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

by

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

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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 H_2 dives. Since there is only limited physiological data on the use of H_2 as a diving gas, direct calorimetry and respirometry were used to determine whether physiological responses to hyperbaric H_2 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 H_2, a H_2-metabolizing microbe (*Methanobrevibacter smithii*) was tested that converts H_2 to H_2O and CH_4. 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 H_2 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 $\bullet$ min^{-1}), and observed for severe symptoms of DCS for 1 h. Chamber gases (O_2, N_2, He, H_2, CH_4) were monitored using gas chromatography throughout the dive. Release of CH_4 in untreated pigs indicated that H_2 was being metabolized by native intestinal microbes and results indicated that native H_2-metabolizing microbes may provide some protection against DCS following hyperbaric H_2 exposure. *M. smithii* injection further enhanced CH_4 output and lowered DCS incidence. A probabilistic model estimated the effect of H_2-metabolism on the probability of DCS, P(DCS), after hyperbaric H_2 exposure. The data set included varying compression and decompression sequences for controls and animals with intestinal injections of H_2 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.

Acknowledgements:

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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 $\dot{V}CH_4$

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

Pa_{in} - alveolar partial pressure

P_{amb} - ambient pressure

P_{blood} - arterial inert gas tension

PH_2O - water vapour pressure

PFC - perfluorocarbon

P_{tis} - tissue tension

$P_{tis}H_2$ - Hydrogen tissue tension

$P_{tis}He$ - Helium tissue tension

Q_C - heat losses by convection across body surfaces

Q_{CD} - heat loss by conduction

Q_Σ - 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

$\dot{D}$ - 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

τ - 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

$\overset{\bullet}{v}$ - flow rate

$\overset{\bullet}{V}CH_4$ - methane release rate

$\overset{\bullet}{V}O_2$ - 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."

Franscisco 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 CO_2, poses little threat to the diver. It has been shown that O_2 can contribute to DCS during rapid decompressions (Lillo, 1988; Lillo and MacCallum, 1991; Parker, *et al.*, 1998). However, O_2 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 O_2 to be metabolized. In contrast, inert gases such as He, N_2, and H_2 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, N_2 has a narcotic effect that increases with the pressure (Bennett, 1989). To avoid this problem, He is commonly substituted for N_2 during deep dives (Table 1.1). The rationale for substituting He for N_2 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 N_2, would be 4 times less narcotic than N_2 (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, H_2 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 H_2 as a diving gas; the most obvious

being its flammability. However, H_2 is not explosive if the O_2 concentration of the gas mixture is 4% or less (Lindén, 1985). Another potential problem with H_2 as a diving gas is the higher thermal conductivity and heat capacity of H_2 compared to He which suggests that heat losses in hyperbaric H_2 will be unusually high (Kayar, *et al.*, 1997a). In addition, the thermal conductivity of H_2, and He are several times higher than N_2 (Lide, 1993-94), which seriously may restrict the use of these gases. Concern has also been raised that the exposure to hyperbaric H_2 could cause incorporation of H_2 into mammalian tissues by saturating lipids (Örnhagen, 1987). However, as early as 1789 it was shown that gaseous H_2 is not metabolized by mammalian tissues at 1 atm (Séguin and Lavoisier, 1789) and a study on tissue slices showed that H_2 was not metabolized or incorporated by mammalian tissues at pressures up to 50 atm (Kayar, *et al.*, 1994).

There are several advantages to using H_2 as the inert component of a breathing gas. The density of H_2 is lower than that of He, and consequently H_2 is easier to ventilate at elevated pressures (Kayar and Parker, 1997). Hydrogen can also be easily made from H_2O 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, *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 O_2 in place of the inert gas (mainly N_2 in air). Since O_2 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 O_2 at pressures exceeding 1.6 atm (normobaric O_2 = 0.21 atm), it is not possible to completely replace the inert gases with O_2 (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 H_2 as the inert gas involves the use of H_2 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 H_2 would in some manner be supplied with a hydrogenase. The hydrogenase would convert some of the dissolved H_2 into H_2O, which would result in a reduction of the tissue H_2 tension ($P_{tis}H_2$). 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 ($\pm$ 5%) has a great impact on the DCS incidence. This is analogous to the large effect on the hemoglobin O_2 saturation by a small shift in the PO_2.

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):

$$4H_2 + CO_2 => CH_4 + 2H_2O \hspace{3cm} \text{Eq. 1}$$

Since CO_2 is 30 times more soluble in tissue fluids than H_2, 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 H_2 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 blood stream (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 H_2 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 H_2 metabolizing microbe into the intestine is able to reduce the $P_{tis}H_2$.

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_2/O_2) as compared hyperbaric heliox (He/O_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_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_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_2-dives will be performed in a hyperbaric chamber (Fig. 1.1) in control pigs and pigs supplemented with H_2-metabolizing microbes. The DCS incidence and the rate of H_2-metabolism, in the form of CH_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 H_2 exposures in general, and offers a predictive advantage over descriptive models. Furthermore, such a model can potentially be used to forecast the benefits of H_2 diving for humans.

Table 1.1. Name of common gas mixtures used for diving, their composition, and approximate pressures at which they are used.

Common Name	Gas Composition	Pressures (atm)
Air	O_2/N_2	< 6
Heliox	O_2/He	6-40
Hydrox	O_2/H_2	10-60

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 H_2 (following NFPA Code 50A

for H_2 at consumer sites).
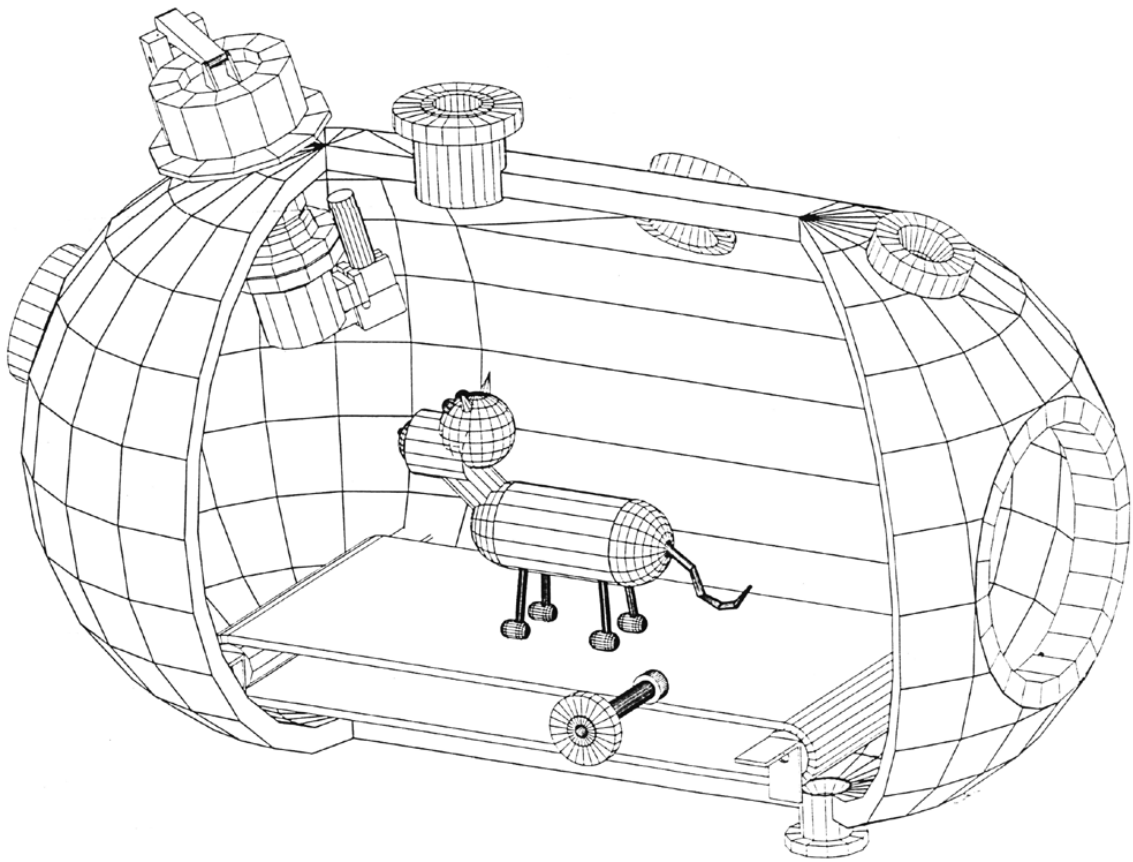
CHAPTER TWO

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 (H_2) during hyperbaria (Brauer, 1987). It was desired to determine if there are different physiological responses to hyperbaric hydrox (98% H_2, 2% O_2) versus heliox (98% He, 2% O_2). 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 (Q_Σ) 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, H_2 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 H_2 as a diving gas include the flammability of some H_2/O_2 mixtures, and its higher thermal conductivity and heat capacity compared to He, which suggests that heat losses in hyperbaric H_2 can be unusually high.

Several studies have measured oxygen consumption rates ($\dot{V}O_2$) of animals in He mixtures at 1 atm (0.1 MPa). While some studies found a reduction in the $\dot{V}O_2$ 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 *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_Σ 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, *Cavia porcellus*, of the Hartley strain (n = 16, mean body weight

= 822 ± 113 g) were housed in a professionally staffed animal care facility and had *ad*

libitium 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

<u>Calorimeter setup</u>

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 incurrent 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 ΔT 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 ΔT 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 H_2, 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 $= 836 \pm 59$ 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_Σ

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 $= 894 \pm 65$ g) or the hydrox group (n = 6, mean body

weight $= 898 \pm 79$ 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 $\bullet$ 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 • 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 ~ 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 ~ 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 $38.0 \pm 0.5°$ 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 $\pm 0.5°$ 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:

$$M = S + Q_\Sigma \qquad \text{Eq. 1}$$

This equation states that the total heat loss rate (Q_Σ) 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_Σ 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}).

$$Q_\Sigma = Q_C + Q_{RC} + Q_R + Q_{EVAP} \qquad \text{Eq. 2}$$

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 kW $\cdot$ s $\cdot$ g^{-1} at 37° C; 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:

$$Q_C = h_c \cdot A \cdot (T_s - T_a) \qquad \text{Eq. 3}$$

where A is the body surface area (cm², T_a and T_s are the ambient and surface

temperatures (°C), respectively. The term h_c is the heat transfer coefficient

(W · m⁻² · °C⁻¹) predicted by:

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

Nu is Nusselt's factor determined by Nu = a · (Gr · 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 (λ, W · m⁻¹ · °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 (1.06·10⁻³ g · mm⁻³, Kayar, et al., 1997a).

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

$$T_s = 0.84 \cdot T_a + 8.64 \qquad \text{Eq. 5}$$

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

The body surface area (A, cm²) was first estimated according to measurements in

1 atm air in guinea pigs (Herrington, 1941), in which:

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

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_Σ for the

hyperbaric experiments.

Heat loss due to radiation was determined by the equation:

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

where σ is Stefan Boltzmann's constant ($5.67051 \cdot 10^{-8}\ \text{W} \cdot \text{m}^{-2} \cdot \text{K}^{-4}$; Lide, 1993-94;

Wissler, 1978) and the temperatures are in degrees Kelvin. The emissivity (ε), 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:

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

where V_E ($\text{cm}^3 \cdot \text{s}^{-1}$) is the respiratory minute volume for guinea pigs estimated by $V_E =$

$\dot{V}_{O_2} \cdot 0.028$ (Blake and Banchero, 1985; Wissler, 1978), ρ ($\text{mol} \cdot \text{cm}^{-3}$) is the density of

the gas mixture, C_p ($\text{J} \cdot \text{mol}^{-1} \cdot {}^\circ \text{C}^{-1}$) 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:

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

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_Σ 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_Σ from the calorimeter experiments. Other models explored here are various

modifications to Eq. 2, as explained below.

<div align="center">**Statistical analysis**</div>

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.

<div align="center">**RESULTS**</div>

Calorimetry measurement of Q_Σ

There was no systematic change in either the Q_Σ ($P = 0.40$) 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_Σ 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_Σ (P

$= 0.61$) measurements. Thus, any changes in $\dot{V}O_2$ or Q_Σ 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_Σ increased

with increasing pressure in heliox and at 10-40 atm in hydrox (Table 2.1). At 60 atm the Q_Σ 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_Σ

For estimating Q_Σ 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_Σ 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_Σ 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_Σ in hyperbaria. Under this

assumption, the measured $\dot{V}O_2$ and Q_Σ 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_Σ 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_Σ 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 $\dot{V}O_2$ 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, Eq. 1), the $\dot{V}O_2/Q_\Sigma$ (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 $\dot{V}O_2/Q_\Sigma$ 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 $\dot{V}O_2/Q_\Sigma$ ratio gave reliable results.

However, in the hyperbaric experiments the measured $\dot{V}O_2/Q_\Sigma$ 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, Eq. 1). This

explanation also describes why Model 0 (data not shown) failed to estimate the Q_Σ 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_Σ 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_Σ 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_Σ 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_Σ 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_Σ 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_Σ 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), ε (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_Σ.

The Q_Σ 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_Σ between hydrox and heliox, based strictly on the thermal properties (TP) of the two gas mixtures (Table 2.4). This approach indicated that Q_Σ in hydrox should be 21-25% higher compared to heliox at all pressures (TP Q_Σ, 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_Σ with pressure (Table 2.1, P < 0.03). From 40-60 atm there was a large increase in the Q_Σ 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_Σ 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_Σ 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 $\dot{V}O_2/Q_\Sigma$ 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 $\dot{V}O_2/Q_\Sigma$ ratio would be expected since helium is believed to be completely biologically inert (Leon, 1960). The substantial decrease in the $\dot{V}O_2/Q_\Sigma$ ratio in both gases made it impossible to be conclusive regarding H_2 metabolism. The simplest explanation for the decrease in the $\dot{V}O_2/Q_\Sigma$ ratio in both gas mixtures is that the $\dot{V}O_2/Q_\Sigma$ 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_Σ 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_Σ 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 $\dot{V}O_2/Q_\Sigma$ 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_Σ at similar T_a 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_Σ 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_Σ of animals in hyperbaric heliox is in part due to HPNS, whereas animals in hyperbaric hydrox allow surface temperature to fall to reduce Q_Σ. 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_Σ), 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.

Gas	Pressure (atm)	$\dot{V}O_2$ $(mL\ O_2 \cdot g^{-1} \cdot h^{-1})$	Q_Σ (W)	T_a (° C)
Air	1	0.720 ± 0.093	3.31 ± 0.32	26.5 ± 3.1
Heliox	10	0.764 ± 0.077	3.81 ± 0.31	32.7 ± 1.5
	20	0.792 ± 0.063	4.43 ± 0.52	33.4 ± 0.7
	40	0.879 ± 0.126	5.08 ± 0.61	34.3 ± 0.2
	60	1.033 ± 0.154*	6.51 ± 0.72*	35.1 ± 0.5
Hydrox	10	0.713 ± 0.102	4.07 ± 0.48	32.8 ± 1.2
	20	0.832 ± 0.199	4.75 ± 0.89	33.2 ± 0.5
	40	0.776 ± 0.040	5.57 ± 0.62	34.5 ± 0.7
	60	0.800 ± 0.048	5.40 ± 0.82	35.3 ± 0.6

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_Σ), oxygen consumption rate ($\dot{V}O_2$), and calorimeter temperature (T_a) for guinea pigs (n = 5) in air at 1 atm during a 6h enclosure in a calorimeter.

Period (h)	Q_Σ (W)	$\dot{V}O_2$ $(mL\ O_2 \cdot g^{-1} \cdot h^{-1})$	T_a (°C)
0-1	3.65 ± 0.67	0.758 ± 0.124	23.4 ± 1.0
1-2	3.27 ± 0.54	0.748 ± 0.102	24.2 ± 0.9
2-3	3.07 ± 0.46	0.720 ± 0.084	24.9 ± 0.8
3-4	3.13 ± 0.37	0.731 ± 0.103	25.2 ± 0.8
4-5	3.10 ± 0.38	0.701 ± 0.082	25.4 ± 0.9
5-6	3.15 ± 0.30	0.734 ± 0.094	25.6 ± 0.9
Overall	3.23 ± 0.48	0.733 ± 0.094	24.8 ± 1.1
P	0.40	0.95	

Table 2.3. Mean (± 1 stdev) heat loss rate (Q_Σ) from direct calorimetry (actual), estimated by a mathematical model (Eq. 2, theoretical), and percent Q_Σ partitioned into various avenues of heat loss as a percentage of Q_Σ (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° C lower in hydrox; Model 2.2: T_{ex} = 2° C lower in hydrox).

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

‡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_Σ) (hydrox/heliox) for a test to compare the difference in Q_Σ between heliox and hydrox based solely on their differing thermal properties (TP Q_Σ), versus their measured values.

Pressure (atm)	Gas	TP Q_Σ (W)	TP Q_Σ Difference (%)	Measured Q_Σ Difference (%)
10	He	7.0		
	H_2	8.5	21.4	6.8
20	He	9.4		
	H_2	11.5	22.3	7.2
40	He	12.8		
	H_2	15.8	23.4	9.6
60	He	15.4		
	H_2	19.2	24.7	-17.1

The following variables were used for both gases at 10-60 atm: $T_a = 32.4°$ 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}.

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Table 2.5. Sensitivity test of model to estimate total heat loss rate (Q_Σ) using Models 0, 1.0, and 2.0 to estimate Q_Σ for 1 atm air, heliox and hydrox, respectively.

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

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.
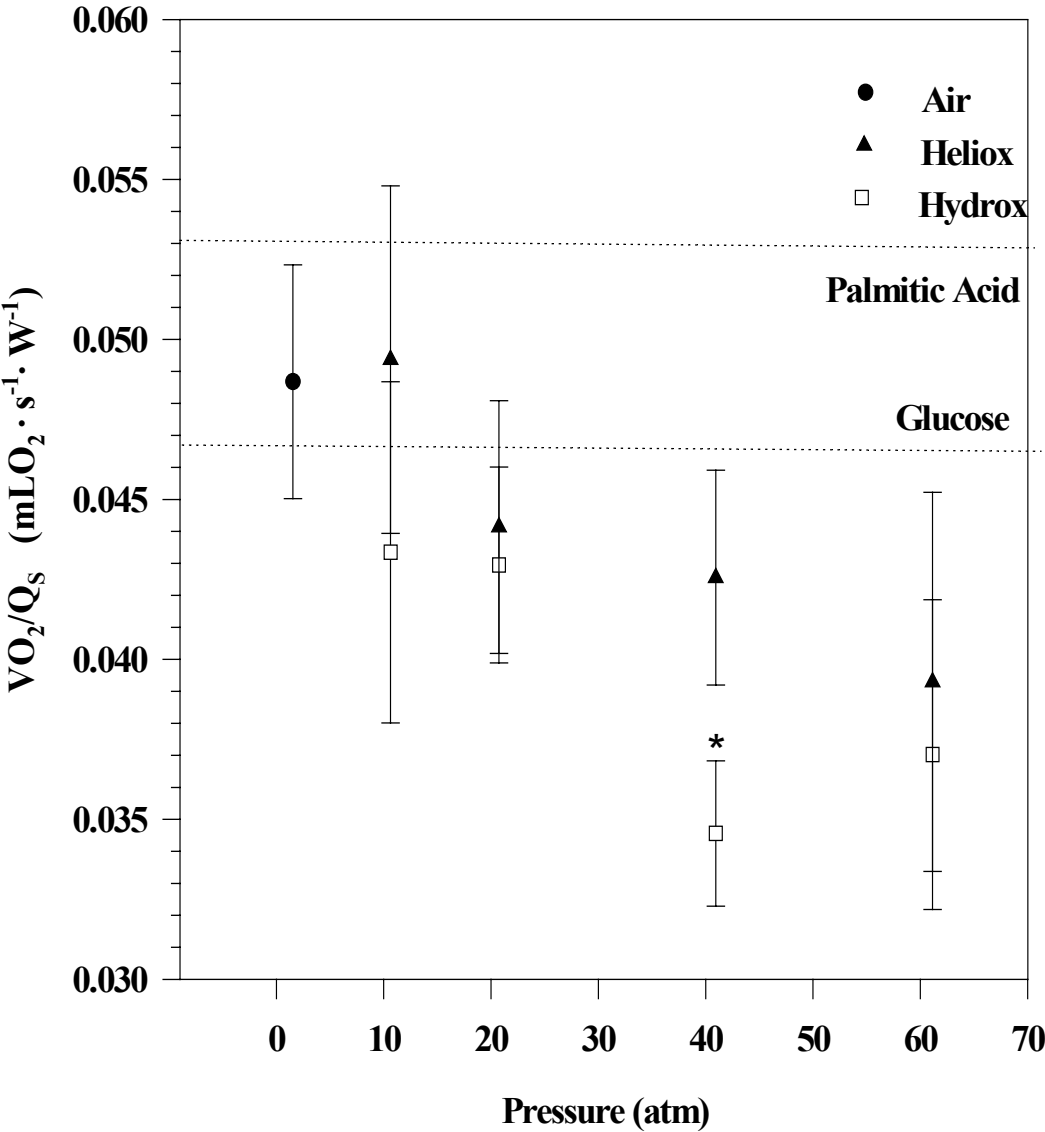
Figure 2.2. Ambient temperature (T_a) versus estimated surface temperature (T_s) for guinea pigs in 1 atm air (Herrington, 1941), and 10-60 atm heliox and hydrox. The regression equations are for temperatures measured in ° C.

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

Heliox, $T_s = 4.74 + 0.91 \cdot T_a$

Hydrox, $T_s = 7.64 + 0.81 \cdot T_a$
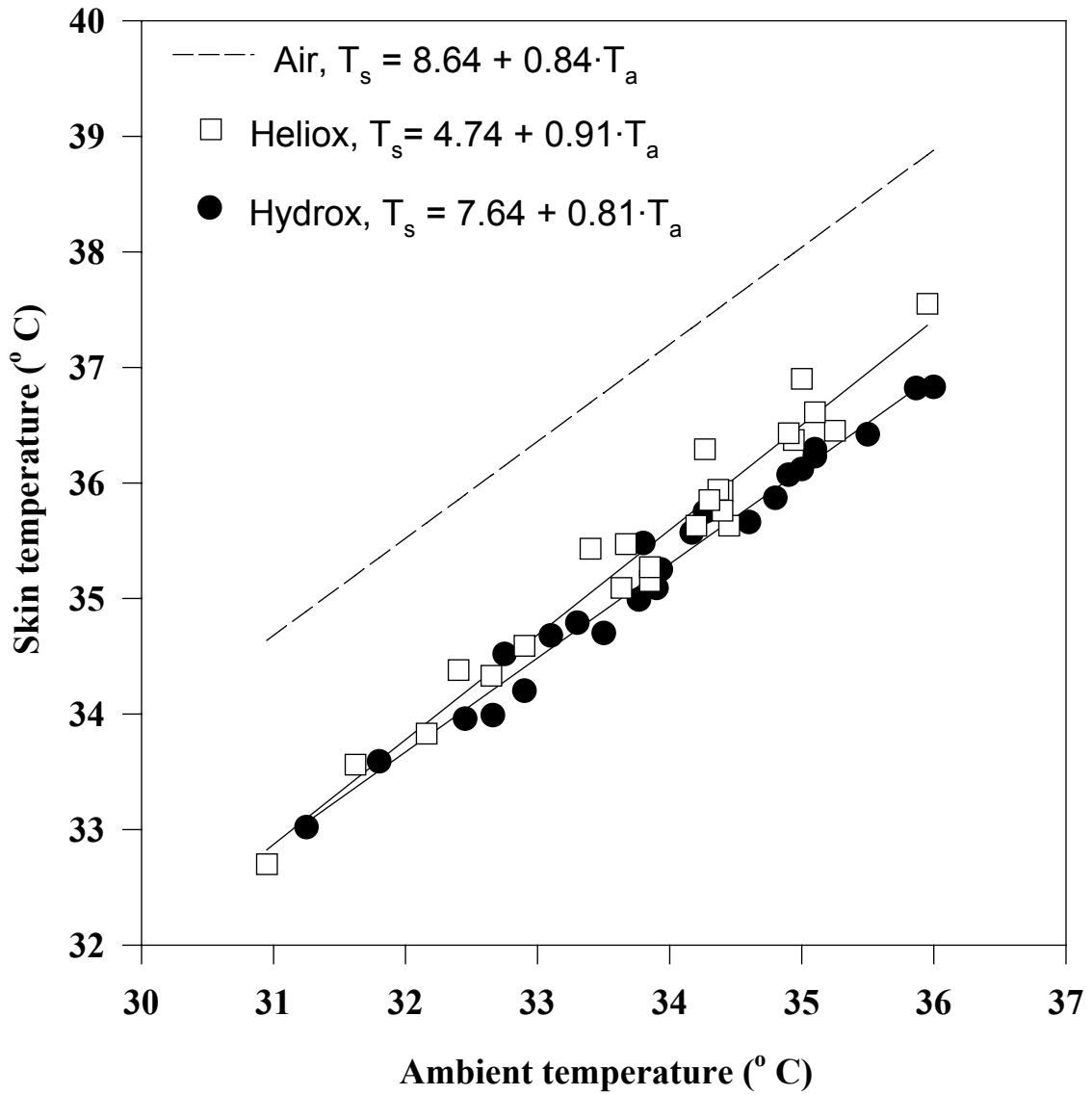
APPENDIX

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_Σ 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 ε ($\varepsilon = 1.0$

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° C (assumed value of T_b), another assuming T_s to be 0.5° 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_Σ for the initial model (Model 0). Model 0 uses the constants for a, A %, ε,

T_{ex} 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

ε has negligible effect on the Q_Σ (Table 2.5). The T_s, however, affects the Q_Σ

substantially, as indicated by the large the percent changes in Q_Σ under various estimates

of T_s (Table 2.5).

CHAPTER THREE

CALIBRATION OF THE PHYSICAL PROPERTIES OF THE HYPERBARIC CHAMBER FOR RELIABLE ESTIMATION OF CH$_4$ RELEASE RATE ($\dot{v}_{CH_4}$)

*"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_2 was recently described by Kayar et al. (Kayar, *et al.*, 1998a).

The premise of this method is the utilization of H_2-metabolizing microbes to consume

some of the H_2 dissolved in the tissues of these animals after exposure to elevated H_2

pressures. The purpose is to convert H_2 into H_2O and CH_4, thereby reducing the amount

of dissolved H_2 gas in the tissues. The microbes, or methanogens, convert H_2 into H_2O

and CH_4 according to the formula:

$$4\ H_2 + CO_2 => 2\ H_2O + CH_4 \hspace{3cm} \text{Eq. 1}$$

where the secondary end product (CH_4) can be used as a non-invasive measure of the

efficacy of the methanogens to metabolize H_2. It is believed that the microbial

metabolism of H_2 increases the washout rate of H_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_2. The microbes were assumed

to receive H_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_4 ($\dot{V}CH_4$) 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 $\dot{V}CH_4$ in the earlier study was performed using the same principles as when measuring the metabolic rates ($\dot{V}O_2$) 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 $\dot{V}CH_4$, 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 ($\dot{D}=\dot{v}/V$) (Steffensen, 1989). The value of $\dot{D}$ 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 $\dot{V}CH_4$ 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 $\dot{V}CH_4$ 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_2, the

$\dot{v}$ in this study was limited to ~115 L • 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 $\dot{v} CH_4$ 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, L) is as follows:

$$V_P = V • P \qquad\qquad\qquad \text{Eq. 2}$$

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_2, N_2, H_2, He, and CH_4

using a gas chromatograph (Hewlett-Packard 5890A, Series II, Wilmington, DE).

Instantaneous measurement of $\dot{V}CH_4$

The method of estimating the $\dot{V}CH_4$ was developed and explained in detail by

Bartholomew et al. (Bartholomew, *et al.*, 1981) for measuring the $\dot{V}O_2$ in a flow-through

chamber. When the changes in [CH4] are rapid relative to $\dot{D}$, it is possible to estimate

the new equilibrium ([CH4]eq) value given the effective chamber volume (V, L), the flow

rate ($\dot{v}$, L • min^{-1}), and the concentration of [CH4] ([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:

$$\frac{dx}{dt} = q - \frac{[x](t) \cdot \dot{v}}{V_P} \qquad\qquad \text{Eq. 3}$$

where q is the rate at which gas [x] is added (ppm • 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 (Δt),

and which can be estimated by:

$$Z = 1 - e^{\frac{-\dot{v}\,\Delta t}{V_P}} \qquad\qquad \text{Eq. 4}$$

or equivalently

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

<div align="right">Eq. 5</div>

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,

$$[x]_{eq} = \frac{[x]_t - [x]_{t-1}}{1 - e^{\frac{-\dot{v}\Delta t}{V_p}}} + [x]_{t-1}$$

<div align="right">Eq. 6</div>

Knowing the $[x]_{eq}$, or in this case the $[CH_4]_{eq}$, it is possible to calculate the $\dot{V}CH_4$ (mol

$CH_4 \bullet min^{-1}$) at standard temperature and pressure (STP) at time t by:

$$\dot{V}CH_4 = [CH_4]_{eq} \bullet \dot{v} \bullet 22.4^{-1}$$

<div align="right">Eq. 7</div>

where $\dot{v}$ is in $L \bullet min^{-1}$ at standard temperature and pressure (STP), and 22.4 the standard

molar volume at $0°$ C ($L \bullet mol^{-1}$).

Thus, the $\dot{V}CH_4$ can estimated from the animal knowing the change in the $[CH_4]$

over a given Δt, 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:

$$V_P = -\frac{\Delta t \cdot \dot{v}}{\ln(1 - Z)} \qquad \text{Eq. 8}$$

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 $\bullet$ min^{-1}, or a $\dot{V}CH_4 \sim 80\text{-}90$ µmol $\bullet$ 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 ~ 380min. 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 Δt 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 Δt, 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_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:

Gas Flow Rate = 119 • [H$_2$] + 82.7 • [He] + (100 – ([H$_2$] + [He]) • 32.7)   Eq. 9

where [H$_2$] 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 • min^{-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 Δt

The data from the bolus injection experiment was used to determine an adequate Δt for generating a reliable estimate of $\dot{v} CH_4$. The $\dot{v} CH_4$ values for the experiment were computed using Eq. 6, Eq. 7 and varying Δt (mean ± stdev, n = 15; Table 3.3). For a Δt of 12 min (two consecutive chromatographic readings) the standard deviation (stdev) was ~0.8 times the estimated mean $\dot{v} CH_4$ (Table 3.3). The stdev of the estimate of the mean

$\overset{\bullet}{V}CH_4$ was plotted against Δt (Fig. 3.2); the stdev decreased exponentially with increasing Δt. At a Δt of ~ 120 min, the change in stdev with increasing Δt leveled off, and did not further change significantly as compared to a Δt of 500 min (Table 3.3, $P < 0.05$, F-test). Therefore, 120 min was determined to be the smallest Δt for reliable estimation of $\overset{\bullet}{V}CH_4$ 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 $\overset{\bullet}{v}$ based on the gas composition and Eq. 9 at each time point, and a Δt of ~ 500 min. This Δt was determined to be sufficiently large to give values with high accuracy, since the mean V values at larger Δt did not change significantly (data not shown). The mean V from this computation was 5718 ± 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 H_2O and CH_4 (Eq. 1). The secondary end product (CH_4) in this conversion can be used as a non-invasive measure to estimate the rate ($\dot{V}CH_4$) microbes efficacy in removing the H_2 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 H_2 and O_2 were injected to keep the chamber at constant pressure. To estimate the $\dot{V}CH_4$, it was necessary to correct the [CH_4] 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 Δt to estimate the $\dot{V}CH_4$ 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 H_2 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 Δt

All values of Δt estimated the $\dot{V}CH_4$ to within the known injection rate. However, a Δt 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 Δt of 500 min (Table 3.3). When the Δt was increased, the stdev became smaller, and at 112 min the change in the stdev with

increasing Δt was insignificant (Fig. 3.2, P < 0.05, F-test). Thus, for the estimation of

$\overset{\bullet}{V}CH_4$ during the study, a Δt 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 $\overset{\bullet}{v}$, and a Δt of 120 min reliably estimates the

$\overset{\bullet}{v}CH_4$ 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% O_2, balance inert gas.
The flow rates are reported in STP.

Ball position (x)	Air	H$_2$	He	Heliox	Hydrox
	($L \cdot min^{-1}$)				
50	13.7	52.2	34.8	30.8	54.6
100	32.7	119.0	82.7	73.0	115.8
150	57.0	200.3	145.0	128.2	184.4
Flow =	= -0.1 + 0.22x + 0.00105x^2	= 0.9x + 0.0029x^2	= 1.37 + 0.524x + 0.00289x^2	= 0.4 + 0.481x + 0.00248x^2	= 1.02x + 0.0014x^2

Table 3.2. Estimated mean washout rate (± stdev, $\dot{v}$) 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 ($i\dot{v}$), and from the flow meter equation ($f\dot{v}$) based on the gas composition after the bolus injection (~8% He, ~21% O_2, ~71% N_2). †There was no difference between the $i\dot{v}$ and the $f\dot{v}$ for either the BI or CI experiments (P > 0.26, and P > 0.06 respectively).

Gas	$i\dot{v}$		$f\dot{v}$	
Experiment	CI	BI	CI	BI
n	5	24	30	24
Mean Washout Rate (L • min^{-1})	27.4 ± 5.5	32.6 ± 12.8	33.9 ± 0.2	36.6 ± 0.5

Table 3.3. Estimated mean $\dot{V}CH_4$ (± stdev, n = 15) using Eq. 6 and Eq. 7 and varying Δt. The estimates are based on washin data, using a V_P = 5665 L • 4 atm, and a $\dot{v}$ of ~ 34 L • min^{-1}. The actual constant injection rate was ~ 85 μmol CH$_4$ • min^{-1}. † Significantly different variance as compared to using a Δt of 500 min (P < 0.05, F-test).

Δt (min)	$\dot{V}CH_4$ μmol CH$_4$ • min^{-1}
12	89.1 ± 68.3†
25	88.6 ± 38.0†
38	87.1 ± 27.8†
62	87.2 ± 20.7†
88	85.5 ± 15.3†
114	83.8 ± 8.9
138	83.2 ± 8.8
176	84.5 ± 8.5
250	84.3± 8.3
400	83.7± 3.1
500	78.8 ± 6.0

Figure 3.1. The change in methane concentration ([CH$_4$], 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 $\dot{D}$ of the chamber setup prolongs the kinetics of this system, making the kinetics appearing linear.
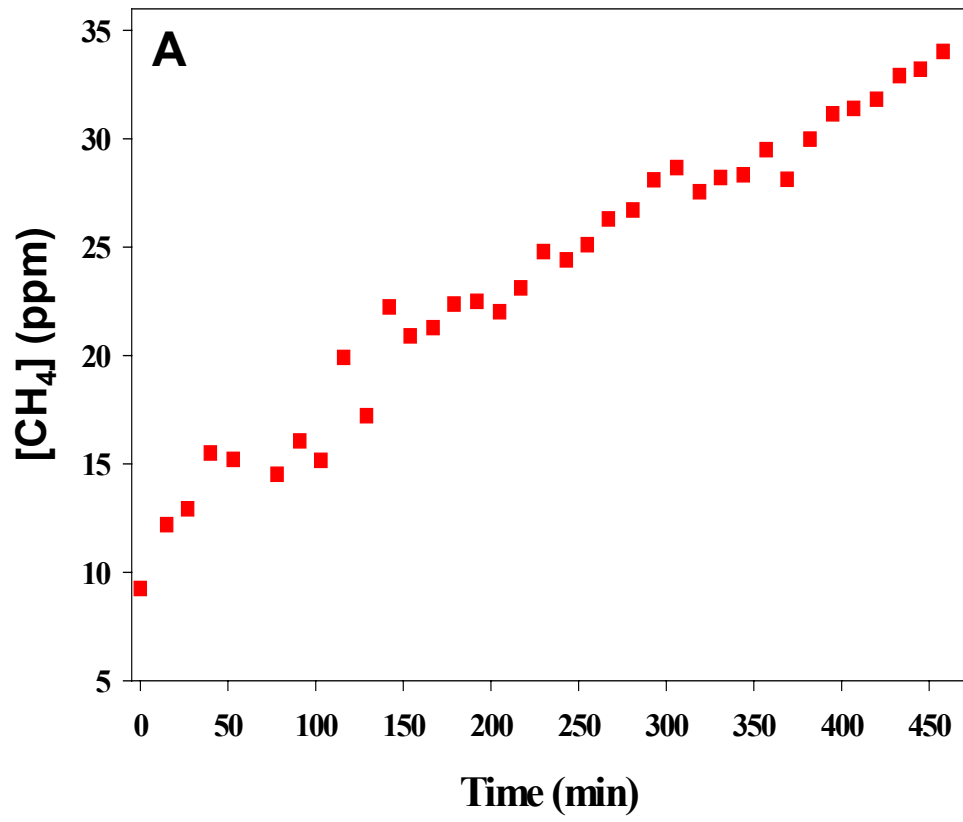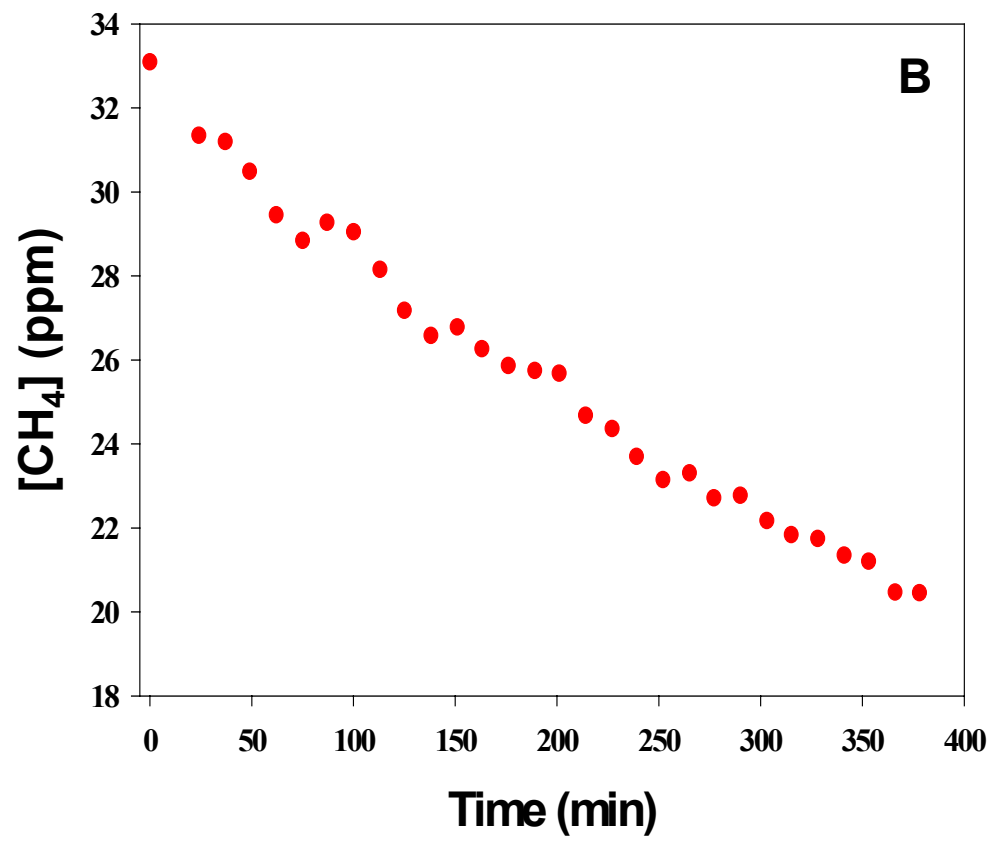
Figure 3.2. Standard deviation (stdev, n = 15) of estimated mean $\dot{V}CH_4$ from the washin phase of the continuous injection experiment versus Δt. The graph shows that the stdev decreases exponentially with Δt, and at a Δt of 112 min the change in stdev does not change significantly with further increase in the Δt ($P <$ 0.05, F-test).
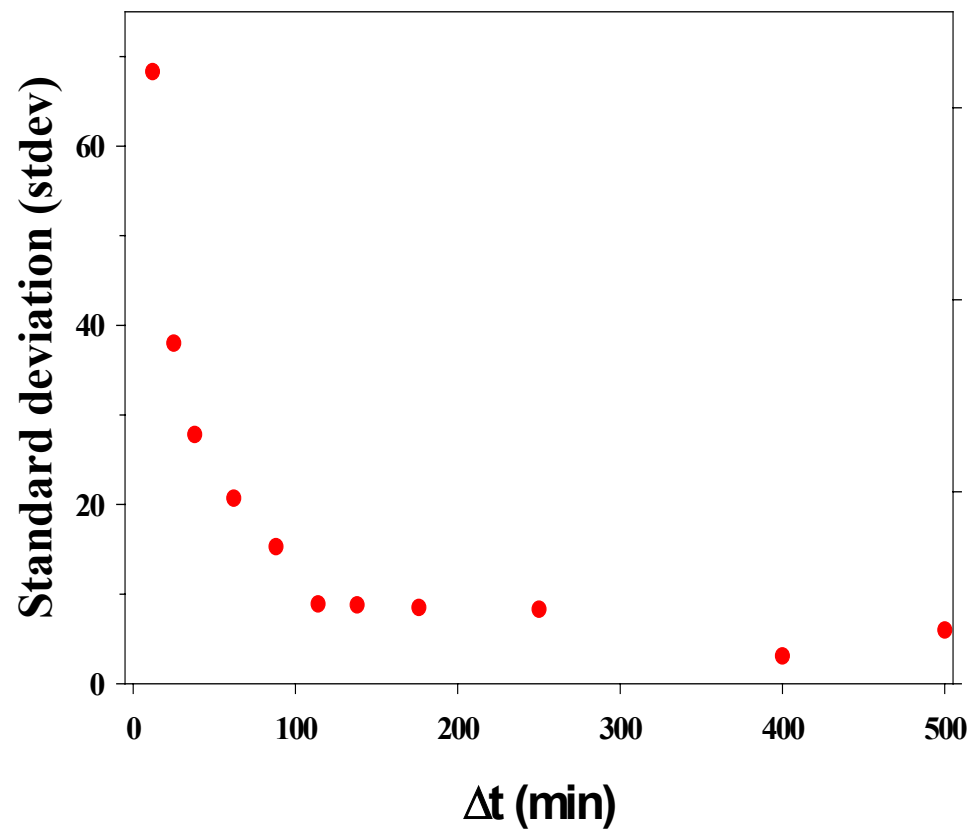
CHAPTER FOUR

REDUCTION IN DECOMPRESSION SICKNESS RISK BY NATIVE H$_2$-METABOLIZING INTESTINAL MICROBES IN PIGS AFTER HYPERBARIC H$_2$ 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 H_2 metabolizing microbe *Methanobrevibacter smithii* had a ~50% lower DCS incidence compared to untreated animals following a hyperbaric exposure to H_2 (Kayar, *et al.*, 1998a). These microbes metabolized H_2 and CO_2 to CH_4 and H_2O, and the efficacy of the H_2 metabolism was measured as the CH_4 release rate ($\dot{V}CH_4$) from the animals. Sample calculations suggested that this 50% decrease in DCS incidence was attributable to microbial reduction of the total H_2 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 H_2 as the inert gas can be advanced for use in human divers, it is necessary to show that the H_2 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

anatomical and physiological similarities to humans (Hannon, 1986; McKiennan, 1986; Swindle, *et al.*, 1992).

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 libitium* 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 H_2, 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 90º angle. Duplicate thermometers allowed continuous monitoring of the chamber temperature (T_{CH}), which was maintained at a seemingly comfortable level for the pigs: $\cong$ 30 °C at 11 atm and $\cong$ 33 °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 libitium* 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 ($\sim$115 L $\bullet$ min^{-1} or $\sim$ 410 L $\bullet$ 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 ($\dot{V}CH_4$, μmoles $CH_4 \bullet min^{-1}$) 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 $\bullet$ 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 $\bullet$ min^{-1} to a pressure of 3.7-5.6 atm and then eventually to 0.45 atm $\bullet$ 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 $\bullet$ min^{-1}, using H_2 again with continuous addition of O_2. There was no difference in H_2 % (P > 0.33) between the 3 experimental groups as determined by one-way ANOVA (Table 4.1).

The desired pressure was maintained ($\pm$ 0.3 atm) for 3 h by continuous addition of H_2 and O_2 to make up for the gas exhausted to the gas chromatograph. Consequently, the H_2 concentration increased slightly (1-2%), while the levels of N_2 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 $\bullet$ min^{-1} to 11 atm. The treadmill was activated and the animal was made to walk (30 m $\bullet$ 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 H_2 flushed out.

Corrected $\dot{V}CH_4$

The change in the chamber CH_4 concentration during the time at constant pressure (c[CH_4], ppm) and during the decompression (d[CH_4], ppm) was corrected to estimate the $\dot{V}CH_4$. The $\dot{V}CH_4$ 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 $\dot{V}CH_4$, n = 5) and for the 1 h observation period at 11 atm (d $\dot{V}CH_4$, n = 3). A Δt of 120 min was used to estimate the c $\dot{V}CH_4$ (Chapter 3), while the largest possible Δt (~ 24 min) was used to estimate the d $\dot{V}CH_4$ 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_4] per chromatographic report for these experiments was 0.5 ppm. Some animals did not release enough CH_4 to accumulate to a concentration of 0.5 ppm after 3 h, and thus are listed as releasing 0 CH_4 (Table 4.1).

The mean flow rate through the chamber ($\dot{v}$) in this study was either 115 L • min^{-1} or 410 L • min^{-1}. These values were mathematically adjusted to a common flow rate of 115 L • min^{-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_2 composition (H_2 %), chamber temperature (T_{CH}), H_2 partial pressure (PH_2), body

weight (Wt), concentration of CH_4 at constant pressure ($c[CH_4]$) and during decompression ($d[CH_4]$), and $\dot{V}CH_4$ at constant pressure ($c\dot{V}CH_4$). 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 χ^2, $P > 0.3$); consequently these two groups were pooled in all subsequent analysis.

$$\dot{V}CH_4$$

Four animals did not release quantifiable levels of CH_4 during the experiment (Table 4.1). The $c[CH_4]$ increased temporally in all other animals (Fig. 4.1). During the decompression phase, the $d[CH_4]$ continued to increase or remained essentially constant (Table 4.1). There was no difference in the $c[CH_4]$ or $d[CH_4]$ between the animals at different pressures ($P > 0.5$, one-way ANOVA).

There was a significantly higher mean $c[CH_4]$ ($n = 22$, 1.40 ± 0.80 ppm CH_4) in animals that did not show symptoms of DCS compared to animals with DCS ($n = 5$, 0.53 ± 0.82 ppm CH_4, $P < 0.05$; Table 4.1). There was also a significantly higher mean $d[CH_4]$ (2.22 ± 1.36 ppm CH_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 $c\dot{V}CH_4$ values are reported in Table 4.1. There was no difference in the mean $c\dot{V}CH_4$ between the animals at three different pressures ($P > 0.5$, one-way ANOVA), and the mean $c\dot{V}CH_4$ at 22.3, 24.1, and 25.7 atm was 30.0 ± 27.2, 30.9 ± 26.2 and 39.7 ± 33.3 µmoles $CH_4 \bullet min^{-1}$, respectively (Table 4.1). Not surprisingly, since $c\dot{V}CH_4$ was computed from $c[CH_4]$, there was a significant correlation between the $c\dot{V}CH_4$ and the $c[CH_4]$ ($P < 0.01$; Fig 4.2A). There was a trend toward a higher mean $c\dot{V}CH_4$ (37.4 ± 27.3 µmol $CH_4 \bullet min^{-1}$) in the animals with no DCS as compared to the animals with DCS (12.6 ± 21.0 µmol $CH_4 \bullet min^{-1}$, $P = 0.07$), and was not significantly correlated with pressure ($P > 0.4$; Fig. 4.3).

During the decompression phase, the $d\dot{V}CH_4$ 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[C H$_4$], 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 H_2 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 CH_4 released by most of the animals, and a clear connection between CH_4 release and DCS incidence (Table 4.1).

The CH_4 in the chamber could potentially come from three sources: from the pig, from the outside air which contains small amounts of CH_4, 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 H_2, any CH_4 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 CH_4, 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 CH_4 in the chamber was released by the pig.

Pigs are reported to have a native intestinal flora of methanogens capable of H_2 metabolism (Robinson, 1989), and increasing levels of CH_4 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 ($P_{tis}H_2$), 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 $c\dot{V}CH_4$ and $d\dot{V}CH_4$ 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 $\dot{V}CH_4$ may reflect the efficacy in H_2

removal from the animal. However, the $\dot{V}CH_4$ 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 $c\dot{V}CH_4$, $c[CH_4]$ 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 $\sim 42\%$ to $\sim 0\%$ (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 $\dot{V}CH_4$ between animals, and it was difficult to identify animals with no detectable $\dot{V}CH_4$ 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 $\bullet$ 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 $\dot{V}CH_4$ 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 ($\dot{V}O_2$) would change the inert gas kinetics. Body weight and ambient temperature are known to affect the $\dot{V}O_2$ (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 (Q_Σ, Chapter 2) increase with pressure. Considering the higher Q_Σ at elevated pressures, keeping the T_{CH} constant but increasing the pressure would eventually lead to a change in the $\dot{V}O_2$ to make up for the lost heat (Chapter 2). Since the mean T_{CH} increased with pressure, the pressure-dependent increase in Q_Σ was counteracted. In addition, the significant correlation between DCS and T_{CH} in this study must be regarded as doubtful because the difference in the mean T_{CH} for animals with and without DCS was ~1° C, a value at the limit for the sensitivity of measurement.

The $d\dot{V}CH_4$ was significantly higher than the $c\dot{V}CH_4$ (Table 4.1, P < 0.01). A similar observation was reported by Kayar et al. (Kayar, *et al.*, 1998a) in rats injected with H_2 metabolizing microbes at a comparable time point in decompression. It is not clear if the increase is from trapped intestinal CH_4 released due to volume expansion during the pressure reduction, or if the increased $\dot{V}CH_4$ indicates elevated levels of H_2 delivered to the methanogens. If the latter is true, one possibility is that H_2 comes out of solution and forms bubbles near the microbes where they have access to higher levels of substrate. There was no correlation between $c\dot{V}CH_4$ and PH_2, as would be expected if the delivery to the microbes was restricted by diffusion. It is also possible that the considerable variability in $c\dot{V}CH_4$ between animals, and the possible error in the computation of $\dot{V}CH_4$, may disguise a correlation between $\dot{V}CH_4$ and PH_2.

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 $\dot{V}CH_4$, it is

possible to get a crude estimate of the total volume of H_2 removed, assuming that all CH_4 comes from metabolism of H_2 (Eq. 1, Chapter 1). Assuming the whole body solubility of H_2 to be 0.02 mL $H_2 \bullet g^{-1}$ 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_2, *i.e.*, 21.6 atm PH_2, should have ~ 8.64 liters of H_2, or ~ 340 mmol H_2 (STP) in solution in its tissues when saturated. The average $\dot{V}CH_4$ in the animals releasing CH_4 was approximately 40 μmol $CH_4 \bullet min^{-1}$ (range 15-75 μmol $CH_4 \bullet min^{-1}$) during the three hours at constant pressure (Table 4.1). Consequently, the methanogens were responsible for releasing approximately 7 mmol CH_4 during the three hour at 24.1 atm, which requiring a consumption of 4 times this amount of H_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_2 in solution in these pigs. Even though this calculation is an oversimplification and contains several assumptions (Kayar, *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_2 body gas burden resulted in a ~ 50% reduction in the DCS incidence (Kayar, *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, *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_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 H$_2$ 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; CH$_4$ concentration in the chamber during the final hour at constant pressure (c[CH$_4$], n = 5 chromatographic readings), mean CH$_4$ concentration in the chamber during the decompression (d[CH$_4$], n = 2-7 chromatographic readings); CH$_4$ release rate in chamber during compression (c$\dot{V}$CH$_4$, µmol CH$_4$ • min^{-1}, n = 5) and mean CH$_4$ release rate after decompression (d$\dot{V}$CH$_4$, µmol CH$_4$ • min^{-1}, n = 3). P-values represent one way ANOVA test comparing the three groups. † Animal not castrated. ‡ The d$\dot{V}$CH$_4$ was significantly higher than the c$\dot{V}$CH$_4$ in each animal (P < 0.001, Paired t-test).

Pressure (atm)	Outcome	H_2 %	T_{CH} (°C)	Weight (kg)	$[CH_4]$ (ppm)		$\dot{V}CH_4$ (µmoles $CH_4 \bullet min^{-1}$)	
					$c[CH_4]$	$d[CH_4]$	$c\,\dot{V}CH_4$	$\ddagger d\,\dot{V}CH_4$
22.3	0†	89.7 ± 0.3	31.9 ± 1.6	20.4	0.60 ± 0.07	0.85 ± 0.13	9.4 ± 6.1	3.3 ± 5.9
	0†	88.3 ± 0.2	32.7 ± 0.6	21.0	2.99 ± 0.36	4.57 ± 0.67	85.0 ± 3.4	98.2 ± 19.0
	0†	87.4 ± 0.2	32.5 ± 0.9	20.7	2.53 ± 0.13	3.72 ± 0.51	62.6 ± 9.1	77.5 ± 21.5
	0†	87.0 ± 0.7	32.6 ± 0.7	19.0	0.96 ± 0.09	1.52 ± 0.22	16.5 ± 1.9	4.7 ± 4.5
	0†	86.1 ± 0.2	32.4 ± 0.4	19.1	1.12 ± 0.18	1.54 ± 0.20	24.7 ± 4.1	31.1 ± 5.1
	0†	84.8 ± 0.3	32.0 ± 0.5	17.9	0.74 ± 0.11	0.80 ± 0.11	8.6 ± 2.9	13.1 ± 14.2
	0	92.8 ± 0.2	30.7 ± 0.8	19.4	1.45 ± 0.25	2.88 ± 0.34	43.2 ± 10.5	43.8 ± 21.7
	0	86.1 ± 0.9	31.9 ± 0.6	18.9	1.20 ± 0.31	1.46 ± 0.11	36.4 ± 12.6	34.1 ± 3.8
	0	90.1 ± 0.9	32.4 ± 0.4	20.0	1.63 ± 0.31	2.56 ± 0.38	43.4 ± 7.3	75.0 ± 5.3
	1	92.4 ± 0.7	30.7 ± 0.7	18.8	0 ± 0	0 ± 0	0 ± 0	
	1	88.3 ± 0.2	30.9 ± 0.3	20.0	0 ± 0	0 ± 0	0 ± 0	
24.1	0	88.8 ± 0.3	32.4 ± 0.6	18.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

	0	86.7 ± 0.2	31.8 ± 0.5	19.0	0.77 ± 0.18	0.94 ± 0.22	22.8±8.7	46.4 ± 8.6
	0	90.9 ± 0.7	33.2 ± 0.9	22.1	1.95 ± 0.25	3.26 ± 0.60	75.3 ± 3.0	81.3 ± 17.1
	0	93.4 ± 0.7	34.1 ± 0.8	18.2	1.78 ± 0.34	2.91 ± 0.25	45.0 ± 4.6	43.6 ± 12.8
	0	88.8 ± 0.2	34.1 ± 0.9	21.0	0.83 ± 0.27	1.83 ± 0.34	16.8 ± 5.8	53.8 ± 25.8
	0	88.8 ± 0.2	32.6 ± 0.5	21.1	1.99 ± 0.20	2.97 ± 0.40	55.2 ± 3.5	73.7 ± 15.5
	1†	91.7 ± 0.2	32.6 ± 0.2	18.0	0 ± 0	0 ± 0	0 ± 0	
	1†	89.6 ± 0.2	31.7 ± 0.8	19.4	0.78 ± 0.11	0.99 ± 0.07	14.7 ± 2.6	
	1	84.2 ± 0.2	32.7 ± 0.3	19.5	1.87 ± 0.27	2.61 ± 0.60	48.4 ± 6.5	
25.7	0†	87.9 ± 0.2	32.9 ± 0.2	18.6	0.51 ± 0.11	0.82 ± 0.11	1.9 ± 4.3	20.3 ± 2.7
	0†	92.2 ± 0.2	32.8 ± 1.2	17.8	3.06 ± 0.42	5.22 ± 0.80	98.0 ± 7.9	126.6 ± 6.2
	0	88.3 ± 0.0	33.8 ± 0.8	19.7	0.68 ± 0.13	0.69 ± 0.11	5.5 ± 5.9	13.6 ± 5.2
	0	93.0 ± 0.2	35.0 ± 1.0	17.9	0.96 ± 0.18	1.94 ± 0.31	33.4 ± 8.3	42.6 ± 28.7
	0	87.9 ± 0.2	33.1 ± 0.7	20.2	1.89 ± 0.29	3.61 ± 1.39	63.4 ± 7.3	67.1 ± 10.0
	0	91.4 ± 0.2	33.1 ± 0.4	17.0	1.33 ± 0.18	2.15 ± 0.45	33.8 ± 4.1	75.4 ± 33.0
	0	91.4 ± 0.5	32.6 ± 0.6	20.1	1.71 ± 0.29	2.61 ± 0.29	41.6 ± 9.7	28.1 ± 25.5
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), H_2 content (H_2 %), chamber temperature (T_{CH}), H_2 pressure (PH_2), animal weight (Wt), mean CH_4 concentration in the chamber during the final hour at constant pressure (c[CH_4]), mean CH_4 concentration in the chamber during the decompression (d[CH_4]) and mean CH_4 release rate at constant pressure (c $\dot{V}CH_4$). Values for the models are parameter estimates ($\pm$ 1 SE), Wald statistics, log likelihood (LL), and likelihood ratio statistics results (P-value).

Variables	β_0	β_1	Wald stat	LL	P-value
Null	-1.48 ± 0.50			-12.94	
H_2 %	-2.44 ± 17.71	0.01 ± 0.20	>0.9	-12.94	>0.9
P	5.00 ± 9.01	-0.27 ± 0.38	0.47	-12.67	>0.5
PH_2	2.76 ± 7.18	-0.20 ± 0.34	0.56	-12.76	>0.5
Wt	2.18 ± 8.06	-0.19 ± 0.42	0.65	-12.83	>0.5
T_{CH}	43.43 ± 22.53	-1.40 ± 0.70	0.05	-10.32	<0.05
c[CH_4]	0.17 ± 0.86	-1.82 ± 0.96	0.06	-10.27	<0.05
d[CH_4]	0.18 ± 0.82	-1.23 ± 0.64	0.06	-9.91	<0.05
c $\dot{V}CH_4$	-0.29 ± 0.72	-0.05 ± 0.03	0.10	-10.78	< 0.05
Multivariate	β_0	β_1	β_2	LL	P-value
c[CH_4] + T_{CH}	32.82 ± 23.48	-1.41 ± 0.99	-1.02 ± 0.74	-9.05	>0.1
d[CH_4] + T_{CH}	34.63 ± 25.32	-0.96 ± 0.64	-1.08 ± 0.79	-8.78	>0.1
c $\dot{V}CH_4$ + T_{CH}	36.64 ± 23.44	-0.04 ± 0.03	-1.15 ± 0.73	-9.18	>0.1
T_{CH} + P	48.23 ± 23.79	-1.93 ± 0.98	0.53 ± 0.59	-9.88	>0.5

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.
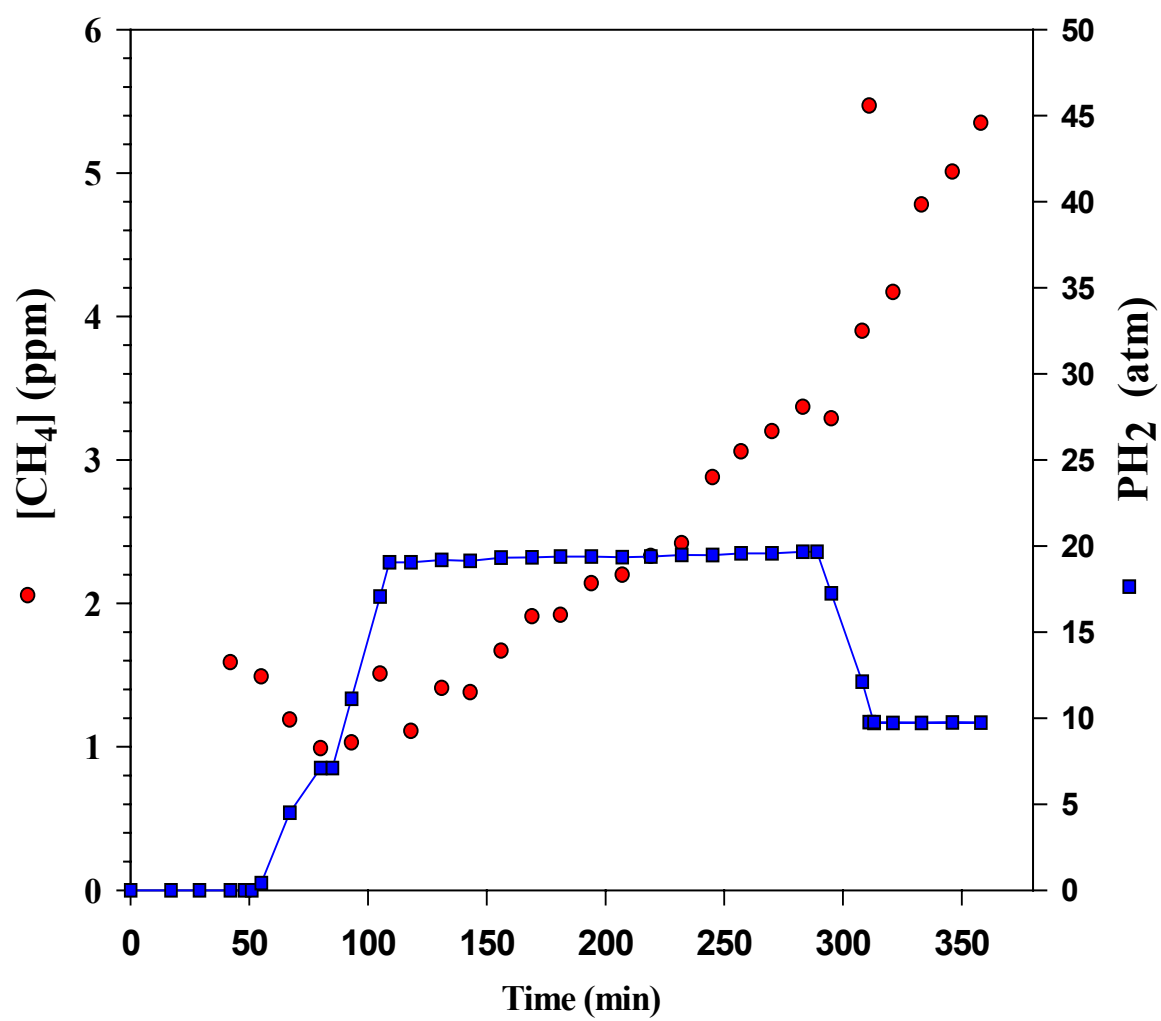
Figure 4.2. Methane release rate ($\dot{V}CH_4$, μmoles CH$_4$ • min^{-1}) versus chamber CH$_4$ concentration ([CH$_4$], ppm) in animals at 22.3, 24.1, and 25.7 atm. A) The c $\dot{V}CH_4$ and c[CH$_4$] are mean values (n = 5 chromatographic readings) at constant pressure. B) The d $\dot{V}CH_4$ and d[CH$_4$] are mean values (n = 3 chromatographic readings) during decompression.
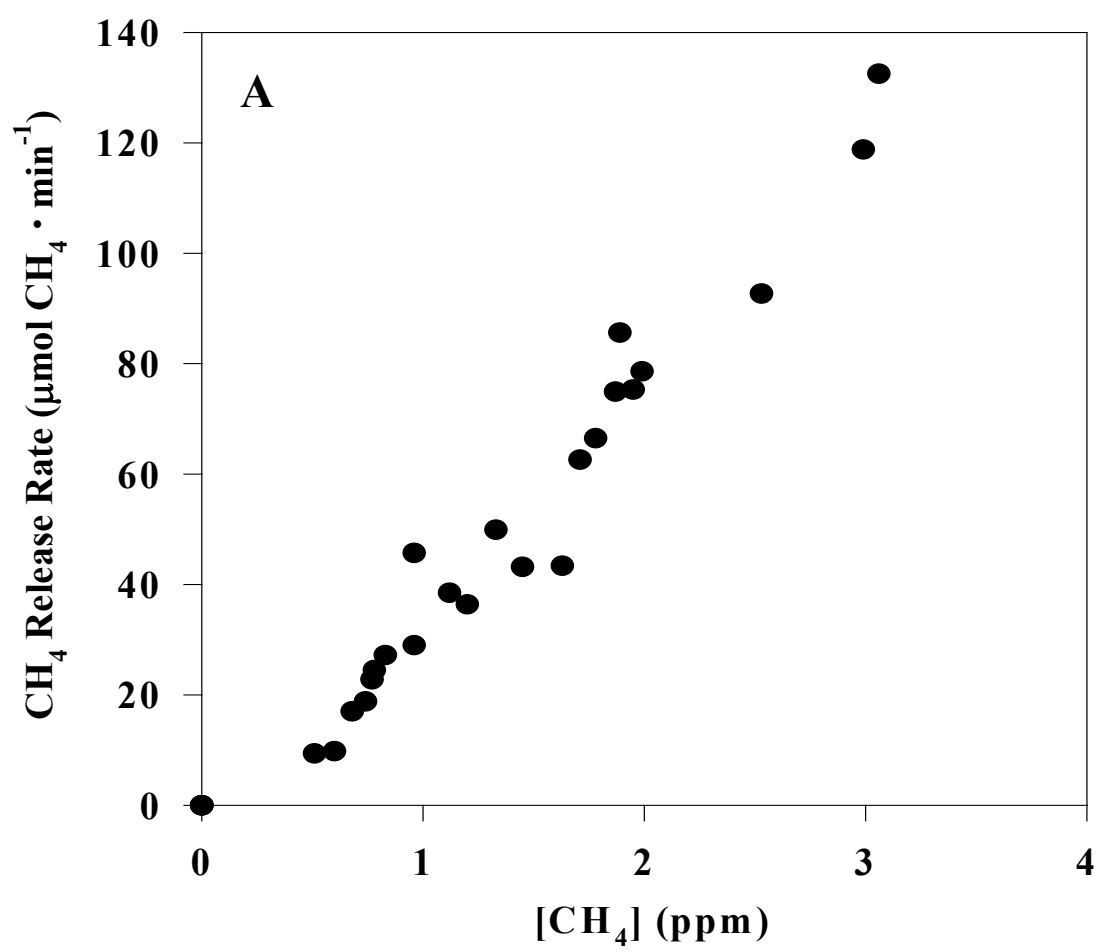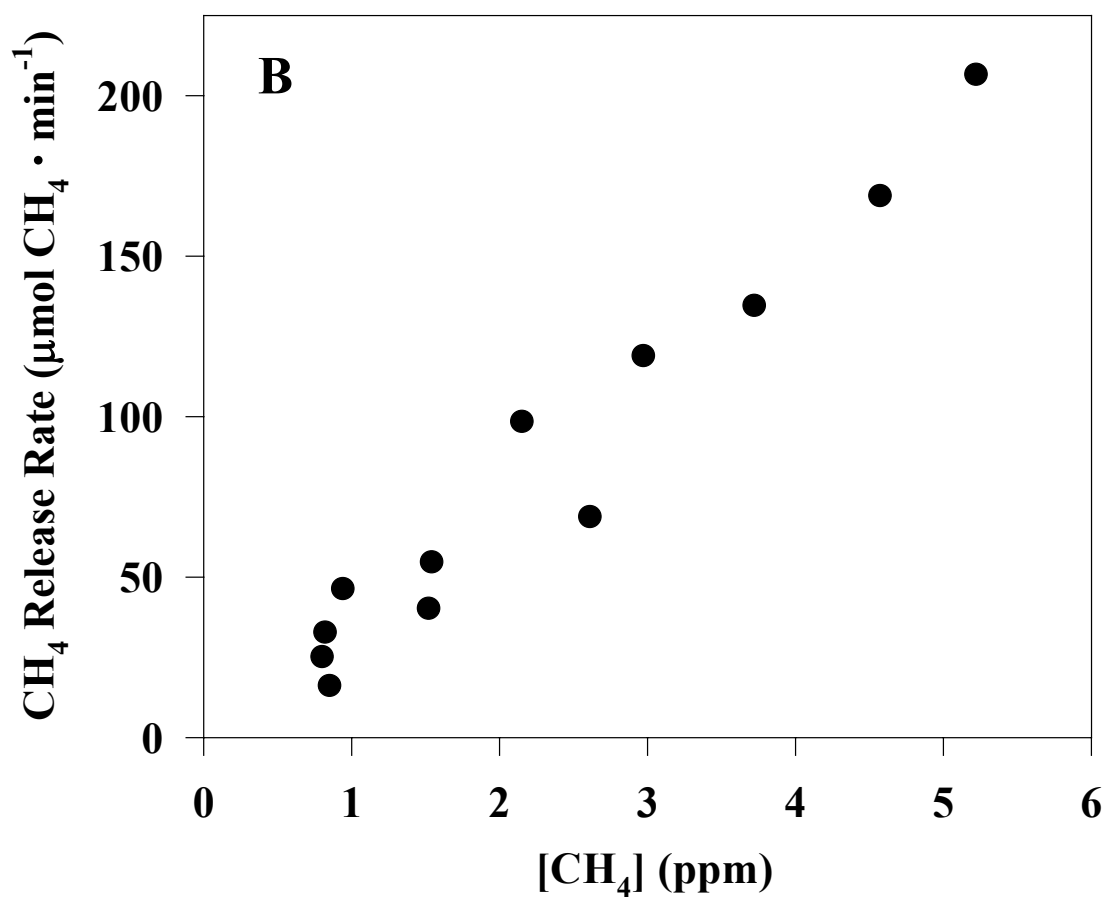
Figure 4.3. Mean CH$_4$ release rate (c $\dot{V}$CH$_4$) for pigs that either displayed or did not display symptoms of decompression sickness (DCS) after hyperbaric exposure versus chamber H$_2$ pressure (PH$_2$). Solid line represents the least squares regression (not statistically significant) for all non-zero values of methane release rate (Y = -29.04 + 3.86 X, P = 0.40, r^2 = 0.03).
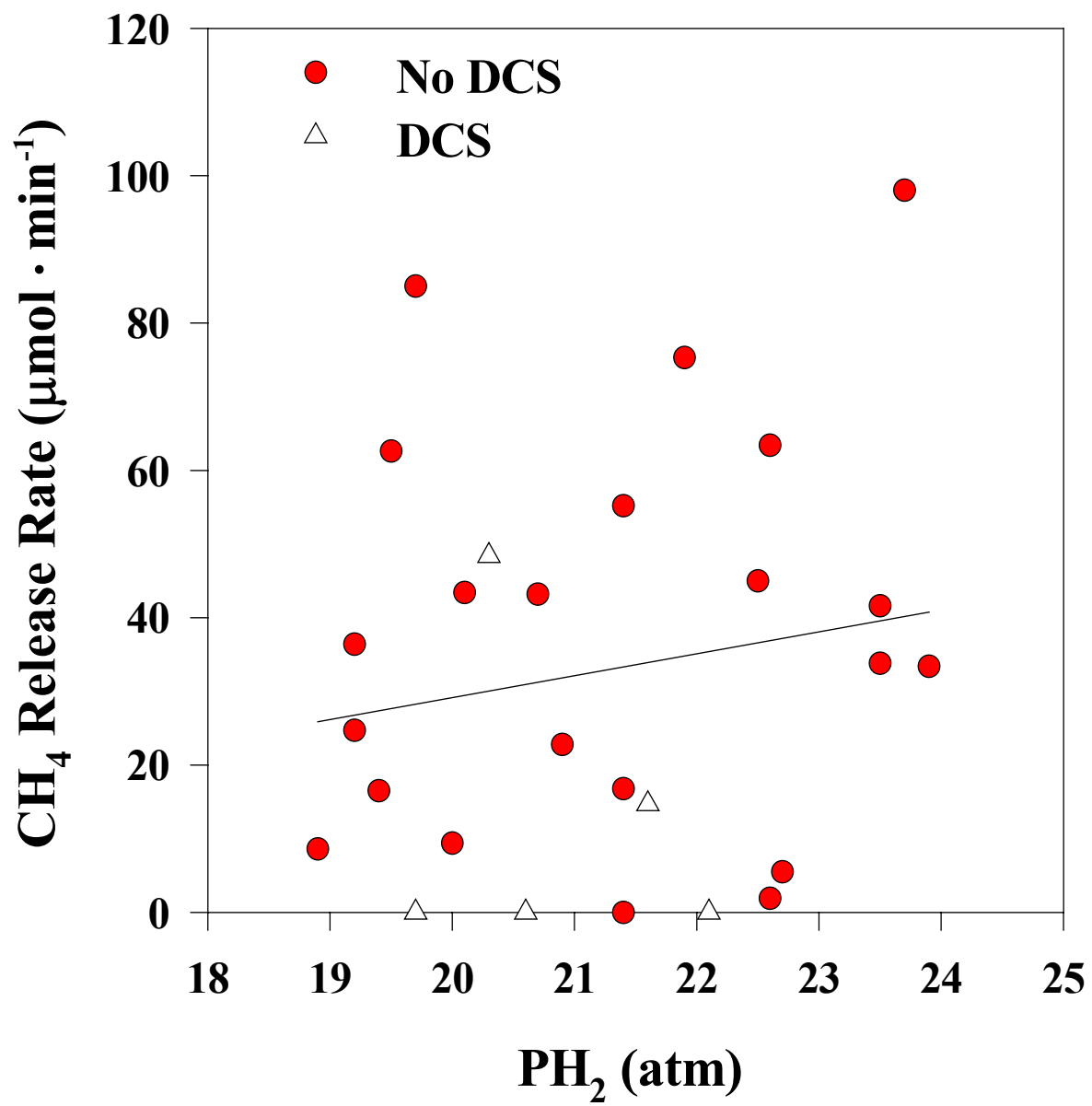
Figure 4.4. Probability of DCS [P(DCS)] versus CH$_4$ release rate (c $\dot{V}$CH$_4$) from pigs with native intestinal flora at 22.3, 24.1, and 25.7 atm hydrox for 3 h. There was a negative correlation between increasing c $\dot{V}$CH$_4$ and DCS incidence, as determined by logistic regression analysis (P < 0.05, log likelihood ratio test).
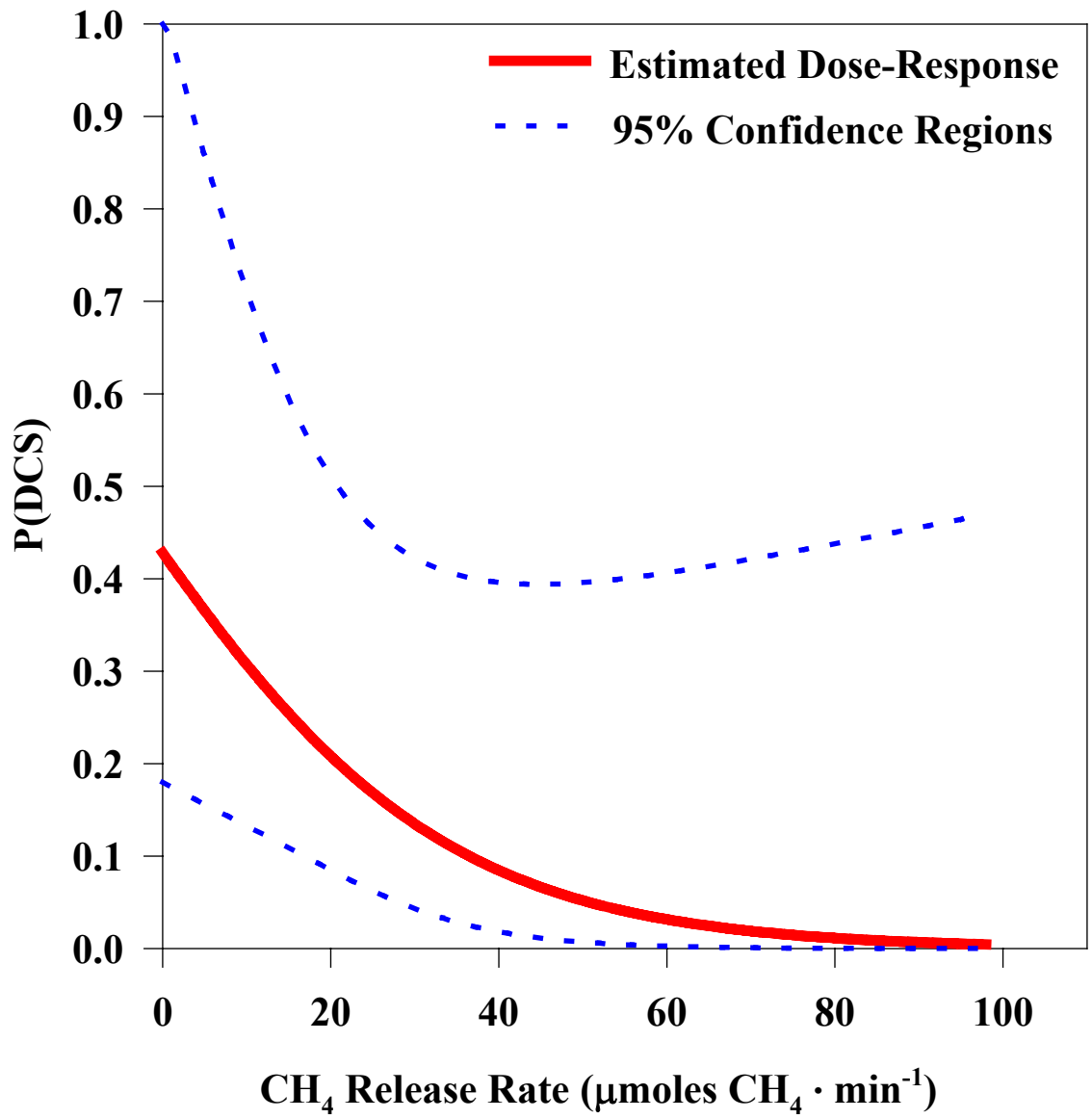
CHAPTER FIVE

INJECTION OF H_2-METABOLIZING MICROBES INTO THE

INTESTINES OF PIGS REDUCES DECOMPRESSION

SICKNESS RISK AFTER HYPERBARIC EXPOSURE TO H_2

"vae victis "

INTRODUCTION

In the previous chapter, a serendipitous finding was reported during a search for a compression and decompression sequence in H_2 that was going to give a high DCS incidence in untreated pigs. It was shown that pigs with higher methane release rate ($\dot{V}CH_4$) during hyperbaric H_2 exposure had a significantly lower risk of decompression sickness (DCS). The release of CH_4 was suggested to be the product of H_2 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 H_2.

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 H_2-metabolizing microbe, *Methanobrevibacter smithii*, were made into the intestines of pigs before hyperbaric exposure to H_2. 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 H_2-metabolizing microbes alone to significantly enhance the H_2 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 H_2-CO_2 (80%/20 %), then stored at 4 °C under 3 atm H_2-CO_2 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 H_2-CO_2 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-fluorane (Aerrane®, Fort Dodge Animal Health, Fort Dodge, IO) balanced with O_2, 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 CO_2-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_2 and given an intravenous injection of Yohimbine (Yobine®, Lloyd Inc., Shenandoah, Iowa, 2 mg • kg^{-1}) to counteract the Xylazine. The animal was closely observed and allowed to recover for 1-3 h before the hyperbaric experiment.

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_2 to keep the chamber atmosphere normoxic ($PO_2 = 0.2 - 0.4$ atm). Upon reaching 11 atm, the chamber was flushed with H_2 for approximately 30 min. The chamber was further pressurized with H_2 to 24.1 atm at a rate of 0.45 atm • min^{-1}. There was no difference in PH_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_2 and O_2 to make up for the gas exhausted to the gas chromatograph (~115 L • min^{-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^{-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: $\cong$ 30 °C at 11 atm and $\cong$ 33 °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 ($c\dot{V}CH_4$) for treated (n = 3) and control animals (n = 1), the chamber pressure was held constant at 24.1 atm for 19 - 24 h. The $c\dot{V}CH_4$ 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.

$\dot{V}CH_4$ at 1 atm

The $\dot{V}CH_4$ 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 µL) were taken with a gas-tight syringe from a sample hole covered with a rubber membrane. A 100 µL 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 µmol 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 CH_4 production rate was calculated as the change in CH_4 concentration over time ($\dot{V}CH_4$, μmol $CH_4 \cdot min^{-1}$). To avoid build-up of CO_2 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 $\dot{V}CH_4$ was taken as the average of the two independent measurements.

Caecal Assay

In a subsample of animals (n = 30), the $\dot{V}CH_4$ 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 $\dot{V}CH_4$

The $\dot{V}CH_4$ 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 Δt of 120 min and a $\dot{v}$ 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(DCS)] using the following independent variables: body weight (Wt), chamber temperature (T_{CH}), partial pressure of H_2 (PH_2), methane release rate at constant pressure (c $\dot{V}CH_4$), 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 ($P < 0.01$, χ^2 one-sided test) and the 70% incidence of the surgical control group alone ($P < 0.05$, χ^2 one-sided test, Table 5.1, Fig. 5.1).

$\dot{V}CH_4$ from animals

The [CH_4] (ppm) increased steadily for the duration of the dive for all animals. The $c\dot{V}CH_4$ (μmol $CH_4 \bullet$ 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 $c\dot{V}CH_4$ was detected (Fig. 5.3). For animals at extended time a constant pressure, regression analysis indicated that the $c\dot{V}CH_4$ increased with time for treated animals (Fig. 5.3).

There was no difference in the $c\dot{V}CH_4$ between the untreated and surgical control groups ($P > 0.8$, Table 5.1), and the average $c\dot{V}CH_4$ from the pooled control animals was 34.5 ± 16.8 μmol $CH_4 \bullet$ min^{-1} (C + SC, Table 5.1). Animals injected with *M. smithii*

released an average of 91.7 ± 36.1 µmol CH$_4$ • 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 CH$_4$ • min^{-1}, with an mean of 978 ± 528 µmoles CH$_4$ • min^{-1}. The $\dot{V}$CH$_4$ increased in animals injected *M. smithii* compared to control animals (Fig. 5.4). There was a significant increase in the c$\dot{V}$CH$_4$ as the total activity injected was increased from less than 500 µmol CH$_4$ • min^{-1} to more than 500 µmol CH$_4$ • min^{-1} (Fig. 5.4, P < 0.001). One animal, injected with a total activity of 240 µmol CH$_4$ • min^{-1}, was compressed to 24.1 atm using He (Table 5.2). This animal had a c$\dot{V}$CH$_4$ of 15.0 µmol CH$_4$ • min^{-1}, which was less than any other treated animal and most control animals (Table 5.1).

During the decompression phase, the CH$_4$ release rate (d$\dot{V}$CH$_4$) 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 $\dot{V}$CH$_4$. For these animals (n = 13), there was a trend toward a lower CH$_4$ release rate during the decompression phase compared to the c$\dot{V}$CH$_4$ at 24.1 atm (P = 0.075, paired 2-tailed t-test, Table 5.3).

$\dot{V}CH_4$ from caecal samples

The caecal assay showed that the *in vitro* $\dot{V}CH_4$ from the animals injected with methanogens was significantly higher than from control animals by two orders of magnitude (Fig. 5.5, $P < 0.01$).

$\dot{V}CH_4$ in 1 atm air versus hyperbaric H_2

The average $\dot{V}CH_4$ in 1 atm air for a subset of untreated animals (n = 45, including animals from Chapter 4, 5, and 6), was 16.6 ± 17.1 µmol $CH_4 \bullet min^{-1}$ (Fig. 5.6). This was significantly less than the mean value of 34.5 ± 16.8 µmol $CH_4 \bullet min^{-1}$ for the pooled control animals (n = 20, Table 5.1, C+SC) in hyperbaric H_2 ($P < 0.01$).

Logistic regression

The univariate analysis suggested that T_{CH}, PH_2, and $c\dot{V}CH_4$ were not predictors of the P(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(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 $c\dot{V}_{CH_4}$ 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 $c\dot{V}_{CH_4}$. This has also been documented in rats injected with methanogens, in which the $c\dot{V}_{CH_4}$ increased with increasing PH_2 (Kayar, *et al.*, 1998a).

Animals injected with methanogens had a significantly higher $c\dot{V}_{CH_4}$ compared to controls (Table 5.1). There was a significant increase in the $c\dot{V}_{CH_4}$ as the total activity injected was increased from < 500 μmol $CH_4 \bullet min^{-1}$ to > 500 μmol $CH_4 \bullet min^{-1}$, supporting a dose response effect (Fig. 5.4). Injection of H_2-metabolizing microbes in a hyperbaric He atmosphere did not increase the $c\dot{V}_{CH_4}$ (Table 5.2), but was comparable to the mean $\dot{V}_{CH_4}$ from untreated animals measured in 1 atm air (Fig. 5.6). Since only the hyperbaric H_2 environment increased the $c\dot{V}_{CH_4}$ in treated animals, this suggests that the increased levels of $c\dot{V}_{CH_4}$ 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 *in vitro* $\dot{V}_{CH_4}$ 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 $c\dot{V}CH_4$. 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 $c\dot{V}CH_4$ 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 *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*, et al.*, 1994; Strocchi*, et al.*, 1991), while others suggest that sulphate reduction (Christl*, et al.*, 1992; Gibson*, et al.*, 1988) or acetogenesis (De Graeve*, et al.*, 1994; De Graeve*, 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*, et al.*, 1990).

Consequently, metabolism of endogenous H_2 is more complex than a simple correlation between the metabolism of H_2 and the $\dot{V}CH_4$ suggests (Eq. 1). In addition, there are several factors complicating the estimation of H_2 metabolism inside the pig based on the $c\dot{V}CH_4$. The kinetics of the release of CH_4 remain speculative, and may not directly correlate with the rate of H_2 metabolism. Furthermore, accurately estimating the $\dot{V}CH_4$ 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_4] continuously increases smoothly throughout the experiment (Fig. 4.1, Chapter 4), correcting the values to a $\dot{V}CH_4$ 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 $c\dot{V}CH_4$ as a significant predictor of DCS risk (Table 5.4).

With extended time at elevated pressure, the tissue PH_2 increases until an equilibrium between the tissues and the ambient environment has been reached, *i.e.* saturation. Prior to saturation, the availability of H_2 to the methanogens in the intestine will continuously increase. Furthermore, *M. smithii* has an *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 $c\dot{V}CH_4$ would increase temporally, primarily as an effect of increasing substrate availability as the tissue PH_2 increased, and secondarily due to an increasing population of *M. smithii*. The large variability in the $c\dot{V}CH_4$ between individual animals during the 3 hours under pressure made it impossible to distinguish any temporal trends in the $c\dot{V}CH_4$. 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 $c\dot{V}_{CH_4}$ 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 $c\dot{V}_{CH_4}$ 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 H_2 pressure (PH_2), animal weight, total methanogenic activity injected into intestines (INJ), mean CH_4 release rate at constant pressure (c $\dot{V}CH_4$), 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.

Group	Outcome	PH_2 (atm)	Weight (kg)	Total activity injected	$c\dot{V}CH_4$	T_{CH} (° C)
				$(\mu mol\ CH_4 \bullet min^{-1})$		
SC	0	21.5	18.9	0	20.9 ± 3.2	32.6 ± 0.7
SC	0	21.2	19.3	0	29.0 ± 10.7	32.6 ± 0.4
SC	0	21.7	19.4	0	52.1 ± 10.6	32.8 ± 0.7
SC	1	21.5	17.4	0	36.0 ± 42.3	32.8 ± 0.5
SC	1	22.4	21.4	0	45.0 ± 19.0	33.1 ± 1.6
SC	1	21.2	21.3	0	29.1 ± 9.6	33.4 ± 0.8
SC	1	21.6	21.9	0	41.3 ± 13.4	32.7 ± 0.8
SC	1	22.8	17.5	0	2.2 ± 6.6	32.8 ± 0.9
SC	1	21.7	18.8	0	33.7 ± 7.0	32.6 ± 0.6
SC	1	21.7	19.7	0	47.4 ± 11.7	32.2 ± 0.3
C	0	21.4	20.7	0	10.6 ± 9.9	33.3 ± 0.9
C	1	21.7	21	0	1.2 ± 8.0	33.8 ± 1.0
C	1	21.5	19.6	0	29.0 ± 11.3	33.3 ± 0.4
C	1	21.9	20.7	0	56.4 ± 43.4	32.6 ± 0.6
C	1	21.9	22.9	0	64.7 ± 9.1	33.8 ± 0.5
C	1	21.1	19.1	0	44.8 ± 34.1	33.1 ± 0.3
C	1	22.6	18.7	0	31.2 ± 10.4	32.9 ± 0.5
C	1	21.2	19.5	0	49.2 ± 10.2	32.4 ± 0.1
C	1	22.5	19.9	0	36.1 ± 8.5	32.8 ± 0.7
C	1	22.0	18	0	29.4 ± 11.0	33.0 ± 0.5
T	0	21.8	18.6	495	79.8 ± 27.0	33.5 ± 0.5
T	0	21.4	18.2	898	95.7 ± 11.5	33.1 ± 0.9
T	0	22.3	18.8	795	106.2 ± 17.7	33.4 ± 0.7
T	0	23.1	16.9	1137	77.8 ± 30.3	32.6 ± 0.7
T	0	21.6	19	1530	85.3 ± 8.1	32.5 ± 0.4
T	0	22.1	19.3	1573	63.4 ± 23.5	32.8 ± 0.4
T	0	21.7	18	1353	82.6 ± 28.1	32.5 ± 0.8
T	0	22.2	20.8	1056	79.3 ± 6.2	31.7 ± 1.3
T	0	21.8	18.1	1223	85.8 ± 20.5	32.0 ± 0.7
T	1	21.9	18.6	210	58.2 ± 9.7	32.4 ± 0.7
T	1	21.5	19.5	400	53.8 ± 13.9	31.9 ± 1.3
T	1	20.7	19.1	1090	91.3 ± 24.6	32.6 ± 0.2
T	1	22.0	19.6	688	107.2 ± 28.2	33.1 ± 0.3
T	1	22.1	18.8	206	61.8 ± 8.9	33.0 ± 0.4
T	1	22.7	19.2	2158	136.5 ± 5.7	33.1 ± 0.5
T	1	21.9	20.0	863	201.8 ± 34.3	32.3 ± 0.4
P		> 0.71	> 0.10		< 0.01	> 0.16

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 (lc $\dot{V}CH_4$), 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.

Group	Outcome	PH_2 (atm)	Weight (kg)	INJ	tc $\dot{V}CH_4$	lc $\dot{V}CH_4$	T_{CH} (° C)
					(μmol $CH_4 \bullet min^{-1}$)		
C-19 h	1	22.1	21.4	0	0 ± 0	21.8 ± 6.8	32.6 ± 0.5
T-24 h	1	21.2	21.1	925	74.7 ± 147.7	262.9 ± 38.6	32.8 ± 0.5
T-24 h	0	22.4	21.0	1168	146.4 ± 17.7	274.2 ± 55.2	31.2 ± 1.1
T-24 h	0	21.2	19.6	253	60.2 ± 8.7	194.0 ± 40.4	32.5 ± 0.5
T-He	0	0.0	20.7	240	$15.0 \pm .9$	$15.0 \pm .9$	32.6 ± 0.5

Table 5.3. CH$_4$ release rate at constant pressure (c$\dot{V}$CH$_4$) and during decompression (d$\dot{V}$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$\dot{V}$CH$_4$ versus d$\dot{V}$CH$_4$ for all animals.

Group	c$\dot{V}$CH$_4$	d$\dot{V}$CH$_4$
	(μmol CH$_4$ • min^{-1})	
T	79.8 ± 27.0	59.1 ± 16.3
T	95.7 ± 11.5	94.6 ± 63.7
T	106.2 ± 17.7	53.3 ± 12.6
T	77.8 ± 30.3	52.6 ± 38.4
T	85.3 ± 8.1	39.5 ± 6.0
T	63.4 ± 23.5	93.3 ± 31.7
T	82.6 ± 28.1	66.8 ± 26.1
T	79.3 ± 6.2	66.5 ± 3.3
T	85.8 ± 20.6	62.8 ± 8.7
SC	52.1 ± 10.6	35.5 ± 17.2
SC	29.0 ± 10.7	50.4 ± 14.3
SC	20.9 ± 3.2	12.7 ± 4.3
C	10.6 ± 9.9	17.1 ± 9.0
Mean T (± 1 stdev)	84.0 ± 11.9	65.4 ± 18.2
Mean SC + C (± 1 stdev)	28.2 ± 17.7	28.9 ± 17.4
Mean All (± 1 stdev)	68.8 ± 29.9	54.2 ± 25.6
P	0.075	

Table 5.4. Logistic regression results for total methanogenic activity injected (INJ, μmol $CH_4 \bullet min^{-1}$), animal weight (Wt, kg), chamber H_2 pressure (PH_2, atm), CH_4 release rate at constant pressure (c $\dot{V}_{CH_4}$), chamber temperature (T_{CH}) versus DCS outcome. Parameter estimates ($\pm$ 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.

Variables	Intercept	Slope	Wald	LL	P
Null	0.57 ± 0.35			-23.546	
INJ	1.33 ± 0.50	$(-1.60 \pm 0.68) \bullet 10^{-3}$	0.019	-20.216	< 0.05
Wt	-9.38 ± 6.24	0.52 ± 0.33	0.112	-22.061	< 0.1
PH_2	0.60 ± 14.94	$(-1.00 \pm 684) \bullet 10^{-3}$	> 0.9	-23.538	> 0.9
c $\dot{V}_{CH_4}$	1.00 ± 0.66	$(-7.04 \pm 9.01) \bullet 10^{-3}$	0.316	-23.019	> 0.1
T_{CH}	-18.79 ± 24.33	0.59 ± 0.74	0.426	-23.222	> 0.1
INJ Wt	-5.97 ± 6.88	$(-1.42 \pm 0.69) \bullet 10^{-3}$ 0.37 ± 0.36	0.040 0.292	-19.610	> 0.1

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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 (χ^2, P < 0.05).***
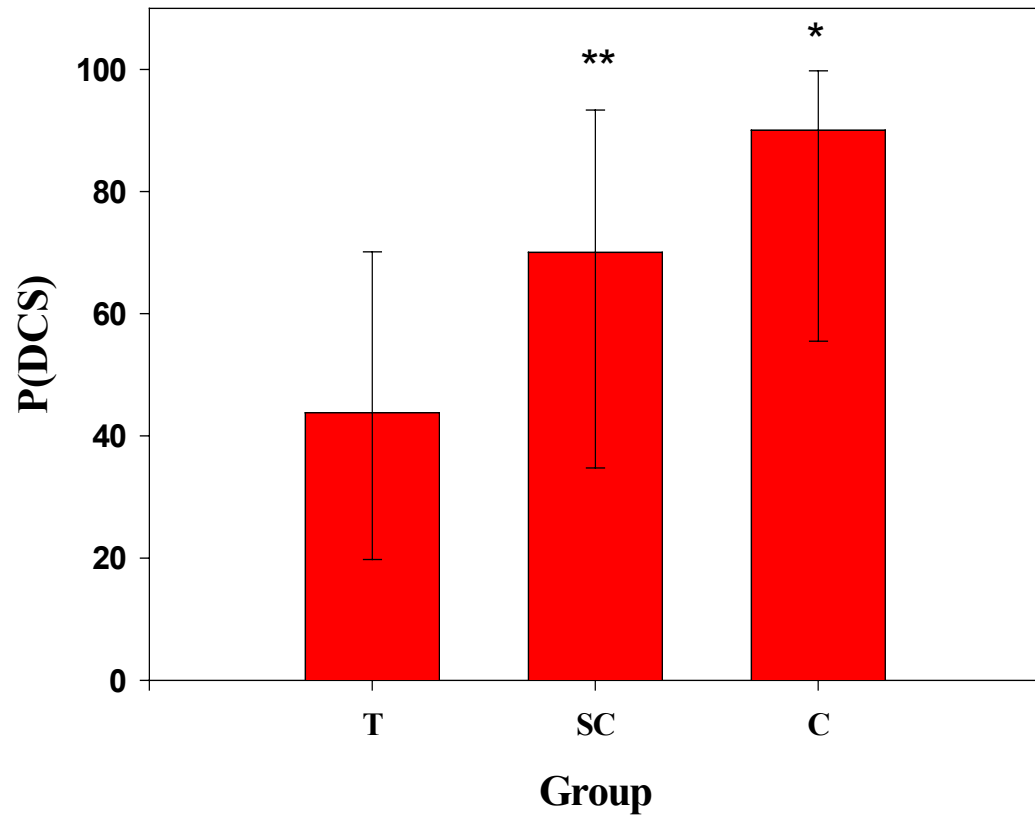
Figure 5.2. Methane release rate (μmol CH$_4$ • min^{-1}) and chamber H$_2$ pressure (PH$_2$) versus elapsed time during a sample dive for (A) a treated animal and (B) a control animal showing the absence of a visible temporal increase in the $\dot{V}CH_4$ during 3 h at constant pressure for either animal.
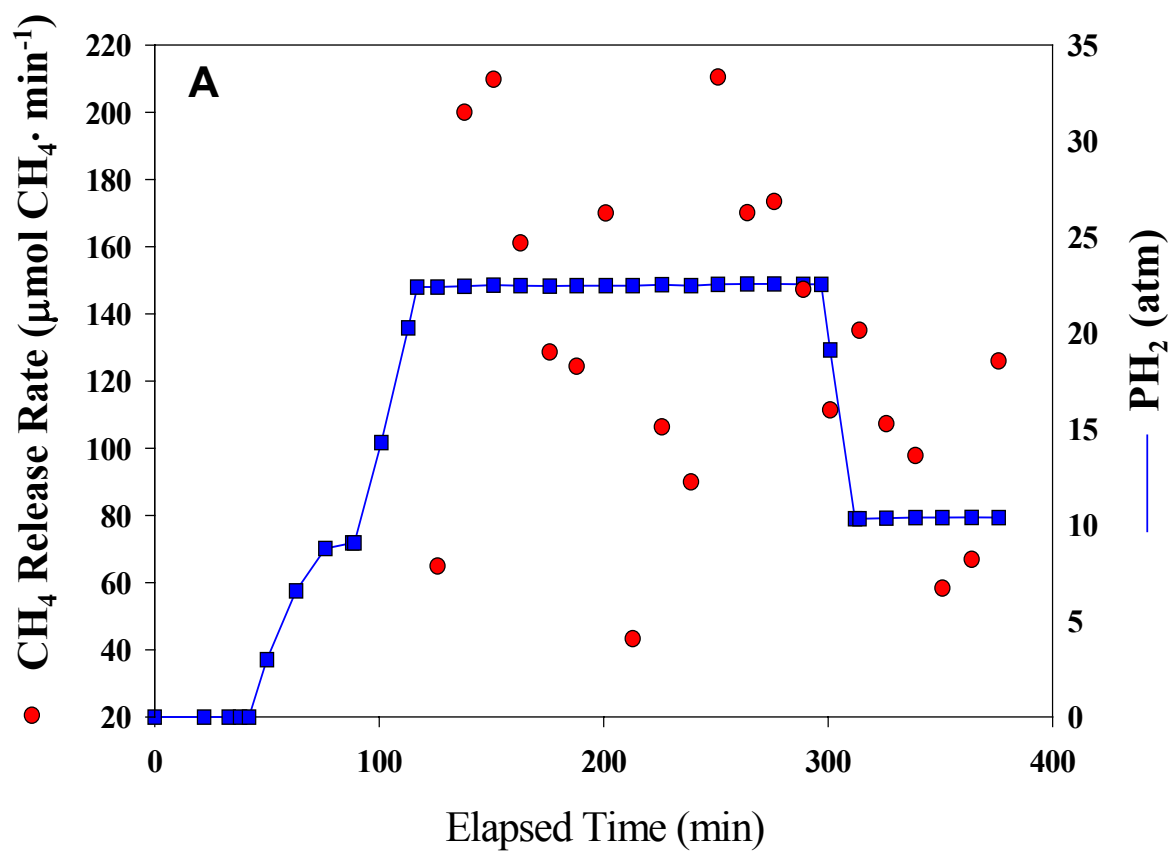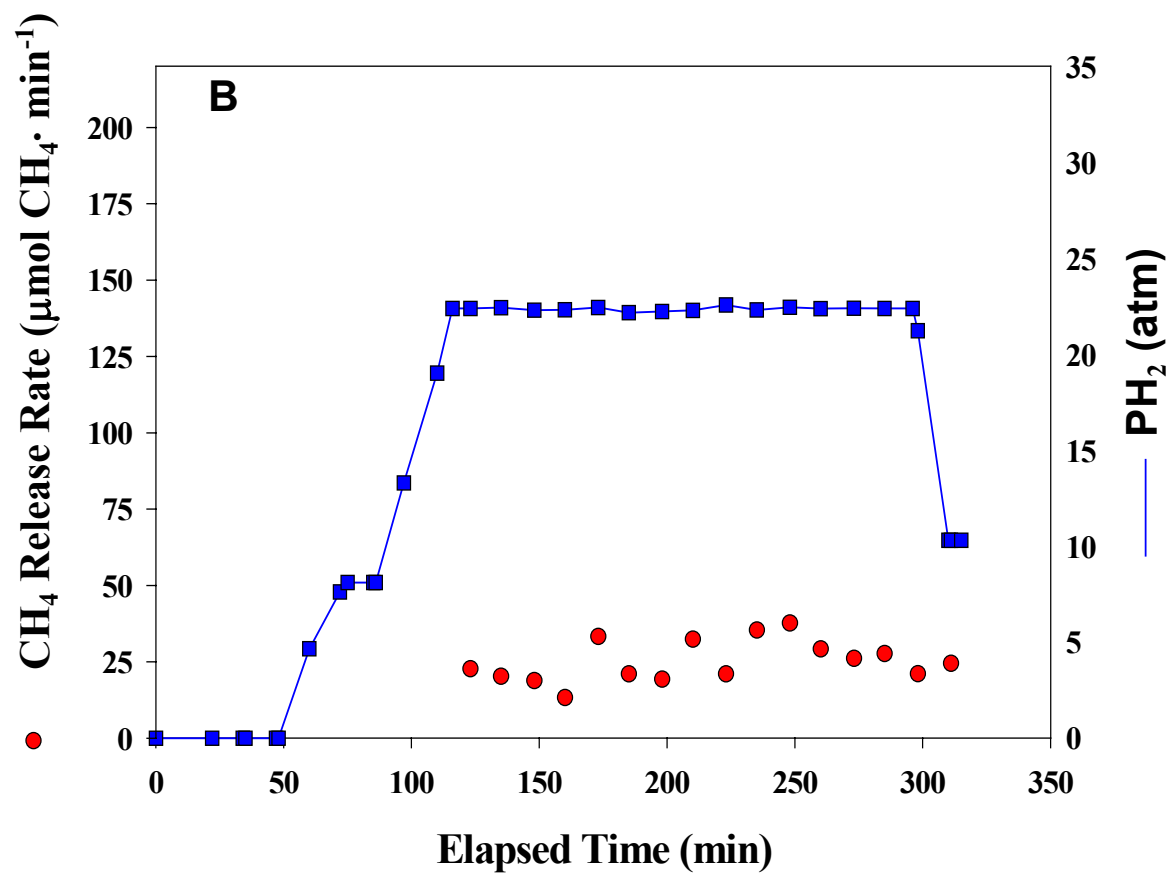
Figure 5.3. Estimated CH$_4$ release rate (c $\dot{V}$ CH$_4$, ± 1 stdev, μmol CH$_4$ • min^{-1}) versus elapsed time for one control animal (A) and three treated animals (B, C, D) during extended time at constant pressure. There was a significant temporal increase in the c $\dot{V}$ CH$_4$ for all treated animals (P < 0.001, linear regression).

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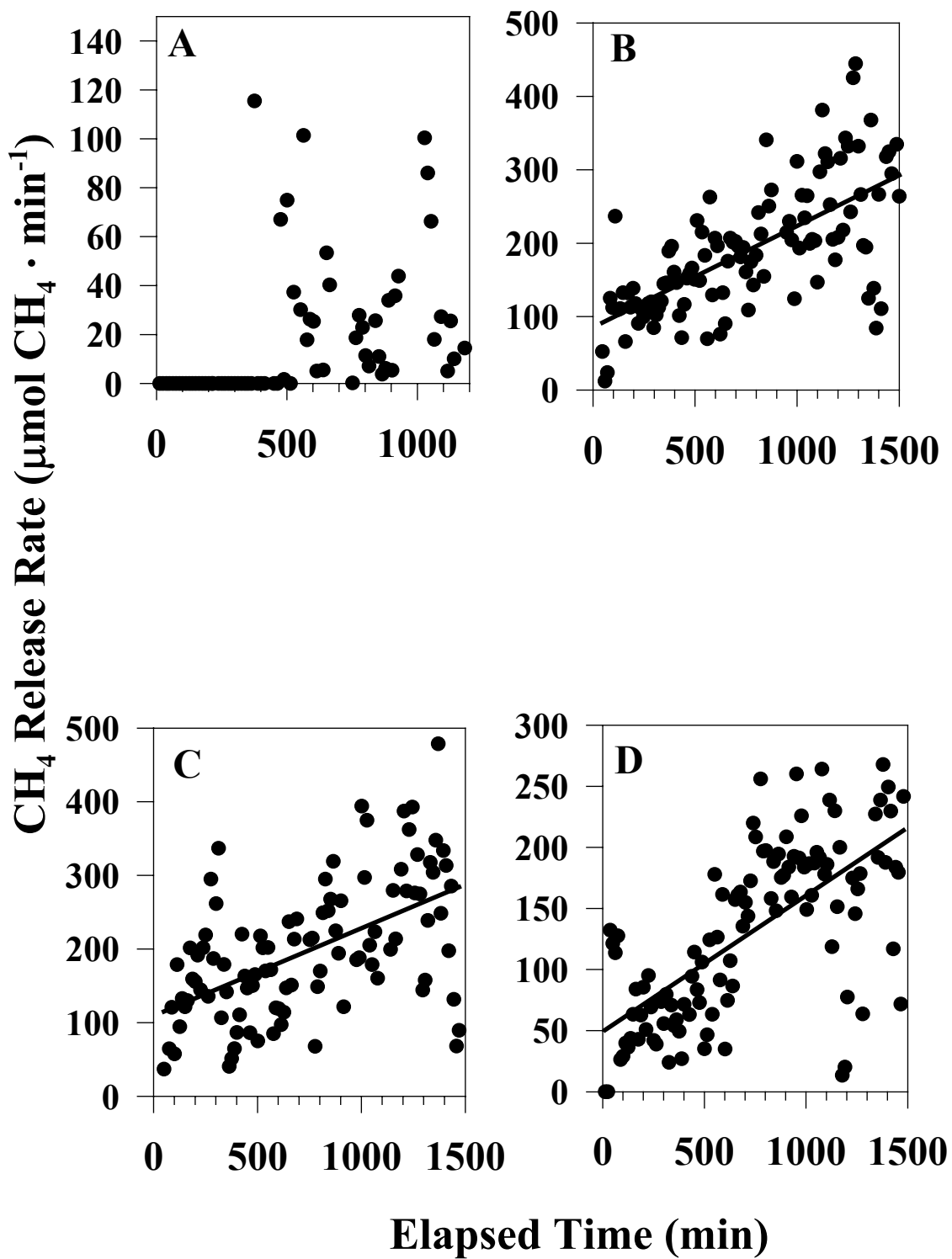
Figure 5.4.   Range of total methanogenic activity injected into the intestines of pigs (μmol CH$_4$ • min^{-1}) versus CH$_4$ release rate (μmol CH$_4$ • min^{-1}) at 24.1 atm. The data are from animals in Chapter 4, 5, and 6 compressed to a maximum pressure of 24.1 atm for 180 min. * There was a significant difference as compared to animals with 0 μmol CH$_4$ • min^{-1} injected ($P < 0.0001$, Mann-Whitney). **There was a significant difference as compared to animals with 500< μmol CH$_4$ • min^{-1} injected ($P < 0.05$, Mann-Whitney).
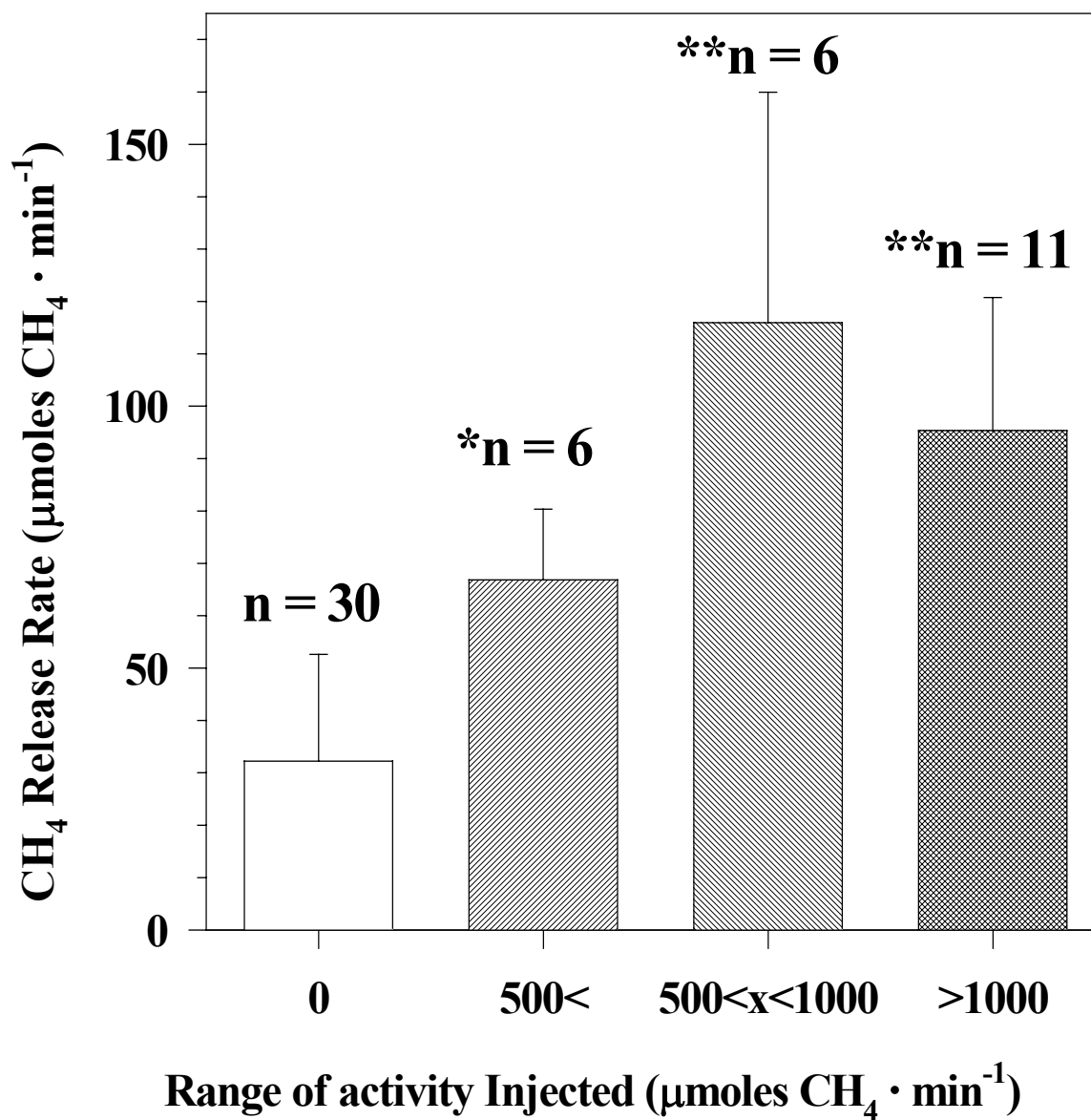
Figure 5.5. *In vitro* methanogenic activity of caecal samples from control (n = 16) and treated animals (n = 14). *Statistically different from treated at P < 0.05 (Mann-Whitney).
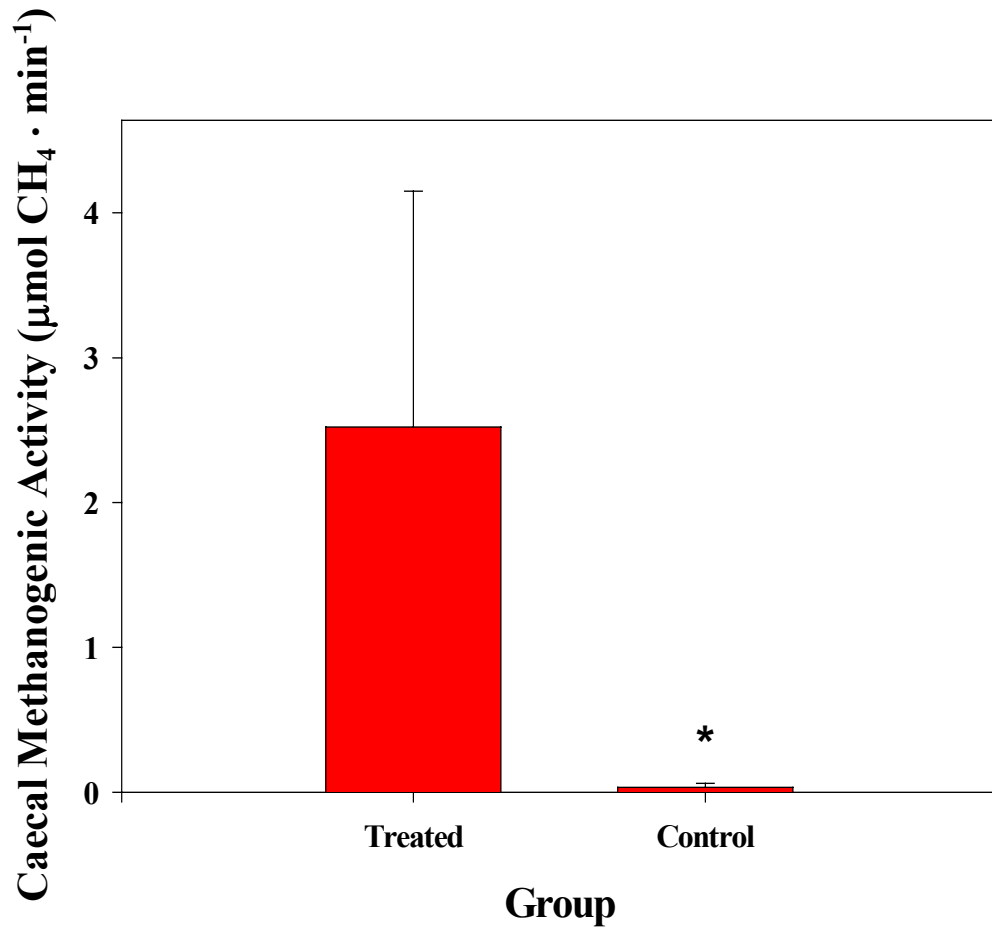
*Figure 5.6. Methane release rate from untreated pigs in 1 atm air (n = 45) versus hyperbaric H_2 (n = 20). ***Statistically different from 1 atm air at P < 0.05 (2 tailed t-test).***
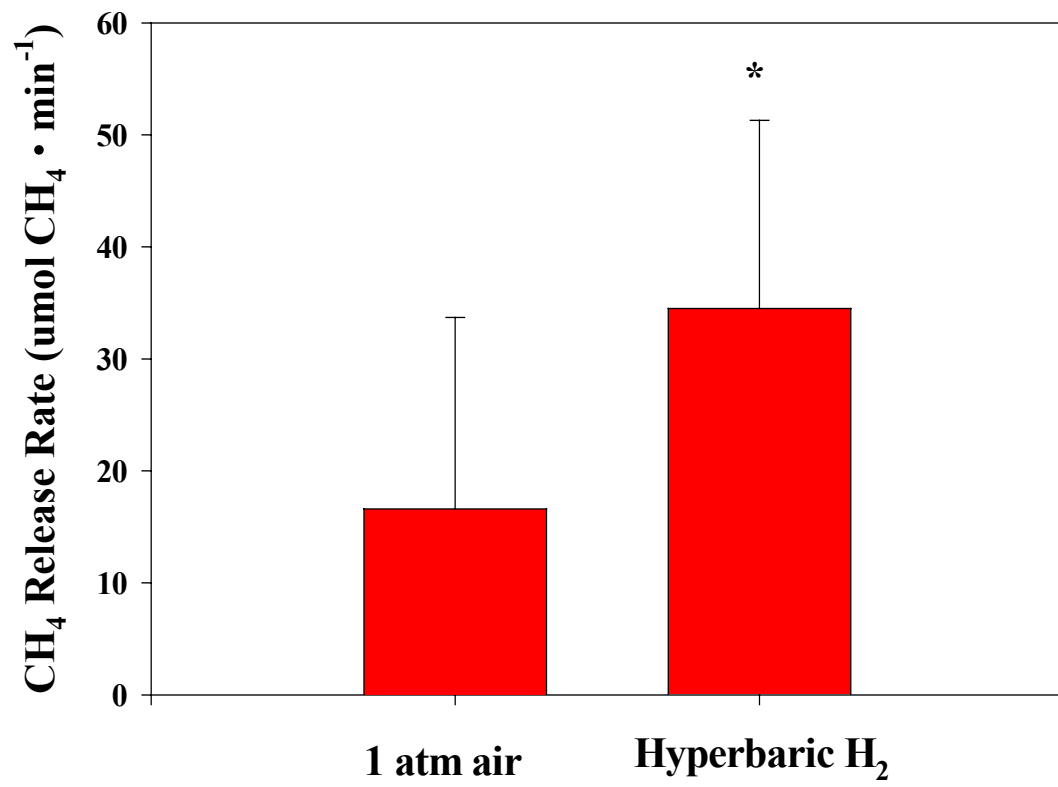
Figure 5.7. Total methanogenic activity injected versus predicted decompression sickness incidence [P(DCS)] as determined by logistic regression (P < 0.05) for treated and control animals at 24.1 atm in H$_2$ for 3 h.
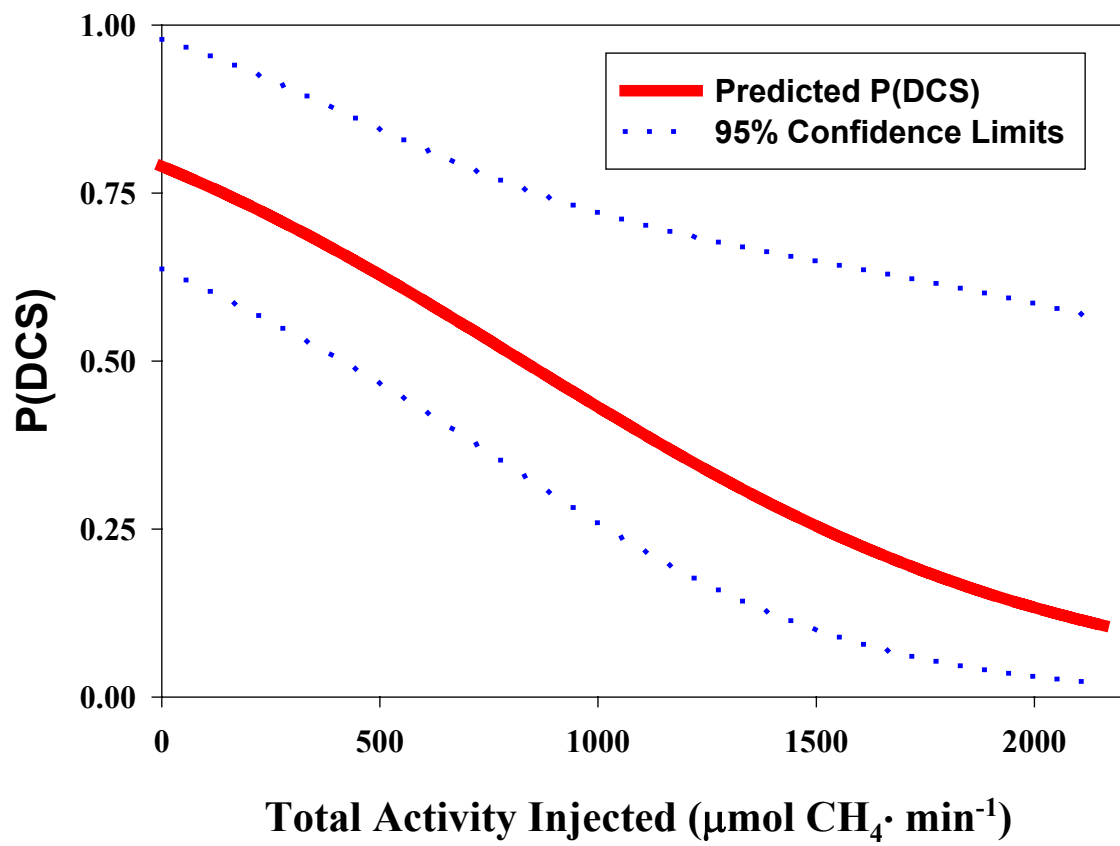
Figure 5.8. The figure shows the current working hypothesis on how H_2 biochemical decompression works as a sink, ultimately reducing the H_2 inert gas tissue tension and enhancing removal of H_2.

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_{blood}). The H_2 in the blood diffuses into the tissues until the blood and tissues are in equilibrium. Saturation is reached when P_{blood}, P_{tis} and P_{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 $P_{tis}H_2$ 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 $P_{tis}H_2$. 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 $P_{tis}H_2$ in the treated animal. The lower overall $P_{tis}H_2$ 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(DCS).

A

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

45 Units H_2

Tissue

50
50
45
70

Mixed Venous
Return

50

70
45

Intestines

50
50

70
70
50
100

Gas Exchange
Surface
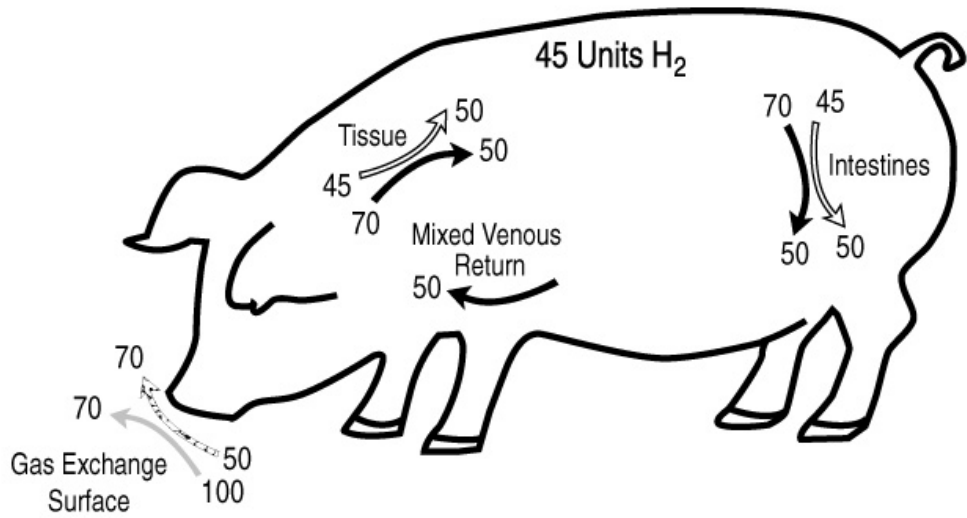
■ Blood
▨ Exterior Gas
▧ Alveolar Gas

Treated Compression

42 Units H_2

Tissue

47
47
42
66

Mixed Venous
Return

46

66
10

Intestines

10
10

66
66
46
100

Gas Exchange
Surface
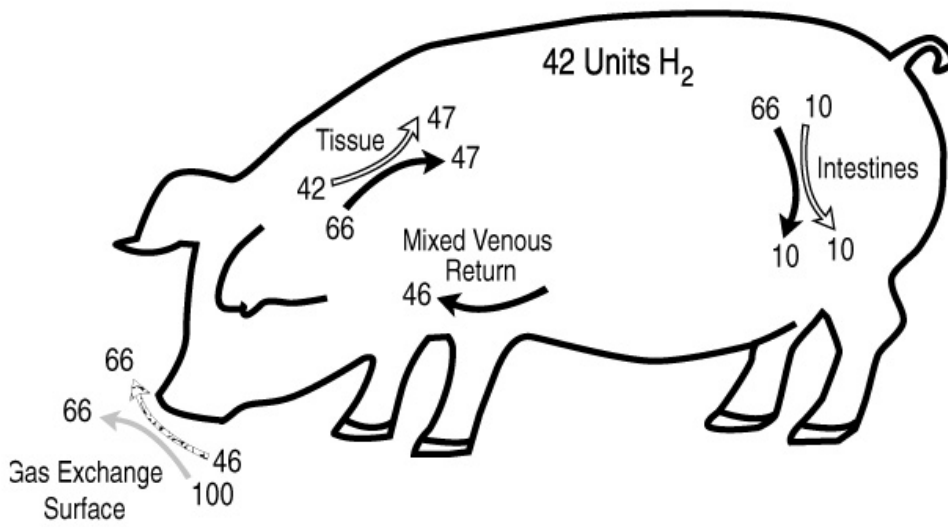
B

Standard Decompression P_{amb} = 50 Units H_2

100 Units H_2

Tissue 99 / 99
100 / 70
Mixed Venous Return 99

70 100 Intestines
99 99

70 70 / 70
Gas Exchange Surface 99 / 50

Blood
Exterior Gas
Alveolar Gas

Treated Decompression

99 Units H_2

Tissue 98 / 98
99 / 65
Mixed Venous Return 95

65 10 Intestines
10 10

65 65
Gas Exchange Surface 95 / 50
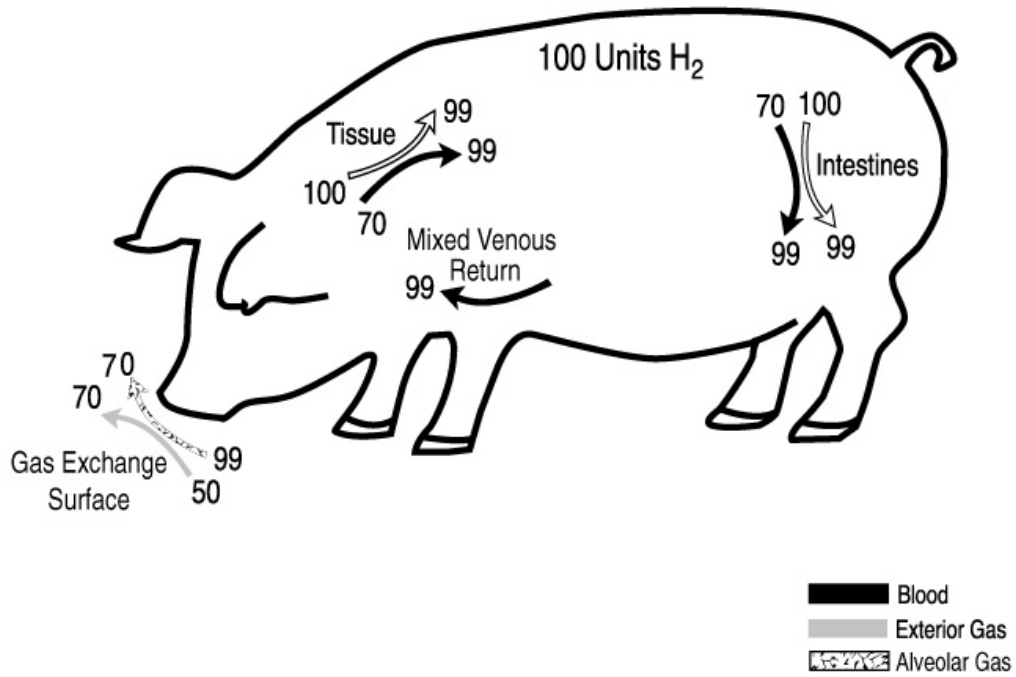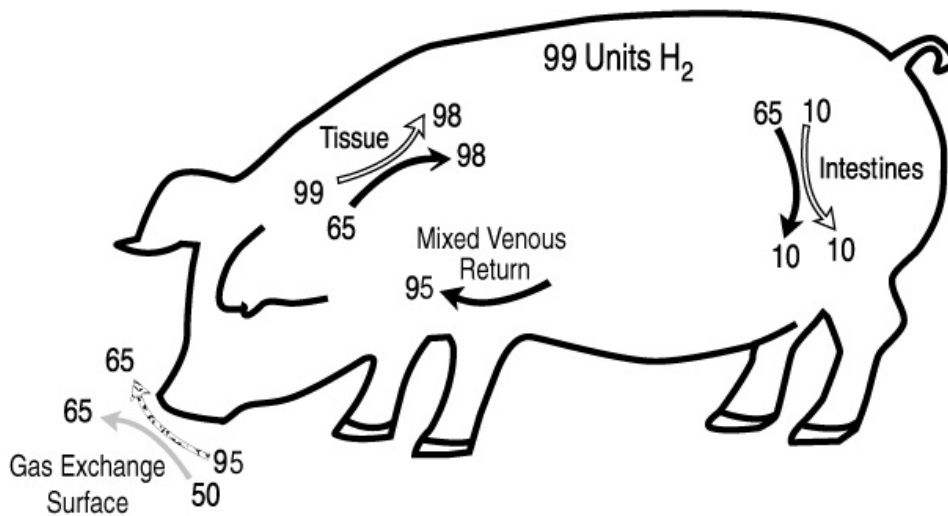
CHAPTER SIX

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 H_2 by injection of H_2-metabolizing microbes into the intestines of pigs during simulated H_2 dives. The metabolism of H_2 in the intestine is readily followed by measuring the release of CH_4, 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(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 H_2 is included. In constructing this model, the microbial metabolism of H_2 was considered to have a direct physiological effect by influencing the gas kinetics. The measure of H_2 metabolism was based on either the total activity injected into the animals (INJ), or as the CH_4 release rate ($\dot{V}CH_4$) from individual animals, assuming that there is a direct correlation between the H_2 metabolized inside the intestines and the release of 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 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:

$$P(DCS) = 1.0 - \exp(-\int_0^t r(x)\,dx) \qquad\qquad\qquad \text{Eq. 1}$$

while freedom of symptoms until time t is defined as

$$P(\text{no DCS}) = 1.0 - P(DCS) = \exp(-\int_0^t r(x)\,dx) \qquad\qquad \text{Eq. 2}$$

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:

$$P(DCS) = [exp(- \int_{0}^{T1} rdt)] [1.0 - exp(- \int_{T1}^{T2} rdt)] \qquad\qquad Eq.\ 3$$

This is the product of the probability of DCS not occurring in the interval [0-T1] and the

probability of DCS occurring in the interval [T1-T2] (Thalmann, *et al.*, 1997;

Weathersby, *et al.*, 1992). T1 is defined as the last known time the diver was definitely

free of DCS symptoms, and T2 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 T1 can be made in various ways as detailed elsewhere (Weathersby,

et al., 1992). Due to subjective aspects inherent in diagnosing DCS, in this study T1 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_{tis}, atm) and the absolute ambient pressure (P_{amb}, atm) above a threshold (Thr) (Thalmann, *et al.*, 1997;Weathersby, *et al.*, 1985;Weathersby, *et al.*, 1992),

$$r_1 = G_1 \bullet (P_{tis} - P_{amb} - Thr) \bullet P_{amb}^{-1} \qquad \text{Eq. 4}$$

where P_{tis} refers to the sum of the tissue tensions for H_2 ($P_{tis}H_2$, atm) and He ($P_{tis}He$, atm), and G_1 is a scaling factor (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 O_2, CO_2, and water vapour were ignored (Weathersby, *et al.*, 1987). In this model, r_1 is constrained to be ≥ 0, and accordingly, r_1 will be set to zero at any time $P_{amb} > P_{tis}$ (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).

$$r_2 = G_2 \bullet \int_0^t (P_{tis} - P_{amb} - Thr) \bullet P_{amb}^{-1} \, dx \qquad \text{Eq. 5}$$

For this model, the relative supersaturation is integrated from the first occurrence that $P_{tis} > P_{amb}$, followed by integration of both positive and negative values. However, the risk cannot be negative, so r_2 is constrained to be ≥ 0. For this model, G_2 is a scaling factor

(min^{-2}) and is expected to be smaller than G_1 due to the second integration (Weathersby, *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_{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, *et al.*, 1990; Novotny, *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, *et al.*, 1997).

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

$$\frac{dP_{tis}}{dt} = \frac{P_{blood}}{\tau} - \frac{P_{tis}}{\tau} \qquad\qquad Eq.6$$

where P_{tis} is the tissue tension of the inert gas (atm), P_{blood} the arterial blood tension of the inert gas (atm) , τ the time constant (min) to be determined from the data, and t is time (min). The time constant (τ) determines the flux of gases in and out of the tissues. The

change in P_{blood} during compression and decompression is assumed to be instantaneous

and therefore equal to the alveolar partial pressure (Pa_{in}) for that inert gas at all times.

The Pa_{in} in turn is assumed to be equal to the ambient partial pressure (P_{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_{blood} =

Pa_{in}):

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

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_{blood} on the P_{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 τ is equal for both He

and H_2. Hence, three parameters need to be determined: τ, 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

H_2 elsewhere in the body. However, a working hypothesis is that the conversion of H_2 into H_2O and CH_4 in the intestine creates a sink that globally affects the arterial H_2 tension ($P_{blood}H_2$, Fig. 5.8, Chapter 5). Therefore, the equation including methanogenesis is:

$$\frac{dP_{tis}H_2}{dt} = \frac{P_{blood}H_2 - BUG * A}{\tau} - \frac{P_{tis}H_2}{\tau} \qquad \text{Eq. 8}$$

where BUG is the rate of H_2 removal as determined by either the measured CH_4 release rate ($\dot{V}CH_4$, mmol $CH_4 \bullet min^{-1}$) or by the total microbial activity injected into the intestines (INJ, mmol $CH_4 \bullet min^{-1}$). A $\dot{V}CH_4$ and A_{INJ} (atm $\bullet$ min $\bullet$ (mmol $CH_4)^{-1}$) are the respective parameters to be determined. This equation assumes that the microbial metabolism of H_2 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 H_2 were made (Fig. 5.3, Chapter 5). Consequently, the removal of H_2 due to microbial metabolism results in up to four parameters that need to be determined for each model; τ, G, Thr and A $\dot{V}CH_4$ or A_{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:

$$P(obs)_i = P(DCS)_i^{X_i} \bullet P(no\ DCS)_i^{(1-X_i)} \qquad\qquad Eq.\ 9$$

The calculation of P(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

$$L(data\ set) = [P(obs)_1] \bullet [P(obs)_2] \ldots [P(obs)_n] \qquad\qquad Eq.\ 10$$

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(DCS) = c. Of the ten observations, there were 3 cases of DCS giving the following likelihood function

$$L = (1\text{-}c) \bullet (1\text{-}c) \bullet (1\text{-}c) \bullet (1\text{-}c) \bullet (1\text{-}c) \bullet (1\text{-}c) \bullet (1\text{-}c) \bullet (c) \bullet (c) \bullet (c)$$

$$L = [(1\text{-}c)^7] \bullet [(c)^3]$$

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

$$(P_{tis} - P_{amb}) \bullet P_{amb}^{-1} = 0 \text{ before decompression begins} \qquad \text{Eq. 11A}$$

$(P_{tis} - P_{amb}) \bullet P_{amb}^{-1}$ = constant after decompression begins.   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_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_{CH},

°C), O_2 %, and elapsed time (min). A gas chromatograph (Hewlett-Packard 5890A,

Series II, Wilmington, DE) measured the chamber gases O_2, H_2, He, N_2, and CH_4 every

12 min. Also included in the history of each experiment was the outcome and temporal

information regarding DCS events (0, T1, T2, and Tend). For animals that were

diagnosed with DCS, data included the time they were last definitely free of DCS signs

(T1), which in this study was set at the start of the decompression, and the time of

diagnosis (T2). For animals without DCS symptoms, the experiment ended after the 1-

hour post decompression observation period at 11 atm (Tend).

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, Tend), or until the animal was declared to have DCS (T2).

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 ($A\dot{V}_{CH_4}$) 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 $A\dot{V}_{CH_4}$ 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 H_2-biochemical decompression in pigs from any compression and decompression sequence in H_2, 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 H_2-metabolism into the models supports the hypothesis that microbial metabolism of H_2 reduces the P(DCS) by an overall reduction in the $P_{blood}H_2$ (Eq. 8). Two terms for H_2 metabolism were tested (Table 6.2): one relating the removal of H_2 to the total activity of H_2-metabolizing microbes injected into the intestines of the animals (A_{INJ}), and the other relating the removal of H_2 to the measured CH_4 release rate ($A \dot{V}_{CH_4}$).

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 $A \dot{V} CH_4$, 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 $A \dot{V} CH_4$ (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 $\dot{V} CH_4$. The estimated fraction removed was ~ 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 $\dot{V} CH_4$ and DCS risk (Table 6.2). Using the parameter for $A \dot{V} CH_4$ 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 $\dot{V} CH_4$ of either 0 (hypothetical control animal without native methanogens) or 40-80 μmol $CH_4 \bullet min^{-1}$ (range for a hypothetical control animal with native methanogens, Chapter 4), or an INJ of either 0 or 1500 μmol $CH_4 \bullet min^{-1}$ (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 $\dot{V} CH_4$ 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_{tis} are close, the difference in the area under the curve between P_{tis} and P_{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(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 $\bullet$ 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 ~ 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 τ for H_2 and He, models with separate

τ 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 H_2-metabolizing microbes injected); for Model 2a: τ, G, INJ, and Thr (threshold), and for Model 2b: τ, G, and $\dot{V}CH_4$ (methane release rate) (Table 6.2). χ^2 is for goodness of fit test comparing observed versus predicted DCS outcome.

Group (ID)	Gas	P (atm)	n	DR (atm $\bullet$ min^{-1})	Time (min)	DCS Observed (#)	DCS Predicted from Model (#)		
							1	2a	2b
C	H$_2$/He	22	13	0.45	180	4	2.9	1.6	1.8
C	H$_2$/He	24	9	0.45	180	3	2.3	1.4	1.8
C	H$_2$/He	26	7	0.45	180	0	1.9	1.1	1.4
C	H$_2$/He	24	20	0.9	180	16	13.0	14.6	13.8
T	H$_2$/He	24	18	0.9	180	7	8.3	7.4	9.5
C	H$_2$/He	24	8	1.8	180	8	5.7	6.5	5.4
T	H$_2$/He	24	2	1.8	180	2	1.3	1.3	1.2
C	H$_2$/He	24	8	0.9	150	3	5.0	5.6	5.0
T	H$_2$/He	24	5	0.9	150	2	2.4	2.2	2.9
C	H$_2$/He	24	5	0.9	120	4	3.3	3.7	3.1
C	H$_2$/He	24	9	0.9	30	2	5.5	6.3	6.0
C	H$_2$/He	24	1	0.9	1100	1	0.4	0.5	0.2
T	H$_2$/He	24	3	0.9	>1100	1	1.3	1.3	0.5
T	He	24	1	0.9	180	0	0.3	0.5	0.2
χ^2							9.24	12.66	15.59
P							> 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). τ, time constant for each model; G, scale factor for each model, A_{INJ} scale factor for the total H_2-metabolizing activity injected for each model; and $A_{\dot{V}CH_4}$ 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).

Model	τ (min)	G (min^{-1})	Thr (atm)	$A_{\dot{V}CH_4}$	A_{INJ}	LL	P
				(atm $\bullet$ min $\bullet$(mmol CH$_4$)$^{-1}$			
1	0.80 ± 0.13	2.23 ± 0.74	++++	++++	++++	-68.90	<0.01
	1.72 ± 2.09	1.15 ± 1.28	0.35 ± 0.76	++++	++++	-68.34	>0.1
	0.76 ± 0.11	2.90 ± 0.96	++++	++++	0.18 ± 0.05	-65.58	<0.01
	0.85 ± 0.16	2.33 ± 0.78	++++	0.94 ± 0.74	++++	-67.99	>0.1
	(min)	(min^{-2})	(atm)	(atm $\bullet$ min $\bullet$(mmol CH$_4$)$^{-1}$			
2	0.75 ± 0.06	0.14 ± 0.03	++++	++++	++++	-90.58	<0.01
	1.91 ± 0.08	0.08 ± 0.04	0.47 ± 0.44	++++	++++	-79.99	<0.01
	0.74 ± 0.06	0.21 ± 0.06	++++	++++	0.16 ± 0.04	-80.35	<0.01
	0.86 ± 0.08	0.17 ± 0.04	++++	1.24 ± 0.38	++++	-84.03	<0.01
	1.73 ± 0.99	0.12 ± 0.06	0.40 ± 0.39	++++	0.33 ± 0.18	-72.91	<0.01
	1.75 ± 1.01	0.10 ± 0.05	0.36 ± 0.38	1.32 ± 0.96	++++	-78.32	<0.1

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 τ (time constant), G (scale factor), and INJ (total activity of H_2-metabolizing microbes injected); Model 2a included parameters for τ, G, INJ, and Thr (threshold); and Model 2b included τ, G, and $\dot{V}CH_4$ (methane release rate) (Table 6.2). χ^2 is for goodness of fit test comparing observed versus predicted DCS outcome.

Group	Observed	Model		
		1	2 a	2b
Treated	12	13.7	12.6	14.7
Control	41	39.7	41.2	38.5
χ^2		0.25	0.03	0.66
P		> 0.6	> 0.8	> 0.4

Table 6.4. Observed versus predicted DCS. Values are observed and mean predicted DCS incidences for each group. Model 1 included parameters for τ (time constant), G (scale factor), and INJ (total activity of H_2-metabolizing microbes injected); Model 2a included parameters for τ, G, INJ, and Thr (threshold); and Model 2b included τ, G, and $\dot{V}CH_4$ (methane release rate) (Table 6.2). χ^2 is for goodness of fit test comparing observed versus predicted DCS outcome.

Group	n	Observed	Model		
			1	2a	2b
Treated	29	12	13.7	12.6	14.7
Control (0.45 atm $\bullet$ min^{-1})	29	7	7.0	4.1	4.9
Control (0.90 atm $\bullet$ min^{-1})	43	26	27.0	30.7	28.0
Control (1.80 atm $\bullet$ min^{-1})	8	8	5.7	6.5	5.4
χ^2			1.17	3.14	2.79
P			> 0.7	> 0.3	> 0.4

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 (τ) 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 • 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.
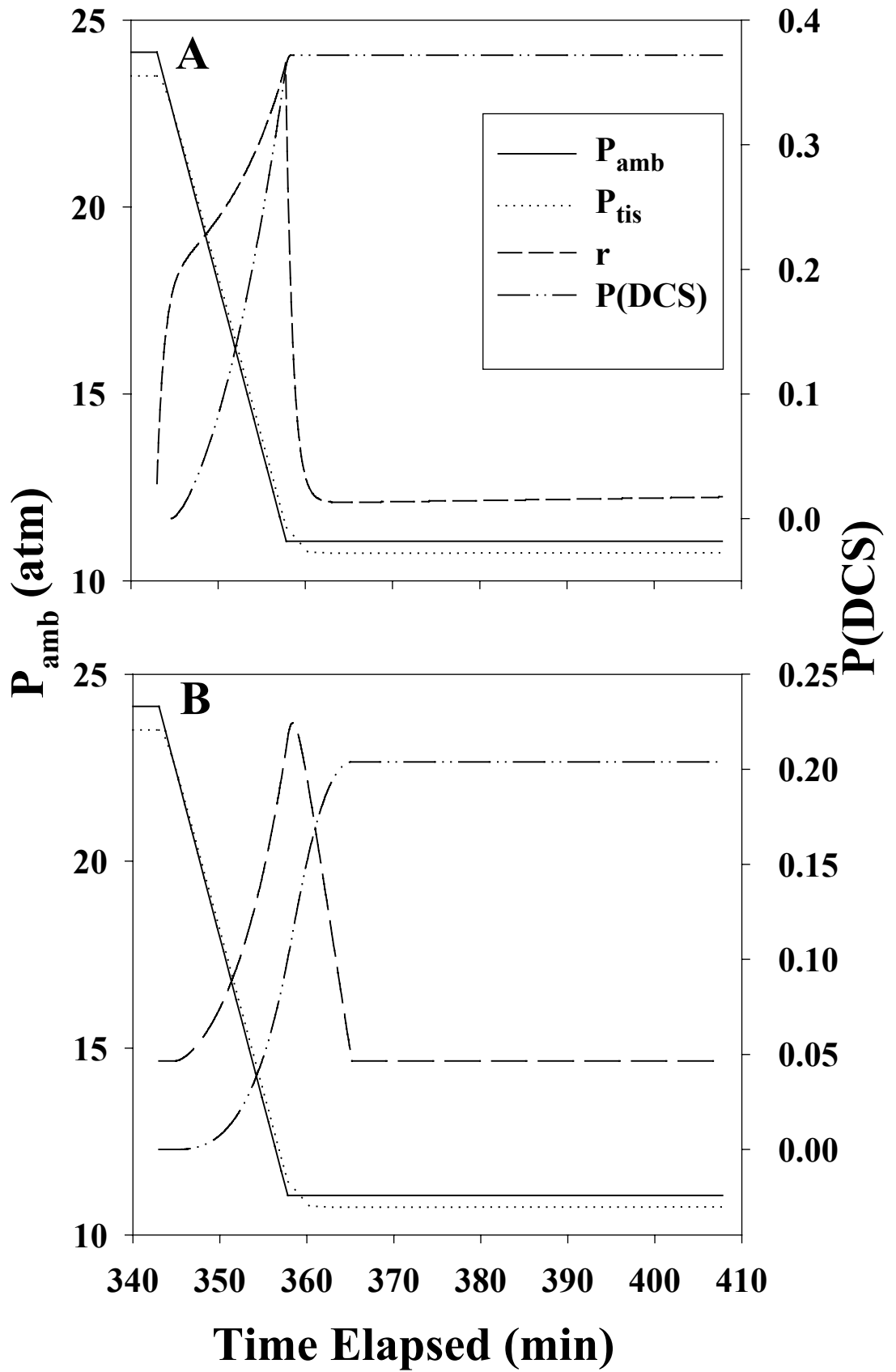
Figure 6.2. Cumulative number of DCS cases with time after reaching the observation pressure (11 atm) for the 53 observed cases of DCS out of 109 observations.
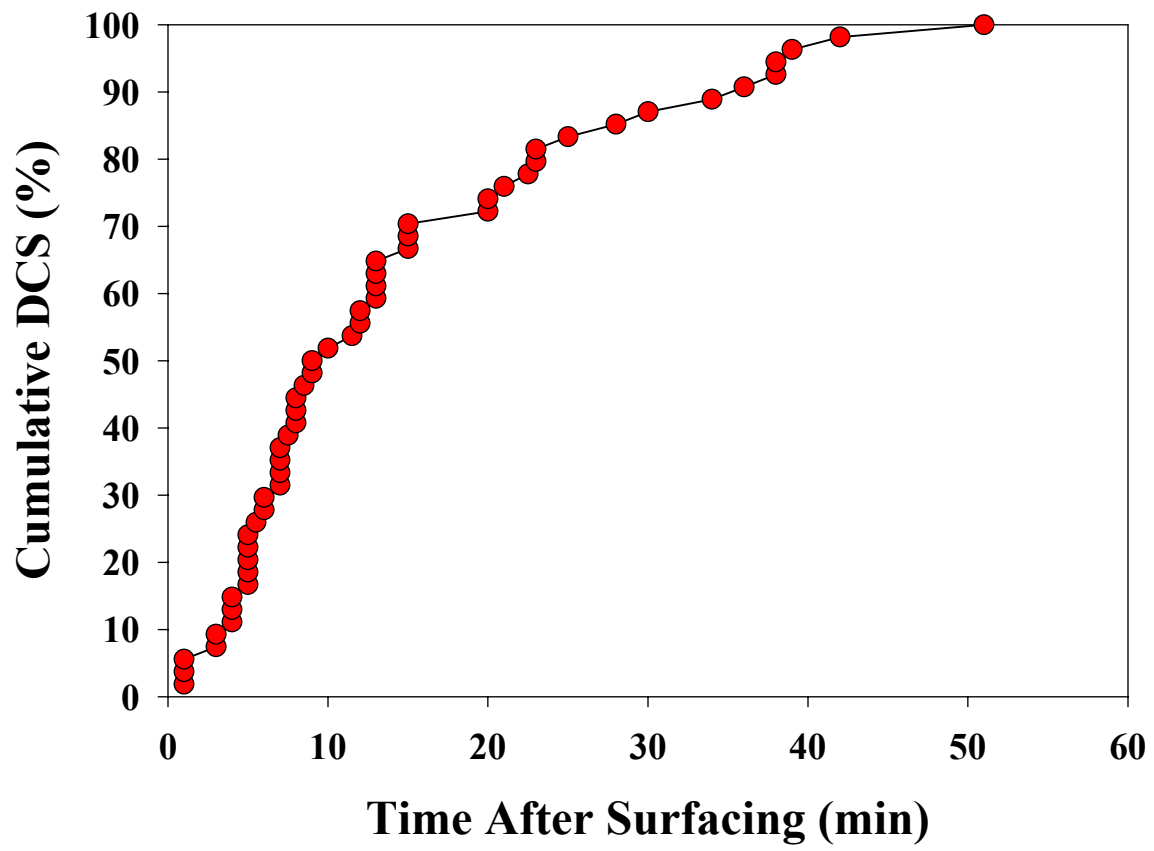
Figure 6.3. Ambient pressure, and inert gas tissue tension (H_2 + He) for a control animal and a treated animal injected with a total methanogen activity of 1500 μmol $CH_4 \bullet min^{-1}$ using the parameters in Model 2 (Table 6.2). The estimated probability of DCS for the control ($P(DCS)_C$) and 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 $\bullet min^{-1}$. 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.
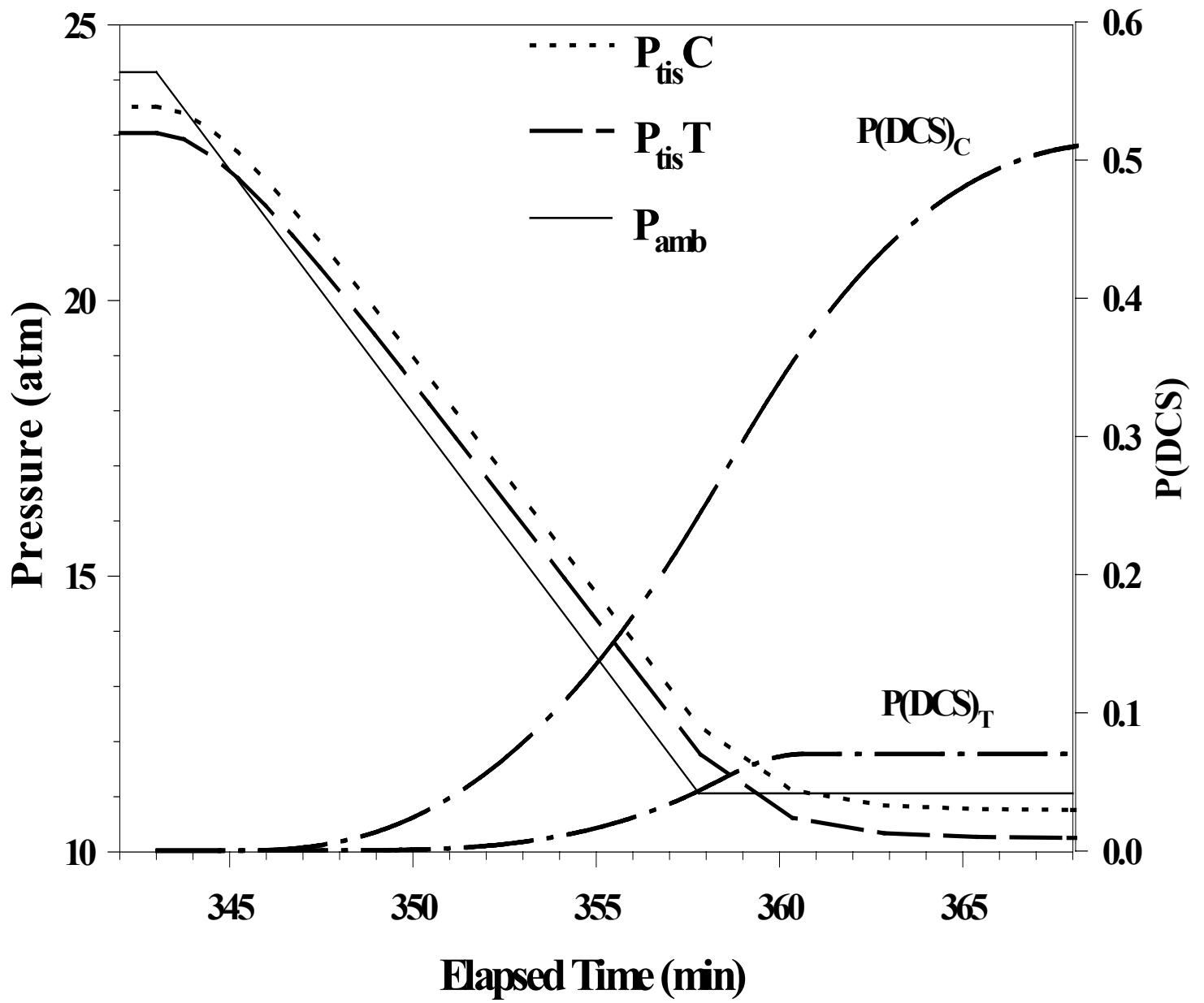
APPENDIX

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 • min^{-1}, or for 180 min at 22.3 or 24.1 atm with a decompression rate of 1.80 atm • 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.

Group	Outcome	P_{H_2} (atm)	Weight (kg)	Total activity injected ($\mu mol\ CH_4 \cdot min^{-1}$)	$c\dot{V}CH_4$	T_{CH} (° C)
VBT-30C	0	20.0	18.9	0	50.9 ± 66.0	27.4 ± 0.6
	0	19.7	18.9	0	46.0 ± 9.0	31.4 ± 0.4
	0	21.0	21.5	0	62.2 ± 22.9	27.4 ± 0.3
	0	21.7	19.8	0	61.8 ± 47.5	31.7 ± 1.9
	0	21.3	19.3	0	24.4 ± 55.4	27.6 ± 0.1
	0	21.6	17.9	0	21.1 ± 20.2	32.1 ± 0.2
	0	21.7	18.3	0	29.2 ± 16.6	32.2 ± 0.0
	1	20.9	18.6	0	31.5 ± 16.6	31.4 ± 0.0
	1	22.6	18.1	0	15.4 ± 5.9	30.6 ± 0.3
VBT-120C	0	20.6	21.0	0	84.5 ± 21.1	32.1 ± 0.8
	1	20.8	21.3	0	137.8 ± 21.6	29.0 ± 0.7
	1	21.7	19.6	0	55.7 ± 58.2	32.5 ± 0.7
	1	20.9	21.9	0	78.4 ± 15.2	30.7 ± 1.1
	1‡	20.9	18.6	0	28.5 ± 21.0	32.6 ± 1.0
VBT-150C	0	21.2	18.6	0	84.2 ± 9.9	32.2 ± 2.1
	0	20.9	18.5	0	101.0 ± 19.6	31.6 ± 1.5
	0	20.8	18.7	0	3.1 ± 13.8	31.3 ± 2.1
	0	21.0	20.2	0	12.5 ± 7.5	31.9 ± 1.9
	0	21.3	21.0	0	13.5 ± 13.2	32.3 ± 0.7
	1‡	21.5	19.2	0	20.3 ± 8.0	32.5 ± 0.6
	1	20.5	18.3	0	38.1 ± 7.3	31.3 ± 0.8
	1	20.9	17.4	0	21.5 ± 7.4	32.0 ± 0.4
VBT-150T	0	22.5	17.8	999	52.8 ± 12.2	32.3 ± 0.6
	0	21.6	18.9	1477	95.5 ± 41.1	32.5 ± 0.8
	0	20.7	19.0	1052	77.1 ± 16.8	33.3 ± 0.4
	1	21.3	21.2	557	117.9 ± 24.1	32.7 ± 0.7
	1	20.6	19.3	469	145.1 ± 95.5	31.4 ± 0.4
VBT-180T	0†	21.5	20.2	405	87.5 ± 22.8	33.5 ± 0.1
VBT-180T	0‡	22.6	17.6	1550	108.9 ± 11.7	31.8 ± 0.3
VDR-0.45C	1¥	19.9	18.4	0	84.4 ± 6.6	33.4 ± 0.5
	1¥	19.6	17.1	0	66.8 ± 1.2	31.9 ± 0.6

Appendix A6.1 continued

Group	Outcome	PH_2 (atm)	Weight (kg)	Total activity injected	$c\dot{V}CH_4$	T_{CH} (° C)
				(μmol $CH_4 \cdot min^{-1}$)		
VDR-1.8C	1	21.6	20.6	0	32.4 ± 9.9	33.4 ± 0.6
	1	20.2	21.2	0	24.4 ± 21.8	34.2 ± 0.5
	1	21.6	19.6	0	60.7 ± 18.7	34.4 ± 0.1
	1	21.0	21.6	0	82.7 ± 14.6	34.4 ± 0.4
	1	21.4	20.9	0	68.8 ± 8.5	33.5 ± 1.9
	1	21.0	20.2	0	3.6 ± 6.6	31.2 ± 1.6
	1	20.4	21.0	0	7.7 ± 13.6	33.6 ± 0.7
	1‡	20.0	23.0	0	37.1 ± 13.6	32.6 ± 1.8
VDR-1.8T	1	22.4	21.6	1240	92.1 ± 21.3	33.4 ± 0.7
	1	21.4	20.8	540	110.3 ± 18.0	33.4 ± 0.4

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 H_2 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 H_2 compared to He in whole animals, calorimetry and respirometry were used (Chapter 2). The study concluded that the physiological responses in hyperbaric He and H_2 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 H_2 could be narcotized and allow their surface temperature to fall to reduce the heat loss rate. Consequently, hyperbaric H_2 is not likely to be as thermally stressful as predicted on the basis of the higher thermal conductivity and heat capacity of H_2 compared to He.

For the experiments on H_2-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 H_2-metabolizing microbes removed some of the H_2 that otherwise would have dissolved in the pig's tissues (Chapter 4). The CH_4 release rate ($\dot{V}CH_4$, Eq. 1, Chapter 1) was used as a measure of the efficacy of H_2 removal by the intestinal microbes, and increasing $\dot{V}CH_4$ was shown to reduce the risk of DCS in the animals (Fig. 4.4, Chapter 4).

When increased activity of H_2-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 $\dot{V}CH_4$ significantly reduced the P(DCS) (Table 6.2 , Model 2, Chapter 6), and the model confirmed that increasing H_2-metabolizing activity delivered to the animals significantly reduced the P(DCS) (Table 6, Chapter 6). Consequently, the hypothesis that enhanced removal of H_2 by

microbial metabolism after a hyperbaric exposure to H_2 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 H_2 atmosphere of 45 atm has approximately 63 L H_2 ($\sim$ 2.5 mol H_2) 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 $\sim$ 124 μmol $H_2 \bullet min^{-1}$ by diffusion- and perfusion-limited processes. In the pig, it was shown that animals supplemented with H_2-metabolizing microbes after 24 hours at 24.1 atm had a rate of H_2 removal of $\sim$ 1 mmol $H_2 \bullet min^{-1}$ (assuming a 4:1 ratio between CH_4 and H_2, Eq. 1, Chapter 1). Since the large intestine in pigs is $\sim$ 2-3.3 times longer compared to that of humans (Barth*, et al.*, 1990; Crouch, 1981; Miller and Ullrey, 1987), H_2-removal rates of at least $\sim$ 250 μmol $H_2 \bullet min^{-1}$ should be feasible in humans. Consequently, the total removal of H_2 by diffusion, perfusion, and active processes could potentially be raised to $\sim$ 374 μmol $H_2 \bullet min^{-1}$ in human divers. This total activity could possibly reduce the decompression time from $\sim$ 14 days to $\sim$ 5 days.

Before testing on H_2-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 H_2 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 N_2 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 (Kooyman, 1989; Kooyman 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_2 as a diving gas

is limited to deep professional or military diving. Biochemical decompression from dives

using N_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_2

biochemical decompression compared to H_2 biochemical decompression is given by the

pressure limit of the use of N_2 to ~ 6 atm. Smaller volumes of gas would have to be

eliminated on a dive using N_2 compared to H_2. However, due to the stable conformation

of the N_2 molecule, reactions that break down N_2 are endergonic and require an energy

source for complete conversion into NH_3, nitrates and nitrites. Therefore, even though

various microbes are capable of using N_2, they more commonly utilize other sources of

nitrogen than the gaseous molecule. Currently known species of enteric N_2-fixing

microbes have such low metabolic efficiency that prohibitively large volumes of

microbes would be needed. In addition, the end products of N_2 metabolism are not

gaseous and would stay in the intestines, in contrast to CH_4, and most are toxic.

Therefore, H_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 N_2-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 N_2 more efficiently and that can convert any toxic end products into innocuous compounds. The production of such enzyme systems will make N_2 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 H_2 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 H_2 biochemical decompression should be a feasible concept for human divers, and N_2 biochemical decompression a possibility in the future.

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