Nitrosation and Nitrosylation of Haemoproteins and Related Compounds. Part 2.[1] The Reaction of Nitrous Acid with the Side Chains of α-Acylamino-acid Esters

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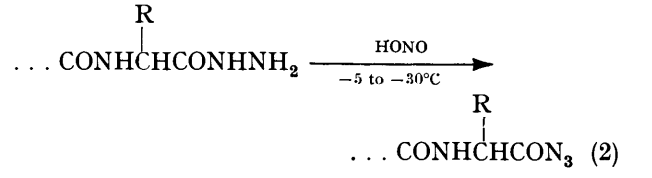
The nitrosation of *N*-acetylcysteine methyl ester with aqueous sodium nitrite and acetic acid furnishes the *S*-nitroso-(thionitrite) derivative as unstable red crystals which decompose in ether to give *NN'*-diacetylcystine dimethyl ester. Under similar nitrosating conditions *N*-acetyltyrosine ethyl ester furnishes the 3-nitro-derivative and an unstable product formulated as the corresponding 3-nitroso-derivative. Neither *N*-acetylhistidine methyl ester nor *N*-acetylmethionine methyl ester are nitrosated under these conditions. At higher acidities the latter compound gives the *N*-nitroso-amide and a polar compound regarded as the *N*-nitrosamide *S*-oxide. *N*-Acetyl-N^1-nitrosotryptophan methyl ester is solvolysed in aqueous methanolic buffer solutions. The reaction is first order in the N^1-nitroso-compound and the rate increases as the pH is lowered in the range 2—8. Solvolysis with n-butyl alcohol gives n-butyl nitrite and *N*-acetyltryptophan methyl ester. Transnitrosation reactions are also observed with the nitroso-thiolcysteine derivative and the N^1-nitrosotryptophan derivative as donors and diphenylamine as the acceptor molecule.

In Part 1 the interaction of haemoproteins, such as myoglobin and haemoglobin, with nitrous acid was introduced and the chemistry of the nitrosation and nitrosylation of porphyrins and haems was investigated.[1] In this paper we turn to the protein part of the molecules and examine the question of side-chain nitrosation.[2]

It is, perhaps, surprising that these reactions are not already well known. Besides an extensive literature on the effect of nitrous acid on enzymes [3] and other proteins,[4,5,6] there are two well known reactions which involve nitrosation of simpler α-amino acid derivatives. However both are carried out in such a way that the present question is not answered. In the van Slyke estimation [7] the reaction mixture containing the information is actually discarded because only the gaseous product—nitrogen from primary amino functions—is required [equation (1)]. In the Curtius coupling

$$NH_2CHCO\ldots \xrightarrow{HONO} N_2 + HOCHCO\ldots (etc.) \quad (1)$$

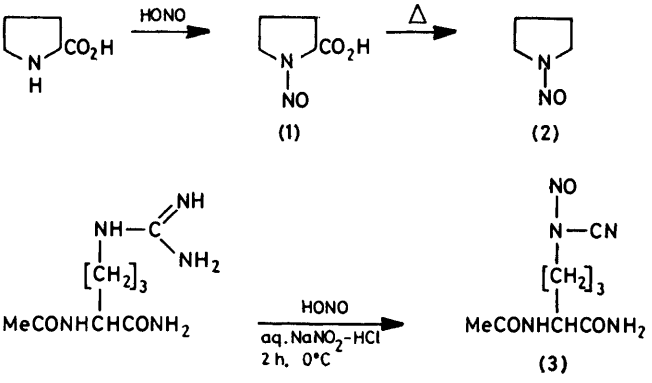
process [8] the azide is deliberately produced at a low temperature, so that usually only the hydrazido-group is affected [equation (2)]. Nitrosation reactions of derivatives of tyrosine [9] and of tryptophan [10,11] have been observed in this context, however.



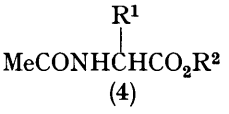
$$\ldots CONHCHCONHNH_2 \xrightarrow[-5 \text{ to } -30°C]{HONO} \ldots CONHCHCON_3 \quad (2)$$

The nitrosations of proline and arginine derivatives have been reported in detail, and are not described here. Proline [12] gives the *N*-nitrosoamine (1) which, although apparently not itself a carcinogen,[13] can decarboxylate to give *N*-nitrosopyrrolidine (2), which is powerfully carcinogenic in animals.[14] *N*-Acetylarginamide gives the *N*-nitrosocyanamide (3), which has mutagenic properties.[15]

Nitrosations have been carried out in our laboratory under mild conditions (aqueous $NaNO_2$–HOAc; 5 °C) using the *N*-acetylamino-acid esters (4) as models. The acyl function is intended to protect the primary amino-



function from deamination, and at the same time to serve as a model for the peptide bond. We have already



$$MeCONHCHCO_2R^2 \quad (4)$$

reported [16] a ^{15}N n.m.r. investigation on the interaction of [^{15}N]nitrous acid with compounds of type (4).

Cysteine.—*S*-Nitrosation has been known for many decades. Tasker and Jones [17] reported the formation of *S*-nitrosothiophenol in 1909, while Vorlander and Mittag [18] isolated trityl nitrosothiol in 1919. Cysteine itself has been reported to give an unstable *S*-nitroso-derivative, but this was inadequately characterised,[19,20] and the method of preparation (aqueous acid, sodium nitrite, cysteine) suggests that (in spite of the greater nucleophilicity of the thiol function compared with amino) [21] some deamination at the α-amino-function may have occurred during the reaction.

Nitrosation of *N*-acetylcysteine methyl ester gave a wine-red reaction mixture from which the *S*-nitroso-derivative (5) was isolated in 64% yield as reddish

needles. The substance was unstable and as a consequence the results of elemental analysis fell outside the

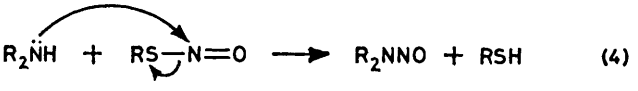
(5) (6)

normally accepted range: even so, the best fit for the experimental values was the empirical formula $C_6H_{10}N_2O_4S$. The presence of the nitrosothiol (thionitrite) function was inferred from (i) the electronic spectrum, $\lambda_{max.}$ 338 (ε 945), 510 (12), and 545 nm (22) [*cf.* $PhCH_2SNO$, $\lambda_{max.}$ 340 (ε 1030), 530 (1), and 560 nm (26)],[22] and (ii) the n.m.r. spectrum, where the triplet at δ 1.55 due to SH in (4; R = CH_2SH) was missing and the methylene multiplet [at δ 3.0 in (4; R = CH_2SH)] had been most strongly deshielded (δ 4.16). In the i.r. spectrum a broad band in the region $\nu_{max.}$ 1 430—1 490 cm^{-1} was observed. This is consistent with the presence of the nitrosothiol function: t-butylnitrosothiol has a broad structured absorption at $\nu_{max.}$ 1 480—1 530 cm^{-1} assigned [23] to the S–N=O function. The i.r. spectrum of (5) also possessed a weak band at $\nu_{max.}$ 1 925 cm^{-1}. Such an absorption has recently been noted (at $\nu_{max.}$ 1 910 cm^{-1}) [24] in both Bu^tSNO and PhSNO. It does not appear to have been assigned. The intriguing possibility arises that this band is due to a valence tautomer with a linear nitrosothiol function (*i.e.* $R-\bar{S}-\dot{N}=O$). The ' normal ' nitrosothiol function (as in R–S–N=O) is expected to be bent (as in *N*-acetyl-*S*-nitroso-DL-penicillamine where S–N–O = 113.2°).[25]

Like most other nitrosothiols, the cysteine derivative (5) is unstable; the S–N bond is weak and nitric oxide, one of the products of homolysis, is a stable radical [equation (3)]. Thus the mass spectrum of (5) showed

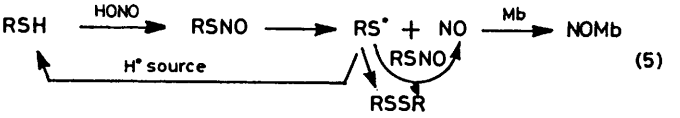
$$RSNO \longrightarrow RS\cdot + NO \qquad (3)$$

no molecular ion, although the fragment $RS^{+\cdot}$ was prominent; and when (5) was kept in ether at room temperature for 6 h the colour faded and the disulphide (6) was isolated in 87% yield.



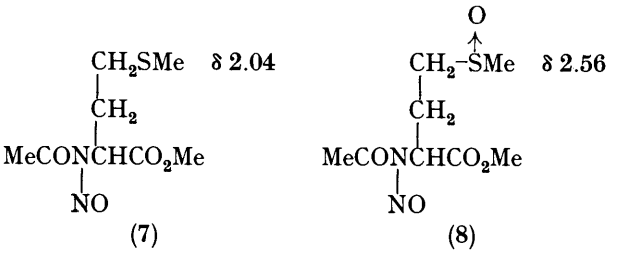
Transnitrosation with (5) as the nitrosating species was demonstrated by a positive Liebermann test, and by the isolation of *N*-nitrosodiphenylamine in low yield when (5) and diphenylamine were kept together in ether under nitrogen.

Thus the nitrosothiol derivative is likely to be formed readily when peptides and proteins containing the cysteinyl residue are nitrosated, but it is also readily destroyed. The limited evidence available suggests that heterolytic and homolytic mechanisms may compete in this decomposition. The heterolytic mechanism could lead to *N*-nitrosamines [equation (4)] while the homolytic process is expected to generate nitric oxide and this appears to be one possible route to nitrosylmyoglobin in the curing process [20,26] [equation (5; Mb = myoglobin)].



Cysteine enhances the antibacterial activity of nitrite,[26,27] inhibits the formation of *N*-nitrosoamines in model food systems,[28] and accelerates the decomposition of certain alkylating *N*-nitroso-derivatives (*e.g.* *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide and *N*-methyl-*N*-nitrosourea).[29] It seems plausible that these processes involve the nitrosothiol as an intermediate.

Methionine.—Nitrosation of *N*-acetylmethionine methyl ester (4; $R^1 = CH_2CH_2SMe$, R^2 = Me) was not observed under mildly acidic conditions (aqueous acetic acid). At higher acidities (aqueous perchloric acid) two products were isolated, albeit in low yields, by preparative layer chromatography. The *N*-nitrosoamide (7) was obtained as a yellow oil which gave a



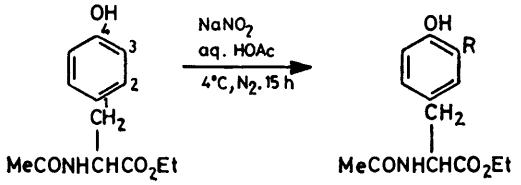
(7) (8)

positive Liebermann test. Spectroscopic properties supported the nitrosoamide structure (see Experimental section). Thus in the i.r. spectrum the amide I band [at $\nu_{max.}$ 1 650 cm^{-1} in (4; $R^1 = CH_2CH_2SMe$, R^2 = Me)] was absent since the nitrosoamide carbonyl stretch (*cf.* Bu-N·NO·COMe $\nu_{max.}$ 1 739 cm^{-1}) now overlapped the ester carbonyl absorption to give a strong broad band at $\nu_{max.}$ 1 740 cm^{-1}, and in the n.m.r. spectrum the protons at the acetamide methyl group and at the α-carbon atom were strongly deshielded. The second product was a polar yellow resin, again giving a positive Liebermann test. It had properties (*e.g.* high resolution M^+; deshielding of SMe group) consistent with its formulation as the sulphoxide (8). Honzl and Rudinger have noted an oxidation related to that postulated here during the treatment of *S*-benzylcysteine derivatives with nitrous acid.[30]

Tyrosine.—In connection with a study of the inactivation of pepsin by nitrous acid, Philpot and Small [31] studied the nitrosation of tyrosine, and found that it reacted less rapidly than did tryptophan, an observation confirmed more recently by spectrophotometric studies on the *N*-acetyl derivatives.[32] Philpot and Small referred to the colorimetric estimation of the copper complex of the 3-nitroso-derivative of tyrosine, and to the formation of diazo compounds. Nitrosation of

bovine serum albumin at gastric pH, followed by hydrolysis, gives 3-nitrotyrosine and dihydroxyphenylalanine derived from tyrosyl residues.[6] Nitrosation of *p*-alkylphenols is well known and gives the *p*-alkyl-*o*-nitrophenols, a reaction thought to proceed by an initial nitrosation, this being the rate-limiting step in aqueous perchloric acid at 0 °C.[33]

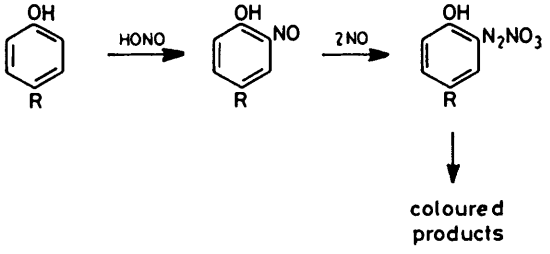
Treatment of L-*N*-acetyltyrosine ethyl ester (9) with sodium nitrite and aqueous acetic acid (4 °C; 15 h) gave a complex mixture of products, including several minor coloured compounds. The major components were separated by preparative layer chromatography to give the starting material (6%), the 3-nitro-derivative (10) (5%; identified with an authentic sample[34]), and a polar compound regarded as the corresponding nitroso-derivative (11) (31%). This substance has not been



(9) (10) R = NO_2 (11) R = NO

satisfactorily characterised and awaits further investigation. It is clearly very reactive, and even the oxidation to the nitro-compound (with H_2O_2–HOAc) is complicated by the formation of orange products believed to arise by oxidative coupling processes. Such substances are also readily formed when (11) is kept on the silica support after t.l.c.

The yellow colour produced when certain proteins (*e.g.* wool, silk, pepsin) are nitrosated has been attributed[5,31] to the formation of diazo-coupling products [equation (6)]. Although the formation of diazonium



(6)

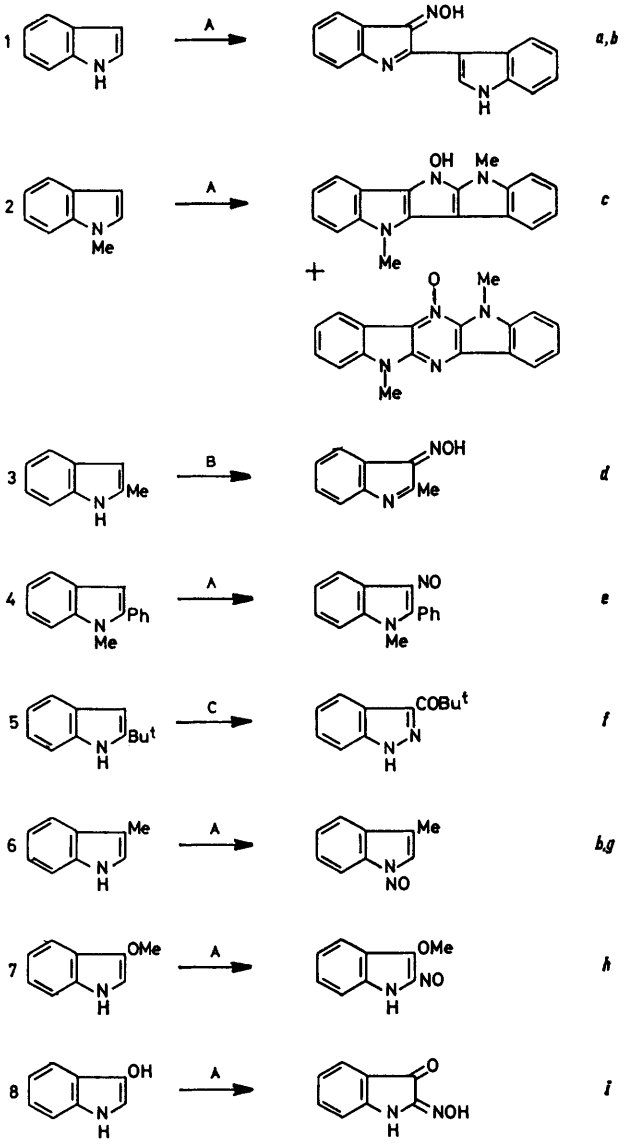
salts along this unusual pathway is seen in several examples,[35] Schnabel and Zahn[9] were unable to detect diazonium salts in the liquor resulting from the nitrosation of *N*-benzyloxycarbonyl-L-tyrosylglycyl-DL-alanine benzyl ester. On present evidence it seems more likely that the pigmentation in nitrosated protein arises from a combination of chromophores[2] of the nitrosation products of the side chains of the amino acids, but particularly N^1-nitrosotryptophanyl, 3-nitrotyrosyl, and the products of the oxidative coupling reactions of 3-nitrosotyrosyl.

Histidine.—Although other electrophilic substitutions such as nitration, have been observed with imidazoles, nitrosation in aqueous acid does not appear to have been recorded,[36] although nitrosation of the more reactive imidazole anion by nitrite esters has been observed.[37]

No nitrosation products were detected when *N*-acetylhistidine methyl ester was treated with aqueous sodium

TABLE 1

Products of nitrosation reported in the indole series



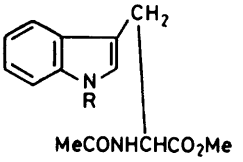
Reagents: A, aqueous $NaNO_2$–HOAc; B, NaOMe–C_5H_{11}-ONO; C, aqueous $NaNO_2$–HOAc–HCl

[a] P. Seidel, *Ber.*, 1944, **77**, 797. [b] H. F. Hodson and G. F. Smith, *J. Chem. Soc.*, 1957, 3546. [c] A. H. Jackson, D. N. Johnston, and P. V. R. Shannon, *J.C.S. Perkin I*, 1977, 1024. [d] W. E. Noland, L. R. Smith, and K. R. Rush, *J. Org. Chem.*, 1965, **30**, 3457. [e] N. Campbell and R. C. Cooper, *J. Chem. Soc.*, 1935, 1208. [f] F. Piozzi and A. V. Ronchi, *Gazzetta*, 1964, **94**, 1248. [g] E. Fischer, *Annalen*, 1886, **236**, 126. [h] W. A. Remers in 'Indoles,' Part I, ed. W. J. Houlihan, Wiley, New York, 1972, p. 84. [i] J. van Alphen, *Rec. Trav. chim.*, 1938, **57**, 911.

nitrite–acetic acid, although some hydrolysis of the ester function (possibly assisted by the imidazole) occurred.

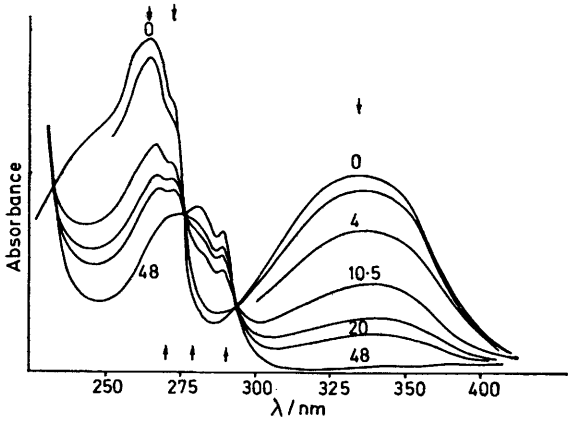
Tryptophan.—In sharp contrast to the imidazole, indoles react readily with nitrous acid, and exhibit a wide variety of reactions (Table 1). In summary, it appears that if C-3 is unsubstituted then nitrosation occurs there (items 3 and 4) at least as a first step (items 1, 2, and 5). Where C-3 is already substituted, nitrosation occurs preferentially at nitrogen (item 6) *unless* the C-3 substituent is a powerful electron donor, when nitrosation at C-2 occurs preferentially (items 7 and 8, the latter molecule being a special-tautomeric example).

N-Acetyltryptophan methyl ester (12) follows the pattern of skatole (item 6) and gives the *N*-nitroso-derivative (13) with aqueous sodium nitrite–acetic acid.[11,38] Compound (13) is a yellow crystalline solid which is stable at room temperature for several months. However, the N–N bond is cleaved in aqueous methanol (Figure) and kinetic studies in dilute solution show that the reaction is first order in the N^1-nitrosoindole (13),



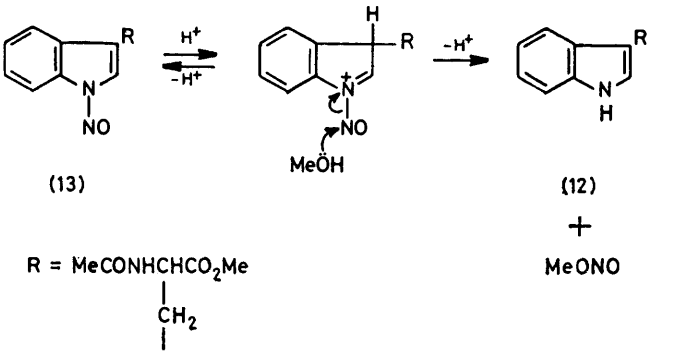
(12) R = H
(13) R = NO

and that the rate increases as the apparent pH is lowered (in the range 2—8). This accords with a mechanism in which the protonated N^1-nitrosoindole is solvolysed, for example by methanol (Scheme). Homolytic mechanisms have also been considered, especially for the reaction in neutral media. Experiments in which the N^1-nitrosotryptophan derivative (13) was heated under the same conditions in light petroleum and in n-butyl alcohol showed that little reaction occurred in the non-polar solvent, arguing against a ready homolytic cleavage



Solvolysis of *N*-acetyl-N^1-nitrosotryptophan methyl ester in water–methanol (1 : 1) containing a small amount of sulphuric acid (to give an apparent pH of 1.8) at 20 °C. The numerals on the curves refer to time in minutes

On the other hand, in n-butyl alcohol (80—90 °C; 45 min) reaction occurred to give *N*-acetyltryptophan methyl ester (12) (62% isolated) and n-butyl nitrite. The latter product was identified by electronic spectroscopy and mixed g.l.c. (g.l.c. comparison with an



SCHEME

authentic specimen): the yield, estimated spectroscopically, was 33%, the relatively low value being ascribed to the volatility of n-butyl nitrite (b.p. 75°). These results support a heterolytic pathway in neutral protic media.

The analogous transfer of the nitroso-group to another *amine* is of interest because in this way N^1-nitrosotryptophanyl residues in a nitrosated protein could eventually lead to carcinogenic *N*-nitrosoamines. The possibility of this reaction was demonstrated when the N^1-nitrosotryptophan derivative (13) was kept in molten diphenylamine (65 °C; 3 h; air or N_2) to give *N*-nitrosodiphenylamine in *ca.* 70% yield [equation (7)].

$$(13) + Ph_2NH \longrightarrow Ph_2N{\cdot}NO + (12) \qquad (7)$$

EXPERIMENTAL

General experimental methods have been described.[38] Infra-red spectra were measured for Nujol mulls, and electronic spectra in methanol, unless otherwise stated. Petroleum means that fraction of light petroleum, b.p. 60—80 °C. Except where otherwise stated, DL-amino acid derivatives were used.

L-*N-Acetyl*-S-*nitrosocysteine Methyl Ester.*—All solvents and solutions were flushed with nitrogen. L-*N*-Acetylcysteine methyl ester[39] (110 mg) in acetic acid (0.35 ml) was treated (ice-bath) with a solution of sodium nitrite (46 mg in 0.1 ml water). The solution became deep red. Dichloromethane (15 ml) was added, and the organic solution was rapidly washed with aqueous sodium bicarbonate, separated, and dried. Removal of solvent under reduced pressure gave reddish needles (82 mg, 64%) of L-N-*acetyl*-S-*nitrosocysteine methyl ester*, m.p. 68—71°, which was recrystallised from dichloromethane–petroleum as pink-red needles in feathery clusters [Found (on sample analysed within 3 h of preparation): C, 35.5; H, 5.05; N, 12.35; S, 15.35. $C_6H_{10}N_2O_4S$ requires C, 34.95; H, 4.9; N, 13.6; S, 15.55%), $\nu_{max.}$ 3 270, 1 925w, 1 740, 1 640, 1 540, 1 490—1 430br, 1 375, 1 220, 1 155, 1 065, 890, 830, and 800 cm^{-1}, $\lambda_{max.}$ 338 (ε 945), 510 (12), and 545 nm (22), *m/e* (40 °C) 176 (77%), 145 (4), 135 (19), 117 (8), 88 (100),

76 (23), and 60 (56), δ 4.96 (m, α-CH), 4.16 (m, CH_2), 3.69 (s, OMe), and 1.97 (s, COMe). The compound gave a positive Liebermann test.

L,L-NN'-*Diacetylcystine Dimethyl Ester*.—L-*N*-Acetyl-*S*-nitrosocysteine methyl ester (75 mg, freshly prepared) was kept in diethyl ether (5 ml, dried) in air at room temperature. During 6 h the red colour became less intense and needles (47 mg, 73%) were formed of L,L-*NN'*-diacetylcystine dimethyl ester, m.p. 131—132° (lit.,[40] 128—129 °C) identical (mixed m.p., t.l.c.) with an authentic sample prepared by aerial oxidation, catalysed by ferrous sulphate,[41] of L-*N*-acetylcysteine methyl ester.

Transnitrosation between N-*Acetyl*-S-*nitrosocysteine Methyl Ester and Diphenylamine*.—Freshly prepared *N*-acetyl-*S*-nitrosocysteine methyl ester (52 mg), diphenylamine (80 mg), and ether (5 ml) were heated in a sealed tube under nitrogen (40—45 °C, 3 h). Preparative t.l.c. (silica gel; petroleum–chloroform 2 : 1) gave unchanged diphenylamine (R_F 0.49), *N*-nitrosodiphenylamine (R_F 0.28), 5.2% yield (estimated spectroscopically), and *NN'*-diacetylcystine dimethyl ester, m.p. 129—130 °C.

Nitrosation of N-*Acetylmethionine Methyl Ester*.—Attempted nitrosation in aqueous $NaNO_2$–HOAc (4 °C, 24 h) led to 82% recovery of the starting material.

N-Acetylmethionine methyl ester [42] (241 mg) in aqueous perchloric acid (8.5M; 1.4 ml) was treated at 4 °C with ice-cold aqueous sodium nitrite (140 mg in 0.8 ml water). After 2 h at 4 °C the orange solution was treated with an excess of saturated $NaHCO_3$ and extracted with ethyl acetate (50 ml) using sodium chloride to facilitate the extraction. The dried organic solution was concentrated and subjected to preparative t.l.c. (EtOAc–Et_2O 1 : 2) to give three major components, the middle one being the starting ester (77 mg, 32% recovery).

The least polar component (R_F 0.73), formulated as N-*acetyl*-N-*nitrosomethionine methyl ester*, was obtained as a viscous yellow oil (35 mg) which did not crystallise (Found: M^+, 234.067. $C_8H_{14}N_2O_4S$ requires *M*, 234.067), $\nu_{max.}$ (neat) 2 260w, 1 740vs, 1 510, 1 440, 1 375, 1 300, 1 240, 1 120, 910s, and 735s cm^{-1}, $\lambda_{max.}$ 241 (ε 6 500), 377infl. (41), 390 (57), 404 (84), and 423 nm (81), *m/e* (37 °C) 234 (3%), 204 (M^+ — NO, accurately measured, 17), 173 (5), 145 (42), and 88 (100), δ 5.45 (m, α-CH), 3.63 (s, OMe), 2.80 (s, COMe), 1.85—2.8 (m, CH_2CH_2), and 2.04 (s, SMe). The compound gave a positive Liebermann test.

The most polar component (R_F 0.08) was obtained as a yellowish resin (30 mg) and is regarded as the sulphoxide derivative of the foregoing compound (Found: M^+, 250.062. $C_8H_{15}N_2O_5S$ requires *M*, 250.062), $\lambda_{max.}$ 242, 387, 403, and 422 nm, *m/e* (46 °C), 250 (4%), 221 (4), 206 (8), 187 (23), 162 (30), 144 (13), 131 (13), 114 (17), 87 (28), 75 (62), and 60 (100), δ 5.33 (m, α-CH), 3.65 (s, OMe), 2.81 (s, COMe), 2.6 (m, CH_2S), 2.56 (s, SMe), and 2.15 (m, CH_2). The compound gave a positive Liebermann test.

Nitrosation of L-N-*Acetyltyrosine Ethyl Ester*.—L-*N*-Acetyltyrosine ethyl ester [43] (500 mg) in acetic acid (2 ml, gentle heat) was treated (N_2, ice–salt-bath, partial freezing) with ice-cold aqueous sodium nitrite (500 mg in 3 ml water). The yellow mixture was kept at 4 °C for 15 h under nitrogen, then treated with excess of aqueous $NaHCO_3$ and extracted with ethyl acetate (400 ml). The extract was dried, concentrated, and subjected to preparative t.l.c. ($CHCl_3$–EtOAc–MeOH, 50 : 15 : 2) to give three main components, the middle one being starting material (6%).

The least polar component (R_F 0.54) was crystallised from ethanol to give yellow prisms (31 mg, 5%) of L-*N*-acetyl-3-nitrotyrosine ethyl ester, m.p. 94—95 °C (lit.,[34] 95—97 °C), identical (m.p., u.v., mixed t.l.c.) with an authentic sample prepared by nitration of the starting material with 28% nitric acid.

The third component was more polar (R_F 0.14) and was obtained as an unstable yellow gum (183 mg, 32%) regarded as the 3-*nitroso-derivative*, $\nu_{max.}$ 3 550, 3 330, 3 160br, 1 880w, 1 720, 1 655, 1 610, 1 600, 1 535, 1 280, 1 230, 1 132, 1 025, 820, and 810 cm^{-1}.

The crude nitrosation product from L-*N*-acetyltyrosine ethyl ester (200 mg) was treated with acetic acid–28% hydrogen peroxide (4 : 5; 1.5 ml) at 40—60 °C for 10 min, and then kept at 4 °C for 24 h. The mixture was treated with excess of aqueous $NaHCO_3$ and extracted with ethyl acetate (90 ml). The extract was dried (Na_2SO_4), filtered, concentrated, and subjected to preparative t.l.c. ($CHCl_3$–EtOAc–MeOH, 20 : 5 : 1) to give L-*N*-acetyl-3-nitrotyrosine ethyl ester (37.5 mg, 16%), L-*N*-acetyltyrosine ethyl ester (55 mg, 28%), and an orange amorphous solid (3 mg), R_F 0.32, regarded as an oxidative coupling product of L-*N*-acetyl-3-nitrosotyrosine ethyl ester.

N-*Acetyl*-N^1-*nitrosotryptophan Methyl Ester. Transnitrosation to Oxygen*.—(a) *Kinetic studies in aqueous methanolic buffers*. *N*-Acetyl-N^1-nitrosotryptophan methyl ester (4 mg) was dissolved in methanol (25 ml) and made up to 50 ml with McIlvaine's citric acid–phosphate buffer of appropriate pH.[44] The loss of absorption at $\lambda_{max.}$ 335 nm with time at 25 °C (±0.5 °C, constant-temperature water-bath) was recorded (Figure 1). First-order rate constants are given in Table 2. At pH 7.00 and 7.94, buffer salts

TABLE 2

First-order rate constants (*k*) for solvolysis of *N*-acetyl-N^1-nitrosotryptophan methyl ester in buffered aqueous methanol at 25 °C

Measured pH of buffer	2.16	2.84	3.65	4.41	5.33	6.08	7.00	7.94
$k/min^{-1} \times 10^4$	370	300	240	120	38	11	(3)	(2)

gradually precipitated, and the values recorded are approximate. The other values are ±5%.

(b) *Isolation studies with n-butanol*. *N*-Acetyl-N^1-nitrosotryptophan methyl ester (108 mg) was dissolved in n-butanol (7 ml, anhydrous) and slowly distilled (N_2 flush) over 45 min until 5.5 ml of distillate had been collected in an ice-cooled receiver. The residue in the distillation flask was diluted with ether, filtered, and evaporated to dryness. Crystallisation from ethyl acetate–hexane gave crystals (60 mg, 62%) of *N*-acetyltryptophan methyl ester.

Mixed g.l.c. (2-m × ⅛-in 15% polypropylene glycol on Chromosorb W, 35 °C, Perkin-Elmer F11) showed that the distillate contained a component indistinguishable from n-butyl nitrite, the yield of which was determined spectrophotometrically (ε_{357} 79, 33%).

Reaction with Diphenylamine.—Diphenylamine (787 mg) and *N*-acetyl-N^1-nitrosotryptophan methyl ester (150 mg) were dried (P_2O_5, 24 h) and heated together (65 °C, 3 h). Preparative t.l.c. ($PhCH_3$–petroleum 12 : 5) gave three products: (i) diphenylamine, R_F 0.69; (ii) *N*-nitrosodiphenylamine, R_F 0.48, identified by u.v. spectroscopy and mixed t.l.c. (yield, calculated spectrophotometrically, 74%); and (iii) *N*-acetyltryptophan methyl ester (R_F 0.10), the identity of which was confirmed by its u.v. spectrum and mixed t.l.c.

A similar reaction under nitrogen gave 67% of *N*-nitrosodiphenylamine.

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