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Production of NO_2^- and N_2O by Nitrifying Bacteria at Reduced Concentrations of Oxygen

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Pure cultures of the marine ammonium-oxidizing bacterium *Nitrosomonas* sp. were grown in the laboratory at oxygen partial pressures between 0.005 and 0.2 atm (0.18 to 7 mg/liter). Low oxygen conditions induced a marked decrease in the rate for production of NO_2^-, from 3.6×10^{-10} to 0.5×10^{-10} mmol of NO_2^- per cell per day. In contrast, evolution of N_2O increased from 1×10^{-12} to 4.3×10^{-12} mmol of N per cell per day. The yield of N_2O relative to NO_2^- increased from 0.3% to nearly 10% (moles of N in N_2O per mole of NO_2^-) as the oxygen level was reduced, although bacterial growth rates changed by less than 30%. Nitrifying bacteria from the genera *Nitrosomonas, Nitrosolobus, Nitrosospira,* and *Nitrosococcus* exhibited similar yields of N_2O at atmospheric oxygen levels. Nitrite-oxidizing bacteria (*Nitrobacter* sp.) and the dinoflagellate *Exuviaella* sp. did not produce detectable quantities of N_2O during growth. The results support the view that nitrification is an important source of N_2O in the environment.

Biological processes exercise a major influence on the composition of the atmosphere. They may function either as sources or as sinks for selected gases and can influence atmospheric composition on time scales ranging from days (NH_3, H_2S) to years (CH_4, CO, N_2O) to millions of years (N_2). This paper is concerned primarily with N_2O, a gas which plays an important role in the chemistry of the stratosphere (29). Until recently it was thought that atmospheric N_2O was formed mainly during denitrification (1, 14). However, Yoshida and Alexander (36, 37) observed production of N_2O by cultures of the nitrifying bacterium *Nitrosomonas europaea,* which obtains its metabolic energy by oxidizing NH_4^+ to NO_2^-. There is a growing body of evidence from field studies to suggest that nitrification may be an important source of N_2O in both soils and aquatic systems (6, 7, 11, 12, 15, 16, 21, 38).

A major fraction of the nitrifying activity in estuaries, streams, and lakes occurs in sediments and in biological films attached to detrital material (13, 28, 32). In these environments nitrification typically occurs at low concentrations of O_2 and high concentrations of NH_4^+. Field studies in aquatic systems (15, 16, 25, 27) suggest that low oxygen or high ammonia concentrations or both may enhance production of N_2O during nitrification. Similar behavior may be inferred from recent work on agricultural soils (6, 7, 18, 19).

The present paper reports laboratory studies on pure cultures of several species of chemoautotrophic nitrifying bacteria. The influence of oxygen concentration on production of NO_2^- and N_2O was examined in detail for a marine bacterium of the genus *Nitrosomonas.*

MATERIALS AND METHODS

The principal organism used in the present studies was an ammonium-oxidizing bacterium of the genus *Nitrosomonas* isolated from the Western Tropical Atlantic Ocean (35). Batch cultures were maintained at 25°C (pH 7.5), and inocula (0.5 ml, $\sim 10^8$ cells) were obtained by centrifuging samples from the culture and resuspending the cells in growth medium. The inocula were introduced into 550-ml distillation flasks through three-way stopcock ports in an arrangement similar to that employed by Barbaree and Payne (2) (Fig. 1). Cell densities in the flasks ($\sim 10^6$ per ml) were comparable to those found in sediments (13, 32) and in soils (3).

The experimental vessel contained 300 ml of buffered growth medium made by addition, to 1 liter of seawater-distilled water (1:1, vol/vol), of: 1.6 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 1 mg of chelated iron, 0.1 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 2 μg of $CoCl_2 \cdot 6H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, 20 μg of $CuSO_4 \cdot 5H_2O$, and 8.7 mg of K_2HPO_4. The medium was buffered with 0.05 mol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pK_a = 7.5 at 25°C) per liter, titrated to pH 7.5 by addition of NaOH. The apparatus was autoclaved with the medium in place.

The medium was continuously stirred, and the temperature was regulated, in a water bath at 26 ± 1°C. The culture was isolated from the atmosphere and

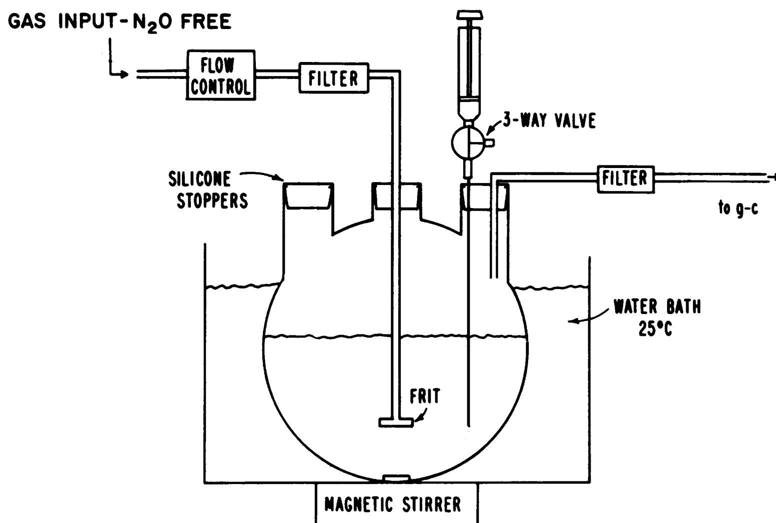

FIG. 1. *Schematic representation of the experimental apparatus.*

ventilated through a fritted disk with a slow flow of N_2O-free synthetic air (~15 ml/min). The oxygen concentration in the flushing gas was varied between 0.005 and 0.2 atm (0.50 to 20.2 kPa) either by mixing O_2 with gas consisting of N_2 and a trace (0.03%) of CO_2, or by use of commercially obtained gas mixtures. The composition of the flushing gas was confirmed by injection into a gas chromatograph equipped with a thermal-conductivity detector. The concentrations of O_2 in the exit streams of active cultures differed insignificantly from concentrations in the inflow, indicating that the flow of O_2 through the flasks greatly exceeded the respiration rates of the cultures.

The headspace of the flask was exhausted directly into the sample loop of an electron-capture detector gas chromatograph (Perkin-Elmer 3920B; see references 16 and 25), permitting accurate determination of N_2O concentrations above a threshold of 20 parts in 10^9 (by volume). Samples were withdrawn periodically through the three-way port and analyzed for cell numbers and concentration of dissolved NO_2^-. Nitrite was measured according to the procedure of Strickland and Parsons (31), and bacteria were counted by staining with acridine orange and counting fluorescent cells (22).

The cultures were tested for heterotrophic contaminants by streaking medium from each flask onto several plates coated with Marine Agar 2216 (Difco). Plates were examined for heterotrophic growth after 4 to 7 days. In a few cases the initial inoculum was found to be contaminated, and the runs were discarded. We observed no contamination during the experiments reported here.

Studies of N_2O production by other nitrifying bacteria were carried out as indicated above, with 20% O_2 in the carrier gas. Cell counts were omitted. Experiments on *Nitrobacter* and *Exuviaella* were carried out in sealed flasks to permit detection of even the most minute production of N_2O. The medium for *Nitrobacter* growth contained 20 mM KNO_2 in place of $(NH_4)_2SO_4$ in the medium. The *Exuviaella* cultures were grown in Guillard f/2 medium with NO_3^- as the only nitrogen source. Optical densities were used to detect growth of *Nitrobacter* and *Exuviaella*, and analyses for NO_3^- were made at the end of each experiment.

RESULTS

Experiments were carried out with two to five replicate flasks. Pilot studies with killed controls ($HgCl_2$ added) showed no production of NO_2^- or N_2O. Results are summarized in Table 1 and Fig. 2 for cultures of *Nitrosomonas* grown at different partial pressures of oxygen between 0.005 and 0.2 atm (0.18 and 7 mg/liter in the medium).

Figure 2a shows the cell number density, concentration of NO_2^-, and exit gas concentration of N_2O for two flasks sampled for a period of 4 to 5 days after inoculation. No distinct lag period was observed. Rates of cell growth were comparable and rather slow, with doubling times between 1.3 and 2.1 days. Considerably less NO_2^- was produced by the cells grown at lower oxygen tension. The quantity of N_2O produced per cell was similar for this particular pair of flasks.

Figure 2b shows the yield of N_2O relative to NO_2^- (moles of N in N_2O per mole of NO_2^-) produced by individual flasks at different oxygen tensions. The production rate for NO_2^- was obtained by differentiating a four-point moving average of the NO_2^- content of the flask. Since the yield is the ratio of differential quantities, instantaneous values are quite sensitive to small systematic errors. Although some variations may be noted over the course of individual experiments, the fluctuations were smaller than the changes observed between different oxygen

TABLE 1. *Experimental results for marine Nitrosomonas* sp.[a]

Date	O_2		N_0 (cells per ml × 10^{-6})	Log growth rate (postlag) per day)	NO_2^- (mM)	ΔCells/ ΔNO_2^- (cells per ml × 10^{-6}/ mM)	N_2O yield[b] (%)	Mean production per cell (mmol per day per cell)	
	%	mg/ liter						N_2O ($\times 10^{-12}$)	NO_2^- ($\times 10^{-10}$)
February 80	1	0.35	0.97	0.38	1.47	3.8	2.5 ± 0.5	2.4	0.95
	1	0.35	0.94	0.24	1.06	2.2	4.15 ± 1.0	4.4	1.1
	1	0.35	1.05	0.37	1.84	3.1	2.8 ± 0.3	3.3	1.2
	20	7.0	1.12	0.26	2.05	0.74	0.29 ± 0.1	1.1	3.7
	20	7.0	1.08	0.20	2.70	0.59	0.3 ± 0.1	1.0	3.4
March 1980	0.5	0.18	0.81	0.28	0.31	5.2	8.1 ± 1.4	4.2	0.52
	0.5	0.18	0.77	0.23	0.26	4.4	9.9 ± 4.0	5.0	0.51
	0.5	0.18	1.26	0.29	0.25	6.0	8.3 ± 1.8	3.9	0.47
	5	1.8	0.81	0.29	1.35	1.3	0.76 ± 0.2	1.7	2.2
	5	1.8	0.70	0.31	1.40	1.2	0.99 ± 0.16	2.6	2.6
October 1979	20	7.0			0.78		0.26 ± 0.10		
	20	7.0			0.56		0.27 ± 0.15		
	10	3.5			1.63		0.52 ± 0.10		
	10	3.5			0.57		0.30 ± 0.07		

[a] Temperature, 26°C; pH 7.5.
[b] Yield defined as moles of N in N_2O produced per mole of NO_2^-.

pressures. The yield of N_2O relative to NO_2^- increased from 0.25% at high oxygen to nearly 10% at low oxygen concentration.

Figure 3 summarizes results from replicate experiments at all oxygen tensions. The yield of N_2O increased sharply relative to NO_2^- at oxygen concentrations below 3 mg/liter (Fig. 3a). Nearly five times as many cells were produced per mole of NO_2^- at the lowest oxygen level (0.18 mg/liter) as compared to higher oxygen levels (2 to 7 mg/liter) (Fig. 3b). However, the rate of cell growth (generation time) changed by only 30% over the entire range of oxygen concentration (Fig. 3c).

Figure 3d shows the mean production rates per cell for N_2O and NO_2^- at different oxygen tensions. The production rate of NO_2^- per cell declined by nearly a factor of 7 as O_2 was reduced from 7 to 0.18 mg/liter, whereas the production rate for N_2O increased by about a factor of 5 over the same range. The sensitivity to oxygen implies that a count of nitrifier numbers in a natural system could not be used as an indicator of the nitrification rate even if all the cells were known to be metabolically active. A similar caveat applies to the use of the ^{14}C incorporation rate as a measure of the rate for nitrification (5).

The yield of N_2O from cultures of autotrophic nitrifiers is surprisingly uniform at atmospheric oxygen levels (0.2 atm partial pressure) for a wide variety of organisms (Table 2). The average yield varied by less than a factor of 3 about a mean value of 0.2% (moles of N in N_2O per mole of NO_2^-) for our studies, which included species from each of the four genera of nitrifying bacteria listed in *Bergey's Manual of Determinative Bacteriology* (8).

Two other organisms were tested for production of N_2O. Chemoautotrophic bacteria which oxidize NO_2^- to NO_3^- (*Nitrobacter* sp.) produced insignificant N_2O during growth. An upper limit of about 2×10^{-7} (moles of N in N_2O per mole of NO_3^-) can be placed on the N_2O yield relative to production of NO_3^-. Assimilatory reduction of NO_3^- has been suggested as a possible source of marine N_2O (21). This suggestion was examined by growing pure cultures of the open-ocean dinoflagellate *Exuviaella* sp. with NO_3^- as the sole nitrogen source. Although the culture grew vigorously, no N_2O production was observed. The experiment allows us to place an upper limit of 4×10^{-6} on the yield for N_2O relative to NO_3^- assimilated by this organism. It is noteworthy that Yoshinari (39) reported no production of N_2O by bacteria (*Vibrio succinogenes*) which reduce NO_3^- to NH_4^+.

DISCUSSION

Earlier studies (9, 10, 20) indicated that *Nitrosomonas europaea* and *Nitrosococcus oceanus* could grow at oxygen tensions as low as 1% of atmospheric. These studies utilized solid media or liquid media containing particulate $CaCO_3$, and it was difficult to control oxygen tension and pH. Total uptake rates of ^{14}C were consistently faster at low oxygen (9, 20), whereas production of NO_2^- was most rapid at oxygen levels near atmospheric (20). In the present work, growth

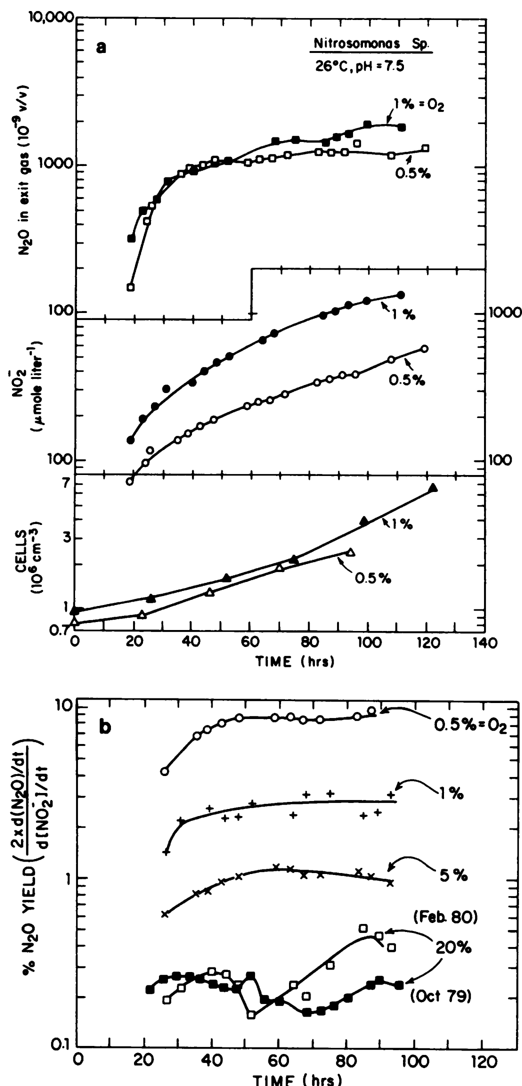

FIG. 2. *(a) Observations for flasks at 0.01 and 0.005 atm partial pressure of oxygen (1% and 0.5% O_2 in flushing gas). Concentrations of N_2O, NO_2^-, and cells of Nitrosomonas (marine) were measured over a 6-day period after inoculation at time zero. Observed levels of NO_2^- and N_2O were below 100 μM and 100 nl/liter, respectively, before time t ≅ 18 h. (See text.) (b) The yield of N_2O relative to NO_2^- produced for the flasks in (a) (1% and 5%) and for flasks at other oxygen tensions. The N_2O concentrations were multiplied by the gas flow rate to determine $d(N_2O)/dt$, and the nitrite content of the flask was differentiated with respect to time to calculate $d(NO_2^-)/dt$. The yield (moles of N per mole of N) is given by $[2 \times d(N_2O)/dt]/[d(NO_2^-)/dt]$.*

rates for cell numbers were slightly larger at reduced concentrations of O_2, but cultures generated much less NO_2^- (per cell) during growth. This finding is consistent with earlier results in both ^{14}C uptake and NO_2^- production and with the original observations of ZoBell (40).

Loveless and Painter (26) studied the growth of *Nitrosomonas europaea* at low oxygen tension. Painter (30) used these data to estimate a Michaelis-Menten half-saturation constant of 0.3 mg/liter for O_2 (the concentration at which growth rate is reduced to 1/2 of its high-oxygen value). Our data would appear to imply a slightly lower value (~0.15 mg/liter). However, the growth rates in Fig. 3a do not appear to fit a simple model with Michaelis-Menten kinetics, since a decline in the rate appears to occur at higher levels of O_2.

Our values for the number of cells produced per mole of NH_4^+ oxidized to NO_2^- (0.65×10^6 cells per μmol) may be compared with earlier results for *Nitrosomonas europaea* (4, 17, 30) and *Nitrosococcus oceanus* (33), about 1.0×10^6 and 0.5×10^6 cells per μmol, respectively. These cell yields are somewhat lower than those reported recently by Belser and Schmidt (3) for soil organisms (~5×10^6).

Yoshida and Alexander (36) observed yields of N_2O between 2 and 25% relative to NO_2^- during 3-h incubations of *N. europaea* (10^9/ml) in sealed Warburg flasks. Most of the N_2O had appeared by the time of the first observation, 1 h after inoculation. The present studies indicate much lower yields of N_2O from nitrifying organisms including *N. europaea*, except at very low concentrations of oxygen. (Cultures contami-

TABLE 2. *Experimental results for other nitrifying organisms (ammonia oxidizers)*[a]

Date	No. of replicates	NO_2^- (mM)	N_2O yield[b] (%)	Organism	Source of isolate
May 1979	4	4.2	0.21 ± 0.08	*Nitrosomonas* sp.	Gulf of Maine (35)
May 1979	6	3.0	0.47 ± 0.1	*Nitrosomonas europaea*	Soils
February 1979	3	2.0	0.26 ± 0.1	*Nitrosococcus oceanus*	Western Atlantic Ocean (33)
May 1979	3	2.7	0.09 ± 0.02	*Nitrosolobus multiformis*	Soils (Surinam) (34)
May 1979	4	1.5	0.11 ± 0.04	*Nitrosospira briensis*	Soils (Switzerland) (34)

[a] Temperature, 26°C; oxygen, 21%; pH 7.5.

[b] Yield defined as moles of N in N_2O per mole of NO_2^-.

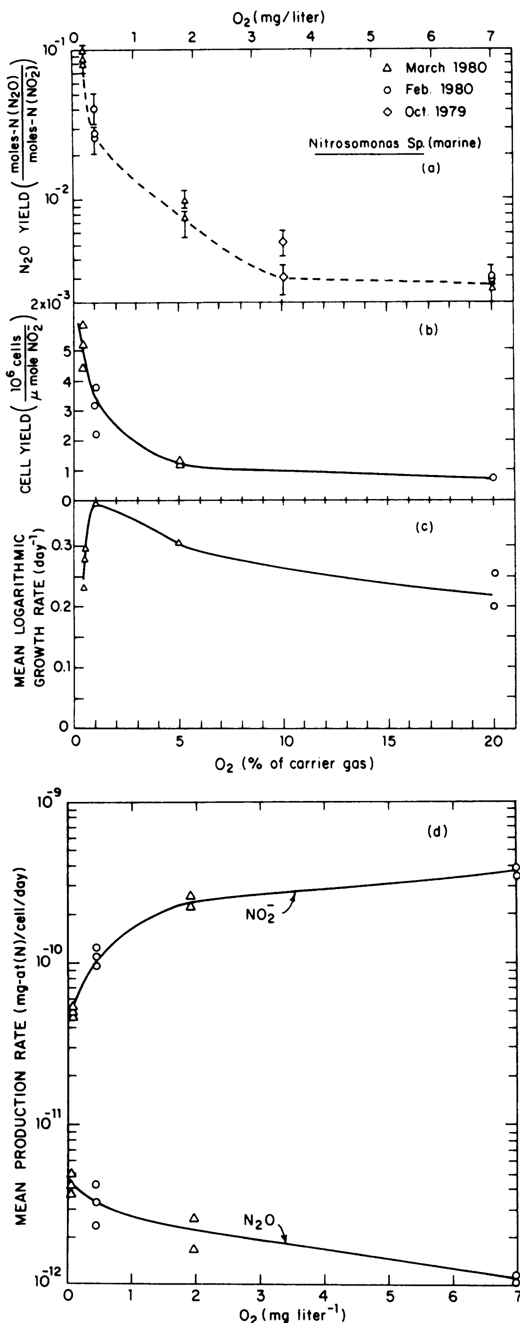

FIG. 3. *Summary of results of replicate flasks of Nitrosomonas sp. (marine) at different oxygen tensions. (a) The N_2O yield increased sharply at low oxygen concentrations. (b) At the lower oxygen tensions more cells were produced for a given quantity of NO_2^-, as compared to higher oxygen treatments. (c) The generation time for cells changed little over the range of oxygen tensions, with a suggestion of a slight optimum near 0.01 atm partial pressure of O_2 (1% O_2 in carrier gas ≃ 0.37 mg of O_2 per liter). (d) The average quantities of N in N_2O and N in NO_2^- produced per unit time by each cell of Nitrosomonas sp. (millimoles of N per day per cell) appeared to depend on oxygen tension. At low oxygen each cell produced substantially less NO_2^- and more N_2O than at high oxygen.*

nated by *Fusarium* sp. were observed to produce large quantities of N_2O.) At the start of each experiment we observed significant N_2O generated by the inoculum during preparation. This N_2O appeared in both live and killed flasks and was flushed out by the gas stream in about 0.5 h. Subsequent production of N_2O paralleled production of NO_2^-, with ratios shown in Tables 1 and 2. It is possible that some of the N_2O observed by Yoshida and Alexander (36) may have been produced during preparation of the cells. It is also possible that the dense cultures employed by Yoshida and Alexander (36) might have consumed the oxygen in the culture medium faster than it could infuse from the headspace. The high yields they observed would in this case reflect the enhanced yield of N_2O at reduced levels of O_2.

The biochemistry of nitrification has been studied in detail by a large number of authors (for a summary, see references 23 and 24). Production of N_2O and small amounts of NO in vitro occurs both during oxidation of NH_2OH and as a by-product of reduction of NO_2^-. These studies utilized extracts from cells grown under reduced oxygen conditions. The present work suggests that isotopic studies on cultures grown under different oxygen tensions could be a significant aid in the determination of the biochemical mechanism for N_2O production by living cells.

Production of N_2O was found to be enhanced at low concentrations of oxygen relative to production of NO_2^-. It rose from 0.002 to nearly 0.1 mol of N in N_2O per mol of NO_2^- as the O_2 concentration was reduced from 7 to 0.18 mg/liter. On a per-cell basis, the production rate of NO_2^- dropped by a factor of 7 over this range of oxygen concentrations, and the production rate for N_2O per cell increased by nearly the same factor. The growth rate varied only slightly (~30%) over the entire oxygen range. Other species of nitrifying bacteria showed similar yields of N_2O and cells (per NO_2^-) at atmospheric concentrations of O_2. Further work is required to define the dependence of rates for N_2O production on species, pH, temperature, substrate concentration, and NO_2^- concentration.

The present results support the view that nitrification is a major source of N_2O in natural systems. Nitrification can proceed in environ-

ments where oxygen concentrations are very low, for example, organic-rich sediments, and under these conditions copious amounts of nitrous oxide may be evolved.

Human activities create high concentrations of oxygen-demanding substances in a variety of environments, such as polluted waters, heavily fertilized soils, or areas where livestock are maintained. Oxidation of reduced nitrogen from decomposition of organic matter or from fertilizer usually is accomplished by nitrifying bacteria. Thus, human perturbations to the environment may increase the global rate of nitrification at low oxygen tension. The result could be release of globally significant quantities of nitrous oxide at rates which might increase nonlinearly should the accumulation of oxygen-demanding wastes also increase.

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