Reaction of Acidified Nitrite Solutions with Peptide Derivatives: Evidence for Nitrosamine and Thionitrite Formation from ^{15}N N.m.r. Studies

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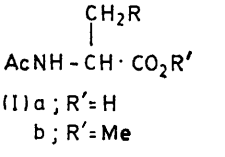
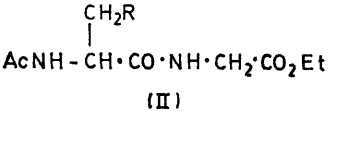
^{15}N N.m.r. chemical shift data are reported for a variety of nitroso-compounds. ^{15}N N.m.r. spectroscopy is evaluated as a method for detecting the formation of *N*-nitroso- and *S*-nitroso-compounds in the reactions of aqueous acidic solutions of sodium [^{15}N]nitrite with model peptides.

EXPERIMENTS with animals have shown that certain nitrosamines have carcinogenic activity.[1] Although nitrosamines have not been proved to cause cancer in man, it is of interest to ascertain the extent to which such compounds can arise under natural, mildly acidic, conditions (such as obtain in the stomach [2]) from traces of nitrite ingested with nitrogenous organic compounds (review [3]). The nitrite may arise naturally (*e.g.* in spinach [4]) or may be an additive (*e.g.* in certain processed meat products [5]). Protein forms a significant part of ingested nitrogen compounds, and we have therefore examined the reaction of nitrite with peptide models of types (I) and (II). The chemical approach has been described for tryptophan derivatives: [6] here we outline the use of ^{15}N n.m.r. spectroscopy to explore the reaction with $Na^{15}NO_2$.



(I) a ; R′ = H
b ; R′ = Me



(II)

^{15}N N.m.r. spectroscopy [7] has certain disadvantages: it is insensitive (*ca.* 0.1% of the sensitivity of ^{1}H n.m.r. spectroscopy even with samples enriched to 100% in ^{15}N)-so that, despite the use of pulsed Fourier methods, concentrated solutions are still desirable. Long accumulation times are frequently required, so that the reaction mixtures being studied may change significantly in

[1] J. M. Barnes and P. Magee, *Brit. J. Ind. Medicine*, 1954, **11**, 167; P. N. Magee, *Biochem. J.*, 1956, **64**, 676.
[2] J. Grieve, *Brit. J. Surg.*, 1961, **39**, 189.
[3] W. Lijinsky and S. S. Epstein, *Nature*, 1970, **225**, 21.
[4] W. E. J. Phillips, *Agric. and Food Chem.*, 1968, **16**, 88.
[5] For a recent review see Proceedings of the International Symposium on Nitrite in Meat Products, Zeist, Holland, 1973, Centre for Agricultural Publishing and Documentation, Wageningen, 1974.
[6] R. Bonnett and R. Holleyhead, *J.C.S. Perkin I*, 1974, 962; 'International Agency for Research on Cancer, Scientific Publications,' 1975, vol. 9, p. 107.
[7] E. W. Randall in 'Nitrogen N.m.r.,' eds. M. Witanowski and G. A. Webb, Plenum Press, London and New York, 1973, ch. 2, p. 41.

composition during the accumulation. The final spectrum therefore is effectively a montage of peaks over the accumulation period in which the intensity of a given signal is governed by the lifetime of the species giving it. In the absence of an CIDNP effects, short-lived (say, with $t_{\frac{1}{2}} < 0.5$ h) species will therefore not be detected. One advantage of ^{15}N studies, however, is that ^{15}N n.m.r. signals are narrow (operationally *ca.* 1 Hz or less). This allows accurate shift values to be obtained, whereas in ^{14}N n.m.r. spectroscopy the signals are often very broad (*ca.* 200 Hz or more). The *in situ* labelling approach has two additional advantages over other methods in the present context: (i) the label can be placed at the site of interest (the nitrite) and only the labelled nitrogens (nitrite and its long lived products) are detected; and (ii) in some cases (*e.g.* the thionitrites) the reaction products are difficult to isolate from aqueous solution, but are nevertheless sufficiently stable to give ^{15}N spectra *in situ*.

The chemical shifts of some model nitroso-compounds prepared for comparison purposes from sodium [^{15}N]-nitrite are collected in Table 1. The samples were examined either as neat liquids or as solutions (generally 0.1—1M). In confirmation of correlations based on ^{14}N spectra,[8] the nitroso-group in nitrosamines resonates at δ *ca.* 500—550 p.p.m., whereas aryl *C*-nitroso- and *S*-nitroso-signals are both at considerably lower field (δ *ca.* 800 p.p.m.). The signals are all sharp ($W_{\frac{1}{2}} < ca.$ 5Hz).

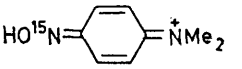
TABLE 1

^{15}N Chemical shifts for some nitroso-compounds [δ in p.p.m. relative to $^{15}NH_4^+$ as nitrate [a] (downfield positive)]

Compound	Solvent	M	δ
N-Nitroso			
PhEtN·^{15}NO	(Neat)		525.8, 522.7
MeN(^{15}NO)·CO·NH_2	EtOH	0.7	545.0
N-(^{15}NO)indoline	$CDCl_3$	1.0	513.4
N(1)-(^{15}NO)-Ac-Trp-OMe	$[CH_2]_4O$	0.07	547.1, 532.1 [b]
N-(^{15}NO)-3-Me-indole	C_6H_6	11.4	544, 528.8
N-(^{15}NO)carbazole	$CDCl_3$	0.2	539 [c]
C-Nitroso			
O^{15}N·C_6H_4·NMe_2-*p*	$CDCl_3$	0.4	783
	N-HCl	0.2	396.9
1,4-Benzoquinone mono-oxime	$(CD_3)_2CO$	0.2	No signal observed
	N-HCl	0.16	390
S-Nitroso			
Ph_3C·S^{15}NO	$CDCl_3$	0.5	785.2 [d]

[a] 5M-$^{15}NH_4NO_3$ (95% enriched) in 2M-HNO_3. The reasons for the adoption of this reference are given in J. M. Briggs and E. W. Randall, *Mol. Phys.*, 1973, **26**, 699. [b] Recorded by Dr. G. E. Hawkes and Miss P. Nicholaidou, whom we thank. [c] This spectrum had an unusually low signal-to-noise ratio. [d] Impurity peaks at δ 559.6 and 372.2 p.p.m.

Certain of the observations call for special comment. *NN*-Dimethyl-*p*-nitrosoaniline showed δ 783 in chloroform but 396.9 p.p.m. in aqueous acid. The shift is ascribed to *O*-protonation which gives the mesomeric cation (III). 1,4-Benzoquinone mono-oxime (*p*-nitrosophenol) did not show a signal in chloroform at 35 °C (possibly because of an awkward rate of tautomerisation



(III)

giving very broad lines which are difficult to detect). In dilute acid, however, a signal appeared at δ 390 p.p.m., *i.e.* at a similar position to that observed for (III).

For three *N*-nitroso-derivatives in Table 1, two peaks were observed in the nitrosamine region for non-hydroxylic solvents. These cases are presumed to be favourable for the detection of geometrical isomerism of the $N^+{:}N{\cdot}O^-$ system. The relative intensities of these peaks for the case of PhEtN–^{15}NO support this conclusion, since the ratio is similar to values found for the isomers by other techniques (*e.g.* ^1H and ^{13}C n.m.r. methods [9]). The magnitude of the shift difference in this case (3.1 p.p.m.) is similar to the differences noted between *cis*- and *trans*-forms of amides (*ca.* 2 p.p.m.),[10] including *N*-formylproline (1.6 p.p.m.).[10,11] In the *N*-nitrosoindole systems, however, the difference observed (*ca.* 15 p.p.m.) is considerably larger. The *N*-nitrosoindoles were rather unstable,[12] especially in alcoholic solution. Thus *N*-acetyl-*N*(1)-nitrosotryptophan methyl ester in [$^2H_4$]methanol (0.35M) was shown (t.l.c.) to be contaminated with the denitrosated compound after 4 h in the spectrometer at *ca.* 35 °C: we suppose that methyl nitrite is formed by transnitrosation, and may account for extra peaks (due to –O–^{15}N=O) in the δ 500—530 p.p.m. region in some runs. The thionitrite examined (Ph_3C·SNO) was also observed to be rather reactive. On re-running the spectrum of the solution in chloroform the peak at δ 785.2 p.p.m. ascribed to –S^{15}NO decreased, and signals at δ 559.6 and 372.2 p.p.m. were observed. These are attributed to reaction products, which deserve further study.

In the absence of chemical reaction, the δ values of nitrosamines appear to be rather insensitive to change of solvent. Thus for diphenylnitrosamine δ increases from 530.1 p.p.m. in the highly polar AcOH to 533.4 in the non-polar CCl_4 (EtOH, 530.7; $CHCl_3$, 531.4; $[CH_2]_4O$, 532.7; Et_2O, 533.4; δ values refer to 0.7M-solutions at 25 °C).

In an attempt to detect nitrosation *in situ*, peptide models [(I) and (II)] at concentrations of *ca.* 0.1—1.0M were treated with sodium [^{15}N] nitrite (95% enrichment, 1—3 mol. equiv.) at pH *ca.* 3, and ^{15}N n.m.r. spectra were accumulated at once. The results are summarised in Table 2. In the absence of peptide substrate, such solutions showed a signal at *ca.* δ 588 p.p.m. ascribed to

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[9] P. S. Pregosin and E. W. Randall, *Chem. Comm.*, 1971, 399.

[10] D. G. Gillies and E. W. Randall, 'Progress in N.M.R. Spectroscopy,' eds. J. W. Emsley, J. Feeney, and L. H. Sutcliffe, Pergamon, Oxford, 1971, ch. 3, p. 141.

[11] E. W. Randall and A. I. White, unpublished results; A. I. White, Ph.D. Thesis, London University, 1972.

[12] H. F. Hodson and G. F. Smith, *J. Chem. Soc.*, 1957, 3546.

nitrite [13] or, at lower pH values, a signal * at δ *ca.* 354 p.p.m.

With *N*-acetylglycylglycine and *N*-acetylmethionine no new signal was detected under the mild conditions employed here. The natural peptide bond seems to be rather sluggish in its reaction with acidified nitrite solutions. Although the hydrolysis of certain glycyl peptides is reported [15] to be catalysed by aqueous nitrous acid, this is not the reagent of choice for the preparation of nitrosamides.[16] Under our conditions no new ninhydrin-positive product could be detected by paper chromatography after the accumulation. However, arguments based on the absence of a signal in the ^{15}N n.m.r. spectrum must be treated with caution. Thus, although a reaction [17] between *N*-acetyltyrosine and acidified nitrite solution certainly occurs (three products detected by t.l.c.) the spectrum of the reaction mixture showed no new signal, possibly because of an inconvenient exchange rate (*cf.* nitrosophenol, above).

On the basis of the appearance of new signals in the δ 500—550 p.p.m. region, *N*-nitrosation was detected in solution for *N*-acetyltryptophan, *N*-acetyltryptophylglycine ethyl ester, and prolylglycine. In each case two sharp signals were observed. The thiol derivatives gave coloured solutions, the ^{15}N n.m.r. spectra of which showed new signals at low field. Thus *N*-acetylcysteine produced a red solution which gave a signal at δ 748.3 p.p.m. ascribed to the thionitrite (Ia) (R = $S^{15}NO$). For comparison, trityl [^{15}N]thionitrite (which is green) showed δ 785.2 p.p.m. (Table 1), and ^{14}N studies have provided chemical shifts in the same region (*e.g.*[18] CF_3SNO, δ 694; EtSNO, δ 764 p.p.m.). Similarly reaction of *N*-acetylpenicillamine gave a green solution, δ 812 p.p.m. ascribed to the tertiary thionitrite [AcNH·$CH(CMe_2{\cdot}S^{15}NO){\cdot}CO_2H$]. Treatment of thioacetic acid (1M-solution; equimolar nitrite) in the same way gave a red solution with δ 738.9 p.p.m., assigned to $O^{15}NS{\cdot}CH_2{\cdot}CO_2H$.

These results suggest that nitrosation at the ring nitrogen atom of tryptophan and *N*-terminal proline, and at the thiol group of cysteine, is possible when polypeptides containing these residues are treated with nitrite in aqueous media under mildly acidic conditions.

The ^{15}N n.m.r. approach, in spite of its limitations, thus appears to have considerable potential in the current intensive study of nitrosation and, probably, in the broader context of chemical and biochemical reactions of nitrogeneous compounds.

TABLE 2

^{15}N Chemical shifts of peptide–[^{15}N]nitrite mixtures

Substance	Solvent	Concentration (M) of peptide	Concentration (M) of $Na^{15}NO_2$	Accumulation time (h)	Accumulation scans (×10^{-3})	δ [a]
Ac-Gly-Gly	Acetate buffer,[b] pH *ca.* 3.5	0.8	1.0	10	220	(353)
Ac-Met (Ia; R = $CH_2{\cdot}SMe$)	Acetate buffer,[b] pH *ca.* 3.5	1.0	1.0	2	39	(355)
Ac-Tyr (Ia; R = *p*-$HO{\cdot}C_6H_4$)	Acetate buffer,[b,c] pH *ca.* 3.5	0.3	1.0	5	110	(587), (355)
Ac-Trp (Ia; R = indol-3-yl)	Acetate buffer,[b] pH *ca.* 3.5	0.1	0.6	5	110	(588), 540.8, 526.1
Ac-Trp-Gly-OEt (II; R = indol-3-yl)	Acetate buffer,[b,c] pH *ca.* 3.5	0.05	0.3	15	330	(588), 528.7, 499.3
Pro-Gly	H_2O, pH *ca.* 0	0.3	1.0	3	66	511.3, 504.7, (354)
Ac-Cys (Ia; R = SH)	aq. HOAc, pH *ca.* 2.5	1.0	1.0	1	25	748.3, (355)
$Me_2C(SH){\cdot}CH(NHAc){\cdot}CO_2H$	H_2O	0.4	0.4	16	102	812

[a] The δ values attributed to nitrite (587) and the products of its acidification (*ca.* 353) are given in parentheses. [b] 0.2N-HOAc–0.2M-NaOAc (4 : 1). [c] With added ethanol.

* The exact position of this is strongly dependent on pH and the signal might reasonably be attributed to an equilibrium involving molecular nitrous acid. We have some evidence, however, that the equilibrium involves nitrate, which is known to arise by disproportionation of nitrous acid. This disproportionation would be expected to be significant at the concentrations and temperatures employed here.[14] The complex reactions in acidified nitrite solutions (referred to as 'aqueous nitrous acid' in the present paper) are being studied further.

EXPERIMENTAL

^{15}N N.m.r. spectra were measured in the Fourier mode on a Bruker HFX 90 spectrometer at 9.12 MHz (2.14 tesla). Free induction decays were accumulated in a Fabritek 1074 CAT instrument employing 4 096 points. Fourier transformation on a Digital PDP8I computer gave magnitude spectra in 2 048 points. The spectrometer employed a time-shared ^2H lock (13.82 MHz), derived from D_2O contained in a 5 mm tube concentric with the 10 mm sample tube. Calibration was achieved by means of an acidified ammonium nitrate sample (5M, in 2M-nitric acid) contained in a 5 mm tube with a D_2O lock sample in the 10 mm tube. The shift difference under these conditions between the NH_4^+ and NO_3^- signals is 354.1 p.p.m. No corrections were made for susceptibility effects.

The labelled *N*-nitroso-derivatives of common secondary

[13] B. M. Schmidt, L. C. Brown, and D. H. Williams, *J. Mol. Spectroscopy*, 1958, **2**, 551.
[14] T. W. J. Taylor, E. W. Wignall, and J. F. Cowley, *J. Chem. Soc.*, 1927, 1923; T. C. Matts and P. Moore, *J. Chem. Soc.* (*A*), 1971, 1632.
[15] P. Cristol, C. Benezech, and A. C. Paulet, *Bull. Soc. chim. France*, 1954, 684; M. Viscontini, *Helv. Chim. Acta*, 1946, **29**, 1491.
[16] E. H. White, *J. Amer. Chem. Soc.*, 1955, **77**, 6008.
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[18] L. O. Andersson, J. B. Mason, and W. van Bronswijk, *J. Chem. Soc.* (*A*), 1970, 296.

amines were prepared from $Na^{15}NO_2$ (95% enrichment) by conventional procedures [19] but with due care to avoid skin contact and inhalation. Thus a solution of $Na^{15}NO_2$ [137 mg in water (0.5 ml)] was added dropwise to indoline (freshly distilled; 0.24 g) in 5N-hydrochloric acid (2 ml) stirred at 0 °C. After 30 min the yellow precipitate was filtered off and crystallised from benzene–hexane to give yellow crystals (184 mg, 62%) of 1-[^{15}N]nitrosoindoline, m.p. 80° (lit.,[20] 83—84°). Nitrosamine residues were destroyed with HBr–HOAc in a fume cupboard.

The less common compounds were prepared as indicated: 3-methyl-1-nitrosoindole,[12] *N*-acetyl-*N*(1)-nitrosotryptophan,[6] *N*-acetyl-*N*(1)-nitrosotryptophylglycine ethyl ester,[6] 1-nitrosocarbazole,[21] and trityl thionitrite.[22]

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