NITRITE AND THE ENVIRONMENT. THE NITROSATION OF α-AMINO ACID DERIVATIVES

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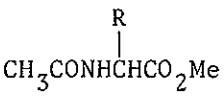
The reaction of nitrous acid with α-amino acid derivatives is reviewed in the context of the environmental hazard of N-nitrosamines. The reaction products from derivatives of proline, tryptophan, tyrosine, methionine, cysteine, arginine and lysine are discussed.

Nitrite occurs naturally in certain foodstuffs such as spinach:[1] it is also an important food additive in meat processing, where it confers colour (through the formation of denatured nitrosylmyoglobin), flavour and, most importantly, resistance to the development of colonies of *Clostridium botulinum*. This microorganism produces a toxin (botulin) which is one of the most poisonous substances known, and its control by nitrite is a considerable benefit to mankind.[2]

However, at the same time, ingested nitrite may be acting in two harmful ways: (i) by promoting the oxidation of haemoglobin

to methaemoglobin (resulting in a lowered capacity of the blood to transport oxygen) and (ii) by leading to the formation of N-nitrosamines. Since the original observation by Barnes and Magee,[3] many N-nitrosamines have been shown to be carcinogenic in experimental animals, and it seems likely that some of these compounds also have this activity in Man.

The question which arises is this. Can the nitrite present in food naturally, or as an additive, react with nitrogenous food constituents at the acid pH of the stomach[4] to generate N-nitrosamines? Several investigators have examined this question in a variety of ways, and the upshot seems to be that such reactions are indeed possible, even likely.[5] Our interest here is a particular reaction - that between the species generated from nitrite under mildly acidic conditions and α-amino acids, peptides and proteins. Deamination will, of course, occur at a free primary amino group (van Slyke estimation, see below) but apart from linkages involving glycine residues,[6] the peptide bonds are rather resistant to nitrosation under mild conditions,[7] presumably because of the operation of steric factors. So it is appropriate to confine our attention to the effect of nitrous acid on the side chains of the common amino acids. Such reactions can be most readily studied by examining N-acyl amino acid esters (1) or N-acyl dipeptide esters (2) which may serve as model compounds for the



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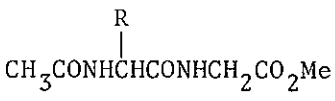

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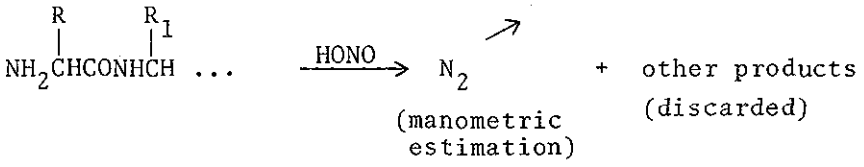
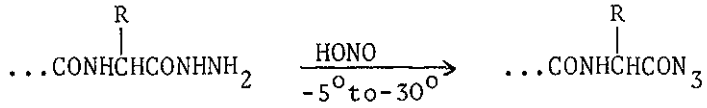
polypeptide situation.

Apart from a fairly extensive literature on the treatment of enzymes[8] and other proteins[9,10] with nitrous acid, there are two long established reactions which involve the exposure of α-amino acid derivatives to nitrosating conditions. It might have been thought, then, that the answer to the present question would be clear long ago. That it is not so is due, in the one case, the van Slyke estimation,[11] to the fact that here it is the gaseous product of the reaction - nitrogen - which is measured to provide an estimate of free primary amino functions.



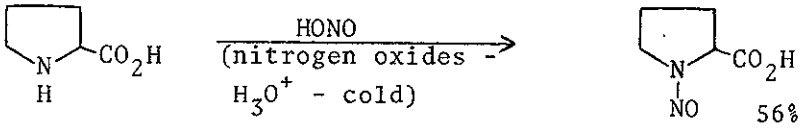
The reaction mixture, which presumably contains products of *nitrosation in the R groups, is discarded.* In the other reaction, the Curtius peptide synthesis,[12] the process is carried out in such a way that usually only the hydrazido function is affected:



With one or two notable exceptions,[13] discussions of the Curtius synthesis have paid little attention to the possibility of reaction in the group R, although nitrosation reactions of tryptophan[14,15] and of tyrosine[16] have been detected in this sort of experiment.

Let us now look at the reactive substrates. The α-imino acids occupy a special place and will be considered first. Amongst the examples of derivatives (e.g. 1,2) of the common α-amino acids it seems likely that tryptophan (R = β-indolyl-CH_2-), tyrosine (R = p-$HOC_6H_4CH_2$-), histidine (R = 4-iminazolyl-CH_2-), cysteine (R = $HSCH_2$-), lysine (R = $NH_2CH_2CH_2CH_2CH_2$-) and arginine (R = $NH_2\underset{\underset{NH}{\|}}{C}NHCH_2CH_2CH_2$-) might provide sufficiently reactive centres to suffer nitrosation under mildly acidic conditions, and these will be considered in turn. In the following, all α-centres have the natural (L) configuration unless otherwise stated. Nitrous acid is not a simple substance, but generates a complex set of species in equilibrium. Moreover nitrosation is catalysed by certain anions (e.g. chloride, thiocyanate). For these reasons the reaction conditions for nitrosation are quoted in some detail under the appropriate arrows.

Proline, hydroxyproline. As secondary amines, these give rise to stable N-nitrosamines such as N-nitrosoproline (3).[17] In a



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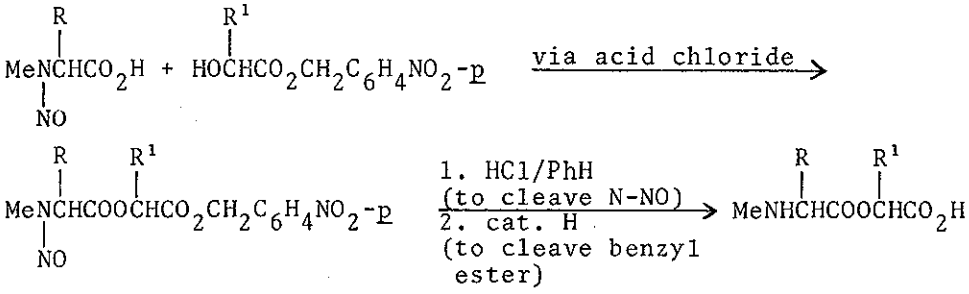
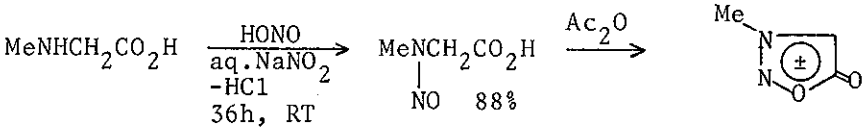
polypeptide, of course, reaction cannot occur in this way unless the proline or hydroxyproline is at the N-terminus. These N-nitroso imino acids do not seem to be carcinogenic

themselves.[18] However, the effect of the electron withdrawing group at the α-nitrogen is expected to increase the ease of thermal decarboxylation. This is one route by which N-nitrosopyrrolidine, a powerful carcinogen in animals, can be formed although the alternative route (proline → pyrrolidine → N-nitrosopyrrolidine) also appears to be important (especially at higher temperatures).[19] It seems possible that the formation of N-nitrosopyrrolidine when bacon is fried may occur in this way.[20] Kinetic studies (and a number of assumptions about process conditions, e.g. 4 hours at 54°) have led to the suggestion[21] that a cured meat prepared under normal conditions should contain less than 0.9 ppb of N-nitrosoproline: nevertheless 0.44 ppm of N-nitrosoproline has been found in samples of bacon.[22]

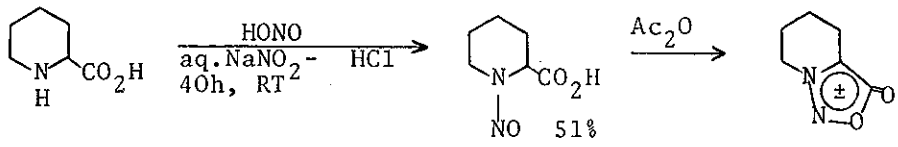
Two applications of such N-nitroso derivatives deserve mention. Firstly, the N-nitroso function has had limited use as a protecting group,[23] for example:[24]



Secondly, the N-nitroso imino acids have featured as intermediates in sydnone synthesis, for example with sarcosine:[25]

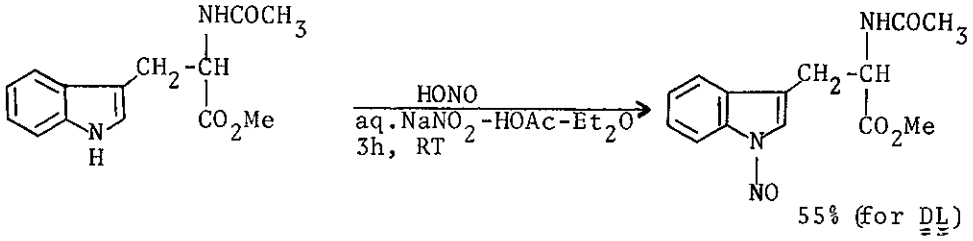


and with pipecolic acid:[25]



Curiously, the sydnone corresponding to proline does not appear to have been made.[23]

Tryptophan. In an early study of this problem,[26] tryptophan was found to react with nitrous acid to give a brick red powder which was regarded as a nitroso-β-indolylacrylic acid. N-Acetyltryptophan methyl ester (4, ≡ 1, R = β-indolyl-CH_2) reacts with sodium nitrite in aqueous acetic acid to give the nitroso derivative (5) in which nitrosation has occurred at the indolic nitrogen.[15,27,28]



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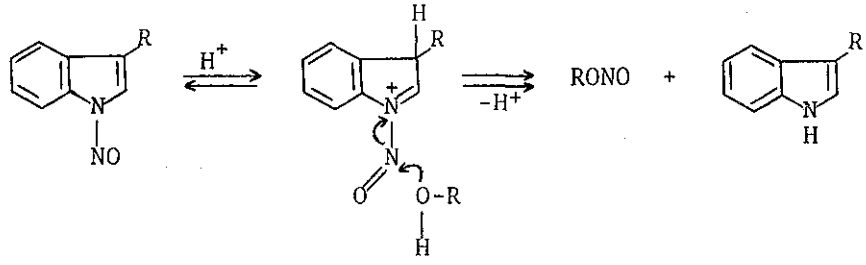
An analogous reaction occurs with skatole.[29] When C-3 of the indole is unsubstituted, nitrosation occurs at that position:[30] but when C-3 is substituted, reaction occurs predominantly on nitrogen even though C-2 is free. Formylation under Vilsmeier-Haack conditions provides another example of this behaviour of indoles with electrophiles of moderate reactivity.[31] The 1-nitrosoindole chromophore of (5) has two maxima at 267 and 274 nm, and a broad but less intense band at 335 nm (see also Table 1). The yellow crystalline solid is quite stable (room temperature, several months) but under acid conditions (e.g. HOAc/HBr) the N-N bond is readily cleaved, and even rather weak nucleophiles appear to attack the 1-nitrosoindole system. Thus *heating (5) in n-butanol regenerates the original tryptophan* derivative (4) with the formation of n-butyl nitrite, readily detected and estimated by electronic spectroscopy and GLC. The progress of such reactions can conveniently be followed by electronic spectroscopy. The reaction is first order in N-acetyl-N^1-nitrosotryptophan methyl ester, and the rate increases as the acidity is increased (over the range pH7-pH2.8). A plausible mechanism involves direct N→O transnitrosation, thus:



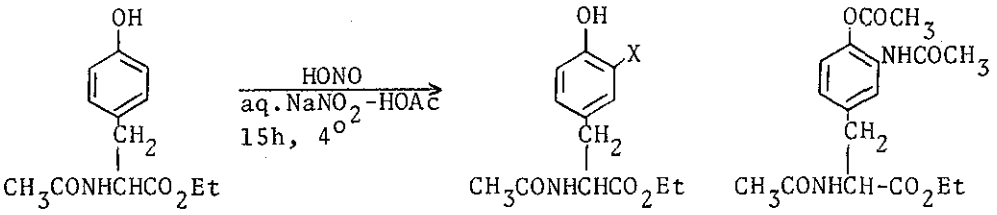
Transnitrosation has also been observed in which the nitroso group is transferred from (5) to other secondary nitrogen functions e.g.

$$\underline{\underline{DL}} - \underline{5} + Ph_2NH \rightarrow Ph_2N.NO + \underline{\underline{DL}} - \underline{4}$$

The N-nitroso derivative (5) is, in its L-configuration, a substrate for α-chymotrypsin: hydrolysis occurs specifically at the ester function to give N-acetyl-N^1-nitroso-L-tryptophan.[32]

Tyrosine. In electrophilic substitution reactions the phenolic group of tyrosyl is one of the most reactive of the side chains of the proteins. Tyrosyl residues may, for example, be specifically nitrated (ortho to the phenolic hydroxyl) under mild conditions using tetranitromethane.[33] Nitrosation of p-alkylphenols is known to lead to 2-nitro compounds, and the reaction here is thought to proceed by an initial nitrosation (the rate limiting step in aqueous perchloric acid at 0°) followed by a rapid oxidation.[34]

Although the reaction of N-acetyltyrosine with nitrous acid is slow compared with that of N-acetyltryptophan,[27] the nitrosation of tyrosyl residues also leads to the nitro derivatives.[16] Under certain conditions a mixture of the nitro derivative and the nitroso derivative appears to be formed. Thus reaction of N-acetyltyrosine ethyl ester (6) with sodium nitrite in aqueous acetic acid led to the isolation of a low yield (5%) of the 3-nitro derivative (7) and a 31% yield of a substance regarded as the 3-nitroso derivative (8).



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7, X = NO_2

8, X = NO

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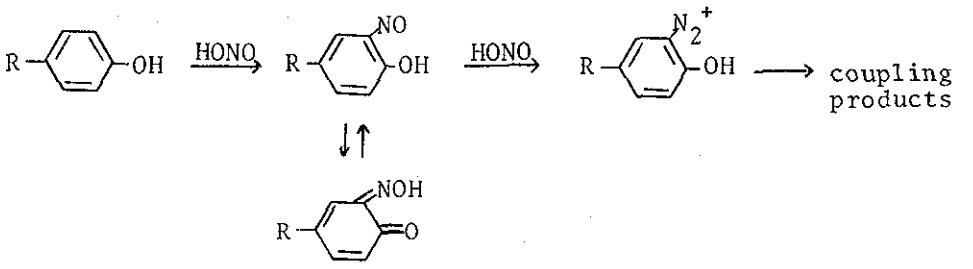
The nitro compound (7) is a yellow crystalline substance and is well characterised. The structure as the nitroso derivative (8) is less well supported: however, oxidation of (8) with H_2O_2/HOAc gives (7), and reductive acetylation of (8) furnishes the triacetyl derivative (9).

Treatment of bovine serum albumin with nitrous acid under simulated gastric conditions, followed by enzymatic hydrolysis, gives 6-hydroxynorleucine (from lysyl residues, see below), 3-nitrotyrosine, and 3,4-dihydroxyphenylalanine (dopa) as the recognised transformation products. The dopa is supposed to arise from the reaction of nitric oxide with 3-nitrosotyrosine to give a 3-diazonium nitrate which hydrolyses to the hydroxy compound.[10] Earlier workers have postulated that the yellow colours produced on treating proteins (e.g. wool, silk, pepsin) with nitrous acid are due to coupling products derived from diazo compounds, formed in the presence of excess nitrous acid.[35,36]



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This proposed scheme seems to go back to an observation by Hepp,[37] but it appears to us that the evidence[35] usually quoted[10] for the formation of a diazo compound from tyrosyl when appropriate proteins are treated with nitrous acid is not satisfactory. Schnabel and Zahn[16] did not observe such a compound when simple tyrosyl derivatives were exposed to nitrous acid during Curtius coupling; and the nitrosation of benzyloxycarbonyl-L-tyrosylglycyl-DL-alanine benzyl ester did not generate products which would couple (as expected for diazonium salts) with dimethylaniline. It seems that the colouration in nitrosated proteins may arise by oxidative coupling reactions at tyrosyl residues and from the nitrotyrosyl chromophore, with a contribution from the other products of nitrosation which have absorption stretching into the visible region, such as those listed in Table 1.

Table 1 Electronic Spectra of Nitrosation Products of α-Amino Acid Derivatives

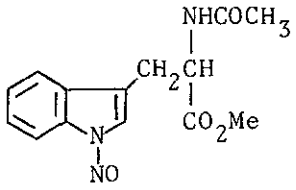
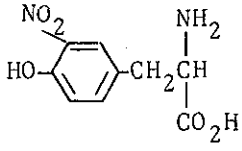
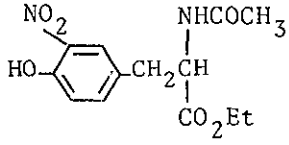
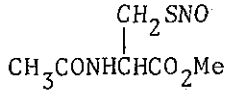
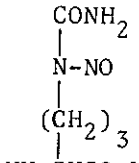
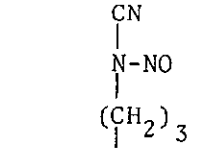
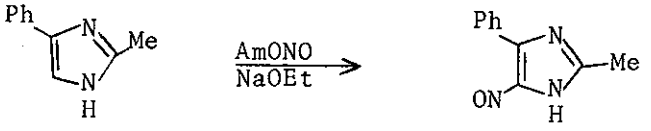
	Substance	Solvent	λ_{max} (ε_{max})	Ref.
1.	 5	EtOH	267 (14,000) 274 (11,500) 335 (7,300)	28
2.		Buffer < pH5 Buffer > pH9	360 (2,790) 427 (4,100)	33
3.	 7	MeOH	358 (2,600) 275 (6,300) 238i (7,400) 217 (17,900)	
4.	 11	MeOH	545 (22) 510 (12) 338 (940)	

Table 1 (continued)

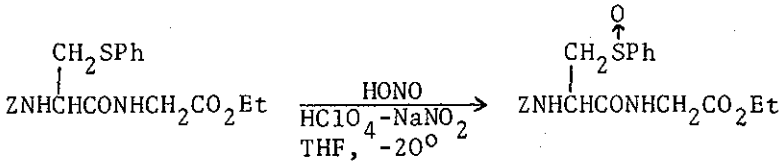
	Substance	Solvent	λ_{max}	(ε_{max})	Ref.
5.		80% EtOH	233	(4,700)	49
			385	(76)	
			399	(99)	
	15		417	(77)	
6.		EtOH	366i		50
			378	(61)	
			392	(88)	
	18		407	(67)	

Histidine. The iminazole anion is reported to be nitrosated by nitrite esters, e.g.[38]

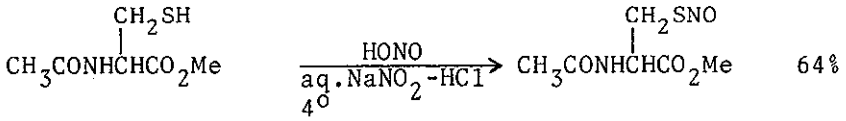


However, attempts to observe a reaction between the iminazole group of histidine derivatives (e.g. N-acetylhistidine methyl ester 1, R = 4-iminazolyl-CH_2-) and sodium nitrite under mildly acidic conditions have been unsuccessful: the starting material is recovered together with some of the free acid. Others have reported similar negative results.[27]

Methionine. Although a reaction between N-acetylmethionine methyl ester (1, R = $MeSCH_2CH_2$-) and sodium nitrite in aqueous acetic acid has not been detected at room temperature,[39] the oxidation of S-benzyl derivatives of cysteine has been observed.[40] The reaction gives the sulphoxide in low yield.



Cysteine. N-Acetylcysteine methyl ester (10 ≡ 1, R = $HSCH_2$-) reacts with nitrous acid to give the S-nitroso derivative or thionitrite (11). This is a rather unstable substance, but it



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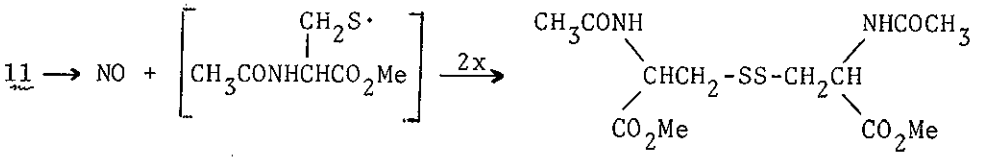
can be obtained as reddish needles (λ_{max} 338, 510, 545 nm, see Table 1) from petroleum. The formation of the thionitrite may also be detected by ^{15}N-n.m.r. spectroscopy, where the -S-^{15}N = O

function appears at characteristically low field (δ 750-800 relative to $^{15}NH_4^+$).[39] The thionitrite function is, perhaps, an unfamiliar one, although long enshrined in the literature.[41] A preparation of the S-nitroso derivative of cysteine itself has been described,[42] but the product was not fully characterised and is, in any case, presumed to have been contaminated with products of deamination. This preparation is reported[43] to have a half life of ca. 50 hours in aqueous solution when generated from sodium nitrite and sulphuric acid in the presence of ammonium sulphamate (to remove excess nitrous acid). The thionitrite (11) gradually decomposes at room temperature with the loss of nitric oxide and the formation of N,N¹-diacetylcystine dimethyl ester (12).



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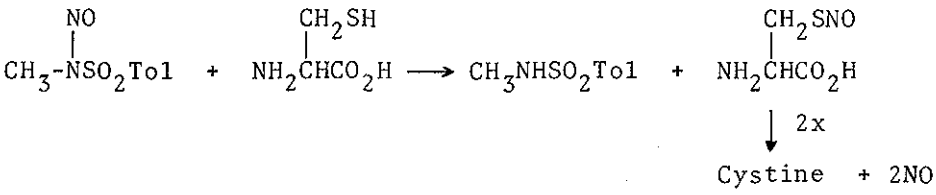
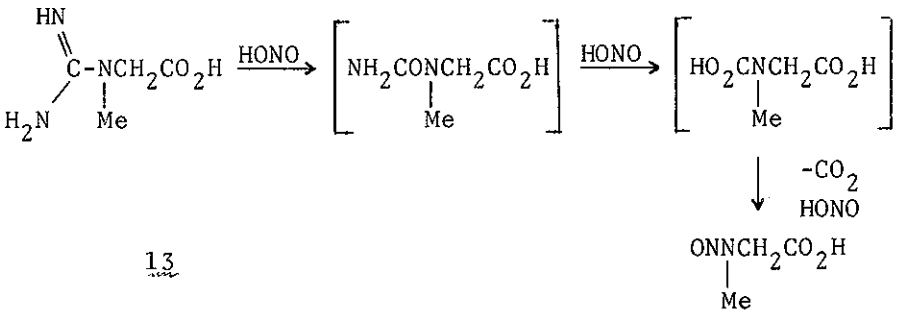
It is pertinent to mention that (i) cysteine inhibits the formation of N-nitrosamines in model food systems,[44] (other reducing agents, e.g. ascorbic acid, also have this effect); (ii) cysteine accelerates the decomposition of certain nitrosamides e.g. N-methyl-N-nitroso-p-toluenesulphonamide and N-methyl-N¹-nitro-N-nitrosoguanidine[45]; and (iii) cysteine enhances the antibacterial activity of nitrite.[46] The involvement of the thionitrite is conceivable in these processes:

there is, indeed, some evidence for such intermediates in the nitrosamide decompositions[45]



and a preparation of cysteine thionitrite is reported to have a somewhat greater inhibitory effect on the growth of Clostridium sporogenes than an equivalent amount of sodium nitrite.[46]

Arginine and Lysine. The two common aliphatic α-amino acids with strongly basic side chains are arginine and lysine, and they owe their basicity to a guanidino function and a primary amino function, respectively. Both groups are expected to react with nitrous acid. Lysine reacts more readily than does arginine: for example, in the treatment of collagen with nitrous acid, 90% of the lysine (and hydroxylysine) residues have reacted in 4 hours, whereas under the same conditions only 13% of the arginine residues have been attacked after 24 hours.[9] Only the α-amino group of arginine itself is reported to be deaminated with sodium nitrite-acetic acid mixtures, whereas with sodium nitrite-hydrochloric acid mixtures (which presumably contain NOCl) all the nitrogen is reported to be removed from the molecule.[47] Creatine (13) contains an N,N-disubstituted guanidino residue: treatment with nitrous acid gives N-nitrososarcosine.[48]



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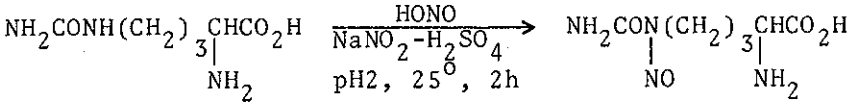
The nitrosation[49] of DL-citrulline (14), the urea analogue of arginine, appears to proceed smoothly to give the δ-nitrosamine (15). Alkylguanidines behave in a more complex



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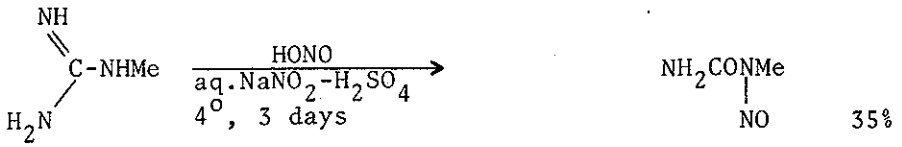
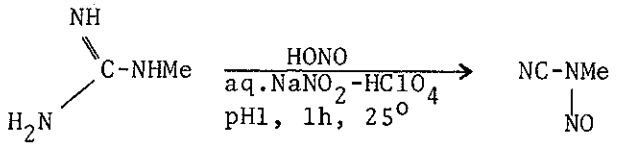
way, and no simple N-nitroso derivative of arginine appears to have been characterised. Nitrosation of methylguanidine under strongly acidic conditions gives N-methyl-N-nitrosourea,



but when this reaction is carried out under less vigorous conditions a product regarded as the nitrosocyanamide (16) has been detected.[49]



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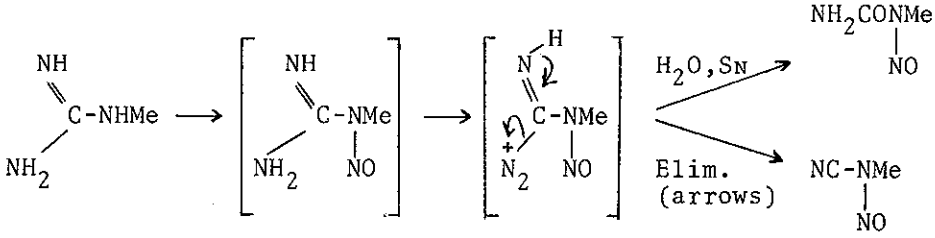
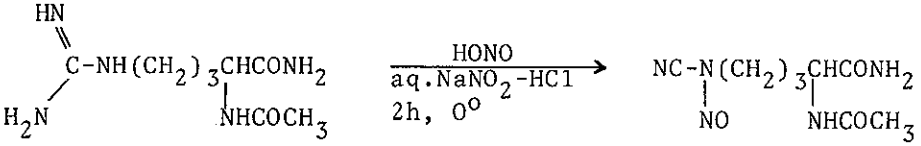
These products presumably arise by alternative reaction pathways from the N-methyl-N-nitrosoguanidine



The N-nitrosocyanamide has been more satisfactorily characterised with arginine derivatives. Thus N-acetylarginin-amide (17) on nitrosation furnishes yellow needles of a substance formulated[50] as (4-acetamido-4-carboxamidobutyl)-N-nitrosocyanamide (18), the electronic absorption spectrum of which shows a weak band with distinct vibrational structure (λ_{max} 366i, 378, 392, 407 nm). It is surprising that the primary amide function survives, but a precisely analogous reaction has been described for



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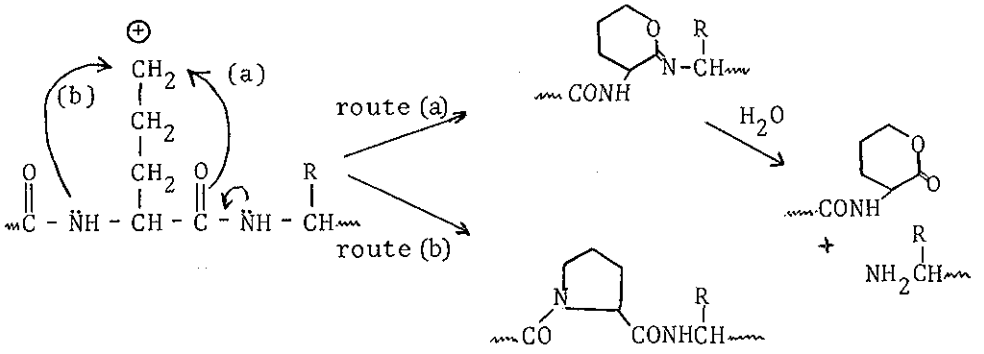
N-benzoylargininamide. The N-nitrosocyanamide (18) and its relatives show a pronounced mutagenic activity.[50]

On a speculative note, it seems plausible that the primary carbonium ion generated on nitrosation of a primary amino function in a side chain (i.e. ornithyl or lysyl) might react intramolecularly with a neighbouring peptide bond. This can be illustrated for the ornithyl case thus:



A similar reaction with lysyl would give chain cleavage with the formation of a seven-membered lactone (route a) or piperidine ring formation (route b).

It must be borne in mind that, in the protein situation, an α-amino acid residue finds itself in a specific chemical matrix of more or less defined geometry, and such features are not represented in simple models (such as 1, 2). Some α-amino acid residues may well be inaccessible in the protein, while others may have enhanced reactivity. Nevertheless a consideration of such models does reveal the type of reactions that are possible, and the conclusion to be drawn is that, with nitrous acid, such

reactions at side chains are more prevalent than has been generally recognised. If nitrous acid is employed to alter the N-terminus of an enzyme to reveal whether or not it is involved at the active site, then the results must evidently be interpreted with some care since several other residues (tryptophan, tyrosyl, cysteinyl, lysyl, arginyl) may suffer chemical change. For example,[51] pepsin has isoleucine as N-terminus: this is rapidly deaminated with nitrous acid, but at the same time one lysine and two tryptophan residues appear to react, and there is a 40% loss in biological activity.

As far as the generation of potentially carcinogenic N-nitrosamines is concerned, arginyl deserves especial attention since arginine derivatives produce N-nitrosocyanamides of demonstrated mutagenic activity. Proline, hydroxyproline, and sarcosine (free, or N-terminal) also appear to be likely candidates, and the N-nitroso derivatives of these three imino acids have been reported in low levels in cured meats.[22] Tyrosine and lysine appear to destroy nitrosating power: but cysteine and tryptophan may serve as a reservoir of nitrosating capacity, since transnitrosation reactions (to diphenylamine) have been observed with the corresponding nitroso derivatives (5) and (11).

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