Spectroscopic Evidence for an Aryldiazonium Salt Formed via the Action of Sodium Nitrite on *N*-Acetyltyrosine and Tyrosyl Groups of Bovine Serum Albumin

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Various spectroscopic methods (^1H, ^{13}C, and ^{15}N NMR; infrared and mass spectrometry) have been used to provide evidence for the formation of an aryldiazonium salt from the action of sodium nitrite on *N*-acetyltyrosine (*N*-AcTyr). Mild pH conditions (4–5.6) were choosen to be compatible with those prevailing in meat products. Mechanisms for the selective synthesis of this compound by transnitrosation of *S*-nitrosocysteine or involving direct reaction of nitrogen monoxide are shown. An homologous aryldiazonium salt was detected in solutions containing bovine serum albumin (BSA) by the comparative use of ^{15}N NMR. The work has been done in a model system and attempts to test reactions that possibly occur in the more complex cured meat system.

Sodium nitrite is an additive used in the curing process of meat products providing color stability, antibacterial protection, and a characteristic flavor. Since Ender and al. (1964) discovered the possible formation of carcinogenic byproducts from nitrite, the fate of this additive in food has been the object of many investigations. It is known that an important decrease of measurable free nitrite during storage takes place and that a substantial amount of nitrite nitrogen can be recovered in a bond form in various meat constituents. Cassens et al. (1977) indeed showed that 20–30% of the nitrite added to a meat product could be bound to proteins. Tyrosine seems to play an important role in this binding since 10–20% of the nitrite incorporated in a protein could be identified as 3-nitrotyrosine via amino acid analysis (Woolford et al., 1976). However, the drastic conditions inherent to this method prohibit detection of labile products.

On the basis of chemical and colorimetric tests, Philpot and Small (1938a) proposed that an aryldiazonium salt is one of the products of the reaction between sodium nitrite and tyrosyl residues. An analogous observation was made on a purified protein by Philpot and Small (1938b). Nevertheless, the formation of such a derivative was contested by Bonnett and Nicolaidou (1977) since attempts to purify this product or to identify it via spectroscopic analysis were unsuccessful. In an experiment involving ^{15}N NMR spectroscopy, Bonnett et al. (1975) observed no peak resulting from a nitrite–tyrosine interaction and suggested that this was due to a rapid exchange between several products. On the other hand, in a later study, Bonnett and Nicolaidou (1979) brought evidence for the formation of a nitro derivative of *N*-acetyltyrosine (*N*-AcTyr) and another (major) product tentatively identified as the homologous nitroso derivative.

This paper further investigates the action of sodium nitrite on *N*-AcTyr and tyrosyl residues of bovine serum albumin (BSA) by spectroscopy, particularly ^{15}N NMR, which is well adapted to trace $Na^{15}NO_2$ in solution. We present evidence for the formation of an aryldiazonium salt in addition to the well-known nitro derivatives.

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EXPERIMENTAL SECTION

Materials. The reagents were purchased from Sigma (*N*-acetyltyrosine, *N*-acetylcysteine, and bovine serum albumin fraction V of Cohn) or CEA, France (sodium nitrite, 95% ^{15}N).

Sample Preparation. The amino acid–sodium nitrite solutions were prepared from the dry reagents in various buffer solutions (pH 1.5, 4, or 5.6) at concentrations ranging from 0.1 to 0.4 M. The transnitrosation reaction was carried out by adding an equivalent amount of dry *N*-AcTyr to a solution of *S*-nitroso-*N*-acetylcysteine (0.2 M, pH 4). The reaction was followed by ^{15}N and ^{13}C NMR before and after *N*-AcTyr addition.

BSA was reacted with sodium nitrite in a molar ratio of nitrite to tyrosyl residues of 5 to 1.

The reaction of NO with the amino acids or with BSA was carried out by the standard procedure under nitrogen. NO was produced by reacting 25% nitric acid with copper.

Nuclear Magnetic Resonance. The NMR spectra were recorded in the pulsed Fourier transform mode on a Bruker WM 250 spectrometer. The conditions used for each nucleus studied were the following.

^{15}N: SW (sweep width) = 22 000 Hz; AT (acquisition time) = 0.36 s; PW (pulse width) = 40°; ν_0 = 25.349 MHz; broad-band decoupling, 2 W; 10 000–50 000 accumulations according to concentration; temperature, 298 K. Chemical shifts are reported relative to a solution of $CH_3{}^{15}NO_2$ in CD_3NO_2 placed in a 4-mm coaxial tube at the center of the 15-mm sample tube.

^{14}N: as for ^{15}N except that ν_0 = 18.068 MHz and 5000–10 000 accumulations according to concentration.

^{13}C: SW = 15 000 Hz; AT = 0.541 s; PW = 35°; ν_0 = 62.896 MHz; broad-band decoupling; 1000–10 000 accumulations according to concentration; temperature, 298 K. Chemical shifts were referenced to a solution of DMSO-d_6 (central peak 39.44 ppm from TMS) in a capillary tube coaxial to the 10-mm sample tube. The same solution was used for the field frequency lock.

^1H: SW = 3000 Hz; AT = 2.736 s; PW = 10°; ν_0 = 250 MHz; 200–500 accumulations; temperature, 298 K. The diazonium salt was dissolved in D_2O after lyophilization of the initial solution; chemical shifts are reported relative to TMS used as internal reference.

Infrared Spectroscopy. The IR spectrum of the aryldiazonium salt was recorded on a Grubb-Parson spectromaster, Model MK 3. The salt was lyophilized, resolubilized in acetone, and dried on a CaF_2 pastille. Thus, the spectrum was recorded between 4000 and 1000 cm^{-1}.

Mass Spectrometry. The mass spectrum was recorded on a double-focalization Varian Mat 112 spectrometer. The ionization energy was 70 eV, and the current in the filament was 1.5 mA. The main peaks observed for the diazonium salt were

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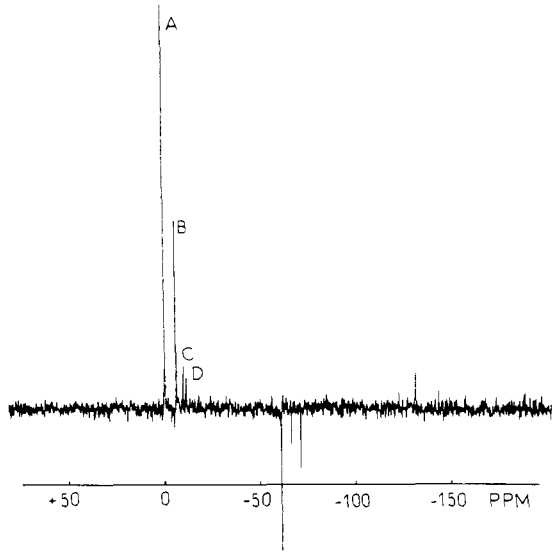


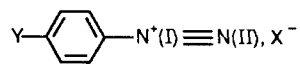
Figure 1. ^{15}N NMR spectrum of a stoichiometric mixture of *N*-acetyltyrosine and $Na^{15}NO_2$ (0.6 M): A = CH_3NO_2 reference; B = NO_3^-; C, D = nitrotyrosines. No other lines observed between +300 and −300 ppm.

m/z 250 (33, M^+), 209 (63), 152 (62), 135 (50), 106 (43), 105 (41), 77 (13), 74 (19), 60 (35), 44 (64), and 43 (base).

RESULTS AND DISCUSSION

N-Acetyltyrosine. *Action of $Na^{15}NO_2$ and NO.* ^{15}N NMR spectra of *N*-AcTyr–$Na^{15}NO_2$ mixtures show many lines; thus, they appear to be different from those previously obtained by Bonnett et al. (1975) in a similar experiment. Figure 1 displays a typical spectrum of such a mixture (0.6 M) after 24-h reaction, in which $Na^{15}NO_2$ has been completely used. Some of the lines may easily be assigned to mono- or dinitro-*N*-acetyltyrosine (≃−6 to −12 ppm) by comparison with commercial homologous *p*-cresol derivatives. Some nitrate (≃−5 to −6 ppm) is also detected, and when the reaction is incomplete, residual nitrite (≃+200 to +230 ppm according to pH) can be seen. Because it is in natural abundance, the nitrogen resonance of the *N*-acetyl group (≃−250 to −255 ppm) may also be observed only after sufficient accumulation. However, the assignment of the other peaks in the −50 to −150 ppm window is rather more difficult.

Interestingly, other groups (Duthaler et al., 1978; Korzeniowski et al., 1981; Olah and Grant, 1975; Casewit et al., 1982; Elofson et al., 1984) reported the detection of nitrogen resonances in this same region, when they analyzed the structure of aromatic diazonium salts via ^{15}N and ^{13}C NMR. Chemical shifts of −130 ppm ≦ δ(N(I)) ≦ −150 ppm and −30 ppm ≦ δ(N(II)) ≦ −70 ppm were reported for compounds of the type

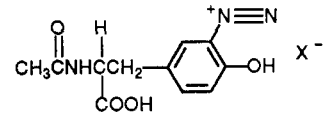


Thus, we considered the possibility of having obtained in our experiment a similar *N*-AcTyr derivative. However, to ascertain this we decided to synthetize this product selectively. The decomposition of nitrous acid may be written as follows:

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$

Assuming that NO was the reacting species leading to the aryldiazonium, we tested this hypothesis by adding it to a *N*-AcTyr solution (see the Experimental Section). The resulting major compound was initially analyzed by ^1H and ^{13}C NMR in the absence of ^{15}N labeling. The spectral characteristics of this derivative are given in Table I. The positions of the ^{13}C chemical shifts match the minority peaks obtained in equimolar *N*-AcTyr–$NaNO_2$ solutions at relatively high concentrations ($C \geqq 0.2$ M) in weakly acidic media (pH ≦ 5).

Comparison of the results given in Table I with the literature (Duthaler et al., 1978; Elofson et al., 1984) and in particular the presence of quaternary carbons at 94 and 168 ppm enable the following diazonium structure to be proposed:



The aromatic carbons were assigned by comparing the proton-decoupled or nondecoupled ^{13}C spectra and by using the data concerning additive parameters of substituants in benzene compounds and the rules relative to the $^nJ_{^{13}C-H}$ coupling constant.

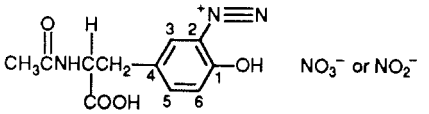
In order to confirm this hypothesis, a mass spectrometric and IR study was undertaken. The only means of obtaining a pure and relatively stable product was the lyophilization of the initial solution. The mass spectrum (Figure 2 and the Experimental Section) gives a molecular peak at *m/z* 250, in agreement with the diazonium structure proposed above.

The IR spectrum, compared to that of *N*-AcTyr, has an additional band to 2220 cm^{-1}, which can only reasonably be assigned to a diazonium-type structure. Any correlation with a band under 1000 cm^{-1} was impossible due to the strong absorption of CaF_2. Further evidence was provided by the ^1H and ^{14}N NMR analyses of lyophilized solutions resolubilized in D_2O. The ^1H NMR spectrum (Table I) shows three aromatic protons whose chemical shifts and coupling constants are compatible with the proposed structure (Korzeniowski et al., 1981). ^{14}N NMR gives two broad lines at −72 and −142 ppm, which may be assigned, respectively, to nitrogen N(II) and N(I) of the aryldiazonium.

The initial assumption that an aryldiazonium was formed in *N*-AcTyr–$NaNO_2$ solutions was thus confirmed. The appearance of a generally weak peak at −66 to −67 ppm in ^{15}N spectra (see Figure 1) was assigned to molecular nitrigen $^{15}N_2$ (Dreher et al., 1981). Since $^{15}N_2$ could be obtained only by decomposition of diazonium, our initial assumption was further supported by this observation.

Action of ^{15}NO: Labeling of the Diazonium by Transnitrosation. It was important to obtain similar results for ^{15}N NMR. But this nonquadrupolar nucleus of spin $^1/_2$ and low natural abundance and sensitivity entails the use of isotopic labeling. $NaNO_2$ is known to act rapidly on *N*-acetylcysteine (*N*-AcCys) to give the *S*-nitroso derivative, which is transformed spontaneously into *N*-acetylcystine accompanied by loss of NO (Bonnet et al., 1975; Bonnett and Nicolaidou, 1977, 1979). The *S*-nitroso-*N*-acetylcysteine derivative was prepared from stoichiometric quantities of $Na^{15}NO_2$ and *N*-AcCys, and the completion of the reaction (unique product and no residual $Na^{15}NO_2$) was verified by ^{13}C and ^{15}N NMR. The introduction of a stoichiometric amount of *N*-AcTyr leads, over a period of time (see the Experimental Section), to ^{15}N spectra showing that the *S*-nitroso-*N*-acetylcysteine derivative disappears (δ +388) and that well-defined lines appear at −6, −72, −132.8, and −255 ppm (see Figure 3). The line at −6 ppm may be assigned to NO_3^-, and that at −225 ppm corresponds to the reso-

Table I. NMR[a] Parameters of the Diazonium Salt



	C1	C2	C3	C4	C5	C6	CH_2	CH	CH_3	C=O	C(O)OH
$\delta(^{13}C)^b$	168.3	94.0	126.4	127.5	143.3	119.3	34.3	52.3	20.6	173.1	172.8
mult	st	st	dt	st	dt	dt	tt	dt	qt	st	st
$\delta(^1H)^c$			7.66		7.64	6.94	2.9 3.12	4.6	1.92		

		N-acetyltyrosine					BSA
		e	*f*	*g*	*h*	*i*	*j*
$\delta(^{15}N)^d$	N(I)	−132.8	−132.5	−132.6	−132.7	−148.4	−146.0
	N(II)	−72.0	−72.0	−72.1	−72.4	−71	−72.4
	NO_3^-	−6.0	−5.9	−6.1	−6.2		
	NO_2^-					+230.8	+227.8

[a] ^1H and ^{13}C chemical shifts are reported relative to TMS. [b] Lyophilized product dissolved in a pH 1.5 buffer. $J_{C_3-H} = 175$ Hz; $J_{C_5-H} = 160$ Hz; $J_{C_6-H} = 172$ Hz. [c] Lyophilized product dissolved in D_2O. $J_{H_5-H_6} = 9$ Hz; $J_{H_3-H_5} = 2$ Hz; $J_{C-H_AH_B} = 14$ Hz; $J_{H_A-CH} = 8.5$ Hz; $J_{H_B-CH} = 5.5$ Hz. [d] ^{15}N chemical shifts are reported relative to CH_3NO_2. Those placed to the right of the reference have negative values. [e] Product prepared by transnitrosation in a pH 4 buffer. [f-h] Product obtained from stoichiometric amounts of $Na^{15}NO_2$ and *N*-AcTyr in acetate buffer (pH 4) at concentrations of 0.2, 0.4, and 0.6 mol L^{-1}, respectively. [i] Product, obtained from $Na^{15}NO_2$ and *N*-AcTyr in relative proportion 50 to 1. [j] Product prepared from BSA (concentration 300 g L^{-1}) and $Na^{15}NO_2$ in an acetate buffer (pH 4). The relative proportion of tyrosyl fragments to nitrite is 1 to 5.

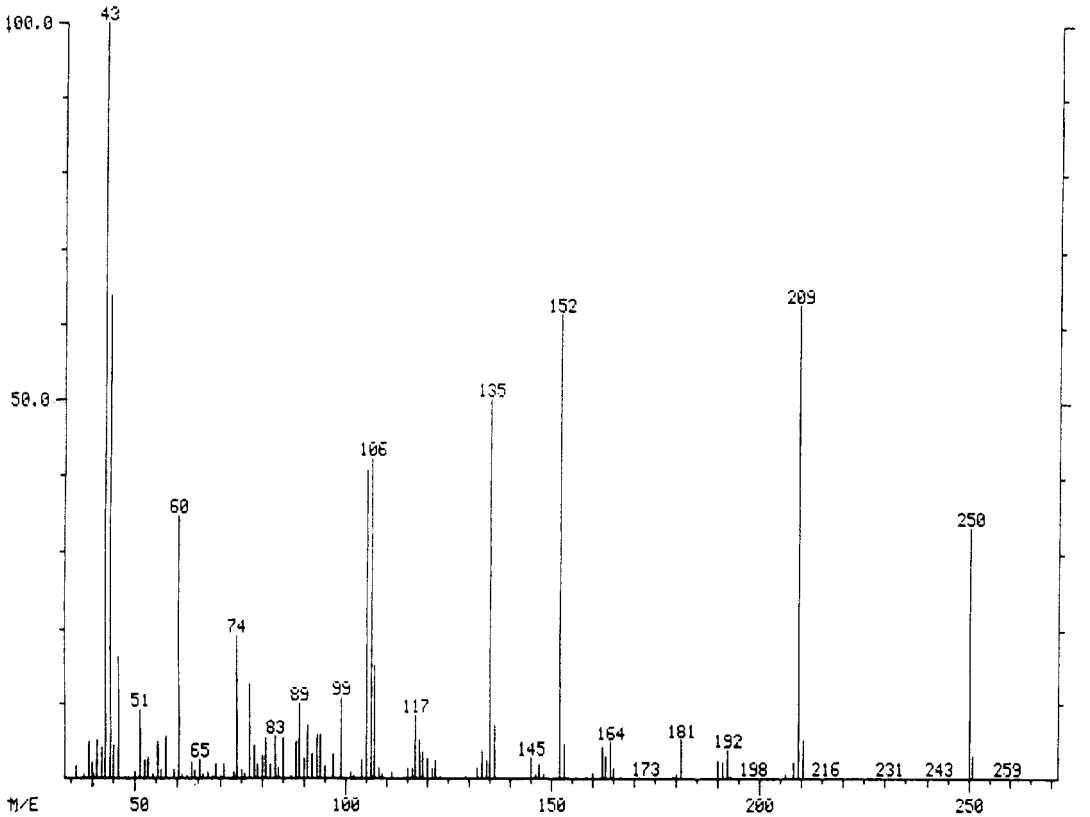
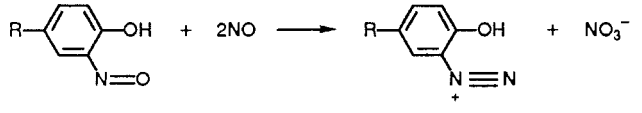


Figure 2. Mass spectrum of the diazonium salt derivative of *N*-acetyltyrosine.

nance of the *N*-acetyl groups present. The two remaining lines, similar to those obtained for ^{14}N and in agreement with the literature (Duthaler et al., 1978; Elofson et al., 1984), clearly indicate the presence of a diazonium salt derivative of *N*-AcTyr.

It should be noted that there is no evidence for the presence of a nitroso derivative or its oxime form. However, a nitroso derivative could react rapidly with NO to give the diazonium salt according to the reaction



Bovine Serum Albumin. BSA was reacted with labeled nitrite under conditions such that the relative concentration of nitrite and tyrosyl residues was close to the one in the *N*-AcTyr–nitrite mixtures (see the Experimental Section). The ^{15}N NMR spectra show three peaks (see Table I): a strong peak at +227 ppm corresponding to unreacted sodium nitrite and two broad bands centered at −72 and −145 to −146 ppm, respectively assigned to N(II) and N(I) nitrogens of the compound described above. The width of these two peaks may be attributed to the viscosity of the medium and the reduced mobility of this macromolecule as well as to the various electronic environments encountered by the aryldiazonium salt within

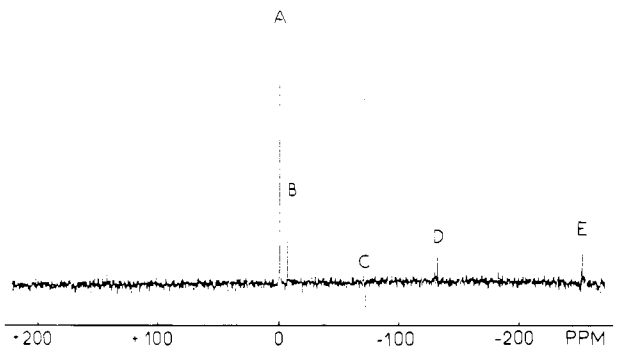


Figure 3. ^{15}N NMR spectrum of the diazonium salt prepared by transnitrosation of *N*-acetyltyrosine: A = CH_3NO_2 reference; B = NO_3^-; C = N(II) and D = N(I) of the diazonium salt; E = amide NH.

the BSA molecule. The nitrogen N(I) chemical shift differs slightly from the one usually observed for the *N*-AcTyr derivative. However, the same difference has been recorded for *N*-AcTyr reacted with a large excess of sodium nitrite (see Table I). This shift may depend on the effect to the counterion available in the solution of the electropositive nitrogen (N(I)).

CONCLUSION

The use of various spectroscopic methods has provided unambiguous proof for the formation of an aromatic diazonium salt during the reaction between sodium nitrite and *N*-AcTyr or the tyrosyl residues of a protein. This reaction takes place under pH conditions close to those generally found in meat products. We also presented evidence for a chemical mechanism leading to the formation of this aryldiazonium salt via transnitrosation of *S*-nitrosocysteine; this reaction gives a single product. Such a reaction probably also takes place during curing. Indeed, several essential conditions for its occurrence seem to be present during this process: the sulfhydryl groups are abundant in meat and they are easily transformed into *S*-nitroso groups, which are efficiently transfered onto tyrosyl residues (considering that three NO seem necessary to give one aryl diazonium). This reaction was also used as a convenient means for the isotopic labeling of a diazonium salt.

Work is currently under way to identify other compounds present in *N*-AcTyr–$NaNO_2$ solutions and particularly the degradation products of the diazonium salt.

ACKNOWLEDGMENT

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Registry No. *N*-AcTyr, 537-55-3; $NaNO_2$, 7632-00-0; NO, 10102-43-9; *S*-nitroso-*N*-acetylcysteine, 56577-02-7; *N*-AcTyr, diazonium derivative, 123963-75-7.

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