BIOORGANIC CHEMISTRY 8, 97–137 (1979)

REVIEW

Some Biochemical Aspects of *N*-Nitroso Compounds

G. A. DIGENIS AND C. H. ISSIDORIDES[1]

Division of Medicinal Chemistry, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40506

Received July 11, 1978

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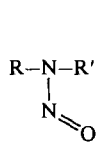
I. INTRODUCTION

A new era in the history of chemical carcinogenesis began in 1956, when Magee and Barnes (*1*) found that *N*-nitrosodimethylamine (**3**) fed to rats produced a high incidence of hepatomas. Since then, a large number of *N*-nitroso compounds has been found to be strongly carcinogenic (*2–9*) in several animal species, including primates (*10*). Every type of animal tested is susceptible to carcinogenesis by *N*-nitroso compounds; and although there is no direct evidence to associate them with human cancer, it is believed that they are probably carcinogenic to man as well (*11*). The present paper is not an exhaustive review of *N*-nitroso compounds but, rather, an outline of some biologically significant aspects of their chemistry.

II. OCCURRENCE AND CARCINOGENICITY

N-Nitroso compounds are conveniently divided into the nitrosamines (**1**) derived from dialkyl, alkaryl, diaryl, or cyclic secondary amines, and the nitrosamides (**2**) derived from simple *N*-alkylamides (X = O, Y = alkyl or aryl), *N*-alkylureas (X = O, Y = NH_2, NHR, NR_2), *N*-alkylcarbamates (X = O, Y = OR), *n*-alkylguanidines (X = NH, Y = NH_2), and related structures.



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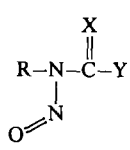

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[1] On leave from American University of Beirut, Lebanon.

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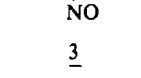
Figure II.1 gives typical examples of compounds whose carcinogenicity is well documented (*4*, *12*, *13*).



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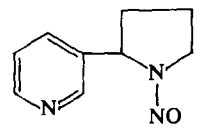
N-Nitrosodimethylamine



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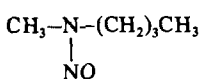
N-Nitrosonornicotine



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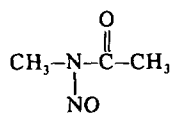
N-Nitrosomethyl-*n*-butylamine



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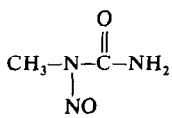
N-Methyl-*N*-nitrosoacetamide



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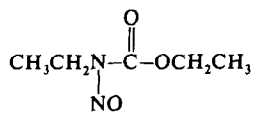
N-Methyl-*N*-nitrosourea



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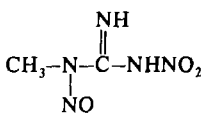
N-Ethyl-*N*-nitrosourethane



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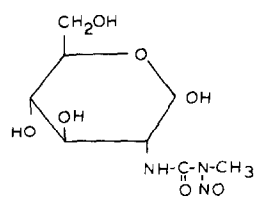
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine
(1-Methyl-3-nitro-1-nitrosoguanidine)

FIGURE II.1

Biological interest in this class is further enhanced by the identification of the broad-spectrum antibiotic streptozotocin as the *N*-nitrosourea **10** (*14*)



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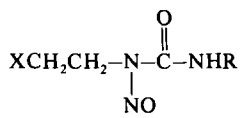
Although carcinogenicity appears to be the rule for *N*-nitroso compounds, some (e.g., *N*-nitrosodiphenylamine) are noncarcinogenic or only weakly carcinogenic (*4*). Furthermore, the recent clinical evidence regarding certain 2-haloethylnitrosoureas (**11**) adds a totally new dimension to the biological action of *N*-nitroso compounds. Despite their

structural similarity to the carcinogenic nitrosoureas, these haloethyl derivatives show great promise as effective antitumour agents (*15–18*).



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It is now believed that most or all carcinogens either are potent electrophilic species or can be converted to such species by chemical or enzymic activation (*19*). Nitrosamides (**12**), for instance, readily undergo heterolysis to give alkylating species via the unstable diazotic acid (alkanediazohydroxide **15**, Fig. II.2). Regardless of what their true nature might be (alkanediazohydroxides, alkyldiazonium ions, carbonium ion, or related ion pairs and triplets), these alkylating species are believed to initiate the carcinogenic process by attacking critical nucleophilic sites of macromolecular constituents of target tissues (DNA, RNA, proteins) (*20*), as depicted in Fig. II.2 for the oxygen atom at position 6 of guanine (*21*, *22*).

Unlike the nitrosamides, which are unstable at physiological pH and decompose nonenzymatically to give reactive species, the nitrosamines, such as **13**, are considerably more stable and are believed to exert their adverse biological effects after enzymic

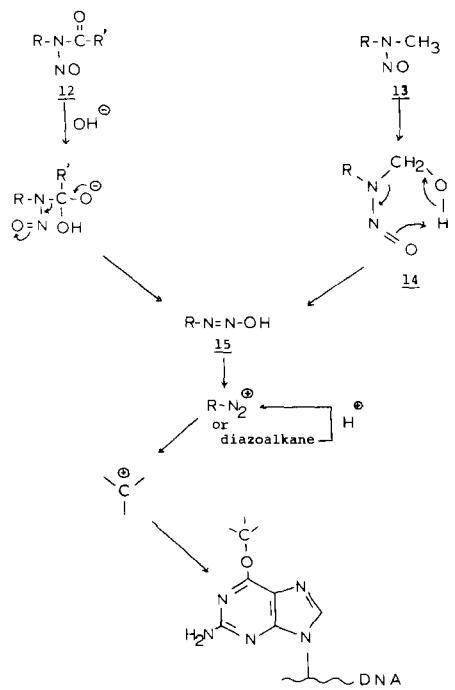


FIGURE II.2

activation by microsomal mixed-function oxidases (*9*). One common activation pathway entails α-hydroxylation to give an intermediate (**14**) which breaks down to alkylating species (Fig. II.2).

The nitrosamines show striking predilection for tumor induction in specific organs (organotropic action). Although this organotropy is not absolute but depends somewhat upon dosage and manner of application, it is clearly structure dependent. For example, animal experiments have shown that symmetrical dialkylnitrosamines (**1**, R=R′) produce cancers predominantly in liver and lung, whereas the unsymmetrical compounds initially attack the esophagus (*23*). The fact that nitrosamines usually produce cancer in certain remote organs rather than at the site of application is consistent with their requiring hydroxylation by specific hydroxylases before they can exert their adverse effects. In this respect nitrosamines, along with many other chemical carcinogens that require enzymic activation, are indirect carcinogens. In contrast, the nitrosamides, which decompose without enzymic activation, may act as direct, topical carcinogens. Consequently they are effective at any site where they are applied (skin) or inserted (colon, stomach) (*10*). Nevertheless, organospecific effects are observed even with the nitrosamides as, for example, with *N*-methyl-*N*-nitrosourea, which selectively produces malignant tumors of the brain (*24*).

N-Nitroso compounds can be readily prepared by nitrosation of secondary and tertiary amines, amides, ureas, carbamates, and guanidines. The components required for this synthesis are ubiquitous in the environment. Several amines occur in foods (*25*), wine, food flavorings, tobacco products (*26*), a number of drugs, and other environmental chemicals. Other nitrosatable substances in the environment include quaternary ammonium salts, guanidines, carbamates, and ureas. The last two are sometimes used as drugs, and because of their anticholinesterase activity, as insecticides (*4*, *27*). With regard to nitrite, it is used in meat and fish curing because it imparts (i) the characteristic pink color of cured meat, through formation of denatured nitrosylmyoglobin, (ii) flavor, and most importantly, (iii) antibacterial activity against *Clostridium botulinum*, a microorganism producing the extremely poisonous toxin botulin (*27*, *28*). Another important source of nitrite in the diet is nitrate, which occurs in some vegetables (spinach, beets, radishes, celery, etc.), in certain water supplies, and in saliva. Normally nitrate becomes toxic only under conditions in which it may be reduced by microorganisms to nitrite, as occurs in saliva (*11*, *29*) and during storage of leftover foods, such as potatoes and spinach, at room temperature (*10*, *30*).

From the foregoing discussion it follows that man may well be exposed to a hazard on two counts: first, by ingesting *N*-nitroso compounds preformed in the environment (for example, by the interaction of nitrite with *N*-nitrosatable constituents of foods); second, by synthesizing *N*-nitroso compounds in the favorable environment of the human stomach (or other site) from nitrite and N-nitrosatable compounds which may be ingested as drugs, food additives, or natural constituents of food. Nitrosamines, for instance, have been found in fish exposed to nitrite and in cured meats (*27*, *31*). Nitrosopyrrolidine has been found in relatively high concentrations in fried bacon (*11*, *27*, *32*) and other *N*-nitrosamines have been found in tobacco and tobacco products (*26*). Moreover, the synthesis of *N*-nitroso compounds from nitrite and secondary or tertiary amines and from amides has been demonstrated *in vitro* with animal and human gastric juice and *in vivo* in animals (*4*, *11*).

III. CHEMISTRY

This section is devoted to some chemical aspects related to the biological properties of *N*-nitroso compounds. For a full account of the chemistry of the nitroso group the reader is referred to several recent reviews (*33*).

A. Formation

Nitrosation of organic nitrogen compounds may be effected by a variety of nitrosating agents. The method of choice depends on the structure of the substrate, its solubility in a particular solvent, and the ease of isolation of the desired product.

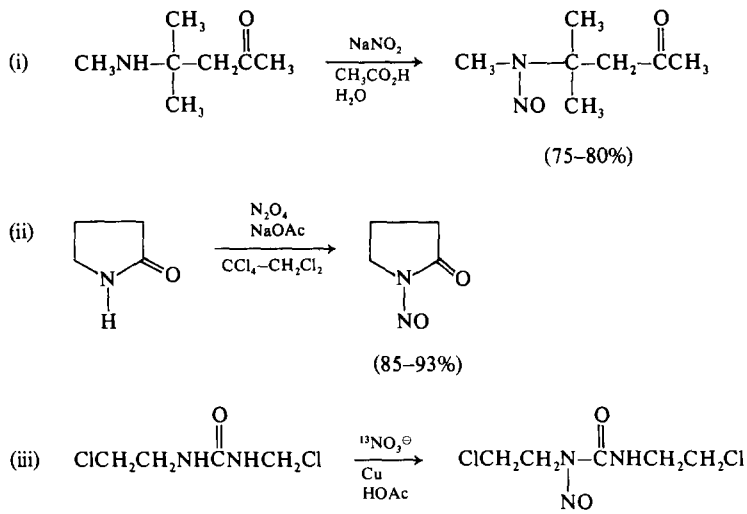


FIGURE III.1

Typical nitrosations (*34*) are carried out with (a) sodium nitrite in aqueous hydrochloric acid, (b) sodium nitrite in acetic acid, (c) nitrous anhydride, N_2O_3, (d) nitrosyl chloride, NOCl (e) dinitrogen tetroxide, N_2O_4, (f) nitrosonium tetrafluoroborate, (g) mixtures of nitrogen dioxide and nitric oxide (formally equivalent to N_2O_3). Examples of nitrosations of secondary amines and of amides are given in Fig. III.1. The second reaction of Fig. III.1 is carried out in the presence of excess sodium acetate to neutralize the liberated nitric acid, since otherwise the reverse reaction of denitrosation would decrease the yield of product (*35*). The last example of Fig. III.1 is of special importance because it illustrates the synthesis of compounds labeled in the nitroso group with the short-lived radionuclide ^{13}N, for *in vivo* scintigraphy (*36*, *37*).

The formation of diazonium salts from primary aromatic amines by reaction with nitrous acid undoubtedly involves transient primary *N*-nitroso compounds as intermediates. Under the same conditions, primary aliphatic amines give similar intermediates which readily decompose by loss of nitrogen to give a variety of products. At low temperatures, however, both aromatic primary nitrosamines and primary aliphatic diazonium ions can be isolated from an ethereal solution of the corresponding amine and nitrosyl chloride. Many of the kinetic characteristics for diazotization have exact

counterparts in aliphatic deamination as well as in the formation of *N*-nitroso derivatives from secondary amines, so that the initial step in the reaction of both primary and secondary amines with nitrous acid is the common one of *N*-nitrosation (*33c*).

Mechanistic studies of N-nitrosation in aqueous solutions are complicated by the fact that nitrous acid (pK_a ~3.37) generates a complex set of species in equilibrium, many of which are effective nitrosating reagents. Furthermore, in the presence of anions such as chloride, bromide, and thiocyanate additional species with the general formula X–NO (X=Cl, Br, SCN) are also formed. Consequently, the nature of the nitrosating reagent depends on the conditions, and in some cases, reaction may occur simultaneously by more than one species. Some of the possible nitrosating agents in aqueous solution are, in order of increasing activity (*33c*):

ON–ONO	Nitrous anhydride (nitrosyl nitrite)
ON–Cl	Nitrosyl chloride
$ON-\overset{\oplus}{O}H_2$	Nitrous acidium ion
$ON^{\oplus}$	Nitrosonium ion

In 0.1 to 0.5 *N* perchloric acid, the main nitrosating species is believed to be nitrous acidium ion (protonated nitrous acid), whereas in more dilute acid (pH 1.5 to 5.0) the nitrosating species is nitrous anhydride (*38*). Increasing solvent acidity favors the formation of the powerful nitrosating reagent nitrosonium ion (NO^+). Sodium nitrite–hydrochloric acid mixtures, which presumably contain nitrosyl chloride, sometimes give results different from those obtained with sodium nitrite–acetic acid (*39*). In its simplest form the nitrosation of amines entails nucleophilic attack by the lone electron pair of the nitrogen atom on the electrophilic nitrosating agent and subsequent deprotonation:

$$R_2NH \rightarrow R_2\overset{\oplus}{N}HNO \rightarrow R_2NNO.$$

The kinetics of N-nitrosation have been reviewed by Mirvish (*40*). Most secondary amines are nitrosated according to Fig. III.2, Eqs. (a) and (b). The nitrosating agent is nitrous anhydride, produced from two molecules of nitrous acid. The rate of nitrosation is proportional to [amine] and $[N_2O_3]$ and hence to $[HNO_2]^2$, and follows rate expressions (c) and (d)[2].

(a) $2HNO_2 \rightleftharpoons N_2O_3 + H_2O$

(b) $RNHR' + N_2O_3 \rightarrow R-\underset{\displaystyle NO}{\underset{|}{N}}-R' + HNO_2$

(c) $\text{Rate} = K_1[RNHR'][HNO_2]^2$

(d) $\text{Rate} = K_2[\text{amine}][\text{nitrite}]^2$

(e) $\text{Rate} = K_3[HNO_2]^2$

FIGURE III.2

Equation (c) refers to [unprotonated RNHR'] and [free HNO_2], and K_1 should be independent of pH, but [RNHR'] and $[HNO_2]$ have to be calculated for each pH. Equation (d) is more convenient to apply, since the total concentrations of amine and nitrite are used, regardless of the actual species present, but here the stoichiometric rate

[2] Name of compound in brackets refers to total stoichiometric concentration, regardless of actual species present; formula of compound in brackets refers to a particular molecular species.

constant K_2 varies with pH. The reaction rate and K_2 show maximum values at pH 3.4 (*40*), which is near the pH of human gastric juices.

The nitrosation of *N*-methylaniline (Fig. III.2, R = Ph, R′ = CH_3) usually follows Eq. (e) because nitrosation is more rapid than N_2O_3 formation, which becomes rate limiting. However, Eq. (d) is followed at higher acidity (pH 1) because [unprotonated $PhNHCH_3$] is so low that the nitrosation becomes rate limiting (*41*). In general the ease of nitrosation, as given by K_2 [Fig. III.2, (d)] increases as the basicity of the amine decreases, i.e., as pK_a decreases (*40*). For example, the rate of nitrosation increases 1000-fold as the basicity of the amine decreases on proceeding from dimethylamine to aromatic amines such as *N*-methylaniline (*42*).

N-Alkylureas and carbamates are rapidly nitrosated in the region of pH 1. For nitrosation of these and other amides the reaction rate increases about 10-fold for each 1-unit drop in pH from 3 to 1. The pK_a for amides is generally less than 1, so that at pH 2 most amides exist as the reactive nonprotonated species. The main nitrosating agent is probably nitrous acidium ion. Unlike the case with amines, there is no simple rule relating ease of nitrosation to other properties of the amides (*40*).

Contrary to the commonly held view, tertiary amines are not inviolate: They, too, undergo nitrosation with elimination of one of the original substituent groups. Moreover, quaternary ammonium compounds and amine oxides, both of which occur in biological systems (*43*, *44*), also undergo nitrosation under appropriate conditions (*43*, *45*). This possibility greatly extends the range of substances which have to be considered as potential precursors of nitroso carcinogens. For some tertiary amines more drastic conditions may be necessary to obtain a significant degree of nitrosation *in vitro*, but *in vivo* reactions may possibly be facilitated by bacterial action (*46*). A plausible mechanism (*47*) for nitrosation of tertiary amines (Fig. III.3) entails initial formation of the *N*-nitrosoammonium ion (**16**) which then eliminates nitroxyl (HNO, nitrosyl hydride) to give the iminium ion (**17**). Hydrolysis of **17** gives a secondary amine, which then undergoes nitrosation in the usual manner. Alternatively the electrophilic iminium ion could react directly with nitrite (a pathway which would be more important at neutral pH) to give intermediate **18**, which then collapses to the nitrosamine (*45*, *48*). The formation, among other products, of the carcinogenic *N′*-nitrosonornicotine (**4**) from the reaction of nicotine (**19**) and sodium nitrite has been postulated to proceed via the cyclic iminium salt **20**, by a similar mechanism (Fig. III.4).

Evidence for the intermediacy of iminium ions in the nitrosation of tertiary amines (Figs. III.3 and III.4) is provided by the failure of quinuclidine (in which the tertiary

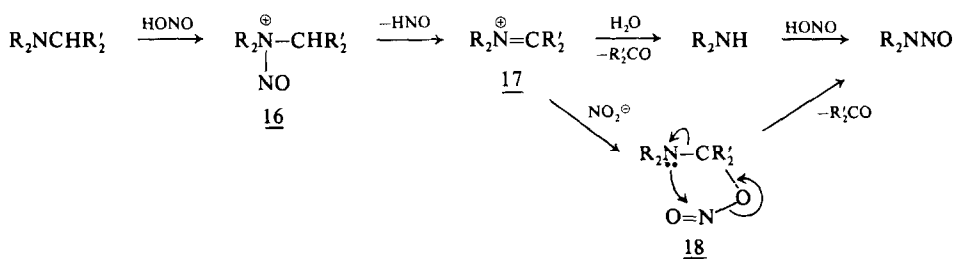


FIGURE III.3

nitrogen is at a bridgehead) to react (*47*) and by the formation of nitrous oxide during the reaction.

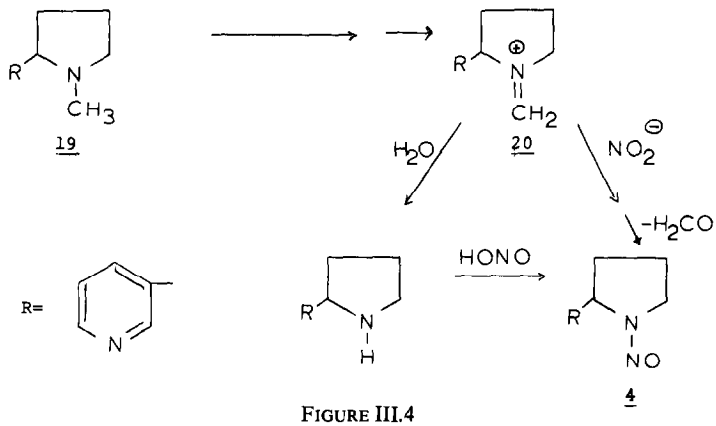
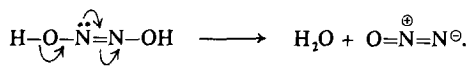


FIGURE III.4

The appearance of nitrous oxide in many reactions is often attributable to dimerization of nitroxyl (*49*), a species which is implicated in the Nef reaction (*50*, *51*), in the photodecomposition of nitrosamines in acidic aqueous solution (*52*), and in the retro-Diels–Alder thermal decomposition of 9,10-di-hydro-9,10-epoxyimino-9,10-dimethylanthracene (*53*). Nitroxyl has an overwhelming tendency to dimerize producing hyponitrous acid ($H_2N_2O_2$) which dehydrates to nitrous oxide in a manner analogous to the formation of diazoalkanes from alkanediazohydroxide precursors:



An alternative route for the nitrosation of tertiary amines *in vivo* is via nitrosative dealkylation under oxidative conditions (*48*, *54*). This route, which does not require the presence of aqueous acid, is outlined in Fig. III.5 (*55*). The first step is removal of an electron from the tertiary amine by an oxidizing agent (Fe III, AgI, oxide of nitrogen, heme protein). The resulting radical cation (**21**) undergoes disproportionation with an adventitious radical (e.g., nitric oxide or oxygen) to give the iminium ion (**17**), which goes on to the product as in the previous mechanism of Fig. III.3.

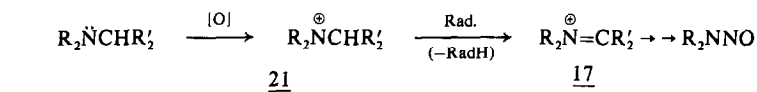


FIGURE III.5

It appears, therefore, that iminium ions are key intermediates in the nitrosation of tertiary amines. The unexpected facility with which the analgesic aminopyrine (**22**) is converted into the carcinogenic *N*-nitrosodimethylamine (**3**) *in vitro* and *in vivo* has been attributed to direct reaction of nitrite ion with the iminium ion (**23**) resulting from protonation of **22** at the enamine carbon (Fig. III.6) (*56–59*). Intragastric nitrosation of aminopyrine presents a possible hazard to people ingesting this analgesic drug (*58*).

The possibility that, in appropriate cases, iminium salts may be in equilibrium with enamines adds another ramification to the reaction of tertiary amines with nitrite. An example is *N*-methylnicotinamide (**29**, Fig. III.7), one of the products arising from

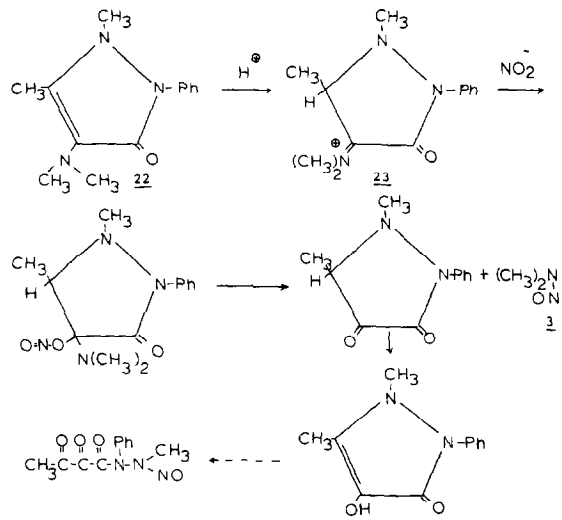


FIGURE III.6

nicotine (**19**) via the iminium ion **24** (isomeric with **20** of Fig. III.4), deprotonation to the enamine **25**, C-nitrosation to **26**, isomerization to the isonitrosoiminium ion (**27**), cleavage to **28**, and hydrolysis to the amide (*12*).

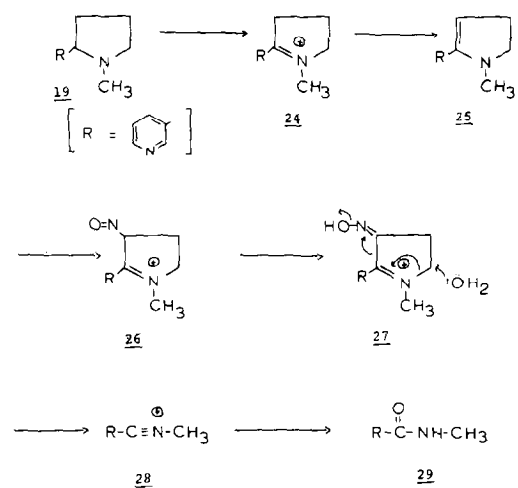


FIGURE III.7

Interestingly, iminium ions analogous to **17** (Fig. III.3) are also involved in the formaldehyde-catalyzed conversion of various secondary amines to nitrosamines in the pH range of 6.4 to 11.0, as discussed in the following section.

It should be noted that nitrite in the environment may effect reactions other than N-nitrosations. Prominent among these are O- and S-nitrosations as well as C-nitrosations on activated aromatic rings or activated aliphatic methylene and methine groups (cf. Fig. III.7). Although the resulting products may well be less hazardous to man than

their *N*-nitroso analogs (*40*, *60*), they may still be of considerable biological significance, depending on whether or not they can participate in transnitrosation reactions (*39*, *61*).

B. Catalysis and Inhibition

Thiocyanate and certain other nucleophilic anions may enhance nitrosation rates markedly (*33o*), and it may well be that the presence of such species in the human alimentary canal may influence profoundly the course of *in vivo* nitrosations (*48*).

Thiocyanate promotes nitrosation by producing the nitrosating agent nitrosyl thiocyanate (ONSCN). The optimum pH for nitrosation at high [$^{\ominus}$SCN] is 2, when nitrite becomes fully protonated. Below pH 2 the rate falls because thiocyanate becomes protonated. Bromide and chloride have effects similar to those of thiocyanate, owing to formation of ONBr and ONCl, but the rate does not fall below pH 2 (*40*). The order of catalytic effectiveness is $^{\ominus}$I > $^{\ominus}$SCN > $Br^{\ominus}$ > $Cl^{\ominus}$. Thiocyanate- and halide-catalyzed nitrosations of amines become especially significant at low pH (<2.5), high thiocyanate (or halide) concentration, and low nitrite concentration (*40*).

There is evidence that the reaction between secondary amines and nitrite in the stomach of the rat is catalyzed by thiocyanate (*63*). Human saliva and gastric contents also contain thiocyanate, and it is known that the amounts of thiocyanate excreted in the saliva and urine of smokers are considerably greater than those in nonsmokers. Furthermore, cigarette smoke contains cyanide which, after inhalation, is converted by the enzyme rhodanase to the nontoxic thiocyanate, the proportion of which in human gastric juice is higher for smokers than for nonsmokers (*62*). It would seem, therefore, that the risk from nitrosamines formed in the stomach would be higher in smokers than in nonsmokers, and that this risk may accentuate the adverse effect of the nitrosamines and other carcinogens present in cigarette smoke (*41*).

It has now been shown by Keefer and Roller (*59*) that formaldehyde catalyzes the formation of nitrosamines from secondary amines and nitrite. That an electrophilic species should catalyze nitrosations is not, in itself, unusual since the simplest known electrophile (the hydrogen ion) readily does so. What is unusual, however, is that formaldehyde should do so in the pH range of 6.4 to 11.0. Hitherto, nitrosamine formation in neutral medium was thought to take place only in the presence of certain bacteria (*63*, *64*), and it was generally assumed that potentially hazardous quantities of carcinogenic *N*-nitroso compounds could be produced only in an acidic medium such as that of the stomach. The work of Keefer and Roller shows that nonenzymatic nitrosation may occur smoothly under neutral and basic conditions, thereby raising the possibility of nitrosations in sites other than the stomach. If, for example, nitrosamines were formed in the mouth, where nitrite is continuously formed via reduction of nitrate by microorganisms, they might be directly absorbed through the oropharyngeal tissues.

In typical experiments (*in vitro*) (*59*), aqueous buffer solutions of diethylamine, sodium nitrite, and formaldehyde led to significant yields of *N*-nitrosodiethylamine at room temperature over the entire pH range studied (6.4 to 11.0). In the absence of formaldehyde, no nitrosamine could be detected above pH 7.5. Formaldehyde catalysis of alkaline nitrosation appears to be a general reaction of unhindered secondary amines. Pyrrolidine, piperidine, dimethylamine, diethylamine, and di-*n*-propylamine (but not diisopropylamine) are smoothly nitrosated at basic pH in the presence of formaldehyde.

Chloral is also active (but less so than formaldehyde) in catalyzing alkaline N-nitrosation, but acetone and 2,2-dimethylpropionaldehyde are not (*48*). The reaction probably occurs by nitrite attack on the highly reactive iminium ion **30** (analogous to **17**, Fig. III.3) resulting from the reaction of the amine with formaldehyde (Fig. III.8).

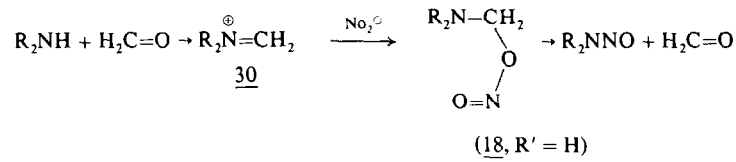


FIGURE III.8

Significant environmental synthesis of *N*-nitroso compounds must now be considered possible in nonacidic media, and the implications become particularly ominous in view of the wide distribution of the catalyst in the environment: Formaldehyde is used as a germicide and fungicide for plants and vegetables, is abundant in smoke used for smoking ham and fish, and is even used in combination with sodium nitrite to preserve fish (*48*).

Recently formaldehyde has been reported (*65*) to catalyze the formation of *N*-nitrosodiethylamine even in the acidic pH range. Acetone has no effect, but hexachloroacetone, hexafluoroacetone, some benzaldehyde derivatives, and the naturally occurring aldehyde pyridoxal also catalyze the nitrosation of diethylamine at pH 4. It has been suggested that the key intermediate for this reaction is the carbinolamine **31**, from which the dialkylaminonitrite **18** (cf. Fig. III.3 and III.8) is formed either directly (attack by a nitrosating agent such as N_2O_3) or after formation of an iminium ion (**30**, Fig. III.9).

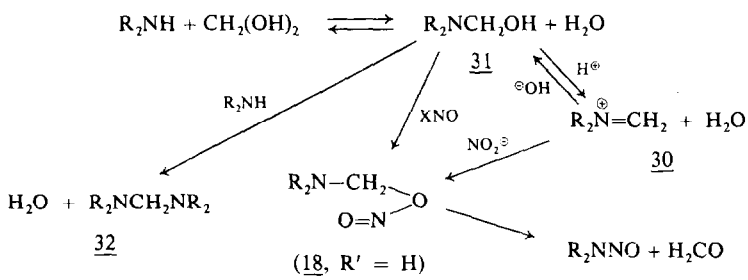


FIGURE III.9

The observed decrease in catalytic rate with increasing amine (morpholine) concentration is attributed to reaction of a further mole of the amine with **31** to give the aminal **32**.

Recently metals have been demonstrated to participate in the formation of *N*-nitroso compounds (*66*).

In 1972, following the discovery that sodium ascorbate blocked *in vitro* formation of *N*-nitroso compounds, the suggestion was made that ascorbic acid be incorporated with readily nitrosatable drugs so as to lessen the risk of *in vivo* nitrosation (*38*). Shortly

thereafter it was reported that induction of lung adenomas in mice by piperazine and nitrite was inhibited when the food contained sodium ascorbate in addition to piperazine (*67*). The recent finding that ascorbic acid inhibits mutagenic activity of nitrite-treated fish extracts is in agreement with earlier reports confirming the protective effect of vitamin C in other systems. It is also consonant with the reported inverse association between gastric cancer on the one hand and the consumption of foods rich in ascorbate on the other (*68*). Interestingly, there is almost total absence of vitamin C in regions with the highest incidence of esophageal cancer (*23*).

A recent study of the effects of several potential inhibitors (ascorbic acid, sodium azide, socium sulfite, sulfamic acid, hydrazine hydrochloride, tannin, gallic acid, cysteine hydrochloride, urea, glutamine, etc.) on the nitrosation of drugs in human gastric juice concluded that ascorbic acid is the most suitable inhibitor because of its pronounced activity at the pH values occurring in the stomach and because of its lack of toxic effects (*69*). Ammonium sulfate and urea, for example, which are as effective as ascorbate at pH 1 and 2, become less effective at pH 3 and 4 (*40*, *67*).

The reaction of ascorbic acid (**33**) with nitrous acid is a general reaction of reductones (enediols stabilized by conjugation with a carbonyl group). It has the characteristics of a typical oxidation–reduction process and gives quantitative formation of nitric oxide and dehydro-ascorbic acid (**36**) according to the stoichiometric equation $C_6H_8O_6 + 2HNO_2 \rightarrow C_6H_6O_6 + 2NO + 2H_2O$. A plausible mechanism entails homolytic decomposition of the nitrite ester (**34**) to the semiquinone intermediate (**35**), which is oxidized to give the final products (*70*, *71*) (Fig. III.10). In perchloric acid at low pH values (<1) the main nitrosating species is nitrous acidium ion

$$NOX, X = {}^{\oplus}OH_2,$$

whereas in more dilute acid (pH 1.5 to 5.0) the nitrosating species is nitrous anhydride (NOX, X = NO_2). In the presence of ions such as chloride or thiocyanate, the corresponding NOX species are the active nitrosating agents.

Ascorbic acid has a pK_a of ca. 4 for the ionizable hydrogen on the hydroxylic group at C-3 (as compared with a pK_a of ca. 12 for that at C-2). Consequently, at pH 3 to 5 a significant proportion of ascorbic acid exists as the reactive monoanion (**33a**), which

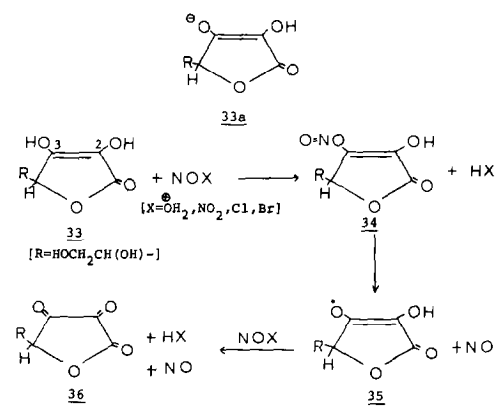


FIGURE III.10

reacts so rapidly with N_2O_3 as to make formation of the nitrosating agent the rate-limiting step in the oxidation (*67*). Between pH 1.5 and 5.0, the nitrosation of secondary amines in the presence of ascorbic acid is described by two competitive reactions (amine + N_2O_3 vs ascorbate + N_2O_3), so that the effectiveness of ascorbate as a nitrosation inhibitor depends on how fast it can scavenge N_2O_3 (*71*). For example, *N*-methylaniline is nitrosated so rapidly that blocking of its nitrosation by ascorbic acid is only partial. Morpholine and piperazine, on the other hand, react less rapidly and their nitrosations can be effectively blocked by ascorbate (*67*).

The amount of ascorbate required to inhibit completely nitrosation of secondary amines such as morpholine depends on whether or not oxygen is present in the system. For example the formation of nitrosomorpholine at pH 4 under anaerobic conditions was completely inhibited when the ratio [asorbate]/[nitrite] was 0.5 (as expected from the stoichiometry) but when pure oxygen was present in the system nitrosation was not prevented even with equimolar concentrations of ascorbate and nitrite. Apparently the nitric oxide produced during the oxidation of ascorbate (Fig. III.10) might have reacted with oxygen to yield additional oxidizing equivalents, or oxygen itself might have directly oxidized the ascorbate semiquinone intermediate, **35** (*71*).

Tannic acid blocks *N*-nitrosodimethylamine formation, and the acid's presence in beer and tea explains why added nitrite disappears rapidly from these beverages (*40*, *72*). With gallic acid (3,4,5-trihydroxybenzoic acid), a component of tannic acid, the situation is less clear in view of the report that it can either catalyze or inhibit the nitrosation of diethylamine depending on relative concentration and pH (*73*).

A reaction which may have some bearing on the mode of action of certain nitrosation inhibitors is the facile nitrosative decarboxylation of benzoic acid derivatives carrying phenolic functions. On adding sodium nitrite to an aqueous alcoholic solution of 3,5-dibromo-4-hydroxybenzoic acid there is an immediate evolution of carbon dioxide (Fig. III.11). The product, 3,5-dibromo-4-hydroxynitrosobenzene, is obtained in quantitative yield (*74*, *75*). Salicylic acid, *p*-hydroxybenzoic acid, 3,4-dimethylsalicylic acid, and 5-methylsalicylic acid also undergo this reaction which apparently is an electrophilic aromatic substitution attended with decarboxylation (Fig. III.11).

The toxicity of some *N*-nitroso compounds may be decreased by substances which can inhibit the metabolism of nitrosamines and a number of other chemical carcinogens.

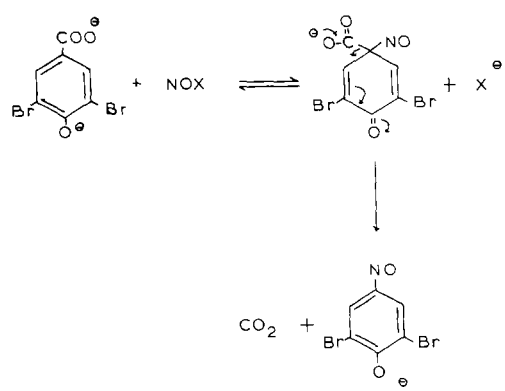


FIGURE III.11

It has been shown, for example, that vitamin A decreases the toxicity of *N*-nitrosomethylbenzylamine (*77*), probably by impairing its metabolism (*78*). A similar inhibition by carbon tetrachloride has been reported for the metabolism of *N*-nitrosodimethylamine (*79*).

C. Some Aspects of Chemical Reactivity

The nitroso group, like the nitro group, is powerfully electrophilic and can activate aromatic rings toward nucleophilic attack as well as it can stabilize α-carbanions (*80*). As an activator in nucleophilic aromatic substitutions the nitroso group is even more effective than the nitro group. In boiling sodium hydroxide solution, for example, 2,4-dinitro-*N*,*N*-dimethylaniline is barely attacked whereas *p*-nitroso-*N*,*N*-dimethylaniline is hydrolyzed to give nearly quantitative yields of dimethylamine and *p*-quinone monoxime. Similarly, *p*-bromonitrosobenzene is more reactive than *p*-bromonitrobenzene toward silver nitrate (*33e*). The nitroso group resembles the carbonyl group as evidenced by the striking analogy between certain reactions of *C*-nitroso and of carbonyl compounds. Azo compounds arise from primary aromatic amines and aromatic nitroso compounds by a facile condensation analogous to the formation of imines from amines and carbonyl substrates [Fig. III.12, (a)] (*81*). Similarly, the Wittig reaction of phosphorus ylides applies as well to *C*-nitroso compounds to give imines [Fig. III.12, (b)] as it does to aldehydes and ketones to give alkenes (*33e*).

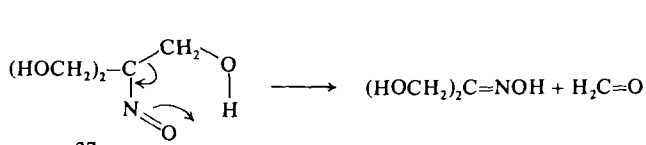
(a) $ArNH_2 + PhN{=}O \rightarrow Ar{-}N{=}NPh + H_2O$

(b) $Ph_3P{=}CR_2 + PhN{=}O \rightarrow PhN{=}CR_2 + Ph_3PO$

FIGURE III.12

There is a significant difference, however, between nitroso and carbonyl functions: The former, unlike the latter, has nucleophilic character by virtue of the nonbonding electron pair on the nitrogen. This nucleophilicity is manifested in the reaction of *C*-nitroso compounds with nitrile oxides to give nitrosonitrones (*33e*), as well as in the conversion of nitroso compounds into nitro compounds by peracids. The latter reaction occurs by attack of the nitroso nitrogen on the electrophilic oxygen of the peracid (*82*).

Compared with *C*-nitroso compounds, *N*-nitroso compounds show greater versatility owing to $n{-}\pi$ conjugation between the lone electron pair on the amino nitrogen and the nitroso group. Nevertheless, similarities between the two classes of compounds do exist, and in some cases, the chemistry of the former has exact counterparts in the chemistry of the latter. One such case is illustrated by the decomposition of the nitroso derivative **37** into the oxime of dihydroxyacetone and formaldehyde, possibly via a redistribution of electrons in a cyclic transition state (*33e*) (Fig. III.13). This reaction, which is reminiscent of the decarboxylation of α-nitrosocarboxylic acids, is analogous to the decomposition of dialkylnitrosamines subsequent to enzymic hydroxylation (cf. **14** → **15**, Fig. II.2).



37

FIGURE III.13

Further similarities between the two classes are also evident in the reactions outlined in Fig. III.14 which demonstrate not only the analogy between *C*-nitroso and *N*-nitroso compounds but also the analogy between the nitroso and the carbonyl functions.

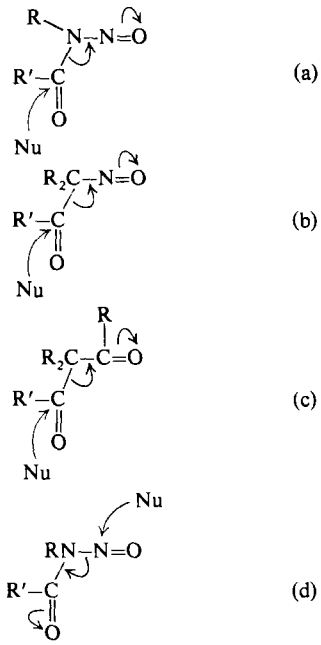
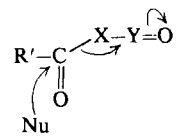


FIGURE III.14

Equations (a) to (c) in Fig. III.14 represent the well-known cleavage by base of *N*-nitrosoamides (*83*), α-nitrosocarbonyl compounds (*33e*), and 1,3-diketones according to the general pattern:[3]



	X	Y
(a)	RN	N
(b)	R_2C	N
(c)	R_2C	RC

Equation (d) represents one of the possible ways by which *N*-nitrosoamides may participate in transnitrosation reactions.

Another striking analogy between nitroso and carbonyl functions is evident in the behaviour of dimethylformamide (**38**, X = CHO, Fig. III.15) and its azalog *N*-nitrosodimethylamine (**38**, X = NO), both of which serve as useful dimethylamination reagents for active halogen compounds (**39**, Fig. III.15) (*84*).

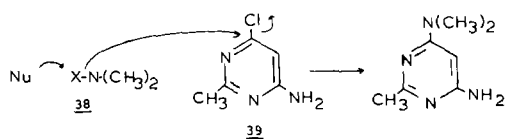
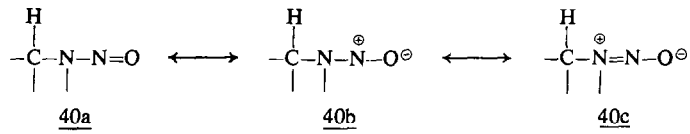


FIGURE III.15

[3] For convenience these reactions are pictured as direct displacements rather than as addition–eliminations.

N-Nitroso compounds, like their *C*-nitroso analogs, are readily converted by peracids into the corresponding nitramines (*33j*). However n–π conjugation in the *N*-nitroso function provides reactive sites that have no counterpart in the *C*-nitroso function (Fig. III.16).



40a 40b 40c

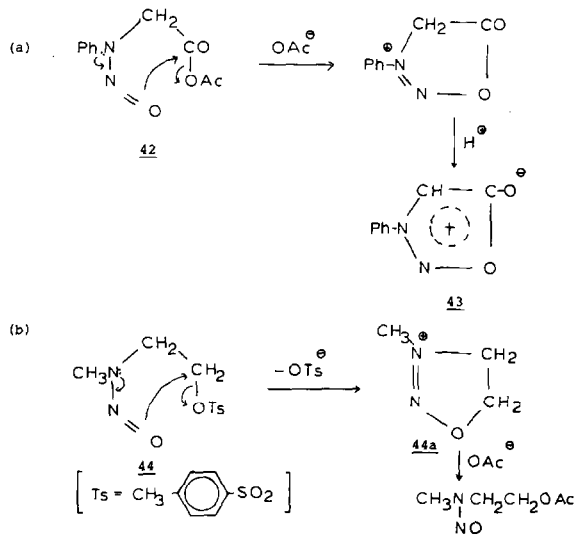
FIGURE III.16

The importance of the dipolar contributing structures is demonstrated by the recent successful resolution of enantiomeric *N*-nitrosamines (**41a** and **b**, Fig. III.17), whose chirality is the result of asymmetry caused solely by restricted rotation (N–NO rotational barrier) about partial double bonds (*85*). The dipolar

41a 41b

FIGURE III.17

structures readily explain the facile cyclizations of appropriately substituted *N*-nitrosamines via nucleophilic attack by the nitroso oxygen atom. Of two such examples outlined in Fig. III.18, the first represents the well-known formation of sydnones (**43**) from suitably substituted amino acid precursors (**42**) (*86*), whereas the second not only explains the exceptionally fast hydrolysis of the tosylate (**44**) but may also have some bearing on the mechanism of carcinogenicity of β-hydroxylated nitrosamines (*87*).



42 43 44 44a

FIGURE III.18

Inspection of the contributing structures (**40a–c**) shows some interesting analogies with carbonyl compounds. Since the nitroso nitrogen carries a partial positive charge, an electron donor reacting with a nitrosamine may act either as a nucleophile (attack on the nitroso nitrogen) or as a base (attack on the α-C–H). In the latter case, the resulting anion (**45**) would be stabilized be delocalization (Fig. III.19) (*88*). Generation of the carbanion **45** has been exploited in a recent method of effecting electrophilic substitutions at the α-carbon atom of secondary amines.

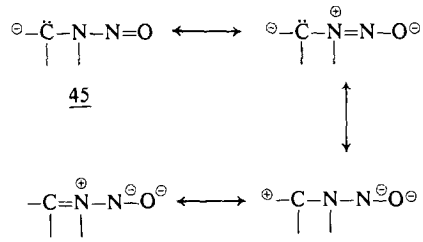


FIGURE III.19

The method consists in nitrosation of a secondary amine (possessing an α-hydrogen), metalation of the resulting nitrosamine by lithium diisopropylamide (LDA) to **46**, reaction with an electrophile ($E^{\oplus}$), and subsequent denitrosation (Fig. III.20) (*88*). Carbanions such as **45** are probably implicated in the decomposition of β-hydroxylated long-chain *N*-nitrosamines into *N*-methyl-*N*-nitrosamines (see Section IV).

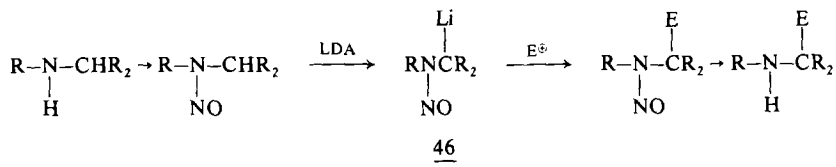


FIGURE III.20

Featuring prominently in the chemistry of *N*-nitroso compounds are the diazotic acids (**15**, alkanediazohydroxides, Fig. III.21) and their conjugate bases, the alkanediazotates (**15a**). These species are central intermediates linking (i) the nitrosative deamination of primary alkylamines

$$(\mathbf{47} \rightarrow \mathbf{48} \rightarrow \mathbf{15} \xrightarrow[\text{Nu}]{-N_2} \text{deamination products});$$

(ii) the decomposition of dialkylnitrosamines after enzymic α-hydroxylation (**49** → **50** → **15**; cf. Fig. II.2: 14 → 15); (iii) the basic cleavage of *N*-nitrosamides (**51** → **15a**); and (iv) the basic decomposition of *N*-nitroso-β-aminoketones (**52**→ **15a**) (*89*). Figure III.21 shows the interrelation of alkanediazotate (**15a**), diazotic acid (**15**), diazoalkane (**55**), alkyldiazonium ion (**53**, the conjugate acid of **55**), and the derived carbonium ion (**54**) (*90, 91*). These species are implicated in the carcinogenicity of *N*-nitroso compounds.

Regarding the generation of diazotic acid (or its diazoester and diazoether derivatives), an important difference exists between *N*-alkyl-*N*-nitrosamides (**56a**) or *N*-alkyl-*N*-nitrosourethanes (**56b**), on the one hand, and *N*-alkyl-*N*-nitrosoureas (**56c**), on

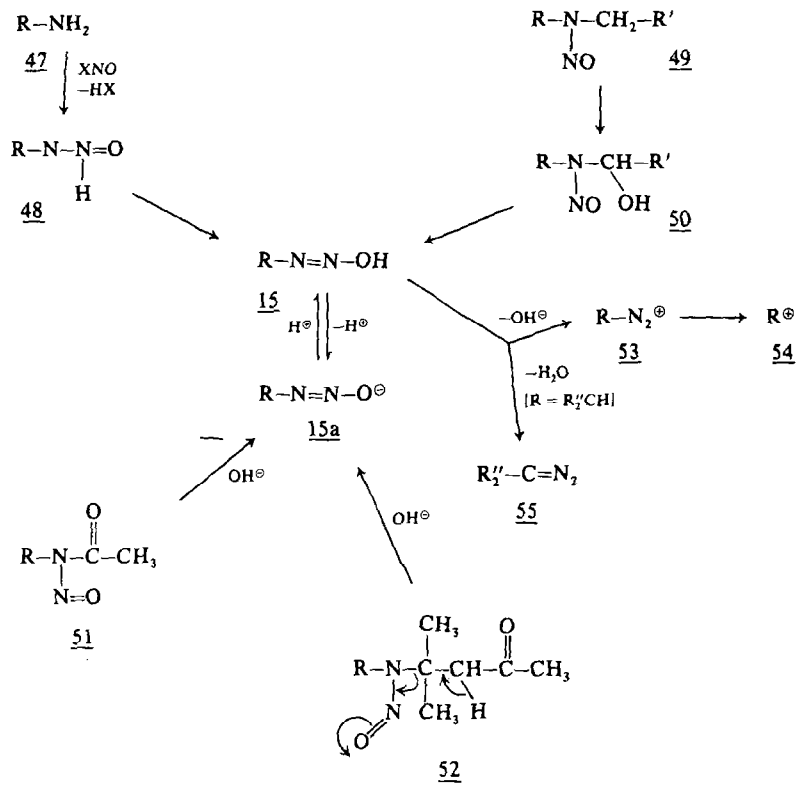
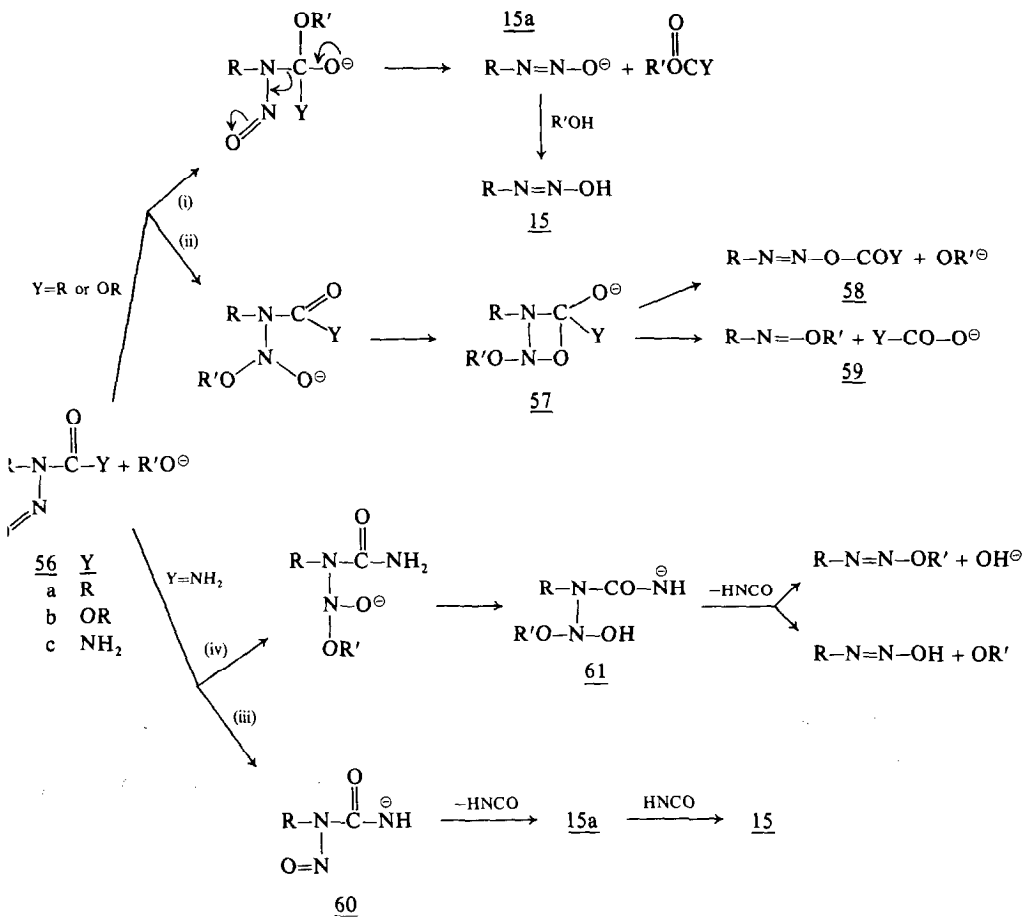


FIGURE III.21

the other (*Scheme 1*). Amides and urethanes undergo competitive attack (*83*) by nucleophiles (alkoxide) on the two electrophilic sites: the carbonyl carbon (Path i) or the nitroso nitrogen (Path ii). Attack on the nitroso nitrogen gives the cyclic intermediate **57**, analogous to the one postulated for the thermal decomposition of nitrosamides to alkyl diazoesters (*33g*). This intermediate collapses to the diazoester (**58**) or the diazoether (**59**), the latter being favored over the former since carboxylate is more readily displaced than alkoxide. The competition between attack at carbonyl and attack at nitroso depends on the nature of the substituents, and as expected, attack at carbonyl decreases relative to attack at nitroso as Y is changed from methyl to phenyl or ethoxy; it also depends on the nature of the base and the solvent, protic solvents favoring attack at the carbonyl (*83*).

In contrast to nitrosamides and nitrosourethanes, *N*-alkyl-*N*-nitrosoureas (**56c**) are not attacked at the carbonyl carbon. Instead, they undergo N–H proton abstraction either directly (Path iii) (*92*) or subsequent to nucleophilic attack at the nitroso nitrogen (Path iv) (*83*) to give conjugate bases (**60** or **61**) from which the products arise by elimination of HNCO. The difference in the decomposition pathways between nitrosamides and nitrosourethanes, on the one hand, and nitrosoureas, on the other (Scheme 1), may be responsible for the observed differences in the biological disposition of these compounds (*93*).



SCHEME 1

Because the negative charge in diazotates is delocalized

$$R{-}N{=}N{-}O^{\ominus} \leftrightarrow R{-}\overset{\ominus}{N}{-}N{=}O,$$

both N–N and N–O linkages are intermediate between single and double bonds. Rotation about N–N is sufficiently restricted to permit *syn–anti* isomerism. The stereochemistry of the diazotates obtained according to Scheme 1 appears to be *syn* (*33b*). *Anti* diazotates, which can be obtained from monoalkylhydrazines (Fig. III.22), are less reactive than their *syn* isomers and can be dissolved in cold water without reaction. Heating, however, promotes diazoalkane and carbonium ion formation, possibly after *anti–syn* isomerization (*33b*).

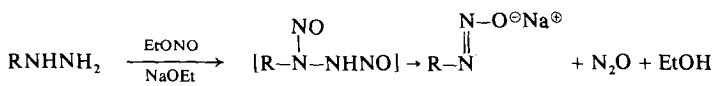


FIGURE III.22

Depending on experimental conditions, alkylation of alkanediazotates occurs either at oxygen giving the unstable diazoether (**62**) or at one or the other of the two nitrogens giving the nitrosamine (**1**) or the azoxyalkane (**63**, Fig. III.23).

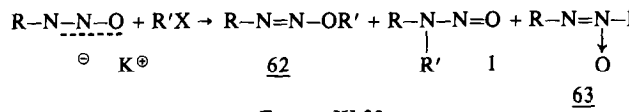


FIGURE III.23

Between **1** and **62**, the former is favored in less polar solvents. The yield of **63** is relatively invariant. These results contrast with acylation or arylation of alkanediazotates (**15a** R = CH_3), which give predominantly products corresponding to **1** (*33b*). Alkylation of alkanediazotates to products **1** and **63** is of biological significance not only in view of the carcinogenicity of dialkylnitrosamines (**1**) but also because of the existence of several naturally occurring carcinogenic azoxyalkanes (**63**; see Section IV).

Since the formation of nitrosamines is a reversible process, reaction from right to left, as in Fig. III.24, represents the nitrosation of water by a nitrosamine (*94*).

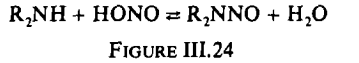


FIGURE III.24

In general, reactions in which nitrosamines act as carriers of the nitroso function, thereby nitrosating other nucleophiles, are referred to as "transnitrosations" (Fig. III.25) and have considerable biological implications.

FIGURE III.25

The rate of transnitrosation depends on the substituents present on the amino nitrogen. Transnitrosations are usually more rapid with arylnitrosamines than with dialkylnitrosamines. The difference in reactivity parallels the expected N–N bond strength and the stability of the amino fragment (*94*). *N*-Nitrosodiphenylamine, with two aromatic substituents on the amino nitrogen, readily transfers its nitroso function to other nucleophiles.

Transnitrosations by secondary *N*-nitrosamines have been observed under two different sets of conditions. In the most common method, the secondary nitrosamine is heated with the nucleophile in an organic solvent. These reactions are homolytic and appear to involve a free radical chain mechanism. In dilute acid, on the other hand, transnitrosations occur by a heterolytic mechanism.

A typical homolytic transnitrosation is illustrated by the reaction of *N*-nitrosodiphenylamine with secondary alkylamines, such as dimethylamine and morpholine, under neutral conditions in an organic solvent (Fig. III.26). Transnitrosation is complete in about 3 days at 50°C (*94*). The initiation step (fission of Ph_2NNO) is rate limiting, and as expected, this step is much easier for arylnitrosamines than for alkylnitrosamines, thus explaining the greater reactivity of the former. The postulated nitroxide radical intermediate (**64**, Fig. III.26) has been confirmed by ESR studies. In neutral organic solvents *N*-nitroso-*N*-methylaniline can also effect transnitrosation to other substrates, but under similar conditions, no transnitrosations are effected by dialkylnitrosamines (*94*).

Under heterolytic conditions in dilute acid, transnitrosations by secondary *N*-nitrosamines may occur either without release of nitrous acid (direct transnitrosation) or via release of nitrous acid (indirect transnitrosation) (*61*).

$$Ph_2NNO \underset{}{\overset{\text{heat}}{\rightleftharpoons}} Ph_2N\cdot + \cdot NO \text{ (initiation)}$$

$$Ph_2N\cdot + R_2NH = Ph_2NH + R_2N\cdot$$

$$R_2N\cdot + Ph_2NNO \rightarrow Ph_2N{-}\overset{\cdot O}{\overset{|}{N}}{-}NR_2$$

64

$$64 \rightarrow Ph_2N\cdot + R_2NNO$$

$$2Ph_2N\cdot \rightarrow \text{termination}$$

FIGURE III.26

Direct transnitrosation is illustrated by the reaction between *N*-nitrosodiphenylamine and *N*-methylaniline in dilute hydrochloric or perchloric acid in 50% aqueous ethanol at 25°C. This reaction (Fig. III.25, R = Ph, Nu = $PhNCH_3$) proceeds readily to equilibrium and its rate, which is independent of $[Cl^-]$, decreases upon addition of diphenylamine to a much smaller extent than would be expected for a reaction proceeding via the liberation of nitrous acid [relative reactivity toward nitrous acid in 0.1 *M* HCl, $K_{(Ph_2NH)}/K_{(PhNHMe)}$ = ca. 200]. The kinetics are consistent with transfer of the nitroso group to PhNHMe directly from the activated complex resulting from protonation of Ph_2NNO (Fig. III.27) (*61*).

$$Ph_2NNO + \overset{\oplus}{H_3O} \rightleftharpoons Ph_2\overset{\oplus}{N}{=}NOH + H_2O$$

$$Ph_2\overset{\oplus}{N}{=}NOH + PhNHCH_3 \rightleftharpoons Ph_2N{-}\underset{\overset{|}{\overset{\oplus}{N}}HCH_3Ph}{\overset{|}{N}}{-}O{-}H$$

$$Ph_2N{-}\underset{\overset{|}{\overset{\oplus}{N}}HCH_3Ph}{\overset{|}{N}}{-}O{-}H \xrightarrow{H_2O} \overset{\oplus}{H_3O} + Ph_2NH + PhN(CH_3)NO$$

FIGURE III.27

With substrates like sodium azide, unlike with *N*-methylaniline, *N*-nitrosodiphenylamine effects transnitrosation via the intermediacy of nitrous acid or nitrosyl halide (indirect transnitrosation). This reaction is slow in 0.1 *M* perchloric acid, unless nucleophilic anions such as $Cl^\ominus$, $Br^\ominus$, or $^\ominus SCN$ are present. The mechanism is outlined in Fig. III.28 (*61*).

$$Ph_2NNO + H^\oplus \rightleftharpoons [Ph_2NNOH]^\oplus$$

$$[Ph_2NNOH]^\oplus + Cl^\ominus \rightleftharpoons Ph_2NH + NOCl$$

$$NOCl + NaN_3 \rightleftharpoons NaCl + ONN_3$$

$$ONN_3 \longrightarrow N_2O + N_2$$

FIGURE III.28

Transnitrosations by *N*-nitrosodiphenylamine to other nucleophiles under heterolytic conditions resemble those of either *N*-methylaniline (e.g., *N*-1-naphthylethylene diamine: direct mechanism) or sodium azide (e.g., hydroxylamine, urea: indirect mechanism). Some nucleophiles, such as aniline and 2-methylindole, undergo heterolytic transnitrosation by Ph_2NNO concurrently by both mechanisms, the products being

$$PhN_2^{\oplus}X^{\ominus}$$

and 3-nitroso-2-methylindole, respectively (*61*).

Transnitrosations involving atoms other than nitrogen are also possible. One such case, involving transfer from nitrogen to oxygen, is illustrated by the nitroso derivative **66**, which can be obtained as a yellow crystalline solid by treatment of *N*-acetyltryptophane methyl ester (**65**) with sodium nitrite in aqueous acetic acid at room temperature (Fig. III.29).

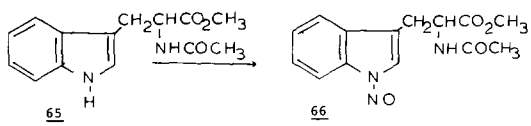


FIGURE III.29

This solid is quite stable at room temperature for several months, but under acidic conditions it readily transfers its nitroso function even to rather weak nucleophiles such as *n*-butanol (Fig. III.30) (*39*).

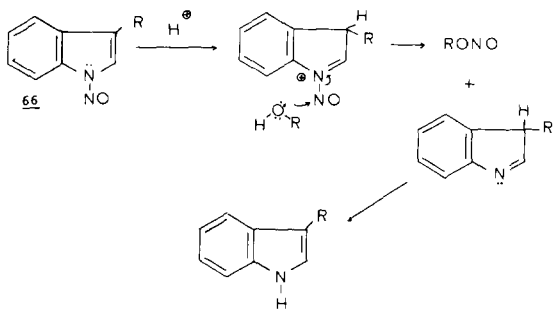
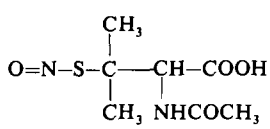


FIGURE III.30

Another case, involving nitroso transfer from sulfur, is illustrated by the unusually stable thionitrite (**67**) obtained by nitrosation of *N*-acetylpenicillamine. This stable solid, under appropriate conditions, can transfer its nitroso group to thiols and amines (*95*).



67

Transnitrosations by *N*-nitrosamides are also possible, but here the transnitrosation path (denitrosation) competes with a deamination path. The point is well illustrated by the decomposition of *N*-*n*-butyl-*N*-nitrosocacetamide in aqueous acid at 25°C (Fig. III.31). Deamination and denitrosation occur concurrently via different conjugate acid intermediates, with denitrosation becoming predominant at high acidity. Deamination, which occurs at a significant rate even in neutral solution, involves rate-limiting attack by H_2O on the O-conjugate acid (**68**) formed in a rapid preequilibrium step by protonation of the carbonyl or nitroso oxygen atoms. In contrast, denitrosation involves rate-limiting formation of the amino-N conjugate acid (**69**), followed by rapid formation of products. The (most unusual) slow proton transfer to nitrogen in this case has been attributed to its low basicity (*96*).

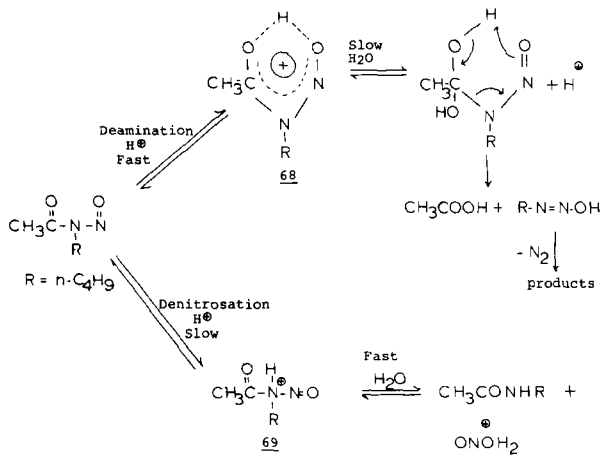


FIGURE III.31

Interestingly, in the decomposition of *N*-nitroso-2-pyrrolidone (**70**) in mildly acidic aqueous solutions, both deamination and denitrosation pathways involve a common rate-limiting step: slow protonation of the amino nitrogen atom. The N-conjugate acid (**71**) rapidly partitions to products via two pathways (Fig. III.32).

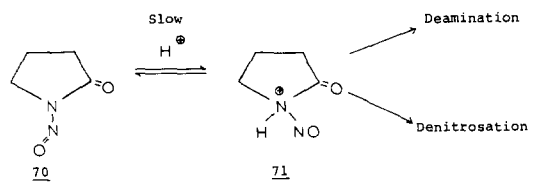


FIGURE III.32

Denitrosation is much more important for *N*-nitroso-2-pyrrolidone as compared with *N*-*n*-butyl-*N*-nitrosoacetamide (*97*).

Even greater reactivity in denitrosation reactions is exhibited by *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, owing to the strong electron-withdrawing effect of the SO_2 group.

Loss of the nitroso function from the conjugate acid (**72**), whether unimolecularly or by reaction with a nucleophile (Fig. III.33), occurs in a rapid step (*98*).

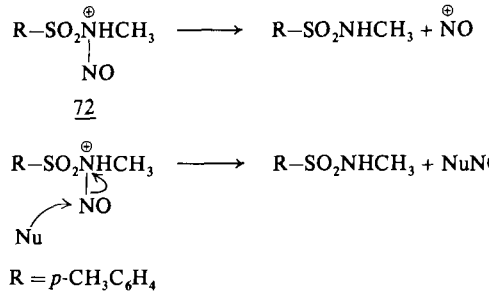


FIGURE III.33

Transnitrosations are biologically important because they provide a pathway for certain *N*-nitroso compounds (which may themselves be noncarcinogenic or weakly carcinogenic) to act as proximate carcinogens by transferring their nitroso groups to other compounds (*61*). In the favorable environment of the stomach, for example, compounds such as *N*-nitrosodiphenylamine and *N*-nitroso-*N*-methylaniline may effect direct or indirect transnitrosations to other substrates and thereby generate potent carcinogens. Moreover, to the extent that transnitrosations may occasionally lead to stable products (e.g., *C*-nitroso compounds), they will profoundly affect the biological disposition of these metabolites. An interesting case of nitroso group transfer from a nitrosamine to an amide has been proposed recently to account for the mutagenicity of a mixture of 2-acetylaminofluorene and dimethylnitrosamine in the presence of bacterial extracts (*99*).

D. Detection and Determination

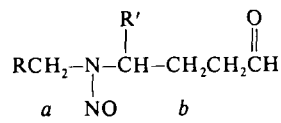
The determination of *N*-nitroso compounds has been reviewed (*27*). Coupled capillary gas chromatography/high-resolution mass spectrometry provides a reliable analytical method for trace amounts of volatile *N*-nitrosamines. The advantages offered by chemical ionization mass spectrometry have been discussed (*100*). The last few years have witnessed increasing use of selective gas chromatography detectors for volatile nitrosamines. Thermal energy analyzers interfaced directly to high-performance liquid chromatographs have proved valuable for the analysis of nonvolatile *N*-nitroso compounds (*101*). Recently, a simple and highly sensitive "frozen animal procedure" has been described for quantitative identification of nitrosamines synthesized *in vivo* from trace levels of their precursors (*102*).

Dialkylnitrosamines in water exhibit absorption in the ultraviolet region at about 340 nm (ε ca. 100) due to $n \rightarrow \pi^*$ transitions and at about 230 nm (ε 5000 or higher) due to $\pi \rightarrow \pi^*$ transitions (*52*). In organic solvents there is a bathochromic shift of about 20 nm for the long-wavelength band, which is split into a triplet (*40*). For nitrosamides, the long-wave length band in water is at about 400 nm (*40*).

Nitrosamines show three relatively intense bands in the infrared region at 1450–1350, 1316–1163, and 1093–1047 cm^{-1}. The first two have been assigned to the vibrations of the N=O bond and the last to the vibrations of the N–N bond (*33j*, *87*).

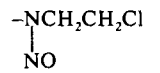
In nitrosamides the nitroso group causes a hypsochromic shift in the carbonyl ir absorption, as exemplified by the nitroso ureas of type **11** (see Section II), which absorb at 1700–1725 cm^{-1} (C=O stretch) as compared with 1620–1635 cm^{-1} for the ureas themselves (*17*).

The nuclear magnetic resonance spectra of nitrosamines may sometimes show splitting attributable to the existence of isomers (*40*). The nitrosaminoaldehydes **73** give nmr spectra which indicate the existence of E and Z isomers. When R = R′ = H, protons *a* give two singlets at δ 3.70 and 3.05 (for the Z and E isomers) whereas

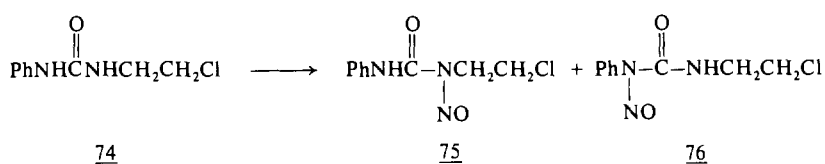


73

protons *b* give two triplets at δ 3.54 and 4.10 (*103*). The direct examination of reaction mixtures using ^{15}N-nmr spectroscopy is a valuable approach to the study of nitrosation reactions. The chemical shift of the $N-{}^{15}N=O$ signal for several nitrosamines falls in the range δ 500–550 ppm downfield from δ $^{15}NH_4^{\oplus}$ = 0 ppm. Nuclear magnetic resonance spectroscopy has been useful in establishing the isomeric purity of nitrosoureas as, for example, in the case of the two isomeric compounds **75** and **76** obtained by nitrosation of 1-(2-chloroethyl)-3-phenylurea (**74**, Fig. III.34): Spectral asymmetry of the $ClCH_2CH_2NH$ group (A_2B_2X) from **76** and symmetry of the



group (A_2B_2) from **75** are clearly seen in the nmr spectra of these compounds (*105*).



74 **75** **76**

FIGURE III.34

The nmr spectrum ($CDCl_3$) of 1,3-bis(2-chloro-1,1-dideuterioethyl)-1-nitrosourea (Section V, **103a**, fully deuterated on the methylene groups adjacent to the amino nitrogens) shows three broadened singlets (δ 7.4, 3.8, and 3.5; relative areas 1:2:2) with no absorption at δ 4.2 for protons next to the nitroso-bearing nitrogen (*106*).

IV. METABOLISM AND CARCINOGENIC ACTION

The existence of a positive correlation between carcinogenicity and mutagenicity in most of the compounds which have been tested probably reflects the operation of a similar or shared mechanism for the initiation of both phenomena. Although some carcinogenic acylating agents are known, most chemical carcinogens produce alkylating or arylating species (*22*).

Alkylation occurs normally within healthy living cells; it is controlled by enzymes displaying strict specificity, and it plays an important role in body chemistry. In contrast, alkylation by carcinogenic alkylating agents, while not entirely at random, is far less selective and may take place at many different nucleophilic sites. Such abnormal alkylations of certain critical sites in biological macromolecules may be responsible for the carcinogenic action. More specifically, the production of alkylated nucleosides at regions within the nucleic acid sequence where they are not normally found may contribute to inhibition of protein and RNA synthesis, lead to misincorporation of nucleosides by nucleic acid polymerase, and induce neoplastic growth. Although the important target appears to be DNA, the alkylation of proteins cannot be excluded from consideration (*107–109*).

The main site of base alkylation in nucleic acids by nitroso compounds, as with other alkylating agents, is the N-7 position of guanine. However, a number of other sites are also attacked, including the N-1, -3, and -7 positions of adenine; the N-1, -3, and O-6 positions of guanine; the N-3 position of cytosine, thymine, and uracil; and the O-4 position of thymine (*9*). Interestingly, there is no correlation between alkylation at the N-7 position of guanine and carcinogenicity or mutagenicity. A much better correlation exists with alkylation at position O-6 of guanine (*107, 110*). 7-Methylguanine appears to pair normally with cytosine (*111*) whereas O-6 alkylation of guanine interferes (*112*) with normal base pairing and is thought to be more important biologically than alkylation at other positions (*113–115*). Alkylation of phosphodiesters to phosphotriesters also takes place, and there is evidence that this is the major, though not necessarily the only, biologically important event when DNA and RNA react with *N*-nitroso-*N*-ethylurea (*116, 117*). This nitrosourea also alkylates ribose oxygens in nucleosides and may thereby affect conformation, enzymatic reactions, or ribosome binding (*118*).

A great variety of compounds (e.g., epoxides, lactones, alkyl sulfates, alkyl sulfonates, dialkylhydrazines, azoxyalkanes, *N*-nitroso compounds) give rise to alkylated nucleic acids after administration to animals. Some of these are powerful carcinogens whereas others are only weakly active or inactive. Most are also mutagenic (*107*). Although most of these agents attack the same sites in nucleic acids, there are important differences in the relative degree of attack on specific sites. All the methylating agents, for instance, give 7-methylguanine as the most abundant alkylated purine, but the formation of the O-alkylated products is much less for dimethylsulfate and methyl methanesulfonate than for the strongly carcinogenic *N*-methyl-*N*-nitrosourea and *N*-nitrosodimethylamine. It has been suggested that agents such as dimethylsulfate, which are predisposed to react by the S_N2 mechanism, attack preferentially the main nucleophilic center (N-7 of guanine) and are less likely to attack the less nucleophilic oxygen sites. On the other hand, agents such as the *N*-nitrosamines and amides, which generate species more likely to react by an s_N1 mechanism, are less selective and thus produce a wide spectrum of products including O-alkyl derivatives (*9, 107, 119*).

Of all the potent chemical carcinogens only the nitrosamides and some alkylating agents are chemically reactive and do not require enzymic activation to exert their noxious effects (*5*). The nitrosamides are unstable in alkaline media and may decompose rapidly under physiological conditions to give reactive, in most cases alkylating species (Fig. II.2 and Scheme 1). Thiol compounds increase the decom-

position rate of some nitrosamides (e.g., *N*-methyl-*N*-nitrosourethane and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) but have no such effect on others (e.g., *N*-methyl-*N*-nitrosourea) (*112, 120*). Cysteine enhances markedly the rate of decomposition of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (**9**, Fig. IV.1) to give the alkylating species ($CH_3N{=}NOH$) and a substantial yield of **77**, together with smaller amounts of cystine (**78**, presumably via the thionitrite), *N*-methyl-*N'*-nitroguanidine (**79**, by denitrosation of **9**), and *S*-methylcysteine (**80**, by alkylation of cysteine) (*112*).

The decomposition of some nitrosamides is also promoted in aqueous solution by heavy metal ions (Cu^{2+}, and to a lesser extent, Ni^{2+}). The discrepancy in the reports concerning the stability of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in aqueous solution may be attributable to the presence of heavy metal impurities in the water (*121*).

In contrast to the nitrosamides, the nitrosamines persist in the body unchanged for much longer periods and require metabolic activation before they can exert their adverse biological effects. It is believed by many workers that the microsomal mixed-function oxidases are primarily responsible for the metabolic activation of the nitrosoamines, although Lake *et al.* (*172, 174*) have suggested that enzyme systems other than the mixed-function oxidases may contribute to the metabolic activation of certain nitrosoamines. These enzymes, which ironically are among those whose primary function is the detoxification and disposal of foreign chemicals (*122*) occur not only in the liver but also in many other organs and tissues and show substrate specificity. The nitrosamines, consequently, produce cancer only in those organs that are capable of metabolizing them (*107*).

The simplest dialkylnitrosamine *N*-nitrosodimethylamine (**3**), is metabolized predominantly in the liver (*123*). Enzymic activation entails α-hydroxylation (Fig. II.2, **13**,

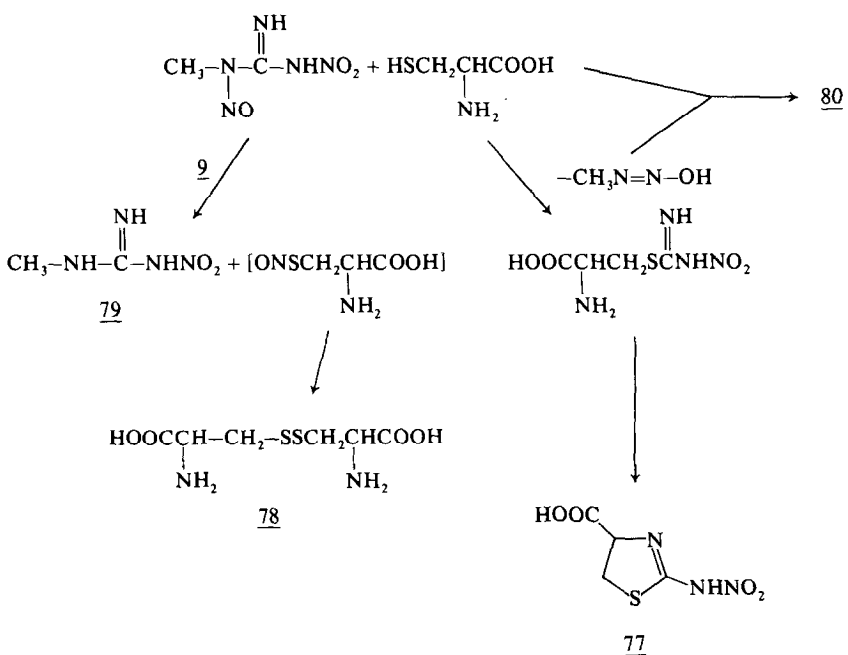


FIGURE IV.1

R = Me), followed by loss of formaldehyde and formation of methanediazohydroxide (**15** R = Me). The ultimate alkylating species from *N*-nitrosodimethylamine and *N*-nitrosodiethylamine are probably the corresponding carbonium ions or precursor species and not the diazoalkanes. This was shown elegantly by Lijinksy *et al.* (*124*, *125*) who found that the N-7-methylguanine or N-7-ethylguanine components of DNA and RNA, isolated from the liver of rats treated with fully deuterated *N*-nitrosodimethylamine or *N*-nitrosodiethylamine, had molecular weights compatible with the transfer of $-CD_3$ or $-CD_2CD_3$ groups, and not of $-CHD_2$ or $CHDCD_3$ that would have been expected had there been diazoalkane intermediates (Fig. IV.2). Similar findings have been reported for *N*-methyl-*N*-nitrosourea *in vivo* (*126*).

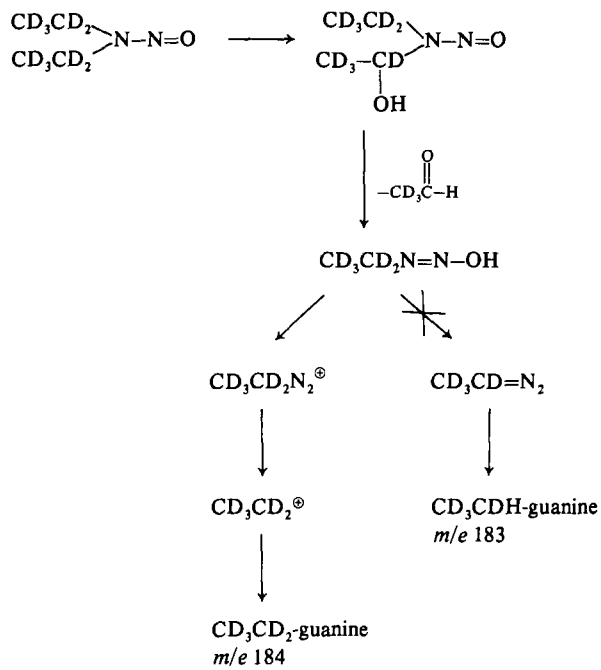


FIGURE IV.2

Convincing as this evidence may be, it does not rule out the possible intermediacy of diazoalkanes in other circumstances. One case in point arises with *N*,*N'*-dinitrosopiperazine (**81**, Fig. IV.3), among whose urinary metabolites is 3-hydroxynitrosopyrrolidine (**83**, 17% yield) (*127*). Formation of this metabolite is best accounted for by ring opening and subsequent ring contraction via the diazoalkane intermediate (**82**). *N*,*N'*-Dinitrosoperhydropyrimidine is also metabolized by similar ring opening and contraction to a five-membered ring (*128*).

Enzymic α-hydroxylation applies as well to higher dialkylnitrosamines such as **84** (Fig. IV.4). Again the resulting α-hydroxynitrosamine (**85**) functions as a transportable form of a potential alkylating agent (*129*, *130*).

Lending further support to the theory of microsomal α-hydroxylation is the observation that several α-acetoxydialkynitrosamines are carcinogenic (*131*) and

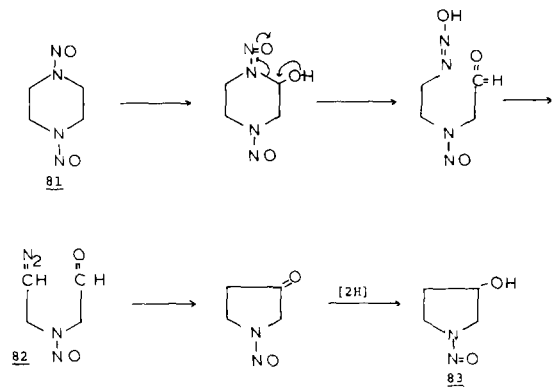


FIGURE IV.3

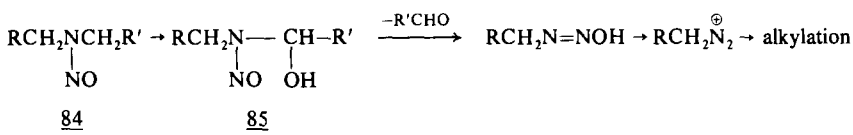
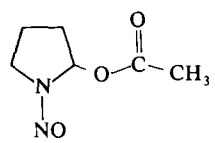


FIGURE IV.4

mutagenic (*132*). Of special interest is α-acetoxynitrosopyrrolidine (**86**) which is a powerful mutagen that requires no activation, in striking contrast to nitrosopyrrolidine which is mutagenic only when incubated with microsomal preparations (*103*).



86

The higher dialkylnitrosamines can be metabolized by pathways other than α-hydroxylation. One of these, β-hydroxylation, has several noteworthy features and is probably responsible for the following finding: *In vivo* application of 1-di-*n*-[^{14}C]-propylnitrosamine (**87**, Fig. IV.5) leads to the formation not only of the expected 7-[^{14}C]propylguanine in the RNA of rat liver but also of 7-[^{14}C]methylguanine (*133*, *134*). Figure IV.5 suggests how the operation of α- and β-hydroxylations may account for the observed results. The cleavage between the α- and β-carbon atoms in going from the oxo compound **89** to **90** generates a methylnitrosamine (**90**) which may ultimately give rise to a methylating species. This cleavage, which readily takes place also *in vitro* by simply stirring **89** with aqueous potassium hydroxide, is reminiscent of the facile cleavage of β-dicarbonyl compounds (*135*) (cf. Fig. III.19 and III.20). Hydroxylation at the β-position may occur also with cyclic nitrosamines such as *N*-nitrosopyrrolidine, one of whose urinary metabolites is 3-hydroxy-1-nitrosopyrrolidine (**91**,

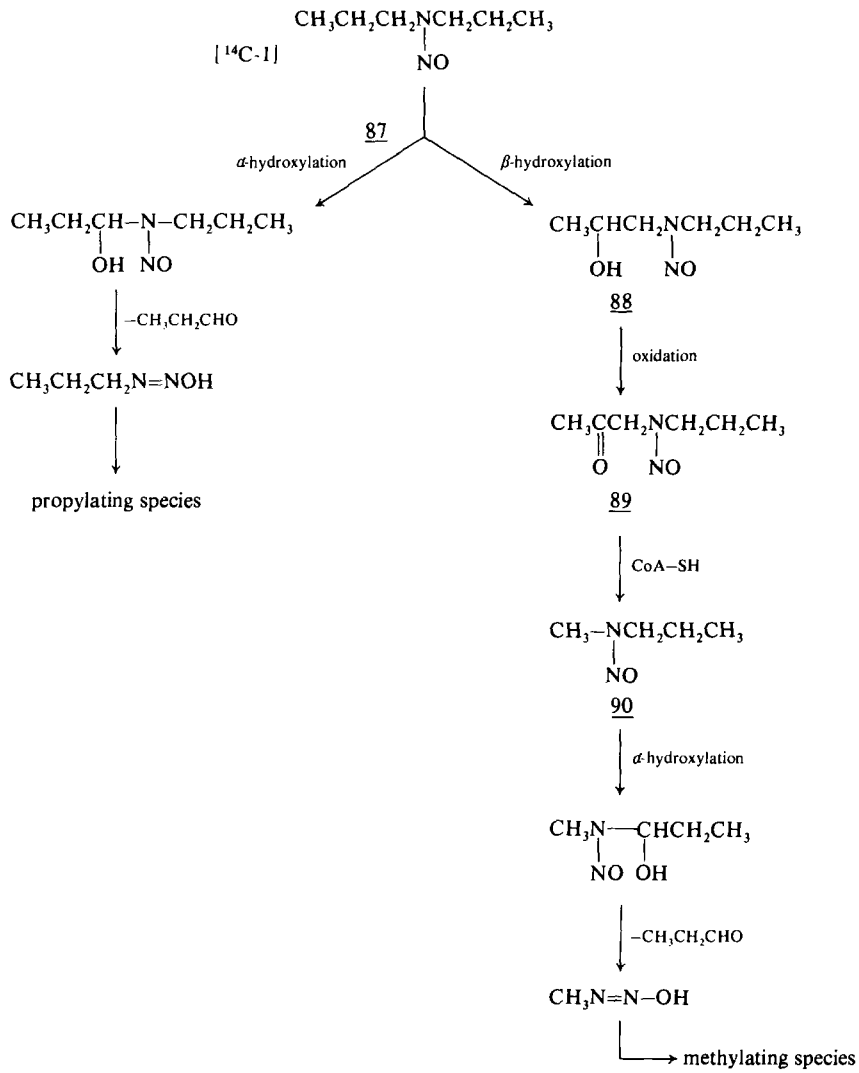


FIGURE IV.5

Fig. IV.6) (*136*). It has been suggested that nitrosamines possessing β-hydroxy substituents may decompose directly into products (**91** → **92**, Fig. IV.6) by a retro-aldol reaction, thereby by-passing the need for prior oxidation to an oxo compound (*103*).

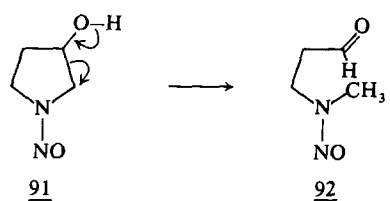


FIGURE IV.6

The recent finding that **44a** (Fig. III.18) reacts readily with a variety of nucleophiles (acetate, thiols, guanine) to give substitution products raises the possibility that sulfate or phosphate conjugates of β-hydroxyalkylnitrosamines could serve as direct-acting *in vivo* alkylating agents (*87*).

The diversity of metabolic activation of the higher dialkylnitrosamines is further evidenced by the occurrence of ω- and (ω − 1)-hydroxylations which, attended with oxidations and chain degradations, are reminiscent of similar transformations in the fatty acid series (*24*, *130*, *137–139*). The point is well illustrated by the carcinogenic butyl(4-hydroxybutyl)nitrosamine (**93**, Fig. IV.7), among whose principal urinary metabolites in the rat are: butyl(3-carboxypropyl)nitrosamine (**94**), butyl(3-carboxy-2-hydroxypropyl)nitrosamine (**95**), and the glucuronide **96** (GA = glucuronic acid residue), along with the minor metabolite butyl(2-oxopropyl)nitrosamine (**97**) (*138*, *140*, *141*). Another striking illustration is provided by *N*-methyl-*N*-dodecylnitrosamine which gives *N*-nitrososarcosine and *N*-methyl-*N*-(3-carboxypropyl)nitrosamine as principal and minor urinary metabolites in the rat (*142*).

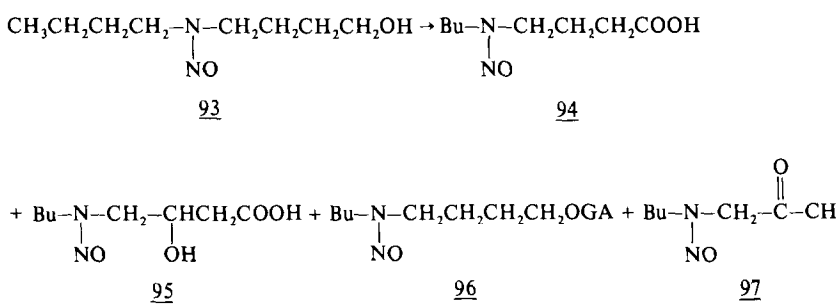


FIGURE IV.7

The multiple carcinogenic effects on different organs of the nitrosamines may be due to the diversity of their metabolic activation (*139*). Furthermore, the noteworthy retention of the *N*-nitroso function in metabolites such as those of Fig. IV.7 has prompted the suggestion that the proximate carcinogenic forms of dialkylnitrosamines are bifunctional metabolic oxidation products which have retained the alkylnitrosamino moiety but have acquired a carbonyl function as a result of ω- or β-oxidation of an alkyl group (*143*). An alternative pathway has also been proposed which establishes a relationship between the nitrosamines and another class of chemical carcinogens, the lactones (*144*). There is also evidence that metabolic pathways other than those leading to the formation of alkylating intermediates may exist for *N*-nitroso compounds (*4*).

Closely related in biological activity to the *N*-nitroso compounds are certain naturally occurring substances containing the azoxy group (Fig. IV.8). Cycasin (**98a**) and macrozamine (**98b**), which have the same aglycone but different sugar moieties, are present in plant species of the *Cycadaceae* family.

Enzymic hydrolysis of these glycosides gives the carcinogenic aglycone methylazoxymethanol (**98c**) whose biological action closely resembles that of *N*-nitrosodimethylamine (*145–147*). Like *N*-nitrosodimethylamine, cycasin is capable of inducing

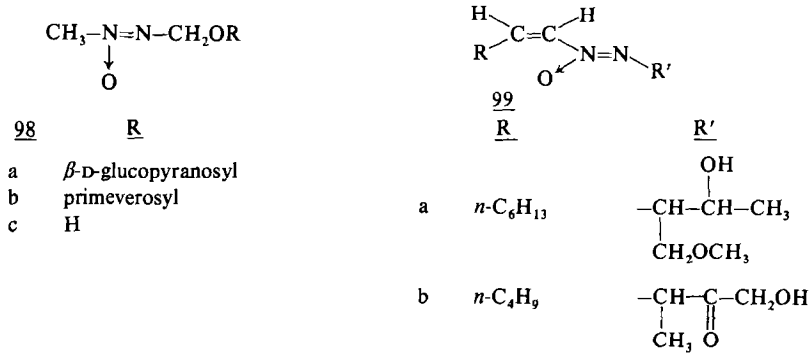


FIGURE IV.8

tumors in rats after a single dose (*148*). The carcinogenicity of **98c** has been attributed to an unstable decomposition product (**100**) (*149*, *150*) or to the metabolic oxidation product methylazoxyformaldehyde (**101**) (*143*). Another possibility includes decomposition via the oxadiaziridine (**102**, Fig. IV.9).

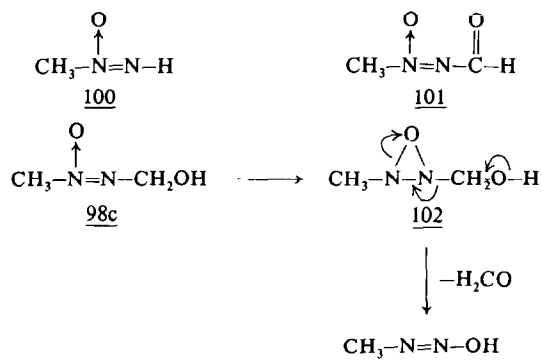
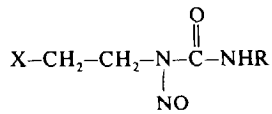


FIGURE IV.9

Elaiomycin (**99a**), a metabolite of the soil organism *Streptomyces hepaticus*, resembles cycasin in its carcinogenic action (*151*). The potent antifungal agent LL-BH872a (**99b**), from *Streptomyces hinnulinus*, is structurally related to elaiomycin (*152*).

V. THERAPEUTIC 2-HALOETHYLNITROSOUREAS

The organ specificity characterizing the nitrosamines and, to some extent, also the nitrosamides suggests that it might be possible to find chemicals which would exert a carcinostatic effect by selectivity affecting cancerous tissue (*88*). This appears to be the case with a group of chemicals known as the 2-haloethylnitrosoureas (**103**, Fig. V.1). These chemicals, as well as some of their metabolites (*153–155*) (CCNU hydroxylated on the cyclohexane ring) and congeners (*156*), show great promise as antitumor agents.



103

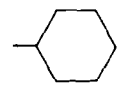
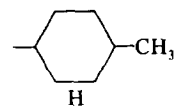
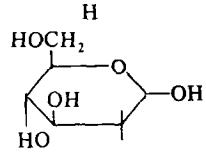
		X	R
a	1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)	Cl	$-CH_2CH_2CL$
b	1,3-bis(2-fluoroethyl)-1-nitrosourea (BFNU)	F	$-CH_2CH_2F$
c	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)	Cl	
d	1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)	Cl	
e	1-(2-chloroethyl)-1-nitrosourea (CNU)	Cl	
f	Chlorozotocin	Cl	

FIGURE V.1

Not only do these drugs show ability to inhibit the growth and spread of many forms of solid tumors in man and animals (*17*, *157*, *158*) but also BCNU and CCNU have been found to rapidly enter the cerebrospinal fluid and control meningeal tumor implants (*159*, *160*). All these compounds are undergoing intense clinical trials and two of them, BCNU and CCNU, have recently been made commercially available. There is evidence that the biological effects of some CCNU derivatives show configurational and conformational dependence (*153*, *161*).

The 2-haloethylnitrosoureas are chemically reactive compounds that may decompose nonenzymatically at relatively rapid rates under physiological conditions. Early studies on the aqueous decomposition of BCNU showed that the course of the reaction depends on experimental conditions. In an aqueous suspension or dilute water solution at room temperature, the products are molecular nitrogen, carbon dioxide, 2-chloroethylamine hydrochloride, and acetaldehyde. The decomposition was postulated to occur (Fig. V.2) (*162*) by loss of the NH proton attended with attack of the resulting anion on the chlorine-bearing carbon atom to give an unstable oxazolidine intermediate (**104**), which breaks down into ethylenediazohydroxide (**105**) and 2-chloroethyl isocyanate (**106**). The ethylenediazohydroxide decomposes to give vinyl alcohol which rearranges to acetaldehyde, while the isocyanate undergoes hydrodecarboxylation to the amine which becomes protonated. It was suggested that the high activity against L1210 leukenia of the 2-haloethylnitrosoureas might result from their *in situ* decomposition into isocyanates which then react with amino groups of some macromolecules, thus interfering with their vital function (*162*). Recent evidence, however, indicates that generation of organic isocyanates is not required for antitumor activity of the 2-haloethylnitrosoureas, although such carbamoylating moieties may contribute to or modify therapeutic effect and toxicity by inhibiting RNA synthesis or DNA repair (*163–165*).

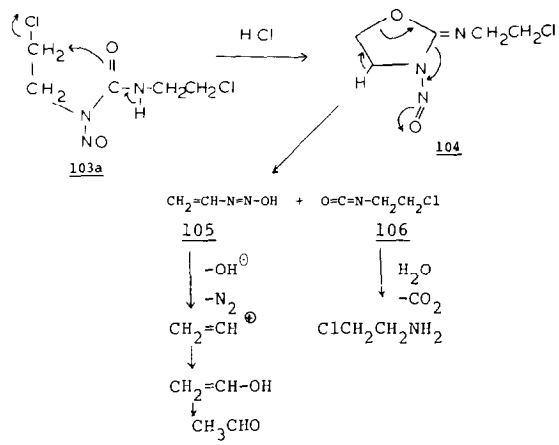


FIGURE V.2

A recent study of the decomposition of several 2-haloethylnitrosoureas showed that BCNU, CCNU, BFNU, and CNU decompose in buffered aqueous solution at physiologic pH to yield 2-haloethanol, vinyl halide, 1,2-dihaloethane, acetaldehyde, and some 1,1-dihaloethane (*106*, *164*). Furthermore, the deuterium distribution in the products obtained from the decomposition of BCNU-α-d_4 and BCNU-β-d_4 was inconsistent with vinyl carbonium ion or diazochloroethane intermediacy but was consistent with a 2-chloroethylcarbonium ion (or a precursor diazohydroxide) intermediate, with some rearrangement to the 1-chloroethylcarbonium ion and the cyclic chloronium ion. The results do not require that the 2-chloroethylcarbonium ion be an intermediate (as is depicted in Fig. V.3) and there is inferential evidence that with BCNU the products could arise predominantly via low-activation energy S_N2 and E2 reactions of the diazohydroxide and via rearrangement concerted with the loss of nitrogen to the chloronium ion or the 1-chloroethylcarbonium ion (*106*, *166*).

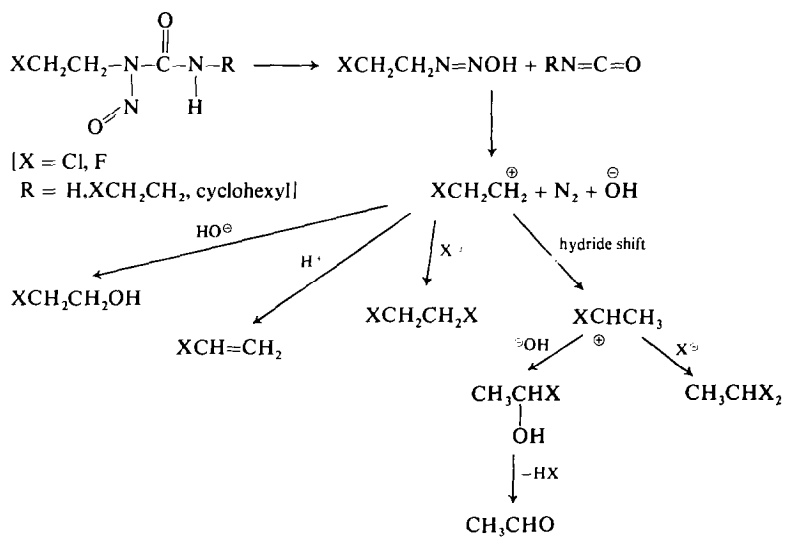
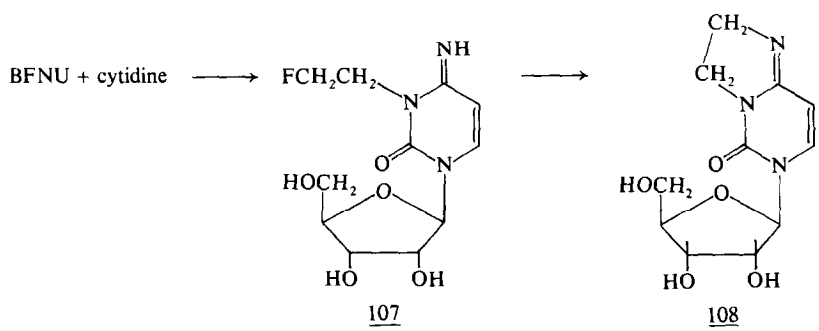


FIGURE V.3

Not surprisingly, with the threo and erythro isomers of 1,3-bis(3-chloro-2-butyl)-1-nitrosourea, the resulting products arise by S_N2 and E2 reactions of the diazohydroxide as well as by S_N1 and E1 reactions of the secondary 3-chloro-2-butylcarbonium ion (*166*). Regardless of whether unimolecular or bimolecular mechanisms are involved, there is one ultimate common feature: delivery of the 2-haloethyl group by the nitrosourea to a nucleophilic site ($^{\ominus}OH$ or $X^{\ominus}$ in Fig. V.3). This feature, rather than carbomoylations arising from the isocyanate moiety, might be the critical event in the biological action of the 2-haloethylnitrosoureas. For example CNU on aqueous decomposition would yield isocyanic acid ($\rightleftarrows$ cyanic acid, $K_a = 2.2 \times 10^{-4}$) which at physiologic pH would be ionized to cyanate and thus be a much poorer carbamoylating agent than the organic isocyanates. Yet, CNU is at least as cytotoxic as BCNU against L1210 cells *in vitro* (*164*). Thus, the relative anti-L1210 effect of the two agents does not correlate with their carbamoylating activity. Both CNU and BCNU, however, act as deliverers of haloethyl group, the former faster than the latter (*164*). Interestingly, with some recently synthesized chloroethyl- and methylnitrosourea analogs of thymidine, neither the alkylating nor the carbamoylating activities correlated with their biological activity (*167*).

There is good evidence that BCNU and related 2-haloethylnitrosoureas react with nucleosides under physiological conditions to form intermediate haloethyl derivatives. One such haloethyl nucleoside, 3-β-fluoroethylcytidine (**107**, Fig. V.4), has been identified as a reaction product of BFNU and cytidine and has been shown to undergo an intramolecular cyclization to form 3,N^4-ethanocytidine (**108**, Fig. V.4).



107 108

FIGURE V.4

The implication of these remarkable reactions is clear: Transfer of the haloethyl group from the nitrosourea to a nucleoside which is already contained in a DNA molecule could impart alkylating ability to the entire molecule! Subsequent intrastrand or interstrand crosslinking could then be largely responsible for the cytotoxicity (*168–170*).

Recently BCNU and related 2-haloethylnitrosoureas were found to covalently crosslink DNA under phsyiological conditions. The rate of the crosslinking increases with increasing pH in the range 4–10 and with the (G + C) content of natural DNA. The reaction leads to stable interstrand crosslinks by a two-step process and is strongly dependent on the 2-halogen in the nitrosourea: Cl $\gg$ Br > F $\gg$ I. This puzzling order of halogen reactivity has been attributed to a possible preference of the 2-bromo- and 2-iodoethylnitrosoureas to decompose via ethylenediazohydroxide intermediates (Fig.

V.2) rather than via 2-haloethane diazohydroxide intermediates (Fig. V.3). Only one 2-haloethyl group is necessary for crosslinking, which is not observed when the halogen is replaced by –OH or $-OCH_3$. Promoting the acidity of the N–H group in the nitrosourea by appropriate aryl substitution increases the rate but not the extent of crosslinking. The position of the halogen is critical since, unlike 1-(2-chloroethyl)-1-nitrosourea, the 3-chloropropyl analog does not induce covalent interstrand crosslinks. Furthermore, a correlation exists between the extent of DNA crosslinking and activity of the nitrosoureas against L1210 leukemia (*171*).

In our laboratory we have been interested in determining the *in vivo* fate of the nitroso moiety by labeling BCNU in the nitroso nitrogen with ^{13}N (a positron emitter) and following the *in vivo* distribution and excretion of the radioactive tag. The labeled BCNU was prepared according to our previously published procedure (*36*) involving the copper reduction of cyclotron-produced $^{13}NO_3^{\ominus}$ to $^{13}NO_2^{\ominus}$ and subsequent reaction with 1,3-bis(2-chloroethyl)urea. The presence of a significant amount of ^{13}N-containing species in the urine of rats treated with labeled BCNU, as well as the prolonged total body retention of the ^{13}N activity, strongly suggests that the pathways involving formation of molecular nitrogen do not represent the sole biologic disposition of the 2-haloethylnitrosoureas (cf. Fig. V.2 and V.3). Consequently, the *in vivo* fate of the *N*-nitroso moiety does not fully parallel its *in vitro* fate (*93*). Whether transnitrosations with amines or other nucleophiles to give stabler species (cf. for example, the retention of the *N*-nitroso function in metabolites such as those of Fig. IV.7) are partly responsible for these findings remains to be established.

ACKNOWLEDGMENTS

This work has been partially supported by National Cancer Institute grant CA 17786 and the University of Kentucky Tobacco and Health Institute, No. 22011. The invaluable assistance of Dr. M. Shambhu and Mrs. Stella Vincent is gratefully acknowledged.

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