Pro-oxidant activity of vitamin C in drinking water: role of copper, iron and bicarbonate

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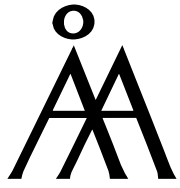

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Everything is poison, it just depends on the dose

Paracelsus

To my family
Gabi and Jacobina

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V.

I. **Measurement of ascorbic acid (vitamin C) induced hydroxyl radical generation in household drinking water.**
Asplund, K.U.M., Jansson, P.J., Lindqvist, C. and Nordström, T.
Free Rad Res. (2002) 36(12), 1271-1276.

II. **Vitamin C (ascorbic acid) induced hydroxyl radical formation in copper-contaminated household drinking water: Role of bicarbonate concentration.**
Jansson, P.J., Asplund, K.U.M., Mäkelä, J.C., Lindqvist, C. and Nordström, T.
Free Rad Res. (2003) 37(8), 901-905.

III. **Oxidative decomposition of vitamin C in drinking water.**
Jansson P.J., Jung H.R., Lindqvist C. and Nordström T.
Free Rad Res. (2004) 38(8), 855-860.

IV. **Effects of iron on vitamin C/copper-induced hydroxyl radical generation in bicarbonate-rich water.**
Jansson P.J., Castillo U.D., Lindqvist C. and Nordström T.
Free Rad Res. (2005) 39(5), 565-570.

V. **Iron prevents ascorbic acid (vitamin C) induced hydrogen peroxide accumulation in copper-contaminated drinking water.**
Jansson P.J., Lindqvist C. and Nordström T.
Free Rad Res. (2005) 39(11), 1233-1239.

CONTRIBUTION OF THE AUTHOR

The experimental work in the thesis was planned and conducted by the author together with docent Tommy Nordström, with the following aid from the co-authors.

Klara U. M. Asplund performed most of the experiments in paper I.

In paper II, Johanna C. Mäkelä assisted with the bicarbonate experiments.

In paper III, Hye R. Jung measured dehydroascorbic acid formation in vitamin C supplemented drinking waters with o-phenylenediamine.

In paper IV, Urko del Castillo helped with hydroxyl radical detection in vitamin supplemented drinking water samples.

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ABBREVIATIONS

Asc	.....	Ascorbate
$Asc^{\bullet}$	.....	Ascorbate radical (protonated)
$Asc^{\bullet-}$	.....	Ascorbate radical (deprotonated, semi-dehydroascorbate radical)
DHA	.....	Dehydroascorbic acid
DTT	.....	Dithiothreitol
EDTA	.....	Ethylenediaminetetraacetic acid
Fe^{2+}	.....	Ferrous iron
Fe^{3+}	.....	Ferric iron
HPLC	.....	High-performance liquid chromatography
H_2O_2	.....	Hydrogen peroxide
MCL	.....	Maximum contaminant level
Milli-Q water	.....	Distilled water, ion-free
NADH	.....	Reduced form of nicotinamide adenine dinucleotide (NAD)
NTA	.....	Nitrilotriacetic acid
$OH^{\bullet}$	.....	Hydroxyl radical
7-OHCCA	.....	7-hydroxycoumarin-3-carboxylic acid
ODS rats	.....	Osteogenic Disordered Shiongi rats
RNS	.....	Reactive nitrogen species
ROS	.....	Reactive oxygen species
SOD	.....	Superoxide dismutase
TRIS base	.....	Tris (hydroxymethyl)-aminomethane

INTRODUCTION

Vitamin C (ascorbic acid) is a water-soluble antioxidant and vitamin, essential for human health. Vitamin C is an important cofactor for many enzymes and vitamin C also increases the absorption of iron from the gut. Humans cannot synthesize or store vitamin C and are therefore dependent on vitamin C through diet. In vitamin C deficiency, collagen is not synthesized normally, which ultimately leads to the symptoms of scurvy.[1]

The antioxidant effects of vitamin C have mainly been reported in *in vitro* studies on cells.[2] In these studies, ascorbic acid has been shown to be a potent free radical scavenger that can protect cells against UV- or radiation-induced apoptosis.[3] Despite the claimed positive effects of vitamin C observed in *in vitro* studies, concerns have been raised due to its pro-oxidant effect in the presence of transition metals. It has been demonstrated that vitamin C (ascorbic acid) can, in the presence of transition metal ions such as Cu^{2+} and Fe^{3+}, function as a strong pro-oxidant.[4-7] Thus, in the presence of transition metals, vitamin C can generate harmful reactive oxygen species (ROS) instead of protecting against them. The relevance of the pro-oxidant activities of vitamin C and the safety of vitamin C intake for humans is still a matter for discussion.[6, 8-11]

Water is necessary for all life and humans must drink water frequently to maintain their fluid requirement. However, drinking water can contain impurities, such as metal ions, from natural and man-made sources. For this purpose, the pro-oxidative properties of vitamin C were studied in drinking water. This is of importance, as increased consumption of vitamin C through vitamin tablets and vitamin supplemented food products might be ingested with metal-contaminated drinking water. The main focus of this study was to look for the physiological milieu that might trigger vitamin C induced formation of ROS (hydroxyl radicals and hydrogen peroxide (H_2O_2)) in drinking water.

REVIEW OF THE LITERATURE

1. Vitamin C – ascorbic acid - ascorbate

Vitamin C is a weak acid and a highly water-soluble molecule. Ascorbic acid ($C_6H_8O_6$) is the trivial name for vitamin C, while the chemical name, very seldom used in the literature, is 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol or 2-(1,2-dihydroxyethyl)-4,5-dihydroxy-furan-3-one (IUPAC). Biologically it is the L-enantiomer of ascorbic acid that is active, while the D-enantiomer shows much lower biological activity.[12] Ascorbic acid is the acidic form of vitamin C having two ionizable –OH groups, with pKa values of 4.25 and 11.8. At physiological pH, the mono-anion is favoured and therefore ascorbic acid is usually referred to as ascorbate (salt) in the literature (Figure 1).

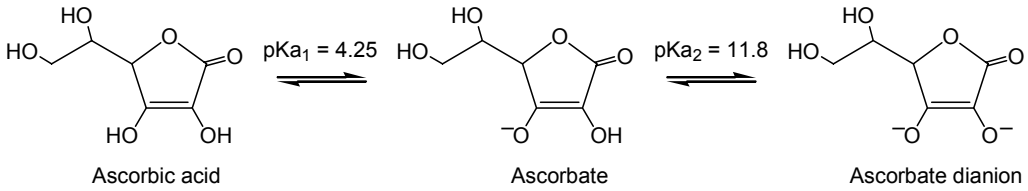

Figure 1. *Forms of vitamin C at various pH*

1.1. Vitamin C deficiency and scurvy

The history of vitamin C is largely the history of the human disease scurvy, one of the first nutritional deficiency diseases recorded.[13-16] The symptoms of scurvy were marked by anaemia, weakness, spongy gums, oedema, often with open sores in the mouth, and loosening of the teeth, bleeding in the mucous membranes, and hard bumps in the muscles of the legs. In 1566, the Dutch physician Ronsseus instructed sailors to consume oranges to prevent scurvy. In 1639, John Woodall, a leading physician in England, recommended lemon juice to treat scurvy. Later a Scottish naval surgeon, James Lind, conducted a controlled trial adding oranges and lemons to the sailors' diet. The positive observed effect of lemons and oranges on scurvy in Lind's trial led to the increased usage of lemons and oranges in the diet of sailors on Captain James Cook's second voyage around the world. However, it was not until 1795 that all sailors in England were ordered to ingest lemon

juice daily. It was later found that vitamin C (ascorbic acid) was the active substance in the oranges and lemons that prevented scurvy. With scurvy also came the belief that vitamin C could function as an antioxidant protecting against oxidation, as the name ascorbate (antiscorbutic) implies. Vitamin C was first isolated from cabbages, oranges and adrenal glands by Szent-Györgyi in 1928.[15, 17, 18]

1.2. Vitamin C - an important enzymatic cofactor

Later it was found that ascorbic acid is a crucial cofactor for many enzymes. Without ascorbic acid, proline hydroxylase and lysine hydroxylase cannot hydroxylate collagen.[1, 19, 20] This leads to reduced formation of collagen fibres, which gives rise to poor wound healing and fragile blood vessels. The collagen fibres strengthen the connective tissues in the blood vessels, skin, teeth and skeleton. Nowadays, scurvy is a very rare condition, as the quality of food intake has improved, i.e., more fruits and vegetables are consumed. In addition, many food products are nowadays fortified with vitamins and vitamin C is a common dietary supplement.[16]

Vitamin C is used as cofactor in the synthesis of noradrenaline and carnitine, tyrosine metabolism and amidation of peptide hormones.[1] In most of these vitamin C-dependent enzymes, vitamin C helps to maintain the enzyme in its active form by keeping the metal (copper and iron) in the active centre in the reduced form.[20]

1.3. Effects of vitamin C supplementation

The body has depots of vitamin C but the vitamin will eventually be depleted. The average adult has a body pool of 1.0-2.0 grams of vitamin C and the average half-life of vitamin C is about 10-20 days.[21, 22] Scurvy can be prevented with a vitamin C intake of only 10 mg/day.[20]

A high intake of vitamin C is generally not believed to be harmful since vitamin C is a water-soluble compound that is not stored in the body and the excess ingested vitamin is excreted in the urine. However, diarrhoea is a directly observable symptom of over-consumption of vitamin C.[11, 23] The Food and Nutrition Board, Institute of Medicine, has set 2000 mg as a tolerable upper limit for vitamin C in healthy adults.[10, 11]

Since vitamin C was identified as the antiscorbutic factor, high hopes have been raised for this compound in the treatment of a variety of diseases, e.g. common colds and cancer. High intake doses of vitamin C for general good health have historically been recommended, but the most attention was drawn to this by Linus Pauling .[24] However, in the mid 80s researchers started to question Pauling's claim that vitamin C might cure cancer,[25] as clinical trials by Moertel *et al.* questioned the beneficial effects of vitamin C on cancer treatment.[26] Nevertheless, the validity of the clinical study performed by Moertel *et al.* was criticized by Pauling.[27] Since then, the benefits of high vitamin C intake have been debated.

Many clinical studies have been undertaken to elucidate whether higher intakes of vitamin C have a positive effect on different complaints and diseases. One such disputed topic has been the role of vitamin C as an aid in preventing and treating the "common cold". So far, no clear-cut conclusion can be made as to whether a higher vitamin C intake is favourable in preventing and treating colds.[28-30] However, studies with higher vitamin C intake during exercise have pointed to beneficial effects in lowering oxidative stress indicators.[31]

Generally, the protective effect of vitamin C has been attributed to its antioxidant properties *in vitro*. Indeed, ascorbic acid has been proposed to have a beneficial effect on many age-related diseases such as atherosclerosis, cancer, and some neurodegenerative and ocular diseases.[32-37]

Concerns have been raised due to the observed pro-oxidant function of vitamin C.[6, 8-10, 38-42] These studies suggest that vitamin C, in some conditions, could expose cells to increased oxidative stress. Furthermore, there have been reports that higher doses of vitamin C could induce haemolysis in glucose-6-phosphate dehydrogenase deficient patients.[43-45]

1.4. Sources and recommended dietary allowance of vitamin C

In plants and most animals, vitamin C (ascorbic acid) is synthesized from glucose. Humans, guinea-pigs and other primates, on the other hand, are not able to synthesize vitamin C because the final enzyme (L-gulonolactone oxidase) of the vitamin C synthesis pathway is missing. Due to the absence of this enzyme, humans are dependent on vitamin C from their diet.[46, 47]

The best sources of vitamin C are foods such as fruits and berries (Table 1). In vegetables, vitamin C is found at high levels in cabbages of every sort, sweet pepper and parsley.

Potatoes contain relatively low amounts of vitamin C, but since humans eat potatoes relatively often and in large quantities, potatoes are generally classified as a good source of vitamin C. However, the vitamin C content of potatoes will decrease during springtime due to the poor storage capability of vitamin C.

Table 1. *Vitamin C content in selected foods*

Food	Vitamin C (mg)/ gram edible portion
Fruits	
Banana	8-16
Apple	3-30
Pineapple	15-25
Orange	30-50
Grapefruit	30-70
Lemon	40-50
Blackcurrant	50-200
Strawberry	40-70
Rosehip	250-800
Vegetables	
Onion	10-15
Tomato	10-20
Radish	25
Spinach	35-40
Cabbage	30-70
Cauliflower	50-70
Broccoli	80-90
Brussels sprout	100-120
Sweet pepper	150-200
Parsley	200-300
Potato	10-12

Adapted from Johnson et al..[48]

The Recommended Dietary Allowance (RDA) for vitamin C is 75 mg/day in the Nordic countries (Finland, Sweden, Norway, Denmark and Iceland) and 90 mg/day for adult men and 75 mg/day for adult women in the USA. A balanced diet contains enough vitamin C to prevent acute scurvy in an average healthy adult. Despite this, vitamin C (ascorbic acid) is

still added as a vitamin and preservative to a variety of food sources (e.g. fruit juices) in high quantities. Intake of supplemental vitamin C has also increased considerably and supplements containing up to 0.5 – 1 grams of vitamin C/tablet can be purchased without a prescription. Thus, the mean daily intake of vitamin C has significantly increased in many countries during the past 20 years.

2. Free radicals, reactive oxygen and nitrogen species

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are collective terms used in the literature that include both oxygen radicals and some non-radical derivates of oxygen (Table 2). There are several definitions in the literature of what a free radical is and if "free" is the right term to use. A simple and broad definition is: a free radical can be of any atomic or molecular species, but it has to have an independent existence and contain one unpaired electron in its outer shell.[49] An unpaired electron is one that occupies an atomic or molecular orbital by itself. A radical is often indicated with a dot after the formula, which indicates that it has one unpaired electron in its outer shell.[50]

Table 2. *Examples of ROS and RNS (radical/non-radical)*

Name	Formula	Radical/non-radical
ROS		
Hydroxyl radical	$OH^{\bullet}$	radical
Superoxide	$O_2^{\bullet -}$	radical
Peroxyl radical	$RO_2^{\bullet}$	radical
Hydrogen peroxide	H_2O_2	non-radical
Singlet oxygen	$^1O_2$	non-radical
Hypochlorous acid	HOCl	non-radical
Ozone	O_3	non-radical
RNS		
Peroxynitrite	$ONOO^-$	non-radical
Nitric oxide	$NO^{\bullet}$	radical
Nitrogen dioxide	$NO_2^{\bullet}$	radical
Nitroxide radical	$RNHO^{\bullet}$	radical

ROS and RNS are normal by-products from mitochondria, peroxisomes, cytochrome P450 metabolism and from activated inflammatory cells (macrophages and neutrophils). However, ROS and RNS can also be generated by UV light, X-rays, γ-rays, pollutants in the atmosphere and in metal catalyzed-reactions.

ROS and RNS play a dual role in biological systems, as they can be either harmful or beneficial.[51] High concentration of ROS or RNS can induce damage to molecules as well as cell structures, such as lipid membranes, proteins and nucleic acids. ROS and RNS have been suggested to be involved in several clinical conditions such as the ageing process, inflammatory diseases, ischemia, brain and nervous disorders just to name a few.[50, 52-55] On the other hand, ROS and RNS can have a beneficial effect in the defence against infectious agents and in cellular signalling. Furthermore, a low concentration of ROS can stimulate the mitogenic response.[51]

This dual role of ROS has been observed, especially with hydrogen peroxide. Although high levels of hydrogen peroxide have been demonstrated to be toxic to cells, low levels of hydrogen peroxide (below or about 20-50 μM) do not affect most cells negatively but instead stimulate cell growth. There is, in fact, a growing body of evidence that hydrogen peroxide could function as a signalling molecule between and inside cells, regulating the signal transduction pathways. Toxic concentration of hydrogen peroxide will activate NF-κB, leading to apoptosis or necrosis.[56-61]

To cope with elevated ROS levels, the human body has non-enzymatic antioxidants and antioxidant enzymes. The non-enzymatic antioxidants include compounds such as vitamin C, vitamin E and reduced glutathione. The enzymatic antioxidant defence systems include enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (one of many peroxidases) and thioredoxin-linked systems to cope with elevated superoxide, hydrogen peroxide concentrations.[62]

2.1. Vitamin C – an antioxidant with free radical scavenging properties

An antioxidant is defined as ”any substrate that, when present at low concentrations compared to those of an oxidizable substrate (proteins, lipids, carbohydrates and nucleic acids), significantly delays or prevents oxidation of that substrate”.[6] Vitamin C has been shown to scavenge ROS and RNS (Table 2), thereby preventing oxidative damage to important biological macromolecules such as DNA, lipids, and proteins.[63-66] The

antioxidant effect of vitamin C is either by regeneration of oxidized substrates, alternatively directly scavenging ROS and RNS.

During physiological conditions, the antioxidant and pro-oxidant properties of vitamin C are basically the chemistry of ascorbate (Figure 1). The deprotonated ascorbate radical (semi-dehydroascorbate radical) ($Asc^{\bullet -}$) alternatively the protonated ascorbate radical ($Asc^{\bullet}$), is the first product when ascorbate is oxidized (Figure 2).

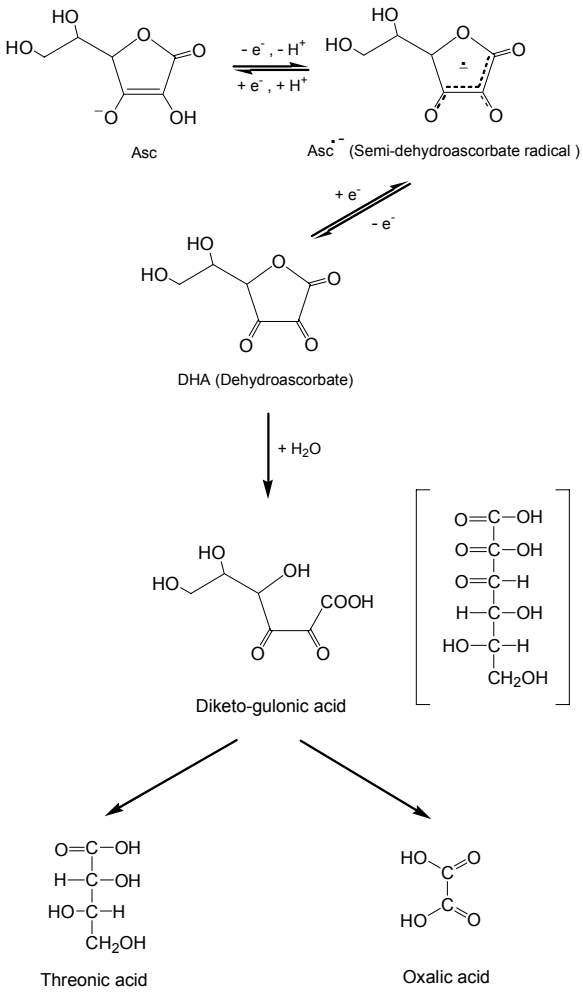

Figure 2. *Degradation/oxidation products of ascorbate*

Asc• is a poorly reactive radical that can be converted back to ascorbate by other, stronger reducing agents, such as NADH-dependent enzymes.[67, 68] Alternatively, Asc• can undergo disproportionation to form DHA.[5, 69, 70] The reactivity of Asc• can account for many of ascorbate's antioxidant effects.[70, 71] DHA on the other hand is very unstable, even in the absence of oxygen,[72] and the lactone ring of DHA can be opened and cleaved into oxalic acid and threonic acid via diketo-gulonic acid (Figure 2).

Vitamin C can reduce an oxidized substrate if the substrate has a lower reduction potential. One of the most well-documented antioxidant effects of vitamin C is to regenerate other oxidized substrates such as vitamin E. Vitamin C has been suggested to be beneficial in atherosclerosis, due to its ability to regenerate oxidized vitamin E (α-tocopherol) in membranes and lipoproteins *in vitro*.[73, 74] The ability of vitamin C to regenerate oxidized substrate is not only seen in vitamin E, as vitamin C has also been shown to regenerate glutathione, β-carotene and urate from their one electron oxidized form *in vitro*.[6, 75, 76] In the case of vitamin E, vitamin C has the capacity to regenerate vitamin E (α-tocopherol) from the α-tocopheryl radical (Figure 3).

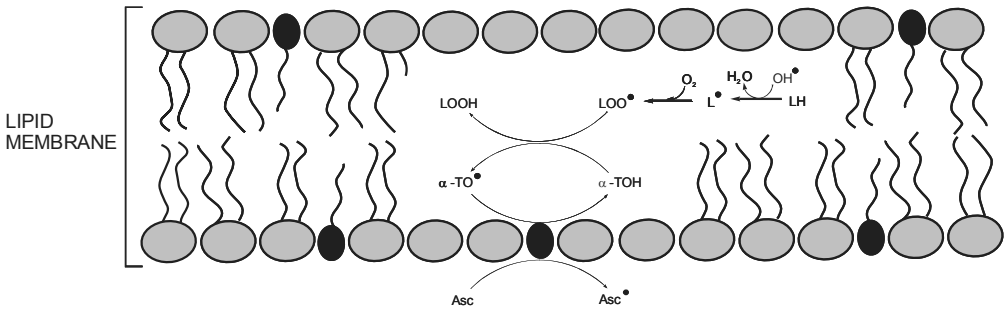

Figure 3. *A schematic illustration of the regeneration of vitamin E (α-tocopherol) by vitamin C (ascorbate) in the aqueous/lipid inter-phase of a lipid bilayer. The illustration also shows a hydroxyl radical (OH•) attack on a lipid (LH) forming a lipid radical (L•). The lipid radical will thereafter form a lipid peroxyl radical (LOO•) in the presence of oxygen (O_2). Vitamin E (α-tocopherol (α-TOH)) will remove the lipid peroxyl radical. Ascorbate (Asc) recycle α-tocopherol (α-TOH) from the α-tocopheryl radical (α-TO•) with the concomitant formation of the ascorbate radical (Asc•). Lipids (LH) are illustrated with grey-coloured circles with two hydrophobic tails and vitamin E with black circles with one hydrophobic tail in the schematic picture of a lipid membrane. Note that in some lipids and vitamin E molecules, the hydrophobic tails are removed for clarity.*

The α-tocopheryl radical is produced as a consequence of vitamin E scavenging of lipid-soluble radicals.[77] Vitamin E is the major chain-breaking antioxidant in membranes and will inhibit lipid peroxidation. Vitamin E will consequently counteract membrane damage and modification of low-density lipoproteins.[32] Thus, regeneration of vitamin E by vitamin C will provide cell membrane stability and protect the cell membrane during oxidative insult. Additionally, vitamin C is of importance as the α-tocopheryl radical can act as a pro-oxidant if not reduced to vitamin E.[77-79] Furthermore, an indirect antioxidant effect of vitamin C on vitamin E *in vivo*, was observed in guinea-pigs or ODS rats (Osteogenic Disordered Shiongi rats) fed on a vitamin C restricted diet. The guinea pigs and ODS rats displayed a decrease of vitamin E content in tissues. This points to an interaction between these two vitamins *in vivo*.[80, 81]

As vitamin C reacts with ROS (scavenging) the ascorbate radical (Asc•) is formed. Asc• can undergo disproportionation to form DHA. Vitamin C depletion during a condition of oxidative stress could imply the ability of vitamin C to act as an antioxidant *in vivo*.[82] DHA can be measured in plasma and the ratio of DHA to total ascorbate *in vivo* has been suggested to be a biomarker of oxidative stress. Indeed, oxidative stress induced by smoking results in elevated DHA levels in comparison to ascorbate in plasma.[83] DHA has also attracted attention since DHA has been shown to be toxic and to generate oxidative stress in various cell systems.[84-86]

Normally, almost no DHA can be detected in body fluids and tissues in a healthy person. Thus, the vitamin C to DHA ratio seems to be kept at a high level in body fluids and tissues during normal health. Interestingly, vitamin C has been shown to stimulate the immune system during infections by enhancing T-cell proliferation.[87] Vitamin C could also block T-cell apoptosis and hence stimulate T-cell proliferation in the presence of pathogens.[88]

Although vitamin C has been reported as a ROS scavenger *in vitro*, (accepts electrons from superoxide, hydrogen peroxide, hypochlorite, hydroxyl radicals and peroxyl radicals) it is still debated whether this is the favoured mechanism of ascorbic acid, since such high concentrations might not be present *in vivo*. For example, hydroxyl radicals react very rapidly with the closest target available.[50] The levels of ascorbate in human plasma lie between (30-100 μM).[50, 89] Other compounds, e.g. glucose (~ 4.5 mM, plasma) and amino acids (40-80 mg protein/ml plasma) have about the same rate constants as ascorbate for reaction with hydroxyl radicals. Uric acid (250-450 μM, plasma) has a lower rate constant for hydroxyl radicals than ascorbate but the concentration of uric acid is probably still high enough in absolute terms to compete successfully as the main scavenger in most

tissues.[50] On the other hand, locally in cerebrospinal fluid (CSF), the tear fluid of the eye, gastric juice and lung lining fluid, even millimolar levels of vitamin C can be detected.[50, 90-92]

2.2. The role of iron and copper in human health

Iron and copper are highly precious metals for the growth and viability of all cells and indispensable for human survival. Iron is primarily required for haemoglobin synthesis, but it has also a crucial role in e.g. DNA synthesis, electron transport and many enzymatic activities throughout the body. Low dietary intake of iron results in iron deficiency and anaemia.[93] On the other hand, iron has also been implicated in the pathogenesis of a variety of neurodegenerative disorders, e.g. Parkinson's disease, Alzheimer's disease, multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE).[94-96] Furthermore, iron has been implicated in diseases such as cancer,[97, 98] diabetes[99, 100] and immune abnormalities.[101, 102]

Proper handling of copper is crucial for the central nervous system. Altered copper homeostasis can be linked to Wilson's disease, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS), among others.[103-105] Impaired binding of copper or absence of copper in the active centres of important enzymes leads to oxidative stress in neurological diseases.[55, 103-105] It has been suggested that oxidative stress, the alternation in copper balance in Wilson's disease, is mediated via mitochondrial copper accumulation. Copper accumulation in patients with Wilson's disease is caused due to a failure in a specific copper pump located in the mitochondrial membrane.[106, 107] Indeed, anti-copper drugs have been tested and suggested for the treatment of diseases with impaired handling of copper.[108, 109]

The toxicity of iron and copper has generally been attributed to their ability to reduce molecular oxygen, thus forming reduced oxygen species. Normally, copper and iron are bound to specific proteins that function as transporting or storage proteins of these metals. Thus, the redox-activity of iron and copper is minimized *in vivo* due to the binding to proteins. Iron enters the circulation bound to the iron transport protein transferrin.[110, 111] In the cell, iron is stored in the ferritin protein. Ferritin can also bind some other metals including copper, but only in trace amounts.[112] Another iron binding protein that resembles transferrin is lactoferrin. Lactoferrin can be found in saliva, seminal fluids, bile, tears and milk (both human and cow).[113]

Copper, will be bound to albumin as it enters the blood. In the liver most of the copper is transferred to the caeruloplasmin protein. Caeruloplasmin with the bound copper is secreted into plasma and distributes the copper to copper-requiring cells. Caeruloplasmin contains more than 95% of the copper found in plasma.[114] Caeruloplasmin can also function as a ferroxidase and facilitate the loading of iron to transferrin as it helps to oxidize iron from Fe^{2+} to Fe^{3+}.[111, 115, 116]

Because of the normally tight binding of iron and copper to metal binding proteins, the pro-oxidant activity of vitamin C *in vivo* has been doubted. If there are no free catalytic metals present, the metals should not redox-cycle with vitamin C.[9, 117], Local acidosis (pH 5.5-7.3) has been reported in inflammation,[118] bone fractures,[119-121] arthritic joints,[122] and surroundings of malignant tumours.[123] During acidic conditions, metal ions may be released from their metal binding proteins and could consequently interact with a reducing agent, e.g. vitamin C.[117, 124, 125]

2.3. Ascorbic acid as a pro-oxidant in the Fenton reaction

Some transitions metals are able to accept and donate single electrons and can therefore catalyze free radical reactions. Metals in the first row of the D-block in the periodic table (iron, copper, chrome, manganese, cobalt, nickel, vanadium, titanium), except zinc, can participate in redox-cycling and radical reactions. Metals having one single oxidation state, such as zinc, and non-transition metals such as aluminium, calcium and magnesium do not promote free radical reactions. It has been shown that ascorbic acid can, in the presence of transition metal ions such as Cu^{2+} and Fe^{3+}, function as a strong pro-oxidant *in vitro*.[4, 5, 7] Thus, vitamin C could generate ROS instead of protecting against them. This pro-oxidant activity of ascorbic acid with transition metals has led to discussions whether this might occur *in vivo*.[6, 8-10]

There is conflicting and confusing information regarding ascorbate and its cytotoxicity in the literature. Some reports clearly show that ascorbate in the presence of copper is highly cytotoxic to cells,[38-42] while others demonstrate that ascorbic acid can protect cells from pro-oxidative insult.[32-36] The toxic effect of ascorbic acid in cell systems has been attributed to hydrogen peroxide formation in the cell culture.[126-128] Hydrogen peroxide triggers apoptosis in cells and therefore hydrogen peroxide levels *in vivo* must be strictly controlled.[129-132]

The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions such as iron and copper, thereby stimulating the formation of ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). Of all the ROS, the cellular damage is mostly caused by the hydroxyl radical. The hydroxyl radical can be directly formed from hydrogen peroxide and Fe^{2+} through the Fenton reaction (Equation 1).[133-135] The Fenton reaction is highly catalyzed if certain metal chelators such as EDTA, NTA and a reducing substance such as ascorbic acid (vitamin C) are present.[117, 136-138] Ascorbate reduces Fe^{3+} back to Fe^{2+}, with ascorbate (Asc) being oxidized to an ascorbate radical ($Asc^{\bullet}$) (Equation 2). The ascorbate radical and other products from ascorbate oxidation (DHA and diketo-gulonate) can further be used to reduce Fe^{3+} to Fe^{2+}.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Fenton reaction)} \qquad \text{Eq. 1}$$

$$Fe^{3+} + Asc \rightarrow Fe^{2+} + Asc^{\bullet} \qquad \text{Eq. 2}$$

Fe^{3+} may also react with hydrogen peroxide, but this reaction is slower than the reaction between Fe^{2+} and hydrogen peroxide at physiological pH. Generation of hydroxyl radicals by a reaction between Fe^{3+} and hydrogen peroxide (H_2O_2) appears to involve superoxide ($O_2^{\bullet-}$). No direct evidence for this chemistry has been obtained, but since SOD can inhibit the reaction between Fe^{3+} and hydrogen peroxide, it seems likely that superoxide participates in this reaction.[139] (Equation 3) .

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+ \qquad \text{Eq. 3}$$

SOD has no effects on hydroxyl radical production in Fe^{2+}/hydrogen peroxide mixtures since SOD catalyzes the dismutation of superoxide. The reactants and the products from the Fenton reaction can also participate in a variety of further chemical reactions (Equations 4-8). The reactions will depend on the experimental conditions, such as pH, concentration of hydrogen peroxide, metal ions and target molecules present.

$$OH^{\bullet} + H_2O_2 \rightarrow H_2O + H^+ + O_2^{\bullet -} \quad \text{Eq. 4}$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad \text{Eq. 5}$$

$$HO_2^{\bullet} + Fe^{2+} + H^+ \rightarrow Fe^{3+} + H_2O_2 \quad \text{Eq. 6}$$

$$OH^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad \text{Eq. 7}$$

$$HO_2^{\bullet} + Fe^{3+} \rightarrow Fe^{2+} + H^+ + O_2 \quad \text{Eq. 8}$$

For example, if hydroxyl radicals are formed and they do not have anything to react with, they can react with hydrogen peroxide, or alternatively, take one electron from Fe^{2+} and oxidize it to Fe^{3+}. However, if hydroxyl radicals are formed *in vivo* many target molecules will be available.

In the laboratory, hydroxyl radicals can be easily generated by UV induced homolytic fission of hydrogen peroxide (Equation 9) or by metal-catalyzed (copper or iron) reduction of oxygen to superoxide that reacts with hydrogen peroxide (Equation 10).[140, 141]

$$H_2O_2 \xrightarrow{UV} OH^{\bullet} + OH^{\bullet} \quad \text{Eq. 9}$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + OH^- \quad \text{(Haber-Weiss reaction)} \quad \text{Eq. 10}$$

2.4. The reactivity of hydrogen peroxide and hydroxyl radicals

The formation of hydroxyl radicals has been studied using the spin trap technique,[142] aromatic hydroxylation of target molecules[143-145] and by studying radical attacks on target molecules such as DNA[4] and deoxyribose[146]. Generally, standards for the final products (e.g. 8-hydroxyguanosine to demonstrate hydroxyl radical attack on guanosine) have been used in order to identify the radicals (ROS or RNS) that are generated. In addition, radical scavenger molecules have been used to confirm the presence of a specific radical. This however, can sometimes be misleading, as some hydroxyl radical generating reactions can be site-specific. In this case radical scavengers fail to scavenge the radicals, but this does not mean that hydroxyl radicals do not form.

Of the ROS, hydroxyl radicals are very harmful due to their high reactivity.[134] Hydroxyl radicals are short-lived and extremely reactive and easily attack any nearby molecule by

taking or giving one electron. Once hydroxyl radicals are formed, they will readily react with DNA, protein or lipids and can therefore induce cellular damage if no protecting hydroxyl radical scavenging molecules are present.[4, 141, 147-151] In contrast, in the absence of metal ions or tightly bound metals, hydrogen peroxide is not very reactive by itself, but imposes a threat due to its ability to easily diffuse through the cell membrane and then participate in metal induced free radical reactions inside the cell.[152, 153]

Generally, the rate constant for a free radical reaction is highly dependent on the radical species and target molecule involved. If two radicals meet and react with each other, a covalent bond will be formed. However, under physiological conditions, the more relevant reaction is that between a free radical and a non-radical target molecule. This occurs in four ways (exemplified with $OH^{\bullet}$ radicals):[50]

1. The non-radical forms a new radical adduct as the free radical adds to the non-radical.

$$OH^{\bullet} + \text{molecule} \rightarrow [\text{molecule-OH}]^{\bullet} \qquad \text{Eq. 11}$$

   An example of this is when $OH^{\bullet}$ adds to guanine in DNA, forming an 8-hydroxyguanine radical.

2. A free radical can function as a reducing agent, donating an electron to a non-radical, thus the recipient will then become a radical

$$OH^{\bullet} + \text{molecule} \rightarrow OH^{+} + \text{molecule}^{\bullet -} \qquad \text{Eq. 12}$$

3. A free radical can also function as an oxidizing agent, accepting an electron from a non-radical. The donation of an electron from a non-radical results in a free electron on the non-radical, making the non-radical a radical.

$$OH^{\bullet} + \text{molecule} \rightarrow \text{molecule}^{\bullet +} + OH^{-} \qquad \text{Eq. 13}$$

4. Alternatively, a free radical can abstract a hydrogen atom from a C-H bond, resulting in a free electron on the carbon atom (Figure 3).

$$\text{R-C-H} + OH^{\bullet} \rightarrow \text{R-C}^{\bullet} + H_2O \qquad \text{Eq. 14}$$

Formation of free radicals during physiological conditions, for example OH • radicals, will set off a chain reaction between newly formed radicals and non-radicals. This chain reaction will not stop until two free radicals meet and react with each other.

3. Drinking water

Water is necessary for all life and human cells consist of 80 percent water. Therefore, humans must drink water frequently to maintain their fluid requirement. Humans need to drink approximately 2 litres (8 glasses) of water every day to replenish the water that is lost from the body through urine, skin and the respiratory tract. However, only 0.3% of the Earth's water supply is safe to drink. Only freshwater originating from rivers, lakes and underground sources can be used for human consumption. In addition, freshwater from many of these sources is unsuitable for human consumption, because of contaminants. The contaminants must be removed or adjusted to accepted threshold values.

3.1. Drinking water impurities and Maximum Contaminant Level (MCL)

Drinking water contains impurities from both natural and man-made sources. Gases, minerals, bacteria, metals and chemicals are examples of contaminants that have to be adjusted to accepted threshold values. This restriction of contaminants in drinking water is necessary to protect public health and ensure a uniform standard for water quality nationwide. Private water wells, on the other hand, are not regulated by drinking water standards. The owner of the well is responsible for testing, and if needed, treating the water himself, all to avoid health risks. The Maximum Contaminant Level (MCL) is the highest amount of a specific contaminant that is allowed in the drinking water. The MCL is normally expressed in milligrams per litre (mg/l).[154, 155]

Contaminants fall into two categories, primary and secondary contaminants. The primary standards serve as threshold value for the contaminant to avoid health problems. Radioactive elements, microbial pathogens and organic/inorganic chemicals are examples of three classes of toxic pollutants that are classified as primary contaminants. Water plants are obliged to follow MCL for primary contaminants.

Secondary contaminants are regarded as contaminants that affect the aesthetic quality of drinking water, such as taste, colour, odour, pH and appearance. Chloride, sulphate, copper, iron, manganese, zinc etc. are all examples of secondary contaminants (Table 3).

Water plants are not forced to fulfil the MCL values for secondary contaminants but serve as guidelines for good-quality drinking water. Thus, if the contaminants in drinking water are lower than the MCL, it is believed that a person can consume the drinking water safely over a lifetime.

Table 3. *Guideline values (MCL) for some cations and anions that are of health significance in drinking water*

	Guideline value (MCL) (mg/l)	
Chemical	**WHO standards 1993**	**EU standards 1998**
Cations		
Aluminium	0.2	0.2
Arsenic	0.01	0.01
Cadmium	0.003 0.005	
Calcium	not mentioned	100
Chromium	0.05	0.05
Copper	2.0	2.0
Iron	not mentioned (1)	0.2
Lead	0.01	0.01
Nickel	0.0	0.02
Magnesium	not mentioned	50
Manganese	0.5	0.05
Zinc	3.0	not mentioned
Anions		
Chloride	250	250
Cyanide	0.07	0.05
Fluoride	1.5	1.5
Sulphate	500	250
Nitrate	50	50
Nitrite	0.5	0.1

The WHO guideline values for cations and anions were adapted from "Guidelines for drinking water quality", Geneva 1993.[154] European guideline values were adapted from the Council Directive 98/83/EC on the quality of water intended for human consumption.[155] Remarks: (1) Desirable 0.3 mg/l

3.2. The role of bicarbonate and pH in drinking water

To prepare safe drinking water, the water plants have to analyse which ions and chemical compounds are present in the water. The most important parameters for drinking water are hardness, pH (alkalinity and acidity) and salinity. The presence and combination of a number of ions will determine these features. Calcium and magnesium ions give the hardness, while bicarbonate, carbonate and hydroxyl ions give the alkalinity in water. Chloride and sulphate ions (as HCl and H_2SO_4) contribute to the acidity and salinity of the water (Table 3, guideline values). Thus, calcium carbonate ($CaCO_3$) contributes both to hardness and alkalinity of water, while calcium chloride ($CaCl_2$) gives hardness and salinity.

The pH of drinking water usually originates from dissolved carbon dioxide (CO_2), which forms carbonic acid (H_2CO_3). Humic acids and other organic acids, which originate from decayed plants, can also make the water acidic. Acidification of the ground water in combination with a bedrock that cannot counteract or buffer acidic particles will lead to acidic water. When the acidity originates from natural sources, the pH of the water is around 3-4. It is not harmful to drink such mildly acidic (pH~ 3-4) drinking water. However, indirectly, such acidic water will cause problems as it can extract metals from the soil and water pipe systems. Acidic water can extract iron, manganese, copper, aluminium and heavy metals such as cadmium and lead. Therefore, water plants adjust the pH to 6.5-9.5 (European guideline values, Council Directive 98/83/EC). Contributing factors in increased copper corrosion from copper pipes are the concentration of sulphate and chloride ions.[156]

Acidic water is usually adjusted to more alkaline pH values with calcium carbonate ($CaCO_3$).[156-158] Alkalinity is a measure of susceptibility for acidification, in other words buffer capacity for acid addition. The higher the alkalinity in the water, the better the water can resist acidification. Bicarbonate, carbonate and hydroxyl ions give different alkalinity to the water. If the pH in drinking water is below 8.3, bicarbonate alkalinity is prevailing. In most drinking water that has a pH between pH 5 and 8, the alkalinity and pH come from bicarbonate ions (Figure 4). Bicarbonate and carbonate alkalinity will exist in drinking water with pH values between 8.3 and 9.4. Corrosive alkalinity (hydroxyl alkalinity from sodium hydroxide, NaOH) can only exist at pH values above 9.4. Furthermore, when water is treated for adjustment in pH the content of other components has to be quantified.

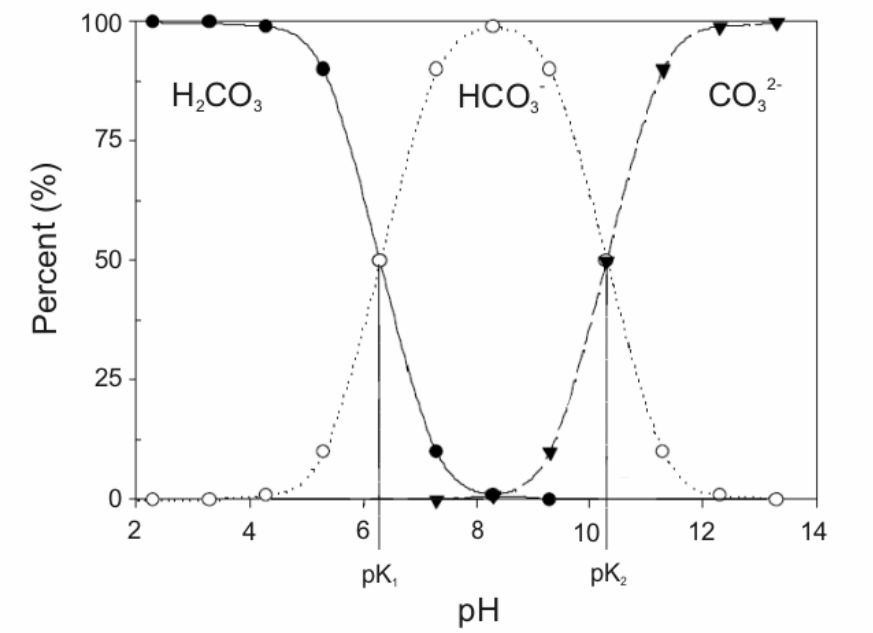

Figure 4. *The distribution of carbonate species as a fraction of total dissolved carbonate in relation to solution pH.*

3.3. Copper intake from drinking water

The daily consumption of copper through diet lies between 1 and 2 mg/day in adults and 0.6 to 0.8 mg/day in two-year-old children. Copper is present in all foods but the highest concentrations can be found in liver, nuts, seeds and chocolate (around 10 mg/kg).[159, 160] Drinking water is not normally included in the daily intake of copper and the total intake of copper can vary greatly depending on the copper concentration in the drinking water consumed.

More than 90 percent of the water pipe system in the USA, Sweden, Great Britain and Norway consists of copper pipes. The proportion of copper pipes in the water system is 40-60 percent for Germany, Spain, France and Italy but only 12 percent in Japan.[161] A survey in Seattle (USA) estimated that the intake of copper from drinking water was approximately 10 times higher among persons with copper pipes (1.3-2.2 mg per day) in their homes than for those having galvanized pipes. Thus, if copper pipes corrode, the concentration of copper in drinking water will rise.[162] The corrosion of copper pipes is influenced by the time drinking water stays in the pipes. Flushing the tap can reduce the

copper concentration in drinking water. Also the length, dimension and the age of the copper pipes will influence how much copper is extracted. The pipes will emit the highest copper concentration in the beginning, until after some time a protecting sheet is formed inside the pipes, minimizing copper extraction.[163]

The copper uptake from the gastrointestinal tract is strictly regulated.[164, 165] Several studies have shown that short-term intake of higher amounts of copper than usual will not affect the status indexes of copper.[165-170] On the other hand, a recent study observed that intake of copper for a longer period of time (4 months) will eventually increase the uptake of copper.[171] Gastrointestinal symptoms have been associated with high copper intake (about 4 mg/day).[172-175] This amount of copper can be obtained by consuming 2 litres of water containing 2.0 mg/l of copper (2.0 mg/l is the MCL for copper).

AIMS OF THE PRESENT STUDY

Vitamin C is nowadays consumed more than ever before. The increased consumption of vitamin C is due foremost to the daily consumption of vitamin C through vitamin tablets and vitamin supplemented food products. Concerns have been raised about the pro-oxidative properties that vitamin C displays in the presence of transition metals. Drinking water could be a source of transition metals needed for pro-oxidation reactions with vitamin C.

The purpose of this thesis was to elucidate:

1. Can vitamin C (ascorbic acid) induce hydroxyl radical formation or accumulate hydrogen peroxide in drinking water?

2. Is there a special milieu that is optimal for the pro-oxidant activity of vitamin C in drinking water?

3. Can a possible pro-oxidant effect of vitamin C be inhibited by ionic species found in drinking water? If so, which ionic species are needed to make vitamin C less pro-oxidative?

4. To which extent is vitamin C degraded in drinking water and what are the degradation products?

EXPERIMENTAL PROCEDURES

1. Materials

Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Coumarin-3-carboxylic was also dissolved in Milli-Q water (18 MΩ cm), and adjusted to pH 8.0 with NaOH. All stock solutions of the reagents used in the experiments were prepared fresh daily. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. Chemicals were obtained from different manufacturers indicated separately in papers I-V.

2. Hydroxyl radical and hydrogen peroxide detection in drinking water

2.1. Measurement of vitamin C induced hydroxyl radical formation with coumarin-3-carboxylic acid as a detector molecule

Hydroxyl radical formation was measured using coumarin-3-carboxylic acid as a detector molecule (Figure 5).[176, 177] When coumarin-3-carboxylic acid is hydroxylated to 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA), a fluorescent product is formed that can be measured with a fluorescence spectrophotometer. The fluorescence of 7-OHCCA is highly pH dependent, showing maximum fluorescence intensity at pH 9.0.[176]

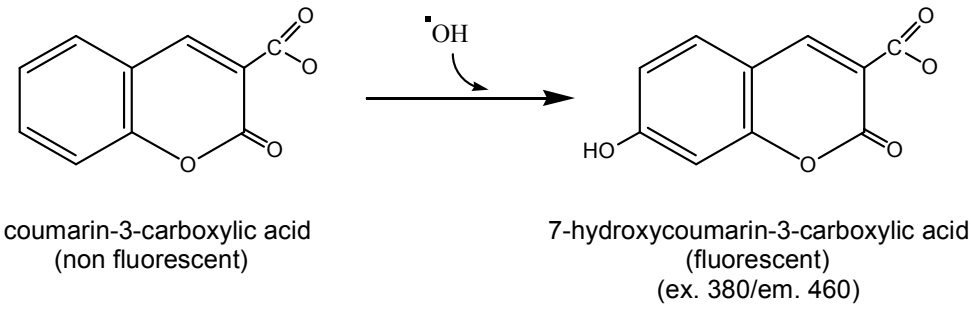

Figure 5. *Hydroxylation of coumarin-3-carboxylic acid to the fluorescent 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA).*

For the assay, 200 µl of the drinking water samples or Milli-Q water supplemented with metals and bicarbonate were pipetted in triplicate onto a microplate. After this, 200 µM coumarin-3-carboxylic acid was added to all wells using an 8-channel multiwell pipette followed by 2 mM ascorbic acid that started the reaction. The microplate was then incubated at room temperature in the dark for various time periods and the reaction was then stopped by pipetting 10 mM TRIS base (pH 9.0) to all wells. Addition of TRIS, a hydroxyl radical scavenger, stopped the reaction and adjusted the pH in the samples to 9.0. The fluorescence was measured with a spectrofluorometer capable of reading the fluorescence from microplates. (Fluoroscan II, Lab systems, Finland). The optical filter set used was excitation 380 nm and emission 460 nm (Figure 6). The fluorescence values were converted into 7-OHCCA formed (nM) from a standard curve where a serial dilution of 7-OHCCA standard in 10 mM TRIS (pH 9.0) was used. All measurements were performed at room temperature.

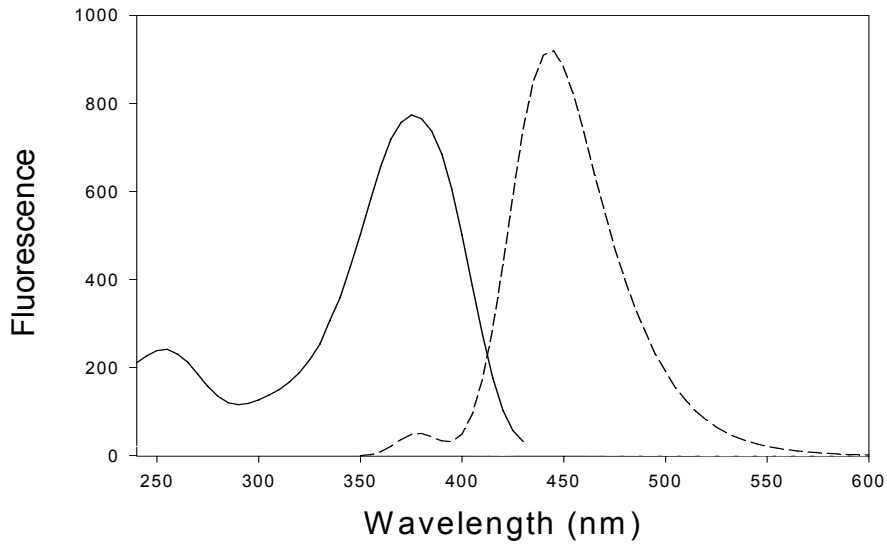

Figure 6. *Excitation (380 nm) and emission (460 nm) wavelengths for 7-OHCCA*

2.2. Measurement of hydroxyl radical formation using plasmid DNA

Hydroxyl radical formation in drinking water was also confirmed using a plasmid DNA assay. The reactions were carried out in a total volume of 10 μl containing 0.3 μg pBluescript DNA in Milli-Q water. After addition of 0.5 mM vitamin C the samples were incubated for 45 min at room temperature and the reaction was stopped by addition of 4 μl sample buffer (50% glycerol, 0.15% bromphenol blue in TRIS-acetate-EDTA buffer (TAE buffer)). The samples were loaded onto a 1% agarose gel in TAE buffer (40 mM TRIS base, 1 mM EDTA, adjusted to pH 7.6 with acetate) and the super coiled (form I) and nicked circular (form II) DNAs were separated at 70 V for 45 min. The gel was then stained with ethidium bromide (10 μg/ml) for 45 min. After washing, the gel was photographed with an AlphaDigiDog gel documentation and image analysis system, Alpha Innotech Corporation, CA.

2.3. Measurement of hydroxyl radical formation by HPLC analysis

Hydroxyl radical generation was also analysed by HPLC with coumarin as a detector molecule. Coumarin readily forms 7-hydroxycoumarin (umbelliferon) when attacked by hydroxyl radicals. An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for the analysis was a Symmetry C18, 250 x 4.6 mm I.D, 10 μm particle size column (Waters). Chromatography was performed using isocratic elution using 150 mM phosphate buffer (KH_2PO_4) containing 30% methanol, pH 3.0 (H_3PO_4). The flow rate was 0.75 ml/min. The peaks were detected at 200 nm and analysed using the Waters Breeze software. For peak identification and calibration we used standards of coumarin, 7-hydroxycoumarin (umbelliferon) in Milli-Q water. All separations were performed at room temperature.

2.4. Measurement of vitamin C induced hydrogen peroxide formation using the FOX assay

Measurement of hydrogen peroxide in metal supplemented Milli-Q water, household drinking water and domestic bottled waters was performed using the FOX assay. In the FOX assay, Fe^{2+} will be oxidized to Fe^{3+} by hydrogen peroxide (oxidizing agent). Fe^{3+} can upon oxidation bind to xylenol orange to give a coloured complex with absorption maximum at 560 nm.[178]

The FOX reagent was prepared by mixing 9 volumes of FOX-1 reagent (4.4 mM 2,6-Di-tert-butyl-4-methanol-phenol in 100% HPLC grade methanol) with 1 volume of FOX-2 reagent (1 mM xylenol orange sodium salt and 2.56 mM ammonium ferrous sulphate in 250 mM sulphuric acid). In the assay, 2 mM of ascorbic acid was added to the different water samples to initiate the reaction. After various time periods, 25 μl samples were withdrawn from the tubes and pipetted into an eppendorf tube containing 750 μl FOX-reagent. The mixture was vortexed for 5 seconds and incubated at room temperature for 30 minutes. After this, 200 μl of the mixture was pipetted in triplicates onto a 96-well microplate and the absorbance of the samples and standards was measured at 560 nm with a Victor plate reader, Wallac. The absorbance values were converted to concentration by comparison with a standard curve where known concentrations of hydrogen peroxide were used.

3. Copper, iron and anion measurements in drinking water samples

3.1. Measurement of copper using diethyldithiocarbamic acid

The presence of trace amounts of copper in the water samples was measured using the copper specific reagent diethyldithiocarbamic acid. For the assay, 200 μl aliquots in triplicate of the water samples were pipetted onto a microplate followed by 300 μM of diethyldithiocarbamic acid. The absorbance of the yellow Cu^{2+}-diethyldithiocarbamic complex was measured at 450 nm with a Victor plate reader, Wallac. The absorbance values were converted to concentration by comparison with a standard curve, generated by adding known amounts of copper chloride to 300 μM diethyldithiocarbamic acid in Milli-Q water.

3.2. Measurement of iron using ferrozine

The iron concentration in the drinking water samples was measured using the iron-specific reagent ferrozine.[179] For the assay, 200 μl aliquots in triplicate of the water samples were pipetted onto a 96-well microplate followed by 400 μM of ferrozine and 100 μM ascorbic acid. Ascorbic acid was used to reduce the Fe^{3+} to the Fe^{2+} form. The coloured Fe^{2+}-ferrozine complex formed was measured at 560 nm using a Victor plate reader, Wallac. The absorbance values were converted to concentration by comparison with a standard curve. The standard curve was generated by adding known amounts of ferric chloride

tetrahydrate, 100 μM ascorbic acid and 400 μM ferrozine to Milli-Q water buffered with 100 mg/l bicarbonate.

3.3. Measurement of bicarbonate and chloride using anion exchange HPLC

An anion exchange column PRP-X100, 150 x 4.1 mm I.D, 10 μm particle size anion exchange column (Hamilton) equipped with a PRP-X-100 guard column (25.0 x 2.3 mm I.D) was used to measure the bicarbonate and chloride in drinking water samples. An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. Chromatography was performed using isocratic elution with 4.0 mM p-hydroxybenzoic acid containing 2.5% methanol, pH 8.5 (NaOH). The flow rate was 2.0 ml/min. The set-up we used for the anion analysis could detect fluoride, bicarbonate, chloride, nitrite, bromide, nitrate, phosphate and sulphate ions. The anions were detected by indirect UV at 310 nm and analysed using the Waters Breeze software. All separations were performed at room temperature.

4. Detection of ascorbic acid and its degradation products in drinking water

4.1. Measurement of ascorbic acid and its degradation products using HPLC

HPLC analysis was used to measure ascorbic acid and its degradation products in drinking water samples, metal-supplemented Milli-Q water and household drinking water. An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for quantization of ascorbic acid and oxalic acid was a Nucleosil C18 150 x 4.1 mm I.D, 10 μm particle size column (Supelco Inc.). Chromatography was performed with isocratic elution using 10 mM phosphate buffer (KH_2PO_4) containing 5% methanol, pH 3.3 (H_3PO_4). The flow rate was 0.5 ml/min. The peaks were detected at 210 nm and analysed using the Waters Breeze software. For the identification and calibration we used standards of DHA, threonic-, oxalic- and ascorbic acid in Milli-Q water. All separations were performed at room temperature.

Using these settings, the peaks for the threonic acid and hydrogen peroxide standards could not be separated with the Nucleosil C18 column. Therefore, to quantitate threonic acid, the anion column PRP-X100 (Hamilton), 150 x 4.1 mm I.D, 10 mm particle size equipped with a PRP-X100 guard column (25.0 x 2.3 mm I.D) was used. Chromatography was performed using isocratic elution using 4.0 mM p-hydroxybenzoic acid containing 2.5% methanol, pH 8.5 (NaOH). The flow rate was 2.0 ml/min. Threonic acid was detected by indirect UV at 310 nm and analysed using the Waters Breeze software.

4.2. Measurement of dehydroascorbic acid with o-phenylenediamine

Due to the difficulty of measuring DHA with HPLC using UV-detection, a more sensitive method was used. To measure DHA formation we therefore used the reagent o-phenylenediamine, a reagent that forms a fluorescent complex with DHA.[180, 181] For the assay, 2 mM ascorbic acid was added to the water samples and incubated at room temperature for various time periods. After this, 2 mM of o-phenylenediamine was added to the tubes followed by 8 mM TRIS buffer. The fluorescent complex was excited at 370 nm, and the emission at 440 nm was measured by a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence values were converted into concentration DHA from a standard curve where known amounts of DHA were used. The actual concentration of DHA in the prepared standards was controlled by the addition of 10 mM dithiothreitol[182-184] followed by HPLC analysis on the ascorbic acid formed. All measurements were performed at room temperature.

RESULTS

1. Vitamin C induces hydroxyl radicals in household drinking water [Paper I]

Vitamin C induced hydroxyl radical generation was studied in drinking water samples by measuring the formation of 7-OHCCA from coumarin-3-carboxylic acid. A marked difference in hydroxyl radical formation was observed over time in the drinking water samples supplemented with 2 mM vitamin C. The highest value, observed among the 22 household tap waters that were screened for hydroxyl radical formation, was around 920 nM of 7-OHCCA as compared to 20 nM of 7-OHCCA in the water sample that gave the lowest signal after a 3 h incubation period with vitamin C. In general, hydroxyl radical formation was lower in waters obtained from private wells as compared to water originating from public water systems. The lowest hydroxyl radical formation was found in domestic sold bottled water.

The hydroxyl radical formation in household drinking water supplemented with vitamin C could also be demonstrated using a DNA nicking assay. The hydroxylation observed in vitamin C supplemented drinking water demonstrated by the coumarin-3-carboxylic acid microplate assay was in good agreement with the DNA nicking assay.

The hydroxyl radical formation in the tap-water samples was strongly dependent on the flushing time before the samples were taken. The formation of 7-OHCCA within 3 h was remarkably decreased when the faucet was flushed for 5 min (from 710 nM to 70 nM). In this water sample the copper concentration decreased from 1.85 mg/l measured in the first drawn to 0.07 mg/l by flushing the faucet for 5 min. On the other hand, flushing did not decrease the formation of 7-OHCCA to the same extent in another sample originating from a different public water system (from around 760 nM to close to 500 nM). In this sample, the copper concentration decreased from 1.24 mg/l to 0.18 mg/l.

2. Ascorbic acid induced hydroxyl radical formation in drinking water is dependent on copper ions and bicarbonate [Paper II]

In paper I we observed that addition of ascorbic acid to some tap water samples contaminated with copper ions could induce hydroxyl radical formation. In paper II it was observed that the amount of copper ions present in the water samples did not always correlate with the amount of hydroxyl radicals generated after ascorbic acid addition.

Therefore, in paper II we wanted to identify the compounds that might be involved in the ascorbic acid induced hydroxyl radical generating reaction observed earlier in drinking water.

To achieve this, we continued our work on two water samples. These samples had been taken in the same way, directly drawn from the tap, but originating from two different municipal water suppliers. Ascorbic acid supplementation led to high amounts of hydroxylated coumarin-3-carboxylic acid in the first sample (above 800 nM) within 3 h. In the other sample, however, hardly any hydroxyl radical formation could be detected after ascorbic acid addition (around 10 nM). When 2 mM ascorbic acid and 200 μM coumarin-3-carboxylic acid were added, the pH in the first drinking water sample decreased rapidly from 7.6 to 5.8 and stabilized at a new pH value close to 6.4. In contrast, when ascorbic acid and coumarin-3-carboxylic acid were added to the other drinking water sample, a much deeper drop in the pH value could be seen. Here, the pH decreased rapidly from 7.2 to 3.8 and remained under pH 4.0.

Anion analysis by HPLC revealed that the drinking water sample generating higher amounts of hydroxyl radicals contained around 90 mg/l of both bicarbonate and chloride. In contrast, the other drinking water sample showed much lower concentrations of bicarbonate and chloride, 30 mg/l and 10 mg/l respectively. No other anions could be detected in the water samples tested (fluoride, nitrite, nitrate, bromide, phosphate or sulphate ions).

The ascorbic acid induced hydroxyl radical formation was highly dependent on the bicarbonate concentration (buffer capacity) in the water samples. This could be demonstrated using Milli-Q water supplemented with 2 mM ascorbic acid, 0.5 mg/l copper and increasing concentrations of bicarbonate. At 50 mg/l bicarbonate a prompt increase in the hydroxyl radical formation was observed. The highest amount of 7-OHCCA was obtained when 100 mg/l bicarbonate was added to the copper supplemented Milli-Q water sample. In the samples that contained 50 mg/l bicarbonate or more, a higher pH value could be measured. In contrast to ascorbic acid, the sodium-, calcium-, and magnesium salts of ascorbic acid could readily trigger hydroxyl radical formation in copper containing Milli-Q water even in the absence of bicarbonate. In the presence of bicarbonate, sodium ascorbate triggered the highest yield of hydroxylated coumarin-3-carboxylic acid at pH 5.0. At higher pH values the signal was lower.

3. Oxidative decomposition of ascorbic acid in drinking water [Paper III]

In paper III we wanted to investigate to what extent ascorbic acid is degraded in copper and bicarbonate containing drinking water. Furthermore, we were interested in the degradation products that are generated from ascorbic acid. In line with paper II, the presence of both copper ions and bicarbonate was required for the degradation of ascorbic acid. This was demonstrated by HPLC analysis on two drinking water samples and a Milli-Q water sample supplemented with 100 mg/l HCO_3^- and 0.5 mg/l Cu^{2+}. As in paper II, the two drinking water samples had been sampled in the same way, directly drawn from the tap, but they originated from two different municipal water suppliers. In the first sample, which contained around 25 mg/l bicarbonate and 0.2 mg/l copper, only about 40% of the added ascorbic acid had been oxidized during the 3 h incubation. On the contrary, when 2 mM ascorbic acid was added to the water sample that contained 150 mg/l bicarbonate and 0.5 mg/l copper, the vitamin was almost completely (93%) oxidized during the 3 h incubation. A similar oxidation process could also be seen when 2 mM vitamin C was added to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper. The addition of 2 mM ascorbic acid to Milli-Q water resulted in a very modest oxidation of the vitamin.

A rapid formation of DHA was observed in the tap water supplemented with ascorbic acid that contained 150 mg/l bicarbonate and 0.5 mg/l copper. About 650 μM of DHA was formed after only 15 min incubation. This rapid formation of DHA was also observed in Milli-Q water supplemented with 2 mM ascorbic acid, 100 mg/l bicarbonate and 0.5 mg/l copper. However, at 15 min, less than 100 μM of DHA had been formed in the sample that contained 25 mg/l bicarbonate and 0.2 mg/l copper. In agreement with this, a very low concentration of DHA could be detected in pure Milli-Q water supplemented with ascorbic acid. Almost no DHA was formed in 4 commercially sold domestic bottled water samples (0-3 μM).

To verify that DHA was formed in our drinking water samples, we used dithiothreitol, a reducing agent that can turn DHA back into ascorbic acid. When dithiothreitol was added to copper and bicarbonate supplemented Milli-Q water that had been incubated with 2 mM ascorbic acid for 3 h, the ascorbic acid peak reappeared in the chromatogram (approximately 680 μM ascorbic acid was formed).

Further analysis revealed that during the 3 h incubation 2 mM ascorbic acid had been oxidized, in bicarbonate-rich and copper-contaminated drinking water, to 710 μM DHA, about 500 μM oxalic acid and 80 μM threonic acid. The oxalic acid and threonic acid were

indicated to be derived from the reaction between DHA and hydrogen peroxide rather than the reaction between ascorbic acid and hydrogen peroxide (see paper III for details). When 2 mM of DHA was added to Milli-Q water supplemented with 4 mM hydrogen peroxide and 100 mg/l bicarbonate, 650 μM oxalic acid and 200 μM threonic acid were formed within 10 minutes. In the absence of hydrogen peroxide, 80% less oxalic acid was formed in a Milli-Q water sample containing 2 mM DHA buffered with 100 mg/l bicarbonate. In contrast, 3 hours were required for 2 mM ascorbic acid to be degraded to the same amount of oxalic acid and threonic acid by hydrogen peroxide in bicarbonate-buffered Milli-Q water.

4. Vitamin C/copper-induced hydroxyl radical formation can be inhibited by iron [Paper IV]

In line with our earlier findings, very low concentrations of copper (0.01-0.05 mg/l) were sufficient to give a detectable hydroxyl radical signal in an ascorbic acid supplemented bicarbonate buffered Milli-Q water sample. On the contrary, neither Fe^{2+} nor Fe^{3+} could support any hydroxyl radical formation in the ascorbic acid supplemented bicarbonate buffered Milli-Q water sample. The same was true for manganese, cadmium, nickel, cobalt, aluminum, magnesium, calcium and zinc (as chloride salts) or gallium (as nitrate salt), as none of these metals could induce hydroxyl radical formation in ascorbic acid supplemented bicarbonate buffered Milli-Q water sample. All these metals were tested using the highest amounts of the contaminants that are allowed in drinking water, MCL (data not shown).

HPLC analysis, with coumarin as a target molecule, was used to elucidate the effects of iron on vitamin C induced hydroxyl radical formation in the presence of copper and bicarbonate. We focused our analysis on one of these hydroxylated compounds, namely 7-hydroxycoumarin. Once again, within 3 h one of the hydroxylation products of coumarin, 7-hydroxycoumarin, could be observed in copper and ascorbic acid supplemented bicarbonate-buffered Milli-Q water. If 0.1 mg/l copper was substituted with 0.2 mg/l ferric iron (MCL of iron in EU), no hydroxylation occurred. Furthermore, 48% inhibition of ascorbic acid/copper-catalyzed hydroxyl radical formation was observed with 0.2 mg/l of iron. Manganese, cadmium, nickel, cobalt, gallium, aluminum, magnesium, calcium or zinc had no inhibitory effect on the ascorbic acid/copper-mediated hydroxyl radical formation.

This inhibitory effect of iron on vitamin C/copper-induced hydroxyl radical formation was also reflected in household drinking water samples. The copper concentration in the

different water samples varied from 0.13 to 0.02 mg/l and the bicarbonate concentration varied between 80 and 130 mg/l. The water samples used in the assay did not contain any detectable iron. Addition of 0.2 mg/l ferric iron to the ascorbic acid supplemented household drinking water samples resulted in a 36 - 45% inhibition in the hydroxyl radical formation. When the household drinking water samples were supplemented with 0.8 mg/l ferric iron the inhibition was even higher, 47 – 60%.

5. Ascorbic acid induced hydrogen peroxide accumulation in household drinking water - interaction between copper, iron and bicarbonate [Paper V]

In this study we evaluated whether ascorbic acid can also stimulate hydrogen peroxide formation in Cu^{2+} contaminated drinking water. To establish this, we again used Milli-Q water as a model solvent for drinking water. The results showed that ascorbic acid induces a substantial increase in the hydrogen peroxide concentration (500 μM) in Milli-Q water supplemented with 0.1 mg/l of Cu^{2+}. This Cu^{2+} concentration is 20 times below the amount of copper that is allowed in drinking water in Europe (MCL). Already after 1-hour incubation, almost 300 μM hydrogen peroxide could be detected. On the contrary, ascorbic acid and Fe^{3+} induced very low concentrations of hydrogen peroxide (45 μM) after 6 hours in Milli-Q water. Other metals tested at MCL levels with ascorbic acid (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} or Al^{2+}) generated between 60 μM and 120 μM of hydrogen peroxide after 6 hours.

Although Fe^{3+} did not induce hydrogen peroxide formation in ascorbic acid supplemented Milli-Q water, the inhibitory effect of iron on hydrogen peroxide formation could be observed in the ascorbic acid/Cu^{2+} supplemented Milli-Q water. The inhibition by 0.2 mg/l ferric iron was very weak during the first hour. However, after 2 h incubation, the hydrogen peroxide production stopped, and the concentration started to decline. After 6 h incubation the hydrogen peroxide level had decreased to about 60 μM as compared to around 500 μM measured in the control sample not containing iron. Other metal ion species that might be present in drinking water, Zn^{2+}, Co^{2+}, Mn^{2+} or Al^{2+} did not have any impact at their MCL levels on the ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation. A modest inhibition in hydrogen peroxide formation could be seen when Mg^{2+} or Ca^{2+} were present. However, the concentrations of Mg^{2+} and Ca^{2+} were 250 and 500 fold higher than the concentration of iron used in the assay.

Oxalic acid was found to be the important factor that assisted the iron inhibitory effect on ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation. Previously, in paper III we established that oxalic acid is one of the degradation products generated when ascorbic acid is oxidatively decomposed in copper-contaminated drinking water. Furthermore, oxalic acid is known to have a high affinity for ferric iron even at very low pH. This was verified when 0.1 mg/l Cu^{2+} and 2 mM ascorbic acid were added to Milli-Q water supplemented with both iron and oxalic acid. When 0.2 mg/l Fe^{3+} and 50 μM oxalic acid were present from the start of the reaction, extremely low concentrations of hydrogen peroxide could be detected in the ascorbic acid/Cu^{2+} supplemented Milli-Q water. Likewise, when 0.2 mg/l Fe^{3+} and 50 μM oxalic acid were added to the ascorbic acid/Cu^{2+}-induced hydrogen peroxide generating reaction after 2 h incubation, the hydrogen peroxide concentration in the sample rapidly decreased. Oxalic acid also directly inhibited the ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation.

The hydrogen peroxide formation was also demonstrated in 40 tap water samples and 8 domestic bottled water samples incubated with ascorbic acid during a 6-hour period. When 2 mM ascorbic acid was added to the low-buffered drinking water samples that were contaminated with copper, hydrogen peroxide was generated. The levels of hydrogen peroxide generated varied between 0 and 500 μM in the water samples. However, in some of the drinking water samples that were contaminated with copper but showed higher buffering capacity, much lower levels of hydrogen peroxide was formed in the presence of ascorbic acid. The impact of bicarbonate on the hydrogen peroxide formation was also demonstrated in ascorbic acid/Cu^{2+} supplemented Milli-Q water that had been supplemented with various concentrations of bicarbonate. The ascorbic acid induced accumulation of hydrogen peroxide in the presence of 0.1 mg/l copper was significantly decreased when the concentrations of bicarbonate was increased.

DISCUSSION

Nowadays, ascorbic acid is added to food and health products more than ever before. This is based on the fact that ascorbic acid can work as an antioxidant. However, ascorbic acid can easily redox-cycle with unbound transition metals such as iron and copper and thus work as a strong pro-oxidant.[5, 6]

The majority of the population of Europe use tap water instead of bottled water as their drinking water. The drinking water leaving the water plants is normally of good quality, rigorously checked for taste and chemical composition. However, the tap water reaching homes through the pipes might be contaminated with minerals such as copper, iron, lead, chromium or arsenic. The degree of contamination is highly dependent on how corrosive the water is, the material used in the pipes and fittings and the time the water has been sitting in the pipes before use.[163] Thus, if a reducing agent is added to a metal-contaminated water of this kind, a free radical generating reaction might occur.

In view of this and the fact that drinking water can be contaminated with copper, we decided to study whether vitamin C can induce hydroxyl radical formation or hydrogen peroxide accumulation in tap water samples. In our studies, 2 mM of vitamin C was used. This equals 70 mg of vitamin C in a 2 dl glass of water, and thus the concentration used is biologically relevant.

1. Vitamin C (Ascorbic acid) induces hydroxyl radicals and hydrogen peroxide in copper-contaminated drinking water

Hydroxyl radical and hydrogen peroxide is formed in drinking water if ascorbic acid is added (Paper I, II, IV and V). The pro-oxidative function of ascorbic acid in drinking water is primarily dependent on trace amounts of copper ions present. None of the other metals tested (iron, manganese, cadmium, nickel, cobalt, aluminium, magnesium, calcium or zinc) could not significantly support ascorbic acid induced hydroxyl radical or hydrogen peroxide formation in drinking water (Paper IV and V).

Our results indicated that there was a large variability in hydroxyl radical and hydrogen peroxide formation in the water samples originating from various municipal water suppliers. In general, hydroxyl radical formation was most evident in household tap water samples as compared to the hydroxyl radical formation in tap water originating from private wells or commercially sold bottled spring water.

Furthermore, we found that the hydroxyl radical and hydrogen peroxide formation in some of the ascorbic acid supplemented tap-water samples was strongly dependent on the flushing time before the samples were taken. The explanation for this is that the first drawn sample can be contaminated with copper from the pipes within the apartment (building).[172, 173] Indeed, the copper concentration in one of the tap-water samples decreased from 1.85 mg/l (measured in the first-drawn sample) to 0.07 mg/l after flushing the tap for 5 min (Paper I).

2. The concentration of bicarbonate and pH regulate whether vitamin C catalyzes hydroxyl radical or hydrogen peroxide formation in copper contaminated drinking water

Anion analysis by HPLC indicated that a buffering anion such as bicarbonate was required for ascorbic acid to redox-cycle copper and generate hydroxyl radicals in the tap water samples. Ascorbic acid stimulated hydroxyl radical formation in a copper-contaminated drinking water buffered with high amounts of bicarbonate (Paper II), and hydrogen peroxide formation in weakly bicarbonate-buffered drinking water (Paper IV).

2.1. Vitamin C catalyzes the formation of hydroxyl radicals in strongly bicarbonate-buffered copper-contaminated drinking water

The importance of bicarbonate and copper ions for the ascorbic acid induced hydroxyl radical formation was studied in detail in a controlled Milli-Q water system (Paper II). As little as 50 mg/l bicarbonate was enough to buffer the pH in Milli-Q water and keep the pH above 4.25 after ascorbic acid addition. The pKa_1 of ascorbic acid is 4.25. When the pH is above 4.25, the ascorbate mono-anion will dominate and this form can be further oxidized with concomitant reduction of Cu^{2+} to Cu^{+}. The reduced copper (Cu^{+}) can in turn react with oxygen and generate superoxide. At higher pH values rapid redox-cycling of copper will generate superoxide, peroxide and hydroxyl radicals via a copper-assisted Fenton reaction (see below: Equation 15-18). At lower pH, the percentage of superoxide in the water also decreases when superoxide anion reacts with a hydrogen proton to form the hydroperoxyl radical ($HO_2^{\bullet}$).

$$Cu^{2+} + Asc \rightarrow Cu^{+} + Asc^{\bullet} \quad \text{Eq. 15}$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -} \quad \text{Eq. 16}$$

$$Cu^{+} + O_2^{\bullet -} + 2H^{+} \rightarrow Cu^{2+} + H_2O_2 \quad \text{Eq. 17}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet} \quad \text{Eq. 18}$$

Our results in paper II stress the importance of the buffering capacity in the water sample for ascorbic acid to stimulate hydroxyl radical formation. Anion analyses by HPLC also detected chloride ions, but the amounts detected in drinking water, as well as the highest amount of chloride ions that is allowed in drinking water, (MCL in Europe) did not affect hydroxyl radical formation. In the presence of bicarbonate, ascorbic acid induced the strongest signal as compared to the sodium-, calcium-, or magnesium salts of ascorbic acid. When the salts of ascorbate were used, the pH in the solution was 7.5 and the hydroxyl radical signal was 35-40% lower. At this pH, the bicarbonate ions (HCO_3^-) are the dominating species in the buffer (Figure 4). Bicarbonate ions present in the solution can rapidly react with the hydroxyl radicals and form bicarbonate- or carbonate radicals.[185]

Vitamin C is one of the most studied antioxidants and it has previously been suggested that vitamin C might inhibit N-nitroso compound-induced gastric carcinoma.[186-189] In this condition, vitamin C has been studied in the absence of transition metals, such as copper ions, and should thus function as an antioxidant. However, one could speculate that bicarbonate *in vivo* in some special compartments, e.g. in the stomach or urinary bladder, could locally increase the pH and thereby assist vitamin C in stimulating hydroxyl radical formation in the presence of transition metals. ROS have been documented as being involved in gastric ulceration and cancer.[153, 190-192] Kadiiska *et al.* reported that installation of high levels of ascorbate with copper ions into the stomach of animals leads to hydroxyl radical formation.[193] Hydroxyl radicals have also been measured in human gastric juice.[194] Thus, bicarbonate and copper could in fact, turn vitamin C (ascorbic acid) into a strong pro-oxidant. In line with this assumption, bicarbonate and vitamin C has been shown to promote (N-butyl-N-(4-hydroxybutyl) nitrosamine) induced bladder carcinogenesis in rats.[195-197] Moreover, it has been shown that alkaline urinary pH promotes bladder carcinogenesis in rat model systems.[198, 199] Interestingly, ascorbic acid in the presence of bicarbonate,[195, 196] or sodium ascorbate,[200, 201] was used in these studies.

The vitamin C induced hydroxyl radical formation that takes place in copper-contaminated drinking water might also explain the recently observed occurrence of benzene in carbonated soft drinks supplemented with vitamin C and benzoic acid. It has been shown that hydroxyl radicals can catalyze decarboxylation reactions and therefore hydroxyl radicals

might turn the preservative benzoic acid into the highly carcinogenic benzene in beverages.[202]

2.2. Vitamin C catalyzes the formation of hydrogen peroxide in weakly bicarbonate-buffered copper-contaminated drinking water

Paper V shows that ascorbic acid can initiate a time-dependent accumulation of hydrogen peroxide in copper-contaminated poorly buffered drinking water. Ascorbic acid induces a substantial increase in the hydrogen peroxide concentration (about 500 μM) in Milli-Q water (bicarbonate free) supplemented with copper ions after 6 hours incubation. Some drinking waters generated close to 500 μM of hydrogen peroxide within 6 hours, reaching close to 300 μM hydrogen peroxide in only 2 hours. Hydrogen peroxide formation occurred at very low concentration of Cu^{2+} ions (0.1 mg/l) (20 times below the amount of copper that is allowed in drinking water, MCL in Europe). Only cobalt (II) generated small amounts of hydrogen peroxide (around 120 μM) during acidic conditions with vitamin C after 6 hours incubation.

The ascorbic acid induced hydrogen peroxide formation was particularly evident in poorly buffered (low bicarbonate concentration) copper-contaminated drinking water samples. When bicarbonate was added to Milli-Q water supplemented with copper, the hydrogen peroxide accumulation was much lower. These results were consistent with the results from study in paper II, where hydrogen peroxide is converted to hydroxyl radicals.

The ascorbic acid induced hydrogen peroxide accumulation in copper supplemented Milli-Q water (bicarbonate free) was significantly higher than the ascorbic acid induced hydrogen peroxide formation earlier observed in cell culture and cell culture medium.[203, 204] However, our experiments were carried out in a much more acidic milieu (pH 3.5) compared to the experiments in the buffered cell culture medium. Furthermore, the copper concentration in our experiments was much higher as compared to the trace amount of copper that normally is present in cell culture medium supplemented with serum. Also, the pH in our vitamin C supplemented drinking water samples was close to or below the pKa_1 value of ascorbic acid (4.25). In more neutral conditions (pH 6-6.5) that were obtained when magnesium ascorbate, calcium ascorbate or sodium ascorbate were added to Cu^{2+} supplemented Milli-Q water, less hydrogen peroxide was generated within 6 hours (data not shown). The results obtained from these experiments are in better agreement with the amounts of hydrogen peroxide that have been reported to accumulate in cell cultures and cell culture medium.[203, 204]

3. Iron inhibits vitamin C (ascorbic acid) induced hydroxyl radical formation and hydrogen peroxide accumulation in drinking water

Iron displays an inhibitory effect on the ascorbic acid induced hydroxyl radical formation and hydrogen peroxide accumulation when copper ions are present in drinking water (Paper IV and V). The study in paper V demonstrates that ascorbic acid-derived oxalic acid is necessary for the inhibitory effect of iron on ascorbic acid/Cu^{2+} induced hydrogen peroxide accumulation. It was confirmed that none of the other metals (manganese, cadmium, nickel, cobalt, aluminium magnesium, calcium or zinc) could inhibit the vitamin C and copper-driven hydroxyl radical and hydrogen peroxide formation. All these metals were tested at MCL.

An interaction between oxalic acid and copper was also observed as oxalic acid decreased the ascorbic acid/Cu^{2+} induced hydrogen peroxide formation in Milli-Q water. Indeed, oxalic acid has a high affinity for Cu^{2+} and forms metal complexes with Cu^{2+} ions as well as with Fe^{3+} ions even at very low pH.[205] The inhibitory effect of oxalic acid on ascorbic acid/Cu^{2+} induced hydrogen peroxide formation in Milli-Q water was confirmed not to be due to Cu^{2+}/oxalic acid catalyzing hydrogen peroxide to hydroxyl radicals. Instead, the inhibitory effect of oxalic acid on ascorbic acid/copper induced hydrogen peroxide formation was implied to originate from oxalic acid's ability to interfere with copper redox-cycling. Interestingly, it has been suggested that oxalic acid could act as an antioxidant in plants, because oxalic acid reduces the rate of ascorbic acid oxidation in the presence of hydrogen peroxide and Cu^{2+}.[206]

The results of the study in paper IV are in agreement with the recent report by White *et al.*, demonstrating that iron can inhibit reductant-mediated copper and hydrogen peroxide generation and neurotoxicity.[207] Moreover, our results are in line with the recent findings by Munday *et al.* showing that copper-catalyzed cysteine oxidation can be partly inhibited by low concentrations of iron salts.[208] In this context, it can also be mentioned that Menditto *et al.* showed that loading seminal plasma with either ferrous or ferric iron up to a concentration of 50 μM only modestly affected the rate of ascorbic acid oxidation.[209] The low oxidation rate of ascorbic acid by iron was also seen in our *in vitro* experiments (data not shown). However, low concentrations of copper, as shown in the study in paper III and IV, induce rapid oxidation of ascorbic acid.[209] One question to be addressed is thus whether simultaneous administration of iron could slow down copper/ascorbic acid-mediated processes.

The inhibition is unlikely to result from an experimental artefact since it is well known that coumarin-3-carboxylic acid can be used to detect iron driven hydroxyl radical reactions.[138, 210, 211] Moreover, our HPLC experiments using coumarin as the target molecule gave the same results. A plausible explanation for the iron-induced inhibition could be that Fe^{3+} reacts with the superoxide generated from the copper/ascorbate redox reaction (Equation 5). Fe^{2+} might also react with hydrogen peroxide and generate water and ferryl ions according to the Bray-Gorin reaction (Equation 19).[212]

$$Fe^{2+} + H_2O_2 \rightarrow [Fe^{4+}O]^{2+} + H_2O \quad \text{(Bray-Gorin)} \qquad \text{Eq. 19}$$

The ability of Fe^{3+} and oxalic acid to regulate ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation during acidic conditions (Paper V), could be of importance *in vivo* when acidic conditions prevail. Thus, when Cu^{2+} ions and vitamin C are present at acidic conditions, the presence or absence of free redox-active iron and oxalic acid will determine how much hydrogen peroxide will be accumulated. In particular, during conditions where catalase is not present or not functioning properly (e.g. in the presence of ascorbic acid and copper),[213, 214] the iron/oxalic acid complex could be of importance in regulating hydrogen peroxide toxicity during acidic conditions in various biological systems *in vivo*. Overall, our results support earlier reports demonstrating that Fe^{3+} can inhibit the cytotoxic effect of hydrogen peroxide in cell systems induced by ascorbic acid.[39, 42, 207]

Hydrogen peroxide has been shown to have strong antibacterial effects.[215] One might speculate whether the ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation and the ability of Fe^{3+} and oxalic acid to regulate hydrogen peroxide formation, as demonstrated here in drinking water, could be involved in controlling the survival of pathogenic bacteria in the human gastrointestinal tract. Higher intake of vitamin C has been associated with lower incidence of gastric cancer, for which *H. pylori* is a significant risk factor.[216]

Normally, catalase will catalyze hydrogen peroxide into water and oxygen. However, substantial quantities of hydrogen peroxide (>100 μM), can be detected in freshly voided human urine.[217, 218] For instance, hydrogen peroxide is surprisingly rapidly formed in freshly prepared coffee and ingestion of coffee containing hydrogen peroxide has been shown to increase the hydrogen peroxide concentration in urine.[219] Thus, a question to be addressed is whether hydrogen peroxide formation in copper-contaminated drinking water, as observed in our study with vitamin C, could result in increased hydrogen peroxide load. Hydrogen peroxide is diffusible within and between cells,[152, 153] and therefore hydrogen

peroxide might have an impact on the urinary tract.[152, 220-222] Interestingly, there have been suggestions that urinary hydrogen peroxide can be a marker of oxidative stress in malignancies.[152, 217, 218, 223, 224] In particular, in the absence of Fe^{3+} ions, ascorbic acid/Cu^{2+}-induced hydrogen peroxide formation in drinking water as demonstrated in our study in paper V, might enhance the total hydrogen peroxide load and hydrogen peroxide induced oxidative stress in some individuals.

4. Rapid decomposition of vitamin C (ascorbic acid) in bicarbonate buffered copper-contaminated drinking water

In paper III we wanted to see how rapidly and to what extent ascorbic acid can be oxidized in a copper-contaminated drinking water sample. In line with paper II, the presence of both copper ions and bicarbonate was required for the degradation of ascorbic acid. Ascorbic acid degradation by copper as a catalyst has been well documented in parenteral mixtures and cooking of vegetables in copper-contaminated water.[225] In our study, approximately one-third of the ascorbic acid added (2 mM) to a sample that contained 150 mg/l bicarbonate and 0.5 mg/l copper had been oxidized after 15 minutes and the vitamin was almost completely (93%) oxidized within 3 hours. On the contrary, in another sample that contained 26 mg/l bicarbonate and 0.2 mg/l copper, only 39% of the added ascorbic acid had been oxidized during the 3 h incubation.

Low concentrations of vitamin C in gastric juice have been associated with *Helicobacter pylori* infection. The *Helicobacter pylori* infection is the main cause of chronic gastritis and increases the risk of gastric cancer.[226-228] Inflammation in the stomach induced by *H. pylori* causes significantly enhanced depletion of vitamin C, but also reduces secretion of the vitamin into the gastric lumen.[229-231] On the other hand, it has not been studied whether vitamin C consumed with a copper/bicarbonate-rich water could be linked to low gastric concentration of vitamin C, and increased susceptibility to *H. pylori* infection.

When ascorbic acid was oxidized in the presence of copper ions, DHA was formed. To verify that DHA was formed in our drinking water samples, we used dithiothreitol as a reducing agent.[183, 184] Dithiothreitol turned DHA in the samples back into ascorbic acid. DHA has previously been shown to be spontaneously decomposed into L-diketogulonate (2,3-DKG) or erythroascorbate.[72, 232, 233] Our results indicated that DHA, and not diketogulonate, was present in the sample. The DHA formation in the bicarbonate-rich water sample contaminated with copper was very rapid, as up to 650 μM DHA was formed within 15 min. The amount of DHA formed within 15 minutes in the various tap water

samples tested was in the range of 100-650 μM. On the contrary, if commercially sold domestic bottled water was used in the assay, a very modest degradation of ascorbic acid took place. Thus, people consuming copper-contaminated drinking water with ascorbic acid will most likely ingest, or generate more DHA in their stomach than people using bottled water.

The impact of long-term intake of DHA on human health has not yet been elucidated. DHA can enter cells via the GLUT glucose transporter.[234] Intracellular reduction of DHA to ascorbic acid by NADPH- and glutathione-dependent reactions decreases the cellular concentrations of NADPH and glutathione in some celltypes.[235-237] In line with these findings, DHA has been shown to cause oxidative stress and apoptosis in pancreatic and neural cells by depleting their intracellular store of reduced glutathione.[238, 239]

In the water samples, DHA was further decomposed into two major metabolites, oxalic acid and threonic acid. Our results indicated that the hydrogen peroxide formed, and not the hydroxyl radicals generated during the reaction, was responsible for the DHA decomposition in the water samples. This is in line with a report by Isbell *et al.* showing that hydrogen peroxide and DHA can react directly at pH 7 to produce oxalic acid and threonic acid.[240]

It has been proposed that high concentrations of oxalic acid (calcium oxalate) can promote the formation of kidney stones.[241-243] It has also been suggested that vitamin C could be a source of oxalic acid in kidney stones,[241] but no reasonable support for such a proposal has been published.[10] One criticism has been that increased oxalate excretion in urine might be an artefact resulting from the spontaneous degradation of vitamin C into oxalate in the urine samples.[244] Oxalate formation during ingestion of vitamin C and copper-contaminated water has not yet been studied, but the results presented in our study (Paper III) might indicate that substantial amounts of oxalic acid could be generated if ascorbic acid is consumed with copper-contaminated bicarbonate-buffered drinking water.

Excess intake of vitamin C is regarded as safe since vitamin C is known to be excreted in urine due to its water solubility.[10, 11] Interestingly, our results show substantial DHA and oxalic acid accumulation from vitamin C in copper-contaminated drinking water. A question that would need to be addressed is whether copper-contaminated drinking water can deplete vitamin C and hence interfere with vitamin C uptake from the stomach and intestine, leading to vitamin C deficiencies. Thus, could our copper pipes, like the copper pans once used in cooking, be linked to vitamin C deficiencies?

CONCLUSIONS

The main focus of this thesis was to study the pro-oxidant effect of vitamin C (ascorbic acid) in metal contaminated drinking water. The main findings of this study are:

1. Bicarbonate and pH regulate the pro-oxidant activity of ascorbic acid in drinking water contaminated with trace amounts of copper. Ascorbic acid will induce hydroxyl radicals in copper-contaminated drinking water buffered with high amounts of bicarbonate (II), and hydrogen peroxide in weakly bicarbonate-buffered drinking water (IV). Of the other metals tested (iron, manganese, cadmium, nickel, cobalt, aluminium, magnesium, calcium or zinc), none could significantly support ascorbic acid induced hydroxyl radicals or hydrogen peroxide formation in drinking water (IV, V).

Ascorbic acid and copper induced hydroxyl radical formation and hydrogen peroxide accumulation could be important modulators of various molecules. The pro-oxidant activity of vitamin C in the presence of copper ions as observed in the bicarbonate-buffered drinking water might be linked to the reported occurrence of benzene in beverages supplemented with vitamin C and benzoic acid.[202] Furthermore, the vitamin C/Cu^{2+}-induced hydrogen peroxide formation during acidic conditions, as demonstrated in poorly buffered drinking water, could be of importance in host defence against bacterial infections. On the other hand, excessive production of hydrogen peroxide could induce increased oxidative stress on tissues, or alternatively be involved in cell activation.

2. Iron displays an inhibitory effect on the ascorbic acid/copper induced hydroxyl radical formation, as well as on hydrogen peroxide accumulation in drinking water (IV, V). Paper V demonstrates that ascorbic acid-derived oxalic acid is necessary for the inhibitory effect of iron on ascorbic acid/Cu^{2+}-induced hydrogen peroxide accumulation.

Bicarbonate and iron might function as important regulators of copper/reductant-induced hydroxyl radical/hydrogen peroxide formation and copper mediated tissue damage. The iron/oxalic acid induced inhibition of hydrogen peroxide formation as demonstrated here in drinking water might also be linked to the protective effect of iron against ascorbic acid (vitamin C) and copper-induced cell toxicity in cell cultures. Our results in paper IV and V also indicate that complete removal of iron from the raw water of the water plants can to some extent increase the redox activity of copper, and the formation of ROS in drinking water.

3. Significant amounts of ascorbic acid are rapidly oxidized into DHA when added to a strongly bicarbonate-buffered copper-contaminated drinking water (III). The results also demonstrate that DHA is further decomposed into oxalic- and threonic acid in bicarbonate-buffered copper-contaminated drinking water. Thus, copper-contaminated drinking water could possibly deplete vitamin C and hence interfere with vitamin C uptake from the stomach and intestine, leading to vitamin C deficiencies. Furthermore, people consuming this type of water will most likely ingest, or generate more DHA in their stomachs than people using bottled water. The impact of long-term intake of DHA and oxalic acid obtained from vitamin C supplemented copper-contaminated drinking water is not known.

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Measurement of Ascorbic Acid (Vitamin C) Induced Hydroxyl Radical Generation in Household Drinking Water

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Ascorbic acid (vitamin C) induced hydroxyl radical formation was measured in household drinking water samples using the hydroxyl radical sensitive probe coumarin-3-carboxylic acid. Vitamin C, a reducing agent that is commonly used as a food additive, triggered a significant hydroxyl radical generating reaction when added to the tap-water samples tested. The capacity of ascorbic acid to trigger hydroxyl radical formation in the tap-water samples was dependent on the flushing time before the samples were taken indicating that the water in the copper piping had been contaminated by copper ions. In line with this, high concentrations of copper were measured in the hydroxyl radical generating first-draw samples. Moreover, a strong correlation was found between the hydroxyl radical generation capacity seen in the coumarin-3-carboxylic acid based microplate assay and the DNA damage seen in an agarose gel assay using the pBluescript plasmid. In the water samples showing high capacity to hydroxylate coumarin-3-carboxylic acid, a rapid formation of the open circular form of the plasmid could also be seen indicating a copper assisted hydroxyl radical attack on the DNA. In conclusion, our results show that addition of vitamin C to household tap water that is contaminated with copper ions, results in Fenton type reactions that continuously generate harmful and reactive hydroxyl radicals.

Keywords: Hydroxyl radical; Water; Coumarin-3-carboxylic acid; Vitamin C; Copper

Abbreviations: $OH^{\bullet}$, hydroxyl radical; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; TRIS, tris(hydroxymethyl)aminomethane; 3-CCA, coumarin-3-carboxylic acid; H_2O_2, hydrogen peroxide; 7-OHCCA, 7-hydroxycoumarin-3-carboxylic acid; TAE, tris-acetate-EDTA

INTRODUCTION

Hydroxyl radicals ($OH^{\bullet}$) are molecules that have one unpaired electron and are therefore very reactive and easily attack any nearby molecule by taking or giving one electron. In the laboratory, hydroxyl radicals can be easily generated by UV-induced homolytic fission of hydrogen peroxide or by metal-catalyzed (copper or iron) reduction of oxygen to superoxide that reacts with hydrogen peroxide (Haber–Weiss reaction: $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + OH^{-}$).[1] Moreover, hydroxyl radicals can directly be generated from hydrogen peroxide by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$.[2,3] This reaction can be highly catalyzed if certain metal chelators such as EDTA, NTA and a reducing substance such as ascorbic acid (vitamin C) are present.[4] Thus, hydroxyl radicals can be generated in a system where oxygen, copper or iron and a reducing agent such as vitamin C are present.[5] The formation of hydroxyl radicals has been studied by using the spin trap technique,[6] aromatic hydroxylation of target molecules[7–9] and by studying radical attack on target molecules such as DNA[10] and deoxyribose.[11]

Tap water might be contaminated to various degrees by copper ions due to corrosion in the pipes. On the basis of this, and the fact that vitamin C is today added in high concentrations to a variety of food sources and drinks, we decided to study

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whether vitamin C can trigger hydroxyl radical generation in tap-water samples. In particular, we wanted to study hydroxyl radical reactions that might take place in tap-water samples originating from plumbing systems that had not been used for a few days "unflushed" drinking water. By using coumarin-3-carboxylic acid as hydroxyl radical indicator we demonstrate that there exists a large variability in vitamin C-induced hydroxyl radical generation in household tap waters originating from different municipal water systems.

MATERIAL AND METHODS

Chemicals

Coumarin-3-carboxylic acid and 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) were from Fluka, Switzerland. Coumarin-3-carboxylic acid was dissolved in Milli-Q water (18 MΩ cm), and pH adjusted to 8.0 with NaOH. Ascorbic acid and cuprous chloride were purchased from Riedel-deHaen, Germany. All other reagents were from Sigma, St. Louis, USA. pBluescript was purchased from Stratagene (La Jolla, CA). SeaKem, LE Agarose was from FMC Bioproducts. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of Vitamin C-induced Hydroxyl Radical Formation in Drinking Water

Hydroxyl radical formation was measured by using coumarin-3-carboxylic acid as detector molecule.[12,13] When this compound is hydroxylated to 7-hydroxycoumarin-3-carboxylic acid, a fluorescent product is formed that can easily be measured with a fluorescence spectrophotometer. The fluorescence of 7-hydroxycoumarin-3-carboxylic acid is highly pH dependent showing maximum fluorescence intensity at pH 9.0.[12]

For the assay, 200 µl of the water samples were pipetted in triplicate onto a microplate. After this, 200 µM coumarin-3-carboxylic acid was added to all wells by using a 8 channel multiwell pipette followed by 2 mM vitamin C that started the reaction. The microplate was then incubated at room temperature in dark for various time periods and the reaction was then stopped by pipetting 10 mM TRIS base (pH 9.0) to all wells. Addition of TRIS, a hydroxyl radical scavenger, stopped the reaction and adjusted the pH in the samples to 9.0. The fluorescence was measured with a spectrofluorometer capable of reading the fluorescence from microplates. (Fluoroscan II, Labsystems, Finland). The optical filter set used was excitation 380 nm and emission 460 nm. The fluorescence values were converted into 7-OHCCA formed (nM) from a standard curve where a serial dilution of 7-hydroxycoumarin-3-carboxylic acid standard in 10 mM TRIS (pH 9.0) was used. All measurements were done at room temperature.

Measurement of Hydroxyl Radical Formation in Household Drinking Water by using Plasmid DNA

The reactions were carried out in a total volume of 10 µl containing 0.3 ug pBluescript DNA in milli-Q water. After addition of 0.5 mM vitamin C the samples were incubated for 45 min at room temperature and the reaction was stopped by addition of 4 µl sample buffer (50 % glycerol, 0.15% bromphenol blue in TAE buffer). The samples were loaded on to a 1% agarose gel in TRIS–acetate–EDTA buffer (40 mM TRIS base, 1 mM EDTA, adjusted to pH 7.6 with acetate) and the supercoiled (form I) and nicked circular (form II) DNAs were separated at 70 V for 45 min. The gel was then stained with ethidium bromide (10 ug/ml) for 45 min. After washing, the gel was photographed with an AlphaDigiDog gel documentation and image analysis system, Alpha Innotech Corporation, CA.

Measurement of Copper by using Diethyldithiocarbamic Acid

The presence of trace amounts of copper in the water samples was measured using the copper specific reagent diethyldithiocarbamic acid. For the assay, 200 µl aliquots in triplicate of the water samples were pipetted onto a microwell plate followed by 300 µM of diethyldithiocarbamic acid. The yellow Cu^{2+}-diethyldithiocarbamic complex was measured at 450 nm with a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve, generated by adding known amounts of copper chloride to 300 µM diethyldithiocarbamic acid in milli-Q water.

RESULTS

Time Course of Vitamin C-induced Hydroxyl Radical Formation in Drinking Water

To measure whether hydroxyl radical formation can be generated in household drinking water we initially tested two water samples obtained from two different municipal water supplies and compared

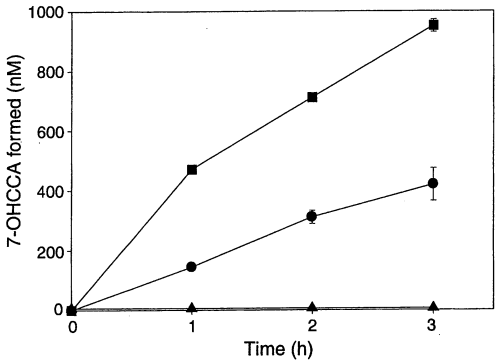

FIGURE 1 The kinetics hydroxyl radical formation in tap-water samples. At time zero, 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C were added to water sample A (●), B (■) or to milli-Q water (▲). After 1, 2 or 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. Data points are mean ± SD of triplicates from one representative experiment out of three conducted. Where absent, bars were smaller than the symbol.

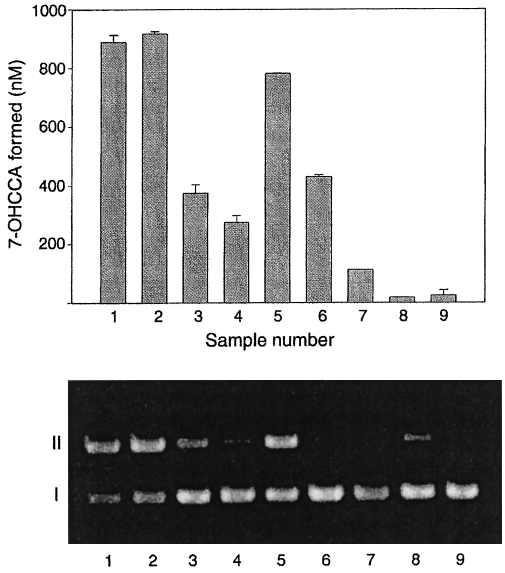

FIGURE 2 Vitamin C-induced hydroxyl radical formation in various tap-water samples. Upper panel: 200 μM coumarin-3-carboxylic acid was added to various tap-water samples followed by 0.5 mM of vitamin C. After 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. The bars show the amount of 7-hydroxycoumarin-3-carboxylic acid formed during the 3 h incubation and represent means ± SD of triplicates. Data are representative of three similar experiments. Lower panel: agarose gel electrophoresis of pBluescript DNA exposed to vitamin C in various tap-water samples. pBluescript DNA was dissolved in tap-water samples and treated for 45 min with 0.5 mM vitamin C at room temperature. The bands seen on the agarose gel are (I) supercoiled DNA and (II) open circular form DNA. The tap-water samples used in the DNA nicking assay are the same ones as used in the microplate assay. One typical gel out of four conducted is shown.

these with milli-Q water. As shown in Fig. 1, addition of 2 mM vitamin C triggered a remarkable time dependent increase in the formation of 7-hydroxycoumarin-3-carboxylic acid in one of the water samples tested (951.3 ± 20.9 nM in 3 h). In the other sample tested, the generation of 7-hydroxycoumarin-3-carboxylic acid was much weaker (419.7 ± 22.0 nM formed in 3 h). In control sample, milli-Q water, no formation of 7-hydroxycoumarin-3-carboxylic acid with time could be seen. Similarly, omission of vitamin C from the assay resulted in no formation of 7-hydroxycoumarin-3-carboxylic acid (data not shown).

Measurement of Vitamin C-induced Hydroxyl Radical Formation in Tap-water Samples Obtained from Various Municipal Water Systems

Having done the time course study, we decided to measure hydroxyl radical formation in a large number of tap-water samples and to standardize the total incubation time in our experiments to 3 h. As shown in Fig. 2 (upper panel), a large variability in vitamin C-induced formation of 7-hydroxycoumarin-3-carboxylic acid could be seen among the water samples initially tested. These samples had been taken directly from the tap without flushing the water system. In some samples, close to 900 nM 7-hydroxycoumarin-3-carboxylic acid was formed while there was hardly any hydroxylation process going on in some of the other water samples tested. The highest value obtained in our assay was 916.7 ± 8.1 nM 7-hydroxycoumarin-3-carboxylic acid formed as compared to 18.1 ± 0.4 nM found in a water sample that gave the lowest signal (Table I). In general, we found that the 7-hydroxycoumarin-3-carboxylic acid formed in tap water originating from private wells were much lower than that formed in tap water originating from public water systems (Table II). The lowest values were found in commercially sold domestic bottled water (Table II). Only one of the commercially sold bottled water samples gave a signal 142.7 ± 4.5 nM comparable with the signals obtained from tap water originating from private groundwater wells.

The hydroxyl radical formation could also be demonstrated in the water samples by using a DNA nicking assay. As shown in Fig. 2 (lower panel) addition of 0.5 mM vitamin C to pBluescript DNA dissolved in milli-Q water showed a major band corresponding to the superhelical form I DNA (lane 9). However, addition of vitamin C to the water samples containing plasmid DNA resulted in

TABLE I Vitamin C-induced hydroxyl radical generation in household tap-water samples originating from various public water systems

Sample No.	Production of 7-OHCCA (nM)
1	888.8 ± 24.8
2	916.7 ± 8.1
3	375.0 ± 28.7
4	275.0 ± 23.2
5	782.7 ± 1.9
6	430.8 ± 5.8
7	112.5 ± 0.3
8	18.1 ± 0.4
9	44.4 ± 22.6
10	786.0 ± 32.6
11	834.6 ± 24.8
12	857.5 ± 1.4
13	501.8 ± 32.6
14	911.7 ± 1.5
15	230.9 ± 24.3
16	640.8 ± 29.8
17	224.2 ± 1.0
18	289.0 ± 16.5
19	214.1 ± 2.5
20	590.0 ± 65.0
21	891.6 ± 49.4
22	85.3 ± 1.0

The reaction was started by addition of 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C. The values shown are the concentration of 7-hydroxycoumarin-3-carboxylic acid formed after 3 h incubation at room temperature and represent the means ± S.D. The results shown are triplicates from one representative experiment out of three conducted.

the appearance of an upper band corresponding to the nicked circular form II of the plasmid. The water samples used in the plasmid agarose gel assay (numbered 1–9) are identical to those used in our coumarin-3-carboxylic acid assay shown in Fig. 2, upper panel. A marked conversion of the closed circular form (form I) to the nicked circular (form II) could be detected in samples 1, 2 and 5 that also gave the highest formation of hydroxylated coumarin-3-carboxylic acid in our microplate assay.

The hydroxyl radical formation in the tap-water samples was strongly dependent on the flushing time before the samples were taken. In Fig. 3, the vitamin C-induced hydroxylation of coumarin-3-carboxylic acid in two different water samples taken either immediately or after 1, 3 or 5 min flushing is shown. As can be seen in the figure, in sample A the formation of 7-hydroxycoumarin-3-carboxylic acid within 3 h was remarkably decreased when the faucet was flushed for 5 min (from 709.5 ± 9.8 to 67.8 ± 19.2 nM). On the other hand, flushing did not decrease the formation of 7-hydroxycoumarin-3-carboxylic acid to the same extent in sample B originating from a different public water system (from 762.0 ± 38.2 to 482.7 ± 49.9 nM).

DISCUSSION

In many countries, the tap water is considered to be of good quality (taste and chemical composition) and the majority of the population use tap water instead of bottled water as their drinking water. However, the tap water reaching the homes through the pipes might be contaminated with minerals such as copper, iron, lead, chromium or arsenic. The degree of contamination is highly dependent on how corrosive the water is, the material used in the pipes and fittings and the time the water is sitting in the pipes before use.[14]

In view of the fact that drinking water can be contaminated with copper, we decided to study whether hydroxyl radical formation can be measured in tap-water samples. To initiate the hydroxyl radical generation reaction we used vitamin C, a commonly used dietary supplement, that easily can redox-cycle transition metals such as iron and copper. Vitamin C is known to be a good antioxidant but during certain circumstances, in the presence of copper or iron, vitamin C can in fact act as a strong pro-oxidant.[15,16] The concentration of vitamin C found to give the highest amount of 7-hydroxycoumarin-3-carboxylic acid in the water samples was 2 mM. Higher concentrations of vitamin C inhibited the signal (data not shown).

TABLE II Vitamin C-induced hydroxyl radical generation in drinking water

	Production of 7-OHCCA (nM)	
Sample No.	Tap water originating from various private groundwater wells	Commercially sold domestic bottled water
1	43.8 ± 22.6	4.3 ± 0.4
2	28.7 ± 0.5	142.7 ± 4.5
3	60.5 ± 22.1	0
4	119.2 ± 10.9	8.7 ± 0.5
5	80.1 ± 5.8	10.8 ± 0.4
6	105.3 ± 1.4	0
7	43.3 ± 0.8	0
8	224.2 ± 1.4	

The reaction was started by addition of 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C. The values shown are the concentration of 7-hydroxy-coumarin-3-carboxylic acid formed after 3 h incubation at room temperature and represent the means ± S.D. The results shown are triplicates from one representative experiment out of three conducted.

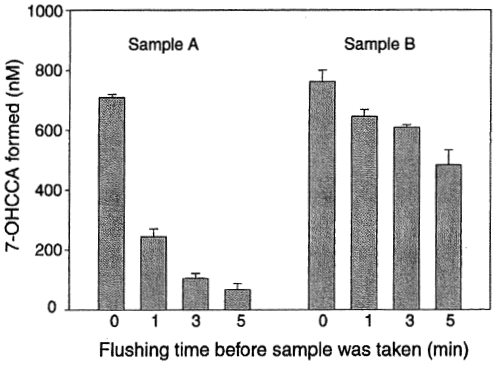

FIGURE 3 Vitamin C-induced hydroxyl radical formation in tap-water samples is dependent on flushing time. 200 μM coumarin-3-carboxylic acid and 2 mM vitamin C was added to tap-water samples taken either immediately or after 1, 3 or 5 min flushing. After 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. The bars show the amount of 7-hydroxycoumarin-3-carboxylic acid formed during the 3 h incubation in two different samples originating from different municipal water systems. The bars shown represent means ± SD of triplicates. Data are representative of three similar experiments.

Our results clearly show that addition of vitamin C to the drinking water samples triggered a hydroxyl radical generating reaction that easily could be measured with coumarin-3-carboxylic acid as detector molecule. The potency of the hydroxyl radicals formed in the same tap water-samples could also be demonstrated with a DNA nicking assay. Hydroxyl radicals are known to easily damage naked DNA, both single stranded[17] and double stranded DNA[18,19] by inducing cleavage of the phosphodiester bonds. Thus, in the presence of a hydroxyl radical generating system, a plasmid DNA can easily be attacked resulting in opening and fragmentation of the DNA plasmid. A close correlation was found between our results obtained in our microplate assay using coumarin-3-carboxylic acid and the plasmid DNA assay verifying that hydroxyl radicals are formed in these water samples after vitamin C addition.

Our results indicated that there was a large variability in hydroxyl radical formation in the water samples originating from various municipal water supplies. In general, hydroxyl radical formation was most evident in household tap-water samples as compared to the hydroxyl radical formation in tap water originating from private wells or commercially sold bottled spring water. Moreover, we found that the hydroxyl radical formation in some of the tap-water samples was strongly dependent on the flushing time before the samples were taken. One explanation for this is probably that the first draw sample can be contaminated with copper from the pipes within the apartment (building).[20,21] In line with this assumption, we found measurable amounts of copper (1.04, 0.71 and 0.42 mg/l) in the samples 1, 2 and 5 used in Fig. 2. Furthermore, in the sample A, shown in Fig. 6, the copper concentration in the tap-water sample decrease from 1.85 mg/l measured in the first draw to 0.07 mg/l by flushing the faucet for 5 min. This might explain the decrease in hydroxyl radical formation seen in this sample. In sample B in the same figure, the copper concentration decreased from 1.24 to 0.18 mg/l. However, fairly high formation of 7-hydroxycoumarin-3-carboxylic acid could still be seen in the 5 min sample. This indicates that relative low concentrations of copper can assist the radical generating process or that other ions (cations or anions) than copper might be involved in the hydroxyl radical generating process in this sample.

In conclusion, we demonstrate that hydroxyl radical formation can occur in drinking water if vitamin C is added. The impact of long-term consumption of hydroxyl radical generating drinking water on human health, e.g. the incidence of ulcer or stomach cancer, remains to be studied.

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Vitamin C (Ascorbic Acid) Induced Hydroxyl Radical Formation in Copper Contaminated Household Drinking Water: Role of Bicarbonate Concentration

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We have previously shown that Vitamin C (ascorbic acid) can trigger hydroxyl radical formation in copper contaminated household drinking water. We report here that the capacity of ascorbic acid to catalyze hydroxyl radical generation in the drinking water samples is strongly dependent on the bicarbonate concentration (buffer capacity and pH) of the samples. We found that at least 50 mg/l bicarbonate was required in the water samples to maintain the pH over 5.0 after ascorbic acid addition. At this pH, that is higher than the pKa_1 4.25 of ascorbic acid, a hydroxyl radical generating redox cycling reaction involving the mono-anion of vitamin C and copper could take place. The ascorbic acid induced hydroxyl radical generating reaction could easily be mimicked in Milli-Q water by supplementing the water with copper and bicarbonate. Our results demonstrate that ascorbic acid can induce a pH dependent hydroxyl radical generating reaction in copper contaminated household tap water that is buffered with bicarbonate. The impact of consuming ascorbic acid together with copper and bicarbonate containing drinking water on human health is discussed.

Keywords: Vitamin C; Hydroxyl radical; Water; Bicarbonate; Copper

INTRODUCTION

Free radicals are reactive oxygen species that has been shown to be involved in a variety of human diseases including stress induced gastric ulceration and cancer.[1–4] Among the various reactive oxygen species known today, the hydroxyl radical (·OH) belong to one of the most reactive form of oxygen. Hydroxyl radicals can easily be generated in the presence of a reducing substance and transition metals such as iron and copper via the Fenton reaction.[5–7] Hydroxyl radicals, once they are formed, will readily react with DNA, protein or lipids and can therefore induce cellular damage if no protecting hydroxyl radical scavenging molecules are present.[8–14]

We have recently demonstrated by using coumarin-3-carboxylic acid that vitamin C (ascorbic acid) can induce hydroxyl radical formation in copper contaminated household drinking water.[15] The hydroxyl radical generating reaction could easily be demonstrated in tap water samples that were contaminated by copper ions released from the pipes due to corrosion. High concentrations of copper, ≥ 1 mg/l can be found in household tap water samples taken from new houses especially if the samples contain first draw water taken in the morning.[16,17] High concentration of copper is toxic and especially infants are prone to copper poisoning. Gastrointestinal symptoms have also been associated with high copper intake.[18–21]

In order to further elucidate the mechanisms involved in the ascorbic acid induced hydroxyl radical generation in household drinking water we measured by HPLC the anion concentrations in tap water samples that generated hydroxyl radicals.

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From these studies and by using Milli-Q water as a model system, we demonstrate here, that ascorbic acid can trigger hydroxyl radical generation in copper contaminated bicarbonate-buffered drinking water.

MATERIALS AND METHODS

Chemicals

Coumarin-3-carboxylic acid[22,23] and 7-hydroxy-coumarin-3-carboxylic acid (7-OHCCA), L(+)-Ascorbic acid calcium salt dihydrate, (+)-Magnesium L-ascorbate and L(+)-Ascorbic acid sodium salt were from Fluka, Switzerland. Coumarin-3-carboxylic acid was dissolved in Milli-Q water (18 MΩ cm), and pH adjusted to 8.0 with NaOH. Ascorbic acid and cuprous chloride were purchased from Riedel-deHaen, Germany. Tris[hydroxymethyl]aminomethane (TRIS base) was from Sigma, St. Louis, USA. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of Vitamin C Induced Hydroxyl Radical Formation in Drinking Water

To measure hydroxyl radical formation in household drinking water, 200 µl of the water samples were pipetted in triplicate onto a 96 well flat-bottomed microwell plate (Greiner). After this, 200 µM coumarin-3-carboxylic acid was added to all wells by using a 8 channel multiwell pipett followed by 2 mM ascorbic acid that started the reaction. The microplate was then incubated at room temperature in dark for 3 h and the reaction was then stopped by pipetting 10 mM TRIS base to all wells. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from a standard curve. The standard curve was prepared by pipetting 200 µl aliquots of a serial dilution of the 7-OHCCA in triplicate onto a 96 well flat-bottomed microwell plate followed by 10 mM TRIS buffer. The fluorescence of the samples and standards were measured with a Fluoroscan II spectrophotometer (Labsystems, Finland). The optical filter set used was excitation 380 nm and emission 460 nm.

Measurement of the Anion Concentration by using HPLC

An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 µl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used was a PRP-X100, 150 × 4.1 mm I.D, 10 µm particle size anion exchange column (Hamilton) equipped with a PRP-X-100 guard column (25.0 × 2.3 mm I.D). Chromatography was performed using isocratic elution using 4.0 mM *p*-hydroxybenzoic acid containing 2.5% methanol, pH 8.5 (NaOH). The flow rate was 2.0 ml/min. The peaks were detected by indirect UV at 310 nm and analyzed by using the Waters Breeze software. All separations were performed at room temperature.

Measurement of pH changes in the Water Samples

pH was measured directly in the microwells by using a Orion pH meter (model 420 A) equipped with a needle tip micro-pH combination electrode. All measurements were done at room temperature.

RESULTS

We have recently shown that addition of ascorbic acid to some tap water samples contaminated with copper ions can trigger an ongoing production of hydroxyl radicals that can be detected by coumarin-3-carboxylic acid.[15] However, in these studies we observed that the amount of copper ions present in the water samples did not always correlate with the amount of hydroxyl radicals generated after ascorbic acid addition. From these studies, we concluded that other ion species might be involved in the ascorbic acid induced hydroxyl radical generating reaction.

We have now continued our analysis on two water samples, here named as A and B. Addition of 2 mM ascorbic acid to the tap water sample A, resulted in high amounts of hydroxylated coumarin-3-carboxylic acid (839.4 ± 4.8 nM) within 3 h. In the sample B, however, hardly any hydroxyl radical formation could be detected after ascorbic acid addition (11.9 ± 0.6 nM). These two water samples had been sampled in the same way, directly drawn from the tap, but they originate from two different municipal water suppliers. The pH in the water sample A and B were 7.60 and 7.15, respectively. When 2 mM ascorbic acid and 200 µM coumarin-3-carboxylic acid were added, the pH in sample A decreased rapidly to 5.78 and stabilized at a new pH value close to 6.4. In contrast, when ascorbic acid and coumarin-3-carboxylic acid were added to sample B, a much deeper drop in the pH value could be seen. Here, the pH decreased rapidly to 3.78 and remained at a value under pH 4.0.

To find out why ascorbic acid rapidly lowered pH in sample B, but not in sample A, we next measured the anion concentrations in the tap water samples by HPLC analysis. The set-up we used for the anion

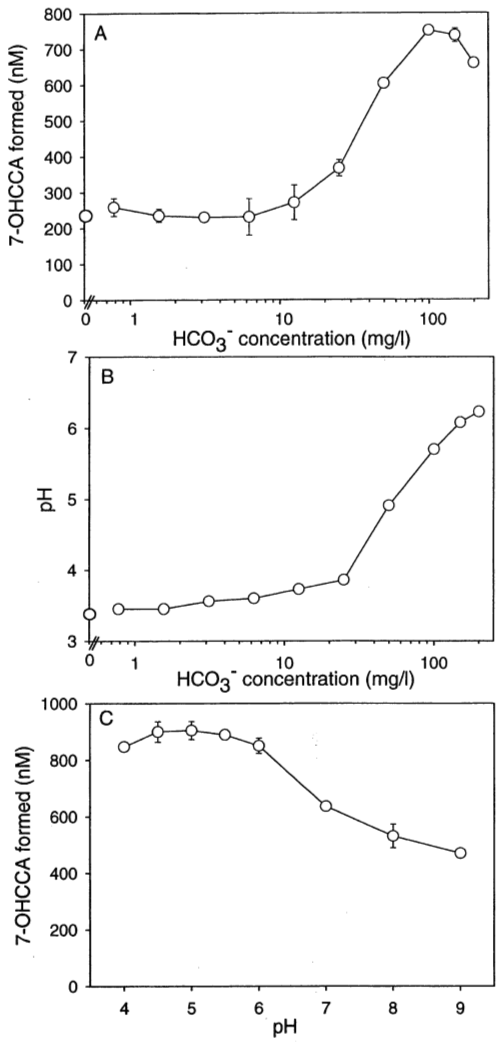

FIGURE 1 Hydroxyl radical formation in Milli-Q water supplemented with copper and various concentrations of bicarbonate. (A) 200 μM coumarin-3-carboxylic acid followed by 2 mM ascorbic acid were added to Milli-Q water samples containing 0.5 mg/l copper and various concentrations of bicarbonate. After 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. (B) pH in the samples. The pH values in the samples used in (A) were measured after a 3 h incubation at room temperature. (C) Sodium ascorbate induced hydroxyl radical formation at various pH values. The pH in Milli-Q water containing 0.5 mg/l copper and 100 mg/l bicarbonate was adjusted to values between 4 and 9 by using HCl or NaOH. 200 μM coumarin-3-carboxylic acid followed by 2 mM sodium ascorbate was added and the samples were incubated at room temperature for 3 h. The reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. Data points in this figure are mean ± SD of triplicates from one representative experiment out of three conducted. Where absent, error bars were smaller than the symbol.

analysis can detect fluoride, bicarbonate, chloride, nitrite, bromide, nitrate, phosphate and sulphate ions. High concentrations of both bicarbonate (88.3 mg/l) and chloride (93.7 mg/l) were found in the water sample A. In contrast, the water sample B showed much lower concentrations of bicarbonate and chloride, 29.8 and 12.5 mg/l, respectively. No other anions could be detected in the water samples tested.

The fact that a high concentration of bicarbonate was present in the water sample A indicated that the pH and the buffering capacity played an important role for the ascorbic acid induced hydroxyl radical formation. Therefore, to study the role of the pH and buffering capacity in the reaction, we next performed additional experiments with Milli-Q water. When increasing concentrations of bicarbonate was added to Milli-Q water containing 0.5 mg/l copper a significant increase in ascorbic acid induced hydroxyl radical formation could also be seen. At 50 mg/l bicarbonate a prompt increase in the hydroxyl radical formation was observed. The highest amount of 7-OHCCA was obtained when 100 mg/l bicarbonate was added to the copper supplemented Milli-Q water sample (Fig. 1A). In Fig. 1B, the pH in the same samples after 3 h incubation is shown. In the samples that contained 50 mg/l bicarbonate or more, a higher pH value could be measured. In contrast to ascorbic acid, the sodium- calcium- and magnesium-salts of ascorbic acid could readily trigger hydroxyl radical formation in copper containing Milli-Q water even in the absence of bicarbonate (Table I). In the presence of bicarbonate, sodium ascorbate triggered the highest yield of hydroxylated coumarin-3-carboxylic acid at pH 5.0. At higher pH values the signal was lower (Fig. 1C).

DISCUSSION

We have previously shown that addition of ascorbic acid to tap water samples contaminated with copper ions results in hydroxyl radical formation in some but not all of the samples.[15] pH measurements and HPLC anion analysis strongly indicated that a buffering anion such as bicarbonate was required for ascorbic acid to redox-cycle copper and generate hydroxyl radicals in the tap water samples. In line with this observation, we found that addition of ascorbic acid to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper also resulted in a hydroxyl radical generating reaction. Already 50 mg/l bicarbonate was enough to buffer the pH in Milli-Q water and keep the pH above 4.25 after ascorbic acid addition, which is the pKa_1 of ascorbic acid. When the pH is over 4.25, the ascorbate mono-anion will dominate and this form can further

TABLE I Hydroxyl radical generation in copper supplemented Milli-Q water

Compound	Production of 7-OHCCA (nM)	
	($-HCO_3$)	($+HCO_3$)
Control	7.8 ± 0.3	10.8 ± 0.3
Ascorbic acid	268.6 ± 5.8	848.1 ± 5.4
Calcium ascorbate	805.9 ± 7.1	505.2 ± 2.9
Sodium ascorbate	944.2 ± 5.8	616.9 ± 2.9
Magnesium ascorbate	673.5 ± 6.0	390.0 ± 6.0

The reaction was started by addition of 2 mM of the indicated vitamin C species to Milli-Q water containing 0.5 mg/l copper and 200 μM coumarin-3-carboxylic acid. The values shown are the concentration of 7-hydroxycoumarin-3-carboxylic acid formed after 3 h incubation at room temperature in the presence or absence of 100 mg/l bicarbonate. The results shown are the means ± SD of quadruplicates from one representative experiment out of three conducted.

be oxidized with concomitant reduction of copper II to copper I. At higher pH levels rapid redox cycling of copper will generate superoxide, peroxide and hydroxyl radicals via a copper assisted Fenton reaction. At lower pH, also the percentage of superoxide in the water decreases when superoxide anion reacts with hydrogen to form the hydroperoxyl radical ($HO_2^{\bullet}$).

The hydroxyl radical generating reaction described here take place in a very simple milieu namely water. Our results stress the importance of both the pH and the buffering capacity in the water sample for ascorbic acid to stimulate hydroxyl radical formation. In the presence of bicarbonate, ascorbic acid induced the strongest signal as compared to the sodium-, calcium-, or magnesium-salts of ascorbic acid (Table I). When the salts of ascorbate were used, the pH in the solution was 7.5 and the hydroxyl radical signal 35–40% lower. At this pH, bicarbonate ions present in the solution can rapidly react with the hydroxyl radicals and form bicarbonate- or carbonate-radicals.[24] In the presence of bicarbonate, sodium ascorbate triggered the highest signal at pH 5.0. At pH 7.5, the signal was approximately 30% lower than the one observed at pH 5.0 due to the ·OH scavenging effect of the bicarbonate ions present at this pH (Fig. 1C).

Vitamin C is one of the most studied anti-oxidants and it has previously been shown that vitamin C protects against gastric cancer by scavenging reactive radical species and inhibits N-nitroso compound induced gastric carcinoma.[25–28] However, one could speculate that bicarbonate *in vivo* in some special compartments, e.g. in the stomach or urinary bladder could locally increase the pH and thereby assist vitamin C to stimulate hydroxyl radical formation. Thus, bicarbonate could in fact, as shown here, turn vitamin C (ascorbic acid) into a strong pro-oxidant. In line with this assumption, bicarbonate and vitamin C has been shown to promote (*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine) induced bladder carcinogenesis in rats.[29–31] Moreover, it has been shown that alkaline urinary pH promote bladder carcinogenesis in rat model systems.[32,33] Interestingly, ascorbic acid in the presence of bicarbonate[29,30] or sodium ascorbate alone[34,35] was used in these studies.

In conclusion, we have shown that ascorbic acid triggers hydroxyl radical formation when added to copper contaminated tap water samples that contain high concentrations of bicarbonate. Our results show that bicarbonate rich water in this regard is safe as long as the water is not contaminated with copper ions. Furthermore, our results support earlier findings that vitamin C also can act as a strong pro-oxidant.[36–39] The impact of long-term consumption of bicarbonate and copper contaminated tap water together with strong reducing substances on human health, e.g. the incidence of ulcer or stomach cancer, remains to be studied.

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Oxidative Decomposition of Vitamin C in Drinking Water

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We have previously shown that vitamin C (ascorbic acid) can initiate hydroxyl radical formation in copper contaminated household drinking water. In the present study, we have examined the stability of vitamin C in copper and bicarbonate containing household drinking water. In drinking water samples, contaminated with copper from the pipes and buffered with bicarbonate, 35% of the added vitamin C was oxidized to dehydroascorbic acid within 15 min. After 3 h incubation at room temperature, 93% of the added (2 mM) ascorbic acid had been oxidized. The dehydroascorbic acid formed was further decomposed to oxalic acid and threonic acid by the hydrogen peroxide generated from the copper (I) autooxidation in the presence of oxygen. A very modest oxidation of vitamin C occurred in Milli-Q water and in household water samples not contaminated by copper ions. Moreover, addition of vitamin C to commercially sold domestic bottled water samples did not result in vitamin C oxidation. Our results demonstrate that ascorbic acid is rapidly oxidized to dehydroascorbic acid and further decomposed to oxalic- and threonic acid in copper contaminated household tap water that is buffered with bicarbonate. The impact of consuming ascorbic acid together with copper and bicarbonate containing drinking water on human health is discussed.

Keywords: Vitamin C; Dehydroascorbic acid; Oxalic acid; Threonic acid; Water; Copper

INTRODUCTION

Ascorbic acid (vitamin C) is a natural antioxidant that is nowadays added as a vitamin and preservative to a variety of food sources, e.g. fruit juices in high quantities. The intake of supplemental vitamin C has also increased considerably and supplements containing up to 1–2 g of vitamin C/tablet can be found in any grocery store. This amount of ascorbic acid is well above the recommended daily intake (RDI) that is 60 mg/day for adult women or men. Thus, the mean daily intake of vitamin C has significantly increased in many countries during the past 20 years. High intake is generally not believed to be harmful since vitamin C is a water-soluble compound that is not stored in the body and the excess ingested vitamin is excreted in the urine.

Numerous studies have shown that the antioxidant, ascorbic acid, has beneficial effect on many age-related diseases such as: atherosclerosis, cancer, some neurodegenerative and ocular diseases.[1–7] However, it has also been shown that, in the presence of transition metals such as copper and iron, ascorbic acid can in fact function as a strong pro-oxidant.[8–10] In line with this, we have recently demonstrated by using coumarin-3-carboxylic acid that ascorbic acid can induce hydroxyl radical formation in copper contaminated, bicarbonate buffered household drinking water.[11] During this process, ascorbic acid is oxidized to dehydroascorbic acid by the copper ions present in the water sample.

Since the oxidized form of ascorbic acid, dehydroascorbic acid, has been shown to be toxic and to generate oxidative stress in various cell systems[12–14] we have here studied the formation and the stability of this compound in tap water samples. We demonstrate here, that ascorbic acid is very rapidly oxidized to dehydroascorbic acid and further decomposed to oxalic and threonic acid in copper contaminated bicarbonate-buffered drinking water.

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MATERIALS AND METHODS

Chemicals

Ascorbic acid, threonic acid and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Tris[hydroxymethyl]-aminomethane (Tris base) and *o*-phenylenediamine dihydrochloride (OPD), oxalic acid, diethyldithiocarbamic acid, dithiothreitol (DTT), *p*-hydroxybenzoic acid, sodium bicarbonate and dehydroascorbic acid were from Sigma, St Louis, USA. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of Copper and Bicarbonate Concentration in the Water Samples

The copper and bicarbonate concentration in the water samples were measured as previously described.[11,15]

Measurement of Dehydroascorbic Acid with *o*-phenylenediamine

In contrast to ascorbic acid, dehydroascorbic acid absorb UV light very poorly. To measure dehydroascorbic acid formation we therefore used the reagent *o*-phenylenediamine, a reagent that form a fluorescent complex with dehydroascorbic acid.[16,17] For the assay, 2 mM ascorbic acid was added to the water samples and incubated at room temperature for various time periods. After this, 2 mM of *o*-phenylenediamine was added to the tubes followed by 8 mM Tris buffer. The fluorescent complex was excited at 370 nm, and the emission at 440 nm was measured by a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence values were converted into dehydroascorbic acid concentration from a standard curve, where known amounts of dehydroascorbic acid was used. The actual concentration of dehydroascorbic acid in the prepared standards was controlled by addition of 10 mM DTT followed by HPLC analysis on the ascorbic acid formed. All measurements were done at room temperature.

Measurement of Ascorbic Acid Metabolites by Using HPLC

An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for quantitation of ascorbic acid and oxalic acid was a Nucleosil C18 150 × 4.1 mm I.D, 10 μm particle size column (Supelco Inc.). Chromatography was performed using isocratic elution using 10 mM phosphate buffer (KH_2PO_4) containing 5% methanol, pH 3.3 (H_3PO_4). The flow rate was 0.5 ml/min. The peaks were detected at 210 nm and analyzed by using the Waters Breeze software. For the identification and calibration we used standards of threonic-, oxalic-, dehydroascorbic- and ascorbic-acid in Milli-Q water. All separations were performed at room temperature.

When the Nucleosil C18 column was used, the peaks for the threonic acid and hydrogen peroxide standards overlapped in the chromatogram. Therefore, to quantitate threonic acid, an anion column PRP-X100 (Hamilton), 150 × 4.1 mm I.D, 10 mm particle size equipped with a PRP-X100 guard column (25.0 × 2.3 mm I.D) was also used. Chromatography was performed using isocratic elution using 4.0 mM *p*-hydroxybenzoic acid containing 2.5% methanol, pH 8.5 (NaOH). The flow rate was 2.0 ml/min. The peaks were detected by indirect UV at 310 nm and analyzed by using the Waters Breeze software.

RESULTS

Ascorbic Acid Oxidation in Drinking Water

Ascorbic acid oxidation in two tap water samples (sample 1 and 2), a Milli-Q water sample supplemented with 100 mg/l HCO_3^- and 0.5 mg/l Cu^{2+} and a control Milli-Q water sample were followed for 3 h by HPLC analysis (Fig. 1A). The two water samples had been sampled in the same way, directly drawn from the tap, but they originated from two different municipal water suppliers. When 2 mM ascorbic acid was added to sample 1, that contained 25.9 mg/l bicarbonate and 0.19 mg/l copper, a very modest degradation of the vitamin occurred with time. In this water sample 38.6% of the added ascorbic acid had been oxidized during the 3 h incubation. On the contrary, when 2 mM ascorbic acid was added to the water sample 2, that contained 150.4 mg/l bicarbonate and 0.53 mg/l copper, the vitamin was almost completely (92.6%) oxidized during the 3 h incubation. A similar oxidation process could be seen when 2 mM vitamin C was added to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper. Addition of 2 mM ascorbic acid to Milli-Q water resulted in a very modest oxidation of the vitamin with time.

Dehydroascorbic Acid Formation in Drinking Water

The formation of dehydroascorbic acid, was measured by using *o*-phenylenediamine. As can

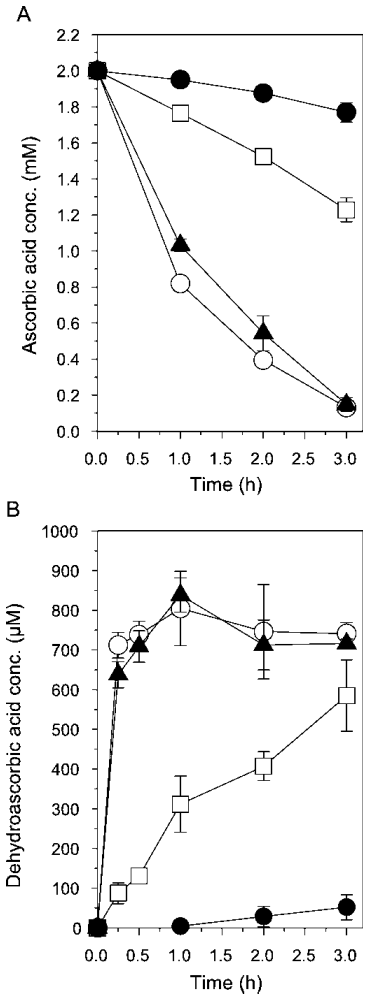

FIGURE 1 Ascorbic acid degradation (A), and dehydroascorbic acid formation (B), in household drinking water 2 mM ascorbic acid was added to two household tap water samples; sample 1 (□) and sample 2 (▲), a Milli-Q water sample (●) and a Milli-Q water sample supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper (○). The concentration of ascorbic acid was measured at indicated time points by using HPLC analysis. Dehydroascorbic acid was measured by using the reagent *o*-phenylenediamine. Data shown are mean ± SD of triplicates from one representative experiment out of three conducted.

be seen in Fig. 1B, dehydroascorbic acid was rapidly formed in the tap water sample 2. After 15 min incubation, 638 ± 33 μM of dehydroascorbic acid had been formed. However, at 15 min, only 87 ± 26 μM of dehydroascorbic acid had been formed in sample 1. Addition of 2 mM ascorbic acid to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper resulted in rapid formation of dehydroascorbic acid. In the control sample where ascorbic acid had been added to Milli-Q water, very low concentrations of dehydroascorbic acid could be detected. The amount of dehydroascorbic acid formed in four commercially sold domestic bottled water samples, after ascorbic acid addition (15 min incubation), was in the range of 0–3 μM.

Dehydroascorbic Acid Reacts with Hydrogen Peroxide and Generate Oxalic Acid and Threonic Acid

A typical chromatogram of a catalytic drinking water sample incubated with 2 mM ascorbic acid for 3 h is shown in Fig. 2A. In this chromatogram, based on retention time, the peak that appeared at 5.6 min was identified as oxalic acid (peak 1). At 5.9 min, a peak appeared that had a retention time similar to the hydrogen peroxide- and threonic acid standards (peak 2). During the 3 h incubation 2 mM ascorbic acid (rentention time 7.9 min, peak 4) had been oxidized, in this bicarbonate rich and copper contaminated drinking water sample to 713 ± 63 μM dehydroascorbic acid (*o*-phenylenediamine assay), 488 ± 28 μM oxalic acid and 77 ± 14 μM threonic acid (quantitated by using an anion column). The peak for dehydroascorbic acid (peak 3) appeared in the chromatogram at 6.8 min together with an unknown metabolite that had a retention time of 7.1 min. In a similar way, addition of 4 mM hydrogen peroxide to 2 mM ascorbic acid in Milli-Q water supplemented with 100 mg/l bicarbonate resulted in 839 ± 72 μM oxalic acid and 192 ± 24 μM threonic acid within 3 h (Fig. 2B). Moreover, very rapid formation of oxalic acid and threonic acid could be obtained when 4 mM hydrogen peroxide was added to 2 mM dehydroascorbic acid in Milli-Q water supplemented with 100 mg/l bicarbonate. A 10-min incubation at room temperature resulted in 640 ± 24 μM oxalic acid and 181 ± 24 μM threonic acid (Fig. 2C). In control experiments, 2 mM dehydroascorbic acid was added to Milli-Q water supplemented with 100 mg/l bicarbonate. In the absence of hydrogen peroxide, 79% less oxalic acid was formed (Fig. 2D).

To verify that dehydroascorbic was formed in our drinking water samples, we used DTT, a reducing agent that can turn dehydroascorbic acid back into ascorbic acid. When DTT was added to copper and bicarbonate supplemented Milli-Q water that had been incubated with 2 mM ascorbic acid for 3 h, the ascorbic acid peak reappeared in the chromatogram (approximately 680 μM ascorbic acid was formed). Dehydroascorbic acid has previously been shown to be spontaneously decomposed to L-diketogulonate

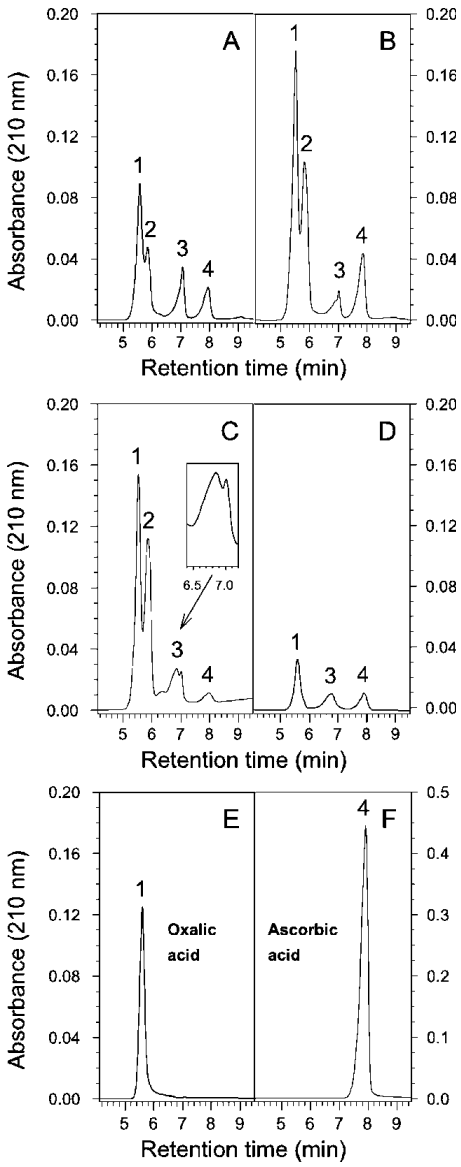

FIGURE 2 HPLC analysis of ascorbic acid metabolites in drinking water. (A) 2 mM ascorbic acid was added to a drinking water sample and incubated at room temperature for 3 h. (B) 2 mM ascorbic acid and 4 mM hydrogen peroxide was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 3 h at room temperature. (C) 2 mM dehydroascorbic acid and 4 mM hydrogen peroxide was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 10 min at room temperature. The inset show the peaks for dehydroascorbic acid (at 6.8 min) and an unknown compound (at 7.1 min). (D) 2 mM dehydroascorbic acid was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 10 min at room temperature. (E) Oxalic acid standard (0.6 mM) in Milli-Q water. (F) Ascorbic acid standard (2 mM) in Milli-Q water. Please note the different scaling in chromatogram F.

(2,3-DKG) or erythroascorbate.[18–20] Our results indicated that dehydroascorbic acid and not DKG was present in the sample.

DISCUSSION

The drinking water reaching the consumer at their homes can easily be contaminated by copper ions due to corrosion in the copper pipes in the house (building). Especially, the first-draw water used in the morning can readily be contaminated by copper ions.[21,22] In light of these facts, and our previous studies showing that ascorbic acid can drive a hydroxyl radical generating process in copper and bicarbonate containing drinking water[11,15] we decided to study how fast and to what extent ascorbic acid is oxidized in a copper contaminated drinking water sample. Our results show that ascorbic acid is oxidized relatively fast in bicarbonate rich water samples that are contaminated by copper ions. Approximately 32% of the vitamin C had been oxidized after 15 min and the vitamin was almost completely oxidized within 3 h (Fig. 1A and B). The oxidation process could easily be mimicked by adding vitamin C to Milli-Q water supplemented with 100 mg/l HCO_3^- and 0.5 mg/l Cu^{2+} because, the oxidation process require copper ions and a pH around 4–5.[15]

When ascorbic acid is oxidized in the presence of copper ions, dehydroascorbic acid is formed (Fig. 1B). The dehydroascorbic acid formation, in the bicarbonate rich water sample contaminated with copper, was very rapid and up to 650 μM dehydroascorbic acid could be formed within 15 min. The amount of dehydroascorbic acid formed within 15 min in the various tap water samples tested were in the range of 100–650 μM. On the contrary, when commercially sold domestic bottled water was used in the assay very modest degradation of ascorbic acid took place. Some of the bottled water samples tested (mineral waters) were buffered with bicarbonate. However, in the absence of copper ions, oxidation of vitamin C cannot take place. This reflects the importance of copper ions in the oxidation process.

HPLC analysis, performed on the water samples that had been incubated with 2 mM ascorbic acid for 3 h at room temperature, clearly indicated that the vitamin had been oxidized into dehydroascorbic acid and further decomposed into two major metabolites, oxalic acid and threonic acid. In the water sample 2, that was contaminated with copper ions and had high concentration of bicarbonate, only 131 ± 13 μM of the added 2 mM ascorbic acid was left after a 3 h incubation. During the 3 h incubation period, the added ascorbic acid had been oxidized to 488 ± 28 μM oxalic acid. High concentrations of oxalic acid (calcium oxalate) are toxic and

can promote the formation of kidney stones.[23,24] Interestingly, calcium oxalate microcalcification has also been observed in benign and malignant breast biopsy specimens.[25,26] Whether the oxalic acid present in the breast tissue originate from the ascorbic acid- or amino acid metabolism is currently not known.

When ascorbic acid is oxidized in the presence of divalent copper, monovalent copper is formed. When the reduced copper is re-oxidized in the presence of oxygen, superoxide is generated. In the presence of a proton donor, the superoxide is further reduced to hydrogen peroxide. Most likely, the hydrogen peroxide formed in the reaction, when ascorbic acid is oxidized by copper, promote the cleavage of dehydroascorbic acid to oxalic acid and threonic acid. In line with this assumption we found that addition of hydrogen peroxide directly to ascorbic acid, in the presence of 100 mg/l bicarbonate, resulted in the formation of oxalic acid and threonic acid during the 3 h incubation (Fig. 2B). However, when hydrogen peroxide was added directly to dehydroascorbic acid in the presence of 100 mg/l bicarbonate, the same amount of oxalic acid and threonic acid could be obtained within 10 min incubation (Fig. 2C). Our results indicate that the hydrogen peroxide formed, and not the hydroxyl radicals generated during the reaction, is responsible for the dehydroascorbic acid decomposition in the water samples.

In conclusion, our results show that significant amounts of either dietary or supplementary ascorbic acid can be rapidly oxidized to dehydroascorbic acid when added to bicarbonate rich (buffered) copper contaminated drinking water. Thus people consuming this type of water will most likely ingest, or generate more dehydroascorbic acid in their stomach, than people using bottled water. Dehydroascorbic acid can enter cells via the GLUT glucose transporter.[27] Intracellular reduction of large amounts of dehydroascorbic acid to ascorbic acid by NADPH- and glutathione-dependent reactions may markedly decrease the cellular concentrations of NADPH and glutathione in some celltypes.[28–30] In line with these findings, dehydroascorbic acid has been shown to cause oxidative stress and apoptosis in pancreatic and neural cells by depleting their intracellular store of reduced glutathione.[14,31,32] The impact of long-term intake of dehydroascorbic acid, the oxidized form of ascorbic acid, on human health, remains to be studied.

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Effects of iron on Vitamin C/copper-induced hydroxyl radical generation in bicarbonate-rich water

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Abstract
The aim of this study was to evaluate whether iron, like copper, could support Vitamin C mediated hydroxyl radical formation in bicarbonate-rich water. By using the hydroxyl radical indicator coumarin-3-carboxylic acid, we found that iron, in contrast to copper, was not capable to support Vitamin C induced hydroxyl radical formation. However, when 0.2 mg/l iron and 0.1 mg/l copper were both added to bicarbonate supplemented Milli-Q water, the Vitamin C induced formation of 7-hydroxycoumarin, as measured by HPLC analysis, was inhibited by 47.5%. The inhibition of hydroxyl radical formation by iron was also evident in the experiments performed on copper contaminated bicarbonate-rich household drinking water samples. In the presence of 0.2 mg/l of ferric iron the ascorbic acid induced hydroxyl radical formation was inhibited by 36.0–44.6%. This inhibition was even more significant, 47.0–59.2%, when 0.8 mg/l of ferric iron was present. None of the other redox-active metals, e.g. manganese, nickel or cobalt, could support ascorbic acid induced hydroxyl radical formation and did not have any impact on the ascorbic acid/copper-induced hydroxyl radical generation. Our results show, that iron cannot by itself produce hydroxyl radicals in bicarbonate rich water but can significantly reduce Vitamin C/copper-induced hydroxyl radical formation. These findings might partly explain the mechanism for the iron-induced protective effect on various copper related degenerative disorders that earlier has been observed in animal model systems.

Keywords: *Vitamin C, water, iron, copper, bicarbonate*

Introduction

The structure of iron and its capacity to vary its oxidation state and bind to different ligands gives iron a unique biochemical role. Iron is a highly precious metal for the growth and viability of all cells and indispensable for human survival. It is primarily required for hemoglobin synthesis, but it has also a crucial role in e.g. DNA synthesis, electron transport and many enzymatical activities throughout the body. Low dietary intake of iron, results in iron deficiency and anemia [1–3].

Iron has also been implicated in the pathogenesis of a variety of neurodegenerative disorders e.g. Parkinson's disease, Alzheimer disease MS and EAE [4–6], liver and heart disease [7–9], cancer [10,11], diabetes [12–13] and immune abnormalities [14,15]. The toxicity of iron has generally been attributed to its ability to reduce molecular oxygen, thus forming reduced oxygen species. One of the most accepted mechanisms by which iron is involved in free radical production is the Fenton/Haber-Weiss reaction cycle. In this reaction, hydroxyl radicals can be easily

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generated by iron-catalyzed reduction of oxygen to superoxide that in turn can react with hydrogen peroxide [16]. Hydroxyl radicals can also directly be generated from hydrogen peroxide by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$ [17,18]. Various iron chelators, such as EDTA and NTA, have also been shown to promote hydroxyl radical generation very effectively via the Haber-Weiss cycle in an ascorbate-driven Fenton reaction [19–22].

Ascorbate (Vitamin C) has been reported, *in vitro*, to mediate hydroxyl radical formation in the presence of iron [23–25]. Based on this, and the known fact that ascorbic acid can redox-cycle with iron, we have here evaluated whether iron, like copper, could have hydroxyl radical formation properties in a drinking water environment. We demonstrate here, that iron cannot by itself produce hydroxyl radicals in such an environment but it has an inhibitory effect on Vitamin C induced hydroxyl radical formation in copper contaminated bicarbonate-rich household drinking water.

Materials and methods

Chemicals

Coumarin-3-carboxylic acid, 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA), coumarin and 7-hydroxycoumarin (umbelliferone) were from Fluka, Switzerland. Ascorbic acid, ferric chloride tetrahydrate, ferrous chloride hexahydrate, calcium chloride dihydrate and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Tris[hydroxymethyl]aminomethane (TRIS base), manganese chloride tetrahydrate, nickel chloride hexahydrate, cadmium chloride anhydrous, gallium nitrate hydrate, zinc chloride, aluminium chloride hexahydrate, cobalt chloride hexahydrate, diethyldithiocarbamic acid, ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) and sodium bicarbonate were from Sigma, St. Louis, USA. Magnesium chloride hexahydrate were purchased from J.T. Baker, Denventer, Holland. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of vitamin C induced hydroxyl radical formation by using coumarin-3-carboxylic acid

To measure hydroxyl radical formation in household drinking water or bicarbonate supplemented Milli-Q water, 200 μl of the water samples were pipetted in triplicate onto a 96 well microtiter plate. After this, 200 μM coumarin-3-carboxylic acid was added to all wells by using a 8 channel multiwell pipett followed by 2 mM ascorbic acid that started the reaction. The microtiter plate was incubated at room temperature in dark for 3 h and the reaction was stopped by pipetting 10 mM TRIS base to all wells. Addition of TRIS buffer adjusted the pH in the samples to 9.0 that maximized the pH dependent fluorescence signal of 7-hydroxycoumarin-3-carboxylic acid. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. All measurements were done at room temperature. The fluorescence of the samples and standards were measured with a Victor plate reader, Wallac, Finland. The optical filter set used was excitation 380 nm and emission 460 nm.

Measurement of iron, copper and bicarbonate concentration in the water samples

The iron concentration in the drinking water samples was measured by using the iron specific reagent ferrozine [26]. For the assay, 200 μl aliquots in triplicate of the water samples were pipetted onto a 96 well microtiter plate followed by 400 μM of ferrozine and 100 μM ascorbic acid. Ascorbic acid was used to reduce the Fe(III) to the Fe(II) form. The colored Fe(II)-ferrozine complex formed was measured at 560 nm by using a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve. The standard curve was generated by adding known amounts of ferric chloride tetrahydrate, 100 μM ascorbic acid and 400 μM ferrozine to Milli-Q water buffered with 100 mg/l bicarbonate. The copper and bicarbonate concentration in the water samples were measured as previously described [27,28].

Measurement of hydroxyl radical formation by HPLC analysis

An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for the analysis was a Symmetry C18, 250 × 4.6 mm I.D, 10 μm particle size column (Waters). Chromatography was performed using isocratic elution using 150 mM phosphate buffer (KH_2PO_4) containing 30% methanol, pH 3.0 (H_3PO_4). The flow rate was 0.75 ml/min. The indicator molecule used in the assay was coumarin that readily forms 7-hydroxycoumarin (umbelliferone) when attacked by hydroxyl radicals. The peaks were detected at 200 nm and analyzed by using the Waters Breeze software. For peak identification and calibration we used standards of coumarin, 7-hydroxycoumarin (umbelliferone) in Milli-Q water. All separations were performed at room temperature.

Results

Copper, but not iron, can support ascorbic acid induced hydroxyl radical formation in bicarbonate-rich water

We have earlier shown that addition of ascorbic acid to tap water samples contaminated with copper ions can trigger an ongoing production of hydroxyl radicals that can be detected by using coumarin-3-carboxylic acid [27,28]. In Figure 1, ascorbic acid (2 mM) was added to bicarbonate buffered Milli-Q water supplemented with different concentrations of either copper or iron. Even very low concentrations of copper (0.01–0.05 mg/l) were sufficient to give a detectable hydroxyl radical signal. On the contrary, when iron was used in the assay, no hydroxyl radical formation could be detected. Neither ferrous nor ferric iron could support any hydroxyl radical formation. Manganese, cadmium, nickel, cobalt, aluminum, magnesium, calcium and zinc (as chloride salts) or gallium (as nitrate salt) did not result in any detectable hydroxyl radical formation (tested by using the highest amount of the contaminants that is allowed in drinking water, Maximum Contaminant Level, MCL, data not shown).

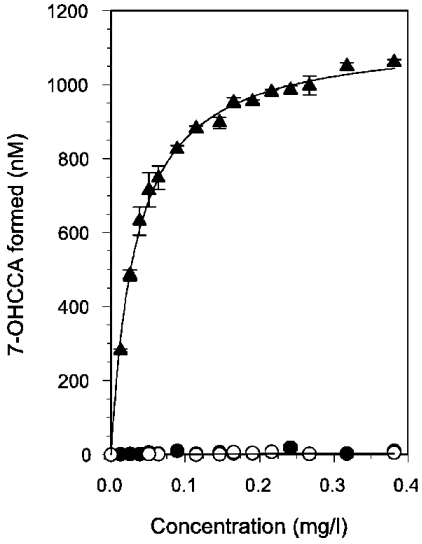

Figure 1. Ascorbic acid induced hydroxyl radical formation in bicarbonate buffered Milli-Q water supplemented with copper or iron. 200 μM coumarin-3-carboxylic acid followed by 2 mM ascorbic acid were added to Milli-Q water samples buffered with 100 mg/l bicarbonate and various concentrations of copper (▲), ferrous iron (•) or ferric iron (○). After 3 h incubation in dark at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. Data points are mean ± SD of triplicates from one representative experiment out of three conducted. Where absent, error bars were smaller than the symbol.

Inhibition of ascorbic acid/copper-catalyzed hydroxyl radical formation by iron

To further elucidate the effects of iron on Vitamin C induced hydroxyl radical formation in the presence of copper and bicarbonate, we used HPLC analysis. For the assay, coumarin was chosen as the target molecule. As shown in Figure 2A, 2 mM ascorbic acid, in the presence of 0.1 mg/l copper and 100 mg/l bicarbonate, promoted the formation of a family of hydroxylated coumarin compounds. We focused our analysis on one of these hydroxylated compounds, namely 7-hydroxycoumarin. Within 3 h, 5.5 μM of 7-hydroxycoumarin was formed. When the copper ion was substituted with 0.2 mg/l ferric iron, no hydroxylated coumarin compounds appeared in the chromatogram (Figure 2B). In our experiments, 0.2 mg/l iron was used since this is the MCL for iron in drinking water in Finland. When 2 mM ascorbic acid was added to Milli-Q water that has been supplemented with 0.2 mg/l iron, 100 mg/l bicarbonate and 0.1 mg/l copper, a 47.5% reduction in the 7-hydroxycoumarin formation was observed (Figure 2C). In these chromatograms, based on the retention time for the standard, the peak that appeared at 22.5 min was identified as 7-hydroxycoumarin (Figure 2D). When manganese, cadmium, nickel, cobalt, gallium, aluminum, magnesium, calcium and zinc salts (chloride salt) were tested at their MCLs no inhibitory effect of the ascorbic acid/copper-mediated hydroxyl radical formation could be seen (data not shown).

Effects of iron on Vitamin C/copper-induced hydroxyl radical formation in household drinking water samples

Next we evaluated whether iron has any impact on ascorbic acid induced hydroxyl radical formation in copper contaminated bicarbonate-rich drinking water samples. The copper concentration in the different water samples varied from 0.13 to 0.02 mg/l. The bicarbonate concentration varied between 77.6 and 130.1 mg/l. The drinking water samples had been sampled in the same way, directly drawn from the tap, but they originated from four different municipal water suppliers. The water samples used in the assay did not contain any detectable iron. When Vitamin C was added to these samples more than 900 nM of 7-hydroxycoumarin-3-carboxylic acid was formed (Table I). When 0.2 mg/l ferric iron was added to the tap water samples the ascorbic acid induced hydroxyl radical formation was inhibited by 36.0–44.6%. When the water samples were supplemented with 0.8 mg/l ferric iron the inhibition was significantly higher, 47.0–59.2%.

Discussion

We have previously shown that ascorbic acid can drive a hydroxyl radical generating process in copper and bicarbonate containing household drinking water

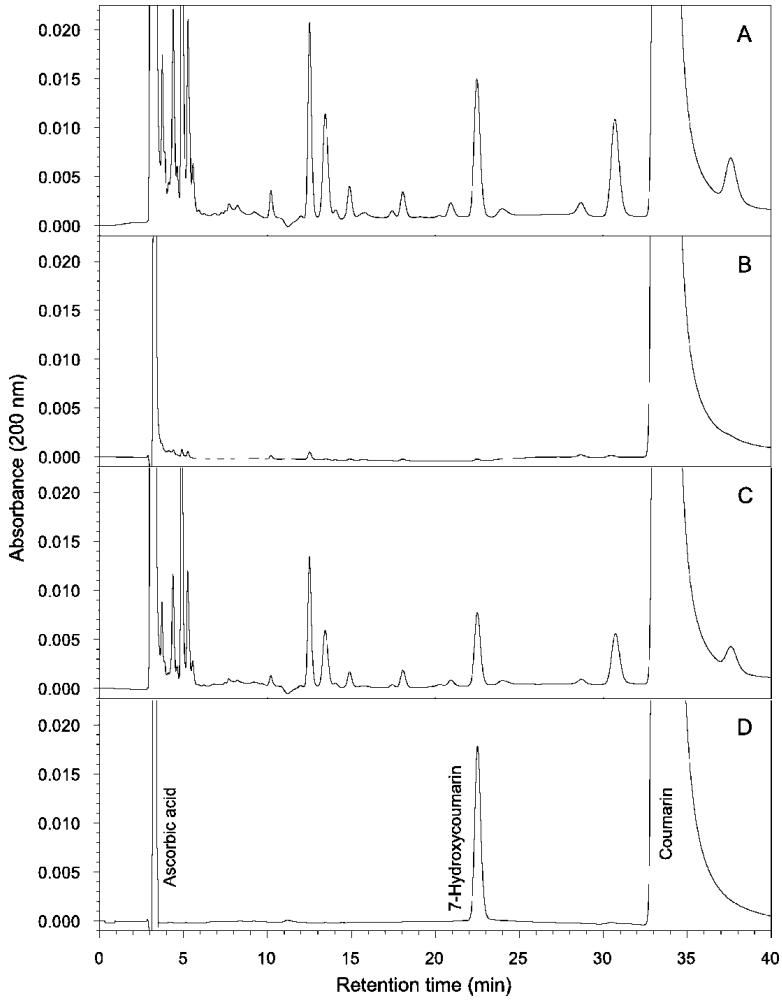

Figure 2. HPLC analysis of ascorbic acid induced hydroxylation of coumarin (100 μM) in the presence of copper or iron. 2 mM ascorbic acid was added to Milli-Q water buffered with 100 mg/l bicarbonate containing (A) 0.1 mg/l copper (B) 0.2 mg/l ferric iron. (C) Both 0.1 mg/l copper and 0.2 mg/l ferric iron present. (D) Ascorbic acid standard (2 mM), 7-hydroxycoumarin standard (7 μM) and coumarin standard (100 μM) in Milli-Q water. Reaction time was 3 h.

[27,28]. Here we show, by using coumarin-3-carboxylic acid as a fluorescent probe for detection of hydroxyl radical formation, that even very low concentrations of copper ($\leq$0.1 mg/l) are sufficient to give a significant hydroxyl radical signal. However, when copper was substituted by iron, ascorbic acid was not capable to stimulate hydroxyl radical formation (Figure 1). This was also demonstrated by using HPLC analysis. The HPLC data clearly showed that coumarin, in Milli-Q water supplemented with 100 mg/l bicarbonate, was strongly hydroxylated by ascorbic acid in the presence of copper ions but not in the presence of 0.2 mg/l iron alone (Figure 2A and B).

Our results demonstrate that iron partly can inhibit the ascorbic acid/copper driven hydroxyl radical formation in a drinking water environment. When 0.2 mg/l iron was added to the Milli-Q water that had been supplemented with 100 mg/l bicarbonate and 0.1 mg/l copper, the ascorbic acid induced formation of 7-hydroxycoumarin was inhibited by 47.5% (Figure 2C). Our results are in agreement with the recent report by White *et al.*, demonstrating that iron can impair reductant-mediated copper and H_2O_2 generation and neurotoxicity [29]. Moreover, our results are in line with the recent findings by Munday *et al.* showing that copper-catalyzed cysteine oxidation

Table I. Effects of iron on Vitamin C/copper induced hydroxyl radical formation in drinking water.

			Iron supplementation				
			7-OHCCA formed (nM)			Percentage inhibition (%)	
Sample No.	Copper (mg/l)	Bicarbonate (mg/l)	0.0 (mg/l)	0.2 (mg/l)	0.8 (mg/l)	0.2 (mg/l)	0.8 (mg/l)
1	0.15 ± 0.00	92.2 ± 2.6	965.8 ± 7.2	593.4 ± 3.6	454.3 ± 9.8	38.6 ± 0.4	52.9 ± 1.0
2	0.20 ± 0.02	130.1 ± 5.7	990.2 ± 58.9	575.5 ± 53.9	428.1 ± 16.4	41.9 ± 5.6	56.8 ± 1.7
3	0.15 ± 0.01	77.6 ± 4.3	1093.8 ± 94.7	699.6 ± 19.6	579.8 ± 4.7	36.0 ± 1.8	47.0 ± 0.4
4	0.13 ± 0.01	101.4 ± 2.1	904.6 ± 20.9	501.2 ± 51.6	369.2 ± 6.8	44.6 ± 5.7	59.2 ± 0.8

Vitamin C (2 mM) induced hydroxyl radical formation was measured in tap water samples (numbered 1–4) supplemented with either 0.2 or 0.8 mg/l of ferric iron by using the coumarin-3-carboxylic acid assay. The values shown are the concentration of 7-hydroxycoumarin-3-carboxylic acid formed after 3 h incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

can be partly inhibited by low concentrations of iron salts [30]. In this context, it can also be mentioned that Menditto *et al.* showed that loading of seminal plasma with either ferrous or ferric iron up to a concentration of 50 μM only modestly affected the rate of ascorbic acid oxidation [31]. The low oxidation rate of ascorbic acid by iron was also seen in our *in vitro* experiments. Low concentrations of copper, however, as shown here, induces rapid oxidation of ascorbic acid [31,32]. Interestingly, it was recently reported that, feeding trace amounts of copper (0.12 mg/l) in drinking water to cholesterol-fed rabbits could induce signs of Alzheimer's disease [33]. Moreover, injection of iron into cholesterol-fed rabbits has recently been reported to cause iron accumulation in the cerebral cortex [34]. One question to be addressed is then whether simultaneous administration of iron could slow down the copper mediated degenerative process.

The data shown in Table I, clearly demonstrate how iron can affect hydroxyl radical formation in copper contaminated, bicarbonate rich household drinking water samples. The formation of hydroxyl radicals in the drinking water samples, in the presence of ascorbic acid, was inhibited by 36.0–44.6% when 0.2 mg/l of ferric iron was present. This inhibition was even more significant, 47.0–59.2%, when 0.8 mg/l of ferric iron was present during the 3 h incubation period with ascorbic acid. Thus, as shown here, iron can to some extent prevent copper/reductant-induced formation of harmful hydroxyl radicals. The exact mechanism by which iron inhibits ascorbic acid/copper-induced hydroxyl radical formation in our water samples is not clear. The inhibition is unlikely to result from an experimental artifact since it is well known that coumarin-3-carboxylic acid can be used to detect iron driven hydroxyl radical reactions. [22,35,36] Moreover, our HPLC experiments using coumarin as the target molecule gave similar results. A plausible explanation for the iron-induced inhibition could be that ferric iron reacts with the superoxide generated from the copper/ascorbate redox reaction. Ferrous iron might also react with hydrogen peroxide and generate water and ferryl ions according to the Bray-Gorin reaction [37].

Iron is an essential micronutrient and the presence of iron in household drinking water is therefore not considered to be harmful. In fact, the intake of iron from drinking water, partly contributes to our daily iron intake. However, due to its offensive taste, color, foaming, odor, corrosion and staining of the drinking water, iron is considered by the water plants as a secondary contaminant. These characteristics are also the reason why excess iron in the drinking water is normally removed or adjusted to very low levels. Our results, however, indicate that complete removal of iron from the raw water in the water plants can to some extent increase the redox activity of copper, and the formation of reactive oxygen species in the drinking water. Moreover, iron deficiency can also increase the intestinal absorption of more harmful metals such as cadmium, lead, and aluminum [38].

In conclusion, our results demonstrate that iron cannot support ascorbic acid induced hydroxyl radical formation in a simple bicarbonate environment but unexpectedly displayed an inhibitory effect on the ascorbic acid induced hydroxyl radical formation process when copper was present. This phenomenon was also evident in our experiments performed in household drinking water samples. Thus, in the presence of bicarbonate, iron might function as an important regulator of copper/reductant-induced hydroxyl radical formation and copper mediated tissue damage. Our results might, to some extent, explain the mechanism for the iron induced protective effect that earlier has been seen in animal model systems.

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Iron prevents ascorbic acid (vitamin C) induced hydrogen peroxide accumulation in copper contaminated drinking water

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Abstract

Ascorbic acid (vitamin C) induced hydrogen peroxide (H_2O_2) formation was measured in household drinking water and metal supplemented Milli-Q water by using the FOX assay. Here we show that ascorbic acid readily induces H_2O_2 formation in Cu(II) supplemented Milli-Q water and poorly buffered household drinking water. In contrast to Cu(II), iron was not capable to support ascorbic acid induced H_2O_2 formation during acidic conditions (pH: 3.5–5). In 12 out of the 48 drinking water samples incubated with 2 mM ascorbic acid, the H_2O_2 concentration exceeded 400 μM. However, when trace amounts of Fe(III) (0.2 mg/l) was present during incubation, the ascorbic acid/Cu(II)-induced H_2O_2 accumulation was totally blocked. Of the other common divalent or trivalent metal ions tested, that are normally present in drinking water (calcium, magnesium, zinc, cobalt, manganese or aluminum), only calcium and magnesium displayed a modest inhibitory activity on the ascorbic acid/Cu(II)-induced H_2O_2 formation. Oxalic acid, one of the degradation products from ascorbic acid, was confirmed to actively participate in the iron induced degradation of H_2O_2. Ascorbic acid/Cu(II)-induced H_2O_2 formation during acidic conditions, as demonstrated here in poorly buffered drinking water, could be of importance in host defense against bacterial infections. In addition, our findings might explain the mechanism for the protective effect of iron against vitamin C induced cell toxicity.

Keywords: *Vitamin C, water, iron, copper, oxalic acid*

Introduction

Ascorbic acid (Vitamin C) is a water-soluble natural antioxidant that has been proposed to have beneficial effects on many age-related diseases such as atherosclerosis, cancer, neurodegenerative and ocular diseases [1–7]. It is believed that ascorbic acid can scavenge reactive oxygen- and nitrogen species and thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids and proteins [8–11]. On the other hand, it has also been shown that ascorbic acid can, in the presence of transition metal ions such as Cu(II) and Fe(III), function as a strong pro-oxidant [12–15].

The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions, and thereby stimulate the formation of reactive oxygen species (ROS) such as superoxide ($O\cdot_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). It is generally believed, that the cellular damage is caused by the hydroxyl radical (OH·). The hydroxyl radical can be directly formed from H_2O_2 and Fe(II) through the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$ [16,17]. This reaction can be strongly catalyzed if certain metal chelators such as EDTA and NTA and a reducing agent such as ascorbic acid are present [18–21]. Thus, in the absence of metal ions, H_2O_2 is not

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very reactive by itself, but imposes a threat due to its ability to easily diffuse through the cell membrane and then participate in metal induced free radical reactions inside the cell [22,23].

There are conflicting and confusing information regarding ascorbate and its cytotoxicity in the literature. Some reports clearly show that ascorbate is highly cytotoxic to cells [24–28], while others demonstrate that ascorbic acid can protect cells from pro-oxidative insult [1–7]. The toxic effect of ascorbic acid in cell systems has been attributed to H_2O_2 formation in the cell culture [29–31].

We have previously shown that ascorbic acid can trigger a pH dependent hydroxyl radical generating process in Cu(II) contaminated bicarbonate-buffered drinking water [32]. We found that this reaction could take place at pH levels above the pK_{al} value 4.25 of ascorbic acid. Here we have studied the chemical reactions that take place when ascorbic acid is added to either Cu(II) supplemented Milli-Q water or Cu(II) contaminated poorly buffered household drinking waters. We have addressed the question whether ascorbic acid can initiate a H_2O_2 accumulation process during acidic conditions (pH below 4.25) that do not support hydroxyl radical formation. The impact of iron on this process is studied in detail.

Materials and methods

Chemicals

Ascorbic acid, oxalic acid, ferrous chloride hexahydrate, ammonium ferrous sulfate, calcium chloride dihydrate and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Manganese chloride tetrahydrate, zinc chloride, cobalt chloride hexahydrate, xylenol orange sodium salt and 2,6-Di-tert-butyl-4-methanol-phenol were from Sigma, St. Louis, USA. Magnesium chloride hexahydrate was purchased from J.T. Baker, Denventer, Holland. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 mL polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of vitamin C induced H_2O_2 formation in drinking water

Measurement of H_2O_2 in household drinking water and domestic bottled waters was performed by using the FOX assay as described earlier [33]. Briefly, the FOX reagent was prepared by mixing 9 volumes of FOX-1 reagent (4.4 mM 2,6-Di-tert-butyl-4-methanol-phenol in 100% HPLC grade methanol) with 1 volume of FOX-2 reagent (1 mM xylenol orange sodium salt and 2.56 mM ammonium ferrous sulfate in 250 mM sulfuric acid). In the assay, 2 mM of ascorbic acid was added to the different water samples to initiate the reaction. After various time periods, 25 μl samples were withdrawn from the tubes and pipetted into an eppendorf tube containing 750 μl FOX-reagent. The mixture was vortexed for 5 s and incubated at room temperature for 30 min. After this, 200 μl of the mixture was pipetted in triplicates onto a 96 well microtiter plate and the absorbance of the samples and standards were measured at 560 nm with a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve where known concentrations of H_2O_2 were used.

Results

Copper, but not iron, can support ascorbic acid induced H_2O_2 formation in Milli-Q water

We have previously shown that addition of ascorbic acid to bicarbonate buffered tap water samples contaminated with Cu(II) ions can generate hydroxyl radicals [34–35]. In this study, we have evaluated whether ascorbic acid has the ability to induce H_2O_2 formation in Cu(II) contaminated drinking water. To study this, we first used Milli-Q water as a model system. As can be seen in Table I, ascorbic acid induced a substantial increase in the H_2O_2 concentration (499.2 ± 5.5 μM) in Milli-Q water supplemented with 0.1 mg/l of Cu(II). This concentration of Cu(II) is 20 times below the amount of copper that is allowed in drinking water in Europe (Maximum Contaminant Level, MCL). The H_2O_2 formation was very rapid and 293.7 ± 5.5 μM H_2O_2 could already be detected after 1 h incubation (Figure 1). On the contrary, when Fe(III), was used in the assay, low concentrations 45.3 ± 8.9 μM of H_2O_2 could be detected after 6 h. When Ca(II), Mg(II), Zn(II), Mn(II), Co(II) or Al(III) were used in the assay, the concentration of H_2O_2 varied between 57.6 ± 3.1 and 120.9 ± 4.7 μM. (Table I). These metal ions were tested by using their highest concentration that is allowed in drinking water (MCL in Europe).

Effects of ferric iron on ascorbic acid/copper-induced H_2O_2 formation in Milli-Q water

We have recently shown that ferric iron can interfere with ascorbic acid/Cu(II)-induced hydroxyl radical formation in drinking water [35]. To examine whether ferric iron also affects ascorbic acid/Cu(II)-induced H_2O_2 formation in Milli-Q water, we next measured H_2O_2 formation in the presence of various divalent and trivalent cations (Table II). When ferric iron, 0.2 mg/l, was present in the assay, a very weak inhibition could be observed during the first hour (Table II). After 2 h incubation the concentration of

Table I. Vitamin C induced H_2O_2 formation in Milli-Q water in the presence of various metal ions.

	H_2O_2 (μM) formation								
	Metal ion added (mg/l)								
Time (h)	None	Cu(II) (0.1 mg/l)	Fe(III) (0.2 mg/l)	Mn(II) (0.5 mg/l)	Co(II) (0.2 mg/l)	Al(III) (0.2 mg/l)	Zn(II) (0.5 mg/l)	Mg(II) (50 mg/l)	Ca(II) (100 mg/l)
0.25	7.1 ± 0.1	139.4 ± 4.0	17.6 ± 0.1	6.4 ± 0.1	8.7 ± 0.1	25.0 ± 2.3	0.9 ± 3.1	6.4 ± 1.6	6.4 ± 0.1
1	18.1 ± 2.4	293.7 ± 5.5	18.4 ± 1.9	10.4 ± 1.4	21.4 ± 0.8	29.0 ± 1.7	12.0 ± 3.1	9.8 ± 1.5	14.8 ± 0.8
2	27.6 ± 1.6	402.6 ± 3.9	27.8 ± 4.9	19.2 ± 0.8	37.6 ± 0.1	32.7 ± 0.6	24.2 ± 1.6	21.4 ± 0.8	23.7 ± 0.8
3	35.9 ± 3.9	455.9 ± 0.8	34.5 ± 1.4	30.3 ± 0.8	57.0 ± 0.8	45.3 ± 1.1	40.3 ± 0.8	28.1 ± 0.8	34.8 ± 2.4
4	50.3 ± 3.9	486.4 ± 3.1	47.3 ± 4.2	40.9 ± 1.6	84.8 ± 2.4	49.4 ± 1.7	55.9 ± 2.4	40.3 ± 2.4	50.3 ± 0.8
5	47.6 ± 6.3	497.0 ± 3.9	45.6 ± 3.2	52.0 ± 1.6	96.4 ± 0.1	60.1 ± 1.3	66.4 ± 6.2	51.4 ± 2.4	49.8 ± 0.1
6	52.0 ± 9.4	499.2 ± 5.5	45.3 ± 8.9	63.1 ± 0.1	120.9 ± 4.7	71.3 ± 0.6	81.4 ± 3.9	57.6 ± 3.1	68.7 ± 3.1

Time dependent formation of H_2O_2 by ascorbic acid (2 mM) in Milli-Q water supplemented with 0.1 mg/l Cu(II). The concentrations of the other metal species used are MCL of the metals that is allowed in European drinking water. The values shown are the concentration of H_2O_2 formed during the incubation in dark at room temperature. Data are expressed as mean ± SD of triplicates of one representative experiment out of three conducted.

Figure 1. Effects of Iron/oxalic acid on ascorbic acid/Cu(II)-induced hydrogen peroxide accumulation in Milli-Q water. (A) 2 mM ascorbic acid was added to Milli-Q water samples containing either 0.1 mg/l Cu(II) (•); 0.1 mg/l Cu(II) and 0.2 mg/l Fe(III) (▲); 50 μM oxalic acid, 0.2 mg/l Fe(III) and 0.1 mg/l copper (△) or 50 μM oxalic acid and 0.1 mg/l copper (□). Where indicated by an arrow, 50 μM oxalic acid and 0.2 mg/l Fe(III) were added to a sample incubated for 2 h with 2 mM ascorbic acid and 0.1 mg/l Cu(II) (○). Data points are mean ± SD of three experiments. If absent, error bars are smaller than the symbols.

H_2O_2 reached 300.9 ± 10.5 μM and after this the concentration of H_2O_2 started to decrease in the water sample. After 6 h incubation, the H_2O_2 level had decreased to 57.0 ± 10.4 μM as compared to 509.5 ± 14.8 μM measured in the control sample not containing iron. Other metal ion species that might be present in drinking water, Zn(II), Co(II), Mn(II) or Al(III) did not have any impact on the ascorbic acid/(CuII)-induced H_2O_2 formation. A modest inhibition in H_2O_2 formation could be seen when Mg(II) or Ca(II) were present. However, the concentrations of Mg(II) and Ca(II) were 250- and 500-fold higher than the concentration of iron used in the assay. This is because the concentration of all the metal ions used in the assay are the amounts of these contaminants that are currently allowed in drinking water (MCL in Europe).

Iron/oxalic acid inhibits ascorbic acid/copper-catalyzed H_2O_2 formation

In our previous work, we have established that oxalic acid is one of the degradation products generated when ascorbic acid is oxidatively decomposed in copper contaminated drinking water [36]. This implies that oxalic acid might participate in the iron catalyzed H_2O_2 decomposition, since oxalic acid is known to have high affinity for ferric iron even at very low pH. To verify this, 0.1 mg/l Cu(II) and 2 mM

Table II. Effects of metal ions on ascorbic acid/copper induced H_2O_2 formation in MQ-water.

	H_2O_2 formation (μM)							
		Metal ions (mg/l) present in copper supplemented MQ-water						
Time (h)	Only Cu(II) (0.1 mg/l)	Fe(III) (0.2 mg/l)	Mn(II) (0.5 mg/l)	Co(II) (0.2 mg/l)	Al(III) (0.2 mg/l)	Zn(II) (0.5 mg/l)	Mg(II) (50 mg/l)	Ca(II) (100 mg/l)
0.25	141.4 ± 3.9	131.4 ± 2.4	144.8 ± 0.8	135.3 ± 1.6	133.9 ± 0.6	119.2 ± 3.9	132.6 ± 2.4	137.0 ± 0.8
1	308.4 ± 17.9	282.8 ± 11.6	320.9 ± 7.9	325.3 ± 6.3	310.5 ± 5.6	300.9 ± 11.0	282.0 ± 12.6	294.8 ± 5.5
2	413.1 ± 12.8	300.9 ± 10.5	399.8 ± 3.1	410.3 ± 5.5	402.0 ± 5.1	385.3 ± 6.3	358.7 ± 4.7	347.6 ± 4.7
3	457.0 ± 8.7	245.6 ± 27.4	440.3 ± 0.8	445.3 ± 3.1	459.0 ± 6.8	439.2 ± 7.1	387.6 ± 1.6	373.1 ± 4.7
4	492.8 ± 11.5	175.9 ± 18.9	471.4 ± 2.4	485.3 ± 4.7	459.8 ± 7.7	466.4 ± 3.1	407.0 ± 0.8	404.2 ± 1.6
5	501.7 ± 8.6	102.3 ± 18.4	493.7 ± 0.8	508.7 ± 3.1	486.4 ± 8.7	488.7 ± 14.1	433.1 ± 7.9	409.2 ± 2.4
6	509.5 ± 14.8	57.0 ± 10.4	502.0 ± 9.4	511.4 ± 5.5	502.0 ± 1.1	500.9 ± 6.3	434.2 ± 0.1	420.3 ± 2.4

Time dependent formation of H_2O_2 by ascorbic acid (2 mM) in Milli-Q water supplemented with 0.1 mg/l Cu(II) and various ion species (concentrations used are their MCL in Europe). The values shown are the concentration of H_2O_2 formed during the incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

ascorbic acid were added to Milli-Q water supplemented with both iron and oxalic acid. When 0.2 mg/l Fe(III) and 50 μM oxalic acid were present from the start of the reaction, extremely low concentrations of H_2O_2 could be detected in the Milli-Q water (Figure 1). Likewise, when 0.2 mg/l Fe(III) and 50 μM oxalic acid were added to the ascorbic/Cu(II) induced H_2O_2 generating reaction after 2 h incubation, the H_2O_2 concentration in the sample rapidly decreased. Furthermore, when oxalic acid alone was present in the assay from the beginning, the ascorbic acid/Cu(II)-induced H_2O_2 formation was also affected.

Ascorbic acid induced H_2O_2 accumulation in household drinking water–The relationship between copper, iron and bicarbonate

Next, we measured the amount of H_2O_2 that was formed during a 6-h incubation when 2 mM ascorbic acid was added to 40 tap water samples and 8 domestic bottled water samples (Table III). The drinking water samples tested had been sampled in the same way (directly drawn from the tap) but they originated from different municipal water suppliers. The observed levels of H_2O_2 generated varied between 0 and 488.4 μM.

When ascorbic acid was added to the low buffered drinking water samples that were contaminated with copper, H_2O_2 was generated. However, in some of the drinking water samples that were contaminated with copper but showed higher buffering capacity, much lower levels of H_2O_2 was formed in the presence of ascorbic acid. Therefore, to study the impact of bicarbonate on the H_2O_2 formation, we next performed experiments in Milli-Q water supplemented with various concentrations of bicarbonate. As shown in Figure 2, the ascorbic acid induced accumulation of H_2O_2 in the presence of 0.1 mg/l copper was significantly decreased when the concentrations of bicarbonate was increased. Consequently, in the presence of bicarbonate, lower concentrations of ferric iron was required to inhibit the hydrogen peroxide accumulation.

Discussion

We have previously shown that addition of ascorbic acid (vitamin C) to tap water samples contaminated with Cu(II) ions can generate hydroxyl radicals [32,34,35]. Here, we have studied whether ascorbic acid can initiate a H_2O_2 accumulating reaction in Cu(II) contaminated poorly buffered drinking water or Cu(II) supplemented Milli-Q water. Our results show that ascorbic acid can initiate a time dependent accumulation of H_2O_2 in copper contaminated poorly buffered drinking water. The H_2O_2 formation occurred at very low concentration of Cu(II) ions

Table III. Vitamin C induced H_2O_2 formation in drinking water.

Household drinking water (from taps)									Bottled water	
Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation
1	417.3 ± 17.4	9	219.5 ± 8.2	17	0	25	282.8 ± 20.3	33	106.7 ± 27.4	41	43.7 ± 12.8
2	20.1 ± 13.2	10	92.0 ± 13.1	18	396.4 ± 7.1	26	62.6 ± 3.5	34	294.5 ± 19.0	42	123.9 ± 2.5
3	252.6 ± 22.9	11	96.4 ± 20.6	19	408.1 ± 10.8	27	398.9 ± 9.2	35	175.9 ± 8.2	43	97.3 ± 13.4
4	85.6 ± 4.3	12	482.0 ± 19.0	20	426.7 ± 16.2	28	361.7 ± 3.9	36	175.9 ± 4.9	44	71.2 ± 42.7
5	481.7 ± 13.6	13	115.9 ± 7.8	21	160.3 ± 3.8	29	142.8 ± 8.8	37	356.7 ± 12.3	45	226.2 ± 11.7
6	488.4 ± 8.8	14	177.6 ± 32.2	22	476.4 ± 30.2	30	440.3 ± 8.7	38	188.9 ± 6.8	46	44.2 ± 8.5
7	488.4 ± 16.6	15	454.2 ± 9.1	23	121.4 ± 5.9	31	252.6 ± 3.9	39	375.9 ± 8.6	47	457.8 ± 12.5
8	219.2 ± 7.1	16	326.4 ± 12.8	24	67.3 ± 6.9	32	305.1 ± 7.4	40	135.6 ± 42.7	48	474.2 ± 4.4

Vitamin C (2 mM) induced H_2O_2 formation in tap water samples (numbered 1–40) and domestic bottle water samples (numbered 41–48). The values shown are the concentration of H_2O_2 formed during a 6-h incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

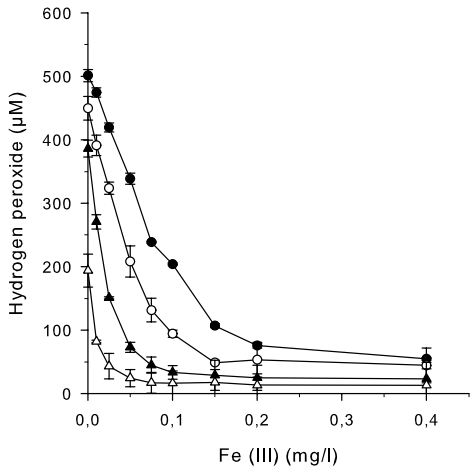

Figure 2. Effect of bicarbonate and iron on ascorbic acid induced hydrogen peroxide accumulation. Ascorbic acid (2 mM) and various concentrations of Fe(III) were added to Milli-Q water supplemented with 0.1 mg/l Cu(II) and 0 mg/l (•); 25 mg/l (○); 50 mg/l (▲) and 100 mg/l (△) HCO_3. The H_2O_2 concentration was measured in the samples after a 6 h incubation at room temperature. Data points are mean ± SD of three experiments.

(20 times below the amount of copper that is allowed in drinking water (MCL in Europe)). Of the other metals tested, only Co(II) generated significant amounts of H_2O_2 (Table I).

The ascorbic acid induced H_2O_2 accumulation in copper supplemented Milli-Q water was significantly higher than the H_2O_2 formation observed earlier in cell culture and cell culture medium [37,38]. However, addition of ascorbic acid to the Milli-Q water model system used in our experiments, resulted in a much more acidic milieu (pH 3.5) than the one normally seen in buffered cell culture medium. Also the pH in our drinking water samples were close to or under the pK_{al} value of ascorbic acid (4.25). During more neutral conditions (pH 6–6.5) that was obtained when magnesium ascorbate, calcium ascorbate or sodium ascorbate were added to Cu(II) supplemented Milli-Q water, less amount of H_2O_2 was generated within 6 h (data not shown). These results are in better agreement with the amounts of H_2O_2 generated in cell cultures and cell culture medium [37,38]. This emphasizes the importance of pH in the ability of ascorbic acid to induce H_2O_2 accumulation in the presence of Cu(II) ions.

In our hands, of the metal ions tested, only Fe(III) was found to strongly affect the ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water during acidic conditions (pH 3.5) (Table II). The inhibition of the H_2O_2 accumulation could be observed after 2 h incubation, and after 6 h incubation the majority of the H_2O_2 formed had been eliminated from the water

sample (Table II, Figure 1). The shape of the ascorbic acid/Cu(II) induced H_2O_2 curve, obtained in the presence of ferric iron, strongly indicated that a metabolite from ascorbic acid might be involved in the H_2O_2 decomposition reaction. Consistent with this assumption, we found that oxalic acid, together with Fe(III) induced a prompt inhibition of ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water (Figure 1). The reason why we used oxalic acid was because we have previously demonstrated that oxalic acid is one of the degradation products when ascorbic acid is oxidatively decomposed in Cu(II) contaminated drinking water [36].

Oxalic acid also decreased the ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water, implying direct interaction between Cu(II) and oxalic acid. The mechanism for the oxalic acid mediated inhibitory effect on ascorbic acid/copper induced H_2O_2 formation in Milli-Q water might be due to its ability to interfere with copper redox-cycling. This is because oxalic acid is known to have affinity for both Fe(III) and Cu(II), even at very low pH. Alternatively, copper/oxalic acid could catalyze H_2O_2 to hydroxyl radicals. However, we could not detect any ascorbic acid induced hydroxyl radical formation when oxalic acid was added to copper supplemented water (data not shown), implying reduced copper redox-activity when oxalic acid was present. Interestingly, it has been suggested that oxalic acid could act as an antioxidant in some systems, because oxalic acid reduces the rate of ascorbic acid oxidation in the presence of H_2O_2 and Cu(II) [39].

The ability of Fe(III) and oxalic to regulate ascorbic acid/Cu(II)-induced H_2O_2 formation during acidic conditions as shown in our experiments could be of importance *in vivo* when acidic conditions prevail. This could emphasize the importance of vitamin C, oxalic acid and iron during certain conditions such as the inflammatory process. Thus, when Cu(II) ions and Vitamin C are present during acidic conditions, the presence or absence of free redox-active iron and oxalic acid will determine the amount of H_2O_2 that will be accumulated. In particular, during conditions where catalase is not present or not functioning properly e.g. in the presence of ascorbic acid and copper [40,41], the iron/oxalic acid complex could be of importance in regulating H_2O_2 toxicity during acidic conditions in various biological systems *in vivo*.

The vitamin C induced accumulation of H_2O_2 could also be demonstrated in 40 household tap water samples and 8 domestic bottled water samples. In 25% of the drinking water samples tested, over 400 μM of H_2O_2 was formed during the 6 h incubation and some drinking waters generated close to 500 μM of H_2O_2 within 6 h (Table III). The kinetics for the H_2O_2 formation in these drinking waters was relative fast, reaching close to 300 μM H_2O_2 in only 2 h (data not shown). The ascorbic acid induced hydrogen peroxide formation was particularly evident in the poorly buffered copper contaminated drinking water samples. When bicarbonate was added to Milli-Q water supplemented with copper, the hydrogen peroxide accumulation was much lower (Figure 2). During these conditions the hydrogen peroxide is converted to hydroxyl radicals [32].

Hydrogen peroxide has been shown to have strong antibacterial effects [42]. One might speculate, whether the ascorbic acid/Cu(II)-induced H_2O_2 formation and the ability of Fe(III) and oxalic acid to regulate H_2O_2 formation, as demonstrated here in drinking water, could be involved in controlling the survival of pathogenic bacteria in the human gastrointestinal tract. On the other hand, our results demonstrating that vitamin C induces H_2O_2 formation in drinking water could, in fact, result in increased oxidative stress *in vivo*. In particular, in the absence of Fe (III) ions, ascorbic acid/Cu(II)-induced H_2O_2 formation in drinking water, as demonstrated here, could strongly enhance the total H_2O_2 load and H_2O_2 induced oxidative stress in some individuals.

In conclusion, our results show that copper, but not iron, can support vitamin C induced H_2O_2 formation in weakly buffered drinking water. Moreover, we show that iron, together with ascorbic acid-derived oxalic acid prevents ascorbic acid/Cu(II) induced H_2O_2 accumulation. Our results strongly indicate that the iron oxalic acid complex might function as an important regulator of ascorbic acid/copper-induced H_2O_2 formation and copper mediated tissue damage. The iron/oxalic acid induced inhibition of H_2O_2 formation, as demonstrated here in drinking waters, might explain the mechanism for the protective effect of iron against ascorbic acid (vitamin C) induced cell toxicity in cell cultures.

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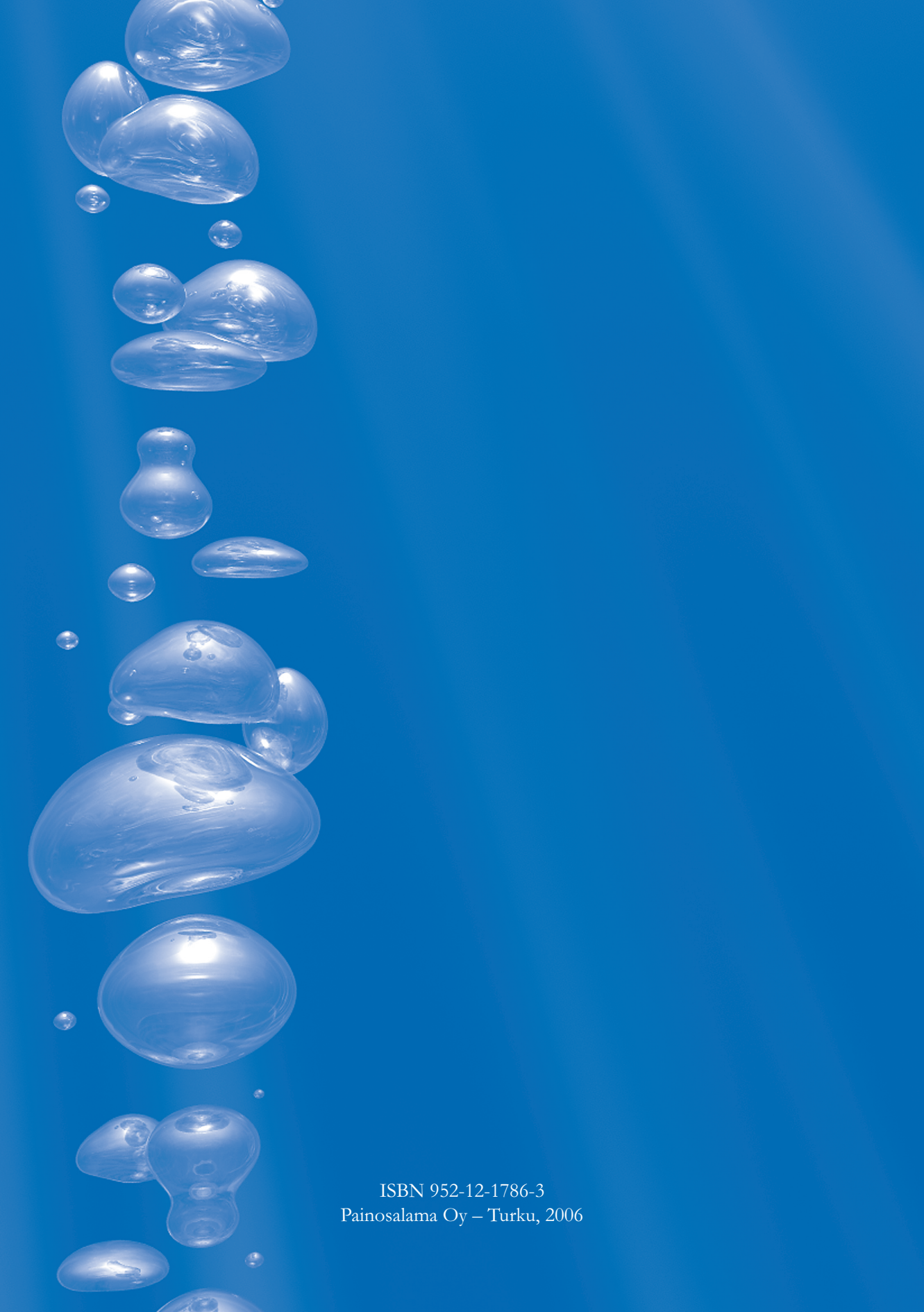

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