Mass Spectrometric Study of Nitrogenous Gases Produced by Silage

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The time course of production of nitrogenous gases from laboratory-scale and full-scale silos has been followed by mass spectrometric analysis. The concentration of NO rises rapidly, reaching a maximum about a day after the silo is filled; after 2 days the concentration of NO again is low. As aseptic soybean tissues ensiled in the laboratory produced NO and nitrogen, plant as well as microbial activity is important in the nitrogenous conversions accompanying silage production.

THE PRODUCTION OF TOXIC GASES in the early stage of silage making has been recognized recently as the cause of a mysterious pulmonary illness suffered by some farmers. Many plants—e.g., corn and oats—accumulate relatively high concentrations of nitrate, especially when a high dosage of nitrogenous fertilizer is applied. Photosynthetic oxygen production stops in ensiled plant materials, but aerobic respiration continues for a short time until it depletes the available oxygen from the silo gases. Under the anaerobic conditions produced, certain enzymes in the plant tissues and in the microorganisms can use nitrate as an electron acceptor. This reduction of nitrate yields the nitric oxide (NO) [nitrogen dioxide (NO_2) by its reoxidation], nitrous oxide (N_2O), and nitrogen observed in silage gases. Peterson and coworkers (*9*) briefly reviewed the pertinent literature on nitrate reduction.

In the current work, the accumulation of nitrogenous gases in silage has been examined under field and laboratory conditions. To aid in defining the respective roles of plant tissues and microorganisms in silage production, some tests have been made with aseptic soybean plants.

Material and Methods

Field Silo. Figure 1 shows a device which was installed in silos on the university farm. Holes were drilled in the pipe at the level of the sintered-glass dispersion tube to admit gases. The tubing assembly was pushed through the vertical pipe and through the horizontal drain pipe which led to a sump outside the silo.

To sample the silo gases, a previously evacuated 500-ml. round-bottomed flask equipped with a Y-stopcock was connected to the small bore plastic tube to withdraw all air from the tube. After removal of the air from the tube, the Y-stopcock was turned 120° to admit gas from the silo to the evacuated sampling bulb attached to the assembly with a 10/30 standard-taper joint; the stopcock on the sampling bulb then was closed. Gas samples were withdrawn from it into the mass spectrometer for analysis. In one experiment, NO_2 was determined by Saltzman's colorimetric method (*10*) to supplement the mass spectrometric analyses.

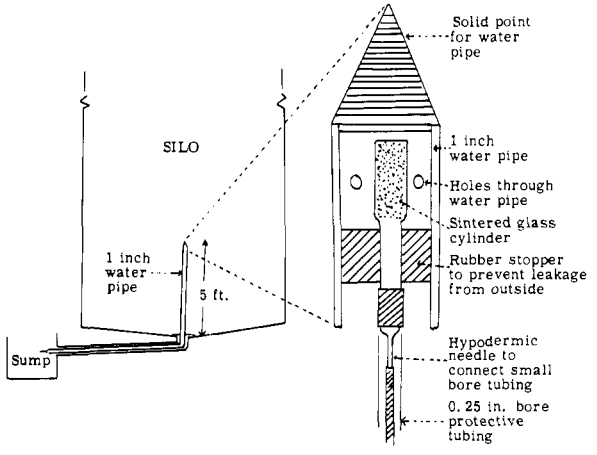

Figure 1. A sampling device installed in the silos used in this study

Laboratory Silo. For the generation of silo gases under controlled conditions, 100 grams of the tops of corn plants grown in the greenhouse were cut about 1 inch long and tightly packed into a 200 × 38 mm. borosilicate glass tube (Figure 2). Treatments were run in duplicate; 1.0 ml. of solution containing 0.166 gram of normal sodium nitrate or 0.166 gram of $NaN^{15}O_3$ (7.5 atom % nitrogen-15 excess) was added to each tube. The tubes then were immersed in a constant temperature water bath at 30° C.; each tube was connected to a T-stopcock. The combination manometers and measuring burets were made from 25-ml. graduated pipets; six burets of this type were connected through a manifold to a common mercury reservoir (Figure 2). The glass system was vacuum tight, but because the crushed plant material foamed, the vessels could not be completely evacuated. Approximately every 6 hours, gas

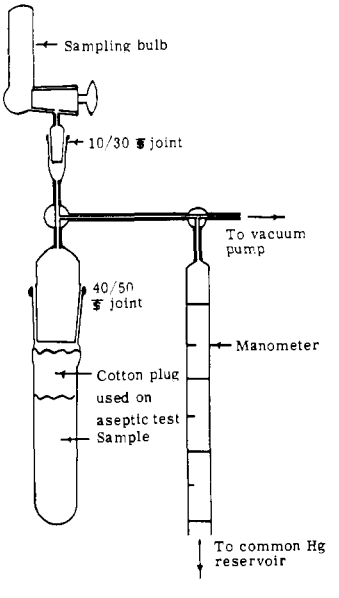

Figure 2. Apparatus to contain ensiled plant tissue to provide for evacuation and gas sampling

samples were taken from each unit, and the samples were analyzed with a Consolidated-Nier mass spectrometer for N_2, NO, oxygen, argon, carbon dioxide, NO_2, and in some cases for N_2O. N_2O was differentiated from carbon dioxide (each has a mass of 44) on the mass spectrometer by determining the proportion of the sum of the 44 and 45 peaks contributed by carbon dioxide and N_2O on the basis of the size of the 22 peak which is produced solely from doubly charged carbon dioxide ions; N_2O splits rather than acquiring a double charge. After the gas samples were withdrawn from the laboratory silos, the systems were re-evacuated to ensure that gas produced in the interval before the next sampling would not be contaminated by previously accumulated gas. Thus, the gases analyzed from the laboratory silos were those produced during the intervals between samples, whereas those from field silos were mixtures of gas originally present and not displaced plus the undisplaced fraction of gases formed after ensiling.

Aseptic Silo. For the aseptic experiments, soybean seeds in a Petri dish were vacuum infiltrated with 75% alcohol in a desiccator and allowed to stand for 10 minutes; they were washed three times with sterile distilled water. The seeds then were treated with sodium hypochlorite (about 0.75% available chlorine) for 10 minutes with vacuum infiltration and again were washed three times with sterile distilled water. The seeds were transferred aseptically into sterilized 15-liter wide-mouthed glass jars containing a 1.5-inch layer of vermiculite nearly saturated with Hoagland-Arnon nutrient solution number 1 (*5*); each jar contained a cotton plugged 200 × 38 mm. silo tube (Figure 2) with added nitrate solution. The jar mouths were covered with 5-ply Aeromat filter to remove microorganisms but to allow passage of air. After 14 days, the plant tops were harvested aseptically in a transfer room and packed into the silo tube in the jar. All manipulations were made inside the wide-mouthed jar, and the materials were handled with a sterilized surgical glove. The cotton plug was replaced in the silo tube above the plant tissue and below the standard taper joint to prevent subsequent contamination. The tubes were taken out of the jars, connected to the evacuation and sampling equipment, and the silage gas was collected and analyzed as described for the laboratory silos. At the end of the experiment, 1 ml. of silage liquid was added to a tube of glucose nutrient broth medium, and the medium was incubated at 37° C. for at least 36 hours. Sterility was checked by examining the medium for turbidity and by observing a stained sample microscopically.

Results

Field Silos. Results reported in Table I and Figure 3 were obtained from a silo on the university farm in 1957; the gas was sampled with the device shown in Figure 1. In general, the results were in good agreement with those obtained with laboratory silos. The sampling device was successful in eliminating contamination of the gas samples by air, for the levels of oxygen in samples were very low. The gradual decrease in the percentage of argon from 0.69% after 6 hours to 0.03% after 66 hours indicates that the air initially trapped inside the silo was gradually displaced by the silage gases. The rapid disappearance of oxygen, only 0.09% remained 6 hours after ensiling, indicates that anaerobic conditions were achieved quickly in the silo. During the experiment, the percentage of oxygen ranged from 0.05 to 0.1%. Carbon dioxide accumulated rather steadily throughout the sampling period and rose from 28.65 to 84.98% of the gas present.

In contrast to the increase of carbon dioxide, the percentage of nitrogen decreased gradually from 68.80 to 13.44%. Nitrogen must have been produced in the field silo, because its fivefold decrease in concentration was far less than the 23-fold decrease in argon concentration from 0.69 to 0.03%. The percentage of NO increased from 0.12% at 6 hours to a maximum of 9.71% at 23 hours and then decreased sharply to approximately the 6-hour level at 42 hours and thereafter. The higher peak values for NO in the laboratory tests than in the field silo can be accounted for on the basis that nitrate was added to plant tissues in the laboratory tests, and the laboratory silos were re-evacuated after each sampling.

The percentage of N_2O reached a peak of 4.35% (54 hours) after the virtual disappearance of NO. The colorimetric determination of NO_2 (Table I) showed about 0.1% NO_2 as a maximum level at 18 hours. Forty-six hours after ensiling, the NO_2 level decreased to 0.001% and remained low afterward. As NO_2 arises from the spontaneous oxidation of NO by oxygen, one would expect that the low level of oxygen in the silo would limit NO_2 formation to the low concentration actually found.

Figure 4 records the compositions of gas mixtures recovered from a silo filled with corn in 1958. The silo was filled over a period of 2.5 days, and the evolution of NO was not as great as in the silo filled rapidly in 1957; the peak concentration of NO did not appear until 30 hours after ensiling. In general, the gas production was much like that observed in 1957 (Figure 3).

Figure 5 indicates the results obtained from a 12 × 40 foot silo on the university farm filled with a mixture of soybeans and sudan grass treated at ensiling with 8 pounds of sodium pyrosulfite ($Na_2S_2O_5$) per ton of plant materials. The gas compositions were distinctly different from those in untreated silos. The air was displaced rather slowly as indicated by the fact that the percentage of argon decreased only from 0.85% at 18 hours to 0.59% at 70 hours. Similarly, the nitrogen decreased at an unusually slow

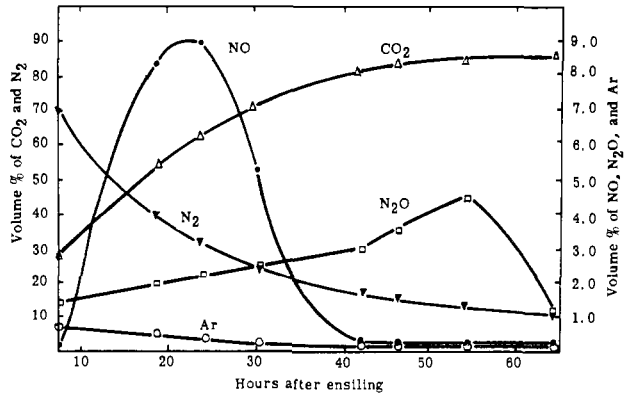

Figure 3. Composition of gas mixtures recovered from a 12 × 40 foot silo on the university farm; filled with corn in September 1957

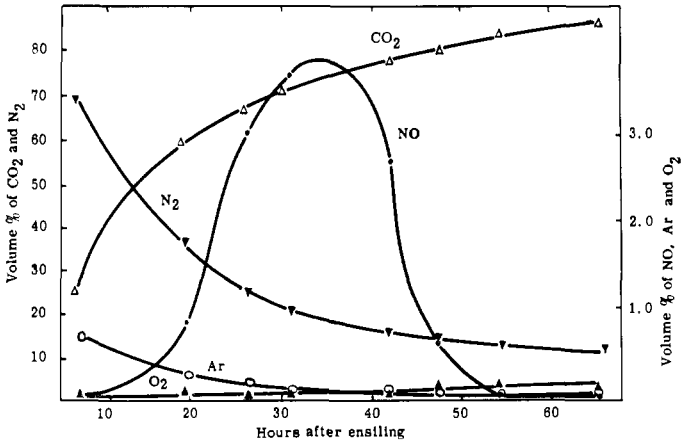

Figure 4. Composition of gas mixtures recovered from a 12 × 40 foot silo on the university farm; filled with corn in the fall of 1958

rate: from 73.7 to 55.5%. Throughout the sampling period, the oxygen concentration was reduced to less than 0.02%. The slow rate of accumulation of carbon dioxide indicated that the plant and microbial metabolism was strongly inhibited by sodium pyrosulfite. The level of NO was less than 0.1% in all but two samples. A normal silage fermentation did not occur in the presence of sodium pyrosulfite, but the plant material appeared to be well preserved.

Laboratory Silos. A number of preliminary experiments were conducted to find a satisfactory laboratory silo before the form described in the section on methods was adopted. The required conditions are that the silo can be operated conveniently on a laboratory scale under controlled conditions, and that it be gas tight to avoid contamination of the silage gases by air.

Table II presents the composition of gases evolved from corn silage in a laboratory experiment. Oxygen was depleted rapidly and remained at a reasonably low level throughout the experiment. As an inert gas, argon gives a good index of contamination of the samples with air. A comparison of the changes in percentages of nitrogen and argon indicates that the nitrogen gas arose partly from plant and microbial metabolism and partly from air contamination. For example, the level of 0.11% of argon in the 41-hour sample indicates that contaminating air constituted approximately 12% of the total gas sample. Such contamination with air should give only about 9.5% of nitrogen in the sample. However, the analysis showed 17.5% of nitrogen, so that nearly half of it was produced by denitrification in the silage.

Nitric oxide accumulated rapidly during the experiment. From 0.81% at 6 hours, NO rose to 47.22% of the total gas volume after 41 hours. This value is much higher than those reported by Peterson and coworkers (*9*) under approximately the same experimental conditions, except that the authors' experimental silos were evacuated initially whereas theirs were filled with air. In one experiment a peak value of 50.60% of NO was found in a 41-hour sample.

No attempt was made to separate N_2O from carbon dioxide in the laboratory silos, and the total gases at masses 44 and 45 were recorded as carbon dioxide; although observations with the field silos indicated that N_2O (mass of 44 like carbon dioxide) was a product of the silage fermentation. Percentages of NO_2 in this experiment were calculated from the peak in the spectrometer at mass 46. These values are rather inaccurate, because NO_2 reacts with the mercury of the pressure adjustor in the mass spectrometer, and the machine must be specially conditioned with NO_2 (*4*) to measure this gas accurately. $C^{12}O^{16}O^{18}$ also contributes to the mass 46 peak. The Saltzman method (Table I) is satisfactory for measuring NO_2 when the color reagent is added directly to the gas sample bulb and shaken vigorously therein.

To study the nitrogen-15-enrichment in each individual gas (nitric oxide, nitrogen dioxide, nitrous oxide, nitrogen), attempts were made to separate these compounds by gas chromatography; however, the method was not perfected and only results of the atom % of nitrogen-15 excess in the nitrogen evolved from corn silage treated with $NaN^{15}O_3$ containing 7.5 atom % of nitrogen-15 excess are given here. The level of nitrogen-15 was 0.157 atom % of nitrogen-15 excess after 6 hours and increased linearly with time to 0.298 after 18 hours, 0.476 after 29 hours, and 0.638 atom % nitrogen-15 excess at the terminal 40-hour sampling. These data give direct evidence that nitrogen-15–labeled nitrate added to ensiled plant material can be converted to molecular nitrogen and lost in this

Table I. Nitrogen Dioxide Found by Colorimetric Analysis of Samples from a Field Silo

Sample No.	Sampling Time, Hours after Ensiling	Volume % NO_2 in Sample
1	6	0.056
2	18	0.104
3	23	0.089
4	30	0.063
5	42	0.016
6	46	0.001
7	54	0.001
8	66	0.001

These samples analyzed by Saltzman's method were aliquots from samples whose analyses are recorded in Figure 3.

Table II. Composition of Silage Gases Produced at 30° C. under Vacuum from Corn Top Tissues in Laboratory Silos

Gas Components	Sampling, Hours after Ensiling: 6	16	22	29	41	49
	Volume % of Gases					
N_2	25.39	16.97	20.42	11.52	17.55	13.04
NO	0.81	2.21	12.99	38.23	47.22	38.84
O_2	0.64	1.39	2.08	0.74	2.14	0.67
Ar	0.16	0.07	0.14	0.03	0.11	0.03
CO_2	72.67	79.00	64.07	49.26	32.78	47.23
NO_2	0.32	0.35	0.28	0.22	0.19	0.19

0.166 gram of $NaN^{15}O_3$ in 1 ml. of solution added to 100 grams of wet weight of tissue.

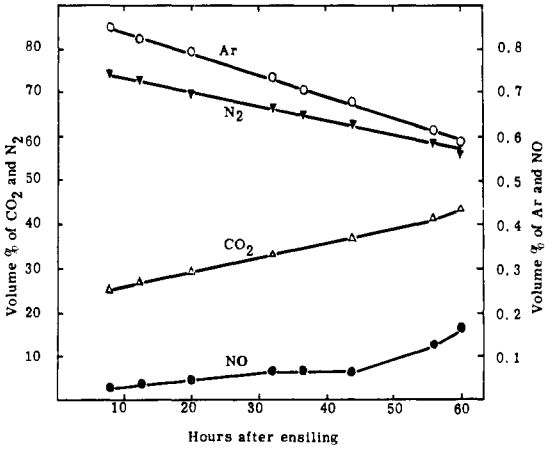

Figure 5. Gases present in a 12 × 40 foot silo filled with a mixture of soybean and sudan grass, October 1958, treated with sodium pyrosulfite

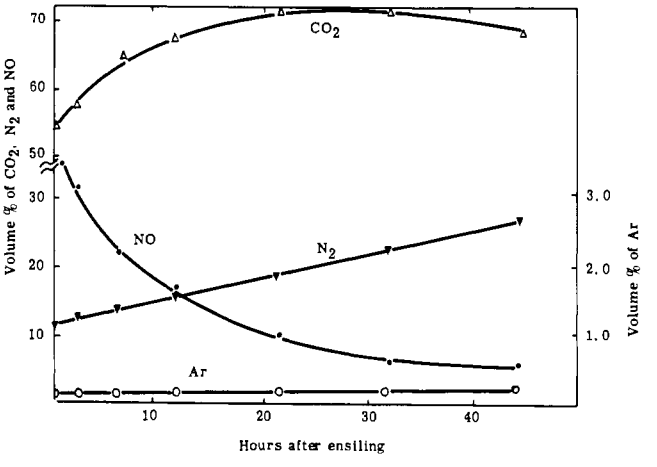

Figure 6. Composition of gas mixtures collected over aseptic soybean tissues

form from silage. These data, as well as the observation that nitrogen decreases in concentration much less rapidly than argon, prove that denitrification does occur in silage.

Aseptic Silage. As normal silage is a fermentation product, it seems improper to speak of aseptic silage, so the qualified term "silage" has been used to indicate that the plant material was packed in laboratory apparatus in the usual fashion except that it was kept aseptic. Exclusion of all microorganisms from the plants posed difficulties because of the necessity for extensive transfer and manipulations of the aseptic plant material during the process. After numerous trials, asepsis finally was achieved by performing all manipulations inside the wide-mouthed glass jar in which the plants were grown. Figure 6 indicates the compositions of the gas mixture collected over aseptic soybean tissues. The rapid rise of carbon dioxide from 54.2 to 71.7% resembled the pattern of carbon dioxide accumulation in a normal silage fermentation. The levels of nitrogen increased with time as the NO decreased. Thirty minutes after ensiling, the first sample contained NO at a concentration of 34.0 volume %. Thereafter the level of NO decreased to a low value of 4.3% at 45 hours. Apparently the plant tissue quickly reduced the added nitrate to NO and with time developed an increased ability to reduce the NO to N_2. These results agree with the finding of Chung and Najjar (*2*) that plant enzymes can reduce nitrate to form NO, but also indicate that plants have the ability to reduce this NO further to N_2.

The claim for the absence of contaminating microorganisms is supported by the pattern of NO formation. If contaminating microorganisms were present, their activity would increase with time as their population increased, and the formation of NO would be expected to increase as it did in many trials in which contaminants were present. The rapid formation of NO by the aseptic plant tissues suggests that, contrary to the general view expressed by Peterson and coworkers (*9*) "that bacteria and not plant enzymes are responsible for the formation of the nitrogen oxides," the plant tissues may have a major role in the production of gases in the silo.

Discussion

Nitrate present in or added to ensiled plant material can be reduced to nitrogen and various compounds intermediate in reduction level between nitrate and nitrogen. These studies have not covered compounds more reduced than nitrogen, but reduction of nitrate to ammonia is accomplished readily by plant tissues (*7*).

The experiments with nitrogen-15 as a tracer indicate that nitrate can be converted to NO, N_2O, and N_2 in silage. Nitrate reductase is widely distributed in plants and microorganisms and its formation of nitrite from nitrate would be the first logical step in NO production in silage. At the acid pH of silage, the next reaction of nitrite could proceed spontaneously as follows:

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$

The nitric acid formed could in turn be reduced by nitrate reductase.

Under the anaerobic conditions in the silo, the reduction of NO logically could continue in the following fashion:

$$2NO + 2H \rightarrow N_2O + H_2O$$

$$N_2O + 2H \rightarrow N_2 + H_2O$$

In the present study, there has been no attempt to establish the mechanism of nitrate reduction in silage, but the products NO, N_2O, and N_2 have been observed consistently. The data of Peterson *et al.* (*9*) indicated that nitrogen arose chiefly by reduction of nitrate, but that some nitrogen was liberated from nitrogen-15–labeled amino acids by their reaction with nitrous acid (Van Slyke reaction). Wijler and Delwiche (*11*) have demonstrated that NO, N_2O, and N_2 are products of denitrification in soil, and Najjar and Allen (*6*) have found that pure cultures of denitrifying bacteria produce these same gases. Evans and McAuliffe (*3*) have observed that anaerobically either ascorbate or DPNH can reduce nitrite to NO, N_2O, and N_2 nonenzymatically. Nommik (*8*) observed no nonenzymatic denitrification in soil but found that the balance among these gases was pH-dependent.

In addition to the denitrification route, undoubtedly a portion of the nitrate yielded ammonia by the usual process of nitrate reduction (*7*); the quantitative importance of the two routes was not established. Burström (*1*) has suggested that bound nitrite normally is reduced to ammonia, whereas free nitrite is denitrified; however, this possibility never has received substantial experimental support.

In the sequence of gas appearance in the silos, NO was produced first and the N_2O and N_2 followed. The appearance of the highest concentration of N_2O was clearly delayed, but the data do not establish the time of maximum nitrogen production. It would be informative to supply nitrogen-15–labeled NO and N_2O to silage and to determine their rate of conversion to nitrogen.

The toxic gas from silos, NO_2, is not a direct product of nitrate reduction but arises readily from the oxidation of NO by oxygen. Its formation is suppressed under the anaerobic conditions in the silo, and its level is low in uncontaminated samples of silo gases.

The data obtained with aseptic "silage" offer direct evidence which may be applicable in the interpretation of the controversy regarding the role of plant tissues *vs.* microorganisms in silage production. The importance of plant enzymes was stressed in many early studies of the changes occurring during silage making, but emphasis more recently seems to have been placed on the microorganisms. The current study indicates that the plant tissues themselves are capable of producing abundant NO and N_2 from nitrate. Although we have not studied the formation of organic acids and the other changes characteristic of silage production, the observed gas metabolism suggests that, under normal conditions, both the plant tissues and microorganisms likely probably contribute substantially in the process of silage production.

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