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Nonenzymatic Microbial Acceleration of Nitrosamine Formation

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Rate enhancements of from 12- to 49-fold occurred when dihexylamine was nitrosated at pH 3.5 in the presence of bacteria and yeast cells at a concentration of 12 mg/mL. Rates were similar in the presence of either boiled or unheated cells. The magnitude of the rate enhancement for nitrosation of other amines depended on the alkyl chain length. A nonenzymatic mechanism involving hydrophobic interactions of the precursor amines and cellular constituents is proposed.

Several authors have described the formation of carcinogenic nitrosamines in the presence of microorganisms (Sander, 1968; Klubes and Jondorf, 1971; Hawksworth and Hill, 1971, 1974; Collins-Thompson et al., 1972; Klubes et al., 1972; Ayanaba and Alexander, 1973; Ayanaba et al., 1973; Thacker and Brooks, 1974; Mills and Alexander, 1976). These reports have primarily concerned the nitrosation of dimethylamine and diethylamine at pH values above 5.0. In several microbial cultures, nitrosamine formation is apparently nonenzymatic (Klubes and Jondorf, 1971; Collins-Thompson et al., 1972; Mills and Alexander, 1976), and some have proposed that the reaction is catalyzed by one or more unidentified metabolic products. Another possibility is the enzymatic catalysis of nitrosamine formation in the presence of microorganisms (Hawksworth and Hill, 1971; Klubes and Jondorf, 1971; Klubes et al., 1972; Ayanaba and Alexander, 1973; Ayanaba et al., 1973; Sherbet and Lakshmi, 1973), but the evidence for such involvement of an enzyme is far from clear.

We have recently reported large rate enhancements for the nitrosation of several amines in the presence of micelle-forming surfactants (Okun and Archer, 1977). The present investigation, as an extension of these findings, was designed to examine the effects of microorganisms on nitrosamine formation at acidic pH. These conditions are of potential relevance to nitrosation of amines in the environment of the stomach, in which microorganisms derived both from food and the oral cavity may be present.

MATERIALS AND METHODS

Dimethylamine, di-*n*-butylamine, di-*n*-pentylamine, di-*n*-hexylamine, and piperidine were purchased from Eastman Organic Chemicals (Rochester, N.Y.), morpholine from Fisher Scientific Co. (Pittsburgh, Pa.), and diethylamine from J. T. Baker Chemical Co. (Phillipsburg, N.J.). All amines were purified by distillation prior to use.

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Di-*n*-butylnitrosamine, diethylnitrosamine, nitrosopiperdine, and nitrosomorpholine were purchased from Eastman Organic Chemicals. Di-*n*-hexylnitrosamine was a generous gift from Dr. Harold Röper of the University of Hamburg. Di-*n*-pentylnitrosamine was synthesized from the purified parent amine according to the method of Druckrey et al. (1967).

Escherichia coli B (ATCC 11303) was purchased from Sigma (St. Louis, Mo.) and *Saccharomyces cerevisiae* was from Standard Brands Inc. (New York, N.Y.). *Saccharomycopsis lipolytica*, a generous gift from L. J. Wickerham (Northern Regional Research Laboratory, Peoria, Ill.), was grown in a medium containing 1% yeast extract, 2% peptone, 3% glycerol, and 0.1% dextrose. *Bacillus brevis* (ATCC 9999) was grown in a medium containing 5% yeast extract, 5% peptone, and 5% glucose. Cells were harvested in the stationary phase by centrifugation. Cell concentrations are in all cases reported as dry weight measurements.

Kinetic runs were performed in stoppered centrifuge tubes. Reaction mixtures (5-mL volumes) contained 20 mM amine and 20 mM sodium nitrite (unless otherwise specified) in citrate–phosphate buffer, pH 3.5, at 25 °C. The buffer was prepared by mixing 0.1 M citric acid with 0.2 M Na_2HPO_4. The microbial cell suspension was first added to a buffered solution of the amine and incubated for about 30 min. Reactions were initiated by addition of nitrite; at various times (0, 10, 20, 30, and 40 min), the nitrosation reactions were stopped by adding an excess of solid ammonium sulfamate. After incubation for about 20 min to insure destruction of nitrite, the contents of the tubes were extracted with an equal volume of methylene chloride. The nitrosamine concentration was then determined by a combined gas chromatography/thermal energy analysis system, as described by Fine and Roubehler (1975). Chromatography was performed on a column ($^1/_8$ in. × 10 ft) packed with 3% OV-17 or 10% FFAP on Chromosorb G. Control reactions in the absence of amine, nitrite, or cells were run in a similar manner.

In order to investigate the distribution of the rate-enhancement effect within the cells, we suspended 1 g of *E.*

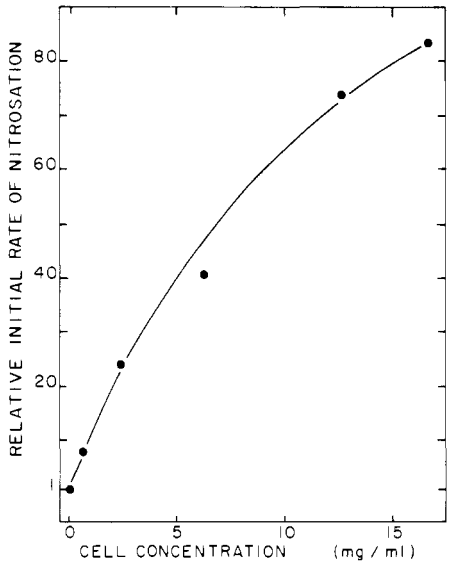

Figure 1. Relative initial rates (+cells/−cells) of nitrosation of dihexylamine as a function of *Saccharomycopsis lipolytica* concentration; conditions: 20 mM dihexylamine and 20 mM nitrite in citrate–phosphate buffer, pH 3.5, at 25 °C. Under these conditions, in the absence of cells, the initial rate of dihexylnitrosamine formation was 13 μM/h.

Table I. Effect of Various Microbial Cells on the Kinetics of Dihexylnitrosamine Formation[a]

Cells	Ratio of initial rates (+cells/−cells)	
	Untreated cells	Boiled cells
Saccharomycopsis lipolytica	49	43
Saccharomyces cerevisiae	12	21
Escherichia coli	35	36
Bacillus brevis	17	

[a] Conditions: 20 mM dihexylamine; 20 mM nitrite; 12 mg/mL cells; in citrate–phosphate buffer, pH 3.5, at 25 °C; cells were boiled for 15 min to destroy enzymatic activity.

coli in 50 mL of buffer. The suspension was sonicated for 3 min, while it cooled on ice, and then centrifuged at 9000*g* for 10 min. After decanting the supernatant fraction, the pellet was resuspended in buffer, sonicated for a further 3 min, and centrifuged as before. The pellet was finally suspended in 50 mL of buffer, to which was added dihexylamine and nitrite to yield final concentrations of 20 mM. The kinetics of dihexylnitrosamine formation was investigated as previously described. It was also examined in the supernatant fraction derived from the first centrifugation.

RESULTS

Figure 1 illustrates the relative initial rates of nitrosation of dihexylamine in the presence of various concentrations of *Saccharomycopsis lipolytica* cells in buffered solution at pH 3.5. As we increased the cell concentration in the reaction mixture, the initial rate of nitrosamine formation increased. At a cell concentration of 15 mg/mL, an 80-fold enhancement of the initial rate was observed.

The effect of several other microorganisms on the rate of nitrosation of dihexylamine at pH 3.5 is shown in Table I. At the same cell concentrations, *Saccharomyces cerevisiae*, *E. coli*, and *B. brevis* all gave substantial rate enhancements. In addition, cells that had been boiled for 15 min to destroy enzymatic activity were investigated for their effect on nitrosation. Table I shows that rate enhancements in the presence of boiled cells were similar to those in the presence of untreated cells.

Table II. Effect of *Saccharomycopsis lipolytica* on the Kinetics of Nitrosamine Formation for Some Secondary Amines[a]

Amine	Ratio of initial rates (+cells/−cells)
Dimethyl	1
Diethyl	1
Dibutyl	7
Dipentyl	12
Dihexyl	74
Morpholine	1
Piperidine	1

[a] Conditions: 20 mM amine; 60 mM nitrite; 11.4 mg/mL *Saccharomycopsis lipolytica*; in citrate–phosphate buffer, pH 3.5, at 25 °C.

Table II illustrates the influence of amine structure on the rate of nitrosation in the presence of *Saccharomycopsis lipolytica*. Dimethylamine and diethylamine showed no increase in rate in the presence of cells. With dibutylamine, however, a rate enhancement of 7 was observed in the presence of cells. As the alkyl chain length of the secondary amine increased further, the magnitude of the rate enhancement for nitrosation also increased. Of the dialkylamines examined, dihexylamine was subject to the greatest acceleration of rate. Nitrosation of the heterocyclic secondary amines, morpholine and piperidine, was not affected by the presence of cells.

In order to investigate the distribution of the rate-enhancement effect within the organism, *E. coli* cells were subjected to sonic disruption, followed by centrifugation. The rate of nitrosation of dihexylamine was measured in both the resuspended particulate material and in the soluble supernatant. The results showed that the particulate fraction was responsible for 80% of the acceleration effect while the soluble cell fraction accounted for the remaining 20% of the rate enhancement.

DISCUSSION

Our results show that the rates of formation of certain dialkylnitrosamines can be accelerated at pH 3.5 in the presence of various microorganisms, including both gram-negative and gram-positive bacteria and also yeast. The magnitude of the rate enhancements were similar for both boiled and untreated cells. The effect thus appears to be nonenzymatic. The increase in overall rate enhancement with increasing alkyl chain length is very similar to the variation in catalytic rate with amine structure that we observed for nitrosation in the presence of micelles (Okun and Archer, 1977). This structure–activity relationship indicated that the acceleration of nitrosation in the presence of cells was caused by hydrophobic interaction of the precursor amine with a cellular constituent, possibly a component of the cell wall, the cytoplasmic membrane, or other intracellular membranous structure. Our finding that most of the rate-enhancement effect was located in the particulate fraction following sonic disruption of cells corroborates this hypothesis. Nitrosation rates would then be increased in an analogous manner to micellar catalysis. The lipid bilayer of cell membranes has recently been shown to be a solvent for small hydrophobic molecules (White, 1976). Thus dissolution of the dialkylamines in membranes is feasible and will lead to a concentration of one of the reactants. In addition, at pH 3.5, regions of the cell surface may be expected to carry a net positive charge (Sherbet and Lakshmi, 1973); hence, one might anticipate a contribution to the rate enhancement occurring from electrostatic

destabilization of the protonated form of the amine with respect to the unprotonated form. This type of electrostatic effect explains, in part, the rate enhancements for nitrosation in the presence of cationic micelles (Okun and Archer, 1977). Moreover, cationic regions of the cell surface would attract nitrite ions, thereby producing high concentrations of the nitrosating agent in the vicinity of the amine.

The effect of amine structure on nitrosation in the presence of cells follows a similar pattern to nitrosamine formation in saliva, which we have previously investigated (Tannenbaum et al., 1977). Thus, the formation of nitrosamines in saliva—at close to neutral pH in this study—may be subject to the same kind of microbial rate acceleration. Similar microbial effects on nitrosamine formation may be expected in other situations of environmental importance, such as in the food itself, in the stomach, or in the intestinal tract. At pH 3.5, we did not observe rate enhancements for formation of dimethylnitrosamine or diethylnitrosamine in the presence of cells. Since microorganism-dependent formation of these products has been reported at higher pHs (Sander, 1968; Hawksworth and Hill, 1971, 1974; Klubes and Jondorf, 1971; Klubes et al., 1972; Thacker and Brooks, 1974; Mills and Alexander, 1976), mechanisms other than the one described here are clearly operative.

Nonenzymatic cellular rate enhancement of chemical reactions facilitated by hydrophobic forces has not previously been described. In addition to its potential importance in nitrosamine formation, it may also be a more general phenomenon for other reactions in which hydrophobic interactions between the cell, reactants, transition states, and products are possible.

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Formation of Bis(hydroxyalkyl)-*N*-nitrosamines as Products of the Nitrosation of Spermidine

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The biologically occurring polyamine spermidine was reacted with nitrite in the presence of acid. Reaction products were isolated and converted to trimethylsilyl (Me_3Si) derivatives. The derivatized products were separated by gas–liquid chromatography using an all-glass, wall-coated, capillary column. Mass spectral data were collected on the chromatographic effluent. Four isomers of bis(hydroxyalkyl)-*N*-nitrosamine were synthesized by combining the appropriate aminopropanol with the appropriate chlorobutanol. Infrared, nuclear magnetic resonance, mass spectral, and thin-layer chromatographic data were collected on the synthetic compounds to assure their structure. Kovat's indices were determined for the Me_3Si derivative of each authentic compound and compared to the retention data collected on the Me_3Si derivatives of the reaction products of spermidine and nitrite. A comparison of the mass spectra of the Me_3Si derivatives of the authentic compounds to the spectra of the reaction products having the same retention time allowed structural assignments to four compounds. The compounds identified, in order of decreasing amounts, were: 4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine, 3-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine, 4-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine, and 3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine.

The polyamine spermidine, $H_2N(CH_2)_3NH(CH_2)_4NH_2$, is ubiquitous in biological materials and is found in many food materials including several cereal germs (Moruzzi and Caldarera, 1964) and soybean flour (Wang, 1972). The levels found in pork and pork products are of special interest. Lakritz et al. (1975) reported concentrations of spermidine as high as 1013 mg/100 g of wet tissue in putrefied pork ham-butt portions. Fresh pork ham-butt portions ranged in concentration from 13.4 to 125 mg/100 g of tissue. Spinelli et al. (1974) reported spermidine

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