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Tissue Residues, Metabolism, and Excretion of Radiolabeled Sodium Chlorate ($Na[^{36}Cl]O_3$) in Rats

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A novel preharvest technology that reduces certain pathogenic bacteria in the gastrointestinal tracts of food animals involves feeding an experimental sodium chlorate-containing product (ECP) to animals 24−72 h prior to slaughter. To determine the metabolism and disposition of the active ingredient in ECP, four male Sprague−Dawley (~350 g) rats received a single oral dose of sodium [^{36}Cl]chlorate (3.0 mg/kg body weight). Urine, feces, and respired air were collected for 72 h. Radiochlorine absorption was 88−95% of the administered dose, and the major excretory route was the urine. Parent chlorate was the major species of radiochlorine present in urine at 6 h (~98%) but declined sharply by 48 h (~10%); chloride was the only other species of radiochlorine detected. Except for carcass remains (4.6% of dose), skin (3.2%), and gastrointestinal tract (1.3%), remaining tissues contained relatively low quantities of radioactivity, and >98% of radiochlorine remaining in the liver, kidney, and skeletal muscle was chloride. Chlorite instability was demonstrated in rat urine and bovine urine. The previously reported presence of chlorite in excreta of chlorate-dosed rats was shown to be an artifact of the analytical methods employed. Results from this study indicate that chlorate is rapidly absorbed and reduced to chloride, but not chlorite, in rats.

KEYWORDS: Chlorate; chlorite; rats; metabolism; pathogen; preharvest food safety; chloride

INTRODUCTION

Contamination of food products with Gram-negative pathogens such as *Escherichia coli* strain O157:H7 and *Salmonella* species is believed to be the cause of tens of thousands of preventable human illnesses per year in the United States (*1*, *2*). Major reservoirs of these pathogens are contained in gastrointestinal (GI) tracts of many livestock species, and these reservoirs may serve as sources of carcass contamination during animal slaughter and carcass processing. A new promising technology for controlling the numbers of *E. coli* O157:H7 and *Salmonella typhimurium* in livestock has been described by Anderson et al. (*3*, *4*). This technology involves the oral administration of an experimental sodium chlorate-containing product (ECP) to animals 24−72 h prior to slaughter. Certain human pathogens such as *E. coli* O157:H7 and *Salmonella* contain respiratory nitrate reductase, which converts dietary nitrate (NO_3^-) to nitrite (NO_2^-). Chlorate (ClO_3^-) is also metabolized by intracellular nitrate reductase to chlorite (ClO_2^-), a chemical species that is toxic to bacteria containing the nitrate reductase enzyme. Previous studies have demonstrated that oral administration of ECP is highly effective at reducing the numbers of *E. coli* O157:H7 and (or) *S. typhimurium* in GI tracts of swine (*3*, *4*), cattle (*5*, *6*), sheep (*7*), and broilers (*8*, *9*). Because fecal contamination of food animal carcasses is a major source of food-borne pathogens (*10*), the use of a sodium chlorate-containing product could have a major impact on food safety for the livestock industry.

Before chlorate may be used as a preharvest food safety tool, the levels of residues remaining in edible tissues of food animals must be determined. To this end, Smith et al. (*11*, *12*) have studied the fate and metabolism of chlorate in cattle. These studies indicated that chlorate was rapidly absorbed and excreted and that chlorate was extensively converted to chloride ion after oral administration. Whether chlorate is converted to chloride primarily in the rumen or after absorption is a current topic of investigation. Smith et al. (*11*, *12*) did not detect intermediate chloroxyanions (i.e., chlorite, hypochlorite) that are presumably formed during the conversion of chlorate to chloride, even though the reduction of chlorate to chloride involves a six-electron transfer. It is unknown whether these metabolic intermediates are formed and are unstable in the reducing atmosphere of the rumen or if they are formed in the rumen, absorbed, and transformed by the beef animal itself.

Previous studies using rats seemed to indicate that at least one intermediate oxyanion, chlorite (ClO_2^-), was formed in tissues and was excreted in sufficient quantities for measurement (*13*, *14*). The fact that chlorite was not formed and excreted in cattle is of food safety importance because the FDA Center for Veterinary Medicine (CVM) considers chlorite to be of toxicological concern. Indeed, the FDA-CVM has established provisional safe tissue concentrations for chlorite in edible

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tissues (personal communication). Several other organizations have used the data of Abdel-Rahman et al. (*13*, *14*) as the model for chlorate and chlorite metabolism in rodents (*15*−*19*). Unfortunately, the data published by Abdel-Rahman et al. (*13*, *14*) concerning chlorate metabolism in rats are fraught with uncertainties. Specifically, methodological descriptions were, at best, ambiguous; recoveries of radiolabeled materials were very poor (∼40%); and variability surrounding the measurement of ^{36}Cl-chloride in fortified plasma was about 20%. In addition, results presented as pharmacokinetic data of chlorate and chlorite were, in reality, pharmacokinetic data of total radiochlorine.

Because of the renewed interest in chlorate and its possible use in animal agriculture and due to uncertainties surrounding the methods (*20*) and results (*13*, *14*) reported by Abdel-Rahman et al. (*13*, *14*, *20*), the objectives of this study were to determine the metabolism and disposition of sodium ^{36}Cl-chlorate in rats. Ion chromatographic methods of analysis were employed in this study, and results were verified using chemical techniques as required.

MATERIALS AND METHODS

Radiolabel. *Sodium [^{36}Cl]chlorate.* Radiolabeled sodium chlorate (Na[^{36}Cl]O_3) with a specific activity of 0.575 mCi/mmol was synthesized by Ricerca Biosciences (Concord, OH). Radiochemical purity of the sodium chlorate stock material, assessed using both paper and ion chromatography, was 94.4% with the impurities (∼5.6%) being sodium [^{36}Cl]chloride and perchlorate (∼0.5%). The ^{36}Cl-chlorate peak was purified using low-pressure liquid chromatography as described by Ruiz-Cristin et al. (*21*). Briefly, 100 μCi of sodium chlorate in water was loaded onto a 1 cm × 30 cm Sephadex G-10 column that was subsequently eluted with 0.1 M ammonium acetate (pH 7.01) at a flow rate of 0.4 mL/min. Fractions were collected every 2 min. [^{36}Cl]-Chlorate-containing fractions were combined to yield a final radiochemical purity of >99.5% as assessed by ion chromatography with radiochemical detection. The remaining radiochemical impurity was chloride. Ion chromatography was accomplished using a Waters (Milford, MA) model 600E controller and pump equipped with Teflon pump heads. Purified [^{36}Cl]chlorate was eluted from Dionex AS 16 HC and AG-16 guard columns (Sunnyvale, CA) with 30 mM NaOH at a flow rate of 1.0 mL/min after injection through a PEEK Rheodyne injector (model 97251 PEEK, Cotati, CA).

The specific activity was determined chromatographically as reported by Smith et al. (*22*). Briefly, a four-point standard curve (0.5−4.1 μg on-column) of unlabeled sodium chlorate was constructed (Dionex AS16 column; 20 mM NaOH isocratic mobile phase) and the relationship between peak area, as determined by conductivity detection (Dionex CD-25; 100 mA, external water mode), and mass was calculated via linear regression. Quintuplicate injections of an unknown mass of purified Na[^{36}Cl]O_3 were made, and the resulting peak area was determined by integration; the corresponding Na[^{36}Cl]O_3 peaks were collected into scintillation vials as they eluted from the column. Radiochlorine captured in the vials was quantified using a liquid scintillation counter (LSC). The specific activity was then determined by dividing the total dpm in each peak by the corresponding mass of Na[^{36}Cl]O_3 injected. The specific activity of the purified radiolabeled chlorate was 12101 ± 34 dpm/μg and was used undiluted for dosing.

Sodium [^{36}Cl]chloride. Sodium [^{36}Cl]chloride (>99% radiochemical purity; 22040 dpm/μg), isolated during the purification of the sodium [^{36}Cl]chlorate dosing material, was used as an analytical standard.

Sodium [^{36}Cl]chlorite. A series of four three-necked round-bottom flasks were used. The first round-bottom flask contained a magnetic stir bar, 0.44 mL of 30% H_2O_2, and approximately 227 μCi of 6.1 M sodium [^{36}Cl]chlorate dissolved in approximately 0.67 mL of water. Nitrogen gas was passed into the center neck of the first flask so that nitrogen bubbled through the reaction mixture. Nitrogen was vented through sequential round-bottom flasks containing 10 mL of 2 M sodium carbonate, 10 mL of 0.61 M NaOH plus 1 mL of 30% H_2O_2, and 10 mL of ice cold water, respectively. An addition funnel containing 2.5 mL of 5 M H_2SO_4 was placed on a side arm of the reaction flask, and the reduction of sodium [^{36}Cl]chlorate was initiated at room temperature by the dropwise addition of the acid to the [^{36}Cl]chlorate. After the addition of sulfuric acid was complete, the temperature of the reaction flask was increased to 50 °C for several hours.

The reaction progress could be followed by the formation of a pale yellow to a yellow-green color in the reaction mixture and the subsequent transport of the evolved chlorine dioxide to the carbonate scrubber (to remove any chlorine gas that might have formed). Chlorine dioxide was transported to the flask containing hydrogen peroxide, where it was quickly reduced to sodium chlorite with an immediate loss of color. The radiochemical purity of the recovered sodium [^{36}Cl]chlorite (approximately 43% yield) was greater than 99% as measured by ion chromatography with radiochemical detection. Sodium [^{36}Cl]chlorite was stored refrigerated in an amber vial as a dilute aqueous solution until use. Because of the propensity of sodium chlorite to decompose, its radiochemical purity was assessed prior to each use.

Animals. Six male Sprague−Dawley rats (349 ± 25.8 g) were obtained from Harlan Sprague−Dawley (Indianapolis, IN). The animals were maintained in accordance with all U.S. Department of Agriculture regulations for the care and use of laboratory animals. Research protocols were approved by the Institutional Animal Care and Use Committee at the ARS Biosciences Research Laboratory. Four were randomly selected for treatment, while the remaining two were used as controls. Animals were housed in hanging stainless steel cages for the prestudy period and were housed in glass metabolism cages for the duration of the study period. Rats were allowed ad libitum access to feed (Purina Mills Rat Chow #5012, St. Louis, MO) and water during the prestudy and study periods.

Dosing and Sample Collection. The target sodium [^{36}Cl]chlorate dose was approximately 1 mg per rat or roughly 3 mg/kg. This dose was nearly 50-fold greater than the dose used by Abdel-Rahmen et al. (*13*, *14*, *23*) but about 10-fold less than sodium chlorate doses (on a mg/kg body weight basis) shown to be effective at reducing pathogens in livestock species [40 mg/kg in cattle (*5*) and 35 mg/kg in swine (*4*)]. Sodium [^{36}Cl]chlorate was formulated in water (2 mg/mL), and 0.5 mL (5.4 μCi) was given by gavage. The actual dose delivered was calculated based on weight of dose delivered and the radioactivity remaining in the syringe. Control rats received 0.5 mL of nanopure water.

Control and treated rats were placed in glass metabolism cages designed for the separate collection of urine, feces, and respired air. Excreta samples were collected at 6 h intervals for the first 24 h (0−6, 6−12, 12−18, and 18−24 h), 8 h intervals for the second 24 h (24−32, 32−40, and 40−48 h), and at 12 h intervals for the last 24 h (48−60 and 60−72 h) of the study. Both urine and feces were weighed at collection and stored frozen (−20 °C). Because of concerns about the stability of some potential chlorate metabolites, a single 5 μL sample of urine from each rat was collected at the 6 h time point for the immediate determination of radiochemical composition by ion chromatography.

Respired air from each cage was bubbled sequentially through two 125 mL flasks containing 1 M NaOH and a third flask containing 250 mL of tap water, respectively, using an air pump. Sodium hydroxide was used to trap gaseous products, either chlorine (Cl_2) or chlorine dioxide (ClO_2), that might form according to the following reactions:

$$Cl_2 + 2OH^- \leftrightarrow OCl^- + Cl^- + H_2O$$

and/or

$$2ClO_2 + 2OH^- \leftrightarrow ClO_2^- + ClO_3^- + H_2O$$

The water trap was used as a final scrubber for gases that might escape the hydroxide traps. Respired gases were collected during the whole collection period, and hydroxide and water traps were sampled at the completion of the 72 h study period.

After the 72 h period, each rat was anesthetized with halothane and exsanguinated via heart puncture. Blood was drawn into heparinized syringes and transferred to heparinized test tubes. A 1 mL aliquot of whole blood was removed and frozen, and the remainder was processed

for plasma (15 min of centrifugation at 730*g*). Rats were dissected, and epididymal adipose tissue, bone (femur), brain, diaphragm, GI tract, heart, kidney, liver, lungs, skin (including tail), spleen, testes, and thymus were removed and weighed. The thyroid gland was not specifically removed. A sample of skeletal muscle (*longisimus dorsii*) of approximately 20 g was removed from each carcass. All remaining tissues were pooled into a "carcass remains" fraction.

Analyses. *Determination of Background Activity and Limits of Quantitation.* For each sample set, quadruplicate aliquots of control matrix (urine, feces, blood, or tissue) were weighed into scintillation vials, solubilized (when appropriate), and/or diluted with scintillation cocktail; background radioactivity was determined by counting each sample for 20 min with the LSC's background set to 0. The background activity was defined as the average value of the replicate control aliquots within a sample set. The limit of detection (LOD) for each matrix or sample set was defined as the mean background dpm plus three standard deviations (SDs) of the mean. Analyzed samples from dosed animals with a mean dpm value below the LOD were considered to have no detectable residues.

Respiratory Gases. Quadruplicate aliquots (1 mL) were removed from the sodium hydroxide and water traps and weighed into 20 mL glass vials, and 15 mL of Ultima Gold liquid scintillation cocktail (Perkin-Elmer Life and Analytical Sciences, Boston, MA) was added. Radiochlorine was quantified using Beckman model 1700 LS (Beckman, Fullerton, CA) or Packard model 1900 or 2500 LSCs (Packard, Meridan, CT). The background activity was determined from replicate 1 mL aliquots of 1 M sodium hydroxide, and water was prepared as described above.

Urine. The radioactivity in urine was determined on 25 μL weighed aliquots (in quadruplicate) plus 250 μL of nanopure water to which 6 mL of Ultima Gold was added. Vials were dark-adapted for 1 h and then counted for 20 min each with a LSC.

Feces. Feces were lyophilized to a constant weight and ground in a mortar and pestle, and quadruplicate 0.2 g aliquots were added and mixed with 8 mL of Carbosorb E (Packard) and then placed into a heated shaking water bath at 60 °C overnight. Vials were brought to room temperature, 12 mL of Permafluor E (Packard) was added and dark-adapted for 1 h, and radiochlorine was quantified using a LSC.

Tissues. Frozen tissues were homogenized in solid CO_2 (*24*) using a Waring Blender (muscle, GI tract) or were blended with a mortar and pestle (adipose tissue, brain, heart, kidney, liver, lungs, spleen, testes, and thymus). Carbon dioxide was then allowed to sublimate in a freezer. Bone was homogenized in liquid nitrogen with a mortar and pestle. Carcass remains were homogenized in a Hobart grinder. Skin was weighed and diluted 1:1 (w:w) in 1 M NaOH and digested for 3 days at 50 °C. Total radiochlorine concentrations in tissues were determined with either triplicate or quintuplicate aliquots of each tissue (200 mg). Weighed aliquots were digested in 8 mL of Carbosorb E for ~16 h at 60 °C. Cooled digests were diluted with 12 mL of Permafluor E, and radiochlorine was quantified by LSC. One milliliter aliquots of solubilized skin samples were weighed, diluted with 15 mL of Ultima Gold, and counted by LSC.

Cage Wash. Each metabolism cage was rinsed with water at the conclusion of the study, and the rinse was collected and labeled "cage rinses". Quantitation of radioactivity in cage rinses was conducted as described for urine, except that the sample aliquot size was either 250, 500, or 1000 μL.

Speciation of Tissue Residues. The methods used to speciate radiochlorine in tissue extracts and urine were those of Smith et al. (*11*). Duplicate sets of partially frozen tissues were weighed (muscle and carcass remains, 5 g; liver, 3 g; and kidney, 0.4 g) and placed in 50 mL polypropylene tubes. Corresponding sets of nonfortified and fortified control tissues were also prepared. Fortified tissues were prepared by adding 25 μL of a solution containing approximately 27800 dpm consisting of [^{36}Cl] as NaCl:$NaClO_3$ (52%:48%, respectively). Fifteen milliliters of water was added to each tissue, homogenized with a Tekmar homogenizer, and centrifuged at 31500*g* for 15 min. Supernatants were decanted into clean tubes, and the pellets were resuspended and homogenized in 10 mL of water. After centrifugation, respective supernatants were combined and 20 mL of ice-cold acetonitrile was then added to precipitate protein. After centrifugation (31500*g*, 15 min) and decanting, acetonitrile in the aqueous phase was evaporated under N_2 at 60 °C. In some cases, a precipitate was formed during evaporation; in such instances, samples were centrifuged at 3750*g* for 15 min and the supernatant was decanted. Aqueous supernatants were applied to conditioned Bakerbond C18 Mega Bond Elut SPE cartridges (J. T. Baker, Phillipsburg, NJ) and the nonretained aqueous phase was collected. Cartridges were rinsed with 5 mL of water, which was pooled with the nonretained phase and assayed for radiochlorine. The C18 eluents were then applied to cation exchange SPE cartridges (LC-SCX; Supelco, Bellefonte, PA), and the nonretained phase was collected. Cartridges were then rinsed with 2.5 mL of water, combined with the bypass, and assayed for radiochlorine content. Recoveries of radioactivity from the C18 and SCX SPE columns, across all tissues, were 94.3 $\pm$ 0.9 and 99.5 $\pm$ 0.9%, respectively. These samples were lyophilized, reconstituted with 1 mL of water, and then chromatographed on the ion chromatography system described above, except that the isocratic mobile phase was replaced by a gradient. Specifically, after 10 min at 10 mM NaOH, a linear gradient from 10 to 32 min to 50% of 100 mM NaOH was used. Fractions were trapped off the detector at approximately 3 min intervals and assayed by LSC (15 mL Ultima Gold).

Speciation of Radiochlorine in Urine. Urine samples collected during the initial 24 h of the study, which contained high concentrations of radiochlorine, were prepared as follows: Duplicate 100−750 μL aliquots were diluted to 2 mL volume with nanopure water. The samples were loaded onto conditioned Bakerbond C18 Mega Bond Elut SPE cartridges (J. T. Baker) and rinsed with water, and the combined bypass/rinse fraction was assayed for radioactivity. The C18 bypass/rinse fraction was then loaded onto a conditioned LC-SCX cation exchange SPE cartridge (Supelco) and rinsed with water, and the combined bypass/rinse fraction was assayed for radioactivity. The SCX eluent was lyophilized, reconstituted with water, and filtered through a syringe filter (0.45 um PTFE, 17 mm, Alltech, Deerfield, IL). Less than 2% of the loaded radiochlorine remained bound to C18 or SCX columns when loaded and rinsed as described. Speciation of radiochlorine in urine extracts post-SPEs was performed using ion chromatography, as described above, using an isocratic mobile phase of 30 mM NaOH. Radiochlorine was detected using a Packard (Meridan, CT) Radiomatic 500TR radiochemical detector controlled by Packard Flo-One software.

Aliquots (750−1000 μL) of urine samples collected from 24 to 72 h contained lower concentrations of radiochlorine, but higher relative concentrations of chloride ion from the endogenous chloride pool. Therefore, it was necessary to remove the chloride ion by application of reconstituted SCX eluants to sequential On Guard II AG and H columns (1 mL; Dionex). Aqueous radiochlorine was eluted into vials containing 500 μL of 10 mM NaOH to reduce the acidity of the sample from the On Guard H column and increase sample stability. Recoveries of radioactivity were greater than 95% for 24−72 h urine samples.

Urinary radioactivity was limiting in samples collected after 48 h so that concentration by lyophilization after elution from the sequential Ag^+ and H SPE columns was required. It was noted that chlorate in fortified samples was converted to chloride ion, presumably due to the acidification of the sample during passage through hydronium columns. Therefore, for 48−72 h urine samples, a 500 μL aliquot of extract, collected after the SCX SPE step, was chromatographed, despite the high chloride content. Because of the large chloride mass injected, the relatively low amount of radiochloride present, and the broad chloride band (i.e., several minutes), the radiochemical detector integrated several "chloride" peaks. The radioactivity of peaks eluting within the broad chloride band was summed and reported as a percentage of total radioactivity detected (which included the [^{36}Cl]chlorate peak, whose chromatography was unaffected by the high unlabeled chloride concentrations). These values were compared to the relative percentages obtained from the Ag^+/H SPE extraction.

Replication of Abdel-Rahman et al. (20) Speciation Procedure. The analytical procedures of Abdel-Rahman et al. (*20*) were repeated with pure standards of [^{36}Cl]chloride, [^{36}Cl]chlorite, and [^{36}Cl]chlorate for two reasons. First, unlike Abdel-Rahman et al. (*20*), we found no evidence that chlorite ion was excreted in rats at the dose provided; second, we believed that the analytical methods of Abdel-Rahman et al. (*20*) were based on faulty assumptions and that the "chlorite"

Table 1. Disposition, Elimination, and Total Recovery of Radiochlorine (% of Dose) in Rats Orally Dosed with [^{36}Cl]chlorate

	animal					
	rat 5	rat 19	rat 26	rat 27	mean	SD
tissues	21.9	4.5	15.4	7.5	12.3	7.9
			feces			
0–24 h	3.0	0.05	0.04	0.6	0.9	1.4
24–48 h	1.5	0.03	0.09	0.3	0.5	0.7
48–72 h	0.6	0.06	0.1	0.5	0.3	0.3
total	5.1	0.1	0.2	1.4	1.7	2.3
			urine			
0–6 h	9.4	45.8	32.5	56.7	36.1	20.4
6–12 h	27.2	32.7	27.9	17.2	26.3	6.5
12–18 h	9.0	5.5	5.1	3.8	5.8	2.2
18–24 h	4.1	2.2	1.9	1.1	2.3	1.3
24–32 h	1.5	1.8	1.2	1.1	1.4	0.3
32–40 h	2.3	1.0	1.5	0.9	1.4	0.7
40–48 h	3.1	0.2	2.5	0.6	1.6	1.4
48–60 h	2.9	0.7	2.8	1.0	1.8	1.1
60–72 h	5.1	0.5	2.6	1.1	2.4	2.1
total	64.6	90.4	78.0	83.5	79.1	10.9
expiratory gases	0.0	0.0	0.0	0.0	0.0	0.0
cage rinse	1.4	0.3	2.2	1.3	1.3	0.8
total recovery	93.0	95.3	95.7	93.7	94.4	1.3

reported by Abdel-Rahman et al. was not, in fact, chlorite. Following the analytical method exactly as outlined by Abdel-Rahman et al. (*20*), fortified standards were processed in water, rat and bovine serum, and rat and bovine urine as matrices. Briefly, 200 μL of each matrix was pipetted into a series of 16 test tubes and quadruplicate tubes were each fortified with either no radioactivity (control), [^{36}Cl]chlorite (72000 dpm), [^{36}Cl]chloride (66000 dpm), or [^{36}Cl]chlorate (78000 dpm). To each tube, 1.0 mL of a 5% $AgNO_3$ solution was added; tubes were subsequently vortexed and centrifuged for 5 min at 2000*g*. The supernatant was removed, the original pellet was washed with 1 mL of 5% silver nitrate, and both supernatants were combined and assayed for radioactivity. Of the four pellets for each matrix−analyte combination, two pellets were dissolved in 2 mL of concentrated NH_4OH and two were dissolved in 2 mL of 2% sodium thiosulfate. Both portions were sonicated in a water bath for 2 min and centrifuged for 5 min at 2000*g*, and supernatants were subsequently assayed for radioactivity. The entire experiment was replicated twice so that a total of eight measurements were made for supernatants of the initial silver nitrate precipitation for each analyte−matrix combination, and four measurements were made for the ammonium hydroxide and sodium thiosulfate fractions for each analyte−matrix combination.

Chlorite Stability in Urine and Serum. Chromatography of control urine samples fortified with chlorite indicated that the chlorite was unstable. Therefore, chlorite stability was evaluated on triplicate aliquots of control rat urine fortified with 250000 dpm (~17.5 μg) of sodium [^{36}Cl]chlorite, held at room temperature for 0, 1, 2, 4, 6, 8, 12, 24, 48, and 96 h postfortification. At the indicated time points, a subsample was removed to which 2 mM NaOH was added to stabilize the sample and then frozen. Subsamples were thawed and injected directly on the ion chromatograph without any cleanup steps, and radiochlorine was detected using a flow-through radiochemical detector. A mobile phase of 20 mM NaOH was used to ensure adequate resolution of chlorite and chloride. The experiment was repeated with bovine serum.

RESULTS

Tissue Disposition. Following a single oral dose of chlorate, only 12.3 ± 7.9% of the radioactivity remained in the bodies of male rats at 72 h (**Table 1**). Tissues with the highest percentages of the administered radiochlorine were carcass remains (4.6 ± 2.9%), skin (3.2 ± 1.9%), and GI tract (1.3 ± 1.1%) (data not shown). No other tissues contained greater than 1% of the administered radiochlorine. When the data were expressed on a concentration basis (fresh tissue weight; chlorate equivalents), the tissue concentrations of radiochlorine were fairly uniform, i.e., less than 1 order of magnitude difference (**Table 2**). The four tissues with the highest concentration of radiochlorine were the plasma (0.94 ± 0.53 ppm), whole blood (0.76 ± 0.43 ppm), testes (0.66 ± 0.36 ppm), and lung (0.61 ± 0.35 ppm). The four tissues with the lowest concentrations of radiochlorine were the carcass remains (0.24 ± 0.13 ppm), bone (0.24 ± 0.13 ppm), adipose tissue (0.17 ± 0.10 ppm), and muscle (0.14 ± 0.08 ppm).

Table 2. Concentrations of Radioactive Residues (ppm Fresh Tissue Weight; Chlorate Equivalents) in Tissues of Rats Dosed Orally with [^{36}Cl]chlorate and Slaughtered 72 h after Dosing

tissue	rat 5 (ppm)	rat 19 (ppm)	rat 26 (ppm)	rat 27 (ppm)	mean	SD
adipose tissue	0.28	0.05	0.08	0.05	0.17	0.10
blood	1.17	0.29	1.09	0.50	0.76	0.43
brain	0.53	0.14	0.52	0.22	0.35	0.20
bone	0.34	0.10	0.35	0.16	0.24	0.13
diaphragm	0.51	0.10	0.36	0.16	0.28	0.19
GI tract	0.61	0.12	0.46	0.20	0.35	0.23
heart	0.53	0.14	0.46	0.23	0.34	0.18
kidney	0.68	0.18	0.67	0.28	0.45	0.26
liver	0.45	0.12	0.40	0.18	0.29	0.16
lung	0.95	0.24	0.86	0.40	0.61	0.35
muscle	0.21	0.06	0.20	0.10	0.14	0.08
plasma	1.43	0.36	1.36	0.62	0.94	0.53
skin	0.62	0.18	0.52	0.27	0.40	0.20
spleen	0.66	0.17	0.56	0.27	0.42	0.23
testes	1.04	0.27	0.90	0.45	0.66	0.36
carcass remains	0.35	0.09	0.34	0.15	0.24	0.13
thymus	0.59	0.17	0.60	0.27	0.41	0.22

Table 3. Speciation of Total Radioactive Residues in Livers, Kidneys, Muscle, and Carcass Remains of Rats Orally Dosed with [^{36}Cl]chlorate and of Tissues from Control Rats Fortified with a [^{36}Cl]chlorite, [^{36}Cl]chlorate Standard[a]

	dosed tissue residue[b]		fortified tissue residue[c]	
tissue	Cl^- (%)	ClO_3^- (%)	Cl^- (%)	ClO_3^- (%)
liver	100	0	59.6	40.3
kidney	100	0	52.5	47.5
muscle	99.8	0.2	53.1	46.9
carcass remains	98.1	1.9	48.1	51.9

[a] Chlorite was not detected in any tissue. [b] Composition of residue recovered from tissues of rats dosed with chlorate; $n = 4$, duplicate analyses. [c] Composition of residue recovered from fortified control tissue (fortification composition was 52.3% chloride, 47.9% chlorate; duplicate analyses per tissue).

Speciation of Tissue Residues. Results from the speciation of radiochlorine present in aqueous extracts of liver, kidneys, muscle, and carcass remains are shown in **Table 3**. The extracts from liver and kidney contained only [^{36}Cl]chloride ion. Except for one replicate analysis from muscle of rat 19, in which 1.9% of the radiochlorine was parent compound, all detected muscle radioactivity was [^{36}Cl]chloride ion. Extracts of carcass remains were, likewise, primarily composed of chloride except for duplicate analyses from rat 19 and one replicate from rat 26, in which 3.0, 2.1, and 0.9% of the radiochlorine, respectively, was parent compound. In no case was chlorite detected in rat tissues. The composition of radiochlorine recovered from muscle, kidney, and carcass fortified with a standard containing 52.3% chlorate and 47.9% chloride was 48.8 ± 2.7% chlorate and 51.2 ± 2.7% chloride, indicating that chlorate was relatively stable during the tissue extraction procedure. In liver, however, the composition of fortified radiochlorine after isolation from fortified tissue was 59.6% chloride and 40.3% chlorate, sug-

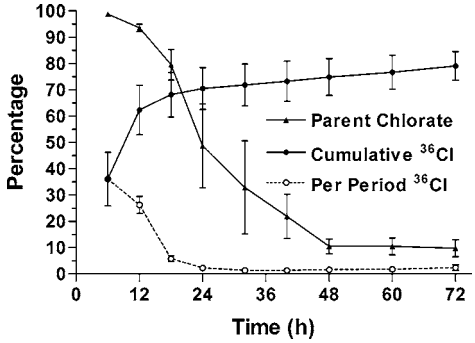

Figure 1. Urinary excretion of radiochlorine in male rats ($n = 4$) following a single oral dose (3 mg/kg) of sodium [^{36}Cl]chlorate is presented in terms of parent chlorate composition, cumulative, and per time period.

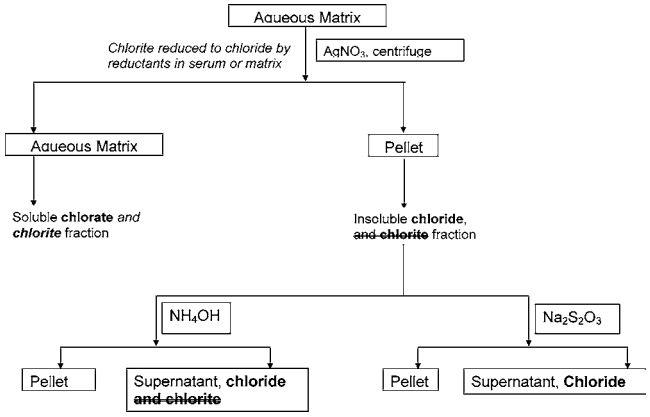

Figure 2. Disposition of chloride, chlorite, and chlorate as proposed by ref *20* vs disposition described by our findings. Italicized fonts represent additions, and strikeouts are deletions from the results of ref *20*.

gesting that some chlorate degradation occurred during sample preparation. In contrast, degradation of chlorate during workup of beef and swine tissues from previous studies has not occurred (*11*, *12*, *25*).

Excreta. Urine was the major route of radiochlorine excretion. The mean, cumulative elimination of radiochorine via the urine was 79% (range 65−91%; **Table 1**). The greatest amount of radiochlorine excreted was generally observed in urine samples collected at the earliest time interval, i.e., 0−6 h (mean 36.1%) and urinary excretion of radioactivity decreased steadily with time thereafter; however, peak elimination of radioactivity for rat 5 occurred during the 6−12 h period. Feces were a minor excretory route of radiolabel with less than 1% was eliminated in feces each day (**Table 1**). The cumulative, mean fecal excretion of radiochlorine was 1.7% of the administered dose, although rat 5 excreted over 5% of the [^{36}Cl]dose in the feces. Cage rinses contained only 0.3−2.2% of the dosed activity (**Table 1**).

Speciation of Urinary Radiochlorine. Parent chlorate was the primary form of radiochlorine excreted at the earliest time points (>98% chlorate at 0−6 h, 94 ± 2.9% at 6−12 h, and 79 ± 11.6% at 12−18 h; **Figure 1**). Sharp declines in the content of radiochlorine present as [^{36}Cl]chlorate occurred beyond 18 h, approaching a mean of 10% of the excreted radiochlorine by 72 h (**Figure 1**). The fractional percentage of chlorate declined with time most rapidly for rats 5, 26, and 27 between 12 and 40 h, while the sharp decrease in the fractional percentage of chlorate the urine of rat 19 occurred later, i.e., 32−48 h, which is why the standard errors at 24, 32, and 48 h are large (**Figure 1**). The only other species of radiochlorine identified in urine samples was [^{36}Cl]chloride. Therefore, as [^{36}Cl]chlorate concentrations in rat urine declined, complementary [^{36}Cl]chloride concentrations increased. Radiochemical analysis of raw urine samples (0−6 h samples) injected onto the ion chromatograph immediately after collection, and without prior cleanup, provided no indication that chlorite was present in urine.

Replication of Abdel-Rahman's Speciation Procedure. In an attempt to verify the accuracy of the methods used by Abdel-Rahman et al. (*20*) samples of water, rat urine and serum and bovine urine and serum were fortified with [^{36}Cl]chlorate, [^{36}Cl]chlorite, or [^{36}Cl]chloride, and Abdel-Rahman's fractionation methods were used exactly as published and diagramed in normal fonts (**Figure 2**). Recovery of radioactivity in the supernatants of chloride-fortified matrices was 0%, indicating that [^{36}Cl]chloride was completely removed from solution by silver nitrate. In contrast, essentially quantitative recovery of [^{36}Cl]chlorate in the supernatants occurred after treatment with silver nitrate indicating that silver chlorate is highly soluble in aqueous matrices. Recoveries of [^{36}Cl]chloride and [^{36}Cl]chlorate in the supernatants were not greatly affected by matrix. In contrast, the recovery of radioactivity in the supernatants of chlorite-fortified matrices varied greatly, ranging from 91.5% for water to a low of 14.1 and 8.6% for rat and bovine serum, respectively. These results suggest that either silver chlorite solubility varies with matrix or that chlorite was not uniformly stable in a given matrix.

The premise of the Abdel-Rahmen (*20*) analytical method was that silver chlorite could be selectively solubilized after precipitation from an aqueous matrix with silver nitrate. The authors assumed that ammonium hydroxide would completely solvate silver chloride and silver chlorite but that sodium thiosulfate would selectively solvate silver chloride. It was reasoned that chlorite content could be calculated by differential solubilization of pellets formed after precipitation with silver nitrate. **Table 4** shows clearly that when chloride-fortified water, urine, and serum samples were precipitated with silver nitrate, the radioactivity precipitated as Ag^{36}Cl from the water and urine samples was quantitatively solubilized with either ammonium hydroxide or sodium thiosulfate. In matrices treated with chlorate, the recovery of radiochlorine from sodium thiosulfate and ammonium hydroxide treated pellets was essentially equal, but in these samples, very little radioactivity was precipitated with silver nitrate. Recovery of radiochlorine from pellets of chlorite-fortified matrices was matrix-dependent but, with the exception of bovine serum, did not generally differ between the ammonium hydroxide-treated and the sodium thiosulfate-solubilized pellets (**Table 4**). In bovine serum, only 66% of the radiochlorine was present in the sodium thiosulfate-solubilized pellets, whereas 82% of the radiochlorine was solubilized in the ammonium hydroxide-treated pellets. Collectively, these data indicate that selective precipitation would be a viable analytical tool to distinguish radiolabeled chlorate and chloride, but selective precipitation and solubilization should not be used to distinguish chlorite from chlorate or chloride. The fact that radioactivity is recovered in the supernatant of all chlorite-fortified matrices indicates that silver chlorite is too soluble for the development of a precipitation-based analytical assay.

Data from the chlorite fortification experiments indicated that the relative amount of radioactivity in the supernatant after silver nitrate treatment was matrix-dependent. Differences between matrices could be due to the solubility of chlorite in the matrix

Table 4. Recoveries (%) of Radioactivity Fortified into Water, Rat Urine, Rat Serum, Bovine Urine, and Bovine Serum as [^{36}Cl]chloride, [^{36}Cl]chlorite, and [^{36}Cl]chlorate after Fractionation According to Ref *20*[a]

	analyte								
	chloride fortified			chlorite fortified			chlorate fortified		
		pellet[b]			pellet[b]			pellet[b]	
matrix	supernatant	NH_4OH	NaS_2SO_3[c]	supernatant	NH_4OH	NaS_2SO_3[c]	supernatant	NH_4OH	NaS_2SO_3[c]
water	0.4 ± 0.5	96.5 ± 4.7	96.8 ± 3.8	91.5 ± 3.3	3.1 ± 1.4	3.3 ± 1.7	100.1 ± 7.7	0.0 ± 0.0	0.1 ± 0.1
rat urine	0.1 ± 0.2	99.3 ± 1.8	99.9 ± 1.8	69.4 ± 7.8	26.6 ± 12.0	25.8 ± 12.4	99.1 ± 6.8	0.6 ± 0.2	0.7 ± 0.3
bovine urine	0.1 ± 0.2	98.7 ± 1.4	92.4 ± 6.9	34.7 ± 12.4	61.9 ± 14.3	58.0 ± 11.5	99.0 ± 2.5	0.8 ± 0.2	1.1 ± 0.4
rat serum	0.3 ± 0.3	95.3 ± 3.1	90.9 ± 4.0	14.1 ± 6.0	80.6 ± 10.1	80.3 ± 16.7	98.1 ± 2.3	4.8 ± 0.3	5.0 ± 0.4
bovine serum	0.0 ± 0.0	86.1 ± 14.4	83.2 ± 9.0	8.6 ± 6.2	81.6 ± 5.3	66.4 ± 25.5	96.9 ± 2.9	6.8 ± 1.7	6.2 ± 1.6

[a] The total recovery of radiochlorine within an analyte and matrix is estimated by summing the supernatant and NH_4OH or NaS_2SO_3 values. Recoveries of radioactivity for some analyte–matrix combinations (i.e., bovine serum chloride and chlorite fortifications) are low because NH_4OH or NaS_2SO_3 did not completely solvate the pellets. [b] Pellets formed after precipitation with silver nitrate were solubilized with NH_4OH or NaS_2SO_3, and radioactivity in the soluble portion was quantified. [c] Sodium thiosulfate pellet was not as easily dissolved as the ammonium hydroxide pellet.

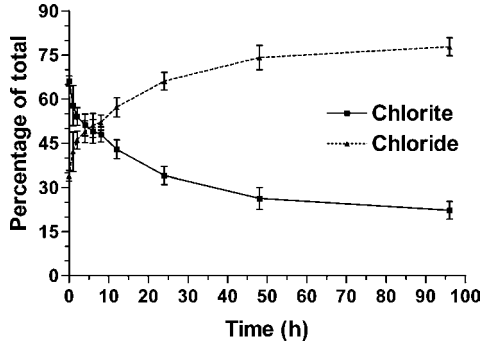

Figure 3. Degradation of chlorite in rat urine as a function of time (n = 3). The initial radiochemical purity of the chlorite fortification solution was 99.3%.

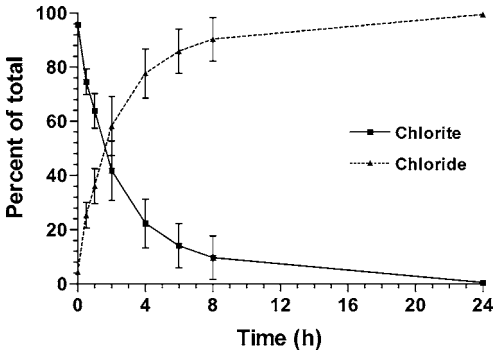

Figure 4. Degradation of chlorite in bovine serum as a function of time (n = 3). The initial radiochemical purity of the chlorite fortification solution was 99.3%.

but, more likely, the stability of chlorite in the matrix. **Figures 3** and **4** show the limited stability of chlorite when fortified into rat urine and bovine serum, respectively. Rapid degradation of chlorite was observed at t = 0 h in rat urine, where less than 67% of the fortified chlorite could be detected, and by 96 h, only 22% remained in urine (**Figure 3**). In rat urine, the half-life of chlorite was only 5 h. Degradation of chlorite was also observed in bovine serum. Despite the fact that nearly 96% of the fortified chlorite could be detected at t = 0 h, by 24 h, only 0.6% remained (**Figure 4**). The half-life of fortified chlorite was only about 2 h in bovine serum. The product of the degradation of fortified chlorite in both rat urine and bovine serum was chloride (**Figures 3** and **4**). No other radiochlorine species were detected.

DISCUSSION

Distribution and Excretion of Radioactive Residues. In general, the distribution of total radioactive residues in this study and the study of Abdel-Rahman were similar, although the total recovery of radiochlorine in this study averaged 94%, whereas recoveries of radioactivity in Abdel-Rahman et al. (*20*) were on the order of 40%.

The body burdens of radiochlorine in male rats 72 h after receiving a single oral dose of [^{36}Cl]chlorate were variable, ranging from 4.5 to 21.9% of the dose. The carcass, skin, and GI tract were the only tissues with mean retention of greater than 1% of the dosed radiolabel. Many similarities were observed when the data were expressed on a concentration basis (chlorate equivalents) and compared to previous results of Abdel-Rahman et al. (*13*, *14*). While none of the tissue concentrations in either study varied by more than seven-fold, plasma and whole blood contained the highest concentrations of radiochlorine at 72 h, while carcass remains, bone, and liver had the lowest concentrations of radiochlorine.

In terms of the overall disposition of an orally administered [^{36}Cl]chlorate dose, our data are in agreement with Abdel-Rahman's in that both studies conclusively show that radioactivity was rapidly absorbed and excreted, mainly in the urine of animals. Abdel-Rahman et al. (*23*) reported that 40% of the total radioactivity was excreted in urine at 72 h, while only 3% of the radiochlorine dose was excreted in the feces. Peak urinary excretion in our study occurred at the earliest sampling periods, i.e., 0−6 and 6−12 h (**Table 3**). This suggests that chlorate is readily absorbed from the intestinal tract. Neither study generated evidence that volatile chlorinated gases (i.e., Cl_2 or ClO_2) were expired. Similar excretion patterns were observed in beef cattle when relatively high chlorate doses were administered for three consecutive days (62.5 and 130.6 mg/kg/day; *11*). Steers eliminated 39 and 47%, respectively, of the two doses in the urine and only 1.7 and 0.4% in the feces.

Speciation of Tissue Residues. Speciation of radiochlorine present in tissues was not performed by Abdel-Rahman et al. (*23*); therefore, comparisons cannot be made with that study. However, speciation of tissue residues in the present study indicated that only chloride ion was present in tissues. These data are in contrast to a study conducted in beef cattle following three consecutive daily doses. The adipose tissue, skeletal muscle, and kidney of steers contained 28−45% chlorate content, while the remainder of the radiochlorine residues was chloride. A short withdrawal period of only 8 h before tissues were harvested and the much greater chlorate dose provided to cattle may help explain these results.

The urinary speciation results of Abdel-Rahman et al. (*13*, *14*) showed that 28% of the 0−8 h urine was chloride, 11% was chlorite, and 60% was parent chlorate. In the 48−72 h urine, 87% of the radiochlorine was chloride and 13% was chlorite, while no chlorate was present. The present rat study contrasts with these results in that >91% of radiochlorine in urine up to 12 h was parent compound, and from 32 to 72 h, the levels of chlorate remained constant at approximately 10%, with the remainder being chloride. No chlorite was detected in any urine sample analyzed, even when samples were analyzed immediately after collection from the rat metabolism cages. The maximal amount of chloride ion in beef cattle urine following three consecutive doses of radiochlorate (*11*) was only 35%, while chlorate ranged from 65 to 98% of urinary radiochlorine.

Replication of Abdel-Rahman's Speciation Procedure. This experiment was conducted in order to verify the results of Abdel-Rahman et al. (*20*) that showed that chlorite is a significant urinary metabolite of chlorate in rats. Our results in ruminants (*11*, *12*) had failed to measure chlorite in the urine or tissues of cattle. Because ruminants have digestive tracts with redox potentials between −250 and −450 mV (*26*), we reasoned that chlorite might be formed and be stable in nonruminants, whereas in ruminants its stability would be precluded by the unfavorable reduction characteristics of the rumen. However, other observations in our laboratory, namely, the absence of chlorite in tissues and excreta of swine (*25*), suggested that Abdel-Rahman's data might be artifactual.

The methods of Abdel-Rahman et al. (*20*) were based on differential precipitation and solubilization of chloride, chlorate, and chlorite, followed by radiochemical analysis of resulting solutions. Fundamental to their analytical method was the premise that both the chloride and the chlorite would precipitate from solution after treatment with $AgNO_3$. Their method suggested that precipitated chloride and chlorite could be distinguished by subsequent extractions (**Figure 2**). An NH_4OH extraction of the pellet was assumed to solubilize both silver chlorite and silver chloride, whereas extraction with a $Na_2S_2SO_3$ would solubilize only the silver chloride. Chlorite could then be determined by the difference between the supernatants.

However, while conducting literature searches of the chemistry behind the NH_4OH and $Na_2S_2SO_3$ extractions, it was discovered that NH_4OH was an effective solvent for the salts of strong acids, e.g., silver chloride, while many different silver salts could be solubilized in $Na_2S_2SO_3$ solution (*27*). Although not specifically mentioning silver chlorite, this hinted at an opposing interpretation of the differential extractions that guided the conclusions of Abdel-Rahman et al. (*20*). Furthermore, numerous gravimetric anion analytical methods indicate that chloride is the only anion that would precipitate with $AgNO_3$ in a chloride, chlorite, and chlorate mixture (*28*). Finally, the CRC Handbook of Chemistry and Physics (*29*) indicates that silver chlorite is over 5000 times more soluble in aqueous solution than silver chloride.

Our results utilizing pure [^{36}Cl]chlorite (>99% radiochemical purity) in water conclusively demonstrate that silver nitrate will not precipitate chlorite and that chloride and chlorite could not be distinguished by previously reported (*20*) methods. Neither extraction of precipitated $Ag^{36}Cl$ pellets with ammonium hydroxide nor sodium thiosulfate provided evidence that differential solubilization was sufficient for a quantitative chlorite assay (**Table 4**). Radioactive pellets from both chlorite and chloride fortifications were equally extractable into NH_4OH and $Na_2S_2SO_3$ solution from urine, serum, or pure water matrices. On the basis of our data, it is now possible to present a flow diagram reinterpreting the results of Abdel-Rahman (*20*; **Figure 2**, our revisions in italics and strikeout fonts). Therefore, we conclude that the quantities of metabolites identified in the studies of Abdel-Rahman et al. (*13*, *14*, *23*) were not accurate.

Chlorite Stability in Urine. Few studies have investigated the stability of chlorite in biological matrices. However, the microbial degradation of chlorate has been investigated, of which chlorite is the two-electron reduction intermediate. Chlorate is chemically stable under many environmental conditions; however, in biological systems, stoichiometric reduction to chloride has been demonstrated. In an experiment designed to measure the influence of electron acceptors on chlorate reduction by microorganisms, van Ginkel et al. (*30*) concluded that chlorate reduction to chloride was facile under anaerobic conditions but ceased immediately under aerobic conditions. Further research has also confirmed these results (*31*−*33*) allowing the conclusion that microbes in anoxic environments, like submerged soils and sediments, can readily reduce chlorate to chloride.

Microbial reduction of chlorate is mediated by chlorate reductase (CR) enzymes, although in some denitrifying microorganisms, nitrate reductase may also catalyze chlorate reduction (*34*). In most microbial studies to date, chlorite has not been detected as an intermediate of chlorate reduction, but instead, only chloride has been observed (*31*, *32*, *35*). It has been demonstrated that chlorate reduction is a two-step process, catalyzed by two distinct enzymes. Chlorate reductase catalyzes the reduction of chlorate to chlorite. A second enzyme has been discovered, which uses chlorite as a substrate and reduces it to chloride in a four-electron transfer (*30*, *31*). Chlorite dismutase (CD) catalyzes the following reaction:

$$ClO_2^- \rightarrow Cl^- + O_2$$

In the presence of both enzymes, the likelihood of detecting chlorite would be very small since the conversion of chlorite to chloride and oxygen is 1000 times faster than the reduction of chlorate to chlorite (*31*, *36*). Chlorate-reducing bacterial isolates exhibiting chlorite dismutase activity are ubiquitous, even existing in pristine environments (*37*), and demonstrate a great diversity within the bacterial world.

Our data demonstrated that chlorite was not stable in either serum or urine (**Figures 3** and **4**). Particularly in urine, a rapid degradation of chlorite was observed, in that less than 70% of a fortified amount of radiochlorite could be detected immediately after fortification. To our knowledge, the microbial CR and CD enzymes discussed above have not been described in mammalian systems, but it can be hypothesized that the lack of stability of chlorite in serum and urine (**Figures 3** and **4**) could be due to the presence of microorganisms within each matrix. However, the probability that bacteria were responsible for the conversion of chlorate to chlorite is remote because all chlorite stability experiments were conducted in an aerobic environment. A greater possibility exists that abiotic processes caused the reduction of chlorite to chloride. The chemical stability of chlorite is known to vary depending upon pH (*15*) with acidified sodium chlorite disproportioning to chloride and chlorate. Typically, chlorite is stable at alkaline pH. Furthermore, chlorite is rapidly reduced to chloride by chemical reductants, such as ferrous iron (*38*, *39*), sulfur dioxide-sulfite (*40*), and pyridoxal 5′-phosphate (*41*). Although studies have not been conducted, it is reasonable to expect that physiologic reductants in serum and urine such as thiols (i.e., glutathione, cysteine), ferrous iron in hemoglobin, and ascorbic acid might also reduce the strong oxidant, chlorite.

Even the fortification results of Abdel-Rahman et al. (*20*) provide evidence that rapid degradation of chlorite was possible in rat plasma. A fortified plasma sample of potassium [^{36}Cl]-chlorite showed 21.5% decomposition to chloride ion in an unspecified amount of time, and a mixture of potassium [^{36}Cl]-chlorite and sodium [^{36}Cl]chloride decomposed completely to chloride ion. Also, plasma samples fortified with either potassium [^{36}Cl]chlorate or potassium [^{36}Cl]chlorate/sodium [^{36}Cl]-chloride showed no evidence for chlorite formation, but a limited amount of chloride was observed (4.2 and 10.9%, respectively).

The purpose of this study was to determine the metabolism and disposition of chlorate in rats. We found that chlorate was metabolized only to chloride in rats and believe that inaccurate analytical methods used previously led to the erroneous conclusion that chlorite was a major metabolite of chlorate in rats. The absence of chlorite in excreta and tissues of rats from this study is consistent with studies in ruminant and nonruminant animals. Because chlorate is being considered for development as a food safety tool to eliminate Gram-negative pathogens in live animals, the absence of chlorite in edible tissues has important implications on the safety of food products from treated animals. Specifically, the absence of chlorite in edible tissues will improve the overall safety of tissue residues should chlorate ultimately be approved for use by the food animal industry. The current study resolves the discrepancy between earlier work in rats indicating that chlorite is a major metabolite of chlorate and work in food animal species in which chlorate could not be identified. In addition, the study suggests that even if chlorite were formed during metabolism of rodents or food animals, it would not survive for appreciable time periods during circulation or in the urine.

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