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Increasing activity of H_2-metabolizing microbes lowers decompression sickness risk in pigs during H_2 dives

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Kayar, S. R., A. Fahlman, W. C. Lin, and W. B. Whitman. Increasing activity of H_2-metabolizing microbes lowers decompression sickness risk in pigs during H_2 dives. *J Appl Physiol* 91: 2713–2719, 2001.—The risk of decompression sickness (DCS) was modulated by varying the biochemical activity used to eliminate some of the hydrogen (H_2) stored in the tissues of pigs (19.4 ± 0.2 kg) during hyperbaric exposures to H_2. Treated pigs ($n = 16$) received intestinal injections of *Methanobrevibacter smithii*, a microbe that metabolizes H_2 to water and CH_4. Surgical controls ($n = 10$) received intestinal injections of saline, and an additional control group ($n = 10$) was untreated. Pigs were placed in a chamber and compressed to 24 atm abs (20.6–22.9 atm H_2). After 3 h, the pigs were decompressed and observed for symptoms of DCS for 1 h. Pigs with *M. smithii* had a significantly lower ($P < 0.05$) incidence of DCS (44%; 7/16) than all controls (80%; 16/20). The DCS risk decreased with increasing activity of microbes injected (logistic regression, $P < 0.05$). Thus the supplemental tissue washout of the diluent gas by microbial metabolism was inversely correlated with DCS risk in a dose-dependent manner in this pig model.

Methanobrevibacter smithii; methanogens; biochemical decompression; decompression illness; hydrogen diving

DECOMPRESSION SICKNESS (DCS) can be the penalty for failing to eliminate some portion of the tissue load of gas acquired while breathing under hyperbaric conditions (3). We report here studies on a novel approach to facilitating gas washout from tissues, thereby reducing the risk of DCS in an animal model. This approach, which we call biochemical decompression, uses microbes to metabolize hydrogen (H_2) inside animals during a hyperbaric exposure with H_2 as the primary gas in the breathing mixture. The conditions of the experiments are intended to simulate deep diving to 20–60 atm (700–2,000 feet of seawater). For such dives, H_2 may be more appropriate than helium or nitrogen as the diluent to O_2. Because of its lower density, H_2 requires less lung ventilatory effort at high pressures (4). H_2 has the additional advantage that it is less narcotic than N_2 at high pressures and that some degree of H_2 narcosis suppresses high-pressure neurological syndrome (4).

The concept of H_2 biochemical decompression has been demonstrated in a rat model (19). Cultures of *Methanobrevibacter smithii* were injected into the proximal end of the large intestines of the animals (19). This microbe, which is native to the human intestinal flora (21), metabolizes H_2 as

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \qquad (1)$$

The microbes convert H_2 to water and to a readily traceable end product (methane; CH_4) that is derived exclusively from this reaction (17, 21). Rats supplied with *M. smithii* had a reduction in DCS risk by approximately half from a chosen compression and decompression sequence in H_2 (19). Likewise, pigs that released detectable quantities of CH_4 were found to have a lower risk of DCS from a chosen exposure to hyperbaric H_2 compared with pigs that did not release CH_4; this CH_4 was generated entirely by microbes (of unknown species) native to the animals' intestinal flora (18). Thus these data demonstrated that the concept of biochemical decompression is valid.

Although most people on a western diet are expected to have *M. smithii* in their gut flora (21), it would not be acceptable to depend on the activity of this highly variable population for all biochemical decompression in human H_2 diving. In order for H_2 biochemical decompression to be useful for humans, the gut flora will need to be supplemented with *M. smithii,* probably by means of enteric-coated capsules of these microbes taken orally.

To advance this work further toward human use, we needed to determine the relationship between the in vitro activity of *M. smithii* delivered, the amount of H_2 these microbes eliminated in vivo, and DCS incidence. In the present study with pigs, intestinal injections of *M. smithii* were made under surgery. We report here the beneficial effect of this microbial activity on DCS incidence after a chosen compression and decompression sequence in H_2.

MATERIALS AND METHODS

Animals and training. Pigs (*Sus scrofa*, neutered male Yorkshires, $n = 36$, mean body mass ± 1 SE = 19.4 ± 0.2 kg;

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Table 1) were used for all experiments. The pigs were housed before experiments in an accredited animal care facility and had ad libitum access to water. The pigs were fed once daily with laboratory animal chow (Harlan Teklad, Madison, WI; 2% by body wt). All procedures were approved by an Animal Care and Use Committee. The experiments reported here were conducted according to the principles presented in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Animals were trained to walk at a moderate pace (50–60 m/min) for intervals of 5 min on a treadmill in the laboratory, separated by intervals of 5 min rest, to acclimate them to a treadmill and to verify that this workload was not excessively strenuous. They were then trained to walk at a slower pace (30 m/min) for intervals of 5 min inside the compression chamber in 1 atm air, on a treadmill that constituted the floor of the chamber. The pigs were acclimated to being left confined and unattended in the chamber, where they were free to lie down. They were trained to rise and walk whenever the treadmill was activated. Treadmill performance was subsequently used to help evaluate an animal's gait for signs of DCS.

Preparation of M. smithii. A sample culture of *M. smithii* (strain PS) was obtained from Dr. Terry Miller (Wadsworth Center for Laboratories and Research, Albany, NY) and was grown in an atmosphere of H_2-CO_2 (80:20 vol/vol; 3 atm) at 37°C at the University of Georgia. Stock cultures were maintained in a complex medium similar to medium 1 (1) except for the following modifications: 3 g/l of yeast extract (Difco, Sparks, MD), 3 g/l of sodium formate, and 3 g/l of sodium acetate·3 hydrate, and 7 g/l trypticase (BBL, Sparks, MD); lipoic acid and folic acid were omitted from the vitamin solution; and $Fe(NH)_2(SO_4)\cdot 6\ H_2O$ was substituted for $FeSO_4$, and $AlK(SO_4)_2$ was omitted from the trace mineral solution.

Growth of *M. smithii* took place in a 14-liter fermentor using 11 liters of the modified medium 1 as described above. The fermentor was sparged with H_2-CO_2. At least 1 h before inoculation, the temperature was set to 37°C, and 10 ml of $Na_2S\cdot 9\ H_2O$ (20% wt/vol) were added. The inoculum size was ~1% vol/vol of the total medium volume. During growth, the gas pressure was maintained at 2.4 atm, with a flow rate of 0.75 l/h of H_2-CO_2. A stirring rate of 240 rpm was used on the first day and 300 rpm on subsequent days. An additional 10 ml of sterile $Na_2S\cdot 9\ H_2O$ were added twice daily until harvesting. Before harvesting, the rate of CH_4 production ranged from at least 7 to 16 μmol $CH_4\cdot min^{-1}\cdot ml\ culture^{-1}$, and the cell absorbance ranged from 2.0 to 3.1 OD_{600}.

Cells were harvested with a Sharples continuous-flow centrifuge (model T-1P; Alfa Lavel, Warminster, PA) at 23,000 rpm. The cell paste was transferred to bottles that were flushed with N_2, and the cells were resuspended with 2.3 ml of a buffer of 5 mM dithiothreitol and 5% wt/vol $NaHCO_3$ per gram of cells. The bottles were flushed with H_2-CO_2 for 15 min before pressurization to 3 atm (absolute pressure). The resuspended cells were stored at 0°C and shipped with gel refrigerant to the Naval Medical Research Center within 12–24 h of harvesting. On arrival, the cell suspension bottles were flushed again with H_2-CO_2 and stored in a refrigerator for use within the next 24–48 h.

Before use, the cultures were assayed for their methanogenic activity by placing 0.2 ml culture and 0.2 ml of resuspension buffer in a 20-ml bottle with 3 atm H_2-CO_2. The bottle was incubated in a 37°C water bath with agitation at 200 rpm. Samples (100 μl) of the gas in the head space were taken every 12 min for 1–1.5 h and analyzed by gas chromatography for CH_4 concentration.

Surgery. Animals were divided randomly into three groups (Table 1): those that were to undergo surgery to be treated with methanogens (treated; $n = 16$), those that were to undergo the same surgical procedure but given intestinal injections of saline (surgical controls; $n = 10$), and untreated animals (controls; $n = 10$). There were no significant differences in the body masses among these three groups of animals (Table 1).

Animals were given an extra meal late in the afternoon of the day before an experiment and were left unfed on the morning of the experiment. Treated and surgical control animals were prepared for surgery by preanesthetizing them with injections of ketamine HCl (20 mg/kg im; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (Rompun 2 mg/kg im; Bayer, Shawnee Mission, KS). Animals were then kept at a surgical plane of anesthesia with inhaled isoflurane (Abbott Laboratories, N. Chicago, IL) and O_2. With use of aseptic technique, a midline incision of ~10 cm was made in the abdomen, and the cecum and spiral colon were exteriorized.

For the treated animals, 1–4 injections of *M. smithii* culture were made, depending on the volume of the microbial culture to be used; this volume ranged from 12 to 116 ml. The injectate was distributed between the cecum and upper, middle and lower spiral colon, with not more than 30 ml in each injection site. Total activity injected ranged from 200 to nearly 2,200 μmol CH_4/min. For the surgical controls, saline that had been deoxygenated by bubbling it with CO_2 was used as the injectate. One to three injections were made, of 60 ml total volume, also distributed between the cecum and various locations in the spiral colon. All needle puncture sites were sealed with a drop of surgical cement (Vetbond, 3M, St. Paul, MN). Postexperiment necropsy never revealed any visible leakage from these punctures. The intestines were moistened externally with saline and returned to the abdomen, and the incision was closed with sutures. As the animal recovered from the anesthesia, yohimbine (2 mg iv; Lloyd Laboratories, Shenandoah, IA) was injected into an ear vein to act as an antagonist to the xylazine. Animals appeared to be fully recovered from the anesthesia in 1–2 h. They were then placed in the compression chamber to commence the experiment. Food and water were freely available in the chamber.

Dive protocol. The compression chamber (5.7 m^3 internal volume, WSF Industries, Buffalo, NY) and the facility in

Table 1. *Data from pigs used as untreated controls, surgical controls, or treated with injections of Methanobrevibacter smithii during hyperbaric H_2 exposures*

	Untreated Controls	Surgical Controls	Treated	*P* values
Body mass, kg	20.0 ± 0.4	19.6 ± 0.5	18.9 ± 0.2	0.09
P_{H_2}, atm	21.7 ± 0.2	21.6 ± 0.2	21.8 ± 0.1	0.70
Activity injected, μmol CH_4/min	0	0	980 ± 132	
$c\dot{V}_{CH_4}$, μmol CH_4/min	35 ± 6	34 ± 5	92 ± 9	<0.001
Time to DCS, min	9.7 ± 1.2	13.9 ± 4.8	12.9 ± 4.9	0.69

Values are means ± 1SE. Data include body mass, maximal partial pressure of H_2 during the experiment (P_{H_2}), activity of *M. smithii* injected, methane release rate while compressed ($c\dot{V}_{CH_4}$), and time to diagnosis of decompression sickness (DCS) after reaching observation pressure. *P* values represent differences among the 3 groups (ANOVA).

which it stood were specially designed for safe handling of high pressures of H_2 (5). View ports on the chamber, as well as a video camera aimed through a view port, allowed ad libitum viewing of the animal.

For each experiment, one pig was placed in the compression chamber, standing on the treadmill. A stream of gas flowed continuously from the chamber to a gas chromatograph (GC; Model 5890A Series II, Hewlett-Packard, Wilmington, DE). Automated analysis occurred every 12 min. The gases analyzed were O_2, N_2, He, H_2, and CH_4. Calibrations were performed before each experiment. The thermal conductivity detector of the GC was calibrated with a certified gas mixture of 2% O_2, 2% N_2, 4% He, and 92% H_2, a mixture that closely approximated the final gas composition in the chamber (ca. 2% O_2, 1% N_2, 9% He, 88% H_2). The flame-ionizing detector of the GC was calibrated with 20 ppm CH_4 in H_2, a concentration that accommodated the full range of CH_4 concentrations measured in the chamber (2–8 ppm). Calibrated values did not vary by more than 5% from standards. A calibration check at the end of each experiment confirmed that the machine did not drift more than 5%. The commercially purchased supplies of O_2, He, and H_2 were certified and confirmed to be below 0.1 ppm CH_4. A gas blower was used to keep the gases in the chamber well mixed, as confirmed by analyzing samples drawn from two spatially separated locations in the chamber and finding not more than 5% difference.

The chamber was pressurized with He to an absolute pressure of 11 atm, adding O_2 as necessary to replace O_2 consumed by the pig and O_2 lost by the ventilation of the chamber. Initial pressurization rate was selected by appearance of comfort for the animal's ears (ca. 0.15 atm/min for the first 2–3 atm, up to 0.45 atm/min at greater pressures). The chamber was then flushed with H_2 while the chamber was maintained at a constant pressure of 11 atm, to a concentration of 60–75% H_2, with O_2 added as necessary to maintain normoxia (0.2 atm P_{O_2}). This initial pressurization with He, followed by replacement with H_2, is necessary for avoiding explosive mixtures of H_2 and O_2 (5).

The chamber was further pressurized with H_2 and O_2 to a final pressure of 24 atm absolute pressure. Animals remained at this pressure for 3 h. The O_2 partial pressure was maintained essentially constant throughout this portion of the dive, at the slightly elevated levels (0.3–0.5 atm P_{O_2}) that are customary for respiration under hyperbaric conditions (8). However, the percent H_2 slowly increased over the period of hours spent at maximal pressure by a few percentage points, as chamber gases containing traces of He and N_2 were replaced with H_2 during chamber ventilation. The partial pressures of H_2 during the final 36 min at maximal chamber pressure were 20.6–22.9 atm H_2 and were not different among the three groups (Table 1).

After 2.5 h, animals were made to walk for 5 min on the chamber treadmill to observe their gait and to check that they appeared to be normal while compressed.

Three hours (± a few seconds) after arrival at maximal pressure, decompression to 11 atm occurred at a rate of 0.9 atm/min, while the animals were observed closely. When the chamber pressure arrived at 11 atm, animals were made to walk at 5-min intervals on the chamber treadmill for up to 1 h or until signs of severe DCS were noted and agreed on by at least three observers. Time of diagnosis was recorded to the nearest minute. The signs of DCS were primarily neurological and included falling, difficulty standing or righting after falling, and seizures. Some animals had labored breathing, which may have indicated cardiopulmonary DCS in addition to neurological DCS. Many animals were also observed to have signs of skin DCS (conspicuous lavender to dark purple mottling of the skin, with or without itching), but these signs alone did not warrant a diagnosis of severe DCS. Mild, transient behavioral changes (agitation or lethargy) were also not considered sufficient for a diagnosis of severe DCS. Once the diagnosis was made, or the hour had passed without evidence of DCS, the animal was euthanized quickly by asphyxiation with He. The chamber was returned to 1 atm after the animal was dead.

Throughout the dive, chamber temperature was thermostatically controlled to values that appeared to be comfortable for the animal (ca. 30–31°C at 11 atm and 32–34°C at 24 atm). Comfort was judged on the basis of absence of shivering with blanched skin or panting with dark pink skin. These temperatures are warmer than one would expect for comfort in 1 atm air, because of the high thermal conductivity of compressed H_2 (10).

Analysis of CH_4 release rate. An accurate estimate of chamber ventilation rate was needed to convert the CH_4 concentration (ppm) in the chamber to a CH_4 release rate ($\dot{V}_{CH_4}$; μmol CH_4/min) from the animals. The flowmeter for analyzing chamber ventilation rate was calibrated with pure He, H_2, O_2, and N_2, and with air, 2% O_2 in He, and 2% O_2 in H_2, using a water spirometer. Ventilation rate estimates were reproducible within 2–4%. Linear interpolation based on relative percentages of gases was used to estimate the ventilation rates of all other gas mixtures. The mean ventilation rate used in these experiments at final chamber gas composition was 115 l/min (STP).

The total volume of gases in the pressurized chamber (136,800 liters STP) was very large compared with the chamber ventilation rate for reasons of economy and safety with H_2. The rate at which an animal was releasing CH_4 in the chamber was therefore not in equilibrium with CH_4 sampling from the chamber in the time intervals we examined. This equilibrium would be reached under the conditions of this experiment only over a period of days (2, 6). Mathematical corrections had to be introduced to estimate the $\dot{V}_{CH_4}$, or actual rate of release of CH_4 from the pigs, after extrapolating to equilibrium conditions. The approach of Bartholomew et al. (2) allows for this correction. The fractional concentration of CH_4 in chamber gases at equilibrium (X_{eq}) was computed from CH_4 concentration at two time points during the experiment (X_i and X_{i-1}) separated by a known time interval (Δt) as

$$X_{eq} = \frac{X_i - X_{i-1}}{1 - e^{-\dot{v}\Delta t/V}} + X_{i-1} \quad (2)$$

where $\dot{v}$ is chamber ventilation rate and V is chamber effective volume (chamber physical volume times total pressure). The $\dot{V}_{CH_4}$ was then computed by multiplying X_{eq} by $\dot{v}$. This method was extensively calibrated and verified in our chamber, as described in detail by Fahlman (6). The $\dot{V}_{CH_4}$ values during the compression phase of the experiment ($c\dot{V}_{CH_4}$) were computed for each animal from a mean of five pairs of gas chromatographic measurements of CH_4 concentration, with one member of the pair from the first hour at constant chamber pressure and the other member from the third hour (Δt = 2 h in each pair).

Statistical analysis. All mean values reported are ± 1 SE.

Multivariate logistic regression techniques (11) were used to determine the probability of DCS, using DCS outcome as the dependent variable and four experimental variables [body mass, chamber partial pressure of H_2 (P_{H_2}), mean $c\dot{V}_{CH_4}$, and injected activity of microbes] as independent variables. Initially, a univariate analysis on each indepen-

dent variable was performed; only those variables with a P value <0.20 (Wald test) were then included in a multivariate analysis. Exclusion of a variable from the multivariate analysis was based on the log-likelihood ratio test at the $P = 0.05$ level (11).

RESULTS

The concentration of CH_4 within the chamber steadily increased throughout the 3-h duration of the compression phase of the experiment for all animals (Fig. 1). This does not, however, necessarily indicate that the animals were detectably increasing the rate at which they were releasing CH_4 in this 3-h time frame. The progressive buildup of CH_4 within the chamber was a consequence of the vast difference between the total volume of gases in the chamber and the sampling rate, as the system slowly approached equilibrium. When the data were mathematically corrected to equilibrium conditions, the $c\dot{V}CH_4$ was best approximated by a single value for the entire 3-h period for each animal. This value was on average higher ($P < 0.01$, Mann-Whitney test) for animals treated with methanogens (92 ± 9 μmol CH_4/min) compared with surgical and untreated controls combined (35 ± 4 μmol CH_4/min; Table 1, Fig. 2).

There was a statistically significant correlation ($P < 0.001$, $R = 0.70$, slope = 0.046; least squares linear regression) between microbial activity injected and $c\dot{V}CH_4$ (Fig. 2). This regression was minimally influenced by seemingly outlying values from two treated animals ($P < 0.001$, $R = 0.74$, slope = 0.039 with these 2 values omitted). The $c\dot{V}CH_4$ was 6–12% of the in vitro activity injected (Fig. 2).

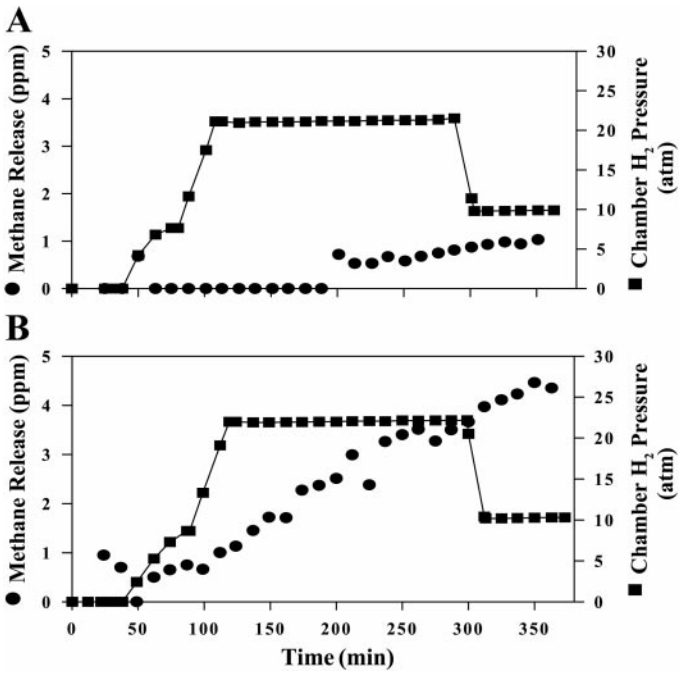

Fig. 1. Sample data from two experiments with pigs in hyperbaric H_2. *A*: untreated control animal. *B*: animal that had received intestinal injections of *Methanobrevibacter smithii*, 795 μmol CH_4/min activity, 1–2 h before the hyperbaric exposure.

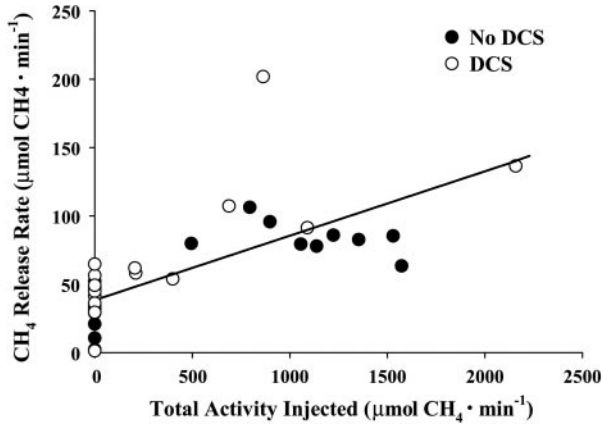

Fig. 2. Activity of *M. smithii* injected into the intestines of pigs vs. mean rate of release of methane from these animals during a 3-h exposure to hyperbaric H_2 [21.7 atm partial pressure of H_2 (P_{H_2})]. Decompression sickness (DCS) outcome is indicated for each animal. The line represents least squares linear regression ($Y = 40.1 + 0.046X$; $R = 0.70$, $P < 0.001$).

With the chosen compression and decompression sequence, 90% (9/10) of untreated control animals and 70% (7/10) of surgical control animals had symptoms of DCS (Fig. 3). The outcomes of these two groups are not significantly different from each other (2-tailed χ^2 test, $P > 0.50$). However, 44% (7/16) of animals receiving intestinal injections of *M. smithii* had symptoms of DCS (Fig. 3). This is significantly different from the DCS incidence for the two control groups combined (2-tailed χ^2 test, $P < 0.05$) and significantly lower than the DCS incidence of the surgical control group alone (1-tailed χ^2 test, $P < 0.05$). The mean time from arrival at 11 atm until the onset of DCS symptoms was 11.9 ± 2.1 min and did not differ significantly among the three groups (Table 1).

Logistic regression was used to determine whether there was a statistical correlation between the incidence of DCS in these animals and one or more variables within the experiments. These variables included body mass, P_{H_2}, $c\dot{V}CH_4$, and injected activity of meth-

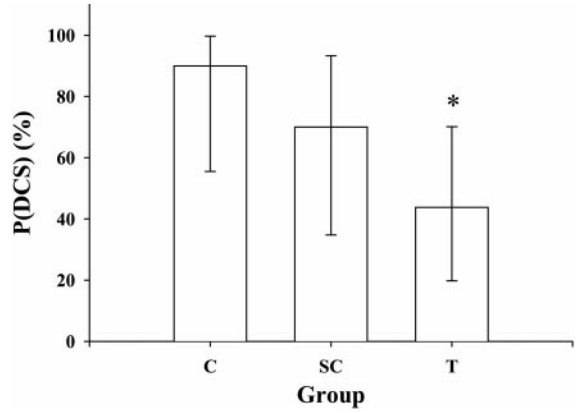

Fig. 3. Probability of DCS [*P*(DCS)] for untreated controls (C), surgical controls (SC), and pigs treated (T) with intestinal injections of *M. smithii*. Error bars represent 95% confidence limits on binomial distributions. *Significantly lower than in pooled control groups ($P < 0.05$, χ^2 test).

Table 2. *Results of logistic regression analysis for methanogenic activity injected, animal mass, chamber H_2 pressure, and CH_4 release rate during $c\dot{V}_{CH_4}$ vs. DCS outcome*

Variables	Intercept	Slope	Wald	LL	*P* value
Null	0.57 ± 0.35			−23.55	
Activity, μmol CH_4/min	1.33 ± 0.50	$(-1.60 \pm 0.68) \times 10^{-3}$	0.02	−20.22	0.02
Mass, kg	−9.38 ± 6.24	0.52 ± 0.33	0.11	−22.07	0.11
Activity + Mass	−5.96 ± 6.89	$(-1.42 \pm 0.69) \times 10^{-3}$ 0.37 ± 0.36	0.04 0.29	−19.61	>0.10
$c\dot{V}_{CH_4}$, μmol CH_4/min	1.00 ± 0.66	$(-7.04 \pm 9.01) \times 10^{-3}$	0.43	−23.23	0.43
P_{H_2}, atm	−1.34 ± 15.20	0.088 ± 0.70	0.90	−23.54	0.90

Parameter estimates (±SE), Wald statistics, log-likelihood (LL), and *P* value for log-likelihood ratio test compared to null model.

anogens. Neither P_{H_2} nor $c\dot{V}_{CH_4}$ was a significant predictor of DCS ($P > 0.40$; Table 2). Body mass and injected activity met the Wald test criteria ($P < 0.20$) for inclusion in a multivariate analysis. Results of this analysis indicated that injected activity was the only one of these variables that was a significant predictor of DCS ($P < 0.05$; Table 2, Fig. 4).

DISCUSSION

Animals receiving intestinal injections of H_2-metabolizing microbes had only roughly half as many cases of DCS as untreated animals after a chosen sequence of compression and decompression with H_2 (Fig. 3). This result is unlikely to be an artifact of the surgical procedure for injecting the microbes, on the basis of similarity in DCS incidence between untreated control and surgical control animals (Fig. 3). It was particularly important to test for effects of surgery, because elicitation of an immune reaction (which could potentially be triggered by a breach in asepsis during the surgical procedure) shortly before decompression has been implicated as a means of reducing DCS risk (16, 26).

There is evidence from these experiments that the reduction in DCS incidence is directly attributable to increased removal of H_2 from within the animals by the injected microbes. The microbial activity given to an animal was a significant predictor of DCS (Table 2, Fig. 4). We confirmed that neither the body mass of the animal, which is a known risk factor in DCS studies with animals (20), nor the small variations between dives in the P_{H_2} were a significant confounding factor in these experiments (Tables 1 and 2). Chamber temperature throughout the experiment, which was controlled within a narrow band, was also recorded and found not to be correlated with DCS incidence (6). Thus these experiments demonstrate that the benefits of microbial treatments in biochemical decompression respond in a dose-dependent manner in a pig model (Fig. 4).

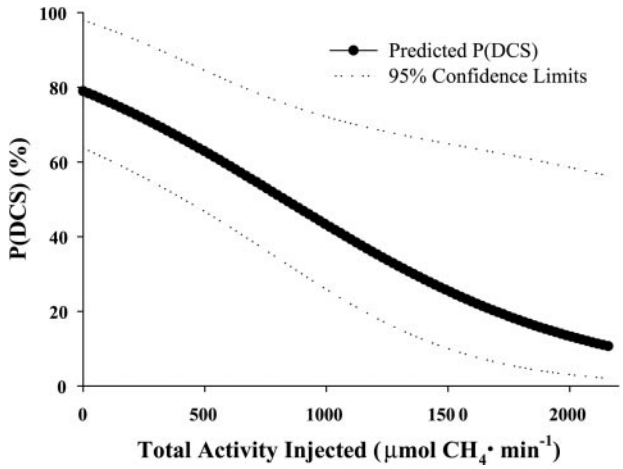

Fig. 4. *P*(DCS) vs. total activity of *M. smithii* injected into the intestines of pigs exposed to hyperbaric H_2 (21.7 atm P_{H_2}), as computed by logistic regression.

Under other circumstances, H_2 would be referred to as the inert gas component of the breathing mixture for these hyperbaric exposures. In this case, it is specifically the fact that H_2, although inert to metabolism by mammalian cells (17, 22), is a highly energetic substrate for metabolism by some microbes that makes biochemical decompression possible. The selected microbe, *M. smithii*, converts some of the H_2 to CH_4 (*Eq. 1*), and there is no other source of CH_4 in the chamber. Thus the release of CH_4 from the animals is a means of tracking the rate of H_2 scrubbing that is occurring within the animal during the hyperbaric H_2 exposure.

Animals given the microbial injections generally released more CH_4 than untreated animals throughout the experiment (Fig. 2). Surprisingly, the $c\dot{V}_{CH_4}$ was not a significant predictor of DCS risk in this study (Table 2), despite a significant correlation between microbial activity injected and $c\dot{V}_{CH_4}$ (Fig. 2). The $c\dot{V}_{CH_4}$ does, however, reach statistical significance as a predictor variable for DCS when we included data from over 100 experiments using other compression and decompression sequences than presented here (7). We interpret this as indicating that, although $c\dot{V}_{CH_4}$ is an invaluable noninvasive index of microbial activity within the animal, it is less than perfect. We do not know the kinetics associated with generating a given molecule of CH_4 within the intestine of a pig vs. sampling that molecule by the gas chromatograph. There are uncertainties in the chromatographic analysis and in the mathematical corrections to equilibrium conditions, which are known to amplify any sampling errors (2). There is also the possibility that, within the complex microbial ecosystem of the large intestine, we have not accounted for all of the H_2 metabolism solely

by following CH_4 release. Although methanogenesis is the primary pathway for microbial H_2 consumption in the intestine (15, 21), there are microbes in the intestinal flora that can metabolize H_2 to other end products such as acetate and sulfide (14).

We were particularly attracted to the two cases in which animals had unusually high $c\dot{V}_{CH_4}$ (Fig. 2). The two animals that released over 130 μmol CH_4/min received microbial injections of 83 and 116 ml, respectively, with the latter the largest volume injected in these experiments. This leads us to speculate that distributing the microbial material over a larger volume of intestinal inner surface may increase the access of the microbes to H_2, thereby increasing in vivo microbial activity. Greater microbial activity places animals at a lower risk of DCS (Fig. 4); nevertheless, these two animals displayed signs of DCS.

In contrast, there was an animal that received an injection of the second highest activity of the study (1,574 μmol CH_4/min) but had a $c\dot{V}_{CH_4}$ that was similar to that of some control animals (Fig. 2). On examining our notes, we discovered that we had failed to give this animal the usual supplemental late afternoon meal on the day before the experiment. We speculate that this animal had a smaller volume of intestinal contents than others in the study, which in turn may have reduced intestinal blood perfusion and therefore H_2 supply to the methanogens. Alternatively, the low intestinal content volume may have altered the internal intestinal environment in a manner that diminished methanogenic metabolism for some reason other than H_2 availability. Low methanogenic activity places animals at a higher risk of DCS (Fig. 4); nevertheless this animal did not display signs of DCS.

This reminds us of a recurring theme in DCS research: some portion of DCS risk always manages to defy attribution to a specific risk factor and remains mathematically approachable only as a random event (28). It is significant that the present research has identified a controllable physiological factor that is predictive of DCS risk (Fig. 4), because DCS research is plagued by disputed attributions of risk to such factors as gender, adiposity, age, and body temperature (9, 13, 30). Consequently, most recent modeling efforts in DCS risk have included only physical aspects of the dive and parameter estimates for the gas kinetics of one or more compartments within animals; these compartments are mathematical constructs rather than physiological or anatomical entities (23, 24, 25).

Three additional experiments were performed in which animals were injected with *M. smithii* and remained at 24 atm in the chamber for 24 h (6). Within this extended time period, the $c\dot{V}_{CH_4}$ increased over time (Fig. 5). After 24 h, the mean $c\dot{V}_{CH_4}$ in each animal was severalfold higher than it had been in the first 3 h (6). We presume that this increasing $c\dot{V}_{CH_4}$ reflects increasing in vivo methanogenic activity over time and is attributable to a combination of microbial reproduction and microbial distribution throughout a greater portion of the large intestine. It should be noted that there was no appearance of discomfort to the animals

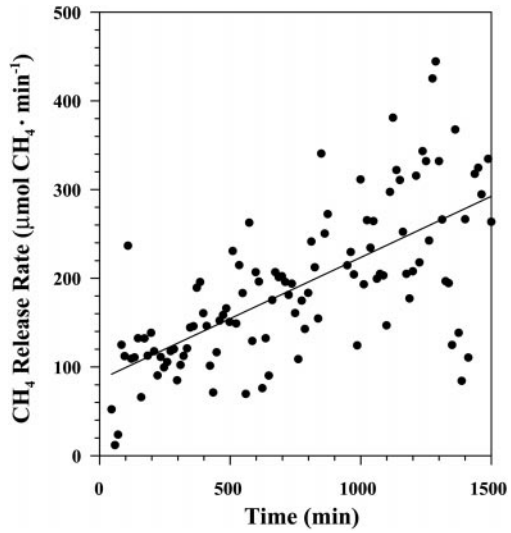

Fig. 5. Sample methane release rate ($c\dot{V}_{CH_4}$) data from one pig treated with intestinal injections of *M. smithii* (925 μmol CH_4/min activity) and exposed to hyperbaric H_2 (21–23 atm P_{H_2}) for 24 h. $c\dot{V}_{CH_4}$ values were computed by use of a time interval of 12 min each.

due to intestinal gases either at the 3-h point or at these higher rates of CH_4 release after 24 h. Only one of these three animals had subsequent symptoms of DCS, but a sample size of three is of course minimally instructive.

Some calculations may be of benefit in analyzing the magnitude of H_2 removed by the microbes compared with the body burden of H_2 dissolved in these animals. If we make the simplifying assumptions that a pig is approximately saturated with H_2 after 3 h (6) and that H_2 solubility throughout the pig can be estimated from the solubility of H_2 in aqueous tissues (0.02 ml $H_2 \cdot g^{-1}$ tissues$\cdot atm^{-1}$ at 37°C) (27), then a 20-kg pig breathing 21.7 atm P_{H_2} (Table 1) for 3 h contains 8.7 liters of H_2, or 340 mmol H_2. The treated animals were releasing an average of roughly 90 μmol CH_4/min for 3 h (Table 1, Fig. 2). This indicated that the methanogens were consuming at least four times that volume of H_2 (*Eq. 1*). Thus nearly 65 mmol H_2 were consumed in this process, i.e., ~19% (65/340) of the total volume dissolved in the animal.

Our laboratory reported previously that a 50% reduction in DCS incidence was achieved by biochemical elimination of an estimated 5% of the total body burden of H_2 in rats (19). An even greater reduction in DCS incidence was found for pigs with native intestinal methanogens that eliminated an estimated 4–17% of their H_2 load (18). It is difficult to make direct comparisons among these experiments and the present one, given the differences in compression and decompression sequences as well as body size. However, the message appears to be one consistent with findings from human studies: small differences in estimated tissue gas loads have a surprisingly large impact on DCS risk (12, 29).

Our laboratory created a mathematical model (7), using the data from the present study and from numerous other H_2 exposures with pigs, that quantifies this relationship between tissue gas loss via biochem-

ical decompression and DCS risk. Qualitatively, we envision that H_2 biochemical decompression works by placing a sink for H_2 within the body to supplement the H_2 lost passively to the environment by diffusion during conventional decompression. So long as the H_2 eliminated in the sink is of some threshold magnitude, it should not matter where that sink is located within the body. A lowering of mixed venous P_{H_2} as a function of perfusing a sink tissue bed should subsequently reduce the alveolar P_{H_2} and therefore reduce P_{H_2} in all perfused tissues. Quantifying the rate or magnitude of gas elimination needed in this sink to have an impact on P_{H_2} in the tissues most critical to DCS risk is precisely the ultimate goal of our research.

We conclude that the benefits of microbial treatments in H_2 biochemical decompression are dose dependent in a pig model. It will be exciting but nontrivial to extend this work to practice with humans, because many factors in dosage, gas kinetics, and optimal microbial conditions remain unknown.

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All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals,* Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

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