Factors Influencing the Rate of Formation of Nitrosomorpholine from Morpholine and Nitrite. II. Rate Enhancement in Frozen Solution

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The reaction of nitrite and morpholine as a model of a nitrosation reaction has been studied in frozen model systems and in frozen milk. The rate was considerably enhanced in frozen systems compared to that expected for a supercooled solution at the same temperature. The degree of enhancement appeared to be a function of overall solute concentration, which in turn controls the amount of unfrozen solution at any temperature. Because this type of nitrosation reaction is third-order, a threefold concentration of reactants leads to a 27-fold increase in reaction rate. A second type of rate enhancement was caused by selective precipitation of sodium phosphate buffers, leading to an increase in the acidity of the solvent and a higher reactant concentration. pH changes under these conditions were determined through the use of indicator dyes. Experiments in frozen milk show the results to be applicable to foods.

Many reactions continue in frozen solutions, sometimes proceeding at a faster rate than in the supercooled liquid state at the same temperature (Pincock, 1969). This does not mean the reaction in solid state proceeds faster than in liquid state. A frozen solution is completely solid only below its lowest eutectic temperature. Above this temperature the system is a matrix of solid material filled with liquid with which the solid is in equilibrium (Paren and Walker, 1971). When an aqueous solution freezes, solute molecules are usually excluded from the ice lattice and concentrated in the liquid phase. For example, at 0.001 *M* initial reactant concentration, a frozen solution is 99.9% solid at −5° and the reactant is 1000-fold concentrated in the liquid phase (Pincock, 1969). Therefore, for the third-order nitrosation reaction of nitrite and morpholine (Fan and Tannenbaum, 1973), a large increase in reaction rate would be expected for a frozen system compared to the unfrozen system at the same temperature. In this paper, the possibility of increased rates of nitrosation at temperatures below the freezing point of water is investigated.

EXPERIMENTAL SECTION

Kinetics of Nitrosation of Morpholine with Nitrite in Frozen System. All chemicals were reagent grade. Solutions of sodium nitrite and morpholine were prepared in buffers (0.1 *M* citric acid and 0.2 *M* disodium hydrogen phosphate buffer was used unless otherwise specified). Two milliliters of each of the solutions was pipetted into a test tube which was incubated in a freezing bath to lower its temperature. After 5 min, the tube was immersed in an alcohol–Dry Ice bath for 40 sec to initiate ice formation. It was returned to the freezing bath; zero time was marked at this time. At 30-min intervals tubes were withdrawn from the freezing bath and ammonium sulfate was added to stop the reaction. The pH of the sample was adjusted to 7 and the concentration of nitrosomorpholine (NMor) was determined by the photolytic procedure of Fan and Tannenbaum (1971). Treatment of the data to determine rate constants and a general treatment of the kinetics of morpholine nitrosation have been previously published (Fan and Tannenbaum, 1973).

Measurement of Concentration of Nitrite in the Liquid Portion of the Frozen Solution at −6°. A 125-ml Erlenmeyer flask containing 100 ml of sodium nitrite solution in pH 3.5 buffer was cooled in a freezing bath at −6° for 20 min. The flask was immersed in the alcohol-Dry Ice bath for 5 min to initiate freezing; it was then equilibrated in the freezing bath at −6° for 30 min. The liquid portion of the frozen solution was extracted with a Pasteur pipet. Concentration of sodium nitrite was measured with the automatic Griess procedure of Fan and Tannenbaum (1971).

Estimation of the pH of Frozen Solutions. Indicator solutions of 0.1% were prepared in distilled water to cover the pH range 3.5–9. Many handbooks list appropriate indicators, their useful pH range, and their color at various pH's.

Ten drops of the indicator solution was added to 4 ml of buffers ranging from pH 2 to 9, each at an increment of one pH unit. Only two drops of indicator solution was added to samples to be frozen which might contain buffer alone or reactants in buffer (morpholine and sodium nitrite each at 2 m*M*). The samples were frozen according to the procedures described above; they were allowed 1 hr to achieve equilibrium. The color of the frozen samples was visually compared with the color of the standards to estimate the approximate pH of the samples in the frozen state. Use of a variety of indicators allowed the estimation of the pH accurate to one pH unit.

Procedure for the Study of Nitrosomorpholine (NMor) Formation in Milk. Dry milk was dissolved in distilled water; the pH was 6.8. Sulfuric acid was added if the reaction was to be conducted at lower pH (perchloric acid was not suitable for pH adjustment here, as it would precipitate proteins of milk). Solutions of morpholine and sodium nitrite at reaction pH were separately diluted by milk to the desired concentration and separately cooled to 0° before mixing. The system was frozen and stored as described for the other model systems. Each reaction time was represented by a single test tube containing a 10-ml reaction volume. Samples were withdrawn at predetermined intervals, and ammonium sulfamate was added to quench the reaction prior to thawing. Perchloric acid was added to lower the pH and to precipitate the protein. The final pH was about 2.5. Samples were centrifuged at 7000 rpm for 20 min; the pH of the supernatant was then brought up to 7 with 50% NaOH. Precipitation reoccurred when the pH rose above 6.5, so samples were again centrifuged at 7000 for 20 min. The concentration of nitrosomorpholine was determined as previously described. When samples spiked with known concentrations of NMor were put through the same procedure, NMor was completely recovered in the supernatant fraction.

RESULTS AND DISCUSSION

Freezing markedly accelerated the rate of nitrosation of morpholine. The nitrosation reaction was studied between +50° and −50° (Figure 1); above 0°, the reaction follows the Arrhenius equation. The rate of nitrosation decreases gradually from 50° to 0°. However, when the temperature is lower than 0°, the reaction rate increases rapidly, reach-

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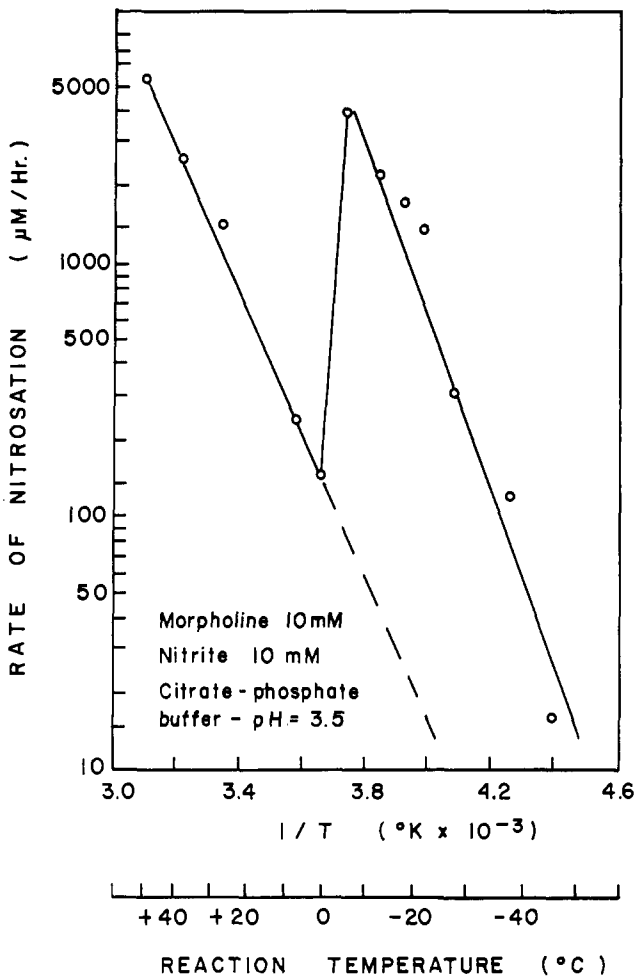

Figure 1. The effect of temperature on the rate of nitrosation of morpholine with nitrite in citrate–sodium phosphate buffer for temperature above and below freezing temperature.

Table I. The Ratio of Reaction Rate in the Frozen State to the Supercooled Solution at Same Temperature at pH 3.5[a]

Temp, °C	Rate ratio
−6°	48.6
−13°	53.5
−18°	60.0
−22°	64.8
−28°	34.7
−38°	25.0
−46°	16.0

[a] Concentration of both nitrite and morpholine is 10 mM.

ing a maximum at −6° for the particular total solute concentration of these experiments. Table I shows the ratio of the reaction rate in the frozen state to the predicted rate in the supercooled solution at the same temperature. The reaction rates for the supercooled solutions are estimated by extrapolation from the reaction rates above freezing temperatures (Figure 1). The ratio reaches a maximum at −22° and drops rapidly at temperatures lower than −28°.

The concentration of nitrite in the liquid portion of the frozen system has been measured, and nitrite concentration was found to increase threefold in frozen solution at −6° and pH 3.5.

Inert solutes were added to the reaction system to study their effect on the reaction rate. Glycerol was found to decrease the nitrosation rate in proportion to its initial concentration. Chloride ion and bromide ion can exert a catalytic effect on the rate of nitrosation (Fan and Tannenbaum, 1973); in the frozen system, however, addition of chloride ion resulted in a marked decrease of the reaction

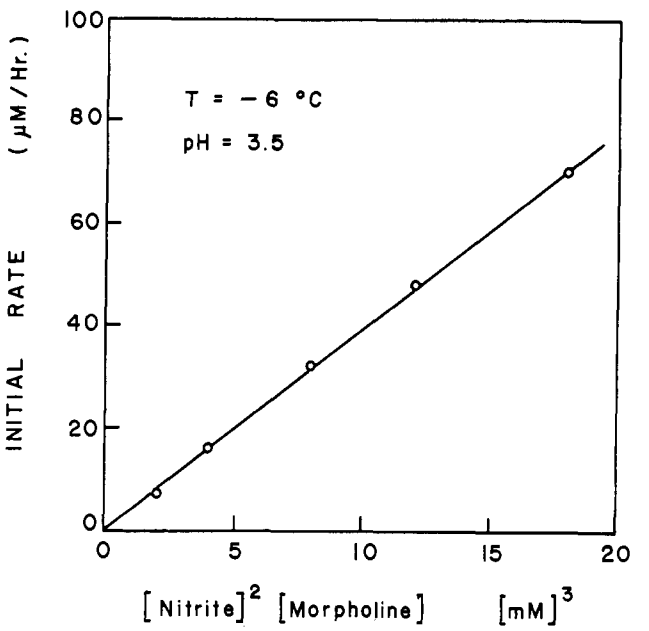

Figure 2. Third-order rate plot for the nitrosation of morpholine with nitrite at −6° and pH 3.5.

Table II. The Effect of Addition of Chloride and Bromide Ion on the Nitrosation Rate of Morpholine at pH 3.5 and −6°[a]

Ion	Concn of ion	Initial rate, μM/hr
NaCl	0.05	2812
NaCl	0.1	188
NaBr	0.05	4250
NaBr	0.1	3375

[a] The reaction concentrations are 10 mM each in 0.1 M of citric acid–0.2 M of Na_2HPO_4 buffer.

rate (Table II). The addition of a low concentration of bromide ion to the frozen system gave a slight increase in reaction rate; however, higher concentrations of bromide ion caused a decrease in reaction rate (Table II). A change in reaction rate was also observed when the concentration of the buffer was changed, with higher concentrations giving lower reaction rates (*e.g.*, an initial rate of 663 μM/hr at 0.3 *M* buffer compared to 7000 μM/hr at 0.05 *M* buffer concentration). Therefore, it is generally observed that an increase in solute concentration results in a decrease of the nitrosation rate, even though the solute may have a catalytic effect in the unfrozen system.

The pH of the various frozen systems was determined with the indicator solutions. The reactants had no effect on the pH of the frozen samples. Since unusually high reaction rates were found for some systems initially at pH 7–8, special attention was paid to measurement of the final pH of these frozen systems. Systems with initial pH between 3 and 6 showed no pH change upon freezing. In systems with pH higher than 6, the final pH depended upon the nature of the buffer system. Thus, citrate–sodium hydroxide and citrate–potassium phosphate buffers do not change pH upon freezing, but citrate–sodium phosphate buffer at pH 8 decreases to pH 3.5 and sodium hydrogen phosphate at pH 9 decreases to pH 5.5 upon freezing.

At subfreezing temperatures the reaction remains second-order with respect to nitrite and first-order with respect to morpholine (Figure 2). When the reaction rate at −6° is compared to that at 25° for the same pH, an approximately constant ratio of rates is observed (Table III). Therefore the mechanism of the reaction in frozen systems appears identical to that in unfrozen solutions.

At −6° there was a 48-fold increase in reaction rate (Table I), which corresponds to a 3.6-fold increase in reac-

Table III. The Ratio of the Reaction Rate of Formation of Nitrosomorpholine in Frozen System at −6° to the Rate in Unfrozen System at 25° for the Same pH[a]

pH	Rate in frozen system at −6°	Rate at 25°	Ratio of rate
2.3	1000	420	2.38
2.5	1600	679	2.36
3.0	3750	1400	2.68
3.3	4000	1500	2.66
3.5	4000	1500	2.66
3.7	2800	1295	2.16

[a] Concentration of both nitrite and morpholine is 10 mM.

tant concentration for a third-order reaction. We found an approximately threefold increase in nitrite concentration at −6°; a similar concentration effect has been found for morpholine under nearly identical conditions (Oakenfull, 1972).

The good agreement between the calculated and measured reactant concentration and the effects of glycerol, chloride and bromide, and buffer concentration indicates that rate increase can be almost entirely accounted for by concentration effects. The results also agree with the unfrozen volumes of similar systems (van den Berg, 1966).

The unusual rate effect found for frozen systems containing sodium phosphate buffers appears to be caused by pH changes in the unfrozen portion of the system; anion catalysis by citrate or phosphate ion was ruled out as frozen citrate–NaOH buffer and buffers of high phosphate concentration showed no rate enhancement. van den Berg (1966) has studied the pH changes in buffers and foods during freezing. In the mixed solution of mono- and dipotassium phosphates, the saturation point for monopotassium phosphate (least soluble) was reached first. This salt precipitated, causing a pH shift toward the alkaline side. In the mixed solution of sodium phosphate, however, the pH changed predominantly to the acidic side due to the insolubility of Na_2HPO_4. We found that the pH in systems containing Na_2HPO_4 shifted to about 3.5 (which is near the optimum pH for nitrosation) from an initial pH 7 or 8. Also, since Na_2HPO_4 precipitated out, the solute concentration of the remaining unfrozen solution decreased. As previously demonstrated, the nitrosation reaction under frozen conditions is faster when it is conducted at lower solute concentration. Therefore, the shift in pH and decrease in solute concentration produce another apparent pH optimum in the reaction system initially at pH 8 with a reaction rate much higher than the reaction system at initial pH of 3.4.

Table IV. Nitrosomorpholine Formation from Morpholine and Nitrite[a] at pH 6 in Reconstituted Milk

Temp, °C	Concentration of dry milk,[b] %	Nitrosation rate,[c] μM/hr
25°	5	10
25°	10	10
−6°	5	44
−6°	10	36

[a] Concentration of both nitrite and morpholine is 20 mM each. [b] Dry milk was reconstituted in distilled water and sulfuric acid was added to adjust pH. [c] At pH 6 and 25°, nitrosation rate in citrate–phosphate buffer was 22 μM/hr at similar reactant concentration.

The extension of the previous experiments to more complex systems was performed in 5 and 10% reconstituted nonfat dry milk. The results are shown in Table IV. Under similar conditions of pH, the reaction rate is 50% slower in milk than in a simple buffer system. However, freezing the milk to −6° causes an approximate fourfold increase in rate relative to 25°, and about twice the rate in buffer at 25° at the same pH.

The application of these results toward the possibility of nitrosamine formation in foods is evident. The reaction rate in our system is approximately the same at −18° (0°F) and at 37° (100°F). 0°F is a common storage condition for frozen foods of all types; the frozen state is obviously not a deterrent to nitrosation reactions. In fact, this phenomenon was observed over 20 years ago for color formation in nitrite-treated meat products (Watts and Lehmann, 1952) wherein color formation progressed under frozen conditions and was even accelerated in the presence of ascorbic acid.

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