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KINETIC STUDY ON THE NITROSATION OF DIBENZYLAMINE IN A MODEL SYSTEM

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Abstract—A kinetic study of the formation of *N*-nitrosodibenzylamine (NDBzA), from the nitrosation of dibenzylamine (DBzA) by sodium nitrite, was performed in a model system under conditions (temperature, pH) that are similar to those encountered in the industrial production of hams processed in elastic rubber nettings. The nitrosation reaction was carried out in a KH_2PO_4 buffer (0.5 M) at pH 5.8 and at a temperature of 69 C. Since DBzA is insoluble in an aqueous buffer system, a non-ionic surfactant, Tween 20, was used as a solubilizing agent. The nitrosation reaction exhibited first-order kinetics with respect to DBzA and second-order kinetics with respect to nitrite. The calculated rate constant was $4.7 \pm 0.5\ M^{-2}$/min. The pH profile of NDBzA formation was also determined. The optimal pH of NDBzA formation, 3.12, was close to the pK_a of nitrous acid (HNO_2, $pK_a = 3.1$).

INTRODUCTION

The presence of nitrosamines has been reported in a wide variety of rubber products such as rubber-stoppered blood collection tubes (Lakritz and Kimoto, 1980), infant pacifiers and baby-bottle nipples (Babish *et al.*, 1983; *Federal Register*, 1984; Havery and Fazio, 1983; Osterdahl, 1983; Sen *et al.*, 1985; Spiegelhalder and Preussmann, 1982), disposable rubber gloves (Fiddler *et al.*, 1985) and orthodontic rubber bands (Fiddler *et al.*, 1992). In 1987, Sen and co-workers (Sen *et al.*, 1987) detected *N*-nitrosodimethylamine (NDMA), *N*-nitrosodibutylamine (NDBA) and *N*-nitrosomorpholine (NMOR) in cured meat products processed in elastic rubber nettings. Sen *et al.* concluded that the elastic rubber netting was the origin of these nitrosamines (Sen *et al.*, 1987 and 1988). The dialkylamine-derived accelerators and stabilizers used in the rubber vulcanization process have been identified as the source of the nitrosamines (Fajen *et al.*, 1979). The high levels of NDBA found in cured meat products processed in elastic rubber nettings led to the reformulation of the rubber used in the nettings. Although hams processed in these new reformulated nettings comply with the maximum allowable level of NDBA, significant amounts of *N*-nitrosodibenzylamine (NDBzA) have been detected in these reformulated nettings (Pensabene and Fiddler, 1993; Sen *et al.*, 1988). NDBzA demonstrated no detectable carcinogenic activity in rats in an earlier study (Druckrey *et al.*, 1967), but recently Boyes and co-workers found it to be genotoxic (Boyes *et al.*, 1990). Therefore, the regulatory agency, the USDA's Food Safety Inspection Service, and the food industry have a keen interest in understanding the mechanism of formation of NDBzA in order to develop means of minimizing its levels in or eliminating it from netted cured meat products.

The nitrosation of secondary amines by sodium nitrite is one of the main causes of exogenous nitrosamine formation (Fan and Tannenbaum, 1973a). Even though nitrosamine formation (Coleman, 1978; Masui *et al.*, 1974; Okany *et al.*, 1974), inhibition (Bailey and Mandagere, 1980; Gray and Dugan, 1975; Gray *et al.*, 1982; Theiler *et al.*, 1984), and catalysis have been studied extensively, the mechanisms of formation of nitrosamines are not fully understood (Archer, 1984). The objective of the study reported here was the kinetic characterization of a food model system with respect to the dibenzylamine (DBzA) nitrosation reaction, since no information is available on the kinetics of NDBzA formation. It is recognized that no model system can fully represent a matrix as complex as food because of the presence in food of compounds that could catalyse or inhibit the nitrosation reaction. However, the temperature (69 C) and the pH (5.8) were selected to increase the usefulness of this study to the food processing industry, since these conditions are similar to those encountered during the processing of cured meat products (Kemp *et al.*, 1974; Kuo and Ockerman, 1983; Wilson *et al.*, 1981).

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Abbreviations: DBzA = dibenzylamine; GC–TEA = gas chromatograph–thermal energy analyser; NDBA = *N*-nitrosodibutylamine; NDBzA = *N*-nitrosodibenzylamine; NDMA = *N*-nitrosodimethylamine; NMOR = *N*-nitrosomorpholine.

MATERIALS AND METHODS

*Materials**

Dibenzylamine (98% pure; Janssen Chemica, Spectrum Chemical Manufacturing Corp., New Brunswick, NJ, USA), Tween 20 (polyoxyethylene 20 sorbitan monolaurate; Sigma Chemical Co., St Louis, MO, USA) and sodium nitrite (99%, Mallinckrodt Inc., Paris, KY, USA) were used without further purification. *N*-Nitrosodibenzylamine was synthesized from its corresponding amine (DBzA) and sodium nitrite according to the general procedure published previously (Pensabene *et al.*, 1972). A KH_2PO_4–NaOH buffer system (0.5 M) was used to maintain the pH of the nitrosation reaction at 5.8.

Methods

DBzA, the most likely precursor of NDBzA, is readily soluble in organic solvents; however, it is insoluble in aqueous systems. In order to perform the kinetics study in a buffer, a solubilizing agent was needed. Tween 20 was selected as a solubilizing agent because it could be used at relatively low concentrations (0.4%, v/v) to form a stable dispersion of DBzA in the buffer. The solubilization of DBzA was desired in order to simulate its solubilization in the fat content of a cured meat product.

The reaction mixture was heated using a silicone oil-bath in a heating mantle. The temperature of the oil-bath (69 ± 2°C) was maintained using a controller (model LC-22, Bioanalytical Systems Inc., West Lafayette, IN, USA). The reaction flask (250 ml) was fitted with a water cooled condenser to minimize losses due to evaporation.

Tween 20 was used to form a stable dispersion of DBzA in buffer. A given amount of DBzA (0.6 g) was mixed with Tween 20 (2.9 g). A stock dispersion of DBzA/Tween 20 in buffer was prepared by transferring the desired amount of the Tween 20/DBzA (5:1, w/w) mixture to a 10.0-ml volumetric flask and diluting it to the final volume with the KH_2PO_4 buffer (0.5 M). The concentration of DBzA in the stock solution was 0.14 M. A stock solution of $NaNO_2$ (2.63 M) was made by dissolving $NaNO_2$ in the buffer.

The buffer was heated at 60°C for 1 hr. The amount of buffer was adjusted in each experiment in order to obtain a total initial reaction volume of 200 ml after all reagents had been added. After 7 hr the pH of the reaction mixture changed only slightly from 5.8 to 5.6. The DBzA/Tween 20 stock dispersion (1.0 ml, 0.14 M) was added to the buffer, which had been temperature equilibrated previously, and after 10–15 min, the nitrosation reaction was initiated by the addition of the appropriate volume of the $NaNO_2$ solution. The initial concentration of DBzA in the reaction mixture was 0.75 mM, and the initial concentration of sodium nitrite was varied (0.053, 0.066 or 0.132 M) in order to obtain a set of curves with different slopes. Aliquots (5.0 ml) were withdrawn from the reaction mixture at predetermined time intervals (2, 10, 20, 25, 30, 40, 50, 60, 120, 180, 240, 300, 360 and 420 min). The aliquots were transferred to 125-ml separatory funnels which contained 3.0 ml 1 N NaOH. The NaOH was used to stop the nitrosation reaction since no further nitrosation is expected to occur at this higher pH (Keefer and Roller, 1973; Okany *et al.*, 1974).

Both DBzA and NDBzA were extracted from the reaction aliquots using 4 × 10 ml dichloromethane. The extracts were dried over anhydrous sodium sulfate, collected in a 250 ml Kuderna–Danish flask with a 4-ml concentrator tube attached, and concentrated to a final volume of 1.0 ml before gas-chromatographic analysis using chemiluminescence detection [gas chromatography–thermal energy analyser (GC–TEA) analysis, 3-μl injections].

The gas chromatograph (model GC-mini3, Shimadzu, Columbia, MD, USA) was fitted with a 2 m × 2.6 mm glass column containing 5% SP-2401-DB on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA, USA). The injector was kept at 22°C (He carrier gas 40 ml/min). The temperature program maintained an initial temperature of 170°C for 5 min, and then the temperature was raised from 170 to 220°C using a 15°C/min temperature ramp. Finally, the temperature was held at 220°C for 6 min. The GC was connected to a nitrogen converter (TEA model 610R, Thermedics Inc., Woburn, MA, USA) through a heated interface kept at 275°C. The nitrogen converter was operated in the nitrogen mode in which the GC effluent was passed through a catalytic pyrolyser kept at 950°C. In this mode of operation, the nitrogen atoms present in DBzA and NDBzA were converted to the nitrosyl radical under a flow of oxygen. The nitrogen converter was coupled to a thermal energy analyser (TEA model 502, Thermedics Inc.), which uses chemiluminescence detection of the nitrosyl radical–ozone reaction. The output of the detector was measured by an integrator (model 3390A, Hewlett-Packard, King of Prussia, PA, USA), and the reported peak area was used to quantify the DBzA and NDBzA. External calibration was used to quantify the DBzA and NDBzA present in the reaction mixture aliquots. All injections were performed in duplicate.

The composition of selected reaction aliquots was verified by GC (model 5890A, Hewlett-Packard) connected to a mass selective detector (model 5971, Hewlett-Packard) equipped with a 30 m × 0.32 mm i.d., 1.0 μm film thickness, DB-5MS fused silica capillary column (J & W Scientific, Folsum, CA, USA). The instrumental parameters used were as follows: 2-μl injections; carrier gas helium 1.2 ml/min; injector set at 220°C; oven temperature program—100°C for 5 min, 100 to 220°C at 8°C/min, 220°C for 20 min; interface set at 300°C; purge on at

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3 min and off at 10 min; scan mode 29 to 240 AMU with a solvent delay of 10 min.

The effect of pH of the amount of NDBzA formed was studied by carrying out the nitrosation reaction at different pH values. Several K_2HPO_4 (0.4 M)–citric acid (0.2 M) buffer systems were used to maintain the appropriate pH between 2 and 7. A total initial reaction volume of 200 ml was used, and the initial concentrations of DBzA and nitrite were 0.025 mM and 0.50 mM, respectively. After 1 hr the reaction was stopped with 120 ml 1 N NaOH. The entire reaction mixture was extracted with dichloromethane (4 × 100 ml), and the extract was dried over anhydrous sodium sulfate. The extracts were concentrated to a final volume of 1.0 ml before GC–TEA analysis. The catalytic pyrolyser was used in the nitroso mode and its temperature was maintained at 500°C. It was advantageous to use this mode of operation for these determinations since only the quantification of NDBzA was of interest, and it provided an enhancement in sensitivity over the nitrogen mode. The interface was kept at 275°C. The GC conditions and parameters used were the same as described earlier.

To compare the nitrosation potentials of dibutylamine (DBA) and DBzA, the nitrosation of the two compounds was performed in separate reaction mixtures using an initial concentration of 0.73 mM and 0.053 M for the amine and sodium nitrite, respectively. The reaction mixture was incubated for 1 hr, and then, the reaction was stopped using 30 ml 1 N NaOH. The nitrosamine formed was extracted from the reaction mixture using 4 × 80 ml dichloromethane. The extracts were dried over anhydrous sodium sulfate, and concentrated to a final volume of 1.0 ml. The amount of nitrosamine formed was determined by GC–TEA using the same experimental conditions described for the determination of NDBzA in the pH dependency study.

RESULTS AND DISCUSSION

Figure 1 shows a GC–TEA chromatogram (3-μl injection) from a 5-ml aliquot withdrawn from the nitrosation reaction mixture 20 min into the reaction.

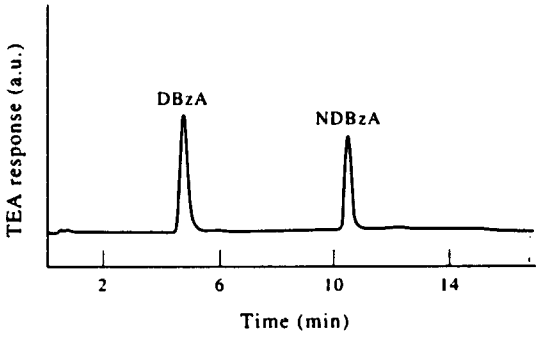

Fig. 1. GC–TEA chromatogram of a 5.0-ml aliquot withdrawn from the reaction mixture (20 min). The peak labelled DBzA corresponds to dibenzylamine, and the peak labelled NDBzA corresponds to *N*-nitrosodibenzylamine.

At the attenuation used for this chromatogram (× 1024), no solvent peak was observed. This GC–TEA method allows the simultaneous determination of the unreacted DBzA and the NDBzA which is formed. Under the conditions used, the DBzA elutes from the column at 4.7 min and the NDBzA 10.3 min.

For a sample containing the same amounts (0.5 mg/ml) of DBzA (2.5 mM) and NDBzA (2.2 mM), the TEA response in the nitrogen mode was twice as great for NDBzA as for DBzA, reflecting the two nitrogen atoms in the former compound versus one in the latter. The composition of selected aliquots was verified by GC connected to a mass selective detector; no nitrosation product other than NDBzA was detected. For DBzA, the mass spectrum showed signals at m/z = 196 (M^+), 120, 106, 91 (base peak), and 65. For NDBzA, the mass spectrum showed signals at m/z = 226 (M^+), 181, 118, 91 (base peak), and 65. For both DBzA and NDBzA, the retention times matched within 0.1 min, and the relative abundance of the five major peaks of each compound matched previously run authentic standards by more than 90% as measured by the computer software.

The reaction was first order with respect to DBzA and second order with respect to nitrite (Archer, 1984; Douglass *et al.*, 1978). The reaction order with respect to each reagent was obtained by the pseudo-order and isolation method (Connors, 1990). In the pseudo-order and isolation method, one of the reactants is added in excess so that the change in its concentration can be neglected. Sodium nitrite was added in excess, and the nitrosation reaction was made pseudo first order. A plot of ln([DBzA]) versus time gave a straight line with a slope equal to k[nitrite]^2, where k is the rate constant, [DBzA] is the concentration of DBzA, and [nitrite] is the concentration of nitrite. The rate constant was calculated by dividing the slope by the square of the initial concentration of nitrite. A direct determination of the reaction order with respect to the nitrite was not feasible because an excess of DBzA was required in order to make the reaction pseudo second order. An excess of DBzA would have been accompanied by an excess of surfactant which would have interfered not only with the kinetics of the nitrosation reaction but also with the determination of nitrite by the Griess reaction (Fox, 1979 and 1985).

The following rate equation describes the nitrosation of DBzA by nitrite

$$v = k[\text{DBzA}][\text{nitrite}]^2$$

where v is the reaction rate, k is the rate constant, [DBzA] is the concentration of DBzA, and [nitrite] is the concentration of nitrite. The calculated rate constant was $4.7 \pm 0.5\ \text{M}^{-2}/\text{min}$. Under mildly acidic conditions, the species that are involved in the rate determining step of the nitrosation reaction are the unprotonated amine and N_2O_3. N_2O_3, the nitrosating species, is formed from two molecules of HNO_2, and

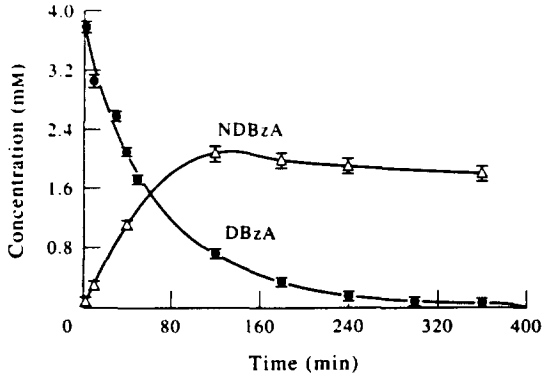

Fig. 2. Plot of the concentration of dibenzylamine (DBzA, ●) and the concentration of *N*-nitrosodibenzylamine (NDBzA, △) versus time ($[DBzA]_0 = 0.75$ mM; $[nitrite]_0 = 0.0527$ M). The error bars represent the 95% confidence level for duplicate injections.

hence, the second order reaction with respect to nitrite. The nitrosamine is formed after electrophilic attachment of the N_2O_3 onto the free electron pair of the amine (Archer, 1984; Douglass *et al.*, 1978).

Figure 2 shows a plot of the concentration of DBzA and NDBzA versus time. The plot that corresponds to DBzA exhibits the behaviour characteristic of a first-order reaction. After 1 hr of reaction, the concentration of the NDBzA reaches a plateau.

The results for the amount of NDBzA formed at a given pH value are shown on Table 1. When a plot of pH versus the amount of NDBzA formed after 1 hr of reaction was constructed, the curve had a maximum around pH 3.12, which is typical for this type of nitrosation reaction (Archer, 1984; Fan and Tannenbaum, 1973b; Mirvish, 1970). Maximum nitrosamine formation occurred at a pH value close to the pK_a of HNO_2 (the temperature dependence of pK_a was not considered). For the pH dependency study, the nitrogen converter was used in the nitroso mode. In the nitroso mode of operation, the TEA does not respond to DBzA since the pyrolytic cleavage of the molecules is not performed in the presence of oxygen (i.e. the nitrosyl radical is not formed).

The pH affects the rate at which a given nitrosamine is nitrosated, not only because it determines the extent of conversion of nitrite to HNO_2, and eventually to N_2O_3, but also because it determines the concentration of the unprotonated amine. In general, the optimum pH for the nitrosation of secondary amines by nitrite is in the range of 3 to 3.5. These pH values are close to the pK_a (25°C) of HNO_2 which is 3.1 (Licht *et al.*, 1988). The two requirements needed for the nitrosation of secondary amines by nitrite are: (1) the formation of HNO_2, which is favoured at low pH; and (2) the presence of the amine in the unprotonated form, which is favoured at high pH (Archer, 1984; Douglass *et al.*, 1978). These factors tend to offset each other.

Table 1. Effect of pH on the amount of NDBzA* formed

pH	Amount of NDBzA formed (mg/ml)†
2.07	2.31 ± 0.14
2.55	6.07 ± 0.24
3.12	7.81 ± 0.33
3.94	4.16 ± 0.24
5.00	1.66 ± 0.12
5.78	0.22 ± 0.02
6.81	0.07 ± 0.01

*Initial concentrations of 0.025 mM and 0.50 mM were used for DBzA and sodium nitrite, respectively.
†After 1 hr.

At a given pH, the lower the basicity of the amine the higher the reaction rate, since the presence of the unprotonated amine is favoured. For example, at pH 3.0, the rate constant for the nitrosation of morpholine ($pK_a = 8.5$, $k = 1400\ M^{-2}/hr$) is about three orders of magnitude larger than the rate constant for the nitrosation of dimethylamine ($pK_a = 10.7$, $k = 5.4\ M^{-2}/hr$) (Archer, 1984; Fan and Tannenbaum, 1973b; Mirvish, 1970). Because DBzA ($pK_a = 8.52$) is a weaker base than DBA ($pK_a = 11.25$) (Dean, 1985); DBzA has a greater potential of nitrosation under the conditions found in a cured meat product. NDBA is the other nitrosamine associated with rubber and rubber products. When the nitrosation of DBA and DBzA was performed using the same experimental conditions, the amount of nitrosamine formed was $11.9 \pm 0.4\ \mu g/ml$ and 1.6 ± 0.1 mg/ml for NDBA and NDBzA, respectively. The rate constant for the nitrosation of DBA is at least two orders of magnitude larger than the rate constant for the nitrosation of DBzA.

Conclusions

The nitrosation of DBzA by nitrite followed a first-order dependence on the DBzA concentration and a second-order dependence on the nitrite concentration. The pH dependency profile revealed that maximum formation of NDBzA is obtained at pH values close to the pK_a of HNO_2 since the formation of the nitrosating species, N_2O_3, is favoured. Although the optimum pH for the nitrosation of DBzA is observed at pH values lower than 5.8, the nitrosation of DBzA at this pH still occurs at a rate that may be conducive to the formation of significant amounts of NDBzA in netted cured meat products.

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