosamines in the body and in the environment has provided additional impetus to an already active field.

The papers delivered at a symposium on nitrosamines (NERM 8, June 26, 1978, Boston) constitute the bulk of this volume. Some of the leading contributors discussed their most recent results in this new frontier encompassing both chemistry and biology. Unfortunately, Prof. Baldwin's paper could not be included in this book. Dr. Wiessler, an invited speaker, was good enough to provide his manuscript even though illness prevented him from participating in the symposium. Dr. Keefer elected to deliver only an oral presentation because he felt that those results of greatest potential importance could be discussed freely, regardless of their preliminary nature at the time; instead of a paper based on his talk, Dr. Keefer has provided a manuscript dealing with a different aspect of *N*-nitrosamines. In addition, Prof. Chow has prepared a review of his most recent investigations of the photochemistry of *N*-nitrosamides, while Prof. Loeppky's contribution deals with a novel reaction of certain *N*-nitrosamines. Finally, I have prepared a brief survey of the organic chemistry of *N*-nitrosamines in order to give perspective to the remaining chapters of the book.

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I would like to acknowledge the help of the Chairman of the morning session, Prof. C. G. Overberger, whose ever gracious willingness to help is never found wanting, despite a very hevy schedule of commitments. The superb cooperation of the Meeting Chairman, Prof. E. I. Becker, made it a pleasure to organize the symposium.

University of Massachusetts at Boston
Boston, Massachusetts
March 27, 1979

JEAN-PIERRE ANSELME

The Organic Chemistry of *N*-Nitrosamines: A Brief Review

JEAN-PIERRE ANSELME

Department of Chemistry, University of Massachusetts at Boston, Harbor Campus, Boston, MA 02125

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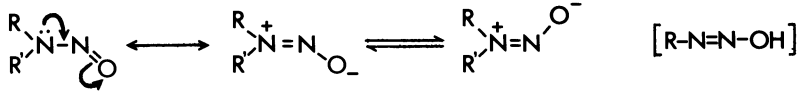
Although N-nitrosamines (I) have been known since the nineteenth century, it was not until quite recently that they became the subject of intensive investigations. This renewed interest was spurred in large part by the report of Magee and Barnes (1) on the carcinogenicity of these compounds. Since then, research into the chemistry and biological properties of N-nitrosamines has accelerated. This brief survey is intended to serve both as a summary of some of the frontiers in this area and as an introduction to the chemistry of N-nitrosamines discussed in the remaining chapters of this Volume.

N-Nitrosamines have the nitroso group attached directly to the amine nitrogen (2-7). As with C-nitroso compounds however, in general only those N-nitrosamines where neither R nor R' is a hydrogen atom, have any existence; when one of the substituents is a hydrogen, the equilibrium favors the tautomeric diazoic acid (A) (8). There are reported cases of stable primary N-nitrosamines;



I I' I" A

it is likely, however, that in those instances these N-nitrosamines exist as the tautomeric N-nitrosimines (9).

Although there is a large body of information on N-nitrosamides (i.e., N-nitrosamines where one of the substituents is either acyl or sulfonyl), this brief survey will deal mainly with N,N-dialkyl- and N,N-aralkylnitrosamines.

Structure

In 1957, Looney, Phillips and Reilly (10) were the first to

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demonstrate that α-hydrogens of N,N-dialkylnitrosamines are magnetically non-equivalent as a result of the large contribution of the dipolar mesomeric structure. The existence and the separation of configurational isomers (I' and I") have been reported (13). These findings confirmed the suggestion that N-nitrosamines are polar and served to usher a major advance in the chemistry of this class of compounds.

Synthesis

The nitrosation of secondary amines with sodium nitrite in the presence of acids is by far still the most widely used method for the preparation of N-nitrosamines. Several variations of this

$$RR'NH + NaNO_2 \xrightarrow{H^+} RR'N\text{-}NO \qquad (1)$$

procedure are known (2-7). Other nitrosating agents such as nitrosyl fluoroborate, dinitrogen trioxide, dinitrogen tetroxide have been utilized. A recent modification (14) involves the nitrosation of the anion of the amines with nitrosyl chloride. Transnitrosation with 3-nitro-N-nitrosocarbazole allows nitrosation to occur in neutral medium (37). Of particular interest is the ability of nitroprusside [$Fe(CN)_5NO^=$] to nitrosate amines under aqueous alkaline conditions (15).

$$(CH_3CH_2)_2NH + Fe(CN)_5NO^= \xrightarrow[H_2O]{OH^-} (CH_3CH_2)_2NN{=}O \qquad (2)$$

A novel method to degrade tertiary amines proceeds through the formation of secondary N-nitrosamines (16).

$$\begin{matrix} R_2NCH_2R' \\ + \\ (CH_3)_2CHNO_2 \end{matrix} \xrightarrow[\text{Pyridine}]{O_2\,,CuCl} R_2NNO + R'CHO + (CH_3)_2CO \qquad (3)$$

Reactions

Reactions of the Nitroso Group. Up until recently, the chemistry of N-nitrosamines was restricted to the reaction involving the nitroso group. It was not until the usefulness of N-nitrosamines in synthesis [concept of "Umpolung" (3)] was demonstrated, that the long-known denitrosation of N-nitrosamines became impor-

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tant. Although numerous methods have been reported for this purpose, the action of mineral acids remains the most common procedure (Eq. 4).

$$R_2NN{=}O + HCl \xrightarrow{H_2O} R_2NH{\cdot}HCl + HONO \qquad (4)$$

There has been some controversy regarding the site of attack of the proton; on the basis of the dipolar structures I' and I", it might be inferred that the proton attack should occur on the oxygen atom; the fact that N-nitrosamines form O-complexes and O-alkylated products would seem to support this view (17). However, it is currently believed that the N-protonated form is the one that leads to denitrosation, irrespective of whether or not initial protonation occurs on oxygen (18).

$$R_2N{-}N{=}O \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} R_2N{-}N{=}O^+{-}H \rightleftharpoons R_2\overset{+}{N}\!<\!\begin{matrix}H\\NO\end{matrix} \xrightarrow{Nu^-} R_2NH + NuNO$$

Scheme 1

One of the oldest-known reactions of N-nitrosamines is their reduction to 1,1-disubstituted hydrazines discovered by Fischer (19). The most common method to perform this transformation has been zinc dust in acid, generally acetic acid; tetrazenes are sometimes formed as by-products (20) and denitrosation can also occur. Several other reducing methods have been investigated; reduction with lithium aluminum hydride and catalytic hydrogenation are sometimes useful. Sodium dithionite reduction of benzyl substituted N-nitrosamines in base can result in fragmentation to

$$\begin{matrix}(PhCH_2)_2NNO\\+\\Na_2S_2O_4\end{matrix} \xrightarrow{OH^-} \left[(PhCH_2)_2N{-}\ddot{N}\right] \xrightarrow{-N_2} PhCH_2CH_2Ph \qquad (5)$$

the hydrocarbons (Overberger-Lombardino reaction). The "abnormal" reduction of N-nitrosamines presumably proceeds *via* the N-nitrene (or N-nitrenoid) which then may extrude nitrogen to yield the observed hydrocarbons (6). N-Nitrosamines can be considered as *N-nitrene N-oxides* and indeed are deoxygenated to the putative N-

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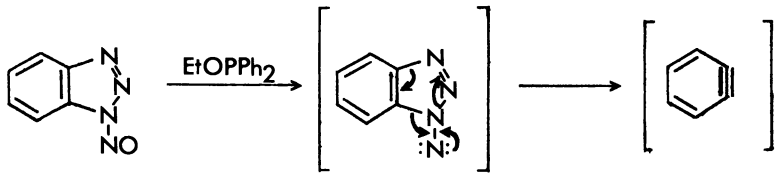
nitrenes with ethyl diphenylphosphinite [Eq. 6] (21), iron penta-



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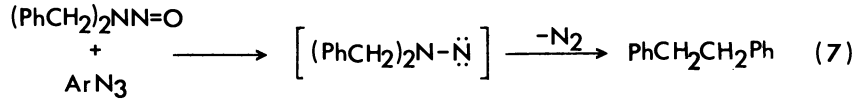


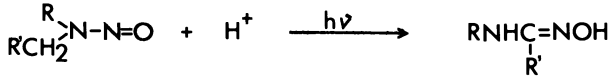
(7)

carbonyl (22,23), aryl azides [Eq. 7] (24) or phenacylcarbonium ions (25).

N-Nitrosamines have been oxidized to the corresponding N-nitramines. Trifluoroperacetic acid and nitric acid/ammonium persulfate are the reagents of choice for this purpose.

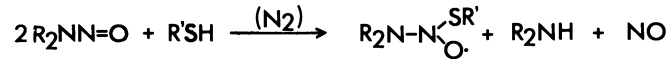
(8)

It has been found that, though stable to light in neutral solution, N-nitrosamines can rearrange to the corresponding amidoximes (4,26,27) in acid solution; other products can also be formed (4).



(9)

A very recent communication (28) reports the addition of thiyl



(10)

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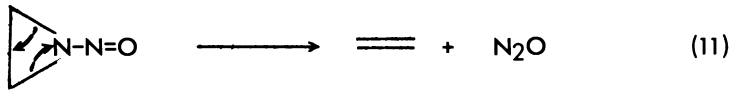
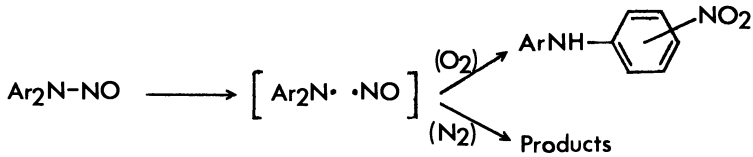
radicals to N-nitrosamines to give the corresponding nitroxide radicals.

The chelotropic elimination of nitrous oxide from N-nitrosoaziridines is a reaction restricted to this class of compounds (29-36).



(11)

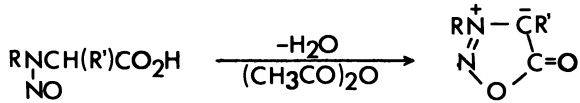
Diarylnitrosamines undergo fission at the nitrogen-nitrogen bond upon heating (37,38). In the presence of oxygen, the ring nitro substituted amines are obtained (37); in the absence of



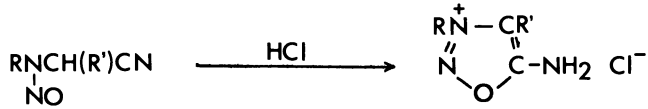
(12)

oxygen, products resulting from the diarylamino radical are formed (38).

A much older reaction of N-nitroso α-aminoacids is their cyclization to the mesoionic sydnones (39,40,41,42); more recently, N-nitroso α-aminonitriles have been cyclized to the corresponding sydnone imines (40,41,42,43).



(13)



(14)

Reactions Caused by the Nitroso Group. A major consequence of the strongly dipolar nature of N-nitrosamines - a fact now firmly established by the work of Phillips (10) and of Karabatsos (11) - has been a better understanding of the chemistry of N-ni-

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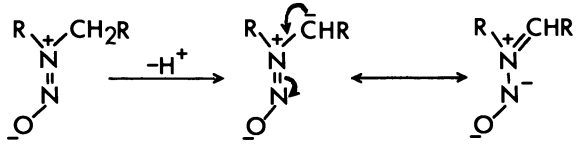
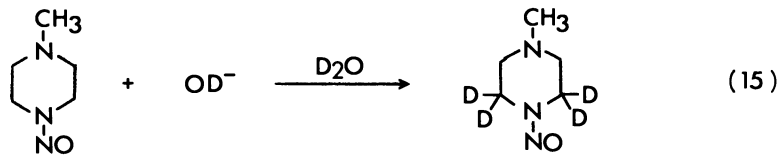
trosamines and the elaboration of new reactions based on the acidic nature of the α-hydrogens of N-nitrosamines. Although resonance stabilization of the negative charge may be difficult to envision (44,46), the experimental data leave little doubt as to the reality of the inductive effect.



Scheme 2

The first indication of the effect of the positive charge on the α-hydrogen came from the isomerization of trans-N-nitroso-2,5-diphenylpyrrolidine to the cis-isomer in aqueous base (45). Keefer and Fodor (46) were the first to demonstrate the deuterium exchange of the α-hydrogens and the alkylation of anions of N-nitrosamines. Seebach and his group (3) have developed this discovery into a major synthetic process; they have coined the word "Umpol-



(15)

ung" to indicate that the nitroso group attached to the amine ni-

Scheme 3

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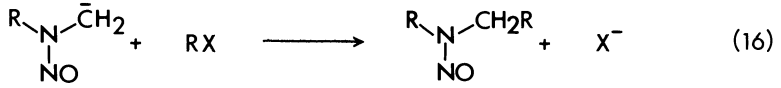
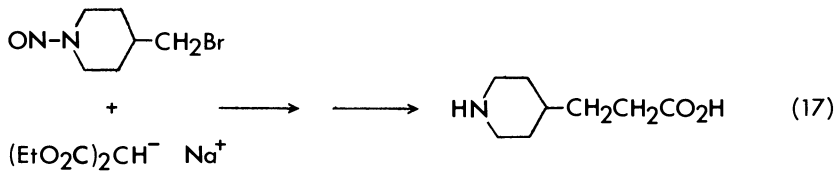
trogen has reversed the normal "polarity" of the α-carbon (Scheme 3). N-Nitrosamino anions have also been added to carbonyl compounds, to α,β-unsaturated ketones and nitroolefins, to nitriles and other



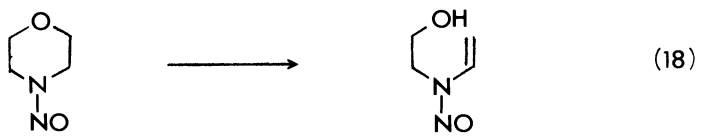
(16)

electrophiles (3). It is of historical interest that as early as in 1944, Koelsch (47) used the N-nitroso group as a removable protective group in the synthesis of 4-(piperidino)propionic acid.



(17)

Some difficulties may be encountered during the metallation of N-nitrosamines. Elimination may occur in either of two ways. For example, N-nitrosomorpholine yields the N-nitrosoenamine while loss



(18)

of [HNO] results in the formation of imines (3). The formation of trans-stilbene from the anion of N-nitrosodibenzylamine may proceed by a mechanism akin to that of the Ramberg-Backlund, at least in its early stages (3).

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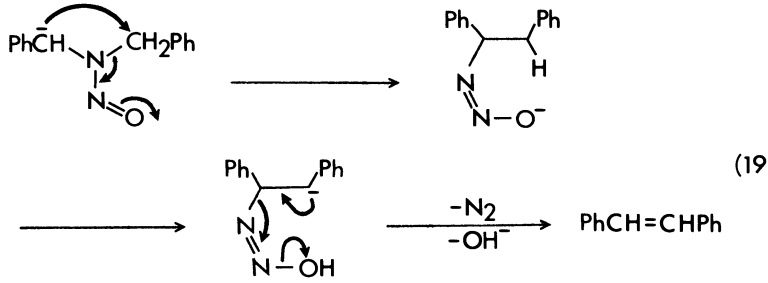
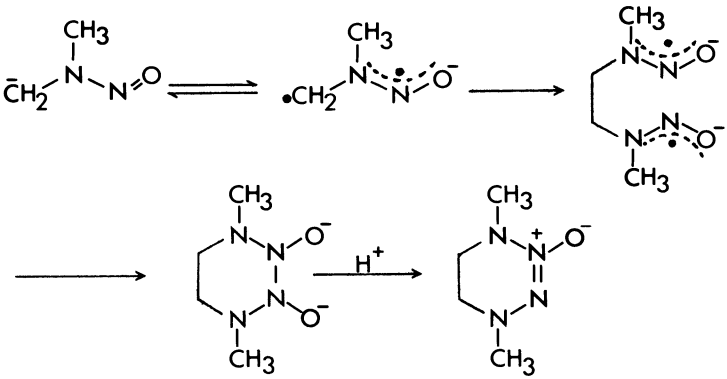


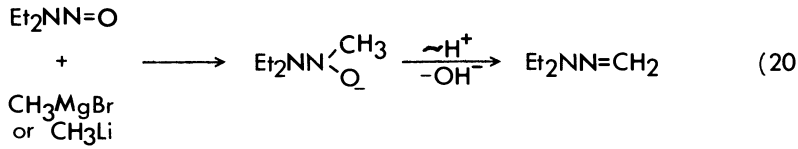
(19)

The interesting "dimerization" of lithiated N-nitrosamines to cyclic tetrazene N-oxides has been described (48) and provides another route to the novel cyclic tetrazenes (49,50). A radical path has been suggested for this reaction [Scheme 4] (3).



Scheme 4

The reaction of organometallics with N-nitrosamines has been investigated in the early part of this century mainly by Wieland and his students (51,52,53,54). More recent work (55,56,57) indicates that Grignard and organolithium reagents may either abstract α-hydrogen or add to the nitroso group. Some of the azomethine imines have been intercepted with 1,3-dipolarophiles.



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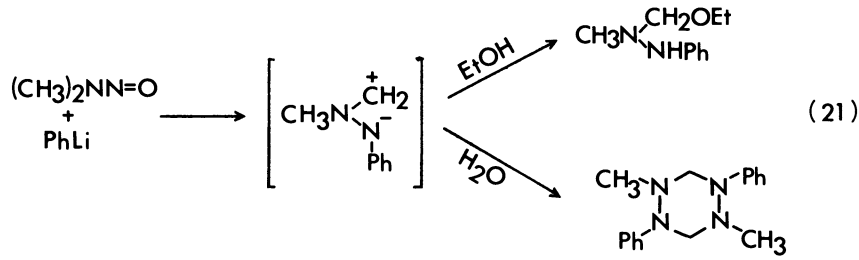
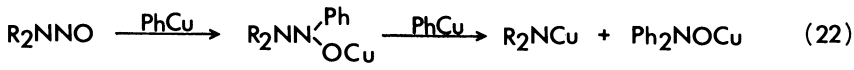


(21)

With phenylcopper (58) or with lithium or magnesium metal (59), denitrosation to the amines is the major reaction path; N,N-diphenylhydroxylamine a significant by-product of the reaction, is believed to arise as shown in Eq. 22 after hydrolysis (58).



(22)

In the short span of twenty-five years, the hitherto rather prosaic N-nitrosamines have revealed themselves to possess a rich and varied chemistry as subsequent chapters of this volume will further demonstrate.

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Chemistry of *N*-Nitrosamides and Related *N*-Nitrosamino Acids

YUAN L. CHOW

Department of Chemistry, Simon Fraser University,
Burnaby, British Columbia, Canada V5A 1S6

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Although the nitrosamide group, R'N(NO)COR, is not frequently encountered in chemistry, its thermal decomposition has attracted much study since the late 1940's, owing to its fascinating molecular rearrangements, convenience in kinetic studies and applicability as a clean method of deamination (*1,2*). Nitrosamides are easily prepared (*3,5*) by treating amides with nitrosating agents such as nitrogen tetroxide, nitrosyl chloride or nitrosyl tetrafluoroborate in the presence of weak tertiary bases, or sodium nitrite in acetic acid-acetic anhydride mixtures. Yellow to orange-yellow colored nitrosamides usually exhibit π-π^* transitions at ca.240 nm (ε 4000 - 6000) and a series of peaks for n-π^* transitions in the 390-430 nm region. Ordinary alkyl nitrosamides have characteristic infrared absorptions at 1720-1730 (C=O) and 1500-1505 cm^{-1} (N=O).

In the early 1960's when nitrosamines, R'RNNO, were recognized as animal carcinogens and were subsequently found widely distributed in environmental samples (*6*), nitrosoamides were generally thought of together with the better-known nitrosamines by most scientists, in spite of their distinctly different chemical behavior. In terms of biological effects, there is neither reliable comparative data on their carcinogenicity nor on their formation and incidence in environmental samples. In view of the abundance of amide linkages in the biosphere, one would expect nitrosamides to form from peptide linkages that come into contact with nitrite-nitrous acid or their precursors. One may suspect that their lack of detection in environmental samples might be due to the instability of the nitrosamide linkage.

Generally, nitrosamides I undergo irreversible thermal rearrangements (at temperatures ranging from ambient to ca.100°) to diazo esters III which themselves decompose even faster under these conditions to give the carboxylic esters or acids and the olefins derived from the R' group (*1,2*). The stability of nitrosamides and the final products from III are very much dependent on the nature of the R' group (primary, secondary, tertiary

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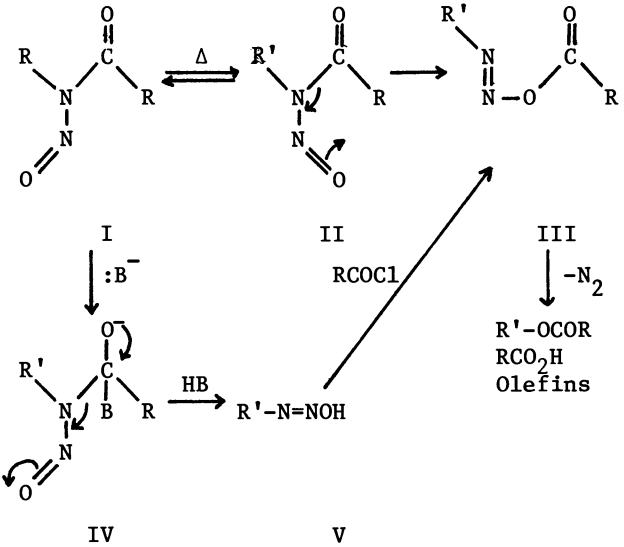
alkyls, phenyl or benzyl groups, etc.) but not so much on the R group. The primary rearrangement step consists of the rotation of the N-NO bond to II and the intramolecular reorganization of the nitrosamide moiety which involved the scission of the N-CO bond to form III (*7,8*). The subsequent fragmentation of diazo esters III is even more sensitive to the nature of the R and R' groups and to the conditions; it occurs by substitution and elimination routes via carbonium ion, free radical or intramolecular migration mechanisms.



Whereas base-induced decomposition of N-nitrosourethanes has been utilized (*9*) as a popular method of generating diazoalkanes, only limited investigations on base treatments of nitrosamides have been reported (*10*). The primary product in the base treatment is assumed, in analogy to better investigate nitrosourethane cases, to be diazo hydroxides V via attack of a base on the carbonyl group as in IV. A diazo hydroxide V has been related to the diazo ester III by a reaction with benzoyl chloride.

Ground State Behavior of Nitrosamides and Nitrosamines

Concurrent with our investigation on nitrosamine photochemistry (*11*), we also initiated an investigation of the ground and excited state chemistry of nitrosamides because of a wide discrepancy in the chemical behavior of these two classes of nitroso compounds. For nitrosamines, the presence of extensive delocalization of the unshared electron pair and the π electrons of the N=O group as in VI and VII has been well supported by i) n.m.r. evidence of the restricted rotation about the N-N bond (*12*), ii) electron diffraction analysis revealing the rather short N-N bond

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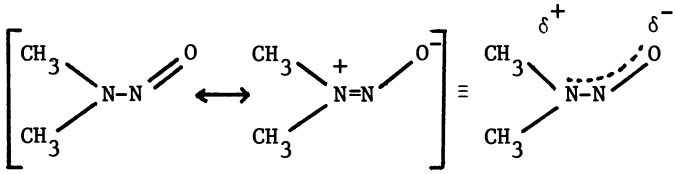
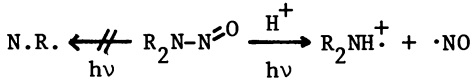
(1.344 Å) for VIII (*13*), iii) theoretical calculations indicating unusually high contributions of the polar resonance form VII (*15*) and iv) association with a proton or metal ion at the nitroso oxygen, the site of the highest electron density (*14*). The structure of nitrosamines is more realistically represented by the resonance hybrid VIII which explains the extraordinary stability of nitrosamines towards acids and bases in contrast to the lability of nitrosamides I under dilute acidic and basic conditions.

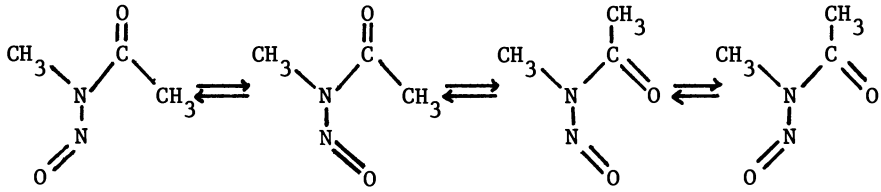


VI VII VIII

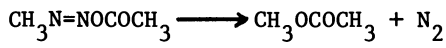


VIII

The n.m.r. spectra of N-nitrosodimethylamine VIII exhibits non-equivalence of the methyl signals at 3.77 and 3.02 p.p.m. at room temperature and the temperature dependent coalescence studies give the energy barrier for the rotation around the partial N-N double bond to be 23.4 Kcal/mol (*12*). In contrast, N-nitroso-N-methylacetamide X shows only two n.m.r. singlets in solution indicating either that the nitrosamide exists as a mixture of rapidly exchanging rotamers X-XIII or that it is frozen in the most stable conformation, probably X in view of the dipole repulsions in other conformations XI-XIII. The nitrosamino group delocalization in X is probably somewhat dampened by the direct attachment of the electron withdrawing C=O group to the amino nitrogen, rendering the unshared electron pair less available for the



X XI XII XIII



IX

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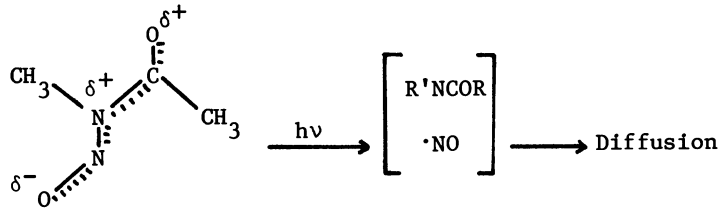

XIV XV

nitroso group conjugation. Similarly, the amide group (N-CO) delocalization in X might be essentially interrupted by the more powerful electron withdrawing nitroso group as suggested by the position of the carbonyl stretching frequency at 1730 cm^{-1}. Thus, nitrosamides might be considered to have the hybrid structure XIV. In view of increased instability with bulkier R' groups, Huisgen has suggested that increasing steric bulk of the R' group in XIV facilitates the change to a conformation, such as XI, which can readily undergo the thermal rearrangement to IX (*7*). These arguments suggest that the ground state nitrosamides should have the conformation X or XIV rather than consist of an equilibrating mixture. Unfortunately, the instability of nitrosamides precludes any possibility of confirming this point by temperature-dependent n.m.r. techniques.

Photochemistry of Nitrosamides

At temperatures low enough to suppress thermal decomposition, a nitrosamide XIV in polar or non-polar solvents is photolytically decomposed to amidyl and nitric oxide radicals (*4,16,17,18*). This is in sharp contrast to *the photostability of nitrosamines in neutral solvents* (including acetic acid) (*11*), although the pattern of photodecomposition is similar to that of nitrosamines in dilute acidic conditions. However, the overall photolysis pattern of nitrosamides is complicated by disproportionation of nitric oxide and existence of a radical pair XV (*20,21,22*).

The primary process following a photoexcitation of nitrosamides XIV is the dissociation of the N-N bond to form a radical pair XV and the ensuing chemical events are the reactions of amidyl and nitric oxide radicals in the paired state or individually in the bulk of solutions. Naturally, secondary reactions, thermal or photolytic, have to be taken into consideration under irradiation conditions (*21*). First of all, the relatively straightforward chemistry of selective excitation in the $n-\pi^*$ transition band (>400 nm) will be discussed, followed by the chemistry of irradiation with a Pyrex filter (>280 nm). As nitric oxide is known to be rather unreactive (*23*), primary chemical processes in the irradiation with >400 nm light under

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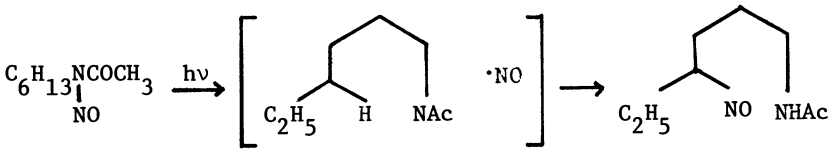
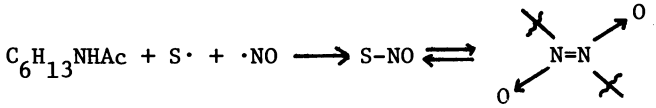
nitrogen are dominated by reactions of the transient amidyl radicals.

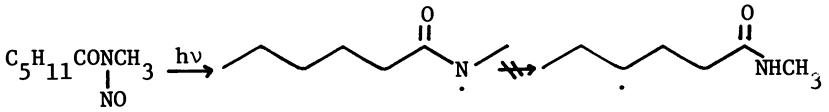


XVI XVII XVIII

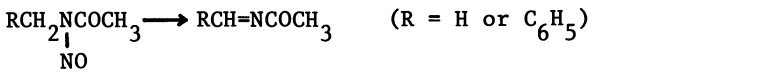
SH



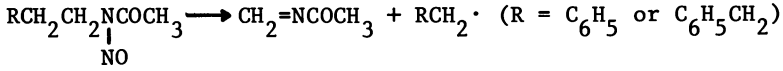
XX XIX



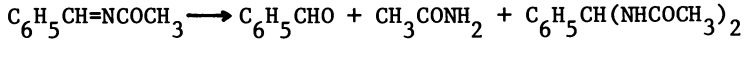
XXI XXII XXIII



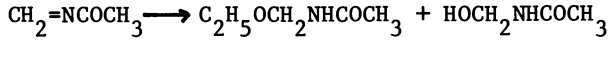
XXIV XXV



XXVI XXVII XXVIII



XXIX XXX



XXVII XXXI XXXII

For amidyl radicals carrying a δ-hydrogen *in the alkyl chain* (e.g., XVII or N-pentyl, N-butyl, N-phenylbutyl analogues) intramolecular abstraction of the δ-hydrogen as in XVII and the collapse to δ-nitroso compound XVIII are very facile and occur preferentially *within the radical pair (vide infra)* over other

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pathways (*20*). Such a specific hydrogen-nitroso group exchange is similar to the mechanism proposed for nitrite photolysis which is commonly known as the Barton reaction (*24*). Alternatively, the amidyl radical in XVII may diffuse away from the pair and performs a similar hydrogen nitroso group exchange intermolecularly with solvent (SH) to afford the corresponding C-nitroso compound XX. The intermolecular H-abstraction can compete favorably in good hydrogen-donating solvents and may dominate the reaction if intramolecular H-abstraction does not operate. The C-nitroso compounds XVIII or XX are generally isolated as the anti-dimer (e.g., XIX) in 50%-60% yields or as the corresponding oxime (e.g., 2-cyclohexenone oxime and 3,5-dimethylbenzaldehyde oxime). The third competing process can be formally represented as β-elimination of amidyl radicals to give N-acylimines XXV and XXVII, the mechanism being not only by a unimolecular but also by a bimolecular reaction, as shown by kinetic studies (vide infra). The β-elimination occurs significantly when either one of the above pathways is blocked either in a poor H-donating solvent or lack of a δ-hydrogen in the alkyl chain and, in particular, when such an elimination is energetically favorable as in XXIV→XXV and XXVI→XXVII. N-Acylimines are susceptible to nucleophilic attacks and generally undergo additions of nucleophiles, hydrolysis and trimerization as shown in XXIX→XXX and XXVII→XXXI→XXXII.

Surprisingly, irradiation of N-nitroso-N-methylhexanamide XXI yields no intramolecular δ-hydrogen nitroso group exchange products indicating the δ-hydrogen abstraction from the acyl chain in XXII is energetically unfavorable in comparison to intermolecular H-abstraction and β-elimination processes; in benzene the latter process dominated to afford N, N', N''-trihexanoylhexahydrotriazine, the trimer of the corresponding acylimine (*16*); a similar result has been observed by Kuhn and the co-workers (*17*). The diminished δ-hydrogen abstraction from the acyl chains is also observed in photolysis of N-bromoamides and is believed to arise from stereochemical controls resulting from a Π electronic configuration of amidyl radicals (*25*).

In contrast to a straightforward and predictable decomposition pattern of photolysis with >400 nm light, irradiation of nitrosamides under nitrogen or helium with a Pyrex filter (>280 nm) is complicated by the formation of oxidized products derived from substrate and solvent, as shown in Table I, such as nitrates XXXIII-XXXV and nitro compound XXXVI, at the expense of the yields of C-nitroso compounds (*19,20*). Subsequently, it is established that secondary photoreactions occur in which the C-nitroso dimer XIX (λ_{max} 280-300 nm) is photolysed to give nitrate XXXIII and N-hexylacetamide in a 1:3 ratio (*21*). The stoichiometry indicates the disproportionation of C-nitroso monomer XVIII to the redox products. The reaction is believed to occur by a primary photodissociation of XVIII to the C-radical and nitric oxide followed by addition of two nitric oxides on XVIII and rearrangement-decomposition as shown below in analogy

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Table I. Photolysis of N-Nitroso-N-hexylacetamide XVI

Conditions	Percentage Yield XIX	XXXIII	A	B	Others (%)
Mesitylene, N_2	24	14	-	53	-†
Mesitylene, O_2	4	53	-	25	XXXV (21), C (10)
Cyclohexane, N_2	26	18	5	48	XXXIV (9), E (-)
Benzene, N_2	37	20	6	33	F (-), G (-)
Benzene, He	45	18	~3	30	H (6), F (-), G (-)
Benzene, O_2	-	60	16	14	F (4), G (4)
Benzene, O_2	-	44	11	22	F (-), G (-)
Benzene*, N_2	58	0	0	42	-†
Benzene*, O_2	0	47	12	26	F (4), G (2)

* These two experiments were run with >400 nm light, but others were run with a Pyrex filter (>280 nm).

† The by-products were not analysed.

$CH_3CH_2CH(NO)(CH_2)_3NHCOCH_3$ (XIX)

$CH_3CH_2CH(ONO_2)(CH_2)_3NHCOCH_3$ (XXXIII)

$CH_3(CH_2)_5N(NO_2)COCH_3$ (A)

$CH_3(CH_2)_5NHCOCH_3$ (B)

$3,6\text{-}(CH_3)_2C_6H_3CH_2ONO_2$ (XXXV)

$3,6\text{-}(CH_3)_2C_6H_3CH{=}NOH$ (C)

$3,6\text{-}(CH_3)_2C_6H_3CHO$ (D)

$(cyclo\text{-}C_6H_{11}NO)_2$ (E)

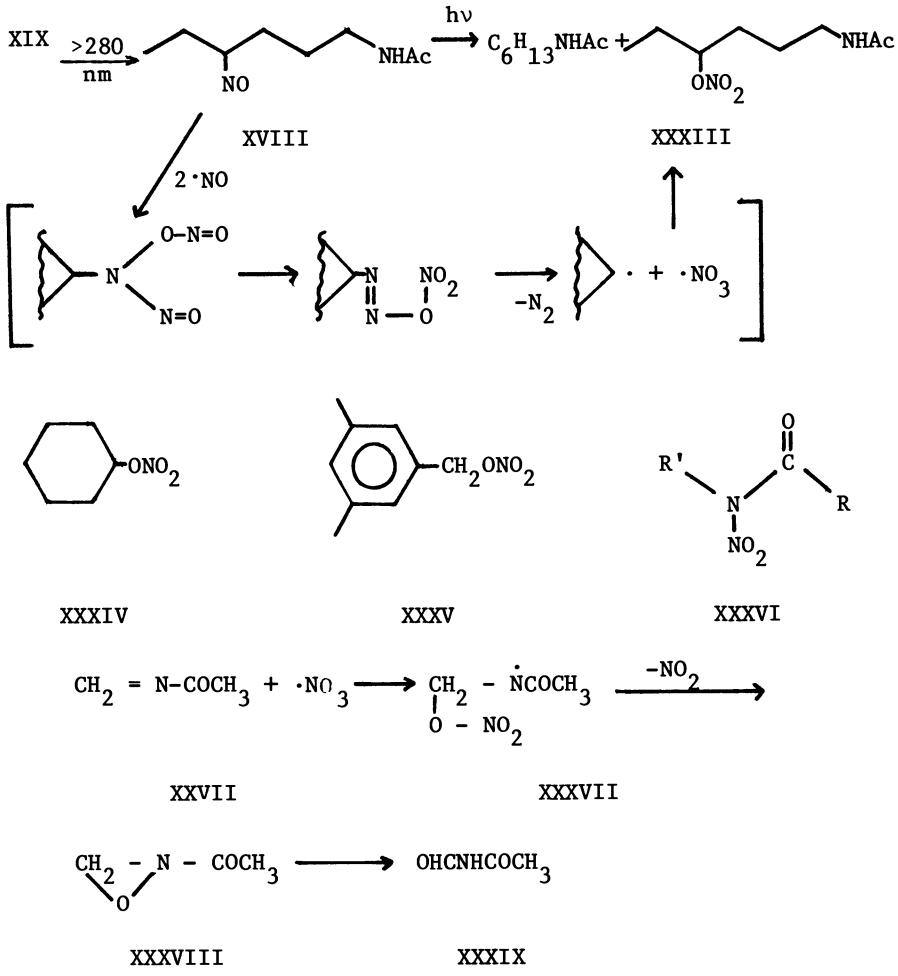
$cyclo\text{-}C_6H_{11}ONO_2$ (XXXIV)

$C_6H_5NO_2$ (F)

$HOC_6H_4NO_2$ (G)

$CH_3(CH_2)_4CH(NACOCH_3)_2$ (H)

In N-Nitrosamines; Anselme, J.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.



with a proposed mechanism (*26,27*). Interaction of $·NO_3$ with ·NO is known to give two $·NO_2$ which might account for the formation of nitramide XXXVI (*23*). Photolysis of nitrosamides XXIV and XXVI in benzene with a Pyrex filter under nitrogen also yields considerable amounts of N-formylacetamide XXXIX which might be formed by $·NO_3$ radical oxidation of XXVII via XXXVII and XXXVIII. That $·NO_3$ and $·NO_2$ radicals are formed in the >280 nm photolysis of nitrosamides is also indicated by the formations of nitrobenzene and nitrophenols when carried out in benzene, as $·NO_2$ and $·NO_3$ are known to attack benzene to give the observed products (*23*).

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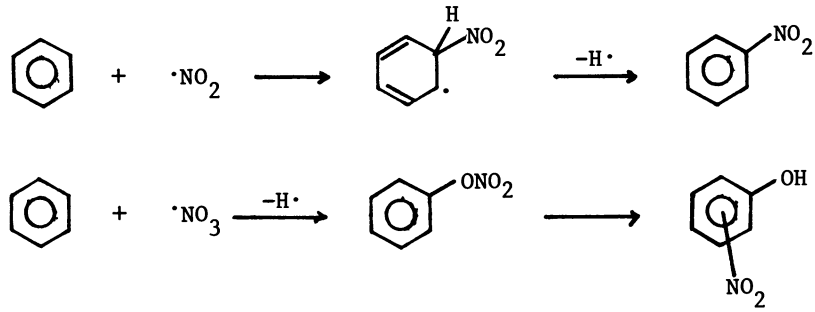

Interaction of externally added nitric oxide with nitrosocyclohexane has been shown to induce the same disproportionation (*26*); a similar reaction between C-radicals and nitric oxide in gas (*28*) or liquid phases (*27,29*) has also been reported. The nitric oxide derived from the C-nitroso compound photolysis (and also that indirectly derived from nitrosamide photolysis with >290 nm light) similarly disproportionates. On the other hand, the nitric oxide generated from nitrosamide photolysis with >400 nm light *does not participate in the disproportionation* and *is obviously different from the ordinary nitric oxide*. Since irradiation of 2-nitroso-2-methylpropane monomer with "red light" (λ ~680 nm) under nitrogen also yields the corresponding nitrate and nitro compounds (*29*), the disproportionation can hardly be ascribed to a "hot" or excited state nitric oxide. We have demonstrated that irradiation of XVI under nitrogen in a nitric oxide saturated benzene solution (opaque up to 380 nm due to NO_2) does not affect the yield of the C-nitroso dimer XXIX, nor does it result in formation of the oxidized product (*4*); this indicates that *an external nitric oxide does not participate in the intramolecular hydrogen nitroso group exchange*, XVII→XVIII. These observations are in agreement with the presence of a tight *radical pair* as shown in XVII.

Irradiation of nitrosamides with either >400 nm or >280 nm light under oxygen gives nitrate or nitro derivatives such as XXXIII-XXXVI without a trace of C-nitroso dimers (Table I) but with a small amount of β-cleavage products such as XLIV and benzaldehyde from XLI. C-nitroso dimer XIX is also cleanly oxidized photolytically to XXIII. The efficient oxidative photolysis of nitrosamides proves that oxygen, instead of quenching excited states of nitrosamides, diverts the radical species to nitrate and nitro compounds. Probable mechanisms are either interception of the C-radical (path a) or the nitric oxide (path b) by oxygen as shown. Recently, pernitrite linkage as in XL has been shown to rearrange to a nitrate group rapidly (*30*). In view of extensive formation of nitrobenzene and nitrophenols when oxidative photolysis is carried out in benzene, path b might be favored. However, since alkyl radicals are commonly scavenged by oxygen with diffusion controlled rates (*31,32*), the pathways are

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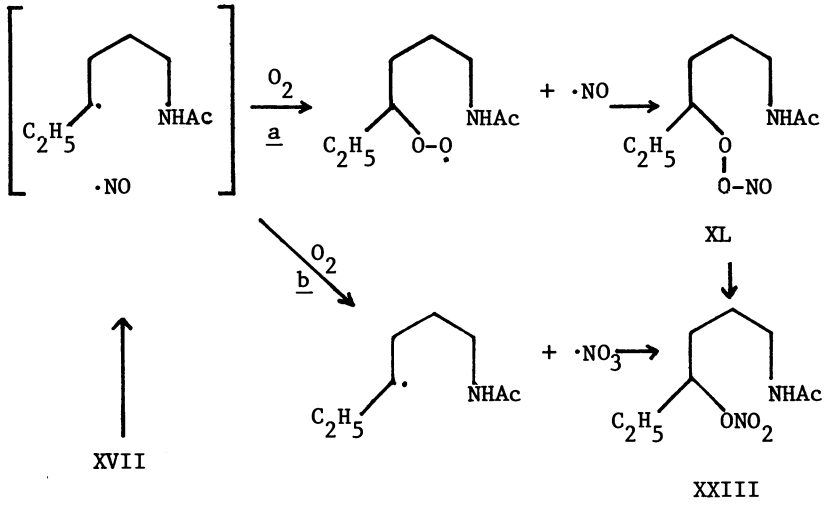
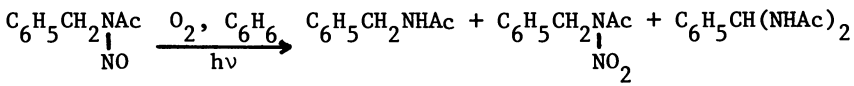


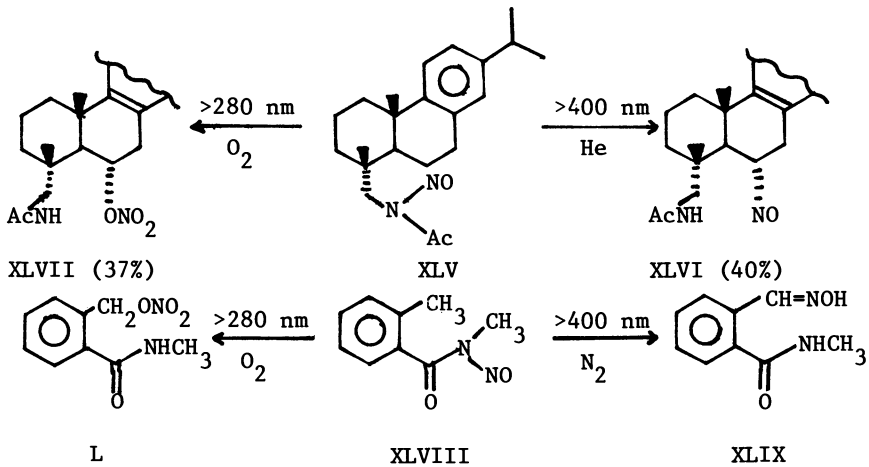


XLI XLII (52%) XLIII (6%) XLIV (10%)

+ C_6H_5CHO (13%) + $C_6H_5NO_2$ + $HOC_6H_4NO_2$

indistinguishable.

Synthetic applications of these non-oxidative and oxidative photolyses of nitrosamides are illustrated below (*22*). Photolysis of N-nitroso-N-actylabietylamine XLV in benzene is a



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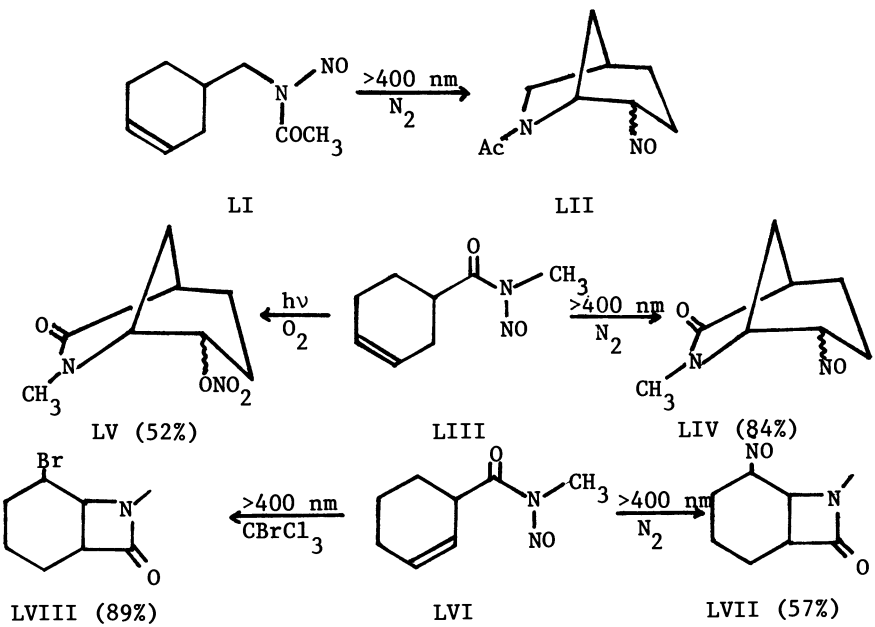
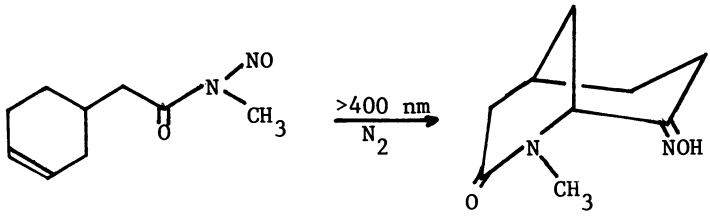
straightforward case but under oxygen a small amount of the anti-dimer of 6α-nitroso compound XLVI is also isolated in addition to 6α-nitrate XLVII. The clean functionalization at the 6α-position reflects stereochemical controls (severe hindrance of the approach of ·NO, O_2 or ·NO_3 radicals from the β-face) on the C-radical reaction but not on the intramolecular H-abstraction in the amidyl radical. Photolysis of N-nitroso-N-methyl-o-toluamide XLVIII proceeds smoothly to give excellent yields of oxime XLIX and nitrate L under the respective conditions. However, both XLIX and L can undergo rearrangements readily during isolation owing to proximity of the functional groups. In contrast to the failure of the intramolecular H-abstraction in XXII, the efficiency shown in the present case might arise from a favorable orientation of reacting centers held rigidly coplanar by a benzene ring. The ease of the intramolecular H-abstraction should be related to the electronic configurations of the amidyl radical center (Σ or Π) and stereochemical effects on orbital overlap in the transition state (*25*).

We have demonstrated that intermolecularly, amidyl radicals preferentially abstract an allylic hydrogen rather than add to a π bond of olefins such as cyclohexene and 1,3-pentadiene (*33*). This reactivity pattern is completely reversed in intramolecular reactions as shown in the following examples of alkenyl nitrosamide photolysis. In every case, the amidyl radicals generated from photolysis preferentially attack the π bonds intramolecu-

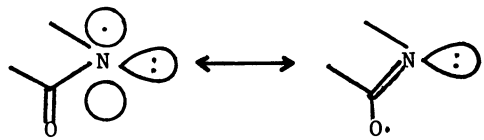




LIX LX (25%)

larly under nitrogen to give the C-nitroso compounds LII, LIV, LVII and LX that are isolated as the corresponding oximes and ketones. Formations of nitrates LV under oxygen and bromide LVIII in the presence of bromotrichloromethane show the variations of synthesis with radical trapping agents. Intramolecular H-abstraction at δ-position might have occurred but only to very minor extents even in photolysis of LIX. It is significant that π bond attacks occur exclusively at the C-5 in the cases of LI and LIII, at the C-4 in LVI and at the C-6 in LIX. Furthermore, intramolecular attacks of π bonds occur at the acyl just as readily as at the alkyl chains, in contrast to severe discrimination of the δ-hydrogen abstraction from the acyl chain in XXII. It should be noted that β-elimination has not been observed in these photolyses.

Slow intermolecular additions of amidyl radicals to π bonds might suggest the presence of repulsive forces between electron clouds as the reacting species approach. However, inversion of reactivity from abstraction to addition in intramolecular amidyl radical attacks might be explained by a better orbital overlap between the p-orbitals than that between the p- and the hydrogen s-orbitals. It is generally accepted that the efficiency of intramolecular reactions is controlled by the extent of orbital overlap available between two reacting centers in the transition state. Orbital overlap requirements are, in turn, controlled by the stereochemistry of the amidyl radicals as well as by the ground state electronic configuration of the nitrogen radical center (*34*). From e.s.r. (*35*) and ^{13}C n.m.r. CINDP (*36*) studies, the ground states of amidyl radicals are more likely to possess the Π electronic configuration rather than Σ.



Π

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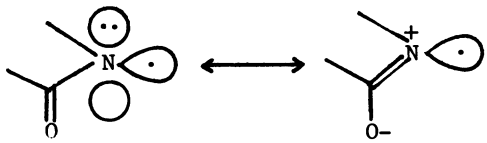

Σ

Stereochemical probes of intramolecular H-abstraction leads to a conclusion not in contradiction with the Π electronic configuration and also reveals that simple amidyl radicals react exclusively as the N-radical but not the O-radical (*25*). Scarcity of data in this area does not allow a definitive discussion on stereochemical controls of amidyl radical reactions that are being studied in our group.

Kinetic Studies

To understand the mechanisms of the nitrosamide decomposition and particularly the behavior of amidyl radicals, kinetic studies were carried out by static irradiation and flash excitation techniques (*4,37*). Quantum yields of the photodecomposition of nitrosamides in benzene vary 0.8~2.5 depending on the R and R' groups, and increase nonlinearly in the concentration range of 10^{-3} - 10^{-1}M. These variations are expected since the major pathway in each nitrosamide is different. Since no detectable emission is observed in photoexcitation of N-nitroso-N-methylacetamide X, the energy level of its lowest singlet state is approximated by the longest absorption peak at 426 nm (E_S 67 Kcal/mol). The energy level of the lowest triplet state is determined by quenching of aromatic triplets generated by flash photolysis by X. The E_T is calculated to be 51 Kcal/mol from the quenching rate constant (k_q) of the pyrene triplet state.

The photodecompositions of nitrosamides can be sensitized by either singlet or triplet sensitizers, such as naphthalene, anthracene and benzophenone (*4*). In photolysis of X, trans-stilbene, an ideal quencher with E_S=89 and E_T=49 Kcal/mol, neither quenches the decomposition nor itself isomerized. Together with the lack of oxygen quenching of the photodecomposition of X, they strongly indicate that, in direct photolysis, the nitrosamide decomposes from the singlet state to form the radicals. This process occurs so rapidly that neither oxygen can quench it nor intersystem crossing can compete with it, resulting in no formation of the triplet state of X.

Direct flash excitation (>400 nm) or the triplet-acetone-sensitization of nitrosamide X in degassed water or benzene solutions gives the amidyl radical transient exhibiting λ_{max} 335-350 nm. This transient is not observed with undegassed solution of X, indicating that oxygen has intercepted the precursor of the amidyl radical at least, with the diffusion controlled rate

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constant, k_{diff} 5 X 10^9 M^{-1} sec^{-1}. Taking the concentration of dissolved oxygen in benzene at 25° as 4.5 X 10^{-2} M (*38*), the upper limit of the lifetime of the precursor (see Eq. 2) can be approximated to be ~10^{-9} sec. The kinetic decay of the transient in benzene solution follows a first-order pattern from which the observed rate constants (k_{obsd}) and lifetime (τ_{obsd}) are obtained from the slopes. The lifetime varies proportionally to the concentration of X at the range of 10^{-3} - 10^{-2} M from 5-50 microseconds. The straight line plot of k_{obsd} *vs* concentration (as in Figure 1) proves that the amidyl radical reacts with X (as in Eq. 5) with a calculated bimolecular rate constant of k_2 = 3.96 (± 0.05) X 10^6 M^{-1} sec^{-1}, and decays by itself (as in Eq. 6) with a unimolecular rate constant of k_1 = 2.09 (± 0.02) X 10^4 sec^{-1} at a high dilution. Both reactions are believed to generate AcN=CH_2 (vide supra).

Flash excitation of nitrosamide X in the presence of hydrocarbon substrates reveals that not only the amidyl radical transient but also the precursor abstract a hydrogen from these substrates, as shown by the decrement patterns of the observed pseudo-first-order rate constants as well as those of the initial optical density of the transient (see Table II). The bimolecular rate constants of the amidyl radical H-abstraction (Eq. 7) from cyclohexane and trans-piperylene are determined to be 1.85 X 10^4 and 2.84 X 10^5 M^{-1} sec^{-1}, respectively. Using Stern-Volmer plots and the lifetime of 10^{-9} sec for the precursor, the rate constants of hydrogen abstraction of the precursor (Eq. 4) from cyclohexane and trans-piperylene are calculated to be 1.87 X 10^8 and 4.04 X 10^9 M^{-1} sec^{-1}. These rate constants are extraordinarily fast, suggesting a highly energetic state of the reacting species. This species is tentatively assigned to either vibronically or singlet excited state nitrosamide in which the N-N bond is stretched to possess radical characteristics. Physically, the bonding electrons are still within the interacting distance and the amido portion resembles the Σ electronic configuration.

Flash excitation of N-nitroso-N-methylhexanamide XXI in benzene registers a similar transient which is assigned to the corresponding radical. Similar studies of N-nitroso-N-pentylacetamide afford a weak transient in the 340 nm region which has lifetimes too short to be analysed with confidence. In comparison to the kinetic pattern of XXI and X, flash photolysis of the latter is in consonance with the occurrence of fast intramolecular δ-hydrogen abstraction from the alkyl chain, which destroys the amidyl radical (and/or its precursor) rapidly. Considering this together with the existence of a tight radical pair, it is suggested that the δ-hydrogen nitroso group exchange reaction as shown (XVI→XVII→XVIII) occurs primarily by an intramolecular process without being affected by addition of an external nitric oxide. The detailed mechanistic interpretation of nitrosamide X is summarized below. Conspicuously missing is the intersystem

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Table II. Reaction of the N-Methylacetamido Radical with Hydrocarbon Substrates.

[Substrate] M	Lifetime, μsec	k_{obsd} X 10^{-6}, sec^{-1}	Initial OD	(OD)o/ (OD)
(1) With Cyclohexane; Concentration of X is 0.001M				
0.00	40.2	0.0249	0.0241	1.00
0.93	16.1	0.0619	0.0212	1.14
1.30	15.9	0.0631	0.0205	1.18
1.85	15.6	0.0643	0.0190	1.27
2.78	11.2	0.0892	0.0153	1.58
4.63	8.6	0.1165	0.0135	1.79
6.48	6.2	0.1608	0.0103	2.34
8.33	5.3	0.1875	0.0099	2.43
(2) With trans-1,3-pentadiene; Concentration of X is 0.004M				
0.00	24.1	0.0415	0.0183	1.00
0.01	22.5	0.0444	0.0176	1.04
0.04	18.5	0.0542	0.0164	1.13
0.10	15.1	0.0664	0.0131	1.40

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$$Ac(CH_3)N\text{-}NO \xrightarrow{h\nu} {}^1[Ac(CH_3)N\text{----}NO] \quad [1]$$

$${}^1[Ac(CH_3)N\text{---}NO] \longrightarrow [AcNCH_3 \cdot NO] \longrightarrow Ac\dot{N}CH_3 + \cdot NO \quad [2]$$

$${}^1Ac(CH_3)N\text{---}NO] + O_2 \longrightarrow (Ac(CH_3)N\text{----}ONO_2) \quad [3]$$

$$[Ac(CH_3)N\text{--}NO] + R\text{-}H \longrightarrow AcNHCH_3 + R\cdot + \cdot NO \quad [4]$$

$$Ac\dot{N}CH_3 + Ac(CH_3)NNO \longrightarrow AcN = CH_2 + AcNHCH_3 + \cdot NO \quad [5]$$

$$Ac\dot{N}CH_3 \xrightarrow[NO]{} AcN = CH_2 + (HNO) \quad [6]$$

$$Ac\dot{N}CH_3 + RH \longrightarrow AcNHCH_3 + R\cdot \quad [7]$$

$$Ac(CH_3)N\text{-}NO + {}^3Sens \longrightarrow {}^3[Ac(CH_3)N\text{-}NO] + {}^0Sens \quad [8]$$

$${}^3[Ac(CH_3)N\text{-}NO] \longrightarrow Ac\dot{N}CH_3 + \cdot NO \quad [9]$$

crossing of the singlet excited state of X to the triplet state which can be generated by a triplet sensitization as in Eq. 8 and which also dissociates to give the amidyl and nitric oxide radicals (Eq. 9).

Primary Photoprocesses of Nitrosamides

Why does thermolysis of nitrosamide cause the scission of the N-CO bond whereas photolysis results in scission of the N-NO bond? The former might be understood in terms of an inbalance in electronegativities of the substituents (vide supra) which weaken the N-CO bond; vibrational energies, therefore, cause the scission of the weakest bond. Intuitively, the dissociation of the N-NO bond with a short bond length due to a partial double bond character is least likely to occur particularly from a singlet state of nitrosamide X, and might be suspected to occur via an intermediate structure more amenable for the N-NO scission. For most photochemical reactions of non-rigid polyatomic molecules with many degrees of freedom, chemical events following excitation are mostly buried in the radiationless transition (electronic and vibrational relaxations) during which a large quantity of electronic energy is converted into some forms of nuclear motions (*39,40*); in other words, a new ground state species may be generated from the transition. Though our knowledge in this respect is awfully inadequate, conceptually an energy diagram such as Figure 2 might be used to trace the chemical events. This has led us to investigate low temperature photolysis of nitrosamide X in the hope of capturing and identifying an intermediate species following a primary excitation (*41*).

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Irradiation of nitrosamide X in EtOH-MeOH (9:1 ratio) solution at -150° with monochromatic light of 405 nm gives a frozen intermediate exhibiting u.v. absorptions at 454, 432, 414 and 396 nm as shown in Figure 3 and i.r. peaks at 1712 and 1555 cm^{-1} for which either conformers XII (or XIII) or the valence tautomer stabilized by hydrogen bonding LXI might be assigned. The intermediate decomposes irreversibly when it absorbs a second photon in the 432-454 nm region at -150°, but relaxes to X completely on warming to -90°. A similar irradiation of X at 25° causes irreversible dissociation to the amidyl radical and nitric oxide. Mechanistically, these results indicate that the singlet excited state of nitrosamide X relaxes to the ground state intermediate and that the biphotonic process at -150° shifts to the monophotonic process at 25°.

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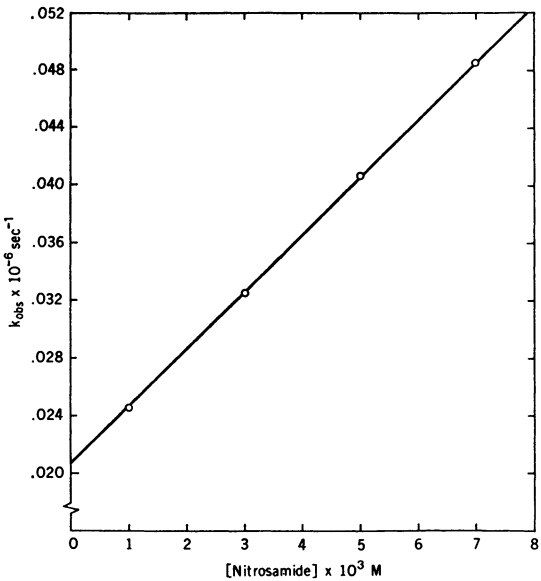

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Figure 1. Observed decay rate of the N-*methylacetamido radical as a function of concentrations of X in benzene*

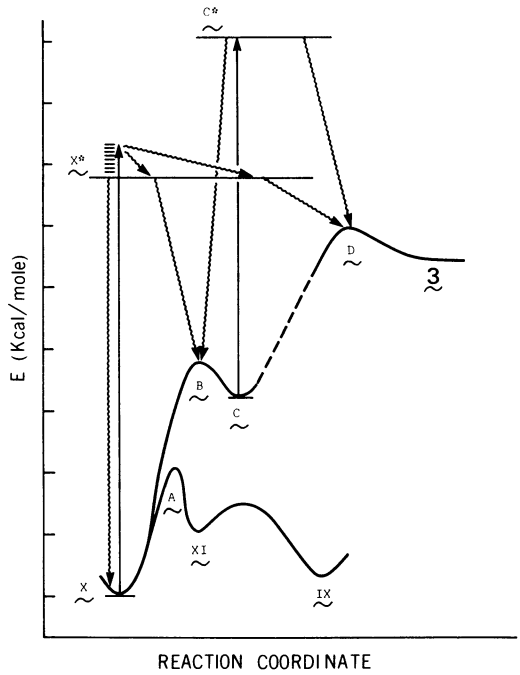

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Figure 2. Potential energy diagram for nitrosamide photoreaction. The letters with asterisks represent the lowest singlet excited states.

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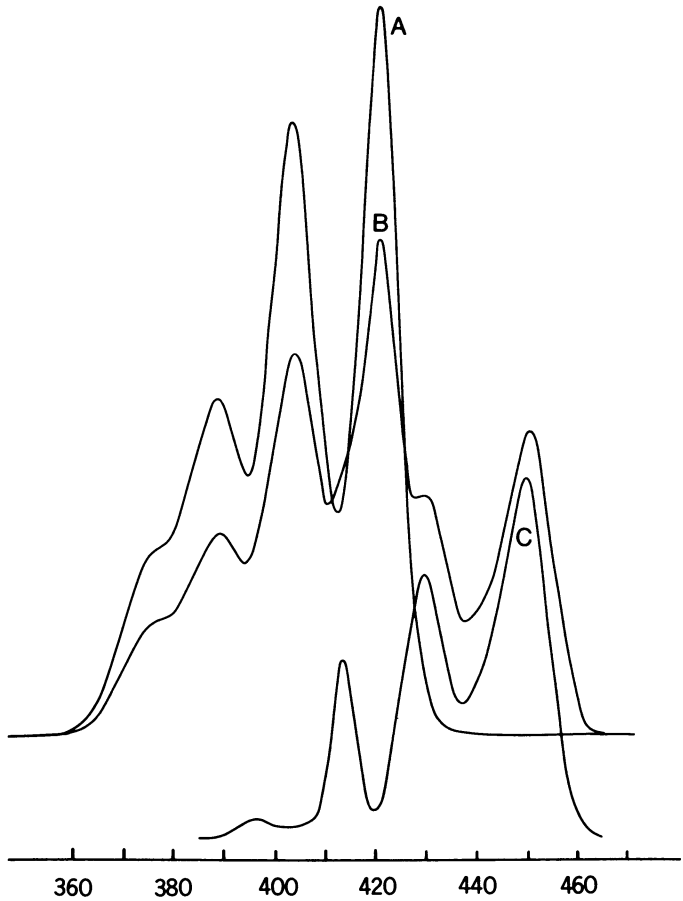

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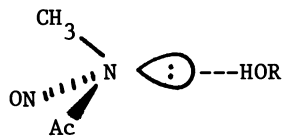
Figure 3. UV spectra of the photolysis of X (~7 × 10⁻³M) in EtOH–MeOH (9:1) mixture at −150° with 405 nm narrow band light. The spectra were monitored with a Cary 14 spectrophotometer: (A) before the irradiation; (B) after 28 min irradiation; (C) UV absorption curve of the intermediate isolated from spectrum B with a Du Pont 310 Curve resolver.

Chemical intuition (*42*) as well as a mathematical model (*40*) based on resonance interaction have predicted that a radiationless transition is biased in favor of the nearest potential energy hypersurface of the lower electronic state provided the two states are not far apart along the reaction coordinate. In the energy diagram Figure 2, the chemical events can be conceptually inferred that the excited state X^*, after electronic transition, preferentially traverses to the nearest minimum C via B rather than to A and XI during losses of vibrational energy at -150°. Absorption of the second photon takes a similar path as in C→C*→D. At 25°, the X*, having progressed farther along the reaction coordinate with an additional vibronic energy, shifts to the hypersurface of dissociation mode D. Thermolysis of X dissipates the vibronic energy by the lowest activation path as in X→A→XI→IX and could never activate X to the state C. In short, photoexcitation enables X to reach *a ground state of a higher energy minimum* C *(intermediate) through electronic transition and non-vertical relaxation processes along vibronic ladders*.

While the structure of the intermediate cannot be decided merely from the available spectroscopic data, the valence tautomer LXI is favored on the following grounds. Firstly, if



LXI

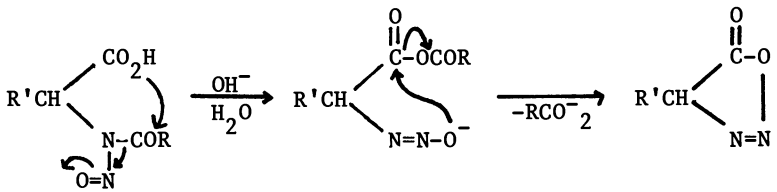
rotamers XII and XIII are involved, some rearrangement might be observed since in the process of rotations, the molecule should have a certain residence time in rotamer XI. Secondly, the observed i.r. stretching frequencies for C=O and N=O groups in the intermediate bear more resemblance to the unconjugated ones than to those of rotamers XII and XIII that are expected to show higher frequencies owing to dipole repulsion. Thirdly, for N-N bond scission to occur, the double bond character should be nullified by interruption of the nitrosamide conjugation. Such a situation might exist in LXI but probably not in rotamer XII and XIII. Evidence that the intermediate is probably the valence tautomer LXI is suggested by the photolysis of X in a hydrocarbon mixture where no hydrogen bond formation is possible. In this irradiation at -150°, the u.v. absorptions of X decrease very little and only feeble absorptions of the intermediate are observed, indicating a limited transformation to the intermediate.

Chemistry of Nitrosamides Derived from α-Amino Acids

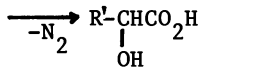
Chemistry of the nitroso derivatives of simple peptides and

N-acylamino acids does not appear to have been studied previously. In solution, N-acyl-N-nitroso-α-amino acids are moderately stable to weak bases, such as triethylamine or sodium carbonate, but are decomposed rapidly at 0° to expel nitrogen on addition of sodium hydroxide (*43*). For example, treatment of N-nitroso-N-benzoyl-D,L-phenyl-alanine LXIIb with an aqueous sodium hydroxide solution at 0° gives benzoic acid and 1-hydroxy-3-phenylpropanoic acid LXVa in a 93% yield. The facile base-catalysed formations of α-hydroxy acids LXV are a general reaction and probably occur by intramolecular attack as shown in LXII→LXIII→LXIV. Oxadiazolone LXIV can decompose by various possible pathways to give LXV among which the carbonium ion pathway is least likely.

In neutral solvent, nitrosamides LXII readily undergo photolysis to give N-acylimines as the primary products which are susceptible to nucleophilic attack due to the conjugated C=N-CO group (*44*). Photolysis of nitrosamide LXIIa in methanol gives LXVII (45%) and LXVIII (23%) obviously derived from the nucleophilic addition of methanol to and proton migration of LXVI, respectively. Photolysis of LXIIa in ether gives LXVIII (27%)



LXII LXIII LXIV

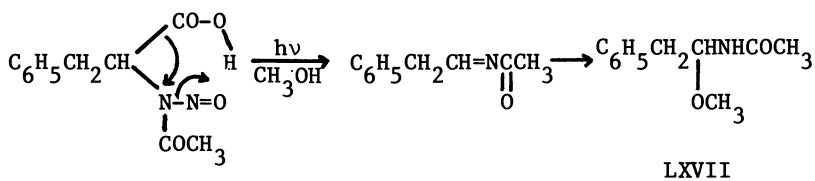


LXV

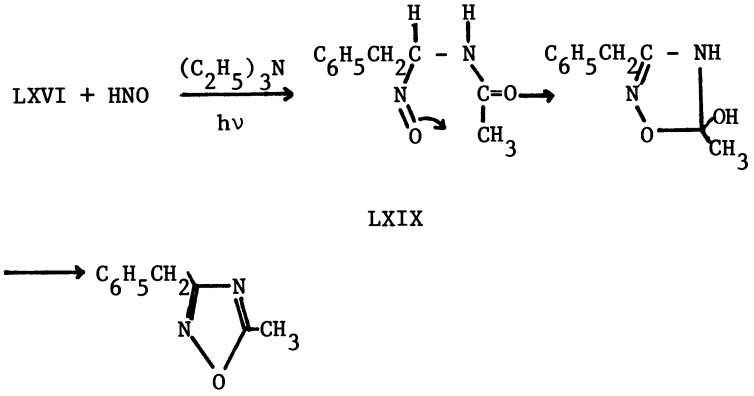
a: $R=CH_3$, $R'=C_6H_5CH_2^-$

b: $R=C_6H_5$, $R'=C_6H_5CH_2^-$

c: $R=CH_3$, $R'=(CH_3)_2CHCH_2^-$



LXIIa LXVI LXVII + $C_6H_5CH=CHNHCOCH_3$ LXVIII



LXIX

and the parent amide (38%). It is tempting to suggest that the primary photoprocess involves a concerted elimination of HNO and CO_2, since even if the amidyl radical is formed, the conjugated elimination of CO_2 is very facile and the net result will be indistinguishable from the concerted elimination.

The lack of the formation of α-oximino amides from these nitrosamide photolyses is in good contrast to an excellent yield of $CH_3NHCH{=}NOH$ from the photolysis of N-nitrososarcosine (*43*) and suggests that NHO is not quite nucleophilic. Photolysis of LXIIa in the presence of a weak base, such as triethylamine, in acetonitoril gives oxadiazole LXX (63%) which is assumed to form by the mechanism shown. The base presumably renders HNO more nucleophilic to give adduct LXIX which undergoes cyclization and dehydration to LXX. The yields of LXX are very sensitive to structures of amino acids and the concentration of base. Photolysis of LXIIc in acetonitrile in the presence of three equivalents of triethylamine give the corresponding oxadiazole in an excellent yield.

Acknowledgement

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Abstract

Nitrosamides and nitrosamines exhibit considerably different reaction patterns when thermolysed or photolysed. The differences are discussed in relation to the ground state electronic configurations of these two classes of nitroso compounds. At -150°,

a singlet excited state of a nitrosamide undergoes radiationless decay to afford a thermally unstable isomer which is readily dissociated by a second photon to the amidyl and nitric oxide radicals. These are generated as a radical pair by monophotonic process at room temperature. The amidyl radicals undergo intramolecular H-abstraction from the alkyl chain, intermolecular H-abstraction or β-elimination; nitric oxide, if diffuses out of the pair, may participate in disproportionation of C-nitroso compounds. The studies of nitrosamide photolysis by flash excitation provide kinetic rate constants of these amidyl reactions, from which the β-elimination is shown to arise from both the bimolecular reaction of the amidyl radical with the nitrosamide and a unimolecular elimination. Base catalysed decomposition and photolysis in the presence and the absence of a weak base of N-nitroso-N-acyl-α-amino acids are described.

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Stereochemical Effects on *N*-Nitrosamine Chemistry

R. E. LYLE, H. M. FRIBUSH, and S. SINGH—Chemistry Department, North Texas State University, Denton, TX 76203

J. E. SAAVEDRA—Department of Chemistry, University of New Hampshire, Durham, NH 03824

G. G. LYLE—Department of Pharmacology, Texas College of Osteopathic Medicine, Denton, TX 76203

R. BARTON, S. YODER, and M. K. JACOBSON—Chemistry Department, Biochemistry Division, North Texas State University/Texas State College of Osteopathic Medicine, Denton, TX 76203

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The electronic arrangement of the nitrosamine function provides an interesting stereochemical consequence which may be as significant in the biochemistry of the metabolism of these compounds as in the chemistry of nitrosamines (1). The electronic interaction and the electronic structure of the nitrosamine is uncertain; however, there are some very definite conclusions that one can draw from the physical properties. The atoms shown in Fig. 1 are all planar, and only the hydrogen or substituents on the alpha carbons are not in the plane of the atoms of the nitrosamine function. The N-O bond of this nitrosamine function is not linear but is angular and therefore leads to Z and E isomerism about the N-N bond. It is clear that the delocalization of the electrons from the amino nitrogen, as indicated in Fig. 1, explains the large rotational barrier to torsion about the N-N bond (2) and leads to a highly polar molecule with a high electron density on oxygen (3). The properties of nitrosamines are consistent with this idea for these compounds have large dipole moments, are soluble in relatively polar solvents (3), and alkylate on oxygen (4).

An unique result of the electronic structure of the nitrosamine function is that it can apparently stabilize a positive or negative charge at the carbon adjacent to the amino nitrogen in a manner similar to the benzylic system. The stereoelectronic control of electrophilic substitutions at the α-position, to be discussed later, requires an orbital interaction of the nitrosamine function with the anionic electrons (5,6,7). The reactivity of electronegative α-substituents has been explained by an electronic stabilization of an electron-deficient carbon, carbonium ion, by the nitrosamine function (8,9).

The large energy barrier to conversion of the Z to E isomer is spoken of casually with respect to rotation about the N-N bond, but it could equally well be considered an inversion of the nitroso nitrogen. The diastereomeric nature of these two forms of unsymmetrically substituted nitrosamines is clearly illustra-

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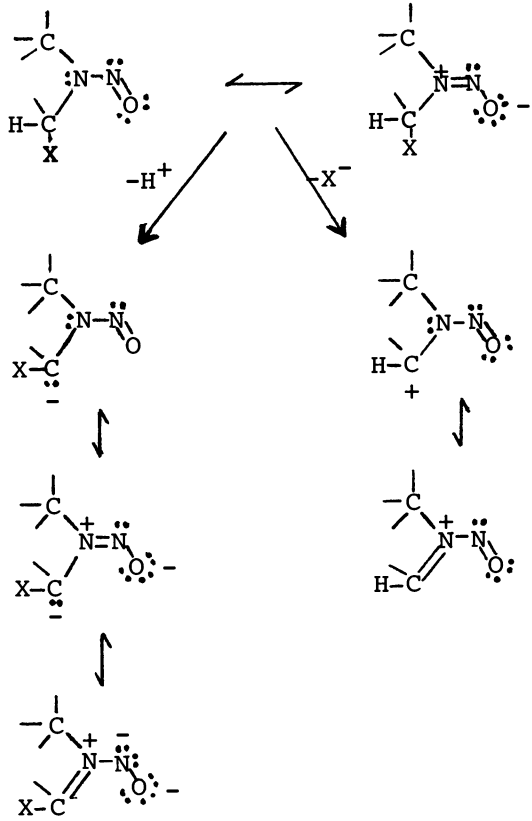

Figure 1. Resonance forms of nitrosamines and the α-carbocations and α-carbanions showing the possible delocalization of charge. The counterions are not shown; however, they may be highly significant in this delocalization.

ted in the difference in physical and chemical properties of the isomers. A recent illustration of the energy barrier to interconversion of these isomeric nitrosamine forms and a clear indication of the stereochemistry of the nitrosamine function were given by the resolution of N-nitrosoisonipecotic acid (I)(Fig. 2) (10). The stereochemistry of the nitroso group destroys the plane of symmetry through the isonipecotic acid molecule and produces, therefore, two enantiomeric forms. By measuring the rate of racemization of these resolved forms, one arrives at an energy barrier of about 23 kcal/mole (10). Another consequence of the stereochemistry of the nitroso function is the fact that the steric interference on the oxygen of the nitroso group provides sufficient destabilization to force an α-methyl group into a conformation perpendicular to the nitrosamine function (6,11). This is most easily demonstrated by the study of the conformation of such compounds as cis-1-nitroso-2,6-dimethylpiperidine (II), in which both of the α-methyls are forced into a diaxial conformation.

Structural Effects on Activity

It has been well established that the nitrosamines in most cases are not carcinogenic in themselves but require oxidative metabolism by some mixed function oxidase to convert them into a carcinogenic form (12). Oxidation has been shown to occur at various sites; however, there is strong evidence that hydroxylation at the α-carbon gives a proximate carcinogen by biological metabolism (13). The ultimate carcinogen is then a carbocation or a diazonium ion which would cause the carcinogenic event (Fig. 3). In order to provide a structure-activity relationship for predicting carcinogenicity and, in particular, a relationship in which the significance of the stereochemistry of the nitrosamine function might be of importance, the several steps from the introduction of the nitrosamine to the reaction of the ultimate carcinogen must be considered (Fig. 4). There are three major stages at which a structural effect might be important. One is the transport of the nitrosamine from the site of introduction to the site of metabolism. The second step is the conversion of the inactive nitrosamine into the active metabolite, which is a reaction with a mixed function oxidase. Finally, the proximate carcinogen that is formed by metabolism will undergo conversion to an electrophile, and the way in which this electrophile undergoes reaction may also be a function of its structure. Of these three steps, the first would seem to be relatively unaffected by stereochemistry. It will primarily be related to the partition between aqueous and lipid layers. It is this form of activity which is most easily correlated by a Hansch relationship based on the transport of the biologically active substrate (13).

The enzymatic metabolism of the pre-carcinogen, i.e., conversion to an electrophile and its reaction with nucleic acid,

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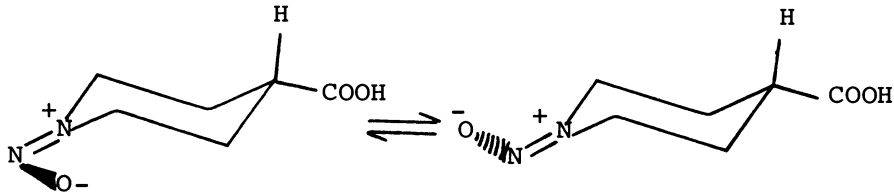

Figure 2. Determination of the energy barrier to rotation about the N—N bond of the nitrosamine by enantiomeric interconversion

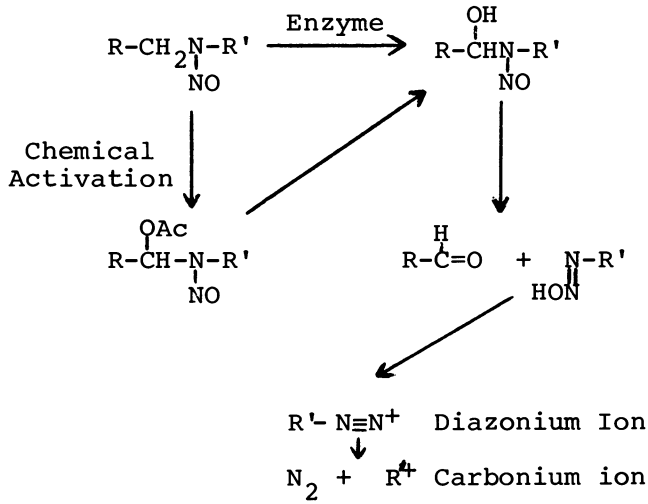

Figure 3. Proposed relationship between metabolic and chemically activated nitrosamines for producing an ultimate carcinogen or mutagen

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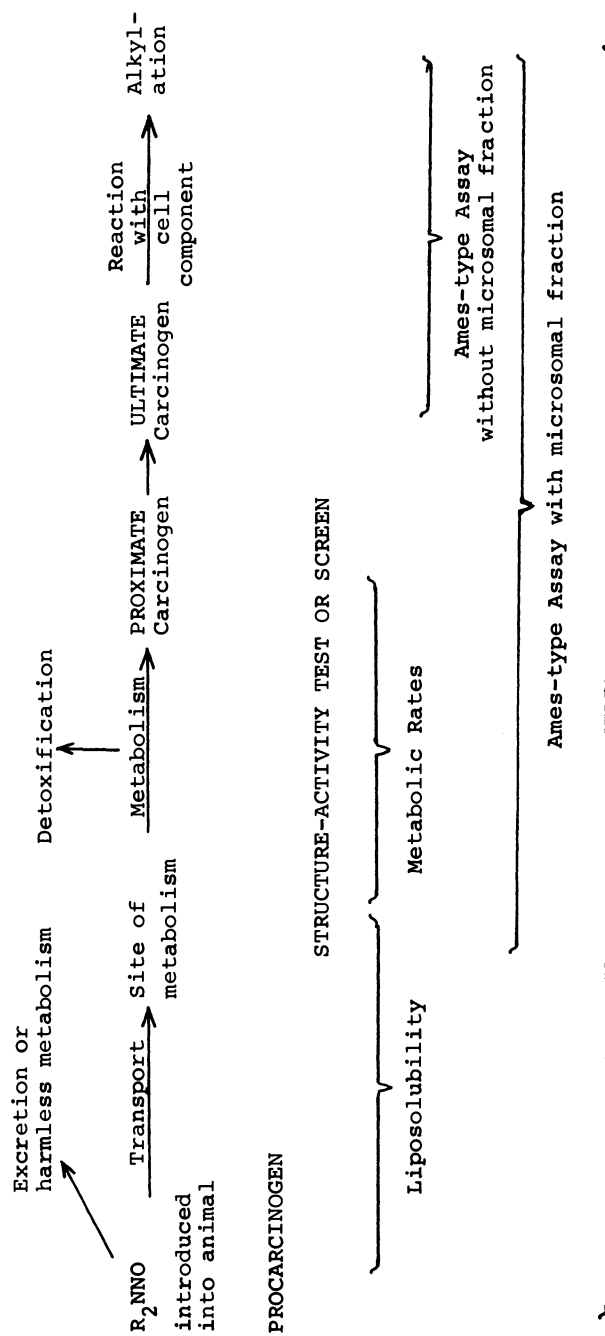

Figure 4. Illustration of the significance of a structure–activity relationship using in vitro metabolism, Ames assay, partition coefficients, or whole animal screens to develop the structure–activity relationship

would be highly affected by structure, and undoubtedly could be affected by the stereochemistry of the nitrosamine group. It might be important at this point to note that the means by which one measures the activity will affect the significance of the structure-activity effects (Fig. 4). For example, the Hansch relationship is largely a measure of the transport properties of the substrate. The Ames assay, and similar bacterial mutagenicity tests are largely a measure of the reactivity of the electrophile with the cell component, for transport and oxidative metabolism are relatively insignificant in this kind of activity. The addition of microsomal extracts enables the metabolism of nitrosamines to be measured in the Ames assay and will reveal the effects of structure (14). Finally, whole animal screens are the measure of all three of these steps and are probably the best models for the predictability of carcinogenicity.

The mechanism by which the mixed function oxidase converts a nitrosamine into a carcinogenic compound is not clearly understood (15). These enzymic oxidations can occur at the alpha, beta, gamma, or omega position of an alkyl chain attached to the nitrosamine function. Regardless of whether or not these other oxidations are significant in carcinogenicity, it has been demonstrated that hydroxylation at the alpha position does indeed produce a carcinogen (13). The discussion which follows will be limited to those factors relative to oxidation at the α-position.

In attempting to relate the biological oxidation of the nitrosamines with a chemical property of the stereochemical forms of nitrosamines, the acidity of the adjacent C-H bonds (5,6,7,16) seemed to provide the most promising property, for the relative acidities of the α-hydrogens are affected by the nitrosamine stereochemistry. Deuterium exchange alpha to a nitrosamine function occurred most rapidly with that hydrogen which was pro-Z and attached by a bond perpendicular with the nitrosamine atoms. This shows that the most acidic hydrogen is that which is Z and perpendicular to the nitrosamine group as illustrated with the conformationally biased system, 1-nitroso-4-*t*-butylpiperidine (III). The four hydrogens (Fig. 5) are non-identical as a result of the stereochemistry of the nitrosamine function. The nuclear magnetic resonance spectrum of this compound shows four distinct signals for the four different α-hydrogens. Thus it is possible to identify that hydrogen which undergoes deuterium exchange most rapidly. In this case, it was the hydrogen which was pro-Z and axial which underwent deuterium exchange first, followed by a slower exchange with the other axial hydrogen (7). No matter how long the deuterium exchange reaction experiment was continued, there was no exchange with the equatorial protons. It is clear that there is stereoelectronic control of the electrophilic or acidic reaction at the α-position.

If the mixed function oxidase reacts by a mechanism involving an electrophilic or radical substitution of oxygen, it is probable that this reaction too would be controlled by the same

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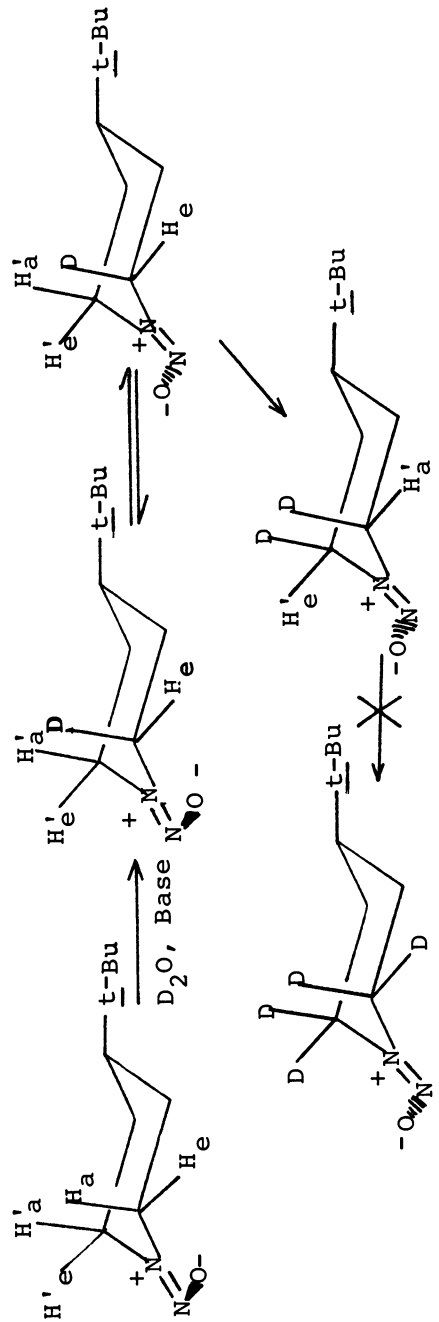

Figure 5. Relative acidity of α-protons as determined by the conformational effect of deuterium exchange on a conformationally biased system

stereoelectronic factors that control the deuterium exchange reaction. This stereoelectronic control of electrophilic substitutions of nitrosamines has been extended by a number of research groups to a variety of systems which have a cisoid arrangement of four atoms which have overlapping p-orbitals containing six electrons (16). This includes such systems as dianions of oximes (16), the monoanion of an oxime ether (17), and the anion of disubstituted hydrazones (18). The recent work of Jung (19), extending the earlier results by Enders and Corey (20), has shown clearly that in the case of the dimethylhydrazone, the anion is formed by the removal of the pro-E hydrogen if there is a difference in steric interference to approach to the α-hydrogens. In the case of the nitrosamines, Fraser (6) has shown that there is a slight difference in acidity between syn and anti protons which are adjacent to the nitrosamine function. Unfortunately, there has not been any clear evidence as to whether or not removal of the anti proton is followed by a rapid inversion of the nitrosamine stereochemistry as has been demonstrated with the dimethylhydrazones (19).

Utilizing information obtained from Lijinsky concerning the carcinogenicity of a series of nitrosamines in a rat screen (21), the relative carcinogenicity of a variety of cyclic systems was related to the presence of an α-pro-Z proton perpendicular to the plane of the nitrosamine function, such as the α-syn-axial proton. The list of some of the compounds forming the basis of this correlation is shown in Fig. 6. For example, 1-nitroso-2-methylpiperidine, which exists in three conformations, has one conformation in which a syn-axial hydrogen is present. The compound should, therefore, be carcinogenic. The rat screen showed 1-nitroso-2-methylpiperidine to be a carcinogen which was weaker than N-nitrosopiperidine, but a definite carcinogen. The other examples also correlate with the postulate that an α-axial hydrogen, i.e., the more acidic hydrogen, is essential for carcinogenicity.

An exception to this postulate is dibenzylnitrosamine which undergoes a rapid exchange with deuterium oxide in base but has been shown in whole animal tests to be non-carcinogenic (22). This compound was found to be non-mutagenic following a procedure of Ames (14) using S. typhimurium tester strains TA100 and TA1535. The lack of activity of this compound, even though there are acidic hydrogens, suggests that at one of the three stages which are shown in Fig. 4, the structure was interfering with transport, metabolism, or reactivity at the electrophilic stage.

It is probable that the transport properties are relatively insignificant in the Ames assay, for the only possibility would be the transport of the agent through the cell wall of the bacterium. Thus it is probable that the dibenzylnitrosamine either does not undergo metabolism or, once the metabolism has occurred, the electrophile does not behave in the usual fashion to give a carcinogenic event. Some evidence has been reported which would

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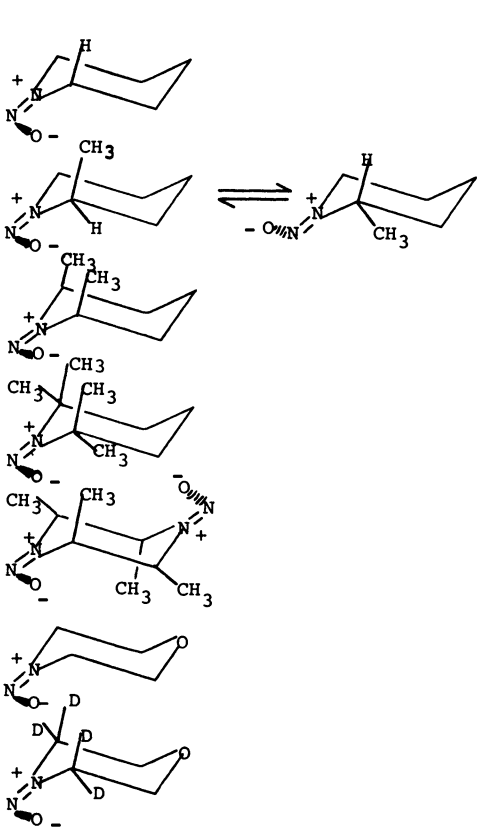

Figure 6. Correlation of the biological activity and the availability of a syn-*axial-proton in cyclic six-membered nitrosamines*

suggest that the stability of the carbocation formed as the ultimate carcinogen was important in determining the lack of carcinogenicity or mutagenicity of these compounds. As shown in Fig. 7, acetoxymethylbenzylnitrosamine (IV) was reported to be non-mutagenic (23). More recent literature shows that acetoxymethyl-t-butylnitrosamine (V) also is not mutagenic (24). Hydrolysis of the esters and decomposition of the α-hydroxynitrosamine give carbonium ions of some stability, the t-butyl or benzyl carbocations. This increased stability of the t-butyl carbocation was offered by the authors (24) as the explanation for the lack of mutagenicity, and a similar relationship could be proposed for the benzyl carbocation.

The chemical activation of dibenzylnitrosamine (VI), that is the chemical conversion to the α-acetoxy derivative, would give an intermediate which on hydrolysis would yield the benzylcarbocation. Thus on the basis of the previous report (23,24) the α-acetoxydibenzylnitrosamine (VII) should be a non-mutagenic, non-carcinogenic nitrosamine.

Dibenzylnitrosamine (VIII) was chemically converted to the α-acetoxy derivative (VII). Following the procedure of Seebach (1), the carboxylation of the anion of dibenzylnitrosamine (VIII) occurred in good yield affording N-nitroso-N-benzylphenylglycine (IX), which on treatment with lead tetraacetate following the procedure of Saavedra (25), gave α-acetoxydibenzylnitrosamine (VII) (Fig. 8) (26). The properties of this synthetic metabolite were consistent with the assigned structure (Fig. 8). This sequence of reactions was used to prepare a number of α-acetoxy nitrosamines and provides a convenient means for synthesis of these pseudo metabolites starting with the parent nitrosamine.

It was surprising and exciting to find that the α-acetoxydibenzylnitrosamine (VII) was a potent mutagen in the Ames test, and since activation by a microsomal fraction was not required for this activity, VII may be an analog of a metabolite (26). The introduction of an activating system, such as an S-9 microsomal fraction, led to a decrease in the potency of VII as a mutagen. Although this was not consistent with the results reported by Camus, Wiessler, Molaveille, and Bartsch (24) for acetoxymethyl-t-butylnitrosamine (V), it did seem to be consistent with the postulate that hydrolysis of the acetoxy group would be catalyzed by the activating S-9 system through an esterase activity. This in turn would deactivate the α-acetoxydibenzylnitrosamine (VII) before it would act as an electrophile with nucleic acids.

The spectral data provide information about the structure of α-acetoxydibenzylnitrosamine (VII). The high frequency (1780 cm^{-1}) for the carbonyl group in the infrared spectrum (Fig. 9) is consistent with this structure (8) and the introduction of the chiral center at the benzylic position causes the methylene of the other benzylic substituent to be diastereotopic and appear in the nmr spectrum as an AB quartet. The center of the quartet

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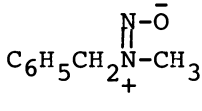
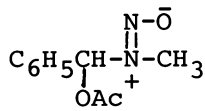
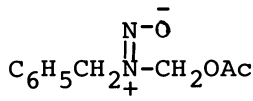


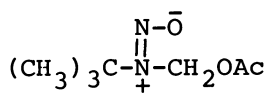
Esophageal Carcinogen



Mutagen



Non-Mutagen



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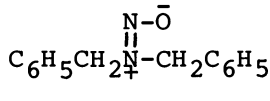
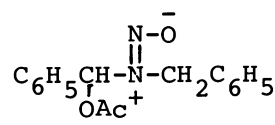
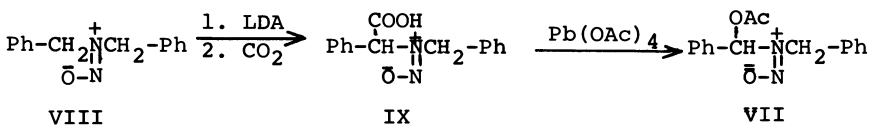


Non-Mutagen



Mutagen

Figure 7. Effect of structure on the mutagenicity of α-acetoxydialkylnitrosamines



VIII IX VII

Figure 8. Synthesis of α-acetoxydibenzylnitrosamine (VII)

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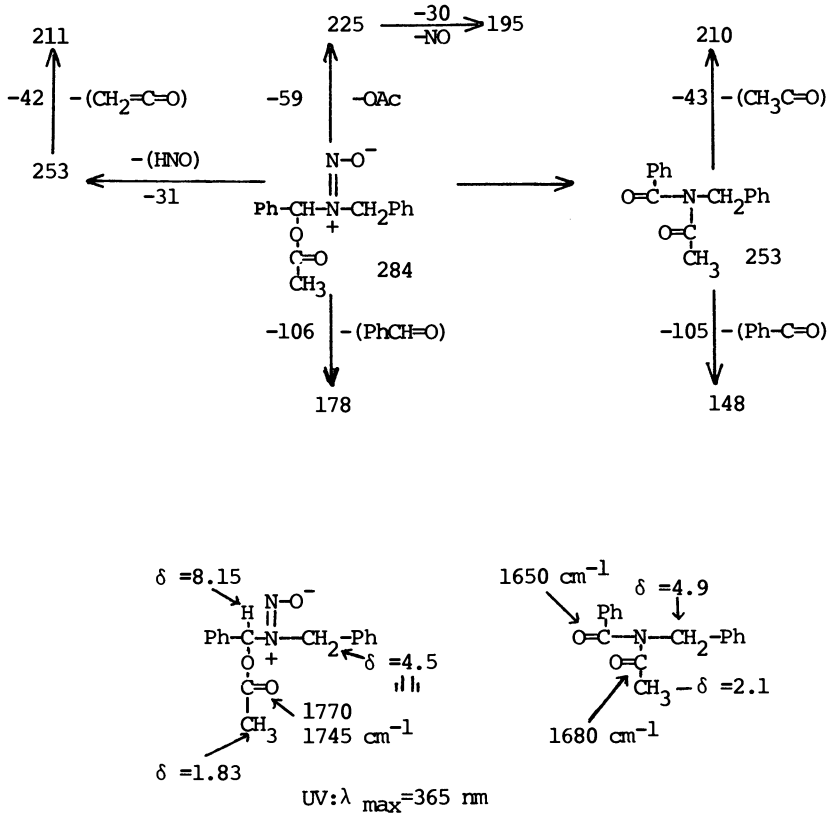

Figure 9. Summary of the spectral data on α-acetoxydibenzylnitrosamine (VII)

(4.5 ppm) is at the same chemical shift as the high field methylene in dibenzylnitrosamine (VIII). This suggests that a single isomer, the E-isomer, of α-acetoxydibenzylnitrosamine (VII) is present. The mass spectrum (Fig. 9) of this compound showed the parent ion at m/e 284, but in addition a very interesting fragmentation peak appeared at P-31 (m/e 253) which appears to result from the loss of HNO (27).

In the synthesis of α-acetoxydibenzylnitrosamine (VII) a significant amount of N-acetyl-N-benzylbenzamide (X) was found in the mixture. Heating α-acetoxydibenzylnitrosamine (VII) in chloroform led to decomposition which ultimately produced N-acetyl-N-benzylbenzamide (X) (Fig. 9). This conversion suggests that the loss of HNO occurs rapidly and thermally to produce the enol acetate (XI) shown in Fig. 10. This enol acetate (XI) would be a potent acylating agent which, undoubtedly, causes intermolecular acylation to form X. XI may be of significance in the mutagenic event resulting from the α-acetoxydibenzylnitrosamine (VII). If this is the case, these α-acetoxy derivatives may not be good models for the metabolites of nitrosamines. The carcinogenesis or mutagenesis of these compounds may result, not from alkylation, as appears to be the case in natural activation, but by acylation.

Induced Circular Dichroism with Nitrosamines

The importance of the stereochemistry of the nitrosamine function in the mutagenicity and carcinogenicity of these compounds has not been demonstrated and yet there is considerable indirect evidence that such a stereochemical dependence may be important. It is evident that the metabolism can be related to a stereochemical factor for unsymmetrical nitrosamines. These, of course, give either of two oxygenated metabolites, and there is evidence that only one of these is mutagenic in some cases (23, 24). Thus, the metabolism may be at least regiospecific, if not stereospecific, relative to the nitrosamine function.

In order to have a better understanding of the stereochemical arrangement of the nitrosamine function a technique which would be sensitive to the interaction of the nitrosamine group with its surroundings was investigated. Induced circular dichroism seemed to provide such a physical property. An achiral nitrosamine would show no Cotton effect or CD curve even though this function shows $n \rightarrow \pi^*$ electronic excitation in the 300-400 nm region. An alcohol which is achiral but has no absorption in the ultraviolet region also would show no Cotton effect or CD curve in this wavelength range. If, however, a complex forms between these chiral-transparent and achiral-UV-absorbing compounds, then an induced Cotton effect should be produced. It should be possible to detect any complex between an achiral nitrosamine and a chiral medium, solvent or biological activating-site, by observing an induced circular dichroism. One of the strongest assoc-

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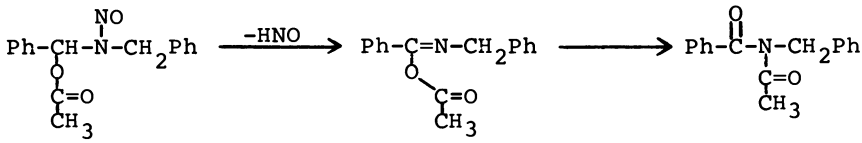

Figure 10. Possible route for the formation of N*-acetyl-*N*-benzylbenzamide (X) from the decomposition of α-acetoxydibenzylnitrosamine (VII)*

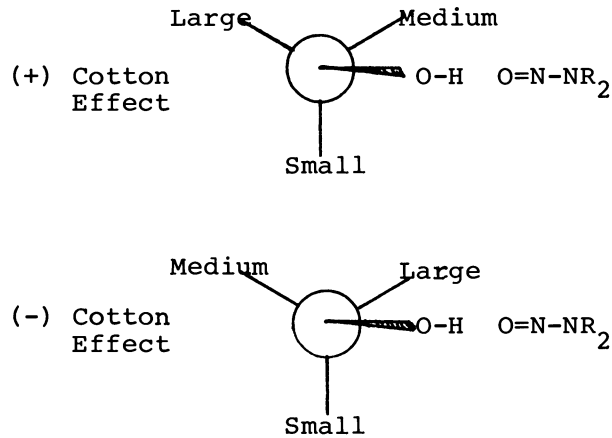

Figure 11. Correlation of the induced Cotton Effect with nitrosamines and chiral alcohols and the configuration of the alcohol

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iations by which the substrate might be complexed with the medium is by way of a hydrogen bond (28). The site of hydrogen bonding between an alcohol and the nitrosamine function is not clear. There is evidence for bonding at the oxygen, the nitroso nitrogen, or the amino nitrogen. The resonance structures suggest that the oxygen would be the predominant site of bonding (4), and it is this position which is supported by the evidence to be presented. The energy barrier to rotation or inversion of the nitroso function has been shown to be reduced by an increase in acidity which supports hydrogen bonding at the amino nitrogen. Similarly, the reversal of the nitrosation reaction also suggests that proton attacks the amino nitrogen prior to loss of the nitroso function (3).

The initial studies (29) of induced circular dichroism were made with the achiral cis-2,6-dimethyl-1-nitrosopiperidine (II) and a series of cyclic alcohols which have large differences in steric size of the groups attached to the carbinol carbon. The CD curve of ℓ-menthol (XII) with cis-2,6-dimethyl-1-nitroso-piperidine (II) in isooctane showed a positive Cotton effect and similar curves were obtained with several cyclic alcohols having the same configuration at the carbinol carbon (Table 1). The opposite configuration of the carbinol carbon gave the enantiomeric Cotton effect. The acyclic 2-octanols did not give observable Cotton effects, probably due to the similarity in size of the methyl and methylene groups and the rotational freedom of the acyclic system. The results in Table 1 can be summarized with the empirical model relating the sign of the induced Cotton effect with the configuration of the carbinols (Fig. 11).

cis-2,6-Dimethyl-1-nitrosopiperidine (II) is a racemic mixture whose isomerism results from the geometrical arrangement of the nitroso group. This was not the source of the Cotton effect since identical results are obtained with N-nitroso-pyrrolidine (XIII).

The fact that there was no great difference in the magnitude of the induced circular dichroism curve of cis-2,6-dimethyl-1-nitrosopiperidine (II) and 1-nitrosopyrrolidine (XIII), suggests that there is no great steric interference introduced by the two axial methyl groups in the cis-2,6-dimethyl-1-nitrosopiperidine (II). This rules out the possibility of hydrogen bonding at the amino nitrogen because there would be a significant difference in the ease of hydrogen bonding of ℓ-menthol (XII) with cis-2,6-dimethyl-1-nitrosopiperidine and with N-nitrosopyrrolidine. The Cotton effect observed between the cis-2,6-dimethyl-1-nitroso-piperidine and ℓ-menthol was found to depend in magnitude on the concentration of both the nitrosamine and the menthol. The concentration of the nitrosamine could not be varied over a very wide range of values because of the ultraviolet absorption of the nitrosamine. The alcohol could be varied over a wide range of concentrations and over this range there was a direct proportionality of the magnitude of the Cotton effect with concentration.

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Table 1. The induced circular dichroism curves with achiral nitrosamines and chiral alcohols in isooctane.

NITROSAMINE / CHIRAL ALCOHOL	1-Nitroso-2,6-dimethyl-piperidine (II)		1-Nitroso-pyrrolidine(XIII)		Dibenzyl-nitrosamine (VIII)	
	Sign	λ(nm)	Sign	λ(nm)	Sign	λ(nm)
R-Menthol	+	358	+	355 (365)	+	355 (365)
S-Menthol	-	356	-	350		
R-Pino-campheol	+	360				
S-Pino-campheol	-	358				
R-Isopulegol	+	355 (365) (380)				
S-Isomenthol	-	360 (370)				

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The generality of the induced circular dichroism with the three nitrosamines and four alcohols (Table 1) indicates that this technique will provide a method for studying interactions of nitrosamines with biological systems such as enzymes. This method furnishes a highly sensitive procedure for detecting an interaction of nitrosamines with any chiral hydrogen bonding donor.

Acknowledgement

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4

Chemistry of α-Substituted *N*-Nitrosamines

M. WIESSLER

Institute of Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, F.R.G.

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Although the mechanism of the activation of dialkylnitrosamines seems to be elucidated (1,2,3), nothing is known about the species whether it be α-hydroxydialkylnitrosamines, diazotates (4,5), monoalkylnitrosamines (6,7) or another as yet undefined molecule which is responsible for the alkylation of nucleic acids and proteins (8) if indeed alkylation is responsible for tumor induction. The purpose of our investigations is to learn about the chemical behavior of the intermediates discussed above, namely to attempt to establish any relation between the chemical reactivity of such species and their biological acitivities.

The present work will first discuss our efforts to synthesize α-functionalized dialkyl N-nitrosamines. The second part will report on the hydrolysis of α-acetoxy N-nitrosamines and the consequenses thereof. Finally the attempted synthesis of α-phosphates of N-nitrosamines and their relationship to the thermal behavior of α-functionalized N-nitrosamines will be discussed.

α-Functionalized N-Nitrosamines

Since 1971, we have been engaged in the synthesis of α-substituted dialkyl N-nitrosamines, namely α-hydroxy N-nitrosamines. Because of the presumed high reactivity of these alcohols, it seemed desirable to have them in a stabilized form, e.g. as esters. In 1970 Franck and his group reported a method to convert tertiary amines into dialkylnitrosamines in the presence of 2-nitropropane, cuprous ion and oxygen (9). If the amine is substituted with different alkyl groups, these groups are cleaved statistically (Eq. 1).

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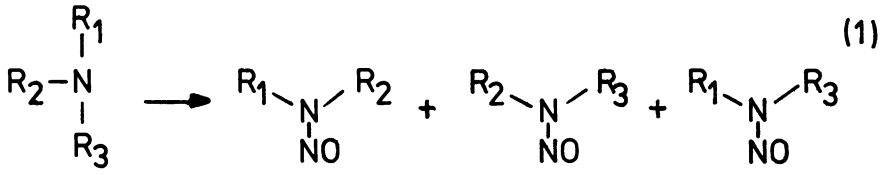
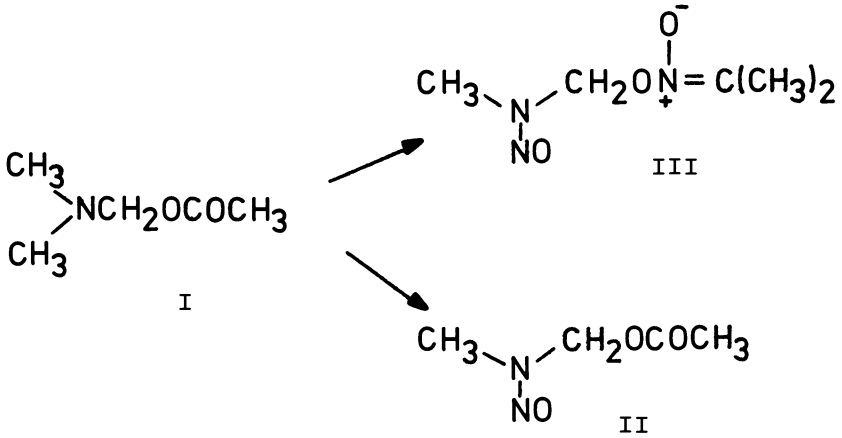


(1)

Application of the Franck reaction to acetoxymethyl dimethylamine I synthesized by Böhme (10),gave a compound identified as the nitronate ester III of hydroxymethyl methylnitrosamine in 20% yield.



III

I

II

In spite of variations in temperature and concentration, there was no evidence for the presence of the α-acetate II.At that time, we have no explanation for the formation of this nitronate ester although we may now have a possible rationalization which will be discussed later.

In 1972, Eiter and his group reported the synthesis of α-alkoxy dialkyl N-nitrosamines (11),which can be obtained easily in 20-50 g quantities. This synthetic scheme works well when formaldehyde was used. In those cases when higher aliphatic aldehydes are used (e.g. acetaldehyde), the yields decreased to 3-5%. The α -alkoxy dialkylnitrosamines always contained the trimeric paraldehyde as impurity. When acetaldehyde and

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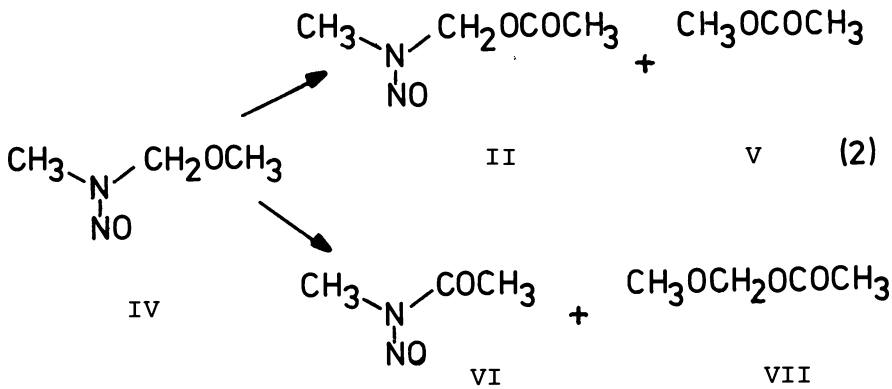
ethylamine are reacted at -30° and the resulting imine is used without further purification, it is possible to isolate the α-ethers without impurities in 5% yield.

Although the conditions used to cleave the ether linkage are vigorous, we decided to attempt this reaction on the α-alkoxy N-nitrosamines. The cleavage of ethers with acetic anhydride in the presence of Lewis acids is well-known in the literature (12,13,14). Reaction of α-methoxy dimethylnitrosamine IV with Ac_2O in the presence of BF_3 etherate at 60° resulted in the cleavage not only of the C-O bond but also the N-C bond (15,16).



The α-acetate of DMN II could be isolated in 10-15% yield after purification. Variations of the Lewis acid used failed to improve the yields of II. All the other cleavage products (Eq. 2) could be isolated except the N-methyl-N-nitrosoacetamide VI. It is well-known from the work of Huisgen and others (4,5,17,18,19) that N-nitrosamides are unstable and rearrange to esters, in our case to V. This was indicated by the high yield (50-60%) of the methyl ester V in contrast to II, the coupled cleavage product. This conclusion was confirmed by the reaction of VIII under analogous conditions which produced V and the corresponding ethyl ester IX as the final product of the rearrangement of X (Eq. 3).

These results were obtained by gas chromatographic analysis of the reaction products.

The use of acetyl chloride instead of acetic anhydride without Lewis acid for the cleavage of the ether bond at 10 to 15° allowed the isolation of the chloromethyl methylnitrosamine XI in 15-20% yields after two distillations (15,16).

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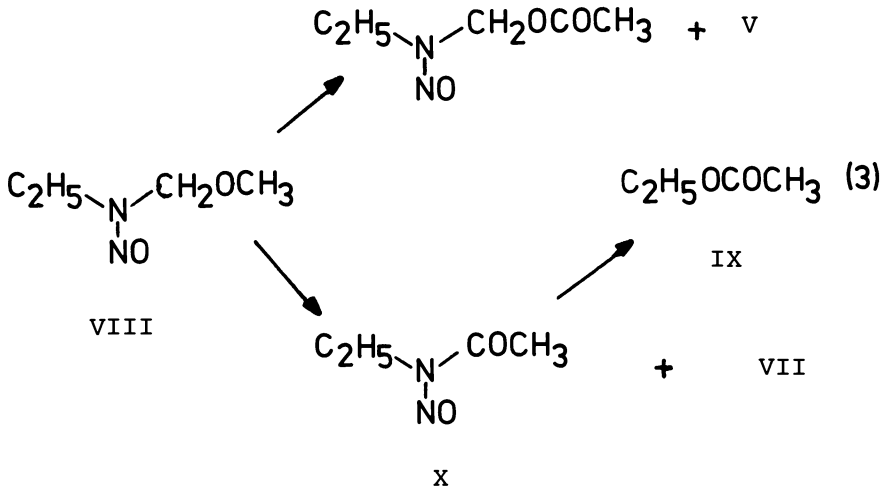
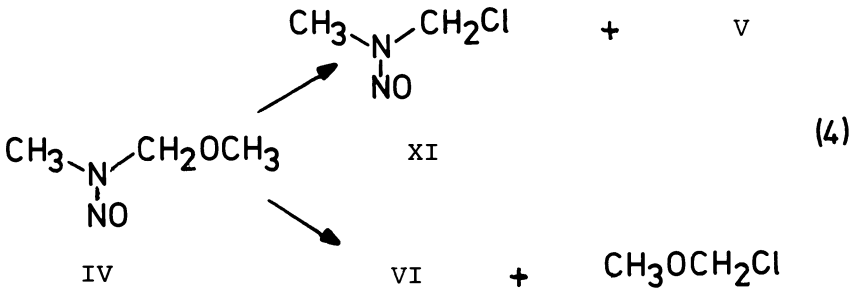
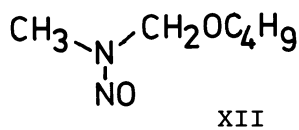


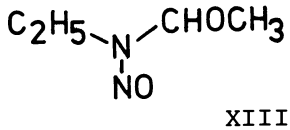
Again all cleavage products could be isolated, except VI; there was no indication that II was formed in this reaction (Eq. 4).



Phosphorus oxychloride (instead of acetyl chloride) was also used with the butyl ether XII (instead of IV) (20), because XI could be separated more easily from the by-products, e.g. tributylphosphate.



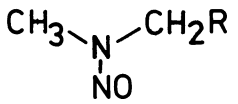
XII



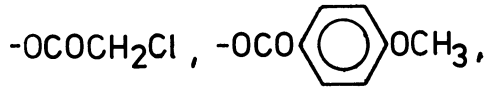
XIII

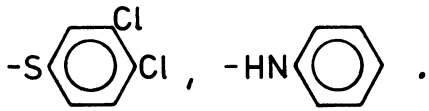
α -Chlorodimethylnitrosamine XI proved to be of great value in the synthesis of other α -substituted dimethylnitrosamines by nucleophilic displacement of chloride (Table 1)

TABLE 1 α-Functionalized Dimethylnitrosamines by Nucleophilic Displacement of Chloromethyl methylnitrosamine XI

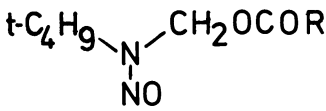


R = $-OCOC_3H_7$, $-OCOC_{17}H_{33}$,



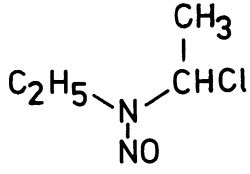


However, these synthetic procedures were useful only in the preparation of primary α-acetoxymethyl alkylnitrosamines and α-chloromethyl alkylnitrosamines. The cleavage of secondary ethers, such as XIII with acetic anhydride or acetyl chloride was so vigorous that no characterizable substances could be isolated. In view of this problem, we looked for another synthetic route which would allow the isolation of secondary derivatives. The first experiment tried, namely that of nitrosyl chloride with N-methylene t-butylamine at -30° was successful (21). The addition of NOCl at this temperature was so rapid that it was complete in a few minutes. Addition of silver acetate gave the corresponding acetoxy derivative XIV in 45% yield.



XIV R = CH_3

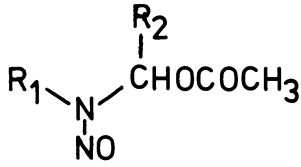
XV R = C_6H_4-p-NO_2



XVI

A series of α-acetates and α-p-nitrobenzoates was prepared by this method in moderate yields (Table 2).

TABLE 2 α-Acetates by Addition of NOCl to Imines followed by Reaction with Silver Acetate (21,22)



R_1	R_2	Yield (%)	Bp (°C) /mm Hg
CH_3	H	30	0.3/56
C_2H_5	H	30	0.3/56
n-C_3H_7	H	30	0.2/55
n-C_4H_9	H	35	0.01/56
i-C_3H_7	H	30	0.1/47
2-C_4H_9	H	35	0.1/60
t-C_4H_9	H	35	0.05/58
c-C_6H_{11}	H	30	0.1/90
CH_3	CH_3	38	0.3/47
C_2H_5	CH_3	52	0.1/42
i-C_3H_7	CH_3	55	0.1/50
t-C_4H_9	CH_3	50	0.1/48
c-C_6H_{11}	CH_3	30	a)
n-C_3H_7	C_2H_5	40	0.3/68
n-C_4H_9	C_3H_7	59	0.2/90
$(CH_2)_3$	$(CH_2)_3$	20	a)
$(CH_2)_4$	$(CH_2)_4$	10	0.1/60

a) short path distillation

An attempt to isolate the α-chlorodialkylnitrosamines gave only the chloromethyl alkylnitrosamines (primary chlorides) which could be purified by distillation (Table 3). Secondary chlorides, e.g. α-chlorodiethylnitrosamine XVI, were stable only in dichloromethane solution as attempted isolation resulted in

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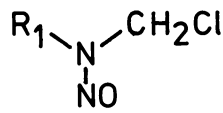
violent decomposition and formation of tars.

TABLE 3 Chloromethyl Alkylnitrosamines synthesized by Addition of NOCl to Imines

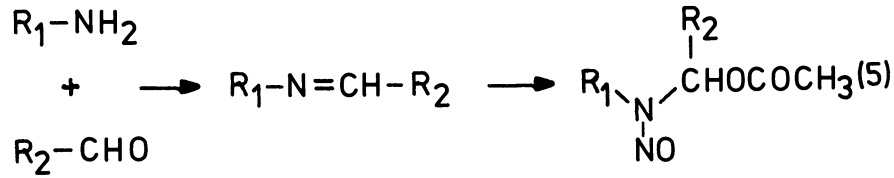


R_1	Bp	Z-isomer (%)
CH_3	0.1/24	8
C_2H_5	0.1/35	22
n-C_3H_7	3/50	27
i-C_3H_7	0.3/37	54
c-$(CH_3)_3$	-	>95

Nitrosyl bromide also added to imines but the yields of α-acetates after the addition of silver acetate were not better than these obtained by the addition of nitrosyl chloride. Because of their high reactivity, it was not possible to isolate α-bromodialkylnitrosamines in either case. Presumably, steric effects may also operate.

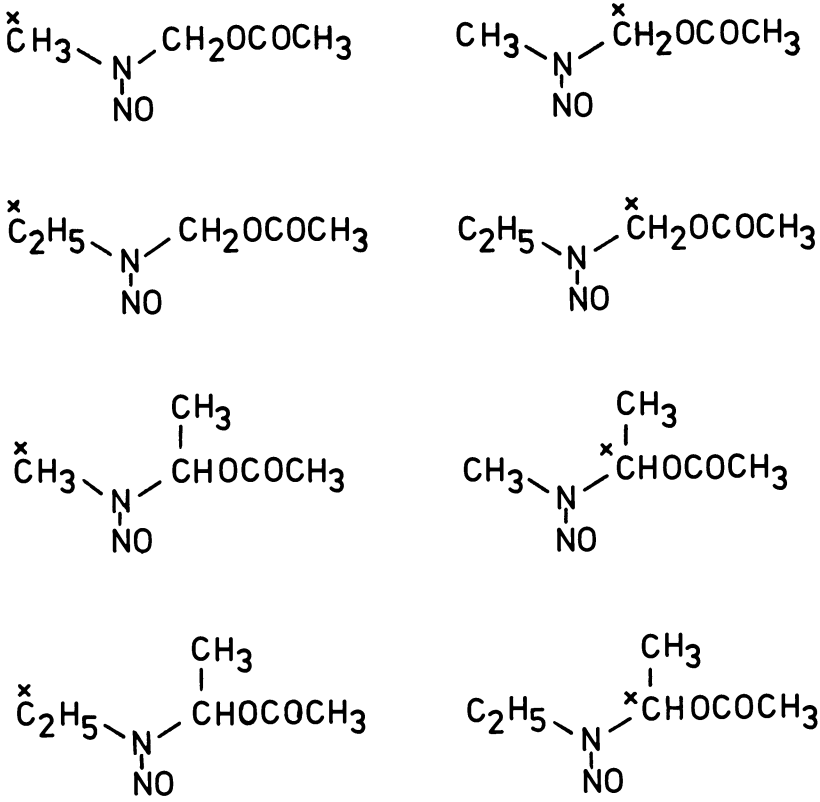
The addition of nitrosyl chloride to imines and the subsequent reaction with nucleophiles provided a route to a wide variety of compounds. Since all operations are easy to carry out in a hood, this synthetic scheme is valuable because of the high carcinogenicity of these α-substituted compounds.

The same synthetic pathway can be used for the synthesis of ^{14}C-labelled α-acetates. For metabolic studies, it was possible to place the ^{14}C-label at different C-atoms by using ^{14}C-labelled amine or aldehyde (Eq. 5).



In this way, we have prepared the ^{14}C-labelled α -acetates listed in Table 4 (23,24)

TABLE 4 ^{14}C-Labelled α -Acetoxydialkylnitrosamines by Reaction of ^{14}C-labelled Imines with NOCl/ $AgOCOCH_3$.



The position of the ^{14}C is marked by an asterisk.

Hydrolysis of α -Acetoxy N-Nitrosamines

Since the α -acetates should produce the corresponding α -hydroxy compounds by hydrolysis of the ester function (20), we investigated the stability of these acetates in aqueous solution by following the UV maximum in the range of 225-235 nm. The results are summarized in Table 5.

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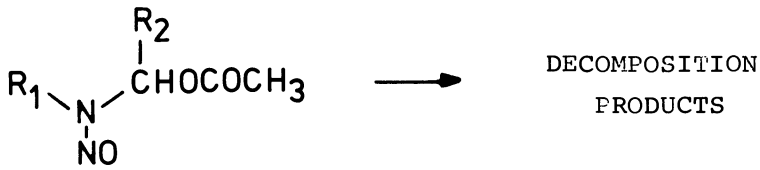
TABLE 5 Half-lifes of α-Acetates in Aqueous Solution (pH 7.0, 37°C)



R_1	R_2	Z-isomer (%)	$t_{1/2}$(min)
CH_3	H	10	3360
C_2H_5	H	10	1680
n-C_3H_7	H	17	1800
n-C_4H_9	H	15	1800
i-C_3H_7	H	35	600
2-C_4H_9	H	40	660
c-C_6H_{11}	H	35	240
$C(CH_3)_3$	H	95	84
CH_3	CH_3	5	9
C_2H_5	CH_3	5	5.7
i-C_3H_7	CH_3	25	2.4
c-C_6H_{11}	CH_3	38	-
$C(CH_3)_3$	CH_3	80	1.9

Two effects must be distinguished in the interpretation of these results. First, there is an anchimeric effect of the N-nitroso group which favors the hydrolysis. This anchimeric assistance can clearly be seen in the dependence of the amount of Z-isomer (the Z/E ratios were obtained from the NMR spectra). Increasing size of the alkyl group resulted in a higher content of Z-isomer and consequently in a higher hydrolysis rate. Primary and secondary acetates showed the same dependence. Recently, Michejda (25,26) showed an anchimeric assistance of the N-nitroso group in the hydrolysis of ß-substituted nitrosamines.

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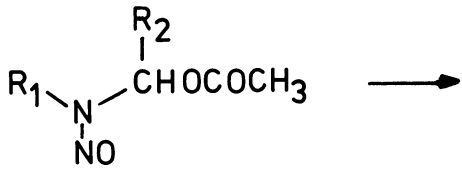
The second effect could be observed by comparison of the hydrolysis rates of primary and secondary acetates (Table 6).

TABLE 6 Comparison of Half-lifes of Primary and Secondary Acetates



R_1	R_2	Z-isomer (%)	$t_{1/2}$ (min)
CH_3	H	10	3360
CH_3	CH_3	5	9
i-C_3H_7	H	35	600
i-C_3H_7	CH_3	25	24
$C(CH_3)_3$	H	95	84
$C(CH_3)_3$	CH_3	80	19

In all the cases, the secondary acetates hydrolyzed faster by factors ranging from 44 up to 350. If a B_{AC} mechanism is operative (the normal hydrolysis mechanism of esters), the hydrolysis rate slows down in comparing the acetates of primary and secondary alcohols. Our results can be interpreted best in terms of the B_{AL} mechanism of ester hydrolysis operating only in those cases when the alcohol is able to form stable carbonium ions. This suggests that in the case of α -acetoxy dialkylnitrosamines, there exists a species such as a contact ion-pair which itself is attacked by a nucleophile, e.g. water (Scheme 1).

These results were supported by the hydrolysis rate at various water concentrations. These experiments clearly showed that the water concentration and the reaction rate of hydrolysis were not proportional. Furthermore, the methanolysis of the α -acetates provided the corresponding α - methoxy nitrosamines (result of C - O bond fission) and not methyl acetate,

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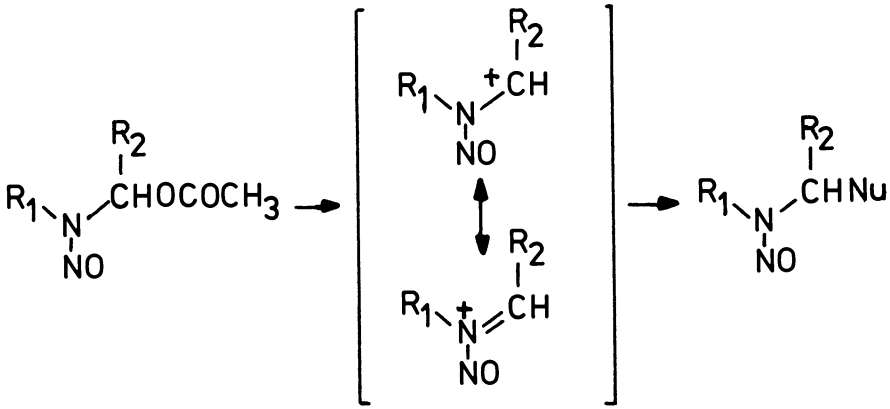
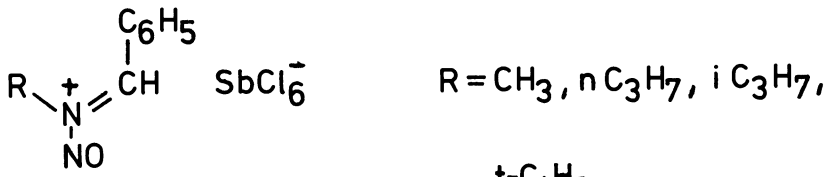


SCHEME 1 Proposed Mechanism for the Nucleophilic Displacement of Acetate in α-Acetoxy dialkylnitrosamines

the product of O-CO bond fission. Naturally the methanolysis rates were faster with secondary α-acetates than with primary acetates, since secondary carbonium ions are more stable than primary ones.

Since N-nitrosoimmonium ions seem to be involved in the hydrolysis of α-acetates, it should be possible to isolate such species as stable salts. For this purpose, we selected a system such as XVII in which the phenyl group should provide further stabilization of such a carbonium ion. After the reaction of nitrosyl chloride with the corresponding imines, addition of antimony pentachloride resulted in the precipitation of pale yellow solids; these could be isolated and stored under nitrogen for several days at room temperature.



XVII

All spectroscopic data (Table 7) are consistent with the proposed structure as N-nitrosoimmonium hexachloroantimonates XVII.

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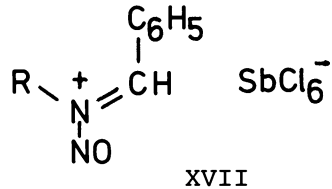
TABLE 7 Spectroscopic Properties of Stable N-Alkyl-N-nitrosoimmonium hexachloroantimonates



XVII

R	λ max (nm), ε		^{13}C
CH_3	272,	18400	172.1
n-C_3H_7	273,	17700	171.6
i-C_3H_7	273,	26400	169.1
tert-C_4H_9	273,	26600	167.1

The isolation of stable crystalline N-nitrosoimmonium salts confirmed the finding that such species are involved in all nucleophilic displacement reactions of α-acetoxy nitrosamines. As will be seen later, the thermal behavior of the α-acetates is also determined by these ions. With these results in hand, it now seems possible that the α-acetate II was formed in Eq. 2 but subsequent displacement by the nitropropane anion resulted in formation of III.It is reasonable to conclude that α -chlorodialkylnitrosamines themselves possess a certain amount of ionic character. In 1968, a Swiss group (27) published a new method for the synthesis of glucosides and glucuronides. They found that reactive halides, such as benzyl chloride and bromide reacted with e.g. triacetyl glucose under BF_3 etherate catalysis to yield the corresponding benzylated triacetyl glucoside. Earlier, a number of experiments designed to synthetisize glucosides or glucuronides of α-hydroxylated nitrosamines by inverse Königs-Knorr reaction failed. This new method was attempted since the α-chloro-N-nitrosamines seem to possess reactivity similar to that of benzyl halides. The compounds synthesized so far are listed in Table 8.

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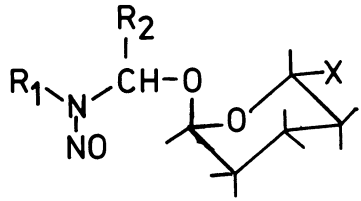
TABLE 8 Protected Glucosides and Glucuronides XVIII



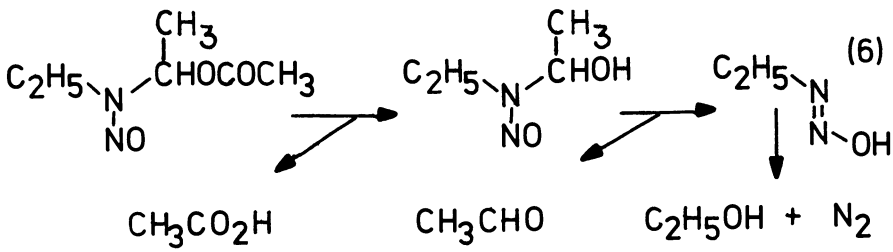
XVIII

X = CH_2OAC:	R_1=CH_3;	R_2=H
	R_1=n-C_3H_7;	R_2=H
	R_1=i-C_3H_7	R_2=H
	R_1=tert-C_4H_9;	R_2=H
	R_1=C_2H_5;	R_2=CH_3
X = CO_2CH_3:	R_1=n-C_3H_7;	R_2=H
	R_1=i-C_3H_7;	R_2=H
	R_1=tert-C_4H_9;	R_2=H

Under identical conditions the acetylated glucosides were formed more easily than the acetylated glucuronides, albeit both derivatives of hydroxymethyl methylnitrosamine XVIII (R_1=CH_3; R_2=H) were obtained only in traces, whereas the corresponding n-propyl compounds are isolable in yields up to 30%.

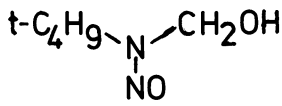
Although a number of problems remains to be solved, the method seems to offer promise for the synthesis of protected glucosides and glucuronides of α -hydroxylated nitrosamines.

The hydrolysis of α -acetates provided the expected products. In the case of α -acetoxy diethylnitrosamine, acetic acid, acetaldehyde and ethanol could be determined by gas chromatography (Eq. 6).

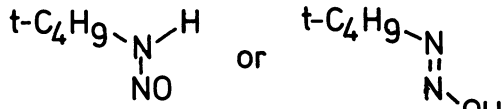


(6)

In the case of XIV, there were indications that XIX and XX were formed as transient intermediates, when an aqueous solution of XIV was monitored by NMR. How-

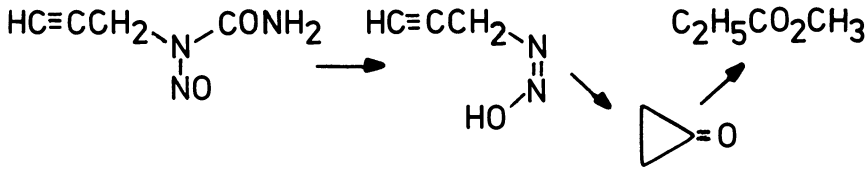


XIX



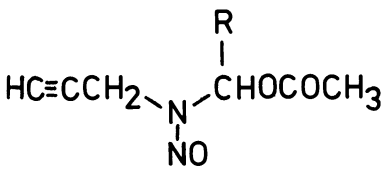
XX

ever, we have so far been unable to isolate a α -hydroxyalkyl nitrosamine or a diazohydroxide. An experiment devised by Kirmse in 1973 to intercept diazohydroxides (Scheme 2) produced by alkaline degradation of nitrosoureas has been initiated (28).

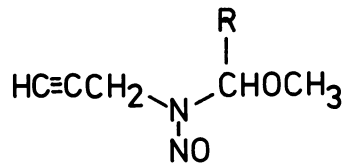


SCHEME 2 Pathway of Methyl propionate Formation from the Alkaline Degradation of N-Propionyl-N-nitrosourea proposed by Kirmse (28)

The α -acetate XXI (R=CH_3) was synthesized in the usual manner in very poor yield and reacted under the conditions of Kirmse. In spite of careful workup, no traces of methyl propionate could be detected. There are at least two reasons why this experiment failed. First,



XXI R = H, CH_3



XXII

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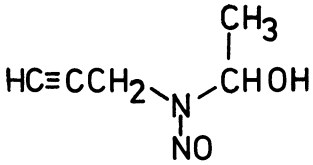
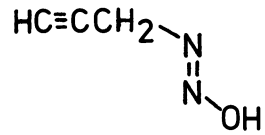
the methanolysis of XXI (R=CH_3) gives exclusively the corresponding α -methoxynitrosamine XXII. That's why we are now investigating the α -acetate XXI (R=H) (18). In the second place, the reaction between the triple bond and the diazotate function requires a syn-diazotate. The breakdown of the α -hydroxy compound XXIII results in the formation of the anti-diazotate. Thus, even if the diazotate function XXIV was formed, it could not react with the triple bond unless a rapid isomerization to the syn-diazotate occurred. Since there exist no exact data about such an isomerisation, a considerable amount of work remains to be done in the field of diazotates (4).



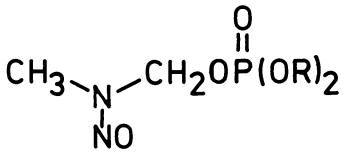
XXIII



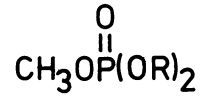
XXIV

Thermal Behavior of α -functionalized Nitrosamines

This section will discuss experiments designed to synthesize phosphates of α -hydroxylated nitrosamines. These investigations shed some light on the thermal stability of α -substituted nitrosamines.



XXV



XXVII

R = $CH_2C_6H_5$

XXVI

XXVIII

R = C_6H_4-p-NO_2

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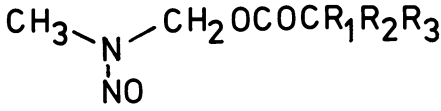
Our aim was to synthesize phosphoric acid triesters XXV and XXVI by the reaction of chloromethyl methylnitrosamine with the corresponding silver salts. Workup allowed the isolation of crystalline substances which were shown to be triesters XXVII and XXVIII. By variation of the N-alkyl group in the nitrosamine from methyl to ethyl, the corresponding phosphoric ethyl ester were obtained. Thus it seems clear that the N-alkyl group was transferred to the oxygen of phosphorus (formally a 1.5 alkyl shift). By working at -20°, we could isolate XXV. Even by working at this temperature, there was no indication for the existence of XXVI. It was not possible to purify XXV, so the hydrogenolytic scission of the o-Benzyl bond by Pd/C doesn't work. The above mentioned rearrangement was observed also in those cases when silver salts of stronger acids were used in the nucleophilic displacement reaction of chloride. In the series of the chlorinated acetoxy derivatives this effect was quite evident (Table 9). Whereas

TABLE 9 Temperature of half-lifes of chlorinated α-Acetoxydimethylnitrosamines measured by NMR



R_1	R_2	R_3	T (°C)
H	H	H	150
H	H	Cl	100
H	Cl	Cl	80
Cl	Cl	Cl	40

the α-acetate was stable up to 130°, the trichloroacetate rearranged even at room temperature in solution and could not be purified by distillation. A mechanistic scheme can be drawn for this rearrangement which involves N-nitrosoimmonium ions (Scheme 3).

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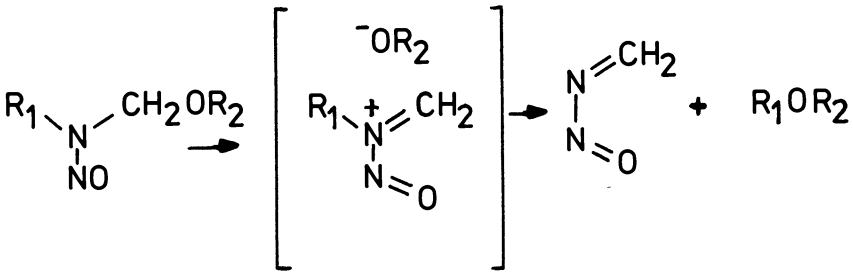

SCHEME 3 Proposed Mechanism for the Thermal Rearrangement of α-Acetates

These ions are presumably so reactive that they are able to alkylate their own anion. The stronger the acid, the better the dissociation and the easier the rearrangement. At present, we are investigating the mechanistic details of this self-alkylation. One can imagine that a stronger nucleophile which comes from outside could be alkylated by the nitrosoimmonium/anion complex (19,20).

These findings hold for the primary acetates. The behavior of the secondary esters is more complicated because these esters tend to hydrolyze more rapidly than primary esters, in general, the rearrangement occurred also.

Conclusion

In summary our study of α-substituted nitrosamines has given us a very interesting view of the chemical reactivity of this class of compounds. We have been able to show that the chemical behavior is governed by the intermediate existence of N-alkyl N-nitrosoimmonium ions in the thermal and solvolytic reactions. The significance of these N-alkyl N-nitrosoimmonium ions in the biological activity of dialkylnitrosamines is not clear at the moment.

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Oxidative Activation of *N*-Nitrosamines: Model Compounds

CHRISTOPHER J. MICHEJDA, MARILYN B. KROEGER-KOEPKE, STEVEN R. KOEPKE, and ROBERT J. KUPPER

Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, MD 21701

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No one really knows what percentage of human cancers are chemically induced. Estimates range from 50 to 90%. Whatever the precise figure, it is clear that chemical carcinogens are among the most dangerous health hazards facing mankind. Several classes of chemicals have been identified as carcinogens in laboratory animals, and by extension, there is a strong likelihood that they are also carcinogenic in man.

It is not known how chemicals cause cancer. A fascinating aspect of the story is that many "carcinogenic" chemicals are in fact, not the culprits responsible for cancer induction. The metabolic processes of the body change the chemicals from relatively innocuous substances into reactive intermediates which in as yet unknown fashion, trigger the chain of events which finally result in tumor formation. In other words, chemical carcinogenesis is an effect of "failed" detoxification.

One of the more important classes of chemical carcinogens are N-nitrosamines. They are important because practically all of the simple nitrosamines are carcinogenic, they are widely distributed in our environment and can be formed in the stomach from secondary and tertiary amines and the ubiquitous nitrite ion. Moreover, nitrosamines are very organ-specific. Thus, a given nitrosamine will produce a liver or an esophageal tumor, regardless of the route of administration of the carcinogen. This fact makes nitrosamines very useful in the study of mechanisms of tumor induction (1).

The work described here has as its goal to contribute to an understanding of how nitrosamines are activated to produce the reactive electrophilic intermediates which interact with cellular components to produce the carcinogenic response.

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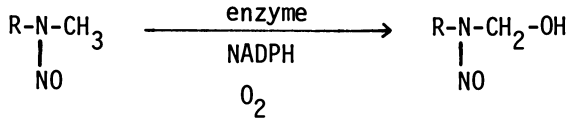
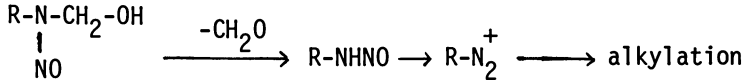
Results and Discussion

α-Oxidized Nitrosamines

It had been suggested a number of years ago that methyl alkyl nitrosamines are hydroxylated enzymatically on the methyl group to give α-hydroxymethylnitrosamines (2, 3).

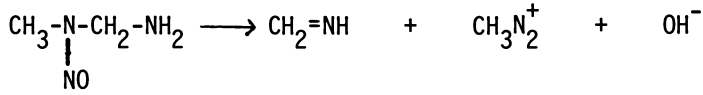


The very unstable hydroxy nitrosamine then loses formaldehyde to form the primary alkynitrosamine, which rapidly rearranges to the alkyl diazonium ion. The latter, being a powerful electrophile, alkylates various cellular nucleophiles, including the nucleic acids.



Thus, it is apparent that the initial α-hydroxylation of nitrosamine constitutes a possibly important pathway to produce the so-called proximate carcinogen. The α-hydroxylated nitrosamines have eluded direct isolation, although derivatives such as esters and ethers have been prepared by various groups (4, 5, 6, 7, 8). These materials, particularly α-acetoxydimethylnitrosamine (9), have been shown to be very potent carcinogens.

After several unsuccessful attempts in our laboratory to prepare authentic α-hydroxydimethylnitrosamine, attention was turned to the preparation of α-aminonitrosamines. The rationale for this work was that these materials might be more stable than the hydroxylated species, but would still lead to the same reactive alkyl diazonium ions.



The α-amino nitrosamines however, also defied isolation in the pure state and, hence, they were isolated as the corresponding ureas. The synthesis of the ureas was accomplished in several steps starting with the ester of an appropriate N-nitrosamino acid (here illustrated by N-nitrososarcosine) (10).

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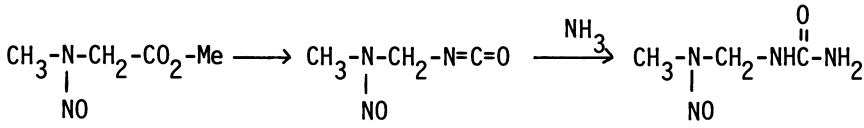
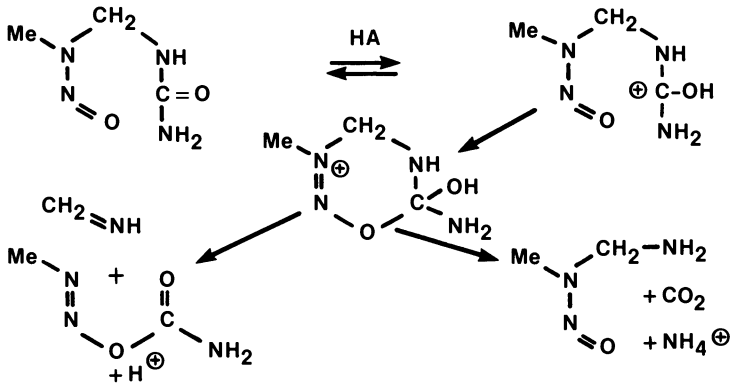


The compound shown in the equation, α-ureidodimethylnitrosamine (DMNU) is currently being tested in a large scale animal experiment in collaboration with Dr. E. Weisburger and her co-workers at the National Cancer Institute. Preliminary experiments have shown that it induces gastric adenomatosis (a precancerous lesion) in male mice, and also causes severe liver necrosis. On the chemical side, a study of the hydrolysis of DMNU and a related compound derived from N-nitrosoproline, α-ureido-N-nitrosopyrrolidine (NPU), has proved to be most interesting (10). A detailed kinetic analysis of the hydrolysis reaction has led to the potentially very important finding that the reaction proceeds through the interaction of the N-nitroso group with the reacting center. In other words, the N-nitroso group acts as an internal catalyst in the reaction.



Chang et al. (11) also recognized the neighboring group properties of the N-nitroso function in their study of the rapid rate of hydrolysis of N-nitroso-2-(methylamino)-acetonitrile.

β-Oxidized Nitrosamines

The discovery of the anchimeric effect of the N-nitroso group led to several exciting possibilities. Krüger (12) postulated that if the side chain of nitrosamines is larger than ethyl, the compounds are degraded to methylating agents by β-hydroxylation. He postulated that the function of this side chain

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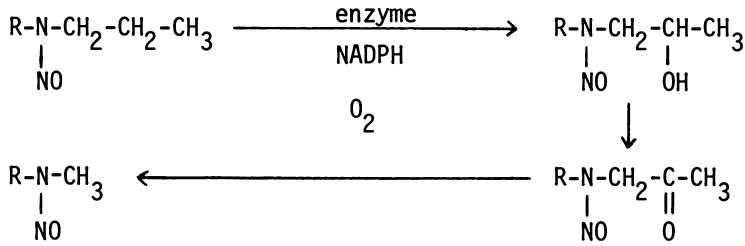
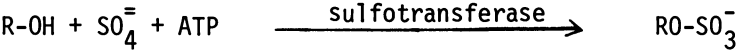
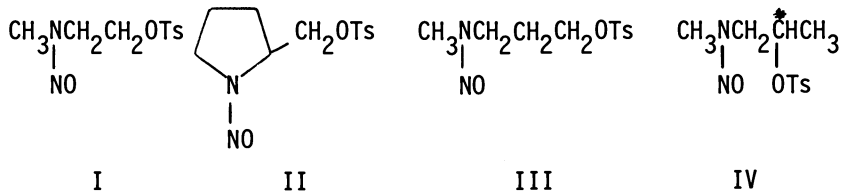


hydroxylation was to shorten the chain, so that, in fact, the end result was a methylnitrosamine. Our discovery of the neighboring group effect of the N-nitroso group led to an alternative postulate. One of the important detoxification reactions in higher life forms (but not in bacteria) is to convert a hydroxyl group to a sulfate (13). One chemical property of sulfate groups is that they are excellent leaving groups in nucleophilic



displacement reactions. Thus, we postulated that the sulfate of a β-hydroxylated nitrosamine might act as direct alkylating agent whose activity would be enhanced by internal assistance of the N-nitroso group. In order to test this idea, the p-toluenesulfonate (tosylate) esters shown below were prepared.



I II III IV

To test the reactivity of these tosylates, the kinetics of the solvolysis reaction in glacial acetic acid were measured. Tosylate II reacted so rapidly that the kinetics could not be measured titrimetrically ($t_{1/2} \sim 30$ sec. at 15°C). At 40° compound III reacted very slowly, but compound I reacted at a convenient rate ($t_{1/2}$ = 7 min. at 40°) and accurate kinetic data were determined for it. The kinetic data for III were obtained at higher temperatures. The results for both compounds are shown in Table 1.

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Table 1

Acetolysis of Methyl-(β-tosyloxyethyl)-nitrosamine (I) and Methyl(γ-tosyloxypropyl)-nitrosamine III

Compound	$k(s^{-1} \times 10^4)$	T(°C±.1)	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (eu)
I	1.66 ± 0.05	15	17.0	-16.6
	2.90 ± 0.1	20		
	4.86 ± 0.2	25		
	9.03 ± 0.3	30		
	15.4 ± 0.6	35		
	16.6 ± 1.1	40		
III	2.28 ± 0.05	60	18.5	-21
	3.17 ± 0.08	70		
	11.9 ± 0.6	80		

It is interesting to compare the rates of tosylates I and III with some representative primary tosylates. These are shown in Table 2.

Table 2

Acetolysis Rates of Selected Primary Tosylates, R-OTs

R	T(°C)	$k(s^{-1} \times 10^7)$	Ref.
CH_3	75	8.52 ± 0.08	14
CH_3CH_2	75	7.39 ± 0.10	14
$(CH_3)_2CH\text{-}CH$	75	2.30 ± 0.06	14
$(CH_3)_3C\text{-}CH_2$	75	0.835	14
Ph CH_2	25	26.1 ± 0.3	15

These rates are some four orders of magnitude slower than tosylate I and even the highly reactive benzyl tosylate is slower by a factor of 200 than I. It is also noteworthy that whereas tosylate I solvolyses substantially more rapidly than III, the solvolysis of the latter is also accelerated significantly. The extremely fast rate of solvolysis for compounds I and II demand that the N-nitroso group be involved in the reaction, which we postulated to take the following course.

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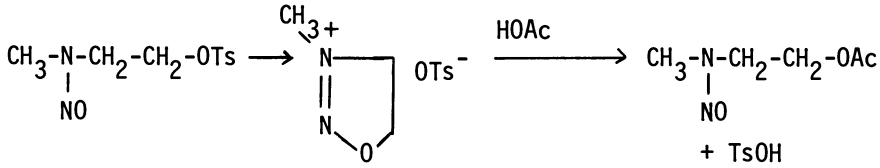
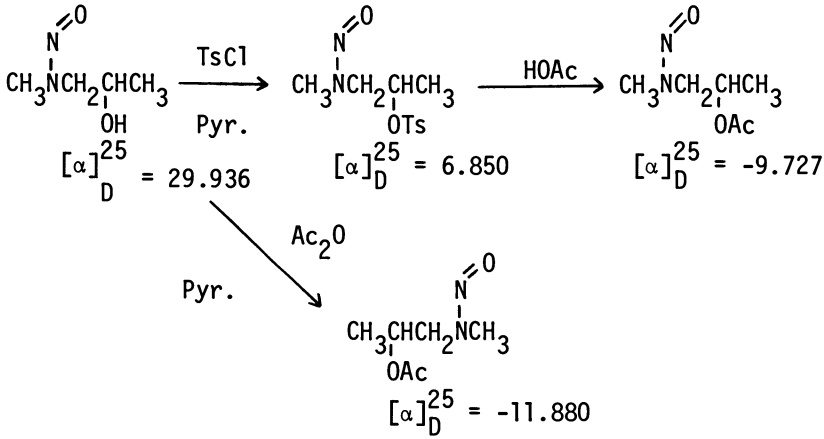


V

Solvolysis of the optically active tosylate IV provided further data to substantiate the intermediate formation of V. If the N-nitroso group did not become involved in the solvolysis, then the product would show either inversion of configuration or racemization. If, on the other hand, V was an intermediate then the product should show retention of configuration. This was indeed found to be the case, the product acetate had exactly the same configuration as the starting material IV. This result is summarized in the following scheme.



The small amount of racemization might be due to a certain degree of internal return and/or direct displacement of the tosylate.

When tosylate I was warmed in a non-nucleophilic solvent, such as methylene chloride, the cyclic intermediate V was formed in essentially quantitative yield. This substance was a relatively stable material which, however, was reactive toward nucleophiles, as was the original tosylate I. The oxadiazolium ion V has two sites for possible nucleophilic attack, as illustrated in the following equation.

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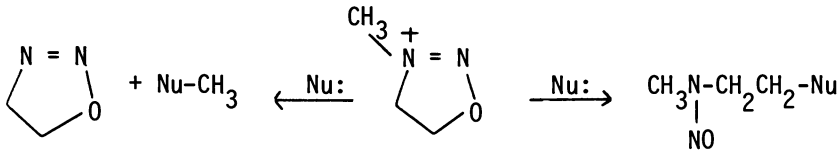
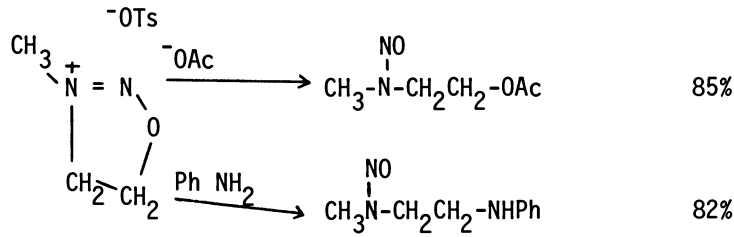
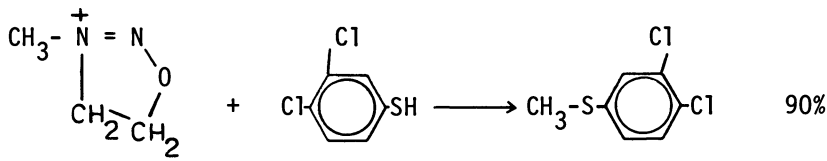


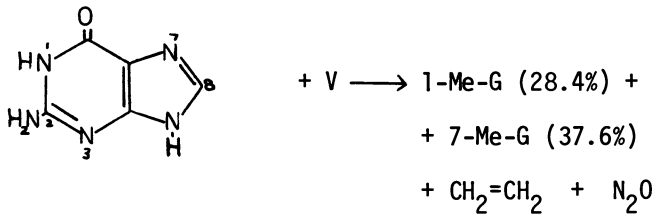
Both reactions were observed. Reaction at the original site of attachment of the tosyl leads to substituted nitrosamines.



The reactions at the methyl lead to methylated products. Thus, reaction with the weak nucleophile 3,4-dichlorothiophenol resulted in the formation of the corresponding thioanisole.



Particularily interesting is the reaction of V with guanine and guanosine. The latter gives exclusively the 7-methylguanosine. Guanine results in methylation at several sites.



The ready alkylation of the bases derived from DNA suggests that it is likely that β-hydroxylation followed by suitable conjugation may in fact, be an activation pathway for nitrosamines. While animal experiments are only now underway, the tosylates have been examined in the Ames mutagenicity test system (16). The tosylate I is strongly mutagenic in the TA 1535 strain of Salmonella typhimurium without microsomal activation. It is interesting to note that the oxadiazolium ion V is also mutagenic

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without activation, with the dose response data indicating the same slope as tosylate I. This suggests that I is transformed into V before the reaction with the genetic material of the bacterium occurs. Tosylate III on the other hand, is not mutagenic without activation and the same is true of the precursor alcohols (17). These data indicate that consistent with the observed chemistry, the tosylate of the β-hydroxylated nitrosamine is capable of altering the genetic material of the bacterium, without enzymatic intervention. Ethanolamines of various kinds are important chemicals of commerce and are present in various environments (cutting fluids, cosmetics, wetting agents, etc.). In these environments, nitrite ion is frequently present also, and therefore, conditions exist for nitrosamine formation. Thus, our data take on added significance, particularly if the animal experiments support the chemical and mutagenicity data.

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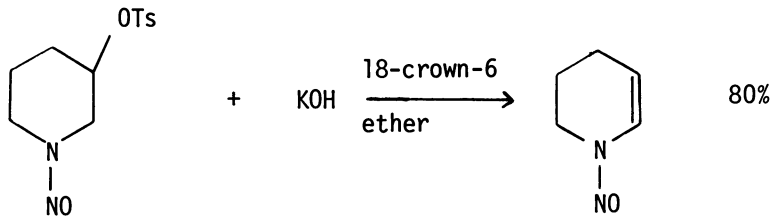
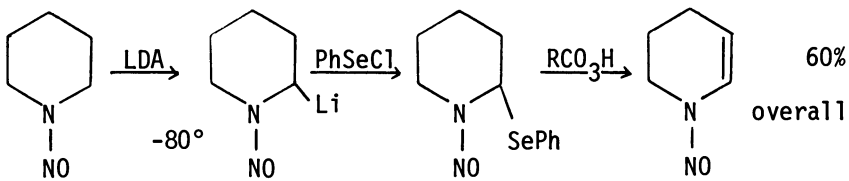
Vinyl Nitrosamines

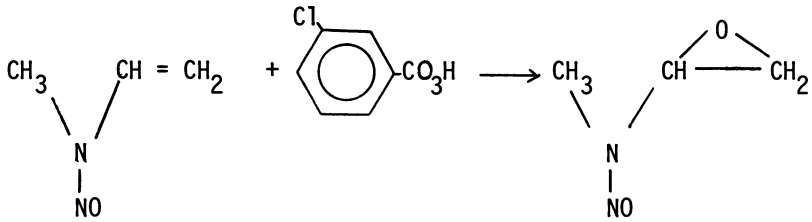
Another interesting class of nitrosamines are the vinyl nitrosamines. Several examples of this group have been described previously. We have however, developed two general, high yield methods, which will make these compounds available for study. The preparation of N-nitroso-2,3-dehydropiperidine is illustrative.



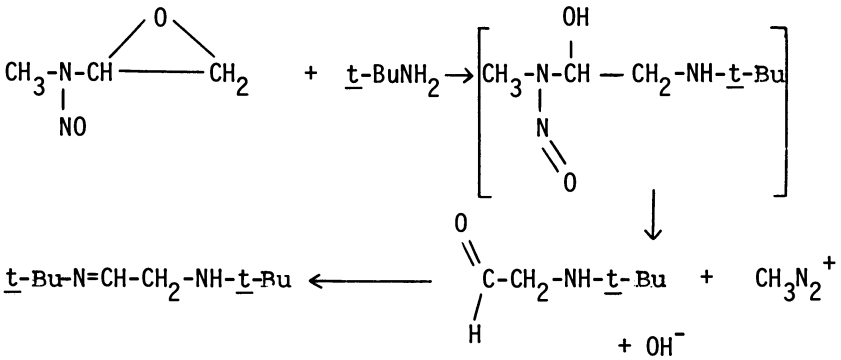
If 3-chloro-N-nitrosopiperidine is used instead of the tosylate, 3,4-dehydro-N-nitrosopiperidine is formed in high yield. Another, perhaps more general, route to the vinylnitrosamines makes use of the acidity of the hydrogens on the α-carbon of nitrosamines (18). The resulting carbanion reacts smoothly with phenylselenyl chloride. The adduct is then oxidized with m-chloroperbenzoic acid (19).



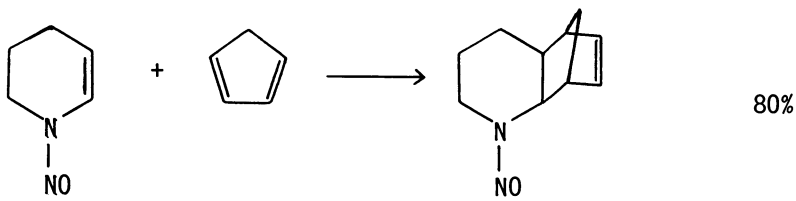
We have just begun to explore the chemistry of these vinyl nitrosamines (which are really N-nitroso enamines) which are extremely interesting synthetic intermediates. One very exciting development has been their conversion to the corresponding epoxides.



These epoxides are very reactive and are excellent alkylating agents. They may be the primary metabolic products of vinyl nitrosamines. One interesting reaction of the epoxides demands the intermediacy of long sought-after α-hydroxynitrosamine.



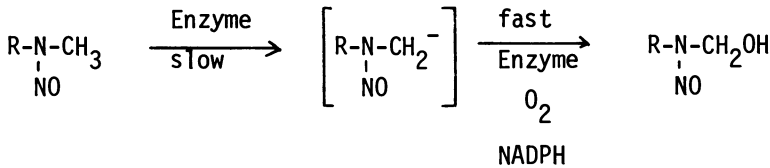
Another, potentially extremely useful reaction, is the Diels-Alder reaction. The reaction is illustrated here with N-nitroso-dehydropiperidine and cyclopentadiene. This reaction is sluggish without catalysis that can be accomplished by addition of various metal salts, such as cupric tetrafluoroborate.



Enzymatic Reactions of Nitrosamines

As has been stated above, the activation of nitrosamines is an enzymatic process. We have made some progress toward the understanding of the mechanism of this important reaction. The study is rendered extraordinarily difficult because there are apparently a number of enzymes involved in the metabolism and the principal ones seem to be membrane bound. This makes the purification of the individual enzymes very hard. Nevertheless, considerable information on the mechanism of the enzymatic processes can be obtained, even with the unpurified preparations.

We have used microsome preparations from rat livers to examine the steady state kinetics of demethylase activity using dimethylnitrosamine (DMN) and phenylmethylnitrosamine (PMN) as substrates. The Michaelis-Menten constants (V_{max} and K_M) have been used to characterize the reactions for both of the nitrosamines and their deuterated analogs (20). It was found that the S-9 fraction of rat liver homogenates, which contains all of the cell contents except for the nuclei, mitochondria and the larger cellular membrane debris, yielded isotope effects of 1.8 and 5.4, respectively. These isotope effects are defined as the ratio of the maximal rates of the undeuterated and deuterated substrates. These data suggest that the breakage of the C-H bond is the rate determining step in the demethylation reaction. One attractive possibility is that the enzymes make use of the acidity of the α-hydrogens of the nitrosamines (18), and that it is carbanion which is oxidized in subsequent steps. The data at hand however, do not distinguish between that possibility and that of homolytic C-H bond clearage.



It has been suggested by others (21, 22) that there is more than one nitrosamine demethylase enzyme. The present kinetic data, together with isotope effect data and experiments involving enzyme "inducers", strongly supports this hypothesis. Two strains of rats, Long-Evans and Sprague-Dawley, were treated with phenobarbital. The kinetics of demethylation of DMN were examined using the microsome (the pellet formed by centrifugation of the S-9 fraction at 105,000 g) and the post-microsomal supernatant. The kinetics showed that while the phenobarbital treatment induced demethylase activity in the microsomal pellet of both strains (by 12% and 31%, respectively), the activity was repressed in the supernatant of the Long-Evans rats (-34%) but

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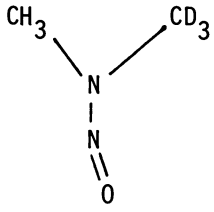
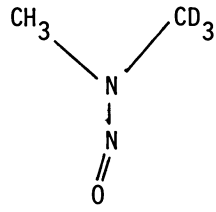
induced in the supernatant of the Sprague-Dawley rats (46%). Phenobarbital is considered to be an inducer of some, but not all, cytochrome P-450-dependent enzyme activity (21, 23). In line with the present results, Jori and Pescador (24) found that the level of cytochrome P-450 in the microsomal pellet from the livers of Long-Evans rats was induced by 47 percent by phenobarbital. Our level of induction was smaller but in the same direction. These data strongly support the presence of more than one nitrosamine demethylase in the liver.

Evidence was also obtained for a non-cytochrome nitrosamine demethylase. This material was best obtained from "pH 5 enzyme" supernatant (25) by precipitation in 33 percent ammonium sulfate. This preparation requires NADPH but in contrast to cytochrome P-450 enzymes (26), it is not inhibited by carbon monoxide.

Nitrosamines have the property of existing in two different stereochemical forms, if the groups on both sides of the nitrogen are different. In collaboration with Dr. Larry Keefer of the National Cancer Institute, we have utilized this property to determine which of the methyl groups in dimethylnitrosamine was hydroxylated. Using the specifically labeled Z-isomer (trideuteromethyl group on the same side as the oxygen) we have determined that it is the methyl on the opposite side



Z - isomer



E - isomer

to the oxygen which is oxidized enzymatically. This was done by comparing the kinetics of demethylation of the unsymmetrical trideuterated DMN, undeuterated DMN and hexadeuterated DMN. The kinetic characteristics of Z-DMN-d_3 were similar to DMN-d_0 and different from DMN-d_6. Equilibrated DMN-d_3 (equal amounts of the Z and E isomers) exhibited kinetics intermediate between DMN-d_0 and DMN-d_6.

Acknowledgment. Support for this work has been provided by grants from PHS(RO1-CA-22110-01), and the NSF(CHE 76-24095) and by the National Cancer Institute contract NO1-CO-75380 to Litton Bionetics, Inc. Part of this work was carried out in the Department of Chemistry, University of Nebraska.

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α-Amino Nitrite Esters and Their Analogues: Possible Reactive Intermediates in *N*-Nitrosamine Formation?

LARRY K. KEEFER

Analytical Chemistry Section, Laboratory of Carcinogen Metabolism, National Cancer Institute, Bethesda, MD 20014

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No one has apparently ever reported observing an α-dialkylamino nitrite ester (I), but I would like to begin this paper by postulating the existence of such species, then try to infer some of their properties from a new look at the literature of nitrosamine formation. Specifically, I would like to show how a diversity of recently investigated reactions might be formulated with the production and fragmentation of α-amino nitrites according to Fig. 1 as their final steps. The list of seemingly disparate nitrosamine-forming reactions which can be mechanistically unified in this way includes: the nitrosative dealkylation of tertiary amines (1,2,3,4,), of amine oxides (3,4) and of tetraalkyltetrazenes (5); catalysis of N-nitrosation reactions by aldehydes (6,7,8) or by aryl nitroso compounds (9,10); the remarkably facile conversion of tertiary enamines such as aminopyrine into dialkylnitrosamines (11,12); nitrosation of secondary amines in methylene chloride solution by means of solid sodium nitrite (13); and the direct reaction of immonium salts with nitrite ion (6,14).

Nitrosative Dealkylation of Tertiary Amines

The first clues that compounds of structure I might be involved in nitrosamine-forming reactions came during the study of tertiary amine nitrosations. Smith and Loeppky had proposed (2) in their detailed, classical investigation of the mechanism of this reaction that the first steps involve nitrosammonium ion formation followed by elimination of nitroxyl (HNO). The resulting immonium ion was postulated to hydrolyze to the secondary amine, which reacted with nitrosating agent to form the observed product. These mechanistic proposals are summarized in Fig. 2a.

In N-Nitrosamines; Anselme, J.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

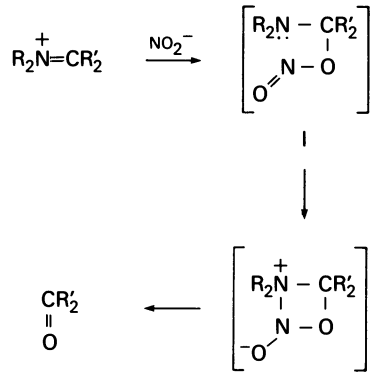

Figure 1. Postulated formation of α-dialkylamino nitrite esters (I) from immonium ion donors and their fragmentation to nitrosamines

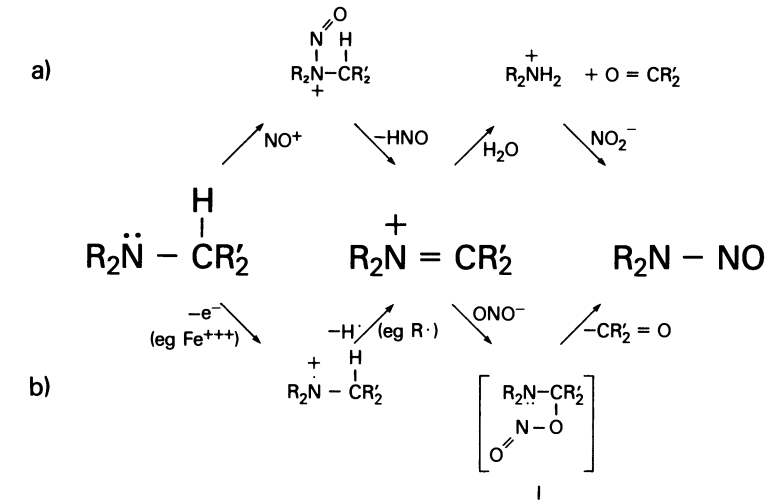

Figure 2. Nitrosative dealkylation of tertiary amines: (a) mechanism postulated by Smith and Loeppky (2); (b) composite of proposals by Lijinsky et al. (3) and Michejda et al. (5).

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The Smith-Loeppky mechanism (2) convincingly rationalized much of what was known about tertiary amine nitrosations. However, there was some evidence in the literature that there might be more to the mechanistic story. For one thing, it was not clear why the nitrosation of tertiary amines should have a higher pH optimum than that of secondary amines (1). Even more troubling was the somewhat controversial later report by Malins et al. (15) that dimethylnitrosamine formed at pH 6 more readily from trimethylamine than from dimethylamine. If that report is correct, then free dimethylamine and the corresponding ammonium ion could not be the only kinetically significant intermediates in the trimethylamine nitrosation.

This led us to propose (3) that the immonium ion of Smith and Loeppky's mechanism (Fig. 2a) might be reacting directly to produce the nitrosamine. If the immonium ion were attacked by free nitrite ion, an adduct of structure I could form and collapse via a four-center mechanism involving intramolecular nucleophilic attack of the amine group on the nitrosyl nitrogen, producing dimethylnitrosamine and formaldehyde via the pathway summarized in Figure 1. The intermediate N,N-dimethylformaldimmonium ion has been reported (16) to be most abundant at pH 10-11 in aqueous solutions, a finding consistent with a higher pH of optimum reactivity for tertiary vs. secondary amines.

A similar mechanism was invoked by Ohshima and Kawabata (4) to account for their results in the nitrosation of tertiary amines and amine oxides. In applying these concepts to the nitrosative dealkylation of tetraalkyltetrazenes, Michejda et al. (5) introduced an interesting variant by suggesting that immonium ions could be formed in two successive one-electron oxidation steps (for example by ferric ion oxidation of tertiary amine to the radical cation followed by radical abstraction of a hydrogen atom from the alpha position), rather than exclusively through the one-step removal of a hydride ion as nitroxyl. The resulting immonium ion was again considered to react directly with nitrite to produce the N-nitroso derivative. These reactions are summarized in Fig. 2b.

Thus the postulate that α-amino nitrite esters could form and fragment to nitrosamines has provided a useful construct for explaining some aspects of the nitrosative dealkylation of tertiary amines and their derivatives.

Formaldehyde Catalysis of Amine-Nitrite Reactions

Assuming that the above rationale for tertiary amine nitrosation was valid, we predicted (3) that the reaction of secondary amines with nitrite at milder pH's should be catalyzed by electrophilic carbonyl compounds, since secondary amines are known to form immonium ions on admixture with appropriate aldehydes and ketones. The prediction turned out to be true. Formaldehyde was shown to promote nitrosamine formation from a

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variety of secondary amines even in neutral or alkaline solutions (6,7,8). The mechanism of Fig. 3 was presumed to account for these results.

Interestingly, there is preliminary evidence that the conversion of primary amines to dialkylnitrosamines is also catalyzed by formaldehyde (S.R. Tannenbaum, unpublished results). It seems likely that a very similar mechanism, as in Fig. 4, might be involved in this transformation.

Nitrosation of Tertiary Enamines

Aminopyrine (II) is a widely used human analgesic which is convertible with surprising facility to dimethylnitrosamine both *in vivo* and in simple chemical model systems (11). The drug gives rise to much higher nitrosamine yields under most conditions than dimethylamine does, implying that the secondary amine cannot be an important intermediate in the nitrosation of II. To account for these remarkable results, we suggested (6) that aminopyrine reacts as a tertiary enamine according to the mechanism shown in Fig. 5a, protonating at the carbon atom *beta* to the dimethylamino group to give an immonium ion (III) capable of reaction with nitrite by a Fig. 1 pathway to form the nitrosamine.

Mirvish *et al.* later investigated the nitrosation of aminopyrine in considerable detail (12), proposing the alternative pathway shown in Fig. 5b, but presenting evidence that the mechanism is actually a great deal more complex than that. Firstly, they identified both a "fast" initial reaction, which was essentially complete within 2-5 min., and a "slow" reaction, which proceeded at a nearly constant rate for 15 min. Secondly, they found that the pH *vs.* rate profile had maxima at both pH 2.0 and pH 3.1. Thirdly, they reported an apparent kinetic order for nitrite which varied considerably under some conditions from the value of 2 required by the mechanism of Fig. 5b, ranging from cleanly first order for the "slow" reaction at pH 2 to as high as 3-4 for the initial reaction at low nitrite concentration (1-6mM).

These seemingly anomalous results suggest that the formation and fragmentation of α-amino nitrite esters could be playing a central role in the nitrosation of aminopyrine. The characterization of both fast and slow reactions, as well as the identification of two pH optima, imply that more than one kinetically significant pathway is involved in the overall transformation. The mechanism of Fig. 5a could well be the first order component the kinetic studies show to be operative under some conditions. It is noteworthy that this pathway also leads directly in its final step to the keto-enol derivative IV, which Mirvish *et al.* have identified as a by-product of aminopyrine nitrosation.

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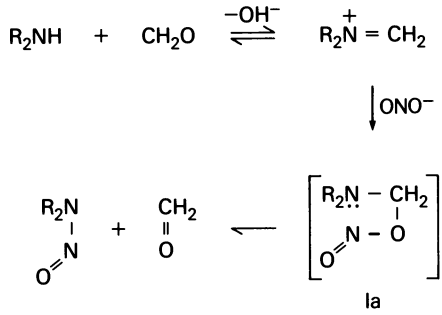

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Figure 3. Proposed mechanism of formaldehyde action in catalyzing secondary amine nitrosations (6)

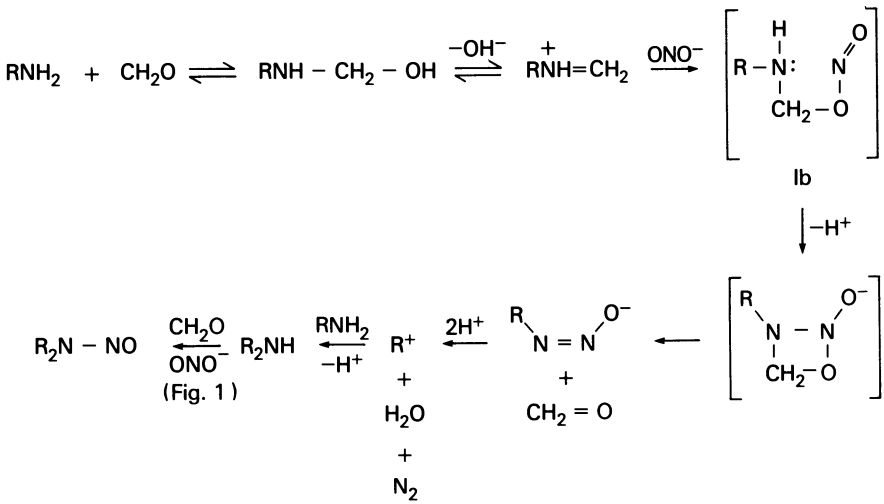

Figure 4. Proposed mechanism of formaldehyde action in promoting conversion of primary amines to N-nitroso derivatives

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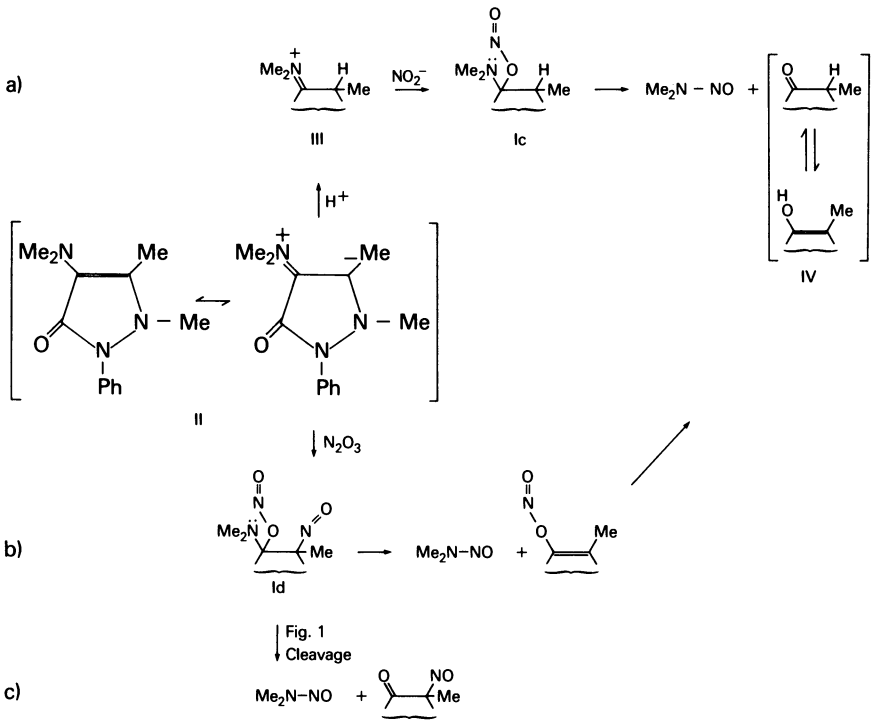

Figure 5. Possible mechanisms of aminopyrine nitrosation: (a) as proposed by Keefer and Roller (6); (b) as postulated by Mirvish et al. (12); (c) an alternative proposed in this paper.

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A second order alternative in which the nitrosamine is produced in a Fig. 1 fragmentation of an α-amino nitrite is shown in Fig. 5c. This mechanism differs from the one proposed by Mirvish et al. (12, summarized in Fig. 5b) only in involving the O-nitroso rather than the C-nitroso function in co-elimination with the dimethylamino group to produce the nitrosamine.

An apparent order in nitrite of 3 or more would also be consistent with α-amino nitrite fragmentation mechanisms if one assumes that nitrite is preferentially consumed in redox or nitrosation reactions elsewhere in the molecule which compete with nitrosation of the dimethylamino group. One such possibility was suggested by Dr. R.N. Loeppky (private communication), as shown in Fig. 6. This mechanism, which postulates the intermediacy of two different α-amino nitrites, Ie and If, should obey third order kinetics, since dimethylnitrosamine is produced only after aminopyrine reacts with the third mole of nitrite. Moreover, this pathway offers a mechanistic explanation for the direct production of nitrosohydrazide V, which has also been reported to be a product of aminopyrine nitrosation (12,17).

Promotion of Amine-Nitrite Reactions by Dihalomethanes

Another observation which at first seemed to constitute quite a different phenomenon but on closer scrutiny appears to involve a very similar mechanism was the finding that secondary amines could be nitrosated by solid sodium nitrite in certain organic solvents (13). Of the solvents studied, methylene chloride and other closely related dihaloalkanes appeared to be uniquely effective. A typical result was that pyrrolidine was nitrosated in 10% yield after only a day and half at room temperature in methylene choride solution, while less than 0.4% yield was detected in chloroform solution after two months under these conditions. Evidence that the solvent was directly participating in the reaction soon emerged in the form of the isolation of bis-(1-pyrrolidyl)methane as a by-product. Support for the conclusion that oxides of nitrogen were not intermediates was found when the nitrosating agent was omitted from the amine-methylene chloride mixture for varying lengths of time; an initial surge of nitrosamine formation was observed in each case when the nitrite was finally added, with the amount formed being roughly proportional to the time the amine and methylene chloride were pre-equilibrated.

In this case too it was postulated that an intermediate of type I was crucial to the methylene chloride mediated reaction, as shown in Fig. 7.

Heteroatom Analogs of I

It is possible that structural analogs of the α-amino nitrite esters might also be intermediates in nitrosamine-forming re-

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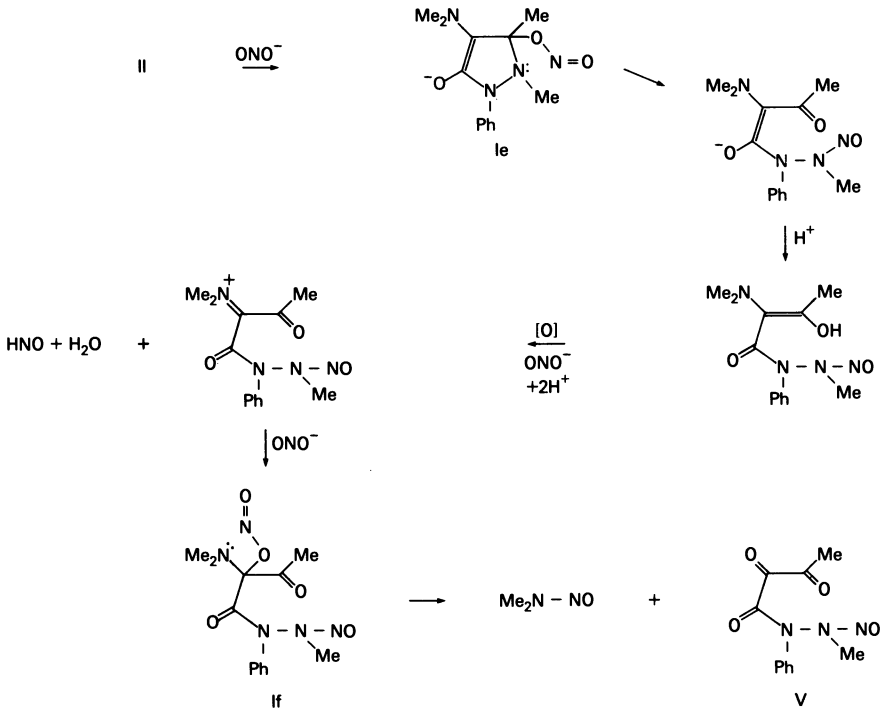

Figure 6. Possible mechanism of aminopyrine nitrosation, proposed by R. N. Loeppky (private communication)

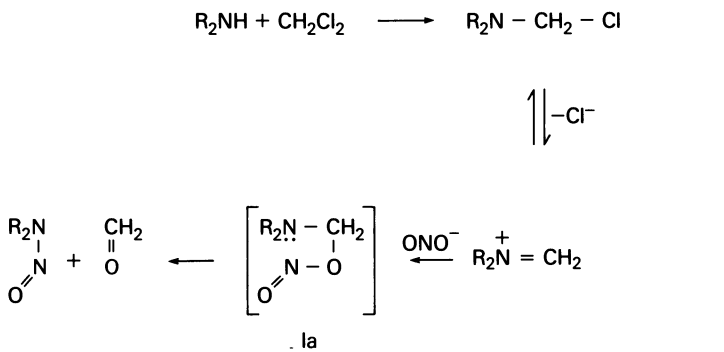

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Figure 7. Proposed mechanism for methylene chloride promotion of secondary amine nitrosations by solid sodium nitrite (13)

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actions. Davies et al. discovered (9,10) that C-nitrosophenols can catalyze nitrosamine formation, and proposed that the nitrite ester of the quinone monoxime was the active nitrosating agent. We suggest, however, that the alternative species Ig could be forming by nitrite attack on "heteroimmonium" ion VI, and that this proposal could equally well account for their results according to the mechanism outlined in Fig. 8. All the aryl nitroso compounds which acted as catalysts in this reaction have functional groups in the ortho and/or para positions which are capable of stabilizing the positive charge of intermediate VI, while those which did not catalyze the reaction have either no such functional group, or else one whose cation-stabilizing capability is diminished by other types of resonance interaction (9,10).

Another possible mechanism for this reaction was suggested by Dr. G.R. Krow (private communication), this one involving a a bona fide α-amino nitrite ester as an intermediate. If the quinone monoxime tautomer of the nitrosophenol were reacting as an electrophilic carbonyl compound with the amine according to Fig. 3, the resulting immonium ion, VII, could attack nitrite to yield the nitrosamine via intermediate Ih with regeneration of the nitrosophenol. This proposal is summarized in Fig. 9.

Direct Reaction of Immonium Ions with Nitrite

We have subjected the mechanistic proposal of Fig. 1 to a direct test. N,N-Dimethylformaldimmonium ion was independently prepared as its trifluoroacetate salt and was mixed with a slight excess of silver nitrite in acetonitrile-d_3. The only product identified was dimethylnitrosamine, which was produced in greater than 90% yield (6). No direct evidence for intermediates of structure I could be obtained, as the reaction was complete within the few minutes required to analyze the mixture by running its NMR spectrum.

Other Decomposition Pathways for α-Amino Nitrites

Nitrosamine formation is not the only conceivable fragmentation mechanism for compounds of structure I. By analogy to the nitrosative dealkylation reactions discussed above, one might predict that such compounds could also undergo cis elimination of nitroxyl in amide-forming reactions. Such a transformation has possibly been observed (14). During an attempt to synthesize the nitrosamino aldehyde VIII from immonium ion IX, Hecht & coworkers were able to isolate only 5-10% of the desired product. The major product proved to be N-methyl-2-pyrrolidone, as shown in Fig. 10. We interpret this as evidence that an intermediate such as Ii can fragment not only by the Fig. 1

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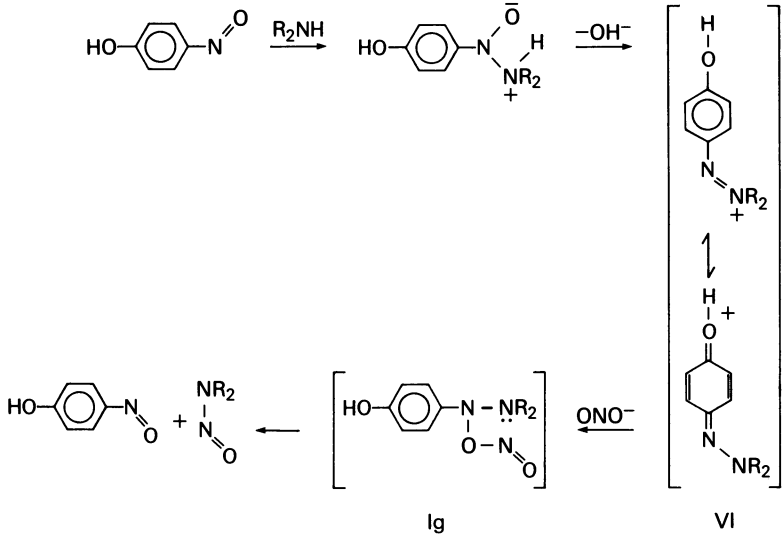

Figure 8. Alternate mechanistic possibility for catalysis of secondary amine nitrosation by aryl nitroso compounds

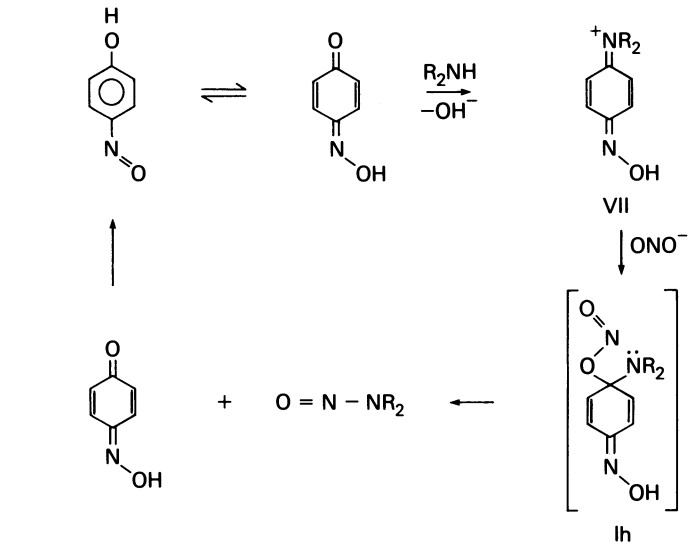

Figure 9. Alternative mechanism for catalysis of secondary amine nitrosation by aryl nitroso compounds, suggested by G. Krow (private communication)

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mechanism discussed extensively above, but also by loss of HNO to form lactams and amides. Under some circumstances, the latter apparently can be the principal course of reaction.

Possible Arguments against the Proposed Mechanism

Ambidence of Nitrite Ion. The postulated nitrosamine-forming mechanism requires that the nitrite ion react with immonium ion donors via its oxygen atom, rather than at nitrogen. The mechanism has been criticized on this basis, since nucleophilic attack by nitrite ion on alkyl halides is well known to furnish one of the most important and general methods for the synthesis of aliphatic nitro compounds, $R\text{-}NO_2$ (18). However, Kornblum et al. have shown that the relative proportion of nitrite ester formed as by-product in these reactions increases with the relative stability of the carbonium ion under attack by nitrite ion, with the alkyl nitrite: nitroalkane product ratio being approximately 2:1 in the reaction of silver nitrite with p-methoxybenzyl bromide (19). Immonium ions are stable enough to be isolated (20), i.e., much more stable than the p-methoxybenzyl cation, thus should show a marked preference for oxygen attack on nitrite. If one further postulates that N-attack is reversible (i.e. that any $R_2N\text{-}CH_2\text{-}NO_2$ which happens to form will dissociate to nitrite and immonium ions again) while products of O-attack rapidly fragment according to Fig. 1, then all the immonium ions present are potentially available for nitrosamine formation regardless of the ambident selectivity of nitrite ion in this system.

The Four-Membered Ring. Another problematical feature of the mechanism of Fig. 1 is the proposed involvement of a four-centered transition state. While the formation of four-membered rings is indeed generally slowest among ring-forming reactions in a homologous series (both 3- and 5-membered rings are formed faster in general than the four-membered homologs (21)), very similar four-centered mechanisms of elimination have been postulated in other synthetically useful transformations (for example the Wittig reaction (22,23)).

It is interesting that a four-membered ring capable of cleaving to a nitrosamine and a carbonyl compound was recently suggested as the probable intermediate in a very different nitrosamine-forming reaction. Schoenhard and colleagues (24) reported the detection of N-benzhydryl-N'-nitrosopiperazine as a contaminant in the commercial production of arylhydrazone X, shown in Fig. 11. The authors proposed that singlet oxygen was involved in a cycloaddition reaction across the hydrazone double bond, producing the four-membered ring compound XI as a transient precursor to the observed nitrosamine contaminant.

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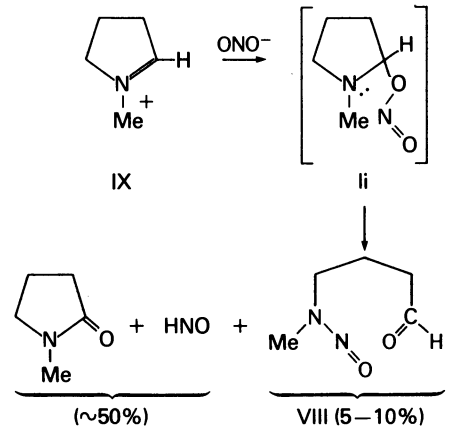

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Figure 10. Multiple fragmentation pathways for immonium ion IX on reaction with nitrite (14)

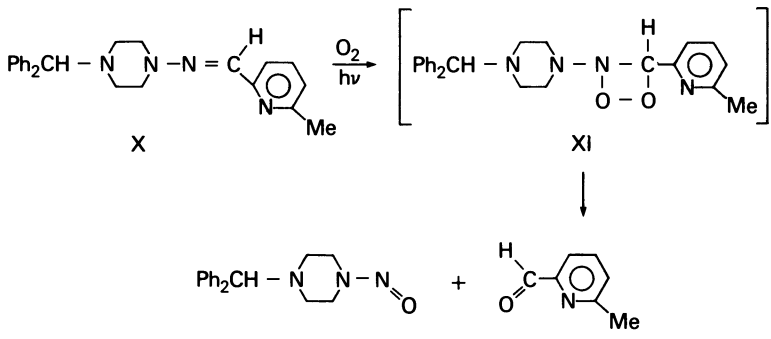

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Figure 11. Proposed mechanism of nitrosamine formation by cleavage of a four-membered ring in the oxidation of hydrazone X (24)

Prediction

One way of assessing the value of the hypothetical intermediates and mechanisms described in this paper is to use them to forecast the outcomes of future experiments. One such prediction which we have made and hope to test soon is that aromatic amines which are suitably activated toward nucleophilic attack can be nitrosated by nitrite ion under non-acidic conditions. Treflan (XII), for example, a widely used commercial herbicide which has been shown (25) to contain significant di-n-propylnitrosamine contamination under some conditions, might be expected to form an α-amino nitrite ester ion analogous to I as shown in Fig. 12. Collapse of this anion to dipropylnitrosamine directly under non-acidic conditions should be possible, with a substituted phenoxide anion being lost as a by-product. Cohen et al. (26) have surveyed nitrosamine contamination in a variety of such pesticides, and have concluded on the basis of preliminary analyses that pesticides containing the five dinitroaniline derivatives studied generally contained N-nitroso impurities. It is possible that a mechanism such as the one depicted in Fig. 12 is responsible for at least some of the observed contamination. A similar mechanism is also conceivable for the s-triazines, although most of the pesticide formulations of this type which were analyzed appeared to be free of N-nitroso contamination (26).

Plans are being made to test this and related proposals in the near future.

Significance to Cancer Prevention

Since most nitrosamines are carcinogenic (27), scientists and administrators involved in the effort to prevent cancer should naturally be focussing on strategies for avoiding formation of these dangerous compounds in the human environment. Based on the considerations outlined in this paper, therefore, we recommend that, whenever possible, nitrite ion be prevented from coming into contact with immonium ion donors. The possible harm from swallowing aminopyrine into the protonating medium of the stomach (potential immonium ion-forming combination) with nitrite-containing saliva (28) has already been widely recognized, and the threat of dimethylnitrosamine exposure in users has led some governments to consider banning the drug.

It is further recommended that amine-nitrite mixtures be kept away from electrophilic carbonyl compounds and gem-dihalides capable of supporting immonium ion formation, for example in the preservation of amine-rich fish products with formaldehyde (6) or in the use of methylene chloride as an aerosol propellant (13). It also seems advisable to avoid storing treflan and related herbicides in the presence of

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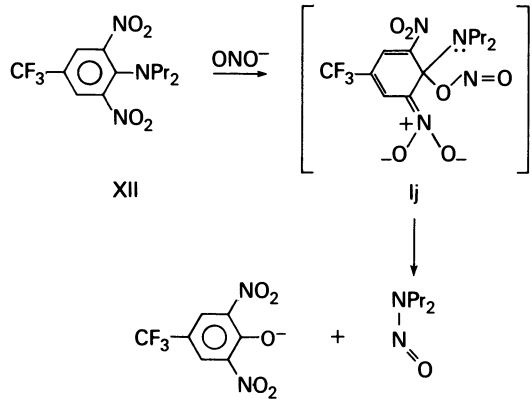

Figure 12. Proposed mechanism of treflan nitrosation

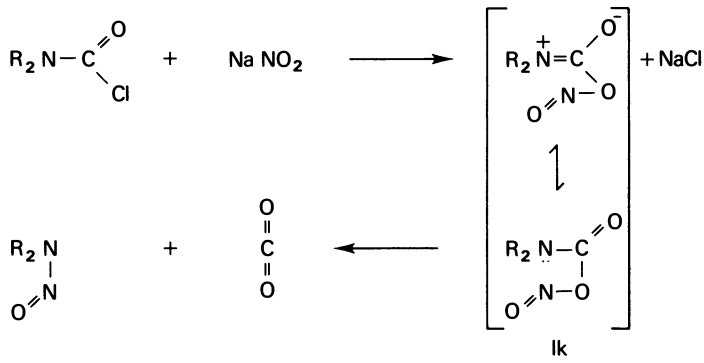

Figure 13. Possible mechanism of nitrosamine formation in the reaction of nitrite ion with N,N-*disubstituted carbamoyl chlorides (suggested by J.-P. Anselme)*

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nitrite, at least until planned experiments testing the viability of the mechanism in Fig. 12 can be completed.

Additional possible public health implications of the concepts presented in this paper will be published as they come to light.

Added in Proof

Thanks are due to the Editor of this volume for suggesting the similarity of N,N-disubstituted carbamoyl nitrites to the α-amino nitrite esters discussed above. When N,N-diphenyl carbamoyl chloride was refluxed with sodium nitrite in acetonitrile solution for 24 hours, N-nitrosodiphenylamine was produced in quantitative yield (M. Nakajima and J.-P. Anselme, unpublished results). The N,N-dibenzyl derivative underwent a similar reaction with nitrite. The mechanism shown in Fig. 13 was postulated to account for these transformations.

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Abstract

The involvement of α-dialkylamino nitrite esters as intermediates in a number of nitrosamine-forming reactions of interest in environmental carcinogenesis is postulated. Once formed, e.g. from nitrite and immonium ions, such species are proposed to fragment to nitrosamines and carbonyl compounds by way of intramolecular interaction between the nucleophilic amino function and the electropositive nitrosyl nitrogen atom. The fact that a variety of seemingly diverse transformations can be mechanistically unified by invoking this pathway is taken as evidence that α-dialkylnitrosamines do in fact have at least transitory existence, and that means of avoiding conditions conducive to their formation should be considered when developing comprehensive strategies for cancer prevention.

Acknowledgments

It is a pleasure to acknowledge the help of Dr. G. W. A. Milne in searching the literature for compounds related to those under discussion in this paper using the Structure And Nomenclature Substructure Search (SANSS) component of the NIH-EPA Chemical Information System (29). Important communications with Drs. J.-P. Anselme, S. S. Hecht, G. R. Krow, R. N. Loeppky, S. S. Mirvish, and S. R. Tannenbaum are also gratefully acknowledged.

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N-Nitrosamine Fragmentation and *N*-Nitrosamine Transformation

RICHARD N. LOEPPKY, C. THOMAS GNEWUCH,
LONNIE G. HAZLITT, and WAYNE A. McKINLEY

Department of Chemistry, University of Missouri—Columbia, Columbia, MO 65211

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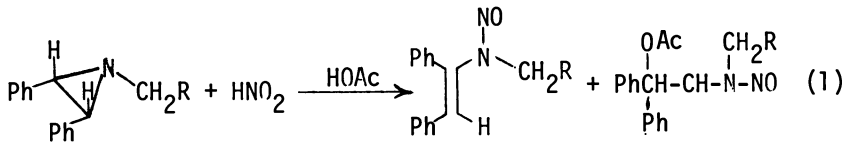
As a result of our previous work on the scope and mechanism of tertiary amine nitrosation (1), we became interested in the behavior of N-alkylaziridines toward nitrous acid. Possible modes of reaction are illustrated in Scheme 1. The operation of either path A or C would be consistent with our previous studies of oxidative dealkylation of tertiary amines (1), while pathway B would be akin to the observed cheleotropic transformation of N-nitrosoaziridines (2).

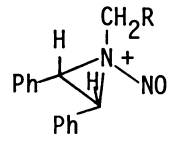
Our study, however, revealed that neither of these processes occurred. The nitrosation of 1-substituted aziridines leads to the nonoxidative ring opening shown in equation 1 (3). The stereo-



(1)

I II III

chemistry of the products II and III indicated that they were formed by either elimination or substitution on the nitrosaminium intermediate IV. While the nitrosation reaction of 1-substituted azir-



IV

idines is unlikely to be of much practical significance, it illustrates that tertiary amine nitrosation can occur by other routes

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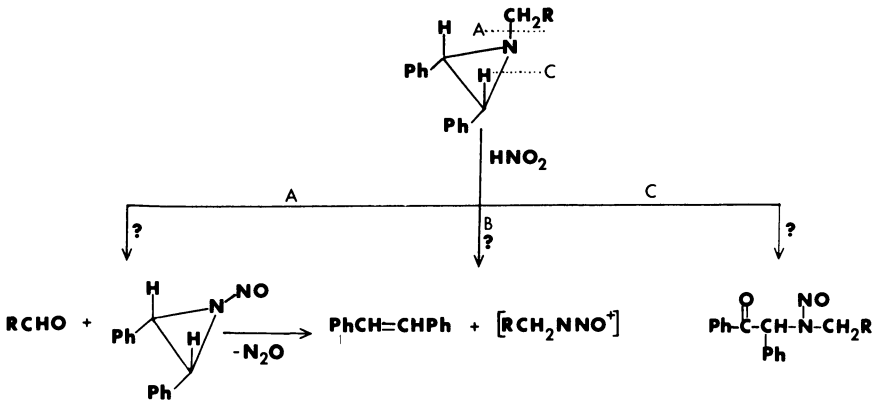
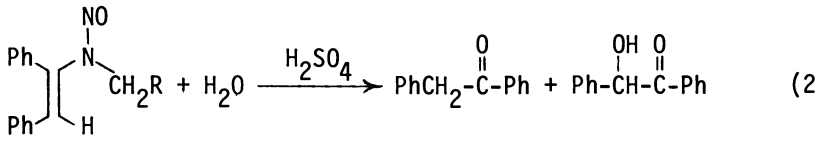


SCHEME 1

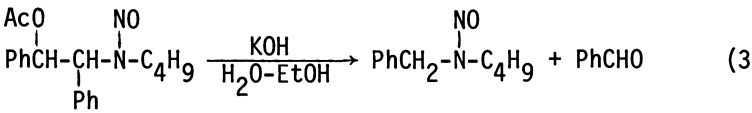
and warrants the attention of those who are interested in mechanisms of nitrosamine formation.

Both the nitrosoenamine II and the nitrosamino acetate III demonstrated some unusual chemistry which led us to the study of nitrosamine fragmentation reactions which will be discussed below. Treatment of the nitrosoenamine II with dilute sulfuric acid led to the formation of benzyl phenyl ketone as anticipated, but the major product from this reaction was benzoin, as is illustrated in equation 2 (3). While this transformation and the proper-



(2)

ties of vinyl nitrosamines are under active study in our laboratory, our principal thrust has been to investigate a reaction discovered during our attempted saponification of the nitrosaminoacetate III. Treatment of compound III (R = n-Pr) with 50% aqueous ethanol produced in high yield benzylbutylnitrosamine and benzaldehyde, as is illustrated in equation 3 (4). This interesting



(3)

IIIa

cleavage reaction is a property of the β-hydroxynitrosamines and a preliminary review of our work in this area is given below. Impetus for this work has been provided by the fact that several β-hydroxynitrosamines are prevalent environmental contaminants and by the possibility that this type of cleavage reaction could either potentiate or diminish the carcinogenicity of these nitrosamines.

Nature of the Reaction

The base-catalyzed cleavage of β-hydroxynitrosamines occurs as is shown in equation 4 to produce a smaller fragment nitrosamine and the carbonyl compound. This reaction is formally analogous to the retroaldol cleavage of a β-hydroxyaldehyde or ketone and as we will see below, appears to be mechanistically related to this transformation. We have demonstrated that this reaction occurs with a wide range of structurally variant β-hydroxynitrosamines (5). Table 1 lists the structure, yield, and reactivity estimate of the compounds that we have studied so far under the same conditions. A variety of conditions have been used to in-

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Table I

β-Hydroxynitrosamine Base Induced Fragmentation:
Products, Yields, Reaction Times, and Half-Lives.

Starting Compound	Product	% Yield	Time (h)	Estimated Half-Life
Va	VII	28	48	172 hours
	VI	8	48	-
Vb	VII	73	6	168 min.
Vc	VII	68	3.5	377 min.
Vd	VII	90	2	24 min.
Ve	VII	74	2	63 min.
Vf	VII	2	48	-
	Va	16	48	-
	VIII	3	48	-
	(IX)	6	48	-

Nitrosamine yields (%) from reactions according to equations (4), (7), and (8) are at specific analysis times. The reported yields are not the maximum yields. The estimated half-life is for the parent nitrosamine (left column) and assumes conversion to VII only. Reactions were conducted in tetrahydrofuran at 70°C. Substrate, 0.42 M; potassium t-butoxide, 0.56 M; t-butyl alcohol, 0.56 M. Half-life estimates are from rate constants obtained from first order plots which are linear in all cases. The word estimate is used because some reactions exhibited some heterogenity.

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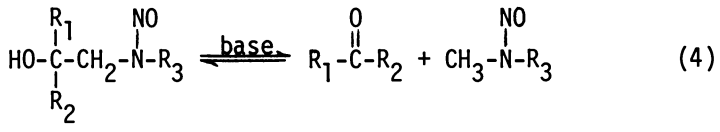


(4)

V

Compound	R_1	R_2	R_3
Va	H	H	CH_3
Vb	C_3H_7	H	CH_3
Vc	C_6H_5	H	CH_3
Vd	CH_3	CH_3	CH_3
Ve	C_6H_5	C_6H_5	CH_3
Vf	H	H	CH_2CH_2OH

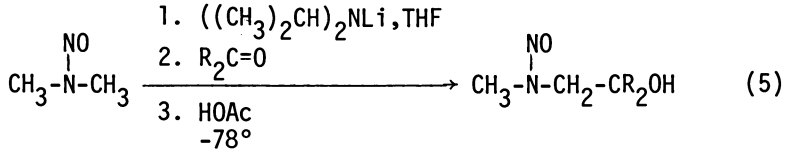
duce the transformations, although we have commonly employed alkoxides and alcohol solvents. Nitrosamine Vc has served as a model compound in evaluating different kinds of conditions which would result in this transformation. Although our study is incomplete at this time, the retroaldol cleavage of this nitrosamine can be brought about by base-solvent combinations ranging from potassium hydroxide in aqueous ethanol to t-butoxide in t-butyl alcohol. Temperatures have ranged from 25° to 150°. The cleavage of Vc did not proceed at a measurable rate in aqueous buffers up to pH 11 at 71°. It is important to note, however, that the coinjection of any of these compounds into a gas chromatographic port with potassium hydroxide brings about instantaneous fragmentation at the port temperature of 200°. This phenomenon can also be observed with much weaker bases. Repeated injection of a potassium acetate alcoholic solution of Ve into a gas chromatograph resulted, after a number of injections, in the fragmentation of this compound. We hypothesize that this occurred through the pyrolysis of the potassium acetate to potassium hydroxide which produced the fragmentation with facility at this temperature (6). We make note of this because at least one β-hydroxynitrosamine Vf (N-nitrosodiethanolamine) is a prominent constituent of water-based metal cutting and grinding fluids where tool-surface contact temperatures are high.

Because the strongly basic media is often hostile to the carbonyl compound produced in these transformations, it has not been characterized in all of our studies. The fate of the carbonyl compound, however, is not unimportant to the nature of the transformation. The fragmentation of Vc produces benzaldehyde which subsequently disproportionates by the Cannizaro reaction to give benzyl alcohol and benzoate (4). This process consumes base and requires the nitrosamine cleavage reaction to be run with a near-stoichiometric quantity of base. The study of the carbonyl compounds produced in the fragmentation of Vb demonstrated that the initially produced butanal entered into several expected and unex-

In N-Nitrosamines; Anselme, J.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

expected base-catalyzed transformations. Under the conditions of the transformation (ethoxide in ethanol at 70°), butanal underwent the expected aldol condensation followed by base-catalyzed dehydration to give the eight carbon, α,β-unsaturated aldehyde which was identified by GC-MS (4). This process is of note because it converts an alkoxide ion into a hydroxide ion. In some of the transformations studied, the hydroxide ion so produced does not appear to be a strong enough base to produce fragmentation of the nitrosamine at an appreciable rate. Thus, the retroaldol cleavage of the β-hydroxynitrosamine proceeds for a time, then slows down and stops as a weaker base is being produced in the medium. Somewhat unexpectedly, we found that butanal was converted in small yield to butanol and butanoate (an unusual Cannizaro reaction) under the conditions which were utilized for the fragmentation of Vb.

In Table 1, we have recorded the yields of fragment nitrosamine produced when the β-hydroxynitrosamine is treated with potassium t-butoxide in tetrahydrofuran (THF) at 70° (5). Reported yields are by no means maximal and the conditions were chosen so that we could compare the rates of cleavage of a number of different β-hydroxynitrosamines. By using an appropriate alkoxide-alcohol system, a nearly quantitative yield of fragment nitrosamine can be produced in this retroaldol type cleavage reaction. It is important to recognize that one of the factors which may affect the yield of these transformations is that the reaction is reversible (in theory). Seebach and Enders have developed an excellent synthesis for β-hydroxynitrosamines that is effectively the reverse of the transformation discussed here (7). The first step of this transformation, shown in equation 5, involves the re-



(5)

moval of the α-hydrogen of the nitrosamine with lithium diisopropylamide at -78°. The appropriate aldehyde or ketone is added at low temperatures and the resulting mixture neutralized with acetic acid also at -78°. This procedure results in the production of the β-hydroxynitrosamine in high yields. We have utilized a variation of this procedure to produce the nitrosaminoalcohols Vc and Ve. The carbonyl compound and dimethylnitrosamine are introduced to a THF solution containing potassium t-butoxide at 0° and stirring for an hour or two effects the condensation of these substances (6). An increase in the temperature of the reaction medium markedly decreases the yield of the condensation product. The reversibility of the retroaldol cleavage reaction of Vc was demonstrated by allowing dimethylnitrosamine and benzaldehyde to

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condense in the THF containing t-butyl alcohol and potassium t-butoxide in a 1:1 ratio. After the presence of Vc was demonstrated in this reaction mixture by HPLC, sodium borohydride was added to reduce benzaldehyde to the corresponding alcohol. Under these conditions there is a complete reversion of the reaction with all of the Vc thus formed being reconverted into dimethylnitrosamine. We have determined an equilibrium constant of 400 M/L for the fragmentation of Ve at 30° in dimethylsulfoxide (6). The reversible nature of this transformation is a matter of considerable further study in our laboratory.

Although our data is of a very preliminary nature, it is evident from an inspection of Table 1 that the rate of fragmentation of β-hydroxynitrosamines is very dependent on the structure of the compound (5). If we consider aliphatic nitrosamines Va, Vb and Vd, the rate of transformation increases markedly as we go from primary to secondary to tertiary alcohol function. This is just opposite of the expected solution acidity of these nitrosaminoalcohols. This behavior is also observed when R_1 and R_2 are phenyl groups. The tertiary alcohol Ve fragments more rapidly than does the secondary alcohol Vc. These results suggest that the cleavage reaction rate may be correlated with the heat of formation (C=O bond energy) of the incipient carbonyl product. Furthur work is underway and should provide a better understanding of this phenomenon.

The nature of the base catalysis of this transformation is also currently under active study in our laboratory. As one might anticipate, the rate constant for the fragmentation of Vd in t-butyl alcohol containing t-butoxide is proportional to the first power of the base concentration (8). On the other hand, treatment of a 0.2 M THF solution of Ve with 0.01 M potassium t-butoxide (no t-butyl alcohol) led to the observation of a base-catalyzed fragmentation, the rate constant of which was not linearly dependent upon the base concentration (6). In contrast to this observation, the relationship between the base concentration and the observed rate constant for the fragmentation of Ve in t-butyl alcohol containing potassium t-butoxide is a complex, nonlinear function of the base concentration with the general observation that the rate constant increases as the base concentration decreases (6). This may be either due to a competitive ionization of the C-H adjacent to nitrogen or an unusual media affect resulting from a high electrolyte concentration in t-butyl alcohol.

Although our investigation of the mechanism of this transformation is incomplete at present, our data are consistent with the view that the fragmentation is an example of the retroaldol cleavage of a β-hydroxynitrosamine as depicted in Scheme 2. Such a hypothesis requires that the α-nitrosamino carbanion possess a stability similar to that of an enolate ion. Keefer and Fodor's discovery of the acidity of the α-hydrogen of the nitrosamino function (9) and Seebach and Ender's extensive utilization of this fact in organic synthesis (7) adequately substantiate this point.

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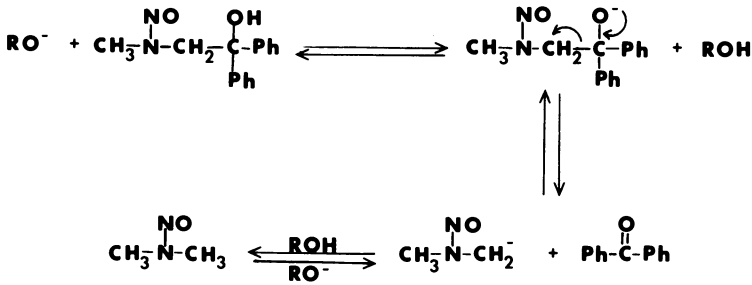
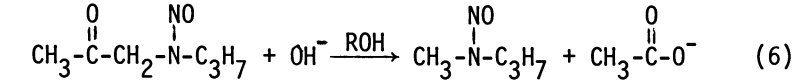


SCHEME 2

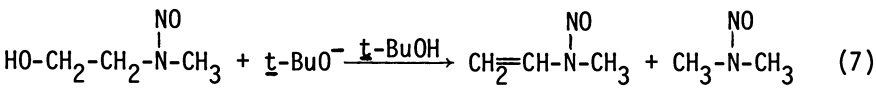
If the fragmentation of the alkoxide is rate-determining and endothermic, one would expect the transition state structure to resemble the products and thence be influenced by the relative stability of the carbonyl fragment. A transformation similar to the retroaldol cleavage of β-hydroxynitrosamines has been observed. Kruger reported that the treatment of 2-ketopropylpropylnitrosamine with refluxing potassium hydroxide in alcohol led to the production of methylpropylnitrosamine as is illustrated in equation 6 (10). This transformation is also under study in our laboratory.



(6)

Side Transformations

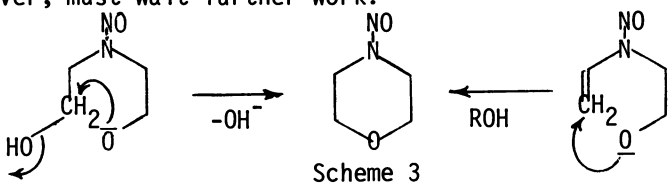
In the course of our study of the fragmentation of β-hydroxynitrosamines, we have encountered several other significant side-reactions of these compounds. Treatment of 2-hydroxyethylmethylnitrosamine Va with potassium t-butoxide and t-butyl alcohol not only gave dimethylnitrosamine but produced methylvinylnitrosamine as well (equation 7) (8). This base-catalyzed elimination of the



(7)

Va VI VII

hydroxide ion is akin to the similar transformation of the β-hydroxycarbonyl compound and undoubtedly results from the acidity of the hydrogen alpha to the nitrosamino function. Although the overall conversion is relatively low (36%), the relative yield of VI is 22%. Treatment of N-nitrosodiethanolamine Vf with potassium t-butoxide in THF led to the formation of N-nitrosomorpholine VIII, and methylvinylnitrosamine VI, in addition to the expected products of the retroaldol fragmentation, Va and VII (equation 8) (11). 2-Hydroxyethylvinylnitrosamine IX could give VI by fragmentation, but conclusive evidence as to its intermediacy is lacking at this date. N-nitrosomorpholine could be formed in this transformation either by nucleophilic displacement of hydroxide or by intramolecular addition through the intermediacy of IX as is depicted in Scheme 3. A clearer understanding of these transformations, however, must wait further work.



Scheme 3

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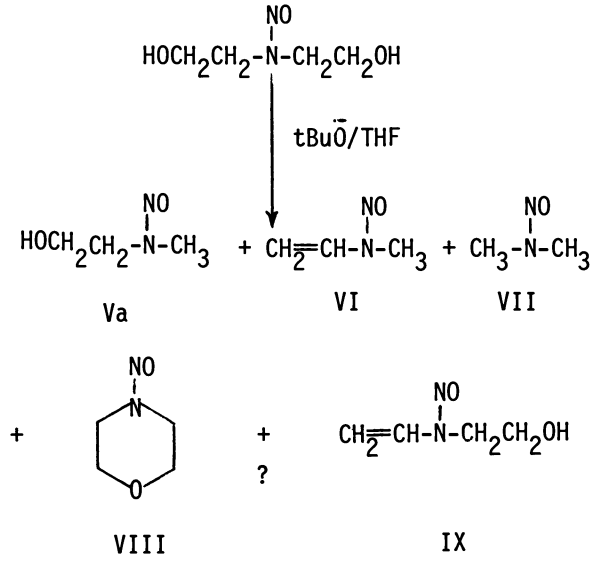

(8)

Va VI VII

VIII IX

Possible Relevance to Nitrosamine Carcinogenesis

Although there have been numerous reports of biochemical transformations of one nitrosamine into a structurally varied nitrosamine, there have been few reports of chemical alterations which change the carbon skeleton of the nitrosamine. A notable exception is the decarboxylation of α-nitrosamino acids (12,13). Such transformations, however, are of considerable importance in nitrosamine carcinogenesis because they may significantly alter the carcinogenicity of a nitrosamine. An example is the conversion of the noncarcinogenic N-nitrosoproline into N-nitrosopyrrolidine upon thermal decarboxylation (12,13). N-nitrosodiethanolamine Vf has been found to occur in metal cutting and grinding fluids in amounts up to 3% (14). Should it undergo retroaldol fragmentation under the conditions of its inadvertent employment in such cutting and grinding fluids, the result would be a significant increase in the carcinogenic hazard. Although N-nitrosodiethanolamine is one of the least carcinogenic of the carcinogenic nitrosamines (15), products from its reactions with alkoxides, namely dimethylnitrosamine and N-nitrosomorpholine, are known to be potent carcinogens (15). Although the carcinogenicity of 2-hydroxyethylmethylnitrosamine has not been reported to date, we may hypothesize from data on structurally similar compounds that it, too, will be more carcinogenic than its progenitor. We have demonstrated that various β-hydroxynitrosamines do fragment at high temperatures with great facility and without the requirement of an alkoxide. We have determined that the reservoir temperature of metal grinding fluids varies between 20° and 100°, depending

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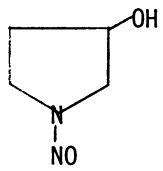
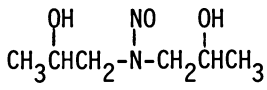
upon the application, and is obviously much higher at the metal cutting surface (11). For this reason, we believe that consideration should be given to the hypothesis that N-nitrosodiethanolamine can be converted to products like those depicted in equation 8 under the conditions of its inadvertent employment in industry. We are currently engaged in a program of analysis of metal cutting and grinding fluids to determine whether substances such as Va, VI, VII, VIII, and IX may be found there in addition to N-nitrosodiethanolamine.

Several other β-hydroxynitrosamines have been or are likely to be found in environmental samples. Among these are N-nitroso-3-hydroxypyrrolidine X (13) (found in bacon), N-nitrosobis(2-hydroxypropyl)amine XI (a potent pancreatic carcinogen in hamsters, the amino progenitor of which is used in many of the same applications as diethanolamine) (16), and the N-nitroso derivatives of the common drugs ephedrine XII (17) and ethambutol XIII (18), both of which have been shown to be carcinogenic.

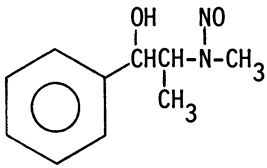
A review of the literature shows that alkaline conditions have often been used in the isolation and analysis of nitrosamines in food stuffs and in other natural samples (19,20). We considered it possible that these conditions which involve digestion of the sample with alcoholic hydroxides could produce fragmentation



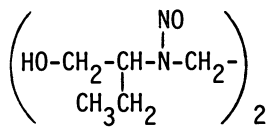
X



XI



XII



XIII

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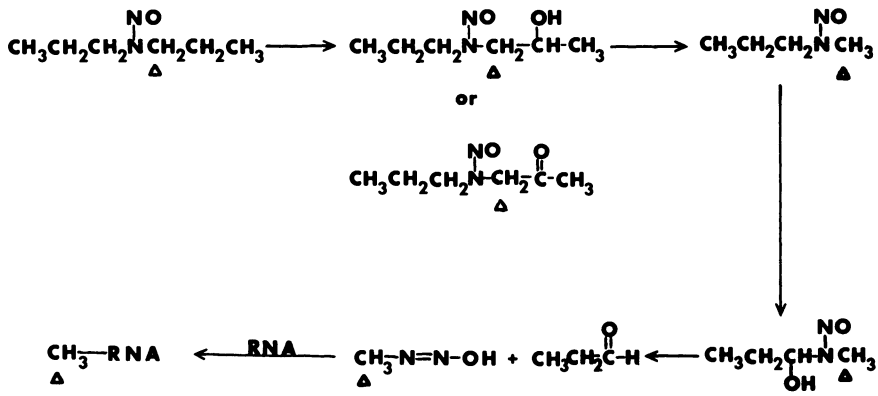

SCHEME 4

of a β-hydroxynitrosamine were it in the sample. We have performed one preliminary experiment in this connection and report it here because of its perceived importance. Treatment of Vc with sodium hydroxide in methanol as described by Havery, et al. (19) for the isolation of volatile nitrosamines from food stuffs resulted in the formation of dimethylnitrosoamine in 5% yield. While further work on the applications and generality of this observation is underway in our laboratory, it is clear that strong basic media should be avoided in the design of analytical procedures for the analysis of nitrosamines of unknown type.

Kruger demonstrated (21) that the RNA isolated from rat liver after feeding of α-carbon labeled dipropylnitrosamine contained methyl groups bearing the radio-label. In further experiments he showed that this result was also obtained when 2-hydroxypropylpropylnitrosamine, a known metabolite of dipropylnitrosamine, was administered to rats (10). Before his death, Kruger hypothesized that this might occur by β-oxidation and cleavage similar to fatty acid degradation. In recent experiments, Preussmann (22) has shown that methyl fragments are the principal nucleic acid alkylating moieties derived from carcinogenic nitrosamines bearing no N-methyl groups. The work of Kruger and Preussmann, as well as our own findings, has led us to propose that there may be a biochemical retroaldol fragmentation type reaction of β-hydroxynitrosamines. This hypothesis is delineated in Scheme 4 which accounts for the origin of methyl fragments in the nucleic acid which have been alkylated as a result of dipropylnitrosamine ingestion. The nitrosamine is first β-hydroxylated by mixed function oxidase. This is followed by a biochemical retroaldol cleavage to yield methylpropylnitrosamine. α-Hydroxylation of the methylpropylnitrosamine at the α-carbon of the propyl group will yield a methylating species which can attack the nucleic acids. Support for this hypothesis is found in the work of Blattmann (23) who recently demonstrated that propanal is produced on the metabolism of dibutylnitrosamine. The validity of this hypothesis is also being investigated in our laboratories.

In conclusion, we demonstrate by this preliminary review of our work that the base-catalyzed fragmentation of β-hydroxynitrosamines is a general transformation. The rate of the transformation is a significant function of structure as well as the catalyst and the conditions. Several other base-catalyzed transformations of nitrosamines have been observed and all of these reactions are expected to be of significance in the area of nitrosamine carcinogenesis.

Note: All nitrosamines not known to be otherwise should be treated in the laboratory as potent carcinogens and handled in accordance with NCI and OSHA guidelines.

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Tobacco Specific *N*-Nitrosamines: Occurrence, Carcinogenicity, and Metabolism

STEPHEN S. HECHT, CHI-HONG B. CHEN, G. DAVID McCOY, and DIETRICH HOFFMANN

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY 10595

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It is now widely accepted that cigarette smoking causes lung cancer (1,2). It is less widely known that smoking is also correlated with an increased incidence of cancer of the oral cavity, esophagus, pancreas and bladder (2,3,4,5,6). Tobacco chewing can also cause oral cavity and esophageal cancer (3,4,7). In fact, oral cavity cancer is a major cancer among men in India, where the habit of chewing the betel quid containing tobacco is widespread (8). Cigarette smoke is known to contain tumor initiators such as the polynuclear aromatic hydrocarbons, and tumor promoters and cocarcinogens, such as catechol (9). These agents can explain many of the observed effects of cigarette smoke condensates in experimental animals and almost certainly are involved in some of the human cancers associated with smoking. However, nitrosamines may also be causative factors in the tobacco related cancers, especially in those organs which are remote from direct contact with tobacco or tobacco smoke. Thus it is known that nitrosamines can cause esophageal, pancreas and bladder cancer in experimental animals, as well as affecting the lung and oral cavity (10,11,12).

Since tobacco and tobacco smoke have specific carcinogenic effects in man, it is tempting to speculate that there may be unique carcinogenic agents in tobacco and tobacco smoke. The tobacco specific nitrosamines are such a group. These nitrosamines are derived from the tobacco alkaloids (see Figure 1). The most prevalent alkaloid is nicotine, which occurs in general in concentrations of 1-2% in commercial tobacco products. Both nicotine and nornicotine could give rise to the prototype of tobacco specific nitrosamines, N'-nitrosonornicotine (NNN). Nicotine could also be nitrosated to form 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)butanal (NNA). In addition, N-nitrosopyrrolidine (NPy) could also be derived from nicotine and nornicotine. Nitrosation of anabasine would give nitrosoanabasine (NAB). The structures of these nitrosamines, which will be considered in this review, are shown in Figure 2. Of course, inspection of Figure 1 reveals other interesting possibilities for nitrosation of the tobacco

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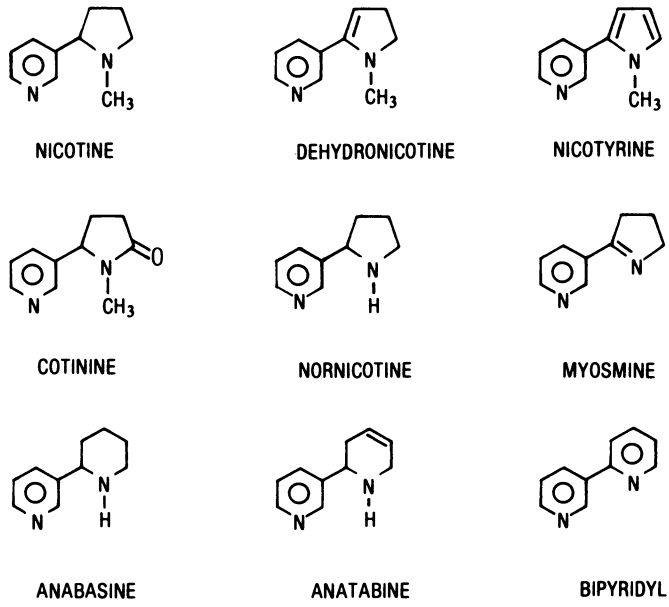

Figure 1. Common tobacco alkaloids in tobacco and/or tobacco smoke

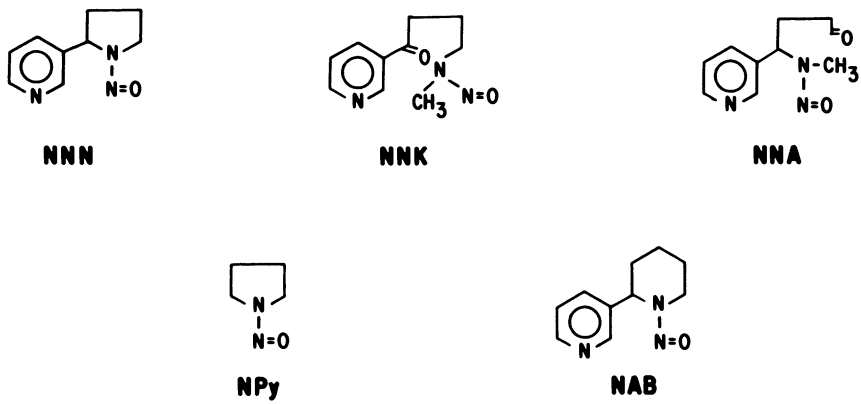

Figure 2. Some nitrosamines which can be derived from the tobacco alkaloids

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alkaloids; some of these will be the subject of future studies.

Occurrence And Formation Of Tobacco Specific Nitrosamines

The prototype of the tobacco specific nitrosamines, NNN, has been detected in both tobacco smoke and unburned tobacco. Various analytical methods have been used including gas chromatography (GLC) (13,14,15,16) combined GLC-mass spectrometry (17), thin layer chromatography (18), high pressure liquid chromatography (HPLC) (19,20), and combined HPLC-thermal energy analysis (21). NNN levels in cigarette smoke typically range from 140-240 ng/cig in a typical American 85mm non-filter cigarette. Surprisingly high levels of NNN were found in unburned tobacco (0.3-9.0 ppm in cigarette tobacco, 3.0-45.3 ppm in cigar tobacco, 3.5-90.6 ppm in chewing tobacco, and 12.1-29.1 ppm in snuff). These levels are among the highest for an environmental nitrosamine in terms of occurrence and human exposure (22). Thus, rather detailed studies were carried out to determine the origins of NNN in tobacco and tobacco smoke.

To study the formation of NNN in tobacco, plants were analyzed at various stages of growth and curing (23). NNN was not detected prior to harvest or in freshly harvested Burley tobacco but only during and after air curing (0.5-1.1 ppm). Since either nicotine or nornicotine could have been a precursor to NNN in tobacco, tobacco leaves were fed nicotine-2'-^{14}C or nornicotine-2'-^{14}C and cured (24). The cured leaves were then analyzed for NNN-2'-^{14}C. The yield of NNN from nicotine was 0.009% and from nornicotine, 0.007%. These results showed that both nicotine and nornicotine could be precursors to NNN in tobacco. However, the greater abundance of nicotine in tobacco leaf (20-100 times the concentration of nornicotine) favored nicotine as the major precursor of NNN in tobacco.

The transfer of NNN from cigarette tobacco to mainstream smoke was studied (20). For this purpose, NNN-2'-^{14}C was added to cigarettes and the smoke was analyzed. The transfer rate was found to be 11.3%. Since, in this experiment, the tobacco column smoked contained 974 ng NNN, 110 ng were transfered to the mainstream smoke. Analysis of the mainstream smoke revealed 238 ng NNN; thus the remaining 128 ng were formed during smoking. Therefore, about 50% of the NNN in mainstream smoke originated by transfer from tobacco while the remainder was formed during smoking.

Either nicotine or nornicotine could be a precursor to NNN formed during smoking. To examine this question, nicotine or nornicotine was added to cigarettes and the smoke was analyzed for NNN (13). In each case, NNN concentration in smoke increased indicating that both alkaloids are precursors to NNN formed during smoking. However, nicotine is considered the more important precursor due to its higher concentration in tobacco. The results of these studies on the formation of NNN during curing, its

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transfer to smoke, and its formation during smoking are summarized in Figure 3.

In tobacco samples examined so far, the levels of NAB were significantly less than those of NNN. In fact, NAB has not yet been detected with certainty in unburned tobacco (15). These findings are in line with the major role of nicotine rather than nornicotine as a precursor to NNN since kinetic studies showed that nornicotine and anabasine were nitrosated at similar rates (25). These rates are relatively high, which suggests that the formation of NNN and NAB could be favored *in vivo*. When chewing tobacco was incubated with human saliva for 3 hours at 37° and the mixture analyzed for NNN, the concentrations of NNN increased by 44% over that in the chewing tobacco, presumably as a result of further nitrosation (15). Thus, *in vivo* formation of NNN and NAB could constitute an additional exposure of smokers or chewers to these tobacco specific nitrosamines.

Since nicotine is the major precursor to NNN in tobacco and tobacco smoke, the reaction of nicotine with sodium nitrite was studied to provide information on formation of other tobacco specific nitrosamines, especially NNK and NNA, which could arise by oxidative cleavage of the 1'-2' bonds or 1'-5' bond of nicotine followed by nitrosation (26). The reaction was investigated under a variety of conditions as summarized in Table I. All three nitrosamines were formed when the reaction was done under relatively mild conditions (17 hrs, 20°). The yields are typical of the formation of nitrosamines from tertiary amines (27). At 90°, with a five fold excess of nitrite, only NNN and NNK were detected. Under these conditions, both NNK and NNA gave secondary products. NNK was nitrosated α to the carbonyl to yield 4-(N-methyl-N-nitrosamino)-2-oximino-1-(3-pyridyl)-1-butanone while NNA underwent cyclization followed by oxidation, decarboxylation and dehydration to give 1-methyl-5-(3-pyridyl)pyrazole, as shown in Figure 4. Extensive fragmentation and oxidation of the pyrrolidine ring was also observed under these conditions. The products of the reaction of nicotine and nitrite at 90° are summarized in Table II.

The formation of NNN, NNK, and NNA from nicotine probably involved the intermediacy of cyclic iminium salts, as shown in Figure 5 (28). These salts can undergo hydrolysis to the free amines which are nitrosated, or at near neutral pH, can be directly nitrosated to give nitrosamines. The formation of nitrosamines from iminium salts under neutral conditions has been demonstrated in at least two studies and is of interest because iminium salts are known to be intermediates in the mammalian metabolism of nicotine (26,29,30,31). The possibility that tobacco bacteria could nitrosate nicotine via this pathway is currently under investigation.

The formation of NNK and NNA from nicotine in these model studies encouraged us to search for these nitrosamines in tobacco and tobacco smoke. In studies undertaken so far, NNK but not NNA

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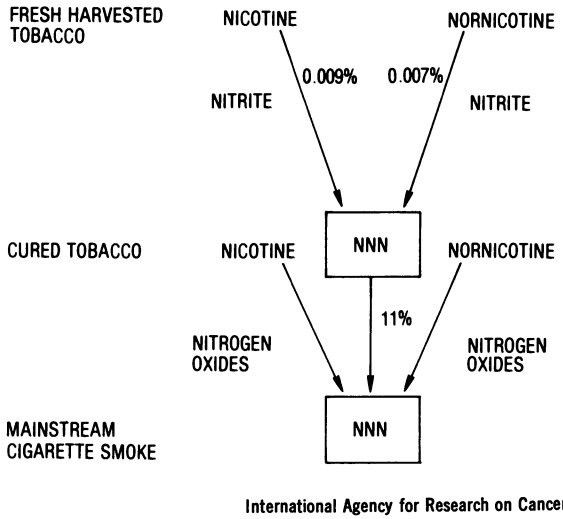

Figure 3. Origins of NNN in tobacco and tobacco smoke (22)

Table I

Formation of NNN, NNK, and NNA from Nicotine and $NaNO_2$

$[NaNO_2]$ / [Nicotine]	Conditions pH[b]	T(°C)	t(hrs)	Yields (%)[a] NNN	NNK	NNA
1.4	2.0	20	17	0.1	ND[c]	0.2
1.4	3.4	20	17	0.5	0.1	2.8
1.4	4.5	20	17	0.5	0.5	2.3
1.4	7.0	20	17	0.2	0.1	0.1
5.0	3.4-4.2	90	0.3	8.0	0.7	ND
5.0	3.4-4.2	90	3.0	8.8	2.3	ND
5.0	3.4-4.2	90	6.0	8.0	1.5	ND
5.0	5.4-5.9	90	0.3	9.0	2.7	ND
5.0	5.4-5.9	90	3.0	13.5	4.3	ND
5.0	5.4-5.9	90	6.0	11.7	2.6	ND
5.0	7.0-7.3	90	0.3	1.3	0.1	ND
5.0	7.0-7.3	90	3.0	4.5	0.2	ND
5.0	7.0-7.3	90	6.0	5.5	0.2	ND

[a]Determined by GC and based on starting nicotine.

[b]Buffer systems: pH2, KCl-HCl; pH 3.4-7, citrate-phosphate.

[c]ND=not detected.

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Table II

Products Formed in the Reaction of Nicotine and $NaNO_2$ [a,b]

Product	Yield (%)[c]	Method of Identification[d]
NNN	8.8	A
NNK	2.3	A
4-(N-methyl-N-nitrosamino)-2-oximino-1-(3-pyridyl)-1-butanone	4.0	C,A
1-methyl-5-(3-pyridyl)-pyrazole	2.1	C
cis and *trans* 3-Pyr-CH=CHCN	19.0	A
3-Pyr-$CONHCH_3$	6.2	B
3-Pyr-COOH	4.0	B
cotinine[e]	0.6	A
3-Pyr-CH=CH-COOH	0.5	B
3-Pyr-$COCH_3$	0.5	B
3-Pyr-CN	0.5	B
3-Pyr-CO_2CH_3	0.3	B
3-Pyr-CHO	0.2	B
myosmine[f]	0.1	A
3-Pyr-CH_2CN	0.1	B

[a] Reaction of 1 equivalent nicotine with 5 equivalents $NaNO_2$ at 90°, 3 h, pH 3.4-4.2

[b] 15-25% nicotine was unreacted.

[c] Based on starting nicotine.

[d] A; comparison of GC or HPLC retention times and mass spectra to independently synthesized standards; B, comparison to commercially available standards; C, spectral properties.

[e] 1-methyl-5-(3-pyridyl)-2-pyrrolidinone.

[f] 2-(3-pyridyl)-1-pyrroline.

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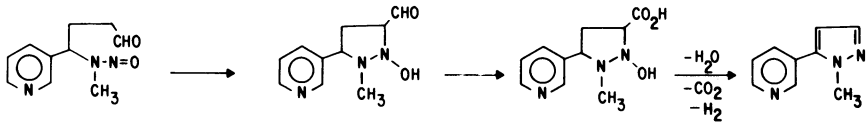

Figure 4. Formation of 1-methyl-5-(3-pyridyl)pyrazole from NNA

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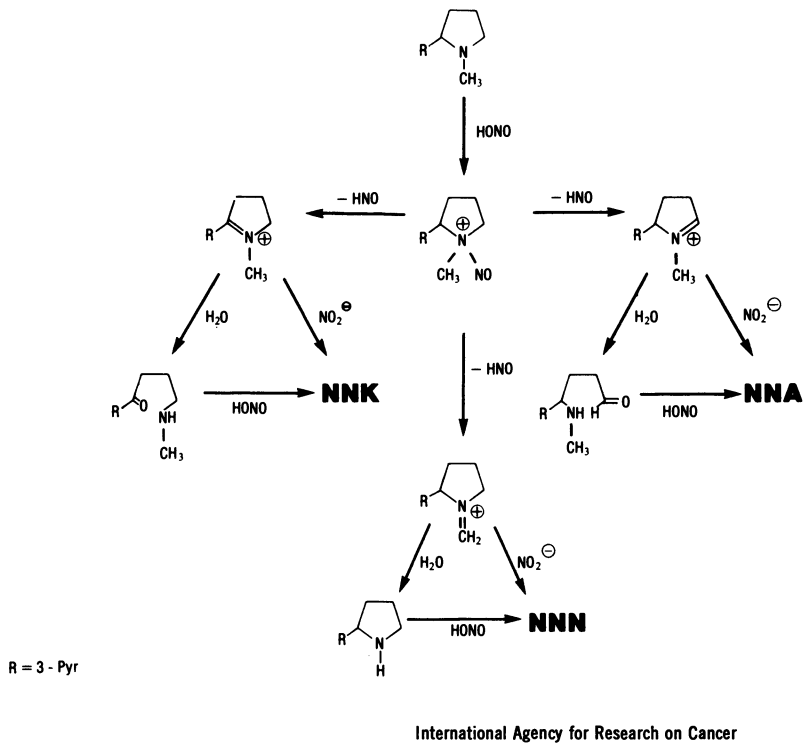

Figure 5. Formation of tobacco specific nitrosamines from nicotine (22)

has been detected. NNK was most readily analyzed by combined HPLC-TEA, although conventional HPLC methods have also been used (21,24). Levels of NNN and NNK in tobacco and mainstream cigarette smoke are summarized in Table III. During these studies by HPLC-TEA, we also identified N'-nitrosoanatabine in tobacco (0.44-3.2 ppm) and mainstream (0.33-4.6 μg/cig)' and sidestream cigarette smoke (0.15-1.5μg/cig). The analytical studies on NNN discussed in this section were done using NNN-2'-^{14}C as internal standard. Tobacco was extracted with aqueous ascorbic acid and smoke was collected in traps containing ascorbic acid to prevent artifactual formation of nitrosamines.

Table III

Non-Volatile N-Nitrosamines in Tobacco And Tobacco Smoke

Product	Mainstream (μg/cig) NNN	NNK	Sidestream (μg/cig) NNN	NNK	Tobacco (ppm) NNN	NNK
Burley,NF	3.7	0.32	6.1	0.66	7.0	N.D.
Bright,NF	0.62	0.42	1.7	0.50	0.22	0.37
Commercial,NF	0.24	0.11	1.7	0.41	1.7	0.74
Commercial,F	0.31	0.19	0.15	0.19	1.4	0.70
Kentucky,1R1,NF	0.39	0.16	0.21	0.24	0.63	0.13
Little Cigar,F	5.5	4.2	0.88	0.81	45.3	35.4
Columbia Cigar (5.7g)	3.2	1.9	16.6	15.7	10.7	1.1

N.D. = Not detected

Carcinogenicity Of Tobacco Specific Nitrosamines

The earliest studies on the carcinogenicity of NAB and NNN were done by Boyland and co-workers, who demonstrated that NAB caused esophageal tumors in rats and that NNN induced lung adenomas in mice (32,33). NAB was administered to rats in the drinking water (total dose, 7.9-11.5 mmoles/rat) and 25 of 32 rats treated developed tumors of the esophagus with the tumors appearing after 50-70 weeks of treatment. NNN was injected in mice (total dose, 0.5 mmol/mouse) and 7 of 40 mice developed pulmonary adenomas, compared to 1 of 30 mice in the control groups.

In our own studies, the carcinogenicity of NNN and NAB was first compared in male Fischer rats (34). Each compound was administered in the drinking water for 30 weeks (total dose; 3.3 mmoles NAB, 3.6 mmoles NNN/rat) to a group of 20 animals. After 48 weeks, the experiment was terminated. In the NNN group, 14 of 20 animals developed tumors; these were mainly esophageal papillomas and carcinomas. One pharyngeal tumor and 3 nasal cavity carcinomas were also observed. By contrast, NAB at this dose gave only 2 of 20 tumor bearing animals. Thus NNN was a moderately

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active carcinogen whereas NAB was only weakly active. The lower tumor yield for NAB in this experiment compared to Boyland's work was probably due to the lower dose of NAB and the shorter lifetime of the animals.

The carcinogenicity of NNN in Sprague-Dawley rats was examined by Singer and Taylor (35). NNN was given in the drinking water for 44 weeks (total dose 8.8 mmoles/rat) to a group of 15 female rats. All the rats were dead by 46 weeks and all 15 animals had adenocarcinomas of the olfactory epithelium. In a parallel study, the carcinogenicity of NPy was examined in male and female rats of the same strain (36). NPy was added to the drinking water for 50 weeks (total dose, 10.0 mmoles/rat). The females were dead after 85 weeks and the males, after 104 weeks. NPy induced hepatocellular tumors in 13 of 14 males and in 14 of 15 females. Thus NNN was a stronger carcinogen than NPy, when judged by time until death. However, the target organs were different in each case.

The tumorigenic activities of NNN and NAB were also compared in Syrian Golden hamsters (37). In this experiment, NNN and NAB were each given by subcutaneous injection for a period of 25 weeks (total dose; 2 mmoles/hamster). Within 83 weeks, 12 of 19 hamsters given NNN developed tracheal tumors and 1 had a carcinoma of the nasal cavity. In the same period, none of the animals treated with NAB developed tumors. Nitrosopiperidine was included as a positive control and induced tracheal tumors in all the animals after a total dose of 1.3 mmole/hamster. Thus, substitution of a pyridine ring adjacent to the ring nitrogen of nitrosopiperidine to give NAB resulted in a significant reduction in carcinogenic activity; this effect was also observed in Boyland's experiments on rats (32). Such an effect was not observed when NNN and NPy were compared (35,36). This is of interest when considering the mechanism of action of these compounds.

The tumorigenic activities of NNN, NNK, and NNA were compared in strain A mice (24). Each compound was injected over a period of seven weeks with a total dose of 0.1 mmole/mouse. For reasons of solubility, NNK was injected as a suspension in trioctanoin while NNA was injected in saline. For comparison, NNN was injected both in saline and trioctanoin. The positive control was urethan. The results are summarized in Table IV. As judged by multiplicity of lung tumors, both NNN and NNK showed significant activity ($P < 0.05$) compared to controls and NNK was significantly more active ($P < 0.05$) than NNN. NNA did not show significant tumorigenic activity. The greater tumorigenicity of NNK than NNN in this strain of mice is indicative of potentially higher carcinogenicity in other rodent species; these bioassays are currently in progress.

Metabolic Studies On NPy, NNN, and NNK

Nitrosamines, like many other classes of chemical carcinogens must undergo metabolic transformation to be converted into elect-

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Table IV

Bioassay in Strain A Mice of Nitrosamines Derived from Nicotine

Experimental Group	Effective No. of Animals	Lung Adenoma Bearing Animals	% Lung Adenoma Bearing Animals	Lung Adenomas per Animal	Others
1. Untreated Control	25	1	4	0.04	
2. Vehicle Control (Saline)	25	3	12	0.24	
3. Vehicle Control (trioctanoin)	24	5	21	0.20	
4. Urethane in Saline	25	25(6)*	100	14.80	
5. NNN in Saline	21	16	76	1.74	Undifferentiated Carcinoma of Salivary Glands 1 (Metastasis: Lungs, Pleura)
6. NNN in Trioctanoin	23	12(1)*	57	0.87	Undifferentiated Carcinoma of Salivary Glands 1, Malignant Lymphoma 1
7. NNA in Saline	25	9	36	0.44	
8. NNK in Trioctanoin	23	20	87	2.61	

*( ) Adenocarcinoma

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rophilic species which can alkylate nucleophilic cellular macromolecules. This process was termed metabolic activation by the Millers who were pioneers in developing these concepts. According to their scheme, an inactive procarcinogen is metabolically transformed to a proximate carcinogen and finally to an ultimate carcinogen; the latter is a reactive electrophile such as a carbonium ion (38). Such a scheme can be applied to dialkyl or cyclic nitrosamines in several ways and various critical initial steps have been suggested including α-hydroxylation, β-hydroxylation, and δ-oxidation (39,40,41). Since the intermediates generated metabolically may be unstable, indirect means have been used to gain evidence supporting the various pathways. Most studies to date on both cyclic and acyclic nitrosamines support the hypothesis that an initial α-hydroxylation is a critical step in carcinogenesis by nitrosamines. For cyclic nitrosamines, substitution at the α-positions often results in decreased carcinogenicity, as demonstrated in studies by Lijinsky, Keefer, and Taylor. For example, 2,5-dimethyl-NPy was significantly less carcinogenic in the rat than an equimolar dose of NPy (36). Similar results were obtained with nitrosopiperidine (42). Substitution of deuterium atoms α- to the nitrosamine function of nitrosomorpholine decreased activity. Thus, 3,3,5,5-tetradeuteronitrosomorpholine was less carcinogenic than nitrosomorpholine (43). This reduction in activity was consistent with the slower rate of C-D bond breaking in α-hydroxylation of the deuterated compound. α-Acetoxynitrosamines have been used as model compounds for unstable α-hydroxynitrosamines in studies by several groups (44,45,46). In the case of NPy, the mutagenicity of α-acetoxyNPy towards *S. typhimurium* provided further evidence supporting α-hydroxylation as an activation step (47). Until recently, however, limited information was available on the metabolic α-hydroxylation of cyclic nitrosamines (48,49,50). This was due, in part, to the inherent instability of the α-hydroxynitrosamines. In our studies on the metabolism of cyclic nitrosamines, we have used model compounds to determine the probable products of metabolic α-hydroxylation and have then searched for these products as metabolites. In this way, metabolic α-hydroxylation of NPy and NNN was demonstrated (51,52).

Our approach for NPy is outlined in Figure 6. α-Hydroxylation of NPy would give α-hydroxyNPy which is expected to undergo spontaneous ring opening to 3-formyl-1-propanediazohydroxide; this intermediate would lose N_2 and hydroxide to give an oxocarbonium ion. This oxocarbonium ion could react with cellular macromolecules as well as being trapped by water to give 4-hydroxybutyraldehyde. The latter exists predominantly as the cyclic hemiacetal, 2-hydroxytetrahydrofuran. To validate this hypothetical scheme, α-acetoxyNPy and 4-(N-carbethoxy-N-nitrosamino)butanal were synthesized as precursors to the unstable intermediates resulting from α-hydroxylation of NPy as shown in Figure 6. α-AcetoxyNPy was prepared according to a previously described procedure (47) and 4-(N-carbethoxy-N-nitrosamino)butanal was synthesized as

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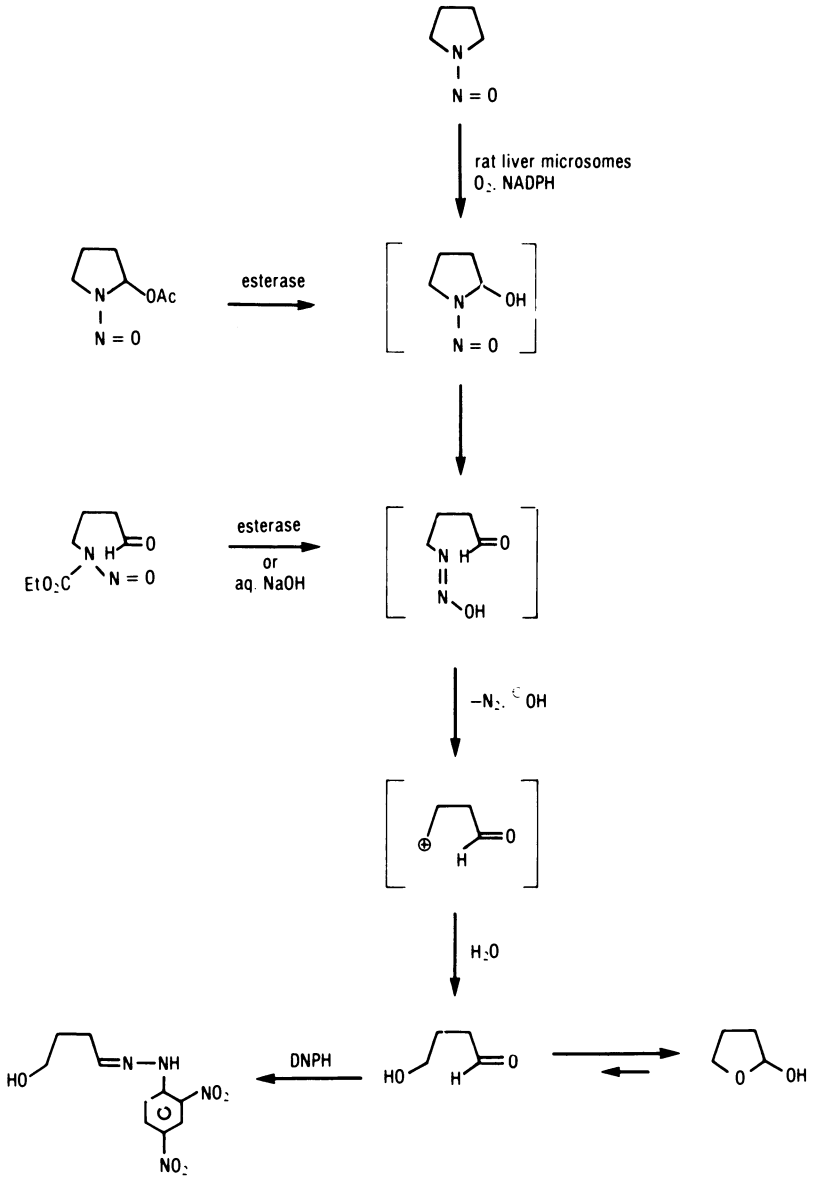

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Figure 6. Intermediates and products resulting from α-hydroxylation of NPy (51)

shown in Figure 7.

The hydrolysis of these model precursors was studied at 37°, with catalysis by hog liver esterase. The major product, isolated in 60-70% yield from the hydrolysis of α-acetoxyNPy, was 2-hydroxytetrahydrofuran. This compound was identified by comparison to a reference sample,prepared by lead tetraacetate oxidation of 1,2,5-pentanetriol (53). Additional evidence was obtained by lithium aluminum hydride reduction of the product to 1,4-butanediol. Minor amounts of butenals were also identified as products of the hydrolysis of α-acetoxyNPy.

When the nitrosourethane, 4-(N-carbethoxy-N-nitrosamino)-butanal, was hydrolyzed at 37°, 2-hydroxytetrahydrofuran was also the major product isolated (40-50%). The latter was also formed when the nitrosourethane was allowed to react with aqueous base, under conditions known to convert nitrosourethanes to diazohydroxides (54). These results are consistent with the intermediacy of an oxocarbonium ion as shown in Figure 6, although direct attack of water on the diazohydroxide intermediate cannot be excluded. In either case, these electrophilic intermediates should be capable of reaction with nucleophilic cellular constituents. The interactions of NPy and α-acetoxyNPy with guanosine and polyguanylic acid are currently being investigated.

The mutagenicity of α-acetoxyNPy and of 4-(N-carbethoxy-N-nitrosamino)butanal towards *S. typhimurium* TA 100 and TA 1535 was also tested, as shown in Figures 8 and 9. As expected, both of these compounds were highly mutagenic without activation. The differences in mutagenicity between the two compounds may be due to differing rates of hydrolysis or other factors. These results are in agreement with previous studies on α-acetoxyNPy and are consistent with activation of NPy via α-hydroxylation (47).

These model studies permitted demonstration of metabolic α-hydroxylation of NPy by isolating 2-hydroxytetrahydrofuran as a metabolite of NPy. This was accomplished by trapping 4-hydroxybutyraldehyde as its 2,4-dinitrophenylhydrazone (DNP) derivative. For *in vitro* studies, NPy-2,5-^{14}C was incubated with rat liver microsomes, O_2, and an NADPH generating system. After the incubation was complete, DNP reagent was added to the mixture and the products were extracted and examined by preparative TLC. A radioactive band corresponding to the DNP of 4-hydroxybutyraldehyde was observed. This band was not present in controls in which NADPH was omitted or in which boiled enzyme was used. The mass spectrum was identical to that of a reference sample. In addition, a minor metabolite with mass spectrum identical to that of the DNP of 2-butenal was also isolated. These results showed conclusively that NPy underwent metabolic α-hydroxylation in this *in vitro* system.

Metabolic α-hydroxylation of NPy was also demonstrated *in vivo*. For this purpose, male F-344 rats were injected with NPy-2,5-^{14}C and the 48 hr urine was collected in vessels containing DNP

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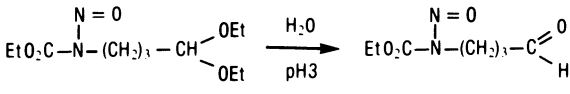

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Figure 7. Synthesis of 4-(N-carbethoxy-N-nitrosamino)butanal (51)

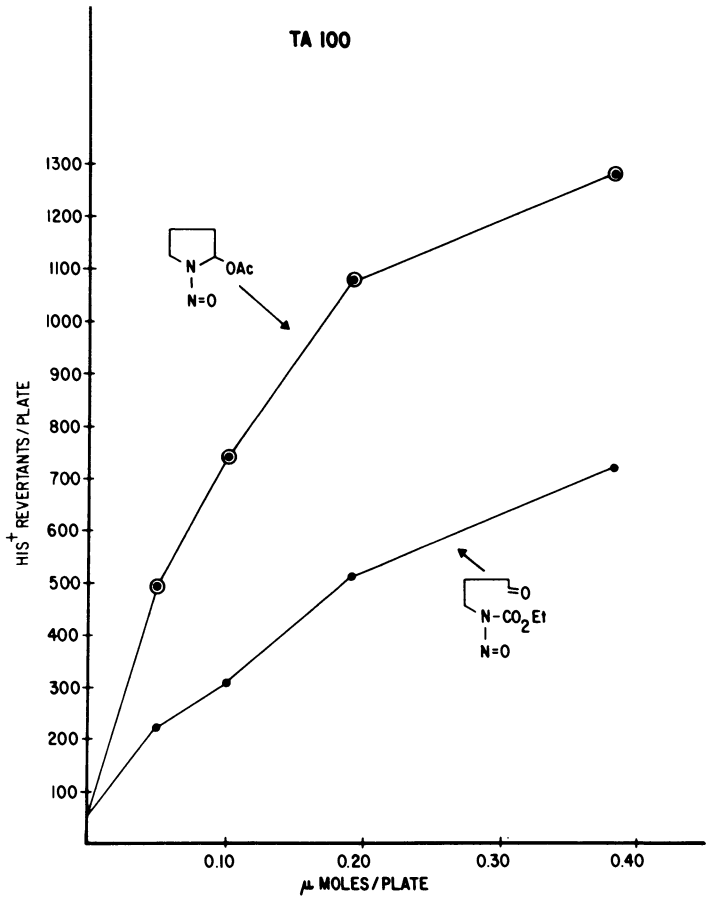

Figure 8. Mutagenicity of α-acetoxyNPy and 4-(N-carbethoxy-N-nitrosamino)-butanal in S. typhimurium *TA 100*

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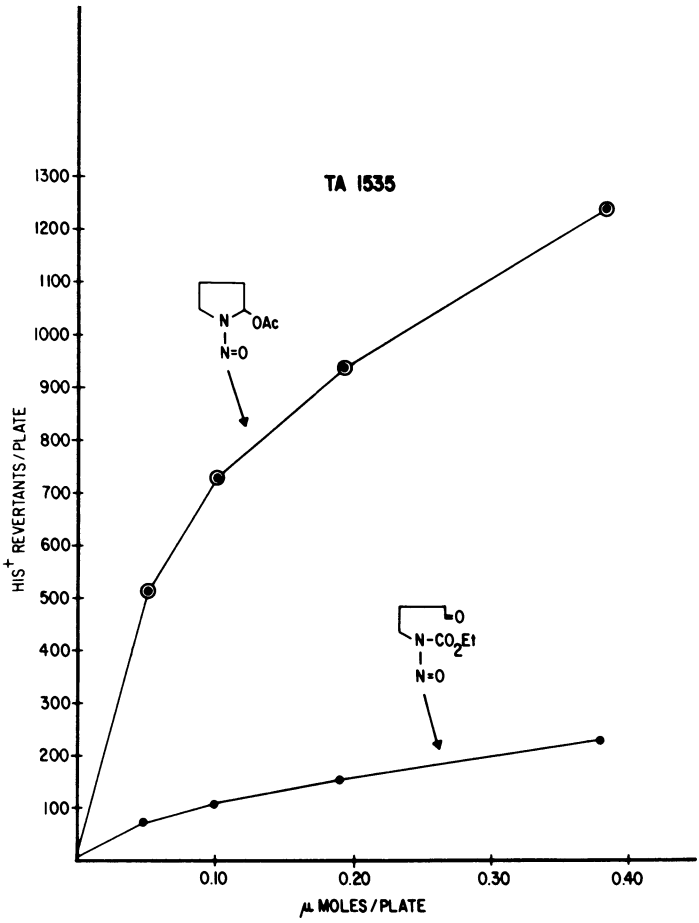

Figure 9. Mutagenicity of α-acetoxyNPy and 4-(N-carbethoxy-N-nitrosamino)-butanal in S. typhimurium *TA 1535*

reagent. The DNP of 4-hydroxybutyraldehyde was identified after extraction of the urine and preparative TLC. The yield was only about 0.1%, presumably because of further oxidation *in vivo*. The major metabolite isolated in this experiment was CO_2, in agreement with previous studies (55).

To allow further studies on the role of *a*-hydroxylation in carcinogenesis by NPy, an *in vitro* assay for *a*-hydroxylation of NPy by isolated hepatic microsomes was developed. Incubation mixtures were added to DNP reagent and analyzed by reverse phase HPLC. A typical chromatogram obtained in this way is shown in Figure 10. The indicated peak was identified as the DNP of 4-hydroxybutyraldehyde (4-OH-BA-DNP) by comparison of its mass spectrum to a reference sample. The other peaks in the chromatogram were also present in control incubations and were therefore not metabolites. Under the optimal conditions for studying *a*-hydroxylation of NPy, as determined by varying enzyme and substrate concentrations, the reaction was linear for at least 90 minutes, as shown in Figure 11. The *a*-hydroxylation of NPy by liver microsomes from male Fischer rats was induced by Aroclor (see Table V), as is typical for the microsomal mixed function oxidase system. Further studies on induction and inhibition of microsomal NPy *a*-hydroxylation are currently in progress.

Table V.

a-Hydroxylation Of NPy by Liver Microsomes From Aroclor-Treated and Control Rats

	Protein (mg/ml)	Cytochrome P-450 (nmoles/mg)	4-OH-BA-DNP (nmoles/min/mg)	Aniline hydroxylase (nmoles/min/mg)
Control[a]	10.5 ± 0.6	0.67 ± 0.03	1.43±0.13	0.88±0.02
Aroclor treated[a]	12.3 ± 0.3	1.54 ± 0.32	3.31±0.63	1.63±0.13

[a]Each value is the average of duplicate determinations on three rats.

Metabolic *a*-hydroxylation of the tobacco specific carcinogen NNN has been studied using a similar approach. Despite the apparent similarity of NNN and NPy, some differences were evident in the model and metabolic studies. Figure 12 summarizes the main features of these experiments. Both 2'-acetoxyNNN and 5'-acetoxyNNN were synthesized as model precursors to 2'-hydroxyNNN and 5'-hydroxyNNN. The syntheses of these compounds are outlined in Figure 13. The ratio of the 2'-thioether to the 5'-thioether was approximately 10 to 1, which made the latter difficult to obtain. This ratio was due to the stabilizing effect of the pyridine ring on a negative change at the 2'-position. The two thioethers were separated by column chromatography and each was

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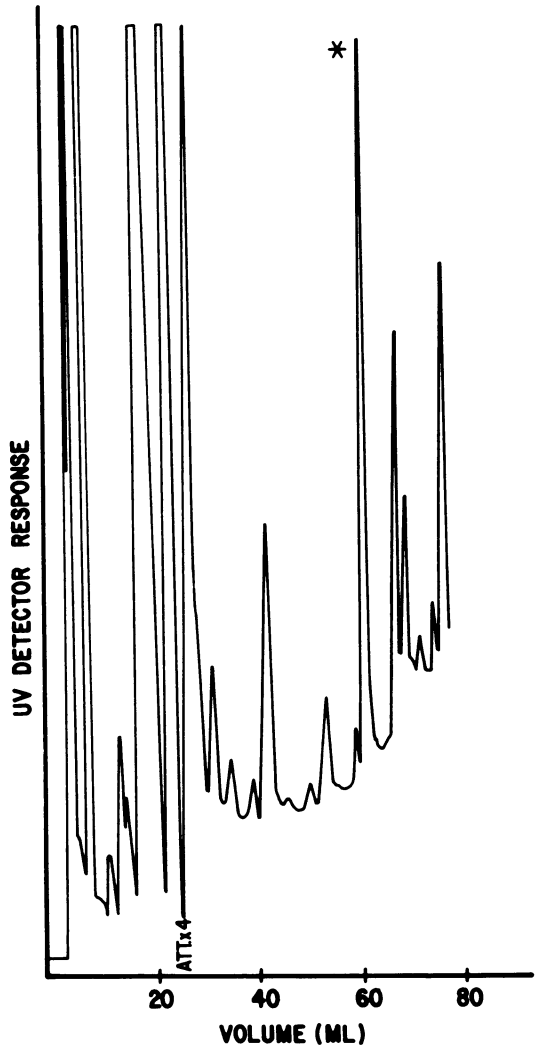

Cancer Research

Figure 10. HPLC trace showing α-hydroxylation of NPy by isolated hepatic microsomes. The indicated peak is the product of α-hydroxylation, 4-hydroxybutyraldehyde-DNP (51).

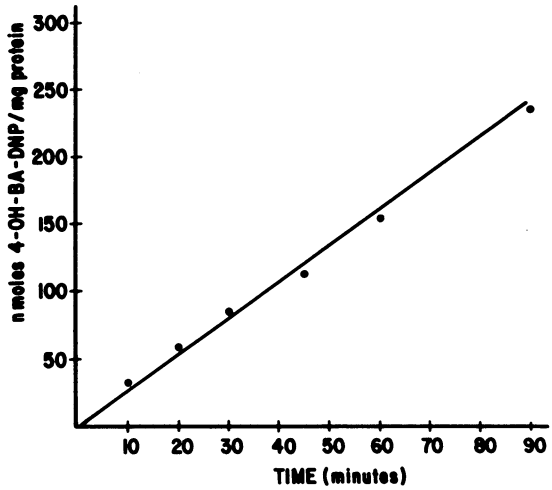

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Figure 11. Kinetics of α-hydroxylation of NPy by isolated hepatic microsomes. The product of α-hydroxylation is 4-hydroxybutyraldehyde DNP (4-OH-BA-DNP). (52)

converted to the corresponding acetate (a mixture of *cis*- and *trans*- isomers in the case of 5'-acetoxyNNN). While the yields in this reaction were satisfactory, purification of 2'-acetoxy NNN was accomplished only with difficulty, due to facile formation of myosmine (2-(3-pyridyl)-1-pyrroline) under the necessary chromatographic conditions. Nevertheless, both acetates were obtained in high purity and were free from traces of the other isomer.

The hydrolyses at 37° of 2'-acetoxyNNN and 5'-acetoxyNNN with catalysis by hog liver esterase, were then studied. The results are indicated in Figure 12. Hydrolysis of 2'-acetoxyNNN gave as the major products, a mixture of myosmine (50-60%) and the keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone (5-10%), whereas, 5'-acetoxyNNN gave predominantly the lactol, 2-hydroxy-5-(3-pyridyl)tetrahydrofuran (60-70%). These products were identified by comparison to reference samples which were synthesized independently.

The mutagenic activity of 2'-acetoxyNNN and 5'-acetoxyNNN was tested in *S. typhimurium* TA 100. Both compounds were mutagenic without activation, but with activity less than that of α-acetoxy NPy. The 5'-acetate showed maximum activity (840 His^+ revertants/plate; control=180) at a dose of 1.70 μmoles/plate. The 2'-acetate was weakly mutagenic (315 His^+ revertants/plate for 1.28 μmoles/plate; control=140). When NNN was tested at these doses in the presence of hepatic supernatants, no activity was observed. NNN was mutagenic at higher doses, however. These results are consistent with involvement of α-hydroxylation as an activation step for NNN.

Evidence for metabolic α-hydroxylation of NNN was first obtained through *in vitro* experiments. NNN-2'-^{14}C was incubated with rat liver microsomes, O_2 and an NADPH generating system. The resulting mixtures were added to DNP reagent and analyzed by preparative TLC or HPLC. The DNPs of the keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone (0.6% from NNN) and of 4-hydroxy-4-(3-pyridyl)butanal (0.3% from NNN) were both identified by comparison of their mass spectra to reference samples. These products, which were not present in controls, resulted from 2'-hydroxylation and 5'-hydroxylation of NNN, respectively (see Figure 12). Another product of 2'-hydroxylation, myosmine (0.6% from NNN) was identi- by GLC-MS analysis of incubation mixtures, after extraction with chloroform.

When male F-344 rats were injected with NNN-2'-^{14}C, 75-95% of the dose was excreted in the 48 hr urine. In one experiment, the urine was collected in vessels containing DNP reagent. However, the DNPs of 4-hydroxy-1-(3-pyridyl)-1-butanone and 4-hydroxy-4-(3-pyridyl)butanal were not detected. Since this was likely due to further oxidation *in vivo*, methods were developed for isolation of their probable oxidation products. This resulted in identification of the lactone, 5-(3-pyridyl)-tetrahydrofuran-2-one (1-2%), the keto acid, 4-(3-pyridyl)-4-oxobutyric acid (1-2%) and the hydroxy acid, 4-(3-pyridyl)-4-hydroxybutyric acid (26-40%) as urinary metabolites. These metabolites resulted,

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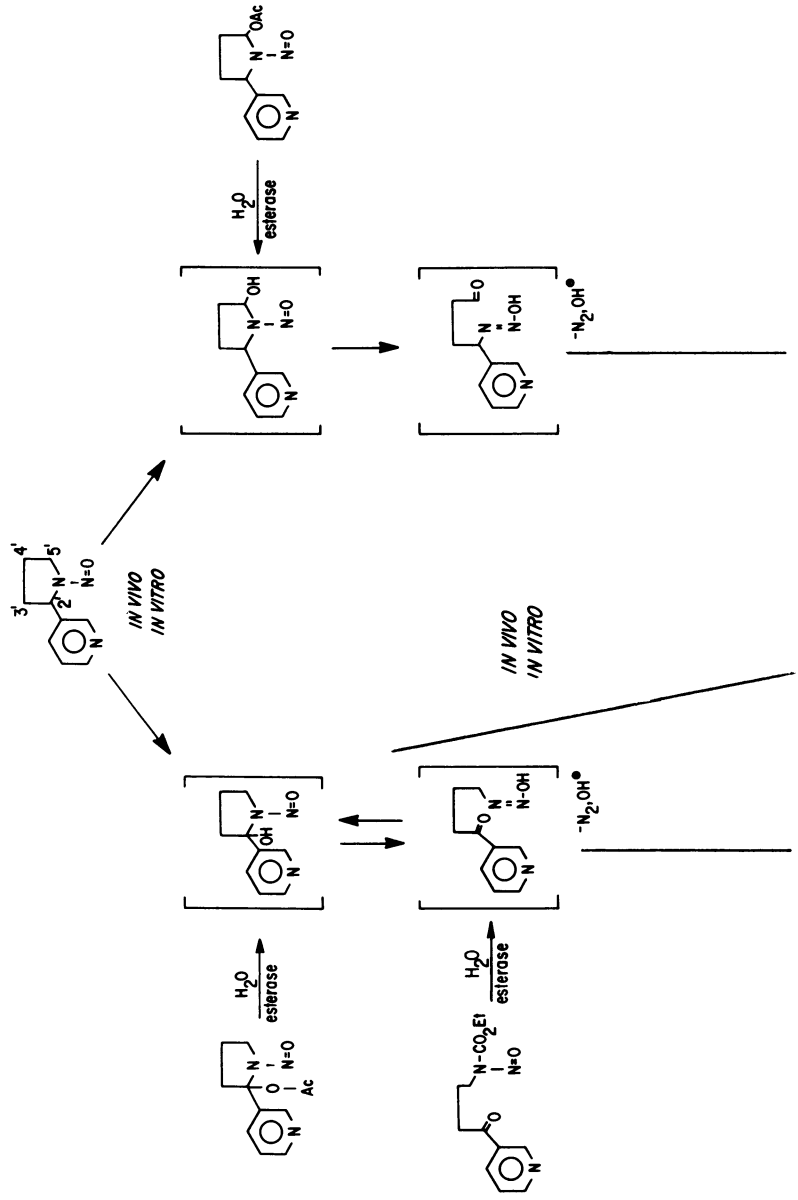

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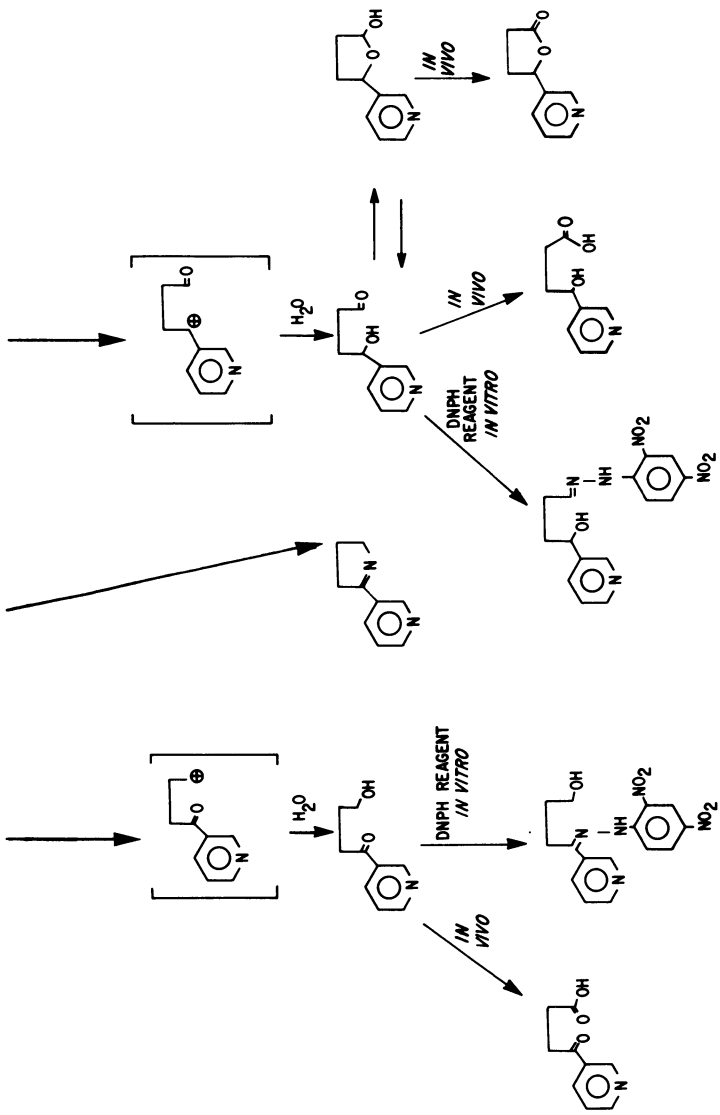

Figure 12. Intermediates and products reulting from α-hydroxylation of NNN (52)

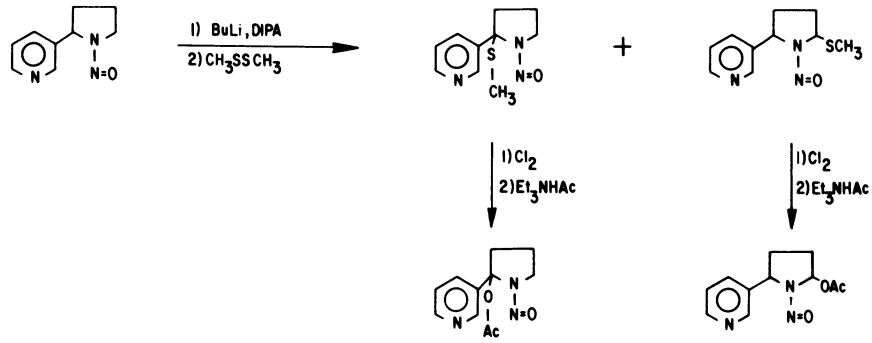

Figure 13. Synthesis of 2′-acetoxyNNN and 5′-acetoxyNNN

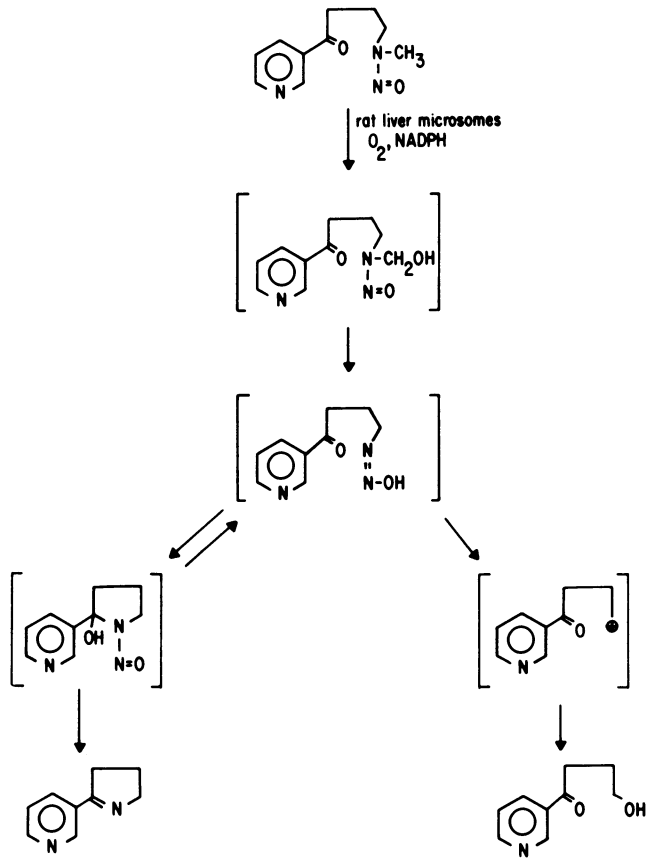

Figure 14. Demethylation of NNK in vitro

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at least partially, from *in vivo* oxidation of the initial products of 2'-hydroxylation and 5'-hydroxylation of NNN, as shown in Figure 12. Since the keto acid, hydroxy acid, and lactone are involved in the metabolism of nicotine (56), pathways other than an initial α-hydroxylation of NNN could be involved in their formation. For example, another metabolite of NNN was the lactam, norcotinine (5-(3-pyridyl)-2-pyrrolidinone); further metabolism of this compound could also give these products.

The results of these *in vitro* and *in vivo* experiments demonstrate conclusivley that NNN undergoes metabolic α-hydroxylation in the rat. The mutagenicity data discussed above are consistent with the involvement of α-hydroxylation as an important step in the metabolic activation of NNN. Further evidence is currently being sought through carcinogenicity studies of α-deuterated NNN derivatives and through studies of the binding of NNN to DNA and RNA.

The metabolism of NNK is also under investigation. When NNK was incubated with rat liver microsomes under the usual conditions and the mixtures extracted and analyzed by GLC-MS, both myosmine and the keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone were indentified as indicated in Figure 14. These results provide evidence for hydroxylation of the N-methyl group of NNK, a metabolic step which produces intermediates common to NNK and NNN. In addition, the formation of myosmine indicates that the diazohydroxide intermediate undergoes cyclization followed by loss of HONO.

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Summary

Tobacco specific nitrosamines may be causative factors in the various cancers associated with tobacco usage. These include cancer of the lung, oral cavity, esophagus, pancreas, and bladder. These nitrosamines are unique to tobacco and tobacco smoke since they are derived from the tobacco alkaloids. The major tobacco specific nitrosamines identified to date are N'-nitrosonornicotine (NNN) and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These compounds occur in mainstream and sidestream tobacco smoke and in unburned processed tobacco, in relatively high concentrations. NNN forms during the curing of tobacco and is transferred to tobacco smoke as well as forming during smoking. Nicotine is the major precursor for both NNN and NNK. Nicotine can also be nitrosated to give 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)butanal (NNA), but this nitrosamine has not yet been detected in tobacco or tobacco smoke. N'-Nitrosoanabasine (NAB) which is derived from the minor tobacco alkaloid anabasine also has not been detected in tobacco or tobacco smoke.

NNN induces esophageal and nasal cavity tumors in rats, tracheal tumors in hamsters, and lung adenomas in strain A mice. NNK is more tumorigenic than NNN in strain A mice and NNA is inactive in this species. NAB is less carcinogenic than NNN in both rats

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and hamsters. A related nitrosamine, nitrosopyrrolidine (NPy) induces hepatocellular carcinomas in rats and appears to be less carcinogenic than NNN.

Metabolic α-hydroxylation of NNN and NPy has been studied. This process may be the activation pathway for NNN and NPy since electrophilic diazohydroxides and carbonium ions are generated upon decomposition of the unstable intermediates α-hydroxyNPy, 2'-hydroxyNNN, and 5'-hydroxyNNN. Synthetic precursors to these intermediates, α-acetoxyNPy, 2'-acetoxyNNN, and 5'-acetoxyNNN were all mutagenic in *S. typhimurium* without activation which is consistent with the role of α-hydroxylation in the metabolic activation of NPy and NNN. The products of hydrolysis of the α-acetoxy compounds were all detected as metabolites of NNN and NPy, which demonstrates that α-hydroxylation is a metabolic process for these cyclic nitrosamines. NNK also undergoes metabolic α-hydroxylation in part to give the same intermediates that arise from α-hydroxylation of NNN. The role of these intermediates in carcinogenesis by NPy, NNN, and NNK is currently being studied by using HPLC assays for α-hydroxylation by hepatic microsomes, and through binding experiments with DNA and carcinogenicity assays of deuterated derivatives of NNN.

Acknowldegements

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Quantitative Aspects of Exposure and Mechanism in *N*-Nitrosamine Carcinogenesis

JOHN S. WISHNOK

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02138

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N-nitrosodialkylamines - nitrosamines - constitute one of the most extensive series of known chemical carcinogens. Most nitrosamines can initiate tumors in at least one animal species, and all animal species which have so far been tested are susceptible to nitrosamine carcinogenesis (1, 2).

These compounds have received increasing attention as it has become apparent that some of them are present in the environment (3, 4, 5, 6), and that they can be readily formed under physiological conditions from amines and nitrite (7, 8, 9).

In addition to these potentially important epidemiological aspects, the nitrosamines are especially interesting in terms of the biochemistry of chemical carcinogenesis. This is exemplified most strikingly, perhaps, in the organ specificity of these compounds (10, 11), and in the wide variations in potency within the series (10, 12).

We have been particularly interested in these potency variations both in terms of environmental exposure and formation and in the context of the mechanism through which nitrosamines initiate cancer.

The important review by Druckrey and Preussmann and their coworkers (10) contains quantitative carcinogenicity data for more than 60 N-nitroso compounds acting on a single animal strain - the BD rat. In this study, the animals were administered a small daily dose of each N-nitroso compound, and the mean total carcinogenic dose (D_{50} expressed as moles/kg body) required for production of tumors in 50% of the animals was then determined. Increasing values for D_{50} represent decreasing carcinogenicity. In our analyses, we usually express carcinogenic potency as $1/D_{50}$ in order to have increased potency represented by increasing numbers (13). For a series of fairly simple nitrosamines the potency among compounds which were observedly carcinogenic varied over a range of nearly one thousand. Some nitrosamines, e.g., N-nitrosoditert-butylamine, were not carcinogenic under the conditions of the experiments (10).

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Relative Risks of Nitrosamines. The mere existence of reproducible variations in carcinogenicity from one nitrosamine to another is directly relevant to the question of potential human health hazards from environmental nitrosamines. The problem of nitrosamines in cooked bacon is a simple and straightforward example.

Several nitrosamines, including N-nitrosopyrrolidine (NP), N-nitrosodimethylamine (NDMA), and N-nitrosodiethylamine (NDEA), have been detected at various concentrations in this food. Table 1 lists some typical values for the concentrations of these compounds in cooked bacon and some other prepared meat products (14). Nitrosopyrrolidine has been generally found, especially in bacon, at much higher levels than NDMA or NDEA, and most attention has consequently been directed toward this compound (15, 16).

Table 1

Concentration of nitrosamines in processed meats, including bacon

Nitrosamine	Typical high values (14,17) ppb	mol X 10^9/g food	Typical low values (14,17) ppb	mol X 10^9/g food
NDMA	25	0.34	3	0.04
NDEA	12	0.12	2	0.02
NP	50	0.50	5	0.05

Table 2 shows the carcinogenic potencies of NP, NDMA and NDEA, expressed as $1/D_{50}$. Interestingly, NDMA and NDEA are more potent (in BD rats, at least) than NP. We have consequently pointed out (17) that the potencies of environmental carcinogens should be considered along with concentrations when assessing the potential hazards of these compounds.

Table 2

Carcinogenic potencies in the BD rat (10,13,17)

Nitrosamine	Daily Dose (mmol/kg)	D_{50} (mol/kg)	$1/D_{50}$	Relative Potency
NDMA	0.05	0.0054	185	7
NDEA	0.05	0.0065	154	6
NP	0.05	0.039	26	1

These ideas can be expressed quantitatively in terms of a relative risk factor:

$$R = PC$$

where C is the concentration and P is the relative potency of a

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given compound. Table 3 shows the relative risks of NP, NDMA and NDEA.

Table 3

Relative carcinogenic risks (17)

Nitrosamine	Relative Risk/g Food	
	High Intake	Low Intake
NDMA	48	6
NDEA	19	2
NP	10	1

From these considerations, it appears that NDMA and NDEA - both of which have received less attention than NP - may actually pose greater hazards than does NP.

This analysis, along with some observations concerning the possible absolute hazards arising from nitrosamines in bacon, has been detailed in an earlier report (17).

Structure-Activity Relationships. More interesting, and - scientifically, at least - more significant, is the question of why one nitrosamine should be more or less carcinogenic than another. In a general sense, the answer is obvious: differences in reactivity within a series are, almost by definition, the result of differences in structure. This concept is one of the cornerstones of physical organic chemistry and, more recently, has been applied extensively to drug systems in a systematic and quantitative way following the initial and continued successes of Corwin Hansch and his coworkers (18-25).

These and other investigators have shown, for many systems where a biological response can be measured quantitatively, that relative biological response can be expressed as functions of various molecular properties by using equations of the same forms as Hammett or Taft relationships:

$$RBR = k_1 + k_2\pi \quad (19) \qquad 1$$

$$RBR = k_1 + k_2\pi - k_3\pi^2 \quad (20) \qquad 2$$

$$RBR = k_1 + k_2\pi - k_3\pi^2 + k_4\sigma \quad (22) \qquad 3$$

RBR is the relative biological response. π is defined as $\log P_S - \log P_H$ where P_H is generally the water-octanol partition coefficient for the parent molecule and P_S is the partition coefficient for the molecule containing substituent 'S'. The σ's are standard Hammett or Taft substituent constants (26, 27).

These relationships have been useful to a certain extent as the basis for rational methods of drug design (24), and have been valuable also as probes for assessing which molecular properties are actually involved in determining drug potency and toxicity (23).

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Despite the widespread use of structure-activity relationships in pharmacology, there have been relatively few attempts to apply this type of data analysis in the area of chemical carcinogenesis, although some very general intuitive structure-activity correlations have often been noted for the nitrosamines. For example, nitrosamines with branching and, consequently, fewer hydrogens at the α-carbon generally have lower carcinogenic potency than their unbranched isomers (10, 28) and unsymmetrical nitrosamines, especially with methyl as one of the alkyl groups, tend to be more specific towards the esophagus (10, 11). Exceptions and overlap, however, have tended to obscure any rigorous systematization of these observations. An interesting quantitative relationship between daily dose and induction time was developed by Preussmann and coworkers (10):

$$dt^n = \text{constant}$$

In this expression d is the daily dose and t is the time required for induction of tumors in 50% of the animals. The exponent n is then characteristic of a given nitrosamine. The value of n is generally about 2 and ranges from about 1 to 4. No systematic association between n and structure, however, has been found and there is consequently no apparent molecular rationale for this relationship.

Quantitative structure-activity analyses in chemical carcinogenicity may also have appeared discouraging because of an intuitive feeling that the biochemical events leading to cancer - since they apparently involve a disruption of the transmission of genetic information - are much more complex than those involved in drug-host interactions, and that there would therefore be little likelihood that carcinogenicity could be described by relationships such as equations 1-3. In addition, there is no generally-agreed-on quantitative criterion for carcinogenic potency; mean carcinogenic dose, D_{50} (10), mean induction time, t_{50} (10), percent-tumor-bearing-animals (29), as well as other criteria (30), have been used by various investigators. Finally, the development of biological structure-activity relationships requires a fairly large sample of internally consistent quantitative data, i.e., the same strain of animal, the same dosing protocol, and the same criterion for potency. This last set of conditions has rarely been met.

The Druckrey-Preussmann review, however, contains reasonably quantitative data for a variety of molecular types including N-nitrosoureas, and cyclic and acyclic N-nitrosodialkylamines. We became interested in whether or not biological structure-activity relationships could be developed for any of these sets of compounds. Only the acyclic nitrosamines appeared to constitute a sufficiently extensive and well-defined series, and we consequently carried out computerized multiple regression analyses on this group of compounds, using log $(1/D_{50})$ as the dependent variable analogous to RBR (12).

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A standard stepwise regression program (31) was used to find the function that would best correlate carcinogenic potency with a set of molecular properties. In this procedure, a linear function is developed by adding in turn the independent variable that yields the highest correlation between the linear function and the dependent variable. The success of the correlation is indicated by the multiple R^2. The increase in R^2, as each independent variable is added, is an indication of the contribution of that variable to the correlation. The standard error of the regression equation, s, is an absolute measure of how well the equation can predict RBR - in this case log $(1/D_{50})$. No correlation analogous to equation 1 or 2 was found, but the inclusion of an electronic factor, σ^*, led to the correlation described by equation 4 [Singer, Taylor, and Lijinsky have recently reported a correlation, for a small series of nitrosopiperazines, with the form of equation 1 (29). Using only partition coefficients, they found no correlation for acyclic nitrosamines (see, however, Discussion section). This is in agreement with our earlier observations based on the Druckrey-Preussmann data (13)].

$$\log (1/D_{50}) = 1.74 - 0.26\pi^2 + 0.92\pi + 0.59\sigma^* \qquad 4$$
$$n = 21 \qquad s = 0.31 \qquad R^2 = 0.84$$

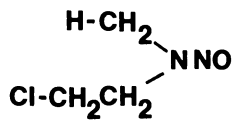
Table 4 shows the development of R^2 for this equation.

Table 4

Results of stepwise regression analysis of log $(1/D_{50})$ vs. π, π^2, and σ^* (12)

Step	Variable	R^2	Increase in R^2
1	π^2	0.36	———
2	π	0.66	0.30
3	σ^*	0.84	0.18

The carbon adjacent to the amine nitrogen (the α-carbon) was considered to be the reaction center, and the compounds were generally considered to have two reaction centers. Thus, for N-nitrosomethyl-(2-chloroethyl)amine, the σ^*'s



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for H (0.49) and $ClCH_2$ (1.05) were used. Table 5 lists a series of nitrosamines along with the values for π, σ^* at each α-carbon, the observed value for log $(1/D_{50})$ and the value for log $(1/D_{50})$ as calculated using equation 4. The compounds designated 'a' were used in the derivative of equation 4 and the log $(1/D_{50})$ values for the remaining compounds were calculated later. A more detailed description of the development of equation 4 is contained in reference 12.

The relationship described by equation 4 indicates that most of the variation in carcinogenicity within the series of acyclic nitrosamines can be associated with water-hexane partition coefficients and electronic inductive effects of substituents on the α-carbons.

This equation is a fairly typical biological structure-activity relationship with a strong dependence on partition coefficients, and it therefore suggests that the nitrosamines, and perhaps other chemical carcinogens as well, are similar - in the pharmacological sense - to analgesics or toxic agents.

The variation of a biological response with variations in solubility properties is usually interpreted in terms of the ability of the active molecules to reach an intracellular site of action (18-22).

In many cases, partition coefficients dominate most other molecular factors in determining variations in biological activity, indicating that transport to the site of action may be the rate-limiting process in the overall sequence of events giving rise to the biological response. In cases of this type, the addition of terms for electronic or steric effects do not lead to significantly improved correlations. The appearance of an electronic term in the nitrosamine carcinogenicity relationship is therefore interesting and potentially informative in terms of the mode of action of these compounds. The mechanism of nitrosamine carcinogenesis has not been firmly established but there is a growing body of evidence which indicates that the initial and biochemical rate-limiting step is enzymatic oxidation at an α-position (2, 10, 32). This is apparently followed by a series of chemical steps leading finally to a highly electrophilic species such as a diazonium ion or carbonium ion which reacts with a nucleophilic site on a macromolecule such as DNA (2, 10, 33, 34).

In our analyses, correlation of carcinogenic potency with Taft σ^* values was obtained only when the α-position was assumed to be the reaction center on the nitrosamine molecule. This observation is consistent with the above hypothesis and, for the first time, directly associates the α-position with carcinogenicity.

It is also interesting to note that this relationship accounts for most of the variation in potency on the basis of properties of the unmetabolized precarcinogen. Specific structural requirements for the ultimate carcinogen at the site of the significant biological interaction thus appear to be relatively unimportant.

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Examination of the observed and predicted values for log $(1/D_{50})$ in Table 5 reveals that there are at least four types of compounds that are apparently not well-described by equation 4. These include compounds with hydroxy groups (e.g. compounds 20, 21, 22), compounds with chemically reactive α-hydrogens (e.g., allylic or benzylic systems, No's 9, 14), and compounds with extensive branching at the α-carbon (No's 4, 11). Acyclic nitrosamines, with one or two apparent exceptions (12), appear to constitute a separate reaction series (29).

In the first three cases the calculated values for log $(1/D_{50})$ are all higher than the observed values. In the cases of the OH-bearing compounds and the allylic and benzylic compounds, this may reflect alternate metabolic pathways in which the molecules are converted to non-carcinogenic or less-carcinogenic metabolites.

The low observed log $(1/D_{50})$'s for compounds with branching at the α-position probably indicate the importance of a steric factor which was not revealed in the regression analysis because of the small number of examples of this structural type.

For a set of cyclic compounds, the N-nitrosopiperidines, Singer, Tayler, and Lijinsky - utilizing the relative number of tumor-bearing animals (RTBA) or the relative mean lifetime (RML) as indices of carcinogenic potency - obtained correlations between potency and partition coefficients as shown below (29):

$$\log \text{RTBA} = 0.098 - 0.08 \log P$$
$$n = 6 \qquad r = 0.940 \qquad s = 0.019$$

$$\log \text{RML} = 0.01 - 0.26(\log P)^2 + 0.2 \log P$$
$$n = 6 \qquad r^2 = 0.99 \qquad s = 0.047$$

Multiple regression analysis on this data (29), with the addition of σ* values, gave no improvement on these relationships. For a series of dinitrosopiperazines, however, for which no correlation was detected with log P values alone, a fair correlation could be generated when σ* was included:

$$\log \text{RTBA} = 0.09 - 0.75(\log P)^2 + 0.762 \log P - 0.62\sigma^*$$
$$n = 5 \qquad R = 0.86 \qquad R^2 = 0.73 \qquad s = 0.11$$

Although additional data would be desirable for all three of these relationships, there seems little doubt that they are real and that structure-activity relationships can therefore probably be generated for additional subclasses of N-nitroso derivatives given sufficient quantitative data.

In summary, it appears that the differences in carcinogenic potency for both cyclic and acyclic N-nitrosodialkylamines are real and systematic, and that the potencies of environmental nitrosamines should be considered in assessing the relative hazards associated with these compounds. In addition, it can be shown that the carcinogenicity of nitrosamines can be associated

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Table 5

Carcinogenic Activity of N-Nitroso Compounds (R_1, R_2N-N=O) (12)

The following compounds, with no observed carcinogenic activity, are not included in the table: N-nitrosodiphenylamine, N-nitrosodicyclohexylamine, N-nitrosodiallylamine and N-nitrosodibenzylamine. Substituent constants (σ*) are based on the following structure:

S_1-CH_2
S_2-CH_2 N-N=O

N-nitroso compound	R_1	R_2	σ^*_1	σ^*_2	π	log(1/D_{50}) Obs.[a]	log(1/D_{50}) Calc.
1[a]	CH_3	CH_3	0.49	0.49	0	2.3	2.3
2[a]	CH_3CH_2	CH_3CH_2	0	0	1.24	3.2	2.5
3[a]	$CH_3(CH_2)_2$	$CH_3(CH_2)_2$	-0.1	-0.1	2.49	2.1	2.3
4	$(CH_3)_2CH$	$(CH_3)_2CH$	-0.49	-0.49	2.24	1.0	1.9
5[a]	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	-0.115	-0.115	3.59	1.6	1.5
6[a]	$CH_3(CH_2)_4$	$CH_3(CH_2)_4$	-0.125	-0.125	4.22	0.6	0.8
7[a]	CH_3	CH_3CH_2	0.49	0[b]	0.60	2.3	2.5
8[a]	CH_3	CH_2=CH	0.49		1.84	2.9	2.8
9	CH_3	CH_2=$CHCH_2$	0.49	0.2	1.17	2.1	2.9
10[a]	CH_3	$CH_3(CH_2)_4$	0.49	-0.125	2.51	2.6	2.6
11	CH_3	cyclo-C_6H_{11}	0.49	-0.71	1.26	3.0	2.4
12[a]	CH_3	$CH_3(CH_2)_6$	0.49	-0.135[b]	3.54	1.5	1.9
13	CH_3	C_6H_5	0.49		4.03	1.6	1.5
14[a]	CH_3	$C_6H_5CH_2$	0.49	0.6	1.92	3.1	3.2
15[a]	CH_3	$C_6H_5CH_2CH_2$	0.49	0.22	3.22	3.0	2.4

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Table 5 (continued)

16[a]	CH_3CH_2	CH_2=CH	0	[b]	2.55	2.6	2.4
17	CH_3CH_2	$(CH_3)_2CH$	0	-0.49	1.15	1.5	2.2
18[a]	CH_3CH_2	$CH_3(CH_2)_3$	0	-0.115	2.52	2.1	2.3
19[a]	$CH_3(CH_2)_3$	$CH_3(CH_2)_4$	-0.115	-0.125	4.17	1.0	0.9
20	CH_3CH_2	$HOCH_2CH_2$	0	0.56	0.57	1.8	2.5
21	$HOCH_2CH_2$	$HOCH_2CH_2$	0.56	0.56	-0.12	0.05	2.3
22	$CH_3(CH_2)_3$	$HO(CH_2)_3CH_2$	-0.115	-0.12	2.92	2.0	2.1
23[a]	CH_3	$ClCH_2CH_2$	0.49	1.05	1.30	3.2	3.4
24[a]	CH_3	$NCCH_2$	0.49	1.25	-0.58	2.2	2.1
25[a]	$NCCH_2$	$NCCH_2$	1.25	1.25	-1.15	1.9	1.8
26[a]	CH_3	CH_3CO	0.49	[b]	0.10	2.3	2.1
27[a]	CH_3	CH_3CH_2OOC	0.49	[b]	0.06	2.0	2.1
28	CH_3CH_2	CH_3CH_2OOC	0	[b]	0.69	2.0	2.3
29[a]	CH_3	CH_3NNOCH_2	0.49	0.49	0.48	2.4	2.7

[a] Compounds included in equation 2.
[b] No "reaction center" on R_2.
[c] From Druckrey et al. (10).

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with fundamental molecular properties via quantitative structure-activity relationships which may prove useful as methods of predicting carcinogenicity and as tools for probing mechanisms of carcinogenicity.

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10

N-Nitrosamines as Environmental Carcinogens

WILLIAM LIJINSKY

Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, MD 21701

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Among carcinogens the N-nitroso compounds are the most broadly acting and among the most potent. They comprise the directly acting nitrosamides and the systemically acting nitrosamines, which require enzymic activation for their carcinogenic action. This difference between the two types of N-nitroso compound is also shown in bacterial mutagenesis. More than a hundred N-nitroso compounds have been tested for carcinogenic activity and most of them have induced tumors in rats; a much smaller number has been tested in hamsters, mice or guinea pigs and, again, most of those tested have been carcinogenic. Among the N-nitroso compounds tested for mutagenesis in bacteria there has been a fairly good qualitative correlation with carcinogenicity, although there have been a number of significant exceptions, particularly in carcinogenic nitrosamines which have not been mutagenic (1, 2).

N-nitroso compounds elicit a varied response from different animals, often giving rise to entirely different tumors in rats compared with hamsters. The organ affected might be different and often the cell type giving rise to tumors is also different. For example, 2,6-dimethylnitrosomorpholine is an esophageal carcinogen in the rat (3), but it induces tumors of the pancreatic duct in Syrian hamsters (4) and only hepatocellular carcinomas in guinea pigs (5). Nitrosoheptamethyleneimine gives rise to squamous lung tumors in rats (6) and in European hamsters (7), but induces tumors of the forestomach in Syrian hamsters (8). Dinitroso-2,6-dimethylpiperazine induces tumors of the esophagus in rats (9) and nitrosomethyldodecylamine induces transitional cell carcinomas of the bladder in both rats (10) and Syrian hamsters (11), but both compounds give rise only to liver tumors in guinea pigs (5). On the other hand, the guinea pig is quite refractory to most other types of liver carcinogens which are effective in rats or mice.

However, the biggest difference lies in the relative effectiveness or potency of nitrosamines, this varying greatly between even structurally closely related compounds. For example, nitrosopyrrolidine is a very much weaker carcinogen than is the homolog nitrosopiperidine (12); nitrosodi-n-propylamine is

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considerably weaker than nitrosodiethylamine (13); 2,6-dimethylnitrosomorpholine is much more potent than nitrosomorpholine (3); 2,6-dimethyldinitrosopiperazine is much more potent than dinitrosopiperazine (9); nitrosobis-(2-methoxyethyl)-amine is considerably more potent than nitroso-bis-(2-ethoxyethyl)-amine (14). There is at present no satisfactory explanation of these differences, which must be added to the long list of unexplained phenomena in the field of chemical carcinogenesis.

These great differences in carcinogenic activity and in carcinogenic effectiveness make it difficult to give leads for epidemiological studies which might relate certain human cancers to exposure to N-nitroso compounds. The major impediment is that, as a group, N-nitroso compounds are able to give rise in some appropriate animal model to almost every type of cancer seen in man. Yet, there are only sporadic reports of the exposure of people to significant concentrations of N-nitroso compounds. For example, nitrosamines have been found in the parts per billion level in some meats cured with nitrite; nitrosodimethylamine has been found in some air samples near factories at the level of micrograms per cubic meter (15); nitroso-di-n-propylamine and nitrosodimethylamine have been found in some herbicide formulations and the very weak carcinogen nitrosodiethanolamine has been found in synthetic cutting oils (16) and at much lower concentrations in some cosmetics (17). While exposure to nitrosamines from these sources undoubtedly adds to the carcinogenic risk of those exposed, it does not seem likely that this increased risk is very large.

It is much more likely that major contributions to the risk of cancer are through formation of N-nitroso compounds by reaction of amines with nitrite *in vivo*. The favored site for nitrosation is the stomach where the acid conditions prevailing are optimal for the reaction of both secondary and tertiary amines with nitrite. Many types of catalyst, such as nucleophilic anions, carbonyl compounds and some phenols, can be present, as also can be inhibitors, such as ascorbic acid and glutathione. The resultant of all of these effects, including the normal mass action effects of concentration and kinetic factors, such as basicity of the amine, is quite unpredictable, even if the contents of the stomach were an homogeneous solution. Considering their heterogeneous nature, in most circumstances it would be possible to miscalculate, one way or the other, by several orders of magnitude in trying to estimate the yield of a particular N-nitroso compound in normal life.

The principal source of nitrite for formation of N-nitroso compounds in the stomach is cured meats, since the concentration of nitrite will be highest because of the rapidity of ingestion of them. The rate of formation of N-nitroso compounds is proportional to the square of the nitrite concentration, so that the extent of formation of these compounds will be greater from the nitrite supplied by cured meats than from nitrite in saliva, even

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though the supply of the latter is continuous. The concentration of nitrite in saliva can be quite high some time after eating a meal high in nitrate containing vegetables, but the secretion of saliva is slow. Other sources of nitrite include the bacterial reduction of nitrate in the infected bladder and in the stomachs of achlorhydrics, as well as the more recently suggested oxidation of amines and ammonia in the intestine (18). Whatever the source of nitrite, it can contribute to the formation of carcinogenic N-nitroso compounds by reaction with secondary and tertiary amines, but the most favored site of these reactions is the stomach with its acid conditions. This is so also when the nitrosating agent is not nitrite, but a nitrosamine, of which several have been found to be effective nitrosating agents in acid conditions (19), particularly in the presence of a catalyst, such as thiocyanate (20). Among the most active of these are several noncarcinogenic nitrosamines, such as nitrosoproline, nitrosohydroxyproline and nitroso-N-methylpiperazine.

Many such studies have been conducted with a variety of amines and have demonstrated that the reactions do take place under simulated gastric conditions of pH and temperature, with formation of the predicted N-nitroso compound. The formation of N-nitroso compounds in the stomach from nitrite or other nitrosating agents and secondary and tertiary amines reflects the results of reactions which can be carried out in simple chemical systems. Kinetic studies have been conducted with a few secondary amines, but no satisfactory kinetic data have been yet obtained with tertiary amines, the mechanism of nitrosation of which is not elucidated, although there have been many studies of it, starting with those of Smith and Loeppky (21). Therefore, while the yields of N-nitroso compounds derived from a particular tertiary amine and nitrite can be measured under certain conditions (22), the theoretical calculation of those yields has not been possible; there has been more success in this regard with secondary amines, although large errors are possible, as suggested above.

The most direct way of testing the possibility that reaction of an ingested amine with nitrite can give rise to sufficient of a carcinogenic N-nitroso compound to induce tumors has been to feed the amine and nitrite simultaneously to animals for most of their lifespan. The first successful experiment of this type was that of Sander and Bürkle (23), using the amine methylbenzylamine, which when fed to rats with nitrite induced esophageal tumors, the same tumor induced in rats by feeding nitrosomethylbenzylamine. Similar experiments were carried out in mice with piperazine (24), and in rats with heptamethyleneimine (25).

In the past few years more attention has been paid to some of those amines which might pose a risk to people because they are commonly ingested by humans. Included are a variety of components of food, food additives, drugs and agricultural chemicals. Following the demonstration that these amines do react with nitrous acid to form N-nitroso compounds, the structure of which has been

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determined, they can be fed with nitrite to rats or mice (which are, in general, less sensitive to carcinogenic nitrosamines than are rats), either mixed with food or dissolved in drinking water. More than 20 such amines have been tested in this way, and several have evoked a positive carcinogenic effect.

Some amines react very rapidly with nitrite in aqueous solution, which limits the interpretation which can be placed on the results of testing the combination in drinking water; such compounds are better tested in food. Other amines are too insoluble in water to ensure administration of an adequate dose to the animals. The doses that can be administered are somewhat restricted, since no more than 0.2% of nitrite in food or water can be given to rats without risking induction of often fatal methemoglobinemia. The dose of amine given simultaneously is such that there is a ratio of amine to nitrite between 1 to 2 and 1 to 4, which favors formation of N-nitroso derivatives and ensures effective utilization of nitrite.

In Table 1 is a list of the environmental secondary and tertiary amines which have been tested by feeding to rats together with nitrite. Of these, several react very readily with nitrite in acid solution, but some, for example phenmetrazine (26, 27), give rise to a noncarcinogenic N-nitroso derivative. On the other hand, aminopyrine reacts extremely readily with nitrous acid, although it is a tertiary amine, and forms the potent carcinogen nitrosodimethylamine in high yield (28, 29). The other amines vary considerably in the extent to which they form N-nitroso derivatives by reaction with nitrous acid, especially at the relatively low concentrations which model human exposure more closely (30).

Table 2 gives the incidence of tumors that can be considered to have been induced by the chronic administration to rats of several of the amines in Table 1 together with nitrite. Those amines which clearly failed to induce a significant incidence of tumors not found in untreated or in nitrite treated controls are omitted, as are those of which the tests are still in progress and at too early a stage for evaluation. It appears that, even under these relatively crude test conditions, several of the amine/nitrite combinations must be considered carcinogenic. On the other hand, because the tests are on a rather small scale, the apparently noncarcinogenic combinations cannot be considered definitive, but only to represent a lower risk than the positive combinations.

Other, more sensitive, tests have been suggested for the evaluation of potential carcinogenic risk of exposure to chemicals. These include the bacterial mutagenesis test devised by Dr. Bruce Ames (31). This test has been applied to many of the amines listed in Table 1, both alone and after reaction with nitrite in weakly acid solution, followed by neutralization and application to the bacteria. In Table 3 are given the results of a number of such tests, together with comparison of the results of chronic

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TABLE 1
AMINES FED WITH NITRITE TO RATS

Aminopyrine	Methapyrilene
Arginine	Methylguanidine
Chlordiazepoxide	Methylbenzylamine
Chlorpromazine	Monuron
Cyclizine	Morpholine
Diethylamine	Oxytetracycline
Dimethyldodecylamine	Piperidine
Dimethylphenylurea	Piperine
Dipyrone	Quinacrine
Disulfiram	Thiram
Heptamethyleneimine	Tolazamide
Hexamethylenetetramine	Tolbutamide
Lucanthone	Trimethylamine-N-Oxide

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TABLE 2

TUMORS INDUCED BY AMINES FED WITH NITRITE TO RATS

Compound (Concentration %)	Nitrite Concentration %	Duration of Treatment (weeks)	No. of Rats	Tumors Induced (% of Animals)
Aminopyrine (0.025)	0.025	50	30	29 (97) liver hemangioendothelial sarcomas
Chlordiazepoxide (0.2)	0.2	50	30	3 (10) nervous system tumors
Dimethyldodecylamine (0.18)	0.2	80	30	3 (10) urinary bladder carcinomas 4 (13) forestomach tumors
Disulfiram (0.1)	0.2	78	40	*15 (37) esophageal tumors
Heptamethyleneimine (0.2)	0.2	28	30	16 (53) lung carcinomas 23 (77) esophagus tumors
Methapyrilene (0.1)	0.2	90	30	9 (30) liver tumors
Methylbenzylamine (0.5)	0.5	12	7	7 (100) esophagus tumors
Morpholine (0.5)	0.5	12	7	7 (100) liver tumors
Oxytetracycline (0.1)	0.1	60	30	5 (17) liver tumors

*Experiment not completed

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TABLE 3

EFFECT OF NITROSATION ON MUTAGENICITY AND CARCINOGENICITY OF DRUGS

COMPOUND	% YIELD OF NITROSAMINE IN CHEMICAL TEST	MUTAGENICITY IN SALMONELLA (1537)				CARCINOGENICITY IN RATS BY FEEDING	
		Plate Test		Liquid Test			
			+ NO_2^-		+ NO_2^-		+ NO_2^-
Aminopyrine	50%	–	+	–	NT*	–	+
Lucanthone	2.4	+	+	NT	+	+	±
Tolazamide	0.6	–	+	–	+	–	–
Oxytetracycline	0.2	–	±	NT	?	–	±
Chlorpheniramine	0.2	–	+	–	+	NT	NT
Quinacrine	0.1	+	+	NT	+	?	?
Disulfiram	0.08	–	+	–	NT	–	+
Methapyrilene	0.08	–	±	–	–	NT	+
Chlorpromazine	0.05	–	+	–	NT	–	–
Methadone	0.04	–	–	–	?	NT	NT
Dextropropoxyphene	0.03	–	–	+	–	NT	NT
Chlordiazepoxide	+	–	+	–	NT	NT	±
Cyclizine	+	–	–	–	+	NT	–
Hexamethylenetetramine	+	–	+	–	NT	–	–

*NT = not tested

administration to rats and of the yields of nitrosamines by reaction with nitrite under standard conditions. It can be seen that several of the amines which, in combination with nitrite, were negative in the rat test gave positive results after nitrosation in the Ames test, which can be considered more sensitive in this regard than the long term animal bioassay. Examples are tolazamide, cyclizine and hexamethylenetetramine. Even more surprising is that several amines including methapyrilene, chlorpheniramine, chlorpromazine and aminopyrine which give rise to nitrosodimethylamine by reaction with nitrous acid give a positive mutagenesis result in the plate test, in which nitrosodimethylamine is negative. This suggests that one of the other possible products of reaction of these tertiary amines with nitrous acid is a nitrosamine positive in the Ames test. Whether or not such nitrosamines are therefore carcinogenic is not certain because of the many discrepancies between carcinogenicity and bacterial mutagenicity among nitrosamines (2). Nevertheless, bacterial mutagenicity is a valuable test for the biological activity of the products of reaction of amines with nitrite and might serve to suggest those biologically useful amines which merit further testing to establish the possible risk to man of products of their nitrosation.

The results obtained so far indicate that there is a carcinogenic risk of unknown extent in ingestion of amines which might react with nitrite from cured meats or in saliva to form carcinogenic N-nitroso derivatives. Furthermore, it is probable that some ranking of the relative risks presented by several such amines can be achieved by examination of the products of their reactions with nitrite in chemical systems and by studies of their mutagenesis, transforming ability *in vitro* and long term carcinogenic effects in animals.

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N-Nitrosamines in Consumer Products and in the Workplace

I. S. KRULL, G. EDWARDS, M. H. WOLF, T. Y. FAN, and D. H. FINE
New England Institute for Life Sciences, 115 Second Avenue, Waltham, MA 02154

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Chemists have long been aware that amines can react with various nitrosating agents, under a variety of conditions, to form a wide array of N-nitroso derivatives (1). It was generally assumed that only secondary amines can effectively form stable N-nitrosamines. However, it has now become apparent that primary and tertiary amines, as well as tetraalkylammonium salts, can all form N-nitroso derivatives under the appropriate reaction conditions (2-10). It has also become apparent that there are several mechanisms possible for the formation of the most common N-nitroso derivatives. Thus, in addition to the more customary reaction of an amine with nitrous acid, N-nitroso derivatives can also form *via* the reaction of an amine with NO_x (NO_2, N_2O_3, N_2O_4) (3). Amines can also be transnitrosated with already formed N-N-nitroso or C-nitro compounds *via* a transnitrosation reaction, they can be converted into their N-nitroso derivatives (6, 8).

In view of the various possible pathways for nitrosation of amines as well as of amine derivatives (amides, ureas, carbamates, etc.), it is not unexpected then for N-nitroso compounds to be found in many different areas of the human environment (11). It is possible that N-nitroso compounds may represent a carcinogenic exposure which most people experience on a daily basis. The list of items that have now been demonstrated to have measurable levels of various N-nitroso compounds present within them has grown considerably over the past decade (2, 11, 12). A portion of this list would include: air, water, soil, cheese, meats, fish, eggs,

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cutting fluids, cigarette smoke, pesticides, cosmetics, shampoos, beauty products, and drugs. It may be expected that additional routes for man's exposure to N-nitroso compounds will be found in the future. Recent advances in methods and instrumentation for the detection of both volatile and non-volatile N-nitroso compounds have enabled new areas of exposure to be determined (13-17).

The widespread interest in the presence of N-nitroso compounds within the environment and consumer products is due to the known carcinogenicity and mutagenicity of many of these chemicals (9, 12, 18-22). Of the approximately 130 N-nitroso compounds that have been tested in various animal species thus far, over 100 of these have been found to be carcinogenic to varying degrees (9, 45). Thus, there is a firm basis to suspect that as yet undiscovered, new N-nitroso derivatives may also share the dubious distinction of being mutagenic and/or carcinogenic in animal species and perhaps in man as well. In view of the very large number of known and possible amine precursors present in the ecosystem, it is to be predicted that a large number of new N-nitroso derivatives remain to be identified in either environmental samples and/or consumer products. The biological properties of such new compounds will have to be determined with regard to their potential threat as carcinogenic risks to man.

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Consumer Products

A. Pharmaceuticals

By comparison with other areas of consumer products, relatively little is known about the possible presence of N-nitroso derivatives in pharmaceuticals. Eisenbrand et al., have reported on the presence of dimethylnitrosamine in all 68 samples of aminopyrine analyzed in Germany (23). They suggested that DMN could have been formed in various drug formulations by two main routes. One was by the in situ reaction of aminopyrine with nitric oxides in the air, and the other involved the synthetic process used in the manufacturing process itself (23). The levels of DMN found ranged from less than 10 ppb to just under 400 ppb. It has been found for several years that animals fed aminopyrine and sodium nitrite in their diets show the formation of malignant tumors (24, 25). In view of the presence of substantial amounts of DMN in the

pharmaceutical itself, and the potential *in vivo* biosynthesis of DMN from this drug, the German government in 1977 curtailed sales of the drug.

Schoenhard *et al.*, reported on the presence of 1-diphenylmethyl-4-nitrosopiperazine in an antibiotic formulation under development, but never released for general use (26). Despite the use of a number of different synthetic routes, the final product always contained varying levels of the N-nitroso contaminant. It was eventually determined that the pure drug reacted with singlet oxygen in the air to form the observed N-nitroso impurity. Thus, even the use of a N-nitroso free synthetic process did not, in this instance, guarantee a final product devoid of any N-nitroso compound.

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Many pharmaceutical products on the market contain primary, secondary, and/or tertiary amines or amine derivatives, and several drugs have been shown to readily form N-nitroso compounds when nitrosated *in vitro* and/or *in vivo* (27-33). With the exception of aminopyrine in Germany (23) and the antibiotic studied by Schoenhard *et al.*, (26), there appears to be no information available with regard to the possible presence of N-nitroso impurities present in pharmaceutical products.

We have completed an initial screen of 73 prescription and over-the-counter drug formulations available in the general Boston area (34). Our initial choice of which drugs to investigate was based on a number of factors: 1) the known structure of the drug itself; 2) the reported usage of the product in the United States (35); and 3) the possibility of an *in situ* reaction of the drug with NO_x in the atmosphere (23, 26). In reporting our findings on N-nitroso impurities in this relatively limited sample of pharmaceutical products, it is not to be implied that this study is comprehensive, or even a necessarily representative sample of all pharmaceutical products. Rather, we hope it will stimulate further interest in this particular area of N-nitroso environmental exposure and distribution.

For the analyses discussed here, we have used Gas Chromatography-Thermal Energy Analysis™ (GC-TEA™) and/or High Pressure Liquid Chromatography-Thermal Energy Analysis (HPLC-TEA). The TEA has been used as the detector

of choice for the determination of trace levels of N-nitroso impurities, whether these are volatile or nonvolatile. It has been found, by various investigators, that the TEA is probably the most sensitive method of detection for this particular class of compounds (36-40).

We have studied 73 pharmaceutical products for the possible presence of N-nitroso contaminants. Over-the-counter items have included some of the popular cold remedies, decongestants, cough syrups, cold syrups, headache pills, and other popular drug products. We have usually surveyed several different products which contain similar types of ingredients, but these ingredients vary widely both in type and amounts present. For the prescription drugs, there generally exist a great number of different formulations, manufactured by different drug companies.

Table 1 indicates our survey of prescription and non-prescription drugs. We have investigated only a single manufacturer's product for each item. Contaminants in three presciption and two over-the-counter formulations were shown to give a positive TEA response. Subsequent chemical tests showed that for the prescription drugs, the impurities were probably N-nitroso compounds, although this has not been confirmed by mass spectrometry. Several other drugs which contained TEA positive materials were demonstrated not to contain N-nitroso compounds by a series of chemical tests used in conjunction with HPLC-TEA.

For the over-the-counter formulations, two of the thirty-nine items tested contained TEA responsive materials. For these two instances, exposure of the organic extracts to glacial acetic acid alone led to the complete disappearance of the TEA responsive materials (34). This observation suggests that the unknown materials are probably not simple N-nitroso derivations, and the results are more compatible with their being O-nitroso compounds (nitrites) (34).

We must stress that in order to confirm these preliminary findings, the material responsible for the TEA peak must be isolated and identified by conventional chemical and spectroscopic techniques. This aspect of our screening program remains to be completed.

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The results imply that for the majority of drug products tested, there does not appear to be a serious problem with regard to the presence of N-nitroso compound contaminants. In those instances where TEA responsive materials were present, the levels were in the low ppb range (40-81 ppb). Should any of these materials be confirmed as real N-nitroso compounds, and if these are known or suspected carcinogens, then there may be a health risk for persons taking them. However, it must be emphasized that we have no evidence at present with regard to the carcinogenic risk of any of the drug product impurities indicated in Table 1.

Table 1.

Survey of prescription and over-the-counter drugs

Drugs	Possible N-nitroso impurities (ng/g)
phenelzine sulfate	81
imipramine. HCl	68
nitrofurantoin	40
31 other amine and/or amide prescription drugs	ND
15 O-T-C cold remedies	ND
5 O-T-C sleeping pills	ND
7 O-T-C headache remedies	ND
5 O-T-C cough syrups	ND
7 O-T-C decongestants	ND
Total	73

B. Cutting Fluids

Recently, we reported on the presence of relatively high concentrations of N-nitrosodiethanolamine (NDE1A) in com-

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mercial cutting fluids (41). Rappe and Zingmark have also demonstrated the formation of nitrosamines in cutting fluids available in Sweden (42). Stephany et al., have reported the presence of N-nitroso-5-methyl-1,3-oxazolidine as an impurity in a commercial cutting fluid used in The Netherlands (43). The levels of NDE1A found in a small sampling of cutting fluids available in the United States ranged from 0.02-3 percent (41). The NDE1A concentrations shown in Table 2, represent the highest levels of a nitrosamine found in any commercial product investigated to date.

Table 2.

Concentration of N-nitrosodiethanolamine in several brands of synthetic cutting fluids (41)

Brand	NDE1A (%)
A	2.99
B	1.04
C	0.42
D	0.25
E	0.18
F	0.06
G	0.06
H	0.02

Many of the "cutting fluids" used today are of the synthetic variety, and usually contain an alkanolamine and sodium nitrite in varying proportions, as well as other ingredients. Most manufacturers have not, until very recently, determined the presence of NDE1A in their products. This situation is changing rapidly. In Canada, the government has moved to ban the importation, sale, and advertisement of products (cutting fluids) which contain any nitrite when diethanolamine or triethanolamine is also present (44).

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Since it has been shown that NDE1A can cause cancer in two species of laboratory animals (22, 45), we believe that its presence at relatively high levels in cutting fluids, may represent a hazardous situation in terms of worker exposure. It is, therefore, suggested that workers who formulate or use cutting fluids (such as machinists) be studied with regard to their cancer incidence, and measures should be taken to reduce their daily exposure to NDE1A.

C. Cosmetics, Skin Lotions, and Shampoos

Fan et al., have recently reported on the presence of N-nitrosodiethanolamine (NDE1A) in a variety of cosmetics, body lotions, and hair shampoos (Table 3) (46). Most of these products were known to contain di- and/or triethanolamine additives; however, the source of the nitrosating agent is unknown. In addition to the presence of NDE1A, a number of these products contained other unidentified TEA responsive materials, sometimes at even higher concentrations. Additional research in this area is obviously needed.

In view of the now demonstrated presence of NDE1A in several types of beauty products, the question arises as to what industrial workers may be inadvertently exposed to NDE1A via this route. There have been at least two major studies regarding an epidemiological relationship between beauticians and cancer (47, 48). However, no firm conclusions can yet be arrived at with regard to the possible role that NDE1A might have on the incidence of certain cancers among these workers. In view of the many different chemicals which beauty care workers handle daily, it is a difficult task to assign a specific role to any one item. The major route of NDE1A entry into the human body would be skin absorption, and there are no literature reports which discuss this phenomenon.

Those groups of industrial workers, other than beauticians, who are involved in the handling of cosmetics, skin lotions, and shampoos, would be people in the formulating process in the cosmetic industry. It is assumed that NDE1A is formed after the cosmetic has been completely formulated; thus it might be expected that workers involved in the final stages of the formulating process could be exposed to varying amounts of NDE1A. Those workers in the cosmetic industry

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who are involved in the packing of the final product might also be exposed to certain levels of NDE1A.

Table 3.

N-nitrosodiethanolamine content of cosmetics, lotions and shampoos (46)

Sample	NDE1A Content (ng/g)
Cosmetics	
C1	100
C2	~40
C3	23,000
C4	49,000
C5	3,700
C6	~1,200
C7	Trace
Lotions	
L1	100
L2	Trace
L3	ND
L6	Trace
L7	Trace
L8	Trace
L9	Trace
L10	Trace
L11	~140
L13	47
L14	22
Shampoo	
S1	260
S3	Trace
S4	100
S5	Trace
S6	ND
S7	70
S8	Trace
S9	68
S10	27

NDE1A = N-nitrosodiethanolamine; ND = not detected (below 1 ng/g); Trace = less than 10 ng/g

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D. Herbicide Formulations

There have been several reports in recent years on the presence of N-nitroso derivatives in agricultural chemicals (49-53). In several instances, these N-nitroso derivatives have also been demonstrated to be carcinogenic in laboratory animals (53-58). We have recently reported that N-nitroso impurities are present in several herbicide formulations that are used by both home gardeners and farmers (Table 4) (59). Most of the herbicides which we examined were formulated as the dimethylamine salt, and all were purchased as aqueous solutions. Dimethylnitrosamine (DMN) was present at 0 to 640 ppm, and dipropylnitrosamine (DPN) was present at 0 to 195 ppm (μg/g of the original salt formulation). It is not known if the DMN and DPN were present at the time of manufacture, or if they formed during storage. It is assumed that both mechanisms for the formation of these volatile nitrosamines may be operative, depending on the particular herbicide in question. Sample 6 of Table 4 contained about 0.06% of DMN in the formulation as purchased from the retail outlet. Such a level of DMN in a consumer product is unusually high, and because of the volatile nature of this nitrosamine, it may pose a health risk to man.

Table 5 is a more comprehensive compilation of the DMN content of some herbicides formulated as the dimethylamine salts (53). Five out of six formulations of 2,4-D contained DMN at levels of between 60 and 370 μg/l. All four formulations of MCPA contained DMN at levels of between 250 and 650 μg/l. DMN levels of between 187,000 and 640,00 μg/l were found in formulations of 2,3,6-trichlorobenzoic acid which had been stored in metal cans to which sodium nitrite had been added. Table 5 represents a comparative study between an EPA laboratory and Thermo Electron. The decrease of the DMN level for any one compound reflects the success of the manufacturers in decreasing the DMN contamination (60).

With regard to worker exposure to nitrosamines present in agricultural chemicals, the volatile nature of these materials suggests inhalation and dermal contact as the major routes of absorption. There is a potential hazard of exposure to field applicators, especially commercial farmers and growers who regularly spray their crops and land. Manufacturers of the formulations may have workers who are exposed to unusually

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Table 4. Determination of nitrosamines in technical herbicides (59)

Sample	Herbicide Formulations	Nitrosamine Studied	mg/L
1	2,4-dichlorophenoxyacetic acid as DMA salt 2-(2-methyl-4-chlorophenoxy) propionic acid as DMA salt 3,6-dichloro-o-anisic acid as DMA salt	DMN	0.30
2	2,4-dichlorophenoxyacetic acid as DMA salt 3,6-dichloro-o-anisic acid as DMA salt	DMN	ND
3	2,4,5-trichlorophenoxypropionic acid as DMA salt 2,4-dichlorophenoxyacetic acid as DMA salt	DMN	ND
4	2,3,6-trichlorobenzoic acid as DMA salt	DMN	187
5	2,3,6-trichlorobenzoic acid as DMA salt	DMN	195
6	2,3,6-trichlorobenzoic acid as DMA salt	DMN	640
7	formulation of a,a,a-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine	DPN	154

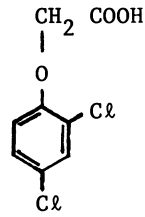
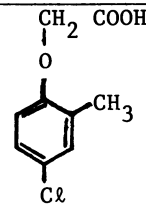
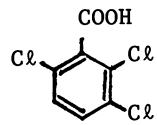
DMN = dimethylnitrosamine
DPN = dipropylnitrosamine
ND = less than 0.05 mg/L

In N-Nitrosamines; Anselme, J.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1979.

Table 5.

N-nitrosodimethylamine Content of Herbicides

Formulated as Dimethylamine Salts (53)

Dimethylamine Salt of Herbicide	DMN Content µg/l
2,4-D 	370 240 200 155 60 <10
MCPA 	650 590 340 255
2,3,6-TCBA 	640,000 353,000 195,000 187,000 29,000 23,000 2,300

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large amounts of the aqueous solutions. There is little available information with regard to exposure levels amongst herbicide formulators as a separate group. Finally, professional gardeners who work with herbicides daily may also be exposed to DMN and/or DPN. Epidemiological studies amongst these particular groups of workers have not yet been undertaken.

E. Samples Analysed by Thermo Electron

Table 6 shows a sampling of recent results divided according to the type of samples, and the specific N-nitroso compounds searched for. Meat products have, in general, exhibited volatile nitrosamines in the low ppb range, whereas certain pesticides have had levels of DMN and DPN well into the ppm range. The most frequently observed nonvolatile nitrosamine was NDELA, which was present in a number of cosmetics, pesticides and cutting fluids at relatively high levels. These analyses for N-nitroso derivatives were performed since publication of our earlier work in these various areas. It is of interest to compare the values in Table 6 with already published data for these groups of customer products indicated in Tables 2-5.

Conclusions

Occupational carcinogenesis is a complex field of investigation (61-64). In the case of N-nitroso compounds, very little information is available with regard to which particular materials various groups of workers may have been exposed to during the past 20 years or so. Thus, it is difficult to arrive at any correlation between current cancer incidences amongst industrial workers. We are only now beginning to collect information regarding what types of N-nitroso chemicals groups of workers are exposed to. This information will take several years to collect, and it is impossible to correlate current exposure to past instances for any given N-nitroso material. Also, the problem is compounded several fold because in most instances of industrial exposure, there are often several different types of chemicals that the same workers may be exposed to at the same time. Thus, it becomes necessary to try and separate possible cancer effects for one group of chemicals from those of another, e.g., N-nitroso compounds. Quite often there may be a co-carcinogenic

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Table 6. Samples Analyzed by Thermo Electron (1977-78)

	Sample Type	N-nitroso Compounds Studied	Levels Found
A.	pesticides	N-atrazine	ND
		DMN	ND 30-9400 ppb
		NDE1A	ND 15-360,000 ppb
		DPN	6000 ppb
		N-morpholine	ND
B.	meat products	DMN	ND 0.3-6.5 ppb
		N-pyrrolidine	NA 0.1-27.6 ppb
		DEN	1-2.2 ppb
C.	cosmetic products	NDE1A	ND 7.5-90,000 ppb
		N-morpholine	2500 ppb
D.	cutting fluids	NDE1A	ND 30-1,400,000 ppb

DMN = dimethylnitrosamine
DEN = diethylnitrosamine
DPN = dipropylnitrosamine
NDE1A = N-nitrosodiethanolamine
N-atrazine = N-nitrosoatrazine
N-morpholine = N-nitrosomorpholine
ND = not detected (below 1 ng/g)

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effect of several different chemicals on the same groups of workers, and it becomes extremely difficult to separate the individual effects due to one particular chemical. However, with regard to N-nitroso compounds, there is virtually no epidemiological data available, and the first step towards obtaining such information seems to be accumulating environmental data on current workers' exposure to this group of chemicals. We have attempted to indicate some areas where such environmental analyses may provide rapid and reliable data.

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