Studies on the Properties and Real Existence of Aqueous Solution Systems that are Assumed to Have Antioxidant Activities by the Action of "Active Hydrogen"

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(Received March 2, 2004; Accepted June 9, 2004)

We evaluated the properties and real existence of an electrolyzed-reduced water, which we prepared, and three commercially purchased water goods, that are advertised to have antioxidant activities by the action of "active hydrogen," on the basis of the results of examinations for inhibitory effects on the oxidative reactions of biomolecules, quantitative analyses of the minerals, and the ESR spectral data in measurement of the scavenging ability for reactive oxygen species. The results suggested that all of the examined aqueous solution systems undoubtedly have antioxidant activities *in vitro* and that such effects are derived from ordinary molecular hydrogen (hydrogen gas) and/or (a) reductive vanadium ion(s). "Active hydrogen" seems to be absent as an effective component of the antioxidant activities of these aqueous solution systems.

Key words —— reduced water, antioxidant activity, oxygen-radical scavenger, ESR spectrometry, hydrogen, vanadium

INTRODUCTION

In recent years, many companies have developed and sold water goods (aqueous solution systems) for health. Some of such solutions are advertised to have antioxidant activities scavenging harmful reactive oxygen species (ROS). It is known that electrolysis of aqueous solutions of electrolytes affords acidic solutions near the anode (electrolyzed-oxidized waters, EOWs) and alkaline solutions near the cathode (electrolyzed-reduced waters, ERWs). Shirahata *et al*. studied the properties of an ERW which they prepared by electrolysis of an aqueous solution of sodium chloride (NaCl), and reported that the examined ERW showed a superoxide dismutase-like activity alleviating the oxidative damage of DNA caused by an L-ascorbic acid (AsA)-Cu^{2+} reaction system.[1] In that paper they also presented a hypothesis that "active hydrogen," namely atomic state hydrogen (not H_2 but H), may be contained stably in the ERW.[1] Shirahata also declared elsewhere that "active hydrogen" may be present in various artificial and natural aqueous solution systems with antioxidant activities.[2,3] Shirahata *et al*. also investigated the biological effects of such aqueous solution systems called reduced waters. Suppression of growth of cultured human cancer cells and acceleration of glucose intake by cells in diabetic rats were reported as the effects of reduced waters.[4] In association with publication of their work, some companies, such as Nihon Trim (Osaka, Japan), Water Institute (Atarashiimizunokai) (Tokyo, Japan) and Hita Tenryosui Kabushikigaisha (Oita, Japan) and others, have developed and sold various water goods for health, advertising that they contain or produce "active hydrogen" which must be a potent scavenger for harmful ROS ["I'm Fine," the electrolyzed-reduced water of Nihon Trim; http://www.kangen-

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water.jp, Water Institute (Atarashiimizunokai); http://www.water-institute.org, Hita Tenryousui Kabushikigaisha; http://www.hitatenryousui.co.jp]. They usually referred to the first paper of Shirahata *et al.*[1] as a proof for confirmation of the presence of "active hydrogen" in the corresponding water goods. We doubted this hypothesis since, if "active hydrogen" is stably present as a solute in aqueous media, conventional physical and chemical knowledge must be essentially denied. Shirahata later proposed a newly-modified theory that "active hydrogen" is accumulated on the surface of metal clusters contained in the corresponding aqueous solution systems not as (a) solute(s) but as (a) nanocolloidal solid component(s).[5] More recently, the Water Institute expressed in the internet home page (HP) that the water of their commercial product contains not "active hydrogen" but molecular hydrogen which is enzymatically degraded in the human body into "active hydrogen" reacting with ROS [Water Institute (Atarashiimizunokai); http://www.water-institute.org]. At present we have little knowledge on the properties and real existence of reduced waters (aqueous solution systems with antioxidant activities), that are assumed to have antioxidant activity by the action of "active hydrogen." In this study, we examined the inhibitory effects of an ERW, which we prepared, and three commercially-purchased water goods, that are advertised to have or produce antioxidant activities by the action of "active hydrogen," on the oxidative reactions of biomolecules. Determination of their mineral components (both metal elements and anions) and measurement by ESR spectrometry of their ability to scavenge ROS were also performed. The results are discussed in connection with possibility of the presence of "active hydrogen."

MATERIALS AND METHODS

Materials —— An ERW was prepared by electrolysis of an aqueous NaCl with an ROX-10WA Electrolyzed-Water Generator (Hoshizaki Electric Co. Ltd., Osaka, Japan). In the electrolysis using this apparatus, the original electrolyte solution obtained by dissolving 140 g of NaCl in 1 l of tap water (TW) is automatically introduced into the chamber to be diluted with a solution prepared by passing TW through ion-exchanging membranes, followed by electrolysis of the mixture in the chamber at 100 V. These procedures are normally operated to produce an acidic EOW with antimicroorganism activities, although an alkaline ERW can also be simultaneously generated. Among them, only the ERW was treated in this study. An aqueous solution system called "I'm Fine" (IF) as a water commercial product, which is advertised to be prepared by electrolysis with a Nihon Trim apparatus of underground water near Mt Fuji ("I'm Fine," the electrolyzed-reduced water of Nihon Trim; http://www.kangen-water.jp), was purchased from Nihon Trim. Mineral-stick water (MSW) was prepared by extraction for 1 hr at room temperature with 1.5 l of deionized water (DW) of a hydrogen promoting mineral (HPM)-stick called Kasseisuisokun (Mr. "Active Hydrogen," in Japanese) purchased from the Water Institute; this procedure is the same as that in the protocol of the Water Institute except for the use of DW instead of TW [Water Institute (Atarashiimizunokai); http://www.water-institute.org]. Hita tenryosui water (HT), which is advertised to be underground water collected in Hita-shi, Oita-ken, Japan (Hita Tenryousui Kabushikigaisha; http://www.hitatenryousui.co.jp), was purchased from Hita Tenryosui Kabushikigaisha. A pooled human serum sample was taken from normal healthy persons.

Examinations for Antioxidant Activities —— Inhibitory effects of aqueous solution systems examined on the oxidative reactions of biomolecules were measured as following (1)–(3). The experiments were performed four times for each of (1), (2) and (3), respectively.

(1) The human serum (0.25 ml each) was diluted to 1.0 ml by adding mixtures (0.75 ml each) of samples to be examined and DW at volume ratios of 0 : 3 (the control), 1 : 2, 2 : 1 and 3 : 0, respectively; samples were ERW (an ERW we prepared), IF, MSW, HT, TW (in Hino-shi, Tokyo, Japan; the solvent of the original electrolyte for preparation of ERW) and an aqueous solution of caffeic acid (CFA) as a known antioxidant with an initial concentration of 100 μM. The reaction was started by further addition of an aqueous solution (50 μl each) of copper sulfate ($CuSO_4$) with an initial concentration of 210 μM. The reaction mixtures thus prepared to contain Cu^{2+} with a final concentration of 10 μM as an oxidant were left at room temperature for 2 hr. Then, the reaction was terminated by addition of Na-ethylenediaminetetraacetate with a final concentration of 50 μM. When the reaction was started and terminated, malondialdehyde (MDA) was determined by the thiobarbituric acid method as described previously,[6] and the lipid peroxide (LPO) quantity

generated in each sample was finally evaluated from the difference of the MDA levels between the start and termination of the reaction.

(2) Reaction mixtures consisting of a 100 mM trishydroxyaminomethane buffer solution (pH 7.4) (0.2 ml each), human serum (0.2 ml each) and samples to be examined (0.6 ml each) were prepared; samples were the same as in (1) except for the use as a known antioxidant of the same concentration of aqueous AsA instead of CFA. The reaction mixtures thus prepared were left at 4°C in the dark, and at 0 hr (when the reaction was started), 17 hr, 41 hr and 65 hr after the preparation, bilirubin (BR) was determined by the diazo method as described previously.[7]

(3) Rutin (= quercetin-3-rhamnoglucoside, Kanto Chemicals, Tokyo, Japan) was dissolved at a concentration of 100 μM in solutions (1.0 ml each) that were prepared by adding 100 mM phosphate buffer solution (pH 6.8) (0.25 ml each) to the mixtures (0.75 ml each) of same samples as in (2) and DW at volume ratios of 0 : 3 (the control), 1 : 2, 2 : 1 and 3 : 0, respectively. The enzymatic oxidation of rutin was started by mixing the above-mentioned rutin solutions with an equal volume of an aqueous solution in which 25 units of polyphenol oxidase (PPO) (mushroom tyrosinase, Sigma, St. Louis, MO, U.S.A.) was dissolved. The reaction mixtures thus prepared to contain PPO as a catalyst for the oxidative reaction and rutin as the substrate were then left at 25°C without shaking. When the reaction mixtures were prepared and 1 hr later, a constant volume each of the reaction mixtures was withdrawn, and heated at 100°C for 5 min in a stoppered-vial to inactivate the enzyme, followed by ultrafiltration with Centricon-10 miniconcentrators (Amicon Japan, Tokyo, Japan) at 2000 × g and 4°C for 30 min to remove the enzyme proteins (only the solutes with molecular weight (MW) below 10000 can penetrate the membrane of this tool). Twenty μl each of the ultrafiltrates were then injected into HPLC, by which rutin can be separated from its oxidation products and determined.[8]

Analyses of the Minerals —— Prior to the analyses, samples to be examined (ERW, IF, MSW, HT and TW) were passed through a filter with a pore size of 0.45 μm. The concentrations of the major metal elements, such as Na, potassium (K), calcium (Ca) and magnesium (Mg), were measured by an atomic absorbance spectrometer (a Perkin Elmer Analyst 800). Other trace metals listed in Table 1 were determined by a microwave-induced plasma mass spectrometer (a Hitachi P-7000). Anions such as chloride (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}) were quantitatively analyzed by ion-chromatography with a Nihon Dionex DX-A.

Measurement of the ROS Scavenging Ability by ESR Spectrometry —— The ESR spectra were taken with a JEOL JES-RE1X using a quartz flat cell designed for aqueous solution, under the conditions of the magnetic field of 336.5 mT, the power of 8.0 mW, the modulation frequency of 100 kHz, the operating frequency of 9.456 GHz, the modulation amplitude of 0.063 mT, the gain of 200, the time scan of 0.5 min, and the time constant of 0.03 sec. Dimethyl sulfoxide (50 μl) and the same volume each of 25 mM sodium hydroxide (NaOH) and sample solution examined (ERW, IF, MSW, HT and distilled water as the reference) were mixed in a disposable plastic tube, followed by addition of 5,5-dimethyll-pyrroline-N-oxide (DMPO) (5 μl) and 30% hydrogen peroxide (H_2O_2) (50 μl). The reaction mixtures thus prepared were sucked into the quartz flat cell and set in the ESR apparatus. Scanning was started at 10 min after the addition of H_2O_2. The signal intensity of detected oxygen radical species-DMPO adducts reached plateaus between 10 and 20 min after the start of the reaction. The signal height of the program was calculated employing a radical analyzing program attached to the apparatus. The calculation was performed for the positive signal of hydroxy radical (•OH)-DMPO adduct and the negative signal of superoxide radical (O_2^-)-DMPO adduct, and the ratios of signal intensity against manganese ion (Mn^{2+}) as the reference were obtained. The ability of the sample solutions to scavenge •OH and O_2^- was expressed as relative values (%) of the above ratios to the reference as 100%.

Examinations for the Antioxidant Activities of Hydrogen Gas —— It is well known that molecular hydrogen (hydrogen gas) is generated when metallic Mg is dissolved in water to form magnesium cation and hydroxyl anion. A reaction mixture of Mg/DW was prepared by adding 200 mg of metallic Mg powder to 100 ml of DW, and allowed to stand for 24 hr at room temperature. At 0 hr (when it was prepared), 1 hr, 2 hr, 3 hr and 24 hr after the preparation, a constant volume each of the solution was withdrawn and passed through a filter with a pore size of 0.45 μm. The filtrates (the aqueous phase of the reaction mixture) obtained at each time were examined for the inhibitory effects on biomolecules, as in (1)–(3) descrived above, and also for the Mg

Table 1. The Concentrations of Minerals in Samples Examined

Elements	ERW[a)]	IF[b)]	MSW[c)]	HT[d)]	TW[e)]
Major cations (mg/l, by AAS[f)])					
Na	304.0	UD[g)]	0.2	20.1	11.8
K	1.1	2.1	0.1	8.6	1.3
Ca	0.3	17.5	1.1	7.5	9.9
Mg	0.1	5.4	15.5	1.9	5.8
Trace Metals (μg/l, by MIP-MS[h)])					
Li	UD[g)]	0.4	0.4	12.0	3.7
Al	2.6	0.9	1.1	3.9	3.9
Ti	0.4	UD[g)]	UD[g)]	0.1	0.1
V	2.8	18.5	UD[g)]	13.2	1.4
Cr	0.1	0.2	UD[g)]	0.9	0.1
Fe	1.3	4.7	0.4	3.0	40.4
Co	0.1	UD[g)]	UD[g)]	UD[g)]	0.4
Ni	1.0	0.7	0.1	0.5	7.3
Cu	UD[g)]	0.2	UD[g)]	UD[g)]	8.3
Zn	UD[g)]	4.3	5.3	UD[g)]	9.8
Ge	UD[g)]	0.2	UD[g)]	0.4	UD[g)]
As	1.8	0.3	0.1	1.3	0.9
Se	0.1	0.3	UD[g)]	1.5	0.3
Rb	0.1	3.1	0.1	23.5	1.3
Sr	0.9	38.2	10.8	56.8	88.6
Mo	0.2	0.3	UD[g)]	1.2	0.3
Pd	UD[g)]	0.2	UD[g)]	0.3	0.4
Cd	0.7	UD[g)]	0.4	0.4	0.6
Sn	1.3	0.6	0.2	UD[g)]	0.2
Ba	UD[g)]	3.2	3.5	12.7	5.4
Pt	UD[g)]	UD[g)]	UD[g)]	UD[g)]	UD[g)]
Pb	0.3	UD[g)]	0.4	0.3	0.6
Anions (mg/l, by IC[d)])					
Cl^-	35.0	4.9	0.5	11.4	13.8
PO_4^{3-}	UD[g)]	UD[g)]	UD[g)]	UD[g)]	UD[g)]
NO_2^-	6.9	UD[g)]	UD[g)]	UD[g)]	UD[g)]
NO_3^-	7.4	5.1	0.8	1.4	8.8
SO_4^{2-}	19.1	11.8	UD[g)]	3.8	22.6

a) Electrolyzed reduced water, *b*) "I'm Fine," *c*) Mineral-stick water, *d*) Hira tenryosui water, *e*) Tap water, *f*) Atomic absorbance spectroscopy, *g*) Under detectable (below 0.1 mg/l for AA and IC and 0.1 μg/ml for MIP-MS), *h*) Microwave-induced mass spectroscopy. Other abbreviations in the text.

concentrations with a commercial kit of Wako Pure Chemicals (Osaka, Japan) (Mg Test Wako-B) which employs the colorimetric method using xylylazo-violet.

Others —— In order to evaluate the degree of contribution of the substance(s) other than (a) volatile component(s), in generation of the antioxidant activities of ERW, IF, MSW and HT, samples of these aqueous solution systems after boiled and re-cooled in an opened container were examined for the antioxidant activities as in (1)–(3). In order to elucidate whether the antioxidant activities of these aqueous solution systems are deribed only from the low MW solutes, their ultrafiltrates, that were obtained by centrifuging of these solutions as described in (3), as well as the control sample centrifuged under the same conditions without ultrafiltration, were also examined for the antioxidant activities as in (1)–(3). Furthermore, two aqueous solutions with approximately the same mineral compositions as in ERW and MSW

(Table 1) were prepared by dissolving 55 mg of NaCl and 16 mg of NaOH in 100 ml of TW (for ERW) and by dissolving magnesium hydroxide ($Mg(OH)_2$) in DW at the concentration of 38 mg/l (for MSW), respectively. The antioxidant activities of these aqueous solutions thus prepared without electrolysis or extraction of a HPM-stick were also checked as in (1)–(3).

RESULTS AND DISCUSSION

The results of examinations for the inhibitory effects of tested aqueous solution systems on the oxidation of biological molecules are summarized in Table 2. In the experiments of oxidation by Cu^{2+} of human serum lipid (1), the mean value and standard deviation (S.D.) of the LPO concentration in the control sample before the reaction was 0.97 nmole/ml and 0.05 nmole/ml ($n = 4$), respectively. After the reaction, it was elevated to be 1.69 ± 0.09 nmole/ml (mean ± S.D., $n = 4$), suggesting that 0.72 ± 0.04 nmol/ml LPO (as MDA) was generated by the action of 10 μM Cu^{2+}. Mean values (± S.D.) for the LPO quantity generated in the samples involving ERW, IF, MSW, HT, TW, and aqueous CFA with various volume ratios to DW in the reaction mixtures were calculated in the same manner, respectively. All of such data are summarized in Table 2a. As shown in Table 2a, the LPO quantities generated in the samples involving only ERW (0.59 ± 0.04 nmole/ml, $n = 4$), IF (0.53 ± 0.03 nmole/ml, MSW (0.56 ± 0.04 nmole/ml, $n = 4$), HT (0.55 ± 0.04 nmole/ml, $n = 4$) and aqueous CFA (0.18 ± 0.02 nmole/ml, $n = 4$) were significantly lower ($p < 0.05$) than that in the control sample (0.72 ± 0.04 nmole/ml, $n = 4$), respectively. Such effects of the four aqueous solution systems and aqueous CFA were reduced depending on decreases in their volume ratios to DW in the reaction mixtures, although the LPO quantities generated in the samples involving all of them with the volume ratios of 2 : 1 and the sample involving aqueous CFA with that of 1 : 2 were also significantly lower ($p < 0.05$) than the control sample (Table 2a). These results suggest that the generation of LPO by Cu^{2+} from human serum lipid is inhibited *in vitro* by the four aqueous solution systems examined which are assumed to have antioxidant activities, although their inhibitory effects are considerably smaller than 100 μM aqueous CFA. On the other hand, the LPO quantities generated in the samples involving TW with various volume ratios to DW tended to be elevated in association with increase in the volume ratios, indicating that the sample involving only TW (0.83 ± 0.05 nmole/ml, $n = 4$) was significantly greater ($p < 0.05$) than the control sample (0.72 ± 0.04 nmole/ml, $n = 4$) (Table 2a). Therefore, TW may act as a weak prooxidant *in vitro* on the human serum lipid. This will be discussed later. In the experiments of oxidation by oxygen in air of human serum BR (2), the BR concentration in the freshly-prepared reaction mixture for the control sample was 1.15 ± 0.07 μg/ml ($n = 4$). At 17, 41 and 65 hr after the preparation, the BR content in the control sample decreased time–dependently to 0.95 ± 0.05 μg/ml ($n = 4$), 0.68 ± 0.06 μg/ml ($n = 4$) and 0.37 ± 0.05 μg/ml ($n = 4$), respectively. In the control sample, the quantities of BR, which disappeared due to oxidation by oxygen in air, in the first 17 hr, the following 24 hr and the last 24 hr, were 0.20 ± 0.02 μg/ml ($n = 4$), 0.27 ± 0.02 μg/ml ($n = 4$) and 0.31 ± 0.03 μg/ml, respectively. Such data obtained for all of sample involving examined aqueous solution systems are summarized in Table 2b. As shown in Table 2b, under the conditions employed, the BR quantities lost by the oxidation in the first 17 hr in the samples involving ERW (0.09 ± 0.02 μg/ml, $n = 4$), IF (0.06 ± 0.01 μg/ml, $n = 4$), MSW (0.08 ± 0.01 μg/ml, $n = 4$), HT (0.07 ± 0.01 μg/ml) and aqueous AsA (0.03 ± 0.01 μg/ml, $n = 4$) were significantly lower ($p < 0.05$) than the control sample (0.20 ± 0.02 μg/ml, $n = 4$), respectively. It was therefore suggested that in the first 17 hr, oxidation by oxygen in air of BR in the human serum was inhibited *in vitro* by ERW, IF, MSW and HT, although their inhibitory effects are considerably smaller than 100 μM aqueous AsA. The antioxidant activities of all of these aqueous solutions decreased time–dependently, and at 41 hr after the start of the reaction, the inhibitory effects on the basis of significant differences in the values from those of the control sample were detected only in the sample involving aqueous AsA (Table 2b). The mechanisms of this phenomenon also will be discussed later. TW exhibited no significant effect on the *in vitro* oxidation of human serum BR, although it tended to promote the oxidation slightly (Table 2b). In the experiments of oxidation of rutin by PPO (3), the rutin concentration in the control sample was 50.3 ± 2.2 μM ($n = 4$) and 13.5 ± 0.9 μM ($n = 4$) at the start and termination of the reaction, respectively. The quantity of rutin oxidatively converted into the reaction products by the action of PPO was 36.8 ± 1.9 μM ($n = 4$) in the con-

Table 2. Effects of Sample Solutions on the Oxidation by Cu^{2+} of Human Serum Lipid (a), by Oxygen on Air of Human Serum Bilirubin (b) and by Polyphenol Oxidase of Rutin (c)

a: The Quantity of LPO (MDA) Generated (nmole/ml) (mean ± S.D., $n = 4$)

	The Volume Ratios to DW			
Samples	0 : 3	1 : 2	2 : 1	3 : 0
Control	0.72 ± 0.04	—	—	—
ERW	—	0.69 ± 0.04	0.65 ± 0.03[a)]	0.59 ± 0.04[a)]
IF	—	0.66 ± 0.04	0.60 ± 0.04[a)]	0.53 ± 0.03[a)]
MSW	—	0.68 ± 0.05	0.63 ± 0.05[a)]	0.56 ± 0.04[a)]
HT	—	0.67 ± 0.03	0.61 ± 0.04[a)]	0.55 ± 0.04[a)]
CFA	—	0.56 ± 0.03[a)]	0.37 ± 0.02[a)]	0.18 ± 0.02[a)]
TW	—	0.71 ± 0.04	0.76 ± 0.05	0.83 ± 0.05[a)]

a) Significantly greater or smaller ($p < 0.05$) than the control. Figures in the parenthesis indicate the values of MDA quantities generated during the corresponding periods.

b: The Content of Bilirubin (μg/ml) (mean ± S.D., $n = 4$)

	Reaction Time (hr)			
Samples	0	17	41	65
Control	1.15 ± 0.07	0.95 ± 0.05	0.68 ± 0.06	0.37 ± 0.05
		(0.20 ± 0.02)	(0.27 ± 0.02)	(0.31 ± 0.03)
ERW	1.18 ± 0.06	1.09 ± 0.06	0.81 ± 0.06	0.49 ± 0.05
		(0.09 ± 0.02)[a)]	(0.28 ± 0.02)	(0.32 ± 0.02)
IF	1.16 ± 0.05	1.10 ± 0.06	0.85 ± 0.06	0.55 ± 0.04
		(0.06 ± 0.01)[a)]	(0.25 ± 0.03)	(0.30 ± 0.03)
MSW	1.13 ± 0.04	1.05 ± 0.05	0.76 ± 0.06	0.45 ± 0.04
		(0.08 ± 0.01)[a)]	(0.29 ± 0.02)	(0.31 ± 0.03)
HT	1.14 ± 0.05	1.07 ± 0.05	0.79 ± 0.06	0.50 ± 0.05
		(0.07 ± 0.01)[a)]	(0.28 ± 0.02)	(0.29 ± 0.03)
AsA	1.15 ± 0.05	1.12 ± 0.05	1.03 ± 0.04	0.91 ± 0.05
		(0.03 ± 0.01)[a)]	(0.09 ± 0.01)[a)]	(0.12 ± 0.02)[a)]
TW	1.17 ± 0.05	0.96 ± 0.06	0.66 ± 0.05	0.33 ± 0.04
		(0.21 ± 0.02)	(0.30 ± 0.03)	(0.33 ± 0.03)

a) Significantly smaller ($p < 0.05$) than the control. Figures in the parenthesis indicate the bilirubin quantities oxidized during the corresponding periods.

c: The Quantity of Rutin Oxidized by the Enzyme (μM) (mean ± S.D., $n = 4$)

	The Volume Ratios to DW			
Samples	0 : 3	1 : 2	2 : 1	3 : 0
Control	36.8 ± 1.9	—	—	—
ERW	—	35.3 ± 1.8	33.2 ± 1.8[a)]	31.3 ± 1.5[a)]
IF	—	33.7 ± 1.6	30.4 ± 1.7[a)]	27.6 ± 1.4[a)]
MSW	—	34.3 ± 2.1	31.8 ± 1.8[a)]	28.9 ± 1.9[a)]
HT	—	35.1 ± 1.9	33.0 ± 1.6[a)]	30.6 ± 1.7[a)]
AsA	—	29.8 ± 1.6[a)]	20.1 ± 1.0[a)]	9.1 ± 0.8[a)]
TW	—	36.9 ± 2.0	37.2 ± 1.9	37.6 ± 2.2

a) Significantly smaller ($p < 0.05$) than the control. Abbreviations in the text.

trol sample. The quantities of rutin enzymatically oxidized by PPO in the samples involving the four aqueous solution systems, aqueous AsA and TW with various volume ratios to DW were obtained in the same manner. They are summarized in Table 2c. As shown in Table 2c, the rutin quantities oxidized in samples involving only ERW (31.3 ± 1.5 μM, $n = 4$), IF (27.6 ± 1.4 μM, $n = 4$), MSW (28.9 ± 1.9 μM, $n = 4$), HT (30.6 ± 1.7 μM, $n = 4$) and aqueous AsA (9.1 ± 0.8 μM, $n = 4$) were significantly smaller

($p < 0.05$) than the control sample (36.8 ± 1.9 μM, $n = 4$), respectively. These data suggest that the enzymatic oxidation of rutin is also suppressed by the four aqueous solution systems, although their effects are not excellent as was observed for 100 μM aqueous AsA. Such effects of these solutions were reduced depending on increases in their volume ratios to DW in the reaction mixtures (Table 2c), as in the case of (1) (Table 2a). It has been known that PPO catalyzes the reaction for production of orthquinones from orthodiphenols including rutin and that the orthoquinones formed are then non-enzymatically polymerized to produce melanin-type pigments.[9] Suppression by these aqueous solution systems of the oxidative transformation of rutin into the corresponding orthoquinone(s) shows that they may act as agents, which reduce (an) orthoquinone(s) to the original substance. Effects of TW on the enzymatic oxidation of rutin were not distinct (Table 2c).

These results clearly suggest that all of the four examined aqueous solution systems, that are assumed to have antioxidant activities, really have such effects *in vitro*, that can be quantitated in comparison with those of known antioxidants. No significant difference in the antioxidant activities detected in the experiments for (1)–(3) were observed between samples of the four aqueous solution samples examined, although IF tended to exhibit the strongest effects among them (Tables 2a–2c).

The results of the analyses of metal elements and anions are summarized in Table 1. As shown in Table 1, the concentrations of such minerals as iron (Fe), copper (Cu), zinc (Zn), and strontium (Sr) and others were considerably higher in TW than in the four aqueous solution systems. It is known that Fe and Cu can act as prooxidants based on the Fenton reaction. The fact that TW slightly accelerated oxidation of serum lipid by Cu^{2+} (Table 2a) may be associated possibly with such properties of these metals. In ERW, Na was the most abundant cation, although a considerable amount of Cl^- was also contained (Table 1) presumably due to unblocked contamination of anions in electrolysis. In IF, MSW and HT, the levels of all of examined anions were lower than in TW, while those of several metal elements were the prominently higher (Table 1). For instance, the concentrations of vanadium (V) and rubidium (Rb) and others in IF, as well as those of lithium (Li), V and Rb and others in HT, were far greater than TW (Table 1). It has been known that among V ions, ones with the lower charge, such as V^{2+} and V^{3+}, generally are reductive cations.[10] It is therefore speculated that the antioxidant activities of IF and HT may be derived at least partly from such reductive V ions. This also will be discussed again later. In MSW, Mg was the most abundant (Table 1).

Table 3. Detection by ESR Spectrometry of the Scavenging Ability for Oxygen Radicals of the Aqueous Solutions Examined (Relative Values to Distilled Water for Manganese)

Samples Examined	Relative intensity of signals for O_2^-	Relative intensity of signals for ·OH
Distilled water	100.0*a)*	100.0*b)*
ERW*c)*	78.2 (−21.8)	> 100.0
IF*d)*	23.8 (−76.2)	91.2 (−8.8)
MSW*e)*	38.6 (−61.4)	> 100.0
HT*f)*	33.4 (−66.6)	> 100.0

Figures in the parenthesis indicate the % values of the intensity of signals for O_2^- and ·OH against the distilled water sample. *a*) and *b*) 6.58 and 12.37 as the values of intensity, respectively. Other abbreviations in the text. *c*) Electrolyzed reduced water, *d*) "I'm Fine," *e*) Mineral-stick water, *f*) Hira tenryosui water.

The results of examinations by ESR on the ROS scavenging ability are presented in Table 3. As shown in Table 3, under the conditions employed, addition of ERW, IF, MSW and HT instead of distilled water gave 21.8 (= 100.0 – 78.2)–76.2 (= 100.0 – 23.8)% reduction of signal intensity for O_2^-, indicating that it was scavenged by the components of added solutions. On the other hand, •OH was not scavenged at all by ERW, MSW and HT or only slightly scavenged by IF (Table 3). It is therefore confirmed by these ESR data that ROS scavengers, such as AsA, tocopherols, polyphenols and "active hydrogen" of which presence was assumed by Shirahata *et al.*,[1] are virtually absent in these examined solutions, since such substances are excellent scavengers not only for O_2^- but also for •OH.[11]

A part of the results of experiments for examination of the boiling and ultrafiltration treatments on the antioxidant activities of the four aqueous solution systems is summarized in Table 4. As show in Table 4, after boiling, inhibitory effects on the Cu^{2+}-induced oxidation of human serum lipid thoroughly disappeared in ERW and MSW, although in IF and HT, a part of the antioxidant activities consistently remained. Similar trends were detected also

Table 4. Effects of Boiling on the Inhibitory Effects of Aqueous Solution Systems Examined on the Oxidation by Cu^{2+} of Human Serum Lipid

Samples	Lipid peroxide generated (% to the values for DW)[a)]		
	Before	⟶	After
ERW	89	⟶	100
IF	79	⟶	93
MSW	86	⟶	100
HT	82	⟶	92

a) The data for the reaction mixtures involving samples and DW at the ratio of 3 : 0 (Reaction conditions in the text). Abbreviations in the text.

in the experiments of oxidation by oxygen in air of human serum BR and of that by PPO of rutin (data not shown). These data suggest that the antioxidant activities of ERW and MSW are derived only from (a) volatile component(s) but those of IF and HT are generated also by (a) non-volatile component(s). The ultrafiltration treatment gave virtually no effect on the antioxidant activities of any of these aqueous solution systems, showing that the effective components are low MW substances which do not bind to macromolecules or are not solid constituents with the larger pore size than that of the solutes with the MW of 10000.

An aqueous solution prepared by dissolving NaCl and NaOH in TW to give the same Na and Cl^- concentrations as in ERW, as well as that prepared by dissolving $Mg(OH)_2$ in DW to afford the same Mg level as in MSW (see MATERIALS AND METHODS), exhibited no antioxidant activity.

The aqueous phase of Mg/DW at 1 hr after preparation of the reaction mixture (see MATERIALS AND METHODS) expressed approximately the same antioxidant activities as MSW in examinations for (1)–(3) (data not shown), and its Mg level by the colorimetric method (see MATERIALS AND METHODS) was 16.3 mg/l, which was roughly equal to that by AAS of MSW (15.5 mg/l, Table 1). The Mg concentration in the aqueous phase of the reaction mixture left at room temperature increased time–dependently to 23.1 mg/l at 24 hr later, while the antioxidant activities were not enhanced. The antioxidant activities in the aqueous phase of the reaction mixture obtained at each time were time–dependently reduced after removal of metallic Mg by filtration (see MATERIALS AND METHODS), and at 24 hr after the treatment, complete disappearance of the activities was found. On the other hand, when a HPM-stick was extracted by 1.5 ml of DW at room temperature for the longer period, the same trends as above (elevation of the Mg level and no change in the antioxidant activities) were confirmed. The saturated concentration of hydrogen gas in an ERW was reported to be 0.75 mM (= *ca*. 1.5 mg/l),[12)] which should be accompanied by dissolution of *ca*. 18.2 mg of metallic Mg into 1 l of DW. These results suggest that hydrogen gas generated in association with dissolution into the aqueous media of metallic Mg at room temperature exhibits the antioxidant activities. So, the HPM-stick of Water Institute, which seems to consist mainly of metallic Mg, may be a product so that the concentration of hydrogen gas dissolved is usually maintained within a narrow range near the saturated state, compensating for the loss by evaporation based on gradual extraction of metallic Mg from the surface of the stick. It is not expected that hydrogen gas (molecular hydrogen) has a potent ability to scavenge ROS directly, although saturation of the aqueous solution with hydrogen gas may prevent dissolution of oxygen molecules, a part of which can be transformed into ROS.

These results suggest that hydrogen gas (molecular hydrogen), which is generated by electrolysis (in ERW) and by dissolving metallic Mg as the major component of HPM-stick (in MSW), is an effective component for the antioxidant activities of these aqueous solution systems. Thus, a part of what is described in the recent HP of the Water Institute (the description that the MSW prepared according to the manual by users contains not "active hydrogen" but molecular hydrogen) [Water Institute (Atarashii-mizunokai); http://www.water-institute.org] has been confirmed to be right. This will be later discussed, again. Molecular hydrogen as an effective component for the antioxidant activities may be contained also in IF and HT. However, even its complete loss by boiling these solutions did not completely remove their antioxidant activities (Table 4). This may possibly be due to the presence of reductive V ion(s) as

(a) solute(s) in these aqueous solution systems (Table 1). The data that the antioxidant activities of IF tended to be greatest among those of the four aqueous solution systems examined (Tables 2a–2c) may be given by the occurrence of both of generated molecular hydrogen and concentrated reductive V ions during the process of electrolysis of the original natural water. Time–dependent reduction in the antioxidant activities of the four aqueous solution systems (Table 2b) may be caused by evaporation of dissolved hydrogen gas from their surface, and also in IF and HT, by oxidation of V^{2+} and/or V^{3+} to V^{4+} and/or V^{5+} having no reductive effects.[10)]

These results indicate that generation of the antioxidant activities in the four aqueous solution systems examined can be explained solely by usual physico-chemical knowledge without assuming the presence of "active hydrogen." As described in the Introduction, Shirahata recently modified the "active hydrogen" hypothesis proposing that "active hydrogen" is accumulated on the surface of microclusters of metals contained as nanocolloidal solid components in aqueous solution systems with antioxidant activities.[5)] A patent by Nihon Trim and Shirahata for a method to prepare aqueous solution systems containing "active hydrogen"-accumulating colloids,[13)] as well as that for a method to determine "active hydrogen" in aqueous media,[14)] has been published. They also revealed the presence of Pt nanoclusters, which was possibly released from the electrodes during electrolysis, in an ERW they prepared.[15)] In this study, as shown in Table 1, we detected only a trace amount (below 0.1 μg/l) of Pt as a solute in the ERW we prepared. This discrepancy may be due to removal by filtration of the solid components in our analytes for determinations of minerals (see MATERIALS AND METHODS) and also caused by difference in the electrolysis conditions to obtain our and their ERWs. It is well known that metal clusters act as catalysts for reductive reactions in aqueous media, especially in those dissolving molecular hydrogen. Therefore, the use of a term of "active hydrogen" may be unappropriate, although we cannot neglect the possibility of the occurrence of such a phenomenon as an enhancing factor for the antioxidant activities of the corresponding aqueous solution systems. The essential fact is that such aqueous solution systems (reduced waters) can generate the antioxidant activities, even in the absence of metal nanoclusters, by the action(s) of hydrogen gas (ordinary molecular hydrogen) and (a) reductive cation(s) dissolved as (a) solute(s). No phenomenon which requires an assumption of the presence of "active hydrogen," occurs in association with the antioxidant activities of these aqueous solution systems.

It has not yet been elucidated whether drinking these aqueous solution systems increases antioxidant activities in human body fluids. This aspect remains to be investigated. However, even if molecular hydrogen dissolved in these solutions is abundantly absorbed into the body of humans who drink them, it may be impossible that "active hydrogen" generated by the action of hydrogenase reacts with ROS in the human body, as recently described in the HP of the Water Institute [Water Institute (Atarashii-mizunokai); http://www.water-institute.org], because humans have not this enzyme except that produced by some enterobacteria including *Escherichia coli* (*E. coli*).[16)] So, even if a trace amount of "active hydrogen" is formed in the intestine, it may be immediately consumed and can not be delivered to the tissues where expression of its ROS scavenging ability is expected.

Acknowledgements The authors express their thanks to Dr. Yuko Amo of Yamagata University for her helpful discussion. This study was aided by the Grants of Japan Skeptics at 2002 and 2003. The publication of this paper is supported by the Science Research Frontier Program from the Ministry of Education, Sports, Science and Technology.

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