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Aqueous Iodine Equilibria in Mammalian Iodination Reactions

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Regulatory activity has been demonstrated in two classes of iodinated organic species: thyroid hormones (T_3 and T_4) and iodinated lipids (ILs), e.g. 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid. The formation of iodinated biomolecules requires iodide oxidation. In mammals iodide oxidation is one of several peroxidase-mediated reactions that serve to reduce hydrogen peroxide. I_2 is one of several reaction products formed by mammalian peroxidases during iodide oxidation. I_2 forms HOI in an aqueous environment which also has the capacity to iodinate organic species. This manuscript examines the potential relationship between the two classes of known mammalian iodinating species. A model describing iodination pathways for organic species in mammals is advanced. The model predicts the formation of ILs under normal dietary intake of iodine. The model was challenged by characterizing the lipids of hogs maintained on a diet containing normal levels of iodine. Iodinated lipids were found to be present in the fatty acids extracted from the thyroid of these hogs.

Introduction

The range of daily dietary iodine intake by humans varies by more than two orders of magnitude. Thyroidal uptake of iodide and synthesis of iodinated organic species (i.e., organification of iodine) varies with intake, yet animals and humans secrete remarkably constant levels of triiodothyronine (T_3) and thyroxine (T_4) regardless of intake. A wide range of adaptive responses to iodide has been demonstrated. Thyroid autoregulation has been extensively studied, but it has not been possible to fashion a cohesive explanation describing the wide range of clinical and experimental observations. It is clear, however, that the organification (i.e., iodination) of iodine plays an important regulatory role in the thyroid exposed to chronic excess iodide.

The reaction products of thyroperoxidase (TPO) are a function of iodide concentration. This is not surprising as iodide reacts with a key intermediate of TPO (Enz-Fe^{IV}-OI) to form I_2 (1). Taurog suggested that formation of molecular iodine (I_2) under elevated iodide concentrations could be responsible for aspects of iodide regulation (2). Taurog's suggestion is intriguing since I_2 has distinctive properties that are not generally appreciated. I_2 is a hydrophobic species that is highly polarizable. I_2 readily partitions into hydrophobic environments and nonspecifically iodinates double bonds; the partition coefficient of I_2 in a chloroform/H_2O mixture is about 100. I_2 is the only species in topical iodine-disinfectants that is able to penetrate into epidermis (3). I_2 has the chemical capacity to nonspecifically iodinate amino acids, proteins, and lipids (4).

The oxidation of iodide is a necessary step to incorporate iodine into bioactive molecules. The oxidation of iodide by reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) has been studied since the early 1920s (5). These reactions yield a complex mixture of different iodine species. One factor that contributes to this complexity is the range of oxidation states associated with iodine species: -1 to $+5$, for example, -1 (iodide: I^-); $+1$ (hypoiodic acid: HOI); $+5$ (iodate: IO_3^-) as shown in Equation 1.

$$I^- \xrightarrow{-0.54V} I_2 \xrightarrow{-1.45V} HOI \xrightarrow{-1.14V} IO_3^- \quad (1)$$

Several of the oxidized iodine species formed from iodide oxidation have the potential to react with both water and I^-. This complex pattern of reactions yields nonlinear reaction kinetics. A variety of approaches have been used to study the kinetic properties of the individual reactions underlying the aqueous reactions formed by iodide oxidation. Gottardi integrated these results into a computer model that calculates the equilibrium distribution of iodine species as a function of both pH and initial reactant (e.g., I^-, I_2) concentrations (6). Several of the reactions that occur under physiological conditions are shown in Equations 2–8.

$$2I^- - 2e \rightarrow I_2$$
formation of molecular iodine (2)

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$$HOI \leftrightarrow H^+ + OI^-$$
dissociation of hypoiodic acid (3)

$$I_2 + H_2O \leftrightarrow HOI + H^+ + I^-$$
hydrolysis of molecular iodine (4)

$$HOI + H^+ \leftrightarrow H_2OI^+$$
protonation of hypoiodic acid (5)

$$H_2OI^+ \leftrightarrow HOI + H^+$$
hydrolysis of iodine cation (6)

$$OI^- + I^- + H_2O \leftrightarrow HI_2O^- + OH^-$$
iodination of hypoiodite (7)

$$I_2 + I^- \leftrightarrow I_3^-$$
triiodide formation (8)

The iodination of organic molecules requires iodide oxidation. TPO-catalyzed oxidation of iodide leads to I_2 formation at iodide concentrations found in the thyroid ($\geq$10 μmol). In addition to biological molecules, I_2 reacts with water and iodide in an aqueous environment. The equilibria described in these reactions would be affected by the interaction of some iodine species (e.g., I_2), with mammalian proteins and lipids as well as the addition of iodide from the action of deiodinases and dehalogenases. Nevertheless, the interactions identified in Equations 2–8 are operative in biological matrices such as the thyroid. I_2 formed within a biological environment is subject to well-defined thermodynamic constraints. The potential impact of the properties of aqueous iodine equilibria on iodine physiology has not been explored. The iodine species that exist at a pH 7.4 are iodide (I^-), triiodide (I_3^-), molecular iodine (I_2), hypoiodious acid (HOI), hypoiodite ion (OI^-), and the iodine anion (HI_2O^-).

Under normal physiological conditions, there are two species that can serve as iodinating agents: HOI and I_2. Both HOI and I_2 iodinate organic species, but they do not exhibit an identical selectivity in this regard. Dunford and Ralston demonstrated that HOI, not I_2, is the species that iodinates tyrosine in aqueous environments that contain both I_2 and HOI (7). The disparity in the capacity of HOI and I_2 to iodinate tyrosine may be due to their distinct physiochemical properties.

Experiments and computer models demonstrate that iodide has a major influence on the ratio of I_2/HOI at pH 7.4 (6). The equations that govern aqueous iodine equilibria exhibit a nonlinear response to iodide with respect to the ratio of I_2/HOI; increased iodide decreases this ratio. A 10^{-3} molar solution of I_2 dissolved in water yields a concentration of HOI that is $10^{-3.9}$ molar. If 10^{-3} or 10^{-2} molar iodide is added to this solution, the concentration of HOI will decrease to $10^{-4.8}$ and $10^{-6.5}$ molar, respectively (8). In comparison, the concentration of I_2 only decreases to $10^{-3.2}$ and $10^{-3.9}$ molar, respectively.

The consequence of introducing increased I^- into a solution of oxidized I^- species is to shift the iodinating capacity away from a water-soluble species (HOI) toward a hydrophobic species (I_2). The concentration and ratio of the unbound iodinating species (HOI/I_2) is therefore a function of I^- concentration.

Iodination—A Model

In mammals, a combination of chemical and biochemical (extra-enzymatic) processes appears to be responsible for the synthesis of iodinated organic species (e.g., lipids, amino acids, proteins). In particular, the chemical equilibria and reactions of I_2 in an aqueous environment may play a role in determining which species are iodinated. The model proposed in this manuscript is outlined in Figure 1.

The first step in the mammalian oxidation of iodide is a two-electron transfer from the TPO to hydrogen peroxide forming water and the intermediate Enz-Fe^V-OH (Compound-I). The Enz-Fe^V-OH intermediate reacts with iodide to form the Enz-Fe^{IV}-OI intermediate.

The first iodination pathway is shown vertically on the left hand side of Figure 1. Three different potential substrates for the Enz-Fe^{IV}-OI intermediate are shown: thyroglobulin (Tg), tyrosine, and mono-iodotyrosine (MIT). The Enz-Fe^{IV}-OI intermediate is thought to directly iodinate the tyrosyl groups on Tg although the precise mechanism is not settled. Tyrosine and MIT also react with Enz-Fe^{IV}-OI to generate free radical intermediates that catalyze coupling of adjacent iodotyrosyl groups on Tg to form Tg-bound thyroxine.

The Enz-Fe^{IV}-OI intermediate exhibits limited specificity and reacts with a variety of different substrates including aromatic phenols, amines, and iodide. The reaction of Enz-Fe^{IV}-OI with iodide leads to the formation of I_2. A second iodination pathway is triggered when I_2 is generated. Some of the I_2 formed is hydrolyzed to HOI; unbound HOI can iodinate tyrosyl groups and contribute to the formation of T_3 and T_4. However, some of the I_2 reacts with lipids and proteins to yield iodinated organic species.

Iodinated lipids are believed to play an important role in thyroid autoregulation (9). Four different iodinated lipids have been identified in mammalian thyroid cells: 6-iodo-5-hydroxy-8,11,14-eicosatrienoic acid (δ-iodolactone), α-iodohexadecanal (α-IHDA), 5-iodo-7,10,13,16,19-docosapenta-enoic acid (γ-lactone), and 14-iodo-l5-hydroxy-5,8,11-eicosatrienoic acid (ω-lactone). Two of these four identified iodinated lipids are known to express biological activity. This suggests a non-specific iodination process that is consistent with the proposed model. However, this model also predicts that I_2 should be

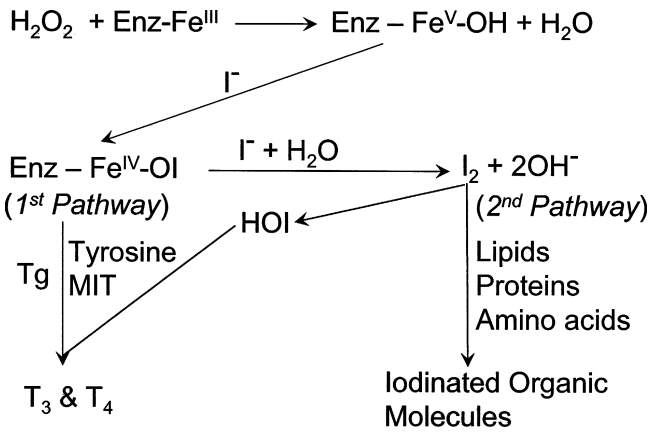

FIG. 1. Model for mammalian iodination. Enz-Fe^{IV}-OI = thyroperoxidase intermediate; I^- = iodide; I_2 = molecular iodine; HOI = hypoiodious acid; T_3 & T_4 = thyroid hormones; Tg = thyroglobulin; MIT = mono-iodotyrosine.

formed under normal physiologic conditions since iodide would be expected to compete for the Enz-Fe^{IV}-OI intermediate with other potential substrates.

This study was conducted to determine whether the thyroid lipids of hogs maintained on an iodine-sufficient diet were iodinated. The term "iodine" in this manuscript refers to all species of iodine, including organified iodine.

Materials and Methods

Hogs in this study were treated in accord with accepted standards of humane animal care. The diet of hogs was supplemented with iodine according to established standards (10). Mineral premix was added to feed to yield an iodine concentration of 0.15 ppm throughout the life cycle of the animals. A survey of nine commercial plants conducted in 1992 observed an iodine concentration in feed that ranged from 0.15 to 2.20 ppm. The bottom of this range was selected because it is more comparable to the recommended intake in humans. The average weight of a slaughtered hog is about 110 kg. Hogs consume about 166 kg of feed over $\approx$ 170 days of growth. Daily feed consumption depends upon body weight. Scaling feed intake linearly with age yields feed consumption of about 1.95 kg at slaughter. An iodine concentration of 0.15 ppm yields about 300 μg of iodine at slaughter for a 110-kg hog.

Hogs were slaughtered at 6 months $\pm$ 2 weeks. Hog thyroids from 15 slaughtered animals were frozen and then minced to 3/8 inch in a refrigerated room. The minced samples were spread uniformly on a stainless steel pan and placed on heat shelf vacuum dryer (71°C) and dried. The dried samples were defatted with heptane, to residual less than 5% fat. Defatted product was run through magnetic separators, positioned in a Fitz Mill and milled to a coarse powder. The samples were ground to a fine powder with a ball mill (porcelain balls) and then blended in a V-mixer. The extracted lipids were stored in a sealed glass bottle at -8°C.

Lipid extraction and washing of the extract were conducted according to the method of Folch *et al*. (11) in the laboratory of Dr. Tilak Dhiman at Utah State University. Extracted fat was derivatized to methyl esters using an alkaline methylation procedure by mixing 40 mg of fat with a sodium methoxide methylation reagent ($NaOCH_3$/MeOH) as previously described (12). After fatty acid (FA) methyl esters (FAMEs) were formed, anhydrous calcium chloride pellets were added and allowed to stand for 1 hour to remove water in the sample. Samples were then centrifuged at 2600 *g* at 5°C for 5 minutes.

Separation of FA was achieved by gas chromatography (model 6800 Series II; Hewlett Packard, Avandale, PA) fitted with a flame ionization detector. Samples containing methyl esters in hexane (1 μL) were injected through the split injection port (100:1) onto CP-Sil 88 fused silica 100 m $\times$ 0.25 mm column, 0.20 μm film (Varian CP-Sil 88 model; Varian, Palo Alto, CA). Oven temperature was set at 80°C and held for 10 minutes, then ramped to 190°C at 12°C/min for 39 minutes. The temperature was then ramped to 218°C at 20°C/min and held for 21 minutes. Injector and detector were set at 250°C. Total run time was 70.57 minutes.

Heptadecadenoic acid was used as qualitative internal standard. Each peak was identified using FA and FAMEs (Nu-Chek Prep, Elysian, MN; Matreya, Pleasant Gap, PA; and Supelco™ 37 Component FAME mix, Supelco, Bellefonte, PA). Percentage of each FA was calculated by dividing the area under the FA peak (minus the area under the peak for heptadecanoic acid) by the sum of the areas under the total reported FA peaks. FAs were reported as g/100 g of FAMEs.

The FA iodine content of FA was determined using inductively coupled plasma (ICP) analysis. Two grams of FA was heated in 20 mL of distilled water (dH_2O) at 95°C. After 30 minutes, 35 mL of a 3% ammonium solution was added and the ICP/MS (m/z 127) analysis was conducted immediately. Serum iodine was also measured with ICP after filtration through a 0.45 μm mixed cellulose ester syringe filter.

Results

Table 1 lists the FA composition of the thyroid tissue. C18 FAs constituted more than 75% (w/w) of the FA present with C18:1 12-t comprising more than 64% of the sample; C18:1 13, 14-t, 6,7,8-c constituted 8.85%, and C18:0 (stearic acid) was 3.72%. The only unsaturated FAs at a concentration above 1% were myristic acid (C14:0; 1.64%) and palmitic acid (C16:0; 18.08%). Overall saturated FAs comprised 25.28% and unsaturated FAs, 74.72%. The assay for iodine yielded a value of 170 mg of iodine per kilogram of hog thyroid lipid. The "average" molecular weight of the FAs in Table 1 is 277.1; the ratio noniodinated to iodinated lipids can be estimated to be 250–275 assuming mono-iodination. The concentration of serum iodine was 38.4 ± 7.1 μg/L.

Samples were analyzed for an additional 28 FAs none of which were detected. This list includes butyric acid, caproic acid, caprylic acid, undecanoic acid, tridecanoic acid, myristoleic acid, oleic acid, vaccenic acid, linoleic acid, arachidic acid, γ linolenic acid, cis-9,t-11-octadecadienoic acid (CLA 1), t-9-t-11-octadecadienoic acid (CLA 2), eicosadienoic acid, behenic acid, homogammalinolenic acid, arachidonic acid, lignoceric acid, cicosapentanoic acid (EPA), heptadecanoic acid, docosapentanenoic acid (DPA), and docosahexaenoic acid (DHA).

Table 1. Fatty Acid Composition of Hog Thyroid

Fatty Acid	*Fatty Acid Name*	*% Composition*
C10:0	Capric acid	0.087
C12:0	Lauric acid	0.997
C14:0	Myristic acid	1.639
C15:0	Pentadecanoic acid	0.643
C16:0	Palmitic acid	18.084
C16:1	Palmitoleic acid	0.514
C17:0	Margaric acid	0.113
C18:0	Stearic acid	3.719
C18:1 6/7/8-t		0.172
C18:1 9-t	Elaidic acid	0.109
C18:1 10-t		0.131
C18:1 12-t		64.132
C18:1 13,14-t, 6,7,8-c		8.849
C18:1 9-c	Oleic acid	0.472
C18:2 9-c, 12-c	Vaccenic acid	0.278
C18:3 9-12-15-c	alpha Linolenic acid	0.060
Total C18:3		0.060
Saturated		25.283
Unsaturated		74.717

Discussion

The thyroid is known to iodinate organic species other than tyrosyl groups in response to elevated dietary iodine intake. The underlying mechanism responsible for the iodination of nontyrosyl groups is an open question, but peroxidases are the only known enzymes with an established capacity to oxidize iodide. Several iodinated lipids have been identified in the mammalian thyroid challenged with elevated iodide levels. Two of these iodinated lipids express an antiproliferative activity and apparently play a role in thyroid autoregulation. Dugrillon (13) speculated about the potential role iodinated lipids play in goitrogenesis and tumorigenesis under normal physiological conditions; however, a mechanism responsible for iodination of organic species, such as lipids, under normal iodine intake has not been advanced.

We examined the FAs of hogs maintained on a diet that contained normal levels of iodine. The results indicate that hog thyroid does iodinate species other than tyrosyl groups during intake of iodine that is considered normal. Iodine comprised 0.017% (w/w) of the FAs of hog thyroid. When viewed from a clinical perspective, this result is surprising since we link dietary iodine intake with formation of T3 and T4. However, oxidized iodide species do not provide a high degree of synthetic specificity, and a range of iodinated organic species is to be expected.

The mechanism of TPO oxidation of iodide has been exhaustively examined, yet basic questions remain open; for example, is tyrosyl iodination of Tg effectuated by an enzyme-bound intermediate or by freely diffusing iodination equivalents? It is clear that TPO can catalyze the formation of I_2 by the interaction of iodide with the Enz-Fe^{IV}-OI intermediate. Iodide competes for the Enz-Fe^{IV}-OI intermediate with other potential substrates like tyrosyl groups. In this model, the formation of I_2 under normal physiological condition would be expected. The amount of I_2 formed is a function of iodide concentration.

The chemical properties of I_2 may limit the role that an enzyme could play in the synthesis of iodine organification. Peroxidases, the only mammalian enzyme known to oxidize iodide, can form a complex with hypoiodite HOI. No enzyme has been identified that forms a stable complex with I_2. I_2 catalyzes a number of reactions of potential relevance to proteins; specifically, it

(i) reacts with amino groups of lysine, histidine, and arginine, as well as the bases of nucleotides;
(ii) oxidizes the sulphydral group of cysteine;
(iii) iodinates tyrosine to form mono- and di-iodotyrosyl groups; and
(iv) adds across double bonds.

The physical and chemical properties of I_2 may be inconsistent with an active-site-directed iodination mechanism based on I_2.

Early life forms used iodide to neutralize ROS (14). Berking *et al*. has shown that iodide and tyrosine are required to protect *Aurelia aurita* (jellyfish) from ROS (15). ROS are neutralized when they oxidize iodide and yield I_2 and HOI. Tyrosine is needed to neutralize the reactive iodinating equivalents (HOI and I_2) formed from the oxidation of iodide. In the course of evolution, the mammalian thyroid has developed more complex strategies to neutralize iodination equivalents not directed to the synthesis of T_3 and T_4. One of these mechanisms appears to be the iodination of lipids. Elevated iodide concentrations in the thyroid favor the formation of I_2 and the chemical logic of using iodinated lipids to modulate thyroid activity follows thereto.

The model proposed in Figure 1 is potentially applicable with all mammalian peroxidases—myeloperoxidase, lactoperoxidase, eosinophil peroxidase, and salivary peroxidase—provided a high enough concentration of iodide can be reached in the relevant tissue. A possible role of extrathyroidal iodine has been discussed in the literature, suggesting that we do not have a complete understanding of iodine physiology. Excluding thyroidal hormones, our understanding of the role of iodinated organic species in mammalian physiology is extremely limited. Some researchers believe that our current level of iodine intake may predispose certain individuals to various disease states (16,17).

There is also indirect evidence that iodinated organic species other than thyroid hormones have biological activity. Several studies have analyzed the comparative effects of oral administration of I_2 versus iodide. Some studies that purport to examine orally administered I_2 suffer due to inappropriate handling of the I_2 species (18). However, five studies have either dissolved I_2 daily before use or generated I_2 at the time of administration by reaction with peroxide. Oral administration of I_2 results directly in the formation of iodinated species in the gastrointestinal tract (19).

The comparative effects of I_2 and iodide in iodine-deficient Sprague-Dawley rats were first examined in 1995 (20). Comparable amounts of orally administered I_2 and iodide caused different histopathological and endocrine patterns in thyroid and mammary glands of iodine-deficient rats. A subsequent study (21) examined the comparative effect of I_2 and iodide on induction and promotion of mammary tumors in Sprague-Dawley rats induced with N-nitroso-N-methyurea. Continuous I_2 administration reduced the incidence of mammary tumors by 70% as compared to 30% for controls and iodide. Tumor suppression was correlated with increased pendrin synthesis and lower levels of lipoperoxidation. Thrall observed that a substantial percentage of the iodine from orally administered I_2 combined with a lipid (22,23) and concluded that I_2 has different pharmacological and toxicological properties than iodide.

The recommendation for the daily dietary intake of iodine has not considered potential health benefits from increased iodine intake; instead, it has understandably incorporated thyroid-related adverse events known to occur under elevated intake. Several categories of individuals experience an increased rate of thyroid-related adverse effects when exposed to elevated iodine. In fact, the suggested recommended dietary allowance (RDA) for iodine (0.125–1.1 mg/day) does not protect some susceptible patients as dietary variations within the RDA are correlated to thyroid-related adverse effects in susceptible individuals. However, controlled studies indicate that acute and sub-acute exposures of high levels of iodine in normal euthyroid individuals are without clinical effect. Entire population in Japan is known to excrete 5–30 mg daily throughout their life (24).

The acute dose of iodide necessary to perturb thyroid-stimulating hormone (TSH) from baseline in normal euthyroid individuals has been used to identify a safe upper

limit for dietary intake. A change in TSH values within the normal range is not an adverse effect, but it has been adopted as a proxy for increased risk of developing clinical hypothyroidism. Critics point to the fact that there is no controlled data supporting this assumption. While true, only epidemiological studies suggest that increased levels of dietary iodine are beneficial to the normal population; at this time, no controlled data support this supposition.

The recommended upper limit suggested as safe for the public has been established mindful of a relatively small subset of the population that is sensitive to iodine. The methodology used to arrive at the recommended upper limit (1.1 mg/day) has not been validated by standards consistent with evidence-based medicine. The percentage of individuals in the United States that are iodine deficient or close to iodine deficient has doubled over the past several decades. It is entirely accurate to say that animals used for feedstock in the United States have their dietary iodine needs met more completely than the corresponding human population. Medicine is said to be a probabilistic activity; a rigorous process would hopefully incorporate a broader range of information than currently used to establish dietary guidelines for iodine.

There is substantial data indicating a relationship between mammary health and iodine intake. Iodine deficiency leads to cystic changes, periductal fibrosis, and lobular hyperplasia in estradiol-treated rats (25). Iodine has a suppressive effect on carcinogen-induced tumors in rats, and suppressed tumors have a significantly higher uptake of iodine (26,27). Several clinical trials have demonstrated that supraphysiological levels of iodine can remediate symptoms associated with chronic clinical mastalgia; two of these trials were randomized and placebo controlled (28–29). There is no evidence that thyroid hormones have any role in these effects.

Diverse nonclinical and clinical observations indicate that iodinated organic species other than T3 and T4 can affect health. Inadequate concentrations of some of these organified species may predispose individuals to different disease states. Cann has postulated that low dietary intake is associated with breast cancer risk (30) and cardiovascular health (31). The hypothesized iodinated species are likely to be present in low concentrations. In addition, organified molecules with no biological activity will almost certainly be present. The challenges inherent in elucidating these species and their clinical relevance are more daunting than those faced with T3 and T4. Nevertheless, an increased understanding of iodine physiology may lead to improved health outcomes in many individuals.

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