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N-Nitrosamides and Their Precursors in Food Systems. 2. Kinetics of the N-Nitrosation Reaction

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The N-nitrosation reaction between sodium nitride and N-substituted amides formed from the interaction of amines and fatty acids or triglycerides was studied as a function of pH and temperature. There was no apparent pH maximum for the reaction, *N*-nitrosamide formation increasing with increasing hydrogen ion concentration. The rates of N-nitrosation decreased rapidly as the pH increased and little reaction occurred above pH 3. A unit drop in pH from 2 to 1 increased the rate of N-nitrosation by a factor of 5–8 times. The rate constants for the reaction remained relatively constant over the pH range 1–3.5, supporting the nitrous acidium ion mechanism. When the temperature of the reaction was increased to 40 °C, the rate constants were almost double those determined at 30 °C, indicating an activation energy of approximately 10 kcal/mol. The rate constants again remained relatively constant as the initial amide and nitrite concentrations were varied between 20 and 40 mM.

There has probably been no topic in the past decade that has generated as much discussion and research as the presence of *N*-nitroso compounds in food systems. The majority of these studies has centered on volatile *N*-nitrosamines such as *N*-nitrosopyrrolidine and dimethylnitrosamine since this group is readily separated for analytical purposes (Issenberg, 1975). On the other hand, there has only been a limited number of studies on the occurrence of the relatively nonvolatile *N*-nitrosamides in food systems. This may be due in part to the instability of *N*-nitrosamides under neutral and alkaline conditions (Mirvish, 1971) or the lack of suitable methods for *N*-nitrosamide analysis (Mirvish, 1977). However, precursors of *N*-nitrosamides have been reported in certain foods. *N*-Methylguanidine has long been considered to occur naturally in various foods including fresh beef (Komarrow, 1929; Kapeller-Adler and Krael, 1930a) and fish (Kapeller-Adler and Krael, 1930b; Sasaki, 1938). However, a recent study by Kawabata et al. (1978) indicated that no appreciable amount or trace amounts of *N*-methylguanidine could be detected in fresh beef, chicken, and various fish and shellfish. These investigators concluded that the high values reported by the earlier workers were, in fact, due to inadequacies in the experimental procedures. Comparatively high concentrations of agmatine, a decarboxylated product of arginine, have been reported in fresh abalone and top-shell muscles (Kawabata et al., 1978). The presence of citrulline in watermelon (30 mg/kg wet weight) has also been reported by Wada (1930).

In the preceding paper, Kakuda and Gray (1980) reported the formation of N-substituted amides in model systems containing free amines and fatty acids or triglycerides. These systems were subjected to thermal stresses commonly encountered in the pan frying of bacon or oven roasting of pork. It was tentatively concluded that these compounds could possibly be present in cooked or processed foods and thus may represent another source of N-nitrosatable compounds available for reaction with nitrite in foods, or in vivo. In this paper, results of a study of the reaction between secondary amides and sodium nitrite are reported.

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EXPERIMENTAL SECTION

N-Nitrosation of N-Substituted Amides. (*i*) *Preparation of N-Nitroso-N-pentylpalmitamide (NOPP).* The N-nitrosation procedure was based on the method described by White (1955a) with a few modifications. A 3.25-g aliquot of recrystallized *N*-pentylpalmitamide (prepared as previously described) was dissolved in a solvent mixture containing glacial acetic acid (50 mL), acetic anhydride (50 mL), and chloroform (95 mL). The mixture was cooled in an ice bath and 15 g of sodium nitrite slowly added with stirring over a 4–5-h period. After reacting overnight at 4 °C, the mixture was carefully poured into ice–water. The $CHCl_3$ phase was collected and the water phase extracted with another 100-mL aliquot of $CHCl_3$. The pooled $CHCl_3$ was washed with water, 5% K_2CO_3 solution and again with water before vacuum evaporation to dryness. The crude *N*-nitrosamide was partially purified by precipitating unreacted amide in cold petroleum ether (4 °C) and vacuum filtering the cold mixture. The clear yellow filtrate was placed on a Supelcosil-ATF 061 column (Supelco, Inc., Bellefonte, PA) and eluted with 60 mL of petroleum ether.

(*ii*) *Preparation of N-Nitroso-N-methylpropionamide (NOMP).* A 25-g aliquot of *N*-methylpropionamide (Eastman Kodak Co., Rochester, NY) was dissolved in 120 mL of glacial acetic acid and 138 mL of acetic anhydride and cooled to 0 °C. Solid $NaNO_2$ (61 g) was added slowly to this mixture over a 4-h period. After allowing the

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mixture to react overnight at 4 °C, the N-nitrosamide was extracted with $CHCl_3$. The $CHCl_3$ extract was washed successively with water, 5% K_2CO_3, and again with water. After drying and removal of $CHCl_3$ by vacuum evaporation, N-nitroso-N-methylpropionamide was purified by two vacuum distillations. The distillation was conducted at 40 °C (15.0 mmHg) and distillates collected in an iced receiving flask. The first and final 5–10 mL of distillate were discarded during each distillation.

Quantitative Study of the N-Nitrosation Reaction. *Stock Buffer Solution.* A 0.05 M H_3PO_4 solution was adjusted to the desired pH with 70% $HClO_4$ or 1 M NaOH.

Acetonitrile/Acetic Acid Buffer. A mixture (1:1) of acetonitrile and 0.2 M acetic acid was adjusted to pH 4.0 with NaOH. This buffer solution was used to dilute aliquots of N-nitroso-N-methylpropionamide taken from the reaction mixture during a kinetic assay. A 0.2 mM solution of N-nitrosamide in this solvent showed no change in absorbance at 240 nm for over 1 h at 25 °C.

Stock Amide Solution. A 1.089-g sample of N-methylpropionamide was dissolved in 10 mL of stock buffer solution.

Stock Nitrite Solution. $NaNO_2$ (0.5 g) and 0.34 mL of 85% H_3PO_4 were dissolved in 80 mL of water. The pH of the solution was adjusted with 70% $HClO_4$ or 1 M NaOH and then diluted to 100 mL. All nitrite solutions were prepared just prior to each kinetic assay. The exact nitrite concentration was determined by the Griess reaction (AOAC, 1970).

Assay Procedure. Each kinetic assay was prepared in triplicate using 25-mL volumetric flasks. The volume of stock $NaNO_2$ solution required to give a 20 or 40 mM solution in 25 mL was calculated and added to each flask. Buffer solution was added to each flask, allowing space for 0.4 or 0.8 mL of stock amide solution. The final amide concentration was 20 or 40 mM, respectively. The nitrite–buffer and stock amide solutions were preincubated at the reaction temperature for 5 min. The reaction was initiated by pipetting the amide into the nitrite–buffer mixture, adjusting the volume to 25 mL if necessary, and mixing throughly. A nitrite blank was prepared by diluting the same volume of stock $NaNO_2$ solution to 25 mL with buffer and incubating this flask along with the samples. The reaction was stopped by diluting 5.0 mL of the reaction mixture to 25 mL with acetontrile/acetic acid buffer. The absorbance of each sample was determined at 240 nm using the incubated $NaNO_2$ solution (5.0 mL diluted to 25.0 mL with acetonitrile/acetic acid) as a blank. At the beginning and end of each kinetic assay, the $NaNO_2$ blank was analyzed by the Griess reagent. The nitrite concentration decreased with increasing incubation time because of nitrous acid decomposition which is especially severe at high acidities. These changes in nitrite concentration were usually less than 5% over the pH range studied. For calculation purposes, an average value was used.

The pH of the reaction mixture was measured at the completion of each kinetic assay. All UV measurements were taken at ambient temperature.

Calculations. The pH-independent third-order rate constants for N-nitrosation of N-methylpropionamide were calculated on the basis of the nitrous acidium ion reaction derived by Mirvish (1971) for N-methylurea:

$$\text{rate} = k_1[\text{amide}][HNO_2][H^+]$$

The second-order rate equation (Frost and Pearson, 1961) was used to calculate $k_1[H^+]$ and hence k_1. The stoichiometric amide concentration was used at all pH values while the nitrous acid concentration was calculated from the averaged sodium nitrite concentration using the Henderson–Hasselbach equation to correct for ionization. A value of 3.36 was used for the pk_a of nitrous acid.

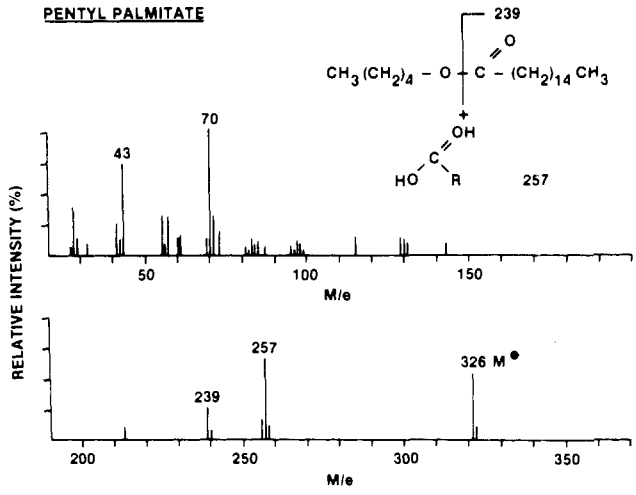


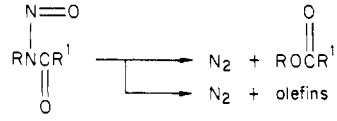
Figure 1. Mass spectrum of pentyl palmitate, a breakdown product of N-nitrosopentylpalmitamide.

Analytical Procedures. The purity of N-methylpropionamide, N-pentylpalmitamide, NOMP, and NOPP was determined by GLC analysis. All compounds were greater than 99% pure. Mass spectra of NOPP and NOMP were obtained as previously described (Kakuda and Gray, 1980).

Ultraviolet and visible spectra of NOMP were recorded on a Unicam SP800 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian 60 MH_Z spectrometer. Adjustments of pH were made with a Radiometer PHM 64 pH meter fitted with a GK 2301C glass electrode.

RESULTS AND DISCUSSION

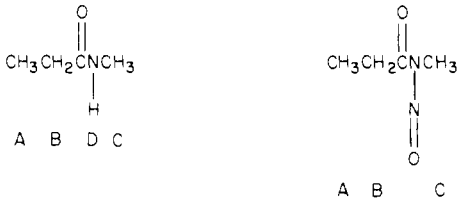
Analysis of N-Nitrosamides. N-Pentylpalmitamide and N-methylpropionamide were N-nitrosated using essentially the method of White (1955a). These amides were chosen as examples of long- and short-chain N-substituted amides, respectively. The mass spectrum of NOPP detected only the corresponding ester produced by thermal elimination of nitrogen. White (1955b) reported the formation of ester and olefins occurred by the following scheme:



The compound detected by GLC–MS analysis of NOPP corresponded to pentyl palmitate (Figure 1), the major ions present being m/e 70, 43, 257, 326 (M^+), and 239. Similarly, only the corresponding ester was detected for NOMP.

Further analyses of the synthesized NOMP were conducted using ultraviolet and visible spectrophotometry and NMR. The ultraviolet and visible spectra of NOMP in 0.1 M acetate buffer (pH 4.0) showed an intense maximum at 237 nm and two weaker bands at 421 and 403 nm. The extinction coefficient at 237 nm and 25 °C was 8.97×10^3 M^{-1} cm^{-1}. In acetonitrile/acetic acid buffer, an extinction coefficient of 7.7×10^3 M^{-1} cm^{-1} was obtained at 240 nm and 25 °C. The NMR spectra of NMP and NOMP were determined on a 60-MHz spectrometer and results were expressed in parts per million (ppm) relative to tetramethylsilyl Table I). The methyl and methylene hydrogens absorb at 1.25 and 3.20 ppm, respectively. The methyl group adjacent to the nitorgen absorbs at 3.01 ppm.

Table I. NMR Data for *N*-Methylpropionamide and *N*-Nitrosomethylpropionamide[a]



A, 1.13 ppm, terminal CH_3 (triplet)	A, 1.25 ppm, triplet
B, 2.30 ppm, quartet	B, 3.20 ppm, quartet
C, 2.75 ppm, doublet	C, 3.01 ppm, singlet
D, 8.16 ppm, broad singlet NH	

[a] Chemical shifts are expressed in ppm relative to tetramethylsilyl.

Table II. Third-Order Rate Constants for the N-Nitrosation of *N*-Methylurea in 0.05 M Phosphate Buffer at 25 °C

[nitrite],[a] mM	[*N*-methylurea], mM	[*N*-nitrosomethylurea], mM	pH	time, min	k_1, M^{-2} min^{-1}
10.6	10	2.03	2.01	5	518
3.8	10	0.97	2.02	5	685
5.3	10	1.08	2.03	5	545

[a] Brackets: molar concentration.

No amide hydrogen absorbance was detected for the *N*-nitroso compound.

Kinetic Study of *N*-Nitrosation Reaction. The reliability and accuracy of our experimental procedure was tested by nitrosating *N*-methylurea (Eastman Kodak Co.) and comparing the rate constants to those reported by Mirvish (1971). The procedure and calculations were identical with the NOMP experiments. The N-nitrosation of *N*-methylurea was carried out at 25 °C in 0.05 M phosphate buffer at pH 2.0. The rate constants ranged from 518 to 685 M^{-2} min^{-1} (Table II). These values agreed reasonably well with the published results (590–850 M^{-2} min^{-1}, pH 2.0, 0.05 M citrate buffer, 25 °C) of Mirvish (1971).

The effect of pH on the rates of N-nitrosation of *N*-methylpropionamide was studied at 30 and 40 °C (Figure 2). There was no apparent pH maximum, just a rapid rise in NOMP formation with increasing hydrogen ion concentration. The rates dropped off rapidly as the pH increased and showed very little reaction above pH 3.0. These trends agree with those reported by Mirvish (1971) for the N-nitrosation of alkylureas, alkylurethanes, and alkylguanidines. Mirvish (1971) reported that the initial N-nitrosation rate was proportional to *N*-methylurea and nitrite concentrations at pH 2 and increased about 10-fold for each 1-unit drop in pH, i.e., a 10-fold increase in hydrogen ion concentration, between pH 1 and 2. In this study, the rate of N-nitrosation of *N*-methylpropionamide increased by a factor of 5–8 times as the pH of the reaction medium was lowered from 2 to 1.

The third-order rate constants were calculated on the basis of the nitrous acidium ion $(H_2NO_2)^+$ reaction. The stoichiometric amide concentration was used for all rate calculations since the amide remained essentially unionized even at low pH values. The nitrous acid concentration, however, was adjusted for ionization. The resulting k_1 values therefore represent the pH independent third-order rate constants.

The rate constants for the N-nitrosation of *N*-methylpropionamide at 30 °C are tabulated in Table III as a function of pH and initial amide and nitrite concentrations. The k_1 values remained relatively constant from pH 1 to 3.5 supporting the acidium ion mechanism. The rate constants ranged from 0.44 to 0.78 M^{-2} min^{-1} with an overall mean of 0.66 ± 0.09 M^{-2} min^{-1} (mean SD). At any given pH, the calculated rate constants remained relatively unchanged despite a change in initial amide and nitrite concentrations that varied between 20 and 40 mM. The k_1 value at pH 1.96 was 0.69 ± 0.05 M^{-2} min^{-1}.

When the temperature was raised to 40 °C, the rate constants were almost double those determined at 30 °C. This is indicative of an activation energy of approximately

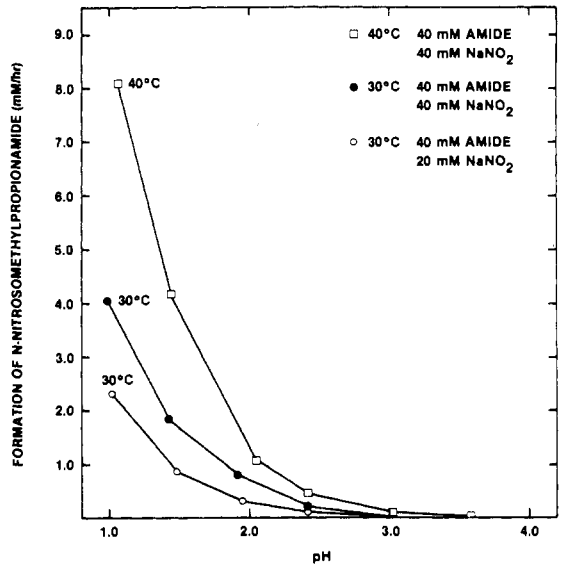


Figure 2. The effect of pH and temperature on the N-nitrosation of *N*-methylpropionamide.

Table III. Third-Order pH-Independent Rate Constants for the N-Nitrosation of *N*-Methylpropionamide at 30 °C

pH	k_1, M^{-2} min^{-1}	[*N*-nitrosamide], mM	[amide],[a] mM	[nitrite],[a] mM	time, min
0.88	0.75	0.548	40	28.3	5
0.99	0.44	0.338	40	39.6	5
1.02	0.50	0.193	20	20.2	5
0.99	0.58	0.208	20	35.8	5
1.42	0.52	0.154	40	38.9	5
1.48	0.56	0.148	40	20.7	10
1.37	0.54	0.188	20	40.9	10
1.47	0.50	0.078	20	22.6	10
1.47	0.52	0.136	20	39.0	10
1.92	0.72	0.208	40	42.4	15
1.94	0.67	0.221	40	18.0	40
1.99	0.64	0.299	40	18.9	60
1.96	0.69	0.086	20	39.9	15
1.99	0.70	0.154	20	18.9	60
2.44	0.74	0.432	40	36.2	125
2.41	0.64	0.271	40	23.6	125
2.44	0.75	0.218	20	36.2	125
2.41	0.61	0.130	20	23.6	125
2.98	0.65	0.143	40	39.4	190
3.06	0.74	0.268	40	62.6	240
3.05	0.78	0.134	20	62.6	240
2.96	0.59	0.063	20	23.6	125
3.03	0.71	0.216	40	35.2	340
3.03	0.58	0.080	40	17.6	314
3.03	0.72	0.110	20	35.2	340
3.03	0.52	0.037	20	17.6	314
3.53	0.74	0.056	40	45.6	363
3.53	0.71	0.024	40	19.8	360
3.53	0.77	0.013	20	19.8	360

[a] Initial concentrations.

Table IV. Third-Order pH-Independent Rate Constants for the N-Nitrosation of N-Methylpropionamide at 40 °C

pH	k_1, M^{-2} min^{-1}	[N-nitrosamide], mM	[amide],[a] mM	[nitrite],[a] mM	time, min
1.02	1.03	0.716	40	23.2	8
1.06	0.95	1.071	40	41.5	8
1.01	1.09	0.385	20	23.2	8
1.06	1.09	0.615	20	41.5	8
1.48	1.26	0.556	40	33.9	10
1.49	1.31	0.275	40	18.9	10
1.44	1.14	0.690	40	43.1	10
1.47	1.34	0.299	20	33.9	10
1.49	1.18	0.143	20	18.9	10
1.45	1.18	0.355	20	43.1	10
1.95	1.26	0.714	40	35.2	38
1.93	1.06	0.476	40	18.2	55
2.05	1.26	0.182	40	42.4	10
1.94	1.22	0.357	20	35.2	38
1.91	1.02	0.238	20	18.2	55
2.05	1.32	0.895	20	42.4	10
2.41	1.28	0.424	40	19.5	125
2.42	1.57	0.567	40	35.5	75
2.41	1.42	0.236	20	19.5	125
2.39	1.56	0.305	20	35.5	75
2.95	1.40	0.225	40	20.9	240
3.02	1.82	0.374	40	39.3	200
2.95	1.39	0.112	20	20.9	240
2.99	1.68	0.184	20	39.3	200
3.58	1.75	0.039	40	23.6	240
3.58	1.95	0.074	40	39.8	244
3.57	1.58	0.017	20	23.6	240
3.58	1.55	0.030	20	39.8	244

[a] Initial concentrations.

10 kcal/mol. The values range from 0.95 to 1.95 M^{-2} min^{-1} over a pH range of 1.06–3.58 (Table IV). Again the rate constants remained relatively constant as the initial amide and nitrite concentrations were varied between 20 and 40 mM. The mean was 1.37 ± 0.23 M^{-2} min^{-1}. At pH 1.97 (average of six determinations) the rate constant was 1.19 ± 0.11 M^{-2} min^{-1}. The k_1 values increased slighly as the pH was increased from 1 to 3.5. This increase may be due to nitrous anhydride (N_2O_3) contributing to the N-nitrosation reaction at the higher pH values (Mirvish, 1971).

A comparison of the rate constants for the N-nitrosation of amides and amines is presented in Table V. N-Methylurea was N-nitrosated much more rapidly than N-methylpropionamide. Proline was N-nitrosated about three times faster, while the secondary amines, dimethylamine and pyrrolidine, have optimum rates slightly less than the simple alkylamide. The short-chain secondary amide was N-nitrosated at rates comparable to the dialkylamines. However, unlike secondary amines, the amide showed no pH maximum over the pH range investigated. The reaction was pH dependent and became quite rapid at high H^+ concentration.

In the previous paper (Kakuda and Gray, 1980), it was reported that long-chain secondary amides could readily form in model systems containing amines and fatty acids or triglycerides under simulated cooking conditions. These amides are readily N-nitrosated in model systems under the appropriate conditions of pH. However, the pH of foods would militate against the occurrence of N-nitrosamides in foods, even if their precussors are present. It is also interesting to speculate that, in the event that N-substituted amides are formed during the processing and cooking of foods, N-nitrosation of these compounds may occur in vivo. The N-nitrosation of these amides requires acid conditions which are found in gastric juices plus sufficient quantities of nitrite. The eating of vegetables and vegetables juices results in increases in salivary nitrite to hundreds of parts per million (Tannenbaum et al., 1976), many times higher than that permitted in any food product. Thus, it is possible that all factors necessary for in vivo N-nitrosamide formation may be present in the stomach during and after ingestion of a meal.

Table V. Rate Constants for N-Nitrosation of Secondary Amides and Amines

amide/amine	k, M^{-2} min^{-1}
N-methylurea	630[a]
N-methylurethan	20[a]
proline	2.2[b]
N-methylpropionamide	0.695[c]
pyrrolidine	0.318[d]
dimethylamine	0.120[e]

[a] Mirvish (1971), pH 2.0, 25 °C. [b] Mirvish et al. (1973), pH 2.5, 25 °C. [c] pH 1.96, 30 °C. [d] Mirvish (1975), pH 3.0, 25 °C. [e] Mirvish (1970), pH 3.9, 25 °C.

NOTE

Because of the extremely hazardous nature of N-nitroso compounds, all work was carried out in efficient fume cupboards whenever possible, and extreme caution was exercised in handling these compounds.

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