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November 11, 2010

Via Federal Express

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notification for Chlorine Dioxide and Withdrawal of FAP 2266; Resonant Biosciences, Inc.; Our File No. RE14070

Dear Sir or Madam:

The purpose of this letter is to request to participate in the pilot program for a Generally Recognized as safe (GRAS) determination for the safe use of chlorine dioxide generated by the notifier's PureMash® system in the production of food grade and non-food grade ethanol and distillers grains for animal feed use (food producing livestock). 75 *Fed. Reg.* 31800 (June 4, 2010). In connection with this GRAS notification, Resonant Biosciences, Inc. (RBS or Notifier) is formally withdrawing Food Additive Petition (FAP) 2266.

The Notifier previously met with your office to discuss residual testing and a submission plan for this product. The potential eligibility for clearance under the Federal Food, Drug, and Cosmetic Act (FFDCA) as GRAS was raised at that time. We trust that our prior discussions fully satisfy the guidance in the *Federal Register* that "FDA strongly encourages potential participants in the animal food pilot program to contact the Division of Animal Feeds" prior to submitting notices. 75 Fed. Reg. at 31802.

We look forward to confirmation that this submission has been accepted and is complete. Should any questions arise, please contact us, preferably by telephone or e-mail, so that we can promptly respond.

Sincerely yours,

Martha E. Marrapese

Enclosures

Generally Recognized As Safe (GRAS) Notification for Chlorine Dioxide

Prepared for:
U.S. Food and Drug Administration
Center for Veterinary Medicine
Division of Animal Feeds (HFV-224)
7519 Standish Place
Rockville, MD 20855

Notifier:
Resonant Biosciences, Inc.
11757 W. Ken Caryl Ave., F-308
Littleton, CO 80127

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I. Introduction

On behalf of Resonant Biosciences, Inc. (RBS or the Notifier), Keller and Heckman LLP submits the enclosed dossier of information in support of this notification that chlorine dioxide produced by the Puremash® system is Generally Recognized as Safe (GRAS) for use as a processing aid in the production of non-food grade and food grade ethanol. Chlorine dioxide is typically added in the first 16 to 24 hours of a fermentation run at a rate of 10-40 ppm per batch, with a maximum application rate of 55 ppm per batch that may be applied on an intermittent basis to treat highly fouled fermentation water.

One of the ethanol fermentation by-products, distillers' grain (DG), will be fed to food producing animals in accordance with good manufacturing or feeding practice. Animal feed is classified as "food" under section 201(f)(3) of the Act. Therefore, residuals in DG associated with the Notifier's GRAS substance require evaluation under the Federal Food, Drug, and Cosmetic Act (FFD&C Act), which broadly prohibits the "adulteration" of food, the statutory term for rendering food unsafe or unfit for consumption.

In addition, because one of the constituents in DG due to the use of chlorine dioxide, sodium chlorate, was the subject of a National Toxicology Program ("NTP"), two-year chronic toxicology study (NTP TR517, December 2005), the safety of chlorate residuals is evaluated using FDA's procedures for addressing the situation in which the use of a substance in a food additive is known to contain minute, but detectable, levels of a presumed carcinogenic impurity. The GRAS substance, chlorine dioxide, is not carcinogenic. These procedures permit the finding that there is no safety risk associated with the chlorate constituent when chlorine dioxide generated by the PureMash® system is intentionally added to the ethanol fermentation process at the levels prescribed.

The determination of GRAS is on the basis of scientific procedures, in accordance with 21 CFR § 170.30(b) and conforms to the guidance issued by the FDA under *proposed* 21 CFR § 570.36, 62 *Fed. Reg.* 18938 (Apr. 17, 1997) and the FDA's Notice of Pilot Program; Substances Generally Recognized as Safe Added to Food for Animals, 75 *Fed. Reg.* 31806 (June 4, 2010). This notification provides supporting information in the following areas:

- Identity of the substance;
- A description of the method of manufacture;
- An estimation of daily intake for all migrants;
- Safety data and safety evaluation; and
- GRAS determination, as a proposed conclusion determined by scientific procedures for use as a processing aid in the production of non-food grade and food grade ethanol.

It is the Notifier's expectation that FDA will concur that the information presented fully supports the determination that the Notifier's chlorine dioxide is GRAS for use as a processing aid in the production of non-food grade and food grade ethanol.

Finally, for purposes of this notification, the GRAS determination evaluates the downstream use of DG as a component of animal feed for food-producing target animals only. This notification does not attempt to assess use in conjunction with DG as a component of food administered to companion/non-food producing animals. It is the Notifier's intention to address the safety for use in food administered to animals such as cats, dogs, and horses independently, once sufficient processing data are generated to proceed with the analysis.

II. Administrative Information

A. Claim Regarding GRAS Status

Chlorine dioxide is GRAS based on scientific procedures for use as a processing aid in the production of non-food grade and food grade ethanol. DG from the ethanol production process will be used in animal feed use for food producing animals in accordance with good manufacturing or feeding practice. Chlorine dioxide is typically added in the first 16 to 24 hours of a fermentation run at a rate of 10-40 ppm per batch, with a maximum application rate of 55 ppm per batch that may be applied on an intermittent basis to treat highly fouled fermentation water. The chlorine dioxide is generated by treatment of an aqueous solution of sodium chlorate with hydrogen peroxide in the presence of sulfuric acid and the generator effluent contains at least 90 percent by weight of chlorine dioxide with respect to all chlorine species.

The use of Chlorine Dioxide from the Puremash® System in this manner has been determined to be exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 301 *et. seq.*)(the Act).

Martha E. Marrapese, Esq., Agent

11/12/10
Date

B. Name and Address of the Notifier

Notifier	Acknowledgement of Receipt of Notification and Inquiries to be Directed to:
Mr. Allen Ziegler President Resonant Biosciences, LLC 11757 W. Ken Caryl Ave., F-308 Littleton, Colorado 80217	Keller and Heckman LLP 1001 G Street N.W. Suite 500 West Washington, DC 20001 ATTN: Martha Marrapese, Esq. marrapese@khlaw.com 202-434-4123 (tel.) 202-434-4646 (fax)

A letter authorizing Keller and Heckman to serve as agent for the Notifier is provided as **Appendix 1.**

C. Common or Usual Name of the Subject Substance

Chlorine Dioxide

Synonyms: ClO_2

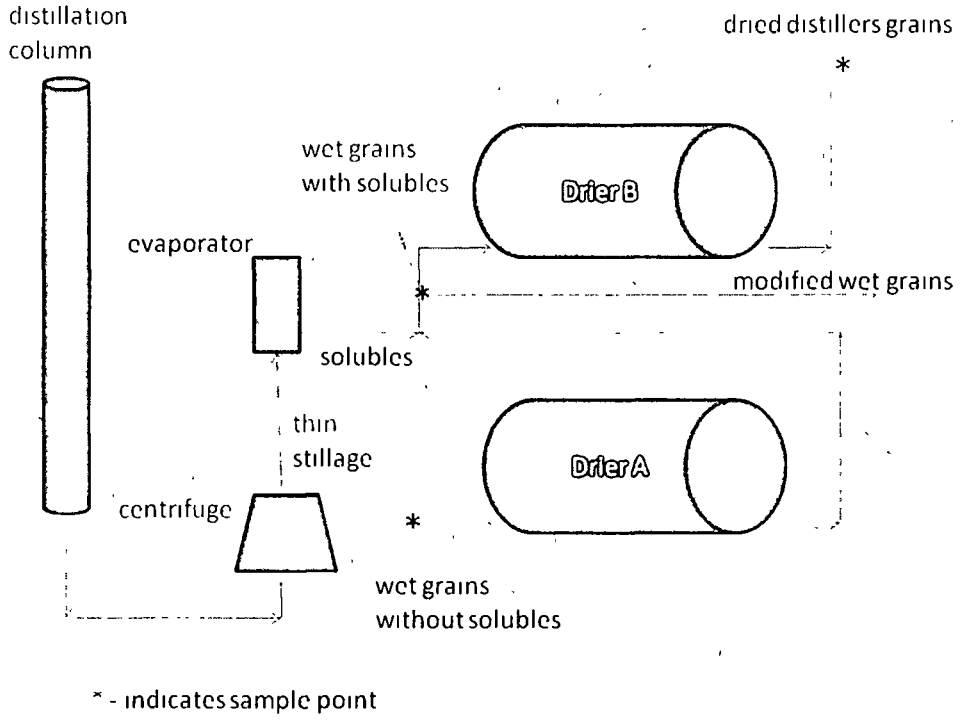
D. Intended Conditions of Use and Technical Effect

Chlorine dioxide generated using the PureMash® system will be used as a processing aid in the production of non-food grade and food grade ethanol. DG from the ethanol production process will be used in animal feed use for food producing animals in accordance with good manufacturing or feeding practice.

This GRAS notification is for DG collectively, including at least four nonfermentable residue byproducts of ethanol fermentation including distiller's wet grains without solubles, distiller's wet grains with solubles, distillers dried grains without solubles, and

distillers dried grains with solubles. For this purpose, data were provided on distillers dried grains with solubles because these data represent the "worst case" for potential residues. The reintroduction of the solubles into the grains will bring any residual that may be in the solubles into the DG, while subsequent drying of the grains will concentrate any residual in the DG. Therefore, residuals will be highest in distillers dried grains with solubles. *See* **Figure 1**.

Figure 1: Ethanol production process.



Chlorine dioxide is typically added in the first 16 to 24 hours of a fermentation run at a rate of 10-ppm per batch, with a maximum application rate of 55 ppm per batch that may be applied on an intermittent basis to treat highly fouled fermentation water, fermentation apparatus and piping. The chlorine dioxide is generated by treatment of an aqueous solution of sodium chlorate with hydrogen peroxide in the presence of sulfuric acid and the generator effluent contains at least 90 percent by weight of chlorine dioxide with respect to all chlorine species.

With respect to the intended technical effect, chlorine dioxide effectively reduces the amount of unwanted bacterial contamination inside the fermentation vessel during

ethanol production (**Appendix 2**). Chlorine dioxide is an oxidizing agent and broad-spectrum antimicrobial agent. It is intended to control bacterial contamination that can grow under fermentation conditions and compete with the growth of the intended yeast, affecting the production of ethanol.

The PureMash® chlorine dioxide technology used for generating the chlorine dioxide is the same as used for generating chlorine dioxide cleared under 21 C.F.R. §§ 173.300(a)(1)(ii) and 173.300(a)(2) where an aqueous solution of sodium chlorate is treated with hydrogen peroxide in the presence of sulfuric acid, and the generator effluent contains at least 90% by weight of chlorine dioxide with respect to all chlorine species.

E. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 570.30, chlorine dioxide has been determined to be GRAS to produce food grade and non-food grade ethanol and distillers grains for food producing target animals on the basis of scientific procedures. The GRAS determination is based upon the publicly available scientific literature pertaining to the safety of the substance, the enclosed residual data, and a dietary exposure assessment, as demonstrated herein.

F. Availability of Information

Much of the data and information that are the basis for the GRAS determination are enclosed with the notification. The Notifier also will retain copies of all of the data and information that form the basis for the GRAS determination, which are available for FDA's review at reasonable times, and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Keller and Heckman LLP
1001 G Street, N.W.
Suite 500 West
Washington, DC 20001
ATTN: Martha Marrapese, Esq.
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202-434-4123 (tel.)
202-434-4646 (fax)

III. Detailed Information about the Identity of the Notified Substance

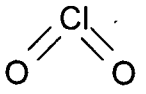
A. Names and Other Identities

Chemical Name: Chlorine dioxide

CAS Registry Number: 10049-04-4

Empirical Formula: chemical formula is O=Cl=O; molecular formula is ClO_2; molecular weight is 67.45 g/mole.

Structural Formula:



A mass spectrum for chlorine dioxide is included in **Appendix 3**.

B. Specification and Product Analysis

The PureMash® technology utilizes two precursors to generate high purity chlorine dioxide. These are MashGuard® One, which consists of 40% sodium chlorate and 8% hydrogen peroxide and 92% sulfuric acid. The product label, material safety data sheet (MSDS), and specifications for use are provided in **Appendix 4**.

C. Analysis of Lots

Resonant Biosciences had the residual levels of chlorate and chlorite measured in several samples of a 4000 ppm concentration chlorine dioxide effluent prepared by the PureMash® process. The analytical report is provided as **Appendix 5.** The results are presented in **Table 1.**

TABLE 1. Chlorite and Chlorate Residual Levels in 4,000 ppm Chlorine Dioxide in Water Effluent Generated by the Resonant Bioscience's PureMash® Chlorine Dioxide Generating Process

Sample	Chlorite (ppm)	Chlorate (ppm)
19G1272-01	335	54.0
19G1272-02	335	41.1
19G1272-03	323	52.1
19G1272-04	325	44.1
19G1272-05	328	39.9
19G1272-06	316	51.9

19G1272-07	324	44.5
19G1272-08	312	43.2
19G1272-09	314	38.4
19G1272-10	310	41.9
Average	**322**	**45**

On the basis of these analytical data, chlorite is present at a level of 8% (322 ppm/4000 ppm x 100% = 8%); chlorate is present at a level of 1.1 % (45 ppm/4000 ppm = 1.1%).

D. Physical Description

In **Table 2**, the physical and chemical specifications of chlorine dioxide such as density, melting point, maximum impurity levels, and solubility in food simulants are provided.

TABLE 2. Physical properties

SPECIFICATION	VALUE
Melting point	-59 °C
Boiling point	11 °C
Solubility in water	3.01 g/L at 25 °C and 34.5 mm Hg

E. Method of Manufacture and Calculated Residual Levels

The method for generating chlorine dioxide uses the PureMash® generator. The PureMash® generation method is based on the reduction of sodium chlorate by hydrogen peroxide in the presence of sulfuric acid. The PureMash® process is a chlorine dioxide technology used for microbial contamination control in the fermentation process in ethanol production. The chlorine dioxide is produced on site within a closed reactor.

The PureMash® generator is an independent unit and the chlorine dioxide solution produced is vacuum-piped into the fermentation stream through the fermenter fill. The process unit generating the chlorine dioxide is fed with two separate solutions: one containing 40 wt.% sodium chlorate and 8 wt.% hydrogen peroxide in water, and the second a concentrated sulfuric acid (93 wt.%) solution. The reactants are fed in controlled proportions into a proprietary reaction chamber that regulates mixing,

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residence time, temperature, and pressure. The finished aqueous solution containing chlorine dioxide in concentrated form, typically 4000 ppm, is automatically drawn out of the reactor and metered into the fermentation vessel through the fermenter fill, typically in the first sixteen (16) to twenty four (24) hours of ethanol fermentation resulting in total batch fermentation treatment concentration levels typically ranging from 10 to 40 ppm in the fermenter, and no more than 55 ppm. Upon reaction with the bacterial contamination (b) (4) [redacted] (b) (4) [redacted] no residual concentrations of chlorine dioxide are expected as the high organic content of the fermentation water and the yeast organisms present in the fermenter will react with any residual levels of chlorine dioxide that has not preferentially reacted with microbial contamination.

The concentration levels anticipated in the fermenter process water are based on the chemical stoichiometry of the Resonant Biosciences' PureMash® chlorine dioxide generator system. The following assumptions are used in the calculations:

- The process will typically require an application of 40 ppm chlorine dioxide for microbial contaminated fermentation water, with a maximum application rate of 55 ppm per batch that may be applied on an intermittent basis to treat highly fouled fermentation water, fermentation apparatus and piping;
- Excess sulfuric acid, approximately 3.7 times the stoichiometric amount, is added to increase the reaction velocity and reaction efficiency; and
- All the hydrogen peroxide is decomposed in the reaction chamber, and none gets into the process water.

Chlorine dioxide is produced according to the following stoichiometric equation:

$$\mathbf{2\ NaClO_3 + H_2O_2 + H_2SO_4 \rightarrow 2\ ClO_2\ (aq) + O_2 + Na_2SO_4 + 2\ H_2O}$$

At 40 ppm, the initial chlorine dioxide concentration [ClO_2] is 5.93×10^{-4} M, as calculated below.

$$\frac{40\ mg\ ClO_2}{L\ water} \times \frac{mole\ ClO_2}{67.45 \times 10^3\ mg} = 5.93 \times 10^{-4}\ M\ ClO_2$$

1. Chlorate (ClO_3^-)

Chlorate (ClO_3^-) is introduced to the fermentation process water in two ways. First, as unreacted sodium chlorate and as a decomposition product of chlorine dioxide. The average level of chlorate in a 4000 ppm chlorine dioxide effluent prepared by the PureMash® technology is 45 ppm; hence a 40 ppm chlorine dioxide concentration will have 0.45 ppm (45 ppm x 40/4000 = 0.45 ppm) of chlorate. For a maximum chlorine dioxide dosing concentration of 55 ppm, the residual levels of chlorate are calculated to be 0.62 ppm (45 ppm x 55/4000 = 0.62 ppm).

2. Chlorite

Chlorite is present as a degradation product in the PureMash® chlorine dioxide generated effluent. The average level of chlorite is 322 ppm in a 4000 ppm chlorine dioxide effluent; hence a 40 ppm chlorine dioxide concentration will have 3.2 ppm (322 ppm x 40/4000 = 3.2 ppm) of chlorite. For a maximum chlorine dioxide dosing concentration of 55 ppm, the residual levels of chlorite are calculated to be 4.4 ppm (322 ppm x 55/4000 = 4.4 ppm).

3. Sulfate, Sodium Salts

Sodium sulfate is one of the primary reaction by-products of Resonant Biosciences' chlorine dioxide generation process, as shown in the stoichiometric equation above. The PureMash® technology uses 3.7 pounds of 93% sulfuric acid to produce 1 pound of chlorine dioxide. The Notifier calculates that 4.7 moles of sulfuric acid is used to generate one mole of chlorine dioxide as follows. In the PureMash® technology, 3.7 g of 93% sulfuric acid reacts with 1 gram of chlorine dioxide.

1.7 g-H_2SO_4 x 93% x (mole/98 g-H_2SO_4) = 0.035 mole H_2SO_4

1 g-ClO_2 x (mole /67.45 g-ClO_2) = 0.015 mole

Since only 0.0075 mole of H_2SO_4 is required to produce 0.015 mole of ClO_2, 0.035/0.0075 = 4.7 moles of H_2SO_4 are used to make two mole of chlorine dioxide.

Based on the stoichiometry and addition rate of sulfuric acid, one mole of Na_2SO_4 and 3.7 moles of SO_4^{-2} are introduced into the process water for every 2 moles of ClO_2 that are generated. None of the SO_4^{-2} introduced as sulfuric acid is consumed in the reaction. Thus, levels of SO_4^{-2} in the process water are:

$$[SO_4^{-2}] = \frac{5.93 x 10^{-4}\ moles\ ClO_2}{L\ water} \times \frac{4.7\ moles\ SO_4^{-2}}{2\ moles\ ClO_2} \times \frac{96.06 x 10^3\ mg}{mole\ SO_4^{-2}}$$

$$[SO_4^{-2}] = 133.9\ mg/L$$

The levels of sodium ion present in the process water result from $NaClO_3$. Thus, a total of 5.93 x 10^{-4} M Na^+ is added to the process water using the PureMash® process.

$$[Na^+] = \frac{5.93 x 10^{-4}\ moles\ Na^+}{L\ water} \times \frac{22.99 x 10^3\ mg}{mole\ Na^+}$$

$$[Na^+] = 13.6 mg/L$$

Below in **Table 3** are calculations for the level of each degradation product and reaction products that will be added to the fermentation process water as a result of the use of Resonant Biosciences'® chlorine dioxide generator.

TABLE 3. Calculated Residuals in DG

CHEMICAL NAME	CAS REG. NO.	TYPICAL RESIDUAL (%)	MAXIMUM RESIDUAL (%)
Sodium ion (the residual percentage is calculated on the basis of an application rate of 40 ppm ClO_2)	17341-25-20	13.6 ppm	---
Chlorate ion (the residual percentage is calculated on the basis of an application rate of 40 ppm ClO_2)	14866-68-3	0.45 ppm	0.62 ppm
Sulfate ion (the residual percentage is calculated on the basis of an application rate of 40 ppm ClO_2)	14808-79-8	134 ppm	---

Chlorite ion (the residual percentage is calculated on the basis of an application rate of 40 ppm ClO_2)	14998-27-7	3.2 ppm	4.4 ppm

F. Self-Limiting Levels of Use

The most advanced feature of the generator is an electronic controller to regulate reactor feed, calculate efficiency, and control output. The System Operation Manual is provided in **Appendix 6.** An ultraviolet (UV) spectrophotometer is used to continuously monitor the system. The controller provides automated control of chlorine dioxide generation. The operator enters the desired production rate of chlorine dioxide, and the unit automatically responds by adjusting reagent flows to maintain chlorine dioxide production with a conversion efficiency of 90 to 98%. The rate of introduction of the chlorine dioxide into the fermentation vessel is controlled by monitoring the chlorine dioxide concentration, and the amount used is dependent on the level of bacterial contamination. (b) (4) (b) (4) but the total additive concentration in the fermentation water will be no more than 55 parts per million (ppm) to meet desired residual levels.

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IV. Detailed Summary of the Basis for Notifier's GRAS Determination[1]

As a consequence of the fermentation, separation, and distillation of the ethanol, the starting materials, chlorine dioxide and its degradation products are not expected to distill with the ethanol, and consequently, will not be present in the final ethanol product. Thus, no residual chlorine dioxide, residual reactants, reduction by-products, or disproportionation products present are expected to be present in ethanol separated from the fermentation process as a result of the intended use of the chlorine dioxide.

Information regarding the safety of chlorine dioxide and its various chlorinated species previously was submitted in Food Additive Petitions ("FAP") 4A4415, 0A4716, 4A4751, and Food Contact Notification Nos. 391, 445, 644, and 645. These data are discussed in

[1] Prepared by Michael T. Flood, Ph.D., William W. Reichert, Ph.D., and Robert A. Mathews, Ph.D., D.A.B.T., Keller and Heckman LLP.

FDA review memoranda related thereto, all of which are incorporated herein by reference. Chlorine dioxide is not a carcinogen and is the subject of numerous GRAS determinations by FDA as documented in **Appendix 7**. As cited in the Agency's FAP review memoranda for chlorine dioxide, FDA has concluded that chlorine dioxide rapidly degrades during use. Due to this degradation, chlorine dioxide *per se* does not raise any toxicological concern for the purpose of a GRAS determination.

DG removed from the fermentation water is expected to contain certain residuals from the use of chlorine dioxide. The fermentation environment of low pH, high organic media, and volume of carbon dioxide (CO_2) is expected to reduce the chlorine dioxide to chloride ion. The chloride ion is then available to interact with other available ions, in this case sodium ions, to form sodium chloride. An additional residual to be expected is sodium sulfate.

Sodium ions are GRAS based on GRAS listings for numerous sodium salts as direct food ingredients, *e.g.*, sodium acetate (21 CFR §184.1721), sodium benzoate (21 CFR §184.1733), and sodium chloride (listed as "salt" in 21 CFR §182.1(a)). Sodium chloride has been in use prior to 1958, and is GRAS on that basis as well under 21 U.S.C. § 321(s).

With respect to sodium sulfate, the WHO Joint FAO/WHO Expert Committee on Food Additives ("JECFA") reviewed available toxicity data on sodium sulfate. JECFA found no toxicity associated with this sodium sulfate to justify establishing an ADI for this substance. Sodium sulfate has an AAFCO listing as a mineral source under 57.109, stipulating only that the minimum sodium and sulfur content must be listed.[2] Sodium sulfate as sulfuric acid is GRAS affirmed for direct addition to food under 21 CFR §184.1095 ("Sulfuric acid"). Sodium sulfate is the sodium salt of sulfuric acid which, if ingested, will result in exposure to sulfate ions and sodium ions.

In addition, chlorine dioxide readily converts to chlorite and unreacted sodium chlorate may be anticipated due to overfeed from the reactor. Thus, in the remainder of this

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[2] *See* Feed Ingredient Definitions of the 2008 Official Publication, Association of American Feed Control Officials Incorporated, p. 316.

notification, the Notifier evaluates the GRAS status of chlorine dioxide based on anticipated residual levels of the chlorite and chlorate residuals.

A. Analysis of Measured Residuals

Analyses were conducted to identify the residual levels of chlorite, chlorate, and chloride in DG. In the ethanol production process, water is recycled in the next fermentation batch, with fresh water added as make-up volume. Analyses were conducted on plant batches that had reached a steady-state operating condition. Chlorine dioxide was administered at a maximum application rate of 55 ppm per batch which is used to treat highly fouled fermentation water, fermentation apparatus and piping. Analyses were performed according to U.S. EPA Method 300.1 which was modified to achieve a minimum detection level (MDL) for chlorite and chlorate of 0.2 mg/kg. The test method and results are provided in **Appendix 8.** Averaged residual levels per lot expressed on a dry weight basis are summarized in **Table 4**.

TABLE 4. Levels of Chlorate, Chlorite, and Chloride in DG

Sample		ClO_2 Dose (ppm)	% Solids	Chlorite (mg/kg)	Chlorate (mg/kg)	Chloride (mg/kg)
(b) (4)		52.5	31.1%	<0.8	14.0	1903
		52.5	32.8%	<0.8	12.8	1783
		52.5	84.9%	<0.2	10.9	1637
		52.5	31.1%	<0.6	13.3	2464
		55	85%	<0.2	8.3	2807
		55	31.1%	<0.6	7.5	2465

Sample		ClO_2 Dose (ppm)	% Solids	Chlorite (mg/kg)	Chlorate (mg/kg)	Chloride (mg/kg)
19J1120-02		55	85%	<0.2	9.5	1543
Control 19L1077-01	-		<0.3	<0.3	1050	

In those cases where the test reports did not report the levels of chlorate, chlorite, and chloride on a dry weight basis – namely for samples 19J0395-01, 19J0395-02, 19J1120-01 and 19J1120-02 – an average solids content of 31.1% was used for wet distillers grains and 85% solids for dry distillers grains to determine the residual levels on a dry weight basis.

Chlorate: The average residual level of chlorate in distiller's grains on a dry weight basis was 10.9 mg/kg, with a maximum residual level of 14 mg/kg reported. Chlorate was not detected at a detection limit of <0.3 mg/kg on a dry basis in distillers grains from fermentation water that had not been dosed with the chlorine dioxide.[3]

Chlorite: Chlorite was not detected at a maximum detection limit of <0.8 mg/kg on a dry basis in any of the distillers grains. Chlorite was not detected at a detection limit of <0.3 mg/kg on a dry basis in distillers grains from fermentation water that had not been dosed with the chlorine dioxide. As worst-case, it was assumed that a maximum of 0.8 mg/kg of chlorite residuals remain in DG separated from ethanol fermentation.

B. Toxicological Evaluation of Chlorate

1. Absorption, Distribution, and Metabolism

Smith *et al.* (2005) dosed cattle daily with sodium [^{36}Cl] chlorate at 62.5 and 130.6 mg/kg bw/day for three consecutive days.[4] For speciation of the tissue metabolites, ion chromatography with a gradient solvent system was used to separate and quantify

[3] These data show higher chlorate residues beyond what would be expected from stoichiometric calculations strictly associated with the proper use of the PureMash® system. It is thought that higher chlorate residuals may have be observed, in part, due to the potential for disproportionation of chlorine dioxide to occur during ethanol production.

[4] Smith, D.J., Anderson, R.D., Ellig, D.A. and G. L. Larsen, "Tissue Distribution, Elimination, and Metabolism of Dietary Sodium [^{36}Cl] Chlorate in Beef Cattle," *J. Agric Food Chem* , 53, 4272-4280 (2005).

chlorate and chlorite. Chlorate is rapidly absorbed and excreted in steers. Apparent absorption of chlorate was 62-68% of the total dose. No chlorite was detected down to a sensitivity (LOD) of 50 ppb or less. The major excretory route for [^{36}Cl] elimination was urine, with the majority of the urine residue being chlorate (65-100%). Chloride was the only other chlorine anion species identified in the urine or tissues. These data demonstrate that when chlorate is administered to cattle, most is eliminated early as chlorate with the rest being chloride; no detectable chlorite is formed. A similar disposition and metabolism of chlorate to chlorite was found in feeding studies for rats, swine, and broilers.[5]

2. Acute toxicity studies

Chlorate at high doses is a well known herbicide.[6] There have been several accidental exposures to humans resulting in death. Helliwell and Nunn (1979) reported on 14 cases on sodium chlorate poisoning.[7] The patients ranged from 3-55 years of age. Symptoms included: methemoglobinemia, cyanosis, abdominal pain, anuria within 24 hours, and death in 64% of the patients. Doses estimated to be in the range of 79 g (as chlorate) were uniformly fatal. One person died after a dose of 15 g or 218 mg chlorate/kg bw. The approximate LD_{50} for humans is thus near 50 g/person or 830 mg chlorate/kg bw. A liter of drinking water containing this dose would be 50g/L or 50,000 ppm. The acute oral LD_{50} for most experimental animals is approximately 1.0 g/kg bw.[8]

3. Repeated Dose Toxicology

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[5] Smith D.J., Anderson, R.C., Huwe, J.K., 2006. Effect of Sodium [^{36}Cl] Chlorate Dose on Total Radioactive Residues of Parent Chlorate in Growing Swine. *J Agric Food Chem* 54, 8648-8653 (2006); Smith D.J., Byrd J.A., Anderson R.C., 2007. Total Radioactive Residues and Residues of [^{36}Cl] Chlorate in Market Size Broilers. *J Agric Food Chem* 55, 5898-5903 (2007); and Hakk H., Smith, D., Shappell, N., 2007. Tissue Residues, Metabolism, and Excretion of Radiolabeled Sodium Chlorate (Na[^{36}Cl]O_3) in Rats. *J. Agric. Food Chem.* 55, 2034-2042 (2007).

[6] *See* http://www.epa.gov/pesticides/reregistration/REDs/inorganicchlorates_red.pdf.

[7] Helliwell, M. and Nunn, J. (1979). "Mortality in Sodium Chlorate Poisoning." *Brit Med J.* 1: 1119.

[8] Heywood, R., Sortwell, R.J., Kelly, P.J., and Street, J.E. (1972). "Toxicity of Sodium Chlorate to the Dog." *Vet Rec.* 90:416-418. Also Clarke, E.G.C. and Clarke, M.L. (1967) *Garner's Veterinary Toxicology*. pp 67-68. Ballière, Tindall & Cassell, London.

Heywood *et al.*,(1972) reported that doses greater than 235 mg chlorate/kg bw/day administered to dogs repeatedly over a 5-day period produced marked clinical symptoms (marked loss of appetite and body weight) and hematological and biochemical changes.[9] At doses below 235 mg/d no clinical symptoms were produced and the hematological and biochemical changes were variable and borderline. Lubbers *et al.*, (1981, 1982, 1984) gave much lower doses of chlorate in drinking water (5 ppm) to human male volunteers for 12 weeks and monitored biochemical parameters.[10] No clinically significant changes occurred during the 12 week period. The authors also tested a small group of subjects with low levels of glucose-6-phosphate dehydrogenase, who are known to be especially susceptible to oxidation stress. There were no obvious undesirable clinical sequellae noted by any of the participating subjects or by the participating medical team. The authors speculated that some observed but clinically insignificant biochemical changes might become significant on longer exposures, but within the limits of the study the safety of oral ingestion of chlorate was demonstrated.

Both chlorate and chlorite produce damage in erythrocytes and produce methemoglobin. Bercz *et al.*, (1982) administered $NaClO_2$ or $NaClO_3$ at doses ranging from 25-400 ppm to African Green monkeys for 30–60 days. The chlorite but not the chlorate induced a dose-dependent oxidative stress on hematopoesis resulting in decreased hemoglobin and erythrocyte count and an increased methemoglobin.[11] Bercz reported that chlorine dioxide caused T4 suppression in the monkeys at doses of 9 mg/kg/day, whereas no such effects were found with $NaClO_3$ or $NaClO_2$ in short-term studies up to doses of 60 mg/kg/day.[12]

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[9] Heywood *et al.* (1972), *see* footnote 5.

[10] Lubbers *et al.* (1981). "Controlled Clinical Evaluations of Chlorine Dioxide, Chlorate and Chlorite in Man." *Fund Appl. Toxicol.* 1: 334-338; (1982) *Environ. Health Perspect* 46.57-62; (1984) The Effects of Chronic Administration of Chlorine Dioxide, Chlorate and Chlorite to Normal Healthy Adult Male Volunteers. *J. Environ. Pathol. Toxicol Oncol* 5(4/5) 229-238.

[11] Bercz, J.P., Garner, L., Murray, D., Ludwig, D.A., and J. Boston (1982). "Subchronic Toxicity of Chlorine Dioxide and Related Compounds in Drinking Water in the Nonhuman Primate." *Environ. Health Perspect* 46:47-188.

[12] Bercz, J.P., Jones, L.L., Harrington, R.M., Bawa, R. and Condie, L. (1986) "Mechanistic Aspects of Ingested Chlorine Dioxide on Thyroid Function: Impact of Oxidants on Iodide Metabolism." *Environ. Health Perspect* 69:249-255.

90-Day Rat Study [13] Male and female Sprague-Dawley rats were exposed to 250, 1,001, and 4,005 mg/L (3.0, 12.0, 48 mM , respectively) of sodium chlorate in the drinking water for 90 days. These concentrations resulted in doses of 30-512 mg/kg/day for males and 42-801 mg/kg/day for females. There were no treatment related deaths, but males and females in the high dose group had significant weight loss. Pituitary gland vacuolization and thyroid gland colloid depletion were present in both sexes in a dose dependent manner. A NOEL of 30 mg/kg/day and 42 mg/kg/day for chlorate was established in male and female rats respectively.

90-day Rat Study[14] The subchronic toxicity of sodium chlorate was evaluated in a 90-day study in which groups of Sprague-Dawley CD® rats (15/sex/group) received 10, 100 or 1,000 mg/kg/day (7 days per week) via gastric intubation for three months. Dose levels were based on a range finding study, which demonstrated a no effect level of 1,000 mg/kg/day (the dose level recommended for use in a 90-day limit test in the FIFRA guidelines of the USEPA). Control animals (15/sex) received the dosing vehicle distilled water. Study animals were observed twice daily for mortality and gross signs of toxicological effects. Weekly examinations for physical signs of local or systemic toxicity, pharmacologic effects and tissue masses were completed. Ophthalmoscopic examinations were completed prior to initiation of the study and at termination. Food consumption and body weight were recorded weekly. Hematology and clinical chemistry evaluations were completed on 10 rats per sex per treatment group, and necropsy of all animals was completed at the termination of the study.

Evaluation of physical observations, food consumption, ophthalmology, clinical chemistry values, and gross and microscopic pathology revealed no evidence of an effect related to treatment. Statistically significant differences in mean terminal organ weights suggestive of a test material related effect were limited to a slight decrease in absolute

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[13] McCauly, P.T , Robinson, M , Daniel, F.B., Olson, G.R. (1995). "The Effects of Subchronic Chlorate Exposure in Sprague-Dawley Rats." *Drug Chem. Toxicol* 18(2&3): 185-199

[14] Bio/Dynamics (1987). "A Subchronic (3 month) Oral Toxicity Study of Sodium Chlorate in the Rat." Via *Gavage Final Report.* Project No.86-3114, December 4, 1987, as cited in NTP Technical Report on the Toxicology and Carcinogenesis Studies of Sodium Chlorate (CAS No. 7775-09-9) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies), NTP TR 517 (December 2005)

adrenal weights for high-dose (1,000 mg/kg/day) males and females. There was no such effect at the mid dose (100 mg/kg bw or 78 mg chlorate/kg bw) which was taken as the study NOEL. The large dose spacing between the mid dose and the high dose and the borderline toxicity observed at the high dose assures a larger than usual margin of safety for an ADI based on the NOEL.

90-Day Dog Study[15] The subchronic toxicity study of sodium chlorate was also evaluated in the dog. Beagle dogs (4/sex/group) were administered 10, 60, and 360 mg/kg/day via oral gavage for 90 days. The dose vehicle, distilled water, served as the control. Test animals were observed twice daily for mortality and gross signs of toxicological effects. Ophthalmoscopic examinations were completed prior to initiation of the study, and at termination. Body weight and food consumption were recorded weekly. Hematology and chemistry parameters were evaluated prior to study initiation, and in weeks 6 and 13 prior to termination. Complete necropsy of all animals was performed postmortem.

All animals survived the study. Body weight gains between control and treated animals were comparable, with the exception of one high-dose male that exhibited a one kilogram weight lose during week 11, which was paralleled by a decrease in food consumption. Evaluation of food consumption, clinical chemistry studies, ophthalmologic observation, organ weights and organ to body weights, and gross and microscopic pathology revealed no evidence of an effect related to treatment. The NOAEL was taken as the highest dose tested (360 mg/kg/day or 282 mg chlorate/kg bw/day).

21- and 90-Day Rat Studies (NTP)[16] These studies complemented for the subsequent 2-year NTP cancer bioassay in rats. Male and female Fischer 344 rats were exposed to 0, 0.125, 0.25, 0.5, 1.0, or 2.0 g/L $NaClO_3$ for 21 days. Additional male rats were exposed

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[15] Bio/Dynamics (1987). "A Subchronic (3 month) Oral Toxicity Study in the Dog." Via Gavage Administration with Sodium Chlorate. Final Report Project No 86-3114, October 19, 1987, as cited in NTP Technical Report on the Toxicology and Carcinogenesis Studies of Sodium Chlorate (CAS No. 7775-09-9) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies), NTP TR 517 (December 2005).

[16] Hooth, M.J., DeAngelo, A.B., George, M.H., Gaillard, E.T., Travlos, G.S., Boorman, G.A. and Wolf, D.C. (2001). "Subchronic Sodium Chlorate Exposures in Drinking Water Results in a Concentration-Dependent Increase in Rat Thyroid Follicular Cell Hyperplasia." *Toxicological Pathology*, Vol. 29 (2): 250-259.

to 0, 0.5, 1.0, 2.0, 4.0, 6.0 g/L of $NaClO_3$ for 90 days. $NaClO_3$ treatment induced a concentration dependent increase in the incidence and severity of thyroid follicular cell hyperplasia. Male rats were more sensitive to the effects of $NaClO_3$ than females.

4. Developmental Toxicity Studies

NTP Study[17] Female NZW rabbits were dosed by gavage with sodium chlorate (100, 250 or 475 mg/kg/day) or with the vehicle (water) on days 6-29. The dose volume was 3 ml/kg. The study was conducted in a two replicate design with 12 naturally mated females per replicate. Sodium chlorate exposure did not significantly affect any endpoints related to prenatal viability. There were no treatment related effects on fetal body weight, average litter size, and on external, visceral, or skeletal malformations. Transient changes in maternal food intake, urinary color, and output were noted at doses > 100 mg/kg/day, but clear evidence of maternal toxicity was observed only at doses greater than 475 mg/kg/day. Sodium chlorate did not cause any significant treatment-related developmental toxicity under the conditions of the study. The maternal and developmental toxicity NOELS were greater than or equal to 475 mg/kg/day.

Biodynamics Study[18] Sodium chlorate was dissolved in distilled water and administered to 24 mated female CD® rats by gastric intubation during the day 6-15 gestation interval. Dose levels were 10, 100 and 1,000 mg/kg/day. All animals were weighed and given detailed in-life physical evaluations at regular intervals during gestation. All animals were sacrificed at day 20 and given a gross postmortem evaluation. Uteri and fetuses were examined according to protocol. No mortality occurred in the treated groups; all females survived to scheduled sacrifice. The mean numbers of corpora lutea, implantations, live fetuses and resorptions per pregnant female were comparable between the control and treated groups. No adverse effects of treatment were evident from

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[17] NTP study. Final Study Report, "Developmental Toxicity Evaluation for Sodium Chlorate Administered by Gavage to New Zealand White Rabbits on Gestational Days 6 through 29." TER-97005. The raw data is available on line from the National Toxicology Program website. http://ntp.niehs.nih.gov/index.cfm?objectid=0731167F-9246-568C-4166DAB9305C0C83

[18] Bio/Dynamics Inc., "A Teratogenicity Study in Rats with Sodium Chlorate." Final Report. Submitted to the Sodium Chlorate Task Force, Sept 24, 1987. Project No. 86-3117, as cited in NTP Technical Report on the Toxicology and Carcinogenesis Studies of Sodium Chlorate (CAS No. 7775-09-9) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies), NTP TR 517 (December 2005).

maternal parameters (pregnancy rates, body weight, weight change, food consumption, physical observation, uterine implantation data or gross post mortem observations). No adverse effects of treatment were evident from evaluations of fetal parameters (sex distribution, external, visceral or skeletal abnormalities) performed on fetuses recovered from treated females. The NOEL in the study was the highest dose tested.

5. Long-term feeding studies

NTP Study[19] Sodium chlorate was the subject of a National Toxicology Program ("NTP"), two-year chronic toxicology study (NTP TR517, December 2005) on sodium chlorate and two NTP genotoxicity studies: an *in vitro* Salmonella mutagenicity test and an *in vivo* micronucleus assay. Sodium chlorate was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA104, or TA1535 at doses of 100 to10,000 μg/plate; all tests were conducted with and without exogenous metabolic activation (induced rat or hamster liver S9 enzymes). No increases in the frequencies of micronucleated normochromatic erythrocytes (NCEs) were seen in peripheral blood samples from male and female B6C3F1 mice exposed to concentrations of 125 to 2,000 mg/L sodium chlorate in drinking water for 3 weeks.

Groups of 50 male and 50 female rats were exposed to drinking water containing 0, 125, 1,000, or 2,000 mg/L sodium chlorate for 2 years (equivalent to average daily doses of approximately 5, 35, and 75 mg/kg per day for male rats and 5, 45, and 95 mg/kg per day for female rats). All study rats in the 1,000 and 2,000 mg/L groups had thyroid gland follicular cell hypertrophy at 3 and 14 weeks. There were positive trends in the incidences of thyroid gland follicular cell carcinoma in male rats and of thyroid gland follicular cell adenoma or carcinoma in males and females. The incidences of thyroid gland follicular cell hypertrophy were significantly increased in all dosed groups of males and in 1,000 and 2,000 mg/L females. Thyroid gland focal follicle mineralization occurred in most 1,000 and 2,000 mg/L female rats. The incidences of hematopoietic cell

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[19] NTP Technical Report on the Toxicology and Carcinogenesis Studies of Sodium Chlorate (CAS No. 7775-09-9) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies), NTP TR 517 (December 2005), *see* http://ntp.niehs.nih.gov/files/517_Web.pdf.

proliferation in the spleen of 2,000 mg/L males and bone marrow hyperplasia in 1,000 and 2,000 mg/L males were significantly greater than those in the controls.

Groups of 50 male and 50 female mice were exposed to drinking water containing 0, 500, 1,000, or 2,000 mg/L sodium chlorate for 2 years (equivalent to average daily doses of approximately 40, 80, and 160 mg/kg per day for male mice and 30, 60, and 120 mg/kg per day for female mice). Survival of exposed mice was similar to that of the control groups. There was a positive trend in the incidences of pancreatic islet cell adenoma or carcinoma (combined) in female mice. Thyroid gland follicular cell hypertrophy was significantly increased in 2,000 mg/L females. The incidences of bone marrow hyperplasia were significantly increased in all exposed groups of females.

Under the conditions of this 2-year study, there was evidence of carcinogenic activity of sodium chlorate in male and female F344/N rats based on the increased incidences of thyroid gland neoplasms. There was no evidence of carcinogenic activity of sodium chlorate in male $B6C3F_1$ mice exposed to 40, 80, and 160 mg/kg bw per day of sodium chlorate. There was equivocal evidence of carcinogenic activity of sodium chlorate in female $B6C3F_1$ mice exposed to 30, 60, and 120 mg/kg bw per day of sodium chlorate based on marginally measured incidences of pancreatic islet neoplasms at these exposure levels.

For a calculation of a cancer unit risk factor ("URF") for thyroid cancer, the Notifier used the thyroid lesion data for rats in the NTP 2-year carcinogenicity study. **Tables 5 and 6** summarize the tumor incidences reported in the study.

TABLE 7. NTP Results Summary/Male Rats

Male Rats: Tumor Site/Type	Dose (mg/kg bw/day)			
	0	5	35	75
Thyroid: follicular cell carcinoma	0/47	0/44	0/43	4/47
Thyroid: follicular cell adenoma	1/47	0/44	0/43	2/47

Male Rats: Tumor Site/Type	Dose (mg/kg bw/day)			
	0	5	35	75
Thyroid: follicular cell adenoma or carcinoma	1/47	0/44	0/43	6/47

TABLE 8. NTP Results Summary/Female Rats

Female Rats: Tumor Site/Type	Dose (mg/kg bw/day)			
	0	5	45	95
Thyroid: follicular cell carcinoma	1/47	0/47	1/43	2/46
Thyroid: follicular cell adenoma	0/47	0/47	0/43	2/46
Thyroid: follicular cell adenoma or carcinoma	1/47	0/47	1/43	4/46

As demonstrated in the above tabulated data, male rats exhibited the highest overall tumor incidence in the NTP study. Therefore, the tumor incidence data for male rats was used to calculate a cancer URF for sodium chlorate. On this basis, the URF for each tumor site may be calculated as the incidence of tumors relative to the dose, as follows:

$$URF_{thyroid} = [(6/47 - 1/47)] - 75 \text{ mg/kg bw/day} =$$

$$0.0014 \text{ (mg/kg bw/day)}^{-1} = 1.4 \times 10^{-3} \text{ (mg/kg bw /day)}^{-1}$$

6. Toxicology Summary for Chlorate

References supporting the discussion above are provided in **Appendix 9**. The results of the repeat-dose toxicology studies discussed above are summarized in **Table 7** below.

TABLE 9. Subchronic and Chronic Toxicology Studies on Sodium Chlorate

Study	Test Animal	Doses (mg/kg/bw)	Effects	NOEL (mg/kg bw/day)
Bio/Dynamics 90-day gavage	Beagle dogs	10, 60, 360	None treatment related.	>360

Study	Test Animal	Doses (mg/kg/bw)	Effects	NOEL (mg/kg bw/day)
Bio/Dynamics 90- day gavage	Sprague -Dawley rats	10, 100, 1000	Some hematological parameters consistent with anemia at the highest dose.	100 (mid dose)
NTP Teratology (abstracts)	NZW rabbits	100, 250, 475	No treatment- related developmental effects. Maternal toxicity observed only in screening studies at higher doses.	> 475
Biodynamics Teratology	Sprague -Dawley rats	10,100, 1000	No treatment related adverse effects either from maternal or fetal parameters.	> 1000
McCauly *et al*	Sprague -Dawley rats	30-512 male 42-801 female	Pituitary gland vacuolization; Thyroid gland depletion	30 (males) 42 (females)
NTP Subchronic 21 and 90 day	Fisher 344 rats	0, 20, 35, 75, 170 and 300 males 0, 20, 40, 75 150, 340 females	Heart weights were significantly decreased in 300 mg/kg males. Incidences of thyroid gland follicular cell hypertrophy were significantly increased in males and females at doses of 75 mg/kg or greater.	NOEL for thyroid follicular cell hypertrophy at 35.
NTP Chronic bioassay	Fisher 344 rats	5, 35, 75 males 5, 45, 95 females	Increased incidence of thyroid gland adenoma and carcinoma combined at 75. Thyroid gland hypertrophy increased in males at 5.	NOEL for tumors at 35. NOEL for decrease in thyroid hormones at 5. NOEL for decrease in hypertrophy in males <5.

Study	Test Animal	Doses (mg/kg/bw)	Effects	NOEL (mg/kg bw/day)
NTP Chronic bioassay	Mice	40, 80, 160 males 30, 60, 120 females	Thyroid follicular cell hypertrophy increased at 120. Bone marrow hyperplasia increase in all exposed females.	NOEL for hypertrophy at 160. NOEL for bone marrow hyperplasia < 30.

C. Dietary Exposure Assessment for Target Animals for Chlorate and Chlorite

Data collected from DG from fermentation water treated with the PureMash® technology indicate that it may contain of <0.8 mg/kg chlorite and as much as 14 mg/kg of chlorate (on a dry basis) when the fermentation water is dosed at up to 55 ppm chlorine dioxide. Therefore, an assessment of dietary exposure at these dosage and residual levels was conducted. These conditions are thought to represent worst case conditions, given that typical dosages range from 10 to 40 ppm and measured residuals included chlorate concentrations that may or may not be fully attributable to the Notifier's technology.

Distiller's grains are typically fed as a portion of daily feed to target animals such as cattle, diary cows, sheep, swine, and broiler chickens. The daily feed diets of cattle, diary cows, sheep, and swine include up to 30% distillers grains on a dry weight basis. The daily feed intake of broiler chickens may include up to 15% by weight dry distillers grains.[20]

Feeding data for animals which the Distillers Grain Technology Council has indicated that DG can be used in daily feed are presented in the table below.[21] Weights and intakes of feed are nominal, meaning that they are representative of populations of animals generally, and may not be specific to particular categories of food animals raised under specific conditions.[22] The quantity of food consumed per day per animal may not be

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[20] Using Distillers Grains in the U.S. and International Livestock and Poultry Industries, *see* http://www.matric.iastate.edu/DGbook/distillers_grain_book.pdf.

[21] Distillers Grains Technology Council, University of Louisville, Lutz Hall Room 435, Louisville, Kentucky 40292: www.distillersgrains.org.

[22] SAX'S Dangerous Properties of Industrial Materials. Ninth Edition (1996). Table 2. Van Nostrand Reinhold Company. New York.

representative of food intakes for a specific period of time during growth, but rather reflect an average that approximates intakes over an expected lifetime.

TABLE 5. Feeding Data for Food-Producing Target Animals

Animal	Weight	Food Consumed	Distillers Grains (dry weight basis) Consumed per Day		
	(kg)	(g/day)	(%)	(g/day)	g/kg bw/day
Beef Cattle	500	10,000	30%	3,000	6
Dairy Cattle	500	10,000	30%	3,000	6
Poultry (broiler)	2.5	190	15%	28.5	11.4
Sheep	60	2,400	30%	720	12
Swine	60	2,400	30%	720	12

The amount of distillers grains consumed on a dry basis for each animal is calculated as follows for cattle:

(10,000 g-food/500 kg bw) x (0.3 g-distillers grains/g-food)

= 6 g-distillers grains/kg bw

The dietary intake of the distiller grains by other is similarly calculated. The maximum distillers grains consumed by beef cattle, on a dry weight basis, is 6 g/kg bw/day.

With a maximum residual level of 14 mg/kg of **chlorate** in distiller's grains on a dry weight basis, a maximum dietary intake for beef cattle is calculated as follows:

6 g-distillers grain/kg bw x (14 mg-chlorate/kg-distillers grains) x (kg/1000 g)

= 0.084 mg chlorate/kg bw/day

The dietary intake of chlorate by other target animals is similarly calculated.

With a maximum residual level of 0.8 mg/kg of **chlorite** in distiller's grain on a dry weight basis, a maximum dietary intake for beef cattle is calculated as follows:

6 g-distillers grains/kg bw x (0.8 mg-chlorite/kg-distillers grains) x (kg/1000 g)

= 0.0048 mg chlorite/kg bw

For chlorite, the dietary intake of chlorite by other target animals is similarly calculated. Estimated daily intakes ("EDIs") of chlorite and chlorate for each type of animal are presented in the table below:

TABLE 6. EDIs for Target animals

Animal	Chlorate	Chlorite
	EDI (mg/kg-bw/day)	EDI (mg/kg-bw/day)
Beef Cattle	0.084	<0.0048
Dairy Cattle	0.084	<0.0048
Poultry (Broiler)	0.16	<0.009
Sheep	0.168	<0.0096
Swine	0.168	<0.0096

D. Safety of Chlorite in Humans and Target Animals

FDA has evaluated the safety data on chlorite in conjunction with the Agency's review of previous notifications and petitions relating to chlorine dioxide. The U.S. U.S. Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) reviewed the available literature on the toxicity of chlorite and established an acceptable daily intake (ADI) of 30 µg/kg bw/day.[23] EPA relied on neurodevelopmental effects reported in a 2-generation reproductive toxicity study of sodium chlorite in drinking water. The NOAEL was determined as 3 mg/kg bw/day, and the ADI was calculated by applying a 100-fold safety factor. The estimated maximum EDI for chlorite in target animals of 0.01 mg/kg bw/day is below the ADI established by EPA.

Published literature has reported that any chlorite that is consumed by animals, such a cattle, swine, and poultry, is not detected in the animal after consumption and is believed to be metabolized to chloride ion. Consequently no dietary exposure to chlorite for humans is expected as a result of the intended use of the chlorine dioxide generated by the PureMash® technology.

Analysis of distillers grain treated with chlorine dioxide did not detect chlorite at a 0.08 mg/kg detection level. Therefore, a maximum of 0.08 mg/kg of chlorite residuals was assumed to remain in the distiller's grain after ethanol fermentation. The maximum EDI for chlorite from this intended use was determined to be 0.01 mg/kg bw/day or 10 µg/kg bw/day. The U.S. EPA IRIS ADI for chlorite is 30 µg/kg bw/day. The estimated EDI of

[23] The acceptable daily intake is the amount of a substance that can be safely orally ingested over a lifetime based on animal toxicology or human studies.

chlorite of 10 µg/kg bw/day is below the ADI established by EPA and is therefore considered safe for humans and target animals.

E. Safety of Chlorate in Target Animals

Sodium chlorate was the subject of an NTP two-year chronic toxicology study (NTP TR517, December 2005) and two NTP negative genotoxicity studies: an *in vitro* Salmonella mutagenicity test and an *in vivo* micronucleus assay. A unit risk of 1.4×10^{-3} $(mg/kg/day)^{-1}$ has been determined for sodium chlorate on the conservative assumption that the compound is a human carcinogen. However, under the conditions of the intended use of chlorine dioxide, because the target animals live out only a fraction of their natural lifespan, the carcinogenicity toxicology endpoint is not appropriate to use to evaluate chlorate residual in this analysis. For example, broiler chickens are typically slaughtered 28 weeks while the normal lifespan for a chicken is about 12 -15 years. Therefore, it is more appropriate to use sub-chronic oral studies to determine a safe ADI for chlorate for the target animals intended for food consumption.

Such an evaluation requires assessing sub-chronic oral studies on sodium chlorate in rats relative to the target animals. Specifically, an ADI for the target animals was calculated by applying a 100-fold safety factor to the NOAEL value obtained in a subchronic rat study as discussed below, **resulting in an ADI of 0.30 mg/kg bw/day.**

For this purpose we used the demonstrated no observed adverse effect level (NOAEL) of 100 mg/kg bw/day for rats for sodium chlorate. This NOAEL corresponds to a NOAEL level of 78 mg/kg bw/day for *only* chlorate ions as reported in the Biodynamics, Inc. study (1987) and a NOAEL of 30 mg/kg bw/day for *only* chlorate ions as reported in the McCauley study (1995). There are some difficulties associated with interpreting the McCauley study results as 10 animals were used in a group, and, although thyroid colloid depletion was reported, the "Methods" section does not state that thyroids were examined. However, the Notifier has selected the lower NOAEL of 30 mg/kg bw for use to determine an ADI, since thyroid effects observed in this study correlate with 2-generation studies where thyroid effects were also observed. The ADI was established by applying a

safety factor of 100 to the results of the McCauley study to allow for interspecies differences.

We think the determined ADI of 0.30 mg/kg bw/day is conservative for ruminants, due to additional reports in the literature that chlorate and chlorite, when dosed to ruminants, such as cattle, diary cows, sheep, and goats, are rapidly reduced to chloride by interaction with ruminant fluids when ingested, prior to absorption into the body or elimination[24]. Chlorate is reduced by interaction with nitrate-reductase-containing bacteria that have the ability to intracellularly convert chlorate to chlorite where the chlorite is rapidly reduced by the presence of dismutase enzymes capable of rapidly metabolizing chlorite to chlo9ride ion.[25] via ruminal bacteria. Oliver *et al* report that in addition to bacteria present in the ruminant of target animals, the chemical environment of the rumen with chemical redox potentials of -200 to -450 mV provides an electrochemical environment for the reduction of chlorate to chloride. Oliver *et al* report that a greater relative fraction of a low chlorate dose is chemically reduced to chloride compared to higher chlorate dose when added in vitro to ruminal fluids. For example, approximately 15% of a 300 mg/L chlorate dose is reduced to chloride over 24 hours compared to a 100 mg/L chlorate dose which is reduced by 60% over a period of 24 hours in the rumen environment. As these dose levels are much greater that the ADI of 0.30 mg/kg bw/day developed by the rat studies, which is equivalent to a dietary concentration of 15 ppm in cattle[26], we would expect a much lower amount of actual chlorate ion absorbed into the ruminants as chlorate would quickly be converted to chloride in the upper digestive tract of these animals. Therefore, the ADI developed from the rat studies is highly exaggerative for ruminant target animals. However, the ADI is applicable for non-ruminant target animals, such as poultry (broilers) and pigs. The referenced papers are provided with **Appendix 9.**

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[24] C.E. Oliver, M.L. Bauer, J. S. Canton, R. C. Anderson, and D.J. Smith, "The *in vitro* reduction of sodium [36Cl]chlorate in bovine ruminal fluid," J. Anim Sci, 85, 2059-2068 (2007).

[25] D.J. Smith, C.E. Oliver, J.S. Canton, and R.C. Anderson, "Effect of Sodium [^{36}Cl] Chlorate Dose on Total Radioactive Residues and Residues of Parent Chlorate in Beef Cattle, J. Agric. Food Chem, 53, 7352-7360 (2005).

[26] 0.30 mg/kg bw/day x 500 kg bw ÷ 10 kg-food = 15 mg/kg or 15 ppm

Next, we employ the ADI and the estimated residual chlorate in DG to benchmark the safety associated with ingestion of the DG as a component of animal feed. Recall that as much as 14 mg/kg chlorate may remain in the DG on a dry weight basis based on the use of the chlorine dioxide. As calculated above, based on this residual level of chlorate in DG fed to target animals, the worst-case EDI for chlorate for target animals (sheep and swine) consuming DG with this level of chlorate residual is 0.17 mg/kg bw/day (0.168 mg/kg bw/day rounded to 0.17 mg/kg bw/day).

The maximum dietary intake of chlorate by target animals is determined to be 0.17 mg/kg bw/day, which is below the calculated ADI for chlorate ingested by target animals of 30 mg/kg bw/day. We conclude that chlorate byproduct from this process can be deemed safe for animals when present at levels generated by the PureMash® system as described.

Table 12. EDI Summary for Chlorite and Chlorate

CHEMICAL NAME	CAS REG. NO.	DC (ppb)	EDI (mg/person/day)
Animal Dietary Exposure			
Chlorate	14866-68-3		0.17 mg/kg bw/day
Chlorite	14998-27-7		0.01 mg/kg bw/day
Human Dietary Exposure			
Chlorate	14866-68-3	0.3 ppb	0.000015 mg/kg bw/day

F. Human Dietary Intake for Chlorate

High doses of sodium chlorate were directly administered to cattle and swine 24 to 72 hours or to broilers 8 hours prior to slaughter and the disposition of the chlorate within the animal was measured. More specifically, sodium chlorate in the form of radioactive $Na^{36}[Cl]O_3$ was dosed to animals and the fate of the chlorine and parent chlorate in animal tissues was measured on the basis of the radioactive markers.

Summarized below are the data associated with the metabolism of chlorate ion, in the form of sodium chlorate, in animals, with the levels of sodium chlorate present in animal tissues 24 hours after dosing for cattle and swine, and 8 hours for broilers.

TABLE 10. Direct Feed Chlorate Residue Data

Animal/Dose	Liver (ppm)	Kidney (ppm)	Muscle (ppm)	Adipose (ppm)
Beef Cattle[27]				
21 mg/kg bw	0.13	0.27	0.05	0.02
42 mg/kg bw	0.10	0.40	0.20	0.13
63 mg/kg bw	0.08	0.04	0.41	0.21
Swine[28]				
20 mg/kg bw	0.01	0.18	0.07	0.19
40 mg/kg bw	0.02	0.20	0.07	0.13
60 mg/kg bw	0.04	0.19	0.18	0.49
Broiler[29]				
164 mg/kg bw	0.063	Not determined	0.088	0.07
292 mg/ kg bw	0.095	Not determined	0.09	0.05
407 mg/ kg bw	0.087	Not determined	0.135	0.129
Rat[30,31]				
3 mg/kg bw	0.00029	0.00045	0.00028	0.00034

The published literature thus indicates that the fate of chlorate in rats is very similar to that of cattle, swine, and poultry. The dose levels of chlorate administered to target animals in these studies were several orders of magnitude greater than the chlorate levels likely to be ingested by animals fed distiller grains from ethanol plants using the PureMash® technology.

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27 Smith, D.J., Oliver, C.E., Caton, J.S., Anderson, R.C. 2005. "Effect of Sodium [^{36}Cl] Chlorate Dose on Total Radioactive Residues and Residues of Parent Chlorate in Beef Cattle " *J. Agric Food Chem.* 53, 7352-7360 (2005).

28 Smith, D.J., Anderson, R.C., Huwe, J.K. 2006. "Effect of Sodium [^{36}Cl]Chlorate Dose on Total Radioactive Residues of Parent Chlorate in Growing Swine." *J Agric. Food Chem* 54, 8648-8653 (2006).

29 Smith, D.J., Byrd, J.A., Anderson, R.C. 2007. "Total Radioactive Residues and Residues of [^{36}Cl]Chlorate in Market Size Broilers." *J Agric. Food Chem* 55, 5898-5903 (2007).

30 Hakk, H., Smith, D., Shappell, N 2007. "Tissue Residues, Metabolism, and Excretion of Radiolabeled Sodium Chlorate (Na[^{36}Cl]O_3) in Rats." *J. Agric. Food Chem.* 55, 2034-2042 (2007).

31 *See* text for the calculation of residual chlorate levels.

There are two published reports of the disposition of single doses of chlorate of 3 mg/kg bw[32] and 0.06 mg/kg bw[33] reported for rats. It is appropriate to use the toxicology data on the fate and metabolism of chlorate when administered to rats at these lower dose levels which are more comparable to a maximum chlorate dietary exposure of 0.17 mg/kg bw/day experienced by animals fed distillers grains treated by the chlorine dioxide. The reports on the lower dose levels of chlorate provide confirmatory information on the disposition of chlorate ingested by animals. There is no accumulation of chlorate; nearly all the chlorate ingested is metabolized to chloride ions. Based on the data reported by Hakk *et al.* (2007), rats dosed with radioactive sodium $[^{36}Cl]O_3$ after 72 hours of exposure. had only 0.2% of the total $[^{36}Cl]$ residues in the muscle tissue attributed to the presence of chlorate ion with the remainder being chloride ion. The amounts of chlorate in the liver and kidney were not detectable. The amounts of chlorate in adipose (fatty) tissue were not reported; however, as seen below, the Notifier calculates that as much as 0.2% chlorate remains of the total chloride content, a level similarly reported for muscle tissue.

The total chloride content, which included both chlorate and chloride ions, in the various tissues were measured for adipose tissue (0.17 ppm), liver (0.29 ppm), kidney (0.45 ppm) and muscle (0.14 ppm), where the ppm amounts are based on chlorate equivalents. The Notifier assumes that as much as 0.2% of the total chloride content is due to the presence of chlorate ion, with the exception of the kidney and liver tissues where chlorate was not detectable; the Notifier uses a residual percentage of 0.1% for chlorate as a worst-case assumption for these latter organs. The residual chlorate levels in the various edible tissues are calculated as follows:

adipose tissue: 0.17 ppm x 0.002 = **0.34 ppb**
liver: 0.29 ppm x 0.001 = **0.29 ppb**
kidney: 0.45 ppm x 0.001 = **0.45 ppb**
muscle: 0.14 ppm x 0.002 = **0.28 ppb**

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32 *See* Footnote 7.

33 M. S. Abdel-Rahman, D. Couri, and R. J. Bull, "The Kinetics of Chlorite and Chlorate in the Rat." *Journal of the American College of Toxicology*, 3(4), 261-267 (1984). In this article dosing levels were reported to be 5 mg/L chlorate in water, and the male rats consumed 3 mL of the treated water. Assuming an average weight of 235 g for a Sprague-Dawley rat, the male rats in this study ranged from 220 to 250 g, the dose levels are calculated to be 3 mL x 5 mg/L x (L/1000 mL) ÷ 0.235 kg = 0.06 mg-chlorate/kg bw.

Confirmation of the order of magnitude of the chlorate concentration in edible tissues is provided by Abdel-Rahman, *et. al.* (1984) where the levels of total chloride [^{36}Cl] ion from both chlorate and chloride residues in male rats dosed with 0.06 mg/kg bw of sodium [^{36}Cl] chlorate were approximately 0.7 ppb in the liver and 1.4 ppb in the kidney. Amounts of chlorate in the adipose tissues and muscle tissue were not measured. However, no residual levels greater than 2 ppb were measured in any tissues of the rat, including plasma and blood. Based on the weight of this evidence, and that no more than 0.2% of the chloride containing species resulting from the ingestion of sodium chlorate remain in the form of chlorate, the Notifier believes that chlorate will not be present at a residual level greater than 0.45 ppb in any of the edible tissues of target animals based on the analysis outlined above. The Notifier also uses this maximum level of 0.45 ppb as a residual level for chlorate in milk and eggs produced from cows and poultry, respectively.

As further support for these residual concentrations of chlorates, it is reported that chlorate is further reduced by animal tissues themselves. For example, chlorate ion was transformed to chloride ion when skeletal muscle samples of cattle were stored at 14 days at 3.1°C.[34] Essentially, chlorate levels ranging from 0.04 to 0.25 ppm in skeletal muscle tissue were reduced to non-detectable levels during this time period. Furthermore, D. J. Smith *et. al.* (2005) report that 10% of the chlorate fortified in fresh skeletal muscle from cattle is converted to chloride within 1 hour; by 4 days, over 25% of the fortified chlorate was converted to chloride. Additional studies indicated that 20-86% of chlorate fortified in human blood, brain, or liver was no longer present after a 60 hour period.[35]

In the studies cited above, there is no evidence of chlorite in excreta or tissue of broilers, swine, cattle and rats due to chlorate ingestion. From a mechanistic point of view, any chlorite produced by the metabolic reduction of chlorate would be followed by a further reduction of chlorite by metabolic reaction to chloride.[36]

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[34] *See* footnote 25.

[35] J. S. Oliver, H. Smith, and A. A. Watson, "Sodium chlorate poisoning," *J. Forensic Sci*, 12, 445-448 (1972).

[36] Hakk, H., Smith, D., Shappell, N. 2007. Tissue Residues, Metabolism, and Excretion of Radiolabeled Sodium Chlorate (Na[^{36}Cl]O_3) in Rats. *J. Agric. Food Chem.* 55, 2034-2042 (2007).

To determine the dietary intake of chlorate by the consumption of edible parts of a species of target animals, FDA assigns consumption values for different edible products of each species, based on the relative amount of each organ or tissue that is consumed by individuals.[37] The consumption value (*i.e.*, grams consumed, per person, per day) is applied to all species of target animals, as it is assumed that an individual will not consume the same full portion of a meat product from a different species, after having consumed the full portion from the first species. These values are used to determine the exposure of chlorate, based on the level of chlorate in each location. The consumption values and the chlorate levels are summarized here:

TABLE 11. Consumption Values for Chlorate

Edible Product	Consumption (g food/day)	Chlorate Level (µg/g tissue)
Muscle	300 g	0.00028
Liver	100 g	0.00029
Kidney	50 g	0.00045
Fat	50 g	0.00034
Milk	1.5 L	0.00045
Eggs	100 g	0.00045

To estimate the dietary exposure of chlorate, the Notifier considered each edible portion of cattle, as well as considering both milk and eggs as individual commodities, as FDA assumes that milk and eggs are consumed in addition to the edible muscle or organ tissues consumed. FDA assumes that on a daily basis a person consumes a full portion of milk in addition to the full portion edible muscle or organ tissue. The intake estimate for milk is 1.5 L. For eggs, the intake estimate will be changed to 100 g. Again, the FDA assumes that on a daily basis a person consumes a full portion of eggs in addition to the consumption of muscle or organ tissue. The Notifier calculated the relative amount of each edible product based on the chlorate level in the edible product, to obtain, in essence, a dietary exposure for individual consumers. The Notifier is submitting separately calculated exposures due to milk as well as eggs, and the sum of all values to obtain the cumulative exposure. The calculations are detailed here:

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[37] As described in *FDA's Guidance for Industry: General Principles for Evaluating the Safety of Compounds used in Food-Producing Animals*; http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052180.pdf.

Muscle:

(0.00028 µg chlorate /1 g muscle) x (300 g muscle/person/day)

= 0.1 µg chlorate/person/day

Liver:

(0.00029 µg chlorate /1 g liver) x (100 g liver/person/day)

= 0.03 µg chlorate/person/day

Kidney:

(0.00045 µg chlorate /1 g kidney) x (50 g kidney/person/day)

= 0.023 µg chlorate/person/day

Fat:

(0.00034 µg chlorate /1 g fat) x (50 g fat/person/day)

= 0.017 µg chlorate/person/day

Total Dietary Exposure to Chlorate for a Person not Consuming Eggs and Milk:

0.01 µg chlorate/person/day (muscle) + 0.03 µg chlorate/person/day (liver) + 0.023 µg chlorate/person/day (kidney) + 0.017 µg chlorate/person/day (fat)

= 0.17 µg chlorate/person/day

Milk:

(0.45 µg chlorate / 1.0 L milk) x (1.5 L milk/person/day)

= 0.68 µg chlorate/person/day

Eggs:

(0.00045 µg chlorate / 1 g egg) x (100 g egg/person/day)

= 0.045 µg chlorate/person/day

Thus, the cumulative exposure to cattle, milk, and eggs is:

0.17 µg + 0.68 µg + 0.045 µg = 0.9 µg chlorate/person/day

Assuming an individual consumes 3 kg of food per day, this calculates to a dietary concentration of 0.9 µg/3 kg = 0.3 ppb per day. The EDI for chlorate is calculated as follows:

EDI (chlorate) = 0.3 µg/kg x 3 kg-food/p/d = 0.9 µg/p/d

Assuming that an average individual weighs 60 kg, the EDI also may be expressed as

0.9 µg/p/d ÷ 60 kg bw = 0.015 µg/kg bw/d.

G. Safety of Chlorate and Humans

Again, a unit risk factor of 1.4×10^{-3} $(mg/kg/day)^{-1}$ was determined for sodium chlorate on the conservative assumption that the compound is a human carcinogen. This is equivalent to a virtual safe dose (VSD) for human dietary exposure of 14 ppb for sodium chlorate, assuming that an average individual weighs 60 kg and consumes 3 kg of food per day.[38] The unit risk factor is adjusted to 1.79×10^{-3} $(mg/kg\ bw/day)^{-1}$ for chlorate ions.[39] Further to account for the presence of only chlorate ion, the VSD is adjusted to 11 ppb (14 ppb x 83.44 g-chlorate/106.44 g-sodium chlorate = 11 ppb).

Based on the maximum residual levels of chlorate that may be present in edible tissues, organs, eggs, and milk that may be derived from animal sources consuming distillers grains from ethanol distilleries that use this product, the Notifier calculated an EDI for humans of 0.015 µg/kg bw/day immediately above.

Based on an EDI of 0.015 µg/kg bw/day, a worst-case upper bound cancer risk of 2.7×10^{-8} for chlorate as a degradation product is calculated (0.000015 mg/kg bw x 1.79×10^{-3} $(mg/kg\ bw/d)^{-1} = 2.7 \times 10^{-8}$). The estimated dietary exposure to chlorate ion associated with the intended use of chlorine dioxide in ethanol production is 0.3 ppb in the human diet. This level is 2.7% of the VSD of 11 ppb, well below 1/10 of the VSD.

Guidance 159 is intended to assess the safety of animal drug residues present in human food and the effect of these residues on human intestinal flora. As outlined above, only chlorate residues are expect to possibly enter the human gut based on any residuals remaining in the tissues, muscles, and fats of livestock. We have determined that the dietary exposure of humans to chlorate in 0.015 µg/kg bw/day. Specifically, we address whether chlorate residues at these levels are microbiologically active against strains of

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[38] $((1 \times 10^{-6})/1.4 \times 10^{-3}$ $(mg/kg\ bw/day)^{-1}) \times 60$ kg bw ÷ 3 kg/day = 0.014 mg/kg = 14 ppb

[39] 1.4×10^{-3} $(mg/kg\ bw/day)^{-1}$ x (106.44 g-sodium chlorate/83.44 g-chlorate) = 1.79×10^{-3} $(mg/kg\ bw/day)^{-1}$

human intestinal flora. It has been reported in the literature that chlorate does not adversely affect the commonsol microflora of gastrointestinal tracts.[40] The presence of chlorate does not adversely affect the commonsol microflora of gastrointestinal tract. We may conclude that the residue is not microbiologically active against human intestinal flora, especially at these very low levels.

As further support to demonstrate that chlorate at a concentration of 0.015 μg/kg bw/day or 0.3 ppb ppm is not microbiologically active against human intestinal flora, we note that people are exposed to sodium chlorate in drinking water. California, for example, has set a notification level for chlorate in drinking water of 800 μg/L (0.8 ppm), and the World Health Organization has set a guideline limit of up to 700 μg/L (0.7 ppm) in drinking water.[41] If one were to drinks 2 liters of water containing 0.7 ppm sodium chlorate, the resulting exposure would be 1, 400 μg chlorate/day. Just as drinking water (and its related potential exposure to sodium chlorate) is not microbiologically active against human intestinal flora, any residual amounts of chlorate occurring in meat and target animals products as a result of the animals being fed DGs is not microbiologically active in the human gut.

In fact, chlorate levels may not be present at all in meat products derived from target animals for rumens such as cattle, diary cows, and sheep. Chlorate (and chlorite) are rapidly reduced to chloride by interaction with chlorate-reducing bacteria present in the rumen. Oliver et. al. report that during studies with in vitro rumenal fluids, that, in addition to being rapidly reduced to chloride, the rate of chlorate reduction to chloride depends on the dose level. A greater relative fraction of a low chlorate is chemically reduced to chloride at a faster rate compared to a higher chlorate dose. For example, approximately 15% of a 300 mg/L chlorate dose is reduced to chloride over 24 hours compared to a 100 mg/L chlorate dose which is reduced by 60% over a period of 24 hours in the rumen environment. As these dose levels are much greater that the chlorate

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[40] R.C. Anderson, S.A. Buckley, L. H. Stanker, R.B. Harvey, and D.J. Nisbet, "Bacterial Effect of Sodium Chlorate on Escherichia coli 0157:H7 and Salmonella Typhimurium DT104 in Rumen Contents in vitro, J. Food Prot., 63, 1038-1042 (2000).

[41] *See* http://oehha.ca.gov/water/pals/index.html and http://www.who.int/water_sanitation_health/dwq/chemicals/chlorateandchlorite0505.pdf.

intake of 0.17 mg/kg bw/day, which is equivalent to a dietary concentration of 8.5 ppm,[42] we would expect only traces, if any at all, of chlorate ion absorbed into the muscles, tissues and fats of ruminants as chlorate would quickly be converted to chloride during the residence time in the upper digestive tract of these animals. Therefore, the daily intact of chlorate into the human digestive tract is highly exaggerated by our calculations.

H. Antibiotic Resistance and Chlorine Dioxide

Chlorine dioxide is used as antimicrobial for the ethanol fermentation process. One of the ethanol fermentation by-products, DGs, will be fed to food-producing animals. As established earlier in this submission (Section IV. A and B), we expect that no chlorine dioxide will be present in the DGs fed to food-producing animals and have determined that residues of an impurity present as a result of the chlorine dioxide generation is present at a level of 14 mg/kg in the DGs.

In this section, the Notifier addresses Guidance 152 ("Guidance for Industry: Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to their Microbiological Effects on Bacteria of Human Health Concern") and CVM Guidance 159 ("Guidance for Industry: Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Establish a Microbiological Acceptable Daily Intake (ADI)"). These guidance documents are for antimicrobial drugs and not feed products. However, the issues raised in these guidance documents are relevant to the extent that chlorine dioxide is used to reduce the level of microbial matter in ethanol fermentation tanks and the presence of chlorine dioxide and chlorate levels in DGs fed to target animals may provide antimicrobial effects in animals similar to antimicrobial animal drugs.

Chlorine dioxide is a chemical disinfectant and is not considered an antibiotic. Guidance 152 assesses the risk to human health resulting from microbial resistance as a result of animal drug applications. We do not expect any introduction of chlorine dioxide

[42] For cattle, 0.17 mg/kg bw/day x 500 kg bw ÷ 10 kg-food = 8.5 mg/kg or 8.5 ppm

into the target animals and thus there is no concern of bacteria developing resistance to the use of chlorine dioxide from this intended use.

Antibiotics function by destroying a specific enzyme within the cell organism, targeting active sites within the organism. Bacterial resistance to antibiotics arises from a genetic mutation that leads to either enzyme deactivation of the target site, a change in the metabolic pathway, or an increase in efflux within the cell.[43]

In contrast, chlorine dioxide effectively kills microbes by breaking down cell walls. This type of cell destruction is not changed by a genetic mutation that allows a microbe to survive the application of chlorine dioxide. Therefore, chlorine dioxide is not expected to induce antibiotic resistance.

We also note that chlorate is present at very low levels as an impurity in the DGS fed to animals. Chlorate ion is not used as a human drug; therefore, there is no concern of any bacteria developing resistance to chlorate as it is not used for treating human bacterial infections.

V. Conclusion

The Notifier is providing supporting information concerning the regulatory framework upon which the foregoing determination of GRAS is based in **Appendix 10**. In summary, based on scientific procedures, chlorine dioxide is GRAS for the Notifier's intended condition of use because:

- Chlorine dioxide added during fermentation is intended to reduce the presence of unwanted organic acids that lower ethanol production yields. Chlorine dioxide is not intended to have a technical effect in ethanol or the DDG byproduct of ethanol production that may be used as a component of animal feed for food producing animals. Chlorine dioxide is highly reactive and is not expected to be present as a result in the DG;

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[43] See http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/ucm134455.htm.

- The maximum levels at which chlorate and chlorite may enter the diet of *animals* is 0.17 mg/kw bw/day and 0.01 mg/kg/bw/day, respectively. The maximum level at which chlorate may enter the diet for *individuals* consuming meat and products produced from animals fed distiller grains treated with the PureMash® technology is 0.015 μg/kg bw/day. No measurable chlorite is expected to enter the diet of these individuals as a result of the use of the chlorine dioxide. Sodium and sulfate ions are generated in the form of sodium sulfate and sulfuric acid and are GRAS at the expected levels at which they may be present from the technology.

- Based on the 2 year NTP carcinogenicity studies, a unit risk factor for a one in a million occurrence of cancer was calculated to be 1.4×10^{-3} $(mg/kg\ bw/day)^{-1}$ for sodium chlorate. This is equivalent to a virtual safe dose for human dietary exposure of 14 ppb for sodium chlorate, assuming that an average individual weighs 60 kg and consumes 3 kg of food per day.[44] To account for the presence of only chlorate ion, the VSD is adjusted to 11 ppb
  (14 ppb x 83.44 g-chlorate/106.44 g-sodium chlorate = 11 ppb);

- The estimated dietary exposure to chlorate ion associated with the Notifier's intended use is 0.3 ppb in the human diet. This level is 2.7% of the VSD, which is well below 1/10 of the VSD. The Notifier's calculations are designed to be extremely conservative and actual exposures due to the use of its technology are likely to be significantly lower.

Based on the documentation provided in this GRAS notification, and as discussed above, the Notifier has concluded that chlorine dioxide is GRAS via scientific procedures for use as a processing aid in the production of non-food grade and food grade ethanol.

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[44] $((1 \times 10^{-6})/1.4 \times 10^{-3}\ (mg/kg\ bw/day)^{-1}) \times 60\ kg\ bw \div 3\ kg/day = 0.014\ mg/kg = 14\ ppb.$

PureMash

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12/9/08

Feed Safety Team
HFV-222
Food and Drug Administration
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: Authorization to Act as Agent for Resonant Biosciences, LLC

Dear Sir or Madam:

This is to advise that the law firm of Keller and Heckman LLP, its employees, associates, and agents, specifically including, but not limited to Scott A. Krygier and Martha E. Marrapese are hereby authorized to act as agents on behalf of Resonant Biosciences, LLC with regard to submissions to the U.S. Food and Drug Administration by Resonant Biosciences, LLC.

This letter is our authorization to you to permit said firm to undertake appropriate communications relevant to making submissions or inquiring as to the status of any and all submissions filed or to be filed by or on behalf of Resonant Biosciences, LLC, including examination of all relevant information including confidential business, proprietary, and trade secret information submitted or developed under the Federal Food, Drug, and Cosmetic Act.

Sincerely,

Allen M. Ziegler
President

PureMash
ANTIBIOTIC-FREE FERMENTATION TECHNOLOGY
Resonant BioSciences, LLC

Internal Report Summary:
10/24/2008
Chlorine Dioxide Use at (b) (4) (b)

CONFIDENTIAL

Executive Summary

A PureMash chlorine dioxide generator was installed at (b) (4) and was utilized to generate chlorine dioxide on site for application to process mash stream. Chlorine dioxide was added to the mash at the fermentor fill at 1000 ppm. **The main objective of the study was to determine if the PureMash technology could replace the current antibiotic addition for control of bacterial contamination with no negative effect on the ethanol fermentation process.** This was demonstrated by a decrease in bacterial cell contamination levels in the chlorine dioxide treated (PureMash) fermentor compared to the untreated control

Background

Chlorine dioxide is a gas which has high bactericidal effect at low dissolved concentration. This system is designed to chemically generate, on site, high purity chlorine dioxide at the point of use. Laboratory work has shown the killing effect to be much greater on bacteria than on yeast. Previous work showed an equivalent kill compared to the effects of antibiotics in whole corn mash. As a dry grind corn ethanol producer looking for non-antibiotic alternatives, (b) (4) agreed to an installation of a PureLine chlorine dioxide generation system for subsequent trial.

Experimental

1. Plate Count Source Site – South Dakota State University
2. Conducted By: Dr. William Gibbons-Professor Industrial Microbiology
3. Treatment Rate/Mg/: 10 mg/l
4. Fermentor Volume: 650,000 Gallons
5. Chlorine Dioxide/lbs: 54
6. Treatment Time: 16 Hours
7. Treatment Regime: Chlorine dioxide applied on fill of fermentor from 0-16 Hours. Chlorine dioxide application discontinued after 16 hours

Methods

For cell counts, mash samples in sterile containers were held refrigerated until they could be delivered to the laboratories at South Dakota State University. Sample aliquots were diluted using sterile saline blanks (10 to 10^6 dilutions) and plated on Petri plates containing MRS medium. Plates were prepared in triplicate and incubated at 37°C for a minimum of 48 hours. Plate counting was done manually and reported in colony forming units per ml (CFU/ml). Cell concentration was determined by choosing the dilution that yielded between 20 and 200 CFU/ml and averaging of the triplicates multiplied by the plate dilution.

Results

Fermentation Fill

Chlorine dioxide was applied through continuous feed at the fermentation fill of mash into the fermentation vessel. The average bacterial contamination increased and remained elevated in the untreated fermentation vessel (see Table 1, Figure 1 and 3), while the chlorine dioxide treated vessel demonstrated significantly lower bacterial counts (see Table 1, Figure 2 and 3).

Table 1: Bacterial Counts in Untreated and Chlorine Dioxide Treated Fermentation Vessels

Untreated		Chlorine Dioxide Treated	
Time	**CFU/g**	**Time**	**CFU/g**
0.67	1.80E+03	1.25	7.67E+02
1.67	3.10E+08	2.25	5.00E+01
5	6.40E+08	3	5.10E+01
7.00	3.70E+08	3.5	2.12E+03
8.00	6.40E+08	4.75	5.70E+01
9.50	1.12E+07	8.50	3.30E+06
10.00	1.49E+09	9.00	3.30E+01
11.50	2.47E+06	12.50	8.27E+03
15.50	5.30E+06	13.00	1.03E+02
16.50	5.80E+07	17.00	2.10E+01
		17.75	3.30E+03

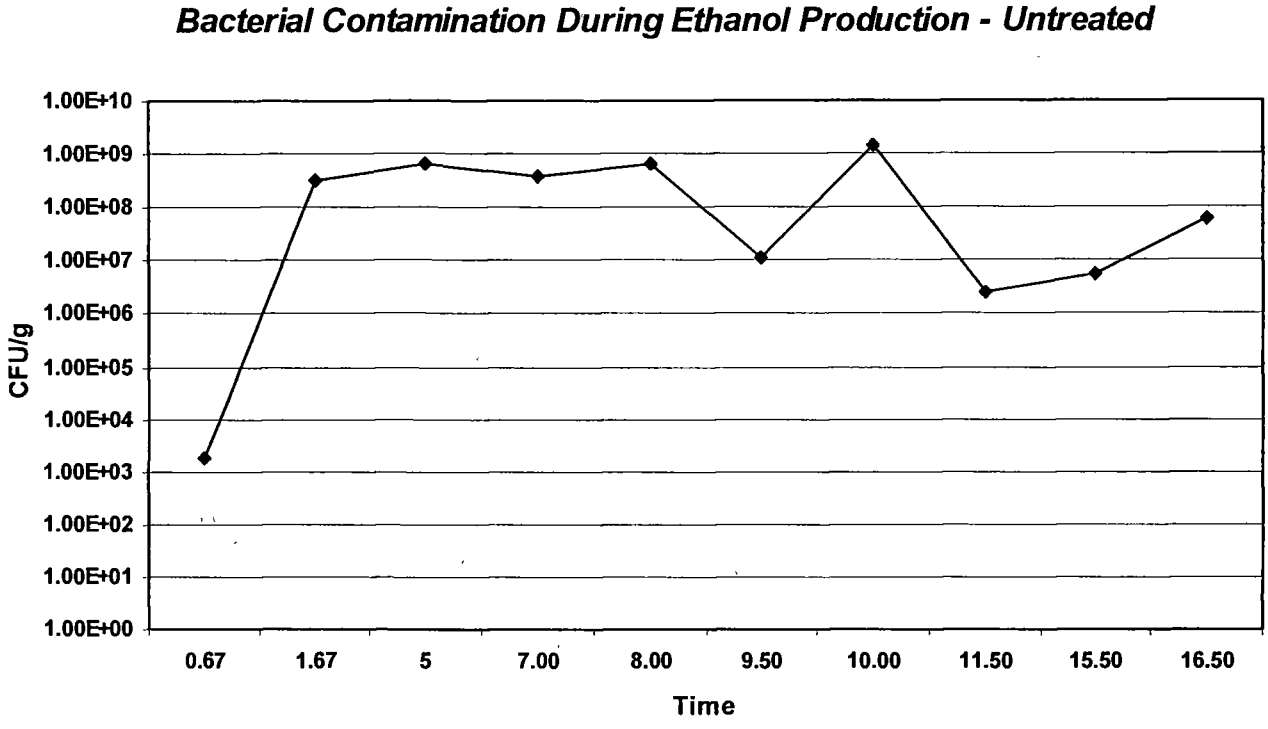


Figure 1: Microbiological samples from an untreated fermentation vessel during ethanol production.

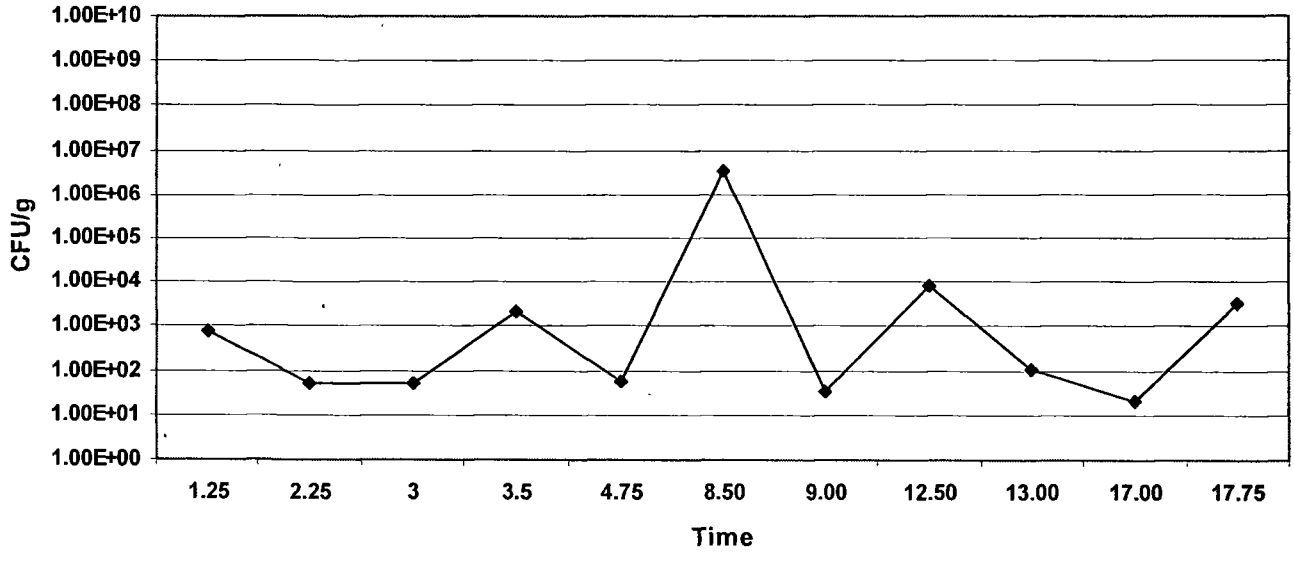


Figure 2: Microbiological samples from a chlorine dioxide treated fermentation vessel during ethanol production.

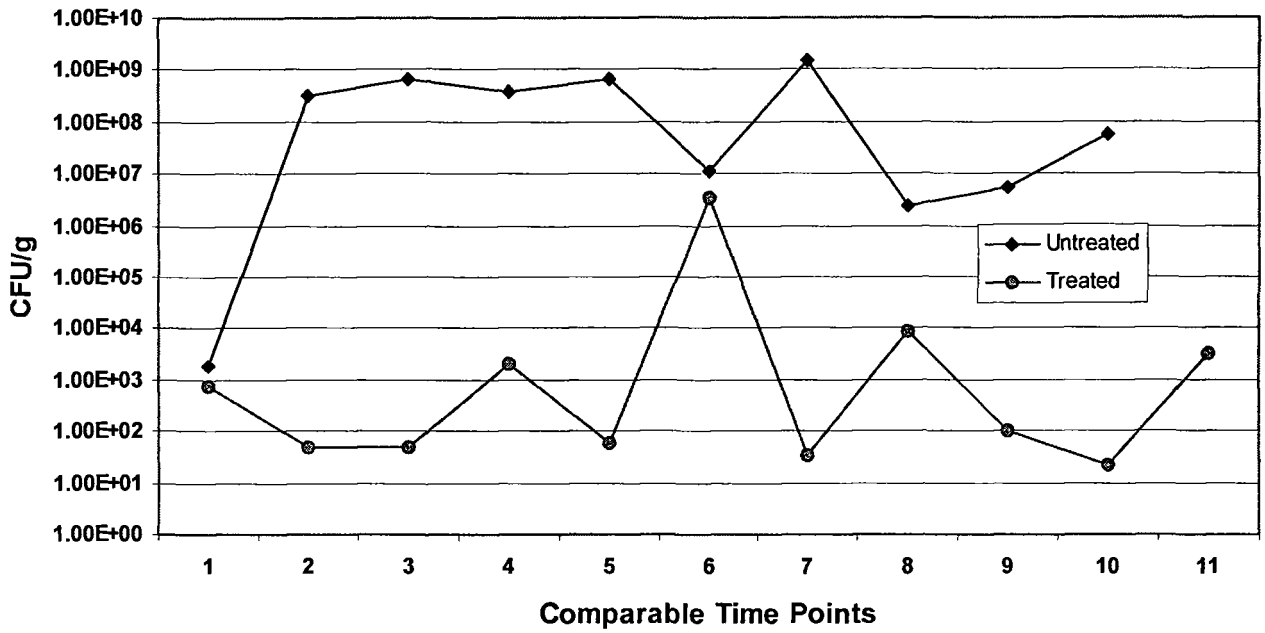


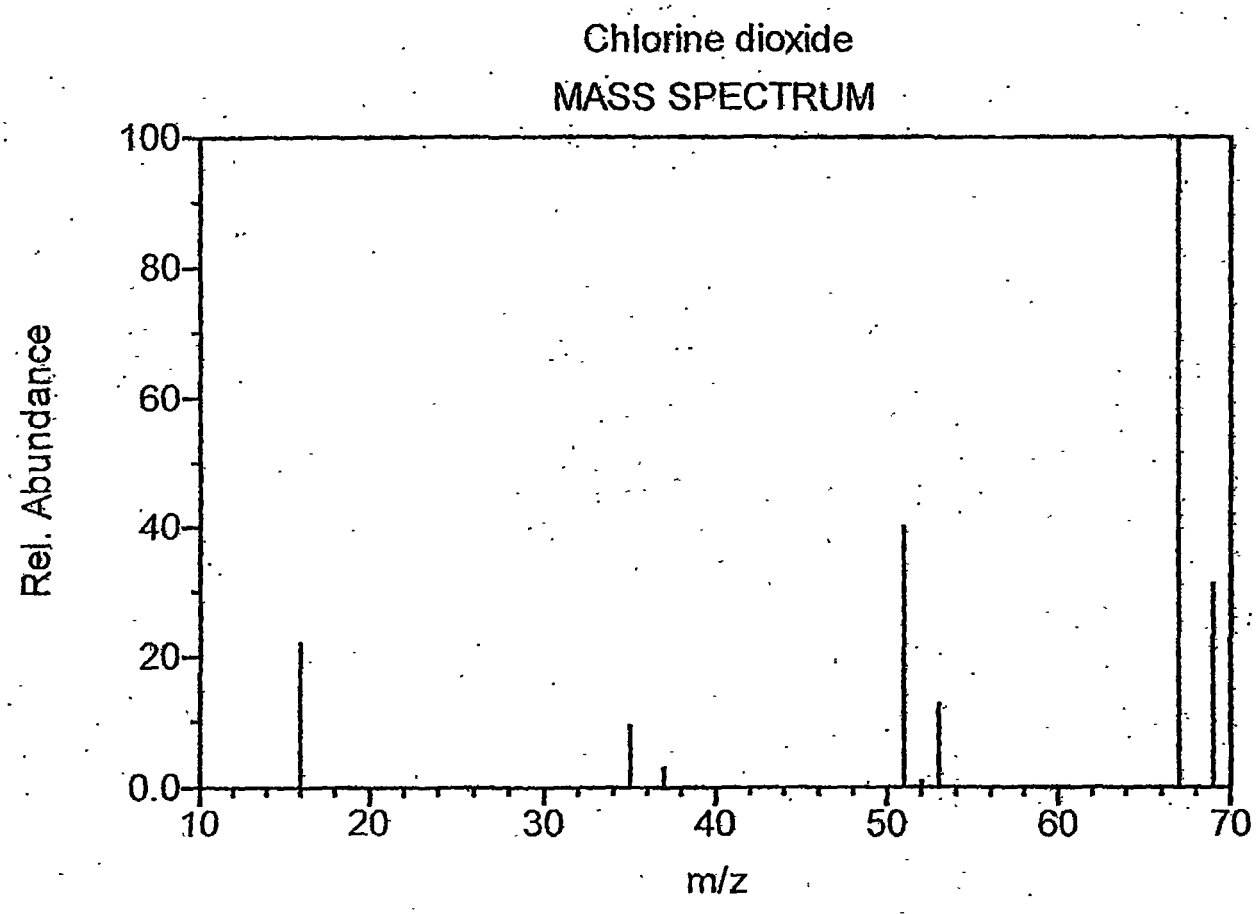
Figure 3: Composite graph of bacterial contamination assuming the time intervals are comparable.

Figure 3 combines the graphs from Figure 1 and Figure 2 making the assumption that the time points tested are comparable. Although the samples were taken at differing times and interval, the important conclusion to draw from the data is that chlorine dioxide treatment decreased bacterial contamination at all time points from a 1-log to a 6/7-log reduction.

Conclusion

Based on the data obtained from the plant trial utilizing the PureMash chlorine dioxide treatment, this high purity chlorine dioxide system is effective at reducing bacterial contamination within the fermentation vessel during ethanol production.

MASS SPECTRUM TYPICAL OF CHLORINE DIOXIDE



MashGuard One™

A Precursor Chemical Solution for Use Only in the PureMash™ Chlorine Dioxide Generator

This chemical solution is for the use only in the PureMash Chlorine Dioxide Generator, a pesticide device that produces Chlorine Dioxide absorbed into water. In addition to this precursor, the PureMash Chlorine Dioxide Generator usually requires a feedstock of 78% sulfuric acid. Please refer to the PureMash Maintenance and Operations Manual to ensure proper activation.

FOR INDUSTRIAL USE ONLY
KEEP OUT OF REACH OF CHILDREN
DANGER

FIRST AID	
IF IN EYES	• Hold eye open and flush with a directed stream of water for 15-20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eyes. • Call a poison control center or doctor for treatment advice.
IF ON SKIN OR CLOTHING	• Take off contaminated clothing. • Rinse skin immediately with plenty of water for 15-20 minutes. • Call a poison control center or doctor immediately for treatment advice.
IF SWALLOWED	• Call a poison control center or doctor immediately for treatment advice. • Have person sip a glass of water if able to swallow. • Do not induce vomiting unless told to do so by a poison control center or doctor. • Do not give anything by mouth to an unconscious person.
IF INHALED	• Move person to fresh air. • If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably mouth-to-mouth if possible. • Call a poison control center or doctor for further treatment.

Hot Line Number

Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-227-5301 for emergency medical treatment information.

Note to Physician: Probable mucosal damage may contraindicate the use of gastric lavage.

Active Ingredient:	
Sodium Chlorate ($NaClO_3$)	40.0%
Other Ingredients	60.0%
Total	100.0%

Distributed by:
Resonant BioSciences, LLC
11757 W. Ken Caryl Ave., F-308
Littleton, CO 80127
866-933-0408

EPA Reg. No. 49620-4-84923
EPA Est. No. 62215-CO-1

Net Contents _____ Gallons

PRECAUTIONARY STATEMENTS

Hazards to Humans and Domestic Animals

DANGER

MashGuard One is corrosive. Causes irreversible eye damage. Causes skin burns. Do not get in eyes or skin or clothing. Wear protective eyewear (goggles or face shield). Wear protective clothing and neoprene gloves. Wash thoroughly with soap and water after handling. May be fatal if inhaled. Remove contaminated clothing and wash before reuse.

ENVIRONMENTAL HAZARDS

This product is toxic to fish and aquatic organisms. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit, and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

CHEMICAL HAZARDS

MashGuard One is a strong oxidizing agent. Do not contaminate with dirt, oils or organic matter of any sort. Contamination may cause violent chemical reactions, fire and explosion. Clean up all chemical spills immediately. Allowing spills to dry or concentrate may cause spontaneous combustion. In case of chemical spills, avoid bodily contact and wear appropriate protective equipment.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling

General Directions

MashGuard One is for use only in the PureMash Chlorine Dioxide Generator, a pesticide device installed to generate chlorine dioxide for the registered uses listed below. Feed rates for MashGuard One are determined by the operator to achieve the desired production rate for chlorine dioxide. As described below, the appropriate production rate will depend on the severity of contamination, the degree of control desired, the size of the system and residual necessary for effective control. For all uses, the point of feed of chlorine dioxide should be below the water level to prevent volatilization of the chlorine dioxide. Chlorine dioxide must be added to the water stream at a point where adequate mixing and uniform distribution can occur.

Drinking Water Treatment

This product is approved for use in water treatment facilities that produce potable drinking water in compliance with the Safe Drinking Water Act. A typical dosage of chlorine dioxide for water systems is between 0.5 and 5 ppm on a continuous basis. MashGuard One has been approved by the National Sanitation Foundation for use in drinking water systems.

Industrial Process Water Uses

This product is approved for the control of microbial, algal and mollusk populations in industrial process or waste water at the sites listed below. The dosage of chlorine dioxide required is dependent on the specific use; see specific directions below. MashGuard One may be used to treat the following aquatic sites:

Recirculating and Non-Recirculating Cooling Water: To control microbial and algal slime in cooling water systems, an intermittent or continuous application may be used. If using a continuous feed, maintain residual chlorine dioxide concentrations between 0.1-1.0 ppm. If using intermittent feed, maintain a residual concentration of 0.1-5.0 ppm. In recirculating systems, chlorine dioxide should be added to the drip pan, cold water well or other points where adequate mixing and uniform distribution can occur. To remove adult mollusks in once-through cooling water systems, an intermittent dose of 0.2-25 ppm is necessary; the exact dose is dependent on the infestation present. If a continuous dose is preferred, apply chlorine dioxide at rates that maintain 0.25-2 ppm in the cooling water. To prevent setting and attachment of the free swimming larvae of mollusks (veligers), apply a continuous feed to achieve a residual of 0.1-0.5 ppm.

Fuel and Industrial Ethanol Fermenters: To prevent or reduce bacterial contamination and to prevent and control the formation of byproducts of bacterial contamination, chlorine dioxide should be added by batch method to achieve an initial dose in the fermenter of 0.1 ppm to 5.0 ppm. Repeat weekly or as signs of bacterial contamination appear. The exact dose of chlorine dioxide will need to be adjusted for levels of contamination, pH and type of contamination.

STORAGE AND DISPOSAL

STORAGE:

Unless delivered in bulk, store in the original container. Store at ambient temperatures from 40°F to 100°F. Store separately from sulfuric acid precursor and all other acids. Store in fire-resistant area separate from incompatible materials such as acids, powdered metals, organic chemicals, combustible materials and dirt. Clean up pills immediately.

DISPOSAL OF WASTES:

Pesticide wastes are toxic. Improper disposal of excess pesticide, spray mixture or rinsate is a violation of Federal law. If these wastes cannot be disposed of by use according to label instructions, contact your State Pesticide or Environmental Control Agency, or the Hazardous Waste representative at the nearest EPA Regional Office for guidance.

CONTAINER DISPOSAL:

Triple rinse (or equivalent) then offer for recycling or reconditioning. If recycling is unavailable, puncture and dispose of container in a sanitary landfill, or by incineration, or if allowed by State and local authorities, by burning. If burned, stay out of smoke.

WARRANTY

RESONANT BIOSCIENCES, LLC warrants that this product conforms to the chemical description on the label and is reasonably fit for purposes stated on such label when used in the PureMash Chlorine Dioxide Generator.

Material Safety Data Sheet
MashGuard One®

Resonant BioSciences

1.0 Chemical Product and Company Identification

Resonant BioSciences, LLC
11757 W. Ken Caryl Ave., F-308
Littleton, CO 80127

24 Hour Emergency Number
US CHEMTREC 1-800-424-9300
CANADA CANUTEC 1-613-996-6666

Product Name
MashGuard One®
EPA Reg. No. 4196-204-84929

Chemical Type
Sodium Chlorate and Hydrogen Peroxide as a stabilized aqueous solution

Intended Use
Reagent feed for PureMash® Chlorine Dioxide generation

2.0 Hazards Identification

Emergency Overview	A clear, faintly blue colored, faintly odored solution which may cause moderate skin irritation and severe irritation of eyes and mucous membranes, including possible blindness. Sodium Chlorate is odorless and very soluble in water. Sodium Chlorate is not listed as a possible carcinogenic by OSHA, IARC or NTP.
Routes of Exposure	Inhalation, skin and ingestion
Potential Health Effects	
Ingestion	Irritation of the gastrointestinal tract, abdominal pain, gas evolution, and red blood cell destruction.
Skin	May cause moderate skin irritation.
Eyes	May cause severe eye irritation, tearing and blurring of vision, with irreversible corneal damage and possible blindness in instances of overexposure.
Inhalation	May cause irritation of the upper respiratory passages; nausea, headache, or weakness.
Target Organs	Skin, eyes, mucous membranes, and renal system.
Chronic Effects	No information.
Medical Conditions Aggravated by Exposure	None documented.

3.0 Composition / Information on Ingredients

Component		CAS #	% Wt/Wt
Hydrogen Peroxide		7722-84-1	< 8%
ACGIH - Threshold Limits Values - Time Weighted Averages (TLV-TWA)	1 ppm TWA		
Sodium Chlorate		7775-09-9	40%
Ingredient Information	Exposure Limits not established for sodium chlorate solution.		

4.0 First Aid Measures

stion	If victim is conscious, give plenty of water to dilute stomach contents. Do not induce vomiting without medical advice. Seek immediate medical attention.
Skin	Wash off immediately with plenty of water for at least 15 minutes. Rinse contaminated clothing with water and launder all clothing prior to use. Call a poison control center or doctor for treatment advice.
Eyes	Immediately flush eyes thoroughly with water for at least 15 minutes. Obtain medical attention if irritation persists. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eyes. Call a poison control center or doctor for treatment advice.
Inhalation	Remove to well-ventilated area. If necessary, give artificial resuscitation and seek medical attention.
Notes to Physician	Sodium chlorate poisoning is rare, but is associated with a high mortality rate with death generally occurring from massive intravascular hemolysis and acute renal failure. Sodium thiosulfate (2 to 5 gm in 200 ml of 5% sodium bicarbonate) is a specific antidote that can be given orally or by I.V. DO NOT treat with methylene blue because of risk of methemoglobinemia. Sodium Chlorate is freely dialyzable and early treatment by peritoneal or hemodialysis is recommended. Direct contact of hydrogen peroxide with the eye is likely to cause corneal damage, especially if not washed away immediately. Careful ophthalmologic evaluation is recommended. Attempts at evacuating the stomach via emesis induction or gastric lavage should be avoided. In the event of severe distention of the stomach or esophagus due to gas formation, insertion of a gastric tube may be required.

5.0 Fire Fighting Measures

...mmable Properties	Non-flammable liquid.

Extinguishing Media

Suitable Extinguishing Media	USE WATER ONLY.
Unsuitable Extinguishing Media	If allowed to evaporate, solid sodium chlorate could be formed. Solid sodium chlorate does not burn, but if exposed to fire it decomposes to give off oxygen which feeds the fire. Consequently, ONLY WATER is effective in cooling and diluting solid sodium chlorate. DO NOT USE CO_2, Halon, dry chemical or powder fire extinguishers, or fire blankets in the event solid sodium chlorate is involved as these are totally ineffective and may confine the heat and create a worse situation.

Protection of Fire Fighters

Protective Equipment for Fire Fighters	Avoid all bodily contact. Wear self-contained breathing apparatus, pressure demand, MSHA/NIOSH approved and full protective gear. Do not allow clothing, shoes, or gloves to become impregnated with sodium chlorate in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of sodium chlorate and hydrogen peroxide solution with plenty of water.
Specific Hazards Arising From the Chemical	DO NOT allow solution to come in contact with any combustible materials. Paper, wood, cloth, and leather impregnated with sodium chlorate solution are highly combustible if allowed to dry, and may be ignited by friction or heat. DO NOT allow the temperature of the storage container to rise above 100 °F (38 °C).

6.0 Accidental Release Measures

sonal Precautions	Protective suit of vinyl, neoprene, PVC or polyethylene; impervious rubber shoes or boots of vinyl or neoprene; safety glasses with side shields or chemical goggles, and hard hat with full face shield when appropriate; rubber gloves of vinyl or neoprene. Isolate area. Keep unnecessary personnel away.
Environmental Precautions	DO NOT ALLOW RELEASES TO ACIDIC DRAINS AS CHLORINE DIOXIDE GAS CAN BE LIBERATED. Contain runoff and contact appropriate local spill response personnel. Do not allow escape into sewers, drains or natural watercourses. Waste disposal in approved chemical disposal area or in a manner which complies with all local, state and federal regulations.
Methods for Containment	Block any potential routes to water systems. Contain spill using noncombustible material such as vermiculite, sand or earth.
Methods for Clean-up	Local authorities should be advised if significant spillages cannot be contained.

7.0 Handling and Storage

Handling Procedures	Prevent possible eye and skin contact by wearing protective clothing and equipment. AVOID PRODUCT CONTACT WITH ACIDIC MEDIA WHICH CAN LIBERATE CHLORINE DIOXIDE GAS.
Storage Procedures	Store in properly vented containers or tanks. Do not block vent. Do not store where contact with incompatible materials could occur, even with a spill. Have a clean water source available for dilution. Keep storage containers out of direct sunlight and away from heat, sparks and flames. DO NOT add any other product to storage container. Never return unused product to storage container.

8.0 Exposure Controls / Personal Protection

Exposure Guidelines	No TLVs have been established for this mixture. The PEL for hydrogen peroxide is 1 ppm. The PEL for sodium chlorate is: Total Dust = 15 mg/m³, Respirable Fraction = 5 mg/m³.
Engineering Controls	Use site specific diking/spill control to avoid uncontrolled releases. Eyewash facility, emergency shower or jump tank should be in close proximity.
Personal Protective Equipment	
Eyes/Face	Wear safety glasses with side shields or chemical goggles. Where appropriate, wear a full face shield. Contact lenses should not be worn when handling this product.
Skin	Use impervious clothing to avoid skin contact. Avoid all bodily contact. Wear self-contained breathing apparatus and appropriate protective equipment. Do not allow clothing, shoes or gloves to become impregnated with sodium chlorate in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of sodium chlorate and hydrogen peroxide solution with plenty of water.
Respiratory	Not applicable under normal conditions of use. For vapor or mist concentration in excess of 10 ppm, a self-contained breathing apparatus should be used. DO NOT USE OXIDIZABLE SORBANTS.
Hygiene Measures	Do not wear leather gloves.

9.0 Physical & Chemical

Appearance

Form	Aqueous Solution	Vapor Pressure	< 0.1 kPa at 40 °C and at 80 °C
Color	Faint Blue to Colorless	Vapor Density	Not Available
Odor	Faint	Specific Gravity	1.37
Odor Threshold	Not Available	Solubility (H_2O)	N/A
Physical State	Liquid	Coefficient of Water/Oil Distribution	Not Available
PH	1.7	Octanol/H_2O Coeff.	Not Available
Melting Point	N/A	Auto Ignition Temperature	Not Available
Freezing Point	Not Available	Decomposition Temperature	Not Available
Boiling Point	104 °C	Viscosity	Water like
Flash Point	Not Available	Bulk Density	1.370 @ 20 °C
Evaporation Rate	> 1 (butyl acetate = 1)	Density	1.37 G/cm³ at 20 °C
Flammability	Not Available		
Upper/Lower Flammability	Not Available		

10.0 Chemical Stability & Reactivity Information

Conditions to Avoid	Avoid heat, flame, strong UV light and other sources of ignition. ELEVATED pH > 7 CAN ENHANCE MORE RAPID DECOMPOSITION OF THE HYDROGEN PEROXIDE.
Incompatible Materials	MashGuard One® may react with acids, organic matter, expanded plastics such as polystyrene or polyurethane, ammonium salts, sulfur or sulfides, phosphorus, arsenic, metals including copper, zinc, aluminum or other metals, manganese dioxide, potassium cyanide, and thiocyanates. MashGuard One is incompatible with soluble metals and their salts (i.e. iron, copper, chromium, vanadium, tungsten, molybdenum, and platinum), reducing agents, organic materials, as well as flammable and combustible materials.
Hazardous Decomposition Products	MashGuard One will react with strong mineral acids liberating chlorine dioxide gas. Contamination from various metals or organic materials may cause rapid decomposition of the hydrogen peroxide, resulting in oxygen gas release and pressure buildup if not properly vented.
Possibility of Hazardous Reactions	Strong mineral acids, organic materials, and powdered metals. Polymerization will not occur.

11.0 Toxicological Information

Acute Effects	The oral LD50 in rats for sodium chlorate is greater than 5000 mg/kg (practically nontoxic). The oral LD50 for a 10% concentration of hydrogen peroxide in rats ranges from 1500 mg/kg to greater than 5000 mg/kg (moderately toxic to practically nontoxic). Ingestion of large doses of sodium chlorate will result in methemoglobinemia and kidney damage.

Component Analysis - LD50	Chromium compounds and perchlorate are created as byproducts in the process for the electrolytic production of chlorates. Hexavalent chromium is a carcinogen present at an average level of < 10 ppm and perchlorate, which can affect the thyroid gland, is present at an average of < 300 ppm.
Inhalation Effects	The LC50 of sodium chlorate is greater than 5.6 mg/l. There was no mortality in rats following a 4 hour exposure to hydrogen peroxide at the minimal attainable concentration of 122 ppm. Acute: Rat LC50 > 5.6 mg/l Lethal Concentration: NOAEL
Irritation to Skin	Sodium chlorate was not irritating to rabbits. Hydrogen peroxide at concentrations of less than 35% is not considered irritating. Acute: Rabbit LD50 > 2000 mg/kg Lethal Dose NOAEL
Irritation to Eyes	Sodium chlorate was mildly irritating to rabbits. Hydrogen peroxide at concentrations greater than 10% is considered severely irritating and corrosive.
Routes of Entry	Hydrogen peroxide at concentrations greater than 10% is considered severely irritating and corrosive.
Sensitization Data	Sodium chlorate was not sensitizing to guinea pigs. Hydrogen peroxide was not sensitizing to guinea pigs at a concentration of 6%.
Carcinogenicity/Mutagenicity Long Term Effects	Sodium chlorate and hydrogen peroxide are not considered carcinogenic.

Rhode Island - Hazardous Substance List

Hydrogen peroxide	7722-84-1	Toxic; Flammable

Neurotoxicity	No data available for this product.
Reproductive Toxicity/Teratogenicity	Sodium chlorate was not teratogenic to rats at doses up to 1000 mg/kg/day during days 6-15 of gestation. Sufficient data is not available for evaluation of hydrogen peroxide.
Epidemiology	No Information.

12.0 Ecological Information

Ecotoxicity

Fish	Rainbow Trout EC50:	> 1000 mg/l, 96 Hours
	Fish NOAEL:	16.4 - 37.4 mg/l, 96 Hours

Aquatic toxicity	The 96 hour LC50 in rainbow trout for sodium chlorate is greater than 1000 mg/l (practically nontoxic). The 96 hour LC50 values for hydrogen peroxide in fish range from 16.4 - 37.4 mg/l (slightly toxic).
Environmental Effects	Hydrogen peroxide occurs naturally as a result of photochemical processes in living organisms.

Persistance/Degradability	Hydrogen peroxide is readily biodegradable and does not bioconcentrate.
Bioaccumulation/Accumulation	Not known.
...bility in Environmental Media	No information.

13.0 Disposal Considerations

Disposal Instructions	In accordance with municipal, provincial, state and federal regulations. D002

14.0 Transport Information

Basic Shipping Description
Material DOT HMR Information

Proper Shipping Name	Sodium chlorate, aqueous solution
Hazard Class	5.1
Subsidiary Hazard Class	
Identification Number	2428
Packaging Group	II
Marine Polutant Identifier	
Severe Marine Polutant Identifier	
Labels Required	Oxidizer

Regulatory Information

US Federal Regulations	Components of this product have been checked against the non-confidential TSCA inventory by CAS Registry Number. Components not identified on this non-confidential inventory are exempt from listing (i.e. as polymers) or are listed on the confidential inventory as declared by the supplier.

CERCLA/SARA - Section 302 Extremely Hazardous Substances TPQs

Hydrogen Peroxide	7722-84-1 1000 lb TPQ (concentration >52%)
OSHA Regulated	Eye/skin irritant as defined in 29 CFR 1910.1200.
SARA 302	Not subject to SARA Section 302.
SARA 311/312	Classified as immediate health hazard and fire hazard. Minimum threshold quantity for reporting is 10,000 pounds.
SARA 313	Not subject to SARA Section 313.
Canada DSL	In compliance.
WHMIS Classification	Class E: Corrosive
General	Not subject to Proposition 65. D002 - RCRA corrosive waste This product contains a chemical known to the State of California to cause cancer or reproductive harm: chromium byproduct Cr(VI) 0.05 mg/m³ ACGIH TLV TWA NTP: Cr(VI) compounds: known human carcinogen IARC: Cr(VI) Group 1 carcinogen.

16.0 Other Information

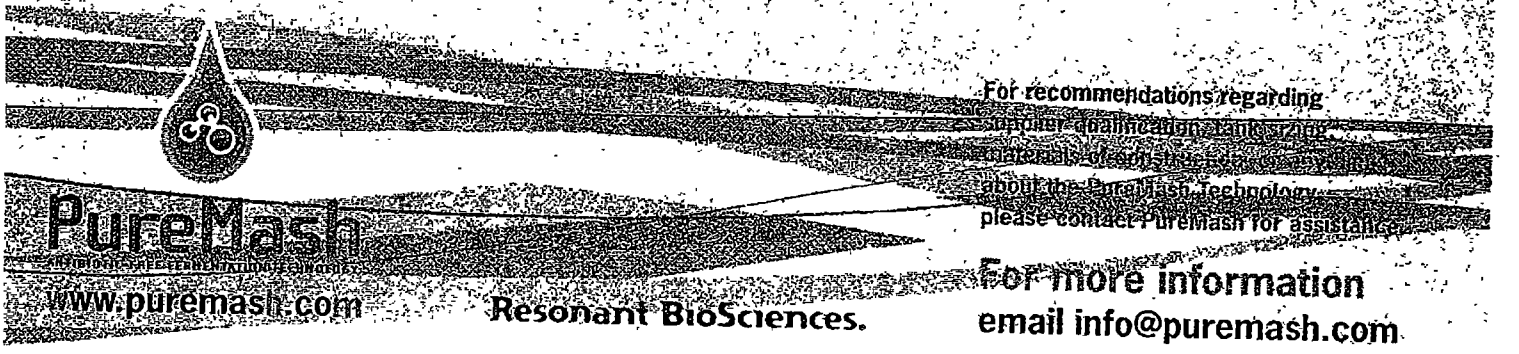
HMIS RATINGS	
Health	2
Flammability Classification	0
Reactivity	2
Pers. Prot	X

NFPA RATINGS	
Health	2
Flammability Classification	0
Reactivity	2
Special Hazards	OXY

Disclaimer

The product is intended for sale only to industrial users. The information in this MSDS is intended to assist these users in determining the suitability of this product for their business applications. Users must inspect and test the product before use to satisfy themselves as to the contents and suitability. Resonant BioSciences, LLC specifically disclaims all warranties express or implied, specifically ALL WARRANTIES AS TO SUITABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR MERCHANTABILITY OF THIS PRODUCT. The exclusive remedy for all proven claims is replacement of our product. In no event shall Resonant BioSciences, LLC be liable for any special, incidental, or consequential damages. The information in this MSDS should be provided by the buyer, transporter or other handlers of this product to all who will use, handle, store, transport or otherwise potentially be exposed to this product. The MSDS has been prepared for the guidance of such persons and Resonant BioSciences, LLC believes this information to be reliable and up to date as to the date of publication, but makes no warranty that it is. If the revision date of this MSDS is more than three years old, then contact Resonant BioSciences, LLC for an updated version.

Issue Date: 20-Aug-2008



Resonant BioSciences, LLC technical personnel experienced in the technology prepared the information presented in this document. It is intended for all who will be involved with the use/implementation of this technology or the use, handling, storage, transport of materials associated with the technology. This information has been prepared for the guidance of engineering, operations and management personnel. While Resonant BioSciences, LLC believes this information to be reliable and up to date, Resonant BioSciences, LLC makes no warranty that it is. BioSciences, LLC makes no warranty or guarantee, express or implied, regarding the accuracy, completeness, applicability, performance at your plant, etc. Prior to implementation, uld make its own independent evaluation of the technology and particularly the implementation of the technology in their facility given their individual circumstances.

Product Specifications

PureMash
ANTIBIOTIC-FREE FERMENTATION TECHNOLOGY

MashGuard One™

A REVOLUTIONARY TECHNOLOGY FROM
Resonant BioSciences, LLC

Description

MashGuard One™ is a stabilized aqueous solution of 40% sodium chlorate and < 8% hydrogen peroxide.

Applications

MashGuard One is a precursor chemical for the production of chlorine dioxide in a PureMash antimicrobial equipment skid. MashGuard One must be used in conjunction with sulfuric acid to produce chlorine dioxide. Chlorine dioxide produced from MashGuard One may be used as a antimicrobial agent in ethanol fermentation, propagation, CIP, heat exchangers and process yeast, mash and water pertaining to the production of ethanol.

Composition

Sodium chlorate	40%
Hydrogen peroxide	8%
Water	52%
Storage temperature limits	5 - 40°C

Physical Properties

Appearance	Clear, faint blue solution
Specific gravity	1.37
Flash point	None
Boiling point	104 °C
Odor	Slight

Shipping Information

USA Customers:
300 gallon tote (IBC)
3800 gallon tank trailer
20 MT ISO container
17,400 gallon railcar

Shipping classification:
DOT: Sodium Chlorate, Aqueous Solution
UN/ID number: UN 2428

International Customers:[1]
1 m³ IBC
20 - 26 MT ISO container

Shipping classification:
UN/ID number: UN 2428

[1] Availability of tank truck and ISO containers varies by region. Contact Resonant BioSciences, LLC for further information pertaining to shipping, etc.

000038

Revised: October 18, 2007

Regulatory Information

It is a violation of Federal Law to use this product in any manner inconsistent with the labeling.

Internationally, regulations vary widely by region and country. Consult local regulatory authorities to determine any local use restrictions or regulatory requirements.

Personal Handling Information

General: Avoid all bodily contact. Wear appropriate protective equipment. Do not allow clothing, shoes or gloves to become impregnated with sodium chlorate in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of sodium chlorate and hydrogen peroxide solution with plenty of water.

Skin: Use impervious clothing to avoid skin contact.

Eyes/Face: Wear safety glasses with side shields or chemical goggles. Where appropriate, wear a full face shield. Contact lenses should not be worn when handling this product.

For more complete information, consult the Material Safety Data Sheet (MSDS) for this product.

Product Identification Numbers

U.S. EPA:	49620-4
CAS:	
Sodium chlorate	7775-09-9
Hydrogen peroxide	7722-84-1

For recommendations regarding supplier qualification, tank sizing, materials of construction, or anything about the PureMash Technology, please contact PureMash for assistance.

PureMash
ANTIMICROBIAL FERMENTATION TECHNOLOGY

www.puremash.com

A REVOLUTIONARY TECHNOLOGY FROM
Resonant BioSciences, LLC

For more information,
call 206.933.0403
or e-mail info@puremash.com

Revised. October 18, 2007

PureMash
ANTIBIOTIC-FREE FERMENTATION TECHNOLOGY

7/8/08

Martha Marrapese, Esq.
Keller & Heckman LLP
1001 G Street N.W.
Suite 500 West
Washington, D.C. 20001

Dear Martha,

RE: PureMash Technology

Overview

PureMash is a chlorine dioxide based antimicrobial technology designed to replace the use of antibiotics currently utilized in propagation/fermentation in fuel ethanol production. (b) (4)

PureMash is "generated" chlorine dioxide utilizing pure, molecular chlorine dioxide. The PureMash technology does not require a low pH media to "activate".

Application Engineering

(b) (4)

The generated chlorine dioxide solution is directed to a batch tank on the skid than further distributed via a pump to the (b) (4)

(b) (4)

(b) (4) The dosage parameters are pre-programmed into the (b) for each plant process. For example, a (b) (4) to a fermentor equates to approximately (b) (4) s of chlorine dioxide applied over a period of (b) (4) depending on the plant metrics. The (b) (4) spectrophotometer located on the skid (b) (4)

(b) (4)

Fermentation Conditions

(b) (4)

Mass Balance – Chlorine Dioxide

The electrochemical half-reaction for the chemical reduction of (b) (4) can be written as:

(b) (4) (1)

From the equation it can be seen that one mole of chlorine dioxide is completely reduced to one mole of chloride. The actual reaction mechanism pathways that lead from (b) (4) likely involve the transient formation of (b) (4).

(b) (4) (2)

(b) (4)

(b) (4)

The typical background chloride concentration in the distillers grains ranges from 40 to 300 mg/l.

Sulfate (SO_4^{2-})

The PureMash technology utilizes (b) (4) sulfuric acid to produce (b) pound of chlorine dioxide. The average dosage (b) (4) of chlorine dioxide per fermentor, therefore, (b) (4) gallons of sulfuric acid.

(b) (4)

Volume of (b)(Required to Treat a Fermentor

Equation 3 is a well-known chemical equation that can be used to determine the volume of a liquid required to dilute a more concentrated liquid:

(b) (4) (3)

(b) (4)

CONCLUSION

This memorandum provides the predicted mass balance based on chemical stoichiometry for the reduction of chlorine dioxide to chloride in a corn mash fermentation study. No other chlorine-containing species were considered in this mass balance equation as none was detected in the IC analytical data provided.

The stoichiometric conversion of (b) (4) will result in the generation of chloride at approximately (the dose of the chlorine dioxide applied. More precisely, for every (b) (4) applied and completely reduced, (b) (4) the by-product.

The PureMash additions to the fermentor consist of chloride and sulfate both of which are minor in concentration when compared the background concentration of these chemical components in both mash and distillers grain.

Best Regards,

Allen M. Ziegler

Allen M. Ziegler
President

CONCENTRATION OF DEGRADATION COMPONENTS IN GENERATED CHLORINE DIOXIDE SOLUTION

The concentration levels anticipated in the fermentor process water are based on the chemical stoichiometry of the Resonant Biosciences' PureMash® chlorine dioxide generator system. Actual measurements of the chlorate and chlorite levels in the chlorine dioxide generator effluent are also presented.

The following assumptions are used in the calculations:

1. The process will typically requires an application of 40 ppm ClO_2 for microbial contaminated fermentation water.
2. Excess sulfuric acid, approximately 3.7 times the stoichiometric amount, is added to increase the reaction velocity and reaction efficiency.
3. All the hydrogen peroxide is decomposed in the reaction chamber, and none gets into the process water.

Additionally, Resonant Biosciences had the residual levels of chlorate and chlorite measured in several samples of a 4000 ppm concentration chlorine dioxide effluent prepared by the PureMash process. The analytical report is attached. The results are presented in Table 1 below:

Table 1: Chlorite and Chlorate Residual Levels in 4,000 ppm Chlorine Dioxide in Water Effluent Generated by the Resonant Bioscience's PureMash® Chlorine Dioxide Generating Process

Sample	Chlorite (ppm)	Chlorate (ppm)
19G1272-01	335	54 0
19G1272-02	335	41.1
19G1272-03	323	52.1
19G1272-04	325	44.1
19G1272-05	328	39.9
19G1272-06	316	51.9
19G1272-07	324	44 5
19G1272-08	312	43.2
19G1272-09	314	38 4
19G1272-10	310	41 9
Average	**322**	**45**

On the basis of these analytical data, chlorite is present at a level of 8% (b) (b) (4) chlorate is present at a level of 1.1 % (b) (4) = 1.1%).

Below are calculations for the level of each degradation product and reaction products that will be added to the fermentation process water as a result of the use of Resonant Biosciences'® chlorine dioxide generator.

Chlorine dioxide is produced with Resonant Biosciences® chlorine dioxide generator according to the following stoichiometric equation:

(b) (4)

At 40 ppm, the initial chlorine dioxide concentration [ClO_2] is 5.93 x 10^{-4} M, as calculated below.

(b) (4)

Chlorate (ClO_3^-)

Chlorate (ClO_3^-) is added to the fermentation process water in (b) ways. First, as (b) (4) . The average level of chlorate in a (b) (4) ppm chlorine dioxide effluent prepared by the PureMash technology is (b) (4) ppm chlorine dioxide concentration will have (b) (4) chlorate. For a maximum chlorine dioxide dosing concentration (b) (4) , the residual levels of chlorate are calculated to be (b) (4)

Chlorite

Chlorite is present as a degradation product in the PureMash chlorine dioxide generated effluent. The average level of chlorite is 322 ppm in a 4000 ppm chlorine dioxide effluent; hence a ( ) ppm chlorine dioxide concentration will have (b) (4) ppm x (b) (4)) of chlorite. For a maximum chlorine dioxide dosing concentration of (b) (4) the residual levels of chlorite are calculated to be (b) (4) ppm x (b) (4)

Sulfate, Sodium Salts

Sodium sulfate is one of the primary reaction by-products of Resonant Biosciences' chlorine dioxide generation process, as shown in the stoichiometric equation above. The PureMash technology uses (b) (4) of (b) sulfuric acid to produce 1 pound of chlorine dioxide. We calculate that (b) moles of sulfuric acid is used to generate one mole of chlorine dioxide as follows:

In the PureMash technology, (b) (4) sulfuric acid reacts with 1 gram of chlorine dioxide.

(b) (4)
(b) (4)

Since only (b) (4) mole of (b) (4) required to produce (b) (4) mole of ClO_2, (b) (4) used to make two mole of chlorine dioxide.

Based on the stoichiometry and addition rate of sulfuric acid, one mole of (b) (4) and (b) (4) $^{-2}$ are introduced into the process water for every (b) moles of (b) that are generated. None of the (b) introduced as sulfuric acid is consumed in the reaction. Furthermore, only sodium and sulfate ions are expected to be present in the fermentation water as reaction by-products of the FCS.

Thus, levels of SO_4^{-2} in the process water are:

(b) (4)

The levels of sodium ion present in the process water result from $NaClO_3$. Thus, a total of (b) (4) added to the process water using the PureMash® process.

(b) (4)

(b) (4)

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(b) (4)

ANALYTICAL REPORT

July 30, 2009

Work Order: 19G1272

Report To	Work Order Information
Allen M. Ziegler	Date Received: 07/23/2009 10:48AM
Resonant BioSciences, LLC	Collector:
11757 West Ken Caryl Avenue, F-308	Phone: (866) 933-0408
Littleton, CO 80127	PO Number:

Project : PureMash

Project Number: (b) (4) ureMash

Container Client Supplied

Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19G1272-01 #1				Matrix:Water	Collected: 07/21/09 10:30		
Chlorite	335 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	54.0 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
72-02 #2				Matrix:Water	Collected. 07/21/09 11:00		
Chlorite	335 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	41.1 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
19G1272-03 #3				Matrix.Water	Collected: 07/21/09 11:15		
Chlorite	323 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	52.1 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
19G1272-04 #4				Matrix:Water	Collected: 07/21/09 13:40		
Chlorite	325 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	44.1 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
19G1272-05 #5				Matrix:Water	Collected: 07/21/09 14:40		
Chlorite	328 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	39.9 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
19G1272-06 #6				Matrix:Water	Collected. 07/21/09 15:05		
Chlorite	316 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	51.9 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8:36	
19G1272-07 #7				Matrix Water	Collected: 07/21/09 15:15		
Chlorite	324 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	44.5 mg/l	1.0	1G93010	300.1	KRM	07/30/09 8 36	
19G1272-08 #8				Matrix.Water	Collected: 07/21/09 16:00		
C	312 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate	43.2 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	

The results in this report apply to the samples analyzed in accordance with the chain of custody document This analytical report must be reproduced in its entirety Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted MRL= Method Reporting Limit

(b) (4)

(b) (4)

Work Order: 19G1272

Analyte		Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19G1272-09	#9				Matrix:Water	Collected:	07/21/09 16:15	
Chlorite		314 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	
Chlorate		38.4 mg/l	1.0	1G93010	300.1	KRM	07/30/09 8 36	
19G1272-10	#10				Matrix:Water	Collected:	07/21/09 16:45	
Chlorite		310 mg/l	1.0	1G93010	300.1	KRM	07/30/09 8 36	
Chlorate		41.9 mg/l	1.0	1G93010	300 1	KRM	07/30/09 8 36	

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes
Batch 19G3004 - 1G93010										
Calibration Check (19G3004-CCV1)				Prepared: 07/29/09 Analyzed 07/30/09						
Chlorite	30 2		mg/l	29 9665		101	90-110			
Chlorate	29 1		"	29 1000		100	90-110			
Calibration Check (19G3004-CCV2)				Prepared 07/29/09 Analyzed. 07/30/09						
Chlorite	28 8		mg/l	29 9665		96 0	90-110			
Chlorate	27 5		"	29 1000		94 6	90-110			
Batch 1G93010 - General Prep HPLC/IC										
Blank (1G93010-BLK1)				Prepared 07/29/09 Analyzed. 07/30/09						
Chlorite	ND	0 1	mg/l							
C ate	ND	0 1	"							
(1G93010-BS1)				Prepared 07/29/09 Analyzed 07/30/09						
Chlorite	11 7	0 1	mg/l	11 8575		98 7	90-110			
Chlorate	26 0	0 1	"	26 7500		97 3	75-125			
Matrix Spike (1G93010-MS1)	**Source: 19G1272-01**			Prepared 07/29/09 Analyzed 07/30/09						
Chlorite	456 4	1 0	mg/l	118 575	335 0	102	67-140			
Chlorate	318 1	1 0	"	267 500	54 0	98 7	75-125			
Matrix Spike Dup (1G93010-MSD1)	**Source: 19G1272-01**			Prepared: 07/29/09 Analyzed 07/30/09						
Chlorite	451 1	1 0	mg/l	118 575	335 0	97 9	67-140	1 18	12	
Chlorate	308 2	1 0	"	267 500	54 0	95 0	75-125	3 16	20	

ND = Non Detect; REC= Recovery; RPD= Relative Percent Difference

Certified Analyses included in this Report

Method/Matrix	Analyte	Certifications

Code	Description	Number	Expires

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted MRL= Method Reporting Limit

Work Order: 19G1272

End of Report

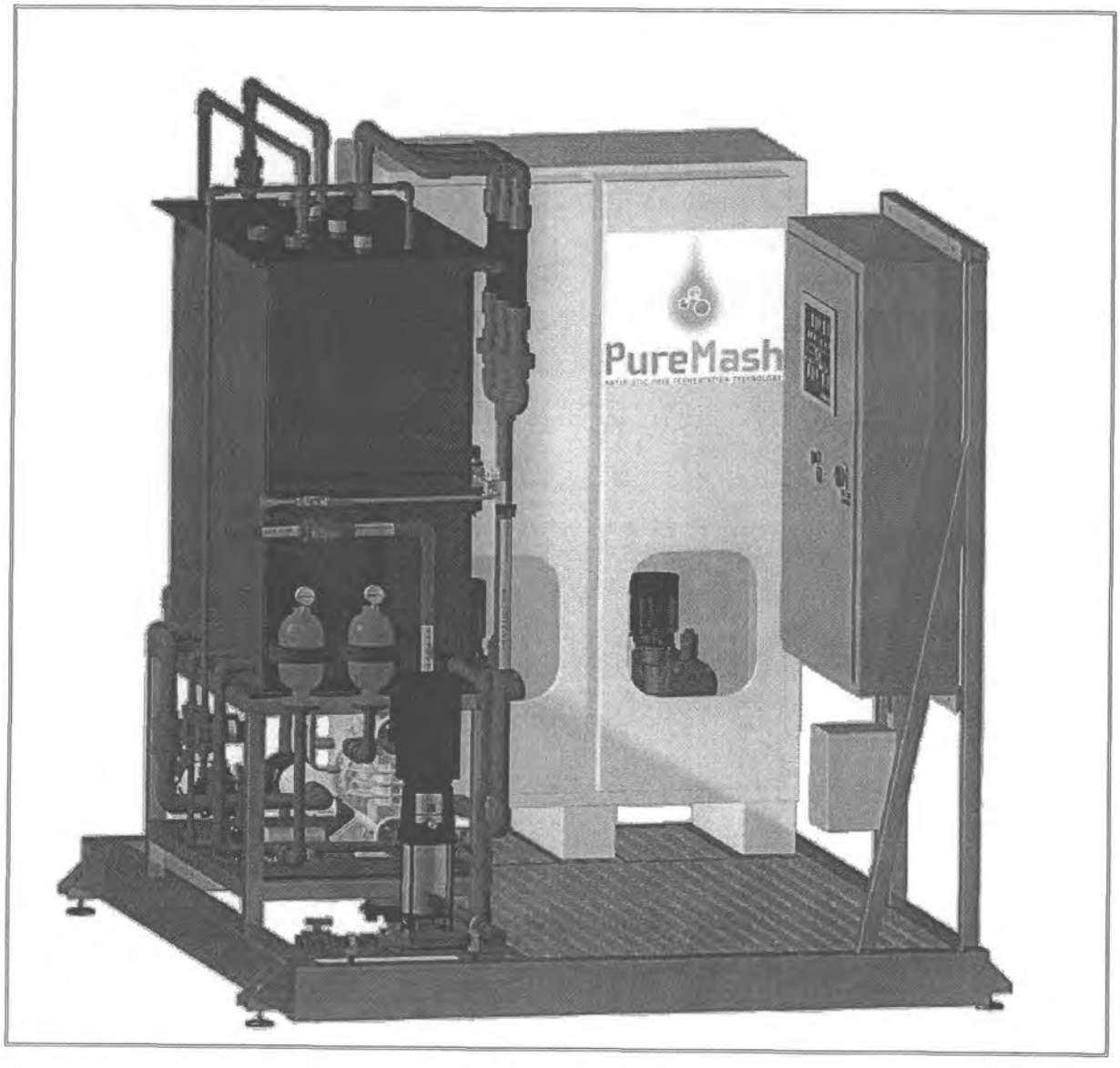
(b) (4)

(b) (4)
Project Manager

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit

PureMash™ System

Operation Manual



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NOTICE AND INTENDED USE

This operating manual and all information, technical advice and recommendations provided herein or otherwise, whether orally or in writing, are intended solely for use by competent, technically-trained personnel with experience and skill in the operation of industrial equipment and the handling of potentially hazardous chemicals. Operators of the PureMash equipment should always consult with their corporate Environmental, Health and Safety (EHS) organization prior to starting the operation of the PureMash equipment to ensure compliance with all of the environmental, health and safety procedures required by your corporation.

Under the terms of our purchase and/or lease agreement, Resonant BioSciences, LLC (RBS) has warranted that the equipment and chemicals supplied by RBS are free from defects in materials and workmanship for a period of one (1) year.

There are no other warranties, representations or conditions, expressed or implied, statutory or otherwise—including, without limitation, warranties, representations or conditions of merchantability or fitness for purpose—relating to the use of this "General Operating Manual" or equipment or chemicals supplied by RBS or their application.

This manual is designed to provide sufficient information and direction to enable site personnel to operate and maintain the PureMash system. While every effort has been made to ensure the accuracy of the contents of this manual, Resonant BioSciences, LLC assumes no contingent liability either for inaccuracies in the content or for uses to which the customer may put the equipment described, with or without the benefit of statements made herein.

Your comments, questions and corrections are appreciated and will help us make the documentation more useful to you. Please send comments to Resonant BioSciences; 1400 16th St. Ste. 400, Denver CO 80202; USA.

THIS MANUAL CONTAINS IMPORTANT SAFETY PRECAUTIONS THAT MUST BE ADHERED TO. PLEASE READ ALL SAFETY INFORMATION THOROUGHLY.

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SAFETY PRECAUTIONS

Safety Warnings

Study this operating manual's safety instructions carefully before installing, operating or servicing the equipment. Become familiar with operating instructions and with the system's automatic shut-offs and alarms. The system will operate efficiently and reliably only if it is properly installed, operated and maintained. Most system-related accidents can be avoided by following the basic safety instructions contained in this manual. In addition, all safety-related regulations, local codes and instructions that appear in this manual or on the equipment must be observed to ensure personal safety and to prevent damage to either the instrument or equipment connected to it. If equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

Within this manual the following terms describe potentially dangerous or harmful situations and have the following meanings:

WARNING! Describes a potentially dangerous situation which, if not avoided, could result in serious physical injury or damage to the equipment and/or system components.

CAUTION! Describes a potentially harmful situation which, if not avoided, may result in physical injury or damage to the equipment and/or system components.

RESONANT BIOSCIENCES' GENERATORS ARE EQUIPPED WITH A NUMBER OF SAFETY FEATURES THAT AUTOMATICALLY SHUT OFF THE SYSTEM IN THE EVENT CERTAIN SAFETY CRITERIA ARE NOT MET. AN OPERATOR SHOULD BE AWARE OF THESE FEATURES AND SHOULD INSPECT THESE FEATURES WHENEVER THE PUREMASH EQUIPMENT IS STARTED.

WARNING! The air vent pipe should not be blocked under any circumstance. Blockage can result in over pressurization, which can lead to a system rupture and the potential release of chlorine dioxide.

WARNING! Only use MashGuard One™ and Sulfuric Acid for the precursor feeds to the system. Utilization of any other chemical can result in reactions that can cause damage to the equipment or produce chemicals for which the system is not rated.

CAUTION! Always use appropriate eye, respiratory and exposed-skin protective equipment required by your EHS department when handling potentially hazardous chemicals. (See the Material Data Safety Sheets in Section 8 of this manual for chlorine dioxide and MashGuard One)

WARNING! Under no circumstances should the chlorine dioxide generation equipment be operated if there is no vacuum indication on the HMI (Human Machine Interface) or the motive booster pump is off. This can result in an over-concentration of chlorine dioxide which can cause auto-decomposition of chlorine dioxide and a potential explosive condition.

CAUTION! Do not attempt to operate the chlorine dioxide generator until any and all chlorine dioxide leaks are located and repaired. (See the Material Data Safety Sheets in Section 8 of this manual for chlorine dioxide)

(Continued on page 5)

WARNING! Electrical Equipment

It is essential that anyone working with the electrical components of the system observe and have knowledge of standard industry safety guidelines regarding electrical equipment.

- No metallic jewelry should be worn while working on the system or moving around the system.
- Wear proper protective clothing. Consult your EH&S department for requirements.
- Voltages or currents in some assemblies are LETHAL. If these assemblies are handled while energized, death or severe personal injury may occur.
- High voltage components should never be placed in areas where they may get wet.
- Standard cautionary practices should be followed when working with the system when the unit power is energized. It houses equipment operating at 460 and/or 230 volts AC and is dangerous. Should the system require maintenance, be sure to disconnect the power either by unplugging it from the power source or using the disconnect at the power source. Using the breaker in the electrical enclosure does NOT fully isolate the equipment electrically.

CAUTION! *High* Pressure Water Sources

The system requires high-pressure water sources. Improper connection or piping may result in failure and exposure to water at dangerously high velocities and/or contact of water with electrical supplies.

CAUTION! Reagents and Samples

Standard laboratory practices should be followed when working with any process water samples and reagents which may be used with the system. Failure to handle reagents properly and safely may result in severe personal injury. It is essential that vessels containing process water or reagents be stored and handled with caution. No liquid should be permitted to come in contact with electrical apparatus. Standard equipment should be used for regulation and dispensing of feed chemicals. Reactors, pump, piping and tubing must be completely flushed with water and drained before working on any internal components.

CAUTION! Personal Protective Equipment

When the potential for exposure exists from one of the reagents, personnel must wear safety goggles, face shields, neoprene or vinyl clothing or aprons, neoprene or vinyl boots. Other protective equipment that the on-site facility may require may include the following: hard hats, escape respirators, acid resistant clothing, ear protection and safety shoes. Familiarize yourself with the facility's personal protective equipment requirements before entering the PureMash system area.

CAUTION! Sulfuric Acid

Health & Safety Conditions

Sulfuric acid solutions are hazardous materials. Only properly trained personnel should be allowed to work in areas where these materials are being stored and/or handled.

WARNING!

- CAUSES SEVERE BURNS.
- REACTS VIOLENTLY WITH WATER.

(Continued on page 6)

- MAY RELEASE EXPLOSIVE HYDROGEN GAS.
- HIGHLY REACTIVE.
- MAY IGNITE COMBUSTIBLE MATERIAL ON CONTACT.
- KEEP AWAY FROM OPEN FLAME, SOURCES OF SPARKS OR IGNITION.

CAUTION! MashGuard One (a mixture containing $NaClO_3$ and H_2O_2)

Health & Safety Conditions

MashGuard One solutions can be hazardous materials if handled improperly. Only properly trained personnel with protective gear should be allowed to work in areas where these materials are being stored and handled.

WARNING!

- MAY CAUSE MODERATE SKIN IRRITATION.
- MAY CAUSE SEVERE EYE INJURY WITH DELAYED EFFECTS AND POSSIBLE BLINDNESS.
- MAY CAUSE IRRITATION OF THE UPPER RESPIRATORY PASSAGES, NAUSEA, HEADACHE, OR WEAKNESS.
- KEEP AWAY FROM OPEN FLAME, SOURCE OF SPARKS OR IGNITION.
- STRONG OXIDIZER—WILL CAUSE FIRE IF IN CONTACT WITH COMBUSTIBLE MATERIALS, INCLUDING LEATHER.
- DRYING ON CLOTHING OR COMBUSTIBLE MATERIALS WILL CAUSE FIRES.

CAUTION! Chlorine Dioxide

Health & Safety Conditions

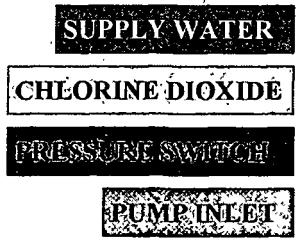
Chlorine dioxide is an unstable material. Only properly trained personnel with protective gear should be allowed to work in areas where these materials are being stored and/or handled.

WARNING!

- CHLORINE DIOXIDE IS AN IRRITANT TO EYES, THROAT AND RESPIRATORY PASSAGE. DEGREE OF IRRITATION IS RELATED TO CONCENTRATION RANGING FROM MILD TO SEVERE.

SYMBOLOGY

Labeling— the PureMash system has been labeled in such a way that operators or maintenance personnel can easily identify a component or pipeline by its name and contents. Label colors represent the process fluid inside be pipeline or component as follows:

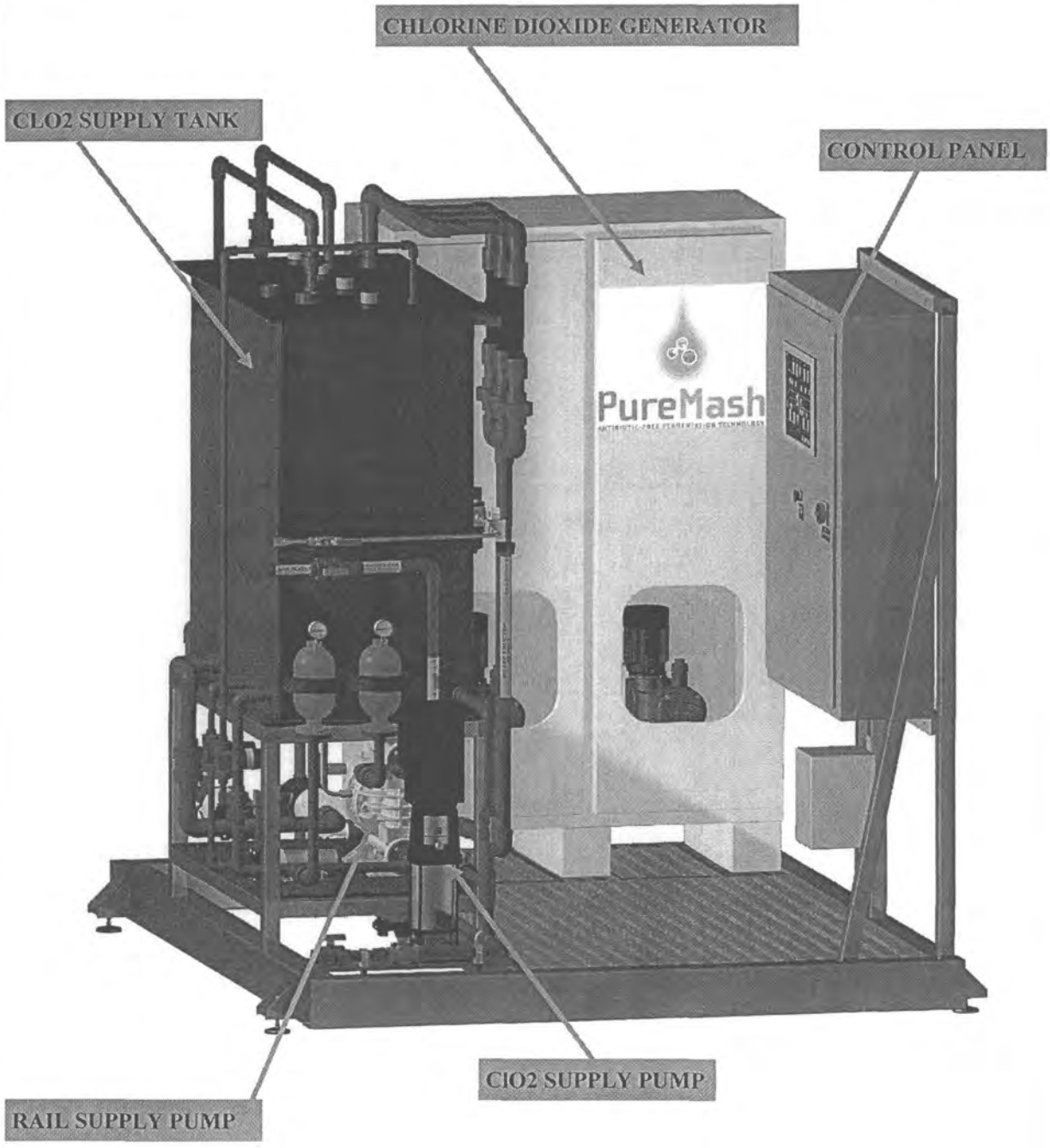


- Process fluid—water
- Process fluid — chlorine dioxide (liquid or gas)
- Process fluid —MashGuard One
- Process fluid — sulfuric acid

In addition to the nomenclature labels there are flow arrows indicating the normal direction of flow within the pipeline. The flow arrow labels will follow the same color pattern as the nomenclature labels.

SYSTEM COMPONENTS

Overall System—*This shows the location and nomenclature of the major components of the PureMash System.*



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CONTROL PANEL—This panel contains the PLCs, VFDs, HMI, DC power supplies and provides for the location of all electrical connections between the system components and the controls. This panel is supplied with 460VAC 3PH power and provides 230VAC 1PH and 24VDC to the system components as necessary.

WARNING! ENTRY TO THIS PANEL IS RESTRICTED TO QUALIFIED PERSONNEL ONLY. RISK OF ELECTRICAL SHOCK IS PRESENT WHILE THE POWER IS ON!

POWER DISCONNECT—Controls all power to the system.

HMI– Provides the ability to operate the system via a 15" color touchscreen. This also provides informational videos and pictures showing how to operate, troubleshoot alarms and perform maintenance functions.

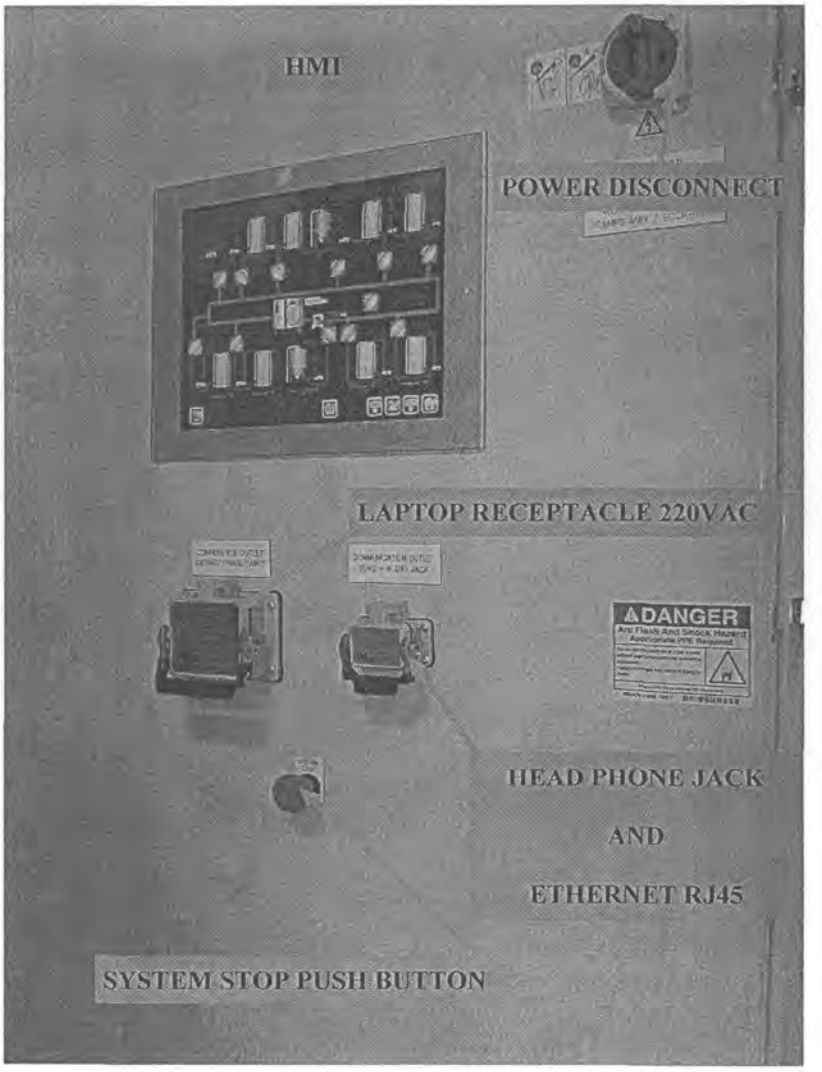
LAPTOP RECEPTACLE—Supplies 220VAC through a European-style receptacle designed for providing power to a laptop.

CAUTION! ENSURE THAT ANY EQUIPMENT CONNECTED TO THIS RECEPTACLE IS RATED FOR 220VAC, OTHERWISE, EQUIPMENT DAMAGE MAY OCCUR.

HEAD PHONE JACK—Allows the user to connect an audio device to allow for listening to narrations that along with video or pictures show how to operate, troubleshoot alarms and perform maintenance functions on the system.

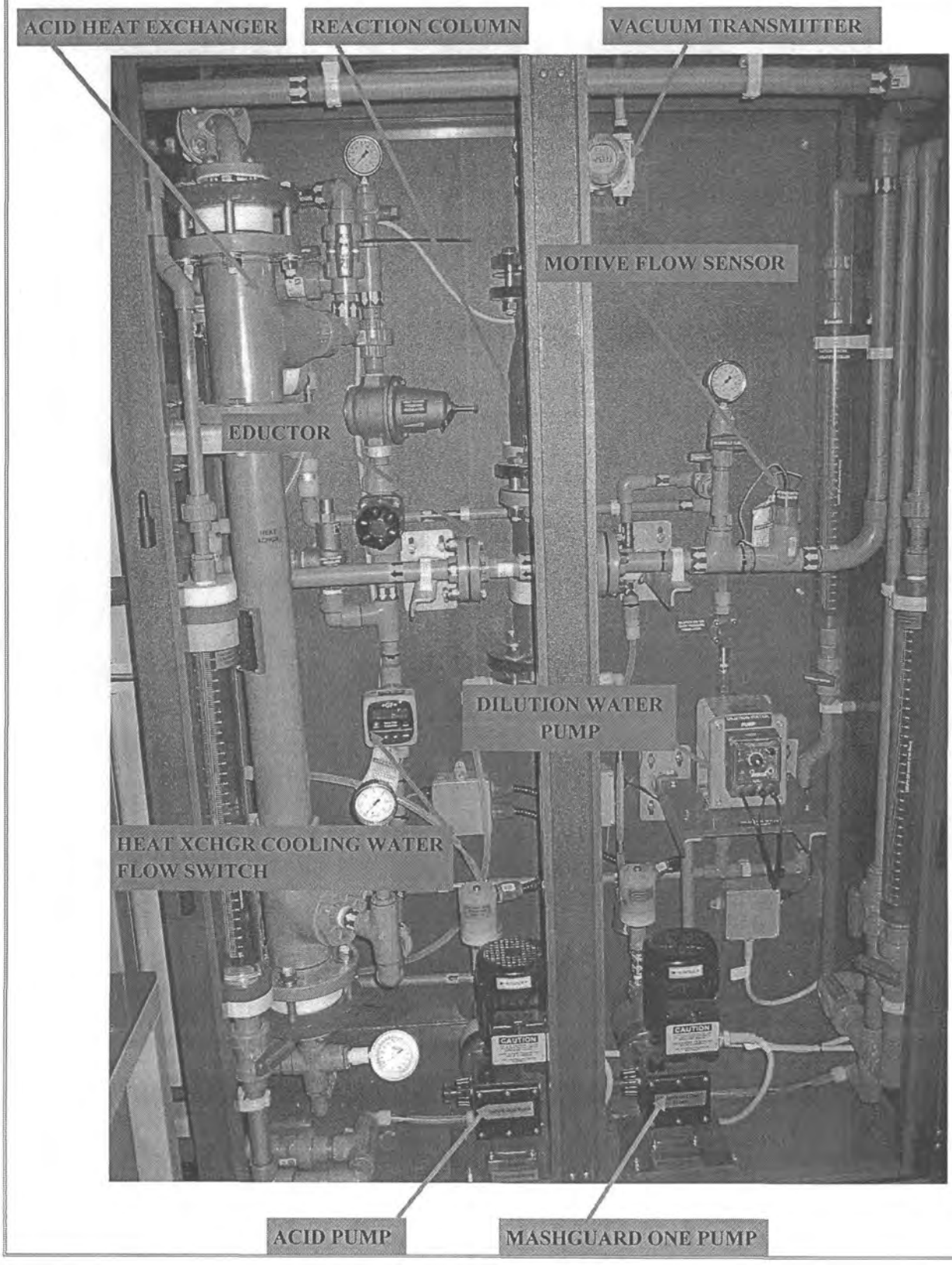
ETHERNET RJ45 –A programming port utilized to connect to a laptop for updating PLC and HMI software.

SYSTEM STOP—Push to operate, pull to release switch that stops all system components while continuing to provide power to the system.



WARNING! POWER IS STILL AVAILABLE TO ALL SYSTEM COMPONENTS THE PLC IS PREVENTING THE COMPONENTS FROM OPERATING. IF MAINTENANCE IS TO BE PERFORMED, PLEASE FOLLOW ALL LOCKOUT PROCEDURES AS PER LOCAL EH&S GUIDELINES.

CHLORINE DIOXIDE GENERATOR



Chemistry

The chlorine dioxide generator uses MashGuard One patented hydrogen peroxide-based chemistry. Sodium chlorate is reacted with hydrogen peroxide and sulfuric acid to produce chlorine dioxide, Equation 1. The advantages of this chemistry as it applies to fermentation is that it provides chlorine-free chlorine dioxide, free of chlorite which will cause problems with the fermentation.

Equation 1.

$$(2NaClO_3 + H_2O_2) + H_2SO_4 \longrightarrow 2ClO_2 + O_2 + Na_2SO_4 + 2H_2O$$

[redacted]

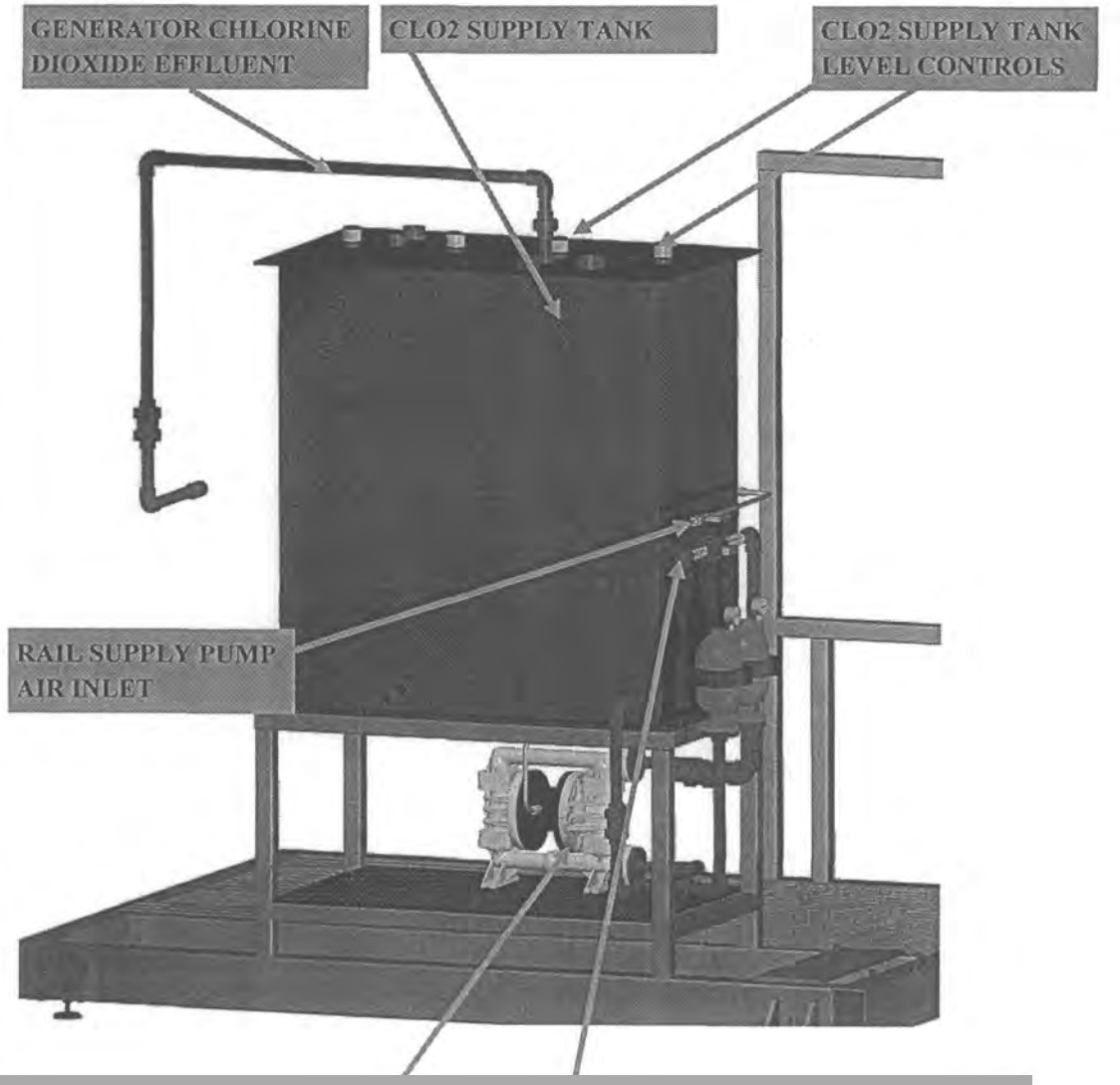
Process Information and Equipment

The unit is fed MashGuard One and sulfuric acid solution. The sulfuric acid concentration required for the reaction is 78%, which is acquired by feeding the system with 93-98% sulfuric acid, delivered via the Acid Pump, and is diluted by the Dilution Water Pump to 78%. This stream is cooled via the Acid Heat Exchanger and is then delivered to the Reaction Column. The MashGuard One pump delivers the MashGuard One precursor to the Reaction Column, where it mixes with the 78% sulfuric acid and produces chlorine dioxide, per Equation 1, under a vacuum. The vacuum is provided by the Eductor, which is fed with Motive Fluid via the CLO2 Supply pump. The Motive Fluid passes through the Eductor, which draws the chlorine dioxide out of the reactor, and is absorbed into the stream forming the Chlorine Dioxide Solution. This Chlorine Dioxide Solution flows to the CLO2 supply tank where it is stored and fed to the injection points via the CLO2 Supply Pump.

The Vacuum Transmitter provides continuous monitoring of the Reaction Column to ensure that a vacuum is always present during system operation. The PLC will shut down the generation of chlorine dioxide if the vacuum of the system is ever less than –3inHg.

Cooling water flow is monitored in the Acid Heat Exchanger during chlorine dioxide production via the Heat XCHGR Cooling Water Flow Switch and a signal is provided to the PLC. The PLC will shutdown production of the chlorine dioxide if the flow is less than 0.5GPM.

RAIL SUPPLY SYSTEM—The PureMash system utilizes a common rail to supply chlorine dioxide solution to the injection points (i.e. Fermentors, Propagators). The chlorine dioxide is created by the generator and delivered to the CLO2 Supply Tank via the Generator Chlorine Dioxide Effluent line. The CLO2 Supply tank level is monitored with two ultrasonic level controls which provide signals to the PLC to both indicate tank level and provide for generator shutdown in the event of a high level or a Rail Supply Pump shutdown in the event of low level. The chlorine dioxide solution is fed from the CLO2 Supply Tank via the Rail Supply Pump to the common rail at a pressure not to exceed 150psi. The pressure of the rail is controlled by the air supply pressure feeding the Rail Supply Pump with a regulator that is mounted on the system.



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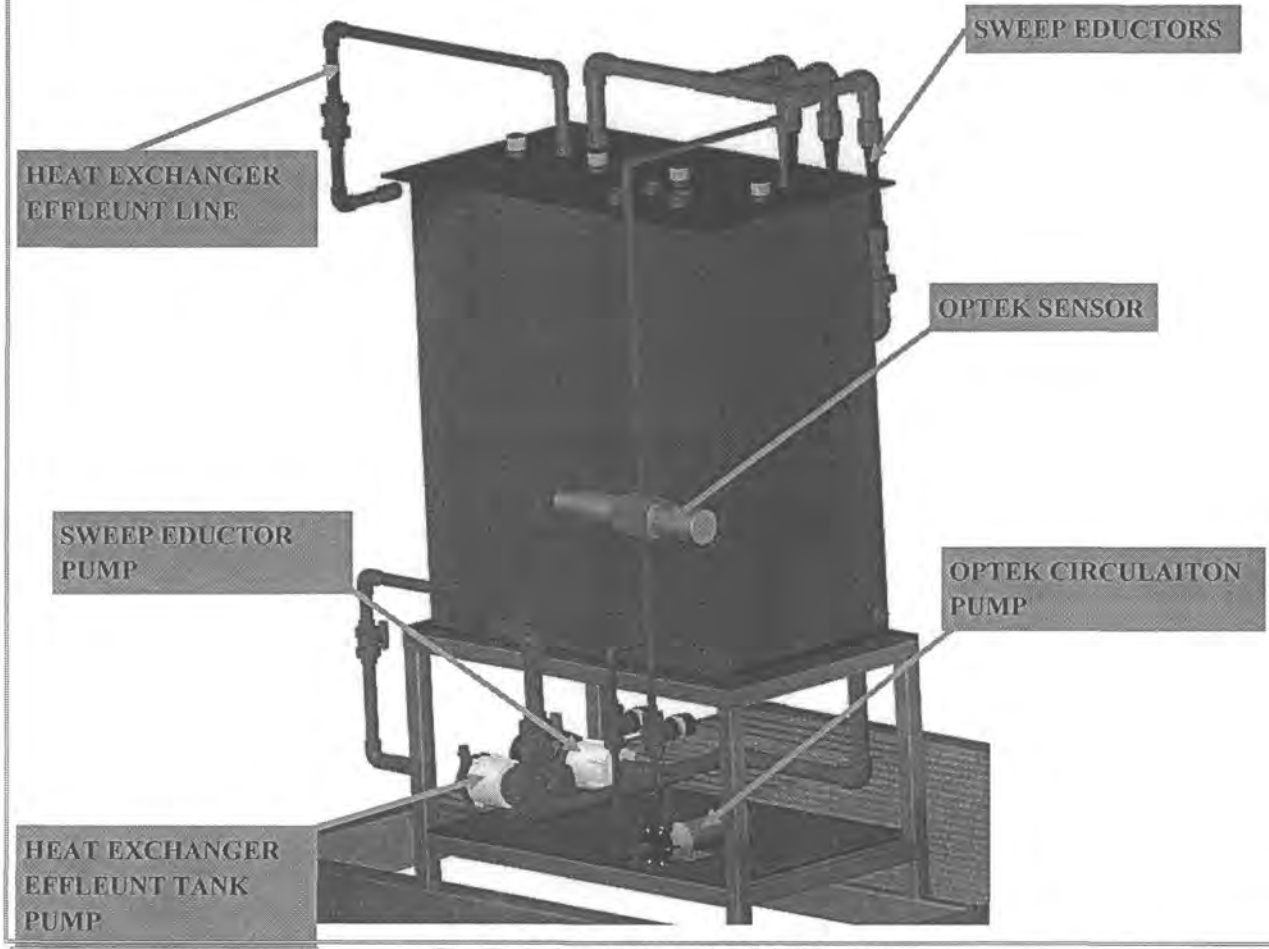
ANCILLARY SYSTEMS—These systems provide support for the PureMash system and are not directly related to the generation of chlorine dioxide nor the supply of it to the rail. These systems are the Heat Exchanger Effluent pump, Sweep Eductor system and the Optek loop. These systems are necessary to operate the PureMash system, provide for accurate dosing of the chlorine dioxide to the injection points and have alarms associated with them that can shut down the generation or delivery of chlorine dioxide.

HEAT EXHANGER EFFLUENT PUMP—Provides a method to remove the acid heat exchanger cooling water from the heat exchanger effluent tank and pump it to the cooling tower return line or any other area where the pressure is less than 10psi.

SWEEP EDUCTOR SYSTEM—This system is comprised of the Sweep Eductor Pump and three eductors . The pump provides motive water from the heat exchanger effluent tank to the three sweep eductors to remove chlorine dioxide vapors from the CLO2 Supply Tank. The vapors are normal due to agitation caused by the generation system and are absorbed in the heat exchanger effluent tank.

OPTEK LOOP—The loop contains the Optek Circulation Pump and the Optek Sensor and is provided as a method to accurately measure the concentration of chlorine dioxide in the CLO2 Supply Tank. The sensor provides a signal to the PLC and this is used to adjust the flow to each injection point as the concentration varies. The PLC will provide an indication if the concentration falls below 2200ppm, at which time the required dose will not be available. If the concentration falls below 1500ppm the Rail Supply Pump will shut down.



PROCESS DESIGN CRITERIA

System

To protect the electronics package in the control cabinet and the plastic components, the unit **MUST** be shielded from direct sunlight and located in a non-condensing environment.

Maximum Temperature: 104F (40C)

Minimum Temperature: 40F(4C)

Electrical Requirements:

Voltage:	460VAC 3PH
Frequency:	50/60 Hz
Amperage:	Approximately 30A
Protection:	Surge protection should be provided.

Streams To/From PureMash System:

Proper water and chemical streams are required for the unit to operate properly. It is important to minimize contaminants from entering the chemical feeds. Upsets such as decomposition can occur when contamination is allowed into the process.

All Chemicals

Maximum Temperature:	104F (40C)
Minimum Temperature:	40F (4C)
Pressure:	Flooded suction to chemical pumps

Acid Specification

Concentration:	98%-78% wt
Density:	1.7043-1.8437 s.g. (per selected concentration)
Max iron:	50ppm
Oxidizable Contaminants:	K number: <5
Insolubles:	5.0ppm maximum
Appearance:	Transparent liquid, no suspended matter
Color:	Colorless to light gray
Odor:	Odorless, free from foreign odor

System Supply Water

Maximum Particle Size:	~850 microns (20 mesh filter)
Temperature:	40F-80F (4C-27C)
Pressure:	40 –100psig. Regulated to 100-150psig (7-10 bar) @ system inlet
Flow Rate:	5-14gpm

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Acid Dilution Water

Quality:	De-ionized or demineralized like. Contaminants in the water could cause upsets in the generator. Care must be taken to use quality water.
Temperature:	Ambient
Pressure:	Regulated to <7psi (0.5 bar) at generator inlet
Color:	Colorless
Appearance:	No suspended matter

Cooling Water Supply

Flow Rate:	1-4 gpm
Pressure:	Greater than 10psig (0.7 barg). Regulated to 10psig (0.7 barg) in generator.
Temperature:	40F-85F (4C-30C)
Quality:	Conditioned to prevent scaling and fouling of heat exchanger.

Air Supply

Flow Rate:	20 SCFM
Pressure:	80 - 100 psig
Quality:	Free of moisture or oil.

CONFIDENTIAL

INSTALLATION AND SETUP

Location of System

The unit should be:

- Located near the proper power supply.
- Easily accessible for operating and maintenance procedures.
- Isolated from open flame and other ignition sources.
- Located on a surface floor that is neither wood nor a combustible material. The area must be clear of combustible materials.
- Located on a flat level surface with drainage to the proper systems suitable for chemical wash downs. Note: the doors may not properly close if not placed on a level surface.
- The unit **MUST** be protected from the elements like rain and direct sunlight.

Location of Chemical Storage

- All chemicals can be stored at ambient temperatures in the range of 40F-104F (4C-40C).
- For safety reasons, MashGuard One storage must be isolated from the acid storage.
- Chemicals should be elevated above the top of the graduated cylinder calibration devices to ensure proper flooding of the lines to make calibration of the unit possible.
- To prevent the possibility of spillage and a reaction between acid and MashGuard One, separate drains or separate containers should be used to catch any overflow from the calibration tubes.
- Proper installation of chemical storage in containment are required. Separate documentation is provided for these requirements. The chemicals must be located apart from one another and not share chemical containment space. The mixing of these chemicals outside the generator can be extremely dangerous.

Piping connections

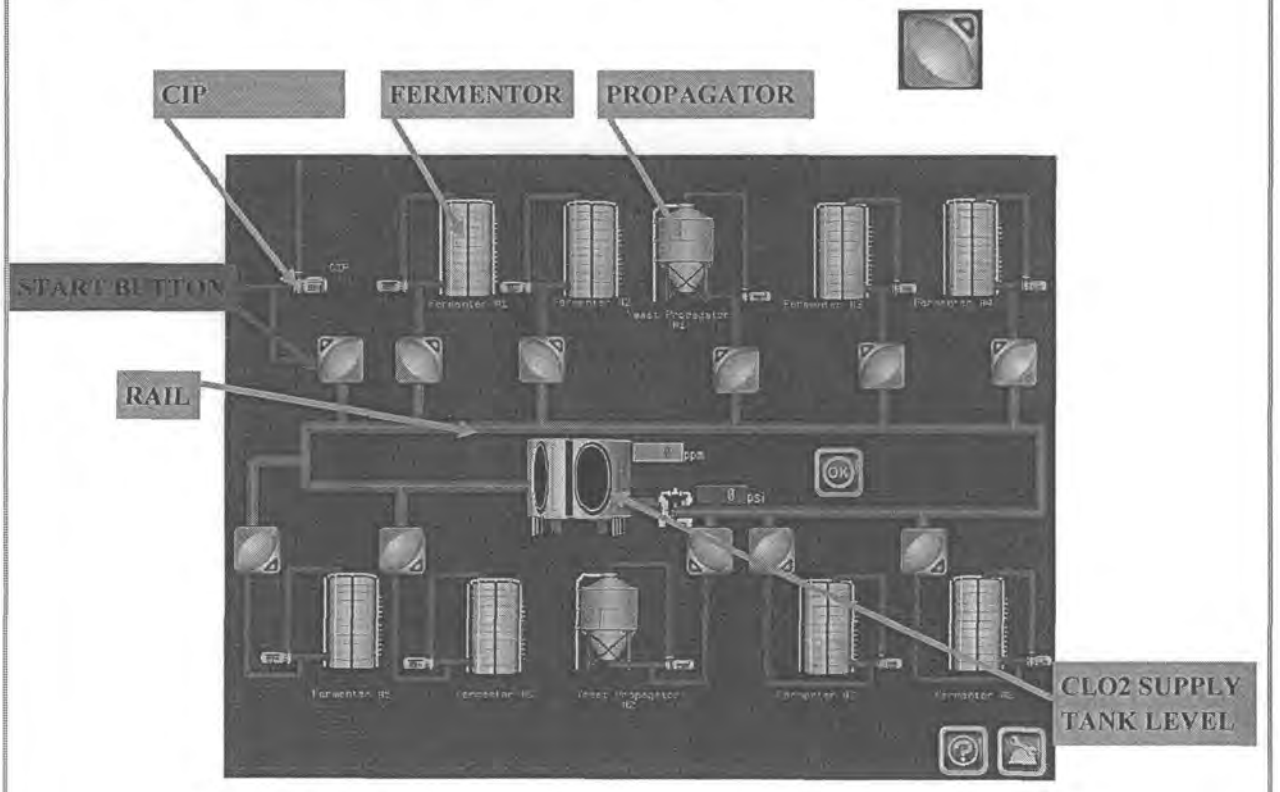
- System supply water — 1 inch PVC schedule 80 female socket
- Cooling supply water — 1 inch CPVC schedule 80 pipe.
- Heat exchanger tank effluent — 1 inch PVC schedule 80 female socket.
- Rail supply pump effluent — 1-1/2 inch PVC schedule 80 female socket.
- Sulfuric acid inlet — 1 inch CPVC schedule 80 pipe.
- MashGuard One inlet — 1 inch CPVC schedule 80 pipe.
- Rail supply pump air inlet — 1/2 inch FPT.

SYSTEM OPERATION

General

While the system is operating, it can be left running unattended for extended periods of time. There are safety features and interlocks built into the system to ensure that it automatically shuts itself down should certain parameters fall out of range. Turning on an injection point, checking chemical levels and routine visual inspections are the only normal activities performed by the operator. The PureMash system may be operated via the HMI on the skid or via the DCS if the PureMash unit is hooked into the facility's control system.

Injection Point Dosing — using the HMI to initiate dosing to an injection point is performed by using the main control panel as shown below. Each injection point is set up with a dosage and time setting via another screen (which will be explained later). The main operation screen shows the injection points (i.e. fermentors, propagators and CIP). Other injection points that are plant specific may also be shown. The screen also shows the level of chlorine dioxide in the CLO2 supply tank, the concentration of chlorine dioxide and the status of which injection points are on. *To initiate flow of chlorine dioxide to an injection point simply press the* **START BUTTON**.



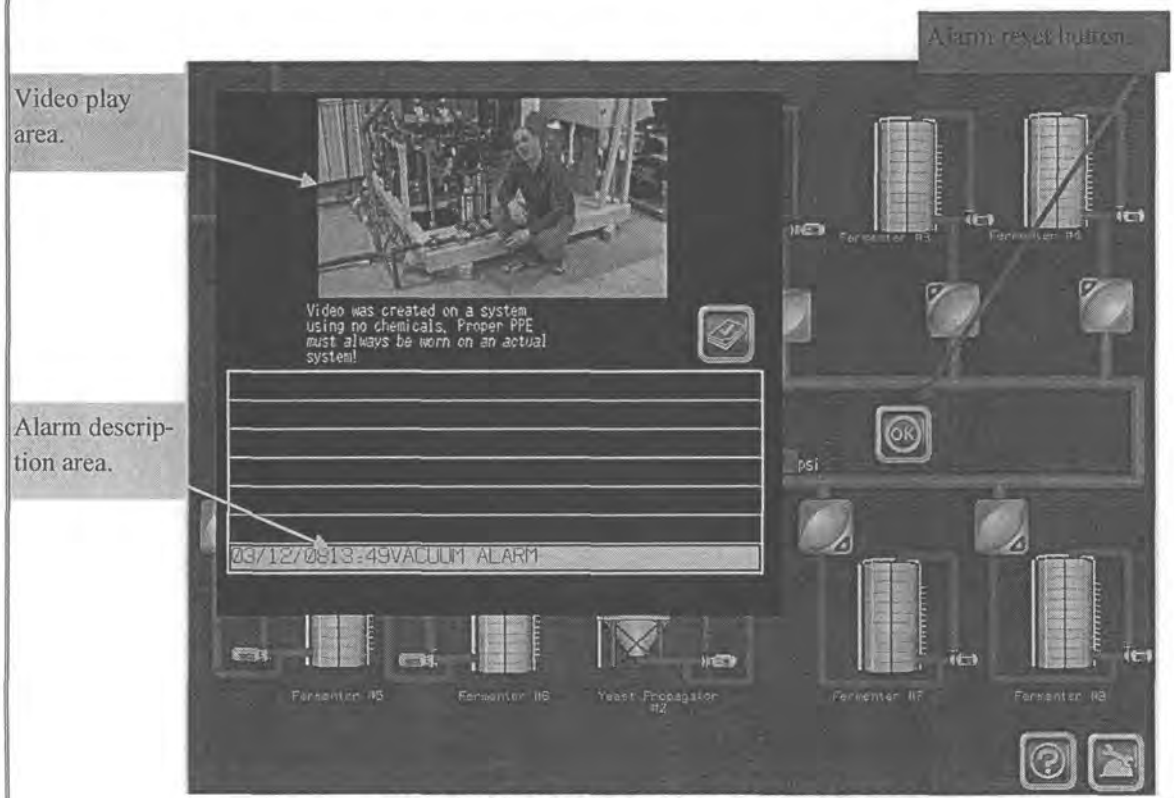
TYPICAL 100 MGY DRY GRIND OPERATIONAL SCREEN

At this point the pipe which is going to be actual injection point should highlight in green, indicating that the flow of chlorine dioxide is occurring. Typical reasons why chlorine dioxide may not be flowing are if (a) the manual stop button is pressed in, (b) there is an active alarm or (c) the set up has not been performed on the injection point.

ALARMS AND NOTIFICATIONS

Viewing Alarm Informational Video

The PureMash system incorporates a number of alarms and notifications to ensure the equipment operates safely without causing any hazardous situations which could cause injury to personnel, damage to the equipment or undesired treatment to the process. When an alarm occurs it is shown on the HMI by the following screen:



EXAMPLE SHOWN IS FOR A VACUUM ALARM

The PureMash system incorporates audio and video files to assist the user in diagnosing the problem and returning the machine to a normal run state as quickly as possible. A headphone jack is located on the front of the control panel to allow personnel to listen to the audio associated with the alarm.

CAUTION! IS RECOMMENDED THAT THE HEADPHONES BEING USED HAVE A VOLUME CONTROL KNOB ATTACHED TO THEM.

Upon touching the alarm description area the appropriate video is shown in the video player area. These videos show the most common problems associated with the alarm and how to repair them. Upon resolving the problem associated with the alarm press the alarm reset button  and the machine should return to its normal run status. The following pages show the alarms associated with the machine, their typical causes and what part of the system they disable.

Alarm Troubleshooting Guide

The alarm troubleshooting guide is designed to show the name of the alarm, the device causing the alarm, the area of the system that the alarm disables and a page number reference to a more descriptive area to guide in resolving the problem.

ALARM NAME	ASSOCIATED DEVICE	DISABLED AREA	DETAILED DESCRIPTION PAGE NUMBER
MANUAL STOP	Manual stop button	All systems	21
CLO2 AIR 1, CLO2 AIR 2, CLO2 AIR 3	Chlorine dioxide air monitor	All systems	21
LOW CLO2 CONC	Optek analyzer	Chlorine dioxide generator, rail supply pump	21
CLO2 TANK LVL LOW	CLO2 TANK level probes 1&2	Rail supply pump	21
ACID PMP PR SWITCH	Acid pump pressure switch	Chlorine dioxide generator	21
MASHGUARD PMP PR SWITCH	MashGuard pump pressure switch	Chlorine dioxide generator	21
HE PR SWITCH	Heat exchanger pressure switch	Chlorine dioxide generator	21
CLO2 TANK LVL HIGH	CLO2 TANK level probes 1&2	Chlorine dioxide generator	21
HE TANK LVL HIGH	HE TANK level probes 1&2	Chlorine dioxide generator	22
LOW MOTIVE PRESS	Motive water pressure transmitter	Chlorine dioxide generator	22
HE TANK LVL LOW	HE TANK level probes 1&2	Sweep eductor pump, heat exchanger effluent pump, chlorine dioxide generator	22
VACUUM ALARM	Vacuum transmitter	Chlorine dioxide generator	22
DECOMP ALARM	Vacuum transmitter	Chlorine dioxide generator	22
HEAT XCHGR FLOW ALARM	Heat exchanger flow sensor	Chlorine dioxide generator	22
ACID PUMP FAULT	Acid pump VFD	Chlorine dioxide generator	22

ALARM NAME	ASSOCIATED DEVICE	DISABLED AREA	DETAILED DESCRIPTION PAGE NUMBER
MG1 PUMP FAULT	MG1 pump VFD	Chlorine dioxide generator	23
STARTUP VACUUM ALARM	Vacuum transmitter	Chlorine dioxide generator	23
VACUUM ERROR	Vacuum transmitter	Chlorine dioxide generator	23

DETAILED ALARM DESCRIPTIONS

MANUAL STOP—The manual stop button is located on the front of the control panel. It is designed to allow a user to quickly stop the entire system in case of errant operation. This is a push-to-activate maintained button which has to be physically pulled out to deactivate. Upon pulling out the push button the machine should restart under normal operating conditions.

CLO2 AIR 1, CLO2 AIR 2, CLO2 AIR 3— These three alarms are associated with the chlorine dioxide air monitors and usually indicate that a leak has occurred somewhere in the system. Each air alarm is associated with an air monitoring zone inside of the plant. Depending on which zone is active, look for a chlorine dioxide leak within that zone. Once the leak has been found and repaired, reset the alarm and the machine should restart normally.

LOW CLO2 CONC—This alarm is triggered by the Optek analyzer when it senses a chlorine dioxide concentration that is too low in the CLO2 supply tank. This is generally caused by improper calibration of one of the chemical feed pumps in the chlorine dioxide generator. This alarm can also be caused if one of the precursor chemicals is not available. Ensure that both chemical precursor tanks are at adequate levels and perform calibration procedures on the chemical feed pumps as well as the acid dilution water pump.

CLO2 TANK LVL LOW— One or both of the ultrasonic tank level probes has indicated to the PLC that the level in the chlorine dioxide tank is low. This is generally due to the chlorine dioxide generator not operating properly. Check for any active alarms on the chlorine dioxide generator and, if all is working properly, check the connections on the ultrasonic level sensors to the PLC.

ACID PMP PR SWITCH— On the discharge side of the acid pump in the chlorine dioxide generator is a pressure switch which is usually set to 100 psi. If this pressure has been exceeded, it is an indication that there is blockage downstream of the pressure switch which could cause pump damage. Inspect the check valve in the reactor column insert to ensure that no blockage exists. If that does not correct the problem, check the wiring from the pressure switch back to the PLC.

MASHGUARD PMP PR SWITCH— On the discharge side of the MashGuard One pump in the chlorine dioxide generator is a pressure switch which is usually set to 100 psi. If this pressure has been exceeded, it is an indication that there is blockage downstream of the pressure switch which could cause pump damage. Inspect the check valve in the reactor column insert to ensure that no blockage exists. If that does not correct the problem, check the wiring from the pressure switch back to the PLC.

HE PR SWITCH— There is a pressure switch located on the back bottom left-hand corner of the chlorine dioxide generator behind the acid heat exchanger which monitors the pressure on the discharge of the heat exchanger. This switch is set to 15 psi, and is designed to protect the heat exchanger from over pressurization. Check the discharge side of the heat exchanger to the reaction column for any blockage especially at the check valve. If no blockages are found, try to restart the unit. If the alarm occurs again, check all wiring from the pressure switch to the PLC.

CLO2 TANK LVL HIGH— One or both of the ultrasonic tank level probes has indicated to the PLC that the level in the chlorine dioxide tank is high. This is a very unlikely alarm and would be an indication that tank level probe number one did not send a stop command to the PLC. Restart the unit and monitor to make sure that the chlorine dioxide generator turns off when the level in the CLO2 tank reaches the stop position.

HE TANK LVL HIGH— One or both of the ultrasonic tank level probes has indicated to the PLC that the level in the heat exchanger effluent tank is high. This could be due to the heat exchanger effluent pump not operating properly or a valve on the discharge side of the pump being closed. Other factors that could cause this would be that the cooling water flow through the heat exchanger is at a higher rate than the heat exchanger effluent pump is taking out of the tank. The cooling water flow through the heat exchanger should be 1-3 gpm. If it is higher than this, reduce the flow with the heat exchanger cooling water control valve.

LOW MOTIVE PRESS— The motive water pressure sensor has indicated to the PLC that the pressure of the motive water is less than 100 psi. Check the pressure of the system supply water and ensure that it is at least 40 psi. Ensure that the booster pump is running when the chlorine dioxide generator is on and that the booster pump is not air bound. Visual verification of the motive pressure is available on the pressure gauge mounted on the inlet side of the eductor in the chlorine dioxide generator. If the gauge is reading between 100 — 140 psi, then the motive water pressure transmitter may be faulty.

HE TANK LVL LOW—The ultrasonic level probes mounted on a heat exchanger effluent tank have indicated to the PLC that the tank level is low. This alarm condition will shut down the sweep eductor pump and the heat exchanger tank effluent pump. This alarm would generally be an indication that ultrasonic level probe #1 did not send the correct signal to the PLC to turn off the heat exchanger effluent pump.

VACUUM ALARM—This alarm is generated if the vacuum in a reaction column is less than –3 inHg. Common reasons for this alarm are lack of motive flow, low motive pressure, high acid strength, impurities in acid, high acid temperature, clogged eductor, high discharge backpressure, or leaks on the system. Motive flow should be between 11 and 13 gallons a minute at 100 — 140 psig. Acid temperature should be below 120F as verified by the acid temperature gauge at the bottom of the acid heat exchanger. Discharge backpressure should always be low unless the check valve is stuck, which would cause an obstruction on the discharge of the chlorine dioxide generator. Verify all bolts are tight on the reaction column and that all tubing fittings are tight on the reaction column insert. Perform a calibration on the acid in dilution water pumps to ensure that they are set correctly which will provide 78% acid to the reaction column. Visually inspect the acid to ensure that it is clear in color with no floating debris.

DECOMP ALARM—The decomposition alarm is related to the vacuum transmitter, but unlike a vacuum alarm it is based on a percentage change of the vacuum over time. This is generally caused by high strength acid or high temperature acid. Verify the acid temperature by using the gauge located at the bottom of the acid heat exchanger to ensure that the acid temperature is below 120F. Perform a calibration on the acid and dilution water pumps to ensure they are set accordingly to provide 78% acid to the reaction column. If neither of these conditions are causing the problem, then the most likely cause is poor acid quality.

HEAT XCHGR FLOW ALARM—The heat exchanger cooling water flow sensor will provide an alarm signal to the PLC if the water flow is less than 0.5 GPM. Check that all valves on the cooling water system are open and that cooling water is available to the system.

ACID PUMP FAULT—The acid pump is controlled by a VFD in the control panel which will provide an alarm signal to the PLC in the event of a VFD fault.

CAUTION! ONLY QUALIFIED PERSONNEL TRAINED IN THE TROUBLESHOOTING OF ELECTRICAL GEAR SHOULD TRY TO RESOLVE THIS PROBLEM.

The control panel must be open while the panel is energized in order to read the full code on the VFD. Please consult the VFD OEM manual to resolve this problem.

MG1 PUMP FAULT—The MashGuard One pump is controlled by a VFD in the control panel which will provide an alarm signal to the PLC in the event of a VFD fault.

CAUTION! ONLY QUALIFIED PERSONNEL TRAINED IN THE TROUBLESHOOTING OF ELECTRICAL GEAR SHOULD TRY TO RESOLVE THIS PROBLEM.

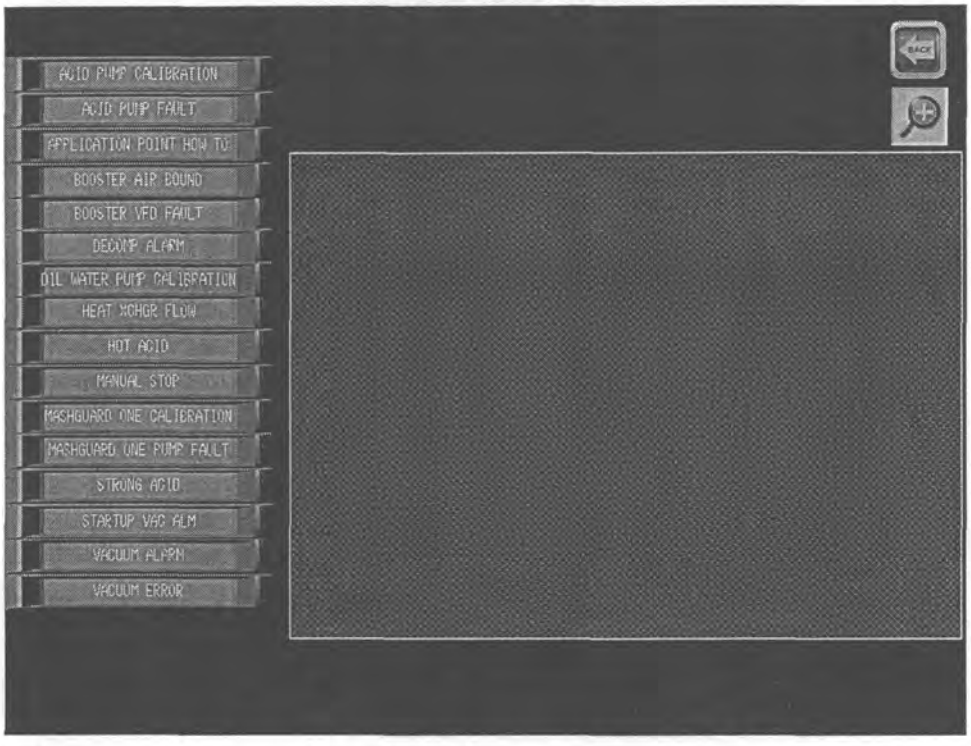
STARTUP VACUUM ALARM—This alarm also occurs while the chlorine dioxide generator is trying to start up and the vacuum is less than –3inHg. Check for proper motive flow, leaks in the system and ensure that the reactor drain valve is CLOSED.

VACUUM ERROR— This is an indication that the vacuum transmitter is not supplying a signal to the PLC. On the chlorine dioxide generator information page on the HMI the vacuum should be reading –38inHg, which indicates loss of signal. Ensure that the connection between a vacuum transmitter and the PLC is not broken and ensure that the vacuum transmitter is operating normally.

HMI SCREENS

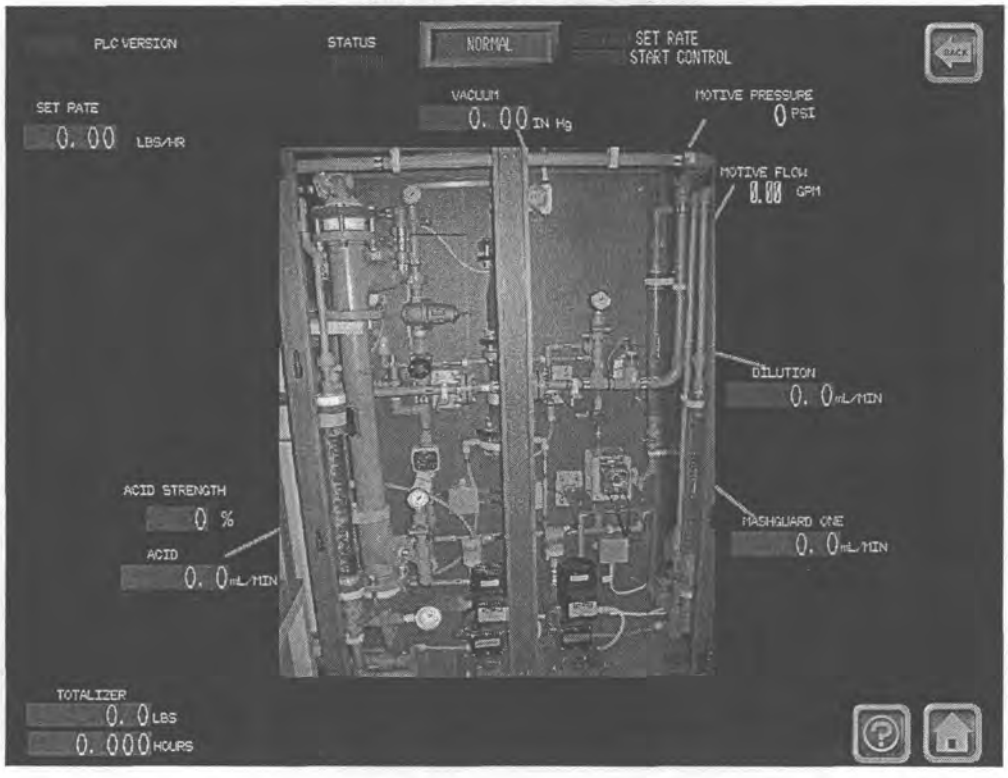
Some additional screens on the HMI that may be useful are the Video Information Screen and the Generator Information Screen.

Video Information Screen



This screen provides access to videos showing how to troubleshoot alarms and perform calibrations on the unit. Utilizing the headphone jack located on the front of the control panel will allow the user to listen to the audio provided with the videos.

Generator Information Screen



This screen provides information about the chlorine dioxide generator such as the motive flow, the vacuum, PLC version, set rate, runtime hours, total pounds produced and motive pressure. Also displayed is the volumetric data for the precursor pumps to serve as a guide for performing calibrations on the pumps.

APPENDIX A

P&ID

MECHANICAL DRAWINGS

ELECTRICAL DRAWINGS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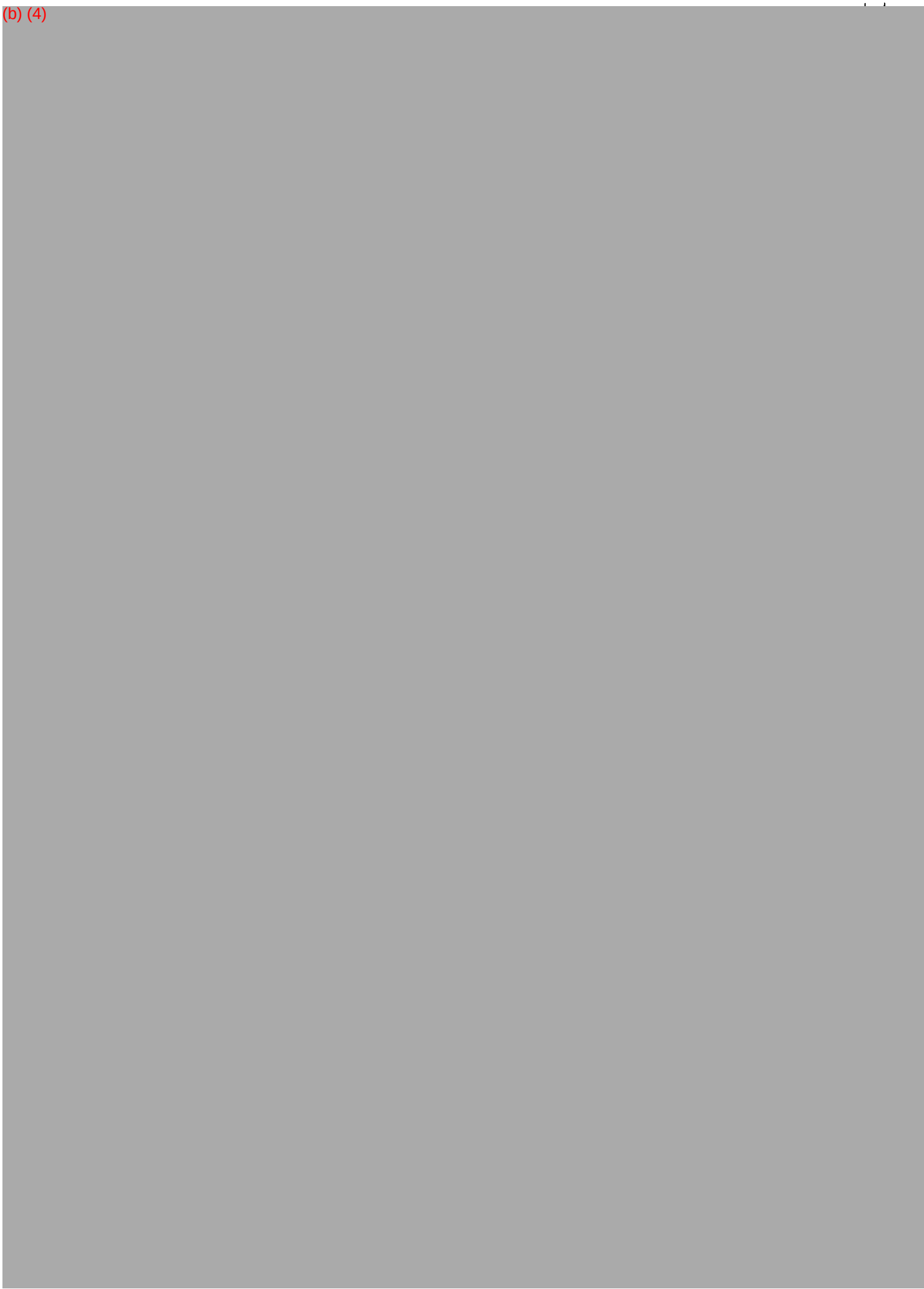

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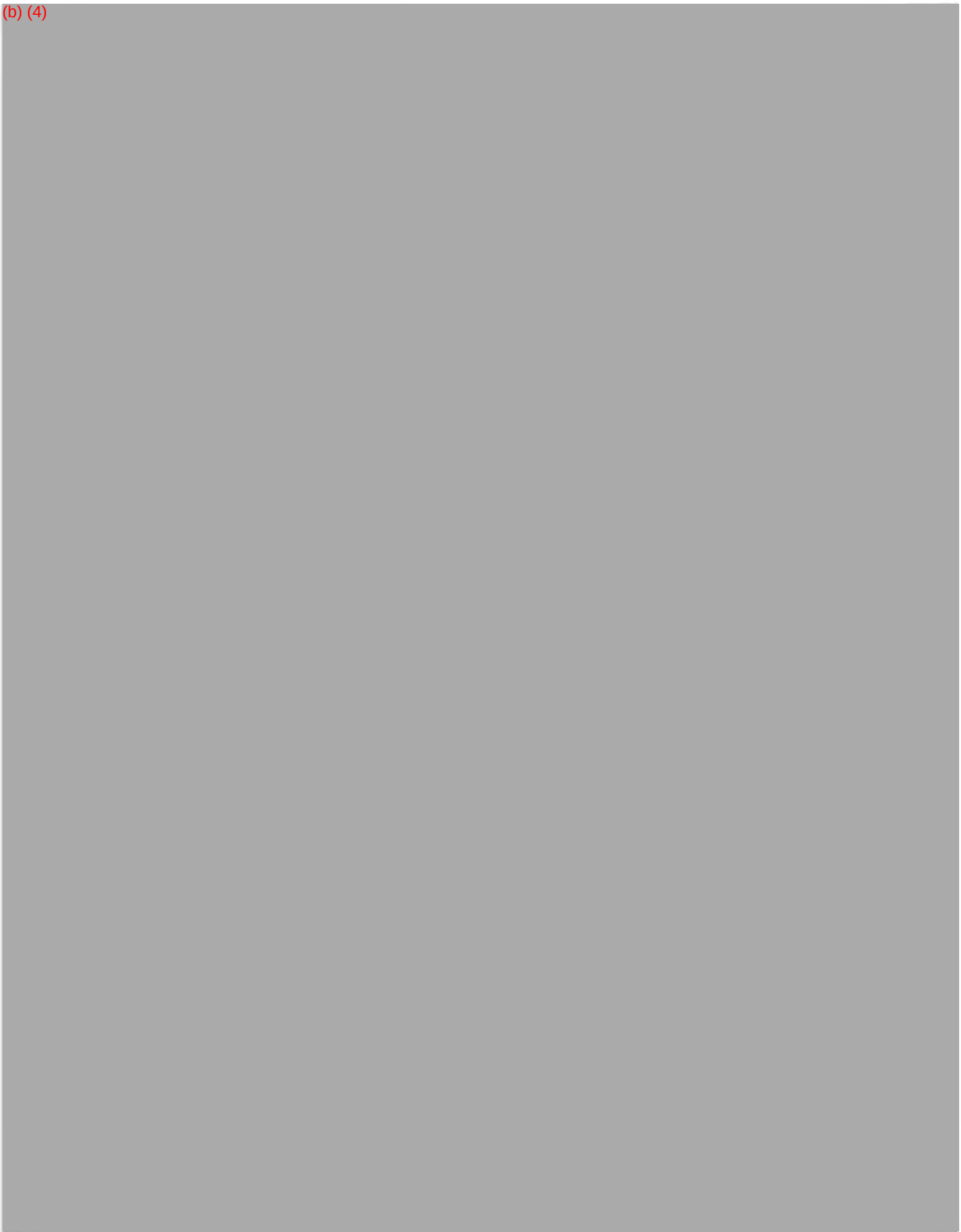

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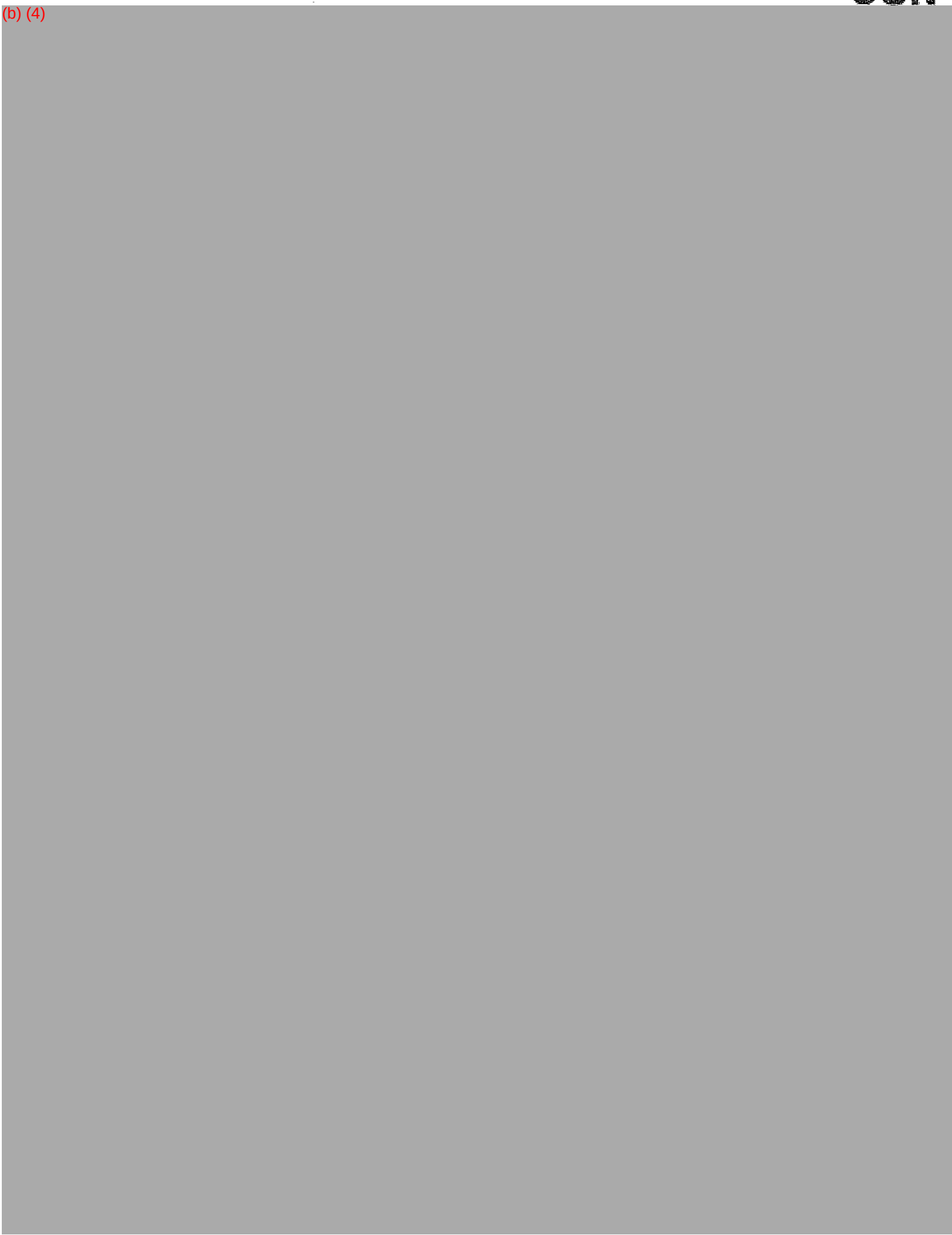

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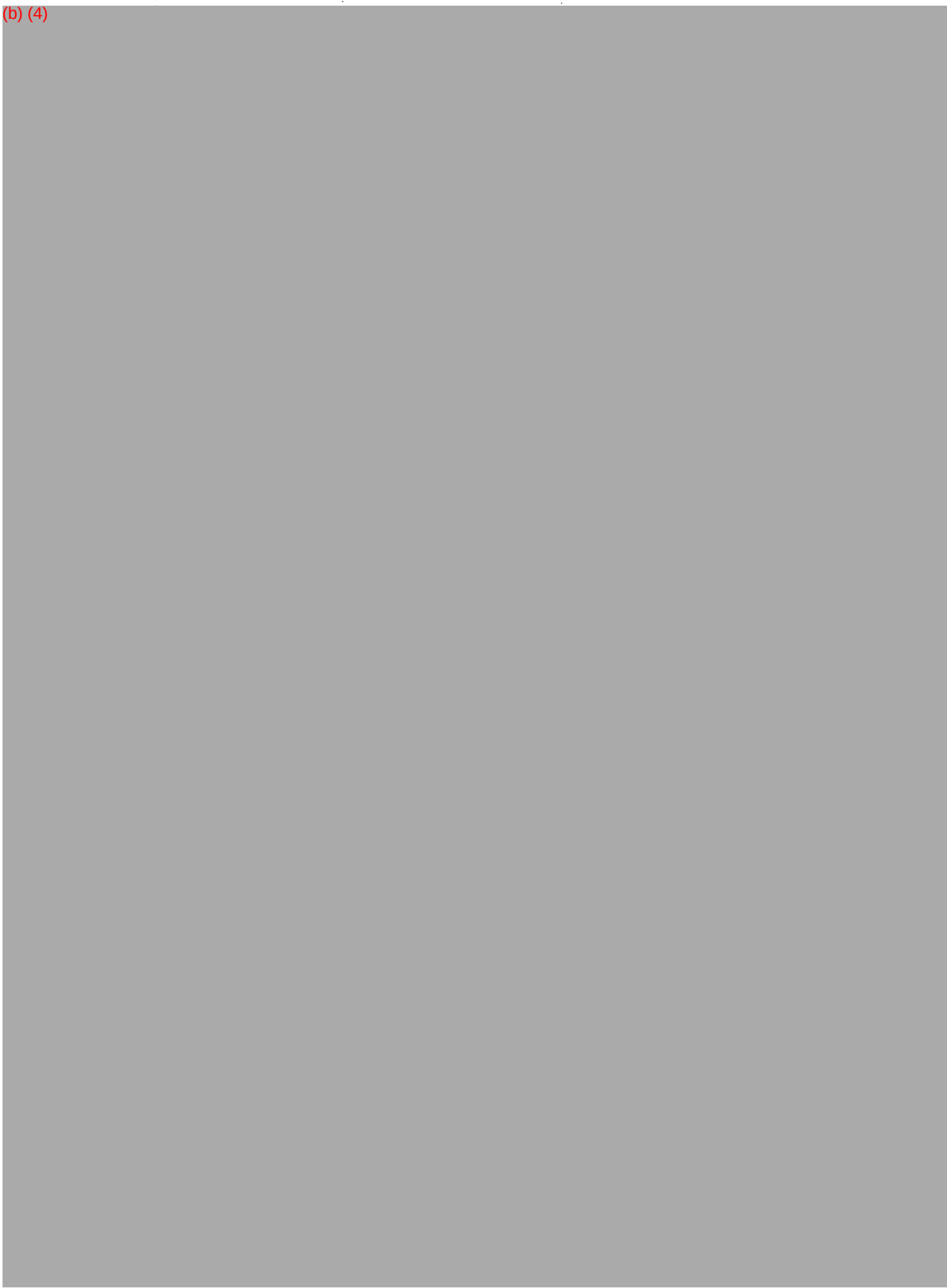

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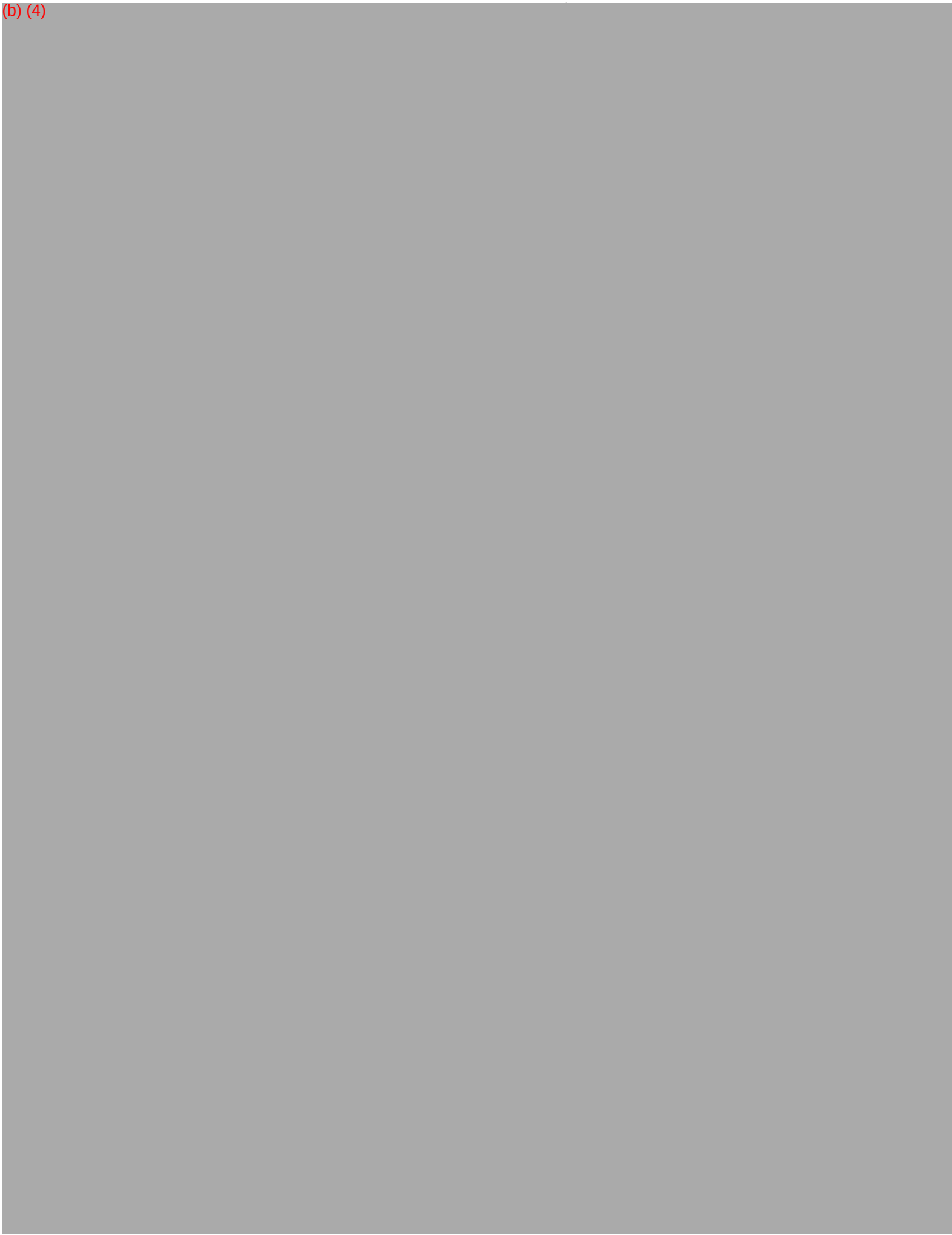

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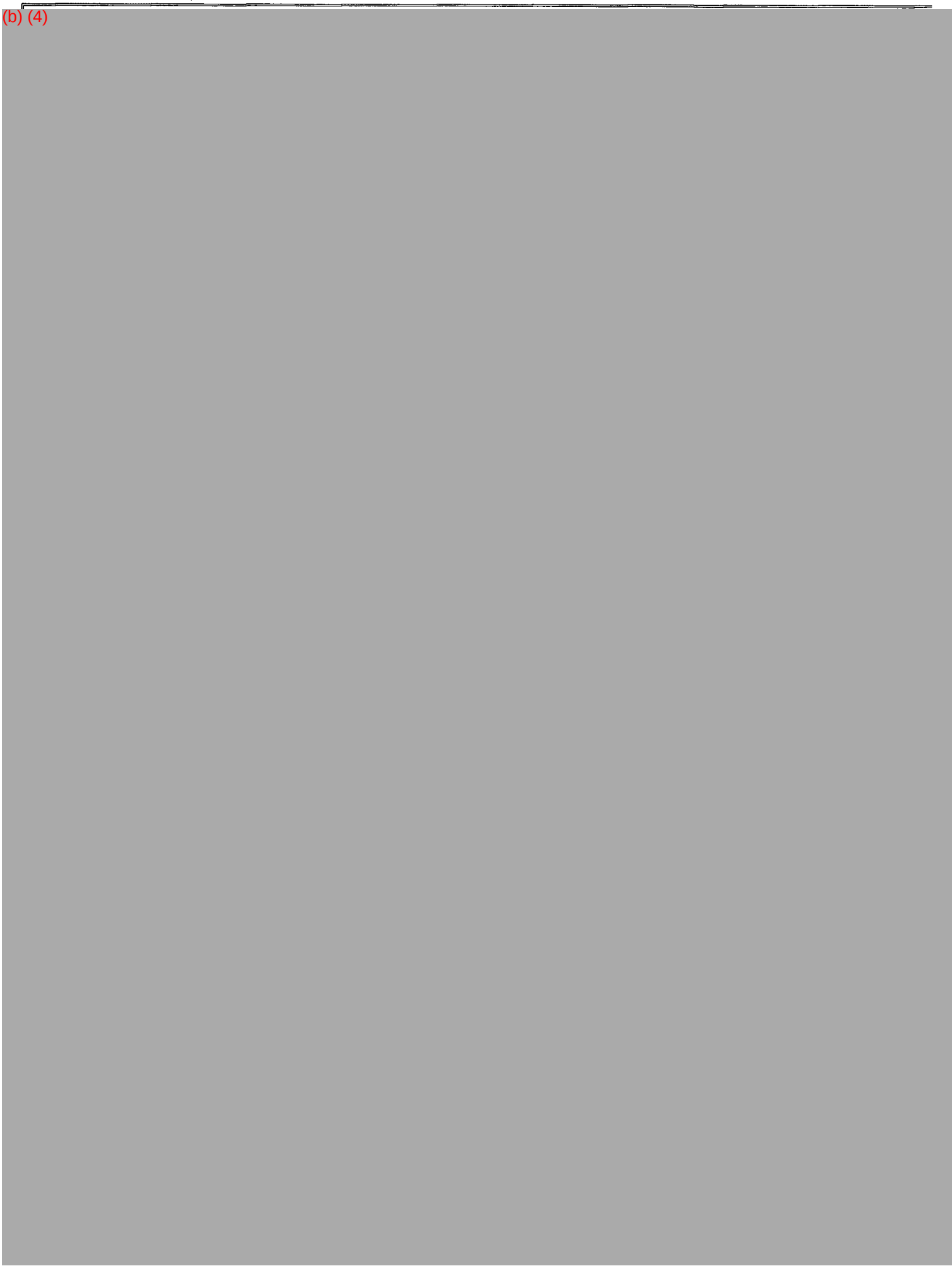

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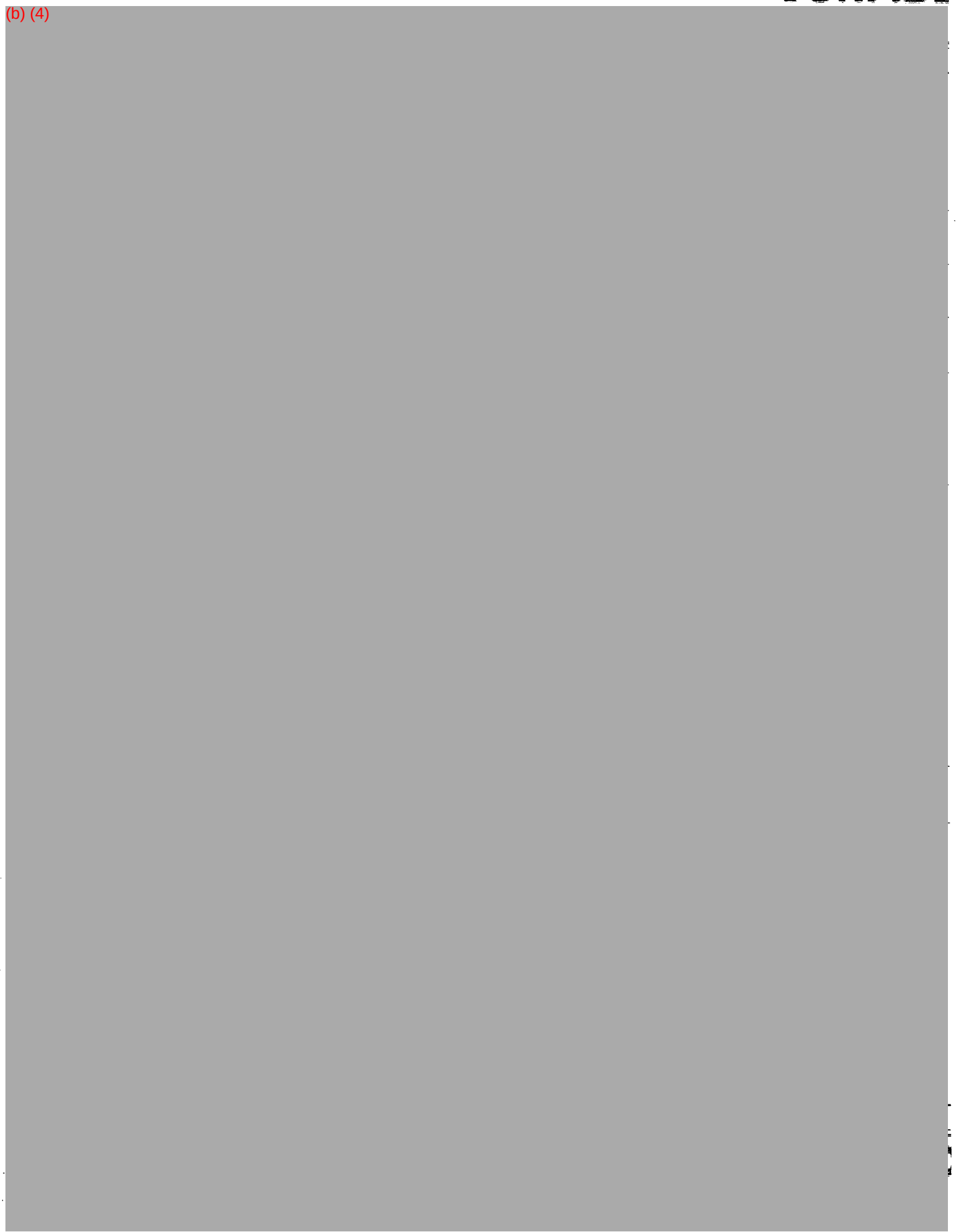

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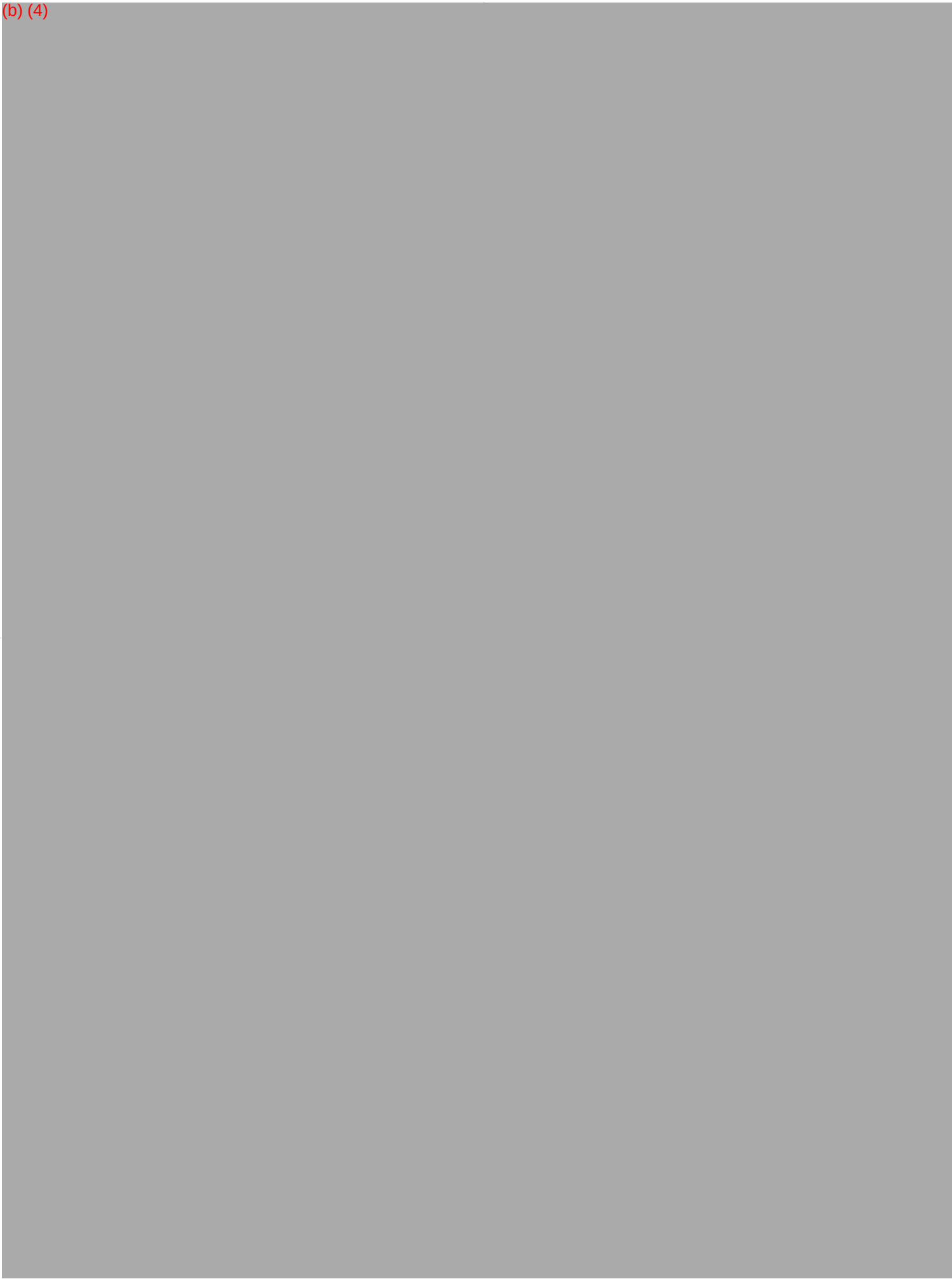

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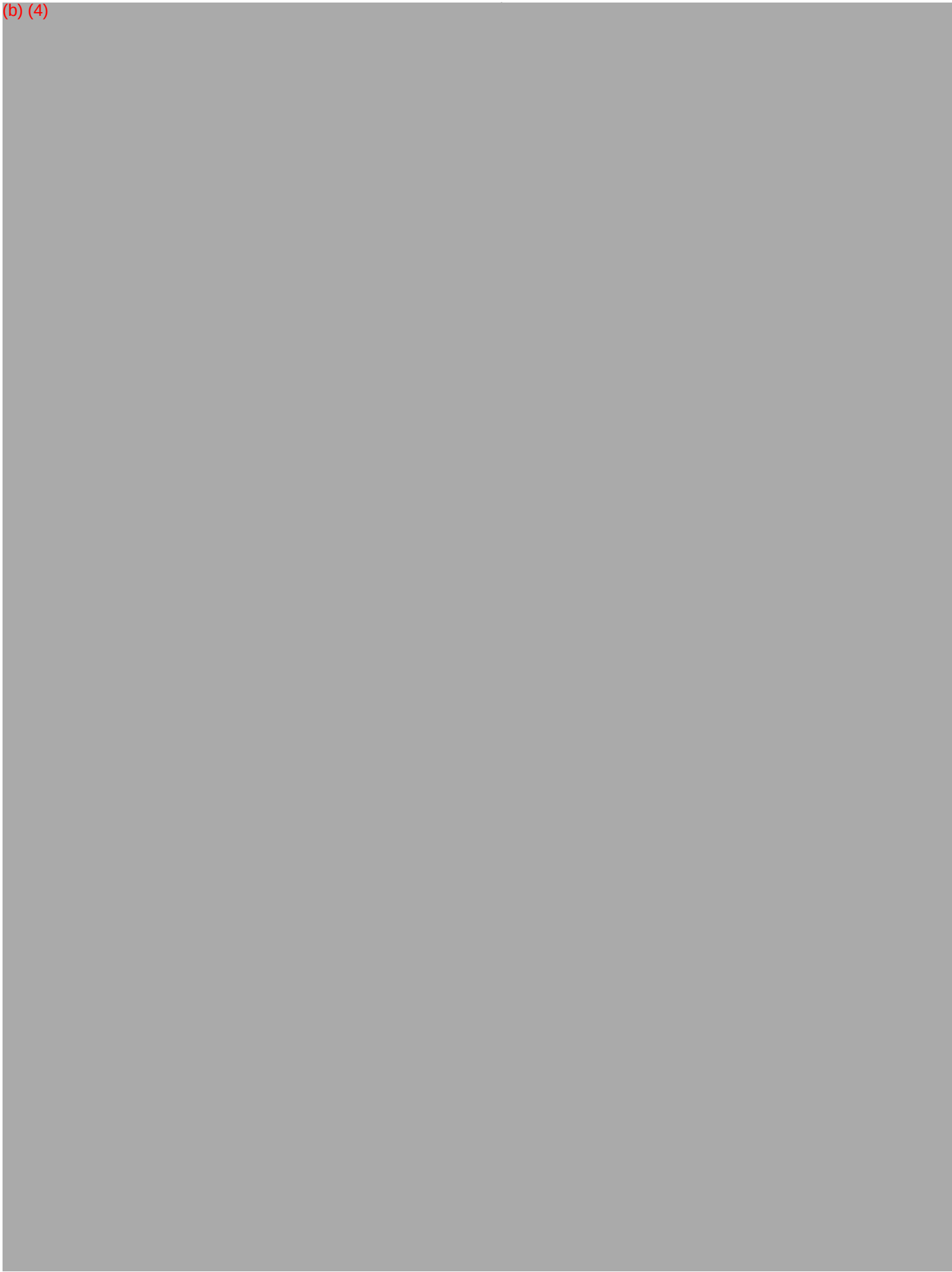

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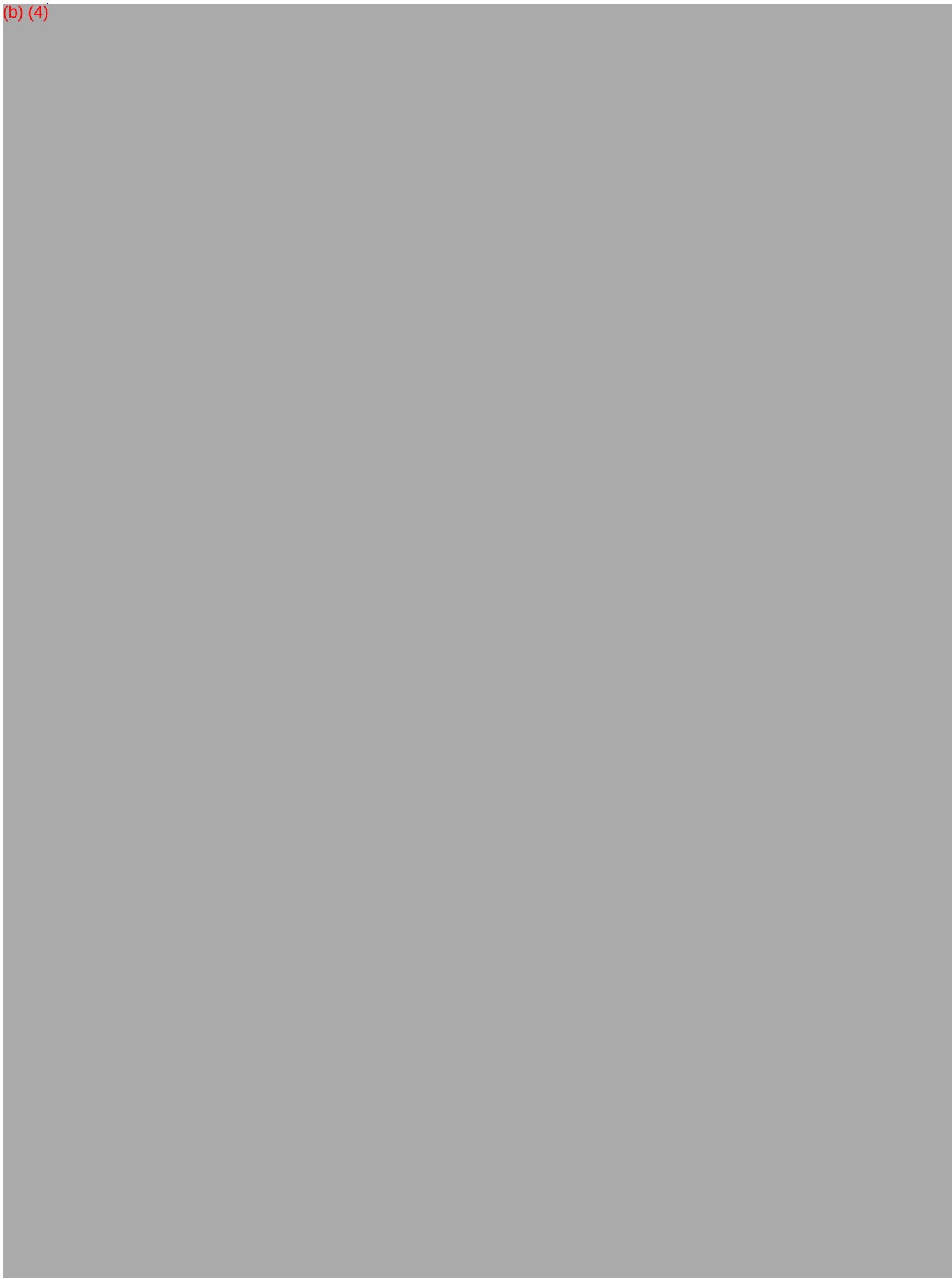

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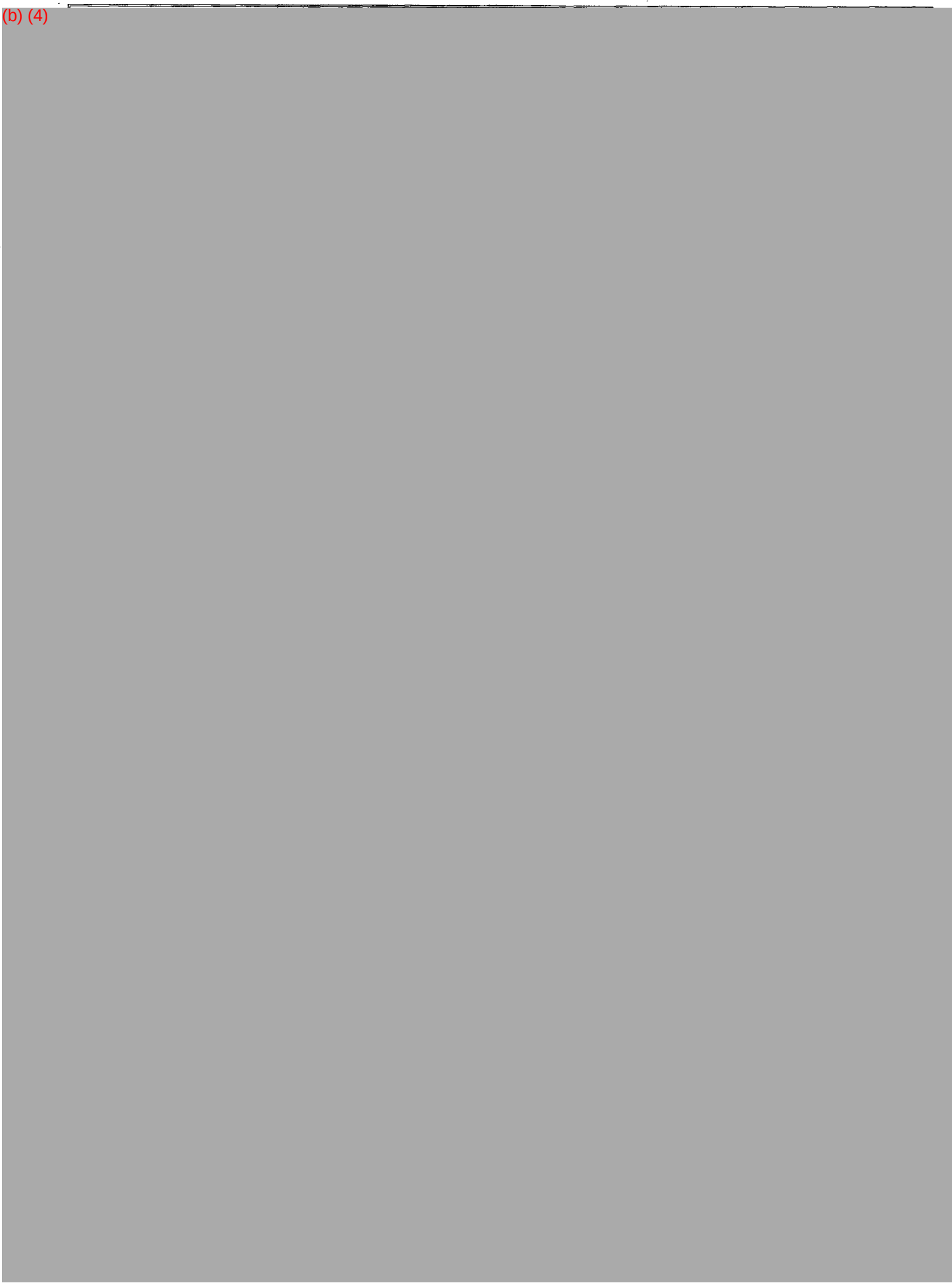

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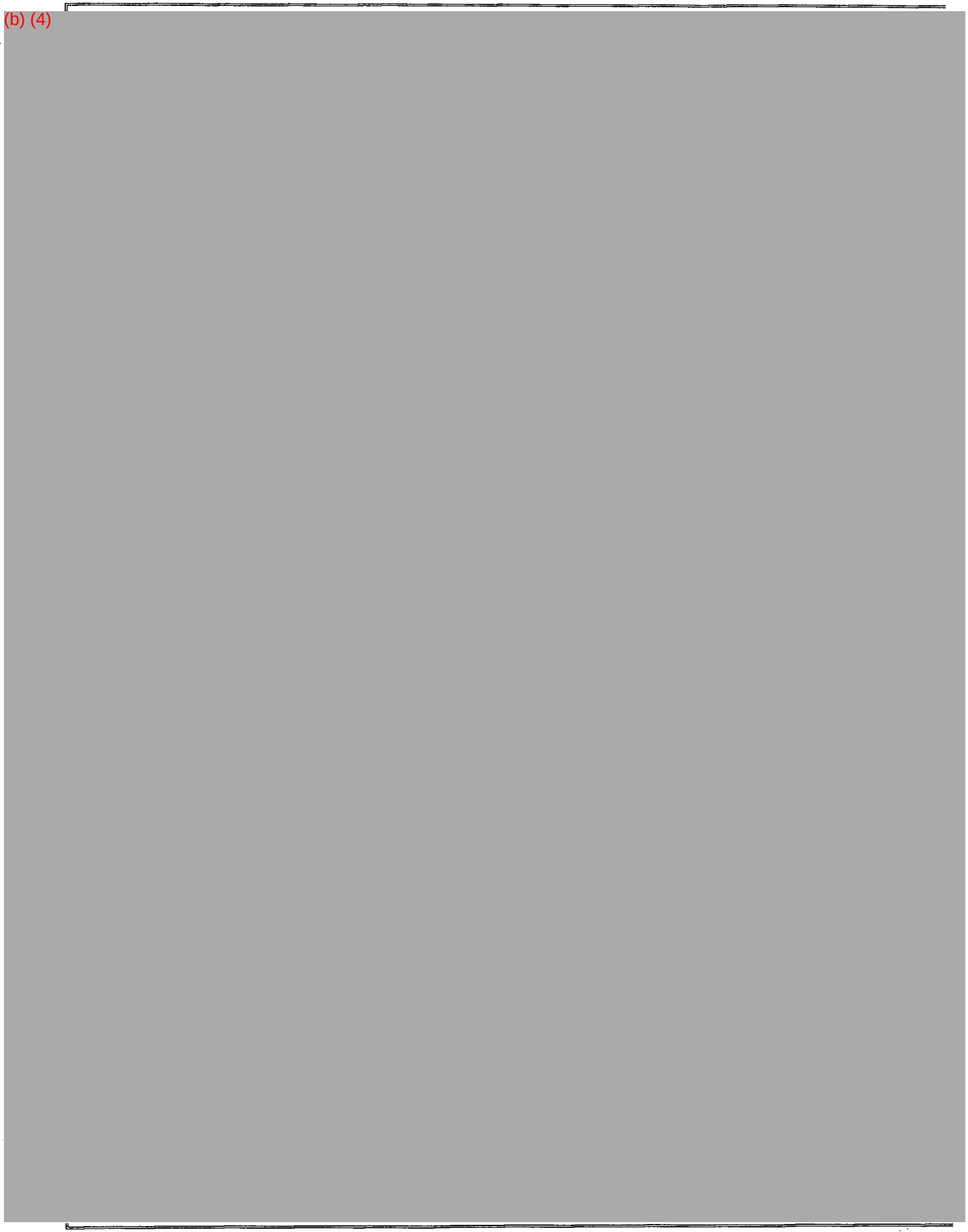

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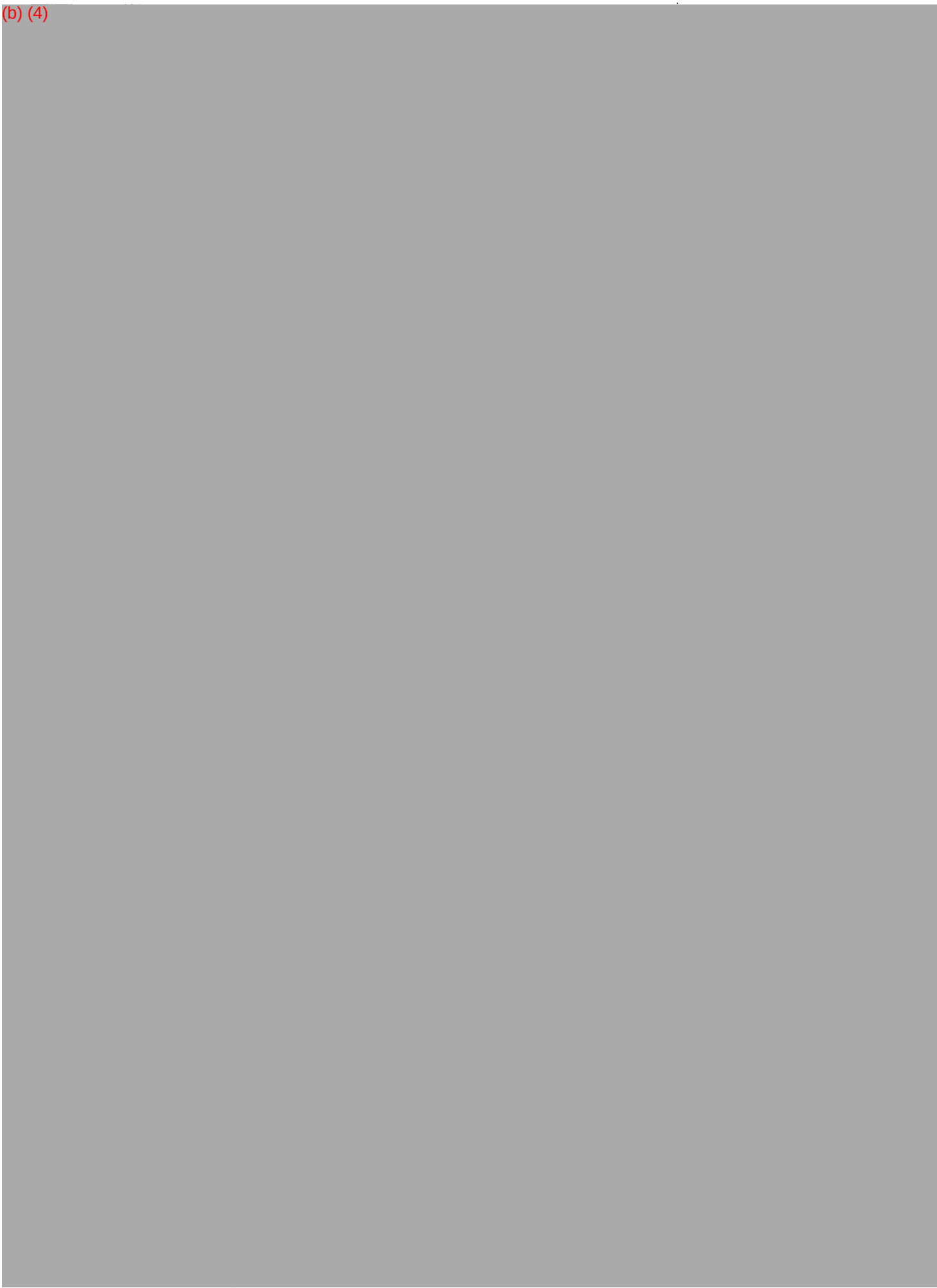


APPENDIX B

MATERIAL SAFETY DATA SHEETS

MASHGUARD ONE

CHLORINE DIOXIDE

RBS Resonant BioSciences

MashGuard One® Mg

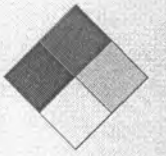
Material Safety Data Sheet

1.0 Chemical Product and Company Identification

Resonant BioSciences, LLC
1400 16th St., Ste. 400
Denver, CO 80202
Toll Free: 866.933.0408
Fax: 303.933.3594
www.puremash.com

Product name: ***MashGuard One®***

Emergency Phone
888.299.3899
CHEMTREC 800.424.9300
CHEMTREC International 1.703.527.3887



Chemical Type
Sodium chlorate and *hydrogen peroxide* as a stabilized aqueous solution.

Intended Use
Reagent feed for PureMash® *chlorine dioxide* generation

2.0 Hazards Identification

Emergency Overview	A clear, faintly blue colored, faintly odored solution which may cause moderate skin irritation and severe irritation of eyes and mucous membranes, including possible blindness. *Sodium chlorate* is odorless and very soluble in water. *Sodium chlorate* is not listed as a possible carcinogenic by OSHA, IARC or NTP.
Routes of Exposure	Inhalation, skin, and ingestion
Potential Health Effects	
Ingestion	Irritation of the gastrointestinal tract, abdominal pain, gas evolution, and red blood cell destruction
Skin	May cause moderate skin irritation
Eye	May cause severe eye irritation, tearing and blurring of vision, with irreversible corneal damage, and possible blindness in instances of overexposure
Inhalation	May cause irritation of the upper respiratory passages; nausea, headache, or weakness
Target organs	Skin, eyes, mucous membranes, and renal system
Chronic effects	No information
Medical conditions aggravated by exposure	None documented

3.0 Composition / Information on Ingredients

Component	CAS #	% Wt / Wt
ogen peroxide	7722-84-1	< 8%
ACGIH - Threshold limits values – Time weighted Averages (TLV–TWA)	1 ppm TWA	
Sodium chlorate	7775-09-9	40%

Ingredient information: Exposure limits not established for *sodium chlorate* solution

4.0 First Aid Measures

Ingestion

If victim is conscious, give plenty of water to dilute stomach contents. Do not induce vomiting without medical advice. Seek immediate medical attention.

Skin

Wash off immediately with plenty of water for at least 15 minutes. Rinse contaminated clothing with water and launder all clothing prior to use. Call a poison control center or doctor for treatment advice.

Eye

Immediately flush eyes thoroughly with water for at least 15 minutes. Obtain medical attention if irritation persists. Remove contact lenses, if present, after the first five minutes, then continue rinsing eyes. Call a poison control center or doctor for treatment advice.

Inhalation

Remove to well-ventilated area. If necessary, give artificial resuscitation and seek medical attention.

Notes to physician

Sodium chlorate poisoning is rare, but is associated with a high mortality rate with death generally occurring from massive intravascular hemolysis, and acute renal failure. *Sodium thiosulfate* (2 to 5 gm in 200 ml of 5% *sodium bicarbonate*) is a specific antidote that can be given orally or by I.V. DO NOT treat with methylene blue because of risk of methemoglobinemia. *Sodium chlorate* is freely dialyzable, and early treatment by peritoneal or hemodialysis is recommended. Direct contact of *hydrogen peroxide* with the eye is likely to cause corneal damage, especially if not washed away immediately. Careful ophthalmologic evaluation is recommended. Attempts at evacuating the stomach via emesis induction or gastric lavage should be avoided. In the event of severe distention of the stomach or esophagus due to gas formation, insertion of a gastric tube may be required.

5.0 Firefighting Measures

Flammable properties

Non-flammable liquid

Extinguishing Media

Suitable extinguishing media

USE WATER ONLY

Unsuitable extinguishing media

If allowed to evaporate, solid *sodium chlorate* could be formed. Solid *sodium chlorate* does not burn, but if exposed to fire, it decomposes to give off oxygen which feeds the fire. Consequently, ONLY WATER is effective in cooling and diluting solid *sodium chlorate*. DO NOT USE CO_2, Halon, dry chemical or powder fire extinguishers, or fire blankets in the event solid *sodium chlorate* is involved as these are totally ineffective, and may confine the heat and create a worse situation.

continued on next page

5.0 Firefighting Measures ...*continued*

tection of Firefighters

Protective equipment for firefighters	Avoid all bodily contact. Wear self-contained breathing apparatus, pressure demand, MSHA / NIOSH-approved and full protective gear. Do not allow clothing, shoes, or gloves to become impregnated with *sodium chlorate* in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of *sodium chlorate* and *hydrogen peroxide* solution with plenty of water.
Specific hazards arising From the chemical	DO NOT allow solution to come in contact with any combustible materials. Paper, wood, cloth, and leather impregnated with *sodium chlorate* solution are highly combustible if allowed to dry, and may be ignited by friction or heat. DO NOT allow the temperature of the storage container to rise above 100° F (38° C).

6.0 Accidental Release Measures

Personal precautions	Protective suit of vinyl, neoprene, PVC or polyethylene; impervious rubber shoes or boots of vinyl or neoprene; safety glasses with side shields or chemical goggles, and hard hat with full-face shield when appropriate; rubber gloves of vinyl or neoprene. Isolate area. Keep unnecessary personnel away.
Environmental precautions	DO NOT ALLOW RELEASES TO ACIDIC DRAINS AS *CHLORINE DIOXIDE* GAS CAN BE LIBERATED. Contain runoff, and contact appropriate local spill response personnel. Do not allow escape into sewers, drains, or natural watercourses. Waste disposal in approved chemical disposal area or in a manner which complies with all local, state, and federal regulations.
Methods for containment	Block any potential routes to water systems. Contain spill using noncombustible material, such as vermiculite, sand, or earth.
Methods for clean-up	Local authorities should be advised if significant spillages cannot be contained.

7.0 Handling and Storage

Handling procedures	Prevent possible eye and skin contact by wearing protective clothing and equipment. AVOID PRODUCT CONTACT WITH ACIDIC MEDIA WHICH CAN LIBERATE *CHLORINE DIOXIDE* GAS.
Storage procedures	Store in properly vented containers or tanks. Do not block vent. Do not store where contact with incompatible materials could occur, even with a spill. Have a clean water source available for dilution. Keep storage containers out of direct sunlight, and away from heat, sparks and flames. DO NOT add any other product to storage container. Never return unused product to storage container.

8.0 Exposure Controls / Personal Protection

ɔsure guidelines	No TLVs have been established for this mixture. The PEL for *hydrogen peroxide* is 1 ppm. The PEL for *sodium chlorate* is: total dust = 15 mg / m^3; respirable fraction = 5 mg / m^3.
Engineering controls	Use site specific diking / spill control to avoid uncontrolled releases. Eyewash facility, emergency shower, or jump tank should be in close proximity.

Personal Protective Equipment

Eye / Face	Wear safety glasses with side shields or chemical goggles. Where appropriate, wear a full-face shield. Contact lenses should not be worn when handling this product.
Skin	Use impervious clothing to avoid skin contact. Avoid all bodily contact. Wear self-contained breathing apparatus, and appropriate protective equipment. Do not allow clothing, shoes, or gloves to become impregnated with *sodium chlorate* in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of *sodium chlorate* and *hydrogen peroxide* solution with plenty of water.
Respiratory	Not applicable under normal conditions of use. For vapor or mist concentration in excess of 10 ppm, a self-contained breathing apparatus should be used. DO NOT USE OXIDIZABLE SORBANTS.
Hygiene measures	Do not wear leather gloves

9.0 Physical and Chemical

ɜarance

Form	Aqueous solution
Color	Faint blue to colorless
Odor	Faint
Odor threshold	Not available
Physical state	Liquid
pH	1.7
Melting point	Not applicable
Freezing point	Not available
Boiling point	104° C
Flash point	Not available
Evaporation rate	> 1 (butyl acetate = 1)
Flammability	Not available
Upper / lower flammability	Not available

Vapor pressure	< 0.1 KPa at 40° C and at 80° C
Vapor density	Not available
Specific gravity	1.37
Solubility (H_2O)	Not applicable
Coefficient of water / oil distribution	Not available
Octanol / H_2O coeff	Not available
Auto ignition temperature	Not available
Decomposition temperature	Not available
Viscosity	Water-like
Bulk density	1370 @ 20° C
Density	1.37 G / cm^3 at 20° C

10.0 Chemical Stability and Reactivity Information

Conditions to avoid	Avoid heat, flame, strong UV light, and other sources of ignition. ELEVATED pH > 4 CAN ENHANCE MORE RAPID DECOMPOSITION OF THE *HYDROGEN PEROXIDE*.
Incompatible materials	MashGuard One may react with acids, organic matter, expanded plastics, such as polystyrene or polyurethane, ammonium salts, sulfur or sulfides, phosphorus, arsenic, metals including copper, zinc, aluminum, or other metals, *manganese dioxide, potassium cyanide*, and thiocyanates. MashGuard One is incompatible with soluble metals and their salts (i.e., iron, copper, chromium, vanadium, tungsten, molybdenum, and platinum), reducing agents, organic materials, as well as flammable and combustible materials.
Hazardous decomposition products	MashGuard One will react with strong mineral acids liberating *chlorine dioxide* gas. Contamination from various metals or organic materials may cause rapid decomposition of the *hydrogen peroxide*, resulting in oxygen gas release, and pressure buildup if not properly vented.
Possibility of hazardous reactions	Strong mineral acids, organic materials, and powdered metals. Polymerization will not occur.

11.0 Toxicological Information

Acute effects	The oral LD_{50} in rats for *sodium chlorate* is greater than 5000 mg / kg (practically nontoxic). The oral LD_{50} for a 10% concentration of *hydrogen peroxide* in rats ranges from 1500 mg / kg to greater than 5000 mg / kg (moderately toxic to practically nontoxic). Ingestion of large doses of *sodium chlorate* will result in methemoglobinemia, and kidney damage.
Component analysis – LD_{50}	Chromium compounds and perchlorate are created as byproducts in the process for the electrolytic production of chlorates. *Hexavalent chromium* is a carcinogen present at an average level of < 10 ppm and perchlorate, which can affect the thyroid gland, is present at an average of < 300 ppm.
Inhalation effects	The LC_{50} of *sodium chlorate* is greater than 5.6 mg / l. There was no mortality in rats following a four hour exposure to *hydrogen peroxide* at the minimal attainable concentration of 122 ppm. Acute: Rat LC_{50}: > 5.6 mg / l Lethal Concentration: NOAEL:
Irritation to skin	*Sodium chlorate* was not irritating to rabbits. *Hydrogen peroxide* at concentrations of less than 35% is not considered irritating. Acute: Rabbit LD_{50}: > 2000 mg/kg Lethal Dose: NOAEL:
Irritation to eye	*Sodium chlorate* was mildly irritating to rabbits. *Hydrogen peroxide* at concentrations greater than 10% is considered severely irritating and corrosive.

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11.0 Toxicological Information ...continued

Routes of entry	*Hydrogen peroxide* at concentrations greater than 10% is considered severely irritating and corrosive
Sensitization data	*Sodium chlorate* was not sensitizing to guinea pigs. *Hydrogen peroxide* was not sensitizing to guinea pigs at a concentration of 6%.
Carcinogenicity / mutagenicity and long-term effects	*Sodium chlorate* and *hydrogen peroxide* are not considered carcinogenic

Rhode Island – Hazardous Substance List

Hydrogen peroxide	7722–84–1	Toxic; flammable

Neurotoxicity	No data available for this product
Reproductive days toxicity / teratogenicity	*Sodium chlorate* was not teratogenic to rats at doses up to 1000 mg / kg / day during 6-15 of gestation. Sufficient data is not available for evaluation of *hydrogen peroxide*.
Epidemiology	No information

12.0 Ecological Information

Ecotoxicity

Fish	Rainbow trout EC50: Fish NOAEL:	 > 1000 mg / l, 96 hours 16.4 – 37.4 mg / l, 96 hours

Aquatic toxicity	The 96-hour LC_{50} in rainbow trout for *sodium chlorate* is greater than 1000 mg / l (practically nontoxic). The 96-hour LC_{50} values for *hydrogen peroxide* in fish range from 16.4 - 37.4 mg/l (slightly toxic).
Environmental effects	*Hydrogen peroxide* occurs naturally as a result of photochemical processes in living organisms
Persistance / degradability	*Hydrogen peroxide* is readily biodegradable and does not bioconcentrate
Bioaccumulation / accumulation	Not known
Mobility in environmental media	No information

13.0 Disposal Considerations

Disposal instructions	In accordance with municipal, provincial, state, and federal regulations. D002

14.0 Transport Information

Basic Shipping Description
Terrestrial DOT HMR Information

Proper shipping name	*Sodium chlorate*, aqueous solution
Hazard class	5.1
Subsidiary hazard class	
Identification number	2428
Packaging group	II
Marine polutant identifier	
Severe marine polutant identifier	
Labels required	Oxidizer

15.0 Regulatory Information

US federal regulations	Components of this product have been checked against the non-confidential TSCA inventory by CAS Registry Number. Components not identified on this non-confidential inventory are exempt from listing (i.e., as polymers), or are listed on the confidential inventory as declared by the supplier.

CERCLA/SARA - Section 302 Extremely Hazardous Substances TPQs

Hydrogen peroxide	7722-84-1 1000 lb TPQ (concentration > 52%)
OSHA regulated	Eye / skin irritant as defined in 29 CFR 1910.1200
SARA 302	Not subject to SARA Section 302
SARA 311 / 312	Classified as immediate health hazard and fire hazard. Minimum threshold quantity for reporting is 10,000 pounds.
SARA 313	Not subject to SARA Section 313
Canada DSL	In compliance
WHMIS classification	Class E: corrosive
General	Not subject to Proposition 65. D002 – RCRA corrosive waste This product contains a chemical known to the State of California to cause cancer, or reproductive harm: chromium by product Cr(VI) 0.05 mg / m^3 ACGIH TLV TWA NTP: Cr(VI) compounds: known human carcinogen IARC: Cr(VI) Group 1 carcinogen.

16.0 Other Information

HMIS ratings	Health: 2 Fire: 0 Reactivity: 2 Pers. Prot: X
NFPA hazard ratings	Health: 2 Fire: 0 Reactivity: 2 Special Hazards: OXY
Special hazards	0 = Insignificant 1 = Slight 2 = Moderate 3 = High 4 = Extreme
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RBS Resonant BioSciences®

For more information email info@puremash.com

Resonant BioSciences, LLC technical personnel experienced in the product prepared the information presented in this document. It is intended for all who will be involved with the use / implementation of this product or the use, handling, storage, transport of materials associated with the product. This information has been prepared for the guidance of engineering, operations and management personnel. While Resonant BioSciences, LLC believes this information to be reliable and current, Resonant BioSciences, LLC makes no warranty that it is. Resonant BioSciences, LLC makes no warranty or guarantee, express or implied, regarding the accuracy, completeness, applicability, performance at your plant, etc. Prior to implementation, the user should make its own independent evaluation of the product and particularly the implementation of the product in their facility given their individual circumstances.

RBS Resonant BioSciences

Chlorine Dioxide Cd

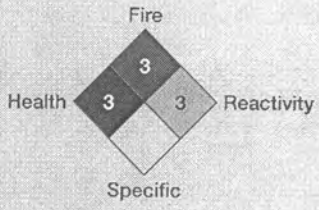
Material Safety Data Sheet

1.0 Chemical Product and Company Identification

Resonant BioSciences, LLC
1400 16th St., Ste. 400
Denver, CO 80202
Toll Free: 866.933.0408
Fax: 303.933.3594
www.puremash.com

Product name: ***Chlorine Dioxide***

Emergency Phone
888.299.3899
CHEMTREC 800.424.9300
CHEMTREC International 1.703.527.3887



Chlorine Dioxide is manufactured by the user as required for use on-site. Equipment and / or raw materials used in its manufacture are made or supplied by Resonant BioSciences, LLC.

2.0 Hazards Identification

gency overview	A greenish-yellow gas with a pungent odor similar to Chlorine. STRONG OXIDIZER. Gas and solutions are severe respiratory irritants. May cause pulmonary edema, which may be delayed in onset. ClO_2 gas partial pressures above 10 volume % can decompose spontaneously with a corresponding pressure pulse or "puff." Decomposes on exposure to sunlight or UV. CORROSIVE to the eyes and skin. Can cause damage to vegetation. Read the entire MSDS for a more thorough evaluation of the hazards.

Potential health effects

General	*Chlorine dioxide* normally exists as a gas at room temperature, and the most important route of exposure is inhalation, followed by eye and skin exposures
Ingestion	Not applicable except for solutions, in which case the symptoms would be expected to parallel those for inhalation
Skin contact	Gas and solutions are highly irritant
Skin absorption	May be absorbed, causing tissue and blood cell damage
Eye contact	Severe irritant. Exposure may cause visual disturbance, (i.e., seeing halos around lights).
Inhalation	Severe respiratory irritant. May cause bronchospasm and pulmonary edema, which may be delayed in onset. May also cause severe headaches. All symptoms may be delayed and long-lasting. Long-term exposure may cause chronic bronchitis. An LC50 value of 500 ppm / $15m^3$ (rat) is quoted in the literature.

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2.0 Hazards Identification ...*continued*

Medical conditions aggravated by exposure	Asthma, bronchitis, emphysema, and other lung diseases, and chronic nose, sinus or throat, and cardiac conditions
Exposure limits	ACGIH 1992–93 TWA 0.1 ppm, STEL 0.3 ppm (0.9 mg / m^3)
Irritancy	Severe irritant
Sensitization	Not available
Carcinogenicity	Not listed by IARC or ACGIH
Mutagenicity	Not available
Reproductive effects	Not available
Teratogenicity and fetotoxicity	Not available
Synergistic materials	May have synergistic effects in conjunction with chlorine, other chlorine oxides, and chlorine fluorine compounds.

3.0 Composition / Information on Ingredients

Hazardous ingredient(s)		**CAS #**	**% Wt / Wt**
Chlorine dioxide		10049–04-4	0 to 5 Vol %
ACGIH – Threshold limits values – Time weighted ages (TLV–TWA)	0.1 ppm TWA		
in Air	0.3 ppm STEL		

WHMIS classification(s): C (Oxidizing material)

D1B (Toxic)

E (Corrosive material)

F (Dangerously reactive)

4.0 First Aid Measures

Ingestion	DO NOT GIVE ANYTHING BY MOUTH OR INDUCE VOMITING IF THE PATIENT IS UNCONSCIOUS. Give large amounts of water to dilute stomach contents. Get medical attention.
Skin	Wash immediately using soap, or mild detergent and water
Eye	Flush immediately with plenty of lukewarm water. Continue to wash for ten minutes, lifting eyelids occasionally. Get medical attention.

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4.0 First Aid Measures ...*continued*

Inhalation	Move the victim to fresh air. If breathing is stopped, commence artificial respiration. Apply artificial respiration if victim is not breathing. Induce artificial respiration with the aid of a pocket mask equipped with a one-way valve, or other proper respiratory medical device. Give cardiopulmonary resuscitation (CPR) if there is no pulse AND no breathing. Symptoms of pulmonary edema can be delayed up to 48 hours after exposure. Obtain medical attention IMMEDIATELY.
Note to physicians	Following exposure, the patient should be kept under medical review for at least 48 hours as delayed pulmonary edema may occur.

5.0 Fire Fighting Measures

Flash point and method	Not applicable
Flammable limits (lower)	Not applicable. See Section 5.
Flammable limits (upper)	Not applicable. See Section 5.
Auto ignition temperature	Not applicable. See Section 5.
Conditions of flammability	*Chlorine dioxide* gas may decompose autocatalytically with a pink / violet flame which may ignite combustible materials. This flame can be extinguished by diluting / cooling with air. *Chlorine dioxide* does not require air for it to burn.
Hazardous combustion products	Chlorine, oxygen, and hydrochloric acid
itivity to mechanical impact	Not applicable
Static discharge sensitivity	Sensitive to electrical discharge or flame
Extinguishing media	When combustibles are burning in the presence of *chlorine dioxide* (or other strong oxidizers), water is the only effective extinguishing medium
Protection of firefighters	
Fire fighting procedures	Apply water from as far a distance as possible, in flooding quantities as a spray or fog. Remove all flammable and combustible materials from the vicinity, especially oil and grease. Use water with caution.
Protective equipment for Firefighters	Use eye protection and impermeable gloves. Use of contact lenses should not be permitted when potentially exposed to this material. Persons in the vicinity of *chlorine dioxide* gas, or solutions should carry a respirator suitable for escape purposes at all times, in case of accidental release of significant amounts of gas.

6.0 Accidental Release Measures

Spills, leaks, or releases	*Chlorine dioxide* and its aqueous solutions should not be discharged to the general environment. Treating a small *chlorine dioxide* solution spill with a dilute *sodium sulfite* or *sodium thiosulfate* solution is recommended. Treating a spill with *sodium hydroxide* will convert *chlorine dioxide* to chlorate and chlorite, stopping release of gas in 15 – 20 minutes. PROPER PERSONAL PROTECTIVE EQUIPMENT SHOULD BE WORN PRIOR TO TREATMENT.
Deactivating chemicals	*Sodium sulfite* or *sodium thiosulfate* solutions; *sodium hydroxide*. See Incompatibles in Section 10.

7.0 Handling and Storage

Handling procedures	Equipment manufacturer's recommendations for design, operation, and maintenance of *chlorine dioxide* generation equipment must be followed. Take all precautions to avoid personal contact. Prevent the release of gas into workplace air. Always ensure adequate ventilation in handling areas. Locate safety shower, and eyewash station close to chemical handling area. Keep away from incompatibles, heat, sparks, flames, and other ignition sources. Locate safety shower, and eyewash station fairly close to chemical handling area.
Storage procedures	*Chlorine dioxide* gas is not stored. Solutions can be stored in light-proof FRP, polypropylene or polyethylene tanks at concentrations below 8 g / l. These tanks should be provided with adequate air-sweep to ensure that explosive concentrations of *chlorine dioxide* do not build up.

8.0 Exposure Controls / Personal Protection

Preventive measures	Recommendations listed in this section indicate the type of equipment which will provide protection against over exposure to this product. Conditions of use, adequacy of engineering, or other control measures, and actual exposures will dictate the need for specific protective devices at your workplace.
Engineering controls	Good ventilation should be provided, so that chlorine dioxide levels are maintained below the TLV at all times

Personal protective equipment

Protective equipment	Use eye protection and impermeable gloves. Use of contact lenses should not be permitted when potentially exposed to this material. Persons in the vicinity of *chlorine dioxide* gas or solutions should carry a respirator suitable for escape purposes at all times, in case of accidental release of significant amounts of gas.
Eye / Face	Use full-face shield, and chemical safety goggles when there is potential for contact. Maintain eye wash fountain, and quick-drench facilities in work area.

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8.0 Exposure Controls / Personal Protection ...continued

Skin	If contact with gas is possible, then use chemical protective gloves, coveralls, boots, and / or other resistant protective clothing. Have a safety shower / eye-wash fountain readily available in the immediate work area. Some operations may require the use of a chemical protective full-body encapsulating suit, and respiratory protection.

Exposure guidelines

Chlorine dioxide (100%)

ACGIH time weighted average (TLV–TWA)	0.1 ppm (0.3 mg / m^3)
ACGIH short-term exposure limit (STEL)	0.3 ppm (0.9 mg / m^3)

9.0 Physical and Chemical

State	Gas at normal temperatures. Normally used dissolved in aqueous solution in water.
Alternate name(s)	*Chlorine peroxide*; "ClO_2"
Chemical name	*Chlorine dioxide*
Chemical family	Inorganic compound
Molecular formula	ClO_2
Molecular weight	67.45
Freezing point	–59° C
Boiling point	11° C
Evaporation rate	Not applicable
Vapor pressure	Not applicable
Vapor density (Air = 1)	2.4 (Air = 1) (for 100% ClO_2)

Appearance

Gas	Greenish-yellow
Solution	Pale to bright yellow
Odor	Similar to chlorine or ozone
Odor Threshold	Characteristic smell very evident at 0.2 – 1 ppm
pH	Not applicable
Specific gravity	Not applicable
Solubility (H_2O)	8 g / l @ 15° C (practical limit for stable solution)
Coefficient of water / oil distribution	Not available
Bulk density	Not applicable

10.0 Chemical Stability and Reactivity Information

Hazardous decomposition products	Chlorine (Cl_2), oxygen (O_2), and hydrochloric acid (HCl)
Chemical stability	*Chlorine dioxide* is a reactive, unstable gas. At ClO_2 partial pressures above about 76 mm Hg (10 Vol %) in air it can decompose spontaneously with a corresponding pressure pulse or "puff" that may be more violent and explosive at higher ClO_2 partial pressures. At partial pressures above 190 mm Hg, explosion relief may be inadequate and rupture of the vessel may occur. These explosions can ignite combustible materials.

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10.0 Chemical Stability and Reactivity Information ...continued

Incompatibility with substances	*Chlorine dioxide* is a powerful oxidizing agent that is incompatible with combustible materials, oxidizable organic vapors, *Hydrogen sulfide*, or metallic dusts. Fire may occur.
Reactivity conditions	Highly reactive on contact with incompatible materials, and will decompose upon exposure to sunlight, ultraviolet light, or heat.
Hazardous polymerization	Will not occur

11.0 Toxicological Information

Product	LD_{50}	LC_{50}
Chlorine dioxide	292 mg / kg (rat, oral)	Not available

Mutagenicity	No human data available
Reproductive effects	No human data available
Teratogenicity and fetotoxicity	No evidence

12.0 Ecological Information

Ecotoxicological information	Not available
Persistence and degradation	Not available. No expected persistence.

13.0 Disposal Considerations

Review federal, state, and local government requirements prior to disposal

Waste control procedures	Contained plant-settling ponds or drains containing organic matter will normally provide an environment in which residual *chlorine dioxide* will be reduced to harmless compounds quickly Do not dispose of waste with normal garbage, or to sewer systems Whatever cannot be saved for recovery or recycling, including containers, should be managed in an appropriate and approved waste disposal facility. Processing, use, or contamination of this product may change the waste management options.
RCRA	Test waste material for corrosivity, D002, prior to disposal

14.0 Transport Information

Basic Shipping Description
Material DOT HMR Information

Proper shipping name	Not applicable — ***shipment FORBIDDEN***
Hazard class	—
Identification number	—
Packaging group	—
Shipping information	***Chlorine dioxide*** **may not be shipped as gas or solution.**

15.0 Regulatory Information

SARA Regulations Sections 313 and 40 CFR 372

CERCLA Section 103 (40CFR302.4)	N
SARA Section 302 (40CFR355.3)	N
SARA Section 304 (40CFR355.4)	N
Section 313 (40CFR372.65)	N
OSHA process safety (29CFR1910.119)	Y 1,000 lbs TQ
California proposition	N
U.S.A. classification	
TSCA status	Y

SARA Hazard Categories

Acute hazard	Y
Chronic hazard	N
Fire hazard	Y
Reactivity hazard	Y
Sudden release hazard	Y

Other regulations / legislation which apply to this product

WHMIS classification(s)

C (oxidizing material)

D1B (toxic)

E (corrosive material)

F (dangerously reactive)

16.0 Other Information

HMIS ratings	Health: — Fire: — Reactivity: —
NFPA hazard ratings	Health: 3 Fire: 3 Reactivity: 3
Special hazards	0 = Insignificant 1 = Slight 2 = Moderate 3 = High 4 = Extreme

Disclaimer

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For more information email info@puremash.com

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APPENDIX C

TECHNICAL BULLETINS

MASHGUARD ONE BULK STORAGE GUIDLINE

REGULATORY STORAGE REQUIREMENTS

PRODUCT SPECIFICATIONS

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PureMash® Pm

Bulk Storage Tank Guidelines

1.0 Introduction: PureMash® Skid

The PureMash Fermentation skid requires MashGuard One as one of the two precursor chemicals to generate *chlorine dioxide*. This bulletin is intended to assist in the selection and installation of a bulk chemical storage tank. It is intended to be used as a guide in conjunction with good engineering practice, and adherence to local codes and standards.

2.0 Site Evaluation

The PureMash bulk storage tank should be located in close proximity to the PureMash Fermentation skid. Consideration must be given to tank truck deliveries, and the need for road clearance and access.

The *hydrogen peroxide* in MashGuard One causes it to be slightly effervescent, thus generating small gas bubbles that accumulate in high points in the piping. The bulk storage area should be designed to allow for proper sloping of the lines to prevent pump air binding. This will be further addressed in Section 9.0 Piping and Gasketing Materials.

3.0 Materials of Construction

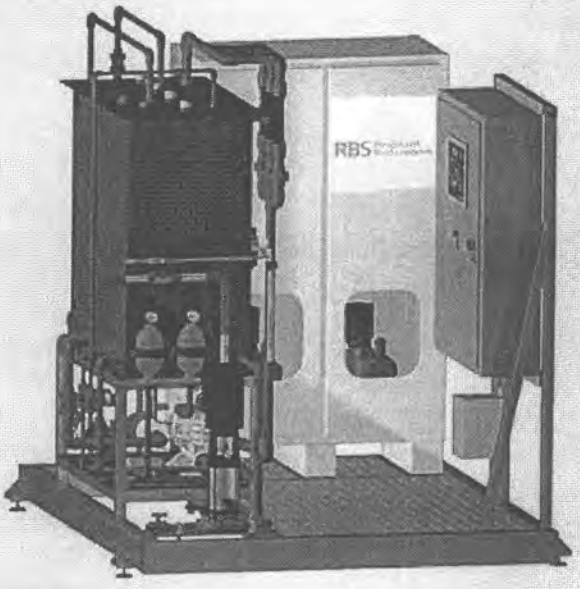
Several materials are suitable for the storage of MashGuard One. The most common is cross-linked high-density polyethylene (HDPE). These tanks are usually readily available in various sizes.

Fiberglass Reinforced Plastic (FRP) tanks are also suitable for the storage of MashGuard One. The resin selection for a FRP storage tank should be Hetron 922, Derakane 411, or approved equal. These tanks are usually custom fabricated to the client specifications.

316L stainless steel is also a suitable material. Stainless steel storage tanks require passivation, which builds an oxide layer on the interior surface of the tank, protecting it from corrosion.

4.0 Tank Capacity and Description

A typical tank truck delivery is 4,000 gallons of MashGuard One. It is recommended in most applications that a minimum of a 5,000 gallon storage tank be installed. This allows a full truck to be unloaded, and gives the user some additional room for inventory.



Several nozzles need to be available on the vessel for level detection, draining, filling, and inspection access. The following two tables outline the required and recommended list of tank nozzles. All nozzles recommended are to be 3", 150#, ANSI type, unless otherwise noted.

4.0 Figure A: Required Tank Nozzles

TOP OF VESSEL	Truck Unloading Connection 2" dia. (with piping insert to within 6" of the tank bottom with siphon breaker)
SIDE OF VESSEL	Tank Vent, one pipe diameter larger than the tank unloading inlet piping. (with 180 deg. bend and screen) Pump Suction
	TANK DRAIN

4.0 Figure B: Recommended Tank Nozzles

TOP OF VESSEL	Tank Level (Ultrasonic)
SIDE OF VESSEL	Tank Manway Access, (HDPE tanks 19" dia., (mfg. Std.), FRP tanks 24" size) Tank Level Bottom (Alternate – Jogler armored level glass)
	Tank Level Top (Alternate – Jogler armored level glass)
	Tank Manway Access

5.0 Weather Protection

MashGuard One should be maintained **between 40° F (5° C) and 100° F (38° C)**. To maintain proper storage conditions, consideration should be given to tank insulation, pipe heat ' g, tank heating, and indoor installation. If the MashGuard U.. temperature drops below 40° F (5° C), the *sodium chlorate* may begin to crystallize and precipitate out of the solution which will lead to operational problems with the unit.

6.0 Tank Unloading Requirements

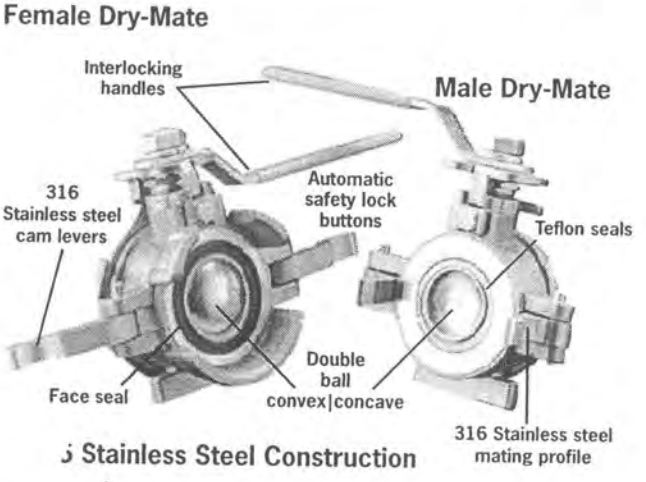
MashGuard One is delivered in dedicated chemical trucks equipped with an unloading pump, and approximately 40 feet of unloading hose. **Under no circumstances should air be used to unload or blow down the unloading hoses or MashGuard One lines.** Plant air typically contains oil, which will adversely react with the MashGuard One, and cause it to decompose.

Only properly trained and approved personnel should unload MashGuard One. **It is critical that no other chemical, especially acids, be unloaded into the MashGuard One tank.** *Sulfuric acid* is the second precursor chemical used to make *chlorine dioxide*. If an acid tank truckload were inadvertently unloaded into a MashGuard One tank, catastrophic equipment damage and possible personnel injury could occur.

It is strongly recommended that the MashGuard One tank un'-ading manual valve be "Locked and Tagged" and that c .e shift supervisor or unloading supervisor have access to the key.

The MashGuard One tank unloading connection is a 2" Banjo Corporation dry-mate ball valve supplied by the chemical provider. This valve will not allow a typical camlock truck connection to be used to unload the incorrect chemical into the MashGuard One tank.

Figure 6.0 Tank Unloading Diagram



The valve is constructed of 316 stainless steel with Viton, EPDM, and Kalrez® face seals. The seats are 100% Teflon. This valve's unique construction has several built-in safety features. When liquid is flowing, the two halves cannot be uncoupled without turning both handles to the closed position. When the dry-mate halves are apart, the handles cannot be turned to the open position (see Figure 6.0).

7.0 Labeling

The MashGuard One unloading station shall be labeled and tagged properly to avoid any confusion by the chemical delivery driver. The MashGuard One storage tank should also be labeled with the appropriate "Oxidizer" labels, and the area should be labeled as a "Non-Smoking" area. An example of a PureMash tank National Fire Protection Agency (NFPA) label is shown below (see Figure 7.0).

8.0 Chemical Containment Requirements

The MashGuard One chemical provider utilizes a Responsible Care® chemical supplier program, which means that before bulk chemical deliveries of MashGuard One will be allowed at a client's site, several critical items must be met.

The MashGuard One tank MUST be installed in its own diked containment area. In the event of a spill or leak, this will prevent the inadvertent mixing of incompatible chemicals, which may cause the MashGuard One to decompose or react. The volumetric capacity of the diked area should not be less than 110% of the greatest amount of liquid that can be released from the largest tank within the diked area, assuming a full tank.

The MashGuard One storage area should be drained to an alkaline or neutral sewer to prevent mixing with acids, thus forming *chlorine dioxide*.

Figure 7.0 Tank Labeling Diagram

9.0 Piping and Gasketing Materials

The recommended piping material is socket welded, or flanged schedule 80 PVC pipe and fittings. Minimize line lengths and the use of threaded connections to prevent leak ... 316L stainless steel pipe and fittings are also suitable ...erials, after passivation.

Piping containing MashGuard One should be protected from temperatures below 40° F (5° C). Installations in areas where the temperature is low for extended periods should be insulated, electrically heat traced, or located indoors.

All piping should be well supported and sloped towards the PureMash Fermentation Skid unit at ¼" per foot. The PVC piping line size should be **2"** for both the sulfuric acid, and MashGuard One chemical feed lines.

The recommended gasket material is 1/8" thick expanded Teflon sheet material cut in a full-face pattern.

10.0 Safety Requirements

The National Fire Protection Association (NFPA), as well as the Occupational Safety and Health Administration (OSHA), should be consulted when designing and / or installing a chemical storage area.

The MashGuard One storage tank should be located in a ...ll-lit area. Organic materials, such as paper, wood, and ...n rags, should not be allowed to accumulate due to their incompatibility with oxidizers like MashGuard One.

Personnel involved in plant operations where oxidizers are stored must receive instruction in handling the material, including manufacturer recommendations.

As with any chemical storage area, a safety shower and eyewash station must be located and easily accessible for personnel working in the MashGuard One storage area. The chemical unloading area should be curbed, and the drain routed to an alkaline sewer to prevent mixing with acids which will form chlorine dioxide.

A water supply must also be available for washing down the equipment and any residual chemical spills that may occur during unloading.

11.0 Pre-shipment Checklist

The following items must be completed before a delivery of MashGuard One will be allowed:

1. Tank materials of construction compatible with MashGuard One
2. Tank must be vented (see 4.0 Figure A)
3. Tank must have top-fill unloading capabilities
4. Tank must have proper labeling
5. Tank level must be visually verifiable
6. Tank unloading line equipped with 2" dry-mate ball valve (supplied with the PureMash Fermentation skid)
7. Tank must be diked, and capable of holding 110% of tank contents
8. Manual shut-off valve located inside the dike area
9. Tank must be located away from reactive chemicals (spills from other tanks into MashGuard One dike area must not occur)
10. Eyewash, and safety shower located at unloading station
11. Site has MSDS sheets
12. Wash-up water available at unloading station

RBS Resonant BioSciences

For more information email info@puremash.com

Resonant BioSciences, LLC technical personnel experienced in the product prepared the information presented in this document. It is intended for all who will be involved with the use / implementation of this product or the use, handling, storage, transport of materials associated with the product. This information has been prepared for the guidance of engineering, operations and management personnel. While Resonant BioSciences, LLC believes this information to be reliable and current, Resonant BioSciences, LLC makes no warranty that it is. Resonant BioSciences, LLC makes no warranty or guarantee, express or implied, regarding the accuracy, completeness, applicability, performance at your plant, etc. Prior to implementation, the user should make its own independent evaluation of the product and particularly the implementation of the product in their facility given their individual circumstances.

RBS Resonant BioSciences

PureMash® Pm

Regulatory Storage Requirements

1.0 PureMash® Summary

The PureMash fermentation technology is a revolutionary antimicrobial system that will provide many benefits over antibiotics, and is regulatory and trouble-free to implement.

Selected key regulations that are applicable to chemicals associated with MashGuard One®, and *chlorine dioxide* are shown in Table 1.

2.0 Process Safety Management (PSM)

Under OSHA Standard 29CFR 1910.119, Process Safety Management (PSM), the threshold quantity (TQ) of *chlorine dioxide* is 1500 pounds.

MashGuard One, and sulfuric acid are **NOT** covered icals under PSM. Even though *chlorine dioxide* is a c. ered chemical, it is generated on site and used immediately, so the TQ is never even approached. It would require 60,000 gallons of 2000 ppm *chlorine dioxide* to reach the TQ reporting requirement.

PureMash systems are NOT subject to the requirements of PSM (see Table 1).

3.0 Risk Management Program (RMP)

The Risk Management Program (RMP) in Section 112(r) of the Clean Air Act requires those affected to go beyond PSM.

Just as for PSM, the PureMash Technology is **NOT** subject to the requirements of RMP (see Table 1).

4.0 Other Regulatory Requirements

While PSM and RMP are the two most demanding regulations associated with the storage and handling of hazardous chemicals, other regulations are pertinent too. The Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) pertains to the reporting of chemical spills. For the PureMash system, only sulfuric acid is covered, and the reportable threshold quantity is shown on Table 1.

The Superfund Amendments and Reauthorization Act (SARA) deal with reporting the use or storage of hazardous chemicals. SARA 313 covers the annual reporting of chemicals used or stored, and requires a simple annual report (see Table 1).

Table 1 — PureMash Products Regulatory Mix

CHEMICAL NAME	CAS #	SARA (all others)	SARA 313	CERCLA	PSM	RMP	NOTES
MashGuard One *is a blend of 40% sodium chlorate and 7% to 10% hydrogen peroxide.*							
Sodium chlorate	7775 – 09 – 9	Chronic, flammable, reactive hazards	NA	NA	NA	NA	MashGuard One is 40% *sodium chlorate*
Hydrogen peroxide	7722 – 84 –1	Acute, chronic, fire hazards	NA	NA	NA	NA	MashGuard One is 7% to 10% *hydrogen peroxide*
Chlorine dioxide	10049 – 04 – 4	NA	No	NA	1,000 lbs	1,000 lbs	
ric acid	7664 – 93 – 9	Acute, and chronic hazard	NA	1,000 lbs	NA	NA	Co-reactant with the MashGuard One to make ClO_2

5.0 SARA: Superfund Amendments and Reauthorization Act

?A 313 covers whether the amount of a chemical used or stored on site during the calendar year must be reported to federal and state authorities annually.

- All "other" sections of SARA (302, 304, 311, 312) cover the hazards associated with a chemical (e.g., is it a fire hazard, an acutely toxic chemical, etc.).

6.0 CERCLA: Comprehensive Environmental Response, Compensation and Liability Act

CERCLA modified the National Contingency Plan (NCP). The NCP provided the guidelines and procedures needed to respond to releases and threatened releases of hazardous substances, pollutants, or contaminants. In other words, it sets the amounts of certain substances that need to be "released" (e.g., spilled) that would require notification to the appropriate authorities.

7.0 PSM: Process Safety Management – OSHA

In 1992, the Occupational Safety and Health Administration (OSHA) developed a program focused on chemical accident prevention as required under Section 304 of the CAA Amendments of 1990. OSHA promulgated a final rule that requires a chemical Process Safety Management (PSM) program for installations and facilities that produce, process, handle, or store hazardous chemicals above specified threshold quantities.

8.0 RMP: Risk Management Program – Section 112(r) of the Clean Air Act

Section 112(r) of the CAA, "Accidental Release Prevention," was signed into law on 15 November 1990 as part of the CAA Amendments of 1990. Under Section 112(r), owners and operators of stationary sources that produced, processed, handled, or stored regulated substances or other extremely hazardous substances had a "general duty" to prevent and mitigate accidental releases, no matter what the quantity of regulated substance at the facility. Essential activities to be undertaken as necessary to satisfy the general duty requirements of the CAA included:

1. Identifying hazards that may result from accidental releases using appropriate hazard assessment techniques
2. Designing, maintaining, and operating a safe facility
3. Minimizing the consequences of accidental releases, if they occur

RBS Resonant BioSciences

For more information email info@puremash.com

Resonant BioSciences, LLC technical personnel experienced in the product prepared the information presented in this document. It is intended for all who will be involved with the use / implementation of this product or the use, handling, storage, transport of materials associated with the product. This information has been prepared for the guidance of engineering, operations and management personnel. While Resonant BioSciences, LLC believes this information to be reliable and current, Resonant BioSciences, LLC makes no warranty that it is. Resonant BioSciences, LLC makes no warranty or guarantee, express or implied, regarding the accuracy, completeness, applicability, performance at your plant, etc. Prior to implementation, the user should make its own independent evaluation of the product and particularly the implementation of the product in their facility given their individual circumstances.

RBS Resonant BioSciences

MashGuard One®

Product Specifications

Mg

1.0 Description

MashGuard One is a stabilized aqueous solution of 40% *sodium chlorate* and < 8% *hydrogen peroxide*.

2.0 Applications

MashGuard One is a precursor chemical for the production of *chlorine dioxide* in a PureMash antimicrobial equipment skid. MashGuard One must be used in conjunction with sulfuric acid to produce *chlorine dioxide*. *Chlorine dioxide* produced from MashGuard One may be used as a antimicrobial agent in ethanol fermentation, propagation, CIP, heat exchangers and process yeast, mash, and water pertaining to the production to ethanol.

3.0 Specifications

Sodium chlorate	40 %
Hydrogen peroxide	8 %
Water	52 %
Storage temperature limits	5° – 40° C

4.0 Physical Properties

Appearance	Clear, faint blue solution
Specific gravity	1.37
Flash point	None
Boiling point	104° C
Odor	Slight

5.0 Shipping Information

USA Customers:
300 gallon tote (IBC)
3800 gallon tank trailer
20 MT ISO container
17,400 gallon railcar

Shipping classification:
DOT: *Sodium chlorate,* Aqueous Solution
UN / ID number: UN 2428

International Customers:[1]
1 m^3 IBC
20 – 26 MT ISO container

Shipping classification:
UN / ID number: UN 2428

[1] Availability of tank truck and ISO containers varies by region. Contact Resonant BioSciences, LLC for further information pertaining to shipping, etc.

6.0 Regulatory Information

It is a violation of Federal Law to use this product in any manner inconsistent with the labeling. Internationally, regulations vary widely by region and country. Consult local regulatory authorities to determine any local use restrictions or regulatory requirements.

7.0 Safety and Handling Information

General: Avoid all bodily contact. Wear appropriate protective equipment. Do not allow clothing, shoes or gloves to become impregnated with *sodium chlorate* in solution, as they will become highly combustible if allowed to dry, and may be ignited by friction or heat. In case of external fire, cool containers of *sodium chlorate* and hydrogen peroxide solution with plenty of water.

Skin	Use impervious clothing to avoid skin contact.
Eye / Face	Wear safety glasses with side shields or chemical goggles. Where appropriate, wear a full-face shield. Contact lenses should not be worn when handling this product.

For more complete information, consult the Material Safety Data Sheet (MSDS) for this product.

8.0 Chemical Registration Numbers

U.S. EPA	49620–4
CAS #	
Sodium chlorate	7775–09–9
Hydrogen peroxide	7722–84–1

Resonant BioSciences, LLC
1400 16th St., Ste. 400
Denver, CO 80202

Toll Free: **866.933.0408**
Fax: **303.933.3594**
www.puremash.com

WARNING: *MASHGUARD ONE* IS NOT INTENDED OR SUITABLE FOR USE IN ANIMAL FEED

Attachment 16

Resonant Biosciences, LLC
Correspondence Index

Date of Correspondence	Correspondent
July 15, 1993	(b) (6)
December 6, 1993	
February 24, 1993	
August 29, 1989	
August 21, 1989	
August 11, 1989	
• May 22, 1989	
April 14, 1989	
April 10, 1987	
March 11, 1987	
March 10, 1987	
February 18, 1987	
February 18, 1986	
July 16, 1984	
• June 4, 1984	
June 4, 1984	
June 1, 1979	
January 4, 1978	
• May 12, 1977	
September 30, 1968	
April 26, 1961	
January 23, 1961	

July 15, 1993

Richard Higby, Ph.D.
Manager, Technical Services
and Regulatory Affairs
Rio Linda Chemical Co., Inc.
410 N. 10th St.
Sacramento, CA 95814

Dear Dr. Higby:

This is in response to your letter of June 16, 1993, in which you requested an opinion on the use of chlorine dioxide to disinfect water used to transport pecans and other nuts. You note that in this application, whole unshelled nuts enter the water system but that the shells are cracked and separated in the water stream to yield the nut "meats" in the transport water.

Please note that FDA advisory opinions are issued only under 21 CFR 10.85. We presume, however, that you are merely seeking guidance concerning whether your proposed use constitutes a food additive situation. Consistent with opinions we have expressed on this subject in the past, we consider the use of chlorine dioxide solution to wash or transport shelled nuts to be an unapproved food additive use. Therefore, you would need to submit a food additive petition for the proposed use in accordance with 21 CFR 171.1.

You are of course free to make your own GRAS self determination. Section 201(s) of the Federal Food, Drug, and Cosmetic Act does not restrict such a determination. However, a company making a GRAS self-determination does so at its own risk that the Food and Drug Administration (FDA) may disagree with the GRAS determination and take regulatory action. A mechanism by which you can request FDA's concurrence that your proposed use is GRAS is submission of a GRAS affirmation petition in accordance with 21 CFR 170.35.

In support of your proposed use, you have stated that the lipophilic nature of nuts prevents aqueous permeation to the interior much in the same manner as the cuticle on fruits and vegetables prevents aqueous diffusion on uncut and unpeeled fruits and vegetables. As you are aware, the agency has stated that it has no objection to the use of water containing chlorine dioxide at 5 ppm to process whole fruits and vegetables

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HFS-206	BERD	7/15/93						

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provided this process is followed by a potable water rinse. This was supported, in part, by data contained in GRASP 3G0020 which showed that oxidative chlorine dioxide reactions are largely restricted to the surface and that, little or no residue of chlorine dioxide or its reaction products remained on the washed fruits and vegetables following a potable water rinse.

Contrary to your assertions, we do not find that the two situations are analogous. First, your proposed use does not allow for a potable water rinse. Secondly, even if chlorine dioxide treatment was followed by a potable water rinse, you have presented no data to indicate that treatment of shelled nuts with a chlorine dioxide solution would leave no residues of chlorine dioxide and its reaction products on the nuts. The articles you enclosed with your letter do not address the issue of possible residues that would be left on nuts following chlorine dioxide treatment.

As you noted in your letter, your firm is currently collaborating with other firms and the National Food Processors Association to petition to broaden approved uses of chlorine dioxide to include use in processing cut fruits and vegetables. As you may recall from a meeting we had with you and your colleagues on November 6, 1992, certain studies designed to address the issue of residues on cut and peeled fruits and vegetables due to chlorine dioxide treatment are underway with the ultimate purpose in mind of supporting a petition for such use. We believe your proposed use will be covered under this forthcoming petition.

We trust the foregoing answers your questions. Please feel free to contact us if we can be of any further assistance.

Sincerely yours,

Nega Beru, Ph.D.
Biotechnology Policy Branch, HFS-206
Division of Product Policy
Center for Food Safety
and Applied Nutrition

cc: HFS-200 HFS-205 HFS-206 HFS-226 HFS-247 HFS-246 GRP 3G0020
R/D:HFS-206:NBeru:254-9519:7/8/93:EOS19671
R/D/Init.:LMTarantino:HFS-206:7/13/93
F/T:HFS-206:NBeru:sdd:7/15/93

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

December 6, 1993

Mr. Eliot I. Harrison
Delta Analytical Corporation
7910 Woodmont Ave.
Suite 1000
Bethesda, MD 20814

Dear Mr. Harrison:

This is in response to your letter of December 1, 1993, concerning the use of chlorine dioxide generated from sodium chlorate rather than sodium chlorite, in the bleaching of paper and paperboard intended to contact food.

In the letter of January 23, 1961, from Mr. Frederick A. Cassidy, which you have attached to your letter, both sodium chlorite and chlorine dioxide are stated to be GRAS for use in the manufacture of paper and paperboard. It is our opinion that chlorine dioxide, both in the case of the earlier letter and your request is the actual additive in the paper making process.

At this time, we know of no reason to revoke our earlier conclusion that the use of chlorine dioxide to manufacture paper and paperboard is GRAS. As the use of sodium chlorite or sodium chlorate to generate the chlorine dioxide would not be material to the decision on the use of the chlorine dioxide as a bleaching agent, the proposed use of sodium chlorate in lieu of sodium chlorite is acceptable.

If we can be of further assistance in this matter, please feel free to call upon us.

Sincerely yours,

Thomas C. Brown
Indirect Additives Branch, HFS-216
Division of Petition Control
Center for Food Safety
and Applied Nutrition

FEB 24 1993

Allen W. Matthys, Ph.D.
Vice President, Technical Regulatory Affairs
National Food Processors Association
1401 New York Ave., NW
Washington, DC 20005

Dear Dr. Matthys:

This is in response to your letter of December 16, 1992, in which you requested our opinion concerning the use of chlorine dioxide to process shelled peas and beans whether blanched or unblanched. Please note that FDA advisory opinions are issued only under 21 CFR 10.85. We presume, however, that you are merely seeking guidance from the Office of Premarket Approval concerning whether such a use is a food additive situation. Thus we offer our comments below.

You correctly point out that the Agency has, in the past, issued letters stating that it has no objection to the use of water containing 5 parts per million (ppm) chlorine dioxide for processing uncut and unpeeled fruits and vegetables provided this is followed by a potable water rinse. As you may know, this was first given in the summer of 1977 based on the users' claim that no residues will result from such use.

This was later supported by data contained in Olin Corporation's GRAS affirmation petition (3G0020: Chlorine Dioxide for the Treatment of Potable Water and Washing of Fruits and Vegetables) which showed that chlorine dioxide primarily works as an oxidant rather than as a chlorinating agent and that, in uncut and unpeeled fruits and vegetables, oxidative chlorine dioxide reactions were largely restricted to the surface. Furthermore, it was shown that little or no residue of chlorine dioxide and its reaction products remained on the washed fruits and vegetables and the nutritional quality of such produce was not significantly affected.

As discussed in the meeting you had with members of my staff on November 6, 1992, we believe that for shelled beans and peas with intact cuticles, treatment with water containing up to 5 ppm chlorine dioxide is unlikely to result in appreciable permeation of chlorine dioxide or its reaction products and little or no residue is expected to remain following the potable water wash. However, please note that this applies to unblanched peas and beans with intact cuticles only. We believe that blanching may change the permeability of the cuticle, thereby rendering it no longer an effective barrier to chlorine

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HFS-206	BFR	2/18/93	HFS-201	Farran	2/4/93			
HFS-206	Tmartin	2/18/93	HFS-200	Rulis	2-23-93			
HFS-205	Park	2/19/93						

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dioxide and its reaction products. Unless we are provided with information to the contrary, we cannot agree that the use of 5 ppm chlorine dioxide on blanched peas and beans would leave no residue after washing.

Additionally we would like to correct one misstatement in your letter. You stated that current FDA policy also permits up to 5 ppm chlorine dioxide in water for washing cut or peeled potatoes provide this is followed by a potable water rinse. While some letters have stated this position, we know of no basis for using a chlorine dioxide solution above 1 ppm for use when processing cut and peeled potatoes followed by a potable water rinse. This concentration has been shown to be sufficient to achieve the intended effect. The use of 5 ppm chlorine dioxide in water to process cut or peeled potatoes is greater than needed to accomplish its effect and, therefore, is not in accordance with good manufacturing practice.

We trust the foregoing answers your questions. If we can be of any further assistance please do not hesitate to contact us again.

Sincerely yours,

/s/

Alan M. Rulis, Ph.D.
Acting Director
Office of Premarket Approval
Center for Food Safety
and Applied Nutrition

August 29, 1989

Joseph M. Kelley, Ph.D.
Director of Operations
International Dioxide, Inc.
136 Central Ave.
Clark, NJ 07066

A.F. 42-330 FILE FC

Dear Dr. Kelley:

This is in regard to your letter of August 21, 1989 concerning the addition of chlorine dioxide to 21 CFR 176.300.

As we noted in our earlier letter, FDA considers chlorine dioxide to be GRAS for use as a slimicide in paper mills. As such, chlorine dioxide, when used in this manner is, by definition, not a food additive and may not be regulated in 21 CFR 176.300 of the food additive regulations. As time and resources permit, this and other unlisted GRAS substances will be affirmed as GRAS under Parts 184 or 186, as appropriate.

We hope this responds satisfactorily to your question.

Sincerely yours,

Thomas C. Brown
Indirect Additives Branch, HFF-335
Division of Food & Color Additives
Center for Food Safety and
Applied Nutrition

cc: HFA-224 HFF-335 HFF-330 HFF-300 HFF-158 HFF-415
HFC-220 MATS No. 2369
R/D: HFF-335:TCBrown:tcb:8/29/89
F/T: HFF-335:TCBrown:srd:8/29/89

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000300

INTERNATIONAL DIOXCIDE, INC.
136 CENTRAL AVENUE, CLARK, NEW JERSEY 07066 · (201) 499-9660

August 21, 1989

Mr. Thomas C. Brown
Department of Health & Human Services
Public Health Service
Food and Drug Administration
Washington, DC 20204

Dear Mr. Brown:

Thank you very much for your letter of August 11, 1989 indicating that chlorine dioxide is GRAS for use as a slimicide in paper mills. Your letter is of great help to the marketing efforts of one of our major distributors.

However, they have several customers who use 21 CFR 176.300 as a bible and would feel comfortable seeing chlorine dioxide listed as a slimicide under that regulation.

I personally realize that GRAS status is all encompassing with respect to use as a slimicide, but our distributor has asked if it would be possible to list chlorine dioxide in the list of slimicides under regulation 21 CFR 176.300.

Is this possible and if so what is required to have chlorine dioxide appear in the listing of slimicides under 21 CFR 176.300?

Thank you for your further assistance in this matter.

Yours very truly,

INTERNATIONAL DIOXCIDE, INC.

Joseph M. Kelley

Dr. Joseph M. Kelley
Director of Operations

JMK.em

August 11, 1989

Joseph M. Kelly, Ph.D.
International Dioxide, Inc.
136 Central Ave.
Clark, NJ 07066

A.F. 42-330 FILE FAC

Dear Dr. Kelly:

This is in regard to your letter of May 22, 1989 concerning the use of stabilized chlorine dioxide as a slimicide in the production of paper and paperboard.

The Food and Drug Administration has issued numerous letters in the past stating that the use of chlorine dioxide is GRAS for use in the white water of paper mills, or for use generally in paper mills, whether for slime control or bleaching. As your process for manufacturing the stabilized chlorine dioxide uses only GRAS materials to stabilized the solution we see no reason to reverse our previous informal opinions on the use of chlorine dioxide in the manufacture of paper and paperboard.

If we can be of further assistance in this matter, please feel free to call upon us.

Sincerely yours,

Thomas C. Brown
Indirect Additives Branch, HFF-335
Division of Food & Color Additives
Center for Food Safety and
Applied Nutrition

cc: HFA-224✓ HFF-335 HFF-330 HFF-300 HFF-158 HFF-415
HFC-220 Letter No. 467
R/D:tcb:8/11/89:467
F/T:HFF-335:TCBrown:srd:8/11/89

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food contact as a slimicide at a concentration of 93 - 186

INTERNATIONAL DIOXCIDE, INC.
136 CENTRAL AVENUE, CLARK, NEW JERSEY 07066 (201) 499-9660

May 22, 1989

Dr. Corbin Miles
Indirect Additive Branch
HFF-335
Division of Food & Color Additives
Food & Drug Administration
200 "C" St.S.W.
Washington, D.C. 20204

Dear Dr. Miles:

According to 21 CFR 186.1750 sodium chlorite is permitted to be used as a slimicide at concentrations of 125 to 250 PPM in paper products to be used in contact with food.

We would like your opinion (and hopefully concurrence) on whether our stabilized chlorine dioxide (trade name ANTHIUM DIOXCIDE, ANTHIUM 200 and CARNEBON 200) would fall under the 186.1750 regulation. We have manufactured this material for some thirty years under the designation of "stabilized chlorine dioxide".

I have enclosed a description of our manufacturing process and a patent which covers the process, for your information.

You will note that we generate a chlorine dioxide gas which is then absorbed in a mixture of sodium carbonate and hydrogen peroxide. Sodium carbonate is used instead of sodium hydroxide so that a carbonate-bicarbonate buffer is incorporated into the stabilized chlorine dioxide solution.

Our solutions are sold as either 50,000 ppm (ANTHIUM DIOXCIDE) and 20,000 ppm, (ANTHIUM 200 and CARNEBON 200) available chlorine dioxide. Therefore, 93 - 186 PPM available chlorine dioxide would be equivalent in concentration to 125 to 250 PPM sodium chlorite.

$$\left(125 \times \frac{67.45}{90.45} \times \frac{\text{MW of } ClO_2}{\text{MW of } NaClO_2} = 93 \text{ ppm}\right)$$

What we would like from FDA as a statement, if you concur, is: "We have examined the data submitted to us on ANTHIUM DIOXCIDE, ANTHIUM 200 and CARNEBON 200. It is our opinion that, when your ANTHIUM DIOXCIDE, ANTHIUM 200 and CARNEBON 200 solutions are added to paper or paperboard destined for food contact as a slimicide at a concentration of 93 - 186 PPM available chlorine dioxide, they will comply with 21 CFR 186.1750 regulation".

Dr. Corbin Miles
FDA Food & Drug Administration May 22, 1989

The EPA Registration numbers of these products are 9150-2, 9150-1 and 9150-3 respectively. They also qualify as Food Additive - Sanitizer under 21 CFR 178.1010.

Thank you for your help in this matter.

Yours very truly,

INTERNATIONAL DIOXCIDE, INC.

Joseph M. Kelley

Dr. Joseph M. Kelley
Director of Operations

JMK.em
Enclosures

000304

APR 14 1989

This is in further response to your submission of 1988 and that of your consultant, dated 1988, in which you requested an opinion regarding the use of calcium hypochlorite in a fruit and vegetable wash in accordance with 21 CFR 173.315.

Substitution of chlorine gas and calcium hypochlorite for sodium hypochlorite for use in washing fresh fruits and vegetables is being considered under FDA's safety review of the regulatory status of chlorine. Because the active ingredient is the same, whether from sodium hypochlorite, calcium hypochlorite or chlorine gas, we offer no objection, pending completion of our review, to the interchangeable use of chlorine gas or calcium hypochlorite for sodium hypochlorite under 21 CFR 173.315 when used in a manner consistent with good manufacturing practices, including following treatment with a potable water rinse.

We trust that this response is satisfactory. If you need additional assistance, please contact us again.

Sincerely yours,

Gerad L. McCowin
Director
Division of Food and Color Additives
Center for Food Safety
and Applied Nutrition

Calcium - Hypochlorite - Fruit and Vegetables - Wash

000305

APR 10 1987

Lawrence J. Lin, HFF-334

Chlorine Dioxide

Manject Singh, HFF-314

Before we respond to these four questions, background information described below would be helpful.

FDA has expressed the opinion since 1977 it has no objection to the use of chlorine dioxide solution (up to 5 ppm) to process unpeeled and uncut fruits and vegetables followed by a potable water rinse. In some of its opinion letters, FDA also indicated no objection to this use on peeled and cut potatoes. A problem has surfaced due to different interpretations of what is meant by "a potable water rinse." The potable water as we understand is a municipal water which contains no chlorine dioxide. But, the potable water these firms (e.g.,] Inc.) claim is a municipal water to which they would add themselves 1 ppm of chlorine dioxide insofar as it meets the EPA's primary drinkg water standards.

Now, we choose to answer these questions as follows:

A. The 5 ppm limit still applies to the unpeeled and uncut fruits and vegetables. The 1 ppm limit stated in Manject Singh's memo (dated 3/10/87) will apply only to peeled and cut potatoes.

B. No approval has been given to peeled and cut fruits and vegetables except potatoes.

C. The majority of municipal water treatment plants do not use chlorine dioxide, therefore virtually all potable water contains no chlorine dioxide. Only two plants in the U.S. has used chlorine dioxide significantly. Even so, the potable water obtained from these plants contain very small amounts of chlorine dioxide because this chemical is reactive and volatile. Therefore, the potable water used for rinsing contains little or no chlorine dioxide.

D. If the product is other than on-site generated chlorine dioxide, the concentration limits apply to the total level of chlorine dioxide, chlorate and chlorite. This is because, when acidified, chlorate and chlorite are converted back to chlorine dioxide.

cc: HFF-334 HFF-330 HFF-300 HFF-158 HFF-458 HFC-220
HFF-314 GRASP 3G0020
R/D:LLin:leb:4/8/87
Final:LLin:leb:4/8/87

MAR 11 '88

Mr. W. O. Hardy
Technical Service
Bio-Cide International, Inc.
P.O. Box 2700
2845 Broce Drive
Norman, OK 73070

Dear Mr. Hardy:

Your letter of December 9, 1986 to FDA's Division of Regulatory Guidance has been referred to us for reply. You requested an FDA opinion concerning the use of chlorine dioxide at one part per million (ppm) in process water to wash cut and peeled fruits and vegetables.

You stated that EPA allows municipal water treatment plants to treat water with chlorine dioxide with a maximum limit of one ppm for total residual levels of combined oxidants of chlorine dioxide. (We agree that the current EPA guidelines allow this maximum limit for the total residual levels of combined oxidants of chlorine dioxide, which include chlorite and chlorate.) You then assumed that we were referring to this limit (in our letter to your firm of July 16, 1984) as a very small amount of chlorine dioxide allowed for by the EPA. We did not refer to this one ppm limit as a very small amount. What we meant as a very small amount was the actual residual concentration of chlorine dioxide in the potable water when it reached a household or food processing plant. We know that chlorine dioxide is reactive and volatile and its concentration in water diminishes during its trip from the municipal water treatment facility to the tap. Therefore, we believe that the potable water obtained from the municipal water supply contains very small amounts, if any, of chlorine dioxide and, without further treatment with chlorine dioxide, would be suitable for rinsing cut and peeled fruits and vegetables.

Concerning the use of plant process water containing one ppm of chlorine dioxide to wash cut and peeled fruits and vegetables, such use is within FDA's jurisdiction. Further, we believe that this use would be in violation of the Federal Food, Drug, and Cosmetic Act, because there are insufficient safety data to support this general use on peeled and cut fruits and vegetables. Such use would then be considered an unapproved food additive use. Therefore, it is necessary to establish the safety of this use via the submission of a food additive petition in accordance with 21 CFR 171.1 (copy enclosed).

Sincerely yours,

(S)

Lawrence J. Lin, Ph.D.
Direct Additives Branch
Division of Food & Color Additives
Center for Food Safety
and Applied Nutrition

Enclosure

cc: HFF-300 HFF-330 HFF-334 HFF-158 HFF-314
HFF-458 HFC-220 HFA-224 HFF-312 fa no 774
R/D:LLin:leb:1/9/87
r/d/intd. RMGryder/HFF-158/2-4-87
KBiddle/HFF-158/2-4-87
DADennis/HFF-334/2-6-87
MBReddoch/HFF-312/2-6-87
JEThomas/HFF-314/2-6-87
EColeman/HFF-334/3/4/87
GMcCowin/HFF-330/3/9/87
redrafted/LJLin/3/3/87
Final:LLin:leb:3/10/87

DEPARTMENT OF HEALTH & HUMAN SERVICES — Public Health Service

Memorandum

Date MAR 10 1997

From CFSAN/Division of Regulatory Guidance (HFF-314)

Subject Chlorine Dioxide Use in Potato Processing Plants

DEP
Initial
C.V.M
3-11

To Donald E. Peterson, CSO
SEA-DO/HFR-0140

In the past, based upon information submitted in the chlorine dioxide petition, we have made an exception on the use of chlorine dioxide for cut and/or peeled potatoes. We have not objected to the use of 5 ppm chlorine dioxide or cut and/or peeled potatoes provided that the use of chlorine dioxide is followed by a potable water rinse.

Recent information, however, shows that only 1 ppm chlorine dioxide is sufficient to achieve the technical effect on cut and/or peeled potatoes. The use of 5 ppm chlorine dioxide on the cut and/or peeled potatoes is therefore, not in accordance with good manufacturing practice.

In our opinion, should be informed that we currently recognize a 1 ppm chlorine dioxide rinse for cut and/or peeled potatoes followed by a potable water rinse to be sufficient to achieve the intended effect. Their use of 5 ppm chlorine dioxide is, therefore in violation of good manufacturing practice.

should be informed that although they are using 1 ppm chlorine dioxide on their cut and/or peeled potatoes, such treatment must be followed by a potable water rinse.

We have no objection to the use of chlorine dioxide on their cut and/or peeled potatoes, which involves a potable water rinse.

Manjeet Singh

Manjeet Singh

DEPARTMENT OF HEALTH AND HUMAN SERVICES

FEB 18 1987

Mr. J. A. Mason
Chemaco International, Inc.
P.O. Box 605
Theodore, AL 36590

Dear Mr. Mason:

This is in response to your letter of December 12, 1986 requesting written approval for the use of "Aqua-Pure", a chlorine dioxide product, as a bleaching agent in the production of pulp for white paper.

We are aware that chlorine dioxide has a long history of use as a bleaching agent for pulp in the production of white paper and continues to be used for such purposes to the present day. It is our opinion that chlorine dioxide when used as a bleaching agent in the manufacture of wood pulp for food packaging is not a food additive by virtue of the fact that it cannot reasonably be expected to become a component of food through this use. Therefore we have no objection to the use of chlorine dioxide as a bleaching agent for the production of pulp for white paper to be used as a food packaging material.

Sincerely yours,

/S/

Gerad L. McCowin
Division of Food & Color Additives
Center for Food Safety and
Applied Nutrition

cc: HFA-224 HFF-335 HFF-330 HFF-300 HFF-158 HFF-458
HFC-220

R/D:ADLaumbach:baw5055:1-14-87
R/V:ADLaumbach:baw5055:2-12-87

Initials: T.C.Brown:HFF-335:1/15/87
M.Flood:HFF-458:1/15/87
S.Grahm:HFF-158:1/15/87
C.Miles:HFF-335:1/20/87
R.W.Gill:HFF-304:1/21/87
H.Parran:HFF-330:1/21/87

000153

FILE

OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE
HFF-335	LaMaster	2/16/87						
225	QCB	2/16/87						
HFF-335	CMiles	2/16/87						

000311

CHEMACO INTERNATIONAL, INC.
P.O. BOX 605
THEODORE, ALABAMA 36590
PHONE 205/653-9060

December 12, 1986

Food and Drug Administration
200 C Street S.W.
Washington, D.C. 20204

Attention: Mr. Tom Brown

Re: Use of chlorine dioxide in the pulp paper industry.

Dear Mr. Brown:

Pursuant to our telephone conversation in November we respectfully request your assistance in obtaining written approval for the use of our product as a water treatment in the pulp and paper industry. We presently generate chlorine dioxide under E.P.A. Number 56135. We market this product under the trade name of "Aqua - Pure". As I am sure you are aware chlorine dioxide is, and has for many years been, in use as a bleach in almost all paper mills making any form of white paper. We have been unable to find any F.D.A. regulations or requirements document stating that this is permissible by F.D.A.

We are currently negotiating with a large mill for the implementation of our product in their plant, and it is necessary that we have something in writing from your agency indicating approval of use of ClO_2 in the paper making process.

It is very important that we receive such correspondence as soon as possible so that we may pursue the above mentioned project.

Your prompt attention to this request will be very much appreciated. I can be reached at the above phone number should you require any further information.

Respectfully,

J. A. Mason,
President

JAM:p

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

FEB 18 1986

This is in reply to your letter dated December 23, 1985 inquiring about the use of a 2% aqueous solution of chlorine dioxide as a rinse for use on fresh fruits and vegetables in grocery stores and restaurants among other uses.

There is no food additive regulation which provides for the safe use of a 2% aqueous solution of chlorine dioxide on fresh fruits and vegetables. Therefore, the use you inquired about is an illegal use.

The agency is in the process of reviewing a GRAS petition concerning the use of chlorine dioxide in wash water to process fresh fruits and vegetable We would have no objection to the use of chlorine dioxide up to 5 ppm in wash water to process whole fruits and vegetables followed by a potable water rinse. We are preparing a food additive regulation which will address the above use and limitations.

The use of a 2% aqueous solution without a rinse is beyond the scope of the GRAS petition and the proposed food additive regulation. Therefore, we would consider the product to be an unsafe food additive, since there is no regulation which provides for its safe use and there is no exemption in effect which provides for such use.

We appreciate you bringing this matter to our attention.

Sincerely yours,

Sandra N. Whetstone

Sandra N. Whetstone
Assistant to the Director
Division of Regulatory Guidance
Center for Food Safety
and Applied Nutrition

Food and Drug Administration
Washington DC 20204

Enclosure "4"

July 5, 1974

RECEIVED JUL 8 1974

Mr. B. C. Danner
Bio-Cide Chemical Co., Inc.
P.O. Box 2700-1111 N. Flood Avenue
Norman, Oklahoma 73070

Dear Mr. Danner:

This is in response to your letter dated June 4 in which you asked several questions concerning the use of chlorine dioxide for rinsing fruits and vegetables.

Your questions are being answered in the sequence they were asked.

1. The basis for making an exception to cut or peeled potatoes is a radiotracer analysis (so called migration) study) submitted in the GRAS Petition Number 360020. A review of this petition indicated that a 5 ppm solution of chlorine dioxide may be safely used to wash cut and peeled potatoes provided that the wash is followed by a potable water rinse.

2. The 5 ppm chlorine dioxide concentration is to be measured before the fruits and vegetables are washed with the water.

3. We would have no objection to a potable water rinse even though the potable water may contain very small amounts of chlorine or chlorine dioxide allowed for by EPA regulations. We have no requirement for measuring residual chlorine dioxide on the fruits and vegetables.

4. FDA's guidelines and regulations pertaining to this use of chlorine dioxide will be published upon completion of the petition review. FDA has not decided at present when it will issue a regulation on chlorine.

5. Assuming that your question 5 refers to the working environment FDA does not have jurisdiction over this area. Perhaps OSHA can answer this question concerning acceptable uses of chlorine, chlorine dioxide (either gaseous or aqueous) in processing environments. If your question refers to food processing uses, they are covered in our responses to other questions.

6. The 5 ppm limit for chlorine dioxide is absolute. Even though chlorine has been used at higher concentrations depending on the demand, chlorine dioxide is distinctly different from chlorine. It is a stronger oxidant and has a stronger odor.

Pending final review of the GRAS petition, chlorine dioxide has not been considered as GRAS by FDA. When used to wash fruits and vegetables it is considered a food additive.

Your question concerning restrictions for protecting public health when chlorine dioxide is used in the food processing environment should be addressed to either OSHA since they have jurisdiction over such matters.

We trust this information is helpful.

Sincerely yours,

Manjeet Singh

Manjeet Singh
Assistant to the Director
Division of Regulatory Guidance
Center for Food Safety
and Applied Nutrition

BIOCIDE CHEMICAL CO. INC.

P.O. Box 2700 - 1111 N. Flood Ave
Norman, Okla. USA 73070

Area Code 405 / 329-5556
Telex (WU) 748-581 BIOFILCO

Enclosure "2"

Manjeet Singh
Assistant to the Director
Division of Regulatory Guidance
Center for Food Safety
and Applied Nutrition
Food & Drug Administration
Washington, D.C. 20204

Ref: Your Letter Received June 4, 1984

Subject: Vegetables & Fruits

Dear Mr. Singh:

Thank you for your response to my letter of May 4, 1984. It is certainly helpful to know that FDA has no objections to using chlorine dioxide for rinsing fruits and vegetables; however, your letter leaves several important issues unanswered, such as:

1. What is the basis for making an exception to cut or peeled potatoes? Is this position addressable? What does your data say?

2. Is the 5 PPM to be measured as the resulting water concentration before or after the wash cycle?

3. The requirement for a potable water rinse may not serve any realistic purpose in as much as the potable water may contain either chlorine or chlorine dioxide as both are approved for potable water use. Would a "no measured residual" requirement serve your objection better and/or does a no chlorine residual requirement exist?

4. What is FDA's plan to issue guidelines or regulations covering the use, differences and limitations for the use of chlorine dioxide vis-a-vis chlorine allowable uses, etc.?

001832

5. What uses of chlorine, chlorine dioxide (either gaseous or aqueous) are acceptable to FDA in food processing environments?

6. Is the 5 PPM limit absolute? Chlorine is apparently used at high concentrations depending on the demand and the same demand may be present to be served by the chlorine dioxide.

These questions are important to us and the food processing industry. The lack of information from FDA in this subject area is causing a considerable economic loss as well as an uncertainty as to what is expected by prudent and concerned businesses. We, for example, are a small business (less than $500,000 per year in product sales). That has limited our capability to pursue petitions as a plausible solution to these questions.

We have understood verbally from FDA regional people in the past that chlorine dioxide was to be placed on the GRAS list some time ago. Is that so; is it under consideration or is there a reason why it should not be considered GRAS?

We have EPA registered labels for the use of our aqueous chlorine dioxide for water treatment and did enjoy, since 1968, USDA F & Z approvals (for process water and equipment sanitation). If the product is safe for consumption, then what restrictions are really necessary to protect the public health when used in the food processing environment?

We will appreciate you efforts to assist us in complying with your requirements and I am sure the industry as a whole would appreciate clarification on the subject.

Sincerely,

BIO-CIDE CHEMICAL CO., INC.

B. C. Danner
President

BCD/lah
cc: Mr. John Thomas
FDA Divi[illegible] Region[illegible]
Harold Popma, Lily Products of Michigan

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administratic
Washington DC 20204

Mr. B. C. Danner
Bio-Cide Chemical Co. Inc.
P.O. Box 2700 - 1111 N. Flood Avenue
Norman, Oklahoma 73070

RECEIVED JUN - 1984

Dear Mr. Danner:

This responds to your letter dated May 4 requesting information on the regulatory status of chlorine dioxide in a rinse for fresh fruits and vegetables.

As stated to you during our telephone conversation of May 2, 1984, pending the publication of a regulation on the above use of chlorine dioxide, FDA is not objecting to the use of a rinse containing upto 5 ppm chlorine dioxide for rinsing uncut unpeeled fruits and vegetables with the exception of cut and peeled potatoes, provided this treatment is followed by a potable water rinse.

We trust this information is helpful.

Sincerely yours,

Manjeet Singh

Manjeet Singh
Assistant to the Director
Division of Regulatory Guidance
Center for Food Safety
and Applied Nutrition

Jun 1, 1979

Dr. Donald Derr
Food Ingredient Assessment Division
Food Safety and Quality Service
U. S. Department of Agriculture
Washington, DC 20250

Dear Dr. Derr:

Pursuant to your telephone request of May 23, 1979, I am writing to you briefly on the current status of chlorine dioxide uses in food processing.

There are no provisions under the Food Additive Amendment to the Federal Food, Drug, and Cosmetic Act which would provide for the use of a substance under conditions where it would be reasonable to expect migration of that substance to food unless regulated as a food additive. The Amendment requires that new food ingredients be regulated for use before entering into interstate commerce. However, the Amendment provides for certain specific exceptions to premarketing clearance, including substances that have been prior-sanctioned by the U.S. Department of Agriculture or Food and Drug Administration (FDA) prior to 1958 and those that are considered generally recognized as safe (GRAS) by the scientific community. General recognition of safety may be established by either (1) scientific procedures, or (2) in the case of a substance used in food prior to 1958, through experience of common use in food. Scientific procedures require published studies which may be corroborated by unpublished studies and other data and information.

Currently, there are no FDA regulations in effect which would provide for the use of chlorine dioxide in water as a disinfectant for food processing. Two GRAS petitions, 3G0020 and 3G0212, have been filed and further reviews are pending upon receipt of additional studies in the near future. The petition 3G0020 requests affirmation that use of chlorine dioxide in water to process fruits and vegetables is GRAS, while the other one 3G0212 requests affirmation that use of chlorine dioxide in water to disinfect freshly slaughtered red meat carcasses is GRAS. But, until such time as the petitioned request is approved and a regulation is established prescribing the safe conditions of use, the use of chlorine dioxide would be in violation of the Food Additive Amendment if its use results in residues in food. However, under the circumstances where no residue of chlorine dioxide is expected

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to remain on food from a specific type of food processing use, that use would not be considered a food additive use. This would be the case with the use of chlorine dioxide in dump and flume water cannery operations when followed by a potable water rinse as has been so determined by the FDA after reviewing process and use data on some raw agricultural commodities.

As mentioned above, additional studies have been requested for both GRAS petitions. In each case, migration and nutrition effect studies will be conducted on each food commodity which is treated with chlorine dioxide. The purpose of migration studies, using labeled chlorine dioxide, is to assure no migration of chlorine dioxide and/or its breakdown products into the treated food commodity. Nutrition studies will be undertaken to determine the extent, if any, of the reactions of chlorine dioxide and/or its breakdown products with natural constituents of the treated food commodity. These additional studies are required to confirm that the proposed conditions of use are GRAS. The petition 3G0020 proposes to use chlorine dioxide at a level of about 1 part per million (ppm) for processing fruits, and 10 ppm for processing vegetables. The petition 8G0212 proposes to use aqueous chlorine dioxide sprays at concentrations of 0.1 to 1.0 ppm and 1.0 to 50 ppm on freshly slaughtered red meat carcasses.

We hope this information is helpful to you, and if you have any questions, please feel free to contact us.

Sincerely yours,

Lawrence J. Lin, Ph.D.
GRAS Review Branch
Division of Food and Color Additives
Bureau of Foods

cc:
HFF-300
HFF-330
HFF-335
GRASP 3G0020 ✓
GRASP 8G0212

LLin:tdc:5-30-79

LJL 5/31/79
Typos initialed on the other petition copy

001451

MEMORANDUM OF CONFERENCE

Date: January 4, 1978

Place: Bureau of Foods, FDA, Washington, DC
GRAS Review Branch, Room 3700, North HEW Building

Participants:

FDA

Joseph W. Lepak, Ph.D., (HFF-122)
John P. Modderman, Ph.D., (HFF-144)
Rong C. Lin, (HFF-414)
Arthur R. Johnson, (HFF-416)
Damon Larry, (HFF-335)
Lawrence J. Lin, Ph.D., (HFF-335)

Subject: Use of Chlorine Dioxide in Water to Process Fruits and Vegetables – GRASP 3G0020

It was indicated that from the microbiological point of view, use of chlorine dioxide presents no problem. The submitted information shows that at a level of 0.4 ppm, chlorine dioxide is effective as a bacterioeide. However, it was suggested that maximum concentration should not exceed 10 ppm, and where possible, lower effective concentrations of less than 10 ppm should be used instead.

Clarification was made that FDA is not going to regulate the use of chlorine dioxide in potable water, but instead we will regulate its uses in in-plant water, flume water and cannery cooling water. GRAS Review Branch has noticed that the Division of Regulatory Guidance responded in the past to inquiries on chlorine dioxide by saying that if no residues will remain under conditions of use described, it would not constitute a food additive situation.

There were discussions on whether surface area of fruit and vegetable could be used as a criterion for determining use on what kind of vegetable constituting a food additive situation. Such a criterion was considered not easily definable and await further assurance that no reaction products are formed on the surface of fruit and vegetable.

It was generally agreed that there are sufficient data in the petition supporting the use of chlorine dioxide in municipal water. However data supporting its use in processing fruits and vegetables are deficient. Since no general agreement has been reached concerning its GRAS status, further action will not be undertaken until DT and DCH have completed their evaluations on the recently submitted supplemental information.

cc: HFF-300
HFF-330
HFF-335
FDA Participants
HFF-195

HFF-335:LJLin:yls:01/16/78:472-4750
Retyped:02/09/78

May 12, 1977

Mr. Richard F. Philpitt
Registration and Regulation Services
Olin Corporation
275 Winchester Avenue
New Haven, CT 06504

Re: GRASP 3G0020

Dear Mr. Philpitt:

This is in reference to your GRAS Petition, GRASP 3G0020, requesting affirmation of GRAS status for chlorine dioxide as a sanitizing agent for water used to process fruits and vegetables.

The data in the petition has been reviewed. As a result of this review it has been concluded that inaccordance with 21 CFR 170.3 the data presented is inadequate and insufficient to affirm the GRAS status of chlorine dioxide for the use you requested. Specifically, a careful review of FDA files and data you submitted to support GRAS affirmation through experience based on common use in food prior to 1958 does not provide a basis for such a conclusion. The limited uses in 1949, 1956, and 1957 by Monmouth Canning Company and the Green Giant Company on corn and peas are not sufficient to comply with GRAS criteria for significant use prior to 1958. Further, the scientific data submitted to date does not provide an adequate base for making necessary judgements of safety for use of chlorine dioxide as a sanitizing agent for water used to process fruits and vegetables.

The continuation of this review will require your submission of scientific data responsive to each of the items that appears in the Federal Register Notice, 41 FR 27856 of July 7, 1976 (enclosure). In addition, the petition should state, with specificity, if the use of chlorine dioxide is intended for treatment of the water and/or fruits and vegetables to reduce bacterial loads only or other effects. In either case the data must demonstrate efficacy as an antimicrobial agent on the microflora expected.

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Please respond within the next thirty (30) days and designate the time you require to provide data responsive to these questions. If you find that these data cannot be provided in a relatively short time, we suggest that you withdraw this petition without prejudice to future filing.

Should you desire further clarification, please do not hesitate to contact us.

Sincerely yours,

Damon Larry
GRAS Review Branch
Division of Food and Color Additives
Bureau of Foods

Enclosure

cc: HFF-144
HFF-152
HFF-300
HFF-330
HFF-335
HFF-416
HFC-20
GRASP-3G0020
DLArry:ign:5-12-77

000551

SBJ
150

Issuance Date 9/30/68

Chlorine dioxide bleaching agent NO

Reference No.

SC-970 Dr. Munsey has raised a question regarding the food additive status of chlorine dioxide when used as a bleaching agent in the manufacture of wood pulp for food packaging.

Does DPT consider chlorine,chlorine dioxide an inorganic alkaline hypochlorites to be GRAS for use as bleaching agents in the production of wood pulp and the other pulps listed as GRAS under § 101(h)? This use is discussed under "wood pulp" in the 7th Edition of The Condensed Chemical Dictionary.

W. F. Randolph

Petitions Control Branch, SC-13

cc: SC-13 SC-440(Dr. Munsey)

WFRandolph:mcs 9/30/68

SC-13 Yes. DPT would consider the use of chlorine dioxide, and inorganic alkaline hypochlorites as for use as bleaching agents in the production of wood pulp and other pulps as GRAS under 101(h).

H. Blumenthal

Petitions Review Branch, SC-970

cc: SC-970
SC-440
HBlumenthal:gr 10-7-68

This form to be used in lieu of yellow Agency Route Slip when requesting comments or recommendations.
Division name only to be used in "To" column. If individual is to be designated, indicate name in body of form to right of division.
Draw double line under conclusion of individual's dated recommendation.

GPO 889-430

000159

000320

April 26, 1961

Mr. Gerald W. Hickey
Cyanamid Chemical Company, Inc.
Woonsocket, Rhode Island

Dear Mr. Hickey:

We regret the delay in replying to your letter of March 30, 1961, in which you suggest a change in the formulation previously described as .

In our letter of March 30, 1960, we indicated that under defined conditions of use we did not consider the product to be a food additive. The proposed change in formulation for the product referred to on March 30, 1961, involves the addition of .
and the replacement of the previously described,
by an manufactured by , the latter is also a

The new formulation now identified as is to be added to the white water in the form of paper and paperboard in a concentration which results in a chlorine dioxide content in the white water of 6.9 part per million.

While our reply should not be construed as an approval of your product, under the described conditions of use, we would not consider your new product to be a food additive as defined by Section 201(s) of the Federal Food, Drug, and Cosmetic Act.

Sincerely yours,

J. K. Kirk
Assistant Commissioner

cc: R
FA DF BP EK

FACassidy:mlo 4-26-61
R/D FAC:jmm 4-19-61/4-25-61 Sgd: JKKirk

JAN 23 1961

AF 30-103

Mr. C. P. Kirchen
Buckman Laboratories, Inc.
Memphis 8, Tennessee

Dear Mr. Kirchen:

This will reply to your letter of December 1, 1960, in which you request the status of five substances with respect to the manufacture of paper and paperboard for food packaging.

It would be our opinion that sodium chlorite and chlorine dioxide when used according with the principles of good manufacturing practice would be generally recognized as safe in the manufacture of paper and paperboard for food packaging use.

Acrolein, 3,5-dimethyltetrahydrothiadiazine-2-thione with isopropanol and 3,5-dimethyltetrahydroxy-1,3,5,2H-thiadiazine-2-thione are substances for which exemptions have not been granted to provide for their use in the manufacture of paper and paperboard nor are they subjects of any regulations for the proposed use purpose.

Sincerely yours,

Frederick A. Cassidy
Food and Drug Officer

Determination of Ions in Distillers Grains by Ion Chromatography

(Based on Method EPA 300.1)

1.0 SCOPE AND APPLICATION

1.1. This method covers the determination of the following inorganic anions in distillers grains. As a result of different specified injection volumes (See conditions in Tables 1A and 1B), these anions are divided between the common anions listed in Part A and the inorganic disinfection by-products listed in Part B. These different injection volumes are required in order to compensate for the relative concentrations of these anions in distillers grains and maintain good chromatographic peak shape throughout the expected dynamic range of the detector. Bromide is included in both Part A, due to its importance as a common anion, as well as Part B due to its critical role as a disinfection by-product precursor.

PART A.-- Common Anions

Bromide	Nitrite
Chloride	ortho-Phosphate-P
Fluoride	Sulfate
Nitrate	

PART B.-- Inorganic Disinfection By-products

Bromate	Chlorite
Bromide	Chlorate

1.2. The single laboratory Method Detection Limits (MDL, defined in Sect. 3.11) for the above analytes are listed in Tables 1A, 1B and 1C. The MDL for a specific matrix may differ from those listed, depending upon the nature of the sample and the specific instrumentation employed..

1.2.1. In order to achieve comparable detection limits, an ion chromatographic system must utilize suppressed conductivity detection, be properly maintained and must be capable of yielding a baseline with no more than 5 nS noise/drift per minute of monitored response over the background conductivity.

1.3. This method is recommended for use only by or under the supervision of analysts experienced in the use of ion chromatography and in the interpretation of the resulting ion chromatograms.

1.4. When this method is used to analyze unfamiliar samples for any of the above anions, anion identification should be supported by the use of a fortified sample matrix covering the anions of interest. The fortification procedure is described in Sect. 9.4.1.

1.5. Users of the method data should state the data-quality objectives prior to

analysis. Users of the method must demonstrate the ability to generate acceptable results with this method, using the procedures described in Sect. 9.0.

1.6. Bromide and nitrite react with most oxidants employed as disinfectants. The utility of measuring these anions in treated water should be considered prior to conducting the analysis.

2.0 SUMMARY OF METHOD

2.1. A small volume of sample, 10 µL for Part A and 50 µL for Part B, is introduced into an ion chromatograph. The anions of interest are separated and measured, using a system comprised of a guard column, analytical column, suppressor device, and conductivity detector.

2.2. The ONLY difference between Parts A and B is the volume of sample analyzed by the ion chromatographic system. The separator columns and guard columns as well as eluent conditions are identical.

3.0 DEFINITIONS

3.1. ANALYSIS BATCH -- A group of no more than 20 field samples (Field sample analyses include only those samples derived from a field sample matrix. These include the initial and duplicate field samples as well as all Laboratory Fortified Sample Matrices). The analysis batch must include an Initial Calibration Check Standard, an End Calibration Check Standard, Laboratory Reagent Blank, and a Laboratory Fortified Blank. Within an ANALYSIS BATCH, for every group of ten field samples, at least one Laboratory Fortified Matrix (LFM) and either a Field Duplicate, a Laboratory Duplicate or a duplicate of the LFM must be analyzed. When more than 10 field samples are analyzed, a Continuing Calibration Check Standard must be analyzed after the tenth field sample analysis.

3.2. CALIBRATION STANDARD (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions and the surrogate analyte. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.2.1. INITIAL CALIBRATION STANDARDS -- A series of CAL solutions used to initially establish instrument calibration and develop calibration curves for individual target anions.

3.2.2. INITIAL CALIBRATION CHECK STANDARD -- An individual CAL solution, analyzed initially, prior to any sample analysis, which verifies previously established calibration curves.

3.2.3. CONTINUING CALIBRATION CHECK STANDARD -- An individual CAL solution which is analyzed after every tenth field sample analyses which verifies the previously established calibration curves and confirms accurate analyte quantitation for the previous ten field samples analyzed.

3.2.4. END CALIBRATION CHECK STANDARD -- An individual CAL solution which is analyzed after the last field sample analyses which verifies the previously established calibration curves and confirms accurate analyte quantitation for all field samples analyzed since the last continuing calibration check.

3.3. FIELD DUPLICATES -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of field duplicates indicate the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.4. INSTRUMENT PERFORMANCE CHECK SOLUTION (IPC) -- A solution of one or more method analytes, surrogates, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.5. LABORATORY DUPLICATE -- Two sample aliquots, taken in the laboratory from a single sample bottle, and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated specifically with the laboratory procedures, removing any associated variables attributed by sample collection, preservation, or storage procedures.

3.6. LABORATORY FORTIFIED BLANK (LFB) -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.7. LABORATORY FORTIFIED SAMPLE MATRIX (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.8. LABORATORY REAGENT BLANK (LRB) – An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.9. LINEAR CALIBRATION RANGE (LCR) -- The concentration range over which the instrument response is linear.

3.10. MATERIAL SAFETY DATA SHEET (MSDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.11. METHOD DETECTION LIMIT (MDL) -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.12. MINIMUM REPORTING LEVEL (MRL) – The minimum concentration that can be reported for an anion in a sample following analysis. This defined concentration can be no lower than the concentration of the lowest calibration standard and can only be used if acceptable quality control criteria for this standard are met.

3.13. PERFORMANCE EVALUATION SAMPLE (PE) – A certified solution of method analytes whose concentration is unknown to the analyst. Often, an aliquot of this solution is added to a known volume of reagent water and analyzed with procedures used for samples. Results of analyses are used to determine statistically the accuracy and precision that can be expected when a method is performed by a competent analyst.

3.14. QUALITY CONTROL SAMPLE (QCS) – A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.15. SURROGATE ANALYTE – An analyte added to a sample, which is unlikely to be found in any sample at significant concentration, and which is added directly to a sample aliquot in known amounts before any sample processing procedures are conducted. It is measured with the same procedures used to measure other sample components. The purpose of the surrogate analyte is to monitor method performance with each sample.

3.16. STOCK STANDARD SOLUTION (SSS) – A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

4.0 INTERFERENCES

4.1. Interferences can be divided into three different categories: direct chromatographic coelution, where an analyte response is observed at very nearly the same retention time as the target anion; concentration dependant coelution, which is observed when the response of higher than typical concentrations of the neighboring peak overlap into the retention window of the target anion; and, ionic character displacement, where retention times may significantly shift due to the influence of high ionic strength matrices (high mineral content or hardness) overloading the exchange sites in the column and significantly shortening target analyte's retention times.

4.1.1. A direct chromatographic coelution may be solved by changing columns, eluent strength, modifying the eluent with organic solvents (if compatible with IC columns), changing the detection systems, or selective removal of the interference with pretreatment. Sample dilution will have little to no effect.

4.1.2. Sample dilution may resolve some of the difficulties if the interference is the result of either concentration dependant coelution or ionic character displacement, but it must be clarified that **sample dilution will alter your Minimum Reporting Limit (MRL)** by a proportion equivalent to that of the dilution. Therefore, careful consideration of project objectives should be given prior to performing such a dilution. An alternative to sample dilution, may be dilution of the eluent as outlined in 11.9.

4.1.3. Pretreatment cartridges can be effective as a means to eliminate certain matrix interferences. Prior to using any pretreatment, the analyst should be aware that all instrument calibration standards must be pretreated in exactly the same manner as the pretreated unknown field samples. The need for these cartridges have been greatly reduced with recent advances in high capacity anion exchange columns.

4.1.4. Extreme caution should be exercised in using these pretreatment cartridges. Artifacts are known to leach from certain cartridges which can foul the guard and analytical columns causing loss of column capacity indicated by shortened retention times and irreproducible results. Frequently compare your calibration standard

chromatograms to those of the column test chromatogram (received when the column was purchased) to insure proper separation and similar response ratios between the target analytes is observed.

4.2. Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that lead to discrete artifacts or elevated baselines in an ion chromatogram. These interferences can lead to false positive results for target analytes as well as reduced detection limits as a consequence of elevated baseline noise.

4.3. Samples that contain particles larger than 0.45 microns and reagent solutions that contain particles larger than 0.20 microns require filtration to prevent damage to instrument columns and flow systems.

4.4. Any anion that is only weakly retained by the column may elute in the retention time window of fluoride and potentially interfere. At concentrations of fluoride above 1.5 mg/L, this interference may not be significant, however, it is the responsibility of the user to generate precision and accuracy information in each sample matrix.

4.5. Close attention should be given to the potential for carry over peaks from one analysis which will effect the proper detection of analytes of interest in a second, subsequent analysis. Normally, the elution of sulfate (retention time of 13.8 min.) indicates the end of a chromatographic run, but, in the ozonated and chlorine dioxide matrices, which were included as part of the single operator accuracy and bias study (See Table 2B), a small response (200 nS baseline rise) was observed for a very late eluting unknown peak at approximately 23 minutes. Consequently, a run time of 25 minutes is recommended to allow for the proper elution of any potentially interferant late peaks. It is the responsibility of the user to confirm that no late eluting peaks have carried over into a subsequent analysis thereby compromising the integrity of the analytical results.

4.6. Any residual chlorine dioxide present in the sample will result in the formation of additional chlorite prior to analysis. If any concentration of chlorine dioxide is suspected in the sample, the sample must be purged with an inert gas (helium, argon or nitrogen) for approximately five minutes or until no chlorine dioxide remains. This sparging must be conducted prior to ethylenediamine preservation and at time of sample collection.

5.0 SAFETY

5.1. The toxicity or carcinogenicity of each reagent used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable.

Cautions are included for known extremely hazardous materials or procedures.

5.2. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data Sheets (MSDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

5.3. The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.

5.3.1. Sulfuric acid -- When used to prepared a 25 mN sulfuric acid regenerant solution for chemical suppression using a Dionex Anion Micro Membrane Suppressor (AMMS).

6.0 Equipment and Supplies

6.1. Ion chromatograph -- Analytical system complete with ion chromatograph and all required accessories including syringes, analytical columns, compressed gasses and a conductivity detector.

6.1.1. Anion guard column: Dionex AG9-HC, 2 mm (P/N 52248), or equivalent. This column functions as a protector of the separator column. If omitted from the system the retention times will be shorter.

6.1.2. Anion separator column: Dionex AS9-HC column, 2 mm (P/N 52244), or equivalent. The microbore (2 mm) was selected in the development of this method as a means to tighten the bromate elution band and thus reduce the detection limit. An optional column (2 mm or 4 mm) may be used if comparable resolution of peaks is obtained, and the requirements of Sect. 9.0 can be met. The AS9-HC, 2 mm column using the conditions outlined in Table 1A and 1B produced the separation shown in Figures 1 through 4.

6.1.2.1. If a 4 mm column is employed, the injection volume should be raised by a factor of four to 40 µL for Part A anions and 200 µL for Part B anions in order to attain comparable detection limits. A four fold increase in injection volume compensates for the four fold increase in cross sectional surface area of the 4 mm standard bore column over the 2 mm microbore column.

6.1.2.2. Comparable results can be attained using the Dionex, AS9-HC, 4 mm column. MDLs for the part B, inorganic

disinfection by-products using this 4 mm column are displayed along with analysis conditions in Table 1C.

6.1.3. Anion suppressor device: The data presented in this method were generated using a Dionex Anion Self Regenerating Suppressor (ASRS, P/N 43187). An equivalent suppressor device may be utilized provided comparable detection limits are achieved and adequate baseline stability is attained as measured by a combined baseline drift/noise of no more than 5 nS per minute over the background conductivity.

6.1.3.1. The ASRS was set to perform electrolytic suppression at a current setting of 100 mA using an external source DI water mode. Insufficient baseline stability was observed using the ASRS in recycle mode.

6.1.4. Detector -- Conductivity cell (Dionex CD20, or equivalent) capable of providing data as required in Sect. 9.2.

6.2. The Dionex Peaknet Data Chromatography Software was used to generate all the data in the attached tables. Systems using a strip chart recorder and integrator or other computer based data system may achieve approximately the same MDL's but the user should demonstrate this by the procedure outlined in Sect. 9.2.

6.3. Analytical balance, ±0.1 mg sensitivity. Used to accurately weigh target analyte salts for stock standard preparation.

6.4. Top loading balance, ±10 mg sensitivity. Used to accurately weigh reagents to prepare eluents.

6.5. Weigh boats, plastic, disposable - for weighing eluent reagents.

6.6. Syringes, plastic, disposable, 10 mL - used during sample preparation.

6.7. Pipets, Pasteur, plastic or glass, disposable, graduated, 5 mL and 10 mL.

6.8. Bottles, high density polyethylene (HDPE), opaque or glass, amber, 15, 20, 30, 125, and 250 mL. For sampling and storage of calibration solutions. Opaque or amber due to the photoreactivity of chlorite anion.

6.9. Micro beakers, plastic, disposable – used during sample preparation.

6.10. Balance able to be tared.

6.11. Sterile spatula – to collect sample.

6.12. Sterile gloves for sample collection.

6.13. Centrifuge capable of maintaining 4°C.

6.14. Filter paper for filtering solution instead of centrifuging.

7.0 Reagents and Standards

7.1. Reagent water: Distilled or deionized water, free of the anions of interest. Water should contain particles no larger than 0.20 microns.

7.2. Eluent solution : Sodium carbonate (CASRN 497-19-8) 9.0 mM. Dissolve 1.91 g sodium carbonate (Na_2CO_3) in reagent water and dilute to 2 L.

7.2.1. This eluent solution must be purged for 10 minutes with helium prior to use to remove dissolved gases which may form micro bubbles in the IC compromising system performance and adversely effecting the integrity of the data.

7.3. Stock standard solutions, 1000 mg/L (1 mg/mL): Stock standard solutions may be purchased as certified solutions or prepared from ACS reagent grade, potassium or sodium salts as listed below, for most analytes. Chlorite requires careful consideration as outline below in 7.3.5.1.

7.3.1. Bromide (Br^-) 1000 mg/L: Dissolve 0.1288 g sodium bromide (NaBr, CASRN 7647-15-6) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.2. Bromate (BrO_3^-) 1000 mg/L: Dissolve 0.1180 g of sodium bromate ($NaBrO_3$, CASRN 7789-38-0) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.3. Chlorate (ClO_3^-) 1000 mg/L: Dissolve 0.1275 g of sodium chlorate ($NaClO_3$, CASRN 7775-09-9) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.4. Chloride (Cl^-) 1000 mg/L: Dissolve 0.1649 g sodium chloride (NaCl, CASRN 7647-14-5) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.5. Chlorite (ClO_2^-) 1000 mg/L: Assuming an exact 80.0 % $NaClO_2$ is amperometrically titrated from technical grade $NaClO_2$ (See Sect. 7.3.5.1). Dissolve 0.1676 g of sodium chlorite ($NaClO_2$, CASRN 7758-19-2) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.5.1. High purity sodium chlorite ($NaClO_2$) is not currently commercially available due to potential explosive instability. Recrystallization of the technical grade (approx. 80%) can be performed but it is labor intensive and time consuming. The simplest approach is to determine the exact % $NaClO_2$ using the iodometric titration procedure (Standard Methods, 19th Ed., 4500-ClO_2.C). Following titration, an individual component standard of chlorite must be analyzed to determine if there is any significant contamination (greater than 1% of the chlorite weight) in the technical grade chlorite standard from any of the Part B components. These contaminants will place a high bias on the calibration of the other anions if all four Part B components are mixed in an combined calibration solution. If these other anions are present as contaminants, a separate chlorite calibration needs to be performed.

7.3.6. Fluoride (F^-) 1000 mg/L: Dissolve 0.2210 g sodium fluoride (NaF, CASRN 768 1-49-4) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.7. Nitrate (NO_3-N) 1000 mg/L: Dissolve 0.6068 g sodium nitrate ($NaNO_3$, CASRN 7631-99-4) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.8. Nitrite (NO_2-N) 1000 mg/L: Dissolve 0.4926 g sodium nitrite ($NaNO_2$, CASRN 7632-00-0) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.9. Phosphate (PO_4-P) 1000 mg/L: Dissolve 0.4394 g potassium dihydrogenphosphate (KH_2PO_4, CASRN 7778-77-0) in reagent water and dilute to 100 mL in a volumetric flask.

7.3.10. Sulfate (SO_4) 1000 mg/L: Dissolve 0.1814 g potassium sulfate (K_2SO_4, CASRN 7778-80-5) in reagent water and dilute to 100 mL in a volumetric flask.

NOTE: Stability of standards: Stock standards (7.3) for most anions are stable for at least 6 months when stored at 4°C. Except for the chlorite standard which is only stable for two weeks when stored protected from light at 4°C, and nitrite and phosphate which are only stable for 1 month when stored at 4°C. Dilute working standards should be prepared monthly, except those that contain chlorite, or nitrite and phosphate which should be prepared fresh daily.

7.4. Ethylenediamine (EDA) preservation solution, 100 mg/mL: Dilute 2.8 mL of ethylenediamine (99%) (CASRN 107-15-3) to 25 mL with reagent water. Prepare fresh monthly.

8.0 Distiller Grains Sample Collection, Preservation and Storage

8.1. Collection of distiller grain samples from the fermentation process:

8.1.1. Wearing sterile gloves, carefully open a sterile bottle into which the sample will be placed. Care must be taken in order to minimize potential contamination of the bag or bottle from other sources.

8.1.2. Place the sterile bottle onto a balance and tare the balance.

8.1.3. Using a sterile spatula, or equivalent, transfer 5g into the sterile bottle. Close the bag or bottle well.

8.1.4. Label the sample with the following information: (1) where in the process the sample was taken (*e.g.*, the centrifuge); (2) moisture content of the sample; (3) date, time, and plant the sample was taken; (4) temperature of the sample when taken (if known); and (5) the amount of ClO_2 used during fermentation.

8.1.5. Let sample cool to room temperature.

8.1.6. Add de-ionized water to each sample to bring up the moisture volume to 10 mls, add preservative as described in 8.3, protect from light, and place in refrigerator. Special sampling requirements and precautions for chlorite:

8.1.6.1. Sample bottles used for chlorite analysis must be opaque to protect the sample from light.

8.1.6.2. When preparing the LFM, be aware that chlorite is an oxidant and may react with the natural organic matter in an untreated drinking water matrix as a result of oxidative demand. If untreated water is collected for chlorite analysis, and subsequently used for the LFM, EDA preservation will not control this demand and reduced chlorite recoveries may be observed.

8.1.7. Ship sample with cold packs.

8.2. Need to separate biomass from water solution for analysis.

8.2.1. Centrifuge or filter samples at 4°C while protecting sample from light.

8.2.2. Remove aqueous phase and store at 4°C protected from light until use.

8.3. Sample preservation and holding times for the anions that can be determined by this method are as follows:

PART A : Common Anions

Analyte	Preservation	Holding Time
Bromide	None required	28 days
Chloride	None required	28 days
Fluoride	None required	28 days
Nitrate-N	Cool to 4°C	48 hours
Nitrite-N	Cool to 4°C	48 hours
ortho-Phosphate-P	Cool to 4°C	48 hours
Sulfate	Cool to 4°C	28 days

PART B.: Inorganic Disinfection By-products

Analyte	Preservation	Holding Time
Bromate	50 mg/L EDA	28 days
Bromide	None required	28 days
Chlorate	50 mg/L EDA	28 days
Chlorite	50 mg/L EDA, Cool to 4°C	14 days

8.4. When collecting a sample from a ethanol plant employing chlorine dioxide, the sample must be sparged with an inert gas (helium, argon, nitrogen) prior to addition of the EDA preservative at time of sample collection.

8.5. All four anions, in Part B, can be analyzed in a sample matrix which has been preserved with EDA. Add a sufficient volume of the EDA preservation solution (Sect. 7.4) such that the final concentration is 50 mg/L in the sample. This would be equivalent to adding 0.5 mL of the EDA preservation solution to 1 L of sample.

8.6. EDA is primarily used as a preservative for chlorite. Chlorite is susceptible to degradation both through catalytic reactions.

9.0 QUALITY CONTROL

9.1. (b) (4) [redacted]

(b) (4)

[redacted]

9.2. INITIAL DEMONSTRATION OF PERFORMANCE

9.2.1. The initial demonstration of performance is used to characterize instrument performance (determination of accuracy through the analysis of the QCS) and laboratory performance (determination of MDLs) prior to performing analyses by this method.

9.2.2. Quality Control Sample (QCS) -- When beginning the use of this method, on a quarterly basis or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS. If the determined concentrations are not within ± 15% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with on-going analyses.

9.2.3. Method Detection Limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of three to five times the estimated instrument detection limit. To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method over at least three separate days. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

$$MDL = (t) \times (S)$$

where, t = Student's *t* value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates].

S = standard deviation of the replicate analyses.

9.2.3.1. MDLs should be determined every 6 months, when a new operator begins work or whenever there is a significant change in the background, or instrument response.

9.3. ASSESSING LABORATORY PERFORMANCE

9.3.1. Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one LRB with each analysis batch (defined Sect 3.1). Data produced are used to assess contamination from the laboratory environment. Values that exceed the MDL indicate laboratory or reagent contamination should be suspected and corrective actions must be taken before continuing the analysis.

9.3.1.1. If conducting analysis for the Part B anions, EDA must be added to the LRB at 50 mg/L. By including EDA in the LRB, any bias as a consequence of the EDA which may be observed in the field samples, particularly in terms of background contamination, will be identified.

9.3.2. Laboratory Fortified Blank (LFB) -- The LFB should be prepared at concentrations similar to those expected in the field samples and ideally at the same concentration used to prepare the LFM. Calculate accuracy as percent recovery (Sect. 9.4.1.3). If the recovery of any analyte falls outside the required concentration dependant control limits (Sect. 9.3.2.2), that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.2.1. If conducting analysis for the Part B anions, EDA must be added to the LFB at 50 mg/L. The addition of EDA to all reagent water prepared calibration and quality control samples is required not as a preservative but rather as a means to normalize any bias attributed by the presence of EDA in the field samples.

9.3.2.2. Control Limits for the LRB

Concentration range	Percent Recovery Limits
MRL to 10xMRL	75 - 125 %
10xMRL to highest calibration level	85 - 115 %

9.3.2.3. These control limits only apply if the MRL is established within a factor of 10 times the MDL. Otherwise, the limits are set at 85% to 115%.

9.3.2.4. The laboratory must use the LRB to assess laboratory performance against the required control limits listed in 9.3.2.2. When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

$$\text{UPPER CONTROL LIMIT} = x + 3S$$
$$\text{LOWER CONTROL LIMIT} = x - 3S$$

The optional control limits must be equal to or better than those listed in 9.3.2.2. After each five to ten new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an on-going precision statement for the level of concentrations monitored. These data must be kept on file and be available for review.

9.3.3. Instrument Performance Check Solution (IPC) -- The Initial Calibration Check Standard is to be evaluated as the instrument performance check solution in order to confirm proper instrument performance. Proper chromatographic performance must be demonstrated by calculating the Peak Gaussian Factor (PGF), which is a means to measure peak symmetry and monitoring retention time drift in the surrogate peak over time. Critically evaluate the surrogate peak in the initial calibration check standard, and calculate the PGF as follows,

$$= \frac{1.83 \times W(1/2)}{W(1/10)} \text{ PGF}$$

where: W(1/2) is the peak width at half height
W(1/10) is the peak width at tenth height

9.3.3.1. The PGF must fall between 0.80 and 1.15 in order to demonstrate proper instrument performance.

9.3.3.2. The retention time for the surrogate in the IPC must be closely monitored on each day of analysis and throughout the lifetime of the analytical column. Small variations in retention time can be anticipated when a new solution of eluent is prepared but if shifts of more than 2% are observed in the surrogate retention time, some type of instrument problem is present. Potential problems include improperly prepared eluent, erroneous method parameters programmed such as flow rate or some other system problem. The chromatographic profile (elution order) of the target anions following an ion chromatographic analysis should closely replicate the profile displayed in the test chromatogram that was shipped when the column was purchased. As a column ages, it is normal to see a gradual shift and shortening of retention times, but if after several years of use, extensive use over less than a year, or use with harsh samples, this retention time has noticeably shifted to any less than 80% of the original recorded value, the column may require cleaning or replacement. Particularly if resolution problems are beginning to become common between previously resolved peaks. A laboratory must retain a historic record of retention times for the surrogate and all the target anions to provide evidence of an analytical columns vitality.

9.4. ASSESSING ANALYTE RECOVERY AND DATA QUALITY

9.4.1. Laboratory Fortified Sample Matrix (LFM) -- The laboratory must add a known amount of analyte to a minimum of 10% of the field samples within an analysis batch. The LFM sample must be prepared from a sample matrix which has been analyzed prior to fortification. The analyte concentration must be high enough to be detected above the original sample and should adhere to the requirement of 9.4.1.2. It is recommended that the solutions used to fortify the LFM be prepared from the same stocks used to prepare the calibration standards and not from external source stocks. This will remove the bias contributed by an externally prepared stock and focus on any potential bias introduced by the field sample matrix.

9.4.1.1. If the fortified concentration is less than the observed background concentration of the unfortified matrix, the

recovery should not be calculated. This is due to the difficulty in calculating accurate recoveries of the fortified concentration when the native sample concentration is so high.

9.4.1.2. The LFM should be prepared at concentrations no greater than five times the highest concentration observed in any field sample. If no analyte is observed in any field sample, the LFM must be fortified no greater than five times the lowest calibration level which as outlined in 12.2 is the minimum reported level (MRL). For example, if bromate is not detected in any field samples above the lowest calibrations standard concentration of 5.00 µg/L, the highest LFM fortified concentration allowed is 25.0 µg/L.

9.4.1.3. Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample. Percent recovery should be calculated using the following equation:

$$R = \frac{C_s - C}{s} \times 100$$

where, R = percent recovery.
C_s = fortified sample concentration
C = sample background concentration
s = concentration equivalent of analyte added to sample.

9.4.1.4. Until sufficient data becomes available (usually a minimum of 20 to 30 analysis), assess laboratory performance against recovery limits of 75 to 125%. When sufficient internal performance data becomes available develop control limits from percent mean recovery and the standard deviation of the mean recovery. The optional control limits must be equal to or better than the required control limits of 75-125%.

9.4.1.5. If the recovery of any analyte falls outside the designated LFM recovery range and the laboratory performance for that analyte is shown to be in control (Sect. 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related,

not system related.

9.4.2. SURROGATE RECOVERY -- Calculate the surrogate recovery from all analyses using the following formula

$$R = \frac{SRC}{SFC} \times 100$$

where, R = percent recovery.
SRC = Surrogate Recovered Concentration
SFC = Surrogate Fortified Concentration

9.4.2.1. Surrogate recoveries must fall between 90-115% for proper instrument performance and analyst technique to be verified. The recovery of the surrogate is slightly bias to 115% to allow for the potential contribution of trace levels of dichloroacetate as the halogenated organic disinfection by-product (DBP) dichloroacetic acid (DCAA) Background levels of this organic DBP are rarely observed above 50 µg/L (0.05 mg/L) which constitutes only 5% of the 1.00 mg/L recommended fortified concentration.

9.4.2.2. If the surrogate recovery falls outside the 90-115% recovery window, a analysis error is evident and sample reanalysis is required. Poor recoveries could be the result of imprecise sample injection or analyst fortification errors.

9.4.3. FIELD or LABORATORY DUPLICATES -- The laboratory must analyze either a field or a laboratory duplicate for a minimum of 10% of the collected field samples or at least one with every analysis batch, whichever is greater. The sample matrix selected for this duplicate analysis must contain measurable concentrations of the target anions in order to establish the precision of the analysis set and insure the quality of the data. If none of the samples within an analysis batch have measurable concentrations, the LFM should be employed as a laboratory duplicate.

9.4.3.1. Calculate the percent difference (%Diff) of the initial quantitated concentration (Ic) and duplicate quantitated concentration (D_c) using the following formula,

$$\%Diff = \frac{(I_c - D_c)}{([I_c + D_c]/2)} \times 100$$

9.4.3.2. Duplicate analysis acceptance criteria

Concentration range	%Diff Limits
MRL to 10xMRL	± 20 %
10xMRL to highest calibration level	± 10 %

9.4.3.3. If the %Diff fails to meet these criteria, the samples must be reanalyzed.

9.4.4. Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

9.4.5. In recognition of the rapid advances occurring in chromatography, the analyst is permitted certain options, such as the use of different columns, injection volumes, and/or eluents, to improve the separations or lower the cost of measurements. Each time such modifications to the method are made, the analyst is required to repeat the procedure in Sect. 9.2 and adhere to the condition of baseline stability found in Sect. 1.2.1.

9.4.6. It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Whenever possible, the laboratory should perform analysis of quality control check samples and participate in relevant performance evaluation sample studies.

10.0 Calibration and Standardization

10.1. Establish ion chromatographic operating parameters equivalent to those indicated in Tables 1A or 1B if employing a 2 mm column, Table 1C if employing a 4 mm column.

10.2. Estimate the Linear Calibration Range (LCR) – The LCR should cover the expected concentration range of the field samples and should not extend over more than 2 orders of magnitude in concentration (For example, if quantitating nitrate in the expected range of 1.0 mg/L to 10 mg/L, 2 orders of magnitude would permit the minimum and maximum calibration standards of 0.20 mg/L and 20 mg/L, respectively.) The

restriction of 2 orders of magnitude is prescribed since beyond this it is difficult to maintain linearity throughout the entire calibration range.

10.2.1. If quantification is desired over a larger range, then two separate calibration curves should be prepared.

10.2.2. For an individual calibration curve, a minimum of three calibration standards are required for a curve that extends over a single order of magnitude and a minimum of five calibration standards are required if the curve covers two orders of magnitude. (For example, using the nitrate example cited above in section 10.2, but in this case limit the curve to extend only from 1.0 mg/L to 10 mg/L or a single order of magnitude. A third standard is required somewhere in the middle of the range. For the calibration range of 0.20 mg/L to 20 mg/L, over two orders of magnitude, five calibrations standards should be employed, one each at the lower and upper concentration ranges and the other three proportionally divided throughout the middle of the curve.)

10.3. Prepare the calibration standards by carefully adding measured volumes of one or more stock standards (7.3) to a volumetric flask and diluting to volume with reagent water.

10.3.1. For the Part B anions, EDA must be added to the calibration standards at 50 mg/L. The addition of EDA to all reagent water prepared calibration and quality control samples is required not as a preservative but rather as a means to normalize any bias attributed by the presence of EDA in the field samples.

10.3.2. Prepare a 10.0 mL aliquot of surrogate fortified calibration solution which can be held for direct manual injection or used to fill an autosampler vial. Add 20 µL of the surrogate solution (7.5) to a 20 mL disposable plastic micro beaker. Using a 10.0 mL disposable pipet, place exactly 10.0 mL of calibration standard into the micro beaker and mix. The calibration standard is now ready for analysis. The same surrogate solution that has been employed for the standards should also be used in the section 11.3.2 for the field samples.

10.4. Using a 2 mm column, inject 10 µL (Part A) or 50 µL (Part B) of each calibration standard. Using a 4 mm column, inject 50 µL (Part A) or 200 µL (Part B) of each calibration standard. Tabulate peak area responses against the concentration. The results are used to prepare calibration curves using a linear least squares fit for each analyte. Acceptable calibration curves are confirmed after reviewing the curves for linearity and passing the criteria for

the initial calibration check standard in section 10.5.1. Alternately, if the ratio of response to concentration (response factor) is constant over the LCR (indicated by < 15% relative standard deviation (RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve,

10.4.1. Peak areas are strongly recommended since they have been found to be more consistent, in terms of quantitation, than peak heights. Peak height can tend to be suppressed as a result of high levels of common anions in a given matrix which can compete for exchange sites. Using peak areas, it is the analyst responsibility to review all chromatograms to insure accurate baseline integration of target analyte peaks since poorly drawn baselines will more significantly influence peak areas than peak heights.

10.5. Once the calibration curves have been established they must be verified prior to conducting any sample analysis using an initial calibration check standard (3.2.2). This verification must be performed on each analysis day or whenever fresh eluent has been prepared. A continuing calibration check standard (3.2.3) must be analyzed after every tenth sample and at the end of the analysis set as an end calibration check standard (3.2.4). The response for the initial, continuing and end calibration check must satisfy the criteria listed in 10.5.1. If during the analysis set, the response differs by more than the calibration verification criteria shown in 10.5.1., or the retention times shift more than ± 5% from the expected values for any analyte, the test must be repeated, using fresh calibration standards. If the results are still outside these criteria, sample analysis must be discontinued, the cause determined and/or in the case of drift, the instrument recalibrated. All samples following the last acceptable calibration check standard must be reanalyzed.

10.5.1. Control limits for calibration verification

Concentration range	Percent Recovery Limits
MRL to 10xMRL	75 - 125 %
10xMRL to highest calibration level	85 - 115 %

10.5.1.1. These control limits only apply if the MRL is established within a factor of 10 times the MDL. Otherwise, the limits are set at 85% to 115%.

10.5.2. SPECIAL CALIBRATION VERIFICATION REQUIREMENT FOR PART B

As a mandatory requirement of calibration verification, the laboratory MUST verify calibration using the lowest calibration standard as the initial calibration check standard.

10.5.3. After satisfying the requirement of 10.5.2, the levels selected for the other calibration check standards should be varied between a middle calibration level and the highest calibration level.

11.0 Procedure

11.1. Tables 1A and 1B summarize the recommended operating conditions for the ion chromatograph. Included in these tables are estimated retention times that can be achieved by this method. Other columns, chromatographic conditions, or detectors may be used if the requirements of Sect. 9.2 are met.

11.2. Check system calibration daily and, if required, recalibrate as described in Sect. 10.

11.3. Sample Preparation

11.3.1. For refrigerated or samples arriving to the laboratory cold, ensure the samples have come to room temperature prior to conducting sample analysis by allowing the samples to warm on the bench for at least 1 hour.

11.3.2. Prepare a 10.0 mL aliquot of surrogate fortified sample which can be held for direct manual injection or used to fill an autosampler vial. Add 20 µL of the surrogate solution (7.5) to a 20 mL disposable plastic micro-beaker. Using a 10.0 mL disposable pipet, place exactly 10.0 mL of sample into the micro beaker and mix. Sample is now ready for analysis.

11.3.2.1. The less than 1% dilution error introduced by the addition of the surrogate is considered insignificant.

11.4. Using a Luer lock, plastic 10 mL syringe, withdraw the sample from the micro beaker and attach a 0.45 µm particulate filter (demonstrated to be free of ionic contaminants) directly to the syringe. Filter the sample into an autosampler vial (If vial is not designed to automatically filter) or manually load the injection loop injecting a fixed amount of well mixed sample. If using a manually loaded injection loop, flush the loop thoroughly between sample analysis using sufficient volumes of each new sample matrix.

11.5. Using a 2 mm column, inject 10 µL (Part A) or 50 µL (Part B) of each sample. Using a 4 mm column, inject 40 µL (Part A) or 200 µL (Part B) of each sample. Tabulate peak area responses against the concentration. During this procedure, retention times must be recorded. Use the same size loop for standards and samples. Record the resulting peak size in area units.

An automated constant volume injection system may also be used.

11.6. The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time can be used to calculate a suggested window size for each analyte. However, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

11.7. If the response of a sample analyte exceeds the calibration range, the sample may be diluted with an appropriate amount of reagent water and reanalyzed. If this is not possible then three new calibration concentrations must be employed to create a separate high concentration curve, one standard near the estimated concentration and the other two bracketing around an interval equivalent to ± 25% the estimated concentration. The latter procedure involves significantly more time than a simple sample dilution therefore, it is advisable to collect sufficient sample to allow for sample dilution or sample reanalysis, if required.

11.8. Shifts in retention time are inversely proportional to concentration. Nitrate, phosphate and sulfate will exhibit the greatest degree of change, although all anions can be affected. In some cases this peak migration may produce poor resolution or make peak identification difficult.

11.9. Should more complete resolution be needed between any two coeluting peaks, the eluent (7.2) can be diluted. This will spread out the run, however, and will cause late eluting anions to be retained even longer. The analyst must determine to what extent the eluent is diluted. This dilution is not be considered a deviation from the method. If an eluent dilution is performed, section 9.2 must be repeated.

11.9.1. Eluent dilution will reduce the overall response of an anion due to chromatographic band broadening which will be evident by shortened and broadened peaks. This will adversely effect the MDLs for each analyte.

12.0 DATA ANALYSIS AND CALCULATIONS

12.1. Prepare a calibration curve for each analyte by plotting instrument response, as peak area, against standard concentration. Compute sample concentration by comparing sample response with the standard curve. If a sample has been diluted, multiply the response by the appropriate dilution factor.

12.2. Report ONLY those values that fall between the lowest and the highest

calibration standards. Samples with target analyte responses exceeding the highest standard should be diluted and reanalyzed. Samples with target analytes identified but quantitated below the concentration established by the lowest calibration standard cannot be reported since the lowest calibrated concentration is the minimum reporting limit (MRL).

12.3. Report results for Part A anions in mg/L and for Part B anions in µg/L.

12.4. Report NO_2^- as N, NO_3^- as N, $HPO_4^=$ as P, Br^- in mg/L when reported with Part A, and Br^- in µg/L when reported with Part B

APPENDIX 8
Residual Levels of Chlorate, Chlorite and Chloride Ions Present in Distillers Grains

The following table summarizes the testing results reported for residual levels of chlorate, chlorite, and chloride ions in distillers grains separated from the fermentation process water which has been treated with the FCS. The residual levels of these by-products are expressed on a dry weight basis.

Levels of Chlorate, Chlorite, and Chloride in Distiller's Grains

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Sample	ClO_2 Dose (ppm)	% Solids	Chlorite (mg/kg)	Chlorate (mg/kg)	Chloride (mg/kg)
(b) (4) [redacted]	52.5	31.1%	<0.8	14.0	1903
	52.5	32.8%	<0.8	12.8	1783
	52.5	84.9%	<0.2	10.9	1637
	52.5	31.1%	<0.6	13.3	2464
	55	85%	<0.2	8.3	2807
	55	31.1%	<0.6	7.5	2465
	55	85%	<0.2	9.5	1543
	-		<0.3	<0.3	1050

In those cases where the actual test reports did not report the levels of chlorate, chlorite, and chloride on a dry weight basis, samples 19J0395-01, 19J0395-02, 19J1120-01 and 19J1120-02, we used an average solids content of 31.1% for wet distillers grains and 85% solids for dry distillers grains.

(b) (4)

ANALYTICAL REPORT

November 04, 2009

Work Order: 19J0395

Report To

Allen M. Ziegler
Resonant BioSciences, LLC
11757 West Ken Caryl Avenue, F-308
Littleton, CO 80127

Work Order Information

Date Received: 10/07/2009 10:40AM
Collector:
Phone: (866) 933-0408
PO Number:

Project : PureMash
Project Number: [none]

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Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19J0395-01 (b) (4) Wet				Matrix:Solid	Collected: 10/06/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**4.2 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**783 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 10:59	
19J0395-01RE1 (b) (4) - Wet				Matrix:Solid	Collected: 10/06/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**4.1 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**734 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 11:14	
19J0395-01RE2 (b) (4) - Wet				Matrix:Solid	Collected: 10/06/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**4.1 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**782 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 11:28	
19J0395-02 (b) (4) - Dry				Matrix:Solid	Collected: 09/29/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**7.4 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**2340 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 11:42	
19J0395-02RE1 (b) (4) - Dry				Matrix:Solid	Collected: 09/29/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**6.9 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**2420 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 11:56	
19J0395-02RE2 (b) (4) - Dry				Matrix:Solid	Collected: 09/29/09 00:00		
Chlorite	<0.2 mg/kg wet	0.2	1K90424	300.1	KRM	11/04/09 14:04	
Chlorate	**6.9 mg/kg wet**	**0.2**	1K90424	300.1	KRM	11/04/09 14:04	
Chloride, soluble	**2400 mg/kg**	**10.0**	1K90436	300.1	KRM	11/04/09 12:10	

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

Work Order: 19J0395

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

Work Order: 19J0395

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

Work Order: 19J0395

End of Report

(b) (6)

Project Manager

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

ANALYTICAL REPORT

November 04, 2009

Work Order: 19J1120

Report To	Work Order Information
Allen M. Ziegler Resonant BioSciences, LLC 11757 West Ken Caryl Avenue, F-308 Littleton, CO 80127	Date Received: 10/21/2009 10:35AM Collector: Phone: (866) 933-0408 PO Number:

Project : PureMash

Project Number: [none]

(b) (4)

Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4) (b) (4) (b) (4) (b) (4)

(b) (4)

(b) (4)

November 04, 2009

Work Order: 19J1120

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes
(b) (4)										

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

05

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

(b) (4)

Littleton, CO 80127

November 04, 2009

Work Order: 19J1120

End of Report

(b) (6)

Project Manager

(b) (4)

(b) (4)

ANALYTICAL REPORT

January 06, 2010

Work Order: 19L1075

Report To	Work Order Information
Allen M. Ziegler Resonant BioSciences, LLC 11757 West Ken Caryl Avenue, F-308 Littleton, CO 80127	Date Received: 12/22/2009 1:45PM Collector: Phone: (866) 933-0408 PO Number:

Project : PureMash

Project Number: [redacted]

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Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19L1075-01 [redacted] Wet				Matrix:Solid	Collected: 12/15/09 14:00		
% Solids	**31.1 %**	**0.1**	1L92905	SM 2540 G	LJG	12/29/09 7:51	
Chlorite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
ate	**13.9 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1840 mg/kg dry**	**32.1**	1A00433	300.1	KRM	01/04/10 15:36	
19L1075-01RE1 [redacted] - Wet				Matrix:Solid	Collected: 12/15/09 14:00		
Chlorite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**14.2 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1960 mg/kg dry**	**32.1**	1A00433	300.1	KRM	01/04/10 15:50	
19L1075-01RE2 (b) (4) - Wet				Matrix:Solid	Collected: 12/15/09 14:00		
Chlorite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**13.9 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1910 mg/kg dry**	**32.1**	1A00433	300.1	KRM	01/04/10 16:04	
19L1075-02 (b) (4) - Wet				Matrix:Solid	Collected: 12/15/09 14:00		
% Solids	**32.8 %**	**0.1**	1L92905	SM 2540 G	LJG	12/29/09 7:51	
Chlorite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**13.2 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1810 mg/kg dry**	**30.5**	1A00433	300.1	KRM	01/04/10 16:19	
19L1075-02RE1 (b) (4) - Wet				Matrix:Solid	Collected: 12/15/09 14:00		
Chlorite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**12.9 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1780 mg/kg dry**	**30.5**	1A00433	300.1	KRM	01/04/10 16:33	
19L1075-02RE2 (b) (4) - Wet				Matrix:Solid	Collected: 12/15/09 14:00		
ite	<0.8 mg/kg dry	0.8	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**12.4 mg/kg dry**	**0.8**	1L93121	300.1	KRM	12/28/09 0:00	

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

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(b) (4)

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Work Order: 19L1075

19L1075-02RE2	(b) (4)	Wet			Matrix:Solid		Collected:	12/15/09 14:00
Chloride, soluble	**1760**	**mg/kg dry**	**30.5**	1A00433	300.1		KRM	01/04/10 16:47

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

(b) (4)

Determination of Conventional Chemistry Parameters - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

Code	Description	Number	Expires
IA-NT	(b) (4)	095	02/01/2010
KS-NT		E-10287	07/31/2010
NELAC		IA001	06/30/2010

End of Report

(b) (6)

ect Manager

(b) (4)

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Sample Recording Sheet

19L1075

Sample Information:

Sample Date: 12-15-09

Plant: (b) (4)

Location Sample was taken: (b) (4)

Is the sample dry or wet distillers grains? Wet

Moisture content of distillers grains: [redacted]% solids

Total amount of chlorine dioxide (ClO_2) used during fermentation: 45 ppm

(b) (4)

Sample Time	Sample Temperature	
2 Pm	140° F	01
2 Pm	140° F	02

Notes: Sample where refrigerated

Print Name (b) (6) Date 12-21-09

Signature

(b) (6)

(b) (4)

ANALYTICAL REPORT

January 06, 2010

Work Order: 19L1077

Report To

Allen M. Ziegler
Resonant BioSciences, LLC
11757 West Ken Caryl Avenue, F-308
Littleton, CO 80127

Work Order Information

Date Received: 12/22/2009 1:45PM
Collector:
Phone: (866) 933-0408
PO Number:

Project : PureMash
Project Number: (b) (4)

CONFIDENTIAL

Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19L1077-01 (b) (4) - Control Sample Mash				Matrix:Solid	Collected: 12/17/09 16:00		
% Solids	**22.3 %**	**0.1**	1L92905	SM 2540 G	LJG	12/29/09 7:51	
Chlorite	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
ate	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1010 mg/kg dry**	**10.8**	1A00433	300.1	KRM	01/04/10 17:01	
19L1077-01RE1 (b) (4) - Control Sample Mash				Matrix:Solid	Collected: 12/17/09 16:00		
Chlorite	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1090 mg/kg dry**	**11.2**	1A00433	300.1	KRM	01/04/10 17:15	
19L1077-01RE2 (b) (4) - Control Sample Mash				Matrix:Solid	Collected: 12/17/09 16:00		
Chlorite	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	<0.3 mg/kg dry	0.3	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1050 mg/kg dry**	**10.6**	1A00433	300.1	KRM	01/04/10 17:29	

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

Determination of Conventional Chemistry Parameters - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

(b) (4)

Work Order: 19L1077

Code Description Number Expires

(b) (4)

End of Report

(b) (6)

(b) (6)

ect Manager

(b) (4)

(b) (4)

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19L1077

Sample Recording Sheet

Sample Information:

Sample Date: 12-17-09

Plant: (b) (4)

Location Sample was taken (b) (4)

Is the sample dry or wet distillers grains? Control Sample Mash

Moisture content of distillers grains: 22.13% Solids

Total amount of chlorine dioxide (ClO_2) used during fermentation: 0

Sample Time	Sample Temperature
4 PM	185°

Notes:

(b) (6)

Print Name

Date 12-21-09

Signature

(b) (6)

(b) (4)

(b) (4)

ANALYTICAL REPORT

January 06, 2010

Work Order: 19L1080

Report To

Allen M. Ziegler
Resonant BioSciences, LLC
11757 West Ken Caryl Avenue, F-308
Littleton, CO 80127

Work Order Information

Date Received: 12/22/2009 1:45PM
Collector:
Phone: (866) 933-0408
PO Number:

Project : PureMash
Project Number: (b) (4)

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Analyte	Result	MRL	Batch	Method	Analyst	Analyzed	Qualifier
19L1080-01 (b) (4) - Grain Pile (Dry)				Matrix:Solid	Collected: 12/15/09 14:30		
% Solids	**84.9 %**	**0.1**	1L92905	SM 2540 G	LJG	12/29/09 7:51	
Chlorite	<0.2 mg/kg dry	0.2	1L93121	300.1	KRM	12/28/09 0:00	
ıte	**11.4 mg/kg dry**	**0.2**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1680 mg/kg dry**	**10.0**	1A00433	300.1	KRM	01/04/10 19:22	
19L1080-01RE1 (b) (4) Grain Pile (Dry)				Matrix:Solid	Collected: 12/15/09 14:30		
Chlorite	<0.2 mg/kg dry	0.2	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**10.6 mg/kg dry**	**0.2**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1620 mg/kg dry**	**10.0**	1A00433	300.1	KRM	01/04/10 19:37	
19L1080-01RE2 (b) (4) Grain Pile (Dry)				Matrix:Solid	Collected: 12/15/09 14:30		
Chlorite	<0.2 mg/kg dry	0.2	1L93121	300.1	KRM	12/28/09 0:00	
Chlorate	**10.7 mg/kg dry**	**0.2**	1L93121	300.1	KRM	12/28/09 0:00	
Chloride, soluble	**1610 mg/kg dry**	**10.0**	1A00433	300.1	KRM	01/04/10 19:51	

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(b) (4)

(b) (4)

Determination of Conventional Chemistry Parameters - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

(b) (4)

Determination of Inorganic Anions - Quality Control

(b) (4)

Analyte	Result	Reporting Limit	Units	Spike Level	Source Result	%REC	%REC Limits	RPD	RPD Limit	Notes

(b) (4)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL = Method Reporting Limit.

(b) (4)

(b) (4)

(b) (4)

Work Order: 19L1080

Code	Description	Number	Expires
(b) (4)			

(b) (6)

End of Report

(b) (4)

(b) (4)

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Sample Recording Sheet 19L1080

Sample Information:

Sample Date: 12-15-09

Plant: (b) (4)

Location Sample was taken: Grain Pile

Is the sample dry or wet distillers grains? dry

Moisture content of distillers grains: > 5%

Total amount of chlorine dioxide (ClO_2) used during fermentation: 45 ppm

#5

Sample Time	Sample Temperature
2:30pm	43° F

Notes:

Print Name (b) (6) Date 12-21-09

Signature

(b) (6)

The results in this report apply to the samples analyzed in accordance with the chain of custody document. This analytical report must be reproduced in its entirety. Samples were preserved in accordance with 40 CFR for pH adjustment unless otherwise noted. MRL= Method Reporting Limit.

(b) (4)

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Standard Operating Procedure

Determination of Chlorate, Chloride, and Chlorite in Distillers Grain by Ion Chromatography

Reference Methods:
EPA 300.1 Part B Modified

SOP #: 13AOE
Author: Kevin McDonald
Date Created: 11/24/2009
Revised Date:
Revised By:

Approval:

Laboratory Manager — Date: 04/27/10

Laboratory Director — Date: 04/27/10

Director of Quality Assurance — Date: 4/27/10

Determination of Chlorate, Chloride, and Chlorite by Ion Chromatography

1.0 Purpose

1.1 The purpose of this standard operating procedure is to establish the process for conducting inorganic anion analysis by ion chromatography.

2.0 Applicability

2.1 This procedure applies to all analysts performing inorganic anion analysis by ion chromatography.

3.0 Summary and General Discussion of the Procedure

3.1 This method is an ion chromatographic procedure used to determine the total concentration of inorganic anions in distillers grain. The inorganic anions applicable are chlorate, chloride, and chlorite.

4.0 Applicable Matrix

4.1 Distillers Grain

5.0 Method Detection Limit

5.1 The method detection limit is determined by analyzing 7 aliquots of a known standard.

5.2 The practical quantitation limit and the method reporting limit are determined from the MDL.

5.3 See SOP 00AAL "Procedure for Determination of Method Detection Limits (MDL), Practical Quantitation Limits (PQL), and Method Reporting Limits (MRL)."

6.0 Scope and Application

6.1 This method is an ion chromatographic procedure used to determine the total concentration of inorganic anions in distillers grain. The inorganic anions applicable are chlorate, chloride, and chlorite.

7.0 Summary of Method

7.1 The sample is extracted with water. A small volume of the extract is injected into an ion chromatograph. The sample is then introduced into a stream of KOH eluent and separated on a system comprised of a guard column, analytical column, and suppressor device. After separation, the conductivity detector determines the analytes of interest.

8.0 Definitions

8.1

IC	Ion Chromatography
EDA	Ethylenediamine
MSDS	Material Safety Data Sheet
BS/BSD	Blank Spike/Blank Spike Duplicate
MS/MSD	Matrix Spike/Matrix Spike Duplicate

9.0 Interferences

9.1 Substances in the samples with similar and overlapping retention times to that of the anion in question present an interference problem.

9.2 Large amounts of an anion may interfere with the resolution of an adjacent anion.

9.3 Method interferences may be caused by contamination in the samples, in glassware, reagents, and other apparatus used during processing of samples.

9.4 Samples that contain particles that are greater than 0.45 microns and eluents that contain particles that are greater than 0.2 microns may damage the instrument columns and flow systems and must be filtered prior to running.

10.0 Safety

10.1 Normal accepted laboratory safety practices should be followed during reagent preparation and instrument operation. No known carcinogenic materials are used in this method.

10.2 Protective eyewear, gloves, and lab coat should be worn.

10.3 Consult the MSDS for all reagents used for appropriate handling procedures.

10.4 Each sample should be treated as a potential health hazard.

11.0 Equipment and Supplies

11.1 Hewlett Packard 35900 Interface

11.2 Chromeleon Software

11.3 AS40 Autosampler

11.4 ICS-2000 Ion Chromatograph

11.4.1 Analytical Column-Ionpac AS19, 4 x 250 mm, Dionex # 062885

11.4.2 Guard Column-Ionpac AG19, 4 x 50 mm, Dionex # 062887

11.4.3 Anion Suppresser-Self Regenerating ASRS-Ultra, 4 mm Dionex #53946

11.4.4 EG40 Eluent Generator

11.4.4.1 EGC II KOH Cartridge, Dionex #058900

11.5 Sample Vials and Filter Caps

11.5.1 5 mL vials with filter caps, Dionex # 038141

11.6 Volumetric Flasks - assorted volumes.
11.7 Microsyringes – various sizes
11.8 Analytical Balance- capable of weighing to the nearest .0001g
11.9 Magnetic Stir Plate
11.10 Magnetic Stir Bars
11.11 EDA preserved 125mL containers, QEC #2223-0004

12.0 Reagents and Standards

12.1

Purified Water (E-Pure)	Having a resistivity of at least 17.5 megohms
Nitrogen cylinder	Gas to run Dionex autosampler and maintain pressurized eluent container between 5 and 6 psi.
Calibration Stock Approximately 1000mg/L Chlorite 1000mg/L Chloride 1000mg/L Chlorate	Weigh approximately 0.1275 g sodium chlorate, 0.1649 g sodium chloride, and 0.1676 g sodium chlorite into a 100 mL volumetric flask, add 250 µL of the preservation solution, and dilute to mark with e-pure water. Stock standard is stored in refrigerator.
Calibration Working Mix Approximately 10mg/L Chorate 10mg/L Chloride 10mg/L Chlorite	Add 1 mL calibration stock standard to a 100 mL volumetric flask, add 250 µL of the preservation solution, and dilute to the mark with e-pure water. Stock standard is stored in refrigerator.
Second Source Standard Approximately 500mg/L Chlorite 500mg/L Chloride 500mg/L Chlorate	Weigh approximately 0.0638g sodium chlorate, 0.0825 sodium chloride, and 0.0838 g sodium chlorite into a 100mL volumetric flask, add 250 µL of the preservation solution, and dilute to mark with e-pure water. Stock standard is stored in refrigerator.
Sodium Chlorate	99+% Aldrich #40316 CAS [7775-09-9]
Sodium Chloride	99+% EMD #SX0420-1 CAS [7647-14-5]
Sodium Chlorite	80% Aldrich #244155 CAS [7758-19-2]

5% Ethylenediamine Preservation Solution	Dilute 10 mL of ethylenediamine(99% [107-15-3]) to200 mL e-pure water.
Dichloroacetate Surrogate Solution Approximately 200mg/L	Weigh approximately 0.065g of dichloroacetate (DCA) acid, potassium salt in a 100 mL flask and dilute to mark with e-pure water. Store in refrigerator

13.0 Sample Collection, Preservation, Shipment & Storage

13.1 The preservation and holding time is dependent on the anion being analyzed. The anion that requires the most preservation treatment and the shortest holding time determines the treatment of the sample.

13.2 The following are the holding times and preservation treatment for each analyte:

Analyte	Preservation	Holding time
Chlorate	freeze if possible	28 days
Chloride	freeze if possible	28 days
Chlorite	freeze if possible	14 days

13.3 Collect sample in a clean Whirl-Pak bags.

14.0 Quality Control

14.1 A batch is defined as no more than 20 samples.

14.2 A method blank (MB), blank spike (BS), matrix spike (MS), and matrix spike duplicate (MSD) must be analyzed with each batch. A BS and BSD may be substituted if there is not sufficient sample for a MS and MSD.

14.3 Analytes will only be reported with concentrations that are between the lowest and highest calibration standards. Any samples falling above this range must be diluted and reanalyzed. Samples falling below this range must be reported either as less than the reporting limit or J-flagged (detected but below the reporting limit, therefore the result is an estimated concentration.)

14.4 A calibration check standard should be run at the beginning and end of each batch, as well as every 10 samples.

15.0 Calibration and Standardization

15.1 External Calibration

15.1.1 Prepare a 5 point calibration curve.

15.1.2 Analyze each calibration standard. If the ratio of response to amount injected (response factor) is constant over the working range (≤20% relative standard deviation, RSD), linearity can be assumed and the average response factor can be used in place of a calibration curve.

15.1.3 If the RSD is >20% and only five standards are analyzed a linear regression curve can be used as long as the coefficient of determination is ≥0.990.

15.1.4 If the RSD is >20% and the curve in non-linear a quadratic regression curve can be used as long as at least six standards are analyzed and the coefficient of determination is ≥0.990.

15.2 The working calibration curve must be verified by analyzing a check standard at the beginning and end of each batch and every ten samples in between. Recovery for these check standards must be between 90-110%.

15.3 A second source standard should also be analyzed to verify the validity of the curve. This standard should be prepared at midlevel calibration concentration. Analyte recovery should be between 90%-110%.

16.0 Procedure

16.1 Extraction- 50 mL of water is added to 1g of sample in a 125 mL EDA preserved container. The slurry is mixed for 10 minutes using a magnetic stirring device. Filter the mixture before injection using a 0.45 µ filter. Samples should be analyzed within 2 days of extraction.

16.2 Set up the instrument according to the parameters for this method.

Flow = 1.5 mL/min
Eluent Concentration = 1 mM
Suppressor Current = 67 mA
Cell Temperature = 40 °C
Column Temperature = 40 °C
Sample Loop = 1000 µL

16.3 Turn on the pump and let the baseline equilibrate.

16.4 Set up and run the analytical sequence.

16.4.1 Analytical Program:

```
Pressure.LowerLimit =       200
Pressure.UpperLimit =       3000
%A.Equate =                 "KOH"
CR_TC =                     On
LoadPosition
Data_Collection_Rate =      5.0
CellTemperature =           30.0
ColumnTemperature =         30.0
Suppressor_Type =           ASRS_4mm
```

```
; Carbonate =                0.0
; Bicarbonate =              0.0
; Hydroxide =                20.0
; Tetraborate =              0.0
; Other eluent =             0.0
; Recommended Current =      50
Suppressor_Current =         50
Flow =                       1.00

-2.300
     Pump_ECD_Relay_1_Closed = Duration=38.00
     Concentration =                10.00
     Curve =                        5
-1.000
                                    Concentration =
     10.00
     Curve =                        5
 0.000
     Pump_ECD.Autozero
     ECD_1.AcqOn
     Pump_InjectValve.InjectPosition
     Duration=30.00

10.000                              Concentration =
     10.00
     Curve =                        5

25.000                              Concentration =
     45.00
     Curve =                        5

31.000                              ECD_1.AcqOff
     Concentration =                45.00
     Curve =                        5

37.000                              Concentration =
     10.00
     Curve =                        5

     End
```

16.5 Compound Identification

16.5.1 Absolute retention times are used for compound identification.

16.5.1.1 Refer to SOP #03AMJ "Procedure for Conducting a Retention Time Window Study."

16.5.2 A sample compound is identified by comparison to the standard chromatogram. Identification of an analyte is positive if the retention time of the sample peak corresponds correctly to the retention time of the standard peak.

16.5.3 Analyst judgement may be used when sample components are not resolved.

17.0 Calculations

17.1 To calculate the percent recovery of a check standard:

$$\frac{\text{Theoretical Value}}{\text{Experimental Value}} \times 100 = \%\ \text{recovery}$$

17.2 To calculate the percent difference (%D) between the initial calibration standard and the continuing calibration standard:

$$\frac{\text{Initial calibration area-continuing calibration area}}{\text{Continuing calibration area}} \times 100 = \%D$$

17.3 Calculate the percent recovery of the MS/MSD:

$$\frac{\text{MS value (mg/L)} - \text{sample value (mg/L)}}{\text{amount of spike (mg/L)}} \times 100 = \text{MS \% recovery}$$

18.0 Method Performance

18.1 Refer to Section 13.0 Method Performance EPA 300.1 and page 19 of the Dionex IonPac AS11-HC Manual Gradient Elution of a Large Number of Inorganic Anions and Organic Acid Anions Using OH Gradient.

19.0 Pollution Prevention

19.1 Reagents and standards will be prepared in volumes consistent with laboratory use to minimize the volume of expired reagents and standards to be disposed.

20.0 Data Assessment and Acceptance Criteria for Quality Control Measures

20.1 The concentration of analyte in the method blank must be lower than the method reporting limit.

20.2 The check standard recovery acceptance criteria is 90% -110%

20.3 The FLB acceptance criteria is 90% - 110%.

20.4 The acceptance criteria for the matrix spike and matrix spike duplicate is 75% - 125% until there is enough data to generate control charts of three standard deviations from the mean.

20.5 The RPD for the MS/MSD is ≤ 20 until there is enough data to generate control charts of three standard deviations from the mean.

20.6 The surrogate acceptance criteria is 90% - 115% until there is enough data to generate control charts of three standard deviations from the mean.

20.7 Calibration criteria see section 15.1.2

21.0 Corrective Actions for Out-of-Control Data

21.1 A corrective action form will be completed whenever unacceptable conditions are identified. The following indicators will be used to determine unacceptable conditions:

- ⇒ QC samples outside of the established acceptance criteria (blanks, standards, duplicates, and spikes).
- ⇒ Equipment failure
- ⇒ Dilutions outside the acceptable range

21.2 Once a problem is identified, an appropriate corrective action will be taken. This table illustrates some of the procedures used for establishing corrective action:

SPECIFIC CORRECTIVE ACTION		
TYPE	**RECOMMENDED ACTION**	**DOCUMENTATION**
Contaminated Method Blank	1. Determine the source of contamination. 2. Eliminate the source of contamination. 3. Obtain water from a difference source	Corrective Action Form IC Log Book
The check standard exceeds the acceptance limits.	1. Check preparation log for errors 2. Check analysis for errors. 3. Check calculations. 4. Remake standard or use a different standard 5. Redo analysis to the last acceptable QA data.	Corrective Action Form IC Log Book
The FLB exceeds the acceptance limits.	1. Check preparation log for errors 2. Check analysis for errors. 3. Check calculations. 4. Remake standard or use a different standard	Corrective Action Form IC Log Book
The matrix spike and/ or matrix spike duplicate exceed the acceptance limits.	1. Check preparation log for errors. 2. Check analysis for errors. 3. Check calculations. 4. Remake spike solution or use a different standard. 5. Redo analysis to the last acceptable QA data. 6. Check for matrix interference	Corrective Action Form IC Log Book

SPECIFIC CORRECTIVE ACTION		
TYPE	RECOMMENDED ACTION	DOCUMENTATION
RSD exceeds the acceptance limits.	1. Check preparation log for errors 2. Check analysis for errors. 3. Check calculations.	Corrective Action Form IC Log Book
Instrument problems	1. Do maintenance of instrument as required by manufacturer. 2. Notify supervisor.	Corrective Action Form IC Log Book
Analyst not following the SOP	1. Provide additional training. 2. Do demonstration of performance. 3. Analyze a PE sample.	Corrective Action Form IC Log Book Analyst Training File.

22.0 Contingencies for Handling Out-of-Control or Unacceptable Data

22.1 Samples will be recollected and reanalyzed if at all possible. If this is not possible, the results will be flagged as being out-of-control.

22.2 If it is determined the validity of the data is compromised; the laboratory will take corrective action and provide written notification to the proper certifying authorities and to the clients affected.

23.0 Waste Management

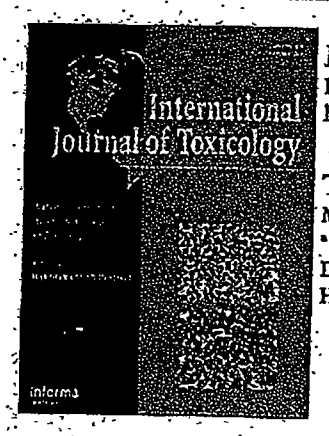
23.1 This analysis does not generate waste that requires special handling.

23.2 Used eluent and other waste generated by this method is poured down the drain then flushed with water.

24.0 References

22.1 Method 300.1, Revision 1.0 *EPA Methods for Chemical Analysis of Water and Wastes.*

22.2 Dionex IonPac AS11-HC Manual Gradient Elution of a Large Number of Inorganic Anions and Organic Acid Anions Using OH Gradient, Page 19-20.



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The Kinetics of Chlorite and Chlorate in the Rat

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The Kinetics of Chlorite and Chlorate in the Rat

M.S. ABDEL-RAHMAN,[1] D. COURI,[2] and R.J. BULL[3]

ABSTRACT

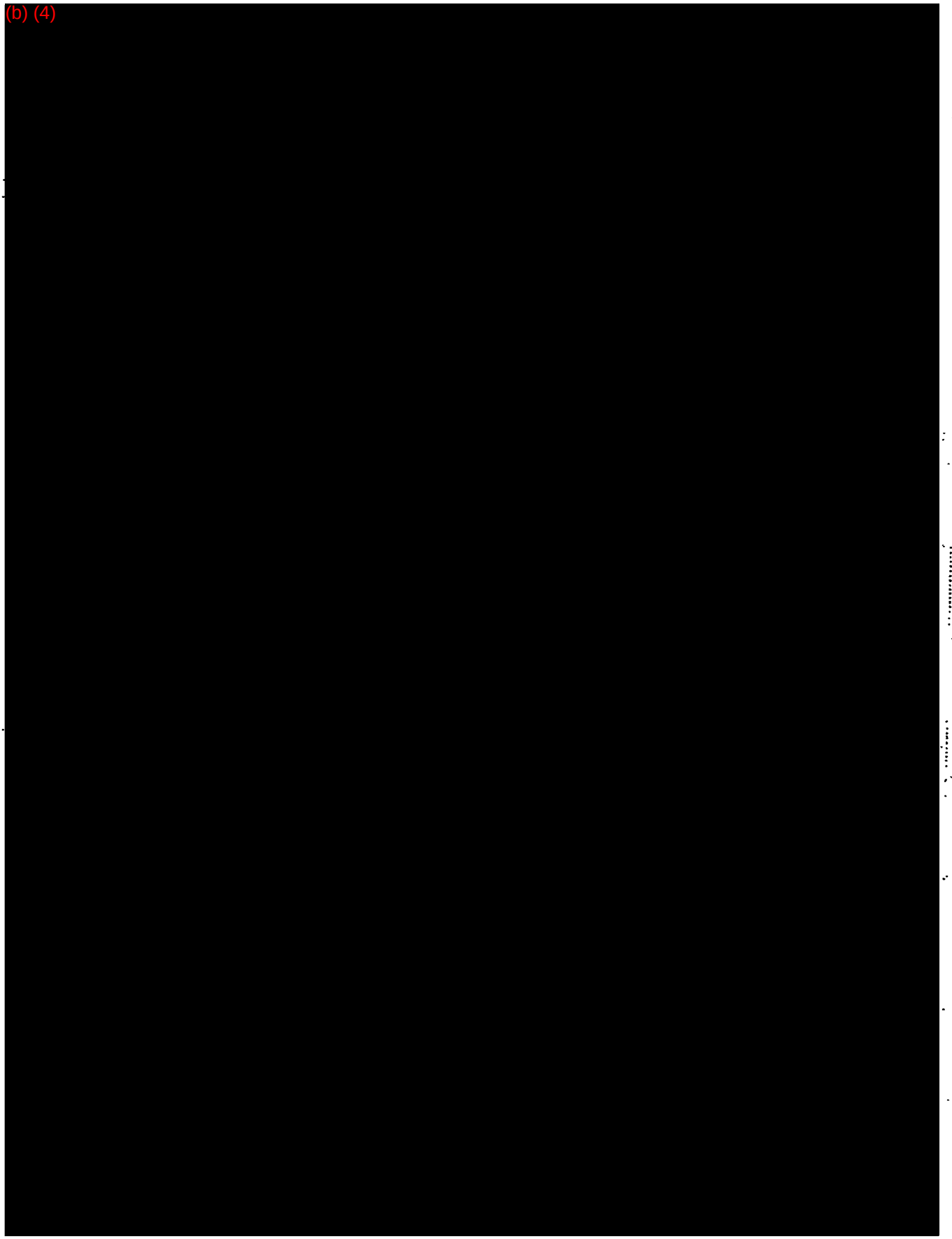
Chlorine dioxide (ClO_2) is under consideration as an alternative to chlorination as a disinfectant for public water supplies. The primary products resulting from ClO_2 disinfection of surface waters are chlorite (ClO_2^-) and chlorates (ClO_3^-). The kinetics of $^{36}ClO_2^-$ and $^{36}ClO_3^-$ was studied in rats. Radioactivity was rapidly absorbed from the gastrointestinal tract following the administration of (0.17 μCi) $^{36}ClO_2^-$ or (0.85 μCi) $^{36}ClO_3^-$ orally, and ^{36}Cl in plasma reached a peak at 2 hours and 1 hour, respectively. After 72 hours, radioactivity was highest in whole blood, followed by packed cells, plasma, stomach, testes, skin, lung, kidney, duodenum, carcass, spleen, ileum, brain, bone marrow, and liver in $^{36}ClO_2^-$ treatment. ^{36}Cl excretion was greatest at 24 hours after the administration of $^{36}ClO_3^-$, but in the $^{36}ClO_2^-$, the excretion most likely represented saturation of the biotransformation and excretion pathways. About 40% of the total initial dose was excreted at 72 hours in the urine and feces in both treatments. No ^{36}Cl was detected in expired air throughout the 72 hours studied.

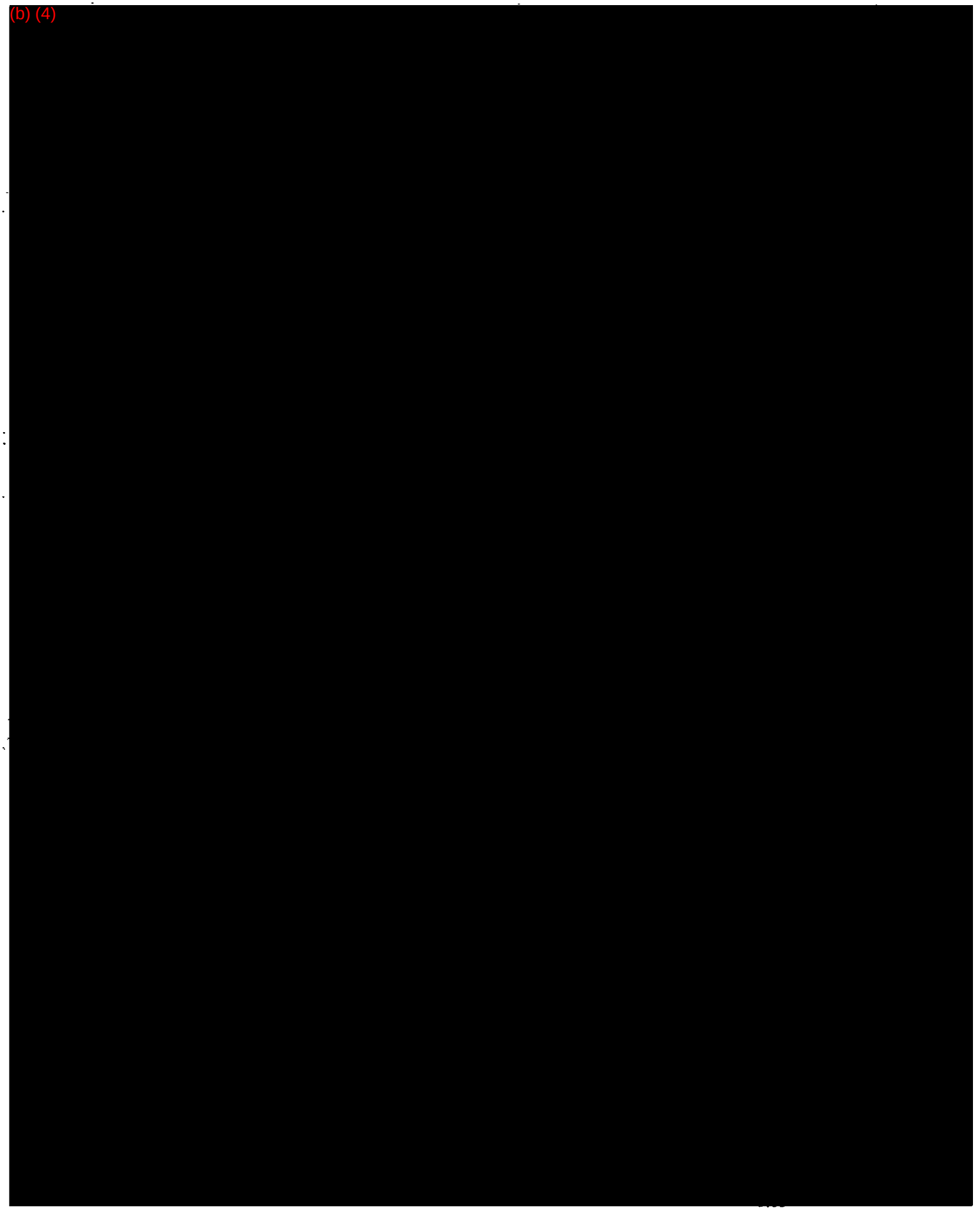
INTRODUCTION

Industrial wastes, domestic sewage, and agricultural runoff all contribute to the problem of water pollution by organic chemicals. Recent studies have demonstrated that the interaction of chlorine with various organic substances in the water results in the formation of halogenated compounds, such as chloroform, bromodichloromethane, dibromochloromethane, and bromoform (Rook, 1976). The awareness of the widespread occurrence of this type of pollution is due, in large part, to recent development in techniques for the identification of trace organic contaminants. Bellar et al. (1974) found that the concentration of trihalomethanes increased each time chlorine was added within the water treatment scheme.

Therefore, the use of chlorine is to be limited and the possible use of other disinfectants promoted. Among them is chlorine dioxide (ClO_2), which does not form trihalomethanes in drinking water (Miltner, 1976). However, the primary products resulting from ClO_2 disinfection of surface waters include chlorites and chlorates, which appear in concentrations 50% and 30% of ClO_2 demand, respectively (Miltner, 1976). Metabolism studies revealed that ClO_2 is converted to chloride, chlorite and chlorate in the rat (Abdel-Rahman et al., 1979b).

[1]Department of Pharmacology UMDNJ–New Jersey Medical School, Newark, New Jersey.
[2]Ohio State University, College of Medicine, Department of Pharmacology, Columbus, Ohio.
[3]USEPA, Health Effects Research Laboratory, Cincinnati, Ohio.

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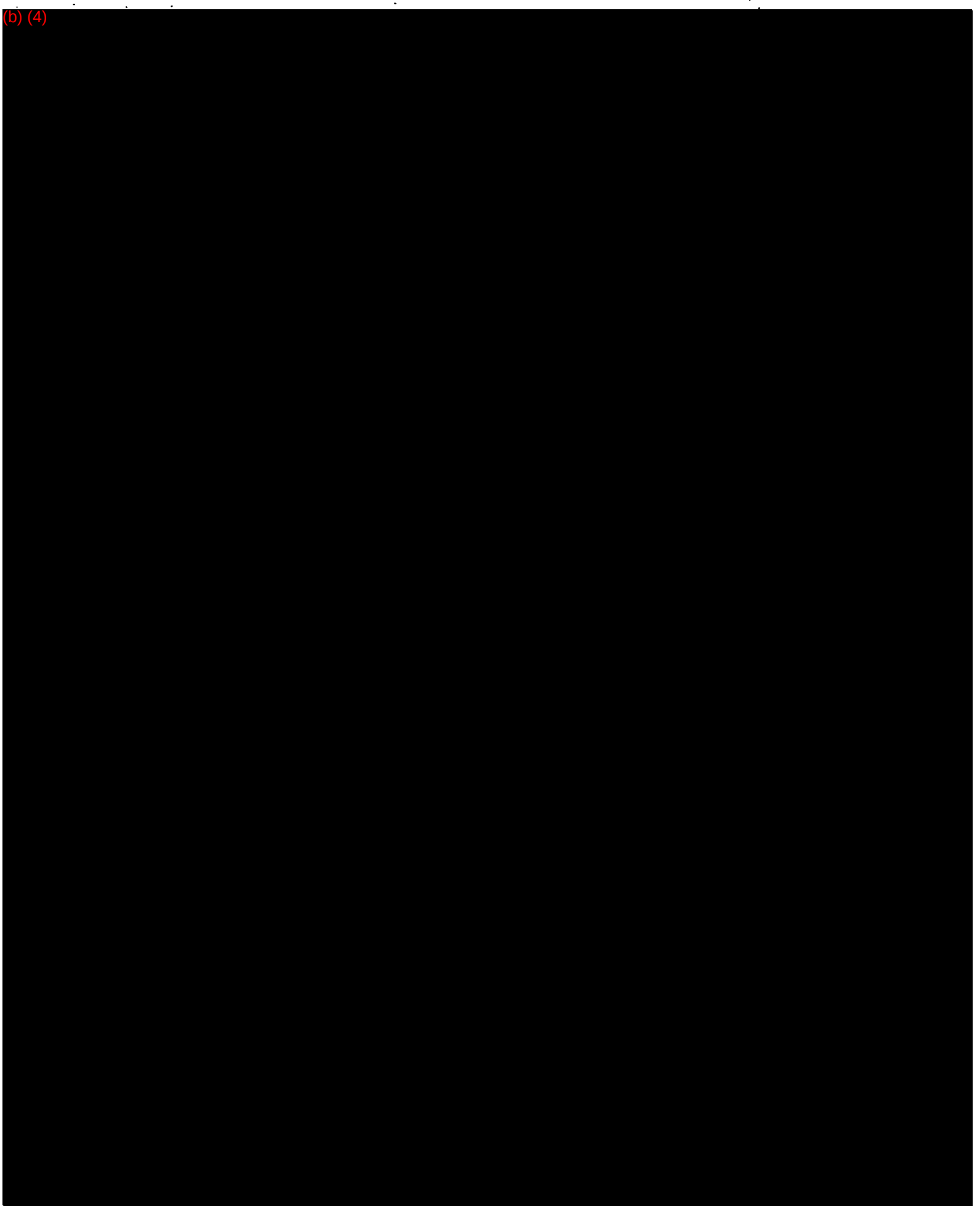

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Effect of Sodium [^{36}Cl]Chlorate Dose on Total Radioactive Residues and Residues of Parent Chlorate in Beef Cattle†

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The objectives of this study were to determine total radioactive residues and chlorate residues in edible tissues of cattle administered at three levels of sodium [^{36}Cl]chlorate over a 24-h period and slaughtered after a 24-h withdrawal period. Three sets of cattle, each consisting of a heifer and a steer, were intraruminally dosed with a total of 21, 42, or 63 mg of sodium [^{36}Cl]chlorate/kg of body weight. To simulate a 24-h exposure, equal aliquots of the respective doses were administered to each animal at 0, 8, 16, and 24 h. Urine and feces were collected in 12-h increments for the duration of the 48-h study. At 24 h after the last chlorate exposure, cattle were slaughtered and edible tissues were collected. Urine and tissue samples were analyzed for total radioactive residues and for metabolites. Elimination of radioactivity in urine and feces equaled 20, 33, and 48% of the total dose for the low, medium, and high doses, respectively. Chlorate and chloride were the only radioactive chlorine species present in urine; the fraction of chlorate present as a percentage of the total urine radioactivity decreased with time regardless of the dose. Chloride was the major radioactive residue present in edible tissues, comprising over 98% of the tissue radioactivity for all animals. Chlorate concentrations in edible tissues ranged from nondetectable to an average of 0.41 ppm in skeletal muscle of the high-dosed animals. No evidence for the presence of chlorite was observed in any tissue. Results of this study suggest that further development of chlorate as a preharvest food safety tool merits consideration.

INTRODUCTION

Contamination of beef carcasses with pathogens such as *Escherichia coli* and *Listeria* during slaughter and processing have led to the annual recall of over 800 000 kg of beef during the past decade (*1*); this average excludes a recall of 10 000 000 kg of beef in 2002. Food-animal products containing undetected pathogens continue to contribute to an unquantified number of foodborne illnesses. In beef cattle, it has been established that hides are a major source of carcass contamination (*2*) and that hide-washing intervention steps effectively reduce subsequent pathogen loads on carcasses (*3*, *4*). Although postharvest sanitation techniques are becoming increasingly efficient, they are in use because no practical methods exist for removing pathogens from live animals prior to slaughter. It has been suggested (*5*) that intervention techniques that eliminate pathogen loads in live animals could have a greater relative impact on food safety than any postharvest intervention strategy known, aside from cooking. In reality, a combination of both pre- and postharvest intervention strategies will likely be employed to minimize risks associated with pathogen-contaminated meats.

Recently, a new preharvest technology that greatly reduces or eliminates the numbers of pathogens inhabiting gastrointestinal tracts of cattle (*6*–*8*), sheep (*9*), swine (*10*–*12*), and poultry (*13*, *14*) has been developed. The technology is based on the feeding of an experimental sodium chlorate-containing product (ECP) 24–72 h prior to the slaughter of an animal. During the chlorate exposure period, bacterial species containing intracellular respiratory nitrate reductase are thought to metabolize chlorate (ClO_3^-) to the bacterial toxin chlorite (ClO_2^-; *15*). Chlorate toxicity is specific to nitrate-reductase-containing bacteria that have the ability to intracellularly convert chlorate to chlorite but which lack chlorite dismutase enzymes capable of rapidly metabolizing chlorite to the chloride ion (*16*, *17*). Use of chlorate does not adversely affect the commensal microflora of gastrointestinal tracts (*6*). Unlike many antibiotics,

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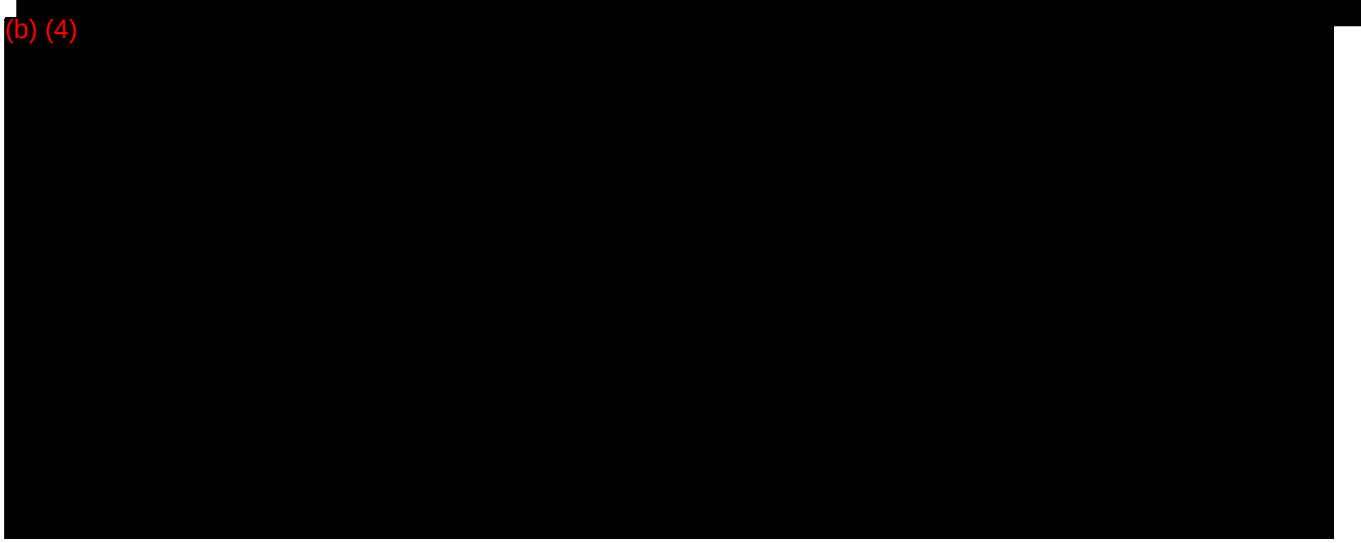

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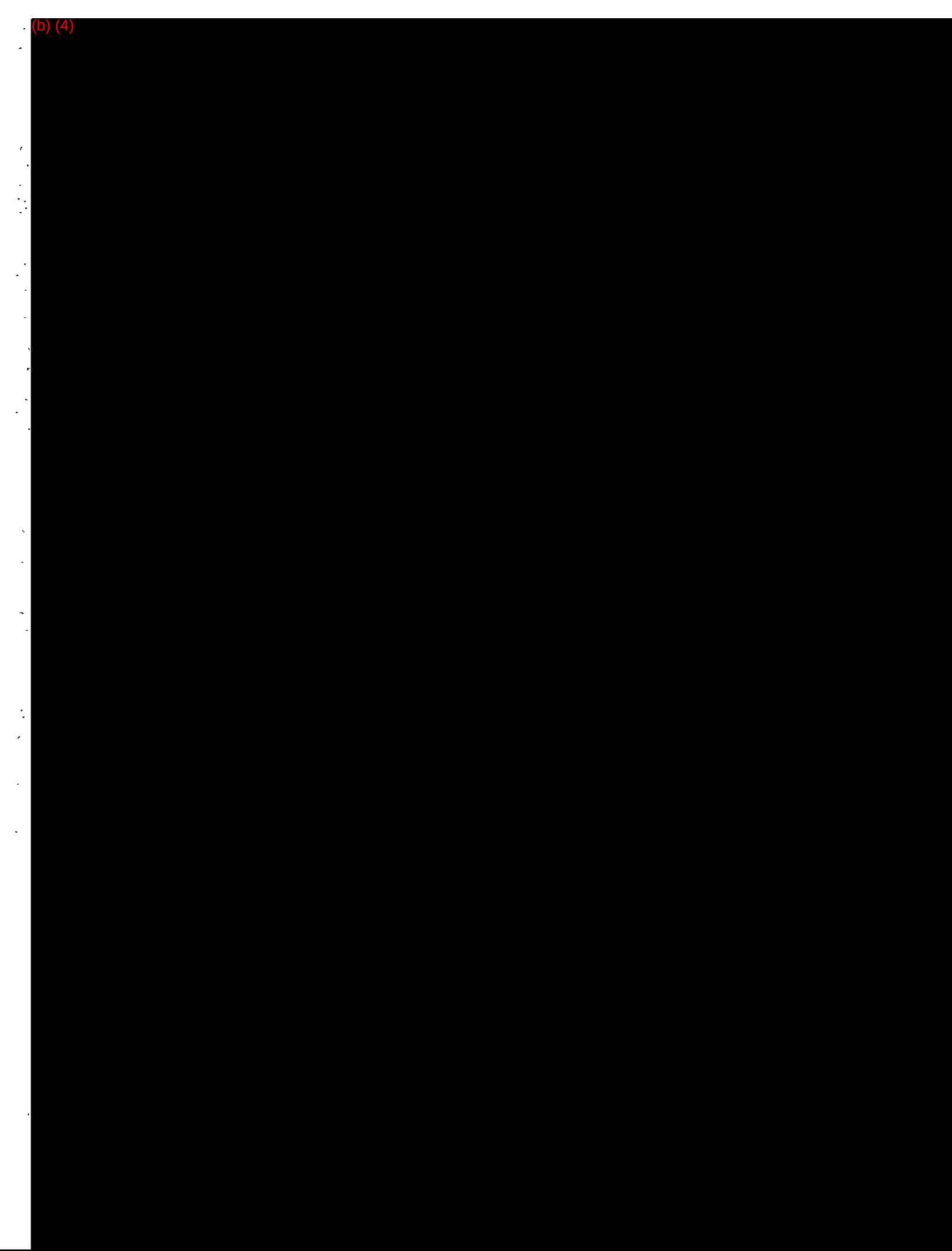
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Tissue Distribution, Elimination, and Metabolism of Dietary Sodium [^{36}Cl]Chlorate in Beef Cattle

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Two steers (~195 kg) were each dosed with 62.5 or 130.6 mg/kg body weight sodium [^{36}Cl]chlorate for three consecutive days. All excreta were collected during the dosing and 8 h withdrawal periods. The apparent radiochlorine absorption was 62−68% of the total dose with the major excretory route being urine. Parent chlorate was 65−100% of the urinary radiochlorine; chloride was the only other radiochlorine species present. Similarly, residues in edible tissues were composed of chloride and chlorate with chloride being the major radiolabeled species present. Chlorate represented 28−57% of the total radioactive residues in skeletal muscle; in liver, kidney, and adipose tissues, chlorate ion represented a smaller percentage of the total residues. Chlorate residues in the low dose steer were 26 ppm in kidney, 14 ppm in skeletal muscle, 2.0 ppm in adipose tissue, and 0.7 ppm in liver. These data indicate that sodium chlorate may be a viable preharvest food safety tool for use by the cattle industry.

KEYWORDS: Sodium chlorate; food safety; residue; cattle; *E. coli* O157:H7

INTRODUCTION

Contamination of beef carcasses with human pathogens including *Escherichia coli* and *Listeria* during slaughter and processing has led to the recall of over 37.8 million pounds of beef during the past decade (*1*). Beef products containing undetected pathogens have contributed to an unquantified number of food-borne illnesses during the same time period. Although beef producers, packers, and retailers are actively seeking pre- and postharvest solutions to eliminate beef-borne pathogens, the problem of carcass contamination remains.

Recently, a new preharvest technology that greatly reduces or eliminates the numbers of pathogens inhabiting the gastrointestinal tracts of cattle (*2*−*4*), sheep (*5*), swine (*6*−*8*), and poultry (*9*, *10*) has been developed. The technology is based on the feeding of a sodium chlorate-containing product (ECP) 24−72 h prior to an animal's slaughter. During the chlorate exposure period, bacterial species containing intracellular respiratory nitrate reductase are thought to metabolize chlorate (ClO_3^-) to the bacterial toxin chlorite (ClO_2^-; *11*). Chlorate toxicity is specific to nitrate reductase-containing bacteria that have the ability to intracellularly convert chlorate to chlorite but which lack chlorite dismutase enzymes capable of rapidly metabolizing chlorite to the chloride ion (*12*, *13*). Use of chlorate does not adversely affect the commensal microflora of gastrointestinal tracts (*2*). Unlike many antibiotics, development of chlorate resistance seems to occur only in pure bacterial cultures and not in mixed culture (*14*).

Although the use of chlorate by the cattle industry seems to be a practical means of reducing the probability of pathogen contamination at slaughter, data demonstrating the absorption, distribution, metabolism, and excretion of chlorate in treated cattle do not exist. Furthermore, the safety of chlorate residues in edible tissues of cattle has not been demonstrated. In rodents, chlorate appears to be rapidly absorbed and excreted (*15*) and chlorate is apparently metabolized to chlorite and chloride ions. The overall goal of this study was to determine if further development of chlorate as a commercial product was warranted from the perspective of the magnitude of residues in edible tissues. The objective of this study was to determine the absorption, distribution, metabolism, and excretion of sodium chlorate in cattle. Because sodium chlorate disposition in ruminants has not been previously studied and because the cost of the test article ($Na^{36}ClO_3$) on a per animal basis was substantial, this communication describes results obtained from a preliminary study in which only two animals were dosed.

MATERIALS AND METHODS

Reagents and Chemicals. Unlabeled sodium chlorate (CAS no. 7775-09-9; 99.96% $NaClO_3$; 0.03% NaCl; 0.01% H_2O) was obtained from EKA Chemicals (Columbus, MS). Radiolabeled sodium chlorate

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($Na^{36}ClO_3$), having a specific activity of 0.575 mCi/mmol, was purchased from Ricerca Biosciences (Concord, OH). The aqueous radioactive sodium chlorate stock solution was stored (<4 °C) until formulated for dosing.

Sodium nitrate was obtained from ICA TriNova, LLC (Marietta, GA). Sodium chloride (VWR, West Chester, PA); heparin, sodium salt (Sigma Chemical Co., St. Louis, MO); sodium hydroxide (50% solution for ion chromatography; Fluka Chemical Corp., Milwaukee, WI); Ultima Gold liquid scintillation fluid, Carbosorb-E, and Permafluor E (PerkinElmer Life and Analytical Sciences, Boston, MA); methylamine (The Matheson Co., East Rutherford, NJ); phenyl mercuric nitrate, phenyl mercuric chloride (Aldrich, St. Louis, MO), and acetonitrile and methanol [high-performance liquid chromatography (HPLC) grade; EM Science, Gibbstown, NJ] were obtained from well-known vendors.

General LSC Techniques. Background radiochlorine and limits of quantitation were determined for individual matrices (i.e., urine, liver, kidney, skeletal muscle, and adipose tissue) as described by Smith et al. (*16*). Individual samples within a matrix set were generally counted for 10−20 min each. Radiochlorine was quantified using Beckman LS1701 (Fullerton, CA) or Packard 2550 (Meriden, CT) liquid scintillation counters. Each instrument was calibrated using a sealed radiochlorine standard (Analytics Inc., Atlanta, GA) prepared in Ultima Gold LSC fluid. A series of nitromethane-quenched vials, constructed with 0.1 μCi of $^{36}Cl^-$ in 15 mL of Ultima Gold, was purchased (Analytics Inc.) and used to construct quench curves for each instrument. Quench was corrected using the H# (Beckman) or tSIE (transformed spectral index of the external standard; Packard) options for each instrument.

Test Article Preparation and Characterization. The radiochemical purity of stock sodium [^{36}Cl]chlorate was assessed using two chromatographic methods. Thin-layer chromatography (TLC) was performed on 5 cm × 20 cm, aluminum-backed Silica Gel 60 F_{254} plates using a solvent system composed of 90% acetonitrile and 10% (v/v) of a 33% (w/v) methylamine solution in water (*17*); radiochlorine was quantified using a Bioscan Imaging Scanner (Bioscan, Inc., Washington, DC).

Duplicate 10 μL injections of the diluted stock chlorate solution were made onto sequential Dionex (Sunnyvale, CA) AG- and AS-11 guard and analytical columns. Solvent (100 mM NaOH in a 60:40 [v/v] mixture of water and methanol) was delivered at a flow rate of 0.5 mL/min using a Waters (Milford, MA) model 600E pump and controller equipped with Teflon pump heads and a Rheodyne (Cotati, CA) model 9725I PEEK injector. Samples were introduced using a Hamilton (Reno, NV) 50 μL syringe. Ions were detected using a Dionex CD 25 conductivity detector (100 mA) equipped with a Dionex ion suppression unit (ASRS Ultra-4 mm) operated in the external water mode. A Waters model 746 data module set at 0.5 cm/min was used to record chromatographic data. Fractions were collected into LSC vials at approximate 2 min intervals; Ultima Gold LSC cocktail was added to each vial, and vials were counted for a minimum of 10 min each. Radiochemical purity of sodium [^{36}Cl]chlorate formulated on its proprietary carrier was also assessed approximately 6 months after its formulation.

Specific Activity Determination. The specific activity of sodium [^{36}Cl]chlorate was determined chromatographically before and after dilution with unlabeled sodium chlorate. Briefly, unlabeled sodium chlorate (0.1370 g) was weighed (Mettler AE100 balance, Mettler Instrument Corp., Heightstown, NJ), dissolved in water, and transferred to a 100 mL volumetric flask. Triplicate 4, 8, 12, and 16 μL aliquots of the chlorate solution were injected onto the ion chromatography system, described for the determination of radiochemical purity, and the respective peak areas were recorded. Quadruplicate injections (10 and 25 μL, respectively) of stock and formulated sodium [^{36}Cl]chlorate were made; peak areas were recorded, and the sodium [^{36}Cl]chlorate peaks were trapped into LSC vials and counted for a minimum of 10 min. The specific activity was determined by dividing the dpm of each trapped peak by its mass (μg), as determined by regression of its peak area on the standard curve generated from the standards.

Animals and Feeding. Two Loala steers (nos. 171 and 172; approximately 166 kg at purchase) were trained to metabolism crates (1.0 m × 2.1 m × 2.7 m; W × H × L) over a 7 week period. Steers were provided a mixture of alfalfa and grass hay (provided on an ad libitum basis) from delivery to the completion of the study. Beginning 21 days prior to study commencement, 0.5 kg of cracked corn was provided daily and this amount was gradually increased to 2.7 kg per day up to the time of the study.

Study Design. At 72, 48, and 24 h prior to sodium [^{36}Cl]chlorate dosing, a proprietary sodium nitrate premix was fed to each steer as a component of the grain. Sodium nitrate is hypothesized to render pathogenic bacteria more susceptible to sodium chlorate (*5*, *10*) and was fed in this study to mimic conditions under which bacterial nitrate reductase might be induced. Sodium nitrate was delivered in the diet at 31 mg/kg body weight before the commencement of chlorate dosing. At 0, 24, and 48 h, each animal was orally dosed with either 63 or 126 mg/kg body weight of sodium [^{36}Cl]chlorate. Time 0 was 24 h after the last sodium nitrate feeding. Fifty-four hours (T54) after the initial sodium [^{36}Cl]chlorate dose and 8 h after the last sodium [^{36}Cl]chlorate dose, each animal was slaughtered and tissues were collected.

The sodium chlorate dose was selected based on the anticipation that the maximum exposure to a chlorate product would be three consecutive days with a preslaughter withdrawal period of 0 days. Therefore, test animals were dosed orally with $Na^{36}ClO_3$ for three consecutive days starting approximately 24 h after the last administration of sodium nitrate. Animal 171 was dosed with 63 mg/kg body weight of sodium chlorate, while animal 172 was dosed with 126 mg/kg body weight of sodium chlorate. These doses represent 1.5× the maximal intended use levels of 42 and 84 mg/kg body wt per day, respectively. A 1.5× dosing level was used to comply with unpublished, but widely known, U.S. Food and Drug Administration Center for Veterinary Medicine (U.S. FDA CVM) guidelines for the conduct of residue studies for compounds having a 0 day withdrawal period (*16*). Approximately 8 h after the last dose administration, each animal was stunned and exsanguinated.

Dose Formulation. Stock sodium [^{36}Cl]chlorate (6.402 mCi; 1.185 g) was combined with 180 mL of water and 118.83 g of unlabeled sodium chlorate. The sodium chlorate was completely dissolved and mixed, and a 100 μL sample was removed for purity and specific activity determinations. Dissolved sodium [^{36}Cl]chlorate was added to a proprietary carrier (280.0 g), allowed to dry, and stored in a labeled amber glass bottle until use.

Capsule Preparation and Administration. Formulated sodium [^{36}Cl]chlorate was weighed into gelatin capsules. The amount of formulated material weighed was based on body weights of 191.8 and 197.7 kg for steers 171 and 172, respectively. Each gelatin capsule held roughly 20 g of the [^{36}Cl]chlorate formulation. Immediately prior to dosing, each capsule was lubricated with vegetable oil, placed in a balling gun, and dosed. On dosing day 2, capsule no. 4 broke in the throat of steer 172 prior to its being released from the balling gun. Some of the contents of the capsule spilled into the head area of the metabolism crate. The capsule and its remaining contents were recovered, repackaged into an additional capsule, and readministered to steer 172. The contents of the spilled capsule were recovered to the extent possible, and radiochlorine in the recovered fraction was quantified by liquid scintillation counting. Dosing was otherwise uneventful.

Collection of Excreta. Urine and feces were collected in intervals of 0−12, 12−24, 24−36, 36−48, and 48−56 h after the initial sodium [^{36}Cl]chlorate administration. Modified incontinent bags (*18*) were fitted to steers to ensure quantitative collection of clean urine. Urine was weighed and mixed thoroughly, and subsamples were collected and stored at −20 °C.

Slaughter and Tissue Collection. Animals 171 and 172 were stunned, elevated, and exsanguinated. Blood was collected into basins that contained approximately 64000 units of sodium heparin and was weighed. Steers were eviscerated, and blood, brain, liver, kidney, adipose tissue, skeletal muscle, lung, spleen, small intestine, large intestine, stomach complex (consisting of the rumen, reticulum, omasum, and abomasum), skin, eyes, heart, bone, diaphragm, remainder of carcass, bile, and adrenal glands were collected. A "remainder of the carcass" fraction was collected that contained tissue scraps and tissue not associated with any one organ; for animal 171, liquid and solid portions were separated and assayed separately. Edible tissues were ground the day of slaughter (before freezing), but other tissues were

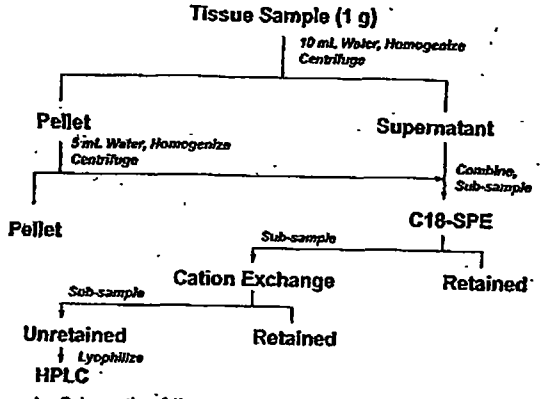


Figure 1. Schematic of tissue processing and analysis.

frozen, partially thawed, and then ground. The summed weights of tissues collected at slaughter were 97.3 and 98.1% of the live weights of steers 171 and 172, respectively.

Cage Wash. Each metabolism crate was washed with water, and the water was collected. Radiochemical analyses were conducted by weighing quintuplicate 1 mL aliquots of cage wash samples into glass LSC vials and diluting with 15 mL of Ultima Gold LSC fluid. Each sample was counted for 5 min on a LSC counter.

Total Radioactive Residues. *Practical Demonstration of Limit of Quantitation of Radioactive Residues.* Blank aliquots (2 g) of liver, kidney, skeletal muscle, and adipose tissue were fortified with $Na^{36}ClO_3$ so that 0.2 g samples would contain nominal levels of 0, 10, 20, 200, and 400 DPM, respectively. Kidney samples were inadvertently fortified with only 5, 10, 100, and 200 DPM per 0.2 g. After fortification, sample vials were vortex mixed at high speed for a minimum of 1 min. Quintuplicate 0.2 g aliquots of each fortified tissue were weighed into glass LSC vials. To each replicate, 8.0 mL of Carbosorb was added and tubes were incubated in a shaking water bath at 60 °C overnight. After the tubes were removed from the incubator and cooled to room temperature, 12 mL of Permafluor was added to each vial, and vials were placed on the LSC, dark adapted for a minimum of 1 h, and were counted overnight for 20 min each.

Liquid Samples. Subsamples from each animal and(or) each time point were thawed, and quintuplicate 1 mL aliquots were removed and weighed into 7 mL LSC vials. Six milliliters of Ultima Gold LSC cocktail was added to each vial, the vials were mixed, and samples were counted for 20 min (or an error rate of 0.2%) on a LSC counter. Background radiochlorine was determined by counting 1 mL blank samples.

Solid Samples. Quintuplicate 0.2−0.25 g aliquots were removed and placed into LSC vials. For fecal samples, 1 mL of water was added after the addition of feces. To each sample, 8.0 mL of Carbosorb E was added. Tubes were placed in a shaking water bath (Dubnoff, Chicago, IL) and were incubated overnight at 60 °C. After the tubes were removed and allowed to cool, 12 mL of Permafluor E was added to each tube. Samples were placed on the LSC and allowed to dark adapt for at least 1 h before the initiation of counting. For some fecal samples, resulting counts were too highly quenched for reliable quantitation, so 2.0 mL aliquots of each sample were removed and placed in new LSC vials, and 10.8 mL of Permafluor and 7.2 mL of Carbosorb were added. Samples were mixed by hand and recounted. Quench values of the recounted samples were well within the limits defined by the quench curve.

Speciation. *Edible Tissues.* Figure 1 illustrates the sample preparation scheme used for speciation of tissue metabolites. Edible tissue samples were run in sample sets consisting of duplicate 1 g subsamples of control tissues, fortified control tissues, tissues from animal 171, and tissues from animal 172. For adipose tissue samples, 2 g subsamples were analyzed. Control tissues were fortified with 30 μL of a standard solution containing 1 μg/μL each of $Na^{36}Cl$ and $Na^{36}ClO_3$ having specific activities of 100 dpm/μg. Samples were weighed into 50 mL polypropylene tubes (Sarstedt, Newton, NC), control tissues were fortified with radioactive standards, and 10 mL of nanopure water was added to each tube. Tissues were homogenized using a Tekmar (Cincinnati, OH) tissue homogenizer and centrifuged for 15 min at 31500g using a Sorvall RC-2 (Norwalk, CT) centrifuge equipped with a SS-34 rotor. Supernates were decanted into weighed glass LSC vials, and the resulting pellets were resuspended in 5 mL of water. The suspended pellets were mixed and centrifuged, and supernates were decanted and combined with their respective supernates. From each pooled supernate, a 2.5 mL aliquot was removed, weighed, and diluted with 15 mL of Ultima Gold LSC cocktail. A 5.0−7.5 mL aliquot of each supernate, depending upon the tissue, was removed and loaded on a previously conditioned (methanol followed by water) C18 Mega Bond Elut (2 or 5 g of sorbent depending upon tissue; Varian Assoc., Harbor City, CA) solid phase extraction (SPE) column. Each column was rinsed with water (5 mL), and the load and rinse fractions were collected into weighed glass LSC vials. A 1 mL aliquot of each "load/rinse" fraction was removed for quantitation of radiochlorine. Depending on the tissue, 5 mL or the entire remaining load/rinse fraction from the C18 SPE tube was loaded onto a preconditioned (methanol followed by water) cation exchange SPE tube (3 mL LC-SCX; Supelco, Bellfonte, CA). Loaded samples were allowed to pass through their respective SPE tubes, and tubes were rinsed with 2.5 mL of water. Aliquots (1 mL) were removed from the combined load/rinse fraction for LSC. The remaining fractions were frozen, lyophilized, reconstituted in 1 mL aliquots of water, and filtered (13 mm, 0.45 μm, PFTE syringe filter; Alltech, Deerfield, IL) in preparation for ion chromatographic analysis.

Ion chromatography was performed using the chromatographic equipment described previously. For speciation of the tissue metabolites, a gradient solvent system was used consisting of 10 and 100 mM NaOH, with Dionex AG- and AS-11 HC guard and analytical columns, respectively: isocratic 10 mM NaOH for 10 min; linear gradient to 50% 10 mM NaOH, 50% 100 mM NaOH from 10 to 30 min; isocratic for 2 min; linear gradient to 100% 10 mM NaOH from 32 to 40 min; reequilibrate for 30−40 min. Each day, prior to injection of samples from a tissue set, a standard containing $^{36}Cl^-$ and $^{36}ClO_3^-$ was injected onto the chromatograph so that recoveries and distribution of radioactivity could be determined. Individual samples were injected, and fractions were collected at approximate 3 min intervals for the first 15 min; thereafter, fractions were collected to minimize the splitting of radioactive peaks into separate vials. Fractions were quantified by LSC using Ultima Gold LSC cocktail. For chromatographic runs, the first four vials were used to assess background levels (initial 12 min of chromatographic run). The limit of quantitation for each run was defined as the mean dpm value of the first four vials plus three standard deviations of the mean. Chromatographic fractions that contained dpm values less than the limit of quantitation were said to have "0" counts.

Reaction with Phenyl Mercuric Nitrate. Phenyl mercuric nitrate (PMN) reacts with aqueous chloride ion under acidic conditions to form water insoluble phenyl mercuric chloride (*19*, *20*). The reaction was used in this study to quantify [^{36}Cl]chloride in tissues and to unambiguously verify the conversion of [^{36}Cl]chlorate to [^{36}Cl]chloride in tissues. Sample sets consisted of $Na^{36}Cl$ and $Na^{36}ClO_3$ fortified control tissues, and tissue samples containing incurred residues from steers 171 and 172. Duplicate 2.5 g samples were weighed into 50 mL polypropylene tubes, control tubes were fortified with [^{36}Cl]chloride or [^{36}Cl]chlorate (95.4% chlorate, 4.6% chloride), 10 mL of water was added to each tube, and tissues were homogenized, mixed, and centrifuged as described above. Respective supernates were combined and weighed, and aliquots were analyzed by liquid scintillation counting. Portions (5.0 mL) of the remaining supernates were placed in 100 mL separatory funnels and acidified to pH 1.5 with 1% nitric acid (~4 mL), and 25 mL of a 0.4 mg/mL aqueous solution of phenylmercuric nitrate was added. To each separatory funnel, 15 mL of chloroform was added (3×) and the layers were mixed. Respective chloroform extracts were removed and combined in 50 mL volumetric flasks; flasks were diluted to the mark with chloroform. Duplicate 5 mL aliquots were placed in LSC vials; the chloroform was allowed to evaporate, and the residue was reconstituted in 1 mL of methanol and then diluted with 15 mL of Ultima Gold LSC fluid. The remaining aqueous phases were placed in

volumetric flasks and diluted to the mark, and aliquots (5 mL) were removed for LSC. Radiochlorine in extracted samples was counted for 10 min each.

Confirmation of phenyl mercuric chloride in chloroform extracts of skeletal muscle was conducted by gas chromatography—mass spectrometry. Samples, dissolved in chloroform, were introduced onto an Autospec mass spectrometer (Micromass, Beverly, MA) using a Hewlett-Packard 5890 gas chromatograph equipped with a HP-7673A autosampler. Samples (1 μL) were cool-on column injected onto a 30 m × 0.25 mm i.d. DB5 MS column (J & W Scientific, Folsom, CA) with a 0.25 μm film thickness. A 1 m retention gap constructed of deactivated fused silica (0.53 mm i.d.) protected the column. Phenyl mercuric chloride eluted at a retention time of approximately 14.5 min using a temperature gradient starting at 150 °C, held for 2 min, followed by ramping to 200 °C at a rate of 5 °C per min, followed by ramping to 320 °C at a rate of 10 °C per min. Total ion chromatograms were generated, and mass spectra were evaluated at the retention time of the phenyl mercuric chloride standard; mass spectra of tissue extract samples were compared to the mass spectrum of the phenyl mercuric chloride standard.

Speciation of Urinary Radiochlorine. Urine was analyzed in sample sets corresponding to collection period and consisting of duplicate replicates each of control samples, fortified control samples, and samples from steers 171 and 172. Urine was thawed, 1 mL aliquots were removed for analysis, fortified samples were spiked with a mixture of [^{36}Cl]chloride and [^{36}Cl]chlorate, and 2 mL of nanopure water was added to each tube. Tubes were vortex mixed, and their contents were loaded onto preconditioned (5 mL of methanol followed by 7.5 mL of water) C18 SPE tubes (Bakerbond, 500 mg of sorbent, 3 mL tube; J. T. Baker, Phillipsburg, NJ). Sample loads from each column were collected and combined with a subsequent 1 mL water rinse of each tube. A 100 μL aliquot was removed, weighed, and subjected to LSC. The remaining load/rinse fractions collected from the C-18 SPE tubes were loaded onto preconditioned (5 mL of methanol followed by 5 mL of water) SCX tubes (LC-SCX, 3 mL; Supelco). Sample loads were collected and combined with 1.5 mL water rinses of each tube. An aliquot (0.25 mL) was removed from each tube and weighed, and radiochlorine was quantified by LSC. About 1 mL of each sample was filtered through a 0.45 μm PTFE syringe filter (17 mm; Alltech) in preparation for ion chromatographic analysis. Aliquots (20—160 μL, depending upon the concentration of radioactivity) were injected onto the HPLC system described for the tissue analysis, and radiochlorine was eluted using the gradient previously described for tissues.

RESULTS AND DISCUSSION

Radiochemical Purity. The radiochemical purity as assessed by HPLC and TLC was 94.5 and 94.3%, respectively. The radiochemical impurity was $Na^{36}Cl$, the starting material for the synthesis of sodium chlorate. During the formulation of the dosing material, the total amount of $Na^{36}ClO_3$ used was adjusted for radiochemical impurity. Radiochemical purity of the formulated $Na^{36}ClO_3$, 6 months after its formulation, was 96.8% as assessed by ion chromatography.

Specific Activity. Unformulated (stock) sodium [^{36}Cl]chlorate had a specific activity of 11573 dpm/μg. Formulated sodium chlorate had a specific activity of 114 dpm/μg.

Live Phase. Animal dosing was without event for steer 171. On dosing day 2, (24 h after the initial dose) capsule 4 broke in the mouth of steer 172 and a portion of the capsule was spilled onto the floor of the metabolism crate. Radioactivity recovered from the floor of the metabolism crate indicated that 139.4 μCi of radiochlorine was present, representing 8.3 g, or 40%, of the formulated material within capsule 4. The 8.3 g of lost material represented 10.0% of the total dose for dosing day 2, and 3.3% of the total 3-day dose of steer 172. Of greater concern was that Steer 172 stopped consuming food on dosing day 2, eating one of its daily grain allotment and only a little forage. Furthermore, steer 172 failed to consume grain on dosing day 3 but did consume some forage. Fecal output for steer 172 stopped entirely during hours 36—48 of the study (12—24 h after capsule breakage).

Table 1. Recoveries of Radiochlorine Fortified into Edible Tissues

	radiochlorine			
tissue	target (dpm/g)	theoretical[a] (dpm/0.2 g)	measured (dpm ± SD)	recovery % ± SD
liver	50	10.4	11.4 ± 1.0	110.3 ± 10.0
	100	20.4	21.4 ± 2.1	104.8 ± 8.2
	1000	202	197.2 ± 7.8	97.6 ± 0.8
	2000	415	392.6 ± 10.5	94.6 ± 1.4
kidney	25	5.2	4.9 ± 1.3	93.0 ± 24.2
	50	9.6	8.4 ± 0.90	86.9 ± 3.7
	500	106	107.5 ± 19.0	101.0 ± 4.3
	1000	205	213.5 ± 13.8	104.4 ± 1.0
skeletal muscle	50	9.7	8.9 ± 2.0	91.9 ± 20.1
	100	20.3	19.4 ± 2.6	95.9 ± 12.9
	1000	206.6	230.8 ± 54.1	111.8 ± 26.2
	2000	409.5	396.4 ± 58.3	96.9 ± 15.1
adipose tissue	50	8.6	10.6 ± 2.9	121.5 ± 17.6
	100	18.8	20.4 ± 5.7	106.8 ± 14.7
	1000	176.6	186.0 ± 54.7	104.6 ± 6.2
	2000	372.3	376.4 ± 66.5	103.4 ± 24.4

[a] Theoretical dpm calculated by: (control tissue wt/fortification dpm) × subsample wt.

Tissue Residues. Radiolabeled chlorate was used so that all chlorate-related residues (parent chlorate and metabolites) could be quantified, so that the degree of chlorate metabolism could be determined, and so that chlorate metabolites could be identified. Detection of 5 dpm of [^{36}Cl]chlorate fortified into blank tissues was possible using Carbosorb solubilization of tissues (Table 1). Recoveries for all tissues were between 91 and 121%. Variation was greatest for low level fortifications and for skeletal muscle and adipose tissue samples in which uniform mixing of the fortified radiolabel was most difficult. These data demonstrate that low levels of radiochorine can be reliably detected in edible tissues using Carbosorb and Permafluor as solubilizers and scintillants, respectively. Other techniques (combustion; Soluene 350 digestion; Soluene 350 with peroxide bleaching; Solubilization with Ultima Gold or Hionic Fluor scintillation fluid) led to low recoveries and greater variation than the technique used for this study (data not shown).

Distribution and Disposition of Radioactive Residues. The excretion of radiochlorine in urine and feces is shown in Table 2, and the overall distribution and recovery of radiochlorine are shown in Table 3. Urine was the major excretory route of radiochlorine with approximately 39—47% of the total administered dose being eliminated in urine by slaughter. Because the last one-third of the total dose was administered only 8 h prior to slaughter, these percentages suggest a fairly rapid excretion of radiochlorine. In contrast, fecal elimination of radiochlorine was minimal with steers 171 and 172 excreting only 1.7 and 0.4% of the total dose, respectively. At slaughter, the small intestine and large intestine collectively contained 8.2 and 5.2% of the total dosed radiochlorine, for steers 171 and 172, respectively, suggesting that absorption or resorption of radiochlorine was occurring in the lower tract. Edible tissues contained a significant fraction of the dosed radiochlorine at slaughter. By virtue of its large proportion of the carcass, skeletal muscle contained the largest fraction of the radiochlorine retained in the body. However, tissues with excretory function such as the liver (bile) and kidney (urine) contained higher concentrations of total residues, also suggesting that the total residue is eliminated rapidly. Total recovery of dosed radio-

Table 2. Excretion of Total Radioactive Residues in Urine and Feces of Steers 171 and 172

		urine radiochlorine		fecal radiochlorine	
animal	time period[a] (h)	concn[b] (ppm)	fraction (%)[c]	concn[b] (ppm)	fraction (%)[c]
171	0–12	1696	7.3	22	0.1
	12–24	1044	2.7	41	0.2
	24–36	4401	8.4	48	0.3
	36–48	3927	7.9	109	0.7
	48–56	4828	10.7	116	0.4
	slaughter	6080	1.6	NA	
		total urine	38.6		1.7
172	0–12	6720	12.8	9	0.0
	12–24	5595	7.0	30	0.0
	24–36	11920	12.5	55	0.1
	36–48	8157	6.0	0	0.0
	48–56	7029	8.4	151	0.2
	slaughter	4521	0.3	NA	
		total urine	47.0		0.4[d]

[a] Animals were dosed at 0, 24, and 48 h. [b] Data are expressed as ppm of sodium chlorate equivalents. [c] Animal 171 received 1993.4 μCi; animal 172 received a total dose of 3976.3 μCi; values are expressed as a percentage of the total dose. [d] Radioactivity excreted in feces (expressed as sodium chlorate equivalents) during time periods 0–12, 12–24, and 36–48 summed to a total of 0.1% of the dose.

Table 3. Concentrations of Radiochlorine, Recoveries of Radiochlorine in Tissues, and Total Recoveries of Radiochlorine in Steers 171 and 172

	steer 171			steer 172		
fraction	ppm	μCi	%	ppm	μCi	%
urine		796.4	38.6		1864.7	46.9
feces		34.8	1.7		14.4	0.4
	edible tissues[a]					
liver	69.6	9.7	0.5	80.7	10.0	0.3
kidney	226.0	5.6	0.3	235.5	8.2	0.2
skeletal muscle	52.9	185.2	9.3	46.9	163.3	4.1
adipose tissue	37.8	NA	NA	29.2	NA	NA
	totals	200.5	10.1		179.5	4.6
	inedible tissues[b]					
brain	76.4	1.4	0.1	59.3	1.2	0.0
lung	171.8	12.0	0.6	163.4	10.0	0.3
spleen	129.4	2.5	0.1	125.6	2.3	0.1
skin	140.7	121.3	6.1	149.2	141.7	3.6
heart	93.0	4.3	0.2	102.7	4.7	0.1
diaphragm	73.5	2.0	0.1	74.8	2.0	0.1
remains of C, solid[c]	73.0	24.7	1.2	116.2	57.8	1.5
remains of C, liquid[c]	99.2	14.9	0.7		0.0	0.0
bone	71.1	130.7	6.6	65.5	128.2	3.2
stomach complex[d]	140.1	215.8	10.8	460.4	836.9	21.0
small intestine	269.0	98.5	4.9	320.7	118.0	3.0
large intestine	245.0	65.5	3.3	312.3	87.5	2.2
blood	187.9	68.5	3.4	244.1	67.7	1.7
bile	190.6	0.3	0	240.3	1.9	0.0
	totals	762.4	38.2		1459.9	36.8
cage wash		4.6	0.2		49.4	1.2
	totals	1771.7	88.8		3567.9	89.9

[a] Traditionally edible tissues in the United States. [b] Traditionally nonedible tissues in the United States. [c] Remains of C, remains of carcass; a liquid portion was collected for animal 171 only. [d] The stomach complex consisted of the rumen, reticulum, omasum, and abomasum.

chlorine was 88.8 and 89.9% for animals 171 and 172, respectively.

For each animal, about 10% of the total radioactivity was accounted for. It is conceivable that some radiochlorine could be lost through respiration, but this is unlikely because the only chlorine species likely to be volatile enough for loss in air would be Cl_2 and ClO_2. Studies by Abdel-Rahman (*15*) and unpublished studies conducted in our laboratory have clearly shown that chlorate and its metabolites are not excreted in expiratory gases of rodents. A more likely cause of the low recovery of radiochlorine was the manner in which the hide was sampled. At slaughter, a sample of the hide was removed from the center of the back where the animal could not lick and where contamination with urine or feces was not an issue. Areas of the hide that were contaminated with saliva, urine, and(or) feces were not sampled; the total amount of radiochlorine present on the hide is almost certainly underestimated.

Summation of the total amount of radiochlorine recovered in nongastrointestinal tissues and in the urine of the steers indicates that 62.1–67.9% of the dosed chlorate was absorbed by steers 172 and 171, respectively. Because cattle were slaughtered only 8 h after the last administration of chlorate (33% of the total dose), these data suggest that chlorate and-(or) its metabolite(s) are rapidly absorbed. Urinary radioactivity represented from 56.9 to 75.5% of the total absorbed radiochlorine in steers 171 and 172, respectively, indicating that radiochlorine was rapidly excreted after absorption. Furthermore, urine collected in the 24 h period after the initial dose contained 30 and 57% of the dosed radiochlorine for animals 171 and 172, respectively.

For edible tissues, total radioactive residues were greatest in kidney (226–236 ppm), followed by liver (70–81 ppm), skeletal muscle (53–47 ppm), and adipose tissue (29–38 ppm). For traditionally nonedible tissues, gastrointestinal tissues generally contained the greatest concentrations of radioactive residue. Ruminal concentrations of radioactivity in steer 172 were about 3.3 times more concentrated than the radioactivity in the rumen of steer 171. In contrast, concentrations of radioactivity in edible tissues of steer 172 matched the concentrations of radioactivity in steer 171 closely, despite the 2-fold dose of chlorate that steer 172 received. If an absorption threshold had occurred, then one might expect higher gastrointestinal concentrations and roughly equal tissue concentrations of radiochlorine between the two animals. However, animal 172 was not consuming normal amounts of feed during the latter portion of the study; if gastrointestinal motility in animal 172 were decreased, then a decreased rate of chlorate absorption would be expected.

Speciation of Tissue Residues. Figure 2 shows an example ion chromatogram of nonradioactive chlorite (ClO_2^-), [^{36}Cl]-chloride, and [^{36}Cl]chlorate and shows the distribution of radioactivity in fractions trapped as solvent eluted from the column. Baseline resolution of the three ions was readily obtained, and the chromatographic distribution of radiochlorine clearly indicates that if radioactive chlorite were present in a tissue sample, it could easily be resolved from either chloride or chlorate.

Figure 3 shows an example chromatogram and a radioprofile of a kidney extract from steer 171. Radioprofiles from other tissues were qualitatively similar to the example radioprofile shown for kidney. Radioprofiles generated from each tissue indicated the presence of [^{36}Cl]chloride and [^{36}Cl]chlorate, but no [^{36}Cl]chlorite-associated radioactivity was detected in any of the tissues.

The speciation of radioactive residues present in edible tissues of cattle is shown in Table 4. For steer 171 (low dose), parent chlorate represented from 1.3 to 28.4% of the total radioactive residue, depending on the tissue. Total radioactive residues in liver were comprised almost entirely of chloride, with the concentration of chlorate residue being 0.7 ppm. Chlorate residue concentrations in skeletal muscle, kidney, and adipose

Table 4. Speciation of Total Radioactive Residues in Edible Tissues of Cattle[a]

	steer 171						steer 172					
	TRR[b]	extractability[c]	chloride		chlorate		TRR[b]	extractability[c]	chloride		chlorate	
tissue	(ppm[d])	(%)	%	ppm[d]	%	ppm[d]	(ppm[d])	(%)	%	ppm[d]	%	ppm[d]
adipose tissue	37.8	99.4	94.9	35.9	5.2	2.0	29.2	74.6	59.8	17.5	40.2	11.7
skeletal muscle	52.9	100.4	73.3	38.8	26.7	14.1	46.9	100.7	56.0	26.3	44.9	21.1
liver	69.6	98.2	98.8	52.3	1.3	0.7	80.7	95.4	98.5	46.2	1.6	1.3
kidney	226	96.4	88.6	200	11.5	25.9	236	97.1	71.6	169	28.4	67.0

[a] Reported values are means from duplicate analyses. [b] TRR, total radioactive residues. [c] Percentage of total radioactive residue extracted into water. [d] Calculated as ppm of sodium chlorate equivalents.

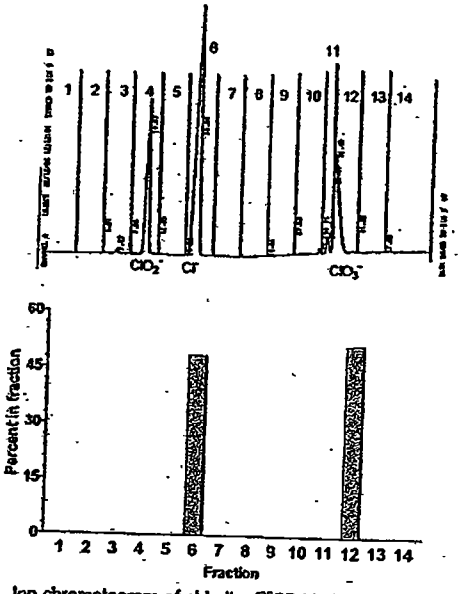


Figure 2. Ion chromatogram of chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate standards. (Top) Ion chromatogram with vertical lines indicating beginning and ending points of numbered fractions. (Bottom) Distribution of radioactivity in collected fractions. Data are expressed as percentage of recovered radioactivity.

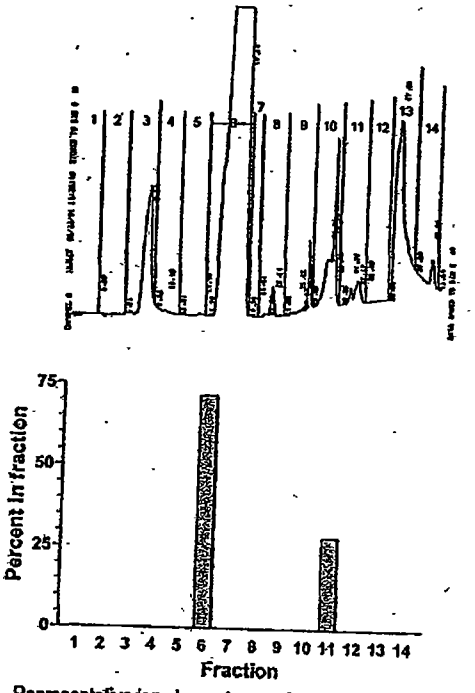


Figure 3. Representative ion chromatogram from a kidney extract of steer 172. (Top) Ion chromatogram with vertical lines indicating beginning and ending points of numbered fractions. (Bottom) Distribution of radioactivity in collected fractions. Data are expressed as percentage of recovered radioactivity.

tissue were 14.2, 25.9, and 2.0 ppm, respectively. For steer 172, chlorate concentrations ranged from 1.3 ppm in liver to 67.0 ppm in kidney. Skeletal muscle and adipose tissue had intermediate concentrations of chlorate at 21.1 and 11.7 ppm, respectively.

Results of the skeletal muscle, liver, and kidney chloride analysis using phenyl mercuric nitrate are presented in Table 5. Reaction with phenyl mercuric nitrate removed over 99% of the chloride from an aqueous extract of [^{36}Cl]chloride-fortified skeletal muscle. In contrast, reaction of phenyl mercuric nitrate with an aqueous extract of [^{36}Cl]chlorate-fortified skeletal muscle removed only 6.6% of the total radioactivity. Because 5.6% of the total radioactivity in the chlorate fortification was chloride ion, it was concluded that reaction of radiochlorine with phenyl mercuric nitrate was specific for chloride. Furthermore, reaction of phenyl mercuric nitrate with radiochlorine extracted from skeletal muscle of steers 171 and 172 indicated that the chloride ion represented 71.9 and 58.1% of the total residue, respectively. These values agree with ion chromatography data indicating that chloride represented 71.6 and 57.0% of the total radiochlorine in skeletal muscle of animals 171 and 172, respectively. In liver, chloride represented an average of 98.7% of the total radioactivity (for animals 171 and 172) as measured by ion chromatography; after reaction with phenyl mercuric nitrate, chloride in livers from animal 171 and 172 assayed at 95.5% of the total residue. Similarly, ion chromatographic analysis of steer 171 kidney indicated that chloride ion represented 88.6% of the radioactive residue, while reaction with PMN indicated that chloride represented 85.1%; the respective values for steer 172 kidney were 71.6 and 70.8%.

Radiochlorine extracted into the chloroform layers was positively identified as phenyl mercuric chloride as evidenced by comparison of mass spectra of authentic phenyl mercuric chloride and peaks eluting at the retention time of phenyl mercuric chloride present in chloroform extracts of animals 171 and 172 (Figure 4). Each mass spectrum shows a multiplet of peaks around *m/z* 314 [M^+]; the multiplet is due to the seven natural isotopes of mercury [^{196}Hg (0.2%), ^{198}Hg (10.1%), ^{199}Hg (16.9%), ^{200}Hg (23.1%), ^{201}Hg (13.2%), ^{202}Hg (29.7%), and ^{204}Hg (6.8%); (*21*)] in combination with the two natural isotopes of chlorine [^{35}Cl (75.77%), ^{37}Cl (24.23%); (*21*)]. The ion cluster around *m/z* 277 represents loss of chlorine, while the base peak at *m/z* 77 represents the loss of both mercury and chlorine. The ion at *m/z* 112 represents a rearrangement whereby mercury is lost and chlorine is retained.

Table 5. Distribution of Radiochlorine in Aqueous (Chlorate) and Chloroform (Chloride) Fractions after Reaction of Tissue Extracts with Phenyl Mercuric Nitrate and Extraction of Phenyl Mercuric Chloride into Chloroform

sample no.	tissue ID	fortification[a]	recovery of radiochlorine			
			aqueous extract[b] (%)	chloroform extract[c] (%)	aqueous layer[d] (%)	total recovery[e] (%)
			skeletal muscle			
1	control	$^{36}Cl^-$	98.9	98.5	1.1	99.6
2	control	$^{36}Cl^-$	100.2	99.6	0.7	100.3
3	control	$^{36}ClO_3^-$	109.9	7.3	93.7	101.0
4	control	$^{36}ClO_3^-$	111.3	5.9	91.8	97.7
5	steer 171	none	93.5	71.9	20.8	92.7
6	steer 171	none	98.5	71.9	23.0	94.9
7	steer 172	none	102.0	58.6	36.6	95.2
8	steer 172	none	97.2	57.5	36.5	94.0
			liver			
1	control	$^{36}Cl^-$	99.7	95.5	0.8	96.3
2	control	$^{36}Cl^-$	97.3	94.8	0.6	95.4
3	control	$^{36}ClO_3^-$	110.4	5.8	93.4	99.2
4	control	$^{36}ClO_3^-$	109.1	5.8	95.9	101.7
5	steer 171	none	101.2	94.4	2.5	96.8
6	steer 171	none	97.5	96.1	9.3	105.4
7	steer 172	none	99.0	94.5	0	93.5
8	steer 172	none	98.8	96.9	1.6	98.6
			kidney			
1	control	$^{36}Cl^-$	98.2	92.0	2.7	94.8
2	control	$^{36}Cl^-$	95.8	93.3	0	93.3
3	control	$^{36}ClO_3^-$	100.4	5.6	85.9	91.4
4	control	$^{36}ClO_3^-$	100.5	5.2	89.4	94.6
5	steer 171	none	100.6	82.1	3.9	85.9
6	steer 171	none	100.7	88.0	7.6	95.6
7	steer 172	none	97.2	75.6	13.2	88.2
8	steer 172	none	102.4	66.0	15.2	81.2

[a] Radiolabeled chlorate contained approximately 5.6% [^{36}Cl]chloride ion. [b] Percentage of total fortified or incurred radiochlorine extracted into water. [c] Percentage of total radiochlorine in aqueous extract removed by chloroform. [d] Percentage of total radiochlorine in aqueous layer not removed by chloroform. [e] Sum of percentage radiochlorine in chloroform and aqueous layers.

Table 6. Speciation of Radiochlorine Excreted into Urine of Steers 171 and 172

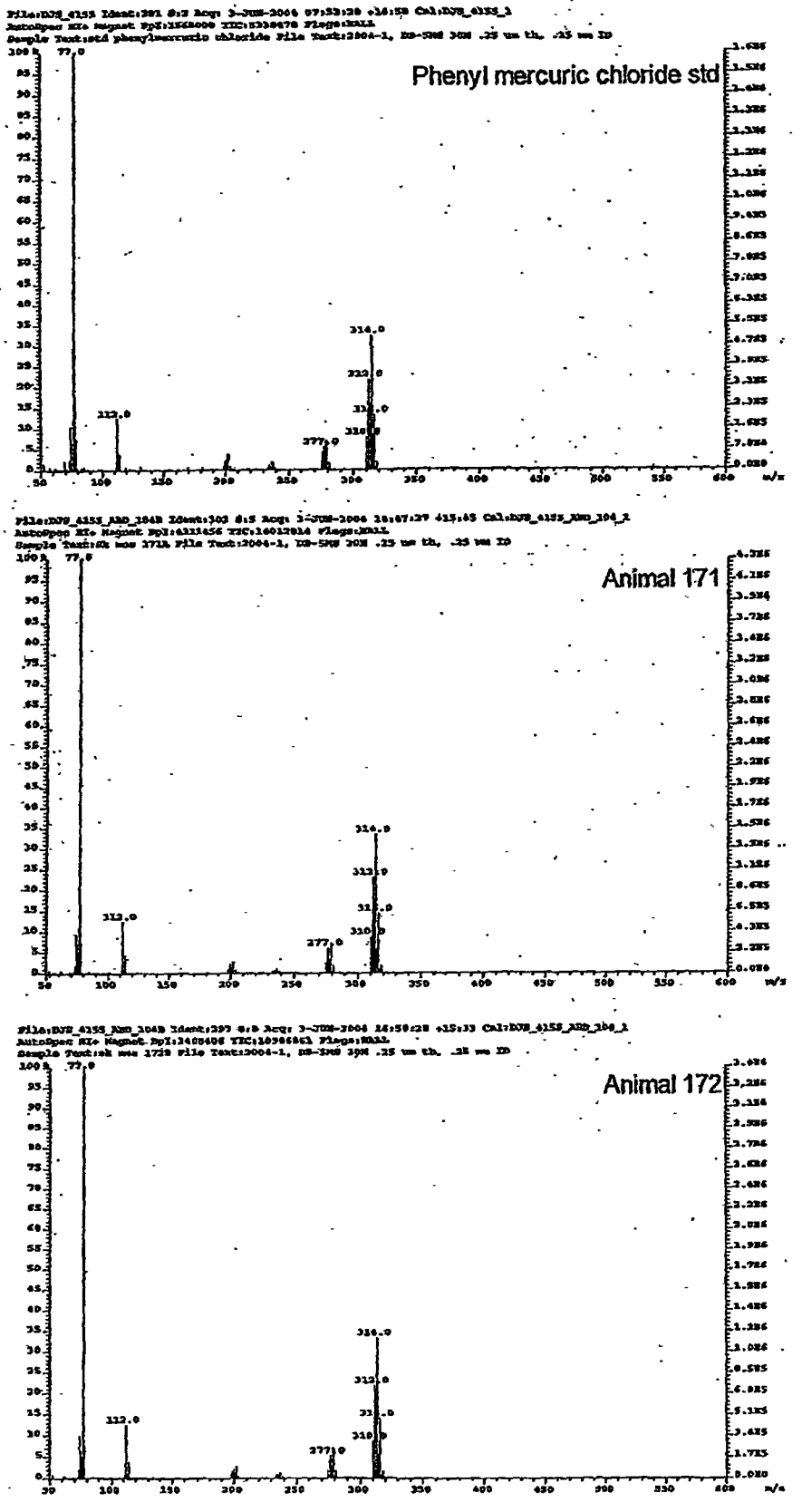
time period (h)	steer 171		steer 172	
	Cl^- (%)	ClO_3^- (%)	Cl^- (%)	ClO_3^- (%)
0–12	9.0	91.1	3.4	96.6
12–24	35.0	65.0	7.5	92.5
24–36	3.1	96.8	1.9	98.0
36–48	1.7	98.3	0.0	100.0
48–56	1.8	98.2	0.3	99.1

The reaction of tissue extracts with phenyl mercuric nitrate provides additional evidence that the very large peak present in ion chromatogram with a retention time similar to chloride was properly identified. Although quantitative data obtained from the chloride analysis using phenyl mercuric nitrate agree with data obtained from ion chromatographic analysis, the assay was executed to verify that the chloride ion is a major product of chlorate metabolism in ruminants.

Speciation of Urinary Residues. The composition of radiochlorine excreted into urine is shown in Table 6. Chlorate was the major radioactive species present in urine with the chloride ion being the only other chlorine species present. Chlorate ranged from 65.0 to 98.3% of the total urinary radioactivity for animal 171 and 92.5 to 100% of the urinary radioactivity for animal 172. For both animals, the largest amount of chloride was excreted during the 12−24 h period after the initial dose. Thereafter, the chloride ion represented a maximum of 3.1% of the urinary radioactivity for both animals. Abdel-Rahman et al. (*15*) reported that 72 h after an oral dose of [^{36}Cl]chlorate, rats had excreted 40% of the total radioactivity in the urine; of this, 62.8% was chloride ion, 25.1% was chlorate ion, and 12.1% was chlorite ion. In cattle, the vast majority of urinary radioactivity was chlorate with little chloride and no chlorite being present. The doses of chlorate administered to cattle in this study were much greater than the dose administered to rats (0.15 mg/kg body weight) by Abdel-Rahman et al. (*15*). In addition, their data were collected over a withdrawal period of 72 h after a single administration, whereas data in this study were collected after three doses and only an 8 h withdrawal period.

In conclusion, chlorate is rapidly absorbed and excreted in steers. Radiochlorine was present in edible tissues primarily as chloride ion, with lesser amounts of chlorate. The proportion of chloride and chlorate was highly tissue dependent. In contrast, chlorate was the major chlorine species present in urine of steers, indicating that the kidney actively excretes chlorate. Because there was a large difference between the proportion of the total residue present as chloride in tissue and urine, it can be concluded that chloride was actively retained, while chlorate was actively excreted.

This study was designed to generate tissue residues after a 3 day chlorate exposure with animals being slaughtered after a practical 0 day withdrawal period. Under this scenario, the doses were high because: (i) chlorate was administered over an extended period even though chlorate efficacy has been demonstrated after a single administration (*3*, *5*); (ii) steers were slaughtered with an 8 h withdrawal period, when efficacy has been measured 24 h after dietary exposure to chlorate (*5*); and (iii) steers were administered 150% of the target dose because it was anticipated that a 0 day withdrawal period might be most useful for cattle producers. With the extended dosing period,



ure 4. Mass spectra of authentic phenyl mercuric chloride and phenyl mercuric chloride present in chloroform extracts of aqueous homogenates prepared from skeletal muscle of steers 171 and 172.

short preslaughter withdrawal time, and an elevated dosing level, two of the four edible tissues (adipose tissue and liver) from the low dose steer (animal 171) contained chlorate residue levels that are thought to be favorable from a food safety point of view. Although no safe tissue concentrations for sodium chlorate have been established by the U.S. FDA CVM, chlorate residues in adipose tissue and liver fell below provisional estimates of safe tissue concentrations (unpublished) provided by the agency. Because chlorate appears to be rapidly metabolized and excreted in steers and because efficacy has been shown for chlorate at lower doses with extended withdrawal periods, further investigation of sodium chlorate at lower doses and a longer withdrawal period is warranted.

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Effect of Sodium [^{36}Cl]Chlorate Dose on Total Radioactive Residues and Residues of Parent Chlorate in Growing Swine

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An experimental chlorate-based product has been shown to be efficacious in eliminating economically important, Gram-negative human pathogens in the gastrointestinal tracts of food animals. Prior to the commercial marketing of such a product, the magnitude and chemical nature of residues remaining in edible tissues must be determined. Thus, the objective of this study was to determine the tissue distribution and elimination of sodium [^{36}Cl]chlorate in orally dosed swine. Three sets of pigs, each consisting of a barrow and a gilt, were orally dosed with a total of 20, 40, or 60 mg of sodium [^{36}Cl]chlorate per kg body weight via the drinking water. Urine and feces were collected throughout the 30 h study. Twenty-four hours after the last exposure to [^{36}Cl]chlorate, each pig was harvested and both edible and inedible tissues were collected. Urine and tissue samples were analyzed for total radioactive residues and for chlorate metabolites. Elimination of radioactivity in urine averaged 81.6, 83.7, and 83.9% of the total dose for the low, medium, and high doses, respectively. Fecal elimination of radioactivity averaged 1.1% of the dosed radiochlorine across all doses. Parent chlorate always represented greater than 97.4% of the urinary radiochlorine with the remaining radiochlorine being excreted as chloride ion. Chlorate represented 39−77% of fecal radioactivity, depending upon dose. Chlorate concentrations in edible tissues ranged from 0.01 to 0.49 ppm, with residues in liver and skeletal muscle generally lower than those in kidney and adipose tissue. Chlorate residues were concentrated in thyroid tissues (7.7−25.4 ppm) relative to edible tissues. No evidence for the presence of chlorite was observed in excreta or in tissues. Results of this study suggest that further development of chlorate as a preharvest food safety tool in swine merits consideration.

KEYWORDS: Sodium chlorate; food safety; pathogens; swine

INTRODUCTION

Respiratory nitrate reductases function in facultatively anaerobic bacteria to capture energy during the conversion of nitrate to nitrite (*1*). Because chlorate (ClO_3^-) is cometabolized by respiratory nitrate reductases to chlorite (ClO_2^-) and because chlorite is toxic to bacteria (*1*), Anderson et al. (*2*) recognized that the metabolism of chlorate by nitrate reductase in Gram-negative pathogens might be exploited for food safety purposes. The vast majority of bacteria present in food animals do not possess nitrate reductase activity; however, economically significant pathogens such as *Escherichia coli* O157:H7 and *Salmonella Typhimurium* express the enzyme when growing under anaerobic conditions (*1*, *3*). Anderson et al. (*2*) hypothesized that when sufficient levels of chlorate are present in the alimentary tract of food animals, pathogens containing nitrate reductase will generate "suicidal" levels of chlorite and will die; those organisms that do not express nitrate reductase were proposed to be unaffected by chlorate.

In vivo studies in both ruminants and nonruminants have validated this hypothesis. For example, chlorate significantly reduced *E. coli O157:H7* populations in gastrointestinal (GI) tracts of cattle and sheep (*4*, *5*) but had little effect on bacterial counts of total culturable anaerobes in ruminal fluid (*2*). Market-age broilers given access to a chlorate-containing product during the 48 h prior to slaughter had significant reductions (40−99%) in crop and cecal *Salmonella* populations (*6*).

In swine, treatment with chlorate is highly effective at reducing populations of both *E. coli* (*7*) and *S. Typhimurium* (*8*−*10*). GI concentrations of *E. coli* were decreased 1.03−2.9 log units (a 62−99.9% reduction, depending on tissue) when sodium chlorate was administered to experimentally infected pigs (*7*) and when the pigs were euthanized 8 h after the last chlorate administration. In weaned pigs artificially infected with *S. Typhimurium* (*8*), animals treated with chlorate contained only about three colony-forming units (CFU) of *S. Typhimurium* per gram of cecal contents, whereas control animals contained

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approximately 1400 CFUs of the pathogen. Consistent with these results are those of Anderson et al. (*9*) who demonstrated that chlorate significantly reduced the incidence of *S. Typhimurium* in lymph, ceca, and recta of finishing pigs.

Collectively, these data suggest that a chlorate-containing product could have several commercial applications, with the preslaughter elimination or reduction of both *E. coli* and *Salmonella* species being of primary importance. The purpose of this study was to determine the magnitude of total and chlorate residues remaining in edible tissues of swine after oral administration, to determine the metabolism of chlorate in swine, and to determine the absorption and elimination of chlorate in tissues and excreta of swine after oral administration.

MATERIALS AND METHODS

Chemicals. Unlabeled sodium chlorate (CAS no. 7775-09-9; 99.96% $NaClO_3$, 0.03% NaCl, and 0.01% H_2O) was provided by EKA Chemicals (Columbus, MS). Sodium chlorate was stored dry at room temperature until use. Sodium chloride (VWR; West Chester, PA); heparin, sodium salt (Sigma Chemical Co.; St. Louis, MO); sodium chlorite (Fluka Chemical Corp.; Milwaukee, WI); sodium hydroxide (50% solution for ion chromatography; Fluka Chemical Corp.); Ultima Gold liquid scintillation fluid, Carbosorb-E, and Permafluor E (Perkin-Elmer Life and Analytical Sciences; Boston, MA); and acetonitrile and methanol (high-performance liquid chromatography grade; EM Science; Gibbstown, NJ) were also used in the study.

Radiolabeled sodium chlorate ($Na^{36}ClO_3$) having a specific activity of 0.575 mCi/mmol and a radiochemical purity of approximately 95% was purchased from Ricerca Biosciences (Concord, OH). Stock sodium [^{36}Cl]chlorate was purified to a radiochemical purity of 99.9% essentially as described by Ruiz-Cristin et al. (*11*). Briefly, a 2.5 cm × 63 cm column of Sephadex G-10 was equilibrated with 0.1 M ammonium acetate (pH 7.0); stock sodium chlorate (0.8 mL aqueous solution; ~1 mCi) was loaded onto the column and was eluted with ammonium acetate at a flow rate of approximately 0.85 mL per min for approximately 24 h. Fractions were collected every 4.7 min (4 mL), and radiochlorine within each fraction was assessed by liquid scintillation counting of 5 μL aliquots. Under the chromatographic conditions used, [^{36}Cl]chloride eluted in fractions 39−46, [^{36}Cl]chlorate eluted in fractions 47−70, and [^{36}Cl]perchlorate eluted as a broad peak in fractions 229−270. Radiochemical purity of the purified [^{36}Cl]chlorate peak was assessed by ion chromatography as described by Smith et al. (*12*).

Dose Preparation. Purified [^{36}Cl]chlorate was diluted with nonradioactive sodium chlorate to a specific activity of 399 ± 1 dpm/μg. The specific activity was determined as described by Smith et al. (*12*). Three dosing solutions (1 L each) containing 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate, respectively, were prepared in aqueous solutions of 2.5 mM sodium nitrate. Sodium nitrate has been shown to increase the efficacy of chlorate in reducing pathogen numbers in live animals (*13*), presumably by inducing respiratory nitrate reductase in nitrate respiring bacteria. The actual chlorate concentrations were 99.9−100.3% of target values. Each dosing solution was transferred to duplicate 1 L plastic water bottles (Kaytee; Kaytee Products, Chicago, IL) so that water bottles contained 498 ± 0.5 g of dosing solution. Sipper tubes, supplied with the water bottles, were attached, and the bottles were stored frozen until dosing.

Animals and Animal Dosing. Three crossbred barrows (9.2 ± 0.4 kg) and gilts (8.2 ± 0.8 kg) were purchased from the North Dakota State University swine herd. Animals were ear tagged and housed by gender in concrete-floored pens during an 18−25 day acclimation period. Pigs were provided with ad libitum access to a swine starter ration (21.6% crude protein, 3.3% fat, and 2.6% fiber; 3204 kcal/kg metabolizable energy; 77.7% total digestible nutrients; North Dakota State University Feed Mill), which they received for the duration of the study. During the acclimation period, pigs were trained to metabolism crates (*14*) and to drink out of 1 L water bottles equipped with stainless steel "sipper tubes".

Table 1. Target and Actual Doses of Sodium [^{36}Cl]Chlorate and Radiochlorine, Dissolved in Drinking Water Delivered to Barrows and Gilts

animal	sex	dose weight (kg)	target dose[a] (mg/kg)	actual dose mg/kg	actual dose μCi	dose duration[b] (h)	withdrawal period[c] (h)
388	gilt	19.1	20	20.8	71	7.7	23.9
390	barrow	19.9	20	19.6	72	5.6	24.0
387	gilt	17.2	40	46.0	143	5.6	24.0
393	barrow	23.1	40	34.4	143	6.0	24.1
389	gilt	19.7	60	60.8	214	5.5	24.0
392	barrow	18.3	60	65.0	215	6.4	24.1

[a] Doses were delivered in approximately 500 mL of 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate in drinking water. [b] Time from initial exposure to [^{36}Cl]chlorate-containing drinking water to complete consumption of the fortified water. [c] Time between the last exposure to [^{36}Cl]chlorate-containing drinking water and slaughter.

Low, medium, and high chlorate doses were each administered to a single barrow or gilt in each of two periods (i.e., within period, a low, medium, and high dose was administered to three swine). Doses were administered by placing the appropriate water bottle containing the frozen chlorate solution on the metabolism cage immediately above the feed tray. Drips from the sipper tube fell directly into the feed and were thus consumed. The pigs drank the chlorate-containing water as it thawed such that the total dose was delivered to pigs within 6.1 ± 0.8 h instead of the 24 h as originally planned. Nevertheless, a 24 h withdrawal period was maintained, and pigs were harvested at 24.0 ± 0.1 h. The target and actual doses, length of exposure to the dosing solutions, and actual withdrawal times are summarized in Table 1.

Collection of Excreta. Swine were housed in metabolism crates that enabled the separate collection of urine and feces (*14*). Urine and feces excreted in the 0−12, 12−24, and 24−30 h time periods were pooled within excreta type for each animal, were weighed, and frozen. At collection, urine and feces were collected as quantitatively as possible. On one occasion, urine from a barrow was excreted beyond the confines of the metabolism crate and urine was recovered from the plastic-backed paper floor covering by placing the contaminated paper in an Erlenmeyer flask, diluting with a known mass of water, and allowing the paper to soak with occasional stirring. Radiochlorine in the water fraction was quantified using liquid scintillation counting.

Animal Harvest and Tissue Collection. Pigs were harvested at the appropriate time by captive-bolt stunning followed by exsanguination into a weighed basin containing 4 mL of heparin (6000 U/mL in physiological saline; Sigma). Pigs were then washed and eviscerated. Traditionally edible tissues (adipose tissue, skeletal muscle, liver, and kidney) and traditionally nonedible tissues (blood, bone, brain, diaphragm, GI contents, GI tract, heart, lung, skin, spleen, thyroid gland, and remainder of the carcass) were collected. Bile was removed using a hypodermic syringe. The GI tract, from the esophagus to the anus, was removed (with pancreatic tissues attached); the GI contents were removed, and the GI tissues and contents were each weighed, the GI contents were subsampled, and both contents and tissues were frozen. Visceral organs, the brain, and the thyroid gland were removed, weighed, and frozen intact. The skin was removed and weighed, and a subsample was removed from the center of the back. Pigs were boned, the bones were weighed, and the scapula was removed as the bone sample. The total muscle was weighed, and a subsample was removed from the *longissimus dorsi*. The remainder of the carcass fraction contained the reproductive tract, trachea, connective tissue, and various tissues not associated with other tissue fractions.

Partially thawed tissues of masses sufficient to pass through a grinder with greater than 50% recovery (brain, diaphragm, GI tract, heart, kidney, liver, lung, and skeletal muscle) were ground; the spleen and thyroid gland were homogenized on dry ice as described by Benville and Tindle (*15*). Adipose tissue was ground with a mortar and pestle after the addition of liquid N_2. Processed tissues were stored frozen. Skin was prepared for total residue analysis by placing 10 ± 0.1 g aliquots into a glass container, adding 90 mL of 1 N NaOH, weighing,

and incubating at 46 °C for approximately 60 h. Under these conditions, the skin was dissolved. Bone was prepared by dissolving approximately one-half of the scapula in 350 mL of 1 N NaOH over 72 h at 90 °C. The solubilized bone solution tended to gel upon cooling; therefore, bone solutions were reheated prior to analysis by liquid scintillation counting (LSC) (described below).

Analytical Methods. LSC techniques, determination of background radiochlorine, and speciation (determination of identity) of total radioactive residues were conducted as described by Smith et al. (*16*) with the following exceptions. Total radioactive residues in skin and bone were determined by placing 0.5 (bone) or 2.0 mL (skin) aliquots of the dissolved tissue in a LSC vial, adding Ultima Gold LSC fluid (15 mL), and counting for 20 min each. The SCX solid-phase extraction step, described by Smith et al. (*16*), was eliminated from the tissue extraction procedure. Briefly, samples were homogenized in water and centrifuged, protein in the resulting supernatant was precipitated with ice-cold acetonitrile, the acetonitrile was evaporated, and the resulting aqueous phase was evaporated under N_2. The remaining aqueous layer was then passed through a C-18 solid-phase extraction cartridge, and the unretained aqueous layer was lyophilized. The dry residue was redissolved in 1 mL of water, and the concentrate was filtered (13 mm, 0.45 μm, PTFE), and subsequently analyzed by ion chromatography as described by Smith et al. (*16*). Urine and tissue sample sets were run with both blanks and blanks fortified with known amounts of [^{36}Cl]chloride and [^{36}Cl]chlorate to determine recovery.

RESULTS

Disposition of Radiochlorine. Table 2 shows the distribution of radiochlorine among edible tissues, nonedible tissues, urine, and feces of dosed swine. Urine contained the greatest portion of the dosed radioactivity. Urine excreted during the first 12 h of the study contained a greater fraction of dosed radiochlorine than any other compartment measured. By the time of slaughter, the cumulative excretion of radiochlorine in urine was 83.1 ± 6% of the total dosed activity. Across all doses, feces contained a cumulative 1.1 ± 1.8% of the administered radiochlorine, an amount equal to the 1.0 ± 0.1% of the dosed radiochlorine remaining in edible tissues at slaughter. Nonedible tissues contained an average of 3.9 ± 0.7% of the dosed activity, with bone, skin, and blood retaining the largest percentage of radiochlorine, largely due to the fairly large masses of these fractions.

Concentrations of total residues are shown in Table 3. As expected from the recovery data, urine contained high concentrations of total residues, ranging from 62 to 2627 ppm depending upon the animal and excretion period. Concentrations of urinary radiochlorine dropped continuously with time periods for all animals. Concentrations of fecal radiochlorine ranged from nondetectable to 524 ppm in gilt 387. Radioactivity in gilt feces was generally greater than concentrations of radiochlorine in barrow feces due to contamination of feces from gilts with urine. In barrows, fecal radiochlorine concentrations ranged from nondetectable to 102 ppm. At slaughter (i.e., 24 h after the last exposure to chlorate containing water), total radioactive residues in feces were 13−215 ppm.

Total radioactive residues in edible tissues generally fell into the following rank order: kidneys > adipose tissue > liver > skeletal muscle. Concentrations of total residues in edible tissues generally showed an apparent dose−response relationship, except for adipose tissue in which residues in tissue of the low and medium dose animals did not appear to differ. Because the pigs were only 17−23 kg, carcasses contained only a small amount of adipose tissue, and the collected adipose tissue samples contained a relatively high proportion of connective tissue. Analysis of the adipose tissue samples indicated that they contained 62.6 ± 7.6% fat, whereas adipose tissue from a market pig would contain approximately 90% fat (*17*).

Table 2. Distribution and Recoveries of Radiochlorine in Tissues and Excreta of Pigs[a]

	low dose[b]		medium dose		high dose	
	gilt 388 (%)	barrow 390 (%)	gilt 387 (%)	barrow 393 (%)	gilt 389 (%)	barrow 392 (%)
edible tissues						
adipose	0.0[c]	0.0	0.0	0.0	0.0	0.0
kidney	0.0	0.0	0.1	0.1	0.0	0.0
liver	0.1	0.1	0.1	0.1	0.1	0.1
skeletal muscle	1.1	1.0	0.8	0.7	0.9	0.7
total in category	1.2	1.1	1.0	0.9	1.0	0.8
inedible tissues						
blood	0.6	0.4	0.4	0.4	0.5	0.4
brain	0.0	0.0	0.0	0.0	0.0	0.0
diaphragm	0.0	0.0	0.0	0.0	0.0	0.0
GI tissue	0.4	0.4	0.3	0.3	0.4	0.5
GI contents	0.6	0.4	0.4	0.3	0.2	0.5
lung	0.1	0.1	0.1	0.1	0.1	0.1
skin	1.3	1.1	0.7	0.9	0.7	1.0
spleen	0.0	0.0	0.0	0.0	0.0	0.0
thyroid gland	0.0	0.0	0.0	0.0	0.0	0.0
heart	0.0	0.0	0.0	0.0	0.0	0.0
bone	2.0	1.8	1.4	1.4	1.2	1.6
bile	0.0	0.0	0.0	0.0	0.0	0.0
remainder of carcass	0.1	0.1	0.1	0.1	0.0	0.1
total in category	5.1	4.3	3.4	3.5	3.1	4.2
urine						
0–12 h	46.6	55.0	62.3	63.0	54.4	44.8
0–12 h spill					10.2	0.7
12–24 h	28.6	25.2	4.0	21.4	18.4	33.4
24–30 h	4.5	3.3	14.3	2.4	1.7	4.1
total in category	79.7	83.5	80.6	86.8	84.7	83.0
feces						
0–12 h	NF[d]	0.0	1.8	NF	0.0	0.0
12–24 h	1.3	NF	1.0	NF	NF	NF
24–36 h	0.4	0.0	1.7	0.1	0.0	0.2
total in category	1.7	0.0	4.5	0.1	0.0	0.2
cage wash	7.7	8.1	8.2	6.3	7.1	8.5
total recovery	95.4	97.0	97.7	97.6	95.9	96.7

[a] Data are expressed as percentages of the total radiochlorine administered. [b] Doses were delivered in approximately 500 mL of 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate in drinking water. [c] Items containing 0% radioactivity did not necessarily have nondetectable residues (see Table 3); generally, the tissues were not of sufficient mass to contain >0.1% of the dosed radiochlorine. [d] NF, no feces were excreted during the indicated time period.

Nature of Residues. *Nature of Urinary and Fecal Residues.* The composition of radiochlorine excreted in urine and feces of swine is shown in Table 4. In no instance was chlorite ion detected in urine or fecal samples. Urinary radiochlorine composition was greater than 97% chlorate, regardless of dose or time of radiochlorine excretion. During the initial 12 h of collection, all radiochlorine detected in the urine was parent chlorate. Radioactive residues present in feces collected in the 6 h period prior to slaughter were composed of both chlorate and chloride ions. Chlorate comprised from 39 to 77% of the total fecal residue. Barrows tended to excrete more parent chlorate in feces than gilts.

Nature of Residues in Tissues. The composition of radioactive residues in edible tissues of swine is shown in Table 5. In contrast to the composition of residues in excreta, tissue residues were composed primarily of chloride ion rather than chlorate ion. In general, radioactive residues were greatest in adipose tissue (0.13−0.49 ppm) and kidney (0.18−0.20 ppm), followed by skeletal muscle (0.07−0.18 ppm). Chlorate residues were always below 0.04 ppm in the liver, regardless of dose. In contrast to edible tissues, a relatively high concentration of parent chlorate was retained by the thyroid gland (Table 5).

Table 3. Concentrations (ppm) of Total Radioactive Residues in Tissues and Excreta of Swine Administered [^{36}Cl]Chlorate in Drinking Water[a]

	low dose[b]		medium dose		high dose	
	gilt 388 (ppm)	barrow 390 (ppm)	gilt 387 (ppm)	barrow 393 (ppm)	gilt 389 (ppm)	barrow 392 (ppm)
edible tissues						
adipose	1.5	1.5	1.6	1.3	2.8	2.8
kidney	2.4	1.7	2.9	2.6	4.5	5.0
liver	0.9	0.7	1.2	1.0	1.7	2.1
skeletal muscle	0.5	0.5	0.9	0.6	1.4	1.3
inedible tissues						
blood	3.5	3.2	4.4	3.9	7.5	7.7
brain	1.4	1.2	2.0	1.5	2.9	3.2
diaphragm	1.2	1.2	1.5	1.1	2.3	2.6
GI tissue	1.6	1.3	2.0	1.9	3.3	4.5
GI contents	1.9	1.0	2.2	1.7	2.8	4.1
lung	2.1	2.0	3.2	2.8	4.5	5.4
skin	1.6	1.4	2.1	2.0	3.2	4.0
spleen	1.4	1.4	2.2	1.7	3.4	4.5
thyroid gland	18.2	19.8	14.0	18.3	17.6	59.8
heart	1.2	1.0	1.9	1.4	2.6	2.9
bone	1.8	1.5	2.6	2.1	3.1	4.1
bile	1.9	1.5	0.0	2.2	4.4	6.1
remainder of carcass	2.0	1.6	3.5	2.2	7.9	5.2
urine						
0–12 h	830	835	2387	1413	2627	1904
12–24 h	423	314	570	412	421	1060
24–30 h	73	86	354	75	62	267
feces						
0–12 h	NF[c]	NDR[d]	524	NF[c]	36	<1
12–24 h	199	NF[c]	307	NF[c]	NF[c]	NF[c]
24–36 h	30	19	215	102	13	55

[a] Data are expressed as sodium chlorate equivalents. [b] Doses were delivered in approximately 500 mL of 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate in drinking water. [c] NF, no feces were excreted during the indicated time period. [d] NDR, no detectable residue.

Table 4. Chlorate Composition of Urinary and Fecal Radiochlorine in Pigs Administered [^{36}Cl]Chlorate in Drinking Water[a]

	low dose[b]		medium dose		high dose	
time	gilt 388	barrow 390	gilt 387	barrow 393	gilt 389	barrow 392
urine						
0–12	100.0	100.0	100.0	100.0	100.0	100.0
12–24	96.2	99.7	98.5	99.1	98.5	98.5
24–30	99.3	99.1	100.0	100.0	97.4	99.5
feces[c]						
24–30	38.8	65.1	50.9	73.1	53.3	76.6

[a] Data are expressed as the percentage of total radiochlorine excreted as parent chlorate; the balance of the radiochlorine was excreted solely as chloride ion. [b] Doses were delivered in approximately 500 mL of 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate in drinking water. [c] Residues in feces were speciated only for the 24–30 h time period.

For example, chlorate residues in the thyroid glands ranged from a low of 3.4 ppm in pig no. 387 to a high of 41.9 ppm in pig no. 392.

DISCUSSION

Data generated in this study clearly demonstrate that chlorate is rapidly absorbed and excreted in the urine of swine. In these pigs, 83.1 ± 2.6% of the dose was excreted in the urine during the 30 h study period with 56.2 ± 8.5% of the dosed radiochlorine excreted during the first 12 h of the study. Overall, 67% of the radiochlorine excreted in the urine was excreted during the first 12 h of the study. This 12 h period included the 6 h dosing period and the subsequent 6 h period after completion of dosing. The rapid absorption and elimination of chlorate clearly indicate that oral delivery of chlorate via the drinking water is an inefficient means to deliver chlorate to the lower GI tract. Presumably, a more efficient delivery of chlorate to the lower GI tract would increase the efficacy at killing pathogens. Nevertheless, even with the inefficient delivery of chlorate to the lower GI tract, numerous studies have demonstrated chlorate's efficacy against *E. coli* and *Salmonella enterica* in swine (*7*–*9*) dosed in a manner similar to the procedure used in this study.

Chlorate concentrations of 1.25 mM (equivalent to 160 ppm) in bovine ruminal fluid were sufficient to cause 3 log unit reductions of *E. coli* O157:H7 and *S. Typhimurium* (*2*). Feces excreted during the 6 h period immediately prior to slaughter contained 13–215 ppm of total radioactive residue, of which chlorate residues ranged from 7 to 110 ppm. Thus, chlorate concentrations in these swine were typically below chlorate concentrations shown to be active against relevant pathogens in vitro. Total radioactive residues in GI contents (whole tract contents) at slaughter were only 1–4 ppm. It is not known if chlorate is active against Gram-negative pathogens at levels below this, but the low GI residues at 24 h might help to explain why chlorate reduced cecal *S. Typhimurium* concentrations about 3 log units 16 h after the last exposure to chlorate but not 24 h after the last chlorate dose (*8*). Anderson et al. (*8*) suggested that the absence of a chlorate effect at 24 h was a function of the kinetics of chlorate in live swine. The current study serves to emphasize Anderson et al.'s point that there is a "need to develop practical administration procedures that optimize delivery and maintenance of effective concentrations of chlorate to the lower gut."

In contrast to previous reports (*18*, *19*) suggesting that chlorate is metabolized to chlorite (ClO_2^-) and excreted as the chlorite ion, no evidence for the existence of chlorite in urine or tissues of swine was generated in this study. In this regard, swine are similar to cattle (*12*, *16*). This finding diverges from studies conducted in the 1980s, which indicated that rats metabolize chlorate to chlorite and that chlorite is excreted as a urinary metabolite in appreciable quantities (i.e., up to 12% of the dosed chlorate). As discussed by Smith et al. (*16*), and as verified by Hakk et al. (submitted for publication) in a replication of Abdel Rahman's rat study, the analytical method used to measure chlorite in rat excreta (*20*) was not adequate, and results from the studies in rats (*18*, *19*) could not be corroborated (Hakk et al., submitted for publication). Subsequent studies in our laboratory utilizing $^{36}ClO_3^-$ in rats demonstrate that chlorite is not present in rat tissues or excreta. The absence of chlorite in tissues of food animals treated with chlorate has important food safety implications because chlorite is a strong oxidizing agent with toxicological concerns (*21*) of its own.

Residues of parent chlorate in edible tissues of these swine were generally less than 1% of the total radioactive residue. As indicated by the urinary chlorate levels, chlorate is apparently actively excreted, presumably because of extremely poor tubular resorption in the kidney. In contrast, little to no radioactive chloride was excreted into urine during the study. Under normal physiological conditions, about 99% of the chloride ion filtered through the glomerulous is resorbed in the proximal and distal tubules (*22*). Suh and Abdel-Rahman (*23*) determined that the half-lives of chloride absorption and excretion in rats are

Table 5. Total Radioactive Residues, Chloride Residues, and Chlorate Residues in Edible Tissues and Thyroid Glands of Swine

			tissue														
			liver			kidney			skeletal muscle			adipose tissue			thyroid gland		
dose (mg/kg)	animal	sex	TRR[a] (ppm)	Cl^{-}[b] (ppm)	ClO_3^{-}[c] (ppm)	TRR[a] (ppm)	Cl^{-}[b] (ppm)	ClO_3^{-}[c] (ppm)	TRR[a] (ppm)	Cl^{-}[b] (ppm)	ClO_3^{-}[c] (ppm)	TRR[a] (ppm)	Cl^{-}[b] (ppm)	ClO_3^{-}[c] (ppm)	TRR[a] (ppm)	Cl^{-}[b] (ppm)	ClO_3^{-}[c] (ppm)
20	390	barrow	0.74	0.74	0.01	1.75	1.68	0.07	0.52	0.45	0.09	1.50	1.23	0.27	19.8	12.9	6.4
	388	gilt	0.92	0.91	0.01	2.40	2.05	0.30	0.53	0.47	0.06	1.51	1.40	0.11	18.2	7.8	10.3
		average		0.82	0.01		1.86	0.18		0.46	0.07		1.31	0.19	19.0	10.3	8.4
40	393	barrow	1.00	0.99	0.02	2.55	2.39	0.17	0.63	0.55	0.08	1.43	1.24	0.17	18.3	6.2	11.9
	387	gilt	1.19	1.18	0.02	2.94	2.69	0.24	0.89	0.83	0.07	1.62	1.52	0.09	14.0	9.9	3.4
		average		1.09	0.02		2.54	0.20		0.69	0.07		1.38	0.13	16.2	8.1	7.7
60	392	barrow	2.11	2.09	0.03	5.03	4.87	0.17	1.28	0.97	0.26	2.83	2.26	0.58	59.8	17.0	41.9
	389	gilt	1.68	1.61	0.05	4.46	4.24	0.22	1.43	1.31	0.10	2.75	2.35	0.40	17.6	8.1	9.1
		average		1.85	0.04		4.55	0.19		1.14	0.18		2.30	0.49	38.7		25.4

[a] TRR, total radioactive residues expressed in parts per million of chlorate equivalents; the sum of chloride and chlorate fractions may not equal TRR due to rounding. [b] Chloride residue calculated by multiplying the percentage chloride in extracted sample by the ppm total radioactive residue. The concentrations of chloride do not reflect the physiological concentration of chloride in tissues, only that fraction of total residue present as radioactive chloride ion. [c] Chlorate residue calculated by multiplying the percentage chlorate in extracted sample by the ppm of total radioactive residues.

approximately 19 and 52 h, respectively. In contrast, the absorption and elimination half-lives of chlorate in cattle were approximately 0.7 and 7.7 h, respectively (*24*). If the kinetics of chlorate and chloride in swine are consistent with measurements taken from cattle and rats, then the preponderance of radioactive residues present as chloride ion in tissues of these swine is easily explained: Chlorate is rapidly eliminated whereas chloride is retained in the body.

From a chemical residue perspective, the chlorate residues remaining in edible tissues of swine, regardless of dose, were always 25% or less than chlorate concentrations provisionally estimated by the U.S. Food and Drug Administration to be safe in edible tissues (unpublished results). The relatively high concentration of chlorate in the thyroid gland would be of no concern to humans because thyroid gland is not a common food. It is also not of concern from a swine health perspective because chlorate is envisioned as a food safety tool to be used during a time just before slaughter.

The sites and mechanism(s) of chlorate conversion to chloride ion within swine are not known. It is likely that some reduction of chlorate to chloride could occur via bacterial reduction. Oliver et al. (unpublished results) have shown that approximately 50% of the [^{36}Cl]chlorate fortified into bovine ruminal fluid (100 ppm) was converted to chloride residue within about 24 h. Not all of the conversion appeared to be related to bacteria, however, as there was some reduction of chlorate in ruminal fluid that had been autoclaved prior to incubation. Thus, enzymatic and nonenzymatic processes are likely occurring. Biotransformation of chlorate after absorption also occurs. Smith et al. (*16*) reported that chlorate residues in skeletal muscle from cattle orally dosed with chlorate were converted to chloride during refrigeration (designed to mimic carcass-aging processes) but that chlorate residues in beef cattle muscle were stable when stored frozen for 6 months. Chlorate degradation during refrigeration (4−6 °C) of fortified skeletal muscle has also been observed (Smith et al., unpublished results). Whether chlorate degradation is due to enzymatic processes or due to the direct reduction by physiologic reducing agents within tissues is not known.

Total radioactive residues in thyroid tissues were clearly greater than total residue levels in other tissues. Although a substantial portion of the radiochlorine was chloride, concentrations of chlorate in the thyroid gland were substantially greater than in the liver, kidney, or skeletal muscle. Perchlorate also accumulates in thyroid tissues of rats (*25*−*27*). Radiochemical analysis of extracted radiochlorine from thyroids of these swine clearly indicated that chloride and chlorate, not perchlorate, were present. Thus, these data suggest that chlorate is similar to perchlorate in that it will accumulate in the thyroid tissues of treated animals. This accumulation may be related to the chronic effects of high dose chlorate on cellular proliferation in the thyroid (*28*).

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Total Radioactive Residues and Residues of [^{36}Cl]Chlorate in Market Size Broilers

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The oral administration of chlorate salts reduces the numbers of Gram-negative pathogens in gastrointestinal tracts of live food animals. Although the efficacy of chlorate salts has been demonstrated repeatedly, the technology cannot be introduced into commercial settings without first demonstrating that chlorate residues, and metabolites of chlorate remaining in edible tissues, represent a negligible risk to consumers. Typically, a first step in this risk assessment is to quantify the parent compound and to identify metabolites remaining in edible tissues of animals treated with the experimental compound. The objectives of this study were to determine the pathway(s) of chlorate metabolism in market broilers and to determine the magnitude of chlorate residues remaining in edible tissues. To this end, 12 broilers (6 weeks; 2.70 ± 0.34 kg) were randomly assigned to three treatments of 7.4, 15.0, and 22.5 mM sodium [^{36}Cl]chlorate dissolved in drinking water (n = 4 broilers per treatment). Exposure to chlorate, dissolved in drinking water, occurred at 0 and 24 h (250 mL per exposure), feed was withdrawn at hour 38, water was removed at hour 48, and birds were slaughtered at hour 54 (16 h after feed removal and 8 h after water removal). The radioactivity was rapidly eliminated in excreta with 69–78% of the total administered radioactivity being excreted by slaughter. Total radioactive residues were proportional to dose in all edible tissues with chloride ion comprising greater than 98.5% of the radioactive residue for the tissue (9.4–97.8 ppm chlorate equivalents). Chlorate residues were typically greatest in the skin (0.33–0.82 ppm), gizzard (0.1–0.137 ppm), and dark muscle (0.05–0.14 ppm). Adipose, liver, and white muscle tissue contained chlorate concentrations from 0.03 to 0.13 ppm. In contrast, chlorate concentrations in excreta eliminated during the 6 h period prior to slaughter ranged from 53 to 71 ppm. Collectively, these data indicate that broilers rapidly convert chlorate residues to an innocuous metabolite, chloride ion, and that chlorate residues in excreta remain fairly high during the time around slaughter. Because the target tissue of chlorate is the lower gastrointestinal tract, the relatively high distribution of parent chlorate to inedible gastrointestinal tissues and low distribution to edible tissues is favorable for the biological activity and for food safety considerations. These data, when used in conjunction with a toxicological assessment of chlorate, can be used to determine a likely risk/benefit ratio for chlorate.

KEYWORDS: Broilers; chlorate; food safety; preharvest; residue

INTRODUCTION

According to statistics compiled by the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS), the baseline rate of *Salmonella* contamination of broiler carcasses within the United States is 20% (*1*), while that of ground poultry meat is 44.6% (*2*). In response to the high rates of poultry product contamination, the U.S. Department of Agriculture FSIS established a series of Hazard Analysis and Critical Control Point (HACCP) rules for "large", "small", and "very small" poultry slaughter establishments that were implemented from 1996 to 2000. Nationwide surveys of broiler carcasses and ground chicken taken since the establishment of the HACCP rules have indicated that rates of *Salmonella* contamination for poultry products (including ground turkey) are typically greater than 50% of the pre-HACCP baseline values (*3*). Indeed, for broiler and ground chicken, rates of *Salmonella* contamination increased each year from 2003 to 2005 (*3*). As a result of these high numbers, FSIS has stated that "FSIS is concerned with increases in *Salmonella* rates observed over the last 3 years (2003–2005) among the three poultry product categories...In

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response, FSIS increased resources allocated to comprehensive Food Safety Assessments in establishments displaying negative performance trends..." (*3*).

Unfortunately, for most poultry-rearing and -processing establishments, there are few affordable technologies available to address the growing concern of carcass contamination by *Salmonella* species. Specifically, few practical technologies other than competitive exclusion (*4*) are available that allow the reduction or elimination of *Salmonella* pathogens in live animals prior to harvest. Unfortunately, even with the availability of competitive exclusion, *Salmonella* remains a problem for the poultry industry (*4*).

A recent innovation in preharvest food safety has been the development of an experimental chlorate product (ECP), which, when provided as a drinking water supplement, significantly reduces the incidence and quantities of *Salmonella* in the crops and(or) ceca of market weight broilers (*5*, *6*). In addition to effects in broilers, other studies have shown that ECP also decreased *Salmonella* incidence and numbers in forced-molt laying hens (*7*) and in other species such as swine (*8*, *9*), cattle (*10*, *11*), and turkeys (*12*).

Prior to the commercial use of a chlorate-based product in food animals, the metabolism of chlorate in target species must be demonstrated, and the magnitude of residues in edible tissues of target animals must be measured. Metabolism and residue studies in cattle (*13*, *14*) and hogs (*15*) have demonstrated that chlorate is metabolized to chloride ion in both ruminant and nonruminant food animals and that under anticipated commercial use situations, chlorate residues remaining in edible tissues were sufficiently low to warrant further development of chlorate as a preharvest food safety tool. The purpose of this study was to determine the metabolism and magnitude of chlorate residues in broiler chickens, a commercially important avian species for which the preharvest control of *Salmonella* would have significant impact.

MATERIALS AND METHODS

Chemicals and Dose Formulation. Chemicals used were essentially those described by Smith et al. (*15*). Sodium [^{36}Cl]chlorate was purified, radiochemical purity was assessed, and the specific activity was determined as described by Smith et al. (*15*). The final specific activity was 404 ± 2 dpm/μg with a radiochemical purity of 99.9%; the radiochemical impurity was [^{36}Cl]chloride ion. Radioactive [^{36}Cl]-chlorite, used as an analytical standard, was synthesized and stored as described by Hakk et al. (*16*).

Three 2 L solutions were prepared to contain 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate, respectively, for delivery to broilers via drinking water. The 15 mM solution corresponds to the "1×" dosing regimen of Byrd et al. (*5*). Dosing solutions also contained 2.5 mM sodium nitrate and 20 mM D,L-sodium lactate. Nitrate and lactate were added to the drinking water solutions to induce bacterial nitrate reductases and to provide readily available reducing equivalents as described by Jung et al. (*6*). It is hypothesized that induction of nitrate reductases in pathogens renders them more sensitive to the bactericidal effects of chlorate (*6*).

Broilers. A detailed animal protocol was approved by the Institutional Animal Care and Use Committee prior to initiating the study. Day-old Jumbo Cornish × Rock cockerels (n = 25) were purchased from McMurray Hatchery (Webster City, IA). Upon delivery, chicks were placed into a battery equipped with 60 or 75 W bulb heating sources and given free access to nonmedicated chick starter ration and to water. At 2 weeks of age, feed was changed from starter ration to a nonmedicated grower ration. At approximately 1 month of age, the birds were transferred from batteries to concrete floored pens covered with pine shavings, where they were provided free access to grower feed and water. Animals were housed in open pens until initiation of experiment. Twelve birds, four per dose level, were selected for inclusion in the residue study, and two birds were selected to provide control tissues. The remaining broilers were used in an unrelated study.

Table 1. Study Timeline, Where "X" Indicates that the Action Defined by the Column Header Was in Effect at the Indicated Time Period[a]

		action				
study day	study hour	feed $NaNO_3$	water Na[^{36}Cl]ClO_3	water lactate/NO_3^-	feed removed	remove water
−5	−120	X				
−4	−96	X				
−3	−72	X				
−2	−48	X				
−1	−24	X				
0	0	X	X[b]	X		
1	24	X	X[b]	X		
	38				X	
2	48				X	X
	54		kill birds, harvest tissues			

[a] Broilers were adapted to metabolism crates 2 days prior to the initiation of sodium nitrate feeding; sodium nitrate was fed throughout the remainder of the study. Birds were provided water containing sodium lactate, sodium nitrate, and sodium [^{36}Cl]chlorate at T0. At 16 h prior to slaughter, feed was withdrawn from the birds. [b] Provided as a 250 mL aliquot; when a bird had consumed the total aliquot of radioactive chlorate, the water bottle was filled with tap water.

Study Design. Seven days (−164 h) prior to dosing with [^{36}Cl]-chlorate containing drinking water, broilers were moved from the group housing of the floor pens to individual cages within suspended wire poultry batteries. Excreta was collected in aluminum trays (33 cm × 45 cm) suspended 6−8 cm below the wire cages. Table 1 summarizes the study timeline. Starting 5 days prior to chlorate administration and continuing until the preslaughter feed withdrawal, birds were provided ad libitum access to a grower ration supplemented with 574 ppm sodium nitrate (*6*). Nitrate-fortified feed (15 kg) was prepared by dripping 250 mL of a 34.4 mg/mL sodium nitrate solution onto feed from a separatory funnel as the feed was mixed in a ribbon mixer. On study hours 0 and 24, each bird was given access to 250 mL of either 7.5, 15, or 22.5 mM sodium [^{36}Cl]chlorate, while nitrate (2.5 mM) and lactate (20 mM) in drinking water were held constant. After consumption of the chlorate-treated drinking water was completed each day, water bottles were filled with tap water. Thirty-eight hours after the first exposure to chlorate (16 h prior to slaughter), feed was removed from each broiler. By 36 h of the study, all chlorate-containing water had been consumed and replaced with tap water. Fifty-four hours after the initial exposure to chlorate, broilers were slaughtered and edible tissues were dissected for residue analysis. Excreta were collected and weighted at periods encompassing 0−12, 12−24, 24−36, 36−48, and 48−54 h of the study.

Slaughter was accomplished by cervical dislocation, followed by exsanguination. Pectoral muscles (white meat; breast), thighs (dark meat), livers, skin with adhering adipose tissue, abdominal adipose tissues, and gizzards were removed and weighed from each bird. Tissues were individually stored in labeled containers and frozen until analysis.

Analytical Methods. Background radioactivity and limits of quantitation of chlorate were determined as described by Smith et al. (*14*). Because total tissue weights were not determined for all tissues (i.e., skin, white meat, dark meat, and adipose tissue), total amounts of radioactivity in each tissue fraction were estimated using literature values for carcass composition of broilers [Richter et al. 1989, as cited by Rose (*17*)], that is, 10.5% skin, 27.6% dark meat (i.e., legs and thigh), 13.9% white meat (breast), and 1.6% abdominal fat.

Speciation (determination of identity) of total radioactive residues (TRRs) in tissues and excreta was conducted as described by Smith et al. (*13*) except that the cation-exchange solid-phase extraction (SPE) step was eliminated from the tissue extraction procedure to reduce the time and expense of the analyses and because the cation-exchange SPE step had little effect on the results of the chlorate analysis. Briefly, samples were homogenized in water and centrifuged, the resulting supernatant was treated with ice-cold acetonitrile to precipitate proteins, and the acetonitrile was evaporated under N_2. The remaining aqueous layer was passed through a C-18 SPE cartridge, and the unretained

Table 2. Chlorate Treatments, Associated Radioactivity, and Doses Delivered to Broilers[a]

nominal dose	chlorate concn (mM)	drinking water[b] (g)	total activity[c] (μCi)	chlorate mass[d] (mg)	broiler wt (kg)	dose (mg/kg)
low	7.5	499 ± 6	74 ± 0.9	403 ± 4.9	2.5 ± 0.5	164 ± 34
medium	15.0	499 ± 5	145 ± 1.5	797 ± 8.4	2.7 ± 0.1	292 ± 9
high	22.5	499 ± 4	215 ± 1.8	1186 ± 9.9	2.9 ± 0.2	407 ± 25

[a] Data are presented as means ± standard deviations of four animals per treatment. [b] Mass of [^{36}Cl]chlorate-containing drinking water consumed. [c] Total amount of radioactivity dosed per bird. [d] Total mass of sodium chlorate in associated drinking water.

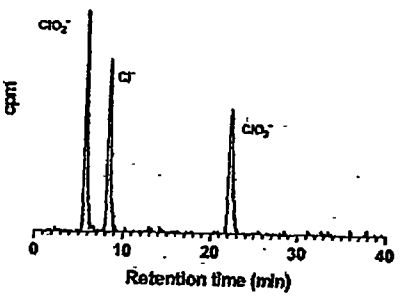

Figure 1. Example chromatogram showing the resolution of [^{36}Cl]chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate with the chromatographic conditions used to speciate radioactive residues in tissues and excreta. Flow-through radiochemical detection was used to separate the radiolabeled standards in this chromatogram; to quantify metabolites in tissue and excreta extracts, fractions were collected during the chromatographic runs and radioactivity was quantified by liquid scintillation counting.

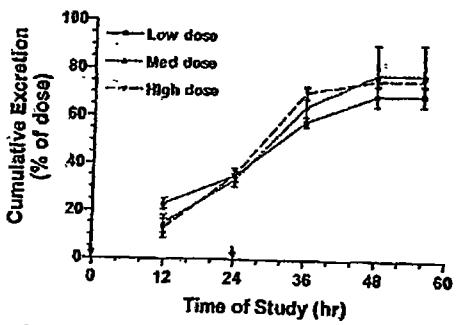

Figure 2. Cumulative excretion of radioactivity by broilers dosed with 7.5 (low), 15 (medium), and 22.5 (high) mM sodium chlorate dissolved in drinking water. Data are expressed as a percentage of the total radioactivity administered to each bird. Arrows indicate exposure to [^{36}Cl]chlorate in drinking water.

aqueous layer was collected and lyophilized. The dry residue was redissolved in 1 mL of water, filtered (13 mm, 0.45 μm, PTFE), and subsequently chromatographed using ion chromatography. Fractions were collected over the entire high-performance liquid chromatography (HPLC) run, the recovery of radioactivity in each fraction was determined by liquid scintillation counting, and the amount of radioactivity in the chlorate fraction was determined. Sample sets included control tissue extracts (blanks), control tissues fortified with known amounts of [^{36}Cl]chloride and [^{36}Cl]chlorate, and unknowns. An example radiochromatogram showing the resolution of a mixture of [^{36}Cl]chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate using the solvent gradient for the tissue analysis is shown in Figure 1.

Differences in extraction efficiency of radioactive residue in fortified control tissues and in tissues with incurred residues were determined using one-way analysis of variance followed by Tukey's multiple comparison test. Significance was set at a probability of 0.05.

RESULTS AND DISCUSSION

fferences ($P \geq 0.4$) in weight gain among the nitrate-orate-exposed birds occurred over a 7 day nitrate exposure period and a 48 h exposure period to chlorate. For the low, medium, and high chlorate exposures, weight gains (mean ± standard deviation) were 0.25 ± 0.17, 0.20 ± 0.19, and 0.37 ± 0.12 kg, respectively. The relatively large variation in gain at the low and medium exposure levels was due to a single broiler in each group having essentially no gain; each of these two birds developed leg problems during the study and did not consume as much feed as the other birds. Chlorate intake was not affected because 100% of the [^{36}Cl]chlorate-fortified drinking water was consumed by each bird. Leg problems were not believed to be related to either nitrate or chlorate treatment but to the rapid growth rates of modern broilers (*18*).

Actual doses of chlorate administered to birds are shown in Table 2. Doses were formulated on the basis of concentration in drinking water and delivered to birds as such. Because body weights of birds within a treatment group varied somewhat, doses delivered on a mg/kg body weight basis varied somewhat as well. For example, the coefficients of variation for the low, medium, and high doses when expressed on a mg/kg body weight basis were 20.7, 2.9, and 6.1%, respectively. Such variation would likely be observed if chlorate salts were to be used in commercial settings.

Figure 2 shows the cumulative elimination of radioactivity in excreta from the treated broilers. When excreta data were expressed as a percentage of the dose, there was little to no proportionality with dose apparent. Recoveries of radioactivity were between 77 and 83% of the dose, as shown in Table 3.

Table 3. Total Recoveries [Mean ± Standard Deviations (SD)] of Radioactivity in Excreta and Edible Tissues of [^{36}Cl]Chlorate-Treated Broilers[a]

	chlorate level		
	7.5 mM	15 mM	22.5 mM
fraction	mean ± SD	mean ± SD	mean ± SD
	excreta		
0–12 h	23.1 ± 4.7	14.8 ± 7.5	12.8 ± 8.0
12–24 h	12.0 ± 5.0	18.4 ± 5.7	22.6 ± 6.6
24–36 h	23.0 ± 3.8	31.1 ± 11.8	35.0 ± 4.6
36–48 h	10.9 ± 6.4	13.3 ± 10.6	4.9 ± 0.4
48–54 h	0.4 ± 0.2	0.2 ± 0.2	0.2 ± 0.1
total	69.4 ± 8.9	77.9 ± 25.8	75.5 ± 4.9
	tissues[b]		
white meat	0.9 ± 0.4	0.8 ± 0.2	0.7 ± 0.3
dark meat	2.8 ± 1.1	1.9 ± 0.6	2.3 ± 0.7
liver	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
skin with fat	3.1 ± 1.2	1.9 ± 0.8	2.6 ± 0.8
abdominal fat	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.1
gizzard	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
total	7.4 ± 2.9	4.8 ± 1.9	6.0 ± 2.0
total	76.9 ± 10.3	82.8 ± 27.3	81.6 ± 6.4

[a] Data are expressed as percentages of the total dose present in each fraction. [b] Percentage recoveries of radioactivity in tissues were calculated by multiplying the TRR (dpm/g) by the product of the bird weight and carcass composition of broilers as reported by Rose (*16*).

Table 4. Concentrations of TRRs, Chloride Residues, and Chlorate Residues in Edible Tissues of Broilers and the Composition of Radioactivity in Excreta Collected during the 48–54 h Time Period[a]

	chlorate level								
	7.5 mM			15 mM			22.5 mM		
tissue	TRR (ppm)	chloride (ppm)	chlorate[b] (ppm)	TRR (ppm)	chloride (ppm)	chlorate[b] (ppm)	TRR (ppm)	chloride (ppm)	chlorate[b] (ppm)
adipose	9.4 ± 3.4	9.4 ± 3.2	0.077 ± 0.045[c]	10.0 ± 2.8	10.0 ± 2.6	0.050 ± 0.034[d]	15.7 ± 7.4	15.6 ± 6.9	0.129 ± 0.159[d]
gizzard	35.6 ± 9.6	35.4 ± 8.9	0.136 ± 0.098	44.7 ± 18.0	44.6 ± 16.7	0.137 ± 0.096[c]	83.8 ± 21.6	83.7 ± 20.0	0.100 ± 0.021[d]
liver	30.0 ± 10.6	29.9 ± 9.8	0.063 ± 0.037[d]	39.8 ± 17.4	39.7 ± 16.1	0.095 ± 0.054[c]	70.6 ± 23.2	70.5 ± 21.5	0.087 ± 0.049[e]
muscle white	10.4 ± 3.3	10.3 ± 3.3	0.068 ± 0.098	12.2 ± 4.5	12.1 ± 4.5	0.090 ± 0.089[c]	22.3 ± 7.1	22.2 ± 7.1	0.030 ± 0.032
muscle dark	15.9 ± 5.0	15.8 ± 4.6	0.053 ± 0.056	19.4 ± 7.4	19.3 ± 6.8	0.097 ± 0.083	33.5 ± 12.2	33.4 ± 11.4	0.135 ± 0.118[c]
skin	45.7 ± 14.4	45.3 ± 13.4	0.329 ± 0.242	54.1 ± 22.8	53.5 ± 21.1	0.570 ± 0.115	98.6 ± 38.5	97.8 ± 35.9	0.819 ± 0.485
excreta, 48–54 h	114.9 ± 66.5	43.6 ± 24.9	70.6 ± 49.2	134.1 ± 66.2[e]	63.6 ± 15.0[e]	70.5 ± 39.1[e]	110.4 ± 49.0	57.3 ± 32.8	53.0 ± 37.2

[a] Data are expressed as means ± standard deviations (ppm) of four broilers per dose level. Chloride residues are expressed in ppm chlorate equivalents and do not represent concentrations of endogenous tissue chloride. [b] Chlorate limits of quantitation were 0.022, 0.017, 0.019, 0.015, 0.019, and 0.021 ppm for adipose tissue, gizzard, liver, white muscle, dark muscle, and skin, respectively. [c] Mean of three broilers; one broiler had no detectable chlorate residues in tissue. [d] Mean of two broilers; two broilers had no detectable chlorate residues in tissue. [e] Mean of two broilers; two broilers did not eliminate excreta during the indicated time period.

Because no attempt was made to measure radioactivity in traditionally inedible carcass parts, total recovery values were not measured experimentally. Values shown in Table 3 represent only the radioactivity recovered in the shown tissues. Assuming a 100% recovery, approximately 18–23% of the dosed radiochlorine remained with carcass tissues not specifically measured. These values are greater than the percentages of radiochlorine remaining in inedible tissues (3–5%) obtained from chlorate-dosed hogs (6 h exposure period; *15*) slaughtered after a 24 h withdrawal period. The percentages are also greater than the percentages of radiochlorine remaining in tissues (~12% of dose) of rats given a bolus dose of [^{36}Cl]chlorate and slaughtered 72 h later (*16*).

A more meaningful metric of chlorate retention is the concentration of parent chlorate relative to that of metabolites. Table 4 shows that TRRs increased numerically with dose for all tissues measured. Chlorate residues were always less than 1 ppm, regardless of tissue. For muscle (dark and white), liver, gizzard, and adipose tissues, mean chlorate residues were always less than 0.150 ppm. Residues of parent chlorate were not always proportional to the chlorate dose except for perhaps dark muscle and skin. The limits of quantitation for [^{36}Cl]chlorate in white skeletal muscle, dark skeletal muscle, skin, adipose, gizzard, and liver were 0.015, 0.019, 0.021, 0.022, 0.017, and 0.019 ppm, respectively. Except for the medium and high dose skin tissues, chloride always comprised greater than 99% of the TRRs present. For the medium and high dose skin tissues, chloride comprised 98.7 and 99.0% of the TRR. These results are not dissimilar to results obtained from cattle, rats, and swine (*14–16*) in which residues of parent chlorate were rapidly excreted whereas chloride residues were retained in tissues for extended periods of time. Retention of chloride ion formed from chlorate is consistent with half-lives for chloride of greater than 20 h or longer in humans and other species (*19–21*).

Residue data in this study were obtained from broilers housed in wire cages where excretory material was not available for reingestion. Because significant quantities of parent chlorate were eliminated in excreta, tissue residues of chlorate determined in this study might not be representative of floor-raised broilers, which could have additional chlorate exposure through litter pecking and scratching activities. The magnitude of chlorate exposure through this activity is unknown but could be significant depending upon the exact production situation and rate of bacterial chlorate reduction in litter itself. Unpublished data in our laboratory indicate that chlorate reduction in cattle waste under either aerobic or anaerobic conditions (20–30 °C) is rapid with chlorate half-lives being less than 1 h.

Likely metabolic intermediates during the sequential reduction of chlorate (ClO_3^-; oxidation state, +5) to chloride would be chlorite (ClO_2^-; +3) and hypochlorite (ClO^-; +1), neither of which were detected in this study. Hypochlorite and chlorite are both strong oxidants, and neither is particularly stable in biological matrices. For example, [^{36}Cl]hypochlorite was stable in untreated water for 30 min but had a half-life of only about 2 min in water containing thawed shrimp parts (*22*); the end product of the reduction was [^{36}Cl]chloride ion (*23*, *24*). In fresh ruminal fluid, the half-life of chlorite was 4.5 min (Oliver et al., in press), and chlorite was rapidly degraded to chloride in rat and bovine serum and urine (*16*). With the exception of a series of studies conducted by Abdel-Rahman et al. (*24–26*), neither hypochlorite nor chlorite has been detected in mammalian systems after animals were dosed with chor-oxyanions (*12–15*). Chlorite or hypochlorite has also not been found in bacterial cultures that respire chlorate and/or perchlorate (*27–30*). Although chlorite is believed to be formed during the bacterial reduction of perchlorate/chlorate, it is quickly reduced to chloride and O_2 by chlorite dismutase (*31*, *32*).

Thus, the absence of either hypochlorite or chlorite in excreta and(or) tissues of broilers used in this study is not surprising and is consistent with previous studies of chlorate metabolism in cattle, swine, and rats (*13–16*). From a mechanistic point of view, the formation of chloride ion from chlorate without the formation of intermediate states is perplexing. It is possible that unstable intermediates may form within tissues but be so short-lived that they are not detectable by HPLC techniques after extraction. Although neither chlorite nor hypochlorite has been measured in tissues or excreta from dosed animals, this does not necessarily preclude their formation. If formed, the problem becomes how the formation of potentially unstable intermediates may be measured. In this regard, a series of studies on the fate of chloroxyanions used as food disinfectants may be instructive. Ghanbari et al. (*22*, *33*) determined that ^{36}Cl from [^{36}Cl]-hypochlorite, and to a lesser extent [^{36}Cl]chlorine dioxide, was incorporated into unsaturated lipids of shrimp when [^{36}Cl]-hypochlorite or [^{36}Cl]chlorine dioxide solutions were used to simulate disinfectant rinses. Chlorine dioxide was proposed to cause chlorination via a chlorite intermediate (*33*). Given the apparent instability of hypochlorite and chlorite, the in situ production of either from chlorate might be indirectly measured via the incorporation of ^{36}Cl into unsaturated lipids (*23*, *33*) or as chloramine adducts in tissue of animals dosed with chlorate.

Table 5. Recoveries (Mean ± Standard Deviation) of Tissue[a] Radiochlorine in Aqueous Extracts of Broiler Tissues

	tissue					
	skeletal muscle					
dose	dark (%)	white (%)	liver (%)	adipose (%)	gizzard (%)	skin (%)
fortified[b]	100.5 ± 2.8 b	96.7 ± 3.6 a,b	101.9 ± 6.5 b	99.0 ± 1.5 b	88.5 ± 1.6 a	102.9 ± 6.7 b
incurred[b]	92.7 ± 4.0 a	90.1 ± 4.9 a,c	100.4 ± 1.6 b	101.4 ± 5.2 b	92.6 ± 2.3 a	95.9 ± 2.9 a

[a] Five grams of tissue was homogenized in 15 mL of water, the homogenate was centrifuged, and the pellet fraction was rehomogenized in 10 mL of water. The aqueous fractions were combined and assayed for total radioactivity. Means are of quadruplicate replicates for fortified controls and for incurred residues. [b] Within a row, means without a common superscript letter differ ($P < 0.05$).

To date, there is little direct evidence that radioactivity from [^{36}Cl]chlorate incorporates into tissues during metabolism. In this study, recovery of radioactivity as chlorate and(or) chloride after extraction from tissues was quantitative (data not shown). However, the extractability of TRRs did vary among tissues (Table 5). For example, in blank tissues fortified with a known composition of [^{36}Cl]chlorate and [^{36}Cl]chloride, radioactivity was quantitatively extracted from liver, adipose tissue, dark skeletal muscle, and skin, but extractability of radioactivity in white muscle and gizzard was less ($P < 0.05$) than quantitative. Across all tissues, extractability of radioactivity fortified into control tissues was greater ($P < 0.05$) than extractability of radioactivity from incurred tissues. Within the incurred tissues, extractability of radioactivity from adipose tissue and liver was quantitative, whereas the extraction efficiency from gizzard, skeletal muscle (dark and white), and skin was less ($P < 0.05$) than quantitative. The less than quantitative extraction of incurred residues from gizzard, muscle, and skin might be explained by the presence of radioactivity trapped within intact cells after incomplete homogenization. Alternatively, lower recoveries in these tissue could be due to the formation of water-soluble chlorination adducts formed from reduction intermediates of chlorate such as chlorite or hypochlorite. Chlorination reactions could be measurable provided that test animals are administered a high specific activity [^{36}Cl]chlorate molecule.

Should chlorination products be found in tissues of animals treated with chlorate, their formation from endogenous sources would have to be ruled out. An endogenous (natural) source of hypochlorite that is capable of chlorinating lipid and other targets molecules (tyrosine, for example) is myeloperoxidase, a major enzyme of leukocytes that catalyzes the formation of hypochlorous acid from chloride and H_2O_2 (*34*, *35*). Myeloperoxidase is released from leukocytes after leukocyte activation, and the formation of chlorinated lipids (chlorhydrins) from myeloperoxidase has been hypothesized to contribute to the development of artheriosclerotic lesions (*36*). We believe that the presence of chlorinated lipids and/or chlorinated amino acids such as 3-chlorotyrosine (*34*) in tissues of [^{36}Cl]chlorate-treated animals would provide good evidence for the in situ formation of relevant amounts of chlorite or hypochlorite. Alternatively, their absence would provide good evidence for the direct reduction of chlorate to chloride ion in chlorate-fed animals, with one caveat. Because chlorate is converted to chloride in large quantities (*13*−*16*) within all species tested and because chlorate-derived [^{36}Cl]chloride might be available to form chlorinated products through activation by myeloperoxidase, the measurement of chlorinated byproducts in [^{36}Cl]Cl$^-$ treated control animals would be necessary.

In conclusion, chlorate-treated broilers converted chlorate to chloride, with no detectable chlorite being formed. Although edible tissues contained fairly substantial quantities of radiochlorine, only a small fraction (1% or less) of the total residue present as parent chlorate, with the remaining residue present as chloride ion. These results suggest that the further development of chlorate as a food safety tool for the poultry industry is warranted.

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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, 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 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 respended and homogenized in 10 mL of water. After centrifugation, active 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 ± 0.9 and 99.5 ± 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 of the administered radiochlorine. When the data were essed on a concentration basis (fresh tissue weight; chlorate

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).

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).

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-

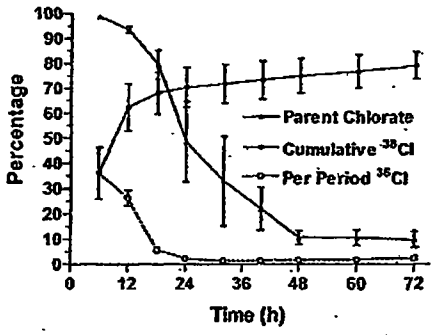

Figure 1. Urinary excretion of radiochlorine in male rats ($n = 4$) following a single oral dose (3 mg/kg) of sodium [^{36}Cl]chlorate is presented in terms of parent chlorate composition, cumulative, and per time period.

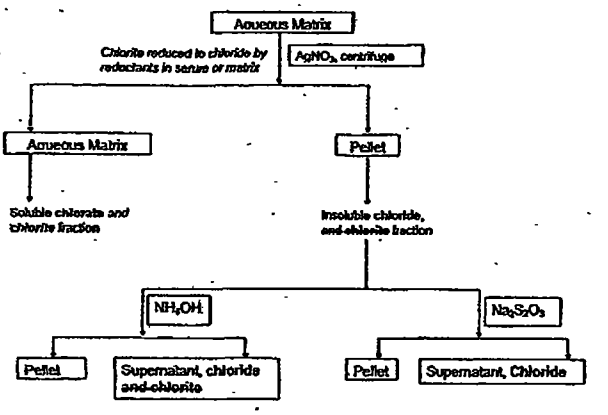

Figure 2. Disposition of chloride, chlorite, and chlorate as proposed by ref *20* vs disposition described by our findings. Italicized fonts represent additions, and strikeouts are deletions from the results of ref *20*.

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, though 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 [^{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.

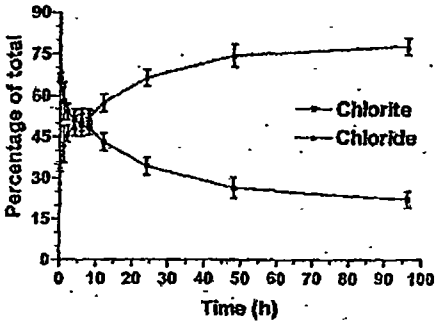

Figure 3. Degradation of chlorite in rat urine as a function of time (n = 3). The initial radiochemical purity of the chlorite fortification solution was 99.3%.

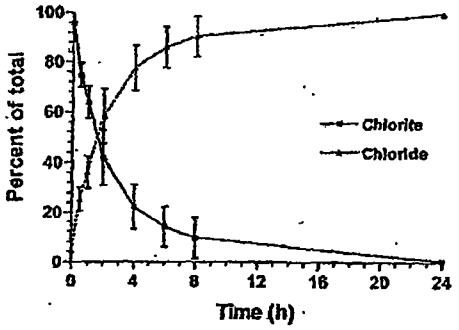

Figure 4. Degradation of chlorite in bovine serum as a function of time (n = 3). The initial radiochemical purity of the chlorite fortification solution was 99.3%.

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% chlorite, and 60% was parent chlorate. In the 48−72 h urine, 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 solution after treatment with $AgNO_3$. Their method tested 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 SO_3 solution from urine, serum, or pure water matrices. ...e 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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SHORT REPORTS

Mortality in sodium chlorate poisoning

Sodium chlorate poisoning is rare but is associated with a high mortality rate,[1] death occurring from massive intravascular haemolysis and acute renal failure. We report the outcome in 14 patients poisoned by sodium chlorate, with special regard to the amount ingested and subsequent management.

Patients, treatment, and results

During 1974-8 we followed up in detail 14 cases of sodium chlorate poisoning referred to the National Poisons Information Service (Guy's Hospital). Data concerning the amount of substance ingested, medical management, and subsequent outcome were obtained (see table). Mortality was high (64%), and death invariably occurred, irrespective of treatment, when the amount of sodium chlorate ingested exceeded 100 g. Early deaths—that is, those within 24 hours—were more common in accidental self-poisoning, when specific antidotes or reducing agents were not administered owing to the delay in diagnosis. Supportive management alone was successful in only one patient (case 12); in this case the ingested amount was less than one-tenth of the stated fatal dose of 20-30 g. In the four other survivors recovery was associated with ingested amounts of 100 g or less of sodium chlorate, prompt administration of sodium thiosulphate or reducing agents, and management of acute renal failure by peritoneal dialysis or haemodialysis. The success of treatment with specific antidotes followed by dialysis is best seen by comparing cases 1 and 14, in which the amounts of sodium chlorate ingested were identical. The clinical features of sodium chlorate poisoning occurred in the following frequency: nausea and vomiting (11 patients; 79%), cyanosis (seven; 50%), abdominal pain (five; 36%), diarrhoea (three; 21%) and dyspnoea (three; 21%). Two patients were admitted in coma and died shortly afterwards. Seven patients became anuric within 48 hours after admission to hospital. Methaemoglobinaemia was found in 13 patients, in two of whom peripheral blood films showed the presence of ghost cells and Heinz-body formation. In the patients who died constant necropsy finding was a "chocolate" discoloration of the blood and sclera due to staining by bilirubin and methaemoglobin.

Comment

Sodium chlorate is a powerful oxidant used extensively as a herbicide. A white crystalline substance, it is applied dissolved in water. The crystals may be mistaken for sugar with fatal results (cases 3 and 8). Poisoning usually results from ingestion but has been reported after inhalation of atomised droplets.[2] Initial symptoms relate to the irritant effect of the chlorate ion on the gastrointestinal mucosa. After absorption haemoglobin is rapidly oxidised to methaemoglobin and intravascular haemolysis results. Cyanosis becomes clinically detectable when the proportion of methaemoglobin exceeds 10%; values above 70% are fatal. Death occurring within a few hours of ingestion is attributed to tissue hypoxia due to severe methaemoglobinaemia or hyperkalaemia resulting from massive haemolysis. Sodium chlorate is nephrotoxic and causes acute tubular necrosis; the ensuing renal failure may be compounded by haemoglobinuria.

In cases presenting early initial management should comprise gastric lavage and administration of activated charcoal. Sodium thiosulphate (2-5 g in 200 ml of 5% sodium bicarbonate) is a specific antidote that inactivates the chlorate ion and may be given by mouth or intravenously. Methaemoglobinaemia is best treated by giving intravenous methylene blue (20-50 ml of a 1% solution), which is superior to ascorbic acid in reducing methaemoglobin to haemoglobin.[3] Oxygen is of no value. Sodium chlorate is freely dialysable, and early treatment of renal failure by peritoneal dialysis or haemodialysis is recommended. Despite isolated reports of success in treating severe sodium chlorate poisoning,[4 5] the mortality rate remains extremely high. Sodium chlorate is freely available and is not listed as a poison: preparations of it are therefore not required to carry any warning to this effect.

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Details of 14 cases of sodium chlorate poisoning showing management and outcome

Case No	Age	Sex	Amount ingested	Deliberate or accidental	Initial management	Further management	Outcome
1	55	F	100 g	Deliberate		Supportive	Died (3 hours)
2	55	M	150 g	"	Methylene blue, ascorbic acid	Mannitol hydrocortisone	Died (36 hours)
3	3	M	Unknown	Accidental		Supportive	Died (6 hours)
4	23	M	100-150 g	Deliberate	Sodium thiosulphate, ascorbic acid	Blood transfusion, hydrocortisone, antibiotics	Died (48 hours)
5		F	30 g	"	Methylene blue, ascorbic acid	Peritoneal dialysis, blood transfusion	Died* (5 days)
6	25	M	50 g	"	Methylene blue	Haemodialysis, exchange transfusion	Died†
7	28	M	300 g	"	Methylene blue	Peritoneal dialysis, exchange transfusion	Died
8	46	F	15 g	Accidental		Supportive	Died (20 hours)
9	48	M	Unknown	Deliberate	Methylene blue, ascorbic acid	Supportive	Died (2 hours)
10		F	45 g	"	Sodium thiosulphate	Peritoneal dialysis	Recovered
11	18	F	30 g	"	Sodium thiosulphate, methylene blue	Peritoneal dialysis	Recovered
12	47	M	1-2 g	Accidental	Sodium thiosulphate	Supportive	Recovered
13	13	M	5 g	"		Peritoneal dialysis, blood transfusion	Recovered
14	18	F	100 g	Deliberate	Methylene blue, sodium bicarbonate	Exchange transfusion, haemodialysis	Recovered

*Necropsy showed portal vein thrombosis. †Fatal cardiac arrest during dialysis. ‡Severe hyperkalaemia present.

Painful gynaecomastia treated with tamoxifen

Unilateral or bilateral gynaecomastia may be a consequence of impaired liver function or lung cancer or occur after certain drugs, notably digoxin, spironolactone, metoclopramide, and cimetidine. In lung cancer gynaecomastia is associated with gonadotrophin-secreting tumours, and there is also an association with hypertrophic pulmonary osteoarthropathy.[1] Histologically the tumour is usually anaplastic large-cell carcinoma, but there are reported cases with squamous-cell carcinoma, adenocarcinoma, and oat-cell carcinoma.[2] Gynaecomastia is usually no more than embarrassing for the patient, but rarely it may be painful. This was the case in the three patients described below. The oestrogen antagonist tamoxifen successfully relieved the pain.

Case reports

Case 1—A 64-year-old heavy smoker presented with weight loss and non-productive cough. Chest radiography showed a right upper lobe opacity with extensive hilar enlargement. Sputum cytology disclosed an oat-cell carcinoma of the bronchus. On presentation he had bilateral gynaecomastia of recent onset. Biochemical liver function values and a liver scan were normal. The gynaecomastia progressed and became intensely painful with little relief from analgesics. Liver function values were abnormal and the serum oestradiol concentration was 455 pmol/l (124 pg/ml) (normal range 55-147 pmol/l; 15-40 pg/ml). He was given tamoxifen 10 mg twice daily. The gynaecomastia regressed and became painless in two weeks. He

Toxicity of Sodium Chlorate to the Dog

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SUMMARY.—Acute and repeated dosage studies with sodium chlorate have been carried out in the beagle dog. The LD_{50} was not defined. Clinical signs, haematological and biochemical changes were induced after administering sodium chlorate at the dosage level of 300 mg. per kg. per day for five days.

Introduction

It is now required that new drugs and chemicals be evaluated for safety before their introduction into man's environment. Some of the older compounds, because of extensive usage and experience, have been found to be of low toxicity, and have not been investigated in the same detail as the newer preparations. From time to time doubt is cast on the safety of some of these older compounds, and sodium chlorate is such a chemical. It has been widely used as a weed killer and is accepted as being of relatively low toxicity. This paper reports some aspects of the toxicity of sodium chlorate in the dog.

Materials and Methods

Tests were carried out on pedigree beagle dogs. The test compound was a commercial preparation of sodium chlorate marketed as a weed killer. The study was divided into three sections, to measure: firstly, acute toxicity by giving sodium chlorate orally as a powder at dose levels of 1 and 2 g. per kg.; secondly, acceptance of the solution containing sodium chlorate at 2 per cent. and 8 to 9 per cent. w/v; and thirdly, the effects of repeated dosages over a five-day period. In the repeated dosage study 50 ml. of 6 per cent. w/v solution (in the animals used this was equivalent to 200 to 326 per kg. per day) was administered by stomach-tube for five days. Haemograms, blood urea levels and methaemoglobin levels were measured during this study; the group size was reduced to four animals after the five-day dosing period, and these animals were then allowed a seven-day recovery period.

(*Continued on page 417*)

Canine Adenovirus Respiratory Disease.—Concluded.

There is little doubt that the viral aetiology of canine respiratory disease is complex, and, although distemper virus probably remains the most important factor, more work is needed to assess the importance of other canine viruses.

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Résumé

Cet article décrit l'isolement d'adéno-virus chez des cas naturels de maladie respiratoire canine. On a pu démontrer que ces virus sont distinct du type A26/61 d'adéno-virus canin, mais non du virus ICH. On a étudié à la microscopie électronique et fluorescente, le développement des nouveaux isolats dans les cultures tissulaires. L'inoculation intraveineuse de ces virus sur des chiots réceptifs, a provoqué une hépatite chronique simple (maladie de Rubarth) mais, administrée par aérosol, ces virus ont provoqué des cas graves de maladie respiratoire.

Zusammenfassung

Die Isolierung von Adenoviren aus spontanen Erkrankungen der Atmungsorgane bei Hunden wird beschrieben. Die Viren liessen sich nicht vom ICH/Virus unterscheiden, waren jedoch verschieden vom A26/61-Stamm des Hunde-Adenovirus. Das Wachstum der isolierten Viren in Gewebekulturen wurde mittels Elektronen- und Fluoreszenz-Mikroskopie untersucht. Intravenöse Injektion der Viren löste bei empfindlichen Welpen akute Hepatitis (Rubarth'sche Krankheit) aus, während Verabfolgung mittels Aerosol zu schwerer Erkrankung der Atmungsorgane führte.

(b) (4)

[redacted]

Discussion

(b) (4)

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Reference

CLARKE, E. G. C., & CLARKE, M. L. (1967). *Garner's Veterinary Toxicology*. pp. 67-68. Ballière, Tindall & Cassell, London.

Résumé

On a étudié l'effet de doses fortes et répétées de chlorate de sodium sur le chien (briquet). On n'a pas défini le LD_{50}. Les signes cliniques, modifications hématologiques et biochimiques apparaissent après l'administration de 300 mg/kg par jour de chlorate de sodium pendant cinq jours.

Zusammenfassung

Zur Feststellung der Toxizität von Natriumchlorat für Spürhunde wurden Experimente mit einmaliger hoher Dosis wie auch mit mehrmaligen Dosierungen vorgenommen. Das LD_{50} wurde nicht bestimmt. Nach fünftägiger Verabfolgung von 300 mg/kg/Tag Natriumchlorat traten klinische Symptome sowie hämatologische und biochemische Veränderungen auf.

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Controlled Clinical Evaluations of Chlorine Dioxide, Chlorite and Chlorate in Man

by Judith R. Lubbers,* Sudha Chauan,* and Joseph R. Bianchine*

To assess the relative safety of chronically administered chlorine water disinfectants in man, a controlled study was undertaken. The clinical evaluation was conducted in the three phases common to investigational drug studies. Phase I, a rising does tolerance investigation, examined the acute effects of progressively increasing single doses of chlorine disinfectants to normal healthy adult male volunteers. Phase II considered the impact on normal subjects of daily ingestion of the disinfectants at a concentration of 5 mg/l. for twelve consecutive weeks. Persons with a low level of glucose-6-phosphate dehydrogenase may be expected to be especially susceptible to oxidative stress; therefore, in Phase III, chlorite at a concentration of 5 mg/l. was administered daily for twelve consecutive weeks to a small group of potentially at-risk glucose-6-phosphate dehydrogenase-deficient subjects. Physiological impact was assessed by evaluation of a battery of qualitative and quantitative tests. The three phases of this controlled double-blind clinical evaluation of chlorine dioxide and its potential metabolites in human male volunteer subjects were completed uneventfully. There were no obvious undesirable clinical sequellae noted by any of the participating subjects or by the observing medical team. In several cases, statistically significant trends in certain biochemical or physiological parameters were associated with treatment; however, none of these trends was judged to have physiological consequence. One cannot rule out the possibility that, over a longer treatment period, these trends might indeed achieve proportions of clinical importance. However, by the absence of detrimental physiological responses within the limits of the study, the relative safety of oral ingestion of chlorine dioxide and its metabolites, chlorite and chlorate, was demonstrated.

Introduction

Chlorine dioxide is currently under serious consideration in the United States as an alternative to chlorine water treatment. Before chlorine dioxide may be used routinely as a water disinfectant, the safety of oral human ingestion of chlorine dioxide and its by-products must be assessed. For this purpose, a controlled clinical evaluation of chlorine dioxide, chlorite and chlorate was undertaken under the auspices of USEPA HERL #CR805643.

The study was conducted in three parts. Phase I was designed to evaluate the acute physiological effects of progressively increasing doses of disinfectants administered to normal healthy adult males. Chronic ingestion by normal male volunteers was studied in Phase II. Phase III assessed the physiological response of a small group of potentially susceptible individuals, those deficient in glucose-6-phosphate dehydrogenase, to chronic ingestion of chlorite.

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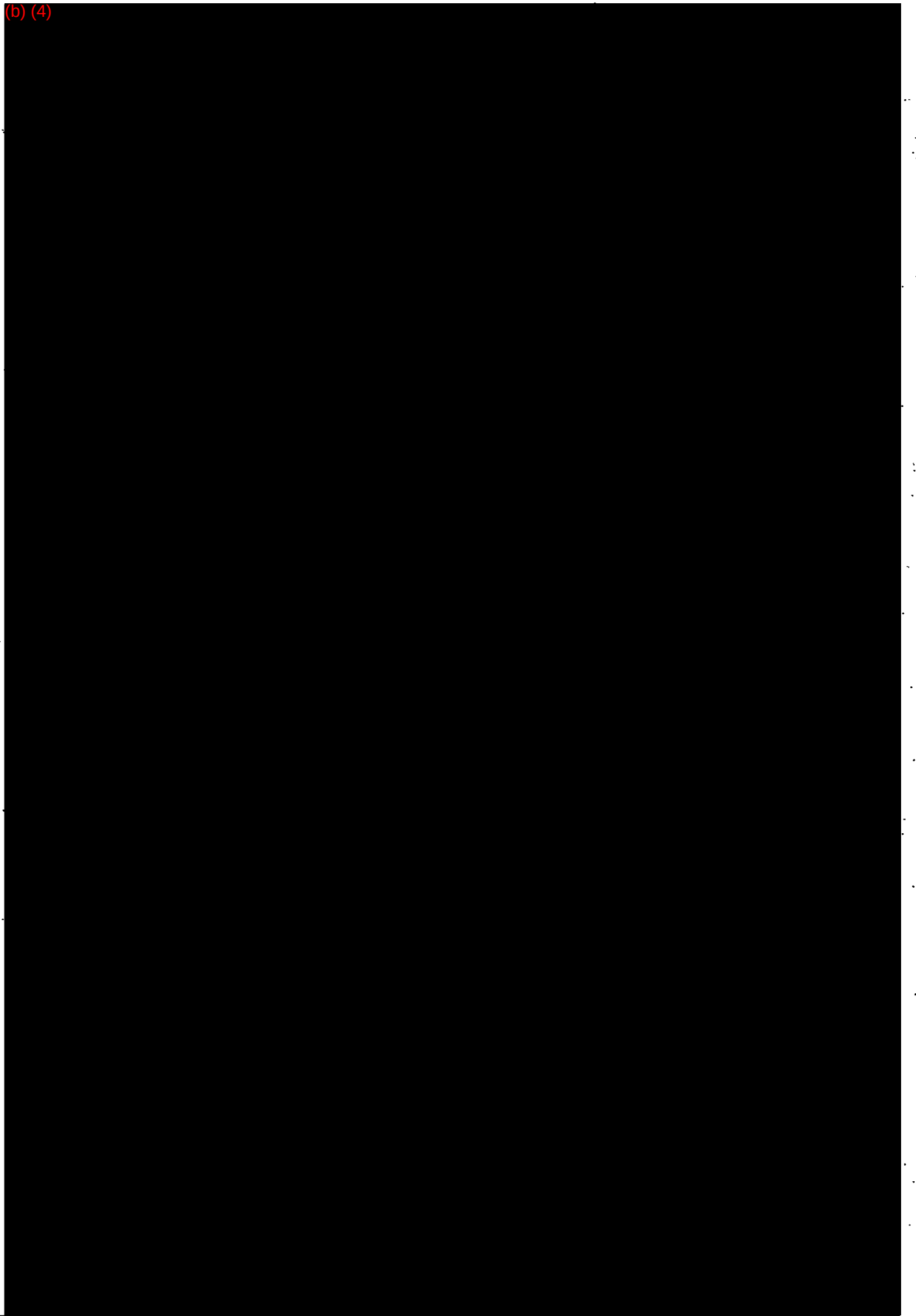
Methods

Subject Selection

For Phase I and for Phase II, normal healthy adult male volunteers were selected. No prospective study participant who exhibited a significant abnormality in the routine clinical serum analysis,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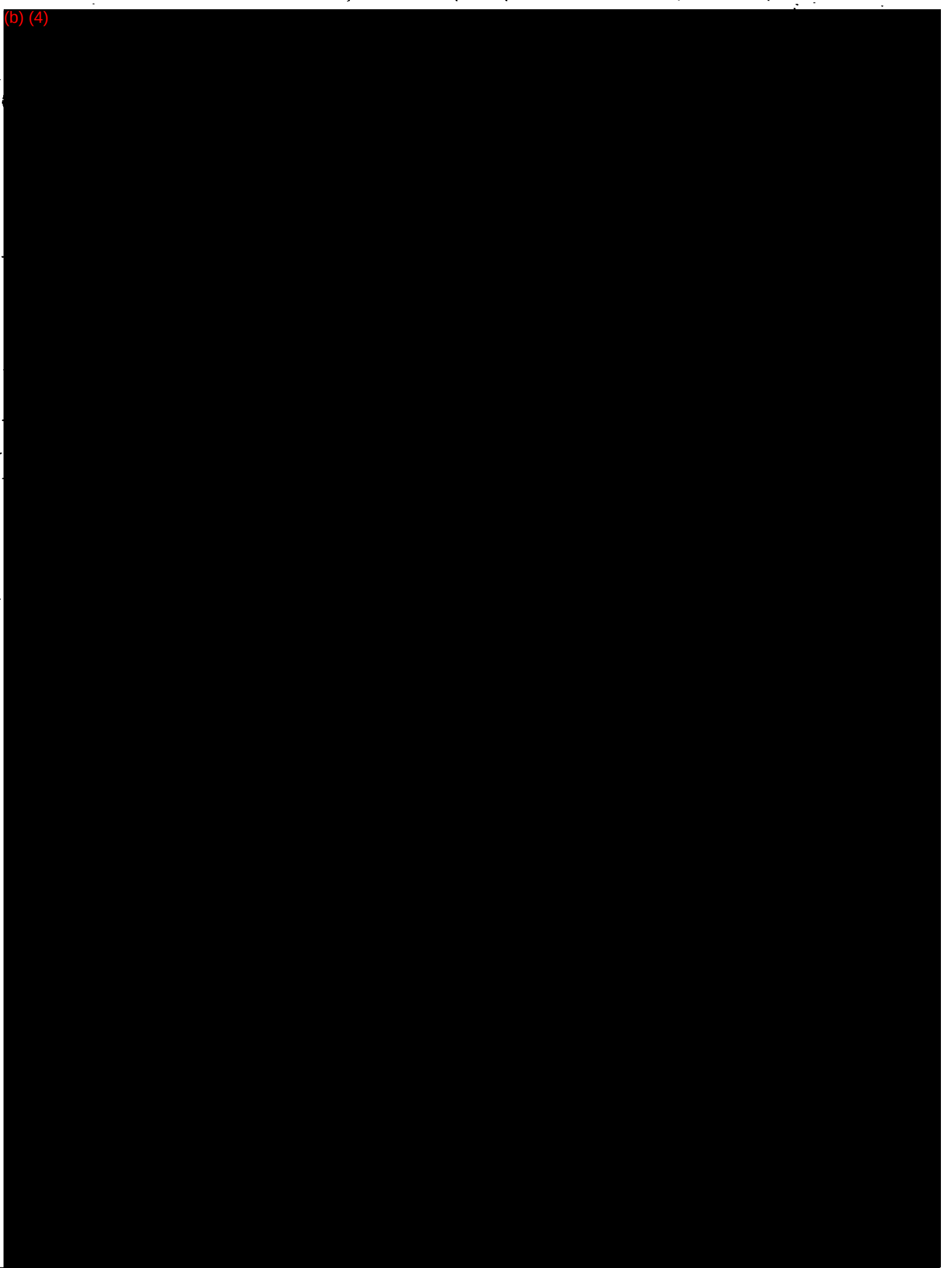
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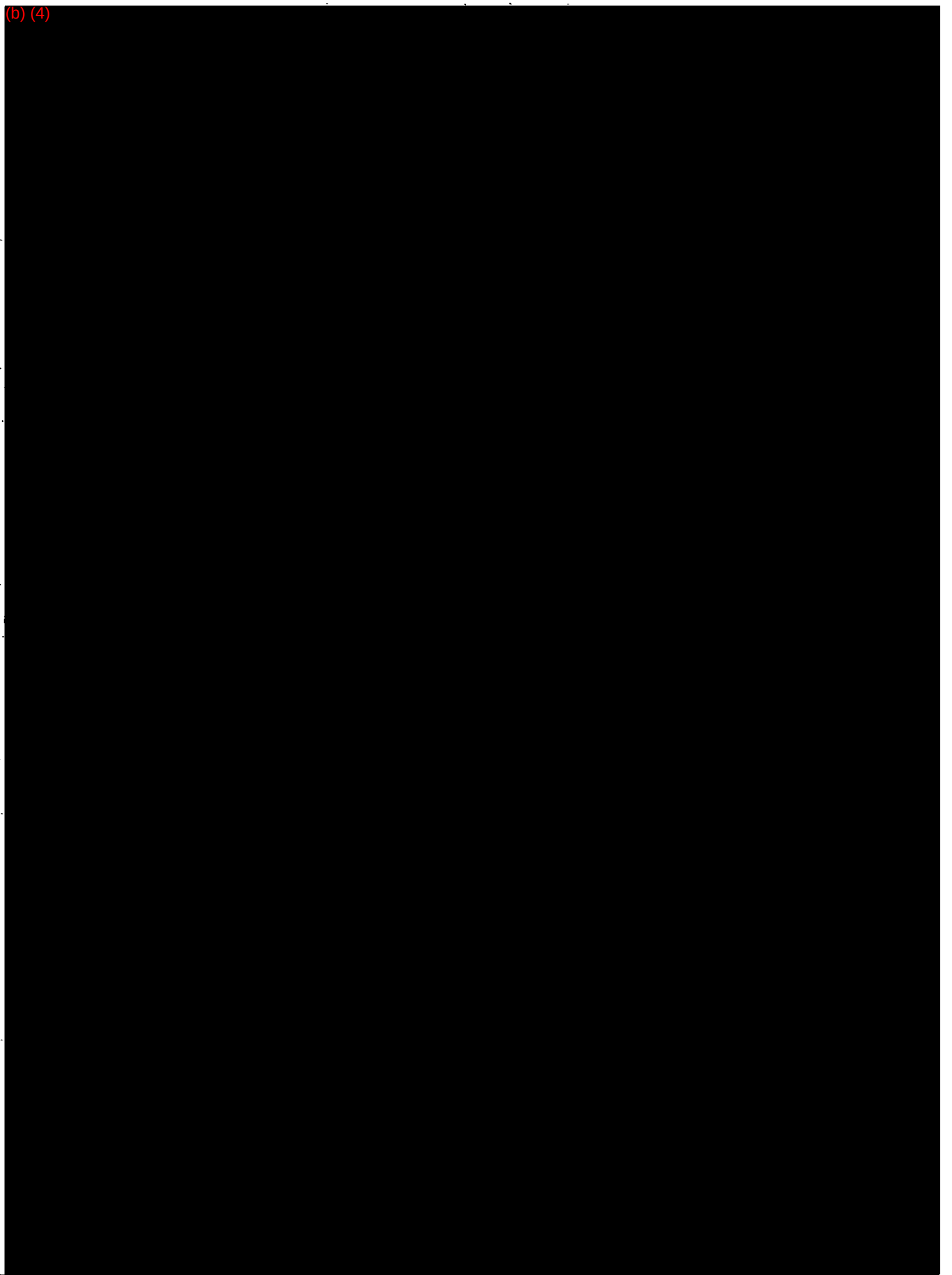
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UBBERS AND BIANCHINE

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MS 1537

THE EFFECTS OF CHRONIC ADMINISTRATION OF CHLORINE DIOXIDE, CHLORITE AND CHLORATE TO NORMAL HEALTHY ADULT MALE VOLUNTEERS

Judith R. Lubbers, Sudha Chauhan, Judy K. Miller, Joseph R. Bianchine

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The physiological impact of chronic 12 week ingestion of chlorine dioxide and its byproducts, chlorite and chlorate, was compared to the effects of chlorine, chloramine and untreated water. The water disinfectant solutions were administered daily (500 ml, 5 ppm) to normal healthy adult male volunteers. An extensive battery of tests was used to evaluate the physiological impact of the ingested water disinfectants. Upon analysis of both quantitative and qualitative parameters it was concluded that the 12 week chronic administration of chlorine dioxide and its byproducts was accompanied by no clinically important physiological effects.

INTRODUCTION

When chlorine is employed as a surface water disinfectant, chlorinated organic compounds are formed (Rook, 1974). Concern about the possible detrimental health effects of the residual chlorinated organic compounds has mounted (Marx, 1974). In contrast, chlorine dioxide in use as a surface water disinfectant agent is not associated with the formation of chlorinated organics. Consequently, chlorine dioxide is currently undergoing serious consideration as a viable alternative to chlorination in the United States.

Before chlorine dioxide can be recommended for routine use, it is imperative that its safety in man be assessed. Animal studies have identified certain areas of potential biological hazard associated with oral ingestion of chlorine dioxide and its water treatment byproducts, chlorite and chlorate (Abdel-Rahman *et al.*, 1979; Couri *et al.*, 1979). A preliminary human study performed in this laboratory (Lubbers *et al.*, 1981) confirmed the safety of the alternative disinfectant and its byproducts over a wide concentration range in acute single dose administration to normal healthy adult male volunteers. The chronic investigation discussed in this report was undertaken to determine the physiological effects of chronic daily administration of chlorine dioxide, chlorite and chlorate to healthy adult males over a 12 week period.

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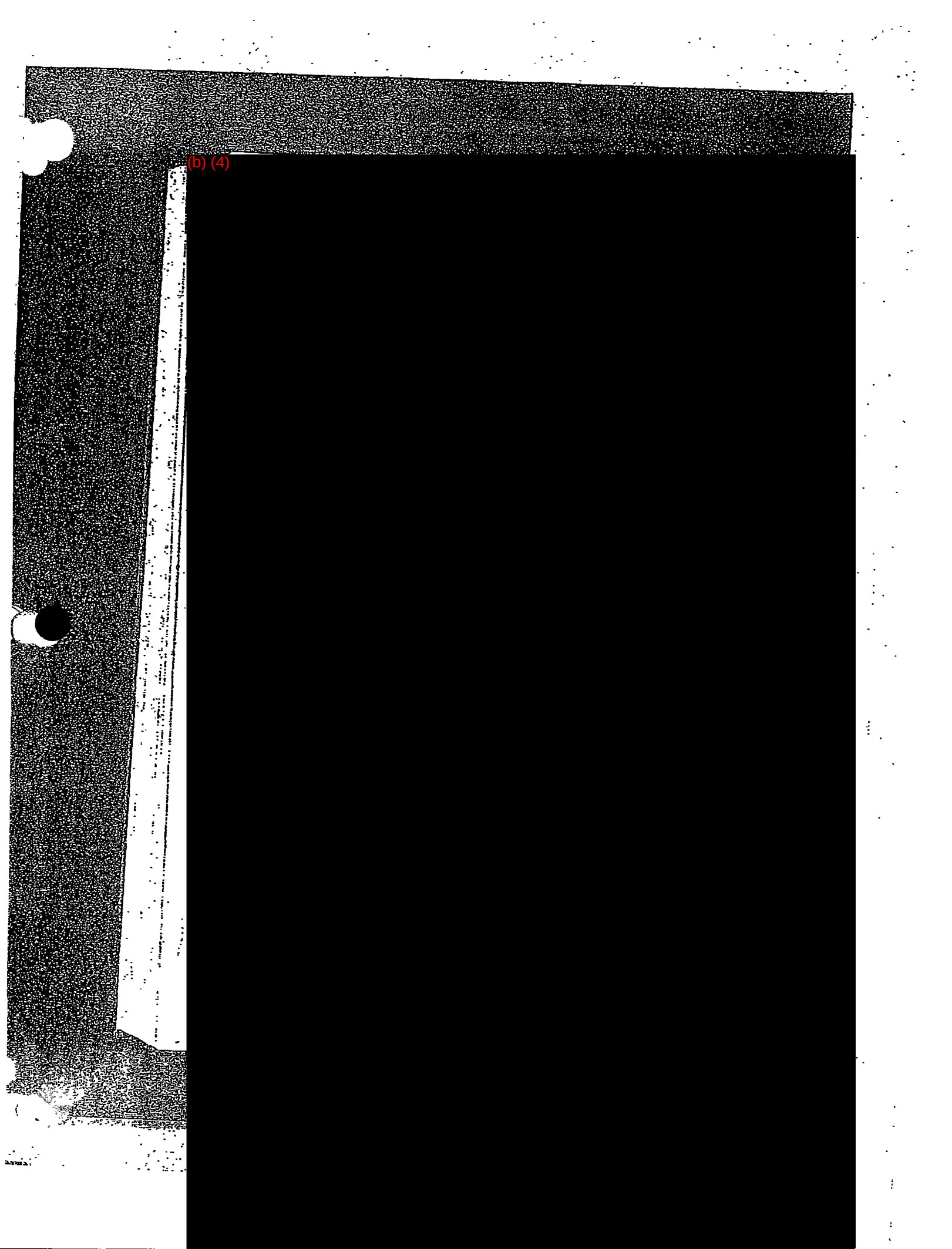
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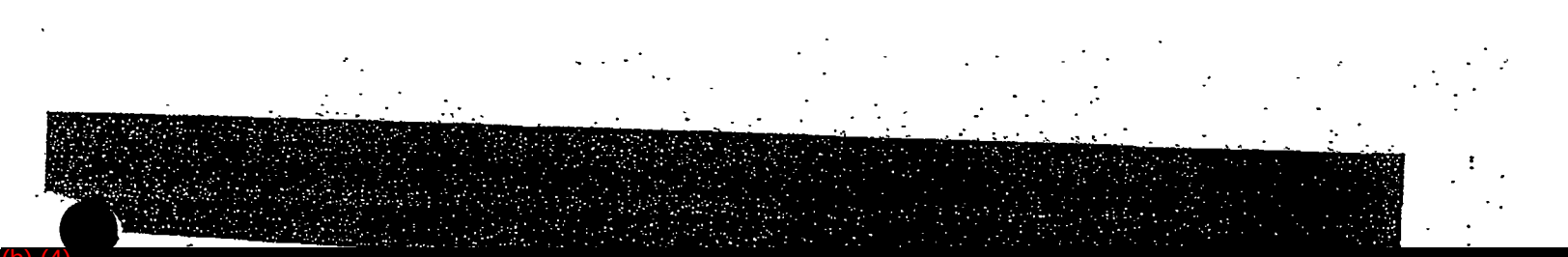

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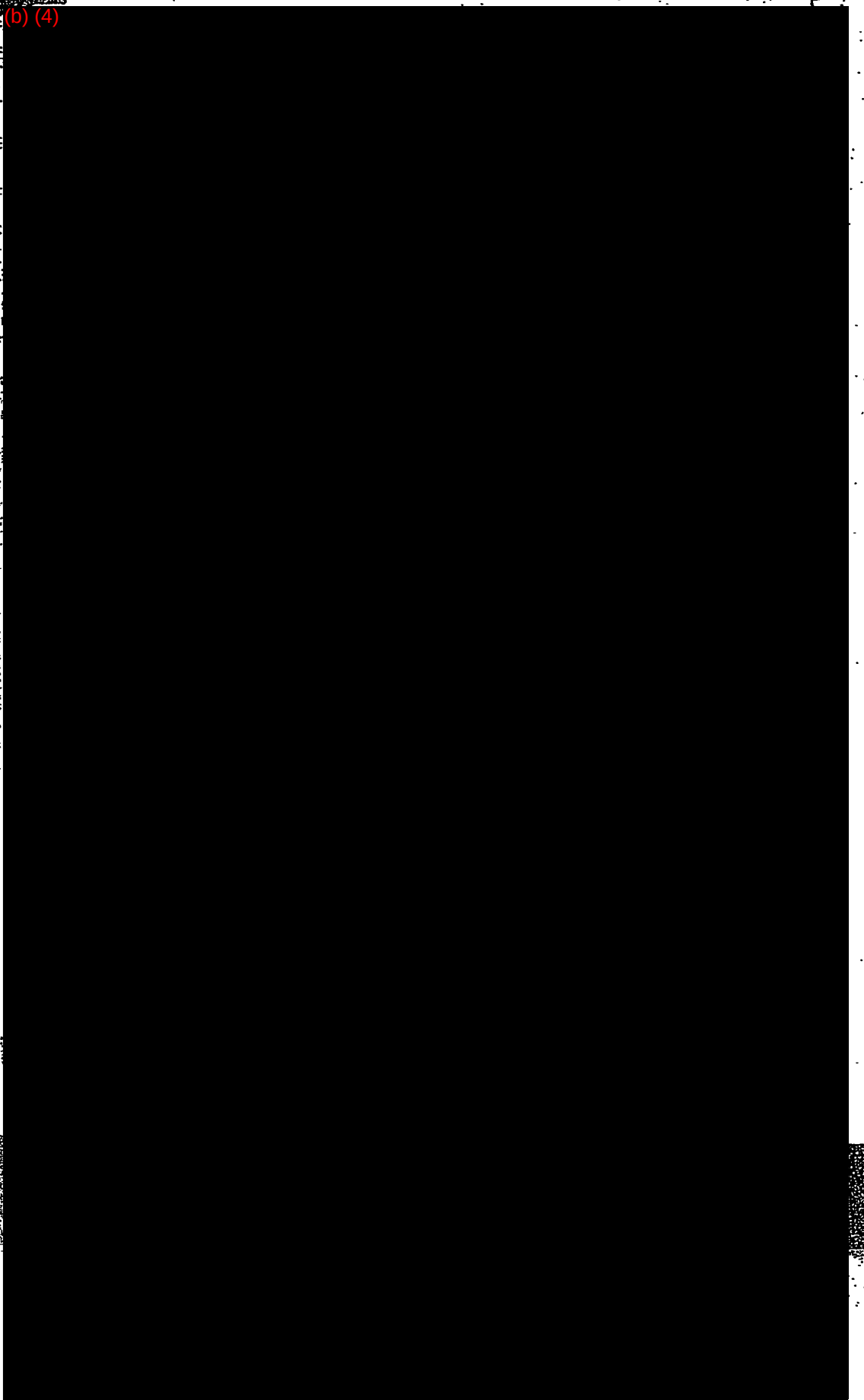

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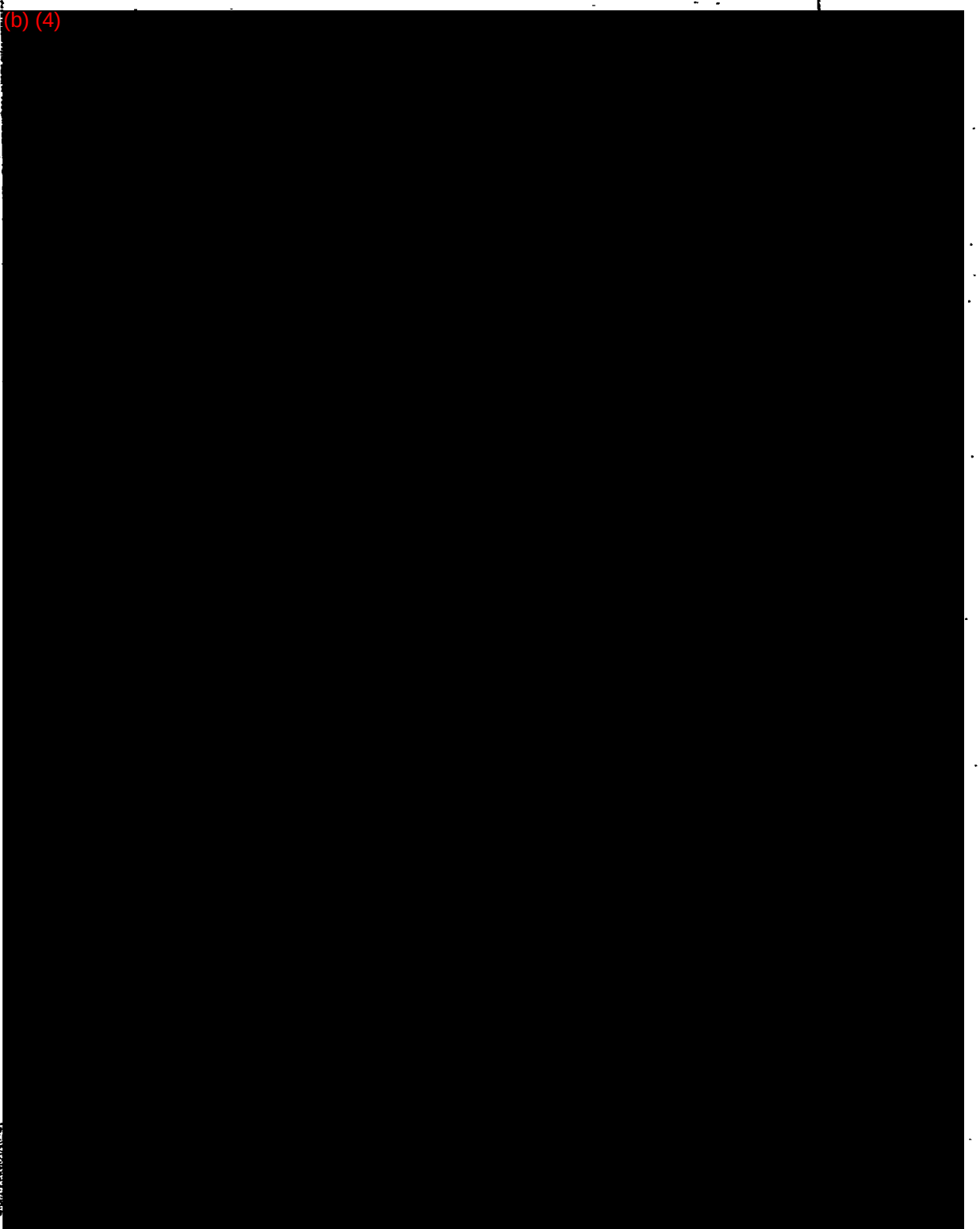

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Environmental Health Perspectives
Vol. 46, pp. 47-55, 1982

Subchronic Toxicity of Chlorine Dioxide and Related Compounds in Drinking Water in the Nonhuman Primate

by J. P. Bercz,* L. Jones,* L. Garner,* D. Murray,* D. A. Ludwig* and J. Boston*

Subchronic toxicities of ClO_2, $NaClO_2$, $NaClO_3$ and NH_2Cl were studied in the African Green monkeys (*Cercopithecus aethiops*). The chemicals were administered in drinking water during 30–60 days subchronic rising dose protocols. The only unexpected and significant toxic effect was elicited by ClO_2; this chemical inhibited thyroid metabolism in the animals at a dose of ca. 9.0 mg/kg/day. A statistically significant decrease of serum thyroxine occurred after the fourth week of exposure to 100 mg/l. concentration. The extent of thyroid suppression was dose dependent in each individual monkey, and was reversible after cessation of exposure. $NaClO_2$ and $NaClO_3$ failed to elicit similar effects in doses up to ca. 60 mg/kg/day. Also, $NaClO_4$ or NH_2Cl did not cause T-4 suppression in doses of 10 mg/kg/day. The selective thyroid effect of ClO_2 was unexplained and it appeared to be paradoxical since ClO_2 was rapidly reduced by the oral and gastric secretions to nonoxidizing species (presumably Cl^-). No evidence of thyroid effects were detected in the serum of human volunteers who ingested ~ 1 mg/l. of ClO_2 in drinking water as a result of routine use in the community water treatment process.

Sodium chlorite induced dose-dependent oxidative stress on hematopoesis, causing decreased hemoglobin and red cell count and increased methemoglobin content. At the same time, serum transaminase (SGPT) levels showed significant subclinical elevation. The hematologic effects of $NaClO_2$ rebounded during exposure indicating compensatory hemopoietic activity taking effect during oxidative stress. Sodium chlorate and chloramine did not induce detectable hematologic changes in the animals.

Introduction

Owing to its excellent microbicidal properties, chlorine dioxide (ClO_2), a water-soluble yellow oxidant gas, has been used in the past for drinking water disinfection. The apparent relative absence of the carcinogenic trihalomethanes (THM) in ClO_2 treated water triggered renewed interest in this compound as a possible alternative to chlorine (*1*), since the latter was shown to generate THMs (reacting) with humic substances (*2*, *3*).

Concomitantly, the toxicology of ClO_2 and its metabolites (ClO_2 and ClO_3) have received wide attention in the recent literature. By using orally administered ClO_2, hematologic changes and inhibition in testicular uptake of ^3H-thymidine was demonstrated in rats by Abdel-Rahman (*4*). Effects of these chlorine oxides on the glucose-6-phosphate dehydrogenase (G6PD)-deficient mouse were reported by Moore et al. (*5*). ClO_2 associated kinetics of red cell GSH depletion and intravascular hemolysis in rats and chickens was reported by Abdel-Rahman et al. (*6*). These workers also described the metabolism of $^{36}ClO_2$ (*7*) in rodents. In addition, the effects of ClO_2 and metabolites, as they effect the cellular GSH system in the rat, mouse and chicken blood, were investigated by Couri et al. (*8*) Oxidative *in vitro* damage to erythrocytes by $NaClO_2$ was reported

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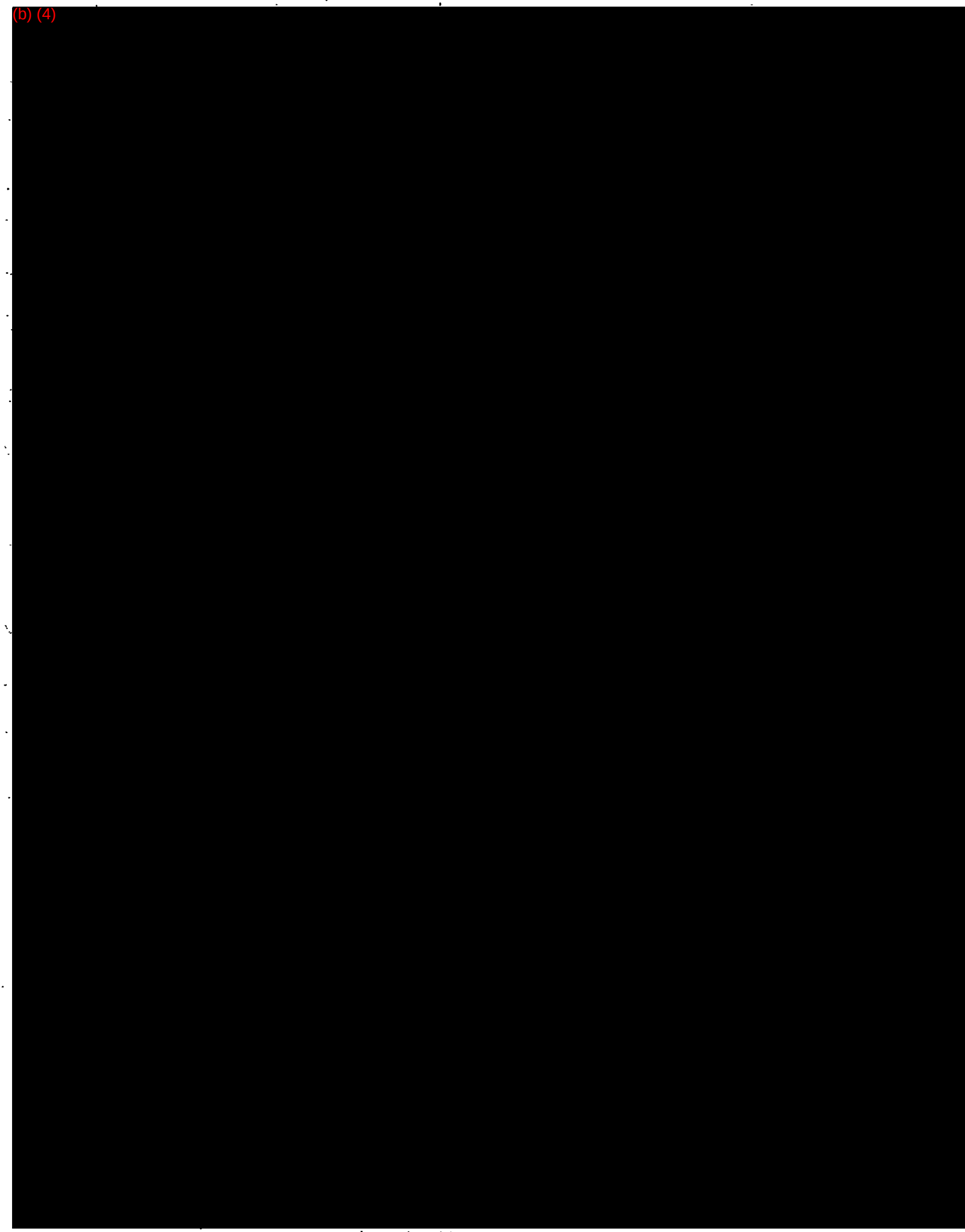
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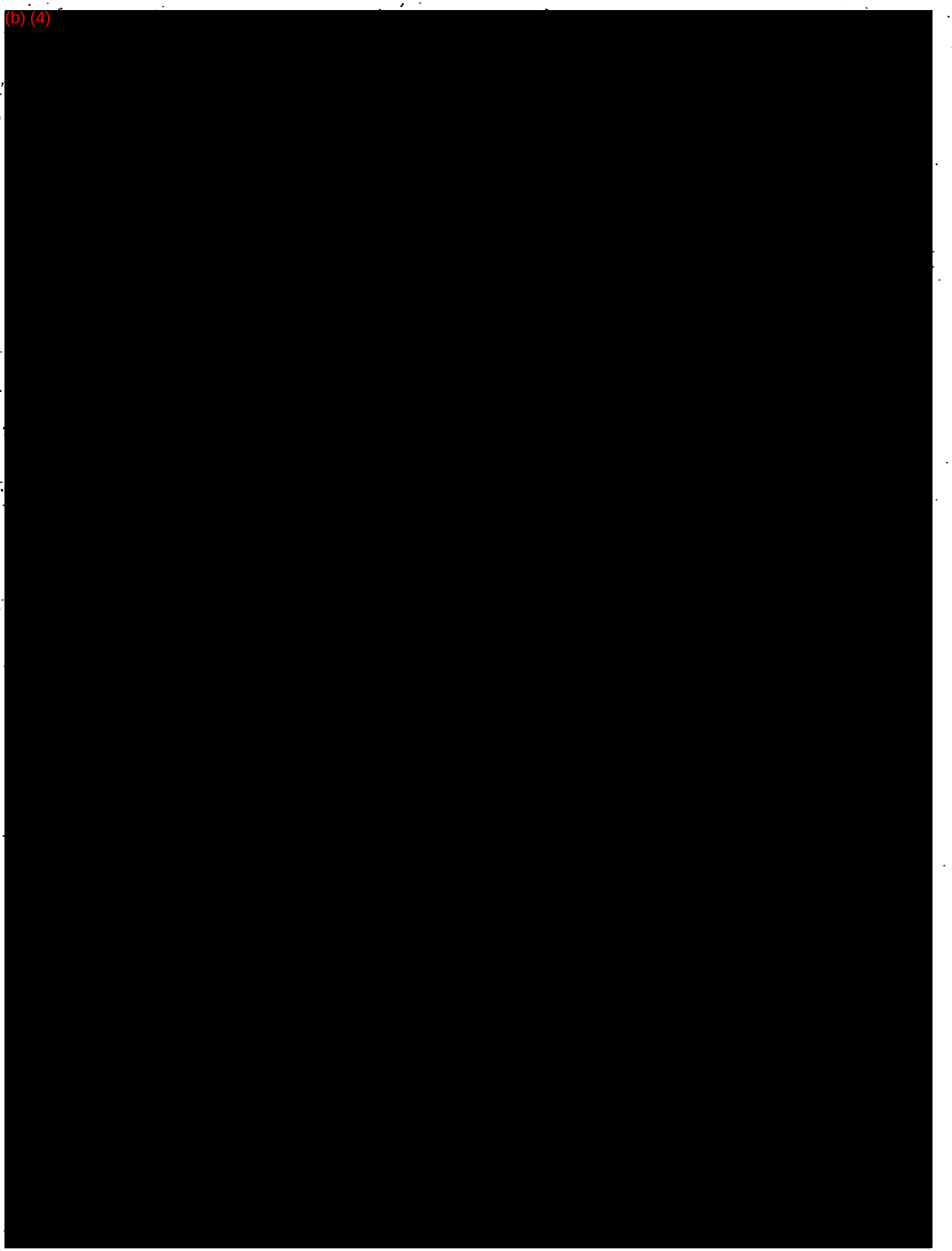
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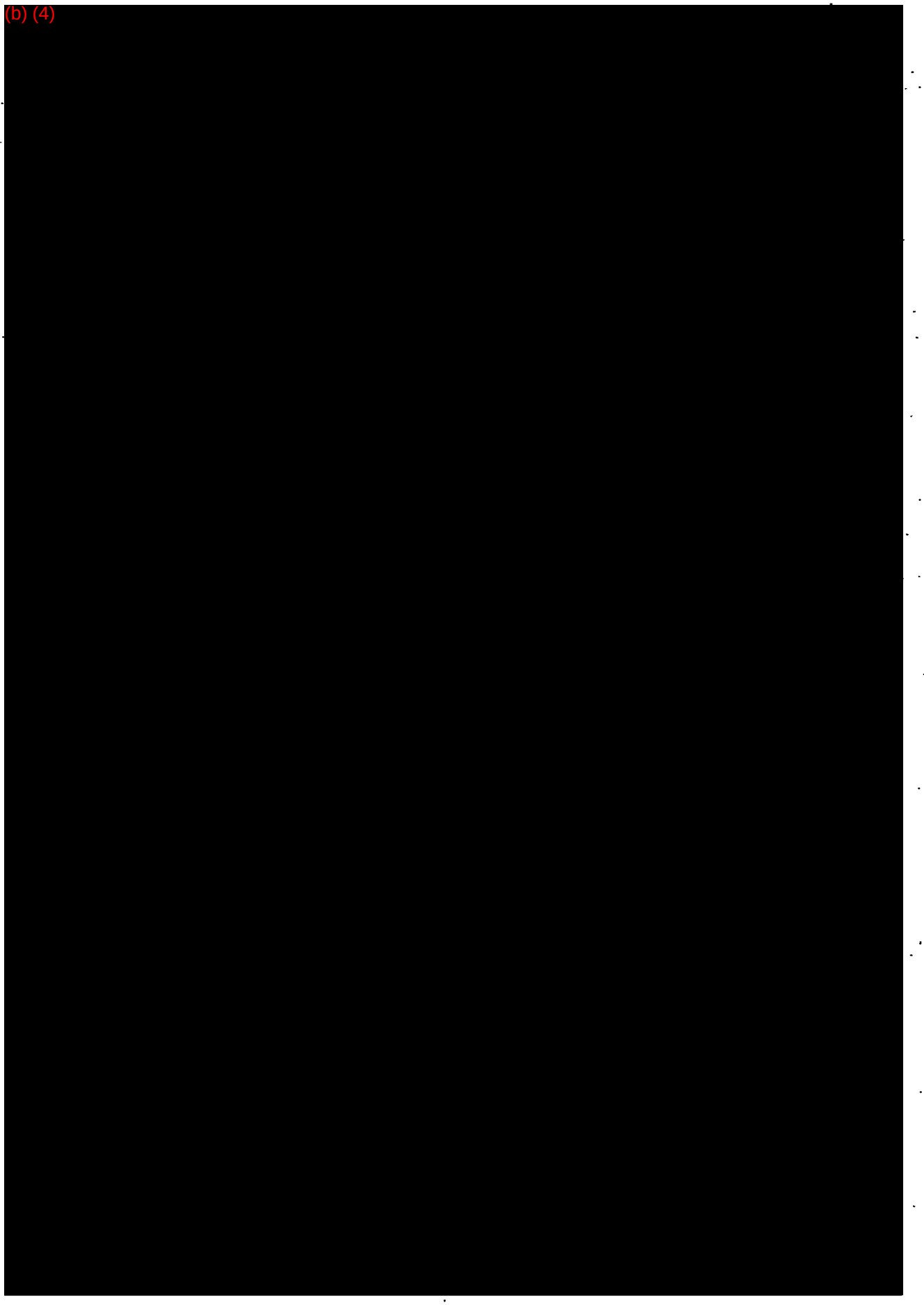
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Vol. 69, pp. 249-255, 1986

Mechanistic Aspects of Ingested Chlorine Dioxide on Thyroid Function: Impact of Oxidants on Iodide Metabolism

by J. Peter Bercz,* Lillian L. Jones,* Robert M. Harrington,* Rohit Bawa,* and Lyman Condie*

Toxicological studies dealing with recent findings of health effects of drinking water disinfectants are reviewed. Experiments with monkeys and rodents indicate that the biological activity of ingested disinfectants is expressed via their chemical interaction with the mucosal epithelia, secretory products, and nutritional contents of the alimentary tract. Evidence exists that a principal partner of this redox interaction is the iodide of nutritional origin that is ubiquitous in the gastrointestinal tract. Thus the observation that subchronic exposure to chlorine dioxide (ClO_2) in drinking water decreases serum thyroxine levels in mammalian species can be best explained with changes produced in the chemical form of the bioavailable iodide. Ongoing and previously reported mechanistic studies indicate that oxidizing agents such as chlorine-based disinfectants oxidize the basal iodide content of the gastrointestinal tract. The resulting reactive iodine species readily attaches to organic matter by covalent bonding. Evidence suggests that the extent to which such iodinated organics are formed is proportional to the magnitude of the electromotive force and stoichiometry of the redox couple between iodide and the disinfectant. Because the extent of thyroid uptake of the bioavailable iodide does not decrease during ClO_2 ingestion, it seems that ClO_2 does not cause iodide deficiency of sufficient magnitude to account for the decrease in hormonogenesis. Absorption of one or more of iodinated molecules, e.g., nutrients, hormones, or cellular constituents of the alimentary tract having thyromimetic or thyroid inhibitory properties, is a better hypothesis for the effects seen.

Introduction

The inhibition of thyroxine (T_4) synthesis in monkeys (*Cercopithecus aethiops*) during subchronic exposure to chlorine dioxide (ClO_2) in drinking water (*1*) was a serendipitous and the only significant finding during the investigation of the so-called oxidative stress caused by ClO_2. Investigators involved with disinfection research (*2,3*) proposed this syndrome to explain methemoglobinemic hemolytic anemia associated with exposure to large doses of disinfectants. According to this hypothesis, disinfectants, when absorbed into the blood stream, deplete red cell glutathione, allowing ferrohemoglobin to be oxidized to ferrihemoglobin (*4,5*).

The morphologic and chemical onset of heme oxidation and erythrocyte membrane damage caused by chlorite *in vitro* (*6,7*) as well as hematologic changes in chickens and rats exposed to up to 1000 mg/L of ClO_2 *ad libitum* in drinking water were demonstrated (*8*). We were unable to elicit *in vivo* hematologic changes in monkeys using ClO_2, since *ad libitum* exposure to this disinfectant above 200 mg/L caused severe taste aversion and dehydration.

The most surprising observation in our studies was that ClO_2 is a relatively potent thyroid inhibitor, showing clear physiologic effects at about 9 mg/kg/day dose in 11 of 13 animals studied (*1*). In this study we also showed that, in monkeys intubated with a gastric tube, ClO_2 does not survive the organic environment of the stomach, and over 98% of the oxidizing capacity of an instilled ClO_2 solution (60 ppm) disappears within a few minutes. In addition, we showed spectroscopically that mixing monkey saliva with ClO_2 solution at various reactant ratios results in the instantaneous reduction of ClO_2. Thus, neither the intact molecule nor chlorite (ClO_2^-) or chlorate (ClO_3^-) is absorbed to any significant degree from the stomach when ClO_2 is consumed.

These products of reduction and hydrolysis of ClO_2, ClO_2^-, and ClO_3^- had no observable effect on the thyroid even at much greater doses (~ 40 mg/kg/day). This observation negated the possibility that such chlorine oxide anions, at the doses used, blocked iodide uptake into the thyroid follicles. Although this pharmacologic property of another chlorine oxide, perchlorate (ClO_4^-), is a recognized therapeutic effect, it can be elicited only with doses high enough to saturate the iodine-concentrating mechanism of the thyroid gland. In contrast to ClO_2, neither hypochlorite (OCl^-) nor monochloramine

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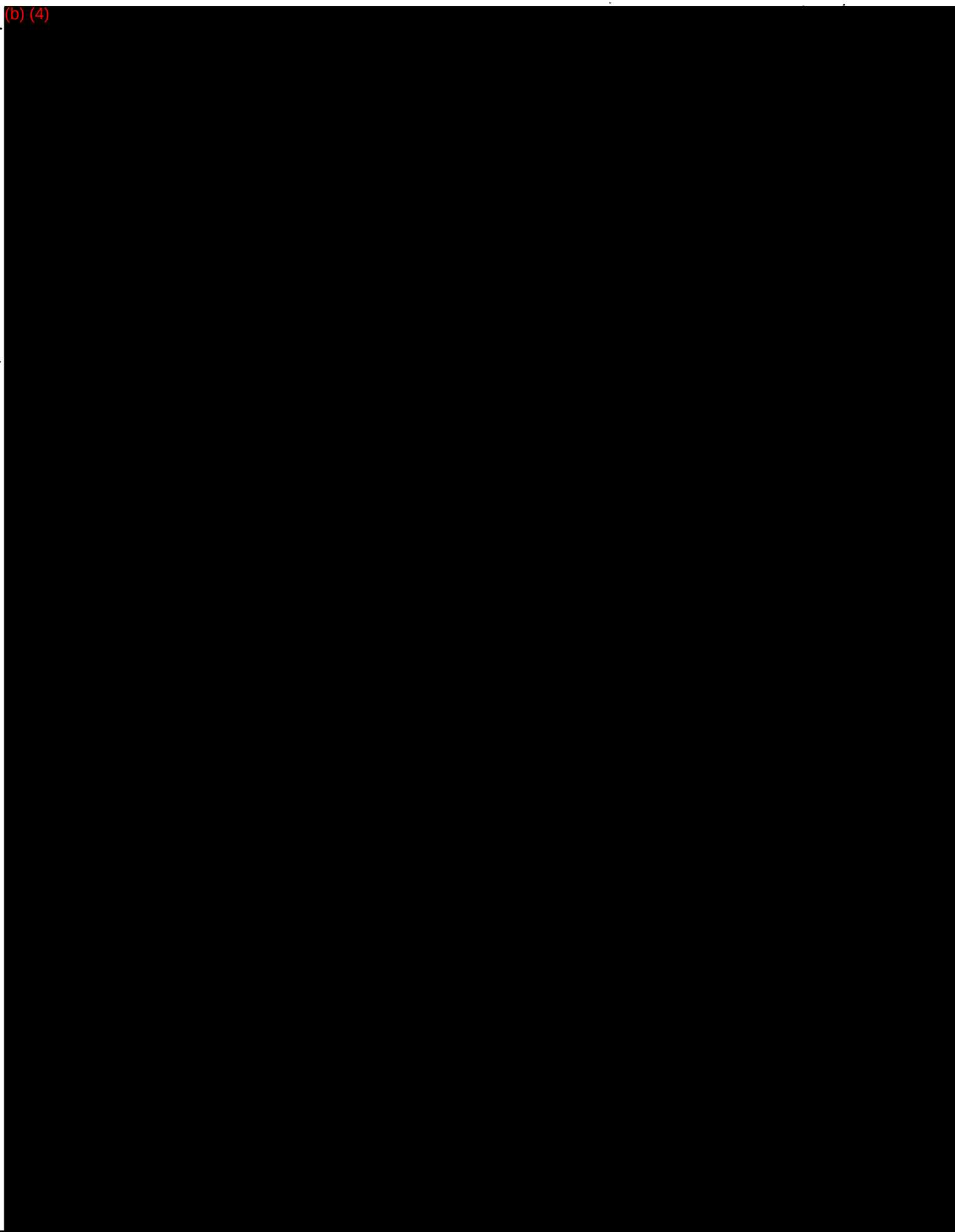

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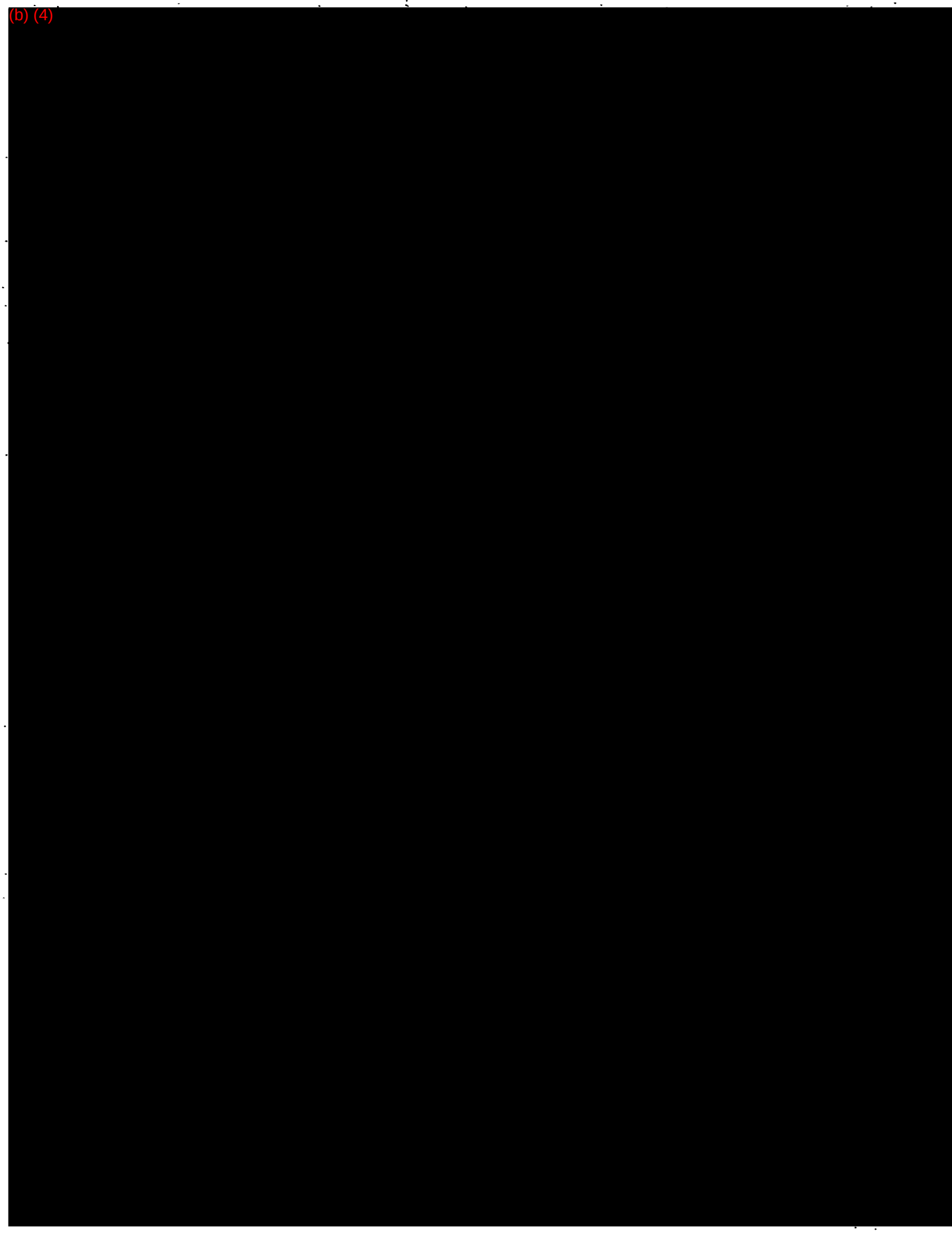

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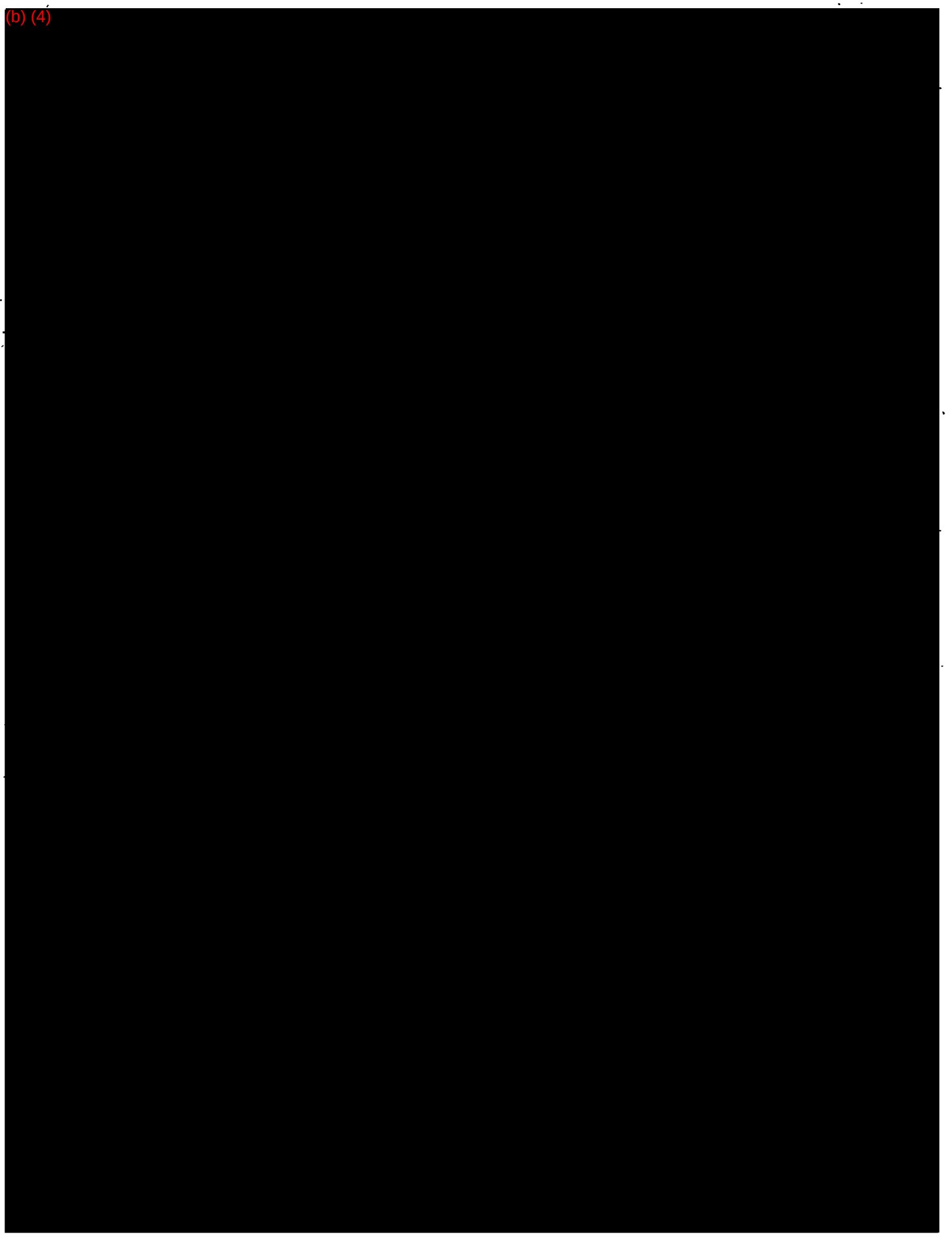

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notive forces for redox couples between iodide and drinking water tants.[a]

sinfectant	E, V	log K_{eq}[b]	EMF, V[c]
$+ 5e^- + 4H^+$	− 0.94	79.7	− 1.07
$+ 2e^- + H^+$	− 0.95	32.3	− 1.01
$I^- + H_2O$	− 0.36	12.9	− 0.42
$+ 2e^- + H_2O$	− 0.21	7.2	− 0.27

, $E_o = 1.45V$; $I_2 + 2e \leftrightarrow 2I^-$, $E_o = 0.54V$; $HOCl + H^+ + 2e \leftrightarrow Cl^- + H_2O$, $NH_2Cl + 2e + H_2O \leftrightarrow Cl^- + NH_3 + OH^-$, $E_o = 0.75V$. Computed medium.

$= (E_o(Ox) + (0.0591/n) \log[Ox]) - (E_o(Red) + (0.0591/n) \log[Red])$.

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Environmental Pathology

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Subchronic Sodium Chlorate Exposure in Drinking Water Results in a Concentration-Dependent Increase in Rat Thyroid Follicular Cell Hyperplasia

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ABSTRACT

Chlorine dioxide (ClO_2) is an effective drinking water disinfectant, but sodium chlorate ($NaClO_3$) has been identified as a potentially harmful disinfection by-product. Studies were performed to describe the development of thyroid lesions in animals exposed to $NaClO_3$ in the drinking water. Male and female F344 rats and $B6C3F_1$ mice were exposed to 0, 0.125, 0.25, 0.5, 1.0, or 2.0 g/L $NaClO_3$ for 21 days. Additional male F344 rats were exposed to 0, 0.001, 0.01, 0.1, 1.0, or 2.0 g/L $NaClO_3$ for 90 days. Female F344 rats were exposed to 0, 0.5, 1.0, 2.0, 4.0, or 6.0 g/L of $NaClO_3$ for 105 days. Thyroid tissues were processed by routine methods for light microscopic examination, and follicular cell hyperplasia was diagnosed using a novel method. Thyroid hormone levels were altered significantly after 4 and 21 days. $NaClO_3$ treatment induced a concentration-dependent increase in the incidence and severity of thyroid follicular cell hyperplasia. Male rats are more sensitive to the effects of $NaClO_3$ treatment than females. Follicular cell hyperplasia was not present in male or female $B6C3F_1$ mice. These data can be used to estimate the human health risk that would be associated with using ClO_2, rather than chlorine, to disinfect drinking water.

Keywords. Colloid depletion; disinfection by-products; drinking water; follicular cell hyperplasia; hormones; sodium chlorate; thyroid.

INTRODUCTION

Under the Safe Drinking Water Act of 1996, the US Environmental Protection Agency (EPA) is mandated by Congress to ensure the quality of drinking water in the United States by setting standards for the control of pathogens and disinfection by-products (DBPs). Special emphasis has been placed on the toxicity and carcinogenicity of chlorine disinfection by-products. Chlorination is the most common water disinfection method in the United States, but a variety of carcinogenic compounds including the trihalomethanes (THMs) and the haloacetic acids are formed when humic material reacts with chlorine. Alternative disinfection methods have been utilized to limit the production of these potentially harmful DBPs. Chlorine dioxide (ClO_2) is more effective than chlorine for killing most microorganisms, produces fewer chlorinated by-products, and does not produce significant levels of THMs (38). However, compounds such as sodium chlorate ($NaClO_3$) have been identified as by-products from ClO_2 disinfection. $NaClO_3$ may be formed by inefficient ClO_2 generation or as a result of the reaction between residual chlorite in finished water and free chlorine in the distribution system (13). Chlorate may be found at levels as high as 2.0 mg/L in finished drinking water (22). Commercially, $NaClO_3$ is used as an oxidizing agent in the tanning and leather industry, in the manufacture of dyes, explosives, and matches, and as a herbicide (31).

The toxicity data for $NaClO_3$ are limited and come primarily from subchronic studies involving administration of chlorate to rats and mice orally, either by gavage, or in the drinking water. Most of the potential adverse health effects of $NaClO_3$ exposure are associated with blood oxidation including increased methemoglobin formation, decreased hematocrit, red blood cell (RBC) membrane damage, and reduction in RBC glutathione levels (1, 2, 6, 19, 45). Other subchronic toxicity tests have identified the rat thyroid as the primary target organ. Bercz et al (4) reported a concentration-dependent decrease in thyroxine (T_4) levels in African green monkeys exposed to ClO_2 in drinking water. A statistically significant decrease in T_4 levels occurred after 4 weeks of exposure to 0.1 g/L (9 mg/kg/day) ClO_2, but changes in T_4 levels did not occur when the animals were exposed to 0.4 g/L (60 mg/kg/day) of $NaClO_3$.

More recently, McCauley et al (31) conducted a subchronic (90-day) study on $NaClO_3$ in male and female Sprague–Dawley rats. Animals were exposed to 250, 1,001,

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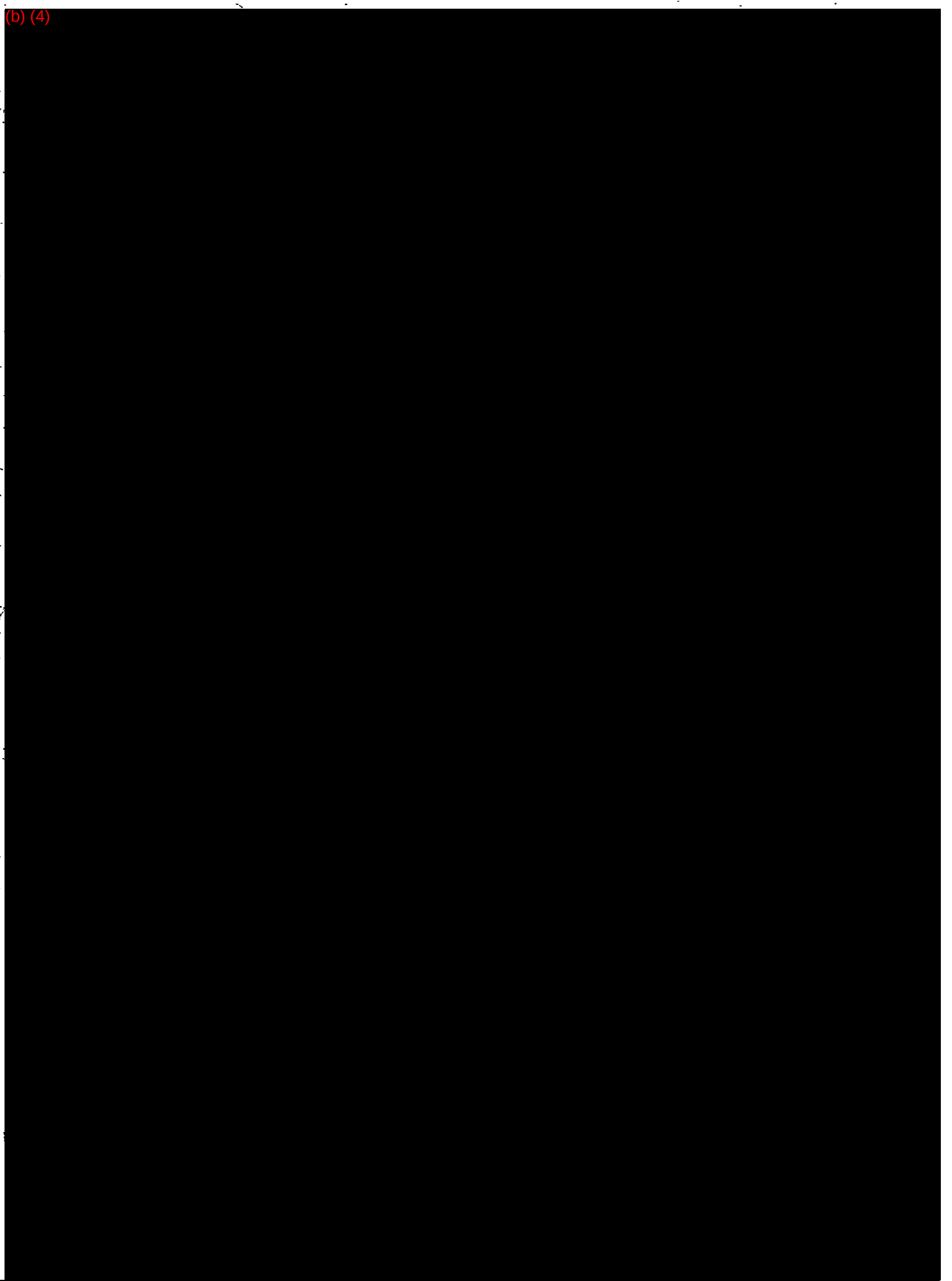
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DRUG AND CHEMICAL TOXICOLOGY, 18(2&3), 185-199 (1995)

THE EFFECTS OF SUBCHRONIC CHLORATE EXPOSURE IN SPRAGUE-DAWLEY RATS

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ABSTRACT

Male and female Sprague-Dawley rats were exposed to drinking water containing 3.0, 12.0 or 48.0 mM sodium chlorate. The mean drinking water consumption varied between exposure groups from 100-200 ml/kg/day. Female exposure groups consistently drank more water (23-42%) than male exposure groups thereby receiving more chlorate/kg/day at every exposure level. There were no compound related deaths; however, both males and females in the high exposure groups had significant weight loss during the 90-day exposure period. Also, in these same groups females had mild but significant decreases in the following relative organ weights; adrenals, thymus and spleen, while the relative brain weight was increased. In males, the heart, kidneys and liver were mildly decreased while the brain and testes were mildly increased. Red blood

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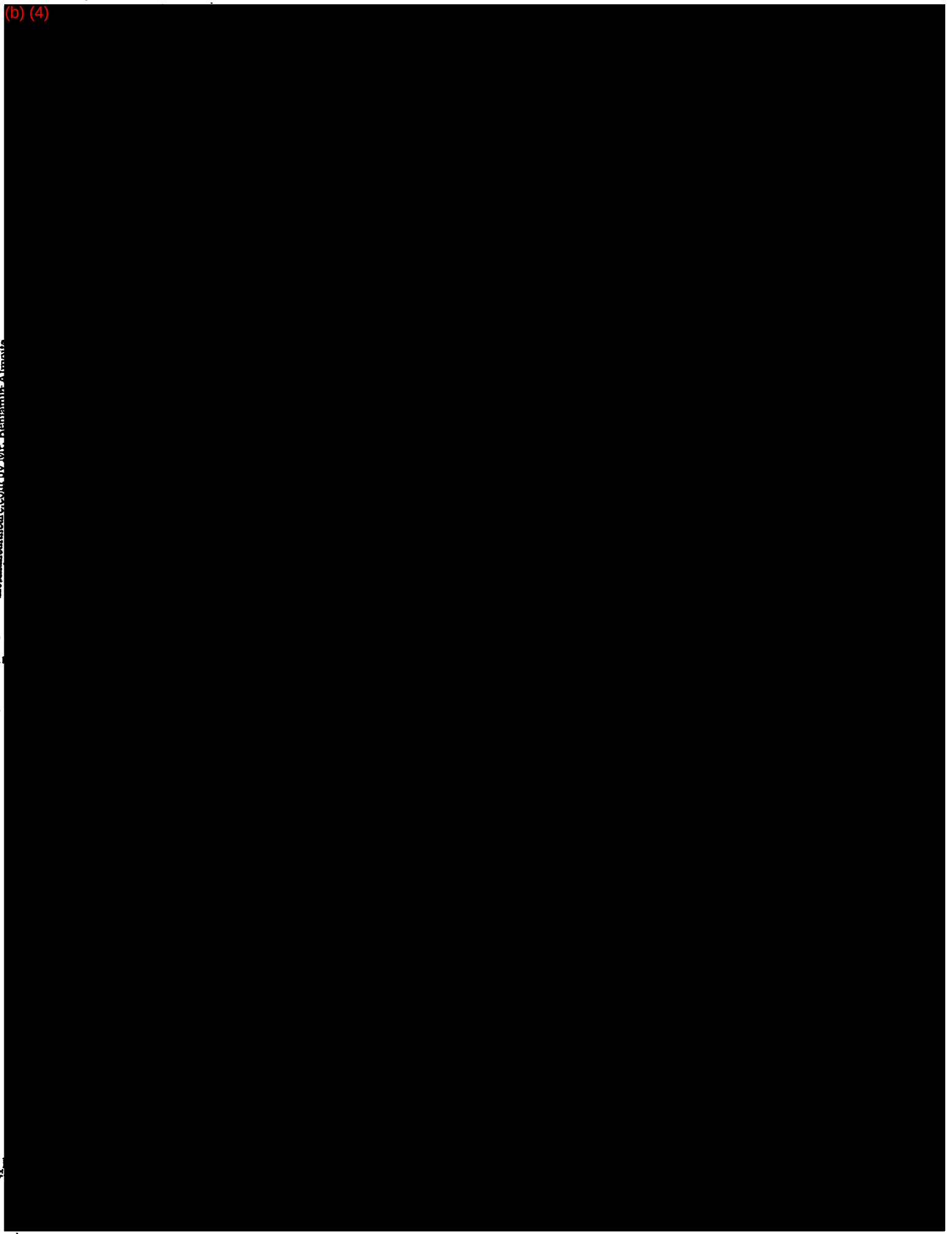

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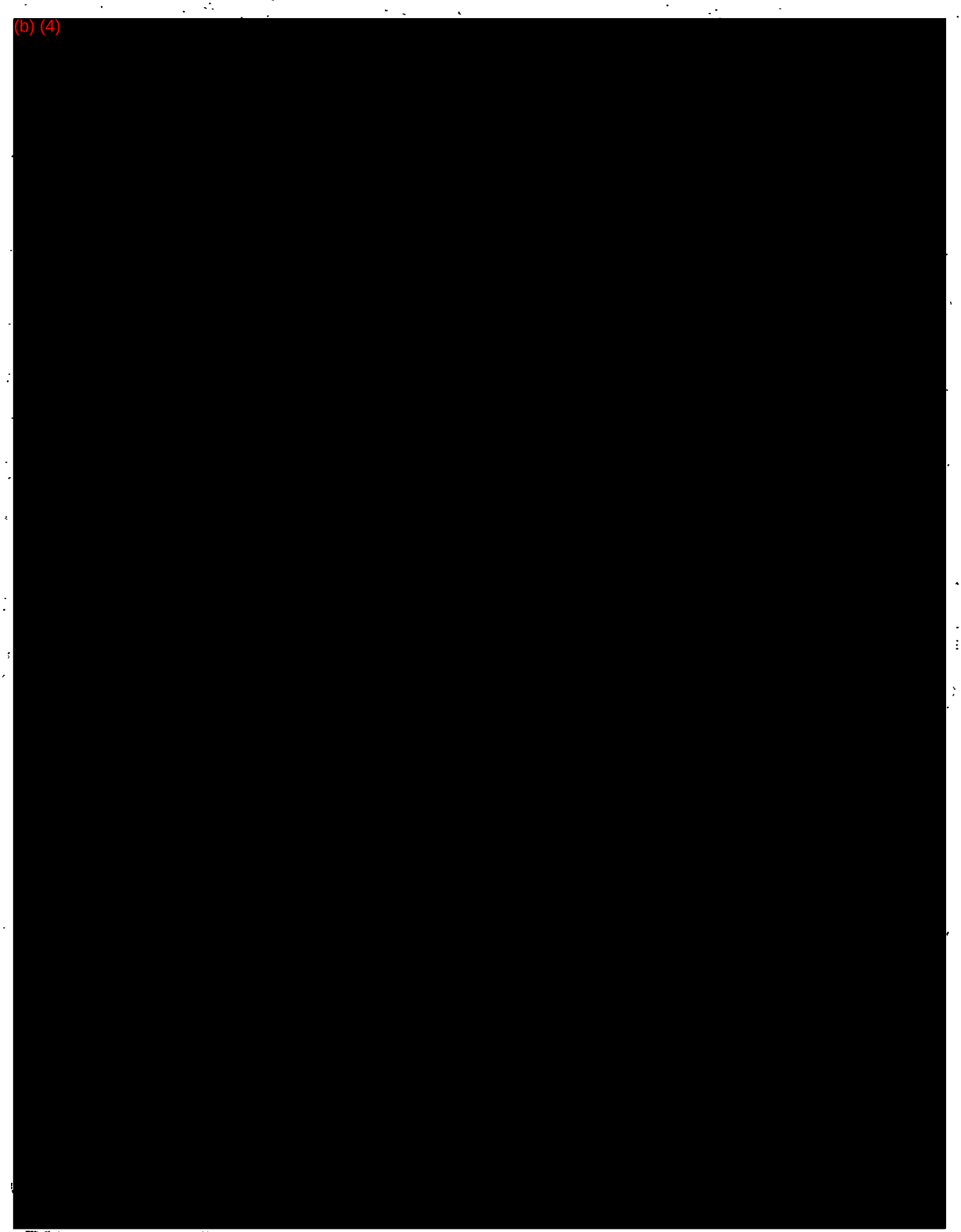

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Bactericidal Effect of Sodium Chlorate on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 in Rumen Contents In Vitro

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ABSTRACT

Escherichia coli O157:H7 and *Salmonella* Typhimurium DT104 are important foodborne pathogens affecting the beef and dairy industries and strategies are sought to rid these organisms from cattle at slaughter. Both pathogens possess respiratory nitrate reductase that also reduces chlorate to the lethal chlorite ion. Because most anaerobes lack respiratory nitrate reductase, we hypothesized that chlorate may selectively kill *E. coli* O157:H7 and *Salmonella* Typhimurium DT104 but not potentially beneficial anaerobes. In support of this hypothesis, we found that concentrations of *E. coli* O157:H7 and *Salmonella* Typhimurium DT104 were reduced from approximately 1,000,000 colony forming units (CFU) to below our level of detection (≤10 CFU) following in vitro incubation (24 h) in buffered ruminal contents (pH 6.8) containing 5 mM added chlorate. In contrast, chlorate had little effect on the most probable number (mean ± SD) of total culturable anaerobes (ranging from 9.9 ± 0.72 to 10.7 ± 0.01 $\log_{10}$ cells/ml). Thus, chlorate was bactericidal to *E. coli* O157:H7 and *Salmonella* Typhimurium DT104 but not to potentially beneficial bacteria. The bactericidal effect of chlorate was concentration dependent (less at 1.25 mM) and markedly affected by pH (more bactericidal at pH 6.8 than pH 5.6).

Escherichia coli O157:H7 and *Salmonella* Typhimurium DT104 are important foodborne pathogens of concern to the beef and dairy industries *(10, 14, 26, 28)*. Like most members of the family *Enterobacteriaceae*, both pathogens possess respiratory (also referred to as dissimilatory) nitrate reductase activity *(6, 25)*. Unlike assimilatory nitrate reductases that function to fix inorganic nitrogen into cell protein, respiratory nitrate reductases function to conserve energy via electron transport phosphorylation *(8, 25)*. Characteristically, respiratory nitrate reductases also reduce chlorate intracellularly to cytotoxic chlorite, and this has traditionally been used to distinguish between the two different types of nitrate reductases *(21, 25)*. An intriguing feature of this characteristic is that bacteria possessing respiratory nitrate reductases, such as *Salmonella* and *E. coli*, are consequently killed by the chlorite, but bacteria not possessing the respiratory nitrate reductase, i.e., many commensal and mutualist (beneficial) bacteria inhabiting the gastrointestinal tract, are not affected. Whereas some ruminal anaerobes such as *Propionibacterium, Selenomonas, Clostridium, Denitrobacterium, Desulfobacterium, Veillonella,* and *Wolinella* (*Vibrio*) possess respiratory nitrate reductase activity *(1, 2, 4, 16, 27)*, most do not. We thus hypothesized that chlorate may selectively inhibit *E. coli* and *Salmonella*. Presently, we report results from in vitro experiments that support this hypothesis, and we discuss potential applications of these results to reduce gut colonization by enteric pathogens.

MATERIALS AND METHODS

In several separate experiments, ruminal fluid was collected from a cannulated cow maintained on pasture (predominantly rye grass) and was mixed 1:1 with 200 mM phosphate buffer (pH 5.6 or 6.8) containing cellobiose, glucose, soluble starch, and xylose (1% wt/vol each). Aliquots of the buffered ruminal fluid were transferred to 18- by 150-mm crimp-top tubes and were incubated anaerobically under O_2-free N_2 gas at 39°C with or without sodium chlorate and either a novobiocin- and nalidixic acid-resistant *E. coli* O157:H7 or *Salmonella* Typhimurium DT104 (DHEP 12362). Both *E. coli* O157:H7 or *Salmonella* Typhimurium DT104 were cultured overnight in tryptic soy broth (Difco, Laboratories Inc., Detroit, Mich.) prior to inoculation into the buffered ruminal fluid. The *E. coli* O157:H7 strain (ATTC 43895) was made nalidixic acid resistant via successive incubations in trytic soy broth (Difco) supplemented with up to 20 μg nalidixic acid/ml. Colony counts for *E. coli* O157:H7 were determined via direct plating on MacConkey agar (Difco) containing 25 μg novobiocin/ml and 20 μg nalidixic acid/ml and for *Salmonella* Typhimurium DT104 via plating on brilliant green agar (Oxoid, Unipath Ltd., Basinstoke, Hampshire, UK) containing novobiocin and chloramphenicol (25 μg/ml each). Most probable number estimates of total culturable anaerobes were determined via a three-tube most probable number test *(5)* using anaerobically prepared (O_2-free N_2 gas) reinforced clostridial medium (Difco) supplemented with 40% (vol/vol) clarified rumen fluid *(13)*, 0.0001% resazurin (wt/vol), and with cellobiose and xylose (0.025% wt/vol each).

Data from each experiment were analyzed using the general linear models procedure of the Statistical Analysis System and

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The in vitro reduction of sodium [36Cl]chlorate in bovine ruminal fluid

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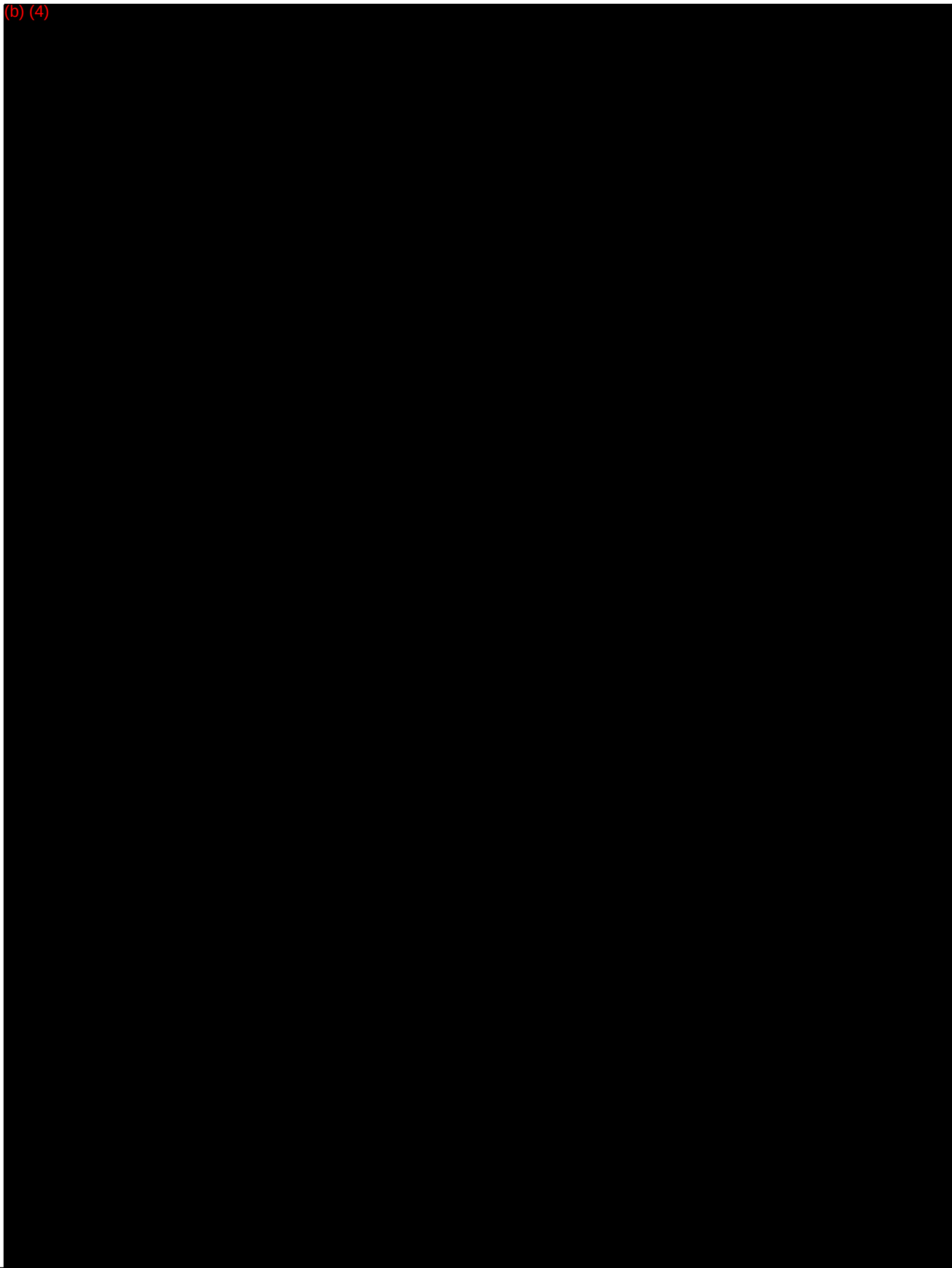
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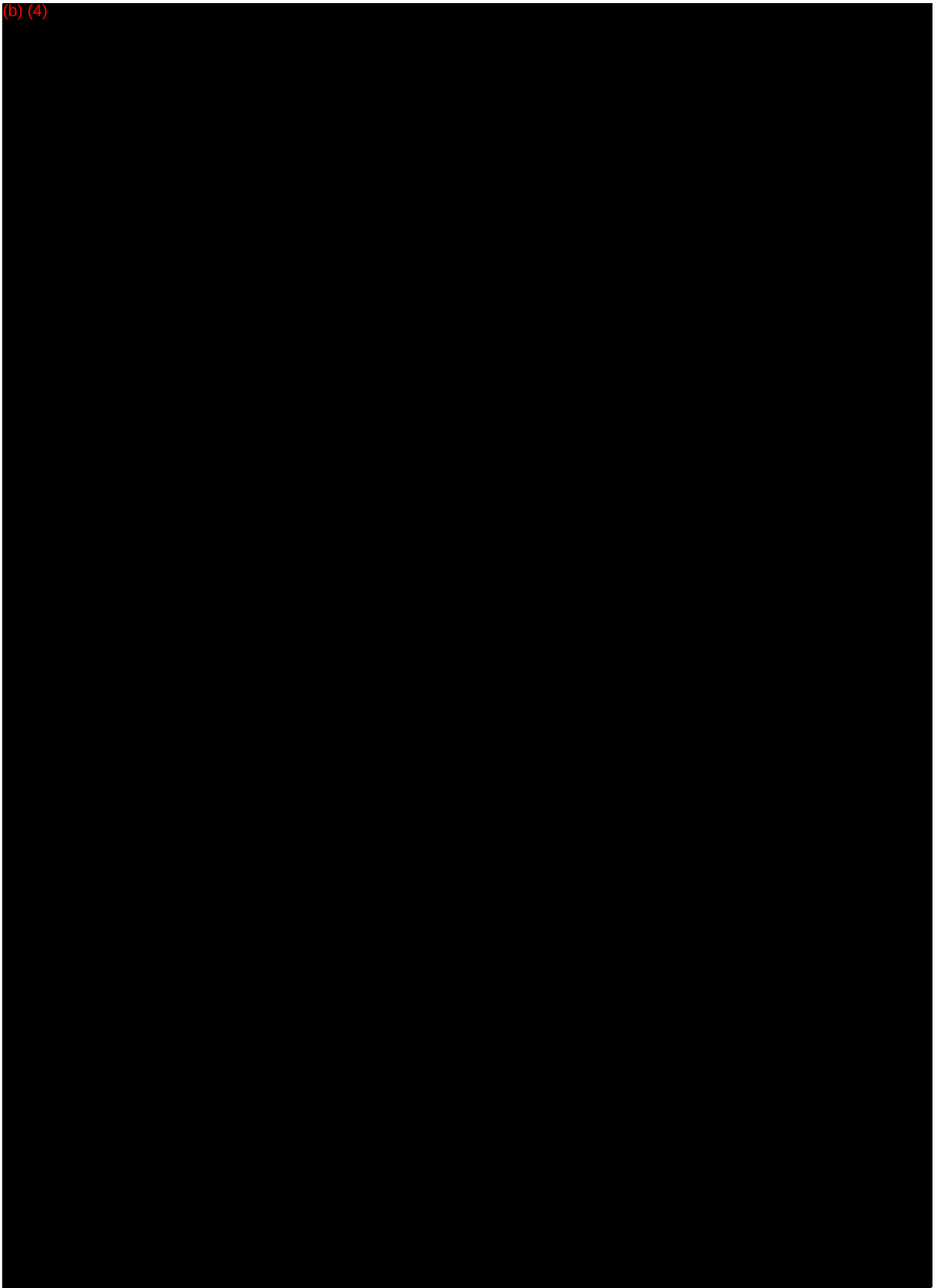
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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Sodium [^{36}Cl]Chlorate Dose on Total Radioactive Residues and Residues of Parent Chlorate in Beef Cattle†

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The objectives of this study were to determine total radioactive residues and chlorate residues in edible tissues of cattle administered at three levels of sodium [^{36}Cl]chlorate over a 24-h period and slaughtered after a 24-h withdrawal period. Three sets of cattle, each consisting of a heifer and a steer, were intraruminally dosed with a total of 21, 42, or 63 mg of sodium [^{36}Cl]chlorate/kg of body weight. To simulate a 24-h exposure, equal aliquots of the respective doses were administered to each animal at 0, 8, 16, and 24 h. Urine and feces were collected in 12-h increments for the duration of the 48-h study. At 24 h after the last chlorate exposure, cattle were slaughtered and edible tissues were collected. Urine and tissue samples were analyzed for total radioactive residues and for metabolites. Elimination of radioactivity in urine and feces equaled 20, 33, and 48% of the total dose for the low, medium, and high doses, respectively. Chlorate and chloride were the only radioactive chlorine species present in urine; the fraction of chlorate present as a percentage of the total urine radioactivity decreased with time regardless of the dose. Chloride was the major radioactive residue present in edible tissues, comprising over 98% of the tissue radioactivity for all animals. Chlorate concentrations in edible tissues ranged from nondetectable to an average of 0.41 ppm in skeletal muscle of the high-dosed animals. No evidence for the presence of chlorite was observed in any tissue. Results of this study suggest that further development of chlorate as a preharvest food safety tool merits consideration.

INTRODUCTION

Contamination of beef carcasses with pathogens such as *Escherichia coli* and *Listeria* during slaughter and processing have led to the annual recall of over 800 000 kg of beef during the past decade (*1*); this average excludes a recall of 10 000 000 kg of beef in 2002. Food-animal products containing undetected pathogens continue to contribute to an unquantified number of foodborne illnesses. In beef cattle, it has been established that hides are a major source of carcass contamination (*2*) and that hide-washing intervention steps effectively reduce subsequent pathogen loads on carcasses (*3*, *4*). Although postharvest sanitation techniques are becoming increasingly efficient, they are in use because no practical methods exist for removing pathogens from live animals prior to slaughter. It has been suggested (*5*) that intervention techniques that eliminate pathogen loads in live animals could have a greater relative impact on food safety than any postharvest intervention strategy known, aside from cooking. In reality, a combination of both pre- and postharvest intervention strategies will likely be employed to minimize risks associated with pathogen-contaminated meats.

Recently, a new preharvest technology that greatly reduces or eliminates the numbers of pathogens inhabiting gastrointestinal tracts of cattle (*6*−*8*), sheep (*9*), swine (*10*−*12*), and poultry (*13*, *14*) has been developed. The technology is based on the feeding of an experimental sodium chlorate-containing product (ECP) 24−72 h prior to the slaughter of an animal. During the chlorate exposure period, bacterial species containing intracellular respiratory nitrate reductase are thought to metabolize chlorate (ClO_3^-) to the bacterial toxin chlorite (ClO_2^-; *15*). Chlorate toxicity is specific to nitrate-reductase-containing bacteria that have the ability to intracellularly convert chlorate to chlorite but which lack chlorite dismutase enzymes capable of rapidly metabolizing chlorite to the chloride ion (*16*, *17*). Use of chlorate does not adversely affect the commensal microflora of gastrointestinal tracts (*6*). Unlike many antibiotics,

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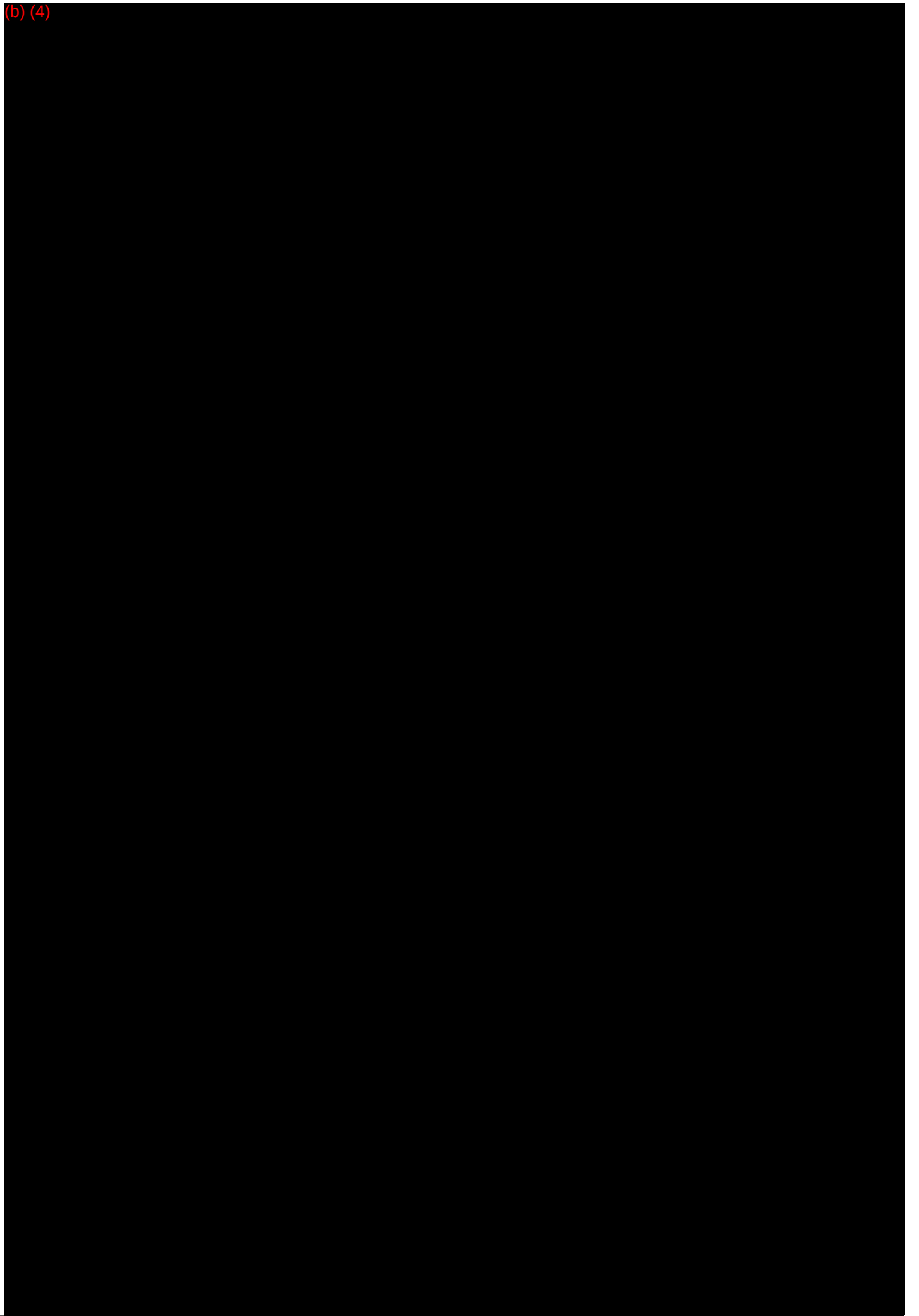
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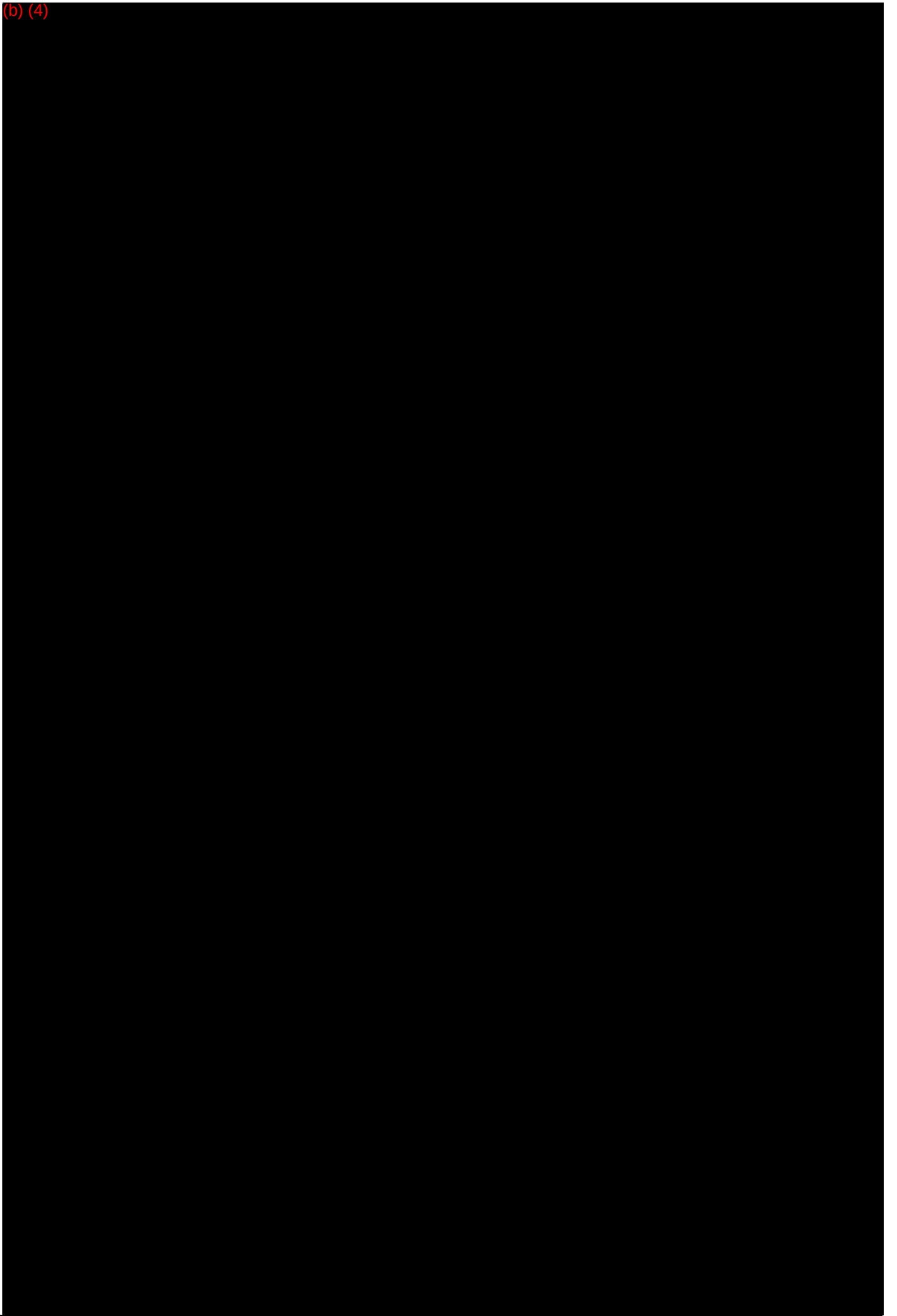
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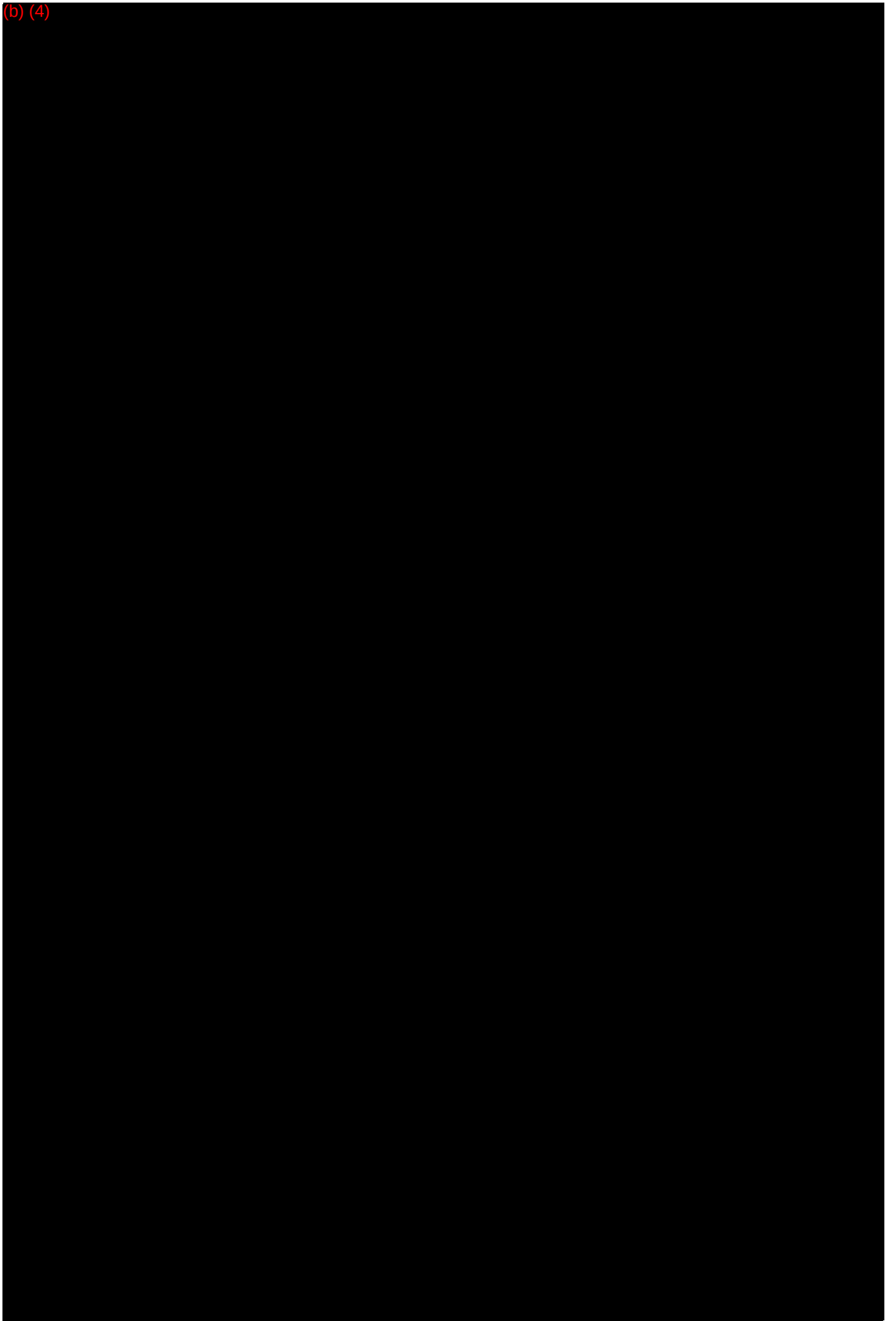
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Supporting Regulatory Analysis for GRAS Determination for Chlorine Dioxide Generated by the Puremash® System[1]

As a starting point, the GRAS concept was introduced with the 1958 Food Additive Amendments to the FFD&C Act to serve an integral role in the newly installed "food additive" regulatory scheme, a rigorous scheme which requires, among other things, that "food additives" receive explicit preclearance by the FDA before being marketed in the United States. In creating the "food additive" regulatory scheme, Congress exempted several classes of substances from the definition of a "food additive," thereby removing these substances from the preclearance requirement and other mandates that apply to food additives. Perhaps the most notable exemption from the "food additive" definition, Section 201(s) of the Act, 21 U.S.C. § 321(s), is the one for substances that are GRAS, *i.e.*, substances that are generally recognized, among experts qualified by scientific training and experience to evaluate their safety, as safe under their intended conditions of use. Section 201(s) defines a GRAS substance as one that is:

> generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use.[2]

The statutory definition for GRAS substances above as found in Section 201(s) establishes three key elements in making a GRAS determination for a substance: (1) there must be a general recognition by experts that a particular substance is safe; (2) the experts must be qualified by scientific training and experience; and (3) the experts must have

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[2] "Safety" means "that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use." 21 C.F.R. § 170.3(i). Safety determinations are based on the following factors: (1) consumption rate, (2) determination of a NOEL from an appropriate animal study, and (3) an appropriate safety factor.

based their safety judgment either on scientific procedures or the fact that the substance was commonly used in foods prior to January 1, 1958.

CVM published regulations describing eligibility requirements for GRAS substances at 21 C.F.R. § 570.30. General recognition of safety requires a "common knowledge" about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food. For substances not widely used in food prior to 1958, general recognition of safety based on "scientific procedures" requires the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. Unlike a food additive petition, however, general recognition of safety is ordinarily based on published studies which may be corroborated by unpublished studies and other data and information. *See, e.g.,* 21 C.F.R. § 170.30(b).

As the GRAS definition implies, and as FDA has recognized on many occasions, the determination as to whether a substance is GRAS is a question of fact, not of law. Thus, ingredient manufacturers and users are free to make a self-determination that their products are GRAS where such a determination is supported by publicly available information. *See* 21 C.F.R. § 182.1(a) (lists of GRAS substances in GRAS regulations are not intended as exhaustive lists of all such substances).

On April 17, 1997, FDA issued a proposed rule (62 Fed. Reg. 18938) that would establish a notification procedure whereby any person may notify FDA of a determination by that person that a particular use of a substance is GRAS. Although this proposed rule has not been finalized, FDA has implemented the program and reviewed over 150 GRAS notifications over the last several years. FDA's responses, which are posted on its web site, have been in one of three categories: (1) FDA does not question the basis of the notifier's GRAS determination, (2) the notice does not provide a sufficient basis for a GRAS determination, or (3) FDA, at the notifier's request, ceased to evaluate the GRAS notice.

On June 4, 2010,[3] CVM published its Notice announcing the establishment of a pilot program for GRAS substances added to animal feed. Similar to what was announced in 1997, this is a voluntary program which permits participants to submit to the Agency notices of claims that a particular use of a substance in animal food is exempt from the statutory premarket approval requirements based on the notifier's determination that such use is GRAS. This program signals CVM's implementation of the 1997 proposed rule and provide an alternative to the food additive petition process for these substances. The agency will implement the pilot program for substances added to animal food in the same manner as the interim policy for substances added to human food and as described above and in section VIII of the 1997 proposed rule.

Because an unintended constituent of chlorine dioxide production, sodium chlorate, was the subject of an NTP two-year chronic toxicology study, the safety of chlorate residuals is being evaluated using FDA's procedures for addressing the situation in which the use of a substance in a food additive is known to contain minute, but detectable, levels of a presumed carcinogenic impurity. The GRAS substance, chlorine dioxide, is not carcinogenic. Relevant to this analysis therefore, the so-called Delaney Clause was added to the FFD&C Act by the Food Additives Amendment of 1958.[4] More specifically, under section 409(c)(3)(A) of the FFD&C Act, no food additive shall be deemed by FDA to be "safe" if the additive is found "to induce cancer when ingested by man or animal, or if it is found, after tests which are appropriate for the evaluation of safety of [the additive], to induce cancer in man or animal."

In recognition of the overall integrity and force of the general safety clause, the legislative record shows that, although the Department of Health, Education and Welfare (HEW)—now the Department of Health and Human Services (HHS)—did not object to the inclusion of the Delaney Clause in the Act, in the view of HEW and FDA, "the [Food

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[3] 75 Fed. Reg. 31800 (Jun. 4, 2010), available at, http://edocket.access.gpo.gov/2010/pdf/2010-13464.pdf.

[4] FFD&C ACT § 409(c)(3)(A), 21 U.S.C. § 348(c)(3)(A).

Additives Amendment] reads and means the same with or without the inclusion of the [Delaney] clause . . . ."[5]

The Department held this view because of the prevalent scientific conviction in 1958 that the state of the art would not permit scientists to establish a tolerance for a carcinogen. Thus, it was impossible to reliably determine a level of exposure to a carcinogen below which one could be assured that there was no significant increase in the risk of developing cancer. In other words, scientists lacked the technology to assess adequately the risk presented by a particular chemical. However, the advent of highly improved analytical methods that made it possible to detect minute levels of a carcinogenic component of a food ingredient, and the increasing number of substances that were being shown in animal studies to be carcinogenic, necessitated a renewed evaluation of the interpretation and application of the Delaney Clause at a certain point in time.

This reevaluation led to the Agency's publication of the so-called "constituents policy," which allows FDA to find the use of food additives safe, even if they contain small amounts of carcinogenic substances as unintended contaminants, as long as the food additive itself is not a carcinogen. *Policy for Regulating Carcinogenic Chemicals in Food and Color Additives,* Advance Notice of Proposed Rulemaking, 47 *Fed. Reg.* 14464 (Apr. 2, 1982). [6]

In the *Federal Register* notice announcing its constituents policy, FDA stated that "[l]ike all chemicals, no food additive . . . can be produced absolutely pure . . . all chemical substances, including those used as additives, contain numerous impurities such as residual reactants, intermediates, manufacturing aids, and products of side reactions and

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[5] S. Rep. No. 2422, 85th Cong., 2d Sess. 10-11.

[6] *See* 47 *Fed. Reg.* 14464 (Apr. 2, 1982). On Nov. 26, 2004 (69 *Fed. Reg.* 68831, 68836), FDA withdrew this advance notice of proposed rulemaking (ANPR) along with approximately 80 other proposed actions and rules that were no longer considered viable candidates for final action. The withdrawal represented an effort by the Agency to reduce its regulatory backlog and focus its resources on current public health issues. The notice states that, "withdrawal of a proposal is not intended to affect whatever utility the preamble statements may currently have as indications of FDA's position on a matter at the time the proposal was published," and further that, "in some cases the preambles of these proposals may still reflect the current position of FDA on the matter addressed." It is understood that the constituent's policy as outlined in this preamble reflects current FDA thinking on the matter. Thus, despite the Agency's withdrawal of the ANPR, the constituents policy outlined in the April 2, 1982 *Federal Register* notice remains a valid policy by which to evaluate minor carcinogenic constituents of food additives.

degradation."[7] To address the increasingly common situation in which a food or color additive was known to contain minute, but detectable, levels of a carcinogenic impurity, FDA revised its procedures for evaluating the safety of such additives. One of the significant decisions at that time was to recognize and distinguish between the additive as a whole separate and apart from its individual "constituents." Under this approach, a food additive is regarded as the "substance that is actually intended for use in food or for food contact," while all "non-functional chemicals present in that substance would be called the 'constituents' of the additive."[8] According to FDA, substances classified as constituents "would include residual reactants, intermediates, and manufacturing aids, as well as products of side reactions and chemical degradation."[9]

Based on this distinction between the "food additive" and its "constituents," the Delaney Clause prohibition is appropriately applied when the food additive substance itself is shown to be carcinogenic. This interpretation has been employed to permit the use of a non-carcinogenic additive if it contains "safe" levels of carcinogenic constituents.

Support for the constituents policy is found in *Monsanto Co. v. Kennedy*, 613 F.2d 947 (D.C. Cir. 1979). In discussing whether a substance that migrates to food is a food additive, the court expressed the view that there is "administrative discretion inherent in the statutory scheme to deal appropriately with *de minimis* [*i.e.*, trivial or insignificant] situations." *Id.* at 955. "If FDA has discretion to disregard low level migration into food of substances in indirect additives because the migration of the particular additive presents no public health concern, then the Agency may also disregard, after appropriate tests, a carcinogenic chemical in a noncarcinogenic food additive or color additive if FDA determines that there is a reasonable certainty of no harm from the chemical."[10]

As noted above, the GRAS concept was introduced with the 1958 Food Additive Amendments to the FFD&C Act to serve an integral role in the "food additive" regulatory framework. As in the case of a non-exempt food additive, the constituent's

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7 *Id.*
8 *Id.* at 14466.
9 *Id.* at 14466-67.
10 *Id.* at 14466.

policy may be legally applied to a GRAS determination. *See* 47 *Fed. Reg.* at 14466 (stating that "FDA may conclude that one, all, or some combination of approaches may be appropriate"). The administrative discretion inherent in the statutory scheme allows FDA to disregard a carcinogenic constituent if the GRAS substance itself is not carcinogenic and if there is a reasonable certainty of no harm from the constituent.

FDA has adopted the use of the following risk assessment to determine whether there is a reasonable certainty that no harm will result from the proposed use of GRAS substance that itself is not carcinogenic due to the presence of a presumed carcinogenic constituent. 47 *Fed. Reg.* at 14468. If the calculated upper-bound lifetime risk from all sources of exposure is less than 10^{-6} (less than one-in-one million), the risk is considered negligible. The dietary concentration of the constituent which gives rise to this level of risk is referred to as the "virtually safe dose" or VSD. When a potentially carcinogenic constituent may enter the diet through more than one source, it is clear that each source cannot be allowed to contribute to the entire VSD. Generally speaking, where several potential sources are involved, a specific application may be considered "safe" if it contributes no more than 10% of the VSD. For those substances whose cumulative dietary exposure already exceeds the VSD, the fraction should be typically on the order of 1% of the VSD to consider the specific application "safe" under the Act.[11]

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[11] Noted as well is Part 500, Subpart E and the agency's interpretation of the DES proviso to the Delaney Clause, which subjects residues in edible tissues to sensitivity of method (SOM) considerations as described in that Subpart. *See* 67 *Fed. Reg.* 78172 (Dec. 23, 2002). This policy was specifically developed for the new animal drug regulations and was not expressly designed to address indirect constituents in the food supply. Nevertheless, the risk assessment required by SOM is essentially the same one used for the constituents policy determination. The long term endpoint is not considered to be appropriate in an analysis for food producing animals. Moreover, based on published reports in the scientific literature, at the estimated levels that are entering the animal diet through use of the Notifier's technology, FDA may conclude that any method to quantify chlorate residues in the target tissue of food producing animals at the level of the VSD in the test animals of 0.3 ppb will result in no detection at that level of detection (LOD).