Magnesium Activated Hydrogen Ions and Biological Activity: Empirical Analyses and Clinical Significance

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ABSTRACT

This study aims to create a scientific foundation for the understanding of the mechanism and associative use between magnesium active hydrogen ions and the parameters of oxidation reduction potential (ORP), biochemical involvement in energy producing intracellular reduction systems and cellular hydration. The first objective is that of direct measurement of ORP, pH and calculated rH. The results had an initial rH reading of 26.46 with a final rH reading of -2.41 after the addition of 225 mg of active hydrogen, demonstrating a significant change in the reduced aqueous environment. The second objective is to measure the correlated reduction of cytochrome *c* and increased oxyhemoglobin production as a function of increased active hydrogen concentration. Overall, a calculated 9.9% +/- 0.23% change in oxyhemoglobin and 45.17% +/- 0.46% increase in reduced cytochrome *c* concentration per 100 μg added of active hydrogen was noted. The third objective is the *in vivo* clinical hydration change measurement with the bioimpedance factors: phase angle and capacitance, through non-segmental methods. Subject data indicate adjusted average increases in phase angle and cellular capacitance of 10% greater than control groups ($P<0.001$ and $P<0.01$), respectively. No negative subjective reports were indicated by any participant. The data collected in this analysis offer "pieces of a puzzle" that as a whole evaluate the effectiveness of the active hydrogen compound.

INTRODUCTION

Silica hydride has been extensively studied for years for its biochemical properties. Silica hydride is a matrix of siliceous, monomeric silsesquioxane cages that act as carriers for negatively charged hydrogen ions [1]. A more appropriate rhetoric to describe and characterize the negatively charged hydrogen ion is that of "active hydrogen", due, in part to its reactivity and ion activity in a solution.

The active hydrogen acts through the donation of an extra electron in the orbital shell of the hydrogen atom. In terms of understanding the reactions and mechanism of active hydrogen, it is important to understand that the atomic structure of the hydrogen anion is not that of a $1s^2$ orbital system, meaning a hydrogen atom with two electrons in its innermost shell, but rather a $1s1s'$ structure, where the $1s'$ electron is in a separate orbital shell orthogonal to the $1s$ orbital making it more loosely held and readily donated [2]. The dynamics of this negative ion allow it to be a significant reducing agent, demonstrating readings of up to -850 mV when a small amount of the compound is added to water [3]. **Figure 1** conceptually demonstrates the mechanism of a reaction involving the electron donation from the $1s'$ orbital shell of active hydrogen to reduce a conjugated bond system.

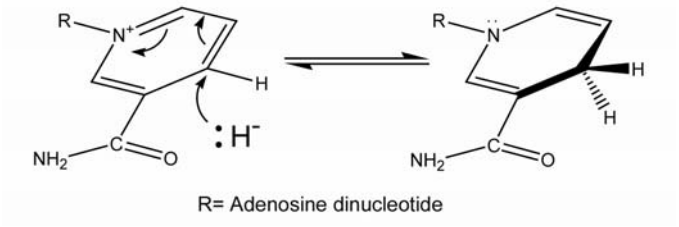

Figure 1. Direct Reduction Capacity of NAD^+. The 1s' orbital orthogonal in the 1s1s' anion is readily donated to the oxidized NAD^+ molecule reducing the compound to its redox pair NADH.

Active hydrogen demonstrates profound abilities, both *in vivo* and *in vitro*. The active

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hydrogen compounds that have been tested demonstrate the ability to completely neutralize reactive oxygen species including hydroxyl radicals [4], superoxide radicals [5] and singlet oxygen species [6]. *In vitro* studies of active hydrogen using Chinese hamster ovary cells and mouse hybridoma cells have indicated twofold increases in ATP production and increased NADH production as determined by capillary electrophoresis [7]. All of the tests performed on active hydrogen indicated a direct correlation of its mechanism with the main metabolic control chains in the body, including the Krebs cycle an electron transport chain. Additional studies performed by Purdy-Lloyd, *et al.* and Hernandez, *et. al.* concur with analyses finding both decreases in lactic acid production after strenuous exercise while consuming active hydrogen [8] and the reduction of Cytochrome P-450 and NADPH in hepatic cells [9], respectively. **Figure 2** demonstrates the general mechanism for the regenerative reduction in the electron transport chain.

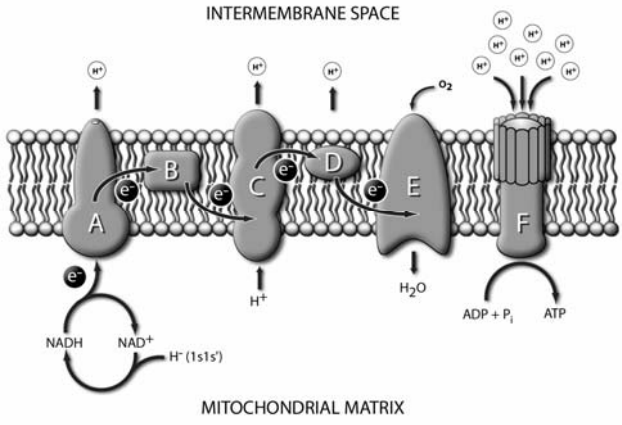

Figure 2. Mechanistic Pathway for the Increased NADH and ATP Production After Treatment with Active Hydrogen. Correlating the decrease in glucose concentration and decreased lactate production suggests an increased glycolytic activity and a post-pyruvate aerobic respiration through the regeneration of NADH as the electron transports into (A) protein complex I, then either directly transfers through (B) ubiquinone or indirectly succinate oxidation, into (C) the cyt b_{566}/b_{562} protein complex III, following the general pathway of (D) cytochrome c into (E) the Cu enriched cytochrome c oxidase complex IV. The resultant increased proton gradient formed in the mitochondrial intermembrane space explains the increased ATP synthase production of ATP in the analysis.

A novel active hydrogen compound based on the silica hydride has been developed that offers unique demonstrations of biochemical activity. This modification of the silicate mineral active hydrogen uses a complex magnesium structure to bind and stabilize the active hydrogen ions. Biomolecular reactions with the novel compound were assayed with the same experimental protocols as used on the silica hydride compound. Assays included comprehensive reactive oxygen species analyses and oxidative stress determination, electron transport chain involvement investigation and metabolic function assays. The results found were consistent with previous publications.

As a means to objectively quantify the biological effects of Magnesium Active Hydrogen, three unique assays were performed to monitor: 1) Reduction potential (Redox) and reaction kinetics with rH analysis, 2) blood porphyrin reduction of Ctyochrome *c* and oxyhemoglobin in whole human erythrocytes and 3) clinical monitoring of intracellular hydration levels via non-segmental bioimpedance spectrometry.

Redox Analysis Prelude

To aid in the evaluation of active hydrogen as a hydride energy source, the use of ORP is one significant indicator of the energy reserves available for use. This is particularly true of liquid environments. Another indicator is through the evaluation of pH responses to the solution. The problem exists, however, that the ORP may be biased by the pH and vice versa. To accommodate for this, Clark reported the idea of computing the absolute reducing potential of a compound by using a variation of the Nernst equation that measures ORP taking into account hydrogen ion activity [10]. The equation developed (1) related hydrogen pressure and reduction potential in units of rH. The use of rH gives a hydrogen proton-unbiased look at the absolute reducing potential of a compound, eliminating the effects of pH in the ORP measurement. It is a true indication of a compound's reduction potential capacity. The

shifts in rH can be used to quantify the reducing ability and energy reserves of the compound as well as illustrating a qualitative comparison between different reagents.

$$E_h = 1.23 - (RT/F)pH - (RT/4F)\ln(1/P_o) \quad (1)$$

where E_h is the measured oxidation-reduction potential, F is the Faraday constant, R is the universal gas constant and T is absolute temperature. The 1.23 references that the potential under one atmosphere pressure of oxygen is 1.23 V greater than the measured potential of hydrogen at one atmosphere of pressure in a solution of the same pH. rH is then defined explicitly as the negative logarithm of the oxygen pressure, P_o(2):

$$rH = -\log P_o \quad (2)$$

Porphyrin Analysis Prelude

Heterocyclic porphyrin derivatives, hemoglobin and cytochrome *c* are two of the most interesting and well studied compounds in human biochemistry, yet some of the least mechanistically understood. Both of these compounds are integral and essential in metabolic function, oxygen transport [11], energy production [12] and allosteric regulation [13]. The structures of both compounds are remarkably similar in that they consist of a ferrous center surrounded by four pyrrole rings linked by methene bridges. These conjugated systems, generally referred to as heme systems, are coordinated by covalent bonds between Fe and N, and shown in **Figure 3**. The main differentiation between hemoglobin and cytochrome *c* is the coordination chemistry where the hemoglobin is covalently bonded to a histine functional group and the cytochrome *c* has a sequence of two cystine and one histine group coupled with side chain amino acid groups [14].

Cytochrome *c* is an octahedrally oriented peripheral membrane protein that is exclusive to eukaryotes. Its main function is that of intramembrane electron transfer. It is located loosely bound to the outer surface of

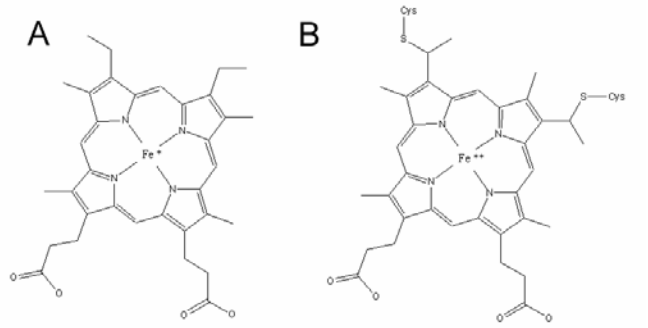

Figure 3. The Comparison of the Two Porphyrin Proteins: Hemoglobin and Cytochrome *c*. The structure of the two compounds is fairly similar with a conjugated seriews of pyrrole rings. The heme systems, as they are known, differ (A) with the hemoglobin having a predominant histine group and a pentagonally oriented coordination to the Fe* atom. Cytochrome *c* (B) in comparison is generally ocatahedrally coordinated bound to the Fe** atom with a series of cystine and histine amino acid functional groups bound to the conjugated pyrrole system. Both proteins are essential for metabolic function, oxygen transport, energy production and allosteric regulation in a cell.

mitochondrial membranes [15]. It is able to alternately bind to the other proteins of the electron transport chain, which include the cytochrome c_1 (residing in complex III) and cytochrome *c* oxidase (complex IV), both of which function to move electrons between them. One of the main functions and responsibilities of cytochrome *c* is to prevent the heme functional groups from transferring electrons nonspecifically to other cellular components. The cytochrome proteins also provide the guidance for electron transfer in eukaryotic cells.

Hemoglobin, a structurally similar compound to the cytochrome proteins, is known as protoporphyrin IX and is pentagonally oriented with respect to its porphyrin ring. Its purpose is transport oxygen, as oxygen solubility in blood plasma is too low (~10^4 M literature) to adequately fulfill our metabolic means. In a reduced state, hemoglobin binds oxygen to form oxyhemoglobin, while in the oxidized state, the compound is known as methemoglobin and does not bind with oxygen, but rather water.

The oxidative reduction potential, known as redox, is an exceptionally important control factor for the rate and direction of electron travel.

Different proteins behave differently under specified redox conditions. Redox controls the geometry and coordination of the proteins, cytosolic gradients, localized ionic charge distribution, electron transfer and hydrogen bonding to other compounds [16]. The redox control of oxyhemoglobin is enzymatic and utilizes the cooperation of cytochrome *c* and compounds such as methemoglobin reductase and cytochrome *c* reductase to carry out its reactions. Current data show that factors of pH, the Bohr effect and redox help to stabilize the electron transfer capabilities of the proteins [17]. Cytochrome *c* and hemoglobin both work together to control the oxygen binding affinity and produce a sigmoidal relationship of oxygen saturation. This relationship provides an interaction between proteins and water and results in an adequate oxygen distribution on micro and macro levels within the organism.

Hemoglobin's affinity for negative ions is an additional redox control and modulates metabolic porphyrin activity. Particularly in erythrocytes, chlorine and carbonate freely permeate the membrane of the cell. The ions bind tightly to the iron group and moderate oxygen saturation within the compound. Additionally, this relationship with negative ions prevents the auto-oxidation of the hemoglobin.

Studies performed on porcine hearts have indicated that the redox state of cytochrome *c* and oxyhemoglobin directly affect changes and blood volume and erythrocyte hydration [18]. Data additionally show that venous oxyhemoglobin saturation and cytochrome *c* redox states are directly related to each other and not limited nor controlled by partial oxygen pressure [19]. Thus, the reduction of cytochrome proteins should be directly proportional to the oxygenated hemoglobin and this reduced state is kinetically a prerequisite for electron transfer and reaction with oxygen in hemoglobin.

It is notable when researching hemoglobin, cytochrome *c* and related heme compounds, that the basic mechanisms and catalysis of substrate proteins will not occur unless in the presence of organic hydrogen [20]. Hydrogen and its associated electrons are the fundamental core components of most metabolic processes. Studies of mitochondrial cytochrome complexes have noted that the proton to electron ratios measured in the reaction kinetics were less than unity (<1:1), indicating the significance and relevance of the electron in the transport mechanisms [21].

It is hypothesized that the redox control and trigger for these reactions may not be entirely enzymatic, but rather redox controlled through electron rich ions. Previously, the biological transfer of an electron was described as that of a proton plus an electron that are simultaneously carried to the oxidized species. Shirahata describes this hydride transfer and provides evidence that the physics is not that of a proton and electron, but rather a direct transfer of a hydrogen anion, known as a hydride ion [22]. Reactions ranging from electrolysis to the reduction of nicotinamide adenine dinucleotide to reactive oxygen species neutralization involve the hydride ion [23]. Shirahata, *et. al.* have contributed to research on hydrogen ions defining the factors of dissolved oxygen, dissolved hydrogen and redox potential as fundamental variables for the evaluation of the ions.

This *in vitro* study aims to investigate the relationship of erythrocyte hydration, increased oxyhemoglobin production and cytochrome *c* reduction through the introduction of active hydrogen as determined spectrophotometrically.

Hydration Prelude

Hydration and its effect on the body have been on the forefront of research and popular media alike. One can hardly look at a periodical or the television without some mention of the need for proper hydration. From the standpoint of this assay, dehydration is specifically defined as an imbalance of water, salt, or both within the body. The external maintenance of proper hydration is significantly important. This is, in part, due to the human body being inefficient at both nutrient assimilation and removing intercellular waste. The buildup of cellular waste or the incursion of a

non-isotonic environment can lead to dehydration. The significance of dehydration is quite profound, as dehydration has been directly linked on a micro level to cellular senescence and death, decreased metabolite production and oxidative stress buildup [24]. On a macro level dehydration has been directly linked to the pathogenesis of numerous conditions including increased thirst, dry mouth and swollen tongue, weakness, dizziness, cardiac palpitations, confusion, inability to sweat, decreased urine production and in severe cases, death [25].

Dehydration is most commonly due to having a hypotonic environment in a cell. When the condition within a cell is hypotonic, an ionic imbalance exists which restricts or inhibits the intermembrane exchange of proteins and adsorption of water. Once dehydrated, cell proliferation and function have a difficult time recovering and cell death generally ensues through necrosis or apoptosis [26].

Current therapies for dehydration range from the administration of saline solutions with added mineral electrolytes, to sipping water, to treatments with magnesium or clotrimazole [27-29] which inhibit the Gardos channel, biochemically controlling the influx of cellular potassium and the efflux of calcium. To prevent dehydration, suggested prophylaxis include drinking adequate amounts of water or drinking water with added electrolytes. To date, the preponderance of preventative measures for dehydration include aqueous-based methods such as drinking water.

This assay attempts to gain insight into the potentials of using active hydrogen as a complimentary treatment for dehydration and as a supplement to increase hydration levels in otherwise healthy subjects.

MATERIALS AND METHODS

Redox Analysis - PART I (Concentration Domain)

An IQ Scientific IQ400 ORP/pH meter was used to monitor the ORP and pH levels of subsequent additions of Active H into 250 mL of distilled water. All readings were taken in a 250 mL Erlenmeyer flask being magnetically stirred. An initial reading of pH and ORP were taken. Cumulative additions of Active H, totaling 5, 10, 15, 20, 25, 50, 75, 125 and 225 mg/250 mL were added to the distilled water in 5 minute increments. At each increment, the pH and ORP values, in mV, were recorded. The water had an initial temperature of 26 ^{o}C.

Redox Analysis – Part II (Time Domain)

The ORP versus time of 250 mg of magnesium active hydrogen added to 250 mL of distilled water was monitored to aid with the insight into the reactivity of the compound. 250 mg of active hydrogen compound was added to 250 mL of distilled water in a 250 mL Erlenmeyer flask. The water, at 26^{o}C, was stirred magnetically. Before adding the active hydrogen, base readings of ORP and pH were taken with an IQ Scientific ORP/pH meter. After the addition of the active hydrogen, ORP and pH reading were taken every 1 minute for a total of 17 minutes.

Blood Porhydrin Analysis (Oxyhemoglobin and Cytochrome C)

All materials used in this assay were of analytical quality and purchased from Sigma Aldrich Corporation, Saint Louis, MO. The type O human erythrocytes used for the assays were additionally purchased from Sigma Aldrich Corp. and suspended in and isotonic buffer consisting of 0.1M phosphate buffer, 0.1M NaCl, 0.2% citrate and 1 mM EDTA adjusted to pH 7.8. All standards and controls for the spectrometer were made with ddH_2O and spectroscopy were performed with a FP-650 spectrophotometer (Jasco, Inc., Easton, MD) with the following parameters: data recording with a linear data array, 1 nm increments and a scan velocity of 400 nm/minute. All experiments were repeated in triplicate.

Determination of Oxyhemoglobin

Solutions were prepared consisting of 2 mL aliquots of 0.1 M and isotonic buffer solution and suspended type O erythrocytes at a concentration of 6 X 10^6 cells/mL. The 2 mL aliquots were added to a 1 cm pass length quartz cuvette. A baseline spectragraph using the fore mentioned parameters was taken blanked to the isotonic buffer solution in a quartz cuvette from 500 to 650 nm. Sample solutions of 25, 50, 100, 500, 1000, 2000 and 5000 mg active hydrogen were mixed into 200 mL aliquots of ddH_2O. Absorbance versus wavelength was plotted to analyze the spectral output of the oxyhemoglobin compound. Absorbance readings at 542 nm and 576 nm were recorded for analysis. As controls, two solutions were made for analysis. One solution contained a 2 mL aliquot of isotonic buffer solution to which the sample solutions of active hydrogen were added. The second control solution consisted of the stock solution of erythrocytes in ddH_2O. Both controls were analyzed with the same parameters as the experimental solutions.

Determination of Reduced Cytochrome c

A visual absorbance difference spectra assay method was used to measure the comparative change between the reduced and oxidized heme iron in cytochrome *c*. Type O human erythrocytes were used in their stock concentration of 6 X 10^6cells/mL in 0.1M isotonic phosphate buffer. A 2 mL sample of red blood cells was prepared for analysis by taking a 2 mL aliquot of erythrocytes and a baseline reading taken blanked and zeroed to a solution of the 0.1 M phosphate buffer in a 1 cm path length quartz cuvette. All spectroscopy was performed with the fore mentioned parameters from 500 nm to 600 nm. Upon recording of the baseline scan, a 2 mg (one very small crystal) of potassium ferricyanide, $K_3Fe(CN)_6$ was added to the cuvette to fully oxidize the cytochrome *c,* at which point a photometric scan was taken. Serial additions totaling 10, 20, 25, 50, 75, 100 and 150 mg of active hydrogen were then added to the 2 mL erythrocyte solution, one aliquot at a time, then scanned. Upon completion of the active hydrogen standard additions, 100 mg of sodium hydrosulphite was added to the mix to fully reduce the mixture and compare to the hydrogen added scans. Absorbance versus wavelength was plotted to analyze the spectral output of the cytochrome *c* compound. Absorbance readings at 520 nm and 549 nm were recorded for analysis.

Bioimpedance Hydration Analysis

Ten healthy subjects (7F, 3M) were recruited for the study. All work was in accordance with the ethics and foundations of the Helsinki Declaration of 1975. The study was developed on a case-control method and administered by the authors and the supervising doctor at an integrative health care clinic. A 50 kHz non-segmental bioimpedance analyzer (Model BIA450, Biodynamics Corporation, Seattle, Washington) was used to measure and record the hydration levels in the subjects. Unit factors of sex, height and weight were considered for the analyses and the outcomes of phase angle and capacitance determined levels of hydration. Prior to the commencement of the study, each subject was asked to discontinue use of nutritional supplements for one week at which time a baseline BIA readings were taken over a three day period. All BIA readings performed were in triplicate and the mean values recorded.

Interventions involved each subject taking two-capsules containing 500 mg of the active hydrogen compound, prepared as previously described [1], three times a day, for 5 days. Each dose was to be taken with 8 oz. of water. After 5 days, the subjects returned to the clinic to have follow-up BIA's performed, again in triplicate. As a blind negative control, one participant received a placebo capsule containing an inert, white powder. As a secondary control, one participant did not ingest any capsules of the active hydrogen product, but consumed 8 oz. of water, three times a day for the duration of the study.

Each participant was additionally asked to indicate and note subjective observations of any variations in health, both beneficial and detrimental.

RESULTS

Redox Analysis- Part I(Concentration Domain)

The initial water reading had an ORP of 146.3 mV and a pH of 7.46. The initial calculated rH was 26.46, indicating a very oxidized environment. The initial addition of 5 mg Active H caused the ORP and pH readings to change to 68.6 mV and 7.89, respectively. The final rH, after the addition of 225 mg of Active H was -2.41, indicating an exceptionally reduced environment. The final ORP reading was -846.1 mV and the pH was 9.51. The plot of the ORP versus the amount of active hydrogen added resulted with a dual, isobestic polynomial relationship, as shown in **Figure 4.** rH values were calculated for the data and are tabulated in **Table 1**.

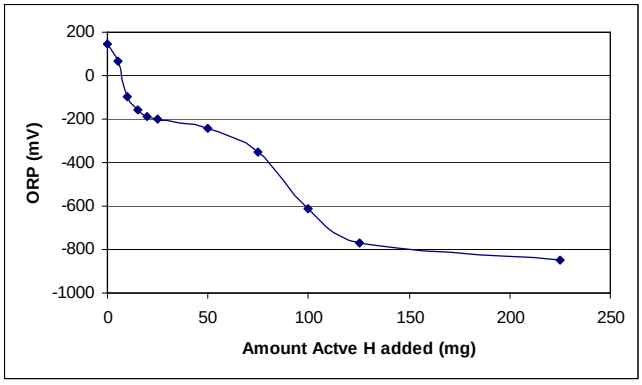

Figure 4. ORP as a Function of Active Hydrogen Concentration. The plot of ORP versus active hydrogen concentration indicates a time-released mechanism of action for the magnesium hydride carbonate compound. The nature of the polynomial decrease in ORP eludes to a second-order kinetic action taking place. Note the initial drop in ORP, followed by a lower rate, then another increased rate. This is very significant for the idea of a consistency in bio-reactivity and the ability of the active hydrogen ions to react in the body over an extended period of time.

AH Added	0	5	10	15	20	25	50	75	125	225
ORP (mV)	146.3	68.6	-101.2	-143.7	-189.3	-198.7	-240.9	-600.1	-775.5	-846.1
pH	7.46	7.89	7.95	8.15	8.45	8.64	8.81	8.90	9.12	9.51
rH	26.46	22.10	19.47	17.82	16.13	15.67	14.43	4.49	-0.63	-2.41
Environment	Hi Ox	Hi Ox	Sl Ox	Sl Ox	Sl Ox	Li Ox	Li Ox	Re	Hi Re	Hi Re

Table 1. An Overview of the Active Hydrogen Redox Characterization. Oxidation reduction potential (ORP), pH, rH (are used to characterize the active hydrogen ions in solution. The data represent mg additions of active hydrogen in 250 mL of distilled water. rH calculations give the overview of how reduced or oxidized the solution is. Environment classifications are: Hi Ox (Highly Oxidized), Sl Ox (Slightly Oxidized), Li Ox (Lightly Oxidied), Re (Reduced), Hi Re (Highly Reduced).

Redox Analysis- Part II (Time Domain)

The data collected for the time domain analysis were tabulated and statistically analyzed. The addition of 250 mg active hydrogen into 250 mL of distilled water resulted in a logarithmic relationship between the decrease in ORP reading and the time, as shown in **Figure 5**:

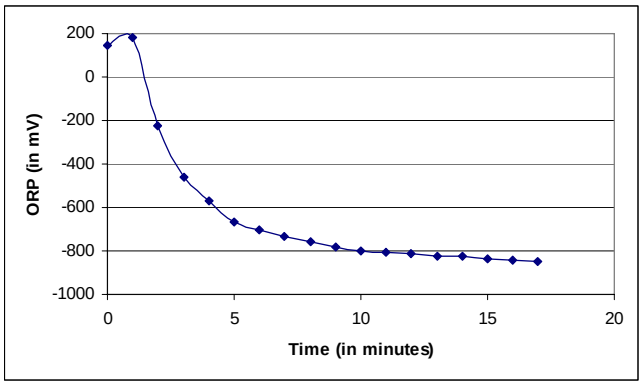

Figure 5. ORP as a function of time with added active hydrogen. The addition of 250 mg active hydrogen into 250 mL of distilled water resulted in a logarithmic relationship between the decrease in ORP reading and the time elapsed. In all replicates a small Gibb's free activation energy had to be overcome before the decrease in ORP.

In all replicates thus far, there has been a small Gibb's energy difference to overcome before the decrease in ORP. Taking the log/log relationship between both axes, a linear regression is formed with a $y = -1.0757x + 3.0997$, $R^2 = 0.99$ equation as depicted in **Figure 6**. Such that each time unit evolved a -1.07 times relative drop in ORP value.

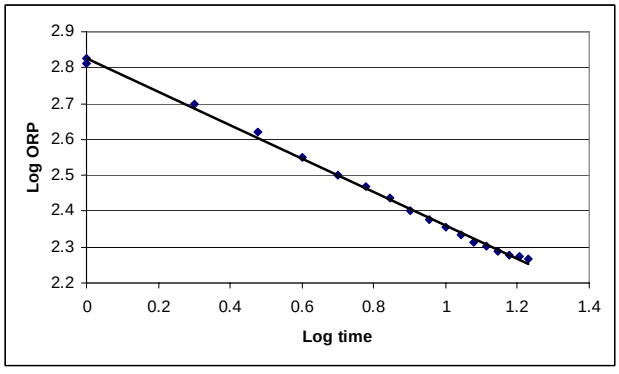

Figure 6. Linear regression of ORP change over time. Taking the log/log relationship between both axes of the change in ORP over a change in time, a linear regression is formed with a $y = -1.0757x + 3.0997$, $R^2 = 0.99$ equation. Such that each time unit evolved a -1.07 times relative drop in ORP value.

Both of these methods of analysis characterize how active hydrogen behaves in solution as a function of time or concentration. These results indicate whether the magnesium active hydrogen formulation reacts instantly, or will be time-released. This method also indicates the ability of the magnesium active hydrogen to successfully react with other compounds, such as free radicals. Oxidation/reduction (redox) potentials measure, in millivolts, the ability of a compound to react. Generally, a lower redox reading means that there is more energy stored in the compound to react. Similarly, the pH of a solution measures the amount of free positive hydrogen ions in solution. Together, redox and pH can be used to calculate a value known as rH. rH is the unbiased determination of the state of reduction or oxidation that a compound is in and is indicative of the overall probability that a compound will react.

Blood Porphydrin Analysis (Oxyhemoglobin and Cytochrome c)
Determination of Oxyhemoglobin

The spectraphotometric results of this assay indicate an increase in oxyhemoglobin as a function of added active hydrogen, as illustrated in **Figure 7**. Using the Beer/Lampert law, the percent change in oxyhemoglobin concentration can be correlated to the concentration of active hydrogen added, which is a 9.86% +/- 0.23% (Standard Deviation 0.013) change. Calculations made for this determination were based on a concentration analysis and 542 nm and 576 nm wavelengths. Calculation of the Pearson's correlation coefficient resulted with a R^2=0.996. Controls for this experiment with consistent

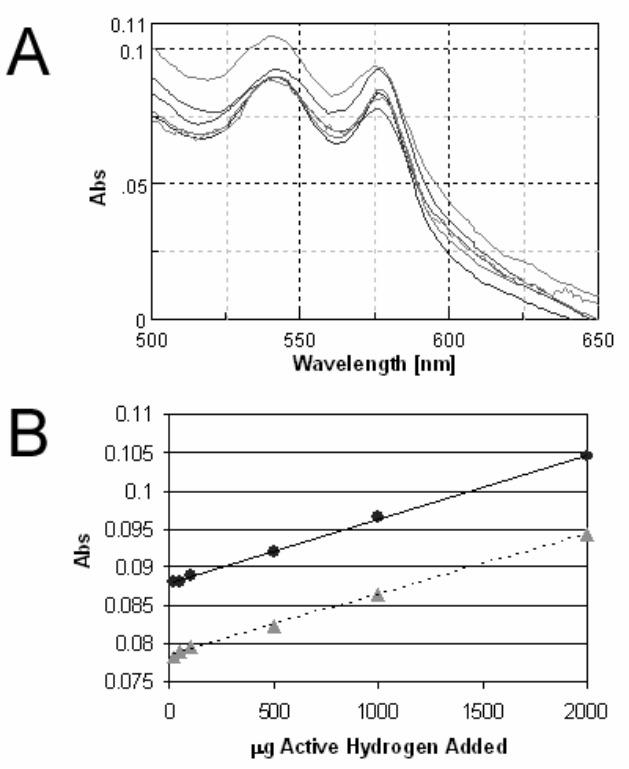

Figure 7. **Oxyhemoglobim response to active hydrogen.** Spectrophotometric results obtained for an investigation of oxyhemoglobn response to active hydrogen ions. The graph of absorbance versus concentration (A) of active hydrogen treated oxyhemoglobin demonstrates significant increases in concentration (as denoted by absobance) when treated. Measurements were extrapolated from the 542 nm and the 576 nm peaks of the oxygenated porphyrin system. The regression analysis of the extrapolated peaks (B) indicate a linearity of R^2=0.996 for both of the trendlines.

with the subject group hypotheses. Control group one indicated no change as active hydrogen was added to the isotonic buffer solution, while the second control group indicated no change in spectra readings with the addition of erythrocytes into the ddH_2O solution.

Determination of Reduced Cytochrome c

The results of the calibration curve creation indicate a linear relationship between concentration and absorbance with a Pearson's correlation coefficient, R^2=0.998. The baseline readings obtained indicated a partially reduced environment. After the addition of the potassium ferricyanide, the spectra readings indicated a fully oxidize environment. The subsequent standard addition of active hydrogen showed a continuing reduction of the iron heme group as a function of hydrogen on concentration. The attempt to fully reduce the heme group by sodium hydrosulfite did not increase the observed absorbance signal, indicating the complete reduction of the cytochrome *c* by active hydrogen. The overall change in concentration of reduced cytochrome *c* was 45.17% +/- 0.46% (Standard Deviation 0.15) per 100 mg of active hydrogen added. **Figure 8** conveys the results of the reduced cytochrome *c* assays.

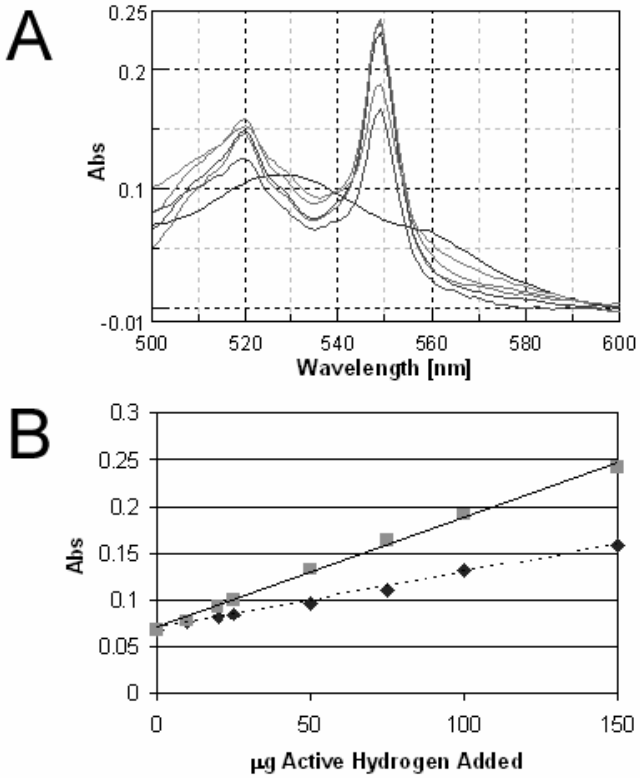

Figure 8. **Cytochrome *c* analysis by spectrophotmetry.** The raw spectra acquired (A) of the cytochrome *c* protein in its fully oxidized state and progression towards full reduction with spectral maxima for the reduced protein at 520 nm and 549 nm. The extrapolation data (B) indicate a linear relation ship (R^2=0.998) between the active hydrogen added and the absorbance.

The cumulative combined statistics convey a correlated 9:2 ratio of oxyhemoglobin to reduced cytochrome *c* per given 100 mg addition of active hydrogen.

Bioimpedance Hydration Analysis

The data obtained from the Bio impedance analyzer were entered into the statistical analysis program, AcaStat (AcaStat Software, Ashburn, VA) for a statistical evaluation. The unit factors of phase angle and cellular capacitance were the predominant variables measured and analyzed, however body resistance, total water capacity, extracellular and intracellular water percentage and total cellular mass were also reported but not used in this study.

All of the participants in the study had increases in phase angle and cellular capacitance. The phase angle changes ranged from a minimum of 0.2 degrees to a maximum of 0.6 degrees. The mean increase for this study was 0.48 degrees +/- 0.005 degrees with a standard deviation of 0.219. The average increase correlated to a 10% increase relative to baseline readings with $P<0.001$ compared to controls.

The individual capacitance readings obtained ranged from changes of minimums of 25 pF (pico Farads) and a maximum change of 322 pF. The mean increase for this study was 98 pF +/- 1.95 pF with a standard deviation of 93.52. This increase in capacitance correlates to a relativistic increase of 10% from the baseline readings with consistency, $P<0.01$, compared to controls.

Control subjects were consistent with the initial study hypotheses. The negative control indicated no statistical change over the course of study. Small fluctuations, corresponding to less than a 0.1% change were acceptable within the tolerance limits of the Bio impedance analyzer instrument. They placebo control similarly indicated no statistical change in study parameters. The overall results are summarized in **Table 2**.

No subjective reports by any of the participants indicated any negative side effects or problems associated with taking the compound. The reports that were received by the participants included descriptions of claims of decreased lethargy and fatigue, relief from headaches and reports in general wellness.

	Mean Change	Confidence Interval	St. Dev.
Phase Angle	0.475	+/- 0.005	0.22
Phase Angle [c]	0.017	+/- 0.001	0.023
Capacitance	98	+/- 1.95	93.52
Capacitance [c]	-0.5	+/- 0.031	0.71

Table 2. Overall Data Results for the Bioimpoedance Experimental. Phase angle and capacitance mean change, 95% confidence interval and standard deviation for the experiment are summarized. Control groups are denoted with (^{c}).

DISCUSSION

The plot of ORP versus active hydrogen concentration indicates a time-released mechanism of action for the magnesium active hydrogen compound. The nature of the polynomial decrease in ORP eludes to a second-order kinetic action taking place. Notice the initial drop in ORP, followed by a lower rate, then another increased rate. This is very significant for the idea of a consistency in bio-reactivity and the ability of active hydrogen to react in the body over an extended period of time. The net change in ORP from an initial reading to a final reading of -992 mV is a profound change in redox value. Consistent with this result, the time-domain assay indicates a clear relationship of time release and ORP drop for the length of the experimentation. The redox rate appears to have slowed after 15 minutes, although still was decreasing to to the end of the observe time window. The ability of a compound to release consistently over time has been a troubling problem in the nutraceutical and pharmaceutical industries. The impact of a consistent reaction mechanism over time is that there is a more generalized, consistent and even distribution of the active ingredient in the solution. The data clearly demonstrate that the sampled magnesium active hydrogen successfully performs three main objectives: 1) creates a reduced environment, 2) Has increased potential for reactivity and 3) reacts evenly over time.

The results of porphyrin analyses clearly indicate that active hydrogen reduces cytochrome

c. Additionally, the data indicate increases in oxyhemoglobin concentration, consistent expected correlations. This correlation offers evidence that active hydrogen concentrations act together with oxyhemoglobin and cytochrome *c*. Mechanistically, the reactions of active hydrogen are presumably as follows (3):

(3)

Where the loosely bound electron on the hydride anion directly transfers to reduce the Fe(III) to Fe(II) and the neutral hydrogen then combines with hydroxyl groups in the aqueous solution to form water.

The data presented represent a non-enzymatic relationship in porphyrins when in the presence of active hydrogen. The relationship of increased active hydrogen concentrations contribute to increases in oxyhemoglobin concentrations and increases reduced cytochrome *c*. This relationship of the changes in porphyrins provide evidence for the support that the mechanisms are redox coupled and are controlled by the donation of the native hydrogen ion in the active hydrogen to a receptor in the heme system.

As indicated by Shirahata, the redox potential and pH should be simultaneously analyzed and used to calculate rH, which is a significant indicator of a compound's accurate reducing potential and provides insight into the mechanism of active hydrogen. Analysis and calculation of rH, in conjunction with the measurement of dissolved oxygen, support mechanistically the electron donation by the hydrogen ion. The rH determination concludes a significantly reduced environment, greater than virtually all comparable compounds. One of the benefits gained from these analyses is the understanding that these mechanisms are consistent with previous studies of active hydrogen which contribute to the collective understanding of negative ions and their significance in biochemical reactions.

In addition to demonstrating oxygen transport ability and available cytochrome *c* for electron transport, porphyrin compounds are also indicators of hydration within the cell system. Specifically, the coupled reactions of reduced cytochrome *c* and increased oxyhemoglobin are additional indicators of erythrocyte hydration. The increased hydration is consistent with previous work on active hydrogen and its relationship to eukaryotic cells. Recent work utilizing bioimpedance analysis of cells treated with active hydrogen indicates increases in hydration through a hypothesized electron transfer mechanism.

The reduction of cytochrome *c* is also directly correlated to the electron transport chain which has been shown to increase metabolite production, including ATP and NADH production, *in vitro*, through the direct transfer electrons between proteins within the chain, consistent with prior published results. These results are a consistent indication of the direct electron transfer mechanism for the porphyrin reactions and provide a non-enzymatic control and allosteric regulation for oxygenated transport within human erythrocytes. The results obtained are promising for the use of active hydrogen as a method to increase oxygenation in blood and to increase cellular energy output and hydration.

As further support of the hydrating effects of active hydrogen, the bioimpedance analysis provides a clinical foundation for the utilization of active hydrogen ions to aid with hydration in the body. Each participant in the study, with the exception of the negative control and the placebo control had significant increases in phase angle and capacitance readings. The phase angle variable is a direct indicator of cellular hydration and health. The objective values of phase angle range on a scale from zero to 14, where zero is a highly diseased individual with poor health and 14 is an Olympic-caliber athlete. The relative change in cellular capacitance is quite significant as well. Increased to cellular capacitance objectively quantifies hydration through cellular membrane integrity and ability of a cell to maintain and hold a charge. Capacitance is also a solid indicator of the ion flux of the Gardos channel, defining

hydration levels and the tonicity of the cell environment.

The results demonstrate increased hydration as measured by this non-segmental means. At the onset of the study some participants had initial readings that were lower for their hydration levels. The relative percent increase for these participants were higher than those individuals who had higher initial readings. The data indicate that active hydrogen may be used as a supplement to compliment existing dehydration treatment protocols or as a cost effective, safe alternative method for increasing hydration in patients. The hydrating abilities *in vivo*, are consistent with *in vitro* results and warrant further detailed investigation including a large scale study.

The magnesium active hydrogen is a biologically active compound with benefits pertaining to hydration, reduction of oxidative stress and in energy production and transport.

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REFERENCES

[1] Stephanson CJ, Flanagan GP: Synthesis of a novel anionic hydride organosiloxane presenting biochemical properties. *Int J Hydrogen E* 2003;28:1243-1250.

[2] Gauyacq J: Dynamics of Negative Ions. World Scientific Lecture Notes in Physics. Vol.15. World Scientific Press, New Jersey, 1987.

[3] Stephanson CJ, Flanagan GP: Non-Toxic Hydride Energy Source for Biochemical and Industrial Venues: ORP and NAD+ reduction analyses. *Int J Hydrogen E* 2004;29:459-464.

[4] Stephanson CJ, Stephanson AM, Flanagan GP: Evaluation of Hydroxyl Radical-Scavenging Abilities of Silica Hydride, an Antioxidant Compound, by a Fe+2-EDTA Induced 2-hydroxyterephthalate Fluorometric Analysis. *J Med Food* 2003;6:249-253.

[5] Stephanson CJ, Flanagan GP: Antioxidant capacity of silica hydride: A combinational photosensitization and fluorescence detection assay. *Free Rad Biol Med* 2003; 35:1129-1137.

[6] Stephanson CJ, Stephanson AM, Flanagan GP: Antioxidant capability and efficacy of MegaH™ silica hydride, an antioxidant dietary supplement, by *in vitro* cellular analysis using photosensitization and fluorescence detection. *J Med Food* 2002;5:9–16.

[7] Stephanson CJ, Flanagan GP: Differential Metabolic Effects on Mitochondria by Silica Hydride Using Capillary Electrophoresis. *J Med Food* 2004;7:79-83.

[8] Purdy-Lloyd KL, Wasmund W, Smith L, Raven PB: Clinical effects of a dietary antioxidant silicate supplement, Microhydrin®, on cardiovascular responses to exercise. *J Med Food* 2001;4:151–159.

[9] Rosales Hernández MC, Ortiz Pérez SS, Aceves JM, Correa-Basurto J, Ramírez Rosales D, Zamorano Ulloa R, Trujillo Ferrara J: Catalytic Activity Of Cytochrome P-450 Using Nadph Reduced By Silica Hydride. J Mol Cat B: Enzymatic

[10] Clark WM. Pub Health Rep 1923;38:666–83.

[11] Clark A, Federspiel WJ, Clark PAA, Cokelet GR: Oxygen delivery from red cells. *Biophys J* 1985;47:171-181.

[12] Mattig S, Knoefler R, Deussen A: Modulation of adenine nucleotide concentrations in human plasma by erythrocytes and endothelial cells. *Thromb Res* 2003;110:195-202.

[13] Zhou GP, Anderson KP, Joiner CH, Gallagher PG: Modification of erythrocyte hydration in the treatment of sickle cell disease. *Blood Cells Mol Dis* 2001;27:65-68.

[14] Voet D, Voet JG: In Biochemistry. 2nd ed. John Wiley and Sons, New York, 1995.

[15] Salemme R: Structure and function of cytochromes *c*. *Annu Rev Biochem* 1977;46:299-329.

[16] Florens L: Structure and functional relationships within the cytochrome c superfamily. *J Cell Biol* 1996;7:222-231.

[17] Perutz MF, Kilmartin JV, Nishikura K, Fogg JH, Butler PJG: Identification of residues contributing to the Bohr effect on human haemoglobin. *J Clin Invest* 1968;47:2652-2656.

[18] Chen W, Zhang J, Eljgelshoven MH, Zhang Z, Zhu XH, Wang Y, Cho H, Merkle H, Ugurbil K: Determination of deoxyhemoglobin changes during graded myocardial ischemia: an in vivo ^{1}H NMR spectroscopy study. *Mag Res Med* 1997;38:193-197.

[19] Arai AE, Kasserra CE, Territo PR, Gandjbakhche AH, Balaban RS: Myocardial oxygenation in vivo: optical spectroscopy of cytoplasmic myoglobin and mitochondrial cytochromes. *Am J Physiol* 1999;277:H683-H697.

[20] Capitanio N, Capitanio G, De Nitto E, Villani G, Papa S: H^+/e^- stoichiometry of mitochondrial cytochrome complexes reconstituted in liposomes. *FEBS* 1991;288:179-182.

[21] Murphy MP, Brand MD: *Eur J Biochem* 1988;173:645-651.

[22] Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H: Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. *Biochem Biophys Res Comm* 1997;234:269-274.

[23] Happe RP, Roseboom W, Pierik AJ, Albracht SPJ: Biological activation of hydrogen. *Nature* 1997;385:126.

[24] Halliwell B, Gutteridge JMC. In: Free radical biology and medicine. 2nd ed. Claredon Press, Oxford, 1993.

[25] Holben DH, Hassell JT, Williams JL, Helle B. Fluid intake compared with established standards and symptoms of dehydration among elderly residents of a long-term-care facility. J Am Diet Assoc 1999;99:1447-1450

[26] Avner ED. Clinical disorders of water metabolism: hyponatremia and hypernatremia. Pediatr Ann 1995;1:23-30.

[27] Goepp JG, Katz SA. Oral rehydration therapy. Am Fam Physician 1993;4: 843-851

[28] Holliday MA, Friedman AL, Wassner SJ. Extracellular fluid restoration in dehydration: a critique of rapid versus slow. Pediatr Nephrol 1999;4:292-297.

[29] Muzyamba MC, Gibson JS. Effect of 1-chloro-2,4-dinitrobenzene on K+ transport in normal and sickle human red blood cells. J. Phys Lon 2003; 547:903-911.