

# Microbial Formation of Nitrosamines In Vitro

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*Mortierella parvispora* and an unidentified bacterium converted trimethylamine to dimethylamine, and the bacterium (but not the fungus) formed dimethylnitrosamine in the presence of nitrite. Dimethylnitrosamine also appeared in cell suspensions of *Escherichia coli* and *Streptococcus epidermidis* and in hyphal mats of *Aspergillus oryzae* incubated with dimethylamine and nitrate. Suspensions of a number of microorganisms produced *N*-nitrosodiphenylamine from diphenylamine and nitrite at pH 7.5, and soluble enzymes catalyzing the *N*-nitrosation of diphenylamine were obtained from two of these organisms. In the presence of these enzymes, several dialkylamines were converted to the corresponding *N*-nitroso compounds.

Nitrosamines have recently attracted considerable interest because members of this group of compounds have been found to be carcinogenic, mutagenic, and occasionally teratogenic. Dimethylnitrosamine (DMNA), for example, is both carcinogenic and mutagenic (7, 8, 14). Although it has been proposed that they may be significant environmental carcinogens (13), whether they do indeed pose a hazard to human health will be determined by the existence of the necessary precursors in nature, the occurrence of the nitrosation reaction, and the exposure of humans to products of the reaction.

The formation of the nitrosamine probably requires the presence of both a secondary amine and nitrite. Secondary amines may be produced from tertiary amines, and nitrite is generated microbiologically from ammonium and nitrate. These nitrogenous precursors are widespread. Not only are nitrate and ammonium naturally present in water, soil, and plants consumed by man, but large quantities of nitrate and nitrite are added to meat products as preservatives and color-enhancing agents (5). Secondary amines are present in urine (3) and in many habitats where microorganisms proliferate (13), and trimethylamine is found in fish (23) and algae (21).

Recent evidence suggests that microorganisms are involved in one or more stages of nitrosamine synthesis. For instance, nitrosopiperidine is formed in the rat's stomach and intestine from added nitrate and piperidine, and microorganisms presumably reduce

the nitrate to nitrite (2). DMNA has been observed in the urine of humans infected with *Proteus mirabilis* (4). Either added trimethylamine or the fungicide thiram and nitrate are converted to DMNA in water and soil, and the indigenous communities convert the tertiary amines to dimethylamine (DMA), nitrate to nitrite, and presumably sometimes serve as nitrosating agents (Ayanaba, Verstraete, and Alexander, unpublished data). It has also been reported that strains of several bacterial species form nitrosamines in vitro (10, 22).

The present study was designed (i) to demonstrate the biosynthesis of nitrosamines by cells maintained under conditions where nonbiological nitrosation would not confuse the interpretation of a presumed microbial role in the nitrosation reaction, and (ii) to obtain an active nitrosating enzyme. No such enzyme has yet been characterized.

## MATERIALS AND METHODS

Six bacterial isolates, designated strains 1 to 6, that utilize nitrate as a nitrogen source were obtained from garden soil by using a medium containing 0.2% KNO<sub>3</sub>, 2.0% glucose, 0.53% KH<sub>2</sub>PO<sub>4</sub>, 1.06% K<sub>2</sub>HPO<sub>4</sub>, 0.01% MgSO<sub>4</sub>, and micronutrients added at the rates used by Powell and Errington (18). The solution without glucose and nitrate was designated solution S. One bacterium, strain B, which utilizes trimethylamine as a carbon and energy source, was isolated from Beebe Lake water enriched with 0.50% of the amine as its hydrochloride, 0.53% KH<sub>2</sub>PO<sub>4</sub>, and 1.06% K<sub>2</sub>HPO<sub>4</sub>. The enriched water was incubated on a rotary shaker at 30 C for 48 h, after which the suspension was streaked on a solid medium of the same composition. To obtain isolates that use DMA as a carbon or nitrogen source, 0.10 g of garden soil or

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fresh cow manure or 1.0 ml of municipal sewage was introduced into media containing 0.50% DMA·HCl, solution S, and either 0.50% glucose, 0.20%  $(\text{NH}_4)_2\text{SO}_4$ , or no addition. Samples from those flasks which became turbid were repeatedly streaked on a solid medium identical to the enrichment solution, and by this means bacterial strains 8 to 11, a fungus (strain 1), and two yeasts were obtained in axenic culture.

To prepare cell suspensions of the bacteria, the organisms were grown in the liquid isolation medium at 30 C on a rotary shaker for 24 to 48 h. The cells were harvested aseptically by centrifugation in the cold and washed twice with sterile 0.1 M phosphate or 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0.

To obtain large yields of bacterial cells, the cultures were grown with constant aeration and agitation in 6 liters of the appropriate medium contained in a 15-liter fermentor maintained at 30 C. The cells were collected by centrifugation during the early stationary phase of growth (after 24 to 48 h) and washed twice in the phosphate buffer. These cells (5 to 10 g wet weight) contained in 15 to 20 ml of Tris or phosphate buffer were introduced into appropriate solutions.

The fungi were grown at 30 C in 500-ml Fernbach flasks containing 200 ml of the medium of Pengra, Cole, and Alexander (17), except that fungus 1 was grown in a medium containing 0.5% glucose and 0.5% DMA·HCl in solution S. After 7 to 10 days the culture filtrates were carefully decanted from the flasks, and the mycelial mats were washed twice with Tris buffer, pH 7.0.

The cells were ruptured with a French pressure cell. The microbial suspension (3 to 5 g wet weight in 30 ml of 0.1 M phosphate buffer, pH 7.5) was passed twice through the instrument, and a pressure of 15,000 lbs/in<sup>2</sup> was used for disruption. The cell debris and unbroken cells were removed by centrifugation at 10,000 × *g* for 15 min, and the supernatant fluid, or crude cell extract, was carefully removed. The crude cell extract was separated into soluble and particulate fractions by centrifugation at 144,000 × *g* for 60 min, and the pellet was suspended in 0.1 M phosphate buffer, pH 7.5. The crude cell extract and the fractions centrifuged at 144,000 × *g* were used within 60 min of centrifugation.

To determine the nitrosating activity of the extracts, 8.0 ml of 0.1 M phosphate buffer (pH 7.5), 0.15 g of NaNO<sub>2</sub>, 0.10 g of glucose, 0.05 g of diphenylamine, and 2.0 ml of either untreated or boiled extracts were introduced into 50-ml Erlenmeyer flasks. The pH values of the reaction mixtures were maintained at 7.5 by additions of NaOH, and the flasks were incubated with shaking at 30 C. (Caution must be exercised in handling samples which contain or are suspected to contain nitrosamines because of their volatility and toxicity.)

The nitrite content of the supernatant fluid remaining after centrifugation at 4,600 × *g* for 10 min was estimated by the method of Montgomery and Dymock (16). Samples to be analyzed for DMA and nitroso compounds were steam-distilled (11), and DMA was estimated by the method of Pribyl and

Nedbalkova (20), whereas a photochemical method (6) was employed to determine nitrosamines quantitatively.

Prior to chromatographic determinations, steam distillates containing DMNA were concentrated in dichloromethane, as described by Howard et al. (12). DMNA and *N*-nitrosodiphenylamine were identified qualitatively by thin-layer chromatography (19).

For the quantitative determination of nitrosamines on chromatography plates, the silica gel thin-layer plates were removed from the chromatography tanks and dried in air for 2 to 3 min. The areas containing the nitrosamines, which were evident on the unsprayed plates as dark spots under an ultraviolet (UV) lamp, were scraped into a 50-ml beaker containing 1.5 ml of a 0.17% Na<sub>2</sub>CO<sub>3</sub> solution. The beakers were placed under a UV lamp for 15 min, and then each received 1.5 ml of the sulfanilic acid and 1.5 ml of the 1-naphthylethylenediamine reagents of Montgomery and Dymock (16). After 15 min, 5.5 ml of water was added to each beaker, and the silica gel was removed by filtration through Whatman no. 30 filter paper. The absorbance of the colored solution was measured at 550 nm. The limit of detection by this procedure was 2.0 μg of nitrosamine. Untreated areas of the silica gel contained 1.0 to 2.0 μg of apparent *N*-nitrosodiphenylamine by this method.

A Varian Aerograph gas chromatograph (model 2740, Varian Associates, Walnut Creek, Calif.) equipped with a flame ionization detector was also used to identify DMNA. The steel column (7.62 m by 4.67 mm) was packed with Porapak Q-S (100–120 mesh; Waters Associates, Inc., Farmingham, Mass.), and the operating temperatures were 225, 180, and 230 C for the injector, column, and detector, respectively. The carrier gas was N<sub>2</sub> flowing at 40 ml/min. The limit of detection by this procedure was 2.0 ng of DMNA.

Authentic dimethyl-, diethyl-, and di-*n*-propylnitrosamine and *N*-nitrosodiphenylamine were obtained from Eastman Organic Chemicals, Rochester, N.Y.

## RESULTS

Cell suspensions (5.0 ml) prepared from cultures of 12 microorganisms were incubated for 36 h in 95 ml of 0.1 M Tris buffer (pH 7.0) containing 0.34 g of trimethylamine·HCl, 0.25 g of NaNO<sub>2</sub>, and 0.10 g of glucose. The pH was kept at 7.0. DMNA was present in distillates of the reaction mixture of bacterium B, and the concentrations of DMNA after 0, 6, 12, 18, 24, and 36 h were 0.0, 0.0, 0.10, 0.20, 0.16, and 0.23 μg/ml, respectively. Cell suspensions of stock cultures of *Aerobacter aerogenes*, *Proteus vulgaris*, *Streptococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Arthrobacter* sp., *Nocardia* sp., *Aspergillus niger*, *Aspergillus oryzae*, *Mortierella parvispora*, and *Zygorhynchus moelleri* contained less than 0.10 μg of DMNA/ml; presumably these organisms were therefore not active. Suspensions of bacterium B and *M.*

*parvispora* produced DMA in significant amounts, however (Fig. 1). The quantity of DMA formed by bacterium B and *M. parvispora* in 36 h was equivalent to 24% of the trimethylamine supplied.

Cell suspensions (5.0 ml) of the same 12 microorganisms were incubated in 95 ml of 0.1 M Tris buffer (pH 7.0) containing 2.0 g of glucose, 2.5 g of  $KNO_3$ , and 0.50 g of DMA-HCl. Any changes in pH that may have occurred during the experiment were not corrected. Cell suspensions of *S. epidermidis* and *E. coli* and mats of *A. oryzae* produced DMNA (Table 1). The pH values of the reaction mixtures containing these microorganisms were 6.7, 6.6, and 6.5, respectively, at 36 h.

The time courses of DMNA formation and nitrite accumulation are shown in Fig. 2, and it is evident that the levels of the two products rose with time. Both *E. coli* and *S. epidermidis* formed large amounts of nitrite. None of the other organisms formed DMNA in amounts appreciably above that noted in microorganism-free solutions. Under the test conditions, *A. aerogenes*, *P. vulgaris*, *B. subtilis*, bacterium B, *Arthrobacter* sp., *Nocardia* sp., *A. niger*, *A. oryzae*, *M. parvispora*, and *Z. moelleri* cultures accumulated a maximum of 4.3, 43, 45, 10, 15, 2.8, 0.3, 352, 5.1, and 7.4  $\mu$ g of nitrite-nitrogen/ml, respectively. Because aseptic conditions

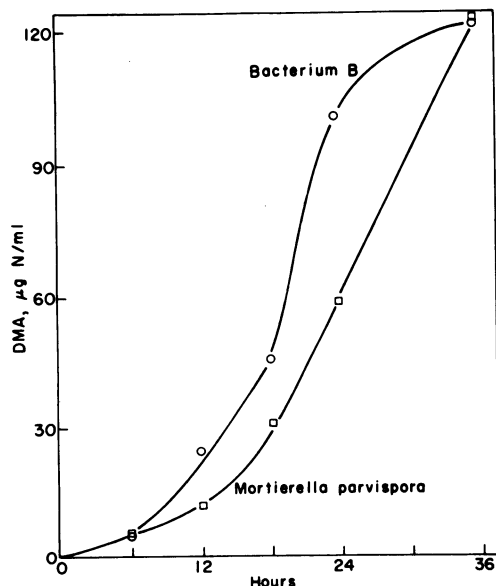


FIG. 1. Formation of dimethylamine (DMA) from trimethylamine by cell suspensions of bacterium B and hyphal mats of *Mortierella parvispora*. Substrate concentrations: 0.34% trimethylamine-HCl, 0.25%  $NaNO_2$ , and 0.1% glucose.

TABLE 1. Formation of DMNA by three microorganisms incubated with DMA and nitrate

| Microorganism                    | DMNA formed ( $\mu$ g/ml) |                |      |      |      |      |
|----------------------------------|---------------------------|----------------|------|------|------|------|
|                                  | 0h                        | 6h             | 12h  | 18h  | 24h  | 36h  |
| None                             | 0.0                       | 0.10           | 0.11 | 0.10 | 0.10 | 0.10 |
| <i>Streptococcus epidermidis</i> | 0.0                       | 0.10           | 0.22 | 0.18 | 0.20 | 0.29 |
| <i>Escherichia coli</i>          | 0.0                       | — <sup>a</sup> | 0.10 | 0.24 | 0.21 | 2.2  |
| <i>Aspergillus oryzae</i>        | 0.0                       | 0.10           | 0.10 | 0.10 | 0.20 | 0.62 |

<sup>a</sup>Not determined.

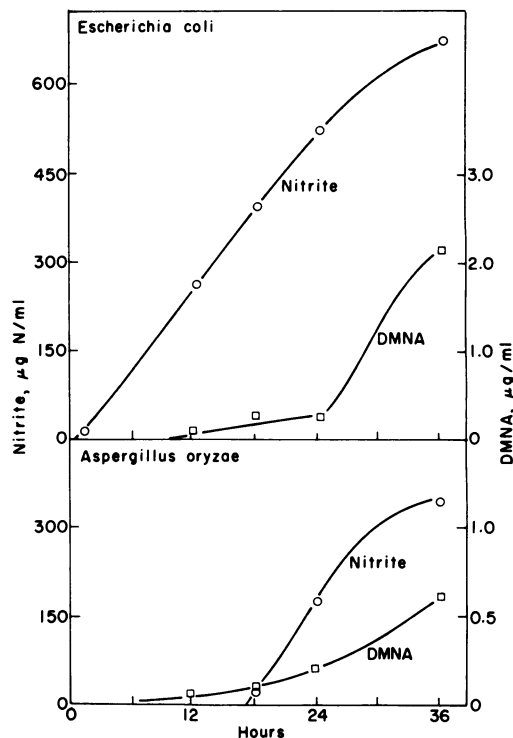


FIG. 2. Formation of dimethylnitrosamine (DMNA) and nitrite by *Escherichia coli* suspensions and *Aspergillus oryzae* hyphal mats.

were used throughout and no organisms appeared in uninoculated vessels, it is likely that the long incubation period required before DMNA began to be produced in solutions containing *E. coli* or *A. oryzae* is not associated with the proliferation of contaminating organisms in the cell suspensions; instead, the long interval without DMNA formation may have resulted from the need for the nitrite level to become appreciable or for induction of a nitrosating enzyme, or both. The identity of the product formed in *E. coli* suspensions and presumed to be DMNA was verified by gas

chromatography. This compound and authentic DMNA have retention times of 342 s.

The presence of DMNA in test systems containing several of the organisms suggested that they could effect the nitrosation of DMA in the presence of nitrite. To investigate this possibility, 10-ml portions of a cell suspension of *E. coli* were incubated with 90 ml of 0.1 M Tris buffer (pH 7.0) containing either (i) 0.50 g of  $(\text{NH}_4)_2\text{SO}_4$  and 0.50 g of glucose; (ii)  $(\text{NH}_4)_2\text{SO}_4$ , glucose, and 0.232 g of DMA·HCl; (iii) glucose and 0.50 g of  $\text{KNO}_3$ ; or (iv) nitrate, glucose, and DMA. One-half of the nitrate-treated vessels were shaken, and one-half were not. Cells incubated with ammonium and glucose or with ammonium, glucose, and DMA did not produce nitrite or DMNA. The pH values in these two treatments fell gradually with time and reached 5.7 after 5.5 h. DMNA was formed in flasks containing DMA, nitrate, and glucose, the level reaching a maximum of 2.2  $\mu\text{g}$  after 5.5 h. The yield was the same whether the flasks were shaken or not. No DMNA was evident in reaction mixtures not provided with DMA. Nitrite was produced from nitrate in the absence of DMA, and the maximum yield was 350  $\mu\text{g}$  of nitrite-nitrogen.

Twenty-five microorganisms were tested for their ability to nitrosate diphenylamine in the presence of nitrite. The bacteria and fungi were grown for 2 to 10 days in either nutrient broth or solution S fortified with 0.50% glucose, 0.20%  $\text{KNO}_3$ , and 0.50% DMA·HCl or trimethylamine hydrochloride in various combinations. The cells were washed, and 3.0-ml portions of the resulting bacterial suspensions were added to 7.0 ml of 0.1 M phosphate buffer, pH 7.5. The fungal mats were suspended in 100 ml of the same buffer contained in Fernbach flasks. The buffer was amended with diphenylamine,  $\text{NaNO}_2$ , and glucose to final concentrations of 0.5, 1.5, and 1.0%, respectively. The bacterial cells were incubated with shaking, whereas the hyphae were not shaken. The pH was maintained at  $7.5 \pm 0.1$ , and the incubation was conducted for 4 h at 30 C.

Because the method for *N*-nitrosodiphenylamine analysis was not sensitive for the detection of less than about 2.0  $\mu\text{g}$  of this compound, values below this amount were deemed negative. The chemicals incubated together for 4 h in the buffer gave values of less than 2.0  $\mu\text{g}$ , as did stock cultures of *A. aerogenes*, *S. epidermidis*, *Arthrobacter* sp., and *Nocardia* sp. grown in nutrient broth, two fungal and two bacterial isolates from soil and one bacterium from sewage grown in solution S containing 0.50% glucose and 0.50% DMA·HCl, five other soil bac-

teria grown in solution S containing 0.50% glucose and 0.20%  $\text{KNO}_3$ , and one additional soil bacterium grown in solution S containing 0.50% DMA·HCl.

However, 10 of the organisms did generate *N*-nitrosodiphenylamine (Table 2). All of the cultures listed contained more than 2.0  $\mu\text{g}$  of the product. The presence of the nitrosamine was established by thin-layer chromatography. The authentic and biologically produced nitrosamine had identical *R<sub>f</sub>* values of 0.64 under the test conditions.

Because *Pseudomonas* sp. and *Cryptococcus* sp. produced *N*-nitrosodiphenylamine spots on silica gel plates with the largest areas and greatest intensities, they were investigated further. The organisms were grown for 40 h in solution S amended with 0.50% DMA·HCl and 0.20% of either  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{KNO}_3$ . The cells were collected and washed, and 3.0 ml of the resulting cell suspension was incubated at 30 C with 7.0 ml of 0.1 M phosphate buffer (pH 7.5) containing 0.1 g of glucose, 0.05 g of diphenylamine, and 0.15 g of  $\text{NaNO}_2$ . Reaction mixtures containing diphenylamine, glucose, and nitrite were also incubated with cells that had been boiled for 30 min.

Suspensions of ammonium- or nitrate-grown cells of the bacterium that were incubated for 4 h with diphenylamine also contained less than 2.0  $\mu\text{g}$  of nitrosamine. However, the yeast and the bacterium were able to bring about a biological nitrosation of this amine (Table 3). Although the nonbiological nitrosation of diphenylamine was appreciable under the test conditions, the yield of the product after 4 h was more than twice as high with unheated than with boiled cells. The data show that the bacterium was as

TABLE 2. Microorganisms forming *N*-nitrosodiphenylamine in cell suspensions or hyphal mats

| Source of microorganism | Microorganism                                       | Growth medium <sup>a</sup>       |
|-------------------------|---|----------------------------------|
| Stock culture           | <i>Escherichia coli</i><br><i>Bacillus subtilis</i> | Nutrient broth<br>Nutrient broth |
| Beebe Lake              | Bacterium B   | Trimethylamine                   |
| Garden soil             | Fungus 1  | Glucose-DMA                      |
|                         | Bacterium 8   | DMA-nitrate                      |
|                         | Bacterium 9   | DMA-nitrate                      |
|                         | <i>Pseudomonas</i> sp.                              | Glucose-nitrate                  |
| Sewage                  | Bacterium 10  | Glucose-DMA                      |
| Cow manure              | Yeast 1   | Glucose-DMA                      |
|                         | <i>Cryptococcus</i> sp.                             | Glucose-DMA                      |

<sup>a</sup> All media except nutrient broth were prepared in solution S.

active in nitrosation whether it was grown on ammonium or nitrate.

Crude enzyme preparations active in nitrosating diphenylamine were made by use of the French pressure cell. The crude extracts and the particle and supernatant fractions resulting from a centrifugation of the extract at  $144,000 \times g$  were incubated in 0.1 M phosphate buffer (pH 7.5) containing 0.5% diphenylamine, 1.5%  $\text{NaNO}_2$ , and 1.0% glucose. Enzymatic nitrosation was readily evident, and extracts of the yeast cells showed the greater activity (Table 4). The soluble fractions exhibited the greatest rate of nitrosation. The activity in the particle preparations may have resulted from soluble cell constituents, because some of the soluble fraction was retained and then mixed with the particles at the bottom of the centrifuge tube. The disappearance of the nitrosamine with time was unexplained but may have resulted from an enzymatic cleavage of the nitrogen-nitrogen bond.

A separate test of the soluble preparation derived from the yeast confirmed that the formation of *N*-nitrosodiphenylamine was linear with time. By using the same substrate

concentrations as in the previous enzyme assays, the nitrosamine concentration rose linearly from  $3 \mu\text{g}$  at zero time to  $27 \mu\text{g}$  at 120 min. Under these conditions, only  $0.5 \mu\text{g}$  of the product, a figure within the experimental error of the method, appeared when an identical enzyme preparation which was boiled for 30 min was incubated with diphenylamine and nitrite for 120 min.

To ascertain which amines could be nitrosated, the yeast was grown for 40 h in a medium containing 0.50% DMA-HCl and 0.20%  $\text{KNO}_3$  in solution S. The soluble enzyme preparation derived from these cells was incubated at 30 C in 0.1 M phosphate buffer (pH 7.5) containing 0.50% of the amine and 1.5% of  $\text{NaNO}_2$ . In a 2-h incubation period,  $4 \mu\text{g}$  of diethylnitrosamine,  $3 \mu\text{g}$  of di-*n*-propylnitrosamine,  $7 \mu\text{g}$  of DMNA, and  $24 \mu\text{g}$  of *N*-nitrosodiphenylamine were produced. In each instance, the apparent nitrosamine present initially was subtracted to obtain the final yield. The soluble preparation incubated with nitrite alone or with each of the amines alone formed  $<2 \mu\text{g}$  of the nitrosamine, a quantity within the experimental error. The yield of nitrosamine was higher with diphenylamine than with DMA as substrate, despite the fact that the yeast was grown on DMA.

TABLE 3. Formation of *N*-nitrosodiphenylamine in cell suspensions of two microorganisms incubated with diphenylamine

| Microorganism           | N source for growth | Nitrosamine formed ( $\mu\text{g}$ ) |                  |
|-------------------------|---------------------|--------------------------------------|------------------|
|                         |                     | 1 h                                  | 4 h              |
| <i>Cryptococcus</i> sp. | Nitrate             | 8.5                                  | 19               |
|                         |                     | 9.0 <sup>a</sup>                     | 9.0 <sup>a</sup> |
|                         |                     | 3.5 <sup>b</sup>                     | 4.5 <sup>b</sup> |
| <i>Pseudomonas</i> sp.  | Ammonium            |                                      | 22               |
|                         | Nitrate             |                                      | 9.0 <sup>a</sup> |
|                         |                     |                                      | 23               |
|                         |                     |                                      | 10 <sup>a</sup>  |

<sup>a</sup>Cell suspension was boiled.

<sup>b</sup>No nitrite was present. All other suspensions were incubated with nitrite.

## DISCUSSION

Because of the toxicity of nitrosamines and the ubiquity of their precursors, information on the mechanisms of *N*-nitrosation and factors affecting the synthesis of such toxicants under natural conditions are of considerable significance. There is ample evidence that secondary amines and nitrite combine nonbiologically in solutions at low pH values, as in the spontaneous formation of di-*n*-propylnitrosamine (9, 22). The nonbiological production of such compounds in acid solutions requires that appropriate controls be included in any study of a possible microbial contribution to the genesis of these hazardous compounds. Although the

TABLE 4. Enzymatic nitrosation of diphenylamine by intracellular fractions from two microorganisms

| Microorganism           | Fraction     | N-nitrosodiphenylamine formed ( $\mu\text{g}$ ) |     |     |     |                  |
|-------------------------|--------------|---|-----|-----|-----|------------------|
|                         |              | 0.5 h   | 1 h | 2 h | 4 h | 4 h <sup>a</sup> |
| <i>Pseudomonas</i> sp.  | Cell extract | <2  | 3   | 3   | 2   | 3                |
|                         | Soluble      | 13  | 10  | 10  | 8   | 3                |
|                         | Particulate  | 3   | 7   | 2   | 3   | 2                |
| <i>Cryptococcus</i> sp. | Cell extract | 11  | 10  | 22  | 14  | 2                |
|                         | Soluble      | 8   | 14  | 33  | 34  | 2                |
|                         | Particulate  | 11  | 11  | 18  | 36  | 3                |

<sup>a</sup>Fraction was boiled for 30 min before being added to reaction mixture.

needed controls are not always reported upon, investigations of axenic cultures (10, 22) and of processes occurring within the gastrointestinal tract of rats (1, 2) suggest a microbial contribution to the formation of *N*-nitroso compounds. Bacterial and enzymatic C-nitrosation has also been established (24).

The present studies demonstrate several microbially catalyzed steps leading to the appearance of nitrosamines. Thus, not only do many microorganisms, including a variety of bacteria and fungi tested here, reduce nitrate to nitrite, but some are also able to convert trimethylamine to DMA. Both bacteria and fungi like *M. parvispora* are active in the dealkylation. The data also show that certain microorganisms contain enzymes that catalyze *N*-nitrosation; this is the first report of *N*-nitrosation by an enzyme preparation, albeit crude, from a micro- or macroorganism.

It is possible, nevertheless, that nitrosamine formation may sometimes result from a nonenzymatic reaction resulting from the microbial formation and concomitant increasing concentration of nitrite, a secondary amine, or both, or from a reaction effected by a cellular constituent other than an enzyme. In the test systems used here in which nitrite was present, its concentration decreased rather than increased with time, making nonbiological nitrosation less rather than more likely. Moreover, although the present data do not rule out the possibility that the cellular constituent is not an enzyme, the findings do show that the responsible cell component is thermolabile, a trait commonly used to suggest, but surely not to prove, that enzyme catalysis is implicated in a particular reaction sequence.

The nitrosating enzyme is particularly active on diphenylamine, and intact cells of *Cryptococcus* sp. likewise formed considerable quantities of *N*-nitrosodiphenylamine. When incubated with DMA, however, yeast suspensions contained little DMNA. These findings are in agreement with other reports that less basic amines are more easily nitrosated than more basic ones (10, 22).

The finding that enzymes of a yeast can catalyze the nitrosation of a secondary amine in the presence of nitrite may be of some practical concern in the alcoholic fermentation. Thus, McGlashan et al. (15) found 1 to 3 ppm of DMNA in alcoholic spirits. Although such amounts of DMNA are small, they are possibly of toxicological significance, and yeasts participating in the fermentation may be responsible for synthesis of the toxicant.

Considerable effort is currently being expended by food and residue chemists to provide a definitive identification of compounds found in natural products and presumed to be nitrosamines. Such firm identifications are essential because the products being tested are complex and highly heterogeneous, and the precursors of the presumed nitrosamine are unknown. In the present investigation, by contrast, the amine and inorganic nitrogen precursors were added, and the test systems were simple and reasonably free of extraneous substances which might necessitate clean-up procedures to remove compounds which might interfere in the identification. The gas and thin-layer chromatograms amply demonstrated the absence of an array of potential interfering chemicals.

These studies suggest that microorganisms may contribute to the production of nitrosamines in nature by synthesizing secondary amines, by producing nitrite, or by forming enzymes which catalyze *N*-nitrosation. In addition, microbial acidification of soil, plant tissues undergoing fermentation, or other substances or habitats may result in conditions favorable to spontaneous nitrosation. In view of the great toxicity of *N*-nitroso compounds, further inquiry into the processes involved and the identification of the types of nitrosamines that can be generated seem warranted.

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