

Perspective

An Interplay of Gases: Oxygen and Hydrogen in Biological Systems

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Abstract: Produced by photosynthesis, oxygen (O_2) is a fundamentally important gas in biological systems, playing roles as a terminal electron receptor in respiration and in host defence through the creation of reactive oxygen species (ROS). Hydrogen (H_2) plays a role in metabolism for some organisms, such as at thermal vents and in the gut environment, but has a role in controlling growth and development, and in disease states, both in plants and animals. It has been suggested as a medical therapy and for enhancing agriculture. However, the exact mode of action of H_2 in biological systems is not fully established. Furthermore, there is an interrelationship between O_2 and H_2 in organisms. These gases may influence each other's presence in solution, and may both interact with the same cellular components, such as haem prosthetic groups. It has also been suggested that H_2 may affect the structures of some proteins, such as globins, with possible effects on O_2 movement in organisms. Lastly, therapies may be based on supplying O_2 and H_2 together, such as with oxyhydrogen. Therefore, the relationship regarding how biological systems perceive and respond to both O_2 and H_2 , and the interrelationship seen are worth considering, and will be discussed here.

Keywords: globins; haem; haemoglobin; hydrogen gas; molecular hydrogen; oxygen; photosynthesis; xenon



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1. Introduction

Since its discovery in the latter part of the 18th century [1], there has been an interest in how oxygen (O_2) has roles in biological systems, including its detection [2] and toxicity [3]. O_2 makes up approximately 21% of the atmosphere and is essential for aerobic life. O_2 is produced by photosynthesis as a by-product of plant physiology and it is used in respiratory processes, being the terminal electron acceptor for the mitochondrial electron transport chain (Complex IV converting O_2 to water). Oxygen has many other uses in normal physiology, too, not least being the starting point of reactive oxygen species (ROS), as outlined in Figure 1.

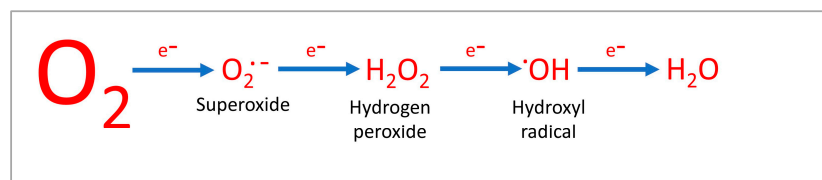
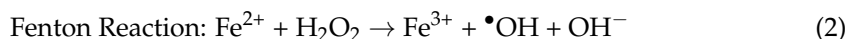
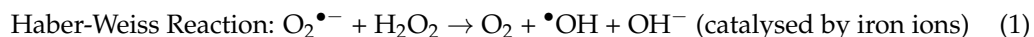


Figure 1. Oxygen can be reduced to reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$). In mitochondrial respiration, O_2 is converted to water by a 4-electron reaction.

Superoxide ($O_2^{\bullet-}$) is regarded as the cardinal ROS as its formation is known to give rise to other ROS including the hydroxyl radical ($\bullet OH$), and also RNS such as peroxynitrite ($ONOO^-$). The dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD) gives rise to hydrogen

peroxide (H_2O_2), which can generate $\bullet\text{OH}$ through downstream reactions noted below in Equations (1) and (2):



The further reduction of $\bullet\text{OH}$ yields water, and it is the four-electron reduction of O_2 by mitochondrial Complex IV which aims to avoid ROS generation. Such reactive molecules are chemically relatively unstable, as many carry unpaired electrons in the outer orbit which have a strong affinity for hydrogen atoms. This can lead to proton extraction from vital biomolecules such as protective lipid membranes, thereby destabilising their structure and altering functionality. ROS/RNS are also able to affect fundamental processes including genetic transcription and mitochondrial activity through the modification of essential proteins and protective membranes and therefore have a significant role in cellular homeostasis. Several excellent reviews on ROS function are available [4–6].

Hydrogen was also discovered in the 18th century, and since then has had a rather on-and-off appearance in biological research [7,8]. However, hydrogen gas has been coming to prominence in the scientific literature more recently, especially since the publication of a *Nature Medicine* paper in 2007 [9], not only as an alternative source of renewable energy but also as a health and lifestyle supplement for humans and animals with potential uses in surgical [10], recuperative [11] and geriatric medicine [12]. Research also suggests incorporating H_2 into the agri-food industry, where it can be used in many ways and can enhance stress tolerance [13,14] and act as a growth stimulant [15] or as a preservative for plants and food products [16,17], for example. Tables 1 and 2 outline the significant effects molecular hydrogen can have in both animals and plants.

Table 1. Examples of the effects seen in animals after molecular hydrogen treatment. HRS: Hydrogen-rich saline; HRW: Hydrogen-rich water MDA: Malondialdehyde; SOD: superoxide dismutase; Nrf-2: nuclear factor erythroid 2-related factor 2; HO-1: haem oxidase-1; NQO-1: NAD (P)H quinone oxidoreductase 1; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; RBC: red blood cell; WBC: white blood cell; COX-2: cyclooxygenase-2; 8-oxo-dG: 8-Oxo-2'-deoxyguanosine; CLDN3: Claudin 3.

Animal	Method of Treatment	Effect Reported	Reference
Chickens	Oral HRW: H_2 (1–1.5 mg/L) (ad libitum)	Increased IgG and IgM antibodies	[18]
Dogs	Oral HRW: H_2 (1.6 mg/L) (800 mL/day)	Improved angiogenesis and dermal thickness Increased SOD, Nrf-2, HO-1, NQO-1, VEGF, and PDGF Reduced MDA	[19]
Dogs	Injected H_2 gas (0.2 mL/kg)	Increased RBC count, Haemoglobin and Haematocrit Reduced haemolysis and WBC infiltration	[20]
Goats	Mg supplement: either $\text{Mg}(\text{OH})_2$ or elemental Mg ($\text{Mg}(\text{s}) + 2\text{H}_2\text{O}(\text{l}) \rightarrow \text{Mg}(\text{OH})_2(\text{s}) + \text{H}_2(\text{g})$)	Altered rumen microflora and fermentation process (increased propionate metabolism); increased methanogenesis	[21]
Guinea Pigs	Oral HRW: H_2 (>1.6 mg/L) (ad libitum)	Attenuated noise-induced hearing loss	[22]
Guinea Pigs	HRS: H_2 (~1.2 mg/L) 10 mL/kg intraperitoneal + 20 μg intranasal	Increased SOD Decreased eosinophils, IgE antibodies mucous production and MDA	[23]

Table 1. *Cont.*

Animal	Method of Treatment	Effect Reported	Reference
Horses	Nasogastric HRW: H ₂ (>1 mg/L) (2L)	Reduced accumulation of reactive oxygen metabolites	[24]
Horses	Intravenous HRS: H ₂ (0.6 mg/L) (2 L)	Elevated antioxidant potential Suppressed oxidative stress	[25]
Pigs	H ₂ gas (2.1% in air/4 h)	Reduced COX-2 expression and 8-oxo-dG	[26]
Pigs	HRW gavage: H ₂ (1.6 mg/L) (10 mL/kg)	Reduced intestinal leakage and toxin-induced apoptosis Restored CLDN3 expression	[27]

Table 2. Examples of the effects seen in plants after molecular hydrogen treatment. APX: ascorbate peroxidase; CAT: catalase; POD: peroxide; SOD: superoxide dismutase.

Plants	Method of Treatment	Effect Reported	Reference
Alfalfa (<i>M. sativa</i> L. <i>victoria</i>)	Seedlings incubated HRW/12 h: H ₂ (0.16 mg/L)	Regulated expression of genes relevant to sulphur and glutathione metabolism; enhanced glutathione metabolism	[14]
<i>Arabidopsis thaliana</i>	HRW and H ₂ -infused growth medium: H ₂ (1.6 mg/L)	Enhanced salinity tolerance via modulation of redox homeostasis and upregulation of serotonin N-acetyltransferase and melatonin expression	[28]
<i>Arabidopsis thaliana</i>	HRW and H ₂ -infused growth medium: H ₂ (0.16, 0.4, 0.8, 1.2 and 1.6 mg/L)	Enhanced salinity tolerance via modulation of redox homeostasis and reduced ion influx through modulation of H ⁺ pump and antiporter activity	[29]
Bananas (<i>Musa</i> spp. AAA cv. <i>Baxijiao</i>)	Soaked in HRW/10 min: H ₂ (0.8 mg/L)	Delayed ripening as a result of repressed ethylene signalling and respiration	[17]
Barley (<i>Hordeum distichum</i> L.)	Soaked in HRW/6 h: H ₂ (2 mg/L)	Increased concentration of vanillic acid, coumaric acid, sinapic acid, Ca and Fe, significantly increasing the germination and growth rates	[15]
Cabbage (<i>Brassica campestris</i> spp. <i>Chinensis</i> L.)	Seeds soaked in HRW/3 h before germination: H ₂ (0.8 mg/L)	Improved Cd tolerance, associated with reduced Cd uptake and increased antioxidant defences	[13]
Chives (<i>Allium tuberosum</i> Rottler ex Spreng.)	H ₂ gas in packaging: H ₂ (1%, 2%, 3%)	Here, 3% H ₂ significantly delayed post-harvest ripening via enhancing antioxidant capacity (APX, CAT, POD, SOD)	[30]
Kiwi fruit (<i>Actinidia deliciosa</i> , cv. Hayward)	Soaked in HRW/5 min: H ₂ (1.2 mg/L)	Alleviated chilling injury through enhanced sugar production and repressed peroxidation	[31]
Rice (<i>Oryza sativa</i> L.)	HNW in irrigation system: H ₂ (1 mg/L)	Increased grain size, weight and yield; decreased amylose content; significantly reduced Cd accumulation	[32]

Table 2. Cont.

Plants	Method of Treatment	Effect Reported	Reference
Strawberries (variety unknown)	H ₂ gas in the packaging atmosphere (10% CO ₂ , 4% H ₂ , 86% N ₂)	Extended the shelf-life (300–500%) by moderating antioxidant responses	[16]

Organisms can be exposed to H₂ in a variety of ways, with several species containing hydrogenase enzymes, which will produce H₂ [33]. Hydrogenase enzymes, some of which are inhibited by O₂ (e.g., group IV NiFe hydrogenases [34]), are responsible for catalysing the reversible oxidation/reduction of H₂ ($\text{H}_2 \leftrightarrow 2\text{H}^+ + \text{e}^-$). Such enzymes are found in all single-celled and many multicellular organisms (e.g., fungi, plants [35]) and can be categorised into specific phylogenetic groups, namely iron only, iron-iron and nickel-iron (Fe, Fe-Fe and NiFe, accordingly). On the other hand, other organisms host an array of H₂-producing microflora in the gastrointestinal tract. Hydrogen can also be naturally occurring, for example in thermal vents [36] and other natural sources [37]. As with O₂, organisms can also be treated with molecular hydrogen which can be applied directly as a gas, or as hydrogen-enriched water (hydrogen-rich water: HRW: [38]), perhaps in the form of nanobubbles (hydrogen nanobubble water (HNW)). Furthermore, organisms can be exposed to O₂ and H₂ at the same time in the form of oxygen/hydrogenated solutions and as a stoichiometric mixture of oxyhydrogen gas (66% H₂/33% O₂) formed through the electrolysis of water.

Although the role of action of O₂ in cells is well established, the way H₂ effects cells is still unclear under many circumstances. As listed in Table 3, there are several mechanisms possible, but it is unlikely that a single action would be able to account for all the cellular responses reported. H₂ was originally thought to be acting as an antioxidant, with many of its effects from scavenging hydroxyl radicals ($\bullet\text{OH}$) but having little or no effect on several significant small reactive signalling molecules such as superoxide ($\text{O}_2^{\bullet-}$), H₂O₂ or nitric oxide (NO). However, as Table 3 exemplifies, several other mechanisms have been proposed, or may at least be possible, and multiple mechanisms are likely to be invoked simultaneously upon rising H₂ concentrations. Several of these mechanisms may involve O₂, and it seems timely to discuss what any possible ramifications of this may be.

Table 3. Some of the possible mechanisms of the action of H₂ in cells and tissues.

Proposed Mechanism	Comment	Reference(s)
Direct scavenging of $\bullet\text{OH}$	$\bullet\text{OH}$ will be derived from O ₂ . Little evidence of other ROS scavenged	[9]
Scavenging of other ROS, e.g., H ₂ O ₂	Evidence suggests that this is not a possible mechanism	[9]
Action of nanobubbles	Could remove $\bullet\text{OH}$, hypochlorite (ClO^-), ONOO ⁻ and $\text{O}_2^{\bullet-}$	[39]
Scavenging of reactive nitrogen species	Evidence suggests that this is not a possible mechanism, e.g., no reaction with nitric oxide (NO) for example.	[9]
Scavenging $\bullet\text{OH}$ in a mechanism that involves haem	Would explain some of the effects reported	[40–42]
Effects mediated by haem oxidase, e.g., HO-1	May involve carbon monoxide (CO)-mediated signalling	[43]
Effects mediated by the redox poise of the H ₂ couple	Suggested as a widely used mechanism, but no evidence	[44,45]
H ₂ spin state	Spin states mediate interactions with other biomolecules. No direct evidence for this	[46]
Acting through protein hydrophobic polypeptide pockets	Globin proteins are a model system, where several inert gases are known to interact	[47,48]
H ₂ and O ₂ interacting	May account for some of the effects seen?	Discussed here

Water (H₂O), composed of hydrogen and oxygen, is a universal biological solvent and is essential for the emergence and continuation of carbon-based life [49]. H₂O is formed through the thermal catalysis of O₂ and hydrogen (H₂) gases, (reaction $2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O}$), but of importance here is that it can also be split to yield O₂ and H₂ in a ratio of 1:2. An inexpensive and relatively simple way to generate H₂ is by electro-hydrolysis, with the H₂ being exploited and the O₂ being vented to waste. However, both gases can be employed together in a mixture known as oxyhydrogen. Oxyhydrogen can be used in many biological arenas, including as a therapy [50]. Such work exemplifies why O₂ and H₂ should be considered together, as discussed further below.

2. Solubility of Oxygen and Hydrogen in Water

When using either O₂ or H₂ in solution, the solubility of the gas has to be considered. The solubility of O₂ in pure water is reported to be between 1.18×10^{-3} and 1.25×10^{-3} mol dm⁻³ (at 25 °C and 1.0 atm of O₂ pressure) [51]. For H₂, the solubility under similar conditions, ~1 atm and 25 °C, is approximately 0.8×10^{-3} mol dm⁻³.

If water is equilibrated with air, the O₂ concentration would be predicted to be approximately 2.56×10^{-4} mol dm⁻³, whilst the concentration of H₂ would be predicted to be negligible—there is little atmospheric H₂ to dissolve at ground level. If the concentration of O₂ or H₂ is increased in water, one of the questions which should be asked is for how long does that elevated concentration last? As can be seen in Figure 2, an elevated level of O₂ in solution starts to decline and if the regression line is extrapolated, compared to the baseline concentration, it gives a half-life of elevated oxygen in solution of approximately 420 min. Therefore, if such a solution is going to be used as a treatment for any organism, be it a plant or animal, the loss of oxygen gas with time needs to be considered—it would be suggested that such solutions are made shortly after being prepared.

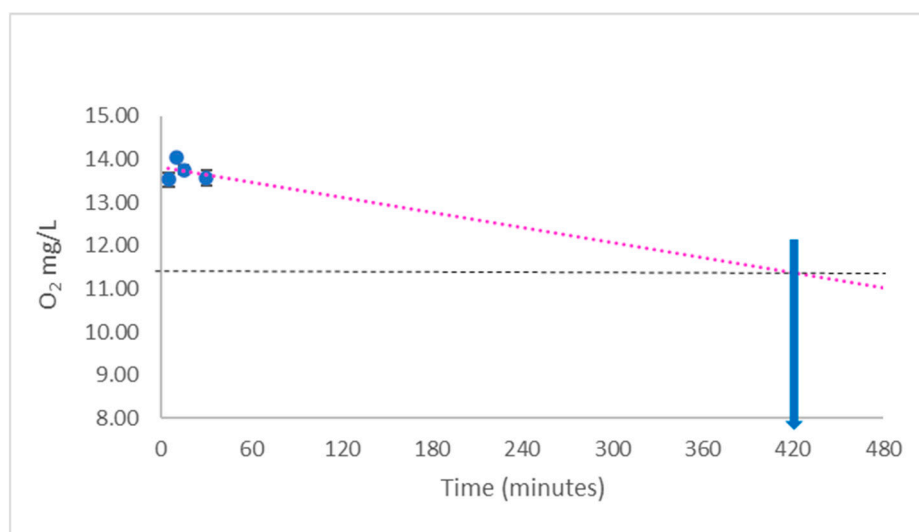


Figure 2. Estimation of the O₂ concentration in solution. On extrapolation, this gives the half-life of concentration elevation. The solution was bubbled with oxyhydrogen solution 450 mL/min for 30 min (HydroVitality™, Wakefield, UK). O₂ was measured with a Clark-type electrode (Hanna Instruments, Bedfordshire, UK. Cat. #H198198). The red dotted line is an extrapolation, whilst the horizontal line represents the half-way point of increase. The arrow indicates an approximate half-life. Data representative of 3 repeats ± SEM.

Similarly, if hydrogen gas in solution is elevated, it will rapidly re-enter the gaseous phase and be lost. Again, representative data are shown (Figure 3), showing that under the conditions used, the half-life of the H₂ in solution is only approximately 32 min (Figure 3). Therefore, long-term storage of such a solution before use is not an option unless kept in a sealed container with little head space. Of critical importance here, such measurements

of O₂ and H₂ in solution should be carried out by researchers using their equipment and solutions before being used in a biological setting.

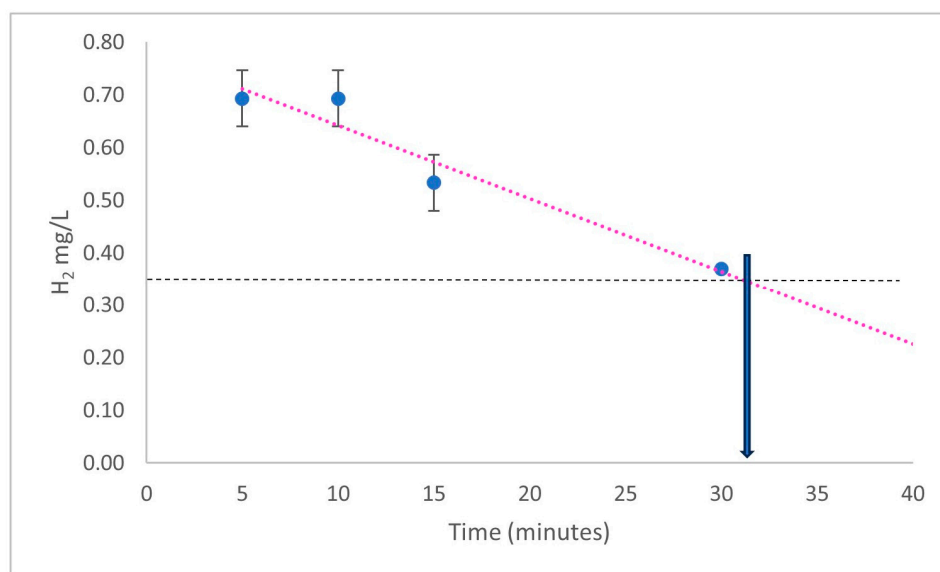


Figure 3. Estimation of the H₂ concentration in solution after bubbling with oxyhydrogen gas 450 mL/min for 30 min (HydroVitality™, Wakefield, UK). H₂ was measured with a methylene blue-based assay system (H₂Blue, H₂ Sciences Inc., Henderson, NV, USA). The horizontal dotted line is halfway between the maximal concentration measured and the minimal concentration expected when the solution is equilibrated with atmospheric air. The red dotted line is an extrapolation, whilst the horizontal line represents the half-way point of increase. The arrow indicates an approximate half-life Data representative of 3 repeats ± SEM.

In a very recent study [52] on the proton exchange membrane (PEM) electrolysis of water (a method that infuses only H₂ into aqueous solutions) from different sources, the increased H₂ in each solution was shown to have a half-life of between approximately 60 and 140 min, which is longer than found in our studies involving alkaline electrolysis that produces and infuses both H₂ and O₂ (~32 min half-life). Interestingly, the results are widely different depending on the source of water used. The shortest half-life was with what the authors describe as “Holy Spring” water, with the longest being tap water. The study also found that the highest H₂ concentration they could obtain was approximately 1.3 ppm. pH rose by about 1 unit over 70 min of electrolysis, and the oxidation-reduction potential (ORP) dropped significantly, from approximately +200 mV to −500 mV. Such work on different waters highlights the need to be careful about knowing what is dissolved in solution and to measure exact working concentrations when reporting data with dissolved gases.

Lastly, it is likely the dissolving of one gas in an aqueous solution (whether pure water, saline or buffer) may affect the presence of other gases. For example, a representative example is shown in Figure 4. Here, it can be seen that the concentration of O₂ in solution drops significantly whilst the magnesium-based tablet produces H₂ (by the Mg catalysis of water hydrolysis), but then the O₂ levels are slowly restored as the oxygen is absorbed from, and hydrogen released into, the atmosphere. Creating a fresh H₂ solution in such a fashion may make a relatively anaerobic solution, and should the creation of such an anaerobic solution not be considered as a mode of action when such solutions are used in agricultural and biomedical research, or as therapies in a medical/sports medicine arena?

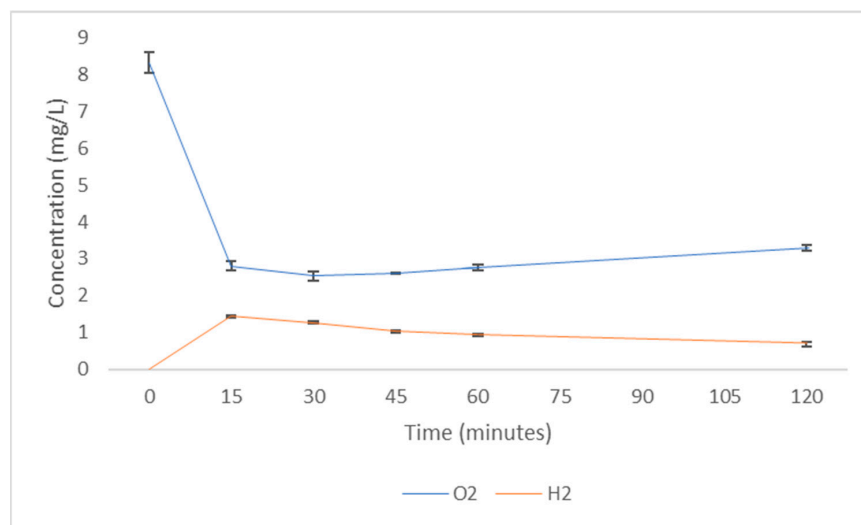


Figure 4. Concentration of O₂ and H₂ in solution. H₂ was produced using a magnesium-based tablet (Drink HRW, Oxnard, USA). The tablet was dropped into deionised water (15 MΩ·cm²) in a non-sealed conical flask (500 mL). H₂ was measured with a methylene blue-based assay system (H₂Blue, H₂ Sciences Inc., Henderson, NV, USA). O₂ was measured using a Clark-type electrode (Hanna Instruments, Bedfordshire, UK. Cat. #H198198). Data are mean 3 repeats ± SEM.

3. Oxyhydrogen

Further to the discussion of O₂ and H₂ solubility above, as already mentioned, the two gases are often used together, in what is referred to as oxyhydrogen gas. Brown's gas, H₂/O₂, HHO and hydroxy are all terms for the same stoichiometric chemical mixture of oxyhydrogen gas (66% H₂ and 33% O₂). Oxyhydrogen can be inhaled as a gas (using a nasal cannula or face mask), applied ingested in aqueous solutions, or supplied as a gas to sealed containers for plants, for example. Clearly, under these conditions, there is significantly increased O₂, but also a very high concentration of H₂. It is known that high H₂ is well tolerated by humans, as long as the O₂ concentration is maintained (something early researchers of gases such as Humphry Davy failed to realise [7]). As a deep-sea diving gas, H₂ has been used since the 1940s [53], and there are no known detrimental health effects. Very early experimentation with what was referred to as "medical gases" did have potentially lethal effects, partly because the researchers were never sure of the purity or exact content of their experimental gases. Humphry Davy nearly killed himself on several occasions [7]. For oxyhydrogen, if such an O₂/H₂ gas mixture is to be used by humans, or in an agricultural or veterinary scenario, it is important to know exactly what is being used, and that there are no erroneous or toxic components present. However, the oxyhydrogen gas mixture, at least in our hands, has a composition as indicated in Table 4. Therefore, there are no significant concentrations of toxic gases or impurities, but both O₂ and H₂ are elevated compared to breathing air (as atmospheric at low altitudes).

Gas analysis such as in Table 4 identifies a little over 10% of N₂ present in oxyhydrogen gas. Although this would not be intuitively expected, N₂ is not toxic and only serves to lower the "working" concentrations of O₂ and H₂, although these are both higher than atmospheric concentrations (29.5% and 60% respectively) in the analysis. Considering that (i) atmospheric air contains 78% nitrogen, (ii) the evaluation of gases is inherently at risk of atmospheric contamination, and (iii) the HydroVitality device is a sealed unit (applying Henry's Law, the amount of air dissolved in a fluid is proportional to the pressure in the system, so dissolved gases such as N₂, O₂ and CO₂ in the reservoir are to be expected) it is assumed that the nitrogen content is probably a result of carryover from the gases originally dissolved in the water used to supply the HydroVitality™ machine.

Table 4. The gaseous and inferred output of alkaline water electrolysis. Gas was prepared from a HydroVitality™ (Wakefield, UK) oxyhydrogen generator, collected in a Tedlar® bag and sent for analysis to SGS Gas Analysis Services (Bristol, UK). N/D means not detected.

Compound	Measured Results (%)	Inferred Output Percentage (%)
H ₂	60.16%	66%
O ₂	29.50%	33%
N ₂	10.32%	Not expected
Methane (CH ₄)	0.01%	0.01%
Carbon dioxide (CO ₂)	0.01%	0.01%
Carbon monoxide (CO)	N/D	N/D

It should also be noted that the adaption of oxyhydrogen-generating machines can yield only H₂, wherein a membrane is used to restrict the diffusion of the gas in the electrolyte, and O₂ is vented as a waste gas. Therefore, this relatively inexpensive and easy-to-use technology can be used to generate oxyhydrogen or H₂, and both can then be used for inhalation or to create an enriched solution for treatments.

When considering the direct inhalation of H₂ and oxyhydrogen gases and the effect of H₂ displacement on O₂ to calculate the percentage of H₂ inhaled, a simple formula can be applied.

$$(\text{mL/s}): \text{H}_2 / (\text{Breath} - \text{H}_2) \times 100$$

To illustrate, the average person (female: height 1.64 m, weight 72.7 kg and BMI 27.1; male: height 1.78 m, weight 86.7 kg and BMI 27.4; UK data) [54] breathes in 500 mL of air with every breath, with inhalation lasting approximately 2 s. Taking the maximum flow rate of the device tested, HydroVitality™ produces 450 mL/min oxyhydrogen. Thus, females will inhale 2.5% H₂ with every breath, whilst males will intake approximately 2%. If the same formula, adjusted for the flow rate and percentage increase (33%) in O₂ consumption, is applied, the values of oxygen intake increase by 1.27% (22.27%, O₂) for females and 1.01% (22.01%, O₂) for males, Table 5.

Table 5. Calculated percentages of gases inhaled by the average female and male (www.worlddata.info; accessed 31 January 2024) when breathing regularly. Normal air is compared to oxyhydrogen and hydrogen-only breathing. To match the flow rate of the HydroVitality™ oxyhydrogen generator, a flow rate of pure H₂, 300 mL/min was used.

	Air (250 mL/s)		Oxyhydrogen (450 mL/min)		H ₂ -Only (300 mL/min)	
(% inhaled)	O ₂	H ₂	O ₂	H ₂	O ₂	H ₂
Female	21%	<0.0001%	22.27%	2.5%	~19%	2.5%
Male	21%	<0.0001%	22.01%	2%	~19.5%	2%

A reduction in O₂ consumption can have unwanted effects on metabolic processes; as mentioned above, O₂ also has effects in biological systems, being the terminal electron acceptor in aerobic respiration and ATP production, and the starting point of ROS production. Therefore, a prolonged decrease can lead to tissue damage.

Due to the addition of O₂, oxyhydrogen gas could alleviate conditions relating to hypoxia and reduce the incidence of hyperoxia as a result of H₂ application. This assumption is supported by clinical data obtained during the COVID-19 pandemic by healthcare professionals in the People's Republic of China, where oxyhydrogen inhalation was shown to reduce airway resistance and improve the serious burden of disease (NCT04336462—Results: [55]). In contrast, for the treatment of chronic obstructive pulmonary disease (COPD) where hyperoxia from pure O₂ inhalation can cause physiological distress, oxyhydrogen was demonstrated to be more effective in reducing breathlessness, cough and sputum. In total, 14.8% fewer adverse events

were also reported in the oxyhydrogen-inhalation group when compared with O₂ inhalation (NCT04000451—Results: [56]). In clinical investigations (NCT02961387—Results: [57]) of tracheal stenosis, a condition that presents with severe inflammation of the airways, treatment with oxyhydrogen was superior to O₂ in reducing inspiration effort. These results suggest that the combination of H₂ and O₂ has a favourable effect in remediating hypoxia, without instigating hyperoxic effects, delineated by the above studies reporting no adverse events.

It is known that H₂ has profound effects on organisms, as exemplified in Tables 1 and 2. Research also has been carried out utilising both H₂- and O₂-enriched water in biological systems. As an example, Shin et al. [18] fed broiler chickens either oxygenated or hydrogenated water (i.e., enriched in O₂ or H₂ respectively) and then measured a variety of characteristics as the chicks developed. A summary of their data is given in Table 6.

Table 6. Examples of some of the effects of oxygenated or hydrogenated water treatments on broiler chickens as they developed (data from Shin et al. [18]).

Treatment Given	Characteristic Measured	Effect Seen
Oxygenated water	Weight	Significantly increased
	Body mass index	Significantly reduced abdominal fat accumulation
	Triacylglyceride	Reduced
	Total cholesterol	Reduced
	LDL cholesterol	Reduced
	IgG antibodies	Significantly increased
	IgM antibodies	Significantly increased
Hydrogenated water	Weight	Increased
	Body mass index	Non-significant reduction
	Triacylglyceride	Reduced
	Total cholesterol	Reduced
	LDL cholesterol	Reduced
	IgG antibodies	Increased
	IgM antibodies	Increased

The data show that drinking H₂ (as a dissolved solution) has a range of effects on the chicks as they develop. This is not a great surprise, considering the data in Tables 1 and 2. However, drinking O₂-enriched solutions also had a similar range of effects. Therefore, on drinking oxyhydrogen-enriched solutions, is it the H₂ or O₂ that is causing the effects seen? Or is it better to have both O₂ and H₂ enriched together? Perhaps, as with oxyhydrogen inhalation detailed above, elevating levels of both gases is better than elevating just H₂, such as through preparation with Mg tablets. Comparative studies of such solutions, where the exact concentration of gases used is recorded, are required to answer this question. After all, it is not just in chickens that the positive effects of oxygenated water have been reported. Handajani et al. [58] found that drinking such a solution was beneficial for diabetes mellitus, although other reports are sceptical. For example, Gruber et al. [59] said that although there were no long-term detrimental effects on the liver, blood or immune system in humans, the presence of radicals in the blood was increased, albeit transiently. Piantadosi [60] says, “Ergogenic claims for oxygenated water cannot be taken seriously”, and then reviews the evidence. All the claims for oxygenated water need to be taken with caution and more work may need to be undertaken. Interestingly, Shin et al. [18] prepared their enriched solutions using bamboo stems, so that the O₂ and H₂ entered the water as nanobubbles. This should lead to better stability for the solutions and may perhaps partly account for their effects. The use of nanobubbles has been successful by others, both in vitro and in vivo [39].

4. Is there an Effect of H₂ on Haem Groups and a Disruption of Function?

It has been well established that O₂ acts as a terminal electron acceptor in cells. As well as accepting four electrons from Complex IV of the mitochondrial electron transport chain (ETC) [45], O₂ is also a terminal acceptor of gp91-*phox* of some NADPH-dependent oxidase (NOX) complexes [61]. Indeed, Kiyoi et al. [62] have described a reduction in both •OH and

ONOO[−] production in murine models when treated by H₂ inhalation. Interestingly, the same study [62] noted that the NADPH oxidase (NOX-1) enzyme, responsible for producing the superoxide anion during cellular stress events, was significantly downregulated in the H₂ group. The expression of critical activation components p40-*phox* and p47-*phox*, however, were unaffected, suggesting that H₂ may influence the expression or action of proteins within the NOX-1 complex. Such enzymes facilitate the conversion of O₂ to O₂^{•−}, utilizing Fe transition metals as catalysts for electron transference, and as such could provide an effectual target for H₂ interactions [63], although empirical data will be required to confirm this. Excellent reviews on the NOX enzymes have been written recently [64,65].

In haemoglobin, the O₂ binds to the sixth coordinate position of the haem in a manner in which O₂ can associate and dissociate depending on the oxygen tension in the solution. Myoglobin has a similar action, but at different O₂ binding constants.

H₂ can also interact directly with the Fe atom at the centre of the haem group. This was found in cytochrome c₃ of *Desulfovibrio desulfuricans* [44], where there was an electron transfer, and the Fe was reduced from Fe³⁺ to Fe²⁺. Such an electron transfer, if it were to take place, would convert methaemoglobin to the haemoglobin (Fe³⁺ to the Fe²⁺) state, and so facilitate O₂ binding as indicated by Singh et al. [66]. Having said that, physiologically, methaemoglobin is not prevalent in the blood, accounting for <10% in healthy adults, although it can be much higher in individuals with haemoglobinopathies and enzymopathies where methaemoglobin is a feature [67]. However, such a reduction of the Fe in other haem proteins would have the potential to enhance their function, as previously argued [45].

Recently, using density functional theory (DFT) calculations, it has been reported that there is a potential for H₂ to interact directly with Fe/haem [40]; in this case, there would also be electron transfer to the haem. The effect on the haem was not the focus, but rather the formation of H[•] radicals and the likelihood of their interacting with such radicals as •OH. Should H₂ with electrons directly interact with the transition metal components of proteins, as described by Hancock and Hancock [46], Kim et al. [40] and Ohta [42], it is possible that the metal group would catalyse the reduction of radicals via reducing the disassociation energy of free H₂ (~4.64 eV~2.35 eV) and the formation of an acceptor/H[•] complex. Logically, the H₂ radicals would readily react with radical oxidants such as •OH, and non-radical species such as peroxyxynitrite, although this would be regulated by the spatial and temporal availability of the perceived radicals. Interestingly, Kim et al. [40] state that, “From the accumulated studies of Fe–O₂ binding, we can expect that H₂ also binds to Fe...”. Does this mean that if H₂ binds to the haem in a manner analogous to O₂, albeit transiently, the binding of O₂ is disrupted? Would the same apply to myoglobin? It has been reported that H₂ treatment can increase blood oxygenation [68], which is contrary to H₂/haem interactions disrupting O₂ ligation.

Jin et al. [41] suggested a similar interaction of H₂ with haem. Fe-porphyrin, both free and bound to protein, could react with H₂, and further they reported the conversion of CO₂ to CO, so implicating CO metabolism as being part of the mechanism mediating H₂ effects (which may also implicate haem oxygenase). They conclude that “Fe-porphyrin is a redox-related biosensor of H₂”. Ohta [42] suggests that a target molecule for H₂, Fe-porphyrin conjugated with the -OH group (PrP-Fe(III)-OH), may mediate the activation of the transcription factor Nrf2, and so alleviates oxidative stress effects [69]. Certainly, the interaction of H₂ with haem needs further work.

Of further consideration are the metal catalytic centres of such antioxidant enzymes as CAT (haem: [70]) and SOD (either Cu/Zn or Mn-based: [71]), which are reported to have enhanced activity after exposure to H₂. It can therefore be surmised that H₂ may also improve the function of such metalloenzymes by preserving the reductive capacity of the catalytic metal elements, which may account for the reports of the effects of H₂ by Zeng et al. [72] in plants and Yu et al. [73] in small mammals. And of course, there are a host of other metal-based enzyme reactions, not least those of the cytochromes and chlorophyll. Are the mechanisms proposed by Kim et al. [40] and Jin et al. [41] only limited to the haem prosthetic groups they studied, or is there potential for a wide use of such mechanisms?

5. Direct Interaction of H₂ with Proteins

Another prevailing theory of H₂ somatic distribution and/or action is one of protein pockets. This hypothesis is based on the identification of discrete hydrophobic channels and surface pockets within protein conformations. Such features are typically lined with amino acids of leucine, isoleucine, alanine or valine, characteristically formed in proteins over 100 amino acids in length (reviewed by Roose et al. [74]). Hydrophobic pockets enable non-covalent/van der Waals interactions with noble gases such as argon (Ar), krypton (Kr), the much larger atom, xenon (Xe) [75], and perhaps molecular hydrogen [48]. Research conducted using X-ray crystallography on one of the largest noble gases, Xe, reports that hydrophobicity and the volume of gas delivered are the primary factors in determining gas–protein binding, occurring via weak London dispersion forces [76]. London dispersion forces, a quantum component of van der Waals interactions, are weak forces that describe a temporary intermolecular attraction between atoms. Such atoms are normally electrically symmetric, resulting in the formation of transitory dipoles in non-polar molecules [77].

Similar to H₂, noble gases have been deemed inert in biological systems due to having filled electron orbitals, meaning they cannot partake in electron exchanges. However, numerous studies show that Ar [78,79], Kr [80], Xe [81,82] and helium (He) [83,84] can have distinct physiological effects. However, how these effects are initiated is yet to be fully understood. Below, the possibility of direct protein/gas interactions is explored.

Studies of metmyoglobin, the oxidized form of the haem-containing protein myoglobin, derived from whale spermatic fluid, observed four discrete ¹²⁹Xe binding sites within the protein [85]. Each ¹²⁹Xe-binding intra-protein channel was situated antithetically from the O₂ binding site adjacent to the prosthetic iron. Two channels by which both O₂ and Xe pass through the protein to reach their respective binding sites have been identified in the syncopated haemoglobin of *Mycobacterium tuberculosis* [86]. Atomistic simulations of dioxygen dynamics recognised Channel 1 as serving as an entry channel for these molecules and nitric oxide (NO), whilst Channel 2 was primarily utilised as an exit passage, although this section was noted to be bidirectional. The authors also demonstrate that O₂ utilises Xenon pockets Xe1a and Xe2 as it traverses through the cavity to the haem interface and docking site (DS2/active site). Interestingly, NO was noted to occupy only the Xe2 site, which may have further effects, conceivably through inhibiting O₂ release through Channel 2.

It was the consideration of the type of research above that allowed the suggestion that H₂, being relatively inert, may act in its interaction with proteins in a manner consistent with the noble and hence inert gases such as Xe [47]. However, another consideration that should be accounted for when assessing the non-chemical effects of protein/molecule interactions includes the size and weight of atoms and molecules, as such physical attributes are likely to have key roles in the effects noted. Examples of these data are summarised in Table 7.

Table 7. The mass and diameter of gaseous substances, noting physical properties, atomic mass and kinetic diameters of biomolecules.

Substance	Chemical Symbol	Molecular Mass (g/mol)	Kinetic Diameter (nm)	Reference
Argon	Ar	40	0.34	[87]
Carbon dioxide	CO ₂	44	0.33	[88]
Carbon monoxide	CO	28	0.37	[89]
Helium	He	4	0.26	[89]
Hydrogen	H ₂	2	0.28	[88]
Krypton	Kr	84	0.36	[87]
Neon	Ne	20	0.28	[87]
Nitrogen	N ₂	28	0.36	[90]
Nitric oxide	NO	30	0.32	[89]
Oxygen	O ₂	32	0.35	[88]
Xenon	Xe	131	0.40	[87]
Water	H ₂ O	18	0.20	[91]

As can be seen, Xe, which is known to have physiological effects including as an anaesthetic [92], magnetoreceptive and neuroprotective agent [82] is considerably larger than H₂, which has been mooted to have a similar mode of action, i.e., using Xe pockets in proteins. However, H₂ is not dissimilar to He, which was suggested to be a therapeutic gas almost 100 years ago [93] and has since been studied for its biological effects [94]. Therefore, this mode of action of H₂ would no doubt be worthy of further investigation, as it would for a range of small—some gaseous—signalling molecules [48]. If such gases as H₂ are interacting with hydrophobic pockets in proteins such as haemoglobin and myoglobin—the model proteins for studying such effects—is this affecting O₂ transport in animals? Certainly, increased O₂ saturation, H₂ effects and disease models have all been considered together [62]. Is this one of the ways H₂ is having biological effects, and could it be mediated by alterations in O₂ metabolism?

6. Future Perspectives and Conclusions

Both O₂ and H₂ were discovered in the 18th century, and early work by a group of eminent scientists such as Antoine Lavoisier, Joseph Priestley, Thomas Beddoes and Humphry Davy were soon examining the biological effects of these and other gases, such as nitrous oxide (discussed by Hancock and LeBaron [7]). Oxygen has become inexorably studied as part of aerobic life, as instrumental to photosynthesis, as well as being linked to host defence in both plants and animals. Lack of O₂ conversion into ROS leads to chronic granulomatous disease in humans, for example [95].

Relatively recently, H₂ has been mooted as a medical therapeutic [96] and as a useful treatment for plants in agriculture [97], and since the paper by Ohta's group in 2007 [9] there has been a resurgence in interest in the biological effects of H₂ gas.

However, there are relatively few works in the literature considering O₂ and H₂ together, and how the actions of each of these gases may interact, or even interfere, with each other. Both gases have been used in solution, and sometimes together as an oxyhydrogen mix, but it is not always considered if the dissolving of one gas alters the measurable concentration of the other gas in solution. H₂ appears to reduce O₂ in solution, and both gases have relatively short half-lives of elevated concentrations in water. This is rarely considered, and such solutions need to be used in a timeframe well before their predicted half-lives of elevated concentrations.

H₂ is thought to have its action, at least in part, by scavenging •OH, and a mechanism to account for this is mediated by H₂ interactions with haem. However, it is known that O₂ also interacts with many haem proteins, including that of the globin family and the ROS-generating NADPH oxidase. Therefore, what is the interaction of H₂ and O₂ here? Does one affect the action of the other? If it does, does this account for the effects seen by H₂ treatments?

Furthermore, H₂ can be considered as an inert gas, and as such does it alter protein function in a manner commensurate with the other inert gases, such as Xe and Ar? Here, the model proteins used for research are the globins, which are so instrumental in oxygen transport and storage. Plants, too, have globins [98]. It has already been suggested that there is an interaction between globins, oxygen and an important signalling gas, that is nitric oxide (NO). Could H₂ be involved here too?

The exact action of H₂ is not well known. The scavenging action of •OH is often suggested to be the mode of action, but several other mechanisms have been suggested, and some of these would impinge on O₂ metabolism. It is suggested here that the interplay between H₂ and O₂ should be further considered as H₂ gas research on biological systems carries on in the future.

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