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Distribution and Metabolism of Ingested NO_3^- and NO_2^- in Germfree and Conventional-Flora Rats

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Germfree and conventional-flora Sprague-Dawley rats were fed sodium nitrate or sodium nitrite in their drinking water (1,000 μg/ml), and various organs, tissues, and sections of the intestinal tract were assayed for nitrate (NO_3^-) and nitrite (NO_2^-) by a spectrophotometric method. When fed NO_3^-, germfree rats had chemically detectable levels of NO_3^- (only) in the stomach, small intestine, cecum, and colon. Conventional-flora rats fed NO_3^- had both NO_3^- and NO_2^- in the stomach, but only NO_3^- in the small intestine and colon. When fed NO_2^-, germfree rats had both NO_3^- and NO_2^- in the entire gastrointestinal tract. Conventional-flora rats fed NO_2^- had both ions in the stomach and small intestine, but only NO_3^- in the large intestine. Conventional-flora rats fed NO_3^- or NO_2^- had lower amounts of these ions in the gastrointestinal tract than comparably fed germfree rats. Control (non-NO_3^- or NO_2^--fed) germfree and conventional-flora rats had trace amounts of NO_3^- (only) in their stomachs and bladders. These results, in conjunction with various in vitro studies with intestinal contents, suggest that NO_3^- or NO_2^- reduction is a function of the normal bacterial flora, whereas NO_2^- oxidation is attributable to the mammalian host. In addition, the distribution of these ions after their ingestion appears more widespread in the body than previously thought.

The distribution and metabolism of ingested nitrate (NO_3^-) and nitrite (NO_2^-) is not well understood. Interest in these ions has expanded in recent years due to their potential toxicity. Under conditions that exist in the human gastrointestinal (GI) tract (6, 18, 35), infected urinary bladder (4, 13), or achlorhydric stomach (29, 30), nitrites (or nitrates after reduction) can react with certain primary, secondary, tertiary, and quaternary amines or amides to produce the corresponding nitrosamines or nitrosamides. This nitrosation reaction is catalyzed either by acid conditions, as found in the normal stomach, or by certain bacteria under more neutral or alkaline conditions (20, 21). Most nitrosamines and nitrosamides are extremely potent and efficient carcinogens (19, 28). They can induce tumors in almost every organ of experimental animals tested. *N*-Nitroso compounds have, therefore, been implicated as causative or contributing agents in certain human tumors.

Since conditions that could allow a nitrosation reaction to occur exist in the human alimentary tract, the key question becomes one of locating where the necessary precursors exist simultaneously and whether they are present in optimal proportions to form the carcinogens. Secondary amines are present throughout the GI tract and bladder, mainly due to host and bacterial metabolism of ingested food (2). Ingested nitrates and nitrites are known to be present in the stomach, bladder, and saliva of humans (12, 32, 33), but were previously considered to be absorbed from the stomach or upper small intestine and excreted by the kidneys (14) and hence not to be present in the lower intestinal tract. Therefore, in vivo formation of *N*-nitroso compounds has been implicated in gastric, esophageal, bladder, and other cancers, but generally not in colon cancer.

The present study was initiated to determine whether ingested NO_3^- or NO_2^- or both could in fact reach the lower intestinal tract where they could react to form potentially carcinogenic *N*-nitroso compounds. To assess the role of the microbial flora in the distribution and metabolism of ingested NO_3^- or NO_2^- or both, conventional-flora (CV) and germfree (GF) rats were used in these experiments.

MATERIALS AND METHODS

Nitrate and nitrite analysis in GI samples. The spectrophotometric method used to assay NO_3^- and NO_2^- (NO_x^-) in intestinal material was a modification of an established method for meat and meat product analysis (3). Only analytical reagent-grade chemicals

and distilled deionized water (DDW), shown to be free of NO_x^-, were used. All glassware and equipment were precleaned in a sulfuric acid-potassium dichromate solution.

For analysis of NO_2^- in portions of "processed" GI tract sections (see below), NO_2^- was first diazotized with a 0.1% sulfanilamide solution (1.0 g of sulfanilamide and 400 ml of 5 N HCl made to 1 liter with DDW) for 3 to 5 min (solution A). It was then coupled with a 0.1% (wt/vol) solution of *N*-1-naphthylethylenediamine dihydrochloride (solution B) for an additional 15 min at room temperature and away from direct sunlight to allow formation of the azo dye. Solutions A and B were stored in brown bottles. The ratio of solution A to B used in the analysis of GI tract samples was approximately 5 to 1.

For analysis of NO_3^- in portions of "processed" GI tract sections, NO_3^- was reduced to NO_2^- via spongy cadmium. Spongy cadmium was made by adding approximately 12 clean zinc rods to 500 ml of a 20% (wt/vol) cadmium sulfate solution. After incubation with occasional stirring for 3 h at room temperature, the cadmium deposited on the zinc rods was scraped into a clean container. The cadmium was washed several times with DDW and transferred to a blender (Waring Products, New Hartford, Conn.), covered with 0.2 N HCl, and macerated in the blender for 15 s or until in the form of fine, spongy crystals. The cadmium was then washed at least six times and stored in DDW until used, when it was "prepared" by regeneration with the 0.2 N HCl solution and again washed six times with DDW until free of chloride ion; a silver nitrite solution can be used to check for residual chloride. A spatula full of the "prepared" cadmium was then added to 11-dram (ca. 42-ml) vials along with 10-ml portions of Carrez-cleaned (1) GI tract samples (see below) in a pH 9.65 buffer (ca. 40 ml of concentrated HCl and 95 ml of concentrated ammonium hydroxide made to 1 liter with DDW) and swirled gently for 30 min to allow complete reduction of NO_3^-. In later experiments, cadmium columns (0.8 by 12 cm) were used to minimize the handling of the cadmium. In either reduction method, approximately 2 to 3 ml of the pH 9.65 buffer was added per 10 ml of GI tract sample. After reduction, NO_3^- was estimated by calculating differences in the concentration of NO_2^- between the reduced and nonreduced (original) GI tract samples. These estimates of NO_2^- (and eventually NO_3^-) were made by comparing corrected absorbance values of the resultant azo dye at 540 nm (A_{540}), using a double-beam spectrophotometer (Beckman model 25), with standard curves obtained by adding known concentrations of these ions to Carrez-cleaned GI tract samples (see below) taken from GF and CV rats ingesting DDW. All ion concentrations were expressed as micrograms of NO_2^- or NO_3^- per gram or milliliter of sample. The lower limit of sensitivity of this analysis ranged from 0.05 μg of ion per g for intestinal tissues to 0.4 μg of ion per g for GF cecum (see Table 1).

Preparation of GI samples for analysis of NO_3^- and NO_2^-. All preparation of GI samples was done at approximately 4°C. The intact GI sections (or tissue and contents, if separated) were either minced with scissors and blended in a Vortex mixer for 2 min at full speed, or they were homogenized in a Waring blender. Samples were then transferred to centrifuge tubes and spun at 17,000 × *g* for 20 min (Sorvall Superspeed, RC2-B), and the supernatant was decanted and saved. The pellet was rediluted with 5 to 10 ml of DDW, blended in a Vortex mixer to ensure a homogenate, and again spun at 17,000 × *g* for 20 min. The pooled supernatants were then deproteinated and clarified with two reagents (1), Carrez I [15%, wt/vol, aqueous $K_4Fe(CN)_6 \cdot 3H_2O$] and Carrez II (30%, wt/vol, aqueous $ZnSO_4 \cdot 7H_2O$) (solution A) to precipitate out the background absorbing (A_{540}) material that remained. Carrez II was added first to the pooled supernatants and blended at full speed for 15 s in a Vortex mixer; Carrez I was then added and blended at full speed for 30 s in a Vortex mixer (NO_2^- will be reduced if this mixing is delayed). Two or three drops of isoamyl alcohol can be added to help reduce foaming that results from this Carrez addition and mixing. The ratio of Carrez II to Carrez I was 0.7 to 1.0 ml. The resultant Carrez-treated suspension was then centrifuged at 17,000 × *g* (4°C) for at least 20 min, and the supernatant was decanted and saved. The pellet was rediluted with 5 to 15 ml of DDW, blended in a Vortex mixer for 2 min, and centrifuged for 20 min at 17,000 × *g* (4°C). The two supernatants were pooled. Finally, all samples were passed through a 0.5-μm Celotate (NO_3^--free) filter (Millipore Corp., Bedford, Mass.) to remove small particles of the precipitate that sometimes remained after centrifugation of the Carrez-cleaned samples. GF cecal and small-intestinal samples sometimes needed a 1.0-μm prefiltration before the 0.5-μm filter cleanup.

In vitro and in vivo studies of NO_3^- and NO_2^- metabolism and distribution. GF Sprague-Dawley rats were supplied by the University of Wisconsin Gnotobiology Laboratory. CV Sprague-Dawley rats were obtained from a local supplier (King Rats, Oregon, Wis.). All rats were 60 to 90 days of age at the start of these experiments.

For in vitro studies of ion metabolism, CV or GF rats were killed with ether, and the various intestinal segments were ligated and excised. The intact intestinal section was weighed, intraluminally injected with NO_3^- or NO_2^- via a 1-ml syringe and a 27-gauge needle, massaged to insure thorough distribution of the ion, and incubated at 4, 37, or 85°C, depending on the experiment. For some experiments, the intestines were separated into tissues and contents. This was accomplished by manually expressing the intestinal material and removing the remaining contents by washing with DDW. After incubation, the samples were then analyzed for NO_3^- and NO_2^-.

For in vivo studies of NO_3^- and NO_2^- distribution, GF or CV rats were given these ions in their drinking water at the concentrations indicated in Tables 1 through 6. When metabolism cages were used, toluene was added to the urine collector to inhibit microbial alteration of the samples. After completion of the feeding experiment, the rats were etherized and bled via cardiac puncture, and the GI tract and bladder were removed. The outer intestinal surfaces were washed repeatedly with DDW before analysis to minimize contamination with extraneous blood. For the

sake of uniformity, all animals, whether GF or CV, were killed between 6 and 7 a.m.

RESULTS

Measurement of NO_x^- in rat GI tract. The complete and consistent quantitative recovery of NO_x^- noted after Carrez treatment of DDW standards (data not shown) indicated that the Carrez treatment itself did not remove these ions. Carrez precipitation, however, did lower the background A_{540} values of the original GF and CV rat GI supernatant samples. As seen by the background A_{540} values in Table 1, CV rat intestinal samples were more effectively clarified than similar sections of GF intestine, whereas the stomach and intestinal tissue of both were clarified to a similar extent. Although the remaining (A_{540}) material in both GF and CV GI tract supernatants did not inhibit or interfere with the quantitation of preformed azo dye and did not seem to break down the formed dye, it did seem to interfere with the azo dye development after NO_2^- (and NO_3^- after reduction) was added to the color reagents. Therefore, as indicated by the corresponding recovery values in Table 1, the ability to detect NO_x^- in Carrez-cleaned GI supernatants was not as good as in DDW. A positive correlation was apparent between the amount of intestinal (background A_{540}) material remaining after Carrez cleanup and the amount of assay interference in these intestinal samples. The interfering compound(s) appeared to be in the intestinal contents rather than the tissues. Since the assay interference was greatest in GF intestinal samples, especially cecal samples, and because NO_3^- recoveries from GF intestinal sections were further reduced in the presence of high amounts of NO_2^-, attempts were made to improve the ion recovery further by removing either the interfering compounds or the ions from these GF samples by the following procedures: passage through Fuller's earth, activated charcoal (Norite, grade A), or cation and anion exchange resins (Bio-Rad Laboratories, Richmond, Calif.); treatment with organic solvents of different polarities; and treatment with trypsin. The Norite treatment cleaned GF intestinal samples to near water quality, but it also removed various amounts of NO_x^-. Cation exchange resins partially clarified the samples, and although anion resins concentrated NO_x^- from water samples, ions could not be consistently recovered from GF intestinal samples. Neither the solvents nor the trypsin treatment improved sample clarity or NO_x^- recovery. In addition, a nitrogen oxide electrode (Orion Research, Cambridge, Mass.) reliably and accurately detected NO_x^- in water samples but proved unsatisfactory in intestinal samples (with or without Carrez treatment). Consequently, even though the "assay interference component(s)" in GF samples could not be easily removed, the limits of ion detection were sufficient for our initial experimental purposes. These NO_x^- estimates varied from as low as 0.08 μg/g (GF and CV stomach and tissues were approximately equal to 0.05 μg/g) in CV large intestine to 0.4 μg/g in GF cecal material.

TABLE 1. *Relationship of background A_{540} values to the recovery of NO_2^- and NO_3^- from GF or CV Carrez-cleaned GI samples*

GI sample	Animal(s)	A_{540}[a]	NO_3^- or NO_2^- recovered[b]
Intact stomach	GF and CV	0.000–0.010	97.0–94.0
Intact small intestine or contents	GF	0.007–0.026	75.0–60.0
	CV	0.000–0.012	94.0–87.0
Intact cecum or contents	GF	0.038–0.115	59.0–36.0
	CV	0.002–0.013	91.0–84.0
Intact large intestine or contents	GF	0.005–0.015	89.0–71.0
	CV	0.000–0.008	97.0–92.0
Tissue only[c]	GF and CV	0.000–0.004	100.0–98.0

[a] Values represent range of background absorbance values obtained by using DDW as blank.

[b] Recoveries (in percent) were calculated by comparing A_{540} values that resulted after the addition of equal concentrations of NO_2^- or NO_3^- with those for DDW and the GI samples and NO_2^- reagents. The original background A_{540} values were substracted for each GI sample.

[c] Representative results of samples from the small intestine, cecum, or large intestine after the contents were "removed."

NO_x^- in CV rats housed in metabolism cages. The distribution of ingested NO_2^- and NO_3^- was first studied by analyzing the urine and feces of CV rats that ingested either tap water (base-line controls) or ion-treated (5,000 μg of NO_2^- or NO_3^-/ml) water. Metabolism cages were altered to prevent the drinking water from contaminating the sample (urine and feces) collectors. Analysis of the latter samples, which were collected separately every 24 h for 3 days, revealed no detectable NO_3^- or NO_2^- in specimens from the control rat (Table 2). On the other hand, except in the feces of the NO_3^--treated rat, which contained only NO_3^-, both NO_3^- and NO_2^- were detectable in all urine and fecal samples collected from the treated rats. The urine ion values of these latter rats (Table 2), besides accounting for most of the ingested ion, suggested that ingested NO_3^- was partially converted to NO_2^- and vice versa.

The observation of NO_3^- and especially NO_2^- in the feces of ion-fed rats was remarkable because it contradicted the generally held opinion that NO_3^- and NO_2^- are rapidly absorbed in the stomach or upper small intestine and excreted

by the kidneys. To further investigate these fecal NO_2^- concentrations and to determine why NO_2^- was measurable only in the feces of the NO_2^--treated rats, all rats were sacrificed after day 3, and the large intestine was removed. Nitrite was undetectable in the large intestine of rats treated with either ion. After verifying the latter observation by sacrificing several other ion-treated CV rats, the metabolism cage studies were abandoned because of the apparent contamination of the fecal pellet with either urinary NO_3^- or NO_2^- as it rolled down the side of the sample collector. Consequently, for all further studies on NO_3^- and NO_2^- distribution in the rats, only samples of freshly excised GI tract and bladder were analyzed.

NO_x^- in intact GI tract sections of GF and CV rats. To establish base-line values of NO_3^- and NO_2^- in the GI tract and bladder of both GF and CV rats, they were fed tap water and standard lab chow and sacrificed after 48 h. NO_3^-, but not NO_2^-, was detectable in the stomach and bladder of both GF and CV rats; ion levels in GF rats were higher than in their CV counterparts (Table 3). The diet seemed responsible for these base-line NO_3^- levels, since this ion was present in the chow and tap water (1 to 2 μg/ml). Neither the diet nor the GI tract and bladder of these rats (GF or CV) contained detectable NO_2^- (Table 3).

Next, CV and GF rats were allowed 48 h to ingest NO_2^-- or NO_3^--supplemented (1,000 μg/ml) water before sacrifice and analysis. GF and CV rats that ingested NO_2^-- or NO_3^--supplemented water had greater concentrations of these ions in their GI tract and bladder than rats ingesting unsupplemented tap water (Table 3). In CV rats, an increase in both ions was noticed

TABLE 2. *Quantitation of NO_3^- and NO_2^- in urine or feces of CV Sprague-Dawley rats ingesting ion-supplemented or control tap water*

Drinking water treatment[a]	Day	Ion recovered[b]			
		NO_2^-		NO_3^-	
		Urine	Feces	Urine	Feces
$NaNO_2$	1	1.8×10^3	50	4.0×10^4	275
	2	800	65	1.9×10^3	180
	3	26	213	2.6×10^3	5
$NaNO_3$	1	150	0	2.6×10^5	175
	2	40	0	2.4×10^4	40
	3	10	0	230	272
Tap water (no ions added)		0	0	0	0

[a] $NaNO_2$ or $NaNO_3$, when present, was at 5,000 μg/ml.
[b] Values for each treatment group represent the total ion recovered (micrograms) from urine or feces collected over a 24-h time period for 3 consecutive days from a single animal that was housed in a metabolism cage.

TABLE 3. *Quantitation of NO_3^- and NO_2^- in the GI tract and bladder of GF and CV Sprague-Dawley rats ingesting tap water or $NaNO_3$- or $NaNO_2$-supplemented drinking water*

Animal	Water treatment[a]	No. of rats	Avg intake/day (ml)	Ion recovered[b]									
				Stomach		Small intestine		Cecum		Large intestine		Bladder[c]	
				NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
GF	Tap	7	60	0.0	13.3 ± 1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.6 ± 0.7
	NO_3^-	5	40	0.0	213.7 ± 13.6	0.0	12.0 ± 1.2	0.0	5.4 ± 0.6	0.0	9.9 ± 0.8	0.0	115.7 ± 52.7
	NO_2^-	5	39	166.0 ± 57.2	71.2 ± 23.2	5.6 ± 2.1	16.4 ± 2.3	3.5 ± 1.3	6.7 ± 1.0	2.9 ± 1.2	16.5 ± 2.7	0.0	72.0 ± 16.9
CV	Tap	7	51	0.0	1.6 ± 0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4 ± 0.7
	NO_3^-	6	31	1.5 ± 0.4	66.5 ± 18.4	Trace	5.6 ± 2.4	0.0	0.0	0.0	0.7 ± 0.3	0.9 ± 0.9	17.9 ± 7.9
	NO_2^-	6	28	4.3 ± 3.0	26.2 ± 10.8	0.2 ± 0.1	1.4 ± 0.6	0.0	0.0	0.0	1.2 ± 0.4	2.9 ± 1.4[d]	12.0 ± 5.9

[a] NO_2^- or NO_3^-, when present, was at 1,000 μg/ml.
[b] Values represent micrograms per gram of sample ± standard error of the mean.
[c] Bladders generally contained little or no urine.
[d] Five animals were analyzed.

in the stomach, small intestine, and bladder, and an increase in NO_3^- (only) was noticed in the large intestine. Gastrointestinal tract NO_x^- concentration increases occurred regardless of which ion was initially ingested; this confirmed that CV rats were able to convert NO_3^- to NO_2^- and vice versa. However, the ion concentration increases over control levels were not consistent throughout the GI tract of CV rats, since both anions were undetectable in the cecum and NO_2^- was not measured in the large intestine of these ion-treated CV rats. Ion supplementation of the drinking water of GF rats, on the other hand, resulted in increases in either NO_3^- or NO_2^- concentrations at all levels of the GI tract and bladder (Table 3). The conversion of NO_2^- to NO_3^-, noted in CV rats, was also evident in the NO_2^--treated GF rats, and this apparently accounted for both these ions being detectable in the GI tract, with the notable exception that NO_2^- was not found in the GF bladder. GF rats ingesting NO_3^-, however, did not convert it to NO_2^- as the CV rats did, since only NO_3^- was detectable in the GF GI tract and bladder. In both GF and CV rats, there was a tendency for slightly higher concentrations of NO_3^- or NO_2^- or both in the lower intestinal tract (i.e., cecum and large intestine) when NO_2^-, rather than NO_3^-, was initially ingested.

Distribution of NO_x^- between intestinal tissue and contents of GF rats. After allowing 48 h to ingest either DDW without added ions (controls) or DDW containing 1,000 μg of NO_3^- or NO_2^- per ml, the small intestine, cecum, and large intestine of these GF rats were excised and separated into tissue and contents. Overall, NO_x^- analysis on GF intestinal tissue and contents (Table 4) agreed with the results of ion analysis in GF intact intestinal sections (Table 3). In addition, these ions were found in both the tissues and the contents (Table 4). When GF rats ingested either NO_2^- or NO_3^-, more of these ions appeared to be in the lumen or contents of the small intestine, whereas in the cecum and large intestine more ion was apparently in the tissues. Also, despite the fact that DDW was ingested by the control rats (Table 4), NO_3^- (though in lower concentration) was again detectable in the stomach and bladder, as was seen in GF rats ingesting tap water (Table 3).

Experiments with excised intestinal segments. The general absence of detectable NO_2^- or NO_3^- in the cecum and large intestine of CV rats ingesting ion-supplemented water (Tables 3 and 4) suggested that either these ions did not reach the lower intestine or they were being altered in some way to chemical forms that were not reactive as NO_2^- or NO_3^- before or after

TABLE 4. *Quantitation of NO_3^- and NO_2^- in stomach, intestinal tissue and contents, and bladder of GF Sprague-Dawley rats ingesting DDW or ion-supplemented drinking water*

Water treatment[a]	Ion recovered[b]													
	Stomach		Small intestine				Cecum				Large Intestine				Bladder	
			Tissue		Contents		Tissue		Contents		Tissue		Contents			
	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
$NaNO_2$	24.9 ± 4.5	50.6 ± 5.3	0.4 ± 0.2	4.2 ± 1.4	2.7 ± 0.8	7.2 ± 1.7	0.5 ± 0.6	4.4 ± 1.5	1.6 ± 0.2	1.7 ± 0.9	0.5 ± 0.6	5.5 ± 1.2	1.9 ± 0.5	2.9 ± 2.0	0.0	23.9 ± 12.3
$NaNO_3$	0.0	100.4 ± 24.7	0.0	2.3 ± 1.1	0.0	3.7 ± 2.9	0.0	4.0 ± 1.0	0.0	1.4 ± 1.6	0.0	4.7 ± 2.4	0.0	1.4 ± 0.8	0.0	10.1 ± 3.5
DDW (no added ions)	0.0	4.8 ± 0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9 ± 0.2

[a] Three GF animals per treatment group. $NaNO_2$ or $NaNO_3$, when present, was at 1,000 μg/ml. Animals ingested approximately the same amount of water per day as similar animals in Table 3.

[b] Values indicate micrograms of ion per gram of sample ± standard error of the mean.

reaching the lower bowel. In an attempt to understand which of these possibilities was occurring in vivo, studies were performed by intraluminally injecting NO_2^- or NO_3^- (500 μg of ion) into the excised (intact) cecum and large intestine of GF and CV rats.

By using these freshly ligated sections of intestine, rather than adding the ions to Carrez-cleaned and centrifuged samples (Table 1), a marked difference was evident between GF and CV rats in the amount of NO_3^- and NO_2^- recoverable after the 30-min incubation (37°C) in vitro (Table 5). Nearly complete recovery of added NO_3^- or NO_2^- was possible from the excised cecum and large intestine of GF rats, whereas the same sections from their CV counterparts showed much lower recoveries (Table 5). Similar results were obtained with GF and CV small-intestinal samples. Interestingly, intestinal sections from gnotobiotic rats (with one nitrate-reducing bacterial species present) manifested ion recovery values that were intermediate between the values obtained in intestinal sections from GF and CV rats (Table 5).

With intact intestinal segments, it was not possible to conclude whether the tissues or the intestinal bacteria were responsible for the metabolism of these ions. This was investigated by separating the ligated intestinal sections into tissue and contents, adding NO_2^- or NO_3^- to the resulting samples, and measuring for these ions after incubation for 30 min at 37°C in vitro. The data in Table 6 indicate that the CV and GF cecal and large intestine tissues were unable to alter added NO_2^- or NO_3^-. However, the contents from both GF and CV lower-intestinal segments gave ion recovery results that mimicked those seen with the intact intestinal segments (i.e., low ion recovery from CV contents and high recovery in GF contents, Table 5). These results strongly indicated that the intestinal bacteria rather than the host intestinal tissues were responsible for the metabolism of NO_3^- and NO_2^-.

TABLE 5. *Recovery of NO_3^- or NO_2^- from ligated intestinal sections of GF, CV, or gnotobiotic Sprague-Dawley rats that were incubated for 30 min at 37°C in vitro*

Intestinal section	Animal	Recovery of added ion[a]	
		NO_2^-	NO_3^-
Cecum	CV[b]	11.5 ± 4.2	7.0 ± 3.2
	GF[b]	97.3 ± 5.9	94.7 ± 7.7
	GB[c]	86	39
Large intestine	CV[b]	44.6 ± 15.2	21.0 ± 10.0
	GF[b]	99.6 ± 2.9	96.3 ± 4.8

[a] Values indicate mean percent ± standard error of the mean; 500 μg of $NaNO_3$ or $NaNO_2$ was added per intestinal section. Neither ammonium ion (NH_4^+) nor opposite anion (i.e., NO_3^- or NO_2^-) was detected upon analysis (qualitatively checked via Nessler's reagent).

[b] Three animals per treatment group.

[c] GB, Gnotobiotic. Two animals colonized with two *Pseudomonas* species; one species was unable to reduce NO_3^-, and the other formed gas from NO_3^- but did not utilize all available NO_3^- as indicated in the NO_3^- test.

TABLE 6. *Effect of microbial status on the recovery of NO_3^- or NO_2^- from intestinal tissue or contents of GF and CV Sprague-Dawley rats*[a]

Animal[a]	Intestinal section	Recovery of added ion[b]	
		NO_2^-	NO_3^-
CV	Cecal tissue	96.0 ± 1.9	99.3 ± 4.3
	Cecal contents	16.0 ± 4.2	14.5 ± 1.9
	Large-intestine tissue	89.6 ± 5.0	92.1 ± 3.2
GF	Cecal tissue	99.0 ± 1.9	95.0 ± 1.2
	Cecal contents	99.3 ± 4.8	96.7 ± 5.4
	Large-intestine tissue	95.0 ± 1.2	98.3 ± 1.8

[a] Three animals per treatment group.

[b] Values represent mean percent ± standard error of the mean; 100 μg of ion per tissue sample or 500 μg of ion per contents sample was added and then incubated at 37°C for 30 min in vitro. Neither ammonium ion (qualitatively checked via Nessler's reagent) nor opposite anion (i.e., NO_3^- or NO_2^-) was detected.

To further study the relationship of the intestinal flora of CV rats and the metabolism of NO_2^- and NO_3^-, the following experiment was performed. Excised ceca from CV rats were either heated at 85°C or cooled to 4°C, or they had no prior treatment for 15 min before the injection of NO_3^- or NO_2^-. After addition of 500 μg of NO_3^- or NO_2^-, the ceca were incubated for various times and temperatures before ion analysis. With no incubation time between injection of NO_2^- and ion analysis, over 50% of the added ion was recovered from ceca that had no prior treatment. Cooling of the ceca to 4°C before NO_2^- injection and during the 30-min incubation period led to an even greater recovery of NO_2^- (67%). Heating the ceca to 85°C before injection of NO_3^- or NO_2^- followed by incubation at 37°C yielded the highest values, which approached a complete recovery of the ion added (86 to 92%). These results again indicated that the metabolism of NO_3^- and NO_2^- was a function of the intraluminal bacteria, and this metabolism exhibited characteristics similar to those of an enzymatically catalyzed reaction.

DISCUSSION

If nitrosamines or nitrosamides are going to be formed in the lower intestinal tract, precursor

amines and amides and NO_3^- or NO_2^- must be present. CV rats given water spiked with 1,000 μg of NO_3^- or NO_2^- per ml for 2 days manifested very low to nondetectable levels of these ions in their intestinal tracts. These low values were considered to be the result of either a rapid absorption and excretion of NO_x^- or the metabolism (host or bacteria) of NO_x^- to a chemically different form(s). Since the Carrez cleanup system allowed quantitation of reasonably low levels of NO_x^- in intestinal samples from CV rats, a rapid metabolism of NO_x^- was assumed to be the cause of these low in vivo ion values. This latter assumption was reinforced by the in vitro studies which showed that large amounts of NO_x^- were metabolized within 30 min by the CV intestinal (cecal) samples. Similar in vitro studies with GF intestinal segments, on the other hand, suggested there was no (or little) metabolism of these ions in the absence of a bacterial flora. When GF rats were fed NO_3^-- or NO_2^--spiked water, they had detectable levels of NO_x^- in the tissues and contents of their intestinal tracts. From these results, it was concluded that the nitrogen of ingested NO_x^- (and at times the intact ion) could consistently reach the large intestine in CV rats. This observation was confirmed with $^{13}NO_x^-$ in other studies (37). A similar finding was noted by C. F. Wang, R. Cassens, and W. Hoekstra (unpublished data), who found up to 19% of the ^{15}N from labeled NO_2^- and NO_3^- (fed as 1.6 and 2.0% of the solid diet, respectively) in CV rat feces 72 h after its ingestion. More than 98% of the ^{15}N in the entire intestine, however, did not react chemically as NO_3^- and NO_2^-. Together, these studies tend to contradict Hill and Hawksworth (14), who attributed the undetectable NO_x^- concentrations in ileostomy samples of patients fed luncheon meat containing NO_3^- to a rapid upper GI tract absorption and subsequent urinary excretion of the ingested NO_3^-. Although the high levels of these ions we noted in the urine of CV rats reinforce the fact that much of the ingested NO_x^- is excreted in the urine, it seems probable that a portion of the NO_x^- not accounted for in urine during metabolism studies of these ions in rats (12), dogs (9), goats, rabbits (16), and humans (26) is probably due to their presence (though chemically altered) in feces. Consequently, an accurate estimation of the amount of ingested NO_x^- that reaches the lower intestinal tract could not be attempted.

The metabolism of NO_x^- noted in these CV (and GF) rats (i.e., NO_3^- to NO_2^-, and NO_2^- to NO_3^- and to unknown intermediates) was also reported by Wang et al. (unpublished data) and others, but they could not determine whether the host or the bacterial flora was responsible for these chemical conversions. With regard to NO_3^-, our in vivo study with GF rats ingesting this ion and our in vitro results with intact and separated intestinal segments of GF and CV rats show that NO_3^- reduction was apparently caused by bacterial nitrate reductases (25), rather than mammalian host enzymes as suggested by others (23, 24). The NO_3^--reducing ability of the normal stomach flora of these CV rats, however, was apparently not as great as in the intestinal tract, since stomach NO_2^- levels in CV rats ingesting NO_3^- were low. A similar lack of NO_3^- reduction in the stomach of the rats was noted by Mirvish et al. (22). The levels of human gastric NO_2^- have also been found to be low until the pH, and so the number of bacteria, increases. The in vitro results with intestinal segments from GF and CV rats indicated that endogenous NO_2^- reduction was also due to the bacterial flora rather than the host.

The end product(s) of these microbial reductions of NO_3^- and NO_2^- was not identified. Other investigators have found bacteria capable of forming *N*-nitroso compounds, from the precursor compounds, both in vitro (5, 20) and in vivo (11); however, the amounts formed were low. This suggests that some other compounds, such as ammonia, could be formed in the CV intestinal tract. The failure to detect significant free amounts of the latter compound (as compared with spiked controls) indicates that other nitrogen derivatives, such as nitrogen gases (7), may have been formed or the NH_4^+ was used by host or bacteria or both to form organic nitrogen-containing compounds. These points remain to be determined.

Although the bacterial flora seemed responsible for NO_x^- reduction, the host appeared responsible for oxidation of NO_2^-. The large amounts of NO_3^- noted in both CV and GF stomachs after NO_2^- ingestion and the lack of measurable NO_3^- after in vitro NO_2^- injection into either GF or CV intestinal sections (intact or separated) suggested that the stomach was the site of this reaction. This assumption correlates with the work of Friedman et al. (8) and Mirvish et al. (22), who also noted oxidation of NO_2^- to NO_3^- in the stomach of CV rats and attributed it to the chemical instability of NO_2^- in acidic (stomach) conditions.

Oxidation of more reduced forms of nitrogen (such as NH_4^+) did not seem to occur in these CV rats. This was seen especially by the undetectable levels of NO_x^- in the intestines of CV rats ingesting tap water or DDW. This finding suggests that nitrifying bacteria (i.e., those capable of oxidizing ammonia to nitrite and then

nitrate) are not a major component of the bacterial flora of these CV rats. This assumption is in contrast with a recent report by Tannenbaum et al. (31), who attributed basal levels of NO_2^- and NO_3^- in intestinal and urinary samples of humans to nitrification (presumably from NH_4^+) rather than to exogenous ingestion of NO_x^-. This is the first recent report of nitrification in humans, although earlier studies alluded to this possibility (10); however, contradictory results have been found (15). The very fact that NO_2^- was detectable in human feces but was undectable in rats given large concentrations of NO_x^- suggests that differences may exist between humans and rats with regard to the presence of either nitrogen-oxidizing (e.g., nitrifying) or nitrogen-reducing (e.g., assimilatory, dissimilatory, and denitrifying) bacteria in their flora. If this is the case, it points to very crucial problems in comparing animal results dealing with endogenous formation of *N*-nitroso compounds with their formation in humans. Even among humans there may be differences in the presence of nitrogen-oxidizing or nitrogen-reducing bacteria or both. This is suggested (i) by the observation that most cases of methemoglobemia have been seen in infants less than 6 months old rather than adults (17), which Focht and Verstraete (7) attribute to greater numbers of nitrate-respiring bacteria in the intestinal tract, and (ii) by very early studies by Whelan, who noted methemoglobinemia only in those persons with constipation (36).

In our study, the cecal and large-intestinal NO_x^- concentrations in GF rats appeared to be the result of direct passage of these ions down the intestine from the stomach. This was suggested by the concentrations of NO_x^- present in the intestinal contents (after separation from the intestinal tissues) of GF rats after ingestion of NO_3^- or NO_2^-. In CV rats, however, the NO_3^- noted in the large intestine did not appear to be the result of a direct NO_3^- (or NO_2^-) intestinal passage because these ions, after ingestion, were never detectable in the CV rat cecum. The colonic NO_3^- in CV rats, therefore, seemed to be bloodstream NO_3^- that was located either in the vascular supply or ion that was secreted into the intestine contents. That this could, in fact, be bloodstream NO_3^- is supported by the fact that NO_3^-, as such, does circulate in the bloodstream and can be found in the saliva (33) and urine (26). Due to its reactivity with components in the blood, such as hemoglobin, it seems unlikely that significant concentrations of NO_2^- circulate freely in the bloodstream. Our observation that only NO_3^- was detectable in the GF rat bladder tends to support this assumption and suggests the NO_2^- levels in the CV rat bladder were the result of a bacterial reduction of NO_3^- before final analysis. These data suggest therefore that ingested NO_3^-, NO_2^-, or their intermediates may reach the lower intestinal tract by passing directly down the intestine or by being secreted from the bloodstream into the intestinal lumen or both. Our further study using ^{13}N indicates that both these mechanisms operate to distribute NO_x^- and metabolites (37). Consequently, a combination of NO_2^- oxidation in the stomach, direct intestinal passage or blood (NO_3^-) distribution or both may explain the concentrations of NO_x^- in the lower intestine of GF and CV rats after ingestion of these ions.

The presence of NO_2^- and NO_3^- in the lower intestinal tract may depend on intricate balances between such things as types and numbers of bacteria at different levels of the intestinal tract, amount of NO_2^- and NO_3^- ingested, the physiological mechanisms that allow these ions (or intermediates) to reach the lower intestinal tract, and the immediate status of the intestine (e.g., constipated, diarrheal, or normal). A recent report by Wang et al. (34) emphasizes the necessity of understanding the above parameters. Human fecal concentrations of *N*-nitroso compounds were suggested to be due to intestinal formation rather than passage of preformed carcinogens from the stomach as suggested by Lijinsky (18). The source of these precursor ions, therefore, is of extreme importance. If NO_2^- and NO_3^- are of endogenous origin (31), intestinal formation of *N*-nitroso compounds would seem unavoidable; if they are of environmental origin, the formation of carcinogens or co-carcinogens may be preventable. Since *N*-nitroso compounds are effective carcinogens in the lower intestine of animals (35) and their action can be enhanced by some compounds (27), a further understanding of the fate and form of ingested NO_2^- and NO_3^- nitrogen is necessary to critically evaluate its relationship to human health.

LITERATURE CITED

1. **Adriaanse, A., and J. E. Robbers.** 1969. Determination of nitrite and nitrate in some horticultural and meat products and in samples of soil. J. Sci. Food Agric. **20:** 321-325.
2. **Asatoor, A. M., M. J. Chamberlain, B. T. Emmerson, J. R. Johnson, A. J. Levi, and M. D. Milne.** 1967. Metabolic effects of oral neomycin. Clin. Sci. **33:**111-124.
3. **Association of Official Analytical Chemists.** 1970. Official methods of analysis, 11th ed., p. 393. Association of Official Analytical Chemists, Washington, D.C.
4. **Brooks, J. B., W. B. Cherry, L. Thacker, and C. C. Alley.** 1972. Analysis by gas chromatography of amines and nitrosamines produced *in vivo* and *in vitro* by *Proteus mirabilis*. J. Infect. Dis. **126:**143-153.
5. **Coloe, P. J., and N. J. Hayward.** 1976. The importance

of prolonged incubation for the synthesis of dimethylnitrosamine by enterobacteria. J. Med. Microbiol. **9**: 211-223.

6. **Fine, D. H., R. Ross, D. Rounbehler, A. Silvergleid, and L. Song.** 1977. Formation *in vivo* of volatile N-nitrosamines in man after ingestion of cooked bacon and spinach. Nature (London) **265**:753-755.
7. **Focht, D. D., and W. Verstraete.** 1977. Biochemical ecology of nitrification and dentrification. Adv. Microbiol. Ecol. **1**:135-214.
8. **Friedman, M. A., E. D. Greene, and S. S. Epstein.** 1972. Rapid gastric absorption of sodium nitrite in mice. J. Pharm. Sci. **61**:1492-1494.
9. **Greene, I., and E. P. Hiatt.** 1954. Behavior of the nitrate ion in the dog. Am. J. Physiol. **176**:463-467.
10. **Grindley, H. S., and W. J. MacNeal.** 1917. Ingested and excreted nitrates and nitrites. *In* Studies in nutrition: an investigation of the influence of saltpeter on the nutrition and health of man with reference to its occurrence in cured meats, p. 9-36. University of Illinois, Champaign.
11. **Hashimoto, S., T. Yokokura, Y. Kawai, and M. Mutai.** 1976. Dimethylnitrosamine formation in the gastro-intestinal tract of rats. Food Cosmet. Toxicol. **14**:553-556.
12. **Hawksworth, G., and M. J. Hill.** 1971. Bacteria and the N-nitrosation of secondary amines. Br. J. Cancer **25**: 520-526.
13. **Hicks, R. M., T. A. Gough, and C. L. Walters.** 1978. Demonstration of the presence of nitrosamines in human urine: preliminary observations on a possible etiology for bladder cancer in association with chronic urinary tract infection. Int. Agency Res. Cancer Sci. Publ. **19**:465-475.
14. **Hill, M. J., and G. Hawksworth.** 1974. Some studies on the production of nitrosamines in the urinary bladder and their subsequent effects. Int. Agency Res. Cancer Sci. Publ. **9**:220-222.
15. **Ishiwata, H., H. Mizushiro, A. Tonimura, and T. Murata.** 1978. Metabolic fate of the precursors of N-nitroso compounds (III). Urinary excretion of nitrate in man. J. Food Hyg. Soc. Jpn. **19**:318-322.
16. **Kameoka, K., and H. Morimoto.** 1953. Utilization of simple nitrogen compounds. Bull. Natl. Inst. Agr. Sci. **7**:147.
17. **Keating, J. P., M. E. Lell, A. W. Strauss, H. Zarkowsky, and G. E. Smith.** 1973. Infantile methemoglobinemia caused by carrot juice. N. Engl. J. Med. **288**:824-826.
18. **Lijinsky, W.** 1977. Nitrosamines and nitrosamides in the etiology of gastrointestinal cancer. Cancer **40**:2446-2449.
19. **Magee, P. N., R. Montesano, and R. Preussmann.** 1976. N-nitroso compounds and related carcinogens, p. 491-625. *In* C. E. Searle (ed.), Chemical carcinogens. ACS monograph no. 173. Washington, D.C., American Chemical Society, Washington, D.C.
20. **Mills, A. L., and M. Alexander.** 1976. *N*-nitrosamine formation by cultures of several microorganisms. Appl. Environ. Microbiol. **31**:892-895.
21. **Mirvish, S. S.** 1975. Formation of N-nitroso compounds: chemistry, kinetics and *in vivo* occurrence. Toxicol. Appl. Pharmacol. **31**:325-351.
22. **Mirvish, S. S., K. Patil, P. Ghadirian, and V. R. C. Kommineni.** 1975. Disappearance of nitrite from the rat stomach: contribution of emptying and other factors. J. Natl. Cancer Inst. **54**:869-875.
23. **Omura, H.** 1959. Intracellular behavior of the nitrate reductase of animal tissues. Enzymologia **20**:270-289.
24. **Omura, H., and H. Takahashi.** 1959. On the nitrate reductase of cattle liver. Bull. Agr. Chem. Soc. Jpn. **23**: 83-88.
25. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409-452.
26. **Radomski, J. L., C. Palmiri, and W. L. Hearn.** 1978. Concentrations of nitrate in normal human urine and the effect of nitrate ingestion. Toxicol. Appl. Pharmacol. **45**:63-68.
27. **Reddy, B. S., K. Watanabe, and J. H. Weisburger.** 1977. Promoting effect of bile acids in colon carcinogenesis in germfree and conventional F 344 rats. Cancer Res. **47**:3238-3242.
28. **Sander, J.** 1971. Untersuchungen über die Entstehung carcerogener Nitrosoverbindungen in Magen von Versuchstieren und ihre Bedeutung für den Menschen. Arzneim. Forsch. **21**:1572-1580, 1707-1713, 2034-2039.
29. **Sander, J., and F. Seif.** 1969. Bakterielle Raduktion von Nitrat im Magen des Menschen als Vrgache eines Nitrosaminbildung. Arzneim. Forsch. **19**:1091-1093.
30. **Segal, H. L., and I. M. Samloff.** 1973. Gastric cancer increased frequency in patients with achlorhydria. Am. J. Dig. Dis. **18**:295-299.
31. **Tannebaum, S. R., D. Fett, V. R. Young, P. D. Land, and W. R. Bruce.** 1978. Nitrite and nitrate are formed by endogenous synthesis in the human intestine. Science **200**:1487-1489.
32. **Tannenbaum, S. R., M. Weisman, and D. Fett.** 1976. The effect of nitrate intake on nitrite formation in human saliva. Food Cosmet. Toxicol. **14**:549-552.
33. **Walters, C. L., M. J. Hill, and W. S. J. Ruddell.** 1978. Gastric juice nitrite, its source and relationship to hydrogen ion concentration. Int. Agency Res. Cancer Sci. Publ. **19**:279-288.
34. **Wang, T., T. Kakizoe, P. Dion, R. Furrer, A. J. Varghese, and W. R. Bruce.** 1978. Volatile nitrosamines in normal human feces. Nature (London) **276**: 280-281.
35. **Weisburger, J. H., B. S. Reddy, and E. L. Wynder.** 1977. Colon cancer: its epidemiology and experimental production. Cancer **40**:2414-2420.
36. **Whelan, M.** 1935. The nitrate content of animal tissues and the fate of ingested nitrate. J. Biochem. **29**:782-787.
37. **Witter, J. P., E. Balish, and S. J. Gatley.** 1979. Distribution of nitrogen-13 from labeled nitrate and nitrite in germfree and conventional-flora rats. Appl. Environ. Microbiol. **38**:870-878.