ABSTRACT

Tanya Moore

Studies into the Mechanisms of Potassium Bromate Induced Thyroid Carcinogenesis.
(Under the direction of Louise Ball, Ph.D, Anthony DeAngelo, Ph.D., Avram Gold, Ph.D)

Bromate (BrO$_3^-$) occurs in finished drinking water as a by-product of the ozonation disinfection process. Potassium bromate, the experimental model compound, has been found to induce thyroid follicular cell tumors in the rat after 100 weeks. Little information exists on the mechanism(s) by which potassium bromate induces thyroid cancer.

Thyroid hormone levels exert a significant effect on cellular metabolism, homeostasis, and central nervous system development. In order to shed light on the possible mechanism(s) of carcinogenesis, the early effects of KBrO$_3$ administration in the drinking water on the serum levels of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxin (T4), thyroid histopathology, follicular cell proliferation, apoptosis, and OH$^\cdot$ guanosine glycosylase (OGG1) repair activity were examined. Animals exposed to KBrO$_3$ displayed increases in T3 and T4 hormones at 30 days, with no change in TSH levels. Colloid depletion was observed within follicular cells in a dose and time dependent manner. Increased apoptosis was observed in the high dose animals (400mg/L KBrO$_3$) at 30 days. No enhanced OGG1 expression was observed in the thyroids from rats treated with KBrO$_3$ for 7 days. It appears that hormonal effects may play an active role in the mechanism of KBrO$_3$ induced thyroid carcinogenesis.
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Bromate (BrO₃⁻) occurs in finished drinking water as a by-product of the ozonation disinfection process. Potassium bromate, the experimental model compound, has been found to induce thyroid follicular cell tumors in the rat after 100 weeks. Little information exists on the mechanism(s) by which potassium bromate induces thyroid cancer. Thyroid hormone levels exert a significant effect on cellular metabolism, homeostasis, and central nervous system development. In order to shed light on the possible mechanism(s) of carcinogenesis, the early effects of KBrO₃ administration in the drinking water on the serum levels of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxin (T4), thyroid histopathology, follicular cell proliferation, apoptosis, and OH₆ guanosine glycosylase (OGG1) repair activity were examined. Animals exposed to KBrO₃ displayed increases in T3 and T4 hormones at 30 days, with no change in TSH levels. Colloid depletion was observed within follicular cells in a dose and time dependent manner. Increased apoptosis was observed in the high dose animals (400mg/L KBrO₃) at 30 days. No enhanced OGG1 expression was observed in the thyroids from rats treated with KBrO₃ for 7 days. It appears that hormonal effects may play an active role in the mechanism of KBrO₃ induced thyroid carcinogenesis.
DEDICATION

To God, the Father, who makes all things possible and to my parents, Richard and Martha Moore, for all their love and support.
ACKNOWLEDGMENTS

I wish to express my appreciation to Mr. Michael George and Mr. Steve Kilburn for their assistance with the study. My thanks to Dr. Julia Carter and Bernedette Stephenson for their assistance with the cell proliferation and apoptosis data. My thanks to Dr. Kevin Morgan, Dr. Lynn Crosby, Lawrence Yoon, and Dr. Michael Jokinen for all of their support. I would also like to thank Dr. Anthony DeAngelo and Dr. Louise Ball for their guidance and patience.
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<td>Aminoethylcarbazole</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>DBP</td>
<td>Disinfection By-Products</td>
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<td>dU</td>
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<td>HAA</td>
<td>Haloacetic acid</td>
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<td>Mean Daily Dose</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>NSB</td>
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<td>OGG1</td>
<td>OH(^6) guanosine glycosylase</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RT-PCR</td>
<td>Real Time polymerase chain reaction</td>
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<td>SD</td>
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<td>T3</td>
<td>Triiodothyronine</td>
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CHAPTER I
BROMATE: A BY-PRODUCT OF THE OZONATION
DISINFECTION PROCESS

In 1974 the Safe Drinking Water Act was enacted in order to protect public health by regulating the U.S. drinking water supply. Congress issued a mandate to the Environmental Protection Agency to identify and regulate contaminants. The law focused on treatment to guarantee safe drinking water. This law was enacted in order to reduce the amount of disinfection by-products (DBP) in the U.S. water supplies. For almost 100 years, the U.S. has utilized chlorination to prevent the transmission of harmful waterborne diseases. Chlorination has reduced the number of cases of chlorea, dysentery, and polio. Although it has decreased the number of epidemics, chlorination results in the formation of DBPs which have raised health concerns (3,6,40).

In the 1970's, researchers found that chlorine combined with organic matter in the water to form by-products with potential risk to human health (3). Several of the DBPs were found to be carcinogenic when tested on laboratory animals. Researchers have taken a progressive approach by searching for other ways to
ozone treatment, which by comparison, has several positive features.

Ozonation is being considered as an alternative because it accomplishes two goals. It kills microbes more efficiently than chlorine and it reduces the levels of trihalomethanes in the drinking water. Unfortunately, there is a negative side to embracing ozonation. Like chlorine disinfection, ozonation also forms by-products. The formation of bromate as a by product of ozonation is of considerable concern. Bromate formation occurs in two ways. First, hypobromous acid may form in surface waters with a high bromide content. Bromide is found in estuarine (brackish) waters, but it is also present in downstream waters near factory utilities. Hypobromous acid reacts with bromine to form bromate. Also, the oxidation of bromide (Br\(^-\)), a naturally occurring ion found in most public waters, forms bromate (3, 5). Two pathways are recognized. Molecular ozone pathway:

1) \( \text{Br}^- + \text{O}_3 \rightarrow \text{OBr}^- + \text{O}_2 \)

\( \text{OBr}^- + 2\text{O}_3 \rightarrow \text{BrO}_3^- + 2\text{O}_2 \)

The bromide is oxidized by ozone forming the hypobromite ion (OBr\(^-\)). It reaches an equilibrium state with hypobromous acid (weak acid) (HOBr). This creates a bromate ion which is pH dependent (5).
Hydroxyl radical pathway

2) \( \text{Br}^- + \cdot \text{OH} \rightarrow \text{BrO}_3^- \)

The second pathway is the hydroxyl radical pathway. Bromide interacts with hydroxyl ions (forms as a product of ozone) to form bromate (5).

In recent studies, Krasner and others measured the concentration of bromide in the surface waters. Results indicated 70-100 \( \mu \text{g/L} \) bromide in the influent waters of U.S. utilities (25). In an early report, Krasner found that 75% of the water supplies contain 40 \( \mu \text{g/L} \) bromate. Also, the World Health Organization has set the limit of bromate in drinking water to 25 \( \mu \text{g/L} \). The EPA has set the limit to 10 \( \mu \text{g/L} \) in drinking water (3). Because 75% of the water supply would contain 40 \( \mu \text{g/L} \) bromate, a risk assessment for ozonation and chlorine disinfection must be carried out. A comparison of the detrimental effects of the toxic by-products must be analyzed and weighted.

In the 1970's, several laboratories conducted studies on chloroform. The biochemical toxicology of chloroform was reviewed in 1979 (36). This DBP is one of the most common trihalomethanes (THM) found in drinking water. The review found that chloroform caused hepatotoxicity and possibly renal toxicity in rodents (36). In addition to chloroform, other DBPs in the water were also studied. Dichloroacetic and trichloroacetic acid studies
ingredient in these products because of its oxidizing property.

Potassium bromate was found to be carcinogenic in the male and female rat (7,26,27). While rodent susceptibility to this compound does not imply a definite health hazard to humans, most studies indicate a reason for concern for potassium bromate induced tumors. Although it is not listed as a human carcinogen, KBrO₃ is a nephro and neurotoxicant in humans (7,26,27). Due to its questionable effects on humans, potassium bromate has been removed from cosmetics and home wave permanent kits.

The EPA has recently investigated rodent and human data involving thyroid follicular cell tumors. The Environmental Protection Agency assessment guidelines for thyroid cancer were implemented in 1988. The guidelines operate under two assumptions. The first guideline makes the following assumption:

1) "Chemically induced cancer in laboratory animals signals potential hazards in humans (15)."
2) For dose-response analyses, use the most biologically appropriate means for dose extrapolation. In the absence of such knowledge, use of default science policy positions should be followed, either low-dose linear, non-linear, or both procedures (15).

For the risk assessment of thyroid cancer, the required data must include: the mode of action information on mutagenicity, increases in follicular cell growth, thyroid gland weight, thyroid-pituitary hormones, site of action, and relationship between the dose
producing thyroid effects which cause cancer and reversibility of effects when dosing stops (17).

In the risk assessment default science policy, the Agency concluded that thyroid follicular cell tumors and thyroid tumors are relevant to human carcinogenicity (2,14,15,17). A change to ozonation disinfection would expose human populations to 40-100 μg/L potassium bromate in water containing 2 mg/L bromide (influent waters) (44).

In order to appreciate the role potassium bromate may play in thyroid carcinogenesis, it is necessary to understand the normal functions of the thyroid-pituitary axis. The thyroid-pituitary axis consists of three main endocrine tissues; these include, the hypothalamus, pituitary and thyroid gland (4,9). The neurons within the hypothalamus produce thyrotrophin releasing hormone (TRH) which activate cells within the anterior pituitary to release thyroid stimulating hormone (TSH). TSH works through two main pathways. TSH activates adenylate cyclase which increases the levels of cAMP. In turn, the cAMP activates phosphatidylinositol/Ca²⁺ signal cascade which turns on phospholipase C. At this point, the pathway diverges into two branches. The first branch includes the activation of inositol triphosphate which releases Ca²⁺ from cell storage. This pathway eventually leads to hydrogen peroxide generation and thyroid hormone synthesis. The second
pathway uses 1,2 diacylglycerol to activate protein kinase C. This signalling cascade activates nuclear transcription factor genes which control cell proliferation and iodide uptake and metabolism (14). Ultimately, the TSH activates follicles within the thyroid to release thyroid hormones. These three operate in a negative feedback system to sustain thyroid hormone homeostasis within the body (4,19,20).

The thyroid gland is located on the trachea. It consists of two lobes connected by a small isthmus of tissue. Blood is supplied by the superior and inferior thyroid artery. 50% of the blood is supplied by capillaries which are adjacent to the thyroid. The thyroid gland is composed of two cell types, the parafollicular cells and the follicles. The follicles are the primary cells which produce the hormones. The follicle consists of a single epithelial layer of cells surrounding a lumen. The lumen contains colloid, the organic storage material which contains the tyrosines which make up T3 (active form) and T4 (less active). The colloid fills the lumen completely in normal cells, until stimulated by TSH, from the pituitary, to release hormones. The follicular cells are cuboidal when active and flattened when at rest. The parafollicle cells (c cells), located in the interfollicular spaces, contain rough endoplasmic reticulum near the base of the cell. This organelle produces polypeptide chains of thyroglobulin. Thyroglobulin is
transferred to the golgi apparatus, followed by transfer to the
surface of the cell by vesicle. These vesicles empty thyroglobulin
into the follicular lumen (4,14,15,19,20).

In order to synthesize T3 and T4, iodination of thyroglobulin
must occur. Thyroperoxidase catalyzes the iodine binding to
tyrosyl residues within the thyroglobulin. The two iodinated
tyrosines are coupled by thyroperoxidase. This reaction forms
molecules of iodothyronines, the precursor to the hormones.
Lysosomes are present and are responsible for the degradation of
the thyroglobulin, which allows hormone release (14).

The extrapolation of rodent data to human data are due to
the following similarities of rodent and human systems. First, the
thyroid-pituitary negative feedback axis is present in both rodents
and humans. Also, the response to disturbances in thyroid function
from antithyroid stimuli is similar in both species. In contrast, the
differences between rodents and humans are cause for caution
when extrapolating data. The size, lifespan, and basal metabolic
rates between the rodents and humans differ. Humans require low
and high affinity protein carriers for thyroid hormones, albumin and
thyroxine-binding globulin. Rodents also use the albumin, but lack
thyroxine-binding globulin. As a result, thyroid hormones have a
shorter half-life in rodents. Humans are not as sensitive to some
stimuli as are rodents. There are no sex differences in humans,
whereas, the rodent, exhibit sex differences. Adult male rats have higher TSH levels than females. Male rats are more sensitive to thyroid stimulation and carcinogenesis. Most important of all, no chemical has been found to be carcinogenic to the human thyroid. Nevertheless, this does not diminish the possibility of a common mechanism for carcinogenesis in the thyroid given the common thyroid-pituitary pathway.

Potassium bromate is a carcinogen in both male and female rats (7,26,44). In drinking water doses are as low as 20 ppm (20mg/l), KBrO₃ was carcinogenic. Renal cell tumors, thyroid follicular tumors, and abdominal mesotheliomas have been observed by several laboratories (26,27,44). The mechanism of carcinogenesis within the rat kidney has been studied thoroughly (1,11,23,26,27,37,38). Potassium bromate induces oxidative damage to the renal proximal tubules, the target site for carcinogenesis within the rat kidney (37,38). Increased levels of lipid peroxidation may be the result of oxidative damage. Also, potassium bromate induced the formation of mesotheliomas on the tunica vaginalis parietalis (located on the attachment between the testis and the epididymis) (44). Recent studies indicate thyroid follicular cell tumors develop as early as 26 weeks after treatment with 10-20ppm KBrO₃ (7,44).

The mechanism (s) of potassium bromate induced thyroid
carcinogenesis is not known. Studies have shown that a direct interference with the thyroid hormones may cause development of tumors(16,18,30,32,33,34). Specifically, decreased triiodothyronine (T3) and thyroxine (T4) levels with increased thyroid stimulating hormone (TSH) may induce the formation of neoplasms (33,34). Neoplasms are abnormal new growths or tumors. Thyroid follicular cell proliferation may also play a role in the development of hyperplasia. Hyperplasia is the nontumorous increase in the number of cells in an organ or tissue(4,16,19,21). This study was designed to investigate the mechanisms by which potassium bromate disrupts the normal function of the rat thyroid. This study was conducted to examine the early effects of KBrO₃ administered in the drinking water on the serum levels of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxin (T4), thyroid histopathology, follicular cell proliferation, and apoptosis.

MATERIALS & METHODS

CHEMICALS

Potassium bromate (99% Purity) was purchased from Sigma Chemical Company (St. Louis, MO) and dissolved into deionized water to achieve the target concentrations of 0.100 mg/L and 0.400 mg/L. The pH was adjusted to 6.8-7.4 with hydrochloric acid. The dosing regimen consisted of 3 groups: (1) (deionized water), (2)
(0.100mg/L KBrO₃), and (3) (0.400 mg/L KBrO₃). Animals were allowed ad libitum access to amber glass bottles fitted with Teflon, double-balled sipper tops. The bottles were filled once a week. Samples from each exposure concentration were collected in 7mL scintillation vials and stored at 5° C. All samples were analyzed by HPLC ultraviolet detection at 215 nm (300mm X 7.8mm anion exclusion column). A constant flow rate of 1ml/min of 1% phosphoric acid was used to determine the potassium bromate concentrations. The results of these analyses are shown in Table 1.

ANIMALS

F344 male rats 21 days old, were purchased from Charles River Laboratories (Portage, MI) and acclimated for 7 days. 28 day old rats were randomly divided into three exposure groups. The rats were housed 2 or 3 per cage in polycarbonate cages. The animals had access to Purina Rodent chow and water ad libitum. Animals were weighed once a week and at the time of euthanasia. The rooms were maintained on a light/dark cycle of 12/12 hours. The temperature and humidity within the animal facilities were maintained at 20 - 22° C and 40-60%, respectively.

NECROPSY

For necropsy, rats were euthanized by carbon dioxide asphyxiation at 4 or 30 days after start of treatment. The liver,
l lung, kidney, thyroid, and testis were removed, weighed, and fixed in 10% neutral buffered formalin.

HISTOTECHNOLOGY

The fixed tissues were processed through a standard series of dehydration for paraffin embedding and sectioned at 5μm. The sections were stained with hemotoxylin and eosin by standard histologic technique for easy observations under a light microscope [Appendix A].

CELL PROLIFERATION

The Zymed Inc. bromodeoxyuridine (BrdU) staining kit was used to determine the BrdU labeling as recommended by Calbiochem Inc. (San Diego, CA). Staining was preceded by antigen retrieval with heat (95°C) for 20 minutes and rinsing with phosphate buffered saline. In order to measure the number of cells actively proliferating, proliferating cell nuclear antigen (PCNA) was measured using the PC10 antibody as recommended by Calbiochem Inc. Sections of tonsil were used as a positive control [Appendix F].

APOPTOSIS

Apoptosis was determined by the TUNEL procedure as described by Gavrieli, et al. with modification. [Appendix G] (12). Thyroids, livers, and kidneys were cut into 5μm sections and mounted on ProbeOn Plus slides (Fisher Biotech Inc.). The
samples were stained by protease K pre-treatment (TUNEL) for apoptosis detection. 5μm sections were exposed to Terminal deoxynucleotidyl transferase (TdT). TdT initiates nick end labeling of biotinylated poly dU. Immediately following labeling, the samples were incubated with avidin-conjugated peroxidase. The techniques identified cell nuclei which were in the process of cell death.

QUANTIFICATION OF CELL PROLIFERATION & APOPTOSIS

Quantitation of the number of S-phase cells in slides of thyroid sections was determined by counting 1000 cells per slide. The 97X oil immersion objective was used to count the total number of nuclei in the epithelium, followed by the total number of labeled cells. 8-12 fields were randomly selected and counted using a double incidence cell counter. Edges and folds were excluded. TUNEL positive, apoptotic bodies within each thyroid section were counted. Apoptotic bodies in the thyroid epithelium were quantitated in the same manner.

SERUM COLLECTION

The collection of the serum was conducted in the morning hours due to the circadian rhythm of the hormones, specifically the thyroid stimulating hormone (TSH). The thyroid hormones fluctuate during the course of the day. Blood was collected by venipuncture in serum separation tubes (Becton Dickinson Cat.# 6511) and
allowed to clot. The blood was centrifuged at 1,000 X g, 4°C for 10 minutes. The serum was carefully removed from the surface and placed into 2 mL microtubes. The samples were labelled and stored in the REVCO -70°C freezer [Appendix C].

HORMONE ANALYSIS

Coat-A-count TSH radioimmunoassay kit was purchased from Diagnostic Products Corporation (Los Angelos, CA) to measure TSH in rat serum (Appendix B). ¹²⁵I-labeled anti-TSH polyclonal antibody in liquid phase and unlabeled monoclonal anti-TSH antibodies were immobilized on the walls of the polystyrene tubes. TSH was bound competitively by immobilized hot and cold antibodies on the surface of the polystyrene walls of the tubes. Free and bound ¹²⁵I labeled anti-TSH antibody was separated by decanting the reaction mixture and washing the tubes to remove unbound antibody. The TSH concentration was determined by using a gamma counter to count the bound portion of the antibody. The TSH concentration was compared to the manufacturer-supplied calibration standards. The calibration range was 0.15 to 60 μIU/mL. All samples were read in the Packard gamma (5000 series) counter for 1 minute (Figure 5).

T3 was measured using the Coat-A-count total T3 Kit purchased from Diagnostic Products Corporation (Los Angeles, CA). This assay measured total serum T3 concentration (Appendix
C). ¹²⁵I-labeled T3 competed with unlabeled T3 in the rat serum sample for antibody sites for a fixed time. The kit supplied polypropylene tubes containing pre-immobilized antibody on the walls of the tubes. Removal of the supernatant from the tube terminated the competition. Human serum-based standards ranging from 20 to 600 ng/dL were used to generate a calibration curve for the concentration of T3. The samples were read as above (Figure 3).

T4 measurements were retrieved by the T4 radioimmunoassay kits purchased from DPC Inc. (Los Angeles, CA). Using the kit, one measures the amount of T4 present in serum as percent of hormone bound to the antibody (see Appendix D). Labeled ¹²⁵I competes with T4 in the rat sample for a fixed time for antibody sites, in the presence of agents that block thyroid hormone-binding proteins. The unbound T4 was removed from the tubes and the remaining bound hormone was counted using a gamma counter (see above). T4 concentration was determined by using human serum-based calibration standards with T4 values ranging from 1 to 24μg/dL (DPC Inc., Los Angeles, CA) and read as above (Figure 4).

RNA ISOLATION FROM THYROID

For the quantitation of OGG1 expression, messenger RNA was collected from frozen tissue samples (-70°C) in order to
measure the repair of oxidative damage within the tissues. RNA was obtained by the protocol of Graveli et al. (12,29) with modifications. The tissues were immediately pulverized under liquid N$_2$ in a mortar and pestle. The powdered tissue was placed into 14mL of Trizol (Gibco, BRL) solution in order to lyse the cells. Chloroform was added to each sample and vortexed. Microcentrifugation for 5 minutes completed the phase separation of this technique. The upper aqueous phase (contains RNA) was removed and placed into cold isopropanol. RNA was precipitated overnight (-70°C) followed by a quick centrifugation to pellet the material. The RNA was washed with 70% ethanol and air dried for 5 minutes. Samples were resolubilized in RNase-free deionized water for 30 minutes. RNA was quantitated using the Beckman DU650 spectrophotometer (readings at 230nm, 260nm, 280nm). Optimal RNA purity was considered to be an absorbance ration of 260/280 nm of 2.0. Integrity of RNA was confirmed by 1% agarose gel electrophoresis for 1 hour at 75 v (constant voltage) containing 4 $\mu$g/$\mu$l ethidium bromide. The gel was visualized using the Kodak camera interfaced to Kodak computer imaging software.

mRNA MEASUREMENT

Any influence of KBrO$_3$ on increasing oxidative damage in the thyroid was examined in male F344/N rats that were exposed to 100mg/L and 400mg/L KBrO$_3$ for 7 days. Thyroid glands and
kidneys were excised and quick frozen in liquid nitrogen (29).

Oxidative damage (8-hydroxy-2'-deoxyguanosine content of DNA) 
was estimated indirectly by measuring the amplification of OH₈ 
guanosine glycosylase mRNA using Real Time PCR (Taqman).

This procedure was preformed on the Perkin Elmer ABI Prism 7700 
Sequence Detector.

The cycling conditions were as follows:

1 cycle of 
  48 °C 30 minutes
  95 °C 10 minutes
followed by 40 cycles of :  
  94 °C 15 seconds
  60 °C 1 minute

1X Taqman PCR reaction buffer; dNTP (300 μM), MgCl₂ (5.5 mM), 
each primer (900 nM), Taqman probe (200 nM), and 5 U/μl 
AmpliTaq Gold. The primer sequences (OGG1) were (forward)- 
CCCTGGCTGGTCCAGAATA; (reverse)- 
TACATAGCGGCGACGGTACC; and (probe)- 
GGCCCAACTTCCTGAGGTGGTCTCT. The Taqman probe was 
designed using Biosource International with 5'-labeled carboxy- 
fluorescein and 3'-labeled with 6-carboxytetramethylrhodamine (22 
). Each quantitation was done from standard curves. Each sample 
was determined in triplicate. This technique is based on the ability 
of Taq (Thermas Aquaticus) polymerase to displace and cut a 
fluorescent-labeled oligonucleotide complementary to an OH₈
guanosine glycosylase DNA probe bound to an internal target sequence during the PCR amplification (22).
CHAPTER II
RESULTS AND DISCUSSION

RESULTS

Potassium Bromate samples were analyzed for target concentrations of 0, 100mg/l, and 400mg/l KBrO₃. Measurements of 0, 100 mg, and 423 mg/l KBrO₃ were observed [Table 1]. During this early period, potassium bromate depressed water consumption in a dose-related manner at 4 days. After 30 days, the water consumption in the 400mg/L treatment group was significantly elevated over the control value (p≤0.05); [Table 1]. MDDs of KBrO₃ are also shown in Table 1. A significant increase in relative kidney weight at 4 days (400mg/L KBrO₃) was noted. No significant alterations in body weight, relative liver and thyroid weight were measured in any of the KBrO₃ treatment groups at 4 or 30 days [Table 2].

HISTOPATHOLOGY

The histopathologic examination of the thyroid gland revealed noticeable alterations within the follicles. When compared to thyroid from control animals, the treated rats displayed mildly dilated follicles that were lined by pale follicular epithelial cells and
filled with pale staining colloid. Minimum hypertrophy was observed in a few follicular epithelial cells. Pale colloid is defined as colloid which stained lightly eosinophilic (pale pink) when compared to the darker eosinophilic staining of the normal colloid (darker pink). Pale follicular cells had clear cytoplasm, as compared with the eosinophilic cytoplasm seen in the follicular cells of control animals. Follicular dilatation consisted of a slight but visible increase in the diameter of the follicular lumen, and follicular cell hypertrophy consisted of a slight increase in the size of the follicular epithelial cells, as compared with the control animals [Figure 2].

At both 4 and 30 days, depletion of colloid within the follicular lumen was significant in both treatment groups versus the control. In addition, smaller follicles were observed in the 30 day high dose group. The intensity of the colloid decreased as the potassium bromate concentration increased. All of these microscopic changes were consistent with glands that were physiologically stimulated. The changes were relatively slight in the treated versus the control follicular cells in all animals and were observed in the thyroid glands of both KBrO₃ treatment groups at both 4 and 30 days. There was a marginal increase in the severity of changes in animals exposed to 400mg/l KBrO₃ when compared with animals receiving the 100mg/l KBrO₃ [Figure 2].

No significant alteration in the TSH, T3, T4 serum levels were
observed at 4 days of KBrO₃ exposure. Normal hormonal function without any implication of chemical interruption continued during this time period. However, after 30 days of exposure to KBrO₃, the T₃ concentration in the 400mg/l treatment group showed a significant increase when compared to the control value (31.5 vs 17.6 ng/dl for 400mg/l KBrO₃ and control respectively; p≤0.05) [Figure 3]. Similarly, there was a significant increase in the serum concentration of T₄ after 30 days of treatment with 400mg/l KBrO₃ (5.47 vs 3.51 µg/dl 400mg/l KBrO₃ and control respectively; (p≤0.05) [Figure 4]. The serum concentrations of TSH were not altered for either KBrO₃ treatment or length of exposure [Figure 5].

Cell proliferation was not significantly increased above the control value after 30 days of KBrO₃ exposure [Table 3]. There was no significant differences in the PCNA labeling indices at 4 days. For the control animals, the percent of cells in apoptosis at 4 days was significantly higher than in 30 day thyroids [Table 3].

Real Time PCR was used to measure OH⁸ guanosine glycosylase in the thyroids. OH⁸ guanosine glycosylase is one of the repair mechanisms used to repair the 8-hydroxy-2'-deoxyguanosine adduct (7,21,27,40,41). This enzyme is induced when mammalian cells are subjected to oxidative stress. In an earlier study, the inducibility of the enzyme was measured by comparing the activity changes within the kidney (27). Activity
changes can be graphically measured by plotting the change in
fluorescence by the cycle threshold. Cycle threshold is defined as
the amount of time it takes to generate detectable copies of mRNA.
The more active the enzyme, the sooner cycle threshold is
reached. In this study, the levels of OH8 guanosine glycosylase
mRNA were measured in the kidneys from rats treated with KBrO3
as a positive control and the untreated kidneys as a negative
control [Figure 4]. The positive kidney control was compared with
control and treated thyroids. A two fold difference was observed in
the positive control kidneys versus the control and treated thyroids.
No significant differences were measured between thyroids from
animals treated with 100 and 400mg/l KBrO3 and those from
control animals.
The diagram above demonstrates the regulatory effect (negative feedback loop) of the TRH, TSH, T3, & T4 hormones.
Figure 2  30 Day Follicular Cells (Control and Treated)
Photomicrograph of rat thyroid treated with 400mg/L KBrO₃ for days. Follicular hypertrophy is evident in the treatment group when compared to control tissue.

30 Day Control (462X)

30 Day 400mg/L KBrO₃ (426X)
Table 1. Dosing Regimens

<table>
<thead>
<tr>
<th>Target KBrO3 concentrations (mg/L)</th>
<th>Control</th>
<th>100mg/L</th>
<th>400mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured KBrO3 concentration (mg/L)</td>
<td>DH2O</td>
<td>100 ± 10</td>
<td>423 ± 30a</td>
</tr>
<tr>
<td>4 Day Exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water consumption (ml/kg/day)</td>
<td>159.68 ± 35.61</td>
<td>137.51 ± 49.96</td>
<td>101 ± 8.00b</td>
</tr>
<tr>
<td>mean daily dose (mg/kg/day)</td>
<td>0</td>
<td>14</td>
<td>43b</td>
</tr>
<tr>
<td>30 Day Exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water consumption (ml/kg/day)</td>
<td>109 ± 51.90</td>
<td>132.84 ± 55.85</td>
<td>145.53 ± 32.00b</td>
</tr>
<tr>
<td>mean daily dose (mg/kg/day)</td>
<td>0</td>
<td>13</td>
<td>62b</td>
</tr>
</tbody>
</table>

N=4

a. Mean ± SD
b. Significantly different (p ≤0.05) from the control group by the two tailed Student’s t-test for unmatched pairs assuming unequal variances.
### Table 2. Body and Organ Weights

<table>
<thead>
<tr>
<th>Target KBrO3 concentrations (mg/L)</th>
<th>DH2O</th>
<th>100mg/L</th>
<th>400mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Day Exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight (g)</td>
<td>77.04 ± 3.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.14 ± 4.57</td>
<td>78.24 ± 4.69</td>
</tr>
<tr>
<td>liver weight (% body weight)</td>
<td>5.06 ± 0.27</td>
<td>5.09 ± 0.36</td>
<td>5.21 ± 0.38</td>
</tr>
<tr>
<td>kidney weight (% body weight)</td>
<td>1.19 ± 0.05</td>
<td>1.31 ± 0.16</td>
<td>1.42 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>thyroid (% body weight)</td>
<td>0.02 ± .004</td>
<td>0.02 ± .003</td>
<td>0.02 ± .006</td>
</tr>
<tr>
<td>30 Day Exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight (g)</td>
<td>165 ± 15</td>
<td>161 ± 9.17</td>
<td>170 ± 6.27</td>
</tr>
<tr>
<td>liver weight (% body weight)</td>
<td>5.74 ± 0.40</td>
<td>5.39 ± 0.21</td>
<td>5.22 ± 0.45</td>
</tr>
<tr>
<td>kidney weight (% body weight)</td>
<td>1.23 ± 0.09</td>
<td>1.35 ± 0.11</td>
<td>1.31 ± 0.08</td>
</tr>
<tr>
<td>thyroid (% body weight)</td>
<td>0.02 ± .002</td>
<td>0.02 ± .005</td>
<td>0.02 ± .003</td>
</tr>
</tbody>
</table>

N=8

<sup>a</sup> Mean ± SD

<sup>b</sup> Significantly different (p ≤0.05) from the control group by the two tailed Student’s t-test for unmatched pairs assuming unequal variances.
Table 3. Apoptosis and Proliferation in the Thyroids of F344 Male Rats Receiving Drinking Water Containing KBrO$_3$ or Distilled Water (Controls)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of Treatment</th>
<th>%Apoptosis</th>
<th>%Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>4</td>
<td>1.06 ± 0.39$^b$</td>
<td>13.46 ±3.08$^a$</td>
</tr>
<tr>
<td>100mg/l KBrO$_3$</td>
<td>4</td>
<td>0.68 ± 0.19</td>
<td>22.88 ± 8.30</td>
</tr>
<tr>
<td>400mg/l KBrO$_3$</td>
<td>4</td>
<td>1.32 ± 0.30</td>
<td>28.65 ± 26.51</td>
</tr>
<tr>
<td>H2O</td>
<td>30</td>
<td>0.46 ± 0.08</td>
<td>20.41 ± 7.45</td>
</tr>
<tr>
<td>100mg/l KBrO$_3$</td>
<td>30</td>
<td>0.43 ± 0.09</td>
<td>25.11 ± 7.45</td>
</tr>
<tr>
<td>400mg/l KBrO$_3$</td>
<td>30</td>
<td>1.69 ± 0.15$^c$</td>
<td>34.74 ± 8.16</td>
</tr>
</tbody>
</table>

N=5

$^a$ Number of PCNA labeled thyroid follicular cells/total number of cells counted.

$^b$ Mean ± SD.

$^c$ Significantly different (p≤0.05) from the 30 day control group by the two-tailed Student's t-test for unmatched pairs assuming unequal variances.
Figure 3.  T3 Concentration (ng/dL) in male F344 rats treated with KBrO₃ for 4 and 30 days

N=8
The T3 concentration in the 400mg/L treatment group increased at 30 Days when compared to the control value (31.5 vs 17.6 ng/dL) for KBrO₃ and control respectively. (p<0.05)
Figure 4. T4 Concentration (μg/dL) in male F344 rats treated with KBrO₃ for 4 and 30 days

![Graph showing T4 concentration (μg/dL) for control, 100mg/L, and 400mg/L conditions.]

N=8
The 30 Day T4 concentration levels showed a significant increase when compared to the control value (5.47 vs 3.51 μg/dL) for 400mg/L KBrO₃ and control respectively; (p<0.05)
Figure 5. TSH Concentration (µIU/mL) in male F344 rats treated with KBrO₃ for 4 and 30 days

N=5
The TSH concentrations were not altered for either KBrO₃ treatment or length of exposure.
Figure 6. OGG1 expression in the F344 rat (kidneys and thyroids)

<table>
<thead>
<tr>
<th></th>
<th>Control (DH2O)</th>
<th>400mg/L KBrO3</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>26.14</td>
<td>25.30</td>
<td>1.84</td>
</tr>
<tr>
<td>Thyroid</td>
<td>27.36</td>
<td>27.13</td>
<td>.23</td>
</tr>
</tbody>
</table>
DISCUSSION

In some systems, the induction of thyroid tumors has been shown to be dependent upon the disruption of the thyroid-pituitary axis which results in hypertrophy and hyperplasia of follicular cells (13,14,15,16). Hypertrophy and hyperplasia are considered to be precursors of adenomas and neoplasms (14). The data from this study demonstrated that KBrO₃ treatment induced hypertrophic changes within the thyroid follicles which appear to be precursors to hyperplasia (14). These effects are characteristic of physiologically stimulated thyroids (15,16) and appear to be dose related. This study also showed increased cell proliferation (although not significant). Decreased apoptosis in the control versus treated 30 day reflect a physiological disruption induced by KBrO₃ (4,18,23), possibly failure to suppress physiologically relevant apoptosis in a time dependent manner. In previous studies, elevation of TSH stimulated the thyroid to exhaust its storage of thyroid hormone. The follicular cells, unable to meet the demand for more hormone, began to divide and hypertrophy. As previously stated, continuous stimulation of the thyroid can lead to hyperplastic nodules and neoplasms (15,16). In this study, evidence of overstimulated follicular cells is shown. Large pale follicular cells lacking colloid were seen in both treatment groups with severity in a dose related manner. Also, hypertrophy and pre-
hyperplastic changes were seen in the high dose group.

Potassium bromate exists as a strong oxidizer which has induced oxidative damage in previous studies; The role of KBrO₃ in the rat kidney has been studied thoroughly (22,25,26,34,35). KBrO₃ induced oxidative damage to the renal proximal tubules. Increased levels of lipid peroxidation are thought to precede formation of oxygen radicals which may cause DNA damage (10,19,43,44,45).

In this study, evidence is lacking for the role of oxidative damage as a mechanism for potassium bromate induced thyroid carcinogenesis. This may indicate that potassium bromate acts through non genotoxic mechanism(s) in the thyroids or other measurements of oxidative stress may be more appropriate to this study. Mechanisms other than oxidative damage may play a role in the early phase of KBrO₃ - induced thyroid cancer.

The mode of action may be indicative of the mechanism of thyroid carcinogenesis. Previous data show that the mode of action for thyroid follicular cell carcinogenesis includes mutagenicity and disturbances in the thyroid-pituitary axis (14,15,16). Mutations result from internal or external exposure to ionizing radiation (30,31,43). Radiation exposure occurrences such as therapeutic purposes, and nuclear reactor meltdowns show specific thyroid cancer cases within humans. The alternative pathway of thyroid
cancer involves thyroid-pituitary disturbances. Several pesticides have been shown to affect thyroid hormonal balance (16). Some potential target sites include iodide pump receptors, various hormonal receptors, and liver enzymes. Antithyroid effects may be produced through many mechanisms: (1) inhibition of active transport of inorganic iodide into follicular cells. (2) inhibition of thyroid peroxidase (3) damage to follicular cells (4) inhibition of hormone release into the blood (5) inhibition of the conversion of T4 to T3, and (6) enhancement of metabolism and excretion of thyroid hormone by the liver (2,9,12,16).

In this study, antithyroid effects induced by potassium bromate were investigated by observing cell proliferation, apoptosis, cell pathology, TSH, T3, T4, and OH8 guanosine glycosylase levels. These effects were measured to determine the mechanism(s) and site of action for potassium bromate induced thyroid carcinogenesis.

Previous studies have investigated the role of pesticides in thyroid carcinogenesis. Thyroid peroxidase inhibitors are one mechanism that has been studied in various pesticides. Only a small group of class of chemicals (aromatic amines) inhibit thyroid peroxidase (e.g. amitrole, ethylene thiourea). Secondly, inhibition of hormone release results from ionizing radiation and polychlorinated biphenyls. Very few chemicals inhibit hormone
release. Also, inhibition of iodide uptake indicates chemical toxicity. This antithyroid effect is uncommon and a small class of pesticides interfere with iodide uptake (anions) e.g. perchlorate, thiocyanate. The Inhibition of the conversion of T4 to T3 by 5' monodeiodinase activity in tissue lacks sufficient pesticide data. Very few chemicals are thought to exert this antithyroid effect. Finally, the bulk of chemicals tested for site of action indicate the liver, outside of the thyroid gland, as a major role player in hormonal imbalance. An increase of uridine diphosphateglucuronosyltransferase activity increases in the liver, indicating metabolism and excretion of thyroid hormone (T4) (14,15, 16).

Bromate may have several potential target sites. Inhibition of the active transport of inorganic iodide into follicular cells may be one mechanism which produces carcinogenic effects. When thyroid hormone levels are low, the hypothalamus releases thyrotropin- releasing- hormone (TRH) to stimulate the pituitary. In response, the pituitary releases TSH. The TSH signals the follicle cells of the thyroid to synthesize hormone (Figure 1) (13,14). The thyroid actively transports inorganic iodide from the capillaries into the lumen. The inorganic iodide is converted from inorganic to organic form and binds with the tyrosyl residues to form the T3 and T4.

Normally iodide is pumped from the plasma to the thyroid.
The iodide accumulates within the follicular lumen before oxidation occurs for hormonal synthesis. Stimulation by TSH results in a 10-20 fold increase of iodide. The iodide pump has several characteristics which may result in disruption to the synthesis of hormones (8). Inhibition of the active transport of iodide may stop production of all hormones. The iodide is driven across an electrochemical concentration gradient which requires energy. Chemicals which interfere with pump action suppress iodide from entering the follicle cell. This prevents the formation of active T3 and T4 hormones. The decrease in T3 and T4, stimulates the TSH. The continued elevation of TSH causes hypertrophy and hyperplasia in cells which cannot meet the demand for more hormones (8,13,14,16).

Disruption of the iodide pump at any of the sites may result in prevention of hormone release. Concentration is very low in the thyroid due to how quickly the iodide is bound to its organic form. A deficiency in iodide may prevent hormone synthesis. According to previous studies, thyroid follicular neoplasia are due to iodine deficiency or excess (40). Further investigations into each of these processes are needed to determine the specific mechanism(s) of KBrO₃-induced thyroid carcinogenesis.

In conclusion, the follicular cell proliferation, histopathology, apoptosis, and thyroid hormonal data show evidence of a non-
genotoxic mechanism(s) of potassium bromate-induced thyroid carcinogenesis in F344 rats. This study supports previous data which indicate thyroid-pituitary disruption as a mechanism of induced thyroid cancer (12,30,31,32,33). The question remains, "How does the collective data relate to human populations?" The relevance to human populations is implied when operating under the current EPA science policy (13,14,16) which states:

1) Any chemically induced rodent thyroid tumors are assumed to be relevant to humans.
2) When interspecies data is not available, assume comparable carcinogenic sensitivity in rodents and humans.
3) Rodent noncancer adverse effects due to chemical induced thyroid-pituitary disruption are assumed to be relevant to humans.
4) Linear dose-response considerations are applied to thyroid cancer induced by chemical substances that either do not disrupt thyroid functioning or lack mode of action information.
5) Non-linear thyroid cancer dose-response considerations are applied to chemicals that reduce thyroid hormone levels, increase TSH and cell division, and are judged to lack mutagenic activity.
6) Non-linear considerations may be applied in thyroid cancer dose-response assessments on a case by case basis for chemicals that disrupt thyroid-pituitary functioning and demonstrate some mutagenic activity. thyroid disruption are assumed to be relevant to humans (14).
CONCLUSION

These data show that follicular cell histopathology, proliferation, apoptosis, and mRNA OH\textsuperscript{8} guanosine glycosylase levels may indicate a non-gentoxic mechanism. Although great strides have been made in determining the mechanism of thyroid follicular cell carcinogenesis, the true mechanism(s) remains unknown for rodents and humans. Currently, EPA science policy guidelines direct researchers to treat chemicals which cause cancers in rodents as potentially hazardous to humans (16). Chemical sensitivity in rodents should not be automatically considered the same for human populations; extrapolation to human populations should be carefully evaluated. Previous data show that humans are less sensitive to chemically induced thyroid-pituitary disruptions in comparison with rodents (13,14,15,16). Humans possess a specific protein carrier which allows hormones remain in the body longer than rats. Rats lack the protein carrier. As a result, T3 and T4 are quickly removed from the body. Currently, ionizing radiation and radioactive fallout are the only known thyroid carcinogens in humans (39). Thyroid follicular cell tumors are usually associated with iodine -deficient areas in humans. Also, research into the roles of epidermal growth factors, insulin-like growth factors, and tyrosine protein kinase interactions in rodents and humans are needed to understand the mechanism
of carcinogenesis (13,38). Currently, little data on the explanation of how these factors interact with the cell cycle to stimulate or inhibit cell division in the thyroid is available (13). In the present study, the results support previous data indicating that potassium bromate induced thyroid carcinogenesis may occur through non-genotoxic mechanism(s) (14,15,16.17,33,34). More investigations into the mechanisms of induced thyroid carcinogenesis in rodents and humans are needed to fill in research gaps which lack other relevant data (13).
REFERENCES


APPENDIX A
HEMATOXYLIN & EOSIN STAINING

1. Place the slides in peroxidase for 10 minutes. Rinse with Phosphate Buffered Saline (PBS) for 2 minutes. Repeat 3X.

2. Place 2 drops of 0.125% trypsin onto the slide. Let incubate in humid atmosphere at 37°C for 10 minutes. Rinse in distilled water for 2 minutes. Repeat 3X. (Essential for Formalin-fixed tissue to dissolve protein cross links)

3. Place 2 drops of 20μg/ml proteinase K onto each section. Incubate 30 minutes at room temperature. Rinse with PBS for 2 minutes. Repeat 3X.

4. Add 100μl of 2% hydrogen peroxide to each section. Incubate 10 minutes at room temperature. Drain or blot off the solution (No rinsing) [Inactivates endogenous peroxidase].

5. Apply 100μl streptavidin-peroxidase to each section. Incubate at room temperature for 10 minutes. Rinse with PBS for 2 minutes. Repeat 3X.

6. Apply 2 or more drops of Diaminobenzidine (DAB) to each section. Incubate for 5 minutes. Rinse well with distilled water. [Stains the nuclei dark-orange color]

7. Counterstain the slides with 100μl of Hematoxylin. Wash the slides in tap water. Place the slides into PBS, until the
slides turn blue. (Approximately 30 seconds). Rinse in distilled water.

8. Dehydrate the slides in a graded series of alcohol and clear water. Add 2 drops of histomount and coverslip.
APPENDIX B

ISOLATION OF RNA FROM SMALL QUANTITIES OF TISSUE
(1-10mg)

HOMOGENIZATION

1. Weigh the tissue samples quickly and pulverize the samples in liquid nitrogen using the sterile, ice-cold ceramic bowls with mortar pestles. Place the bowls over dry ice to prevent RNA degradation.

2. Add 800µl Trizol reagent (Life Technologies Cat.#15596026) containing 200µl of glycogen [final concentration:250µg/ml] followed by vigorous vortexing.

3. At room temperature, cap the vials and vortex at high speed for 10 seconds.

4. Shear the genomic DNA by passing the sample twice through 26-gauge needle connected to 1ml syringe. Using the sterile syringe, transfer the sample to a sterile 1.5ml microcentrifuge tube.

PHASE SEPARATION

5. Add 160µl chloroform to each sample and vortex up to 30 seconds.

6. Spin at maximum speed in the microcentrifuge 5 minutes to separate the phases.

HARVEST RNA
7. Transfer the upper aqueous phase to a fresh tube and add 400µl ice-cold isopropanol. (Keep on ice to avoid degradation)

RNA PRECIPITATION

8. Allow the samples to precipitate at -70°C overnight.

9. Pellet the RNA by centrifugation at maximum speed in the microcentrifuge 15 minutes at room temperature.

10. Decant the supernatant.

RNA WASH

11. Wash the pellet in 200µl of 70% ethanol and spin again for 10 minutes at maximum speed.

12. Decant the supernatant, removing as much as possible without disturbing the pellet.

DRYING THE RNA (removal of ethanol)

13. Air dry the samples for 3-5 minutes.

REDISSOLVING THE RNA

14. Resolubilize the pellet in 30-50µl RNase free deionized water or DEPC-treated water.

15. Vortex the samples up and down to ascertain that the pellet is resolubilized fully.

16. Store at -70°C.
APPENDIX C

THYROID STIMULATING HORMONE RADIOIMMUNOASSAY

Diagnostic Products Corporation, Los Angeles, CA (Cat.# IKTS1)

1. Label sixteen TSH ab-coated tubes A (nonspecific binding) and B through H (maximum binding) in duplicate. Label additional tubes in duplicate for controls and samples.

<table>
<thead>
<tr>
<th>Calibrators</th>
<th>μIU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(Nonspecific)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0.15</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
</tr>
<tr>
<td>D</td>
<td>1.5</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>15</td>
</tr>
<tr>
<td>G</td>
<td>30</td>
</tr>
<tr>
<td>H(Maxium Binding)</td>
<td>60</td>
</tr>
</tbody>
</table>

2. Pipet 200μl of each calibrator, control, and samples serum sample into the tubes.

3. Add 100μl of 125I TSH Ab to all tubes.

4. Shake at room temperature on a rack shaker for 2 hours (set shaker for 200 strokes per minute).

5. Decant thoroughly, add 2 ml buffered wash solution (saline)
to all tubes. Wait 1 to 2 minutes, decant the wash. (Repeat)

6. Remove the wash and remove all visible moisture from the tubes. Turn the tubes upside down and allow to drain for 2-3 minutes.

7. Count for 1 minute in a gamma counter
APPENDIX D
TOTAL T3 RADIOIMMUNOASSAY
Diagnostic Products Corporation, Los Angeles, CA (Cat.#TKT31)
1. Label four plain 12 X 75mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate. Label twelve Total T3 Ab-coated tubes A (maximum binding) and B through F in duplicate. Label additional Ab-coated tubes for the controls and the samples.

<table>
<thead>
<tr>
<th>Calibrators</th>
<th>ng/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (MB)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
</tr>
<tr>
<td>E</td>
<td>200</td>
</tr>
<tr>
<td>F</td>
<td>600</td>
</tr>
</tbody>
</table>

2. Pipet 100μl of the zero calibrator A into the NSB and A tubes, and 100μl of each remaining calibrator, control, and sample into the appropriate tubes.

3. Add 1 ml of \(^{125}\text{I}\) Total T3 to all tubes. Vortex gently.

4. Incubate the tubes for 2 hours at 37°C in the waterbath.

5. Decant thoroughly, remove all visible moisture by draining the tubes on absorbant towels.

6. Count for 1 minute in a gamma counter.
APPENDIX E
TOTAL T4 RADIOIMMUNOASSAY
Diagnostic Products Corporation, Los Angeles, CA (Cat.#TKT41)

1. Label four plain 12 X 75mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate. Label twelve Total T4 Ab-coated tubes A (maximum binding) and B through F in duplicate. Label additional Ab-coated tubes in duplicate for controls and samples.

<table>
<thead>
<tr>
<th>Calibrators</th>
<th>µg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (MB)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>16</td>
</tr>
<tr>
<td>F</td>
<td>24</td>
</tr>
</tbody>
</table>

2. Pipet 25µl of the zero calibrator A into the NSB and A tubes, and 25µl of each calibrator, control, and sample into the appropriate tubes.

3. Add 1.0ml of $^{125}$I Total T4 to all tubes. Vortex.

4. Incubate for 1 hour at 37°C in the waterbath.

5. Decant thoroughly, remove all visible moisture by draining the tubes on an absorbant towel. Allow the tubes to dry for
2-3 minutes.

6. Count for 1 minute in a gamma counter.
APPENDIX F
IDENTIFICATION OF PCNA IN ARCHIVAL RAT AND MOUSE TISSUE (FIXED IN 10% NEUTRAL BUFFERED FORMALIN FOR GREATER THAN 24 HOURS)

1. Retrieve 5μm Paraffin embedded samples mounted on poly-l-lysine coated slides.

2. Remove paraffin by placing the slides into xylene. Place in xylene bath for 5 minutes. Repeat 2X. (Organic solution dissolves fatty substances (paraffin)

3. Hydrate through a grades series of ethyl alcholol : 96% ethanol bath for 3 minutes (2X), 90% ethanol bath 1minute, 80% ethanol bath for 1 minute, and double-distilled water for 1 minute. (Formalin-fixed tissue require trypsin digestion)

4. Quench endogenous peroxidase by placing slides In 3% hydrogen peroxide for 15 minutes.

5. Rinse and wash slides in distilled water for 5 minutes.

6. Place slides in plastic coplin jars filled with deionized water.

7. Microwave on high power (700 watts) for 2 minutes. Check solution levels again after 1 minute and repeat high power microwave for 2 minutes. Remove and fill to volume with deionized water.

8. Allow slides to cool for 15 minutes at room temperature in
coplin jars.

9. Discard deionized water and rinse 2 times in distilled water.

10. Rinse in 1X Automatic Buffer for 5 minutes.

11. Apply monoclonal antibody anti-PCNA (Calbiochem, Los Angeles, CA Cat.# NAO3). Incubate 30 minutes at room temperature.

12. Rinse in 2 changes of 1X Automatic buffer.

13. Apply the secondary antibody, Goat anti-mouse IgM Mu chain-specific biotin conjugated (Jackson Immunoresearch Laboratories, Inc., West Grove, PA Cat.#115-065-020)

14. Rinse in 2 changes of 1X Automatic buffer.

15. Apply the label antibody, Streptavidin peroxidase Super sensitive predilute antibody. Incubate at 30 minutes at room temperature. (Biogenex laboratories, San Ramon, CA cAT.#hk330-5k)

16. Rinse slides in 2 changes of 1X Automatic buffer for 5 minutes each.

17. Apply Diaminobenzidine (DAB) chromagen for 6 minutes in dark. (Add 1 drop of DAB per ml of substrate)

18. Rinse in tap water for 3 minutes.

19. Counterstain with modified Harris Hematoxylin for 30 seconds.

20. Rinse in tap water until water is clear.
21. Place slides in 1X Automatic buffer for 1 minute with gentle agitation to blue slides.

22. Dehydrate the slides: 95% ethanol for 3 minutes, 100% ethanol for 3 minutes (3X), xylene for 5 minutes (2X)

23. Place coverslip on for reading under the light microscope.
APPENDIX G
TUNEL PROTOCOL

1. Place 20μg/ml proteinase K for 15 minutes at room
temperature (strips nuclei of tissue sections from proteins)
2. Wash slides in double-distilled water for 2 minutes (4X)
3. Quench endogenous peroxidase by covering the sections
with 2% hydrogen peroxide for 5 minutes at room
temperature.
4. Rinse with double-distilled water and immerse in Terminal
deoxyribonucleotidyl transferase (TdT) buffer [30mM Trizma
base, pH 7.2; 140mM sodium cacodylate, 1mM cobalt
chloride]
5. TdT (0.3e.u./μl) and biotinylated dUTP in TdT buffer should
be added to the cover slips of the samples (detects nicks in
DNA).
6. Incubate at 37°C for 60 minutes. (Waterbath)
7. Terminate the reaction by transferring slides to TB buffer
[300mM sodium chloride, 30mM sodium citrate] for 15
minutes at room temperature.
8. Rinse slides with double-distilled water.
9. Cover slides with 2% aqueous solution of bovine serum
albumin for 10 minutes at room temperature.
10. Rinse in double-distilled water and immerse in phosphate buffered saline for 5 minutes.


12. Wash in double-distilled water and immerse in phosphate buffered saline for 5 minutes.

13. Stain with Aminoethylcarbazole(AEC) for 30 minutes at 37°C.
APPENDIX H

REAL-TIME REVERSE TRANSCRIPTASE PCR OF OH8 GUANOSINE GLYCOSYLASE mRNA

1. Retrieve pre-quantitated RNA samples (200ng/sample) from freezer storage (-70°C).
2. Place samples on ice and determine the concentration of RNA: [10μL buffer, 2 μl Dnase 1, 49μl RNA, 41μl Rnase free-H2O] 102μl/sample
3. Incubate for 30 minutes at 37°C.
4. Incubate for 5 minutes at 75°C [heat will kill the DNA]
5. Preform the Ribogreen assay
   490μl TE buffer to standard (1000)
   250μl TE buffer for the rest of the standards: 500,250, 125, 62.5, 31.25, 0).
6. Place 10μl of the known for 1000μl standard. Use 3μl of unknown (dilution 1:100 for unknowns). Place into 96-well plate. (Each well should contain 100μl of unknown sample and 100μl ribogreen dye (dilution for dye 1:200)
7. Prepare the Master mix for 7700 RT-PCR quantitation
   Target Final Concentration volume(μl)
   25mM MgCl2 5.5mM 11
   10X Taqman Buffer A 1X 5
dNTP (10mM) 300µM 1.5
Rnase inhibitor (20U/µl) 1
MuLV R'tase (50U/µl) 0.25
Amplitaq Gold (5U/µl) 0.25
Forward primer (9µM) 900nM 5
Reverse primer (9µM) 900nM 5
Taqman Probe (2.0µM) 200nM 5
Template 50ng 5
dH2O 11
Total of 50µl

8. Use 30µl of mastermix, 15µl of probe/primer mix, 5µl of template. Place 45µl of master mix into the 96-well plate. Place samples on top.

9. Centrifuge the 96-well plate to remove RNA from the side of the wells.

10. Cycling conditions:

   Room temperature 48°C 30 minutes (cDNA present)
   95°C 10 minutes (Taq RT activation)
   Actual PCR cycling begins: 40 cycles of : 94°C 15 seconds
   60°C 1 minute.

11. Retrieve results from 7700 Sequence Detector.