

## ACKNOWLEDGMENT

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## Factors Influencing the Rate of Formation of Nitrosomorpholine from Morpholine and Nitrite: Acceleration by Thiocyanate and Other Anions

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The kinetics of nitrosation of morpholine, a weak base and possible food contaminant, have been investigated. Temperature, pH, and composition of the reaction medium have been explored as experimental variables, and certain anions have been shown to influence strongly the reaction rate. The extent of the effect of thiocyanate, a

strong promoter of nitrosation, is dependent upon pH and relative reactant concentrations. pH-independent rate constants have been calculated for two nitrosation mechanisms, and mathematical expressions have been derived which accurately predict overall rates of nitrosation.

The toxicity and carcinogenicity of nitrosamines are well known, and the subject has recently been reviewed (Lijinsky and Epstein, 1970; Wolff and Wasserman, 1972). Morpholine is a particularly interesting compound for the study of nitrosation reactions for a variety of reasons, including the fact that as a weak base it will nitrosate very rapidly compared to compounds like dimethylamine. Morpholine is also a potential inadvertent food contaminant, since it is a permitted corrosion inhibitor in steam boilers. Also, a variety of substituted morpholine structures are naturally occurring compounds. Finally, *N*-nitrosomorpholine is a known carcinogen for the rat (Druckrey *et al.*, 1967).

Previous investigations on nitrosation kinetics (Mirvish, 1970, 1972) have only briefly considered morpholine, and the accelerating influence of thiocyanate on morpholine nitrosation was first demonstrated by Boyland (1972) and Boyland *et al.* (1971). This latter investigation neglected the influence of pH on reaction rate. In this paper, pH-independent rate constants are calculated for morpholine nitrosation under a variety of reaction conditions, and the temperature-dependency of the reaction is also analyzed.

## MATERIALS AND METHODS

All chemicals were reagent grade. Solutions of amine and sodium nitrite were separately adjusted to the reaction pH with perchloric acid and mixed immediately prior to incubation. Anions were used as their potassium salts

and were preincubated with the amine solution. After initiation of the reaction, samples were taken at fixed intervals and quenched by the immediate addition of a large excess of ammonium sulfamate. At reaction pH's above 2, it was necessary to heat the solution to 65° for 5 min after ammonium sulfamate addition. When the pH was above 3.5, perchloric acid was added to reduce the pH to below 3.0. A zero time sample, to which ammonium sulfamate was added immediately following the mixing of nitrite and morpholine, was included in each kinetic run.

The concentration of *N*-nitrosomorpholine (NM) was measured spectrophotometrically at 260 nm using an absorbance of 3540 l. cm<sup>-1</sup> mol<sup>-1</sup>. Although  $\lambda_{\max}$  for NM is at 235 nm, the substantial absorption of the parent amine at this wavelength makes it simpler to use 260 nm. NM solutions obey the Lambert-Beer law at this latter wavelength over the concentration range studied. Nitrite was measured using the automatic procedure of Fan and Tannenbaum (1971).

Rate constants were determined from initial rates, since nitrite concentrations in open vessels are inconstant upon prolonged incubation (Table I). The method used was the Method of Initial Rates: the NM concentration was plotted as a function of time, the best curve fitted with a French Curve, and the best straight line fitted with a straight edge between the origin ( $t = 0$ , NM = 0) and the French Curve. This straight line was the tangent to the curve, and its slope was the initial reaction rate. A typical run had the following reaction conditions: nitrite and morpholine were each 5 to 10 mM, temperature held constant at 25°, and the reaction was followed for 30 to 60 min. The yield of nitrosomorpholine varied from 0.5 to 5% of the theoretical maximum.

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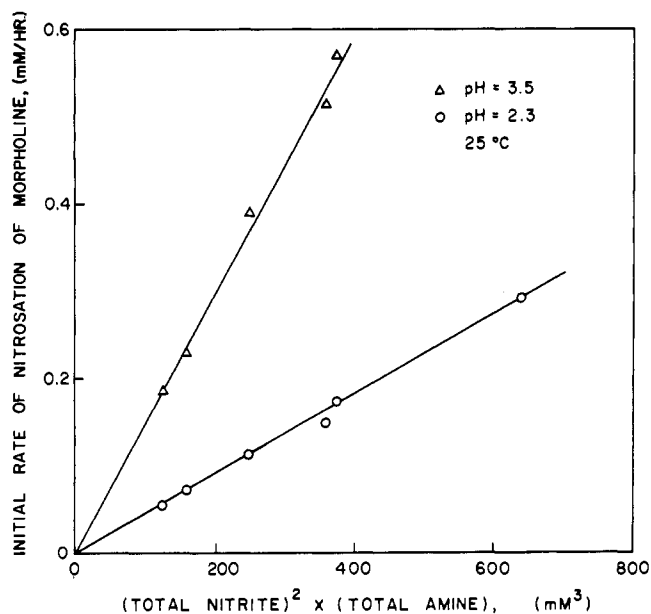


Figure 1. Third-order rate plot for nitrosation of morpholine.

## RESULTS

**Reaction of Morpholine with Nitrite.** At constant pH, the rate of nitrosation is dependent upon the square of the total nitrite concentration and the morpholine concentration. As shown in Figure 1, the rate is proportional to pH, since the reaction takes place between the free basic form of morpholine and nonionized nitrous acid (Morrison and Boyd, 1966). The complete pH-rate profile is shown in Figure 2, along with the influence of bromide and thiocyanate. In the absence of added anions, the rate maximum occurs in the region of pH 3.4, which is also the approximate  $pK_a$  for  $HNO_2$ . From the data in Figure 2, a pH-independent rate constant ( $k'_n$ ) can be calculated from the following equation, where  $k_n$  is the rate constant at any pH.

$$k'_n = k_n [HNO_2 / \text{total nitrite}]^{-2} \times [\text{free amine} / \text{total amine}]^{-1} \quad (1)$$

Both  $k_n$  and  $k'_n$  are tabulated as a function of pH in Table II, from which the constancy of  $k'_n$  and the validity of eq 1 can be ascertained.

**Effect of Anions on Nitrosation.** The effect of various anions on the rate of nitrosation can be demonstrated either by the use of various acids for pH adjustment (Table III) or the addition of a variety of salts to a single acid (Table IV). The extent of the effect depends upon the nature of the anion and its concentration. Thiocyanate is by far the most effective promoter of nitrosation among com-

Table I. Loss of Nitrite<sup>a</sup> as a Function of Incubation<sup>b</sup> pH and Temperature

pH	% recovery at	
	23°	50°
1	59	26
2	62	31
3	76	48
4	100	90
5	100	94
6	100	100
7	100	100

<sup>a</sup> Initial concentration, 10 mM. <sup>b</sup> Incubation time, 3 hr.

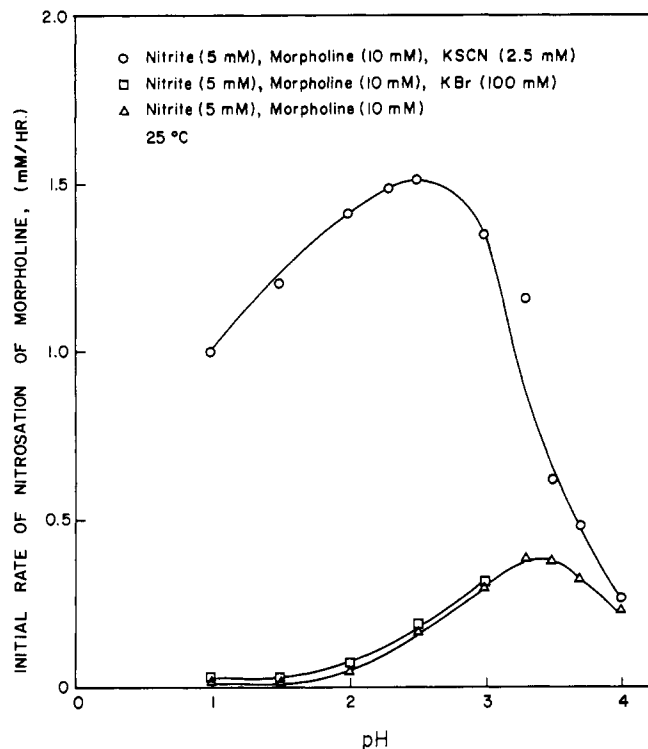


Figure 2. Initial rate of nitrosation of morpholine with nitrite. pH dependency and influence of certain ions.

pounds tested (Figure 2), with the order of effect being  $SCN^- \gg Br^- \gg Cl^- > SO_4^{2-} = ClO_4^- = H_2PO_4^- = RCO_2^-$ . Other changes in the nature of the nitrosation reaction also occur upon the addition of anions, including a pronounced shift in the pH optimum for the reaction (Figure 2) and a change in the influence of reactant concentration on the rate of nitrosation (Figure 3). This latter effect, which will be further elucidated in the Discussion section, demonstrates that although reaction rate increases as a result of an increase in nitrite concentration, a net decrease in rate can actually result from a relative increase in thiocyanate concentration.

**Effect of Temperature on Reaction Rate.** The effect of temperature on reaction rate is accurately described by an Arrhenius plot, as shown in Figure 4. The activation energy in the absence or presence of thiocyanate is approximately 10 kcal/mol.

## DISCUSSION

The rate of nitrosation of morpholine in the absence of an anionic promoter is given by eq 2.

$$d[NM]/dt = k'_n [HNO_2]^2 [R_2NH] \quad (2)$$

Table II. pH-Dependent ( $k_n$ ) and Independent ( $k'_n$ ) Rate Constants for the Nitrosation of Morpholine<sup>a</sup>

pH	$M^{-2} \text{ hr}^{-1}$	
	$k_n$	$k'_n$
2.3	$4.2 \times 10^2$	$9.1 \times 10^8$
2.5	$6.8 \times 10^2$	$7.0 \times 10^8$
3.0	$1.4 \times 10^3$	$7.7 \times 10^8$
3.3	$1.5 \times 10^3$	$8.6 \times 10^8$
3.5	$1.5 \times 10^3$	$7.7 \times 10^8$
3.7	$1.3 \times 10^3$	$8.6 \times 10^8$
4.0	$8.9 \times 10^2$	$8.5 \times 10^8$

<sup>a</sup> 25°.

**Table III. Influence of Different Acids on the Rate of Nitrosation of Morpholine<sup>a</sup>**

Acid used to adjust pH	Initial rate of nitrosation, $\mu\text{M}/\text{hr}$
Hydrochloric acid	199
Perchloric acid	31
Sulfuric acid	28

<sup>a</sup> 50°, pH 0.5, all reactants 10 mM.

The  $pK_a$ 's of nitrous acid and morpholine are known to be 3.36 and 8.5, respectively (Weast, 1968), and the exact concentrations of the reactive species can be calculated for any pH by the Henderson-Hasselbach equation.

$$\text{pH} = pK_a + \log[A^-]/[HA] \quad (3)$$

By substitution, therefore, the rate of nitrosation becomes

$$d[\text{NM}]/dt = k'_n \{[\text{antilog}(3.36 - \text{pH})] / [1 + \text{antilog}(3.36 - \text{pH})]^2 \{[\text{antilog}(\text{pH} - 8.5)] / [1 + \text{antilog}(\text{pH} - 8.5)]\} \} \quad (4)$$

The maximum rate will be found by differentiating eq 4 with respect to pH and setting it equal to 0. This can be shown to give eq 4a, where the pH for maximum reaction rate is  $\text{pH}^*$ .

$$10^{3.36 - \text{pH}^*} - 2[10^{\text{pH}^* - 8.5}] = 1 \quad (4a)$$

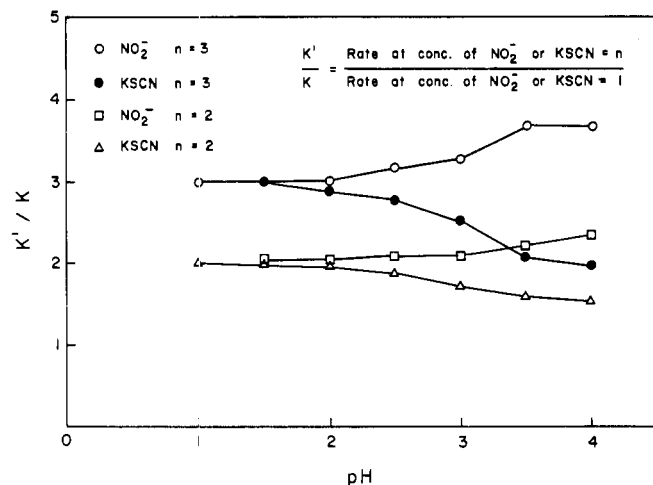
Since the term  $10^{\text{pH} - 8.5}$  would be a very small number for values of pH 5 or less, 4a simplifies to

$$10^{3.36 - \text{pH}^*} = 1$$

and

$$\text{pH}^* = 3.36 \quad (4b)$$

Therefore, the nitrosation of morpholine or any other amine which has a  $pK_a$  greater than 5 will proceed at the fastest rate at pH 3.36 or the corresponding  $pK_a$  for nitrous acid at the actual reaction temperature. This effect was found for the case of nitrosation of dimethylamine (Mirvish, 1970) but the same author found a rate maximum at pH 3.0 for piperidine, morpholine, and mononitrosopiperazine (Mirvish, 1972). Since the reaction conditions were not completely specified in the latter publication, it is not possible at this time to account for this discrepancy. However, since the pH maxima for these reactions are broad (Figure 2), an insufficient number of observations could easily lead to an inaccurate value for the maximum.



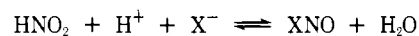
**Figure 3.** The effect of changing nitrite and KSCN concentration on nitrosation of morpholine.

**Table IV. The Effect of Addition of KCl and KBr on the Rate of Nitrosation of Morpholine<sup>a</sup>**

Ion used	Initial rate of nitrosation, $\mu\text{M}/\text{hr}$
Control	28
KCl, 100 mM	60
KCl, 300 mM	97
KBr, 100 mM	1473
KBr, 300 mM	3220

<sup>a</sup> 50°, pH 0.5 adjusted with  $\text{H}_2\text{SO}_4$ , reactants 10 mM.

Boyland *et al.* (1971) and Boyland (1972) have previously demonstrated that several anions could accelerate the rate of nitrosation of morpholine and *N*-methylaniline. They did not present any detailed analysis of the reaction kinetics. It is apparent from Figure 3 that the mechanism of nitrosation is different in the absence and presence of thiocyanate, since the proportionality of rate to reactant concentration changes as a function of pH. A probable mechanism for anion participation is given by the reactions proposed by Hughes *et al.* (1958a,b) and Turney and Wright (1959).



The new rate expression for nitrosation by this mechanism would be

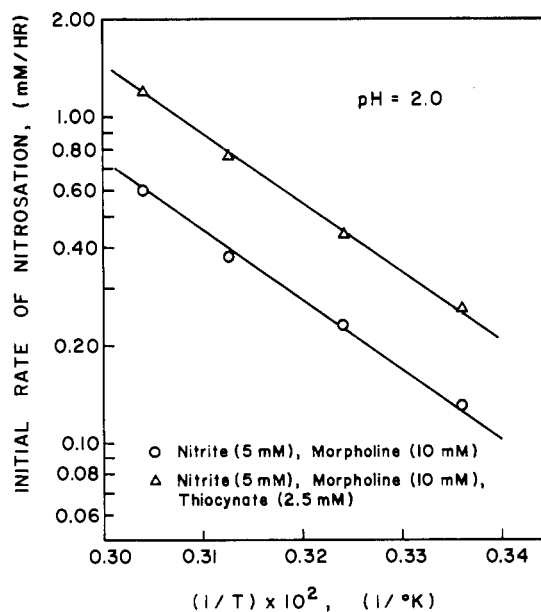
$$d[\text{R}_2\text{NNO}]/dt = k'_x [\text{HNO}_2][\text{H}^+][\text{X}^-][\text{R}_2\text{NH}] \quad (6)$$

If both mechanisms were to operate simultaneously, the overall rate would be

$$d[\text{NM}]/dt = k'_n [\text{HNO}_2]^2 [\text{R}_2\text{NH}] + k'_x [\text{HNO}_2][\text{H}^+][\text{X}^-][\text{R}_2\text{NH}] \quad (7)$$

which can be rearranged to

$$d[\text{NM}]/dt = [\text{HNO}_2][\text{H}^+][\text{R}_2\text{NH}] \{ k'_n [\text{NO}_2^-] / K_{\text{equil. nitrite}} + k'_x [\text{X}^-] \} \quad (8)$$



**Figure 4.** The effect of temperature on the nitrosation of morpholine.

**Table V. pH-Independent Rate Constants<sup>a</sup> for the Nitrosation of Morpholine in the Presence of Anions**

pH	$k'_n$ ( $M^{-2} \text{ hr}^{-1}$ )	$k'_x$ ( $M^{-3} \text{ hr}^{-1}$ )	
		Thiocyanate	Bromide
0.5	<i>b</i>	<i>c</i>	$7.5 \times 10^9$
1.0	<i>b</i>	$2.6 \times 10^{12}$	$8.0 \times 10^9$
1.5	<i>b</i>	$3.2 \times 10^{12}$	$7.4 \times 10^9$
2.0	<i>b</i>	$3.7 \times 10^{12}$	$8.8 \times 10^9$
2.5	$8.0 \times 10^8$	$3.4 \times 10^{12}$	$8.0 \times 10^9$
3.0	$8.7 \times 10^8$	$3.7 \times 10^{12}$	$7.1 \times 10^9$
3.5	$9.0 \times 10^8$	$3.7 \times 10^{12}$	<i>c</i>
4.0	$7.6 \times 10^8$	$3.4 \times 10^{12}$	<i>c</i>

<sup>a</sup> Rate constants correspond to eq 7 for a temperature of 25°. <sup>b</sup> Contribution to overall rate too small to be evaluated. <sup>c</sup> Not evaluated.

The values of  $k'_n$  and  $k'_x$  for the nitrosation reaction in the presence of thiocyanate and bromide are given in Table V. The constancy of the pH-independent rate constants over most of the pH range and the good agreement between the  $k'_n$  values in Tables II and V demonstrate the veracity of eq 7.

The results in Figure 3 can be explained by eq 8. At pH values below 2

$$k'_x[X^-] \gg k'_n[\text{NO}_2^-] \quad (9)$$

and the rate of nitrosation is first-order with respect to nitrous acid, amine, and  $X^-$ . At pH's greater than 2,  $k'_n[\text{NO}_2^-]$  increases and both nitrosating mechanisms become operative. Therefore, conditions may exist when the overall reaction rate is between first- and second-order for nitrite, and less than first-order for  $X^-$ .

The effectiveness of various anions as activators would appear to be approximately related to their relative nucleophilicity, as given by Hine (1956). The experimental proportionality of the  $k'_x$ 's for thiocyanate, bromide, and chloride equals 15,000:30:1. The values of their nucleophilic constants would predict ratios of 54:7:1 and thus nucleophilicity alone does not explain their catalytic effectiveness.

The velocity of formation of NOX is approximately similar for NOCl, NOBr, and NOCNS (975, 1170, and 1460  $M^{-2} \text{ sec}^{-1}$ , respectively; Dahn *et al.*, 1960). The equilibrium constants are different, however (Turney and Wright, 1959), possibly providing some of the difference in effectiveness of various anions as reaction promoters.

In Figure 2, the optimum pH was shifted from 3.4 to 2.3 in the presence of thiocyanate. However, according to eq 7, the overall pH optimum will be determined by the relative value of each term in the equation and will therefore depend upon anion concentration. The equilibrium constants for dissociation of HX and formation of NOX will also be important.

$$K_{\text{equil}} = ([\text{NOX}]a_x)/([\text{HNO}_2][X^-]a_{\text{H}^+}) \quad (10)$$

There is an implicit assumption in deriving eq 7 from eq 5 that the actual concentration of NOX is sufficiently small so that  $\text{HNO}_2$  is not depleted. If, however, large concentration ratios of anion to nitrite are employed, one might expect a depression in reaction rate. This has been actually demonstrated for KBr, where reactant excesses of bromide more than 20-fold excess can cause no increase or even cause a decrease in reaction rate.

In eq 6 and 7, the product of  $[\text{H}^+]$  and  $[\text{R}_2\text{NH}]$  is constant at any pH, while  $[\text{X}^-]$  is constant only if HX is fully ionized at all values of pH investigated. The rate of nitrosation by NOX is also dependent on  $[\text{HNO}_2]$ , which increases when pH decreases until it approaches its maximum concentration at about pH 2. Nitrosation by  $\text{N}_2\text{O}_3$  has already been shown to have a maximum rate at pH 3.36. The optimum pH in the presence of anions will therefore occur between pH 2 to 3.36, with higher concentrations of anions shifting the optimum pH closer to pH 2.

Since the rate of nitrosation by  $\text{N}_2\text{O}_3$  below pH 2 is negligible compared to the rate of nitrosation by NOX, eq 6 would predict a constant nitrosation rate if HX is fully ionized. The pH rate curve for thiocyanate shows a steady rate decrease between pH 2-1, while bromide remains approximately constant over the same range (Figure 2). In Table V it can also be seen that the rate constants, calculated on the basis of complete ionization of HX, decrease with pH below pH 2 for thiocyanate but remain constant down to pH 0.5 for bromide. It is probable that these results can be explained by the fact that HBr is indeed a strong acid, fully ionized at all values of hydrogen ion activity investigated, while HSCN is only weakly ionized below pH 2.

It is expected that the improved ability to predict nitrosation rates afforded by these studies will enable a more accurate assessment of the extent of nitrosomorpholine formation in foods and in the living animal. Attempts to correlate these findings with feeding studies in animals will be reported at a future date.

#### ACKNOWLEDGMENT

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