On the Physiology of Hydrogen Diving and Its Implication for Hydrogen Biochemical Decompression

by

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B.Sc. Hawaii Pacific University, 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario Canada

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The undersigned hereby recommend to the Faculty of Graduate Studies and Research acceptance of this thesis,

“On the Physiology of Hydrogen Diving and Its Implication for Hydrogen Biochemical Decompression”

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in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract:

Biochemical decompression, a novel approach for decreasing decompression sickness (DCS) risk by increasing the tissue washout rate of the inert gas, was tested in pigs during simulated H₂ dives. Since there is only limited physiological data on the use of H₂ as a diving gas, direct calorimetry and respirometry were used to determine whether physiological responses to hyperbaric H₂ and He are different in guinea pigs. The data suggested that responses in hyperbaric heliox and hydrox cannot be explained solely by the thermal properties of the two gas mixtures. To increase the washout rate of H₂, a H₂-metabolizing microbe (Methanobrevibacter smithii) was tested that converts H₂ to H₂O and CH₄. Using pigs (Sus scrofa) comparisons were made between untreated controls, saline-injected controls, and animals injected with M. smithii into the large intestine. To simulate a H₂ dive, pigs were placed in a dry hyperbaric chamber and compressed to different pressures (22.3-25.7 atm) for times of 30-1440 min. Subsequently, pigs were decompressed to 11 atm at varying rates (0.45-1.80 atm • min⁻¹), and observed for severe symptoms of DCS for 1 h. Chamber gases (O₂, N₂, He, H₂, CH₄) were monitored using gas chromatography throughout the dive. Release of CH₄ in untreated pigs indicated that H₂ was being metabolized by native intestinal microbes and results indicated that native H₂-metabolizing microbes may provide some protection against DCS following hyperbaric H₂ exposure. M. smithii injection further enhanced CH₄ output and lowered DCS incidence. A probabilistic model estimated the effect of H₂-metabolism on the probability of DCS, P(DCS), after hyperbaric H₂ exposure. The data set included varying compression and decompression sequences for controls and animals with intestinal injections of H₂ metabolizing microbes. The model showed that
increasing total activity of *M. smithii* injected into the animals reduced P(DCS). Reducing the tissue concentration of the inert gas significantly reduced the risk of DCS in pigs, further supporting the hypothesis that DCS is primarily caused by elevated tissue inert gas tension. The data provide promising evidence for the development of biochemical decompression as an aid to human diving.
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Sist men inte minst, tusen tack till min familj som har stött mig hela vägen. Detta arbete är tillägnat Kurt.
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List of Abbreviations

A - parameter for BUG

atm - atmosphere absolute, pressure unit equal to 1.01 bar or 0.1 MPa

BUG - variable for INJ or \( \sqrt{CH_4} \)

C - control

DCS - Decompression Sickness

G - scaling factor

\( h_c \) - heat transfer coefficient

HPNS - high pressure nervous syndrome

INJ - total \( H_2 \) metabolizing activity injected

M - metabolic heat production rate

\( P(DCS) \) - Probability of DCS

P - pressure, measured in atm

\( P_{\text{alveolar}} \) - alveolar partial pressure

\( P_{\text{amb}} \) - ambient pressure

\( P_{\text{blood}} \) - arterial inert gas tension

\( P_{\text{H}_2} \) - water vapour pressure

PFC - perfluorocarbon

\( P_{\text{tissue}} \) - tissue tension

\( P_{\text{tissue}} H_2 \) - Hydrogen tissue tension

\( P_{\text{tissue}} He \) - Helium tissue tension

\( Q_C \) - heat losses by convection across body surfaces

\( Q_{\text{CD}} \) - heat loss by conduction
$Q_S$ - total heat loss rate

$Q_{EVAP}$ - heat loss by evaporation

$Q_R$ - heat loss by radiation

$Q_{RC}$ - heat loss by convection by respiration

$Q_{RE}$ - heat loss due to evaporation from the respiratory tract

$Q_{SE}$ - heat loss due to evaporation from the skin

$D$ - dilution factor

$r$ - risk function

$S$ - storage of body heat

$SC$ - surgical control

$STP$ - standard temperature (0°C) and pressure (1 atm)

$T$ - animal injected with $H_2$ metabolizing microbes

$t$ - time

$\tau$ - time constant

$T1$ - last time at which diver was definitely free of signs of DCS

$T2$ - time at which definite signs of DCS occurred

$T_{amb}$ - ambient temperature

$T_{CH}$ - chamber temperature

$Thr$ - Threshold parameter

$Tend$ - time for end of observation period

$UC$ - untreated control

$V$ - volume
\( v \) - flow rate

\( \dot{V}_{CH_4} \) - methane release rate

\( \dot{V}_{O_2} \) - oxygen consumption rate

Wt - weight

Z - transformation of fraction towards new steady state
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CHAPTER ONE

GENERAL INTRODUCTION

“My body and my spirit are ready.  
My mind is focused.  
I can only see my objective:  
to conquer the great blue.”

Frascisco Ferreras “Pipin”
Underwater exploration involves stressful exposure to elevated pressures, and the return to the normobaric environment has been recognized as one of the most dangerous parts of diving due to the risk of the so-called “bends,” or decompression sickness (DCS). DCS is not only limited to diving, but is also a problem for caisson workers and during aerospace flights, or any other exposure to changing atmospheric pressures. For free diving marine mammals, physiological adaptations such as atelactic lungs are part of the solution that enable them to perform repetitive dives without apparent problems. However, these animals live with elevated inert gas tissue tension that would initiate DCS in humans, and most of the adaptations how these animals avoid DCS are still unknown (Kooyman, 1989; Kooyman and Ponganis, 1999).

During exposure to elevated pressure, increased levels of gas dissolve in the tissues of air-breathing animals. The amount of gas dissolved in the tissues is a function of the pressure and duration of the changed pressure. The tissue tension of dissolved gas continues to increase until equilibrium with the environment occurs, at which time the organism is said to be saturated. Oxygen, which is utilized and exhaled as CO₂, poses little threat to the diver. It has been shown that O₂ can contribute to DCS during rapid decompressions (Lillo, 1988; Lillo and MacCallum, 1991; Parker, et al., 1998). However, O₂ is not considered a potent gas for DCS in recreational divers since, under most normal circumstances, decompressions are slow and allow sufficient time for the O₂ to be metabolized. In contrast, inert gases such as He, N₂, and H₂ accumulate in the tissues, resulting in elevated concentrations after a hyperbaric exposure. If the hyperbaric exposure is long enough, the elevated levels will exceed the solubilities of the individual gases during the decompression phase, a term called supersaturation. Some level of
supersaturation may be tolerated, but current research suggests that any hyperbaric exposure has a finite probability of DCS (Weathersby, et al., 1984; Weathersby, et al., 1992). When the gases become supersaturated in the tissues, they begin to come out of solution. Decompression sickness is believed to be caused by an excessively rapid pressure decrease leading to the evolution of gas bubbles.

The bubbles may form in all tissues, but in certain critical tissues such as the central nervous system (CNS), the damage can cause serious lesions. In the blood, the bubbles may embolize blood vessels leading to severe ischemic damage (Hills, 1977).

Even though the probability of DCS has been investigated for decades, it is not clear why there are differences in susceptibility to this problem between individual divers and between tissues. Haematological studies have given birth to the idea that the circulating bubbles might induce activation of the clotting cascade, leading to the formation of a free-floating fibrin clot that would cause an embolus (Boussuges, et al., 1998; Holland, 1969; Philp, 1974; Philp, et al., 1979; Philp, et al., 1972). In addition, activation of an acute immune response (Ersson, et al., 1998; Kayar, et al., 1997b; Stevens, et al., 1993; Ward, et al., 1990; Yamashita, et al., 1994) and the complement cascade (Stevens, et al., 1993; Ward, et al., 1986; Ward, et al., 1987; Ward, et al., 1990) have been proposed to be involved in the development of DCS. These effects may help explain some of the variability in DCS susceptibility between individuals and why symptoms may develop several hours after return to the surface. Nevertheless, it seems that the level of supersaturation is the primary factor that triggers DCS, since longer stays at great pressures with rapid ascent are more likely to give rise to DCS than are short stays at lower pressures with slower ascents (Hills, 1977; Weathersby, et al., 1992).
Therefore, if the tissue inert gas tension could be reduced by metabolism of the gas or more efficient removal during the decompression phase, it seems reasonable to suggest that the risk for DCS should also decrease.

**Gases used in diving**

Air is commonly used as a breathing gas for recreational and shallow dives (Table 1.1). For dives exceeding 40 meters, N₂ has a narcotic effect that increases with the pressure (Bennett, 1989). To avoid this problem, He is commonly substituted for N₂ during deep dives (Table 1.1). The rationale for substituting He for N₂ is based on the Meyer-Overton theory, which proposes that there is a correlation between the hydrophobicity of an anaesthetic and its narcotic potency. That is, increased lipid solubility increases the anesthetic effects. According to this theory, He which has roughly one-fourth the lipid solubility of N₂, would be 4 times less narcotic than N₂ (Bennett, 1989). However, the low narcotic potency of He allows the expression of High Pressure Nervous Syndrome (HPNS), a condition marked by tremor, nausea, and sometimes convulsions and death at depths beyond 200 meters (21 atm, Jain, 1994). According to Hallenbeck (Hallenbeck, 1981), it is generally thought that HPNS originates from perturbations of excitable membranes with pressure changes, either through direct effects on the resting- and action-potential, or through secondary effects by affecting synaptic transmission or neurotransmitters.

For dives exceeding 100 meters, H₂ is currently being investigated as an alternative inert gas, and research in Europe has been quite successful (Imbert, 1989, Table 1.1). There are some disadvantages with H₂ as a diving gas; the most obvious
being its flammability. However, H\textsubscript{2} is not explosive if the O\textsubscript{2} concentration of the gas mixture is 4% or less (Lindén, 1985). Another potential problem with H\textsubscript{2} as a diving gas is the higher thermal conductivity and heat capacity of H\textsubscript{2} compared to He which suggests that heat losses in hyperbaric H\textsubscript{2} will be unusually high (Kayar, \textit{et al.}, 1997a). In addition, the thermal conductivity of H\textsubscript{2}, and He are several times higher than N\textsubscript{2} (Lide, 1993-94), which seriously may restrict the use of these gases. Concern has also been raised that the exposure to hyperbaric H\textsubscript{2} could cause incorporation of H\textsubscript{2} into mammalian tissues by saturating lipids (Örnhagen, 1987). However, as early as 1789 it was shown that gaseous H\textsubscript{2} is not metabolized by mammalian tissues at 1 atm (Séguin and Lavoisier, 1789) and a study on tissue slices showed that H\textsubscript{2} was not metabolized or incorporated by mammalian tissues at pressures up to 50 atm (Kayar, \textit{et al.}, 1994).

There are several advantages to using H\textsubscript{2} as the inert component of a breathing gas. The density of H\textsubscript{2} is lower than that of He, and consequently H\textsubscript{2} is easier to ventilate at elevated pressures (Kayar and Parker, 1997). Hydrogen can also be easily made from H\textsubscript{2}O by hydrolysis, whereas He is expensive and sometimes difficult to obtain (Kayar and Parker, 1997). Hydrogen also has a slight narcotic potency that may help alleviate HPNS at extreme pressures (Abraini, \textit{et al.}, 1994; Bennett and Rostain, 1993; Kayar and Parker, 1997).

**Avoiding DCS**

Currently the only method of avoiding DCS is to carefully control the ascent according to published guidelines for dive depth, duration, and rate of decompression to avoid critical supersaturation. Nevertheless, this strategy is not always successful. Even
when the tables are followed, divers sometimes get DCS. It is possible to substitute more
O₂ in place of the inert gas (mainly N₂ in air). Since O₂ is metabolized, it poses little
threat for generating DCS symptoms if the decompression rate is kept low (Lillo and
MacCallum, 1991; Parker, et al., 1998). However, due to the toxic effect of O₂ at
pressures exceeding 1.6 atm (normobaric O₂ = 0.21 atm), it is not possible to completely
replace the inert gases with O₂ (Bennett and Rostain, 1993). Due to the poorly-
understood physiological responses and kinetics of inert gases, it seems that the process
of decompression is inherently dangerous. All diving scenarios could benefit if the
decompression could be made safer.

**Biochemical Decompression - An innovative method for safer decompression**

A novel approach for safer decompression from dives that employ H₂ as the inert
gas involves the use of H₂ metabolizing microbes (methanogens). The concept was first
postulated by Dr. Lutz Kiesow in 1963, then a scientist at the Naval Medical Research
Institute (NMRI) in Bethesda, MD (Kayar, personal communication). The theory was
based on the idea that the diver breathing H₂ would in some manner be supplied with a
hydrogenase. The hydrogenase would convert some of the dissolved H₂ into H₂O, which
would result in a reduction of the tissue H₂ tension (PₜᵢₛH₂). The reduced burden of
excess inert gas in the body could potentially reduce the incidence of DCS.

Research on DCS in humans (Weathersby, et al., 1986), pigs (Dromsky, et al.,
2000a; Kayar and Fahlman, 1999a), rats (Kayar, et al., 1998a; Lillo, et al., 1997), and
sheep (Ball, et al., 1999) suggest that removal of even a small fraction of inert gas will
have a surprisingly large impact on the DCS risk. It is easy to understand this when
observing the sigmoidal dose-response graphs on DCS outcome versus total saturation pressure for rats (Lillo, *et al*., 1997), and pigs (Dromsky, *et al*., 2000a), where a small change in the total saturation pressure (± 5%) has a great impact on the DCS incidence. This is analogous to the large effect on the hemoglobin O₂ saturation by a small shift in the PO₂.

**Experimental design**

For biochemical decompression to be useful for human diving, the hydrogenase must be situated in a highly specialized location. An electron acceptor must be located nearby and at abundant levels. Any end product must not cause a toxic or immunogenic response. Initial studies at NMRI used the colonic microbe *Methanobrevibacter smithii*, the rationale for using this species was two-fold. First, this methanogen is native to the human gut flora and is non-pathogenic (Miller, 1991; Miller, *et al*., 1982; Weaver, *et al*., 1989). Second, the hydrogenase used by this methanogen catalyses the reaction (Miller, 1991; Weaver, *et al*., 1989):

\[
4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \text{Eq. 1}
\]

Since CO₂ is 30 times more soluble in tissue fluids than H₂, and is in high concentrations in most animal tissues, it is an ideal electron acceptor (Kayar, 1999; Lango, 1996; Weathersby and Homer, 1980). The large intestine appeared to be the obvious location to place the methanogen, since this location is anaerobic, a requirement of *M. smithii*. The intestine is also highly perfused, which would guarantee that high levels of H₂ would be transported to this location. Furthermore, injection of a microbe into the intestine would
not cause a systemic immune response, as would, for example, injection into the bloodstream (Kayar, et al., 1997b).

Initial studies of this form of biochemical decompression used rats which had methanogens injected into their intestines (Kayar, et al., 1998a). *Methanobrevibacter smithii* has been reported to be native to the rat intestine (Lin and Miller, 1998), but the levels of methanogenesis were too small to be detected from the native intestinal microbes in the rat (Kayar, et al., 1998a). However, the study showed that the DCS incidence could be reduced by as much as 40% compared to control animals without apparent harmful effects on the rats by the microbes (Kayar, et al., 1998a). To determine whether biochemical decompression might be a viable concept for humans, the initial studies on rats required further investigation using a mammal that is more similar to humans in both its intestinal flora, its general metabolic make-up, and its physiological responses to hyperbaric pressure. The study undertaken in this thesis aims to test the hypothesis that H2 metabolism by a methanogen significantly reduces the DCS incidence in a pig model. Furthermore, a physiologically-based mathematical model will propose a mechanism by which placement of a H2 metabolizing microbe into the intestine is able to reduce the P_tisH2.

**Declaration of DCS in swine**

Studies of DCS are at a disadvantage due to the subjective elements of the diagnosis of the disease. This problem is intensified during animal studies, since only the most noticeable symptoms can be clearly detected (Ball, et al., 1999). However, animal research allows testing of compression and decompression sequences that are at such
high risk of severe symptoms that they are not ethically possible in humans. In addition, for this study, the animals were observed during a period of treadmill training, giving the observers the possibility to familiarize themselves with the behaviour of each animal. Throughout the study, only cases of severe DCS were considered for a positive declaration of DCS, such as neurological and cardiopulmonary symptoms.

Much experience exists in use of the pig as an animal model for DCS research, and past data suggest that this animal is a good model for human applications (Broome and Dick, 1996; Buttolph, et al., 1998; Dromsky, et al., 2000a; Dromsky, et al., 1999).

**Aims and objectives**

**Aim 1.** A mathematical analysis of whole body calorimetry data will compare thermoregulation during a hyperbaric exposure to hydrox (H\textsubscript{2}/O\textsubscript{2}) as compared to hyperbaric heliox (He/O\textsubscript{2}), the more commonly used inert gas mix for deep dives. This is important because only a limited number of studies have examined the physiological effects of hyperbaric H\textsubscript{2} on whole animals (Brauer, 1987; Kayar and Parker, 1997; Kayar, et al., 1998b; Kayar, et al., 1997a). For this study the guinea pig was chosen as the model animal due to its small size and placid nature.

**Aim 2.** The study will demonstrate that biochemical decompression is a viable concept for human use by testing if metabolism of H\textsubscript{2} will reduce the DCS incidence in a pig model. The pig was chosen due to its anatomical and physiological similarities to humans. Simulated H\textsubscript{2}-dives will be performed in a hyperbaric chamber (Fig. 1.1) in control pigs and pigs supplemented with H\textsubscript{2}-metabolizing microbes. The DCS incidence and the rate of H\textsubscript{2}-metabolism, in the form of CH\textsubscript{4} release (Eq. 1), will be compared.
between the two groups to determine if biochemical decompression is able to reduce the DCS incidence in a large animal model.

Aim 3. The physiological data will be used to create a probabilistic model to show the advantage of metabolism of the inert gas during the decompression. Such a model will be able to predict the advantage of biochemical decompression for hyperbaric H₂ exposures in general, and offers a predictive advantage over descriptive models. Furthermore, such a model can potentially be used to forecast the benefits of H₂ diving for humans.
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Gas Composition</th>
<th>Pressures (atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>O₂/N₂</td>
<td>&lt; 6</td>
</tr>
<tr>
<td>Heliox</td>
<td>O₂/He</td>
<td>6-40</td>
</tr>
<tr>
<td>Hydrox</td>
<td>O₂/H₂</td>
<td>10-60</td>
</tr>
</tbody>
</table>

Table 1.1. Name of common gas mixtures used for diving, their composition, and approximate pressures at which they are used.
Figure 1.1. The hyperbaric chamber used throughout this study. The chamber had an internal volume of ~ 5665 L (WSF Industries, Buffalo, NY) and was placed within a facility especially built for safe handling of high pressure H₂ (following NFPA Code 50A for H₂ at consumer sites).
CHAPTER TWO

CALORIMETRY AND RESPIROMETRY IN GUINEA PIGS

IN HYDROX AND HELIOX AT 10-60 ATM

“It is not every truth that recommends itself to the common sense.”

Thoreau
INTRODUCTION

Body heat loss is a serious problem in diving. For simulated human dives in a dry hyperbaric chamber, the major avenues for heat loss are due to convection and radiation, and the heat loss due to the former increases with the increased density of the gases.

Whereas helium (He) has been used extensively as a major component of a breathing gas for human diving, there is only a limited amount of data on the physiological and thermal effects of using hydrogen (H₂) during hyperbaria (Brauer, 1987). It was desired to determine if there are different physiological responses to hyperbaric hydrox (98% H₂, 2% O₂) versus heliox (98% He, 2% O₂). If so, can the differences be explained by the physical characteristics of the gases alone, or are there additional factors contributing to the total heat loss rate (Qᵣ) in hyperbaric heliox versus hydrox?

Hydrogen was first introduced as a diving gas in 1943 by Arne Zetterström of the Swedish Royal Navy (Lindén, 1985). Hydrogen has the advantage of being lighter than He and thus easier to ventilate with increasing pressure. In addition, at elevated pressures, H₂ has a narcotic effect that ameliorates symptoms of high pressure nervous syndrome (HPNS) in some subjects; however, it induces psychosis in others (Fructus, 1987). Disadvantages of H₂ as a diving gas include the flammability of some H₂/O₂ mixtures, and its higher thermal conductivity and heat capacity compared to He, which suggests that heat losses in hyperbaric H₂ can be unusually high.

Several studies have measured oxygen consumption rates (\(\dot{V}O₂\)) of animals in He mixtures at 1 atm (0.1 MPa). While some studies found a reduction in the \(\dot{V}O₂\) in rats
(Clarkson, et al., 1972), others measured an increase in the \( \dot{V}O_2 \) in mice in a 1-atm He environment (Cook, et al., 1951) compared to animals breathing normal air. It was suggested that the greater heat transfer coefficient \( (h_c) \) of He compared to air led to increased rates of heat loss in the helium environment and this caused an increase in the metabolic rate (Leon, 1960). Increasing the ambient temperature \( (T_a) \) at 1 atm, which decreased the driving force for heat loss, caused a decrease in the difference in the \( \dot{V}O_2 \) of rats in air versus He (Leon, 1960). Although there seems to be an effect of gas mixture on animal metabolic rate, many other reports of metabolic disturbances in studies with tissue slices (Maio, 1967), insects (Cook, 1950), microorganisms (Schreiner, et al., 1962), whole-animal studies in mammals (Clarkson, et al., 1972; Kayar and Parker, 1997; Kayar, et al., 1997a), and inhibition of enzyme activity \textit{in vitro} (Schreiner, 1968) suggest other possible mechanisms.

In this study, measurements were made of the \( \dot{V}O_2 \) by respirometry and the \( Q_c \) by direct calorimetry in guinea pigs at 10, 20, 40, and 60 atm in heliox and hydrox. In addition, a mathematical model was created for partitioning the various paths for heat loss in guinea pigs in 1-atm air and 10-60 atm hydrox and heliox.

METHODS

Animal protocol

Male guinea pigs, \textit{Cavia porcellus}, of the Hartley strain (\( n = 16 \), mean body weight \( = 822 \pm 113 \text{ g} \)) were housed in a professionally staffed animal care facility and had \textit{ad libitum} access to food and water before experiments. The animals were fed a standard guinea pig chow (Prolab, Agway Inc., Syracuse, NY) with crude protein \( > 18\% \), fat \( > 4\% \)
and fiber > 15%. All animals were used for calorimetry experiments at 1 atm in air and a subset (n = 12) was subsequently assigned randomly to one of two experimental groups: hydrox or heliox.

**Experimental procedure for direct calorimetry**

**Calorimeter setup**

Direct calorimetry was performed with a Seebeck envelope gradient layer calorimeter (Thermonetics Corp., San Diego, CA), with internal dimensions of 15 cm x 15 cm x 30 cm. The calorimeter was equipped with a system of ducts in the walls for holding water to increase the thermal inertia of the walls, but no further temperature regulation of the calorimeter was used in any experiment. Under ordinary circumstances, the water would be circulated through a temperature-controlled water bath. However, safety regulations in a hydrogen environment prohibited the use of a heating element or pump in the chamber.

Animals were placed inside a wire mesh cage of sufficient size for the animals to turn and walk (11 cm x 13 cm x 27 cm). The mesh cage was placed inside the calorimeter with a tray of sand underneath to collect urine and feces. The customary tray of oil, which is used to cover the urine and feces to prevent them from releasing water vapour, was not permitted in the dive chamber due to the combustibility of hydrocarbons.

The calorimeter was equipped with a temperature sensor (ΔT sensor) measuring the temperature difference between the incumbent and excurrent gas. This made it possible to account for heat lost by the animal due to warming of the gas flowing through the calorimeter. Calibration and correction factors for the density and molar heat capacity of
the gas mixtures were provided by the manufacturer of the ∆T sensor. A thermistor (#402YSI Co. Inc., Yellow Springs, OH) was used to record the ambient temperature inside the calorimeter.

The calorimeter door was sealed, leaving the only entry and exit for gases via the ports of the ∆T sensor. A constant stream of gas passed through the calorimeter. The gas flow rate was measured with a floating ball-type flow meter that had been calibrated using air, heliox, and hydrox. The \( \dot{V}O_2 \) of the animal was calculated from the difference between \( O_2 \) content of the incurrent and excurrent gas, and the gas flow rate, once steady state was attained. All incurrent gases used were dry, and the \( \dot{V}O_2 \) corrected to standard temperature and pressure dry (STPD). The rate of \( CO_2 \) production to \( \dot{V}O_2 \) was assumed to be 1.0, with an error of less than 2% in the predicted \( \dot{V}O_2 \) estimate if this ratio was as little as 0.7 (Kayar, et al., 1997). For the 1-atm air experiments, the \( O_2 \) content was measured using a paramagnetic oxygen analyzer (Servomex 540A, Sybron, Boston, MA). For the hyperbaric experiments, the chamber gases were analyzed with a gas chromatograph (Shimadzu GC-9A, Columbia, MD) to monitor the \( O_2 \), He, \( H_2 \), and \( N_2 \) content of the chamber and of the excurrent gas from the calorimeter.

Water vapour was scrubbed from the excurrent gas with an absorbent (anhydrous \( CaSO_4 \), Drierite®, W.A. Hammond Co., Xenia, OH) and the water vapour loss was estimated by weighing the Drierite canister before and after each experiment.

For hyperbaric experiments, the calorimeter was placed in a hyperbaric chamber (Bethlehem Corp., Bethlehem, PA), and the same chamber was used for all measurements. The facility housing the chamber was designed to comply with the safety
regulations for work with H₂, as described in detail elsewhere (Kayar and Parker, 1997; Kayar, et al., 1997a).

**Calibration of the calorimeter**

The calorimeter was calibrated in 1 atm air by placing a small heater inside the instrument. A regulated direct current power supply (BK Precision Model 1601, Maxtec International Corp., Chicago, IL) was used to generate power in the heater from 1 to 15 watts (W), with a random sequence to the step changes in power input. When the calibration was completed, average power output measured by the calorimeter was within 2% of the power input to the heater, with time to stable calorimeter reading of approximately 30 min. The calibration was repeated with the calorimeter placed in the dive chamber at 10, 20, 40, and 60 atm heliox. Average calorimeter output was within 3% of the power input from the heater, without the need to make corrections for pressure or thermal properties of the gas. However, the time to reach stable calorimeter readings was increased to 45-60 min, due to the increased time needed to stabilize the thermostatically controlled temperature inside the chamber. Safety regulations did not allow the calibration to be repeated in the presence of hydrogen, and it was therefore assumed that if no correction was necessary in heliox, this would also be the case for the hydrox mixture.

**Calorimeter measurements**

Guinea pigs (n = 16) in 1 atm air served as controls. The calorimeter was near room temperature, with a range of values between 19.3 and 34.0°C. Animals were placed in the calorimeter for 1 h and data were collected during the second half-hour
when the calorimeter output was constant. Animals were removed from the calorimeter for at least 1 h or up to a few days, before a replicate measurement was made. The repeated measurements were scheduled such that one was performed in the morning and one in the afternoon to determine any diurnal effects. A subset of 5 randomly selected animals from the control 1-atm air group (mean body weight = 836 ± 59 g) were kept in the calorimeter for 6 h continuously in 1 atm air. The extended exposure was set to 6 h since this was the approximate length of the hyperbaric experiments. The $Q_{\Sigma}$ and $\dot{V}O_2$ were measured for each hour to test for systematic temporal changes in heat output during extended confinement without food or water.

At a later date (1-89 days), 12 of the original 16 animals were used for the hyperbaric experiments, and these animals were randomly assigned to either the heliox group (n = 6, mean body weight = 894 ± 65 g) or the hydrox group (n = 6, mean body weight = 898 ± 79 g). The animals were placed in the calorimeter, which was put inside the hyperbaric chamber. The chamber was pressurized as described earlier for hydrox and heliox experiments (Kayar and Parker, 1997; Kayar, et al., 1997a) to 10, 20, 40, and 60 atm and will be briefly described here. In hydrox, the chamber was pressurized at 1-2 atm • min$^{-1}$ with pure He to 10 atm. Consequently, the $O_2$ concentration fell to 2%, but the $PO_2$ remained near 0.2 atm from the 1-atm air initially in the chamber. The initial pressurization using He was necessary to avoid explosive mixtures with $H_2$; nonexplosive limits for $H_2/O_2$ mixtures are 0-4% $O_2$ in $H_2$, or 0-4% $H_2$ in $O_2$ (Eichert and Fischer, 1986). The chamber was then flushed with a mixture of 2% $O_2$ in $H_2$ (hydrox) until the $N_2$ content of the chamber dropped to < 0.5%, and the He content was < 4%, as measured by a gas chromatograph (Shimadzu GC-9A, Columbia, MD). After ~1 h at 10 atm, the...
chamber was pressurized at 1-2 atm \textbullet\ min$^{-1}$ using hydrox to 20, 40 and 60 atm. Since the hydrox contained 2\% O$_2$, the PO$_2$ increased throughout the experiment to 0.4, 0.8, and 1.2 atm, respectively. Each pressure was maintained for $\sim$ 1 h to measure the heat output and \dot{V}O$_2$. Heat output from the animal was computed from a mean of the readings from the last 10-15 min of the 1 h spent at each pressure, when the calorimeter output was stable. If stable measurements had not been achieved in 45 min, the total time under pressure was prolonged up to 15 min. The dive profile for the animals in the He mixture (2\% O$_2$ in He, Heliox) was identical to that used for animals in hydrox. The chamber was initially pressurized to 10 atm using pure He. The chamber was then flushed using heliox until the N$_2$ content fell below 0.5\%. Animals spent $\sim$ 1 h at 10, 20, 40 and 60 atm in heliox (0.2, 0.4, 0.8, and 1.2 atm PO$_2$, respectively). The temperature of the chamber was the same for the two gases at each pressure (Table 2.1) and selected such that the animal could maintain a rectal temperature at or near 38.0 $\pm$ 0.5$^\circ$ C, as determined from an earlier experiment (Kayar and Parker, 1997). At equilibrium, the temperature difference between the inside and the outside of the calorimeter was usually not more than $\pm$ 0.5$^\circ$ C.

At the end of the experiment, animals were rapidly decompressed within 1-2 min to 10 atm and immediately euthanized using 2 atm CO$_2$. It was necessary at this point to euthanize the animals to prevent them from asphyxiating, since the chamber PO$_2$ dropped below 0.2 atm at chamber pressures < 10 atm. Death at 10 atm also prevented the animals from suffering from decompression sickness. After euthanasia the chamber was further depressurized and, in the case of hydrox dives, flushed with pure He for several minutes before opening to ensure safe elimination of H$_2$. 
Mathematical analysis

Model

The model of theoretical heat loss used the following heat balance equation:

\[ M = S + Q_{\Sigma} \quad \text{Eq. 1} \]

This equation states that the total heat loss rate \( Q_{\Sigma} \) plus the rate of storage of body heat \( S \) is equal to the metabolic heat production rate \( M \). Unless otherwise stated, the above thermal variables are expressed in watts (W). A negative sign for \( S \) indicates a net loss of heat from the animal’s body (i.e., heat loss exceeds heat production). The \( Q_{\Sigma} \) from the animal’s body is due to the sum of heat losses by convection across body surfaces \( (Q_C) \), convection by respiration \( (Q_{RC}) \), radiation \( (Q_R) \), conduction \( (Q_{CD}) \), and evaporation \( (Q_{EVAP}) \) from the skin \( (Q_{SE}) \) and from the respiratory tract \( (Q_{RE}) \).

\[ Q_{\Sigma} = Q_C + Q_{RC} + Q_R + Q_{EVAP} \quad \text{Eq. 2} \]

For this study, \( Q_{CD} \) (which depends on the heat loss due to direct contact with a surface) was assumed to be negligible based on the criteria described by Clarkson et al. (Clarkson, et al., 1972) and therefore excluded from Eq. 2. The \( Q_{SE} \) and the \( Q_{RE} \) were estimated together by measuring the total amount of water in the absorbent canister and converting to its evaporative energy equivalence \( (Q_{EVAP}, 2.4 \text{ kW} \cdot \text{s} \cdot \text{g}^{-1} \text{ at } 37^\circ \text{C}; \text{Lide, 1993-94}) \).

Convective heat loss was determined from techniques for estimating heat loss from a horizontal cylinder (Kreith and Bohn, 1997; McAdams, 1954). Following the criteria of Clark et al. (Clark, et al., 1981), the \( Q_C \) is characterized by natural convection, and predicted by:

\[ Q_C = h_c \cdot A \cdot (T_s - T_a) \quad \text{Eq. 3} \]
where $A$ is the body surface area (cm$^2$), $T_a$ and $T_s$ are the ambient and surface tempera
tures (°C), respectively. The term $h_c$ is the heat transfer coefficient
$(W \cdot m^{-2} \cdot °C^{-1})$ predicted by:

$$h_c = Nu \cdot \lambda \cdot L^{-1}$$  \hspace{1cm} \text{Eq. 4}$$

$Nu$ is Nusselt’s factor determined by $Nu = a \cdot (Gr \cdot Pr)^{0.25}$, where $a$ is a geometry-
dependent constant approximated to be 0.53 in this case in which the shape of a small mammal is approximated by a horizontal cylinder (Kreith and Bohn, 1997; McAdams, 1954), and $Gr$ and $Pr$ are the dimensionless Grashof and Prandtl numbers, respectively (McAdams, 1954). The thermal conductivity ($\lambda$, $W \cdot m^{-1} \cdot °C^{-1}$) was determined for each gas mixture and temperature according to equations for binary gas mixtures (Reid, et al., 1987). The shape factor ($L$, cm) was estimated as 1/3 of the animal’s volume (Mitchell, 1976). The latter was assumed to be equal to the animal’s weight divided by the density of mammalian muscle ($1.06 \cdot 10^{-3} \text{ g \cdot mm}^{-3}$, Kayar, et al., 1997a).

The $T_s$ (°C) was estimated using the following formula (Herrington, 1941):

$$T_s = 0.84 \cdot T_a + 8.64$$  \hspace{1cm} \text{Eq. 5}$$

where $T_a$ was measured by a thermistor located inside the calorimeter.

The body surface area ($A$, cm$^2$) was first estimated according to measurements in 1 atm air in guinea pigs (Herrington, 1941), in which:

$$A = 9 \cdot m^{2/3}$$  \hspace{1cm} \text{Eq. 6}$$

where $m$ is the animal’s weight (g). However, the body surface area available for heat loss, expressed as a percentage of total body surface area (Gonzalez, 1988) was adjusted such that the model would fit the measured values for heat loss in 1 atm air. The adjusted value or effective area for heat loss was assumed to be independent of
pressure and temperature, and the same value was used to estimate the $Q_\Sigma$ for the hyperbaric experiments.

Heat loss due to radiation was determined by the equation:

$$Q_R = A \cdot \varepsilon \cdot \sigma \cdot (T_s^4 - T_a^4), \quad \text{Eq. 7}$$

where $\sigma$ is Stefan Boltzmann’s constant ($5.67051 \times 10^{-8} \text{ W} \cdot \text{m}^{-2} \cdot \text{K}^{-4}$; Lide, 1993-94; Wissler, 1978) and the temperatures are in degrees Kelvin. The emissivity ($\varepsilon$), which is reported to vary between 0.95 and 1.00 for small mammals (Campbell, 1977), was set to unity for this study.

The portion of the convective heat exchange from the respiratory tract was determined by:

$$Q_{RC} = V_E \cdot \rho \cdot C_p \cdot (T_{ex} - T_{in}), \quad \text{Eq. 8}$$

where $V_E$ ($\text{cm}^3 \cdot \text{s}^{-1}$) is the respiratory minute volume for guinea pigs estimated by $V_E = \dot{V} \text{O}_2 \cdot 0.028$ (Blake and Banchero, 1985; Wissler, 1978), $\rho$ (mol $\cdot$ cm$^{-3}$) is the density of the gas mixture, $C_p$ (J $\cdot$ mol$^{-1} \cdot \circ C^{-1}$) is the heat capacity of the gas mixture, $T_{in}$ is the temperature of inhaled gas and assumed equal to $T_a$, and $T_{ex}$ is the temperature of the exhaled gas (Wissler, 1978). For estimating $T_{ex}$, equations for cats in 1 atm air and hyperbaric heliox were used (Naraki, 1983), which estimate the temperature of exhaled gas as:

$$T_{ex} = a + b \cdot T_{in} \quad \text{Eq. 9}$$

where $a = 15.8$ and $17.4$, and $b = 0.68$ and $0.56$ for 1 atm air and hyperbaric heliox, respectively. The calculation for hyperbaric hydrox used the same value as heliox.

The $Q_\Sigma$ was computed using the mathematical model (Eq. 2) assuming that the animals were in thermal equilibrium ($S = 0$). These values were compared to the
measured $Q_\Sigma$ from the calorimeter experiments. Other models explored here are various modifications to Eq. 2, as explained below.

**Statistical analysis**

All estimates of variance are reported as standard deviations, and all mean comparisons are based on 2-tailed t-tests with acceptance of differences at $P < 0.05$, unless stated otherwise. Group sample sizes are indicated in Tables 2.1 and 2.2.

**RESULTS**

**Calorimetry measurement of $Q_\Sigma$**

There was no systematic change in either the $Q_\Sigma$ ($P = 0.40$) or the $\dot{V}O_2$ ($P = 0.95$) with length of exposure in 1-atm air, as determined by repeated measures single factor ANOVA (Table 2.2). The $\dot{V}O_2$ and the $Q_\Sigma$ measurements for the 1-hour exposure in 1-atm air were not statistically different from the 6-hour exposure ($P > 0.62$; Table 2.1 and 2.2). Furthermore, there were no diurnal differences in the $\dot{V}O_2$ ($P = 0.83$) and the $Q_\Sigma$ ($P = 0.61$) measurements. Thus, any changes in $\dot{V}O_2$ or $Q_\Sigma$ in hyperbaria are not expected to be due to diurnal effects or the experimental duration.

During the hyperbaric exposure, there was an increase in the $\dot{V}O_2$ with pressure for animals in heliox but not in hydrox (Table 2.1), with a significantly higher $\dot{V}O_2$ in heliox at 60 atm ($P < 0.03$, 2 sided Mann-Whitney test) compared to hydrox. The $Q_\Sigma$ increased
with increasing pressure in heliox and at 10-40 atm in hydrox (Table 2.1). At 60 atm the \( Q_{\Sigma} \) was significantly higher in heliox compared to hydrox (\( P < 0.03 \)). The \( \dot{V}_{O_2}/Q_{\Sigma} \) ratio for the hydrox and heliox mixtures at 10 atm was close to the value in air, but the ratio decreased with increasing pressure in both gas mixtures (Table 2.1 and Fig. 2.1). At 40 atm, the ratio was significantly lower in hydrox (Table 2.1, \( P < 0.03 \), 2 sided Mann-Whitney test) than in heliox or 1-atm air. At all pressures in hydrox and at 20, 40, and 60 atm in heliox, the \( \dot{V}_{O_2}/Q_{\Sigma} \) ratio was out of the range of biological fuel sources (Fig. 2.1). This suggests that the animals were not in thermal equilibrium and lost stored heat (\( S \neq 0 \)). This speculation will be discussed in detail below.

**Mathematical model to estimate \( Q_{\Sigma} \)**

For estimating \( Q_{\Sigma} \) in 1 atm air, the model (Eq. 2) was evaluated in three separate trials, using one randomly chosen result for each animal from either the duplicate 1-hour trial at 1 atm, or the mean value of the 6-hour run (Model 0, Table 2.3). Model 0 used Eqs. 5 and 9 to estimate \( T_{s} \) and \( T_{ex} \), respectively. Using the assumption that the animals were in thermal equilibrium with their environment, i.e., \( M = Q_{\Sigma} \) and \( S = 0 \) (Eq. 1), the model predicted values for heat loss in 1 atm air that were similar to the measured values (\( P > 0.60 \) for all 3 trials, Table 2.3, Model 0).

The estimated \( Q_{\Sigma} \) from the hyperbaric experiments were as much as 211% higher than the measured values in both gas mixtures when using the same model as in 1 atm air (Model 0; data not shown). Since the deviation in the \( \dot{V}_{O_2}/Q_{\Sigma} \) ratio could potentially be attributed to the loss of stored heat (\( S \neq 0 \), Eq. 1, Fig. 2.1), it was speculated that \( S \neq 0 \) was the source of the failure of Model 0 to estimate \( Q_{\Sigma} \) in hyperbaria. Under this
assumption, the measured $\dot{V}O_2$ and $Q_2$ from the calorimeter was used to fit the equations to estimate new values for the $T_s$. Since an independent measurement was done on each animal at each pressure, this resulted in 4 independent values for each animal and experiment (Draper and Smith, 1981). These values were regressed against $T_a$ (Fig. 2.2) and found to be significantly lower in the hydrox mixture than in heliox at all pressures ($P < 0.05$, student t-test comparing slopes, Zar, 1996). The regression equations were used to estimate the $Q_2$ in heliox and hydrox at elevated pressures (Model 1 and Model 2, Table 2.3).

The $Q_C$ and $Q_{RC}$ were predicted to account for 63-71% and 4-15% of the total heat loss, respectively. In both gas mixtures, these contributions tended to increase with pressure at 10-60 atm (Table 2.3, Model 1.0, and 2.0). The contribution to the $Q_2$ from $Q_R$ and $Q_{EVAP}$, on the other hand, tended to decrease with increasing pressure and were approximately 23-9% and 10-6%, respectively, at 10-60 atm (Table 2.3, Model 1.0, and 2.0).

DISCUSSION

The average $\dot{V}O_2$ measured in air at 1 atm was similar to values reported in other studies with guinea pigs (Blake and Banchero, 1985; Herrington, 1941; Kayar, et al., 1997a). For the hyperbaric experiments, the $\dot{V}O_2$ measured in this study were compared with the values measured by Kayar et al. (Kayar, et al., 1997a) at 10-60 atm in guinea pigs in hydrox and heliox. The $\dot{V}O_2$ values in the earlier study were higher by 28-46% and 30-73% in heliox and hydrox, respectively. This disparity can be attributed to
methodological differences between the experiments. The lower \( \dot{VO}_2 \) of the animals in the present study might be due to their calmer state during confinement in a dark calorimeter, and without the use of an invasive rectal probe as in the earlier study.

When animals are in thermal equilibrium (\( S = 0, \text{Eq. 1} \)), the \( \dot{VO}_2/Q_\Sigma \) (mL O\(_2\) \cdot s\(^{-1}\) \cdot W\(^{-1}\) STPD) ratio has been shown to vary within narrow limits depending on the fuel source utilized (Dejours, 1975). Thus, by referring to the energetic equivalents of O\(_2\) for a pure glucose substrate (0.0469 mL O\(_2\) \cdot s\(^{-1}\) \cdot W\(^{-1}\)) and a pure palmitic acid substrate (0.0531, mL O\(_2\) \cdot s\(^{-1}\) \cdot W\(^{-1}\)) an estimate of the fuel source used during the calorimeter experiment could be obtained (Dejours, 1975). The stable \( \dot{VO}_2/Q_\Sigma \) ratio in 1-atm air of 0.049 ± 0.0037 mL O\(_2\) \cdot s\(^{-1}\) \cdot W\(^{-1}\) (Fig. 2.1) suggested aerobic metabolism (Hardewig, et al., 1991), using a fuel source with an energetic equivalent between protein and glucose (Dejours, 1975). An earlier study (Herrington, 1941) reported similar fuel sources utilized when measuring the RQ value of guinea pigs during a 6-hour confinement, suggesting that the present method of using the \( \dot{VO}_2/Q_\Sigma \) ratio gave reliable results.

However, in the hyperbaric experiments the measured \( \dot{VO}_2/Q_\Sigma \) ratio was much lower than could be accounted for by changing fuel sources (Fig. 2.1). The most likely reason for the deviation is that the animals were not in thermal equilibrium (\( S \neq 0, \text{Eq. 1} \)). This explanation also describes why Model 0 (data not shown) failed to estimate the \( Q_\Sigma \) in animals in hyperbaria. Following this speculation, the \( T_s \) for animals in hyperbaric heliox and hydrox was adjusted (Model 1 and 2, Table 2.3).

When computing the theoretical heat loss in 1 atm air using the mathematical model, the method of estimating surface temperature (\( T_s \)) by Herrington (Eq. 5;
Herrington, 1941) to calculate $Q_C$ and $Q_R$ was used. The theoretical $Q_\Sigma$ values for the hyperbaric experiments were considerably larger than the measured values, and it was speculated that Eq. 5 was not valid for the hyperbaric experiments. Consequently, the actual $Q_\Sigma$ and $\dot{V}O_2$ were used to fit the model (Fig. 2.2), which made it possible to estimate $T_s$ for each animal. The estimate assumes that all factors except $T_s$ remain constant at elevated pressures and temperatures. This is probably an oversimplification since a change in the body posture, and thus the area available for heat loss (Eq. 3 and Eq. 7), or a change in the $T_{ex}$ (Eq. 8) might alter this value. Nevertheless, the estimated values for $T_s$ were similar to the measured values at 1-60 atm in cats in heliox (Naraki, 1983). Since $T_s$ has been shown to vary linearly with $T_a$ in small mammals (Gonzalez, et al., 1971), $T_s$ was regressed on $T_a$ and the resulting equations were used to estimate the $T_s$ (Fig. 2.2) for estimation of $Q_\Sigma$ in hyperbaria (Model 1 and 2, Table 2.3).

An earlier study showed that the $T_{ex}$ in humans is approximately 2°C lower in hydrox than in heliox at 21 atm, at a $T_a$ range of 10-31°C (Burnet, et al., 1990). To estimate this effect on the model, the $Q_\Sigma$ and $Q_{RC}$ were recalculated for hydrox by lowering the $T_{ex}$ by 1° (Model 2.1) and 2° (Model 2.2) compared to heliox (Table 2.3). Animals with a 2° C lower $T_{ex}$ in hydrox than in heliox (Table 2.3, Model 2.2) had a predicted heat loss from $Q_{RC}$ that represented only a 1.3-2.7% contribution to the $Q_\Sigma$ at 10-40 atm, and a predicted heat gain at 60 atm (negative $Q_{RC}$, Model 2.2, Table 2.3). It is unlikely for the animals to be losing peripheral heat while gaining heat from respiration (Table 2.3, Model 2.2). Humans have been reported to have a loss due to $Q_{RC}$ of 10% at 30.8 atm in heliox (Timbal, et al., 1974), which is better approximated by the hydrox models 2.0 or 2.1 (Table 2.3). Thus, it appears from these calculations that the $T_{ex}$ is
probably the same or only 1° lower in hydrox than for animals breathing heliox (i.e., Models 2.0 and 2.1 are more realistic than Model 2.2).

Upon appropriate adjustment, the model was able to estimate $Q_\Sigma$ values in both gas mixtures that were not significantly different from the values measured by direct calorimetry. Furthermore, the predictions made by the model for the various sources of heat loss in guinea pigs (Table 2.3) corresponded relatively well with the predictions made for humans in heliox mixtures at 0-150 atm (Flynn, et al., 1974). Additionally, it appears that the model (Eq. 2) is relatively stable when using a range of realistic values for $a$ (Eq. 4), $\varepsilon$ (Eq. 7), and the percent of total surface area available for heat loss (Eqs. 3 and 7; see Appendix for sensitivity test). The value for $Q_{EVAP}$ was probably overestimated since some of the water vapour could have been derived from evaporation of the urine and feces collected in the sand. The only variable with a large impact on the model was $T_s$; using a plausible range of values for $T_s$ (Eq. 5) caused a large change in the $Q_\Sigma$.

The $Q_\Sigma$ values at 10, 20, and 40 atm were between 7 and 9% higher in hydrox compared to heliox (Table 2.1), but the difference was not statistically significant ($P > 0.2$). At 60 atm the heat loss was significantly higher in heliox than in hydrox ($P < 0.03$). To investigate this, an evaluation of the model was performed (Eq. 2) to determine the expected difference in $Q_\Sigma$ between hydrox and heliox, based strictly on the thermal properties (TP) of the two gas mixtures (Table 2.4). This approach indicated that $Q_\Sigma$ in hydrox should be 21-25% higher compared to heliox at all pressures (TP $Q_\Sigma$, Table 2.4). Thus, the thermoregulatory responses to hydrox as compared to heliox at elevated
pressures appear to be different from those predicted solely on the basis of the thermal properties of the gases.

Earlier studies showed that there are different physiological responses in guinea pigs to hyperbaric heliox and hydrox (Kayar and Parker, 1997; Kayar, et al., 1998b; Kayar, et al., 1997a). It was suggested that the differences could be due partly to HPNS in animals breathing hyperbaric heliox, while the hydrox animals could be narcotized. In hyperbaric heliox, there was a positive correlation between $\dot{V}O_2$ and $Q_c$ with pressure (Table 2.1, $P < 0.03$). From 40-60 atm there was a large increase in the $Q_c$ that might indicate that the animals in heliox were suffering from HPNS, with increased muscular activity leading to increased convective heat loss. In these experiments, the animals were confined to a calorimeter and could therefore not be observed. However, during earlier experiments with guinea pigs at 40-60 atm in heliox, animals were clearly seen to be agitated and had tremors consistent with a diagnosis of HPNS (Kayar, et al., 1997a). In hydrox, on the other hand, there was no simple correlation between $\dot{V}O_2$ and $Q_c$ with pressure (Table 2.1). Hydrogen has anesthetic and narcotic properties at elevated pressures (Fructus, 1987). Anesthesia and narcosis are known to cause a lower body temperature ($T_b$), loss of perception of cold, and a lower $T_b$ before the onset of thermogenesis (Crawshaw, et al., 1992). Hydrogen narcosis could explain why the animals in hydrox did not defend the increasing $Q_c$ by an increase in their $\dot{V}O_2$ at 60 atm and therefore lost stored heat.

Hydrogen has been shown to be biologically inert in isolated tissues at 1-50 atm (Kayar, et al., 1994) and in intact multicellular animals at 1 atm (Séguin and Lavoisier, 1789). However, whether gaseous $H_2$ is biologically inert in intact animals in hyperbaria...
is unknown. If metabolism of hydrogen occurred at elevated pressures in the mammalian body, this was expected to be detected as a deviation in the $\dot{V}O_2/Q_S$ ratio from the value in 1 atm air in the direction of more heat released than could be attributed to aerobic metabolism. In heliox, on the other hand, no change in the $\dot{V}O_2/Q_S$ ratio would be expected since helium is believed to be completely biologically inert (Leon, 1960). The substantial decrease in the $\dot{V}O_2/Q_S$ ratio in both gases made it impossible to be conclusive regarding $H_2$ metabolism. The simplest explanation for the decrease in the $\dot{V}O_2/Q_S$ ratio in both gas mixtures is that the $\dot{V}O_2/Q_S$ ratio can be used to suggest metabolic pathways only when an animal is in thermal equilibrium, with no loss or gain of stored heat, a condition not attained in the hyperbaric animals. However, the possibility that animals in hydrox received a contribution of heat from $H_2$ metabolism by microbes resident in the intestine cannot be excluded (Kayar and Fahlman, 1999).

Non-shivering thermogenesis has been reported in neonatal guinea pigs as a mechanism for defending core temperatures during absences of maternal heat, but in the adult animal, shivering thermogenesis replaces non-shivering thermogenesis (Hill, 1976). In this study adult guinea pigs was used, and as a consequence of that, heat production from brown adipose tissue was therefore not further considered.

Not surprisingly, it was found that the major avenue for $Q_S$ in hyperbaric heliox and hydrox is through convection ($Q_C$ and $Q_{RC}$, Eq. 2). Since $Q_C$ and $Q_{RC}$ are exceedingly dependent on the difference between the $T_a$ and $T_s$ (Table 2.5), one would expect that animals in hyperbaria would regulate the latter to minimize the density-induced increase in $Q_S$ at elevated pressures. This could be done by the commonly found regulatory
vasoconstriction of peripheral appendages, reducing heat loss from exposed areas and with an overall lowering of the $T_s$ (Schmidt-Nielsen, 1990). This was supported by the model, which predicted that animals in hyperbaria more strictly regulated their $T_s$ to minimize the gradient for heat loss than did animals in 1 atm air (Fig. 2.2). The finding that animals in hyperbaria had increasing difficulties with maintaining a normal core temperature (i.e., $S < 0$), as suggested by the decreasing $\dot{V}O_2/Q_s$ ratio, supports the conclusion of others who state that thermal failure in human divers is most likely due to insufficient heating of the chamber (Flynn, et al., 1974). Thus, it is predicted that human divers in hydrox will find that the usual strategies of adjusting environmental temperature and layers of clothing will suffice to maintain stable core temperature. Indeed, given the additional problems of HPNS and narcosis at high pressures, human divers at 60 atm may find that hydrox is not as thermally stressful as heliox.

In this study, I wanted to determine if physiological responses to hyperbaric heliox and hydrox could be explained based on their thermal properties alone. A model was created that predicted the $Q_s$ at similar $T_s$ to be approximately 20% higher in hydrox compared to heliox at 10-60 atm, based solely on the thermal properties of the gas mixtures. Total heat loss rates were measured by direct calorimetry and showed that the $Q_s$ was no more than 9% higher at 10-40 atm, and was 17% lower at 60 atm in hydrox than in heliox. It is suggest that the elevated $Q_s$ of animals in hyperbaric heliox is in part due to HPNS, whereas animals in hyperbaric hydrox allow surface temperature to fall to reduce $Q_s$. Finally, it is concluded that the physiological responses in hyperbaric heliox and hydrox cannot be explained solely by the thermal properties of the two gas mixtures.
Table 2.1. Mean (± 1 stdev) oxygen consumption rate (\(\dot{V}O_2\)), mean total heat loss rate (Q\(_\Sigma\)), and average calorimeter temperature (T\(_a\)), for guinea pigs in heliox (n = 6) and hydrox (n = 6) at 10-60 atm, and in air (n = 16) at 1 atm, as measured by respirometry and calorimetry.

<table>
<thead>
<tr>
<th>Gas</th>
<th>Pressure (atm)</th>
<th>(\dot{V}O_2) (mL O(_2) · g(^{-1}) · h(^{-1}))</th>
<th>Q(_\Sigma) (W)</th>
<th>T(_a) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>1</td>
<td>0.720 ± 0.093</td>
<td>3.31 ± 0.32</td>
<td>26.5 ± 3.1</td>
</tr>
<tr>
<td>Heliox</td>
<td>10</td>
<td>0.764 ± 0.077</td>
<td>3.81 ± 0.31</td>
<td>32.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.792 ± 0.063</td>
<td>4.43 ± 0.52</td>
<td>33.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.879 ± 0.126</td>
<td>5.08 ± 0.61</td>
<td>34.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.033 ± 0.154*</td>
<td>6.51 ± 0.72*</td>
<td>35.1 ± 0.5</td>
</tr>
<tr>
<td>Hydrox</td>
<td>10</td>
<td>0.713 ± 0.102</td>
<td>4.07 ± 0.48</td>
<td>32.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.832 ± 0.199</td>
<td>4.75 ± 0.89</td>
<td>33.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.776 ± 0.040</td>
<td>5.57 ± 0.62</td>
<td>34.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.800 ± 0.048</td>
<td>5.40 ± 0.82</td>
<td>35.3 ± 0.6</td>
</tr>
</tbody>
</table>

*Indicates a statistically different value (P < 0.05) between hydrox and heliox at the specified pressure.
Table 2.2. Mean (± 1 stdev) of average heat loss rate ($Q_\Sigma$), oxygen consumption rate ($\dot{\text{V}} O_2$), and calorimeter temperature ($T_a$) for guinea pigs (n = 5) in air at 1 atm during a 6h enclosure in a calorimeter.

<table>
<thead>
<tr>
<th>Period (h)</th>
<th>$Q_\Sigma$ (W)</th>
<th>$\dot{\text{V}} O_2$ (mL O$_2$·g$^{-1}$·h$^{-1}$)</th>
<th>$T_a$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>3.65 ± 0.67</td>
<td>0.758 ± 0.124</td>
<td>23.4 ± 1.0</td>
</tr>
<tr>
<td>1-2</td>
<td>3.27 ± 0.54</td>
<td>0.748 ± 0.102</td>
<td>24.2 ± 0.9</td>
</tr>
<tr>
<td>2-3</td>
<td>3.07 ± 0.46</td>
<td>0.720 ± 0.084</td>
<td>24.9 ± 0.8</td>
</tr>
<tr>
<td>3-4</td>
<td>3.13 ± 0.37</td>
<td>0.731 ± 0.103</td>
<td>25.2 ± 0.8</td>
</tr>
<tr>
<td>4-5</td>
<td>3.10 ± 0.38</td>
<td>0.701 ± 0.082</td>
<td>25.4 ± 0.9</td>
</tr>
<tr>
<td>5-6</td>
<td>3.15 ± 0.30</td>
<td>0.734 ± 0.094</td>
<td>25.6 ± 0.9</td>
</tr>
<tr>
<td>Overall</td>
<td>3.23 ± 0.48</td>
<td>0.733 ± 0.094</td>
<td>24.8 ± 1.1</td>
</tr>
<tr>
<td>$P$</td>
<td>0.40</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Mean (± 1 stdev) heat loss rate ($Q_\Sigma$) from direct calorimetry (actual), estimated by a mathematical model (Eq. 2, theoretical), and percent $Q_\Sigma$ partitioned into various avenues of heat loss as a percentage of $Q_\Sigma$ (Eq. 2).

Model 0 (n=16) is specific for 1 atm air and uses Eq. 5 and 9 to estimate the surface ($T_s$) and exhaled ($T_{ex}$) temperatures. Model 1 (n=6) and 2 (n=6) are for hyperbaric heliox and hydrox, respectively, and use the regression equations in Figure 2.2 to estimate the $T_s$. Model 2.1 and 2.2 are modifications of Model 2, using different values for the $T_{ex}$. (Model 2.1: $T_{ex} = 1^\circ$ C lower in hydrox; Model 2.2: $T_{ex} = 2^\circ$ C lower in hydrox).

<table>
<thead>
<tr>
<th>Gas</th>
<th>Pressure (atm)</th>
<th>Actual $Q_\Sigma$ (W)</th>
<th>Theoretical $Q_\Sigma$ (W)</th>
<th>Model #</th>
<th>$Q_C$ (%)</th>
<th>$Q_R$ (%)</th>
<th>$Q_{RC}$ (%)</th>
<th>$Q_{EVAP}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>1</td>
<td>3.31 ± 0.32</td>
<td>3.30 ± 0.22‡</td>
<td>0</td>
<td>32.4</td>
<td>55.9</td>
<td>1.5</td>
<td>10.2</td>
</tr>
<tr>
<td>Heliox</td>
<td>10</td>
<td>3.81 ± 0.31</td>
<td>3.53 ± 0.48‡</td>
<td>1.0</td>
<td>63.6</td>
<td>22.8</td>
<td>4.7</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.43 ± 0.52</td>
<td>4.41 ± 0.38‡</td>
<td></td>
<td>68.4</td>
<td>17.6</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.08 ± 0.61</td>
<td>5.66 ± 0.38‡</td>
<td></td>
<td>70.6</td>
<td>13.1</td>
<td>10.2</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.51 ± 0.72</td>
<td>6.57 ± 0.60‡</td>
<td></td>
<td>69.9</td>
<td>10.9</td>
<td>13.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Hydrox</td>
<td>10</td>
<td>4.07 ± 0.48</td>
<td>3.84 ± 0.75‡</td>
<td>2.0</td>
<td>65.1</td>
<td>18.9</td>
<td>5.5</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.75 ± 0.89</td>
<td>4.98 ± 0.68‡</td>
<td></td>
<td>66.1</td>
<td>13.8</td>
<td>9.3</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.57 ± 0.62</td>
<td>5.53 ± 0.89‡</td>
<td></td>
<td>67.5</td>
<td>10.6</td>
<td>12.4</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.40 ± 0.82</td>
<td>5.77 ± 0.90‡</td>
<td></td>
<td>66.5</td>
<td>8.9</td>
<td>15.1</td>
<td>9.5</td>
</tr>
<tr>
<td>Hydrox</td>
<td>10</td>
<td>4.07 ± 0.48</td>
<td>3.87 ± 0.70‡</td>
<td>2.1</td>
<td>66.8</td>
<td>19.3</td>
<td>3.6</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.75 ± 0.89</td>
<td>4.97 ± 0.63‡</td>
<td></td>
<td>68.9</td>
<td>14.3</td>
<td>6.0</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.57 ± 0.62</td>
<td>5.50 ± 0.85‡</td>
<td></td>
<td>72.4</td>
<td>11.3</td>
<td>6.8</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.40 ± 0.82</td>
<td>5.70 ± 0.86‡</td>
<td></td>
<td>73.7</td>
<td>9.7</td>
<td>7.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Hydrox</td>
<td>10</td>
<td>4.07 ± 0.48</td>
<td>3.87 ± 0.66‡</td>
<td>2.2</td>
<td>68.3</td>
<td>19.6</td>
<td>1.8</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.75 ± 0.89</td>
<td>4.92 ± 0.58‡</td>
<td></td>
<td>71.6</td>
<td>14.8</td>
<td>2.7</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.57 ± 0.62</td>
<td>5.43 ± 0.80‡</td>
<td></td>
<td>77.3</td>
<td>11.9</td>
<td>1.2</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.40 ± 0.82</td>
<td>5.56 ± 0.81‡</td>
<td></td>
<td>80.9</td>
<td>10.5</td>
<td>-1.3</td>
<td>9.9</td>
</tr>
</tbody>
</table>

‡Estimated values not statistically different from measured values ($P > 0.60$).
2.4. Pressure, gas mixture, and percent difference in total heat loss rate ($Q_\Sigma$) (hydrox/heliox) for a test to compare the difference in $Q_\Sigma$ between heliox and hydrox based solely on their differing thermal properties (TP $Q_\Sigma$), versus their measured values.

<table>
<thead>
<tr>
<th>Pressure (atm)</th>
<th>Gas</th>
<th>TP $Q_\Sigma$ (W)</th>
<th>TP $Q_\Sigma$ Difference (%)</th>
<th>Measured $Q_\Sigma$ Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 He</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td>8.5</td>
<td>21.4</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>20 He</td>
<td>9.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td>11.5</td>
<td>22.3</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>40 He</td>
<td>12.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td>15.8</td>
<td>23.4</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>60 He</td>
<td>15.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td>19.2</td>
<td>24.7</td>
<td>-17.1</td>
<td></td>
</tr>
</tbody>
</table>

The following variables were used for both gases at 10-60 atm: $T_a = 32.4^\circ C$ in Eq. 5, $a = 17.4$ and $b = 0.56$ in Eq. 9, $W = 863g$, $\dot{V}_O_2 = 0.184 mL \cdot s^{-1}$. 
Table 2.5. Sensitivity test of model to estimate total heat loss rate ($Q_\Sigma$) using Models 0.0, 1.0, and 2.0 to estimate $Q_\Sigma$ for 1 atm air, heliox and hydrox, respectively.

<table>
<thead>
<tr>
<th>Gas</th>
<th>Pressure (atm)</th>
<th>$Q_\Sigma$ (W)</th>
<th>Model #</th>
<th>$a = 0.60$</th>
<th>$a = 0.45$</th>
<th>$A = 65%$</th>
<th>$A = 100%$</th>
<th>$\varepsilon = 0.9$</th>
<th>$T_s = 38.4$</th>
<th>$T_s = T_a - 0.5$</th>
<th>$T_s = 0.84 \cdot T_a + 8.64$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>1</td>
<td>3.30 ± 0.22</td>
<td>0</td>
<td>3.0</td>
<td>-6.1</td>
<td>-21.2</td>
<td>15.2</td>
<td>-6.1</td>
<td>79</td>
<td>-30</td>
<td></td>
</tr>
<tr>
<td>Heliox</td>
<td>10</td>
<td>3.53 ± 0.48</td>
<td>1.0</td>
<td>8.5</td>
<td>-9.6</td>
<td>-21.0</td>
<td>14.2</td>
<td>-2.3</td>
<td>284</td>
<td>-66</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.41 ± 0.38</td>
<td>1.0</td>
<td>9.1</td>
<td>-10.2</td>
<td>-20.9</td>
<td>14.1</td>
<td>-1.6</td>
<td>248</td>
<td>-65</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.66 ± 0.38</td>
<td>1.0</td>
<td>9.4</td>
<td>-10.6</td>
<td>-20.3</td>
<td>13.6</td>
<td>-1.2</td>
<td>191</td>
<td>-62</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.57 ± 0.60</td>
<td>1.0</td>
<td>9.1</td>
<td>-10.5</td>
<td>-19.6</td>
<td>13.2</td>
<td>-1.1</td>
<td>135</td>
<td>-59</td>
<td>112</td>
</tr>
<tr>
<td>Hydrox</td>
<td>10</td>
<td>3.84 ± 0.75</td>
<td>2.0</td>
<td>8.6</td>
<td>-9.9</td>
<td>-20.6</td>
<td>13.8</td>
<td>-1.8</td>
<td>320</td>
<td>-62</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.98 ± 0.68</td>
<td>2.0</td>
<td>8.8</td>
<td>-9.8</td>
<td>-19.5</td>
<td>13.1</td>
<td>-1.4</td>
<td>295</td>
<td>-58</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.53 ± 0.89</td>
<td>2.0</td>
<td>8.9</td>
<td>-10.3</td>
<td>-19.2</td>
<td>12.7</td>
<td>-1.1</td>
<td>251</td>
<td>-52</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.77 ± 0.90</td>
<td>2.0</td>
<td>8.8</td>
<td>-10.1</td>
<td>-18.4</td>
<td>12.3</td>
<td>-0.9</td>
<td>206</td>
<td>-46</td>
<td>191</td>
</tr>
</tbody>
</table>

Values are expressed as a percentage of change compared to the original model estimate.
Figure 2.1. Mean (± 1 stdev) ratio of oxygen consumption rate to heat loss rate versus pressure for air, hydrox and heliox, as measured by direct respirometry and direct calorimetry. The upper and lower dotted lines indicate the \( \dot{V}O_2/Q_\Sigma \) ratio for a pure palmitic acid and glucose substrate, respectively (Dejours, 1975). *Indicates a significant difference between hydrox and heliox at the specified pressure.
Figure 2.2. Ambient temperature ($T_a$) versus estimated surface temperature ($T_s$) for guinea pigs in 1 atm air (Herrington, 1941), and 10-60 atm heliox and hydrox. The regression equations are for temperatures measured in °C.
Ambient temperature (°C)

Skin temperature (°C)

- Heliox, $T_s = 7.47 + 0.91 \cdot T_a$
- Hydrox, $T_s = 8.64 + 0.84 \cdot T_a$

Air, $T_s = 8.64 + 0.84 \cdot T_a$
APPENDIX

A2.1 A sensitivity test was performed of the model (Eq. 2) by changing the assumed constants and variables one at a time, and compared the estimated heat loss rates with the $Q_\Sigma$ from the original model (Table 2.5).

The constant $a$, used to calculate $Q_C$ ($a = 0.53$ in original model; Eq. 3), was changed to 0.60 and 0.45, which is the range of values most commonly used for this constant (Campbell, 1977; Clark, et al., 1981; Kreith and Bohn, 1997; McAdams, 1954). The body surface area available for heat loss ($A\%$, 85% in original model) was changed to 65 and 100% which is the range reported for humans (Gonzalez, 1988), and $\varepsilon$ ($\varepsilon = 1.0$ in original model; Eq. 7) was changed to 0.9 as an extreme to the values reported (Campbell, 1977; Gonzalez, 1988). $T_s$ was changed three times; one value assuming $T_s$ to be equal to $38.4^\circ C$ (assumed value of $T_b$), another assuming $T_s$ to be $0.5^\circ C$ greater than $T_a$, and the third using the measured $T_s$ values from guinea pigs in 1 atm air (Herrington, 1941). The results are reported as a percentage change compared to the estimated $Q_\Sigma$ for the initial model (Model 0). Model 0 uses the constants for $a$, $A\%$, $\varepsilon$, $T_{ea}$ and $T_s$ as described in the results section.

It can be seen that $Q_C$ (Eq. 3) is directly affected by a change in the constant $a$ (Table 2.5). The surface area available for heat loss affects both $Q_C$ and $Q_R$. A change in $\varepsilon$ has negligible effect on the $Q_\Sigma$ (Table 2.5). The $T_s$, however, affects the $Q_\Sigma$ substantially, as indicated by the large the percent changes in $Q_\Sigma$ under various estimates of $T_s$ (Table 2.5).
CHAPTER THREE

CALIBRATION OF THE PHYSICAL PROPERTIES OF
THE HYPERBARIC CHAMBER FOR RELIABLE
ESTIMATION OF CH₄ RELEASE RATE (\dot{V}_{CH₄})

“He who does not know the supreme certainty of mathematics is wallowing in confusion.”
- Leonardo Da Vinci
INTRODUCTION

A new method for safer and faster decompression in test animals after a hyperbaric exposure in H\textsubscript{2} was recently described by Kayar et al. (Kayar, et al., 1998a). The premise of this method is the utilization of H\textsubscript{2}-metabolizing microbes to consume some of the H\textsubscript{2} dissolved in the tissues of these animals after exposure to elevated H\textsubscript{2} pressures. The purpose is to convert H\textsubscript{2} into H\textsubscript{2}O and CH\textsubscript{4}, thereby reducing the amount of dissolved H\textsubscript{2} gas in the tissues. The microbes, or methanogens, convert H\textsubscript{2} into H\textsubscript{2}O and CH\textsubscript{4} according to the formula:

\[
4 \text{H}_2 + \text{CO}_2 \rightarrow 2 \text{H}_2\text{O} + \text{CH}_4 \quad \text{Eq. 1}
\]

where the secondary end product (CH\textsubscript{4}) can be used as a non-invasive measure of the efficacy of the methanogens to metabolize H\textsubscript{2}. It is believed that the microbial metabolism of H\textsubscript{2} increases the washout rate of H\textsubscript{2} from the animal during the decompression phase, and therefore decreases the incidence of decompression sickness (DCS, Kayar, et al., 1998a).

In a previous study, the microbes were injected into the intestines of rats (Kayar, et al., 1998a). The rats were then exposed to hyperbaric H\textsubscript{2}. The microbes were assumed to receive H\textsubscript{2} by diffusion across the intestinal wall, primarily supplied by the local blood flow (Kayar, et al., 1998a). Animals injected with methanogens had a 50% lower incidence of DCS compared to control animals at the selected hyperbaric compression and decompression sequence (Kayar, et al., 1998a). The rate at which the rats released CH\textsubscript{4} (\dot{V}\text{CH}_4) was measured, and according to Eq. 1, expected to be directly proportional
to the efficacy of the microbes in reducing the tissue burden of H$_2$ (Kayar, et al., 1998a). In addition, a simplified calculation suggested that total H$_2$ load could be reduced by ~5% in the rat (Kayar, et al., 1998a).

The estimation of the $\dot{V}_{\text{CH}_4}$ in the earlier study was performed using the same principles as when measuring the metabolic rates ($\dot{V}_{\text{O}_2}$) of animals using a flow-through system (Kayar, et al., 1998a; Steffensen, 1989). In a flow-through system, a stream of gas with known composition is injected into the chamber at a known flow rate ($\dot{v}$). The gas composition of the excurrent gas, having the same flow rate as the incurrent gas, is measured. With a change in the $\dot{V}_{\text{CH}_4}$, the concentration of CH$_4$ ([CH$_4$]) in the excurrent gas will approach a new steady state. The time to this equilibrium depends entirely on the volume of the chamber (V) and $\dot{v}$, and can be expressed as a dilution factor ($D = \dot{v}/V$) (Steffensen, 1989). The value of $D$ is used to determine the time required to reach a certain fractional transformation to the new steady state (Steffensen, 1989). In the study by Kayar et al., (Kayar, et al., 1998a), the time to reach 95% transformation was ~9 min. With sampling of the gas composition every 12 min, reliable estimates of the $\dot{V}_{\text{CH}_4}$ could be made on the basis of the difference in the [CH$_4$] between any two gas samples.

For this study, I followed the same principle of correlating the $\dot{V}_{\text{CH}_4}$ from the animals supplied with methanogens in their intestines to the H$_2$ metabolism of the methanogens (Eq. 1). The study was undertaken with pigs to determine whether biochemical decompression might be a feasible concept in humans. Consequently, the
initial studies in rats required further investigation in a larger animal model more similar to humans. To work with pigs, the chamber for these experiments had to be much larger than the chamber used for the rat study to allow researchers access to work with the pig. Due to safety regulations and to the economics of working with large volumes of H\textsubscript{2}, the \textit{v} in this study was limited to \( \approx 115 \text{ L} \cdot \text{min}^{-1} \), resulting in a small \( \dot{D} \). The small \( \dot{D} \) was predicted to require 2.5 days to reach 95\% transformation. Experiments in this chapter were performed to validate the method of computing \( \dot{V}_{\text{CH}_4} \) under conditions temporally distant from equilibrium.

**MATERIALS & METHODS**

**Hyperbaric Chamber Setup**

For the hyperbaric experiments, a dry hyperbaric chamber was used (WSF Industries, Buffalo, NY) with a physical volume (V) of 5665 L at 1 atm. The same chamber was used for all hyperbaric experiments described in this and future chapters. For the calibration experiments, the chamber was pressurized to 4 atm with air. Since the effective volume of the chamber is pressure-dependent, the resulting volume at pressure \( (V_P, \text{ L}) \) is as follows:

\[
V_P = V \cdot P \quad \text{Eq. 2}
\]

where P is the pressure (atm). Air at low pressure was used for economical reasons. A stream of gas was continuously exhausted from the chamber. A sample of this gas was taken every 12 min to determine the chamber composition of O\textsubscript{2}, N\textsubscript{2}, H\textsubscript{2}, He, and CH\textsubscript{4} using a gas chromatograph (Hewlett-Packard 5890A, Series II, Wilmington, DE).
**Instantaneous measurement of \( \dot{V} \text{CH}_4 \)**

The method of estimating the \( \dot{V} \text{CH}_4 \) was developed and explained in detail by Bartholomew et al. (Bartholomew et al., 1981) for measuring the \( \dot{V} \text{O}_2 \) in a flow-through chamber. When the changes in [\text{CH}_4] are rapid relative to \( \dot{D} \), it is possible to estimate the new equilibrium ([\text{CH}_4]_{eq}) value given the effective chamber volume (\( V, \text{L} \)), the flow rate (\( v, \text{L} \cdot \text{min}^{-1} \)), and the concentration of [\text{CH}_4] ([x], ppm) in the chamber at two time points, t and t-1. In the general case, the temporal change in concentration of gas [x] in the chamber can be described using a first order differential equation:

\[
\frac{dx}{dt} = q - \left[ \frac{[x](t) \cdot \dot{v}}{V_p} \right]
\]  
Eq. 3

where \( q \) is the rate at which gas [x] is added (ppm \( \cdot \) min\(^{-1} \)), and [x](t) is the content of gas [x] (ppm) at time \( t \) (min).

Bartholomew et al. (Bartholomew et al., 1981) introduced the Z transformation, which is the fraction toward the new steady state reached in a given time interval (\( \Delta t \)), and which can be estimated by:

\[
Z = 1 - e^{-\frac{\dot{v} \Delta t}{V_p}}
\]  
Eq. 4

or equivalently
\[ Z = \frac{[x]_t - [x]_{t-1}}{[x]_{eq} - [x]_{t-1}} \]

**Eq. 5**

where \([x]_t\) and \([x]_{t-1}\) are the concentrations of gas \(x\) at time \(t\) and \(t-1\), respectively, and \([x]_{eq}\) is the concentration at equilibrium. Combining Eq. 4 and 5 and solving for the equilibrium value \([x]_{eq}\) gives,

\[ [x]_{eq} = \frac{[x]_t - [x]_{t-1}}{-\frac{\Delta v}{\Delta t}} + [x]_{t-1} \left(1 - e^{-\frac{v}{V_p}}\right) \]

**Eq. 6**

Knowing the \([x]_{eq}\), or in this case the \([\text{CH}_4]_{eq}\), it is possible to calculate the \(\dot{V} \text{CH}_4\) (mol \(\text{CH}_4 \cdot \text{min}^{-1}\)) at standard temperature and pressure (STP) at time \(t\) by:

\[ \dot{V} \text{CH}_4 = \text{[CH}_4]_{eq} \cdot \dot{v} \cdot 22.4^{-1} \]

**Eq. 7**

where \(\dot{v}\) is in L \(\cdot\) min\(^{-1}\) at standard temperature and pressure (STP), and 22.4 the standard molar volume at 0\(^\circ\) C (L \(\cdot\) mol\(^{-1}\)).

Thus, the \(\dot{V} \text{CH}_4\) can estimated from the animal knowing the change in the \([\text{CH}_4]\) over a given \(\Delta t\), given that accurate values for \(V\), \(\dot{v}\), \([x]_t\), and \([x]_{t-1}\) can be obtained.

**Measurement of \(\dot{v}\)**

The \(\dot{v}\) from the chamber was determined by a flow meter. The flow meter (Brooks Instruments, Hatfield, PA, flow meter: R-6-15-B) was initially calibrated with pure He, H\(_2\), O\(_2\), N\(_2\), and air, and with 2\% O\(_2\) in 98\% He and 98\% H\(_2\), using a water
spirometer. The pressure of the excurrent gas was adjusted with a regulator to be constant (1.3 atm) for all chamber pressures. The volume of gas injected into the chamber to keep it at constant pressure was used to confirm that the flow meter gave reliable estimates.

**Effective Volume**

Having confirmed that the flow meter gave consistent results, it was possible to estimate $V$. The chamber manufacturer stated that the floodable volume of the chamber was 5665 L. Additional tubing and equipment inside the chamber may have changed this. Furthermore, according to Bartholomew et al. (1981) the effective $V$ is not always equal to the physical volume (Bartholomew, et al., 1981). By rearrangement of Eq. 6, $V$ can be calculated by computing $V_P$:

$$V_P = \frac{\cdot \Delta t \cdot v}{\ln(1 - Z)}$$

**Hyperbaric Experiments**

Two experiments were performed to determine the physical characteristics of the chamber. During the first experiment, a continuous stream of a gas mixture of 1 % CH$_4$ in He was injected at a known rate (~ 2.0 L • min$^{-1}$, or a $\cdot$CH$_4$ ~ 80-90 µmol • min$^{-1}$) for ~ 450 min. Following this, the CH$_4$ injection was terminated, and the washout of CH$_4$ and He from the chamber were measured for an additional ~380 min. During the second experiment, a bolus injection of CH$_4$ was completed while the chamber was held at constant pressure of 4 atm. Next, the washout of CH$_4$ and He were measured for >1400 min. The $V$ was determined from the washout data from the bolus injection experiment.
**Determination of \( \dot{V} CH_4 \)**

From Eq. 6, it can be shown that the estimation of \([x]_{eq}\) is heavily dependent on the measured values of chamber \([CH_4]\): \([x]_t\) and \([x]_{t-1}\). The time between two gas chromatograph samples in these experiments was 12 min. If the change in the \([CH_4]\) between two consecutive samples was very small, or if mixing of the chamber was not thorough, this could cause an error in the estimation of the \( \dot{V} CH_4 \) (Steffensen, 1989). Therefore, the washin data from the constant injection experiment described above were used to determine an appropriate \( \Delta t \) to be used in the computation of the \( \dot{V} CH_4 \) in the following chapters. In other words, the constant injection of the gas could be viewed as a “pseudo-pig”, releasing \( CH_4 \) at a known, constant rate (\( q \) in Eq. 3). Based on the data from the gas chromatograph, it is possible to estimate the \( \dot{V} CH_4 \), and with an adequate \( \Delta t \), the estimated washin rate should equal the known injection rate of \( CH_4 \).

**RESULTS**

The change in \([CH_4]\) during the constant injection experiment is shown in Fig. 3.1. The increase in \([CH_4]\) during the washin phase (Fig. 3.1A) and the decrease during the washout phase (Fig. 3.1B) both appear to change linearly with time (\( P < 0.0001 \), linear regression), but an exponential change fit the data equally well (\( P < 0.0001 \), exponential regression).
Flow meter

The flow rate of the pure gases for air, pure H\textsubscript{2}, and He, and the resulting regression lines are reported in Table 3.1. The reproducibility of the flow rate estimates was 2-4%. The regression equation for all other mixtures was a combination of the fractional composition of the mixture assuming that the flow meter ball was set at 100 units. The resulting equation was as follows:

\[
\text{Gas Flow Rate} = 119 \cdot [H_2] + 82.7 \cdot [He] + (100 - ([H_2] + [He]) \cdot 32.7)
\]

Eq. 9

where [H\textsubscript{2}] and [He] are the fractional composition of each of the two gases in the mixture, and assuming the balance to be air. The gas flow rate is given in L \cdot min\textsuperscript{-1} (STP).

The volume of gas injected into the chamber to keep it at constant pressure was used as a second means of estimating the \( \dot{V} \) during the washout of both experiments. This value was not significantly different from the mean value given by Eq. 9 for the given gas mixture (Table 3.2, 2-tailed t-test for unequal variances).

An appropriate \( \Delta t \)

The data from the bolus injection experiment was used to determine an adequate \( \Delta t \) for generating a reliable estimate of \( \dot{V} \text{CH}_4 \). The \( \dot{V} \text{CH}_4 \) values for the experiment were computed using Eq. 6, Eq. 7 and varying \( \Delta t \) (mean \pm \text{stdev}, n = 15; Table 3.3). For a \( \Delta t \) of 12 min (two consecutive chromatographic readings) the standard deviation (stdev) was \( \sim 0.8 \) times the estimated mean \( \dot{V} \text{CH}_4 \) (Table 3.3). The stdev of the estimate of the mean
\( V_{CH_4} \) was plotted against \( \Delta t \) (Fig. 3.2); the stdev decreased exponentially with increasing \( \Delta t \). At a \( \Delta t \) of \( \sim 120 \) min, the change in stdev with increasing \( \Delta t \) leveled off, and did not further change significantly as compared to a \( \Delta t \) of 500 min (Table 3.3, \( P < 0.05 \), F-test). Therefore, 120 min was determined to be the smallest \( \Delta t \) for reliable estimation of \( V_{CH_4} \) between two chromatograph samples, [x]_t and [x]_{t-1}.

**Effective Volume**

The estimation of \( V \) depended on the relative difference in [CH_4] at the two time points. If the interval between the two samples is short, there may be very little difference between the measured [CH_4] values, leading to low precision in the estimated \( V \). Therefore, \( V \) was estimated using the data from the bolus injection, with a \( \Delta t \) of \( \sim 500 \) min. This \( \Delta t \) was determined to be sufficiently large to give values with high accuracy, since the mean \( V \) values at larger \( \Delta t \) did not change significantly (data not shown). The mean \( V \) from this computation was 5718 \( \pm \) 480 L (\( \pm \) stdev, \( n = 66 \) chromatographic readings that were 500 min apart), suggesting that the \( V \) was identical to the floodable volume of the chamber, which was 5665 L according to the manufacturer.

**DISCUSSION**

The main objective of the subsequent chapter is to show that removal of H\(_2\) from the tissues of pigs during a hyperbaric exposure to H\(_2\), by microbial H\(_2\) metabolism, will significantly reduce the DCS incidence. The microbial metabolism converts the H\(_2\) gas
into H₂O and CH₄ (Eq. 1). The secondary end product (CH₄) in this conversion can be used as a non-invasive measure to estimate the rate (\dot{V}CH₄) microbes efficacy in removing the H₂ gas within the animal.

The hyperbaric chamber used throughout this and subsequent studies (Chapter 3, 4, 6) was utilized as a flow-through respirometer, in which a constant stream of gas was exhausted for analysis, and H₂ and O₂ were injected to keep the chamber at constant pressure. To estimate the \dot{V}CH₄, it was necessary to correct the [CH₄] readings to account for the dilution factor in the flow-through chamber (Bartholomew, et al., 1981; Steffensen, 1989). Consequently, the main objective of the experiments in this chapter was to find a value for V, a reliable method for estimating \dot{V}, and an adequate \Delta t to estimate the \dot{V}CH₄ from an animal during the hyperbaric experiments.

**Chamber flow rate (\dot{v})**

The calibration of the flow meter showed that there were differences in the flow rates between the different gases (Table 3.1), and an increasing percentage of H₂ led to a higher \dot{v} (Table 3.1). Since the chamber gas composition was different between hyperbaric experiments, a composite formula was created that would estimate the appropriate \dot{v} (Eq. 9). It was shown that the formula gave similar \dot{v} when compared with the total volume of gas injected during the experiments (Table 3.2). Consequently, the flow rates estimated by Eq. 9 gave consistent values and this formula was used throughout the study to compute \dot{v}. 

Effective volume (V)

As can be seen in Eq. 7, it is necessary to know \( \dot{v} \) to be able to determine \( V \). Consequently, after having confirmed that Eq. 9 gave consistent values for \( \dot{v} \), it was possible to estimate \( V \). The results from the washout experiment suggested that the \( V \) was approximately equal to the physical volume of the chamber times the chamber pressure. The actual physical volume of the chamber also included plumbing to and from the gas supply, a system for safe addition of \( O_2 \), and plumbing to a sanitary tank. The additional plumbing would have added physical volume to the chamber, possibly resulting in a larger \( V \) than the floodable volume of the chamber itself. The interior of the chamber was also equipped with a treadmill with a volume that may have counteracted the chamber volume added by the plumbing. Therefore, it was reasonable that \( V \) was similar to the floodable volume times the chamber pressure. Accordingly, the floodable volume of the chamber (5665 L) was used for \( V \) in estimating \( \dot{V} CH_4 \) throughout the study.

Finding an adequate \( \Delta t \)

All values of \( \Delta t \) estimated the \( \dot{V} CH_4 \) to within the known injection rate. However, a \( \Delta t \) of 88 min or less had a standard deviation for these intervals that was significantly different from the stdev for the mean \( \dot{V} CH_4 \) using a \( \Delta t \) of 500 min (Table 3.3). When the \( \Delta t \) was increased, the stdev became smaller, and at 112 min the change in the stdev with
increasing $\Delta t$ was insignificant (Fig. 3.2, $P < 0.05$, F-test). Thus, for the estimation of

$\dot{V}_{CH_4}$ during the study, a $\Delta t$ of 120 min was used whenever possible.

In summary, the data in this chapter have shown that using the floodable volume of the chamber for $V$, Eq. 9 to estimate $\dot{V}$, and a $\Delta t$ of 120 min reliably estimates the $\dot{V}_{CH_4}$ from the hyperbaric chamber. Consequently, by measuring the temporal change in the $[CH_4]$ during the hyperbaric experiments, a consistent method of estimating the $H_2$ metabolism by the intestinal methanogens has been defined.
Table 3.1. Flow meter calibration. The flow meter was calibrated for each of 3 pure gases, and for a Heliox and Hydrox mixture with 2% O₂, balance inert gas. The flow rates are reported in STP.

<table>
<thead>
<tr>
<th>Ball position (x)</th>
<th>Air</th>
<th>H₂</th>
<th>He</th>
<th>Heliox</th>
<th>Hydrox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(L • min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>13.7</td>
<td>52.2</td>
<td>34.8</td>
<td>30.8</td>
<td>54.6</td>
</tr>
<tr>
<td>100</td>
<td>32.7</td>
<td>119.0</td>
<td>82.7</td>
<td>73.0</td>
<td>115.8</td>
</tr>
<tr>
<td>150</td>
<td>57.0</td>
<td>200.3</td>
<td>145.0</td>
<td>128.2</td>
<td>184.4</td>
</tr>
<tr>
<td>Flow =</td>
<td>-0.1 + 0.22x + 0.00105x²</td>
<td>0.9x + 0.0029x²</td>
<td>1.37 + 0.524x + 0.00289x²</td>
<td>0.4 + 0.481x + 0.00248x²</td>
<td>1.02x + 0.0014x²</td>
</tr>
</tbody>
</table>
Table 3.2. Estimated mean washout rate (± stdv, \( \dot{v} \)) from the hyperbaric chamber during the constant injection (CI) and the bolus injection (BI) experiments. The \( \dot{v} \) was estimated based on the total volume of gas injected into the chamber to keep it at constant pressure (\( \dot{i}v \)), and from the flow meter equation (\( \dot{f}v \)) based on the gas composition after the bolus injection (~8% He, ~21% O\(_2\), ~71% N\(_2\)). †There was no difference between the \( \dot{i}v \) and the \( \dot{f}v \) for either the BI or CI experiments (P > 0.26, and P > 0.06 respectively).

<table>
<thead>
<tr>
<th>Gas</th>
<th>( \dot{i}v )</th>
<th>( \dot{f}v )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>CI</td>
<td>BI</td>
</tr>
<tr>
<td>( n )</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Mean Washout Rate (L ( \cdot ) min(^{-1}))</td>
<td>27.4 ± 5.5</td>
<td>32.6 ± 12.8</td>
</tr>
</tbody>
</table>
Table 3.3. Estimated mean $\dot{V}_{CH_4} (\pm \text{stdev}, n = 15)$ using Eq. 6 and Eq. 7 and varying $\Delta t$. The estimates are based on washin data, using a $V_P = 5665 \text{ L} \cdot 4 \text{ atm}$, and a $v$ of $\sim 34 \text{ L} \cdot \text{min}^{-1}$. The actual constant injection rate was $\sim 85 \mu\text{mol CH}_4 \cdot \text{min}^{-1}$. † Significantly different variance as compared to using a $\Delta t$ of 500 min ($P < 0.05$, F-test).

<table>
<thead>
<tr>
<th>$\Delta t$ (min)</th>
<th>$\dot{V}_{CH_4}$ $\mu\text{mol CH}_4 \cdot \text{min}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>89.1 ± 68.3†</td>
</tr>
<tr>
<td>25</td>
<td>88.6 ± 38.0†</td>
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<tr>
<td>38</td>
<td>87.1 ± 27.8†</td>
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<tr>
<td>62</td>
<td>87.2 ± 20.7†</td>
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<td>88</td>
<td>85.5 ± 15.3†</td>
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<tr>
<td>114</td>
<td>83.8 ± 8.9</td>
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<td>138</td>
<td>83.2 ± 8.8</td>
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<td>176</td>
<td>84.5 ± 8.5</td>
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<td>250</td>
<td>84.3 ± 8.3</td>
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<tr>
<td>400</td>
<td>83.7 ± 3.1</td>
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<tr>
<td>500</td>
<td>78.8 ± 6.0</td>
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</tbody>
</table>
Figure 3.1. The change in methane concentration ([CH₄], ppm) in the chamber during the (A) washin and (B) washout phases of the constant injection experiment. The data show that the concentration changes almost linearly throughout the time of the experiment, which is in contrast to the single exponential function that commonly describes washin and washout from a through flow system. Consequently, the small $D$ of the chamber setup prolongs the kinetics of this system, making the kinetics appearing linear.
B

Time (min)

[CH₄] (ppm)
Figure 3.2. Standard deviation (stdev, n = 15) of estimated mean $\dot{V}$ CH$_4$ from the washin phase of the continuous injection experiment versus $\Delta t$. The graph shows that the stdev decreases exponentially with $\Delta t$, and at a $\Delta t$ of 112 min the change in stdev does not change significantly with further increase in the $\Delta t$ ($P < 0.05$, F-test).
CHAPTER FOUR

REDUCTION IN DECOMPRESSION SICKNESS RISK BY NATIVE H₂-METABOLIZING INTESTINAL MICROBES IN PIGS AFTER HYPERBARIC H₂ EXPOSURE

“What we know is a drop-
What we don’t know is an ocean.”

Newton
INTRODUCTION

Biochemical decompression is a term used for the protective effect against decompression sickness achieved by removing by biochemical means some of the inert gas dissolved in a diver’s tissues after a hyperbaric exposure (Kayar, et al., 1998a). It has been shown that rats given intestinal injections of the H2 metabolizing microbe Methanobrevibacter smithii had a ~50% lower DCS incidence compared to untreated animals following a hyperbaric exposure to H2 (Kayar, et al., 1998a). These microbes metabolized H2 and CO2 to CH4 and H2O, and the efficacy of the H2 metabolism was measured as the CH4 release rate (\(v_{CH_4}\)) from the animals. Sample calculations suggested that this 50% decrease in DCS incidence was attributable to microbial reduction of the total H2 tissue load by ~ 5%. An earlier study reported a similar decrease in the DCS incidence by reducing the hyperbaric exposure from 23.7 atm to 22.5 atm, i.e., by 5% (Lillo, et al., 1997).

The gas flux kinetics in an animal are believed to be governed by the solubility of the gas in the different tissues of the animal and the blood flow rate perfusing the different tissues. Based on the fact that the metabolic rate of smaller animals is generally higher than that of larger animals (Calder, 1984; Schmidt-Nielsen, 1984), it is expected that net gas exchange in a rat will be faster than in a human. Consequently, before biochemical decompression using H2 as the inert gas can be advanced for use in human divers, it is necessary to show that the H2 metabolism by the methanogen, M. smithii, is sufficient to reduce the DCS incidence in a large animal model of more similar size to a human. The pig was chosen as the large animal, based on its documented

Before experiments in which pigs were treated with methanogens, it was necessary to find a baseline pressurization and depressurization sequence that gave a high DCS incidence in control animals. This would assure the detection of a significant reduction in DCS incidence for treated animals. Early results showed that something unanticipated was taking place: DCS incidence did not increase with increasing pressure as is commonly found in DCS research. This chapter will report these observations and suggest a reasonable explanation.

MATERIALS & METHODS

Animals

Male Yorkshire pigs, *Sus scrofa*, (n = 27, mean body weight = 19.4 ± 1.2 kg, range 17.0 - 22.1 kg, castrated or non-castrated) were used for all experiments. Animals were assigned randomly to one of 3 pressure groups: 22.3, 24.1, or 25.7 atm absolute pressure (mean body weight ± 1 stdev 19.6 ± 0.9 kg, n = 11; 19.6 ± 1.5 kg, n = 9; 18.8 ± 1.3 kg, n = 7, respectively ). There was no difference in individual body weight (P > 0.34) among the 3 groups, as determined by one-way ANOVA. The animals were housed in an accredited animal care facility and were fed once daily in the morning with laboratory animal chow (Harlan Teklad, Madison, WI: 2% of body weight); water was available for *ad libitum* drinking. On the day prior to an experiment, the animal was fed a second time in the afternoon. On the day of the experiment, no food was available to the animal until entry into the chamber. All experimental procedures were approved by an Animal Care and Use Committee, and the experiments reported were conducted
according to the principles presented in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

**Treadmill training**

Each animal was trained to walk on a treadmill 1-3 days before the hyperbaric exposure. Initially, the animal walked on a treadmill in 1 atm air in the laboratory at a speed of 66 m • min\(^{-1}\) for 5 min, followed by at least 5 min rest, then walked again for 5 min. Next, the procedure was repeated on the treadmill housed inside the chamber in 1 atm air, at a speed of 24 m • min\(^{-1}\). This second phase of training acclimatized the animal to the chamber environment. Walking on the treadmill allowed the observers to note each animal’s gait prior to the hyperbaric exposure, to more clearly identify symptoms of DCS during the hyperbaric experiment.

**Pressurization and depressurization trial process**

To identify a pressurization and depressurization sequence that gave a high DCS incidence in control animals, a trial process with proven efficiency was selected (Dromsky, *et al.*, 2000a). A constant duration at maximum pressure (3 h) and a constant compression and decompression rate (0.45 atm • min\(^{-1}\)) were initially chosen. A small number of untreated animals were exposed to a series of pressures in order to identify a pressure yielding a DCS incidence between 70% and 90%. This pressure selection procedure has been shown to be successful in DCS research and has been described in detail elsewhere (Dromsky, *et al.*, 2000a).
Dive protocol

For the hyperbaric experiments, one animal was placed in a dry hyperbaric chamber (WSF Industries, Buffalo, NY), and the same chamber was used for all experiments. The facility housing the chamber was designed to comply with the safety regulations for work with H2, as described in detail elsewhere (Kayar and Parker, 1997; Kayar, et al., 1997a). Observation ports and a video camera allowed continuous monitoring of the animal from each 90° angle. Duplicate thermometers allowed continuous monitoring of the chamber temperature ($T_{CH}$), which was maintained at a seemingly comfortable level for the pigs: $\approx 30^\circ C$ at 11 atm and $\approx 33^\circ C$ at 22.2, 24.1, and 25.5 atm.

The animal was confined to a space on the treadmill of 45 x 45 x 100 cm using a plexiglass box. The enclosure was large enough for the animal to stand up and move a few steps. In the front of the enclosure there was a trough filled with an ample supply of food and water for ad libitum access throughout the experiment.

On one side of the plexiglass confinement box there was a sample port through which a stream of gas was continuously exhausted from the chamber (~115 L • min$^{-1}$ or ~410 L • min$^{-1}$) via a flowmeter. A gas sample was taken every 12 min to determine the composition of O$_2$, N$_2$, H$_2$, He, and CH$_4$ using a gas chromatograph (Hewlett-Packard 5890A, Series II, Wilmington, DE). An external gas blower circulated the chamber gases to keep them well-mixed. Thorough mixing was confirmed by replicate gas chromatographic analyses of samples drawn from the pig’s enclosure and from a second, relatively distant location in the chamber. The flow meter (Flowmeter:R-6-15-B, Brooks Instruments, Hatfield, PA) was calibrated with He, H$_2$, O$_2$, N$_2$, air and 2% O$_2$ in 98% He, or 2% O$_2$ in 98% H$_2$, using a water spirometer (Chapter 3). The flow rates of all other
gas mixtures were estimated by linear interpolation of the fractional composition of the
gases (Eq. 9, Chapter 3). In addition, the volume of gas injected into the chamber to keep
it at constant pressure during an experiment confirmed that the flow rates were reliably
estimated. The careful calibration of the flow meter was necessary for accurate
determination of the CH$_4$ release rate ($\dot{\nu}$CH$_4$, $\mu$moles CH$_4$ • min$^{-1}$) from the chamber.

The chamber was pressurized from 1-11 atm using He, at a rate that allowed the
animal to equalize the pressure in its ears. Most commonly, the rate was 0.15 atm • min$^{-1}$
to a pressure between 2.5-3.0 atm. If the animal had no problems, the rate was increased
to 0.3 atm • min$^{-1}$ to a pressure of 3.7-5.6 atm and then eventually to 0.45 atm • min$^{-1}$
unless the animal exhibited signs of discomfort. The pressurization of the chamber was
discontinued at any time during the pressurization phase if the animal exhibited signs of
nausea, nystagmus, problems with balance, or shaking or abnormal holding of the ears.
On these occasions, the chamber was held at constant pressure for a few minutes; if the
animal continued to exhibit signs of discomfort, the chamber was decompressed
approximately 0.2-0.3 atm. When the animal appeared to have recovered, the
compression gradually continued.

The O$_2$ level was monitored continuously throughout the dive by an O$_2$ analyzer
(Teledyne Analytical Instruments, % O$_2$ Analyzer Model 327R, City of Industry, CA).
This made it possible to keep the chamber atmosphere normoxic at all times (PO$_2$ = 0.2-
0.4 atm).

Upon reaching 11 atm, the chamber was flushed with H$_2$ with concomitant
addition of O$_2$ to keep the chamber atmosphere normoxic. The chamber flush was
continued for approximately 30 min, or until the H$_2$ content had reached between 60 and
70%. Following the flush, the chamber was further pressurized to the desired pressure of 22.3, 24.1, or 25.7 atm at a rate of 0.45 atm • min⁻¹, using H₂ again with continuous addition of O₂. There was no difference in H₂ % (P > 0.33) between the 3 experimental groups as determined by one-way ANOVA (Table 4.1).

The desired pressure was maintained (± 0.3 atm) for 3 h by continuous addition of H₂ and O₂ to make up for the gas exhausted to the gas chromatograph. Consequently, the H₂ concentration increased slightly (1-2%), while the levels of N₂ and He decreased continuously. After 2.5 h at constant pressure, the treadmill was activated for 5 min to judge the animal’s ability to walk before the decompression.

Following 3 h at constant pressure, the chamber was decompressed at 0.9 atm • min⁻¹ to 11 atm. The treadmill was activated and the animal was made to walk (30 m • min⁻¹) for 5 min every 10 min, either until the animal was judged to have DCS, or until 1 h had passed. Severe symptoms of DCS included walking difficulties, fore and/or hind limb paralysis, falling, respiratory distress and seizures. Some animals showed signs of skin DCS (Buttolph, et al., 1998), appearing as light to dark purple spots with or without apparent itching, but these symptoms of DCS alone were considered as a positive diagnosis of DCS. In addition, mild beahvioural changes in the form of lethargy or agitation did not warrant diagnosis of DCS. Declaration of DCS was made by the consensus decision of at least 3 observers. The animal was then euthanized in the chamber by rapid asphyxiation. Following euthanasia, the chamber was returned to 1 atm and the H₂ flushed out.

Corrected $\cdot VCH_4$
The change in the chamber CH\textsubscript{4} concentration during the time at constant pressure (c\text{[CH\textsubscript{4}]} ppm) and during the decompression (d\text{[CH\textsubscript{4}]} ppm) was corrected to estimate the \text{V CH\textsubscript{4}}. The \text{V CH\textsubscript{4}} was estimated according to the method of Bartholomew et al. (Bartholomew, et al., 1981, Chapter 3), and the values reported are the mean value (± 1 stdev) at constant pressure (c \text{V CH\textsubscript{4}}, n = 5) and for the 1 h observation period at 11 atm (d \text{V CH\textsubscript{4}}, n = 3). A Δt of 120 min was used to estimate the c \text{V CH\textsubscript{4}} (Chapter 3), while the largest possible Δt (∼ 24 min) was used to estimate the d \text{V CH\textsubscript{4}} giving at least 3 estimates at 11 atm for animals that were observed for a full hour without displaying signs of DCS. The lowest quantifiable [CH\textsubscript{4}] per chromatographic report for these experiments was 0.5 ppm. Some animals did not release enough CH\textsubscript{4} to accumulate to a concentration of 0.5 ppm after 3 h, and thus are listed as releasing 0 CH\textsubscript{4} (Table 4.1).

The mean flow rate through the chamber (\text{v}) in this study was either 115 L • min\textsuperscript{−1} or 410 L • min\textsuperscript{−1}. These values were mathematically adjusted to a common flow rate of 115 L • min\textsuperscript{−1}.

Mathematical analysis

It was desired to determine which variables had a significant effect on the DCS outcome. Since the dependent variable (presence or absence of DCS) is dichotomous, and multiple independent variables were involved, multivariate logistic regression techniques were used (Hosmer and Lemeshow, 1989). The model selected determined the probability of DCS [P(DCS)] using the following independent variables: pressure (P), H\textsubscript{2} composition (H\textsubscript{2} %), chamber temperature (T\textsubscript{CH}), H\textsubscript{2} partial pressure (P\text{H\textsubscript{2}}), body
weight (Wt), concentration of CH\textsubscript{4} at constant pressure (c[CH\textsubscript{4}]) and during decompression (d[CH\textsubscript{4}]), and \( \cdot V CH\textsubscript{4} \) at constant pressure (c \( \cdot V CH\textsubscript{4} \)). Initially it was determined which subset of variables to include in the multivariate model by performing univariate analysis on each independent variable and including only those variables with a P < 0.20 (Wald test). Variables passing this test were used in the multivariate analysis, where exclusion or inclusion of a variable was based on the likelihood ratio test (Hosmer and Lemeshow, 1989).

**Statistical analysis**

All estimates of variance are reported as standard deviations, and all mean comparisons are based on 2-tailed t-tests with acceptance of differences at P < 0.05, unless stated otherwise.

**RESULTS**

**DCS outcome**

Animals at 22.3, 24.1, and 25.7 atm had a DCS incidence of 18%, 33%, and 0% respectively (Table 4.1). There was no difference in the DCS incidence between castrated and non-castrated animals (2-tailed \( \chi^2 \), P > 0.3); consequently these two groups were pooled in all subsequent analysis.

\( \cdot V CH\textsubscript{4} \)
Four animals did not release quantifiable levels of CH\textsubscript{4} during the experiment (Table 4.1). The c[CH\textsubscript{4}] increased temporally in all other animals (Fig. 4.1). During the decompression phase, the d[CH\textsubscript{4}] continued to increase or remained essentially constant (Table 4.1). There was no difference in the c[CH\textsubscript{4}] or d[CH\textsubscript{4}] between the animals at different pressures (P > 0.5, one-way ANOVA).

There was a significantly higher mean c[CH\textsubscript{4}] (n = 22, 1.40 ± 0.80 ppm CH\textsubscript{4}) in animals that did not show symptoms of DCS compared to animals with DCS (n = 5, 0.53 ± 0.82 ppm CH\textsubscript{4}, P < 0.05; Table 4.1). There was also a significantly higher mean d[CH\textsubscript{4}] (2.22 ± 1.36 ppm CH\textsubscript{4}) in animals with no signs of DCS as compared to the animals with DCS (0.72 ± 1.14, P < 0.05; Table 4.1).

The corrected mean c \text{\dot{V}} CH\textsubscript{4} values are reported in Table 4.1. There was no difference in the mean c \text{\dot{V}} CH\textsubscript{4} between the animals at three different pressures (P > 0.5, one-way ANOVA), and the mean c \text{\dot{V}} CH\textsubscript{4} at 22.3, 24.1, and 25.7 atm was 30.0 ± 27.2, 30.9 ± 26.2 and 39.7 ± 33.3 μmoles CH\textsubscript{4} • min\textsuperscript{-1}, respectively (Table 4.1). Not surprisingly, since c \text{\dot{V}} CH\textsubscript{4} was computed from c[CH\textsubscript{4}], there was a significant correlation between the c \text{\dot{V}} CH\textsubscript{4} and the c[CH\textsubscript{4}] (P < 0.01; Fig. 4.2A). There was a trend toward a higher mean c \text{\dot{V}} CH\textsubscript{4} (37.4 ± 27.3 μmol CH\textsubscript{4} • min\textsuperscript{-1}) in the animals with no DCS as compared to the animals with DCS (12.6 ± 21.0 μmol CH\textsubscript{4} • min\textsuperscript{-1}, P = 0.07), and was not significantly correlated with pressure (P > 0.4; Fig. 4.3).

During the decompression phase, the d \text{\dot{V}} CH\textsubscript{4} could only be reliably estimated using the animals that did not show DCS symptoms and were kept for 1 hour at 11 atm.
For these animals (n = 22; Table 4.1), there was a significant correlation between the  
\( d \dot{V}CH_4 \) and the \( d[CH_4] \) (P < 0.01; Fig 4.2B). Furthermore, there was a significantly  
higher \( d \dot{V}CH_4 \) compared to the \( c \dot{V}CH_4 \) for each animal (P < 0.01, paired 2-tailed t-test,  
Table 4.1).

There was a significant difference in the mean \( T_{CH} \) between the 3 groups (P < 0.05, one-way ANOVA, Table 4.1). The mean \( T_{CH} \) between animals at 22.3 atm (31.9 ± 0.8° C, n = 11) versus animals at 24.1 (32.8 ± 0.9° C, n = 9) and 25.7 atm (33.3 ± 0.8° C,  
n = 7) was significantly different (P < 0.05), while there was no difference in the mean  
\( T_{CH} \) between the animals at 24.1 and 25.7 atm (P > 0.2).

**Logistic regression**

The univariate logistic regression analysis showed that \( P, Wt, \) and \( H_2 \% \) were not  
significant contributors to \( P(DCS) \) (Table 4.2). The \( c(CH_4), d(CH_4), c \dot{V}CH_4, \) and \( T_{CH} \)  
were significantly correlated with \( P(DCS) \). The multivariate analysis demonstrated that  
no combination of variables significantly improved the fit to the data (Table 4.2).
DISCUSSION

This study was initially aimed at identifying a compression and decompression sequence that would yield a high incidence (70%-90%) of DCS in control animals. This sequence would then be used to compare DCS incidence between control animals and animals injected with H₂ metabolizing microbes (see Chapter 5). While searching for this high risk compression and decompression sequence, it was discovered that the DCS incidence did not increase with increasing pressure. This trend is contrary to what is commonly found in DCS research (Boycott, et al., 1908; Hills, 1977; Weathersby, et al., 1984). In addition, there were substantial levels of CH₄ released by most of the animals, and a clear connection between CH₄ release and DCS incidence (Table 4.1).

The CH₄ in the chamber could potentially come from three sources: from the pig, from the outside air which contains small amounts of CH₄, and from the commercially purchased gases used to pressurize the chamber. Since the chamber was pressurized from 1 atm to 11 atm with He and then flushed with H₂, any CH₄ trapped in the chamber at the start of the experiment would have been removed or reduced to undetectable levels by the flushes. The commercially purchased gases were guaranteed to contain < 0.1 ppm CH₄, levels too small for reliable detection with the gas chromatograph used in this study. The purity of the gases was confirmed in preliminary gas chromatography tests. Therefore, it was concluded that all CH₄ in the chamber was released by the pig.

Pigs are reported to have a native intestinal flora of methanogens capable of H₂ metabolism (Robinson, 1989), and increasing levels of CH₄ in the chamber for most animals confirmed this (Fig. 4.1). Methane release from mammals is strictly a process of
CH$_4$ production by methanogenic symbionts in the intestines. The normal source of the H$_2$ used by these microbes is the H$_2$ produced as a metabolic end product by other intestinal microbes (Miller, 1991). Consequently, the release of CH$_4$ suggests that some of the H$_2$ that otherwise would have been dissolved in the pigs’ tissues during the hyperbaric exposure was being converted into CH$_4$. The microbial removal of H$_2$ should in turn have led to reduced H$_2$ tissue tensions ($P_{\text{tis} \text{H}_2}$), and therefore potentially reduced DCS incidence (Kayar, et al., 1998a).

To use the change in [CH$_4$] as an inter-animal comparative measure for the activity of H$_2$ metabolism, it is important that the same time interval is used for computing the mean [CH$_4$]. Even though the time at constant pressure was 3 hours ($\pm$ 30 seconds) for all animals, the accumulation of CH$_4$ in the chamber begins at the end of the H$_2$ flush at 11 atm, at which time the [CH$_4$] should be $\sim$ 0 ppm. Since there was a slight difference in the rate of compression to the final pressure between experiments ($34.2 \pm 7.4$ min), the c[CH$_4$] and d[CH$_4$] between animals could vary slightly. However, this error should have been minimal because variation in this time was miniscule compared to the 3 h at constant pressure.

The corrected \( \dot{V} \) CH$_4$ will not contain the error described above. Due to the large volume of the chamber and the low ventilation rates (due to strict safety and restrictive economical issues) the time to equilibrium between perturbation by the pig and sampling rate would take several days (Steffensen, 1989; Eq. 6, Chapter 3). The values used for computing \( \dot{V} \) CH$_4$ are projections to equilibrium values (Eq.5, Chapter 3) that would be reached at a temporally distant time point, and therefore subject to an error of unknown magnitude. Since the calibration of the physical characteristics of the chamber showed
that reliable values are achieved if the Δt is large enough (Fig. 3.2; Chapter 3), a Δt of
120 minutes was chosen. The corrected $c \cdot V CH_4$ and $d \cdot V CH_4$ were necessarily highly
correlated with the $c[CH_4]$ and $d[CH_4]$, respectively (Fig. 4.2A and 4.2B), and it is
reasonable to assume that both the $[CH_4]$ and $\cdot V CH_4$ may reflect the efficacy in H$_2$
removal from the animal. However, the $\cdot V CH_4$ gives a quantitative measure of the H$_2$
removal rate, while the $c[CH_4]$ and $d[CH_4]$ are only qualitative in nature.

The $c \cdot V CH_4$, $c[CH_4]$ and $d[CH_4]$ were significantly higher in pigs that did not
show symptoms of DCS compared to pigs with DCS after the hyperbaric exposure to H$_2$
(Fig. 4.3). These measures of methanogenesis suggest that animals with a more active
H$_2$-metabolizing gut flora have a lower risk of DCS caused by the reduced P$_{tis}$H$_2$.

The results from the logistic regression analysis indicated that there was a
negative correlation between increasing levels of microbial H$_2$ metabolism and DCS
incidence (Table 4.2; Fig. 4.4). These data suggest that if the microbial activity is large
enough, the DCS incidence can be reduced from ~ 42% to ~0% (Fig. 4.4). However, the
potential reduction of DCS incidence to 0% must be considered with genuine doubt, since
some aspects of DCS appear to be random. Current models that predict DCS consider
that any dive has a finite probability of DCS and the only way to eliminate DCS risk is
not to dive at all (Weathersby, et al., 1984; Weathersby, et al., 1992). Yet, the results of
the present study suggest that removing even a small amount of the extra inert gas
dissolved during a hyperbaric exposure has a beneficial effect on DCS risk (Weathersby,
et al., 1986).
There was a large variability in the $V\cdot CH_4$ between animals, and it was difficult to identify animals with no detectable $V\cdot CH_4$ prior to the hyperbaric exposure. In an attempt to remove the native methanogenic flora, a group of animals ($n = 8$) was given chloramphenicol (50 mg $\cdot$ kg$^{-1}$ twice daily per os, Lyphomed, Deerfield, IL) 3-5 days prior to the experiment. Of the animals treated with this broad-spectrum antibiotic, 62% (5/8) still had detectable levels of $V\cdot CH_4$ after treatment, suggesting that chloramphenicol is not sufficiently specific for these microbes (Miller, 1989). In addition, some of the side effects of chloramphenicol are haematological in nature, including anemia, leukopenia, thrombocytopenia, and reticulocytopenia (Lyphomed, Deerfield, IL). Since there is reason to believe that the etiology of DCS may in part be caused by haematological changes (Bove, 1982; Catron and Flynn, 1982; Catron, et al., 1987; Moon, et al., 1992), and since the antibiotic was unable to effectively eliminate the native methanogens, these animals were not included in this study.

It is believed that blood flow and solubilities of gases in tissues govern fluxes of inert gas in the tissues of a diver (Parker, et al., 1998; Weathersby, et al., 1984). Consequently, it is reasonable to expect that changes in the metabolic rate ($\dot{V}\cdot O_2$) would change the inert gas kinetics. Body weight and ambient temperature are known to affect the $\dot{V}\cdot O_2$ (Schmidt-Nielsen, 1984; Schmidt-Nielsen, 1990). It would be expected that changes in either of these variables could change the risk of DCS. The logistic regression analysis suggested that $T_{CH}$ is a significant predictor of DCS (Table 4.2). This was unexpected since the $T_{CH}$ was adjusted to be more or less equal between experiments. Based on visual observation of the animals’ apparent comfort, the $T_{CH}$ could have been
selected to be slightly higher at elevated pressures since body heat losses (QΣ, Chapter 2) increase with pressure. Considering the higher QΣ at elevated pressures, keeping the TCH constant but increasing the pressure would eventually lead to a change in the VO2 to make up for the lost heat (Chapter 2). Since the mean TCH increased with pressure, the pressure-dependent increase in QΣ was counteracted. In addition, the significant correlation between DCS and TCH in this study must be regarded as doubtful because the difference in the mean TCH for animals with and without DCS was ~1°C, a value at the limit for the sensitivity of measurement.

The dVCH4 was significantly higher than the cVCH4 (Table 4.1, P < 0.01). A similar observation was reported by Kayar et al. (Kayar, et al., 1998a) in rats injected with H2 metabolizing microbes at a comparable time point in decompression. It is not clear if the increase is from trapped intestinal CH4 released due to volume expansion during the pressure reduction, or if the increased VCH4 indicates elevated levels of H2 delivered to the methanogens. If the latter is true, one possibility is that H2 comes out of solution and forms bubbles near the microbes where they have access to higher levels of substrate. There was no correlation between cVCH4 and PH2, as would be expected if the delivery to the microbes was restricted by diffusion. It is also possible that the considerable variability in cVCH4 between animals, and the possible error in the computation of VCH4, may disguise a correlation between VCH4 and PH2.

The total gas burden eliminated by the intestinal methanogens is a crucial link between the inert gas tissue tension and the DCS risk. Using the estimated VCH4, it is
possible to get a crude estimate of the total volume of H\textsubscript{2} removed, assuming that all CH\textsubscript{4} comes from metabolism of H\textsubscript{2} (Eq. 1, Chapter 1). Assuming the whole body solubility of H\textsubscript{2} to be 0.02 mL H\textsubscript{2} • g\textsuperscript{-1} tissue per atm at 37 °C (Weathersby and Homer, 1980), a 20-kg pig at 24 atm total pressure with an atmosphere with 90% H\textsubscript{2}, \textit{i.e.}, 21.6 atm PH\textsubscript{2}, should have ~ 8.64 liters of H\textsubscript{2}, or ~ 340 mmol H\textsubscript{2} (STP) in solution in its tissues when saturated. The average \( \dot{V}_{CH_4} \) in the animals releasing CH\textsubscript{4} was approximately 40 µmol CH\textsubscript{4} • min\textsuperscript{-1} (range 15-75 µmol CH\textsubscript{4} • min\textsuperscript{-1}) during the three hours at constant pressure (Table 4.1). Consequently, the methanogens were responsible for releasing approximately 7 mmol CH\textsubscript{4} during the three hour at 24.1 atm, which requiring a consumption of 4 times this amount of H\textsubscript{2} (Eq. 1, Chapter 1). The estimated removal of the gas volume is therefore ~ 8% (28/340; range 4-16%) of the total gas volume predicted to be the total gas body burden of H\textsubscript{2} in solution in these pigs. Even though this calculation is an oversimplification and contains several assumptions (Kayar, \textit{et al.}, 1998a), it gives an estimate of the gas volumes involved.

The previous study on biochemical decompression in rats reported that an estimated 5% reduction in the total H\textsubscript{2} body gas burden resulted in a ~ 50% reduction in the DCS incidence (Kayar, \textit{et al.}, 1998a). A similar observation has been made in human DCS research, where it has been discovered that small changes in the decompression time can reduce the DCS risk by 50% or more (Weathersby, \textit{et al.}, 1986). The overall implication appears to be that elimination of relatively small fractions of dissolved gas may have a surprisingly large impact on the DCS incidence.

In summary, it has been shown that the native intestinal flora of pigs is able to protect against DCS following a simulated H\textsubscript{2} dive. This animal model has shown that by
removing a portion of the inert gas dissolved in the tissues after a hyperbaric exposure, it is possible to reduce the DCS incidence. The magnitude of the possible reduction in DCS incidence is explored in the next chapter by injecting methanogens directly into the intestines.
Table 4.1. Outcome of decompression sickness (1 = DCS, 0 = no DCS) and values representing mean (± 1 stdev) for H₂ content (n = 3 chromatograph readings) in the chamber during the final 36 min at constant pressure; chamber temperature (T_{CH}, n = 3-15 temperature readings); animal weight; CH₄ concentration in the chamber during the final hour at constant pressure (c[CH₄], n = 5 chromatographic readings), mean CH₄ concentration in the chamber during the decompression (d[CH₄], n = 2-7 chromatographic readings); CH₄ release rate in chamber during compression (c \cdot \dot{V}_{CH₄}, \mu mol CH₄ • min⁻¹, n = 5) and mean CH₄ release rate after decompression (d \cdot \dot{V}_{CH₄}, \mu mol CH₄ • min⁻¹, n = 3). P-values represent one way ANOVA test comparing the three groups. † Animal not castrated. ‡ The d \cdot \dot{V}_{CH₄} was significantly higher than the c \cdot \dot{V}_{CH₄} in each animal (P < 0.001, Paired t-test).
<table>
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<tr>
<th>Pressure (atm)</th>
<th>Outcome</th>
<th>H₂ %</th>
<th>T&lt;sub&gt;CH&lt;/sub&gt; (°C)</th>
<th>Weight (kg)</th>
<th>[CH₄] (ppm)</th>
<th><em>V&lt;sub&gt;CH₄&lt;/sub&gt;</em> (μmoles CH₄ • min⁻¹)</th>
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<tr>
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<td></td>
<td></td>
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<td>c[CH₄]</td>
<td>d[CH₄]</td>
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<td>Value 2 ± Error 2</td>
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<td>Value 4 ± Error 4</td>
<td>Value 5 ± Error 5</td>
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| P    | > 0.33            | < 0.05            | > 0.34            | > 0.5             | > 0.5             | > 0.5             | > 0.7             |
Table 4.2. Logistic regression results for DCS outcome versus pressure (P), H₂ content (H₂ %), chamber temperature (T<sub>CH</sub>), H₂ pressure (P<sub>H₂</sub>), animal weight (Wt), mean CH₄ concentration in the chamber during the final hour at constant pressure (c[CH₄]), mean CH₄ concentration in the chamber during the decompression (d[CH₄]) and mean CH₄ release rate at constant pressure (c V CH₄). Values for the models are parameter estimates (± 1 SE), Wald statistics, log likelihood (LL), and likelihood ratio statistics results (P-value).

<table>
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<tr>
<th>Variables</th>
<th>β₀</th>
<th>β₁</th>
<th>Wald stat</th>
<th>LL</th>
<th>P-value</th>
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<td>Null</td>
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<td>-12.94</td>
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<tr>
<td>H₂ %</td>
<td>-2.44 ± 17.71</td>
<td>0.01 ± 0.20</td>
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<td>-12.94</td>
<td>&gt;0.9</td>
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<td>P</td>
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<td>PH₂</td>
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<td>-0.20 ± 0.34</td>
<td>0.56</td>
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<td>Wt</td>
<td>2.18 ± 8.06</td>
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<td>0.65</td>
<td>-12.83</td>
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<tr>
<td>T&lt;sub&gt;CH&lt;/sub&gt;</td>
<td>43.43 ± 22.53</td>
<td>-1.40 ± 0.70</td>
<td>0.05</td>
<td>-10.32</td>
<td>&lt;0.05</td>
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<tr>
<td>c[CH₄]</td>
<td>0.17 ± 0.86</td>
<td>-1.82 ± 0.96</td>
<td>0.06</td>
<td>-10.27</td>
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<tr>
<td>d[CH₄]</td>
<td>0.18 ± 0.82</td>
<td>-1.23 ± 0.64</td>
<td>0.06</td>
<td>-9.91</td>
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<tr>
<td>c V CH₄</td>
<td>-0.29 ± 0.72</td>
<td>-0.05 ± 0.03</td>
<td>0.10</td>
<td>-10.78</td>
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</table>

<table>
<thead>
<tr>
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<th>β₀</th>
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<th>β₂</th>
<th>LL</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c[CH₄] + T&lt;sub&gt;CH&lt;/sub&gt;</td>
<td>32.82 ± 23.48</td>
<td>-1.41 ± 0.99</td>
<td>-1.02 ± 0.74</td>
<td>-9.05</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>d[CH₄] + T&lt;sub&gt;CH&lt;/sub&gt;</td>
<td>34.63 ± 25.32</td>
<td>-0.96 ± 0.64</td>
<td>-1.08 ± 0.79</td>
<td>-8.78</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>c V CH₄ + T&lt;sub&gt;CH&lt;/sub&gt;</td>
<td>36.64 ± 23.44</td>
<td>-0.04 ± 0.03</td>
<td>-1.15 ± 0.73</td>
<td>-9.18</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>T&lt;sub&gt;CH&lt;/sub&gt; + P</td>
<td>48.23 ± 23.79</td>
<td>-1.93 ± 0.98</td>
<td>0.53 ± 0.59</td>
<td>-9.88</td>
<td>&gt;0.5</td>
</tr>
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</table>
Figure 4.1. Chamber CH$_4$ concentration ([CH$_4$]) and chamber H$_2$ pressure (PH$_2$) versus time for a sample dive to 22.3 atm.
Figure 4.2. Methane release rate (\( \dot{\text{V}}_{\text{CH}_4} \), \( \mu \text{moles CH}_4 \cdot \text{min}^{-1} \)) versus chamber CH\(_4\) concentration ([CH\(_4\), ppm] in animals at 22.3, 24.1, and 25.7 atm. A) The c \( \dot{\text{V}}_{\text{CH}_4} \) and c[CH\(_4\)] are mean values (n = 5 chromatographic readings) at constant pressure. B) The d \( \dot{\text{V}}_{\text{CH}_4} \) and d[CH\(_4\)] are mean values (n = 3 chromatographic readings) during decompression.
A graph showing the relationship between \([\text{CH}_4]\) (ppm) and \(\text{CH}_4\) Release Rate (\(\mu\text{mol CH}_4 \cdot \text{min}^{-1}\)).
Figure 4.3. Mean $\text{CH}_4$ release rate (c $\dot{V} \text{CH}_4$) for pigs that either displayed or did not display symptoms of decompression sickness (DCS) after hyperbaric exposure versus chamber $\text{H}_2$ pressure (PH$_2$). Solid line represents the least squares regression (not statistically significant) for all non-zero values of methane release rate ($Y = -29.04 + 3.86 \times X$, $P = 0.40$, $r^2 = 0.03$).
CH$_4$ Release Rate ($\mu$mol · min$^{-1}$)

PH$_2$ (atm)

No DCS

DCS
Figure 4.4. Probability of DCS [P(DCS)] versus CH$_4$ release rate ($c \dot{V}_{CH_4}$) from pigs with native intestinal flora at 22.3, 24.1, and 25.7 atm hydrox for 3 h. There was a negative correlation between increasing $c \dot{V}_{CH_4}$ and DCS incidence, as determined by logistic regression analysis (P < 0.05, log likelihood ratio test).
CH$_4$ Release Rate (µmoles CH$_4$ · min$^{-1}$)

Estimated Dose-Response

95% Confidence Regions
CHAPTER FIVE

INJECTION OF H₂-METABOLIZING MICROBES INTO THE INTESTINES OF PIGS REDUCES DECOMPRESSION SICKNESS RISK AFTER HYPERBARIC EXPOSURE TO H₂

“vae victis“
INTRODUCTION

In the previous chapter, a serendipitous finding was reported during a search for a compression and decompression sequence in H₂ that was going to give a high DCS incidence in untreated pigs. It was shown that pigs with higher methane release rate (V CH₄) during hyperbaric H₂ exposure had a significantly lower risk of decompression sickness (DCS). The release of CH₄ was suggested to be the product of H₂ metabolism by native hydrogenase-containing microbes in the intestinal flora, since this has been reported in domestic pigs (Robinson, 1989). The theory behind this observation was that the reduced DCS risk was due to microbial reduction of the body burden of H₂.

In this chapter, the possibility that enhancing the native gut flora of methanogens could further reduce the DCS risk is explored. Injections of live cultures of a H₂-metabolizing microbe, *Methanobrevibacter smithii*, were made into the intestines of pigs before hyperbaric exposure to H₂. The compression and decompression sequence used in this chapter differs from the previous chapter, resulting in a higher DCS incidence in untreated animals. The high-risk sequence used in this chapter makes it harder for the native flora of the H₂-metabolizing microbes alone to significantly enhance the H₂ removal. As described previously, Yorkshire pigs were chosen as the model animal for their many similarities to humans in cardiovascular and digestive anatomy and physiology (Lumb, 1966; Miller and Ullrey, 1987; Swindle, 1992).
MATERIALS & METHODS

Animals and treadmill training

Castrated male Yorkshire pigs (Sus scrofa, n = 36, mean body weight = 19.4 ± 1.3 kg, range 16.9 - 22.9 kg) were used for all experiments. The animals were randomly assigned to one of three groups: control (C), surgical control (SC), or treated (T). There was no difference in body weight between groups (one-way single factor ANOVA, P > 0.1). The animals were housed in an accredited animal care facility, fed once daily in the morning with a laboratory animal chow (Harlan Teklad, Madison, WI: 2% of body weight), and had an unlimited supply of water. On the day prior to an experiment, the animal was fed a second time in the afternoon. On the day of the experiment, no food was available to the animal until entry into the chamber. Experimental procedures were approved by an Animal Care and Use Committee, and the experiments reported were conducted according to the principles presented in the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 1996). Each animal was trained to walk on a treadmill prior to the hyperbaric exposure as described in Chapter 4. Treadmill performance was used to diagnose DCS (Chapter 4).

Culturing of the methanogens and activity assay

Cultures of M. smithii were prepared at the Department of Microbiology, University of Georgia, Athens, by Mr. W. Lin and Dr. W.B. Whitman, and shipped 1-3 days before use in the experiments. Upon arrival, each culture bottle was flushed for 2 min with H₂-CO₂ (80%/20 %), then stored at 4 °C under 3 atm H₂-CO₂ until the following morning when the microbes were injected into the animal. The in vitro
methanogenic activity was measured in duplicate; each assay done in a 15 mL assay bottle pressurized to 3 atm with H₂-CO₂ containing 0.2-0.3 mL bicarbonate-dithiothreitol (0.005M, DTT). A 100-300 µL subsample from the methanogen culture was injected into the assay bottle and incubated on a platform shaker (200 excursions • min⁻¹) at 37°C for 1 hour. The methane concentration in the gas phase was determined by a gas chromatograph (Hewlett-Packard 5890A, Series II, Wilmington, DE)(Pavlostathis, 1988), and the rate of gas production was calculated as an average for the hour.

Surgery for injection of *M. smithii* or saline

Animals were fed the afternoon before surgery and then fasted until surgery the following morning. Animals were pre-anesthetized by intramuscular injection with a mixture of ketamine (Ketaset®, Fort Dodge Animal Health, Fort Dodge, Iowa, 20 mg • kg⁻¹) and Xylazine (Rompun®, Bayer Co., Shawnee, MI, 2 mg • kg⁻¹). During surgery, anesthesia was maintained by gas inhalation using 1-4% iso-flurane (Aerrane®, Fort Dodge Animal Health, Fort Dodge, IO) balanced with O₂ by an automated anesthesia machine (A.D.S 1000, Engler Engineering Company, Hialeah, FL). Using aseptic surgical techniques, a midline abdominal incision of approximately 10 cm was made to expose the intestines. Injections (range 12-116 mL) of microbial culture or CO₂-bubbled saline (60 mL) were made into the middle and lower spiral colon and the caecum of each animal. The total volume injected was divided into equal parts for injection into the three different regions. Each injection puncture was closed using surgical cement (Vetbond, 3M, No. 1469, St Paul, MN). The intestines were moistened with sterile saline and returned to the abdominal cavity. The incision was closed using common suturing
techniques. Following surgery the animal was allowed to breathe 100% O\textsubscript{2} and given an intravenous injection of Yohimbine (Yobine\textsuperscript{®}, Lloyd Inc., Shenandoah, Iowa, 2 mg • kg\textsuperscript{-1}) to counteract the Xylazine. The animal was closely observed and allowed to recover for 1-3 h before the hyperbaric experiment.

\textit{Dive protocol}

For each hyperbaric experiment, one animal was placed in a dry hyperbaric chamber (5665 L internal volume, WSF Industries, Buffalo, NY), and the same chamber was used for all experiments. The animal was compressed as detailed in chapter 4.

The hyperbaric chamber was pressurized to 11 atm using pure He, with concomitant addition of O\textsubscript{2} to keep the chamber atmosphere normoxic (PO\textsubscript{2} = 0.2 - 0.4 atm). Upon reaching 11 atm, the chamber was flushed with H\textsubscript{2} for approximately 30 min. The chamber was further pressurized with H\textsubscript{2} to 24.1 atm at a rate of 0.45 atm • min\textsuperscript{-1}. There was no difference in PH\textsubscript{2} (P > 0.71) at 24.1 atm between the 3 experimental groups as determined by single factor ANOVA (Table 5.1).

Experimental pressure was maintained constant (± 0.3 atm) for 3 h by continuous addition of H\textsubscript{2} and O\textsubscript{2} to make up for the gas exhausted to the gas chromatograph (~115 L • min\textsuperscript{-1}). After 2.5 h at constant pressure, the treadmill was activated and the animal made to walk for 5 min to judge the animal’s normal gait before decompression.

Following the 3 h (± 30 sec) at constant pressure, the animal was decompressed at 0.90 atm • min\textsuperscript{-1} to 11 atm, a rate twice that used in Chapter 4. The animal was observed for 1 h at 11 atm for severe symptoms of DCS as previously described (Chapter 4).
The chamber temperature ($T_{CH}$, °C) was held at a seemingly comfortable level for the pigs: \(\approx 30^\circ\text{C}\) at 11 atm and \(\approx 33^\circ\text{C}\) at 24.1 atm. There was no difference in the $T_{CH}$ ($P > 0.16$) between the 3 groups, as determined by single factor ANOVA (Table 5.1).

**Extended time at pressure**

To determine any temporal changes in the CH$_4$ release rate during compression ($c \cdot \text{CH}_4$) for treated ($n = 3$) and control animals ($n = 1$), the chamber pressure was held constant at 24.1 atm for 19 - 24 h. The $c \cdot \text{CH}_4$ was regressed against time at constant pressure, allowing changes in the release rate to be quantified. Following the extended time at constant pressure, the animals were decompressed and observed as described above.

$\dot{V} \text{CH}_4$ at 1 atm

The $\dot{V} \text{CH}_4$ in 1 atm air was measured in a subset of untreated animals ($n = 45$), from the complete study (Chapters 4, 5 and 6). One animal at a time was placed in an airtight box with internal dimensions of 90 cm x 60 cm x 55 cm ($\approx 300$ L) made of plexiglass. Gas samples (approximately 500 \(\mu\text{L}\)) were taken with a gas-tight syringe from a sample hole covered with a rubber membrane. A 100 \(\mu\text{L}\) sample of the gas was injected into a gas chromatograph (HP 5890 Series II, Hewlett–Packard Co., Wilmington, DE) and analyzed for CH$_4$ concentration ([CH$_4$], ppm). The concentration of CH$_4$ was converted to \(\mu\text{mol CH}_4\) by correcting for the volume of the box, assuming that the animal displaced a volume (L) equal to its body weight (kg), and converting the values to
standard temperature and pressure. The CH₄ production rate was calculated as the change in CH₄ concentration over time (\( \dot{V} \text{CH}_4, \mu\text{mol CH}_4 \cdot \text{min}^{-1} \)). To avoid build-up of CO₂ in the box, the experiment was limited to 30 min, after which the lid of the box was taken off and the air in the box exchanged using a fan. Each experiment was made in duplicate and the \( \dot{V} \text{CH}_4 \) was taken as the average of the two independent measurements.

**Caecal Assay**

In a subsample of animals (n = 30), the \( \dot{V} \text{CH}_4 \) from the caecum was measured following the hyperbaric experiment. At this time, an abdominal incision was made to expose the intestines. The caecum was located, opened, and its contents emptied into a glass jar and covered with a lid. The total volume of the sample was measured by weighing the jar before and after collection of the sample. If necessary, the sample was diluted with bicarbonate-DTT, to allow 0.2 µL to be drawn and injected into an assay bottle. Duplicate samples were assayed in a fashion similar to the *in vitro* assay of *M. smithii* described above.

**Corrected \( \dot{V} \text{CH}_4 \)**

The \( \dot{V} \text{CH}_4 \) was estimated according to the method of Bartholomew et al. (Bartholomew, *et al.*, 1981); for a detailed explanation, see Chapter 3. The correction used a \( \Delta t \) of 120 min and a \( v \) determined by a flow meter as described earlier (Chapter 3).
Mathematical Analysis

It was desired to determine which variables had a significant effect on the DCS outcome. Since the dependent variable (presence or absence of DCS) is dichotomous, and multiple candidate independent variables were involved, multivariate logistic regression techniques were used (Hosmer and Lemeshow, 1989). The model determined the probability of DCS \[ P(\text{DCS}) \] using the following independent variables: body weight (Wt), chamber temperature (T\text{CH}_\text{CH}) , partial pressure of H\text{2} (P\text{H}_\text{2}) , methane release rate at constant pressure (c V CH\text{4}) , and total microbial activity injected (INJ). Initially it was determined which subset of variables to include in the multivariate model by performing univariate analysis on each independent variable and including only those variables with a P-value < 0.20 (Wald test). Variables passing this test were used in the multivariate analysis, where exclusion of a variable was based on the likelihood ratio test (Hosmer and Lemeshow, 1989).

Statistical Analysis

All estimates of variance are reported as standard deviations, and all comparisons of means are made with acceptance of differences at P < 0.05.
RESULTS

DCS outcome

Untreated control animals had a DCS incidence of 90% (9/10), which was not significantly different from the incidence of the surgical control group of 70% (7/10, P > 0.29, Fisher’s Exact test). Untreated and surgical control animals were therefore pooled in some analyses. Animals injected with methanogens had a 43% (6/14) DCS incidence which was significantly lower than the 80% (16/20) DCS incidence of the pooled control animals (P < 0.01, $\chi^2$ one-sided test) and the 70% incidence of the surgical control group alone (P < 0.05, $\chi^2$ one-sided test, Table 5.1, Fig. 5.1).

$\cdot \sqrt{CH_4}$ from animals

The [CH$_4$] (ppm) increased steadily for the duration of the dive for all animals. The $c \sqrt{CH_4}$ ($\mu$mol CH$_4$ • min$^{-1}$) estimated for each animal showed no apparent trends, and seemed to be constant for all animals during the 3 hours at 24.1 atm (Fig. 5.2a and b). During the dives with extended time at pressure, a temporal increase in the $c \sqrt{CH_4}$ was detected (Fig. 5.3). For animals at extended time a constant pressure, regression analysis indicated that the $c \sqrt{CH_4}$ increased with time for treated animals (Fig. 5.3).

There was no difference in the $c \sqrt{CH_4}$ between the untreated and surgical control groups (P > 0.8, Table 5.1), and the average $c \sqrt{CH_4}$ from the pooled control animals was $34.5 \pm 16.8$ $\mu$mol CH$_4$ • min$^{-1}$ (C + SC, Table 5.1). Animals injected with *M. smithii*
released an average of 91.7 ± 36.1 µmol CH₄ • min⁻¹ during the 3 hours at elevated pressure, which was significantly different from the pooled control animals (Table 5.1, P < 0.01 Mann-Whitney).

Treated animals received injections of *M. smithii* of varying total activity, ranging from 200-2200 µmol CH₄ • min⁻¹, with an mean of 978 ± 528 µmoles CH₄ • min⁻¹. The  \( \cdot \dot{V} \) CH₄ increased in animals injected *M. smithii* compared to control animals (Fig. 5.4). There was a significant increase in the  \( c \cdot V \) CH₄ as the total activity injected was increased from less than 500 µmol CH₄ • min⁻¹ to more than 500 µmol CH₄ • min⁻¹ (Fig. 5.4, P < 0.001). One animal, injected with a total activity of 240 µmol CH₄ • min⁻¹, was compressed to 24.1 atm using He (Table 5.2). This animal had a  \( c \cdot V \) CH₄ of 15.0 µmol CH₄ • min⁻¹, which was less than any other treated animal and most control animals (Table 5.1).

During the decompression phase, the CH₄ release rate (\( d \cdot V \) CH₄) could only be reliably estimated using animals that were kept for 1 hour at 11 atm; *i.e.* animals that did not show DCS symptoms. Animals with DCS were euthanised immediately upon diagnosis, not allowing enough data to be collected to accurately estimate the  \( \cdot V \) CH₄. For these animals (n = 13), there was a trend toward a lower CH₄ release rate during the decompression phase compared to the  \( c \cdot V \) CH₄ at 24.1 atm (P = 0.075, paired 2-tailed t-test, Table 5.3).
\[ \dot{V} \text{CH}_4 \text{ from caecal samples} \]

The caecal assay showed that the \textit{in vitro} \[ \dot{V} \text{CH}_4 \] from the animals injected with methanogens was significantly higher than from control animals by two orders of magnitude (Fig. 5.5, \[ P < 0.01 \]).

\[ \dot{V} \text{CH}_4 \text{ in 1 atm air versus hyperbaric H}_2 \]

The average \[ \dot{V} \text{CH}_4 \] in 1 atm air for a subset of untreated animals (\( n = 45 \), including animals from Chapter 4, 5, and 6), was \( 16.6 \pm 17.1 \) \( \mu \text{mol CH}_4 \cdot \text{min}^{-1} \) (Fig. 5.6). This was significantly less than the mean value of \( 34.5 \pm 16.8 \) \( \mu \text{mol CH}_4 \cdot \text{min}^{-1} \) for the pooled control animals (\( n = 20 \), Table 5.1, C+SC) in hyperbaric \( \text{H}_2 \) (\( P < 0.01 \)).

\textit{Logistic regression}

The univariate analysis suggested that \( T_{\text{CH}_4}, PH_2, \) and \( c \text{VCH}_4 \) were not predictors of the \( P(\text{DCS}) \) (Wald statistics, \( P < 0.20 \)). The variables considered for a multivariate model were INJ and Wt. Using the log-likelihood ratio test (LL), only total activity injected was a significant predictor of \( P(\text{DCS}) \) (\( P < 0.01 \), Table 5.4, Fig. 5.7).
DISCUSSION

The animals injected with methanogens (T) had a significantly lower DCS incidence following the chosen compression and decompression sequence, compared to either untreated control (C) or surgical control (SC) animals. There was no significant difference in DCS incidence between the C and SC animals, suggesting that the effect of the treatment was not an artifact caused by the surgical procedure. It has been suggested that immune activation plays a pivotal role in the etiology of DCS (Ersson, et al., 1998; Kayar, et al., 1997b). Therefore, it was important to show that the surgery was not responsible for the treatment effect. Even though the difference in the DCS incidence between the C and SC animals was not statistically significant, a surgical effect with the small sample sizes of the control groups (n = 10 per group) in this study cannot be completely excluded. However, because of the significant decrease in the DCS incidence between T and SC animals, any surgical effect should not affect the conclusion that injection of methanogens significantly reduced the DCS incidence.

The release of CH$_4$ most likely indicates that the injected microbes metabolized H$_2$, because mammalian tissues do not metabolize H$_2$ (Kayar, et al., 1994; Smith, et al., 1953). It is possible that the microbes utilized fuel sources other than H$_2$ in the CH$_4$ production, but H$_2$ is the preferred fuel source of H$_2$-metabolizing microbes (Jones, 1991; Miller, 1991). Yorkshire pigs have a native flora of methanogens capable of H$_2$ metabolism (Robinson, 1989), and substantial levels of CH$_4$ were released from animals in 1 atm air, suggesting that their methanogens have an endogenous source of H$_2$. This endogenous H$_2$ is usually produced by other species of intestinal microbes (Miller, 1991;
Miller and Wolin, 1973). The \( \dot{V} \text{CH}_4 \) was significantly higher for control animals in hyperbaric H\(_2\) compared to 1 atm air, showing that an increased access to H\(_2\) increased the \( \dot{V} \text{CH}_4 \). This has also been documented in rats injected with methanogens, in which the \( \dot{V} \text{CH}_4 \) increased with increasing PH\(_2\) (Kayar, et al., 1998a).

Animals injected with methanogens had a significantly higher \( \dot{V} \text{CH}_4 \) compared to controls (Table 5.1). There was a significant increase in the \( \dot{V} \text{CH}_4 \) as the total activity injected was increased from \(< 500 \, \mu\text{mol CH}_4 \, \text{min}^{-1}\) to \(> 500 \, \mu\text{mol CH}_4 \, \text{min}^{-1}\), supporting a dose response effect (Fig. 5.4). Injection of H\(_2\)-metabolizing microbes in a hyperbaric He atmosphere did not increase the \( \dot{V} \text{CH}_4 \) (Table 5.2), but was comparable to the mean \( \dot{V} \text{CH}_4 \) from untreated animals measured in 1 atm air (Fig. 5.6). Since only the hyperbaric H\(_2\) environment increased the \( \dot{V} \text{CH}_4 \) in treated animals, this suggests that the increased levels of \( \dot{V} \text{CH}_4 \) in hyperbaric H\(_2\) are due mainly to microbial metabolism of inhaled H\(_2\), whereas a fraction may be from endogenous sources.

The post-dive caecal assays showed that the \textit{in vitro} \( \dot{V} \text{CH}_4 \) was significantly higher by two orders of magnitude in the animals receiving injections of methanogens compared to untreated animals (Fig. 5.5). It was not possible to use the caecal assays to quantify the methanogenic activity during the experiment because samples were taken only from the caecum, whereas injections were made to both the caecum and along the large intestine. Furthermore, peristalsis more than likely transported a large part of the injected microbes down the intestines during the hyperbaric experiment. However, it was
important to measure caecal \( \dot{V}CH_4 \) to demonstrate that viable methanogens were still residing in the intestines after the experiment.

It was hypothesized that the reduction in DCS incidence in treated animals would correlate with the methanogenic activity injected into the animal (INJ). To test this hypothesis, logistic regression techniques were applied. The multivariate analysis indicated that microbial activity injected into the intestines of the pigs (INJ) was significantly correlated with DCS incidence (Table 5.4). Thus, injection of a H\(_2\) metabolizing microbe appears to decrease DCS incidence in a dose-dependent trend during simulated H\(_2\) dives in a pig model (Fig. 5.7).

There was a significant correlation between the activity injected and the \( \dot{V}CH_4 \). If all H\(_2\) metabolism is a product of methanogenesis (Eq. 1), and if there is a good correlation between H\(_2\)-metabolism and CH\(_4\) release from pigs into the chamber, then \( \dot{V}CH_4 \) should be correlated with DCS outcome. The logistic regression analysis did not support this correlation (Table 5.4). The daily variability in the pre-dive \( \dot{V}CH_4 \) (unpublished observation), and the potential that not all H\(_2\) metabolism was due to methanogenesis by \textit{M. smithii} are factors that may mask a correlation between \( \dot{V}CH_4 \) and DCS outcome. Some studies have shown that methanogenesis (Eq. 1, Chapter 1) is the major route of H\(_2\) metabolism in the human intestine (Strocchi, \textit{et al.}, 1994; Strocchi, \textit{et al.}, 1991), while others suggest that sulphate reduction (Christl, \textit{et al.}, 1992; Gibson, \textit{et al.}, 1988) or acetogenesis (De Graeve, \textit{et al.}, 1994; De Graeve, \textit{et al.}, 1990) may predominate in the human or animal intestine depending on the feed (Jouany, 1994; Zawadzki, 1986; Zawadzki, 1986), and the intestinal pH (Gibson, \textit{et al.}, 1990).
Consequently, metabolism of endogenous H\textsubscript{2} is more complex than a simple correlation between the metabolism of H\textsubscript{2} and the \( \dot{\text{V}} \text{CH}_4 \) suggests (Eq. 1). In addition, there are several factors complicating the estimation of H\textsubscript{2} metabolism inside the pig based on the c \( \dot{\text{V}} \text{CH}_4 \). The kinetics of the release of CH\textsubscript{4} remain speculative, and may not directly correlate with the rate of H\textsubscript{2} metabolism. Furthermore, accurately estimating the \( \dot{\text{V}} \text{CH}_4 \) during the dive is complicated by the large volumes of chamber gas (> 135000 L at 24.1 atm), and the necessarily slow exhaust rate of the chamber (see Chapter 3). Even though the [CH\textsubscript{4}] continuously increases smoothly throughout the experiment (Fig. 4.1, Chapter 4), correcting the values to a \( \dot{\text{V}} \text{CH}_4 \) may result in large variability (Fig. 5.2A and 5.2B). Any or all of these factors could explain why the logistic regression analysis did not distinguish c \( \dot{\text{V}} \text{CH}_4 \) as a significant predictor of DCS risk (Table 5.4).

With extended time at elevated pressure, the tissue PH\textsubscript{2} increases until an equilibrium between the tissues and the ambient environment has been reached, \textit{i.e.} saturation. Prior to saturation, the availability of H\textsubscript{2} to the methanogens in the intestine will continuously increase. Furthermore, \textit{M. smithii} has an \textit{in vitro} generation time of approximately 8 hours in monocultures with optimal growth conditions (Dr. W. Whitman, personal communication). Consequently, it was expected that the c \( \dot{\text{V}} \text{CH}_4 \) would increase temporally, primarily as an effect of increasing substrate availability as the tissue PH\textsubscript{2} increased, and secondarily due to an increasing population of \textit{M. smithii}. The large variability in the c \( \dot{\text{V}} \text{CH}_4 \) between individual animals during the 3 hours under pressure made it impossible to distinguish any temporal trends in the c \( \dot{\text{V}} \text{CH}_4 \). To further
investigate temporal trends, experiments were done with time at pressure prolonged up to 24 hours. Based on these results, it was shown that the methanogens remained viable and increased their $c\dot{\nu}CH_4$ when exposed to hyperbaric H$_2$ up to at least 24 hours (Table 5.2).

There is another possible explanation for the temporal increase in $c\dot{\nu}CH_4$ over 24 hours. *Methanobrevibacter smithii* tend to keep the H$_2$ concentration low in their immediate vicinity; neighboring microbes thus suppress each others’ metabolism (W. Whitman, personal communication). During an extended time under pressure, the injected suspension would spread over a larger intestinal surface area, possibly giving the methanogens more access to H$_2$. This, in turn, would increase the efficacy of H$_2$ removal over time, possibly further reducing the $P_{tis}H_2$.

The significant decrease in DCS incidence in treated versus control animals and the dose-response effect of the injected methanogens demonstrate the feasibility of biochemical decompression. A study using rats reached the same conclusion observing approximately the same magnitude of reduction in DCS (Kayar, et al., 1998a).

Nonetheless, the physiological process behind the reduction of DCS by biochemical decompression is open to debate. A working hypothesis is that the microbial H$_2$ metabolism in the intestines operates as a sink continuously removing H$_2$, resulting in a lower $P_{tis}H_2$ in the treated animals compared to untreated animals (Fig. 5.8A and B treated versus control). This hypothesis will be explored in the following chapter, in which a probabilistic model was created to include parameters on risk assessment, gas kinetics, and H$_2$ metabolism. This model allows a measurable advantage of biochemical
decompression to be predicted for hyperbaric H$_2$ exposures. Furthermore, this measurement will be based on a fundamental physiological process.
Table 5.1. Outcome (1 = DCS, 0 = No DCS), chamber H₂ pressure (PH₂), animal weight, total methanogenic activity injected into intestines (INJ), mean CH₄ release rate at constant pressure (c \( \dot{V} \text{CH}_4 \)), and mean chamber temperature (T_{CH}) for untreated control (C), surgical control (SC), and treated animals (T) at 24.1 atm. P-values represent single factor ANOVA analysis for the three groups.
<table>
<thead>
<tr>
<th>Group</th>
<th>Outcome</th>
<th>PH₂ (atm)</th>
<th>Weight (kg)</th>
<th>Total activity injected</th>
<th>Rate cV CH₄ (µmol CH₄ • min⁻¹)</th>
<th>T CH (°C)</th>
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<td>20.9 ± 3.2</td>
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<td>32.6 ± 0.4</td>
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<tr>
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<tr>
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<td>33.1 ± 1.6</td>
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<tr>
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<tr>
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<tr>
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<td>&gt; 0.10</td>
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<td>&gt; 0.16</td>
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Table 5.2. Outcome (1 = DCS, 0 = No DCS), chamber \( H_2 \) pressure (PH\(_2\)), animal weight, total methanogenic activity injected into intestines (INJ), mean \( CH_4 \) release rate during the first three hours at constant pressure (tc \( \dot{V}_{CH_4} \)), or during the last three hours at constant pressure (le \( \dot{V}_{CH_4} \)), and mean chamber temperature (T\(_{CH}\)) for untreated control (C), and treated animals (T) at 24.1 atm. The groups are either animals at extended time at constant pressure (19 or 24 h) or in an He atmosphere with no \( H_2 \).

<table>
<thead>
<tr>
<th>Group</th>
<th>Outcome</th>
<th>PH(_2) (atm)</th>
<th>Weight (kg)</th>
<th>INJ</th>
<th>tc ( \dot{V}_{CH_4} ) (µmol CH(_4) • min(^{-1}))</th>
<th>le ( \dot{V}_{CH_4} ) (µmol CH(_4) • min(^{-1}))</th>
<th>T(_{CH}) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-19 h</td>
<td>1</td>
<td>22.1</td>
<td>21.4</td>
<td>0</td>
<td>0 ± 0</td>
<td>21.8 ± 6.8</td>
<td>32.6 ± 0.5</td>
</tr>
<tr>
<td>T-24 h</td>
<td>1</td>
<td>21.2</td>
<td>21.1</td>
<td>925</td>
<td>74.7 ± 147.7</td>
<td>262.9 ± 38.6</td>
<td>32.8 ± 0.5</td>
</tr>
<tr>
<td>T-24 h</td>
<td>0</td>
<td>22.4</td>
<td>21.0</td>
<td>1168</td>
<td>146.4 ± 17.7</td>
<td>274.2 ± 55.2</td>
<td>31.2 ± 1.1</td>
</tr>
<tr>
<td>T-24 h</td>
<td>0</td>
<td>21.2</td>
<td>19.6</td>
<td>253</td>
<td>60.2 ± 8.7</td>
<td>194.0 ± 40.4</td>
<td>32.5 ± 0.5</td>
</tr>
<tr>
<td>T-He</td>
<td>0</td>
<td>0.0</td>
<td>20.7</td>
<td>240</td>
<td>15.0 ± 9</td>
<td>15.0 ± 9</td>
<td>32.6 ± 0.5</td>
</tr>
</tbody>
</table>
Table 5.3. CH$_4$ release rate at constant pressure ($c\,\dot{\nu}\,CH_4$) and during decompression ($d\,\dot{\nu}\,CH_4$) for surgical control (SC), untreated control (C), and treated (T) animals showing no symptoms of DCS. P-value represents paired t-test of $c\,\dot{\nu}\,CH_4$ versus $d\,\dot{\nu}\,CH_4$ for all animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>$c,\dot{\nu},CH_4$ ($\mu$mol CH$_4$ • min$^{-1}$)</th>
<th>$d,\dot{\nu},CH_4$ ($\mu$mol CH$_4$ • min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>79.8 ± 27.0</td>
<td>59.1 ± 16.3</td>
</tr>
<tr>
<td>T</td>
<td>95.7 ± 11.5</td>
<td>94.6 ± 63.7</td>
</tr>
<tr>
<td>T</td>
<td>106.2 ± 17.7</td>
<td>53.3 ± 12.6</td>
</tr>
<tr>
<td>T</td>
<td>77.8 ± 30.3</td>
<td>52.6 ± 38.4</td>
</tr>
<tr>
<td>T</td>
<td>85.3 ± 8.1</td>
<td>39.5 ± 6.0</td>
</tr>
<tr>
<td>T</td>
<td>63.4 ± 23.5</td>
<td>93.3 ± 31.7</td>
</tr>
<tr>
<td>T</td>
<td>82.6 ± 28.1</td>
<td>66.8 ± 26.1</td>
</tr>
<tr>
<td>T</td>
<td>79.3 ± 6.2</td>
<td>66.5 ± 3.3</td>
</tr>
<tr>
<td>T</td>
<td>85.8 ± 20.6</td>
<td>62.8 ± 8.7</td>
</tr>
<tr>
<td>SC</td>
<td>52.1 ± 10.6</td>
<td>35.5 ± 17.2</td>
</tr>
<tr>
<td>SC</td>
<td>29.0 ± 10.7</td>
<td>50.4 ± 14.3</td>
</tr>
<tr>
<td>SC</td>
<td>20.9 ± 3.2</td>
<td>12.7 ± 4.3</td>
</tr>
<tr>
<td>C</td>
<td>10.6 ± 9.9</td>
<td>17.1 ± 9.0</td>
</tr>
<tr>
<td>Mean T (± 1 stdev)</td>
<td>84.0 ± 11.9</td>
<td>65.4 ± 18.2</td>
</tr>
<tr>
<td>Mean SC + C (± 1 stdev)</td>
<td>28.2 ± 17.7</td>
<td>28.9 ± 17.4</td>
</tr>
<tr>
<td>Mean All (± 1 stdev)</td>
<td>68.8 ± 29.9</td>
<td>54.2 ± 25.6</td>
</tr>
<tr>
<td>P</td>
<td>0.075</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4. Logistic regression results for total methanogenic activity injected (INJ, \(\mu\text{mol CH}_4 \cdot \text{min}^{-1}\)), animal weight (Wt, kg), chamber H\(_2\) pressure (PH\(_2\), atm), CH\(_4\) release rate at constant pressure (\(c \sqrt[4]{\text{CH}_4}\)), chamber temperature (T\(_{\text{CH}}\)) versus DCS outcome. Parameter estimates (± SE), Wald statistics, log likelihood, and P-value for log likelihood ratio test compared to null model. The analysis was made on animals at a similar compression and decompression sequence; i.e. the animals in Table 5.1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Intercept</th>
<th>Slope</th>
<th>Wald</th>
<th>LL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>0.57 ± 0.35</td>
<td></td>
<td></td>
<td>-23.546</td>
<td></td>
</tr>
<tr>
<td>INJ</td>
<td>1.33 ± 0.50</td>
<td>(-1.60 ± 0.68) (\times 10^{-3})</td>
<td>0.019</td>
<td>-20.216</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Wt</td>
<td>-9.38 ± 6.24</td>
<td>0.52 ± 0.33</td>
<td>0.112</td>
<td>-22.061</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>PH(_2)</td>
<td>0.60 ± 14.94</td>
<td>(-1.00 ± 684) (\times 10^{-3})</td>
<td>&gt; 0.9</td>
<td>-23.538</td>
<td>&gt; 0.9</td>
</tr>
<tr>
<td>(c \sqrt[4]{\text{CH}_4})</td>
<td>1.00 ± 0.66</td>
<td>(-7.04 ± 9.01) (\times 10^{-3})</td>
<td>0.316</td>
<td>-23.019</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>T(_{\text{CH}})</td>
<td>-18.79 ± 24.33</td>
<td>0.59 ± 0.74</td>
<td>0.426</td>
<td>-23.222</td>
<td>&gt; 0.1</td>
</tr>
<tr>
<td>INJ</td>
<td>-5.97 ± 6.88</td>
<td>(-1.42 ± 0.69) (\times 10^{-3})</td>
<td>0.040</td>
<td>-19.610</td>
<td>&gt; 0.1</td>
</tr>
</tbody>
</table>
Figure 5.1. Decompression sickness (DCS) incidence in animals injected with methanogens (T; n = 16), surgical controls (SC; n = 10), and untreated controls (C; n = 10), with 95% binomial confidence limits. * C significantly different from T, and ** SC significantly different from T ($\chi^2$, $P < 0.05$).
Figure 5.2. Methane release rate ($\mu$mol CH$_4$ • min$^{-1}$) and chamber H$_2$ pressure (PH$_2$) versus elapsed time during a sample dive for (A) a treated animal and (B) a control animal showing the absence of a visible temporal increase in the $\dot{V}$CH$_4$ during 3 h at constant pressure for either animal.
Graph B shows the relationship between elapsed time (min) and CH₄ release rate (µmol CH₄·min⁻¹). The release rate increases sharply initially, peaks around 150 minutes, and then decreases gradually. The PH₂ atm values are also indicated on the graph, though their specific relationship to time and CH₄ release rate is not clearly shown in the provided data.
Figure 5.3. Estimated CH$_4$ release rate ($c \cdot V_{CH_4}, \pm 1$ stddev, $\mu$mol CH$_4$ • min$^{-1}$) versus elapsed time for one control animal (A) and three treated animals (B, C, D) during extended time at constant pressure. There was a significant temporal increase in the $c \cdot V_{CH_4}$ for all treated animals ($P < 0.001$, linear regression).
Figure 5.4. Range of total methanogenic activity injected into the intestines of pigs (µmol CH₄ • min⁻¹) versus CH₄ release rate (µmol CH₄ • min⁻¹) at 24.1 atm. The data are from animals in Chapter 4, 5, and 6 compressed to a maximum pressure of 24.1 atm for 180 min. * There was a significant difference as compared to animals with 0 µmol CH₄ • min⁻¹ injected (P < 0.0001, Mann-Whitney). **There was a significant difference as compared to animals with 500< µmol CH₄ • min⁻¹ injected (P < 0.05, Mann-Whitney).
Range of activity Injected (µmoles CH$_4$ · min$^{-1}$)

<table>
<thead>
<tr>
<th>Activity Range</th>
<th>CH$_4$ Release Rate (µmoles CH$_4$ · min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>500&lt;</td>
<td>50</td>
</tr>
<tr>
<td>500&lt;x&lt;1000</td>
<td>100</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>150</td>
</tr>
</tbody>
</table>

**n = 6**

**n = 11**

*n = 6*

*n = 30*
Figure 5.5. *In vitro* methanogenic activity of caecal samples from control (n = 16) and treated animals (n = 14). *Statistically different from treated at P < 0.05 (Mann-Whitney).
Caecal Methanogenic Activity (µmol CH\textsubscript{4} · min\textsuperscript{-1})

Group

- Treated
- Control
Figure 5.6. Methane release rate from untreated pigs in 1 atm air (n = 45) versus hyperbaric H₂ (n = 20). *Statistically different from 1 atm air at P < 0.05 (2 tailed t-test).
CH₄ Release Rate (umol CH₄ • min⁻¹)

1 atm air  Hyperbaric H₂

*
Figure 5.7. Total methanogenic activity injected versus predicted decompression sickness incidence [P(DCS)] as determined by logistic regression (P < 0.05) for treated and control animals at 24.1 atm in H$_2$ for 3 h.
Total Activity Injected (µmol CH₄· min⁻¹)

Predicted P(DCS)
95% Confidence Limits
Figure 5.8. The figure shows the current working hypothesis on how $H_2$ biochemical decompression works as a sink, ultimately reducing the $H_2$ inert gas tissue tension and enhancing removal of $H_2$.

During exposure to a hyperbaric $H_2$ environment of 100 units, $H_2$ is delivered to all regions of the body via the arterial blood supply. The units in these figures are hypothetical.

The uptake or removal of $H_2$ in the various regions of the body is dependent on the local perfusion rate and the difference in the arterial and tissue concentration of $H_2$. During compression (A) in an untreated animal, the arterial blood is loaded with $H_2$ at the gas exchange surface. The alveolar $PH_2$ is somewhat less than the ambient $PH_2$, until the tissues have reached saturation. The $PH_2$ of alveolar gas is determined by a balance of two processes: the removal of $H_2$ by the capillary blood to the tissues and the replenishment by alveolar ventilation. By the time the blood leaves the lungs, the alveolar $PH_2$ is in complete equilibrium with the blood ($P_{\text{blood}}$). The $H_2$ in the blood diffuses into the tissues until the blood and tissues are in equilibrium. Saturation is reached when $P_{\text{blood}}$, $P_{\text{tis}}$, and $P_{\text{amb}}$ are all equal. In the treated animal, the process is slightly different. Microbial metabolism of $H_2$ in the intestines works as a sink, resulting in a mixed venous return that will have a lower $PH_2$ compared to the untreated animal. As the venous blood reaches the lung, the alveolar $PH_2$ will be somewhat lower than in the untreated animal, assuming that the ventilation is the same in the treated and untreated animal. The result is a slightly lower arterial $PH_2$ compared to the untreated animal. The overall magnitude is dependent on the efficacy of the metabolism of $H_2$. The result is a prolonged time to equilibrium, and a somewhat lower $P_{\text{tis}}H_2$ at equilibrium and a chronically subsaturated state.

During decompression (B), the fluxes of inert gases are reversed. In the case of the untreated animal, $H_2$ is transported from the tissues to the blood and via the blood to the gas exchange surface. This leads to a continuous decrease in the $P_{\text{tis}}H_2$. In a treated animal, there is a dual removal of $H_2$; one from the intestines and one from the gas exchange surface. This enhances the reduction of the $P_{\text{tis}}H_2$ in the treated animal. The lower overall $P_{\text{tis}}H_2$ of the mixed venous blood in the treated animal may result in a lower risk of bubble formation as compared to the untreated animal, thereby reducing the $P(\text{DCS})$. 
A

**Standard Compression** $P_{amb}=100$ Units $H_2$

45 Units $H_2$

Tissue

Mixed Venous Return

Intestines

Gas Exchange Surface

Blood

Exterior Gas

Alveolar Gas

**Treated Compression**

42 Units $H_2$

Tissue

Mixed Venous Return

Intestines

Gas Exchange Surface

Blood

Exterior Gas

Alveolar Gas
**Standard Decompression** $P_{amb} = 50$ Units $H_2$

**Treated Decompression**
CHAPTER SIX

ON THE LIKELIHOOD OF DECOMPRESSION SICKNESS DURING H$_2$ BIOCHEMICAL DECOMPRESSION

“Every science, as it grows towards perfection become mathematical in its ideas.”

Alfred North Whitehead
INTRODUCTION

Modeling of decompression sickness (DCS) risk has been confounded by the inability to correlate physiological variables with DCS risk. Some studies have tried to find a correlation between DCS risk and variables such as body temperature, body weight, exercise, gender, obesity, age, serum cholesterol, sensitivity to complement activation, Doppler bubble grades and patent foramen ovale (Broome, et al., 1995; Germonpre, et al., 1998; Jain, 1990; Lillo, et al., 1985; Lillo, 1988; Lillo, et al., 1997; Robertson, 1992; Vann, 1990). However, where some studies have found a correlation, others refute those results (Jain, 1990). The only physiological variable that has been successfully correlated with DCS risk in rats is body weight (Lillo, et al., 1985; Lillo, 1988; Lillo, et al., 1997). Since reliable physiological correlates are lacking (Broome, et al., 1995; Germonpre, et al., 1998; Jain, 1990; Robertson, 1992; Vann, 1990), researchers have used a variety of models based solely on the physical history of the compression and decompression sequence to find variables that can predict the probability of DCS (Thalmann, et al., 1997; Tikuisis, et al., 1990; Tikuisis, et al., 1988; Weathersby, et al., 1985; Weathersby, et al., 1984; Weathersby, et al., 1986; Weathersby, et al., 1992).

The DCS risk assessment in this study builds upon previously published models used in DCS research (Thalmann, et al., 1997; Weathersby, et al., 1985; Weathersby, et al., 1984; Weathersby, et al., 1992). The goal is to estimate the beneficial effects on DCS risk by the active removal of tissue H2 by injection of H2-metabolizing microbes into the intestines of pigs during simulated H2 dives. The metabolism of H2 in the intestine is readily followed by measuring the release of CH4, the metabolic end product (Eq. 1, Chapter 1), from the pig. As a result, this study is using a measured physiological
variable together with the physical history of the hyperbaric exposure to predict the DCS incidence.

**Model**

A probabilistic model was defined, connecting the instantaneous risk to the probability of DCS \( P(\text{DCS}) \). The risk is described based on the pressure history of the dive (Weathersby, *et al.*, 1985; Weathersby, *et al.*, 1984; Weathersby, *et al.*, 1992), and uses the method of maximum likelihood to identify the best-fitting parameters (Collett, 1994; Weathersby, *et al.*, 1984). In these models, the outcome data for each hyperbaric exposure were used to estimate the model parameters. As a result, the parameters are a mathematical composition and have only limited physiological value (Ball, *et al.*, 1999).

The model presented here differs from earlier models in that a parameter for the microbial metabolism of \( \text{H}_2 \) is included. In constructing this model, the microbial metabolism of \( \text{H}_2 \) was considered to have a direct physiological effect by influencing the gas kinetics. The measure of \( \text{H}_2 \) metabolism was based on either the total activity injected into the animals (INJ), or as the \( \text{CH}_4 \) release rate (\( V_{\text{CH}_4} \)) from individual animals, assuming that there is a direct correlation between the \( \text{H}_2 \) metabolized inside the intestines and the release of \( \text{CH}_4 \) from the intact animal (Eq. 1, Chapter 1). This model will be able to quantitatively predict the advantage of biochemical decompression for hyperbaric \( \text{H}_2 \) exposures in general, and offers a predictive advantage over the descriptive logistic regression model that was derived earlier (Fahlman, *et al.*, 1999b).
**DCS risk assessment**

Experience has shown that elevated pressure, longer exposure to elevated pressure, and increasing decompression rate all increase the risk of DCS (Hills, 1977; Weathersby, *et al.*, 1992). However, the occurrence is seldom either a certainty or zero for any hyperbaric exposure (Weathersby, *et al.*, 1992). Therefore, researchers have used probabilistic models to predict the P(DCS) in dives with varying compression and decompression profiles (Tikuisis, *et al.*, 1988; Weathersby, *et al.*, 1985; Weathersby, *et al.*, 1984; Weathersby, *et al.*, 1992). The probabilistic models previously used were constructed to be well-behaved in the sense that the P(DCS) was predicted to be 0 when no pressure reduction occurred, and increased with increasing pressure reduction (Weathersby, *et al.*, 1992).

The probability of having DCS symptoms at a time t, during or after a hyperbaric exposure, is defined as:

\[
P(DCS) = 1.0 - \exp\left(-\int_0^t r(x) \, dx \right) \tag{1}
\]

while freedom of symptoms until time t is defined as

\[
P(no\ DCS) = 1.0 - P(DCS) = \exp\left(-\int_0^t r(x) \, dx \right) \tag{2}
\]

where \(r\) is the instantaneous risk (Collett, 1994; Elandt-Johnson and Johnson, 1980; Kalbfleisch and Prentice, 1980), and \(r \geq 0\). The \(r\) depends on the theory used to describe the mechanism of DCS, and can be one of several measures integrated over the dive and post dive period.

The probability of developing DCS for a particular hyperbaric exposure is defined as the integrated \(r\) over the exposure period through the end of the post decompression
observation period (Tend). Early research using these models for estimating P(DCS) from human data sets integrated \( r \) for all observations in the data set for approximately 24 hours (Weathersby, et al., 1985). In other words, no concern was given to the time of symptom onset for the cases where DCS occurred. This approach was shown to be insensitive to the shape of \( r \) and unable to predict the time DCS is likely to occur. The improvement involved incorporating the time of DCS into the model (Weathersby, et al., 1992). Consequently, if DCS is observed, the following formula was used:

\[
P(DCS) = \left[ \exp\left(\int_0^{T_1} r \, dt\right) \right] \left[ 1.0 - \exp\left(\int_{T_1}^{T_2} r \, dt\right) \right]
\]

Eq. 3

This is the product of the probability of DCS not occurring in the interval \([0-T_1]\) and the probability of DCS occurring in the interval \([T_1-T_2]\) (Thalmann, et al., 1997; Weathersby, et al., 1992). \( T_1 \) is defined as the last known time the diver was definitely free of DCS symptoms, and \( T_2 \) is the time the diver was declared to have definite signs of DCS (Thalmann, et al., 1997; Weathersby, et al., 1992). At the start of the hyperbaric experiment (time 0, Eq. 1-3) every animal is definitely free from DCS. For an animal with no DCS, Eq. 1 is used to estimate the P(DCS) with the upper limit for integration at the end of the post decompression observation period (Tend). For an animal displaying DCS, Eq. 3 is used and the integration is performed until the time the animal definitely shows signs of DCS (T2).

**Choice of T1**

The choice of \( T_1 \) can be made in various ways as detailed elsewhere (Weathersby, et al., 1992). Due to subjective aspects inherent in diagnosing DCS, in this study \( T_1 \) is set to the start of the decompression. This approach is conservative and may result in
some loss of temporal information. However, since T1 is often difficult to determine, this approach seems warranted.

**Definition of r**

It is widely accepted that the presence of gas bubbles in tissues leads to DCS (Hills, 1977). Given that premise, it may be most appropriate to use a risk function in which the greatest $r$ occurs immediately after a decompression step (Weathersby, *et al*., 1992). Alternatively, if one prefers the theory that DCS develops after the bubbles have grown to a certain size (Van Liew and Hlastala, 1969), or that they trigger an immune or haematological response (Kayar, *et al*., 1997b; Shinomiya, *et al*., 1999; Stevens, *et al*., 1993; Zhang, *et al*., 1991), $r$ may slowly rise to a maximum and finally decrease. Unrelated to the theory used, when $r$ is zero there should be no occurrence of DCS, and as $r$ increases so should the DCS incidence (Weathersby, *et al*., 1992).

**Models**

Two models were tested, both describing $r$ from a single tissue. One model used the speculation that the maximum $r$ develops immediately after a decompression step (model 1, Eq. 4, Fig. 1A). The second model (model 2, Eq. 5, Fig. 1B) used the hypothesis that $r$ slowly increases to a maximum value after which it decreases.
METHODS

Model 1

The instantaneous risk \( r_1 \) is defined as the relative difference between the tissue tension \( P_{\text{tis}}, \text{atm} \) and the absolute ambient pressure \( P_{\text{amb}}, \text{atm} \) above a threshold \( \text{Thr} \) (Thalmann, et al., 1997; Weathersby, et al., 1985; Weathersby, et al., 1992),

\[
    r_1 = G_1 \cdot (P_{\text{tis}} - P_{\text{amb}} - \text{Thr}) \cdot P_{\text{amb}}^{-1} \quad \text{Eq. 4}
\]

where \( P_{\text{tis}} \) refers to the sum of the tissue tensions for \( \text{H}_2 \) \( (P_{\text{tis}}_{\text{H}_2}, \text{atm}) \) and \( \text{He} \) \( (P_{\text{tis}}_{\text{He}}, \text{atm}) \), and \( G_1 \) is a scaling factor \( (\text{min}^{-1}) \) to be determined from the fitting procedure. The inclusion of a threshold parameter has been shown to improve the fit for some human data sets (Weathersby, et al., 1985), while this parameter has not been very successful in describing others (Thalmann, et al., 1997). The contributions of \( \text{O}_2, \text{CO}_2 \), and water vapour were ignored (Weathersby, et al., 1987). In this model, \( r_1 \) is constrained to be \( \geq 0 \), and accordingly, \( r_1 \) will be set to zero at any time \( P_{\text{amb}} > P_{\text{tis}} \) (Weathersby, et al., 1985; Weathersby, et al., 1992).

Model 2

The second model has the same structure as model 1, but here the relative supersaturation is integrated (Weathersby, et al., 1992).

\[
    r_2 = G_2 \cdot \int_0^1 (P_{\text{tis}} - P_{\text{amb}} - \text{Thr}) \cdot P_{\text{amb}}^{-1} \, dx \quad \text{Eq. 5}
\]

For this model, the relative supersaturation is integrated from the first occurrence that \( P_{\text{tis}} > P_{\text{amb}} \), followed by integration of both positive and negative values. However, the risk cannot be negative, so \( r_2 \) is constrained to be \( \geq 0 \). For this model, \( G_2 \) is a scaling factor.
and is expected to be smaller than \( G_1 \) due to the second integration (Weathersby, \textit{et al.}, 1992).

The maximum value of \( r_1 \) is reached just after the decompression step (Fig. 1A), whereas \( r_2 \) increases slowly to reach its maximum value later (Fig. 1B).

**Tissue inert gas tension (\( P_{\text{tis}} \))**

A single exponential kinetics model was used to describe the tissue tensions of the inert gases assuming a single compartment. In other words, the animal was considered to be composed of a single tissue, with a single perfusion rate, and tissue gas solubility. A more complex model is required to describe real tissues, in which the diver is assumed to be composed of several tissues with varying perfusion rates and gas solubilities (Homer, \textit{et al.}, 1990; Novotny, \textit{et al.}, 1990). For this study, the models assumed that the gas uptake and elimination followed symmetrical exponential kinetics, although models using exponential uptake followed by linear elimination have proven to correlate better with some human data (Thalmann, \textit{et al.}, 1997).

The differential equation used to describe the inert gas tissue tension was as follows:

\[
\frac{dP_{\text{tis}}}{dt} = \frac{P_{\text{blood}}}{\tau} - \frac{P_{\text{tis}}}{\tau}
\]

where \( P_{\text{tis}} \) is the tissue tension of the inert gas (atm), \( P_{\text{blood}} \) the arterial blood tension of the inert gas (atm), \( \tau \) the time constant (min) to be determined from the data, and \( t \) is time (min). The time constant (\( \tau \)) determines the flux of gases in and out of the tissues. The
change in $P_{\text{blood}}$ during compression and decompression is assumed to be instantaneous and therefore equal to the alveolar partial pressure ($P_{\text{al}}$) for that inert gas at all times. The $P_{\text{al}}$ in turn is assumed to be equal to the ambient partial pressure ($P_{\text{amb}}$) for that gas. Earlier human models for air (Thalmann, et al., 1997) corrected the arterial inert gas partial pressure by using the alveolar gas equation (Antonisen and Fleetham, 1987), assuming a respiratory quotient of 1.0. In this study, a constant inspired fraction of 2% $O_2$ was used, resulting in the following equation for arterial inert gas tension ($P_{\text{blood}} = P_{\text{al}}$):

$$P_{\text{blood}} = (P_{\text{amb}} - PH_2O) \cdot (1 - 0.02) = (0.98 \cdot P_{\text{amb}} - 0.06) \quad \text{Eq. 7}$$

At pressures of 22.3, 24.1, and 25.7 atm, this correction is negligible. This small physiologic correction has therefore been omitted and will be contained within the Thr parameter.

The effect of a change in $P_{\text{blood}}$ on the $P_{\text{tis}}$ is computationally complicated, and has been described in detail elsewhere (Thalmann, et al., 1997; Weathersby, et al., 1985). The inert gas flux during the hyperbaric experiment was calculated by dividing the compression and decompression sequence into pressure-time ramps (Weathersby, et al., 1985; Weathersby, et al., 1992). For this study, it is assumed that $\tau$ is equal for both He and $H_2$. Hence, three parameters need to be determined: $\tau$, $G$, and Thr.

**Effect on $H_2$ tissue tension by $H_2$ metabolism**

Animals injected with *Methanobrevibacter smithii* into the intestines had a significantly lower incidence of DCS as compared to control animals (Chapter 5). It is unclear how the reduction of $H_2$ in the caecum and large intestine affects the presence of
H₂ elsewhere in the body. However, a working hypothesis is that the conversion of H₂ into H₂O and CH₄ in the intestine creates a sink that globally affects the arterial H₂ tension (P\text{bloodH₂}, Fig. 5.8, Chapter 5). Therefore, the equation including methanogenesis is:

\[
\frac{dP_{\text{inH}_2}}{dt} = \frac{P_{\text{bloodH}_2} - \text{BUG} \times A}{\tau} - \frac{P_{\text{inH}_2}}{\tau} \tag{Eq. 8}
\]

where BUG is the rate of H₂ removal as determined by either the measured CH₄ release rate (\(\dot{V}_{\text{CH}_4}, \text{mmol CH}_4 \cdot \text{min}^{-1}\)) or by the total microbial activity injected into the intestines (INJ, mmol CH₄ • min⁻¹). A \(\dot{V}_{\text{CH}_4}\) and A\text{INJ} (atm • min • (mmol CH₄)⁻¹) are the respective parameters to be determined. This equation assumes that the microbial metabolism of H₂ is constant throughout the hyperbaric exposure. This simplification appears justified, since only a limited number of measurements on the temporal changes of the metabolism of H₂ were made (Fig. 5.3, Chapter 5). Consequently, the removal of H₂ due to microbial metabolism results in up to four parameters that need to be determined for each model; \(\tau\), G, Thr and A \(\dot{V}_{\text{CH}_4}\) or A\text{INJ}.

The parameters need to be determined from the data by fitting the estimated P(DCS) to the actual outcome. Consequently, a P(DCS) must be estimated for each hyperbaric exposure using Eq. 1 or 3. If the animal did not suffer from DCS at the end of the 1-hour post decompression observation period, the hyperbaric exposure was considered to be safe through the end of the observation period (Tend).
For the parameter search, a Marquardt non-linear parameter estimation routine using maximum likelihood was used to search for best-fitting parameters (Weathersby, et al., 1984). The likelihood ratio test was used to determine significance of parameters compared to the constant hazard models (Weathersby, et al., 1984) and between nested models (Collett, 1994). A P < 0.05 was used to indicate significant differences.

Maximum likelihood parameter search

Suppose we have a comprehensive data series of compression and decompression sequences from a set of animals, and their decompression response. It is assumed that each exposure is independent of all others, and as such constitutes a separate observation. Each hyperbaric exposure is assumed to have a certain probability of DCS [P(DCS)], which is determined by the specific compression and decompression sequence as described in Eq. 1. The probability of no signs of DCS after a hyperbaric exposure is described by Eq. 2. Upon examination of the data, where the outcome variable $X_i$ is one (1) if DCS occurred and zero (0) if no DCS occurred, the probability of the observed outcome for a certain hyperbaric exposure (i) is:

$$P(\text{obs})_i = P(\text{DCS})_i^{X_i} \cdot P(\text{no DCS})_i^{(1-X_i)}$$  \hspace{1cm} \text{Eq. 9}

The calculation of $P(\text{obs})$ is repeated for all exposures in the data set. Next, the likelihood function (L) is calculated which gives the theoretical outcome of the data set. The $L$ of the outcome for the whole data set is defined as the product of probabilities of each observation

$$L(\text{data set}) = [P(\text{obs})_1] \cdot [P(\text{obs})_2] \ldots [P(\text{obs})_n]$$  \hspace{1cm} \text{Eq. 10}
The likelihood function is the product of many numbers less than one, and the natural logarithm of L, or log-likelihood (LL), is therefore most commonly used. The LL is an assessment of how well the physiological model defined in \( r \) fits the entire data set. Consequently, the search routine adjusts the model parameters to maximize the LL based on the data outcome and the theory of the model (Kendall and Stuart, 1979).

The following simple example will explain the creation of a likelihood function from a data set with ten observations. It assumes that all the compression and decompression sequences in the data set are similar and therefore have the same constant risk of DCS, \( i.e. \) \( P(\text{DCS}) = c \). Of the ten observations, there were 3 cases of DCS giving the following likelihood function

\[
L = (1-c) \cdot (1-c) \cdot (1-c) \cdot (1-c) \cdot (1-c) \cdot (1-c) \cdot (c) \cdot (c) \cdot (c)
\]

Letting \( c \) vary over its possible values from zero to one, it can be shown that the maximum LL occurs at \( c = 0.3 \). Any other choice of \( c \) will lower the LL and is therefore an inferior choice. For this example the calculation can be performed by hand, but for the more complex problems described in this chapter, a modified Marquardt nonlinear least-square search routine was used to find the maximum LL (Bailey, 1976; Homer, 1977).

**Constant hazard models**

A special case of the risk function model is a more general and simplified model in which there are no explanatory variables. This model considers the instantaneous risk to be constant at all times

\[
(P_{\text{tis}} - P_{\text{amb}}) \cdot P_{\text{amb}}^{-1} = 0 \text{ before decompression begins} \quad \text{Eq. 11A}
\]
\[(P_{\text{tis}} - P_{\text{amb}}) \cdot P_{\text{amb}}^{-1} = \text{constant after decompression begins.} \quad \text{Eq. 11B}\]

The scaling factor G is set to one and not considered a parameter. This model is therefore referred to as the constant hazard model, and will be used to make comparisons with the LL for more complex models.

**Data set and case description**

The data set contains 109 well-documented hyperbaric exposures (Table 4.1, 5.1 and A6.1 in the appendix), with 53 DCS cases. All animals were juvenile males, either castrated (n = 98) or non-castrated (n = 11). There was no difference in DCS incidence between the castrated and non-castrated animals (Logistic regression analysis, P > 0.3). Consequently, these two groups were pooled for all subsequent analyses.

The experiments were carried out over a period of 30 months (May 97 - Oct 99), and include a variety of pressurization and depressurization sequences (Table 6.1). The animals can be divided into 3 groups: untreated control (UC, n = 69), surgical control (SC, n = 11), and treated (T, n = 29). Treated animals had H\textsubscript{2}-metabolizing microbes injected into the large intestine, a procedure requiring major surgery (Chapter 5). Therefore, a surgical control group was added to show that the significant decrease in the DCS incidence was not an artifact of the surgery (Chapter 5). Since DCS incidence was indistinguishable between the SC and C groups, the two groups were pooled (P > 0.29, Fisher Exact test, Chapter 5) and will be referred to as control animals (C = UC + SC, n = 80).

The data set consists of 109 single simulated dives, performed in a dry hyperbaric chamber; *i.e.* each animal performed only one hyperbaric exposure as previously
described (Chapter 4). The hyperbaric chamber was driven by a computer that recorded
the dive data to a file, including the ambient pressure (atm), chamber temperature (T\textsubscript{CH},
°C), O\textsubscript{2} %, and elapsed time (min). A gas chromatograph (Hewlett-Packard 5890A,
Series II, Wilmington, DE) measured the chamber gases O\textsubscript{2}, H\textsubscript{2}, He, N\textsubscript{2}, and CH\textsubscript{4} every
12 min. Also included in the history of each experiment was the outcome and temporal
information regarding DCS events (0, T\textsubscript{1}, T\textsubscript{2}, and T\textsubscript{end}). For animals that were
diagnosed with DCS, data included the time they were last definitely free of DCS signs
(T\textsubscript{1}), which in this study was set at the start of the decompression, and the time of
diagnosis (T\textsubscript{2}). For animals without DCS symptoms, the experiment ended after the 1-
hour post decompression observation period at 11 atm (T\textsubscript{end}).

DCS research is plagued by subjectivity of symptoms and the problem is
compounded for animal studies, since only the most overt symptoms can be detected
clearly (Ball, et al., 1999). However, animal research allows testing of compression and
decompression sequences that are not ethically possible in humans. For this study, the
animals were observed before the hyperbaric exposure during a period of treadmill
training, giving the observers a familiarization with the behaviour of each animal.
Throughout the study, only cases of severe DCS were considered for a positive
declaration of DCS (Chapter 4).

Each animal was closely observed from the start of decompression through one
hour at 11 atm (67-90 min, T\textsubscript{end}), or until the animal was declared to have DCS (T\textsubscript{2}).
Most animals had some signs of skin DCS, presenting as itchiness or pink to purple spots
(Buttolph, et al., 1998). However, skin DCS alone was not considered a DCS case for
the purpose of this study. Severe DCS symptoms were of two types: cardiopulmonary or
neurological (Chapter 4). The hyperbaric experiments in this study were not blinded, resulting in the possibility of biased diagnosis. However, the rigorous compression and decompression sequences used throughout this study made it possible in most cases to diagnose the animal with obvious signs of DCS. Every diagnosis of DCS was determined by a consensus of at least 3 observers.

**RESULTS**

The general trends of these data indicate that DCS incidence increased with increasing decompression rate and with time under pressure, but not with increasing pressure (Table 6.1). This unusual finding has been discussed (Chapter 4), suggesting that an elevated activity of the native flora of H$_2$-metabolizing microbes with increasing H$_2$ availability masked the effect of increasing DCS risk with increasing pressure.

The cumulative distribution of the 53 cases of DCS versus time is shown in Fig. 6.2. The data show that the occurrence of DCS is tightly clustered within the first half-hour after reaching the observation pressure (11 atm, Fig. 6.2). No cases of DCS were observed during the transition to lower chamber pressure (Fig. 6.2).

The LL for the constant hazard models were –122.36 and –176.79 for Model 1 and 2, respectively. The results for the entire data set using two parameters (τ and G) are summarized in Table 6.2. Both model 1 (Eq. 3 and 4) and model 2 (Eq. 3 and 5) showed improvement compared to the constant hazard models (Table 6.2). Therefore, incorporating a specific description of the dive history significantly improved the fit to the data.
The results when three or more parameters were added to the models are summarized in Table 6.2. Addition of a threshold parameter (Thr) to the risk function improved the fit only for Model 2 (Table 6.2). Inclusion of the parameter for H$_2$-metabolism injected into the animals ($A_{INJ}$) improved the fit for both models compared to the two-parameter model (Table 6.2). The parameter for methane release rate ($\dot{A}_{CH_4}$) improved the fit only for Model 2 (Table 6.2), and only Model 2 could be fitted using four parameters. This model included both Thr and $A_{INJ}$ as best fit parameters (Table 6.2). There was a trend for a significant improvement when including both $\dot{A}_{CH_4}$ and Thr to Model 2 ($P < 0.10$, Table 6.2).

The observed versus predicted number of cases of DCS for both models were tested by separating the data into groups by treatment and varying compression and decompression sequences (Table 6.1, 6.3, and 6.4). Both models could satisfactorily predict the DCS incidence when the data set was divided into 2 groups (control vs. treated, $P > 0.4 – 0.8$, Table 6.3), 4 groups (treated vs. control at 3 different decompression rates, Table 6.4, $P > 0.3 – 0.7$), or 14 different groups (treatment vs. control and different dive sequences, Table 6.1, $P > 0.2 – 0.7$).

**DISCUSSION**

In DCS research in humans, symptoms may emerge as late as 20 h after return to normobaria (Ball, et al., 1999; Weathersby, et al., 1992). Cases as late as several days have also been reported but their validity is viewed with skepticism (D. Temple, personal communication). In this study, most DCS cases occurred within the first 20 minutes (Fig. 6.2). This is similar to other studies on DCS in pigs (Dromsky, et al., 2000a), sheep
(Ball, et al., 1999), and rats (Lillo, personal communication). The prolonged latency observed in humans but not found in other animals may be an experimental artifact, since only obvious and early signs of DCS are observable in other animals. Furthermore, other animals have only limited ability to report symptoms.

Probabilistic models for DCS have been successfully used to model human and sheep data (Ball, et al., 1999; Parker, et al., 1998; Thalmann, et al., 1997; Tikuisis, et al., 1988; Tikuisis, et al., 1991; Weathersby, et al., 1985; Weathersby, et al., 1984; Weathersby, et al., 1992). Physiological interpretation of the parameters must be made with care, since the fitted values are made from the observed outcome, and the physical history of the hyperbaric experiment and are not actual physiological measurements. Consequently, the parameters are mathematical constructs and have limited physiological value. In contrast, the proposed mechanism of biochemical decompression has been incorporated into the models presented in this study, based on a measured physiological variable. This will permit an evaluation of the beneficial effects of H₂-biochemical decompression in pigs from any compression and decompression sequence in H₂, and will be one of the first demonstrations of a mathematical model using a measured physiological variable to predict DCS.

The successful incorporation of a parameter for H₂-metabolism into the models supports the hypothesis that microbial metabolism of H₂ reduces the P(DCS) by an overall reduction in the P_{blood}H₂ (Eq. 8). Two terms for H₂ metabolism were tested (Table 6.2): one relating the removal of H₂ to the total activity of H₂-metabolizing microbes injected into the intestines of the animals (A_{INJ}), and the other relating the removal of H₂ to the measured CH₄ release rate (A_{\dot{V}_{CH₄}}).
The parameter $A_{INJ}$ improved the fit to the data for both models compared to models with only two parameters (Table 6.2). The parameter $A_{VCH_4}$, on the other hand, improved the fit only for Model 2. Based on the log-likelihood ratio, inclusion of both Thr and $A_{INJ}$ significantly improved the fit for Model 2, and there was a trend for significant improvement using Thr and $A_{VCH_4}$ (Table 6.2).

In Chapter 4, a rough estimate was made of the fraction of total gas burden removed by the native methanogens based on the $\dot{VCH_4}$. The estimated fraction removed was $\sim 8\%$, with a range between 4-16\%. In this chapter, a physiologically-based model estimated the inert gas tissue tensions of the animal throughout the dive based on the outcome data. Model 2 was able to show a correlation between $\dot{VCH_4}$ and DCS risk (Table 6.2). Using the parameter for $A_{VCH_4}$ and $A_{INJ}$, it is possible to estimate the $P_{tis}$ for an animal on a similar compression and decompression sequence as the calculation used in Chapter 4, with a $\dot{VCH_4}$ of either 0 (hypothetical control animal without native methanogens) or 40-80 $\mu$mol CH$_4$ $\cdot$ min$^{-1}$ (range for a hypothetical control animal with native methanogens, Chapter 4), or an INJ of either 0 or 1500 $\mu$mol CH$_4$ $\cdot$ min$^{-1}$ (injection comparable to those used in Chapter 5). Based on these computations, it can be shown that the fraction removed of the total gas burden is between 0.5-2\% when using $\dot{VCH_4}$ and 2-5\% when using INJ as a predictor. A graphical example of the possible reduction in the $P_{tis}$ using INJ and Thr with and without a high dose of H$_2$-metabolizing microbes is shown in Fig. 6.3. Since risk of DCS is the area under the curve between $P_{tis}$ and $P_{amb}$, where $P_{tis} > P_{amb}$, the beneficial effects of biochemical
decompression are clearly illustrated (Fig. 6.3). Even though the two curves for $P_{\text{tis}}$ are close, the difference in the area under the curve between $P_{\text{tis}}$ and $P_{\text{amb}}$ for untreated and treated animals is appreciable for DCS risk. This illustrates how elimination of relatively small fractions of dissolved gas may have a surprisingly large impact on the DCS incidence.

The models described here can be used to predict the overall benefit from $H_2$-biochemical decompression for any specified compression and decompression sequence. Based on the parameter estimates, it is possible to predict the change in the $P(\text{DCS})$ with varying $\dot{V}_{CH_4}$ or INJ for any given hyperbaric sequence. For example, the most tested compression and decompression sequence in this study was to a total pressure of 24.1 atm for 3 hours with a decompression rate of 0.9 atm $\cdot$ min$^{-1}$ ($n = 38$, Table 6.1). The observed DCS incidence was 80% and 39% for control and treated animals, respectively, which is a reduction in the DCS risk of $\sim 51\%$. The predicted values for Model 2 with inclusion of $\dot{V}_{CH_4}$ were 69% and 55%, and using INJ and Thr they were 73% and 41% for control and treated animals, respectively (Table 6.1). Thus, the estimated reduction in the DCS risk for an animal with enhanced removal of $H_2$ is 44% and 19% for INJ and $\dot{V}_{CH_4}$, respectively. The reduction in DCS risk predicted by Model 2 with inclusion of INJ is fairly close to the observed value. Using $\dot{V}_{CH_4}$, although significant to model 2, has lower predictive power, stemming from the problems in accurately estimating this variable as explained in Chapter 5.

Other models were tested and not found significant. Among these were models with more than one tissue, models with a separate $\tau$ for $H_2$ and He, models with separate
During compression and decompression, models with a T1 set by the observers at 11 atm, and models incorporating body weight and chamber temperature.

In conclusion, a hypothesis of how biochemical decompression works (Fig. 5.8, Chapter 5) has been postulated and incorporated into a physiologically-based probabilistic model (Eq. 8). The model has quantified a description of how microbial metabolism of H$_2$ significantly reduces the DCS risk after a hyperbaric H$_2$ exposure in pigs. The model can be used to evaluate the beneficial effects of biochemical decompression from any compression and decompression sequence in hyperbaric H$_2$. The physiological interpretation of the model gives a foundation for further research including other physiological measurements. The model supports the hypothesis that removal of H$_2$ by microbial metabolism, and a possible reduction in the body tissue gas burden, reduces the DCS risk by as much as 50%. This is one of the first successful demonstrations in which a measured physiological variable is able to predict DCS risk.
Table 6.1. Group type, inert gas components of the gas mixture (Gas), maximal ambient pressure (P), number of animals in each group (N), decompression rate (DR), time held at maximum pressure (Time), and number of decompression sickness cases in each group (DCS). The groups are untreated and surgical control animals (C), and animals injected with methanogens into the intestine (T). DCS predicted from the models used the following parameters for Model 1: τ (time constant), G (scale factor), and INJ (total activity of H₂-metabolizing microbes injected); for Model 2a: τ, G, INJ, and Thr (threshold), and for Model 2b: τ, G, and \( \dot{\text{CH}_4} \) (methane release rate) (Table 6.2). \( \chi^2 \) is for goodness of fit test comparing observed versus predicted DCS outcome.
<table>
<thead>
<tr>
<th>Group (ID)</th>
<th>Gas</th>
<th>P (atm)</th>
<th>n</th>
<th>DR (atm • min⁻¹)</th>
<th>Time (min)</th>
<th>DCS Observed (#)</th>
<th>DCS Predicted from Model (###)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>22</td>
<td>13</td>
<td>0.45</td>
<td>180</td>
<td>4</td>
<td>2.9, 1.6, 1.8</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>24</td>
<td>9</td>
<td>0.45</td>
<td>180</td>
<td>3</td>
<td>2.3, 1.4, 1.8</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>26</td>
<td>7</td>
<td>0.45</td>
<td>180</td>
<td>0</td>
<td>1.9, 1.1, 1.4</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>24</td>
<td>20</td>
<td>0.9</td>
<td>180</td>
<td>16</td>
<td>13.0, 14.6, 13.8</td>
</tr>
<tr>
<td>T</td>
<td>H₂/He</td>
<td>24</td>
<td>18</td>
<td>0.9</td>
<td>180</td>
<td>7</td>
<td>8.3, 7.4, 9.5</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>24</td>
<td>8</td>
<td>1.8</td>
<td>180</td>
<td>8</td>
<td>5.7, 6.5, 5.4</td>
</tr>
<tr>
<td>T</td>
<td>H₂/He</td>
<td>24</td>
<td>2</td>
<td>1.8</td>
<td>180</td>
<td>2</td>
<td>1.3, 1.3, 1.2</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>24</td>
<td>8</td>
<td>0.9</td>
<td>150</td>
<td>3</td>
<td>5.0, 5.6, 5.0</td>
</tr>
<tr>
<td>T</td>
<td>H₂/He</td>
<td>24</td>
<td>5</td>
<td>0.9</td>
<td>150</td>
<td>2</td>
<td>2.4, 2.2, 2.9</td>
</tr>
<tr>
<td>C</td>
<td>H₂/He</td>
<td>24</td>
<td>5</td>
<td>0.9</td>
<td>120</td>
<td>4</td>
<td>3.3, 3.7, 3.1</td>
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<td>H₂/He</td>
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</tr>
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<td>0.9</td>
<td>1100</td>
<td>1</td>
<td>0.4, 0.5, 0.2</td>
</tr>
<tr>
<td>T</td>
<td>H₂/He</td>
<td>24</td>
<td>3</td>
<td>0.9</td>
<td>&gt;1100</td>
<td>1</td>
<td>1.3, 1.3, 0.5</td>
</tr>
<tr>
<td>T</td>
<td>He</td>
<td>24</td>
<td>1</td>
<td>0.9</td>
<td>180</td>
<td>0</td>
<td>0.3, 0.5, 0.2</td>
</tr>
</tbody>
</table>

\[ \chi^2 \quad 9.24, 12.66, 15.59 \]

\[ P \quad > 0.7, > 0.4, > 0.2 \]
Table 6.2. Parameter estimates (± SE) and log-likelihood (LL) for Model 1 and 2 for the whole data set (n = 109, Table 6.1). $\tau$, time constant for each model; G, scale factor for each model, $A_{\text{INJ}}$, scale factor for the total H$_2$-metabolizing activity injected for each model; and $A_{\dot{V}_{\text{CH}_4}}$ scale factor for the CH$_4$ release rate for each model. The P-value based on log-likelihood ratio testing between nested models (Collett, 1994).
<table>
<thead>
<tr>
<th>Model</th>
<th>$\tau$ (min)</th>
<th>G (min$^{-1}$)</th>
<th>Thr (atm)</th>
<th>$A_{V,CH_4}$ (atm $\cdot$ min $\cdot$ (mmol CH$_4$)$^{-1}$)</th>
<th>$A_{INJ}$ (atm $\cdot$ min $\cdot$ (mmol CH$_4$)$^{-1}$)</th>
<th>LL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.80 ± 0.13</td>
<td>2.23 ± 0.74</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>-68.90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>1.72 ± 2.09</td>
<td>1.15 ± 1.28</td>
<td>0.35 ± 0.76</td>
<td>++++</td>
<td>0.18 ± 0.05</td>
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</tr>
<tr>
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<td>2.90 ± 0.96</td>
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<td>++++</td>
<td>0.94 ± 0.74</td>
<td>-67.99</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.75 ± 0.06</td>
<td>0.14 ± 0.03</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>-90.58</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>1.91 ± 0.08</td>
<td>0.08 ± 0.04</td>
<td>0.47 ± 0.44</td>
<td>++++</td>
<td>0.16 ± 0.04</td>
<td>-79.99</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>0.74 ± 0.06</td>
<td>0.21 ± 0.06</td>
<td>++++</td>
<td>1.24 ± 0.38</td>
<td>0.33 ± 0.18</td>
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<td>&lt;0.01</td>
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<tr>
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<td>0.86 ± 0.08</td>
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<td>1.32 ± 0.96</td>
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<td>-84.03</td>
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<tr>
<td></td>
<td>1.73 ± 0.99</td>
<td>0.12 ± 0.06</td>
<td>0.40 ± 0.39</td>
<td>++++</td>
<td>0.33 ± 0.18</td>
<td>-72.91</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>1.75 ± 1.01</td>
<td>0.10 ± 0.05</td>
<td>0.36 ± 0.38</td>
<td>1.32 ± 0.96</td>
<td>++++</td>
<td>-78.32</td>
<td>&lt;0.1</td>
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</table>
Table 6.3. Observed versus predicted DCS. Values are observed and mean predicted DCS incidences for treated (n = 29) and control (n = 80) animals. Model 1 included parameters for \( \tau \) (time constant), G (scale factor), and INJ (total activity of \( \text{H}_2 \)-metabolizing microbes injected); Model 2a included parameters for \( \tau \), G, INJ, and Thr (threshold); and Model 2b included \( \tau \), G, and \( \dot{V}_\text{CH}_4 \) (methane release rate) (Table 6.2). \( \chi^2 \) is for goodness of fit test comparing observed versus predicted DCS outcome.

<table>
<thead>
<tr>
<th>Group</th>
<th>Observed</th>
<th>Model</th>
<th></th>
<th></th>
<th></th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>2 a</td>
<td>2b</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>12</td>
<td>13.7</td>
<td>12.6</td>
<td>14.7</td>
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Table 6.4. **Observed versus predicted DCS.** Values are observed and mean predicted DCS incidences for each group. Model 1 included parameters for $\tau$ (time constant), $G$ (scale factor), and INJ (total activity of H$_2$-metabolizing microbes injected); Model 2a included parameters for $\tau$, $G$, INJ, and Thr (threshold); and Model 2b included $\tau$, $G$, and $\nu$CH$_4$ (methane release rate) (Table 6.2). $\chi^2$ is for goodness of fit test comparing observed versus predicted DCS outcome.

<table>
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<td>12</td>
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Figure 6.1. Model behaviour showing the ambient pressure ($P_{amb}$), estimated inert gas tissue tension for H$_2$ and He ($P_{tis}$), the probability of DCS [$P(DCS)$], and the integrated risk ($r$) for Model 1 (A) and 2 (B) using time constant ($\tau$) and a scale factor ($G$) for a sample dive to 24.1 atm. Constant pressure was maintained for 3 h followed by decompression at 0.9 atm \text{ min}^{-1} to 11 atm. Model 1 (A) used $\tau = 0.80$ and $G = 2.23$ while Model 2 (B) used $\tau = 0.75$ and $G = 0.14$ (Table 6.2). The units for $r$ are inverse of time but the scaling has been chosen for convenience and are not shown on the graph.
Figure 6.2. Cumulative number of DCS cases with time after reaching the observation pressure (11 atm) for the 53 observed cases of DCS out of 109 observations.
Figure 6.3. Ambient pressure, and inert gas tissue tension ($H_2 + He$) for a control animal and a treated animal injected with a total methanogen activity of 1500 $\mu$mol CH$_4$ • min$^{-1}$ using the parameters in Model 2 (Table 6.2). The estimated probability of DCS for the control ($P(DCS)_C$) and treated animal ($P(DCS)_T$) is also shown. The hyperbaric exposure was to a total pressure of 24.1 atm for 3 h with a decompression rate of 0.9 atm • min$^{-1}$. The total activity injected into the intestines of the treated animal was a relatively high dose (Fig. 5.4 and 5.7, Chapter 5). The units for $r$ (arbitrary for convenience) are inverse of time, and the scale is the same for the control and treated animals.
APPENDIX

A6.1. Outcome (1 = DCS, 0 = No DCS), chamber H$_2$ pressure (PH$_2$), animal weight, total methanogenic activity injected into intestines (INJ), mean CH$_4$ release rate during the last hour at constant pressure (c $\dot{V}$CH$_4$), and mean chamber temperature ($T_{CH}$) for untreated (C), and treated animals (T). The table shows the data for the animals that have not been reported in earlier chapters, but were included in the full data set used for probabilistic modeling. The experimental groups were either compressed to a maximum pressure of 24.1 atm for varying times at constant pressure (VBT, 30, 120, 150 min) with a decompression rate of 0.90 atm $\cdot$ min$^{-1}$, or for 180 min at 22.3 or 24.1 atm with a decompression rate of 1.80 atm $\cdot$ min$^{-1}$ (VDR-0.45 and VDR-1.80 respectively).

Animals were either treated with intestinal injections of *Methanobrevibacter smithii* (T), or untreated control animals (C). †VBT-180T censored observation. Animal fell out of observation range in chamber after 38 min of observation at 11 atm. ‡ Modified compression and decompression sequence. ¥ Diagnosed with cardiopulmonary DCS.
<table>
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<tr>
<th>Group</th>
<th>Outcome</th>
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### Appendix A6.1 continued

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CHAPTER SEVEN

GENERAL DISCUSSION

“Get your facts first, then you can distort them as much as you please.”

Mark Twain
Extensive research has been performed on the prevention and treatment of decompression sickness (DCS), but the basic physiology of the disease process still remains largely unknown. It is universally recognized that longer stays at higher pressures with rapid decompressions result in more DCS than shorter stays at lower pressures with slow decompression (Weathersby, et al., 1985; Weathersby, et al., 1986; Weathersby, et al., 1992). This supports the notion that the level of inert gas supersaturation plays a major role. Therefore, if the level of supersaturation can be reduced, it is reasonable to believe that the risk of DCS will also be reduced. Biochemical decompression is one way to actively reduce the body burden of inert gas. It was the objective of this study to determine whether biochemical decompression is a viable concept for humans.

Conclusions from the study

The use of H₂ as a diving gas is relatively new (Lindén, 1985) and only limited research has been performed on the physiology of this gas in hyperbaria (Brauer, 1987). To evaluate the physiology of hyperbaric H₂ compared to He in whole animals, calorimetry and respirometry were used (Chapter 2). The study concluded that the physiological responses in hyperbaric He and H₂ could not be explained solely by the thermal properties of the two gas mixtures. It was suggested that the elevated heat loss rate in animals exposed to hyperbaric He could be in part due to high pressure nervous
syndrome (HPNS), whereas animals in hyperbaric H₂ could be narcotized and allow their surface temperature to fall to reduce the heat loss rate. Consequently, hyperbaric H₂ is not likely to be as thermally stressful as predicted on the basis of the higher thermal conductivity and heat capacity of H₂ compared to He.

For the experiments on H₂-biochemical decompression, 20-kg pigs were used. These animals have similar cardiovascular physiology to humans (Lumb, 1966; Miller, 1987), and extensive research has established that the pig model is a good substitute for human studies on DCS (Broome, 1996; Broome and Dick, 1996; Buttolph, et al., 1998; Dromsky, et al., 2000a; Dromsky, et al., 2000b; Fahlman, et al., 1999a; Fahlman, et al., 1999b; Kayar and Fahlman, 1999). The initial experiments suggested that the intestinal flora of H₂-metabolizing microbes removed some of the H₂ that otherwise would have dissolved in the pig’s tissues (Chapter 4). The CH₄ release rate ( \( \dot{V}_{\text{CH}_4} \), Eq. 1, Chapter 1) was used as a measure of the efficacy of H₂ removal by the intestinal microbes, and increasing \( \dot{V}_{\text{CH}_4} \) was shown to reduce the risk of DCS in the animals (Fig. 4.4, Chapter 4).

When increased activity of H₂-metabolizing microbes was delivered to the animals, the probability of DCS [P(DCS)] was further reduced in a dose-dependent manner (Fig. 5.7, Chapter 5). For the compression and decompression sequence studied most extensively, the DCS incidence was reduced by 50% using microbial treatment (Chapter 5). The probabilistic model showed that increasing \( \dot{V}_{\text{CH}_4} \) significantly reduced the P(DCS) (Table 6.2, Model 2, Chapter 6), and the model confirmed that increasing H₂-metabolizing activity delivered to the animals significantly reduced the P(DCS) (Table 6, Chapter 6). Consequently, the hypothesis that enhanced removal of H₂ by
microbial metabolism after a hyperbaric exposure to H₂ will reduce the DCS incidence in a pig model has been successfully demonstrated. In addition, a mathematical model of DCS risk showed a significant improvement by including $\dot{V}_{CH_4}$ or injected microbial activity (INJ), and this is one of the first reported incidences of a measured physiological variable that has successfully been able to predict DCS.

**Significance and importance for human diving**

The successful demonstration of biochemical decompression in the pig makes it a likely concept for human divers. From the information given by these experiments, it is possible to forecast the improvements that could be possible for a human diver. A 70-kg man in a hyperbaric H₂ atmosphere of 45 atm has approximately 63 L H₂ (~ 2.5 mol H₂) dissolved in his tissues at saturation. The decompression from such a hyperbaric exposure takes approximately 14 days (Bennett and Rostain, 1993), giving a washout rate of ~ 124 $\mu$mol H₂ • min⁻¹ by diffusion- and perfusion-limited processes. In the pig, it was shown that animals supplemented with H₂-metabolizing microbes after 24 hours at 24.1 atm had a rate of H₂ removal of ~ 1 mmol H₂ • min⁻¹ (assuming a 4:1 ratio between CH₄ and H₂, Eq. 1, Chapter 1). Since the large intestine in pigs is ~ 2-3.3 times longer compared to that of humans (Barth, *et al.*, 1990; Crouch, 1981; Miller and Ullrey, 1987), H₂-removal rates of at least ~ 250 $\mu$mol H₂ • min⁻¹ should be feasible in humans. Consequently, the total removal of H₂ by diffusion, perfusion, and active processes could potentially be raised to ~ 374 $\mu$mol H₂ • min⁻¹ in human divers. This total activity could possibly reduce the decompression time from ~ 14 days to ~ 5 days.
Before testing on H₂-biochemical decompression could commence in humans there are several questions left to answer in the pig. The apparent dose-response effect by injection of increasing activity into the animals on the H₂ removal (Fig. 5.4, Chapter5) must be further explored to determine the maximum dose the animals are able to support and that gives the greatest effect. The residence time of the microbes needs to be tested, to determine the best point in time to administer the microbes. Even though *M. smithii* is native and non-pathogenic to both pig and human intestine, it needs to be determined if elevated levels of these microbes cause any immediate or chronic problems. In addition, a non-invasive vehicle for the administration of the live cultures of methanogens needs to be tested, such as freeze-dried cultures in an enteric capsule.

**Other pathological processes to consider in DCS**

It is widely accepted that increased supersaturation leads to the formation of bubbles during decompression, and the presence of gas bubbles in tissues or the circulatory system leads to DCS (Hills, 1977; Lee and Hairston, 1971; Newton, *et al.*, 1944; Thorsen, *et al.*, 1993). The results from this study further support this hypothesis, and also suggest that enhanced inert gas removal reduces DCS risk. However, all DCS problems cannot be explained by gas kinetics alone, and there are other aspects of the disease process that are still unexplained. Several researchers have proposed that DCS may in part result from hematological or immunological interactions at the blood-gas interface.

The role for an inflammatory component in DCS in terrestrial animals has been suggested for years, but the establishment of a causative role for such a multifactorial
process has only recently begun to be investigated (Buttolph, et al., 1998; Ersson, et al., 1998; Kayar, et al., 1997b). At present there are several theories relating DCS and immune function in terrestrial animals. Two general areas are activation of the immune system and/or tissue ischemia/reperfusion injury; both primarily caused by the bubbles. While these two areas explain different aspects of observed symptoms and outcomes in DCS, the cellular and molecular mechanisms are not well determined. There are known biochemical and morphological changes that occur in circulating immune cells, and blood cells in a hyperbaric environment in humans and other animals. These changes include decreases in red cells, white cells, hematocrit, and platelets and changes in T cell subsets (Barnard and Weathersby, 1981; Philp, 1974; Philp, et al., 1972; Shinomiya, et al., 1999). These events could prime immune functions, which, during subsequent decompression, cause the activation of one or more immune cascades.

It appears as if DCS is mainly a phenomenon that occurs in terrestrial animals subjected to hyperbaria, while free diving marine mammals appear to be unaffected by DCS. This cannot be explained merely by the absence of lung ventilation under pressure in marine mammals. Some of the adaptations that have been reported, such as atelactic lungs, peripheral vasoconstriction, and bradycardia, would reduce the amount of N₂ available to equilibrate with the tissues. Still, current estimates indicate a continuous tissue nitrogen tension of 3-4 atm, which would predominate for most of the animal’s life (Kooymman, 1989; Kooymman and Ponganis, 1999). These nitrogen levels give an estimated 30% severe DCS incidence in similar-sized terrestrial animals (Dromsky, et al., 2000a). Thus, the adaptations necessary to prevent DCS in diving mammals could be
valuable in answering how we can allow for safe diving practice in humans without the detrimental symptoms of DCS.

Future prospects

The work presented in this thesis has demonstrated that biochemical decompression is a feasible concept in a large animal model. The results show that removing a relatively small fraction of the tissue burden of inert gas can potentially have a surprisingly large effect on the DCS outcome. However, the use of H\textsubscript{2} as a diving gas is limited to deep professional or military diving. Biochemical decompression from dives using N\textsubscript{2} as the inert gas would be the most useful, given its potential wide application for professional and recreational diving and aerospace flight. A second advantage of N\textsubscript{2} biochemical decompression compared to H\textsubscript{2} biochemical decompression is given by the pressure limit of the use of N\textsubscript{2} to \(\sim 6\) atm. Smaller volumes of gas would have to be eliminated on a dive using N\textsubscript{2} compared to H\textsubscript{2}. However, due to the stable conformation of the N\textsubscript{2} molecule, reactions that break down N\textsubscript{2} are endergonic and require an energy source for complete conversion into NH\textsubscript{3}, nitrates and nitrites. Therefore, even though various microbes are capable of using N\textsubscript{2}, they more commonly utilize other sources of nitrogen than the gaseous molecule. Currently known species of enteric N\textsubscript{2}-fixing microbes have such low metabolic efficiency that prohibitively large volumes of microbes would be needed. In addition, the end products of N\textsubscript{2} metabolism are not gaseous and would stay in the intestines, in contrast to CH\textsubscript{4}, and most are toxic. Therefore, H\textsubscript{2} was chosen to test the hypothesis that improved removal of the inert gas significantly reduces the risk of DCS, and that biochemical decompression is a feasible
concept in a large animal model. The proof of concept suggests that a similar approach would work using N₂-metabolizing microbes for reducing the DCS risk during air diving and aerospace flight. Genetic engineering in the future may create enzymes that are able to metabolize N₂ more efficiently and that can convert any toxic end products into innocuous compounds. The production of such enzyme systems will make N₂ biochemical decompression possible.

The hypothesis that increased rates of inert gas removal will reduce the DCS incidence can further be tested by the use of injectable compounds that have a large carrying capacity for gases. This is an approach currently explored at the Naval Medical Research Center. Perfluorocarbons (PFC) are compounds with high gas solubility. When injected into the circulatory system, they are able to hold in solution a significantly greater number of inert gas molecules than can be held in plasma. These compounds can therefore be used to increase the rate of inert gas removal from tissues. Reduction of DCS by the use of PFCs has been tested successfully in rats (Lutz, 1984), hamsters (Lynch, 1989), and pigs (Dromsky, et al., 2000b). The successful results with biochemical decompression, and the promising results using PFCs warrant further studies. These new methods represent the future for DCS treatment and avoidance.

In summary, this thesis has presented a new way of reducing the risk of DCS from hyperbaric H₂ exposures in a pig model. This is one of the first presentations of a measured physiological variable that is able to predict the DCS incidence in a mathematical model. The success of this project suggests that H₂ biochemical decompression should be a feasible concept for human divers, and N₂ biochemical decompression a possibility in the future.
REFERENCES


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