Prevention of Artifact Formation during the Analysis of *N*-Nitrosamines in the Rumen Fluid of Cows

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Various procedures were tested for correct extraction of nitrosamines from nitrate-amended rumen fluid of cows. It was found that artifact formation during the preparation of the extracts for analysis persisted even when inhibitors were added and when the bacteria were killed or removed by passing the liquid through a 0.22-μm filter. A rapid direct extraction with an extraction cartridge proved to be the most convenient method.

Research is being carried out in our Institute on nitrate poisoning in cattle fed with nitrate-rich feed (Kemp et al., 1977, 1978). In the rumen nitrite was produced which entered into the blood and caused methemoglobinemia (Vertregt, 1977).

Following nitrate ingestion, nitrite in the rumen may increase temporarily to about 4 mmol/L.

The question arose whether this may lead to the formation of nitrosamines as a second aspect of the risks. Nitrosamines are carcinogenic substances, and the formation and presence of these products in the environment are of much concern.

In the presence of certain microorganisms nitrosamines may be formed at pH values above 5.0 (Archer et al., 1978). In the rumen of a goat formation of nitrosamines was established under extreme conditions by Juszkiewicz and Kowalski (1976), and earlier they demonstrated that ingested volatile nitrosamines may enter into the blood and milk of lactating animals (Juszkiewicz and Kowalski, 1974).

Terplan et al. (1978) found only in two of their rumen content samples some evidence for the presence of diethylnitrosamine. van Broekhoven and Stephany (1978) showed the temporary presence of nitrosamines in the rumen fluid of a cow after administration of nitrate at levels up to 0.5 μg/kg. However, there remained the possibility of the artifact nitrosamine formation during storage and working-up procedures. The distinct relation between nitrosamine and nitrite concentrations caused distrust as nitrite and secondary amines were in the 1 mg/kg range while only about 0.5 μg/kg of nitrosamine could be detected. In general, samples containing only very low concentrations of nitrosamines and relatively high amounts of their precursors should be examined with care for the likelihood of false positives.

Antioxidants like ascorbic acid or sulfamic acid added to prevent nitrosamine formation have been applied earlier by many authors (Archer et al., 1978; Mirvish et al., 1972; Tanaka et al., 1978; Douglass et al., 1978; Williams, 1978; Gray and Dugan, 1975). But complete inhibition was not attained even when applied at the proper pH. Even after the microorganisms are killed, the formation of nitrosamines may continue (Archer et al., 1978).

Recently, Krull et al. (1978) presented methods to detect and prevent positive and negative artifact formation during analysis. They stated that serious considerations should be given whenever nitrosamines are found in unknown types of samples toward the possibility of false positives.

The present study has been aimed at developing a safe working-up procedure for biological samples like rumen fluids.

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Several methods have been used to suppress artifact formation. Attempts were made to stop microbial activity or to inhibit chemical reactions by removing nitrite. Also, combinations were tried out. Finally, a simple and quick working-up procedure was developed.

MATERIALS AND METHODS

Reagents and Instrumentation. *N*-Nitrosamines were obtained from the following sources: *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR), ICN–K+K Laboratories, Inc., Plainview, NY; *N*-nitrosodipropylamine (NDPA) and *N*-nitrosopiperidine (NPIP), Schuchardt, Munich, Federal Republic of Germany; *N*-nitrosodiethylamine (NDEA), Eastman, Kodak, Rochester, NY. Dichloromethane (DCM) (J. F. Baker Chemical Co., Phillipsburg, NJ) was distilled before use. All other reagents were of analytical grade and used without further purification. A Millipore filter system with 0.22-μm (MF, Millipore Co., Bedford, MA) filters was used. Preptube cartridges type 117 were obtained from Thermo Electron Corp. (Waltham, MA).

The nitrosamine analyses were performed on a GC/TEA combination. A Therman Energy Analyzer (TEA) Model 502 LC (Thermo Electron Corp.) was coupled to a Packard-Becker Model 427 gas chromatograph (Delft, the Netherlands). The instrument parameters were as follows. GC: injection port, 220 °C; column, stainless steel, length 3 m, 3.2 mm o.d., packed with 10% Carbowax 20M on Chromosorb WHP (80/100 mesh) or packed with 15% FFAP on Chromosorb W/AW DMCS (80/100 mesh); oven temperature, 160 or 200 °C isothermal or temperature programmed (Carbowax column), initial 120 °C (4 min), 5 °C/min, final 180 °C (4 min); carrier gas, argon; inlet pressure, 0.3 MPa (3 atm). TEA: pyrolyzer temperature, 450 °C; reaction chamber pressure, 133–200 Pa (1–1.5 mmHg); cold trap (liquid N_2–isopentane slurry), −160 to −145 °C.

Sampling Procedures. For the experiments cows fitted with a rumen fistula were used as described by Kemp et al. (1977). The cows were fed with hay low in nitrate and habituated for at least 1 week to the daily administration of approximately 7.5 g of nitrate/100 kg body weight through the rumen fistula (Kemp et al., 1977). On the sampling day a quantity of 15–20 g of nitrate/100 kg body weight was usually added. The rumen liquid was sampled through the fistula as described by Kemp et al. (1977).

Preparation of Samples. For the determination of nitrite a 40-mL sample of the rumen fluid was added immediately after sampling to 10 mL of a saturated lead acetate solution (about 30% w/v) in order to stop microbial activity. The samples were stored at 4 °C until analyzed. During the course of this study, the samples were prepared in various ways.

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(a) Samples of 200 or 20 g were mixed with 50 or 5 mL, respectively, of a saturated lead acetate solution and stored at −20 °C until analysis. The 20-g samples were submerged in liquid nitrogen before storage. (b) Samples of 20 g were mixed with lead acetate, and sulfamic acid was added to a final concentration of about 10 mmol/L. (c) Samples were prepared similarly to (b) with subsequent acidification to pH 2. (d) Samples of 20 g were treated with lead acetate and filtered over a 0.22-μm filter. The resulting solution was directly extracted 3 times with successive 20-mL portions of DCM. (e) Samples of 20 g were treated with sulfamic acid or ascorbic acid (final concentration 10 mmol/L), subsequently adjusted to pH 2, quickly frozen, and stored in dry ice before analysis. (f) Samples of 200 and 20 g were made alkaline (pH 8), frozen, and stored in dry ice before analysis. (g) Samples of 60 g were treated with ascorbic acid and extracted 1 time with 50 mL of DCM within 5 min after sampling. The emulsion was broken by filtration over diatomaceous earth, and the DCM layer was separated and passed through a filter with Na_2SO_4 and stored with Na_2SO_4 at 4 °C before analysis. (h) Samples of 15 g were put on a Preptube prewetted with 10 mL of DCM and eluted by pouring 4 × 10 mL of DCM into the cartridge.

As a control for artifact formation, duplicate samples were mixed directly after sampling with a 50-mmol nitrite solution and enough of a solution containing 100 mg/L dimethylamine and piperidine was added to attain final concentrations of nitrite and amines similar to these expected to be already present, which were about 3 mmol/L nitrite and 0.1 mmol/L amines. These samples were worked up as described above. The amines have been tested for the absence of nitrosamines. Recovery experiments were performed by adding a mixture of NDMA, NDEA, NDPA and NPIP in the 1 μg/kg range to a number of duplicate samples. Routinely NDPA (200 ng) was added to all samples as the internal standard.

Nitrite Analysis. Determination of nitrite was performed after removal of the excess lead acetate with sodium sulfate solution in an automatic analysis system with sulfanilamide and naphthylethylenediamine (Vertregt, 1977).

Nitrosamine Analysis. The 200-g samples were steam distilled (Stephany et al., 1978). The 20-g samples prepared as mentioned above under procedures a, b, c, e, and f were analyzed by the mineral oil distillation procedure (MOT) (Fine, 1978; Stephany and Schuller, 1978). After distillation, extraction with DCM, and concentration in a Kuderna-Danish concentrator (T = 50 °C), the samples were taken in 2 mL of hexane or isooctane. The extract was further concentrated under a stream of nitrogen to a volume of 0.5 mL. As an aid to confirmation of positive GC/TEA peaks as nitrosamines, the method of Doerr and Fiddler (1977), who irradiated samples with UV light in order to decompose the nitrosamines, was employed. Blanks were performed throughout the experiment.

RESULTS AND DISCUSSION

In the rumen samples only small amounts of nitrosamines (1 μg/kg or less) were found with NDMA as the main compound. In some cases small peaks of NDEA and NPYR were present. The results were confirmed by the UV light treatment. As no NPIP was found in the original samples, the occurrence of NPIP in the samples to which nitrite, dimethylamine, and piperidine were added was regarded as an indication for possible artifact formation.

For the various methods of isolation applied, percentage recoveries are given in Table I for the N-nitrosamines added to the various samples at the 10- or 1-μg/kg level.

Table I. Percentage Recoveries and Limits of Detection of N-Nitrosamines Added to Rumen Fluid Samples

method of isolation	spike, μg/kg	n[a]	mean recovery/SD (both %) NDMA	NDEA	NDPA	NPIP
mineral oil distillation	10	3[b]	60/7	58/2	58/10	69/5
extraction	1	5	18/9	32/11	42/13	45/13
Preptube	1	2[c]	70/10			
limit of detection[d]			0.1			0.4

[a] n = number of samples. [b] n = 20 for NDPA. [c] Only NDMA was added. [d] For the mineral oil distillation and the Preptube methods. Volume of extract injected was about 10 μL.

Table II. Contents of N-Nitrosamines in Rumen Fluid Samples As Determined by the Steam Distillation Method[a]

NO_2^-, mmol/L	NDMA, μg/kg I	NDMA, μg/kg II	NPIP, μg/kg I	NPIP, μg/kg II
0.0	0.0	2.8	0.0	2.3
1.9	0.3	5.4	0.0	3.7
1.8	0.3	2.3	0.0	2.0

[a] The results were not corrected for recovery. I, original samples; II, with added nitrite and amines; 0.0, below detection limit (NO_2^-, 0.01 mmol/L; NDMA, 0.03 μg/kg; NPIP, 0.06 μg/kg).

Table III. N-Nitrosamine Contents of a Rumen Fluid Sample after Different Treatments[a]

pretreatment	method of analysis	NO_2^-, mmol/L	NDMA, μg/kg I	NDMA, μg/kg II	NPIP, II
(a) lead acetate	MOT	2.7	3.0		
(b) lead acetate/ sulfamic acid	MOT	2.7	1.5		
(c) as (b), pH 2	MOT	2.7	0.1	0.3	+
(d) lead acetate/ filtration	extraction	2.7	2.0		

[a] Only treatment c was duplicated after addition of nitrite and amines. The NPIP content was not quantified but only used as an indication for artifact formation. MOT, mineral oil technique; I, original samples; II, with added nitrate and amines; +, present above detection limit (see Table I).

Except for the results in Table II, all the results were corrected for recovery.

In the first experiment the samples were prepared in the same way as described (van Broekhoven and Stephany, 1978). The results are shown in Table II. The addition of nitrite, dimethylamine, and piperidine gave an enhancement of the corresponding nitrosamine concentrations. The low level of NDMA in the original samples could be explained by a low level of dimethylamine in the rumen fluid. Although no recoveries were determined in these experiments, the results clearly show the possibility of formation of artifacts.

Therefore, an additional treatment of the lead acetate added samples was tried out by filtering off the precipitate that retained the microorganisms on a 0.22-μm filter (d) or by removing nitrite with sulfamic acid at pH 7 (b) and pH 2 (c). For this experiment one rumen fluid sample was taken and divided into five portions. Therefore, the results after the different treatments should have been the same. As shown by Table III, the amount of NDMA decreased slightly as a result of filtering (d) compared with (a) and a little more after addition of sulfamic acid at pH 7 (b) but considerably after addition of sulfamic acid at pH 2.

Table IV. N-Nitrosamine Contents Found in Rumen Fluid Samples after Different Treatments[a]

pretreatment	method of analysis	NO_2^-, mmol/L	NDMA, μg/kg I	NDMA, μg/kg II	NPIP, II
(e) sulfamic acid/pH 2	MOT	1.0	0.5	6.0	+
(e) ascorbic acid/pH 2	MOT	1.0	0.8	7.0	+
(f) pH 8	MOT	1.0	0.1	0.8	+
(g) ascorbic acid	extraction	2.5	0.4	0.6	0.0
(h)	Preptube	2.2	0.4	0.5	0.0

[a] MOT, mineral oil technique; I, original samples; II, with added nitrite and amines; +, present above detection limit (see Table I); 0.0, below limit of detection.

Method c seemed the most promising. But, although the increase after addition of nitrite and amines is not significant, the presence of NPIP in the sample to which the nitrite and amines were added made it suspect.

The results of the further treatments are shown in Table IV. Direct treatment of a sample (without lead acetate) with sulfamic acid or ascorbic acid at pH 2 (e) was attempted to study the effect of the lead acetate treatment. The results were less than those of treatment c. Conservation at pH 8 (f), as was used with urine samples by Hicks et al. (1978), under our conditions also showed considerable risk of artifact formation of nitrosamines.

Storage at −20 °C or even −40 °C did not prevent artifact formation. At this stage in the experiments it dawned that none of the precautions were sufficient to warrant artifact-free samples. Owing to the high selectivity of the TEA (Fine et al., 1975), the number of analytical steps could be kept small to minimize the possibility of artifact formation. In the present material the only measure that remained was to speed up the procedure. The treatment described under (g) under Material and Methods was performed within 5 min from sampling, resulting in a DCM extract. A single extraction was applied because pilot experiments with precursors dissolved in water at neutral pH and extracted directly after addition had shown that repeated shaking with DCM in the presence of relatively high concentrations of nitrite and secondary amines also led to nitrosamines formation. No good recoveries could be obtained in this way (NDMA, 20 ± 10%) (Table I). Complications concerning the way and time of extraction, the undue emulsifications, and the poor recovery made this method unsatisfactory.

With the Preptube extraction cartridge as an alternative extraction method these disadvantages were avoided. This extraction column contained an inert packing material with a large surface area.

Directly after being sampled the rumen fluid was added to the column and extracted with DCM. Added nitrite, dimethylamine, and piperidine produced no sign of artifact formation, and experiments showed a recovery for NDMA of 70% (Table I). The results are presented in Table V. It could be concluded that for the present material the Preptubes provided the most reliable and convenient method. Compared with the only alterative in this study, method c, the Preptube was preferable because of the speed and simplicity. Because the concentrations measured in the rumen liquid were comparable to those observed after incubation of a solution of comparable concentration of nitrite and amines in water, the formation of nitrosamines in the rumen must be a chemical process

Table V. Determination of N-Nitrosamines in Rumen Fluid Samples with Preptube Cartridges[a]

sample	NO_2^-, mmol/L	NDMA, μg/kg I	NDMA, μg/kg II	NPIP, II
1	2.2	0.4	0.5	0.0
2	1.6	0.6	0.7	0.0
3	3.1	0.8	0.6	0.0
4	0.0	0.0	0.0	0.0

[a] I, original samples; II, with added nitrite and amines; 0.0, below limit of detection (see Tables I and II).

rather than a result of microbiological activity. However, the possibility remains that microorganisms are involved for both formation and degradation, resulting in a low equilibrium level.

Rowland and Grasso (1975) reported the metabolization of nitrosamines by intestinal bacteria, but Tate and Alexander (1976) found that nitrosamines resisted microbial degradation in soil. A measurable influence of the small amounts of nitrosamines found in the rumen on the concentration in the blood or the milk of the cow seems unlikely. Juszkiewicz and Kowalski (1974) found that only 0.14–0.85% of the nitrosamines administered to the rumen of a goat had moved to the blood and the milk.

Further investigations are in progress in this Institute to study the influence of different feeds and feeding regimes on the level of nitrosamines in the rumen of a cow.

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