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Distribution of Nitrogen-13 from Labeled Nitrate and Nitrite in Germfree and Conventional-Flora Rats

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The in vivo distribution of physiological concentrations of NO_3^- and NO_2^- labeled with ^{13}N was studied in germfree and conventional-flora Sprague-Dawley rats after gastric intubation (gavage), intravenous (cardiac or tail vein), or intraluminal (intestinal) injection. Some in vitro studies were performed to determine the influence of the bacterial flora on ion distribution. After gavage with $^{13}NO_3^-$, essentially all of the label passed into the upper small intestine, where most was absorbed; however, up to 24% of the ^{13}N could reach the ileum within 1 h. Gavage with $^{13}NO_2^-$ resulted in some gastric absorption of the label, but most seemed to exit the stomach via passage into the duodenum. The exit of $^{13}NO_2^-$ from the stomach was slower, and less ^{13}N appeared to be absorbed from the small intestine than with $^{13}NO_3^-$. Movement of label through the gastrointestinal tract could be enhanced by inducing diarrhea. Absorbed ^{13}N was either excreted in the urine, reentered the gastrointestinal tract at various points, or was temporarily stored in the eviscerated carcass. The bacterial flora, either by incorporation or chemical alteration, appeared to have some influence on the distribution of ^{13}N from $^{13}NO_3^-$ or $^{13}NO_2^-$.

The presence of nitrate (NO_3^-) and nitrite (NO_2^-) in the alimentary or urinary tract of mammals could constitute a threat to their health because of an in vivo chemical (10, 15) or bacterial (2, 5) nitrosation of primary, secondary, tertiary, or quaternary amines or amides to form the corresponding carcinogenic *N*-nitroso compounds (12, 18). Although others have determined that these precursor amines are present throughout the gastrointestinal (GI) and urinary tracts (1, 8), the disposition of ingested NO_3^- and NO_2^- (NO_x^-) in the body is not as well understood. In several studies, urinary (7, 16, 21) or salivary (20, 22, 24) concentrations of these ions have been assayed after ingestion of NO_x^-; however, no study has elucidated the distribution of these ions or their metabolites in vivo.

Attempts to determine the distribution of NO_3^- and NO_2^- in rodents colorimetrically, with a special emphasis on the lower-intestinal tract, were complicated by chemical and bacterial alteration of these ions in conventional-flora (CV) rats and by chemical conversion and the presence of assay-interfering compounds in intestinal contents of germfree (GF) rats (29). We therefore used the longest-lived radioisotope of nitrogen, ^{13}N, to study the absorption, body distribution, and secretion of gavaged or intravenously (i.v.) injected $^{13}NO_2^-$ or $^{13}NO_3^-$. Despite its half-life of only 10 min, experiments of up to 60 to 90 min duration were possible with ^{13}N, and the detection of physiological concentrations of these labeled ions, and their metabolites, could be rather easily and sensitively traced in rats.

MATERIALS AND METHODS

$^{13}NO_3^-$ and $^{13}NO_2^-$ production and measurement. The tandem accelerator at the University of Wisconsin was used to produce "carrier-free" ^{13}N as $^{13}NO_3^-$ (26). Chemical determinations showed that less than 0.1% of the ^{13}N was in the form of $^{13}NO_2^-$ or $^{13}NH_4^+$. About 30 mCi was made by bombarding an aerated water target for 20 min with a 3-μA beam of 11-MeV protons (28). Passage of $^{13}NO_3^-$ through regenerated spongy cadmium columns was used to make $^{13}NO_2^-$ (29).

Samples were measured for radioactivity by placement inside an NaI (Tl) crystal "well" detector. The carcass (i.e., the rat without the internal organs specified in the tables), however, was counted at a fixed distance from the well. Corrections to compensate for this change in counting geometry were made on these carcass values to allow comparison with other samples. All samples were counted at least twice for a 10-s interval, and values were decay-corrected to the start of ^{13}N administration with the equation $C_t = C_0 \cdot e^{-\lambda t}$, where C_t = the measured counts at various times (t) after the start of dissection, C_0 = the counts corrected to the start of dissection, and $\lambda = \ln_2/\text{half-life of } ^{13}N$.

Animals. GF Sprague-Dawley rats were supplied by the University of Wisconsin Gnotobiology Labora-

tory. CV Sprague-Dawley rats were obtained from a local supplier (King Rats, Oregon, Wis.). Since previous experimentation with dyes had indicated a faster stomach exit if rats were prestarved, GF or CV rats were starved for approximately 18 h (unless otherwise specified) before use to facilitate a rapid distribution of label.

^{13}N distribution experiments. Rats were lightly anesthetized with ether, and 1 to 2 ml (generally with an activity of 1 to 2 mCi) of carrier-free $^{13}NO_3^-$ or $^{13}NO_2^-$ was placed in the stomach by gavage or injected i.v. via cardiac puncture or tail vein injection. At designated time intervals after gavage or injection, the rats were etherized and bled via cardiac puncture and GI tract segments, and all viscera, as indicated in Tables 1 through 7, were removed and washed with distilled deionized water to minimize contamination with extraneous blood. In some experiments, the intestinal segments were manually separated into tissues and contents. In other experiments, intestinal contents were separated into supernatant and pellet by centrifugation at 17,000 × *g* for 5 min (Sorval Superspeed-RC2B).

Surgical manipulations. Rats were injected intraperitoneally with 0.2 to 0.5 ml of a 2.5% sodium thiopental solution or were anesthetized with ether. After a small incision was made into the abdomen, the pyloric valve or the ileocecal valve was tied off by using surgical thread. The $^{13}NO_3^-$ was then injected into the GI tract of the rats.

Pharmacological manipulations. To study $^{13}NO_3^-$ secretion by the salivary glands, 9 mg of pilocarpine-nitrate (Sigma Chemical Co., St. Louis, Mo.) was injected i.v. along with 1 ml of $^{13}NO_3^-$. Prostaglandin E_2 (PGE_2; 2 mg/ml; Kurt Miller, Department of Veterinary Science, University of Wisconsin, Madison) or 2 ml of castor oil was used to induce diarrhea.

RESULTS

Distribution of gavaged $^{13}NO_3^-$ in GF and CV rats. The general pattern of label distribution in both GF and CV rats was that the activity in the stomach decreased with time and concomitantly increased primarily in the small intestine (especially the jejunum), carcass, and bladder. The bedding was another indicator of bladder ^{13}N content, since it captured any urine excreted between gavage and sacrifice. Feces excreted during the 60-min time period contained no significant levels of radioactivity. The liver, kidney, cecum, large intestine, and blood of all rats, on the other hand, had moderate to low (and relatively consistent) amounts of activity present throughout the experimental time period (Table 1). Although the overall ^{13}N absorption and distribution appeared similar in GF and CV rats 45 min after gavage, some major differences were evident when the amounts of label in the stomachs, carcasses, and to a lesser extent, the ilea and bladders were compared 60 min after $^{13}NO_3^-$ gavage (Table 1). GF rats tended to have higher

TABLE 1. *Distribution of gavaged $^{13}NO_3^-$ (2 ml) in prestarved GF and CV Sprague-Dawley rats*

Time[a]	Animal type	% Distribution of ^{13}N from $^{13}NO_3^-$[b]											
		Blood (per ml)	Stomach	Intestines[c]					Carcass	Liver	Kidney	Bladder	Bedding
				Du	Je	Il	CC	LI					
15–30	GF (2)	0.7	20.5	1.1	10.8	0.9	1.9	0.8	59.4	2.8	ND	0.4	0.0
	CV (2)	0.2	77.9	1.4	0.7	0.2	0.4	0.3	15.9	2.3	ND	0.1	0.1
45	GF (4)	0.6 ± 0.3	38.2 ± 16.0	2.3 ± 0.9	10.1 ± 5.5	1.0 ± 0.6	1.1 ± 0.3	0.4 ± 0.1	35.6 ± 10.0	1.6 ± 0.6	1.3 ± 0.7	4.2 ± 1.4	1.3 ± 0.8
	CV (5)[d]	0.6 ± 0.3	30.5 ± 8.1	4.5 ± 1.6	11.1 ± 3.3	0.8 ± 0.3	0.7 ± 0.2	0.8 ± 0.2	42.8 ± 4.4	3.4 ± 0.8	1.7 ± 0.4	1.2 ± 0.5	0.4 ± 0.3
60	GF (4)	0.3 ± 0.1	34.4 ± 2.9	1.3 ± 0.4	12.8 ± 1.5	3.6 ± 2.5	0.8 ± 0.2	0.3 ± 0.1	33.8 ± 3.3	1.5 ± 0.4	1.1 ± 0.3	0.6 ± 0.1	11.5 ± 5.7
	CV (4)	0.3 ± 0.2	10.0 ± 6.5	1.0 ± 0.2	15.3 ± 5.2	8.4 ± 5.9	0.7 ± 0.2	0.5 ± 0.1	52.9 ± 12.7	1.8 ± 0.4	1.2 ± 0.2	4.0 ± 2.4	3.4 ± 2.9

[a] Approximate time (minutes) elapsed between gavage of $^{13}NO_3^-$ and sacrifice of animals.

[b] Each value represents mean for two rats, or mean ± standard error of the mean for four or five rats, as indicated in parentheses at left, respectively. ND, Not done.

[c] Du, Duodenum; Je, jejunem; Il, ileum; CC, cecum; LI, large intestine.

[d] Separation of the intestinal tract into tissue and contents of one rat revealed the following percentages of the total ^{13}N in the tissue component: duodenum, 25%; jejunum, 58%; ileum, 86%; cecum, 50%; and large intestine, 86%.

amounts of ^{13}N in the stomach and bladder, and lower amounts in the jejunum and ileum, than their CV counterparts. These observations suggested that GF rats were able to excrete in urine the ^{13}N absorbed from the stomach or intestines or both more rapidly than CV rats. However, ^{13}N did reach the lower small intestine of both GF and CV rats within an hour of $^{13}NO_3^-$ gavage (Table 1). Absorption and distribution of ^{13}N was particularly rapid and diverse in the GF rats sacrificed 15 to 30 min after gavage; these animals were starved approximately 6 h longer than the other GF and CV rats listed in Table 1. In contrast to the long starvation times, one CV rat gavaged with $^{13}NO_3^-$ within 2 h after eating had approximately 67% of the ^{13}N remaining in its stomach 32 min later. In the case of all these GF and CV rats, the weight of the stomach measured at the time of sacrifice correlated in a general way with the ^{13}N stomach exit results obtained, in that rats with lighter stomachs (suggestive of having less food contents) retained the label for the shortest amount of time.

Distribution of gavaged $^{13}NO_2^-$ in CV rats. A similar gavaging experiment was performed with $^{13}NO_2^-$ in CV rats (Table 2). The emptying of the stomach and resultant increase of ^{13}N in the small intestine and carcass with progression of the label down the GI tract with time was similar to the $^{13}NO_3^-$ data. Liver, kidney, cecum, large intestine, and blood again had moderate amounts of activity. At 45 min after gavage, however, greater amounts of label persisted in the stomach of the $^{13}NO_2^-$-gavaged rats (61%; Table 2) than in the $^{13}NO_3^-$-gavaged rats (30%; Table 1), whereas the bladder plus bedding did not show the ^{13}N increase previously seen with $^{13}NO_3^-$.

Effects of pyloric ligation or intestinal injection (without a ligation) on the distribution of $^{13}NO_3^-$. Gastroduodenal ligation was performed on starved CV rats before $^{13}NO_3^-$ gavage to determine whether absorption directly into the bloodstream or passage into the duodenum was more important for movement of ^{13}N from the rat stomach. Ligation caused the retention of ^{13}N in the stomach (Table 3), indicating that body distribution of $^{13}NO_3^-$ was mainly due to the passage of stomach contents into the duodenum. When $^{13}NO_3^-$ was injected into the unligated lower intestine (i.e., ileum, cecum, and large intestine) of CV rats, approximately 80% of the ^{13}N remained there 60 min after injection (Table 3). However, a similar injection of $^{13}NO_3^-$ into the unligated cecum of a GF rat showed that, at 30 min, only 60% of the label remained (data not shown). It did not appear that these stomach-emptying and intestinal-absorption re-

TABLE 2. *Distribution of gavaged $^{13}NO_2^-$ (2 ml) in prestarved CV Sprague-Dawley rats*

Time[a]	No. of animals tested	% Distribution of ^{13}N from $^{13}NO_2^-$[b]										
		Blood (per ml)	Stomach	Intestines[c]				Carcass	Liver	Kidney	Bladder	Bedding
				Du	Je	Il	CC	LI					
30	2	0.7	57.9	1.2	15.7	2.5	0.8	0.6	16.8	2.1	0.3	0.4	0.2
45	3	0.4 ± 0.2	61.2 ± 23.8	1.9 ± 1.6	5.6 ± 1.2	3.2 ± 2.9	0.7 ± 0.6	0.5 ± 0.3	22.8 ± 18.9	2.1 ± 1.7	0.2 ± 0.3	0.2 ± 0.1	0.3 ± 0.3

[a] Approximate time (minutes) elapsed between gavage of $^{13}NO_2^-$ and sacrifice of animals.
[b] Each value is expressed as the mean (two rats), or the mean ± standard error of the mean (three rats).
[c] See Table 1, footnote *c*.

TABLE 3. *Absorption and distribution of $^{13}NO_3^-$ from the stomach or cecum of CV Sprague-Dawley rats*

Treatment	No. of rats	Time[a]	% Distribution of ^{13}N from $^{13}NO_3^-$[b]								
			Blood (per ml)	Stomach	Intestines[c]				Carcass	Liver	Kidney	Bladder
					Du	Je	Il	CC	LI			
Stomach (pyloric) ligation[d]	3	45	0.04 ± 0.006	94.6 ± 3.6	0.2 ± 0.1	0.3 ± 0.4	0.2 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	4.0 ± 2.4	0.2 ± 0.1	ND	0.1 ± 0.2
Cecal $^{13}NO_3^-$ injection[e]	1	30	ND	0.0	0.2	0.4	32.8	48.4	12.2	5.2	0.4	0.0	0.0
	3	60	0.07 ± 0.1	2.1 ± 1.6	0.8 ± 0.3	1.1 ± 0.2	0.8 ± 0.3	71.6 ± 6.1	7.8 ± 4.2	12.3 ± 4.5	2.3 ± 0.4	ND	1.2 ± 0.7
Surgical (sham-operated) control[d]	2	30–45	0.4	47.4	6.9	8.4	0.9	1.2	1.0	28.1	3.2	0.6	1.1

[a] Approximate time (minutes) elapsed between gavage of $^{13}NO_3^-$ and sacrifice of animal(s).
[b] Each value is expressed as the mean (two rats) or mean ± standard error of the mean (three rats). ND, Not done.
[c] See Table 1, footnote *c*.
[d] A 2-ml amount of $^{13}NO_3^-$ was given by gavage.
[e] A 1-ml amount of $^{13}NO_3^-$ was injected into the terminal ileum, cecum (primarily), and proximal colon.

sults were significantly influenced by the surgical manipulation and anesthesia, because the sham-operated controls in Table 3 showed ^{13}N distributions comparable to those of "normal" rats (Table 1).

Bacterial influence on $^{13}NO_3^-$ after injection into excised ceca. The less efficient absorption of ^{13}N from the lower-intestinal tract of CV than GF rats suggested that the bacterial flora was involved in the metabolism or retention of $^{13}NO_3^-$. To investigate this possibility, excised ceca of GF and CV rats were injected intraluminally with $^{13}NO_3^-$ and incubated at 37°C for 15 min. They were then homogenized and separated into pellet and supernatant fractions via centrifugation (Table 4). This procedure did not allow a quantitative separation of intestinal material into supernatant and pellet components, but the data in Table 4 indicate that bacteria of CV rats were able to retain some ^{13}N because the amount of pelleted ^{13}N decreased when CV cecal contents were heated before the addition of $^{13}NO_3^-$. This same heat treatment did not appear to influence the distribution of ^{13}N in GF cecal fractions. These results suggest that ^{13}N would be more available for absorption from the GF than the CV intestine in vivo.

Distribution of label from $^{13}NO_3^-$ after administration by cardiac (i.v.) puncture. Up to 50 min after i.v. injection, most of the injected ^{13}N had not been excreted, and large amounts of label were present in both the GF and CV carcass (Table 5). The carcass values seemed to be inversely related to the bladder ^{13}N values; the GF animals showed much more urinary ^{13}N than did the CV animals (20.9 versus 2.0%), and they retained less label in the evis-

TABLE 4. *Effect of the microbial flora on the distribution of $^{13}NO_3^-$ in the supernatant and pellet fraction of cecal homogenates*

Cecal treatment	Animal[a]	Mean % of $^{13}NO_3^-$ added[b]	
		Supernatant	Pellet
Heated (85°C for 15 min) before adding isotope	GF	51.9	48.2
	CV	40.3	59.7
No pretreatment before adding isotope	GF	51.3	49.3
	CV	27.1	73.4

[a] Two animals per treatment group.
[b] Supernatant and pellet were obtained by centrifuging homogenized cecal samples at 17,000 × *g* for 10 min.

cerated carcass (67.2 versus 83.7%). GF and CV rats, however, showed minor differences in the amount of label in their GI tracts (Table 5). Throughout the 50-min period after i.v. $^{13}NO_3^-$ injection, the GF stomach tended to have more ^{13}N than did the CV stomach. Conversely, the intestines of CV rats, especially the jejunum, cecum, and large intestine at 50 min (Table 4), had more ^{13}N activity than those of GF rats. The amount of ^{13}N present in the GI tract of all rats generally decreased with time, especially in GF rats (Table 5). The cardiac puncture did not appear to influence the bloodstream distribution of ^{13}N, since the injection of $^{13}NO_3^-$ into the tail vein of one CV rat gave a comparable ^{13}N distribution pattern 20 min after injection (data not shown).

Distribution of ^{13}N between intestinal tissue and contents. To determine whether $^{13}NO_3^-$ could pass from the bloodstream to the lower-intestinal lumen, the ileocecal valve was ligated (to block any potential passage of stomach, biliary, or pancreatic ^{13}N), and $^{13}NO_3^-$ was injected i.v. via cardiac puncture. The cecum and large intestine were then excised and counted for ^{13}N activity at various times after $^{13}NO_3^-$ injection. After the intact intestinal segments were counted, they were separated into tissue and contents, and the activity in the resulting fractions was assessed. The total amount of ^{13}N in the intact, ligated ceca of GF and CV rats was greater than that in the ceca of rats without ligation (Tables 5 and 6), whereas the amount of label in the large intestine of ligated and nonligated rats was similar. In both the ceca and large intestines of the ligated CV rats the contents accounted for more of this ^{13}N with time after injection. The ceca of ligated GF rats also exhibited the latter pattern; however, the activity in the contents of the large intestine of these GF rats decreased with time after the injection and initial accumulation of label. These observations indicated that ^{13}N could, in both GF and CV rats, be passed into the intestinal lumen from the blood. The lower ^{13}N values in the GF large intestine indicated either a slower passage into the intestinal lumen or more movement of ^{13}N from the bacteria-free intestinal contents back into the bloodstream. Injection of $^{13}NO_2^-$ i.v. into a CV rat with a prior ileocecal ligation again indicated that ^{13}N could be secreted from the bloodstream to the intestinal lumen (data not shown).

Excretion of ^{13}N in saliva after i.v. $^{13}NO_3^-$. Because salivary NO_3^- and NO_2^- (after bacterial reduction) levels have been attributed to concentration and excretion of bloodstream NO_3^- in saliva (23), the submaxillary salivary glands of the rats used in the experiment of Table 6 were removed and analyzed for ^{13}N. The pancreas, which histologically resembles a salivary gland, was also removed and counted, since it is possible that ^{13}N is passed into the duodenum by the pancreatic ducts. In all five rats in Table 6, the salivary glands contained close to 0.4% of the administered ^{13}N; pancreatic ^{13}N levels per animal ranged from 0.4 to 0.9%. A further study of the ^{13}N content of the saliva itself was conducted, using two other CV rats (not shown). Pilocarpine-nitrate, which was used to induce salivation, was injected simultaneously with 1 ml of $^{13}NO_3^-$ into the heart. The resulting saliva in one rat contained 1.0% and that in the other rat contained 3.0% of the label per milliliter at 6 and 9 min, respectively, after $^{13}NO_3^-$ injection. These pilocarpine-induced salivary ^{13}N levels, however, probably did not represent "normal" salivary ^{13}N concentrations because the amount

TABLE 5. *Kinetics of $^{13}NO_3^-$ distribution after i.v. (cardiac puncture) injection into GF and CV Sprague-Dawley rats*

Animal	Time[a]	% Distribution of ^{13}N from $^{13}NO_3^-$[b]											
		Blood (per ml)	Stomach	Intestines[c]					Carcass	Kidney	Liver	Bladder[d]	Bedding
				Du	Je	Il	CC	LI					
GF	20 (2)	0.8	11.9	1.6	2.8[e]	1.2	1.6	0.8	64.7	1.5	4.0	7.3	0.0
	35 (2)	0.7	11.0	0.8	2.7[e]	1.3	1.1	0.8	71.4	2.0	2.8	3.5	0.0
	50 (1)	0.2	3.1	0.4	1.3[e]	1.1	0.7	0.3	67.2	2.0	1.7	20.9	0.0
CV	20 (2)	1.6	7.9	1.5	2.1	1.0	1.7	1.2	70.3	2.0	3.7	0.5	0.3
	35 (2)	1.1	7.5	1.2	1.9	1.0	2.3	1.6	74.8	1.6	4.9	0.1	1.5
	50 (1)	0.6	3.6	0.5	2.9	0.4	1.4	1.3	83.7	1.5	1.4	0.5	1.7

[a] Approximate time (minutes) between cardiac puncture and sacrifice of animal(s).
[b] Each value is either expressed for one rat, or is the mean of two rats, as indicated in parentheses at left.
[c] See Table 1, footnote *c*.
[d] All GF rats had noticeable amounts of urine in the bladder.
[e] Separation of jejunal sections into tissue and contents yielded from two to eight times more label in tissue over contents.

of pilocarpine used (9 mg) altered the normal physiology of these rats. This was seen by the serous-like lacrimation and salivary foaming in both rats and the watery stools in one rat (this watery stool, at 29 min, contained 0.4% of the injected $^{13}NO_3^-$).

Influence of diarrhea on distribution of ^{13}N from gavaged $^{13}NO_3^-$. To investigate the extent to which diarrhea might affect the processing, distribution, or transit time of NO_x^- (or their metabolites), castor oil, a well-known cathartic, and PGE_2, which has been implicated in certain types of bacterial diarrhea (13), were gavaged into CV rats at various times before $^{13}NO_3^-$ gavage. Castor oil was effective in inducing watery stools in many of the CV rats (Table 7). The small-intestine contents of most of these rats were also very liquid and loose. These bowel alterations resulted, in some instances, in a dramatic increase in ^{13}N passage down the GI tract within 60 min after $^{13}NO_3^-$ administration. For example, the 720/15 (diarrhea induction time per time after ^{13}N gavage) rat had 2.9% of the ^{13}N in the large intestine 15 min after ^{13}N gavage,

TABLE 6. *Effect of ileocecal ligation on the distribution of i.v.-injected $^{13}NO_3^-$ in the tissues and contents of the ceca and large intestines of GF and CV rats*

Animal[a]	Time[b]	% Distribution of ^{13}N from $^{13}NO_3^-$[c]						
		Blood (per ml)	Cecum			Large intestine		
			Intact	Tissue	Contents	Intact	Tissue	Contents
CV[d]	20	1.1	5.5	4.1	1.4	1.0	0.8	0.2
	30	0.4	4.7	2.9	1.8	1.8	1.0	0.8
	45	0.2	4.4	1.6	2.8	0.8	0.4	0.4
GF[e]	35	0.2	6.0	2.3	3.7	0.5	0.35	0.15
	60	0.2	4.3	0.9	3.4	0.4	0.3	0.1

[a] One animal used per time period.
[b] Minutes from $^{13}NO_3^-$ injection to time of sacrifice.
[c] Values represent the percentage of the total ^{13}N injected.
[d] All other GI tract, kidney, liver, etc., results not listed for these rats agreed to within ±1% of the ^{13}N values for similar time (i.e., 20-, 35-, and 50-min) period rats presented in Table 1, except for the liver at 45 min, which had 2.7% more, and the carcasses at 30 and 45 min, which had 3.5 and 2.8%, respectively, less ^{13}N than rats in Table 1.
[e] There was good general agreement with rats in Table 1, except for the stomach at 35 min, which had 7.8% less and the bladder at 60 min, which had approximately 20% less than rats in Table 1.

TABLE 7. *Effect of castor oil on the distribution of gavaged $^{13}NO_3^-$ (2 ml) in CV Sprague-Dawley rats*

Treatment	Time (min) from:		Watery stool[a]	% Distribution of ^{13}N from $^{13}NO_3^-$[b]										
	Treatment to $^{13}NO_3^-$ gavage	$^{13}NO_3^-$ gavage to sacrifice		Stomach	Intestines[c]					Carcass	Kidney	Liver	Bladder	Bedding
					Du	Je	Il	CC	LI					
Castor oil (2 ml)	720	15	+	1.7	3.0	8.2	2.8	2.4	2.9	65.2	4.0	6.7	2.2	0.9
	260	30	+	15.9	2.6	4.8	2.7	3.8	2.0	52.9	2.9	5.2	2.6	0.0
	210	45	++	34.8	1.7	2.0	0.5	1.4	0.6	36.8	1.2	3.1	0.1	16.5
	160	60	−	48.3	1.5	12.6	2.6	0.6	0.6	26.7	1.2	2.8	0.7	1.9
	60	30	++[d]	10.4	24.7	8.0	1.7	1.2	1.3	45.2	0.9	2.8	2.1	0.0
	60	30	−	72.2	4.3	1.2	0.5	0.2	0.1	20.3	0.2	0.6	0.1	0.0
	60	60	—	41.2	19.1	4.9	2.6	0.9	0.6	26.7	0.5	2.1	0.1	1.2
Control (2 ml of water)	60	60	−	25.5	1.3	12.6	4.1	1.4	0.3	44.7	1.3	1.8	3.1	3.0

[a] +, Watery stool was evident between treatment time and injection of radioisotope; ++, watery stool was evident between injection of radioisotope and sacrifice; −, no watery stool obvious; —, rat urinated on bedding.
[b] Each control value represents the mean of two animals per group; each of the other values in the table applies to a single animal.
[c] See Table 1, footnote c.
[d] Watery stool had 2.0% of total label at 11 min after $^{13}NO_3^-$ gavage (bedding was changed after this); no urine was noted in the bladder of this animal.

a 60/30 rat passed 2% of the label through the GI tract and into the watery stool that was excreted 11 min after ^{13}N gavage, and a 210/45 rat excreted 16.5% of the label in the watery stool (no urine was noted) within 45 min after gastric intubation of $^{13}NO_3^-$ (Table 7). In other CV rats that did not have watery stools, however, there was a much slower ^{13}N stomach exit and distribution (i.e., the 160/60 and 60/30 rats; Table 7). These latter results correlated with the observation that castor oil was still present in their stomachs. Except for the large amount of ^{13}N noted in the intestines of one 60/30 rat, and the 60/60 rat, this oil-induced diarrheal state did not seem to remarkably influence the absorption of gavaged $^{13}NO_3^-$ or its metabolites.

PGE_2 gavaged 60 min before $^{13}NO_3^-$ gavage into four CV rats also produced mixed results (data not shown). One rat showed high ^{13}N values in the cecum (8.6%) and throughout the intestinal tract (i.e., 36% compared with 29%, which was the highest previous value noted for one of the CV rats in a "normal" state in Table 1). However, PGE_2 seemed also to slow down stomach exit of ^{13}N, since over 49% of the label remained in the stomach at 60 min after $^{13}NO_3^-$ gavage. All PGE_2-treated rats had only slight (if any) fluidity of contents, and no PGE_2-treated rat passed watery stools during the treatment or isotope study periods.

DISCUSSION

From chemical results it is generally accepted that ingested NO_x^- are rapidly removed from the upper GI tract and excreted by the kidneys in the urine (8). Our results with GF and CV rats gavaged or injected with $^{13}NO_3^-$ or $^{13}NO_2^-$ suggest that the distribution of these ions or their metabolites is more widespread in the body. Furthermore, NO_3^- and NO_2^- distribution appears to be a complex and dynamic process that is influenced by such factors as the bacterial flora, the clinical state of the GI tract at the time of ion administration, and which ion is initially ingested.

For intragastric nitrosation to be significant, ingested NO_x^- must not disappear too rapidly from the stomach. Since little (29) or no (15) gastric reduction of NO_3^- seems to occur in the normal CV or GF rat, the increased retention of gavaged $^{13}NO_3^-$ in full stomachs, as opposed to those empty of food, presumably reflects the contents acting mainly as a physical barrier to exit, although gastric pH changes due to the presence of food (and bacteria) may also influence $^{13}NO_3^-$ exit. The situation in humans appears similar, since the time after a meal influenced stomach exit of $^{13}NO_3^-$ (28) and since little NO_3^- seems to be reduced in the normal human stomach (26).

On the other hand, when CV rats were gavaged with $^{13}NO_2^-$, label left the stomach more slowly than when $^{13}NO_3^-$ was given to CV rats that were starved for the same amount of time. This difference suggests that $^{13}NO_2^-$ reacts with material (i.e., contents or bacterial or both) in the stomach, perhaps by diazotization, nitrosation, or conversion to oxides of nitrogen which can occur at the low gastric pH (14, 15) to produce chemical forms which cannot exit the stomach as quickly as $^{13}NO_3^-$. The slow exit of $^{13}NO_2^-$ (or derivatives) is supported by the results of Mirvish et al. (15), who fed NO_2^--supplemented food to CV rats and found that NO_2^-, as such, persisted in their stomachs for up to 5 h. In similar experiments with nonstarved mice, however, Friedman et al. (3) found that 85% of gavaged NO_2^- was lost within 10 min. The latter authors attributed most of the gastric loss of NO_2^- to direct absorption into the bloodstream, since pyloric valve ligation did not affect the rate of exit from the stomach. When $^{13}NO_2^-$ was gavaged into a CV rat (data not shown) with a pyloric ligation, approximately 10% left the stomach after 35 min. Hence, $^{13}NO_2^-$ appears to pass slowly from the rat stomach into the bloodstream, presumably as $H^{13}NO_2^-$ as previously suggested (14), but apparently much more slowly than from the mouse stomach (3). Consequently, in agreement with Mirvish et al. (15), we conclude that most of the ingested NO_x^-, and derivatives, leave the stomach via the pyloris.

The bacterial flora appears to influence the distribution of ^{13}N in the intestinal tract. Whether $^{13}NO_3^-$ was gavaged (Table 1) or intraluminally injected (Table 3) into the ceca, GF rats absorbed and excreted the ^{13}N faster than comparably treated CV rats. Since GF rats seem unable to metabolize NO_3^- to NO_2^- (29), this suggests that the microbial flora of the CV rats metabolized the $^{13}NO_3^-$ to chemical forms that were unable to pass the intestinal barrier as easily as NO_3^-. It also could indicate that the microbial flora of the CV rats was able to incorporate the ^{13}N from $^{13}NO_3^-$; that such incorporation does occur is indicated by the data in Table 4. The incorporated nitrogen is then presumably excreted in the feces, although the half-life of ^{13}N was too short to allow this to be observed. Bacterial incorporation may also explain our inability to chemically assay NO_2^- and NO_3^- throughout most of the intestinal tract of CV as opposed to GF rats (29).

The accelerated transit of ^{13}N induced by PGE_2 or castor oil shows that in diarrhea, NO_x^-

or their metabolites can move rapidly from the stomach to the lower intestines. Such passage could both increase exposure of the large intestine to carcinogens formed in the stomach as suggested by Lijinsky (11) or allow the nitrosation reactions themselves to more readily occur in the lower GI tract as suggested by Wang et al. (27).

Most of the gavaged $^{13}NO_3^-$ or $^{13}NO_2^-$ which left the stomach was rapidly absorbed into the bloodstream from the upper small intestine but was not rapidly excreted in the urine. Experiments where $^{13}NO_3^-$ or $^{13}NO_2^-$ was injected i.v. showed that ^{13}N can reenter the GI tract at several points. From a chemical standpoint, as reported by others with NO_x^- (17, 26), we noted that ^{13}N from $^{13}NO_x^-$ appeared in the saliva and thus in the stomach. The ^{13}N in the lower intestinal contents that increased with time after i.v. injection of label into rats with ileocecal ligations demonstrated that ^{13}N is secreted through the intestinal walls and into the contents. It is also possible that some label passed into the small intestine in bile or in pancreatic secretions, since much of the ^{13}N in the jejunum after i.v. injection was in the lumen. Although we did not do experiments to measure the significance of these secretions to the appearance of ^{13}N into the intestine, the histological similarity of the pancreas to the salivary (parotid) glands suggests that the pancreas may play the more important role in this regard.

It is reasonable to assume that some of the ^{13}N in the blood after gavage, and especially after i.v. injection, was in the form of $^{13}NO_3^-$, since studies by ourselves (29) and others, as previously noted, show that only NO_3^- is normally excreted in the urine after ingestion of either NO_3^- or NO_2^-. If further studies confirm this assumption, it would suggest that the ^{13}N which accumulated in the carcass of both CV and GF rats after oral or i.v. administration was also partly $^{13}NO_3^-$, since mammalian tissues seem unable to metabolize NO_3^- in vivo (29). More ^{13}N was found in the carcasses than could be accounted for by blood-borne label so that the tissues generally must be considered to retain NO_3^-. This is supported by an early observation that the NO_3^- level in ascites fluid matches that in blood (9). The capacity of the body to retain ingested NO_x^- may also be influenced by the amount ingested, since studies with urinary excretion of NO_3^- in humans (6) and dogs (4) noted that the percentage of this ion recovered in the urine increased as the amount of ingested NO_3^- increased. The possibility exists, therefore, that bacteria in infected tissues, as well as in the stomach, bladder, and intestinal tract, have access to NO_3^- which they could use to form *N*-nitroso compounds if the necessary precursor amines or amides are also present. Antibiotics are one possible source of such precursors (19).

A recent report by Tannenbaum et al. (23) demonstrates the importance of thoroughly understanding the distribution and metabolism of these ingested ions. They attributed NO_3^- and NO_2^- values in urinary, fecal, and ileostomy samples of persons on restricted-protein diets or restricted-NO_3^- and -NO_2^- diets, or both, to be the result of bacterial nitrification, presumably from ammonia, in the small intestine. Our ^{13}N results suggest that passage of ingested NO_3^- or NO_2^- down the GI tract and secretion from the bloodstream into the intestinal lumen, rather than bacterial nitrification may account in part for the results observed by Tannenbaum et al. (23). The temporary storage of NO_3^- in the extravascular spaces of the body, as suggested by our ^{13}N data, may also account for the fluctuation seen in the levels of these compounds in human urine (23). The reports by Wang et al. (27) of volatile *N*-nitroso compounds and by Varghese et al. (25) of bacterial mutagens presumed to be nonvolatile nitrosamines in human feces further emphasize the importance of determining the distribution and metabolism of ingested NO_2^- and NO_3^-. If these nitroso compounds were formed in the lower intestine, as has been suggested, it is necessary to determine whether the precursor NO_2^- and NO_3^- ions are of endogenous or environmental origin. If they are endogenous, this would represent an apparently unavoidable exposure of the large intestine to endogenously formed carcinogenic compounds.

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LITERATURE CITED

1. **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.
2. **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.
3. **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.
4. **Greene, I., and E. P. Hiatt.** 1954. Behavior of the nitrate ion in the dog. Am. J. Physiol. **176**:463-467.
5. **Hashimoto, S., T. Yokokura, Y. Kawai, and M. Mutai.** 1976. Dimethylnitrosamine formation in the gastro-in-

testinal tract of rats. Food Cosmet. Toxicol. **14**:553–556.
6. **Hawksworth, G., and M. J. Hill.** 1971. Bacteria and the N-nitrosation of secondary amines. Br. J. Cancer **25**: 520–526.
7. **Hawksworth, G., and M. J. Hill.** 1974. The *in vivo* formation of N-nitrosamines in the rat bladder and their subsequent absorption. Br. J. Cancer **29**:353–358.
8. **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.
9. **Keith, N. M., M. Whelan, and E. G. Bannick.** 1930. The action and excretion of nitrates. Arch. Intern. Med. **46**:797–832.
10. **Klein, D., N. Gaconnet, B. Poullain, and G. Derby.** 1978. Possibility of nitrosation during human digestion. I. Evaluation of precursors and affecting factors *in vivo*. II. Effect of food intake on the reaction. Int. Agency Res. Cancer Sci. Publ. **19**:485–494.
11. **Lijinsky, W.** 1977. Nitrosamines and nitrosamides in the etiology of gastrointestinal cancer. Cancer **40**:2446–2449.
12. **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. American Chemical Society, Monograph no. 173, Washington, D.C.
13. **Matuchansky, C., and S. Coutrot.** 1978. The role of prostaglandins in the study of intestinal water and electrolyte transport in man. Biomedicine **28**:143–148.
14. **Mirvish, S. S.** 1975. Formation of N-nitroso compounds: chemistry, kinetics and *in vivo* occurrence. Toxicol. Appl. Pharmacol. **31**:325–351.
15. **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.
16. **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.
17. **Ruddell, W. S. J., L. M. Blendis, and C. L. Walters.** 1977. Nitrite and thiocyanate in the fasting and secreting stomach and in saliva. Gut **18**:63–77.
18. **Sander, J.** 1971. Untersuchungen über die Entstehung carcerogener Nitrosoverbindungen in Magen von Versuchstieren und ihre Bedeutung für den Menschen. Arzneimittel-Forsch. **21**:1572–1580, 1707–1713, 2034–2039.
19. **Scheunig, G., and D. Ziebarth.** 1976. Formation of nitrosamines by interaction of some drugs with nitrite in human gastric juice. Int. Agency Res. Cancer Sci. Publ. **14**:269–277.
20. **Spiegelhalder, B., G. Eisenbrand, and R. Preussman.** 1976. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of N-nitroso compounds. Food Cosmet. Toxicol. **14**:545–548.
21. **Stephany, R. W., and P. L. Schuller.** 1978. The intake of nitrate, nitrite and volatile N-nitrosamines and the occurrence of volatile N-nitrosamines in human urine and veal calves. Int. Agency Res. Cancer Sci. Publ. **19**: 443–460.
22. **Tannenbaum, S. R., M. C. Archer, J. S. Wishnok, and W. W. Bishop.** 1978. Nitrosamine formation in human saliva. J. Natl. Cancer Inst. **60**:251–253.
23. **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.
24. **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.
25. **Varghese, A. J., P. C. Lund, R. Furrer, and W. R. Bruce.** 1978. Nonvolatile N-nitroso compounds in human feces. Int. Agency Res. Cancer Sci. Publ. **19**:257–264.
26. **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.
27. **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.
28. **Witter, J. P., S. J. Gatley, and E. Balish.** 1979. Distribution of nitrogen-13 from labeled nitrate ($^{13}NO_3^-$) in humans and rats. Science **204**:411–413.
29. **Witter, J. P., and E. Balish.** 1979. Distribution and metabolism of ingested NO_3^- and NO_2^- in germfree and conventional-flora rats. Appl. Environ. Microbiol. **38**: 861–869.