LITERATURE CITED

Bonnett, R.; Nicolaidou, P. *Heterocycles* **1977**, *7*, 637.
Challis, B. C.; Hopkins, A. F.; Milligan, J. R.; Mitchell, R. C.; Massey, R. C., presented at the Eighth International Meeting on *N*-Nitroso Compounds: Occurrence and Biological Effects, Banff, Canada, Sept 5–9, 1983, sponsored by the International Agency for Research on Cancer, Lyon, France.
Hansen, T.; Iwaoka, W. T.; Archer, M. C. *J. Labelled Compd.* **1974**, *10*, 689.
Hertz, H. S.; Hites, R. A.; Biemann, K. *Anal. Chem.* **1971**, *43*, 681.
Kakuda, Y.; Gray, J. I. *J. Agric. Food Chem.* **1980**, *28*, 584.
Magee, P. N.; Montesanto, R.; Preussmann, R. In "Chemical Carcinogenesis"; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1976; ACS Monogr. No. 173, p 491.
Outram, J. R.; Pollock, J. R. A., presented at the Eighth International Meeting on *N*-Nitroso Compounds: Occurrence and Biological Effects, Banff, Canada, Sept 5–9, 1983, sponsored by the International Agency for Research on Cancer, Lyon, France.
Pollock, J. R. A. In "Nitroso Compounds: Occurrence and Biological Effects"; Bartsch, H.; O'Neill, I. K.; Castegnaro, M., Eds.; International Agency for Research on Cancer: Lyon, 1982; IARC Sci. Publ. No. 41, p 81.
White, E. H. *J. Am. Chem. Soc.* **1955**, *77*, 6008.

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Kinetics of Nitrosation of Four Dipeptides N Terminal in Proline

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The kinetics of nitrosation for the imino nitrogen of dipeptides N terminal in proline were studied. The pH optima for nitrosation of four dipeptides were, for Pro-Gly, pH 2.7, for Pro-Hyp, pH 2.7, for Pro-Ser, pH 2.9, and, for Pro-Glu, pH 3.0. On the basis of a determination of the initial rates of nitrosation, the pH-dependent rate constants at optimal pH and 25 °C were 0.26 M^{-2} s^{-1} for Pro-Gly, 0.19 M^{-2} s^{-1} for Pro-Hyp, 0.29 M^{-2} s^{-1} for Pro-Glu, and 0.18 M^{-2} s^{-1} for Pro-Ser.

Recent studies from our laboratory have shown that the nitrosation products from dipeptides N terminal in proline were *N*-nitroso dipeptides with the nitroso group on the imino nitrogen, (Kubacka et al., 1984). Mirvish et al. (1973) examined the nitrosation of prolyglycine and found that the nitrosation kinetics obeyed the rate expression

$$\text{rate} = k_1[\text{amine}][\text{nitrite}]^2 \quad (1)$$

According to eq 1, the pH-dependent rate constant k_1 is a function of the total concentrations of amine and nitrite. Mirvish et al. (1973) did not synthesize the nitroso derivative of Pro-Gly; rather, they used the molar absorptivity for *N*-nitrosoproline in their investigation. On this basis they reported a pH-dependent rate constant for *N*-nitrosoprolylglycine of 0.25 M^{-2} s^{-1} at pH 3.0, which is approximately 7 times larger than the pH-dependent rate constant for proline. This suggests that dipeptides N terminal in proline might undergo nitrosation more rapidly than free proline.

The purpose of this study was to determine the nitrosation kinetics of four dipeptides that are N terminal in proline: prolylglycine, prolylglutamic acid, prolylhydroxyproline, and prolylserine.

EXPERIMENTAL SECTION

Chemicals. L-Proline (Pro), L-prolylglycine (Pro-Gly), and L-prolylhydroxyproline (Pro-Hyp) were purchased from Aldrich Chemical Co. L-Prolylglutamic acid (Pro-Glu) and L-Prolylserine (Pro-Ser) were obtained from Bachem, Inc. Ammonium sulfamate was obtained from J. T. Baker Chemical Co. Seventy percent acid and sodium nitrite were from Mallinckrodt Chemical Works. Nitrosoproline (NPro) was synthesized according to Hansen et al. (1974). All reagents were analytical grade.

Decomposition Rate of HNO_2. An aqueous solution 0.4 M in sodium nitrite was adjusted to pH 3.0 with 70% perchloric acid and placed in a 25 °C water bath. After 0, 5, 10, 15, 30, 45, 60, 90, 120, and 180 min, 2-mL aliquots were transferred to 1 cm path length quartz cells for absorption readings at 359 nm. The experiment was performed twice, and the rate of nitrous acid decomposition was calculated by using a first-order kinetic equation.

Standard Curve for NPro. The following aqueous solutions of NPro were prepared: 6.0, 4.0, 3.0, 2.0, 1.5, 0.5, and 0.25 (all 10^{-3} M). Absorbance readings of the solutions were obtained at 340 nm by using a 1 cm path length quartz cell. The experiment was repeated twice, and a linear regression analysis produced eq 2 with a coefficient

$$y = 110.8x + 15.4 \quad (2)$$

of determination of 0.99. Our experimental design assumes that the molar absorptivities of nitrosated dipeptides that are N terminal in Pro are essentially the same as the molar absorptivity for NPro. Accordingly, the relationship between absorbance and NPro concentration as expressed by eq 2 was used to determine concentrations of the nitrosated dipeptides.

Assay Procedure. Solutions of sodium nitrite were prepared immediately before use to minimize decomposition. Solutions of dipeptides and sodium nitrite were adjusted to the desired pH with 70% perchloric acid or 1 M sodium hydroxide. The reaction was initiated by pipetting sodium nitrite solution into a reaction vial containing the dipeptide solution. The initial dipeptide concentration at the initiation of the reaction was 0.02 M while the concentration of sodium nitrite was 0.01 M. The reaction was carried out in a constant-temperature bath

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Table I. Amount of Nitrosated Dipeptide at Different pHs[a]

pH	NPro-Gly, M × 10^{-3}	pH	NPro-Hyp, M × 10^{-3}	pH	NPro-Glu, M × 10^{-3}	pH	NPro-Ser, M × 10^{-3}
1.06	0.07	1.12	0.11	1.12	0.13	1.12	0.23
1.66	0.26	1.57	0.13	1.57	0.19	1.57	0.26
2.22	0.93	2.16	0.47	2.16	0.66	2.16	0.58
2.37	1.16	2.36	0.62	2.36	0.95	2.36	0.80
2.57	1.24	2.55	0.73	2.51	1.05	2.51	0.89
2.74	1.27	2.73	0.79	2.68	1.14	2.68	1.03
2.87	1.16	2.88	0.74	2.87	1.26	2.87	1.08
3.05	1.05	3.02	0.71	3.02	1.29	3.02	1.05
3.25	0.93	3.25	0.63	3.25	1.18	3.25	0.97
3.44	0.79	3.45	0.54	3.44	1.01	3.44	0.86
4.00	0.33	4.01	0.26	4.00	0.60	4.00	0.44

[a] 25 °C; 30 min; [dipeptide] was 0.01 M; [nitrite] was 0.02 M. All data represent mean values from two repetitions.

at 25 ± 1 °C. After the desired time, the reaction was stopped by adding 0.5 M ammonium sulfamate in 2.5 M perchloric acid and absorbance at 340 nm was immediately determined.

RESULTS AND DISCUSSION

Decomposition of Nitrous Acid. Initial experiments were conducted to estimate the rate of decomposition of the acidified nitrite solutions that were used to study the kinetics of dipeptide nitrosation. The nitrite loss was evaluated by using a first-order kinetic equation. Calculated in this way, the rate constant was 6.4×10^{-5} s^{-1}. This value is approximately 3×10^4 times lower than the rate constants obtained for the nitrosation of the dipeptides under study. It was concluded therefore that the relatively small amount of nitrite loss would not seriously interfere with the study of the nitrosation kinetics of the dipeptides, especially over relatively short reaction times.

Influence of pH on Nitrosation Rate. Table I shows the relative amounts of nitrosated derivative obtained from the dipeptides as a function of pH. The pH optima for nitrosation of the dipeptides were as follows: pH 2.7 for Pro-Gly and Pro-Hyp, pH 2.9 for Pro-Ser, and pH 3.0 for Pro-Glu. These pH optima were used in subsequent experiments to determine the rate of nitrosation for the different dipeptides. Mirvish et al. (1973) had previously reported that the optimum nitrosation of Pro and Pro-Gly occurred at pH 2.5 and pH 3.0, respectively.

Rate of Nitrosation for Pro-Gly, Pro-Glu, Pro-Hyp, and Pro-Ser. Table II shows the amounts of the nitrosated dipeptides observed at various time intervals up to 24-h reaction time. As expected, the rate of nitrosation appeared to decrease for all of the dipeptides as the reaction time increased. At relatively short reaction times however, a linear relationship was observed between reaction time and amount of nitrosated product. Equations resulting from linear regression analysis and the corresponding coefficients of determination obtained for 5-, 10-, and 15-min reaction times are as follows:

NPro-Gly: $y = 0.063x + 0.008$ $R^2 = 0.999$

NPro-Hyp: $y = 0.034x + 0.076$ $R^2 = 0.999$

NPro-Glu: $y = 0.061x + 0.057$ $R^2 = 0.998$

NPro-Ser: $y = 0.055x - 0.028$ $R^2 = 0.991$

These data verify a linear relationship between reaction time and amount of nitrosated dipeptide for the first 15 min of the reaction. Accordingly, data collected after 5-min reaction times were used to estimate the pH-dependent rate constants for the nitrosation of the four dipeptides. Table III contains the amount of nitrosated dipeptide obtained after a reaction time of 5 min for 10 replicate

Table II. Amount of Nitrosated Dipeptide at Different Reaction Times

time	NPro-Gly, M × 10^{-3}	NPro-Hyp, M × 10^{-3}	NPro-Glu, M × 10^{-3}	NPro-Ser, M × 10^{-3}
5 min	0.316	0.244	0.357	0.231
10 min	0.648	0.425	0.686	0.551
15 min	0.944	0.587	0.971	0.779
30 min	1.58	1.00	1.61	1.43
45 min	2.02	1.34	2.05	1.84
60 min	2.42	1.63	2.43	2.16
90 min	3.12	2.05	3.12	2.88
2 h	3.53	2.38	3.65	3.36
2.5 h	3.84	2.61	4.02	3.71
3 h	4.04	2.74	4.34	3.99
3.5 h	4.28	2.95	4.58	4.32
4 h	4.52	3.11	4.74	4.46
5 h	4.78	3.24	5.06	4.67
6 h	5.00	3.31	4.99	4.85
7 h	5.19	3.68	5.28	4.96
8 h	5.28	3.81	5.46	5.03
9 h	5.36	3.92	5.48	5.04
10 h	5.42	4.01	5.40	5.18
11 h	5.45	4.05	5.54	5.21
12 h	5.44	4.06	5.58	5.25
24 h	5.45	4.09	5.91	5.55

Table III. Amount of Nitrosated Dipeptide after a Reaction Time of Five Minutes[a]

NPro-Gly, M × 10^{-4}	NPro-Hyp, M × 10^{-4}	NPro-Glu, M × 10^{-4}	NPro-Ser, M × 10^{-4}
3.39	2.35	3.61	2.13
3.07	2.40	3.48	2.22
3.30	2.31	3.57	2.22
3.43	2.31	3.48	2.31
3.03	2.26	3.39	2.31
3.30	2.31	3.39	2.26
2.94	2.26	3.48	2.26
3.03	2.44	3.34	2.26
3.16	2.53	3.39	2.13
3.03	2.26	3.39	2.13
mean: 3.17	2.34	3.45	2.22
SD: 0.17	0.08	0.08	0.07
CV:[b] 5.47	3.81	2.54	3.19

[a] 25 °C; optimal pHs. [b] CV = coefficient of variation.

experiments. The mean values from Table III were used to calculate initial reaction rates, which were in turn used in eq 1 to calculate the pH-dependent rate constant for each dipeptide. The pH-dependent rate constants for the four dipeptides are as follows: for Pro-Gly, 0.26 M^{-2} s^{-1}; for Pro-Hyp, 0.19 M^{-2} s^{-1}; for Pro-Glu, 0.29 M^{-2} s^{-1}; for Pro-Ser, 0.18 M^{-2} s^{-1}.

Using the Student's t test (Dixon and Massey, 1969), it was determined that all of the pH-dependent rate constants were significantly different from each other (P =

0.005). It is interesting that both dipeptides that contain hydroxyl groups, Pro-Hyp and Pro-Ser, had lower rate constants than Pro-Gly and Pro-Glu. Perhaps the lower rate constants for the dipeptides containing hydroxyl groups are due to competition of the hydroxyl groups with the imino nitrogen of Pro for the nitrosating agent.

Mirvish et al. (1973) has previously studied the nitrosation of Pro-Gly and reported a pH-dependent rate constant of 0.25 M^{-2} s^{-1}. Our results for Pro-Gly are in excellent agreement with the previous report since we find a rate constant of 0.26 M^{-2} s^{-1}. It is interesting to note that the pH-dependent rate constants for the four dipeptides N terminal in Pro examined in this study were substantially larger than the rate constant of 0.037 M^{-2} s^{-1} reported by Mirvish et al. (1973) for the free amino acid Pro. This of course suggests that the dipeptides would be expected to form *N*-nitroso derivatives in food, or in the stomach, more readily than Pro. Additional research will be required to determine whether nitrosated dipeptides N terminal in Pro form in food or in the stomach. Particularly pertinent to this question is a recent report by Ohshima and Bartsch (1981). These investigators reported that NPro, an *N*-nitroso compound that has not shown carcinogenic activity in experimental animals, can form endogenously and is excreted quantitatively in the urine. Perhaps a reasonable approach in our future investigations would be to determine the urinary excretion products of nitrosated dipeptides that are N terminal in Pro.

Registry No. Pro-Gly, 2578-57-6; Pro-Glu, 67644-00-2; Pro-Hyp, 18684-24-7; Pro-Ser, 71835-80-8.

LITERATURE CITED

Dixon, W. J.; Massey, F. J. "Introduction to Statistical Analysis"; McGraw-Hill: New York, 1969; p 117.
Hansen, T.; Iwaoka, W. T.; Archer, M. C. *J. Labelled Compd.* **1974**, *10*, 4.
Kubacka, W.; Libbey, L. M.; Scanlan, R. A. *J. Agric. Food Chem.* **1984**, preceding paper in this issue.
Mirvish, S. S.; Sams, J.; Fan, T. Y.; Tannenbaum, S. R. *J. Natl. Cancer Inst. (U.S.)* **1973**, *51*, 1833.
Ohshima, H.; Bartsch, H. *Cancer Res.* **1981**, *41*, 3658.

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Determination of Suspected Toxic Impurities in Firemaster FF-1 and Firemaster BP-6 by High-Resolution Gas Chromatography–High-Resolution Mass Spectrometry

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The analysis of Firemaster FF-1 (or BP-6) for minor and suspected toxic impurities has been accomplished by high-resolution GC–selected ion monitoring at medium and high resolution, the mass spectrometer being operated under a computer-controlled peak-matching system. Analysis for polybrominated naphthalenes reveals the presence of various components having an exact mass corresponding to the elemental compositions of tetra-, penta-, and hexabromonaphthalenes. The search for the corresponding polybrominated methylnaphthalenes or methylbromonaphthalenes results in a series of signals that have similar but incorrect exact masses. The analysis of the flame retardant mixture has also been extended to the measurement of the exact mass of some monochloropentabromobiphenyl isomers reported recently by Domino and Domino (1980) and to the screening for tetrabromobiphenylene isomers.

Firemaster FF-1 (or BP-6) is a complex mixture of highly brominated biphenyls that has been of environmental importance since the accidental contamination of animal feed in Michigan less than a decade ago (Kay, 1977). The presence of polybrominated aromatics other than the bromobiphenyls as trace impurities in the mixture has been the object of several studies and is of concern from a toxicological point of view (Taylor, 1979). Polybrominated naphthlenes, benzenes, and a possible methylbrominated furan have been reported by Hass et al. (1978). O'Keefe has independently reported polybrominated naphthlenes (O'Keefe, 1978, 1979). More recently, a monochloropentabromobiphenyl has been added to the list of detected impurities (Domino and Domino, 1980.

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The chemical analysis of the flame retardant for these polybrominated aromatic contaminants has so far been limited to low-resolution characterization. We report here the results obtained for the direct screening of Firemaster (FF-1 or BP-6) by high-resolution gas chromatography (HRGC) at medium and high resolution of the mass spectrometer, MRMS and HRMS, respectively, the latter being under a computer-controlled peak-matching mode of operation (Harvan et al., 1982; Tondeur et al., 1983). The major advantage of this method is that it enables one to observe the peak profile of any signal falling in the preset mass window (typically selected to be twice the peak width at 5% peak height) scanned by the electric sector and, thereby, allowing the measurement of its exact mass. Application of this technique to the study of Firemaster FF-1 (or BP-6) for polybrominated naphthalenes, polybrominated methylnaphthalenes or methylbromonaphthalenes, monochloropentabromobiphenyls, and tetrabromobiphenylenes will illustrate its usefulness for the determination of minor components in complex mixtures.