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Microbial Formation of Secondary and Tertiary Amines in Municipal Sewage

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Dimethylamine was formed in municipal sewage that was amended with creatinine, and trimethylamine was formed from choline or phosphatidylcholine. The maximum level of product that accumulated was equivalent to 0.13, 0.096, and 6.7% by weight, respectively, of the added chemicals. No dimethylamine or trimethylamine was detected in sewage amended with betaine, and no dimethylamine was found in sewage that was amended with methylamine and methionine.

Nitrosamines, many of which are carcinogenic and teratogenic (4, 10), are formed in the presence of a secondary amine and nitrite. Because both organic and inorganic precursors are ubiquitous, the potential exists for widespread nitrosamine formation. Microorganisms may contribute to the production of nitrosamines inasmuch as it has been shown that the toxicants can be formed by cultures of microorganisms (1, 5) and in samples of natural environments amended with the precursors (2, 3). Microorganisms may also generate the precursors of the nitrosamines (2).

The structures of creatinine, choline, phosphatidylcholine, and betaine suggest that they may be transformed to yield the immediate precursors for *N*-nitrosation. Creatinine is excreted in the urine of humans and animals, choline and phosphatidylcholine are common in plant and animal tissues, and betaine is found in plants. Because sewage receives urine and breakdown products of plant and animal tissues, a study was conducted to establish whether these four compounds could be converted to dimethylamine (DMA) or trimethylamine (TMA) in sewage.

MATERIALS AND METHODS

To test the aerobic metabolism of creatinine, choline chloride, and betaine hydrate (Eastman Kodak Co., Rochester, N.Y.), 12.5-ml samples of sewage (initial pH from 7.1 to 7.3) from the Ithaca, N.Y., treatment plant were incubated on a rotary shaker (120 rpm) in the dark at 29°C in 25-ml Reacti-Flasks (Pierce Chemical Co., Rockford, Ill.) that were sealed with silicone rubber septa and open-top screw caps (Pierce Chemical Co.). Enough oxygen was present in the solution and headspace to allow for complete degradation. Triplicate samples of amended, unamended, and autoclaved (20 min) amended sewage were analyzed at regular intervals by a modification of the method of Larsson et al. (8). A 3.0-ml portion of the sample was pipetted into 10-ml Reacti-Flasks containing 1.5 g of anhydrous Na_2SO_4, and approximately 0.4 g of NaOH was added. The flasks were then immediately sealed with silicone rubber septa and open-top screw caps. The samples were heated for 10 min at 75°C and then were vigorously shaken for 20 s. Portions (2.0 ml) of the headspace were injected into the gas chromatograph.

In studies of the anaerobic metabolism of phosphatidylcholine (Eastman Kodak Co.), 400 ml of sewage contained in 500-ml Erlenmeyer flasks was amended with 500 μg of cysteine per ml as a reducing agent. High-purity N_2 was bubbled through each flask for 3 h. With N_2 still bubbling through the liquid, phosphatidylcholine was added to two flasks, which were then sealed with serum stoppers. A third flask received no amendment, and a fourth contained sterile sewage and substrate and was flushed with sterile N_2. The flasks were incubated at 29°C in the dark without shaking. Three replicates were withdrawn regularly from each flask for analysis. Essentially identical procedures were used in studies of methylamine, except that the liquid was amended with 200 μg of methylamine per ml with and without 100 μg of DL-methionine per ml, and duplicate samples were taken for analysis; in this instance, the chemicals were sterilized by filtration.

The values reported represent the average concentrations in the amended sewage above those found in amended sterile sewage and in unamended sewage. The gas chromatograph (model 3920B; The Perkin-Elmer Corp., Norwalk, Conn.) was equipped with a flame ionization detector. The injector and detector were held at 225°C, and the flow rate of the carrier gas, N_2, was 30 ml/min. In studies of creatinine metabolism, the samples were injected onto Chromosorb 103 (80/100 mesh; Johns-Manville, Denver, Colo.) packed in a 2-m column. For studies of creatinine breakdown, a Teflon-lined, stainless-steel column with an inner diameter of 2 mm was used, and the column temperature was 150°C; the retention times of TMA and DMA were 1.35 to 1.40 and 1.17 to 1.23 min, respectively. In studies of the other compounds, a glass column with an inner diameter of 2.5 mm was used, and it was programmed from 80 to 170°C at a rate of 16°C per min; the retention times for methylamine,

DMA, and TMA were 2.73 to 2.92, 3.62 to 3.75, and 3.85 to 4.03 min, respectively. The products were identified and quantified by comparing retention times and peak heights with standards.

RESULTS

In sewage amended with 200 μg of creatinine per ml, DMA was detected at 24 h, the concentration was highest at 30 h, and DMA was no longer found at 72 h (Fig. 1). The concentration of DMA at 30 h, 259 ng/ml, is equivalent to 0.13% by weight of the added creatinine. The maximum rate of DMA formation was 40 ng/ml per h at 30 h (Table 1).

In sewage (initial pH, 7.1) amended with 300 or 500 μg of betaine per ml, TMA formation was not observed. The detection limit for TMA was 60 ng/ml. The samples were taken at 24 h and at regular intervals thereafter.

Sewage amended with 200 μg of choline per ml contained 182 ng of TMA per ml at time zero. The amount increased to 374 ng/ml and then rapidly fell to 0 ng/ml (Fig. 1). No TMA was found in samples taken after 30 h and up to 84 h. The maximum accumulation of TMA was 0.096% by weight of the choline added; this value was calculated from the concentration at 24 h minus that at 0 h. In sterile sewage amended with 500 μg of filtered-sterilized choline per ml, the concentration of TMA did not change with time.

In sewage amended with 1.0 mg of phosphatidylcholine per ml, 1.6 μg of TMA per ml was found at 24 h, and the level increased at a rate of 2.45 μg/ml per h until a maximum accumulation of 67 μg/ml was reached at 78 h (Fig. 1). The concentration then fell to about 63 μg/ml.

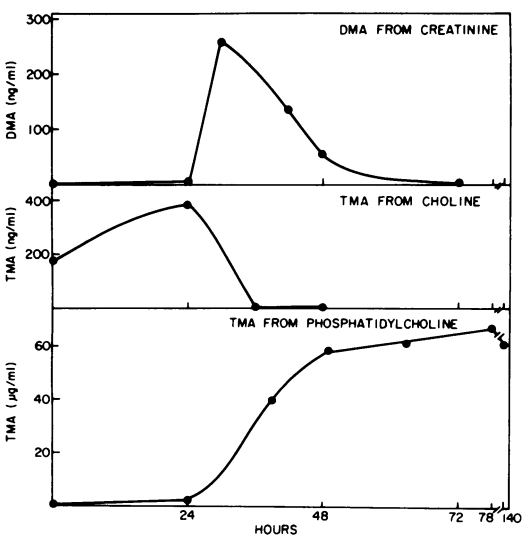

FIG. 1. *Formation and disappearance of DMA and TMA in the metabolism of creatinine, choline, and phosphatidylcholine. The three substrates were tested separately.*

TABLE 1. *Accumulation of TMA or DMA in amended sewage*

Substrate (μg/ml)	Maximum rate of accumulation (ng/ml per h)	Maximum product yield (ng/ml)
Creatinine (200)	40[a]	259[a]
Choline (200)	11[b]	192[b,c]
Phosphatidylcholine (1,000)	2,450[b]	67,000[b]

[a] DMA.
[b] TMA.
[c] Corrected for the amount at time zero.

In tests of the possible methylation of methylamine under anaerobiosis, samples were removed for analysis every 12 h for 8 days. However, no DMA was detected in the amended wastewater, the sensitivity of detection being 40 ng/ml.

DISCUSSION

These data demonstrate that creatinine is converted to DMA, and choline and phosphatidylcholine are cleaved to yield TMA. Sarcosine is a possible intermediate in the conversion of creatinine to the alkylamine. Because it is known that dimethylnitrosamine can be produced in sewage from DMA or TMA (2, 3), the formation of DMA and TMA in sewage from compounds as common in nature as those tested in this investigation may be important from the standpoint of environmental pollution. The accumulation of TMA or DMA in aerobic sewage is unlikely, because both were rapidly metabolized in sewage amended with creatinine or choline.

Evidence exists for the anaerobic conversion of choline to TMA (6). In this study, it was observed that the TMA that was produced aerobically was metabolized, but it accumulated under anaerobiosis. Even this anaerobic accumulation may be important because TMA can be nitrosated directly, at least under nonphysiological conditions (9).

The lack of accumulation of DMA and TMA in sewage amended with betaine may reflect the functioning of a pathway that involves intermediates other than these alkylamines. The data of Jones et al. (7) indicate that sarcosine and glycine are formed from betaine in the corn rhizosphere, and G. G. Gottschalk (personal communication) observed that betaine was converted anaerobically to dimethylglycine, acetate, and butyrate by *Eubacterium* sp. Even though TMA

and DMA may not accumulate during betaine metabolism in sewage, its conversion to dimethylglycine may still be important because dimethylglycine can be nitrosated (4).

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