Old Herborn University Seminar Monograph

21. THE BIOLOGICAL SIGNIFICANCE OF GASEOUS BIOMARKERS FROM THE MICROBIOTA IN THE ALIMENTARY TRACT

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WHAT DO WE KNOW FROM GERMFREE LIFE? BASIC KNOWLEDGE ABOUT MICROBES AND GAS PRODUCTION

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SUMMARY

The gases within the alimentary lumen reflect the composition and volume of swallowed respiratory air or gases, the release of gases from food, the kinds and amounts of non-respiratory gases produced within the alimentary tract by its microbiota, and the rate of gaseous exchange between the alimentary lumen and surrounding blood vessels and tissue. Basic information about microbial produced gases was obtained some decades ago. The physiological and pathophysiological influences that these gases might have on the host will be outlined in detail in the following chapters. On-going and future technological improvements will give us valuable tools for studying these continuously ongoing host-microflora cross-talks.

INTRODUCTION

All multi-cellular organisms with an alimentary tract have gas in their tract. Principally, its origin may be from the following sources:
1. Swallowed as air,
2. Derived from gaseous compounds in the diet,
3. Diffusion into the tract from blood vessels and surrounding tissue, and
4. Being produced by the alimentary microbiota.

During this seminar other speakers focussed upon microbial production of gases. In order to put this production into its physiological and pathophysiological frame, some general comments and introduction of some terms will be given. From a historical point of view, the basic differences between conventional and germfree animals with regard to alimentary gas production was worked out in the 1960-ties due to one simple reason: Man was entering outer space and recognized that this caused some abdominal discomfort. Great efforts were made to overcome this discomfort and germfree animal laboratories in several countries became involved. The present list of references and Figure 1 reflect to some extend the interest and efforts of 40 years ago.

COMPOSITION OF ALIMENTARY GAS

Comparative studies in germfree and conventional animals have clearly shown that the microbiota alone are responsible for presence of hydrogen,

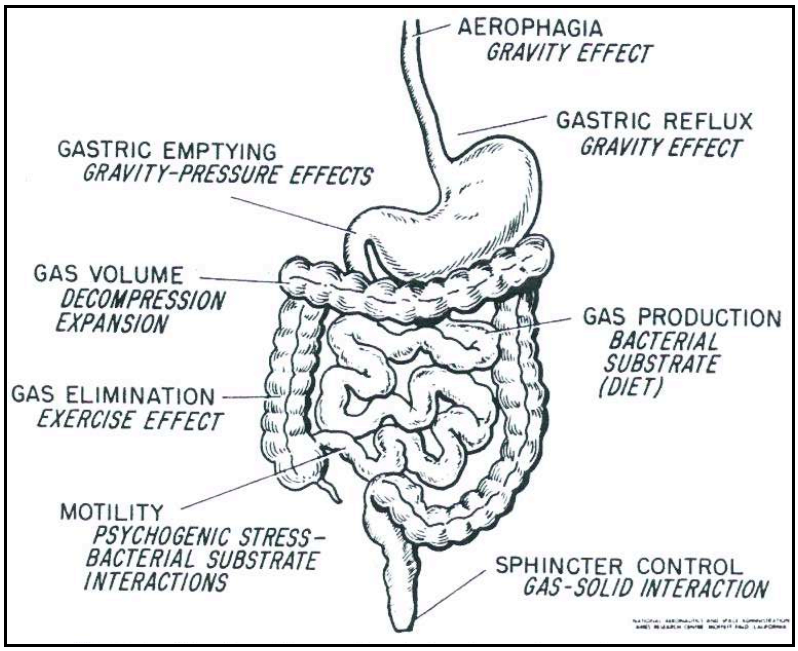

Figure 1: Alimentary tract problems that man encounters in space.

methane, hydrogen sulphide, some of the volatile fatty acids and amines as indole, skatole etc. The major differences between the microbial produced gases and the others that might be present in the GI tract is that the former reach their highest concentration within the lumen and will always diffuse out from the lumen, most often to be removed to the atmosphere from the transporting blood as it flows through the lung. Consequently, measurements of any of these gases in respired air reflect an alimentary production and this can be of diagnostic importance. The absolute and relative amounts of the microbial produced gases vary profoundly with the types and abundance of microorganisms present in the various compartments of the alimentary tract and with the substrate provided for their growth.

The other gases, regardless whether they are derived *per os* or via the blood stream may diffuse both into and out of the lumen and their presence in respired air does not reflect a microbial alimentary origin.

The net sum of all these processes yields a rest of gases to be expelled as flatus. A simple rule of the thumb says that around 2/3 of the gases in flatus originates from the host whereas 1/3 is of microbial origin. In the following, attempts will be made to follow the gas on its way through the alimentary tract.

UPPER ALIMENTARY TRACT

Air swallowing, aerophagia, is a universal phenomenon in all mammals. In man, the relative amount varies considerably. However, there is considerable evidence that swallowed air accounts for more than half of the gastro-

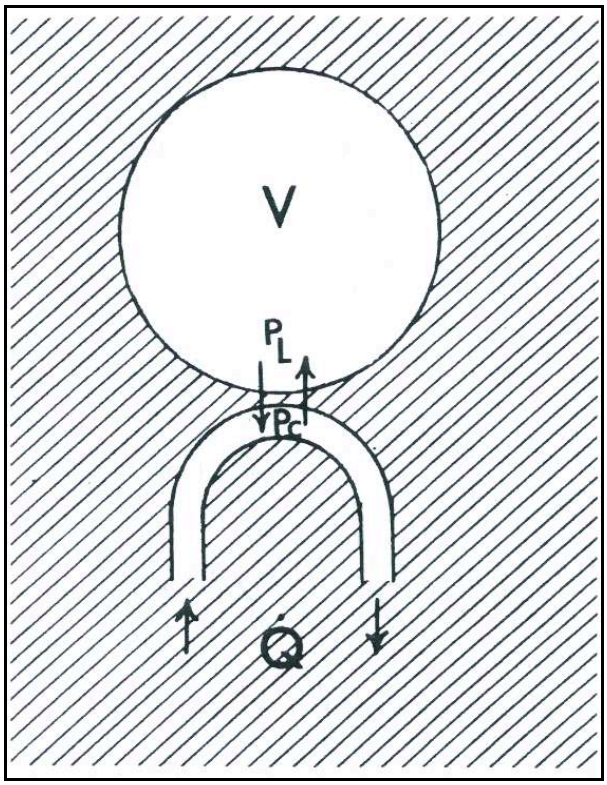

Figure 2: Exchange of gas between lumen and tissue.
Q = tissue, V = volume of gas in the gut. P_L = partial pressure of a gas in the lumen; P_C =Partial pressure of same gas in venous blood/surrounding tissue.

intestinal "gas" in man. Increased aerophagia is sometimes referred to as *eructatio nervosa*. This designation implies that frequent eructation may occur on a nervous or psychogenic basis. Whatever the amount of swallowed air might be, it can either be eructated from the oesophagus/stomach, absorbed by the host, utilized by his alimentary microbiota or expelled per rectum as flatus. In the past in our Western culture (it still is in some other cultures) eructation was looked upon as an audible expression of appreciation of the host's culinary accomplishment. Anyhow, eructation may account for a lesser part of swallowed air. Within its way down the tract, the swallowed air equilibrates or approaches equilibrium with in the gases dissolved in blood perfusing the intestines or locally produced or utilized by the microbiota. In any compartment it will attain a volume determined by the relative rates of gas inflow, formation, exchange and expulsion.

Passage of swallowed air and other gases within the alimentary tract is faster than passage of content. Already for more than 75 years ago, it was demonstrated that swallowed air can move from the stomach to the caecum in 6 to 15 minutes and to the rectum in 36 minutes (*Magnusson*, 1931). These times are too short for a complete exchange between some alimentary gases and blood/tissue to take place.

VISIBLE GAS IN THE INTESTINE OF GERMFREE ANIMALS

It is an everyday experience for people working with germfree animals that gas can be seen in both the small and large intestine, especially in the caecum. Depending of where in the alimentary tract the gas bubble is located, the relative content of gases might vary, ending up as mostly N_2 in flatus. The mathematical background for this can be worked out using rather complicated mathematical equations (*Forster*, 1968), but basically can be depicted as done in Figure 2**.**

Any time - and at any place - the content in a gas bubble will be regulated by influx/efflux, new production and utilization. The relative rate of influx/efflux for the gases of non-microbial origin will always be related to corresponding values in surrounding blood vessels/tissue. Some basic efflux data are given in Table 1. The data are taken from different publications and based upon studies in various animals species and alimentary compartments but is nevertheless of some value.

In the germfree animal there is no microbial production or utilization of gases. Consequently, the relative difference between N_2, O_2 and CO_2 are of importance. As is evident from the table, N_2 will diffuse far more slowly than O_2 and CO_2, yielding more and more N_2 in the gas bubble on its rapid passage to the anus. Further, the high values of CO_2 might be due to other factors than diffusion, and might vary from species to species (*Rasmussen* et al., 2002). At least in rats, the amounts of carbonic anhydrase enzymes do not seem to be influenced upon by the microbiota (*Lonnerholm* et al., 1988)

The data in Table 1 might also be used when explaining the old clinical experience that it may help postoperatively to give the patients some oxygen. Inhalation of air with an increased amount of oxygen will denitrogenate blood and tissue, thereby widening the gradient for N_2 across the intestinal mucosal barrier. As a result, intraluminal nitrogen enters the blood stream more rapidly and leaves the body via the lungs, and the intestinal gas volume will decrease concomitantly (*Pogrund* and *Steggerda*, 1948).

Another way to obtain the same result might be to increase the environmental pressure thereby reducing the intraluminal gas volume proportionally and obtaining a rapid relief in discomfort. This works out efficiently in experimental models (*Cross* et al., 1953; *Cross*, 1965) and has been tried on patients with some limited results (*Stewart* et al., 1964). However, a hyperbaric chamber, suitable for treatment of seriously ill patients, is usually not close enough when it is urgently needed.

Following an establishment of small intestinal strangulation obstruction, germfree rats will be alive somewhat longer than their conventional counterparts (*Midtvedt*, 1984). One reason for this difference might be a relatively larger deficiency in oxygen supply to the enterocytes in conventional animals. Intraluminal administration of pure oxygen at one atmosphere of absolute pressure protects three-inch length of ischemic small intestine in conventional rats from gross and histological damage for up to 6 hours (*Gottfried* et al, 1963). By contrast, frank necrosis appeared within some few minutes in control animals. However, these promising results could not be repeated in dogs (*van Zyl*, 1966).

Previously it has also been reported that alteration in environmental pressure and composition of inhaled gas, may influence upon intestinal motility.

Table 1: Relative rates of diffusion from the lumen

Nitrogen	1
Methane	4
Hydrogen	8
Oxygen	11 - 14
Hydrogen sulphide	69
Carbon dioxide	160

These data are derived from experiments on isolated intestinal segments of different animal species, mostly referred to by *Saltsman* and *Sicker*, 1968.

DIETARY INFLUENCE UPON COMPOSITION OF INTESTINAL GAS

"It must be something that I have eaten" has been – and still is – a general explanation for many gastrointestinal disturbances. Out of the many subjective hypotheses, probably the most accurate, is the relationship that exists between the ingestion of some vegetables, as beans, peas, etc, and the production of intestinal gases. This relationship is based upon an influence of the alimentary microbiota upon dietary compounds with a subsequent production of large amounts of various gases. Basis information of this relationship was worked out when man went into space in the 1960-ties, and a simple construction for quantitative and qualitative analysis of flatus was established. Shortly, a catheter was inserted into the rectum some cm beyond the anal sphincter. The end inserted into the rectum was perforated with a number of holes approximately one cm apart and the other end was attached to a cylinder containing an acidified sulphate solution. The volume of flatus passed was recorded by measuring the displacement of fluid in the cylinder. At the end of the collection period, the composition of the gas was analysed. The collection period could vary and so could also their relationship to meals. Data from one out of several experiments are as follows. On a basal diet, the volume of flatus collected was 15 cm^3/h, and percentage of methane was 7.3, i.e. around 1 cm^3/h. After a meal consisting of pork, beans or peas, the total volume increased more than tenfold and the percentage of methane more than doubled i.e. 30 - 40 cm^3/h of methane was produced hourly. It is well known that ruminants produced considerable amounts of methane (hundreds of cm^3 hourly after a meal), which is a gas around 30 times more "toxic" for global warming than CO_2. Consequently, presence of a number of "holy cows "in some countries have been questioned. Probably, methane production by man should also be taken into some consideration. In Sweden, pea soup is traditionally served every Thursday in nearly all restaurants. It goes back some hundred years, since one of their kings (Erik XIV) was killed by eating poisoned pea soup, and the Swedes want to demonstrate that they were innocent. Assuming that more than a million Swedes have pea soup meal every Thursday, the production of human produced methane in Sweden will be some thousands cubic meter of methane, outnumbering many holy cows.

A LIFE WITHOUT ANAEROBIC ALIMENTARY MICROBIOTA

"Life is not possible without bacteria!" That statement, expressed by L Pasteur more than a century ago, is contradicted by the life of germfree mammals. However, a life together with microbiota, but without the anaerobes and only with aerobic metabolism, should be something very special. Firstly, the ruminants would have difficulties to exist, since their yield of energy is based solely on an anaerobic breakdown of cellulose and an anabolic building-up of energy-rich organic acids, to be utilized by the host. In an omnivore as man, very large amounts of air, i.e. oxygen would be needed to serve the aerobic microbiota. In short, we would need to have a respiration as a 100-meter runner just finished. The major endproduct of carbohydrate metabolism would be CO_2 and water. There would be little, if any, need for alterative ways of getting rid of excess of H^+ or electrons, i.e. no methane, H_2 or hydrogen sulphide would be formed, and the need for a recirculation of nitrogen would be diminished and more products would have to be excreted in the urine. In short: More intake of food and air, much more production of urine and flatus. Thus, 1-2 kg of anaerobes has a dramatic influence upon our living.

FUTURE RESEARCH ON GASEOUS BIOMARKERS DERIVED FROM THE MICROBIOTA IN THE ALIMENTARY TRACT

As outlined above, studies on the amounts and composition of alimentary phases may be somewhat troublesome and not always easy to perform. For many years, röntgenology has been a suitable method in clinical medicine to study presence of gas in the alimentary tract (*Felson*, 1968). However, there might be good reasons to believe that even more specific methods are on their way. Future developments in nuclear magnetic resonance (NMR) spectroscopy will allow us to follow gases specific produced by the microbiota on their way from the alimentary tract to their possible places for influences on the host – wherever it might be (intestinal wall, blood vessels, nerve cells etc.). A better understanding of the many gaseous cross talks between a host and his intestinal microbiota represent future challenges and germfree - specifically contaminated ex-germfree - and conventional animals are valuable tools for overcoming these challenges.

Another challenge for the future is microbial utilization of N_2 in swallowed air. Probiotics with that property might reduce the increasing amounts of N_2 all the way down in the alimentary tract, and may reduce the symptoms in some patients with irritable able bowel syndrome.

Assuming that lack of nitrogen reduces a microbial breakdown of cellulose and other carbon-rich molecules, genetically modified mircroorganisms with N_2-fixating properties present in the rumen, would solve the problem. Similarly, probiotics with that property would reduce the demand for proteins in developing countries.

CONCLUSION

Presence of gas in the alimentary tract is a challenge, in physiology as well as pathophysiology. A better understanding of the many microbe/microbe and microbe/host cross-talks that are governed by gaseous molecules throughout the alimentary tract will create possibilities for interventions. Alimentary gases are far more than just flatus.

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GASTROENTEROLOGIST´S DILEMMA: PATIENTS WITH GAS RELATED PROBLEMS IN THE CLINICAL PRAXIS

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SUMMARY

Gas related problems such as belching, burping, halitosis and flatulence are common causes giving rise to consultation. Rarely, these persons have a serious underlying disease.

The cause of belching is often that these patients swallow too much air into the stomach, especially in situation when they are in a hurry or under stress during food intake (*Conchillo* et al., 2007). Furthermore and interestingly, some of these patients drink carbonated beverages, without considering the impact of the intake on the symptoms. If these patients have a medical history of heartburn it could be of value to perform a gastroscopy. Otherwise these patients are suggested a change in behaviour regarding the intake of food and beverages. However, people who repeatedly eructate can usually be shown to aspirate air into the hypopharynx before each belch. Chronic eructation is always a "functional disorder" and further examination should be reserved for patients with additional complaints.

Another gas related problem from the mouth is halitosis or bad breath. These persons have previously often consulted a dentist before they see a gastroenterologist. Conditions like diabetes and liver disease must be excluded. These patients often propose a gastroscopy since they think the problem is basically related to the stomach. If these persons do not suffer from heartburn or regurgitation the problem is not related to the gastrointestinal tract. It is rather related to dry mouth, which facilitates bacterial fermentation of food particles. These patients are advised to brush the tongue, cheeks and the roof of the mouth, which will remove the bacteria (*Tonzetich*, 1977).

The most common patient with gas related problem is the one with bloating and/or flatulence. Many of these patients fulfil the criteria for irritable bowel syndrome (IBS) (*Thompson* et al., 1999).

However, conditions like coeliac disease (*Sanders* et al., 2001) and difficulties in digesting lactose must be excluded (*Böhmer* and *Tuynman*, 1996). If the patient has a history of changes in bowel movement pattern a further examination with colonoscopy must be considered. Rarely this procedure discloses any specific disorder. The cause of bloating is probably complex, some patients suffer from visceral hypersensitivity (*Mertz* et al., 1995) while other patients have increased gas retention in the gut primarily due to an abnormal fermentation of fibre rich food (*Dear* et al., 2005; *Francis* and *Whorwell*, 1994; *King* et al., 1998). From my own clinical experience, many of these patients report noticeably less bloating and distension with a diet reducing gas production, i.e. a low intake of fermentable fibres. Some of these patients have noticed the relation between the

intake of “healthy food” such as fibres and their symptoms of bloating, distension and abdominal pains but believed that they have to suffer for a “healthy life”.

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INFLUENCE OF DIET ON MICROBIAL PRODUCTION AND UTILIZATION OF H_2, CH_4, H_2S IN THE COLON: PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONSEQUENCES

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SUMMARY

The intestinal microflora is capable of degrading and fermenting complex oligosaccharides and amino acids generating short chain fatty acids (mostly acetate, propionate, butyrate). The colonic microflora also produces copious amounts of H_2 and CO_2 during fermentation of carbohydrates. Varying amounts of H_2S and CH_4 are produced by the intestinal microflora and in humans appear to be strongly correlated with dietary habits. A diet high in red meat favours the formation of increased amounts of H_2S. In contrast, a diet low in meat and high in resistant starch favours the formation of CH_4 and increased amounts of short chain fatty acids. A high meat diet also shifts the bile acid pool in man to primarily taurine conjugation. Taurine is another source of colonic H_2S. Sulphate reducing bacteria have been shown to out-compete methane producing bacteria for available H_2 both *in vitro* and *in vivo*. In humans, *Desulfovibiro vulgaris* and *Methanobrevibacter smithii* are believed to be the bacterial species most responsible for the formation of H_2S and CH_4, respectively. H_2S, in high concentrations (mM) in the colon, appears to be highly toxic and carcinogenic to colonocytes and may contribute to the pathogenesis of inflammatory diseases and colon cancer. In high concentrations, H_2S inhibits the mitochondrial terminal respiratory chain and decreases the metabolism of butyrate by colonocytes. However, intestinal mucosal cells have the ability to detoxify H_2S by oxidative metabolism in the mitochondria and conversion to thiosulphate by rhodanese. H_2S can be synthesized endogenously from cysteine in mammalian cells and may function as a gasotransmitter molecule controlling the opening of K_{ATP} channels in smooth muscle cells, neurons, cardiomyocytes and pancreatic β-cells. The formation of H_2S by intestinal bacteria and metabolism by host cells provides an excellent example of how the body flora and diet can impinge on host physiology and pathophysiology.

INTRODUCTION

The first life on earth evolved in an anoxic environment. For 2.5 billion years, microorganisms have successfully exploited niches devoid of molecular oxygen (*Brocks* et al., 1999). In fact, it is interesting to note that one of the most densely inhabited environments on earth is the mammalian large

intestine (>10^{11} bacteria/gram wet weight faeces) (*Moore* et al., 1974; *Whitmann* et al., 1998). To put this in perspective, the adult human body contains an order of magnitude more prokaryotic cells (10^{14}) than mammalian cells (10^{13}). The collective bacterial genomes (termed "microbiome") of the human microflora encode an estimated 2-4 million genes, surpassing the human genome by a staggering 100-fold (*Bäckhed* et al., 2005). Intestinal bacteria can carry out hundreds, if not thousands, of enzymatic reactions not carried out by host cells. Hence, the human body should be regarded as a complex ecosystem of interacting prokaryotic and eukaryotic cells balanced by selective pressures from both the "top-down" (host) and the "bottom-up" (gut flora) (*Ley* et al., 2006). *Relman* and *Falkow* (2001) have called for the task of sequencing this microbiome, which they have termed "the second human genome project". We will, however, focus only on a small fraction of the genes within this "microbiome", and illustrate the magnitude of effect these few metabolic pathways have on human well-being and how diet appears to control them.

A few species of eubacteria and archaea have evolved the ability to utilize hydrogen gas and carbon dioxide waste products of colonic bacterial fermentation. Metabolism of molecular hydrogen by the microbial community improves the thermodynamics of colonic fermentation, which in turn provides the host with more energy and comfort through reduced gas volume. Three pathways exist in colonic bacteria to oxidize hydrogen, including: acetogenesis, methanogenesis, and sulphidogenesis. An important observation gathered from review of the current literature on the physiology of hydrogen sulphide is that concentration determines whether this gas functions as a toxic compound or "gasotransmitter". Hydrogen sulphide has come to be a recognized "gasotransmitter" along with CO and NO produced by the host for normal physiological processes. This was in fact quite a surprise given the toxic nature of sulphide. However, the gastrointestinal flora produces large amounts of hydrogen sulphide in some individuals. Given appropriate dietary and endogenous sulphur sources, the gastrointestinal flora can tip the balance toward disease states through copious production of H_2S in the human large bowel. In this review, we will examine the importance of diet on routes of hydrogen metabolism in the human colon in relation to colon cancer incidence and examine the native African case study as a model for diet, colonic bacterial metabolism and colorectal cancer risk.

THE GASTROINTESTINAL ECOSYSTEM

The human colon is a hollow organ roughly 1.5 m in length, 6.5 cm in diameter with a volume of 540 ml (*Wilson*, 2005). The colonic epithelium secretes mucin, a sulphated glycoprotein, which polymerizes into a viscous gel increasing in thickness from ±107 μM (proximal colon) to ±155 μM (rectum) along the length of the large bowel. The mucosal surface functions largely as an innate defence against microbial colonization of the epithelium, binding toxic metabolites, and provides lubrication that aids in peristaltic movement of luminal contents (*Wilson*, 2005). The colon functions to absorb water and recover energy from undigested dietary material through bacterial me-

tabolism. From the ileum, a mixture of undigested dietary material, biliary and pancreatic secretions known collectively as chyme empty into the caecum. As chyme enters the caecum, a sharp decrease in pH relative to the ileum (from pH 7.5 in the ileum to pH 5.7 in the caecum) occurs due to rapid bacterial fermentation of undigested carbohydrates and production of short chain fatty acids (SCFA; see next section). pH rises along the length of the colon to roughly 6.8 in the rectum as a result of absorption of SCFA by the colonic epithelium and buffering by bicarbonate secretion. Unlike the lumen of the small intestine, which supports mainly facultative anaerobes (10^6 to 10^8 bacteria.ml^{-1}), the lumen of the colon is strictly anaerobic with redox potentials ranging from -200 mV to -300 mV. For this reason bacterial biotransformations in the colon are largely restricted to hydrolytic and reductive reactions.

The host is sterile before birth, though bacteria are acquired during and shortly after birth (*Savage*, 1977) initially from maternal contact (*Mandar* and *Mikelsaar*, 1996; *Conway*, 1995) followed by continuous environmental exposure which persists throughout life. The composition of the gut flora appears to be assembled through factors such as environment, host genetics and stochastic events or "historical contingencies" such as colonization order (*Dethlefsen* et al., 2006). The adult flora is largely established during weaning, once the diet shifts to solid food (*Conway*, 1995). Factors such as redox potential, bile salt concentration, pH, transit time, available dietary substrates and host genotype relating to binding sites and secreted host factors produce strong selective pressures on potential bacterial colonizers. This is evident in light of recent comprehensive 16S rDNA sequencing and comparison of the human gut flora between individuals (*Eckburg* et al., 2005, *Ley* et al., 2006). Of the 50 bacterial phyla represented, only two major lineages, the Firmicutes and the Bacteroidetes are significantly represented in the gut flora of humans (>98% of the sequences) (*Eckburg* et al., 2005, *Ley*, 2006). In addition, only a single Archaean, *Methanobrevibacter smithii*, was represented in 1524 archaeal 16S rDNA sequences (*Eckburg* et al., 2005). This archaean has been shown to be the predominant methanogen in human faeces in other molecular based (*Lin* and *Miller*, 1998) and culture based studies (*Miller* and *Wolin*, 1982, 1983, *Miller* et al., 1984). The human colonic Firmicutes and Bacteroidetes lineages terminate in broad, shallow radiations comprising an estimated 800 species and likely thousands of strains (*Backhed* et al., 2005, *Dethlefsen*, 2006, *Ley*, 2006).

The majority of gut inhabitants are metabolically versatile generalists as a result of both microbe-microbe competition and synthrophy (*Dolfing* and *Gottschal*, 1996) as well as "top-down" selection favoring stable communities with functional redundancies that provide the host a steady supply of nutrients despite variations in diet (*Ley* et al., 2006). The 'normal' flora protect against infection through saturating binding sites that otherwise could be exploited by pathogens (*Ley* et al., 2006). Metabolic cross-feeding sets up competitive as well as cooperative interactions between intestinal microbes (*Flint* et al., 2007). For instance, species of *Roseburia* produce hydrogen and butyrate during acetate consumption. In co-culture with an acetogen, hydrogen is consumed, producing acetate which is then metabolized to butyrate, which is absorbed by the host or further metabolized by colonic bacteria (*Chassard* and *Bernalier-Donadille*, 2006).

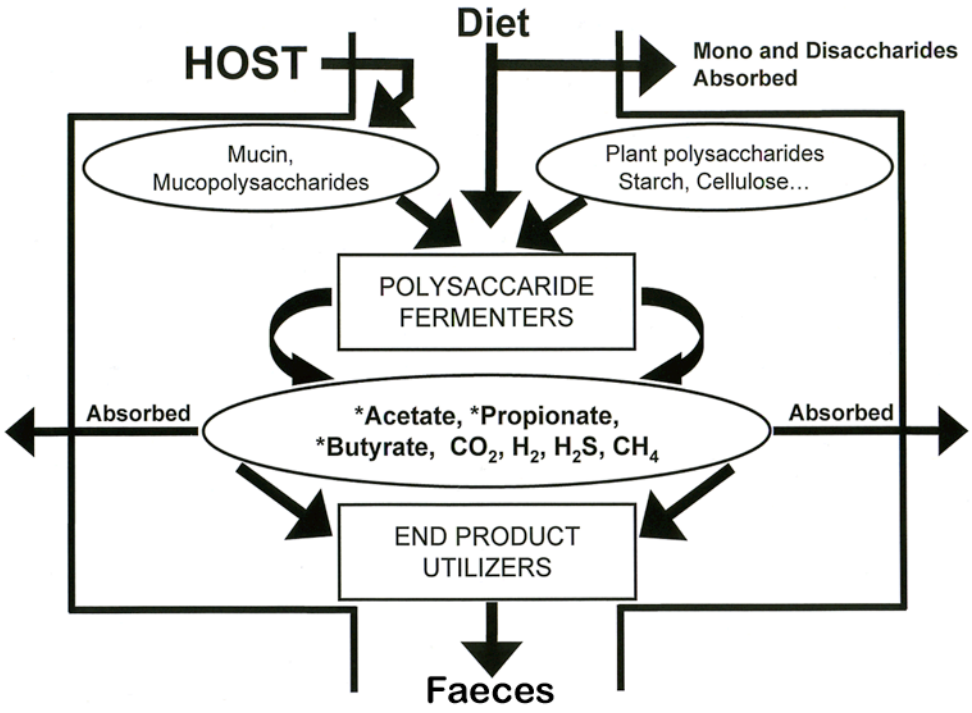

Figure 1: Growth substrates for colonic bacteria. Sloughed intestinal epithelial cells (100-200 g/day wet weight), plant polysaccharides and resistant starches are major substrates for colonic bacteria. Colonic bacteria produce short chain fatty acids (acetate, propionate, and butyrate 5:3:1 ratio) and various gases (CO_2, H_2, CH_4) from these substrates. Short chain fatty acids are absorbed from the colon and are metabolized by various tissues in the body. The amounts of different gases produced are influenced by dietary habits.

METABOLISM BY THE 'SECOND HUMAN GENOME'

Carbohydrates are the major and preferred carbon and energy source for the gut microflora. Indeed, the genus most represented in the human large intestine, the *Bacteroides* (*Wilson*, 2005), appears to dominate due to their arsenal of glycosylases (*Sonnenburg* et al., 2006). In fact, *Bacteroides thetaiotaomicron* alone contains 128 more glycoside hydrolases than the human host genome (*Xu* et al., 2003). Fermentation of complex dietary and endogenously produced carbohydrates is an emergent property of the microbiota (Figure 1). Breakdown of complex polysaccharides produces oligosaccharides and monosaccharides substrates which can be fermented by other members of the flora resulting in a chemical food web (*Hudson* and *Marsh*, 1995). Fermentation end products include the major short chain fatty acids (SCFA) acetate, propionate and butyrate in roughly a 5:2:1 ratio as well as the gases CO_2 and H_2 (Figure 1) (*Hoverstad* et al., 1984; *Miller* and *Wolin*, 1979). SCFA account for 5-8% of the total caloric intake/day in humans, which may seem irrelevant given the problem of "overeating" in many industrialized nations; however, the colonic epithelium derives an estimated 70% of its energy

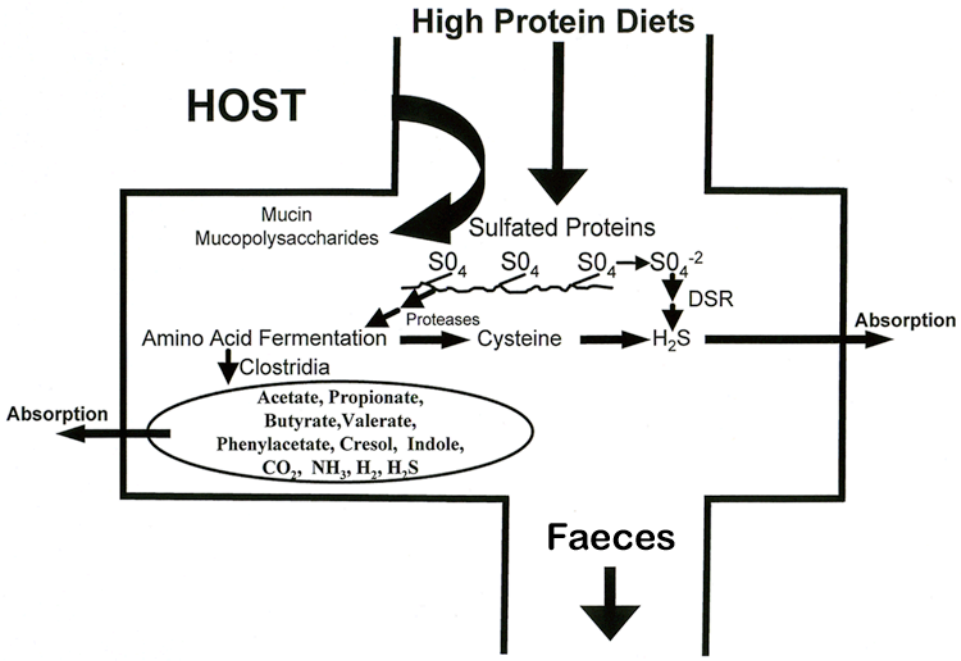

Figure 2: Effect of high protein diets on colonic bacterial metabolism. Increased input of protein into the colon increases the amount of hydrogen sulphide produce by colonic bacteria and selects for dissimilatory sulphate reducing (DSR) bacteria. High protein diets may also select for amino acid fermenting bacteria i.e. *Clostridium species*. The types and amounts of short chain fatty acids produced are altered by high protein diets.

from butyrate oxidation (*Bergman*, 1990). Indeed, this is an interesting example of co-evolution in which colonocyte gene regulation is geared toward β-oxidation of a microbial fermentation product. In addition, the amount of each fatty acid produced can be altered by many factors, including dietary habits. For example, a diet high in resistant starch produces more total SCFAs and more butyrate (*Scheppach* et al., 1988).

Metabolism of dietary protein generally occurs in the distal colon as carbohydrates become limiting. Proteolysis also represents a significant source of SCFA, H_2 and CO_2 production in the colon (*Macfarlane* and *Macfarlane*, 1995). Unlike saccharolytic fermentation, many additional and potentially harmful metabolites are produced from the fermentation of amino acids by clostridia via the Stickland reaction. This pathway of energy metabolism involves the oxidation/reduction of pairs of amino acids. During this process, ATP is generated via substrate level phosphorylation (*Lengeler* et al., 1999). Reaction products resulting from amino acid fermentation include 3-methylbutyrate, 4-methylvalerate, phenylacetate, cresol, indole acetate, indole, formamide, amines, and hydrogen sulphide (*Macfarlane* and *Macfarlane*, 1995) (Figure 2). In this regard, diets high in animal fat and protein have been linked to several cancers including breast, prostate and colon cancer (*Bingham*, 1999).

Additional substrates for the gut flora include the approximately 100-200 grams of sloughed intestinal

epithelial cells that enter the human colon each day along with bile components (Figure 1). Indeed, breakdown of host mucin supports a diverse colonic flora through production of oligosaccharides, monosaccharides, SCFA and free SO_4^{-2} (*Macfarlane* et al., 1989; *Gibson* et al., 1988; *Corfield* et al., 1992). Some members of the flora specialize in use of alternative electron acceptors i.e. carbon dioxide (methanogens and acetogens), free sulphate from diet and mucin degradation (sulphate reducing bacteria), cholesterol, bile acids (7α-dehyroxylation), steroid hormones, bilirubin and others. Indeed, specialized functions have been targeted to achieve a clinical outcome. The most familiar example is the use of prebiotics to increase the levels and activity of *Bifidobacteria, which* specifically metabolize fructo-oligosaccharides (*Gibson* et al., 1995). Thus, bacteria have evolved to utilize a wide range of host dietary and endogenous substrates with considerable functional redundancy, cross-feeding as well as specialization.

HYDROGENOTROPHIC METABOLISM

Hydrogen gas represents a key metabolite whose partial pressure regulates the SCFA profile through thermodynamic control of substrate oxidation (*Hungate*, 1967; *Wolin* and *Miller*, 1983). Hydrogen gas is formed as a means of disposing of reducing equivalents during fermentation, and at elevated partial pressures inhibits oxidation of reduced NADH, a coenzyme essential for hexose catabolism (*Gibson* et al., 1990b). Therefore, removal of molecular hydrogen is important both to the flora and the host as higher energy yields are derived through more efficient fermentation (*Gibson* et al., 1993). H_2, unlike SCFA's, is not metabolized by host cells, and instead the host expels 50-60% through breath and flatus (*Christl* et al., 1992). It has been observed that far less hydrogen is excreted than would be expected theoretically during fermentation of carbohydrates, suggesting that interspecies hydrogen transfer is the predominant mechanism of hydrogen gas disposal (*Christl* et al., 1992).

In aquatic ecosystems, hydrogen is consumed through two primary pathways: Methanogenesis and dissimilatory sulphate-reduction (DSR). Numerous studies in anoxic natural environments have shown that given adequate sulphate, methanogens are effectively out-competed for hydrogen (*Widdel*, 1988; *Cappenburg*, 1974a,b; *Martens* and *Berner*, 1974; *Winfrey* and *Zeikus*, 1977; *Oremland* and *Taylor*, 1978). Hydrogenotrophic dissimilatory sulphate-reduction proceeds through the following equation: $SO_4^{-2} + H^+ + 4H_2 \rightarrow HS^- + H_2O$ ($\Delta G^{\circ\prime} = -152$ kJ/mol HS^-). Hydrogenotrophic methanogenesis proceeds as follows: $CO_2 + 4 H_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G^{\circ\prime} = -131$ kJ/mol CH_4) (*Lengeler* et al., 1999). Sulphate reducing bacteria can outcompete methanogens as DSR by hydrogen gas yields more cellular energy than methanogensis. In the absence of sulphate, methanogenesis predominates (*Cappenberg* et al., 1974a,b) as under normal conditions, copious amounts of CO_2 and molecular hydrogen are present.

Similar to other natural anoxic environments, reduction of H_2 gas volume in the colon is carried out by a small group of hydrogenotrophic methanogenic archaea (MA) which are predominantly represented by *Methanobrevibacter smithii* (*Miller* and *Wolin*,

1983; *Miller* et al., 1984; *Lin* and *Miller*, 1998; *Eckburg* et al., 2005) as well as hydrogenotrophic sulphate-reducing bacteria (SRB) including *Desulfovibrio spp.* and *Desulfotomata spp.* (*Postgate* and *Campbell*, 1966; *Holdeman* et al., 1976; *Gibson* et al., 1988b,c; *Gibson* et al., 1990a; *Willis* et al., 1997). *In vitro* and *in vivo* studies have convincingly demonstrated a competitive relationship between human faecal populations of SRB and MA (*Gibson* et al., 1988a,b,c; *Gibson* et al., 1990a; *Christl* et al., 1992). Indeed, both groups of organisms have been shown to predominate in the distal gut, thus their overlapping spatial distribution should provide competitive microenvironments for molecular hydrogen. *Gibson* et al. (1988b) constructed a three-stage continuous culture system (TSCCS) to mimic the dilution rate, pH and substrates found in various regions of the colon to test the hypothesis that methanogenesis could be inhibited by sulphate sources. Previous observations in sudden death victims suggested MA preferentially inhabit the distal colon (*Macfarlane* et al., 1992). Indeed, methanogenesis was detected predominantly in vessel 3 of the TSCCS (distal colon) as expected due to considerations of pH. However, upon addition of 5.8 g/day porcine gastric mucin, methanogenesis was strongly inhibited with a concomitant 100-fold increase in DSR rates and an increase in SRB levels by several $\log_{10}$ colony-forming units. Once mucin addition was terminated sulphide production and SRB counts decreased and methanogenesis recovered to baseline. *Christl* et al. (1992) studied the *in vivo* effect of sulphate consumption on methanogenesis and sulphidogenesis in healthy humans placed on a low-sulphate diet. Hydrogen metabolism in 3 of 6 methanogenic individuals changed in response to addition of sodium sulphate to the diet. Breath methane decreased significantly along with methanogen colony counts, while SRB increased from undetected to 10^3 g^{-1} wet weight and sulphide production rate increased 3-fold. These observations changed the perception that methanogenesis was stable and independent of diet (*Bond* et al., 1971). It appears that methanogenesis can be rapidly and steadily inhibited through competition for molecular hydrogen with SRB and that dietary and endogenous sulphate sources regulate these relationships *in vivo*. The amount of sodium sulphate given in the *Christl* et al. (1992) study was 1.6 mmol; however, western dietary intake is estimated to vary between 1.5 and 16.0 mmol (*Florin* et al., 1993). Therefore, significant amounts of sulphide are routinely consumed in the western diet which may result in sulphidogenic hydrogen metabolism.

It has been observed by several studies that SRB and MA are not mutually exclusive in methanogenic individuals, so the question arises how SRB maintain growth rates to prevent washout. Desulfovibrios are found both in the lumen as well as mucosal associated, and may be able to subsist at low growth rates in the mucus (*Fite* et al., 2004). A second possibility suggests that rather than competing, methanogens may actually facilitate the persistence of SRB in the absence of adequate sulphate. A syntrophic relationship exists between *Methanobrevibacter smitthii* and *Desulfovibrio* spp. in the human colon in the absence of sulphate (*Stolyar* et al., 2007). In the absence of sulphate sources, *Desulfovibrio spp.* will ferment substrates such as lactate, which under high partial pressures of hydrogen, are extremely energetically unfavourable (*McInerney* and *Bryant*, 1981). In this regard, methanogenesis reduces hydrogen par-

tial pressure and makes Desulfovibrio fermentation of organic acids thermodynamically favourable. Indeed, levels of sulphate-reducing bacteria in some "methanogenic" individuals is low (~10^2 SRB.gram^{-1} wet weight) vs. 10^6 to 10^8 SRB.gram^{-1} wet weight in sulphidogenic populations (*Gibson* et al., 1988c). In this sense, the sulphate-reducer, in order to maintain status as a "resident" (autochthonous) member of the flora, must compete for organic acids with other secondary metabolizers (*Willis* et al., 1997). Methanogenesis may thus permit carriage of SRB. However, input of a selective agent (sulphate in this case) appears to break the synthrophy between the methanogen and SRB and allows H_2 dependent growth of *Desulfovibrios* with significant and rapid increases in abundance-especially if significant quantities of sulphate persists (*Gibson* et al., 1988b; *Christl* et al., 1992). While this has been shown both *in vitro* and *in vivo*, recent data have shown that some individuals harbour high levels of both SRB and MA (*Pitcher* et al., 2000; *Levine* et al., 1998; *Lewis* and *Cochrane*, 2006). Therefore, the relationship may be more complicated in certain situations and may depend on the composition of the gut flora, hydrogen partial pressures and possibly unidentified factors.

Acetogenesis is a third form of hydrogen disposal in the gut of mammals which forms acetate from H_2 and CO_2 through acetyl-CoA (*Lengeler*, 1999). Acetogenesis follows this equation: $4H_2 + 2\ CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ ($\Delta G^{\circ\prime} = -94.9$) (*Lengeler* et al. 1999). This pathway requires the same molar ratio of H_2 as methanogenesis and sulphidogenesis but yields the least amount of energy and will therefore be out-competed by both methanogenesis and sulphate reduction at the near neutral pH values in the distal colon. There have been some reports of acetogenesis in human faeces (*Lajoie* et al., 1988; *Bernalier* et al., 1996; *Leclerc* et al., 1997). Addition of $^{13}CO_2$ to faecal suspensions of three human subjects resulted in $^{13}CH_3COO^-$ only in the low methanogen (<10^2 g^{-1} dry weight) and non-methanogenic faeces. However, methanogenesis and sulphidogenesis appear mainly in the distal colon where pH is optimal. At acidic pH, in the caecum and ascending colon, acetogenesis may occur (*Gibson* et al., 1995). However, these same organisms are also saccharolytic, and would likely ferment sugars over acetogenesis for energetic reasons. While playing a role in hydrogenatrophic metabolism in some individuals, the majority of humans produce either methane or sulphide. The route of interspecies hydrogen transfer may have significant consequences for human health.

SULPHIDE AND DISEASE: A HYPOTHESIS

Several lines of evidence implicate sulphide production and SRB in the pathogenesis of the inflammatory bowel disease ulcerative colitis (UC). Faecal sulphide has been shown to be significantly elevated in UC patients with active disease (*Christl* et al., 1995; *Pitcher* et al., 1995; *Gibson* et al., 1991; *Roediger* et al., 1997) while other studies reported no significant increase (*Moore* et al., 1998). Stool measurements likely do not reflect total production of sulphide but rather binding capacity of chelators in stool. Roughly 95% H_2S is estimated to be absorbed through the colon (*Levitt* et al., 2002). Thus, faecal measurements underestimate the amount of sulphide

exposed to and detoxified by the colonic mucosa. Indeed, due to multiple dissociation states (HS^- and H_2S) and pH values in the colon, once H_2S is formed about 2/3 dissociates into the HS^- anion. However, H_2S, and to a lesser degree HS^-, are permeable to the plasma membrane of colonocytes. Because the relatively high concentration of H_2S in the colon (mM) as compared to blood (50 μM) there is a natural diffusion gradient into colonocytes.

Sulphide has been shown in several studies to inhibit butyrate oxidation, the preferred energy pathway for the colonic epithelium both *in vitro* (*Roediger* et al., 1980; *Roediger* and *Nance*, 1986; *Babidge* et al., 1998) and *in vivo* (*Roediger*, 1980). Indeed, breath CO_2 and luminal bicarbonate were significantly reduced in UC patients as compared to controls after rectal instillation of butyrate (*Roedinger* et al., 1984; *Den Hond* et al., 1996). In addition, sulphide increased proliferation in upper colonic crypts by 54% in one study, and this proliferation was reversed by butyrate (*Christl* et al., 1996). *In vivo* evidence demonstrates the effectiveness of butyrate enemas on the clinical symptoms of UC (*Harig* et al., 1989; *Sheppach* et al., 1992). This data strongly suggests that UC is an "energy deficiency" disease whose pathology is rooted in impaired β-oxidation (*Roedinger*, 1980). *Roedinger* et al. (1997) pointed out that impairment of butyrate metabolism would inhibit processes critical to maintaining epithelial cell barrier function. A noted risk factor for relapse into active UC is high meat intake, which provides substrate for SRB and thus sulphide production (*Tragnone* et al., 1995; *Magee* et al., 2000). In addition, SRB numbers and activity are significantly upregulated during active vs. quiescent disease (*Pitcher* et al., 2000). Treatment of UC with 5-aminosalicyclic acid (5-ASA) containing drugs including sulphasalazine resulted in inhibition of sulphide production in the colon and improvement of symptoms (*West* et al., 1974; *Pitcher* et al., 2000). In addition, animals fed sulphated polysaccharides (carageenan or dextran sodium sulphate) developed colitis, which could be inhibited by antibiotic treatment (*Onderdonk* et al., 1978) and fails to induce colitis in germfree animals (*Onderdonk* et al., 1977) suggesting the importance of the colonic flora in UC. However, while this is a compelling hypothesis, it has yet to be determined whether the increase in H_2S in patients with inflammatory bowel disease precedes the disease or is an alteration of the normal microflora as a result of chronic inflammation.

Inflammatory bowel disease significantly increases risk of developing colon cancer (*Mayer* et al., 1999; *Dincer* et al., 2007; *Tanaka* et al., 2006). Colonocytes have protective enzymes (rhodanese and thiomethyltransferase) which are expressed at the mucosal surface functioning to detoxify H_2S produced by colonic bacteria. Rhodanese (thiosulphate sulphurtransferase) can catalyze the formation of thiosulphate from H_2S and SO_3^{-2} and appears to be the main enzyme involved in detoxification (Figure 3). Studies from *Levitt* and co-workers (1999) showed that the addition of $H_2{}^{35}S$ to colonic mucosa resulted in the formation of primarily $^{35}S^{35}SO_3^{-2}$. The effect of H_2S on colonocyte physiology and pathophysiology is a function of concentration. At low concentrations (μM), H_2S can be oxidized by the mitochondria electron transport chain via H_2S:quinone oxidoreductase, an enzyme originating in eubacteria exposed to sulphidic environments (*Theissen* et al., 2003; *Goubern* et al., 2007). The input of electrons increases the electrochemical gradient of the cytoplasmic

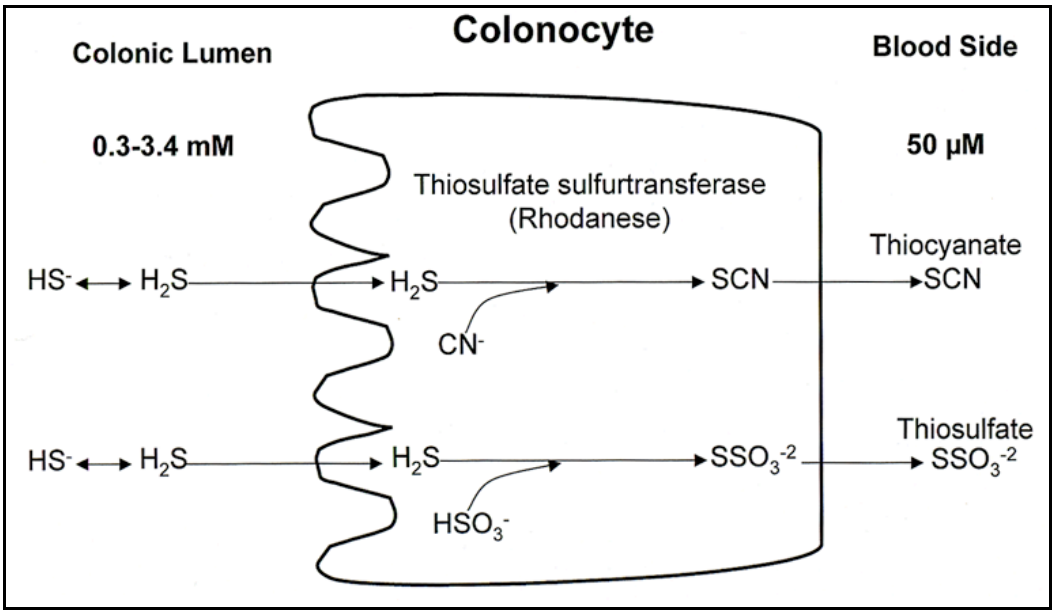

Figure 3: Effect of high meat vs. high resistant starch diets on colonic bacterial metabolism. High meat diets increase the amount of total protein and taurine conjugated bile acids (BA) entering the colon which serve as substrates for hydrogen sulphide formation by gut bacteria. Diets high in resistant starches (RS) and low in meat produce more methane and less hydrogen sulphide (H_2S) than high meat diets. In addition, diets high in RS produces more short chain fatty acids (SFA) as compared to high meat diets.

membrane of mitochondria and can increase ATP synthesis. The oxidation of H_2S generates various oxidation products of sulphur including: $S_2O_3^{-2}$, HSO_3^-, SO_4^{-2}. Enzymatic metabolism of H_2S and SO_3^{-2} by rhodanese generates thiosulphate, which is eliminated in urine (*Morris* and *Murer*, 2001). In contrast, at high concentrations (mM) of H_2S there is marked inhibition of cytochrome oxidase and a decrease of the mitochondria electrochemical gradient inhibiting basic cellular physiology including production of oxidized sulphur for sulphide detoxification by rhodanese.

Interestingly, recent data suggests loss of sulphide-detoxification possibly occurs in active UC and CRC (*Ramasamy* et al., 2006) and functional genetic polymorphisms of rhodanese (*Billaut-Laden* et al., 2006) coupled with dietary habits in some individuals (*Magee* et al., 2000) may saturate and overwhelm rhodanase enzymatic activity, leading to persistent conditions conducive to genotoxicity which lead to genetic changes and ultimately CRC. Indeed, recent data suggests that hydrogen sulphide is involved in the carcinogenesis process either through direct DNA damage or through upregulation of signalling pathways leading to proliferation, loss of apoptosis and tumour vascularization (*Attene-Ramos* et al., 2006, 2007; *Deplancke* et al., 2003; *Rose* et al., 2005).

HYDROGEN SULPHIDE AS A GASOTRANSMITTER

Hydrogen sulphide appears to be the third gasotransmitter synthesized in mammalian cells along with nitric oxide (NO) and carbon monoxide (CO). NO and CO are formed by NO synthase and haeme oxygenase, respectively. Hydrogen sulphide can function as a neuromodulator, smooth muscle relaxant and can play a role in protecting the intestinal mucosa (*Distrutti* et al., 2006; *Fiorucci* et al., 2006). Mammalian cells synthesize H_2S primarily by the activity of two highly regulated enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). These two pyridoxal phosphate-dependent enzymes are differentially expressed in tissues throughout the body. For example, the brain contains large amounts of CBS, whereas CSE is highest in peripheral tissues. The levels of H_2S in tissue and blood are highly regulated and are in the micromolar range (30-160 μM). Unlike the high concentrations (mM) of H_2S formed in the colon by intestinal bacteria, the levels of H_2S form in tissues are probably not toxic under normal physiological conditions. However, there is a small concentration range where H_2S functions as a regulatory gas that is not toxic.

Hydrogen sulphide appears to evoke many different physiological responses in mammalian cells (for reviews see *Wang* 2002, 2003; *Lowicka* and *Beltowski*, 2007). The cellular and molecular mechanisms regulated by H_2S are beginning to be elucidated. Hydrogen sulphide has been reported to regulate various cell signalling cascades and specific ion channels. The effect of H_2S on ATP-sensitive potassium channels (K_{ATP}) has been well studied. Many cellular effects of H_2S can be mimicked by drugs (pinacidil) that are agonist for K_{ATP} channels and these effects can be blocked by K_{ATP} channel antagonists (glibenclamide). H_2S appears to play a protective role against gastric injury caused by anti-inflammatory non-steroidal drugs (NSAIDs) (*Fiorucci* et al., 2005). NSAIDs were found to reduce cystathione-γ-lyase (CSE) mRNA and protein resulting in a decrease in H_2S formation in gastric mucosa. The addition of exogenous NaHS restored protection of the mucosa by increasing blood flow, inhibiting leukocyte adherence to endothelial cells and repressing pro-inflammatory cytokine formation induced by NASIDs. The protective effects of NaHS were through its effects on K_{ATP} channels.

Hydrogen sulphide has been reported to regulate various cell signalling pathways in mammalian cells including: ERK (stimulation and inhibition), iNOS (stimulation and inhibition), increase in intracellular Ca^{2+}, stimulation of adenylate cyclase among others (*Lowicka* and *Beltowski*, 2007, *Zhi* et al., 2007). There is no evidence that H_2S activates guanylyl cyclase as does NO. Moreover, H_2S can chemically react with reactive oxygen and nitrogen species, which may also alter different, cell signalling pathways. There is evidence that there may be cross-talk between H_2S and NO generation in mammalian cells (*Lowicka* and *Beltowski*, 2007).

COLORECTAL CANCER: THE NATIVE AFRICAN CASE STUDY

In the United States, colorectal cancer (CRC) is expected to be the third leading cause of cancer death in both men and women in 2007 in the United

States (*American Cancer Society*, 2007). However, patients classified as high-risk for colon cancer including those with familial adenomatous polyposis (FAP), hereditary non-polyposis CRC or inflammatory bowel disease comprise only 5-15% of all CRC incidence (*O'Shaughnessy* et al., 2002). The majority of CRC incidence is thus nonhereditary and sporadic; suggesting the importance of environmental influences. Epidemiologically, CRC incidence is primarily found in "Western" nations who consume a diet high in animal fat and protein (*Parkin* et al., 1992). In particular, native black Africans have been observed to have extraordinarily low colon cancer rates (*Burkitt*, 1971) as compared to African Americans who have higher incidence than Caucasian Africans or Caucasian Americans (*American Cancer Society*, 2005; *O'Keefe* et al., 1985, 2007). *Berg* (1973) made the observation that CRC risk increased significantly in descendents of immigrant populations from low-risk nations (including native Africans) living in developed nations who adopted a "Western" diet. It was originally thought that the low incidence of CRC observed in native black Africans was due to dietary fibre (*Burkitt*, 1971). However, adoption of a more 'Westernized' diet has not led to an increase in CRC despite lower non-starch fibre intake than the majority of 'Western' nations (*O'Keefe* et al., 1985; *Segal*, 2002). Within the same region, whites, despite better overall nutrition, including high non-starch fibre intakes have approximately a 10-fold higher rate of CRC (*Sitas* and *Parcella*, 1989). Recent data has called into question the hypothesis that non-starch fibre intake is associated with low CRC risk (*Park* et al., 2005; *Fuchs* et al., 1999). In fact, two large prospective studies demonstrated that dietary fibre supplementation failed to significantly reduce adenomatous polyp recurrence (*Schatzkin* et al., 2000; *Alberts* et al., 2000). Therefore, a closer look at the behaviour of the African population is required. Two important observations have been made in native African populations:

1) Native Africans consume large quantities of maize, which is high in resistant starch rather than non-starch fibre; and three times less red meat than South African whites (*O'Keefe* et al., 1999), and

2) This population absorbs carbohydrates in the small bowel with decreased efficiency as compared to other ethnic groups resulting in increased resistant starch reaching the large bowel (*Segal* et al., 2002; *O'Keefe* et al., 1999).

In addition, and as noted above, this population has the highest rates of methanogenesis (90%) as compared to Caucasian British (30%) and low sulphidogenesis in native Africans (15% vs. 70% in British) (*Gibson* et al., 1988c), and some of the lowest rates of diverticular disease and inflammatory bowel disease. The consumption of foods high in resistant starch and low in animal protein and fat was concluded to be a contributing factor to low incidence of colon cancer in native Africans relative to South African whites and indeed the remainder of the Western world (*O'Keefe* et al., 1999). The question remains as to a potential mechanism, an explanation of this data that may hold the simple key to reducing the risk of colonic disease. A recent animal model of high meat vs. resistant starch diet in mice coupled with genome-wide transcriptome analysis of *Bacteroides thetaiotaomicron* in a gnotobiotic animal model may help to explain the Native African data in terms of diet and our microbiome.

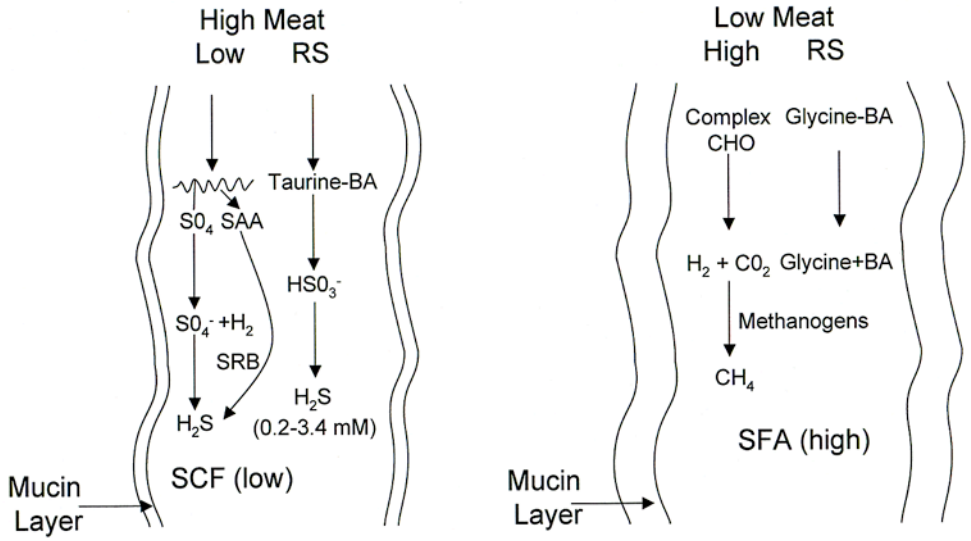

Figure 4: Metabolism of hydrogen sulphide by coloncytes. Hydrogen sulphide (H_2S) produced by gut bacteria can be taken up by colonocytes and metabolized to thiosulphate and thiocyanate by rhodanese. High levels of rhodanese protein and activity are found in colonic tissue.

CONSILIENCE

The title of this section is derived from the classic work of Edward O. Wilson entitled *Consilience,* which he expressed as "a unity of knowledge". A disease as complex as colon cancer is clearly multifactorial and requires a unity of knowledge from several fields. In this review the data has led us to examine aspects of the biology in all three domains of life; the eubacteria, the archaebacteria and the eukarya. We will now provide a synthesis of this analysis in light of exciting recent studies.

The observations gathered from studies in South Africa led Toden and co-workers to develop an animal model in order to determine the link between red meat, resistant starch and colorectal cancer (*Toden* et al., 2006, 2007). These studies demonstrated that resistant starch prevented DNA damage and mucin depletion in a dose-dependent manner. In the absence of RS, mucin was depleted 40% in rat colons on a meat diet and colonocyte DNA damage was nearly twice as great in the absence of RS as compared to its presence. Mucin may be important for binding toxic molecules, whose depletion may lead to cellular damage. DNA damage correlated negatively with butyrate production (*Toden* et al., 2007). Butyrate is the preferred source of energy for the colonocyte and regulates normal cellular functions (*Bergman*, 1990). Two possible explanations exist for the observed decrease in colonic mucus in meat fed rats. The first possibility is that SCFA increase mucin secretion through intestinal epithelial MUC2 gene expression (*Willemsen* et al., 2003), however, other studies have reported conflicting results (*Tarrerias* et al., 2002). A second interpretation is induction of mucin degrading genes by intestinal bacteria. A gnotobiotic mouse model of glycan foraging by *Bacteroides thetaiotaomicron* addressed genome-wide transcriptional

changes and metabolic products of this bacterium during abrupt changes in the carbohydrate composition of the mouse diet (complex polysaccharides vs. simple sugars). In the presence of complex dietary carbohydrates, *B. thetaiotaomicron* expressed genes involved in the attachment, uptake and metabolism of these compounds. However, when the diet shifted to simple carbohydrates that are absorbed by the host, and thus do not reach the colon, the microbe shifted its transcriptome to genes involved in degrading host mucin (*Sonnenburg* et al., 2005). These data suggest that in the absence of sufficient dietary carbohydrates, host mucus provides a stable source of carbohydrates (*Ridlon* and *Hylemon*, 2006) (Figure 4). Mucus thickness exists in equilibrium between host production and losses due to mechanical abrasion and bacterial metabolism.

As noted previously, utilization of host mucin results in release of large amounts of free sulphate, which the *Bacteroides spp*. cannot utilize. Thus along the intestinal tract, sulphate levels begin to increase until reaching the left colon where *Desulfovibrio spp.* reside. Their levels can rapidly rise in response to this substrate resulting in production of cytotoxic and potentially carcinogenic hydrogen sulphide. Lifelong exposure to high colonic sulphide levels due to diets low in complex polysaccharides and high in meat may decrease mucin thickness, increase distal colon putrifaction and secondary bile acid levels resulting in constant exposure of the colonic epithelium to molecules which up-regulate cell-turnover and intracellular production of DNA damaging reactive oxygen species. Conditions may be set up to generate mutations in colonocytes that may lead to colon cancer; possibly more rapidly if the sulphide-detoxifying pathway is impaired as is observed in UC and CRC (*Ramasamy* et al., 2006). The native African population appears to maintain a stable colonic environment through consumption of complex carbohydrates in the diet resulting in a steady supply of butyrate, precluding mucin degradation by supplying colonic bacteria with dietary carbohydrates, down-regulation of bacterial sulphate-reduction and lowering production of carcinogenic nitrogen compounds through their method of cooking and low animal protein and fat consumption.

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NITRIC OXIDE IN THE GASTROINTESTINAL TRACT: ROLE OF BACTERIA

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ABSTRACT

Nitric oxide is produced by numerous cell types along the GI tract where it serves to regulate a variety of physiological processes including gut motility, secretions, mucosal blood flow and immunity. Classically, NO is produced from L-arginine and molecular oxygen by specific enzymes- the NO synthases, but more recently a fundamentally different pathway for NO generation was described. This involves stepwise reduction of the higher nitrogen oxides nitrate and nitrite to form NO. In this process commensal bacterial in the GI tract play a key role. Dietary nitrate (mainly provided for by vegetables) accumulates in saliva and the oral microflora reduces this nitrate to nitrite. Nitrite then enters the stomach where it is reduced to NO by the acid. A picture is now emerging suggesting an important role of entero-salivary circulation of nitrate and serial reduction to NO in regulation of gastric function. Intriguingly, the nitrite that survives gastric passage is absorbed and can later recycle to NO in blood and tissues via several enzymatic as well as non-enzymatic pathways. Such systemic NO generation is likely involved in regulation of cardiovascular function and tissue homeostasis, especially in response to ischaemia and hypoxia.

INTRODUCTION

NO is generated in our bodies from the oxidation of L-arginine and this reaction is catalysed by specific enzymes, the NO synthases. A tremendous amount of data generated over the past two decades show that NO regulates vital physiological as well as pathophysiological processes ranging from vasoregulation, neuromodulation and regulation of platelet function to host defence and immunity (*Ignarro*, 2002). Here, a previously unrecognized and fundamentally different pathway for the generation of NO in humans is discussed. This pathway is NO synthase-independent and utilizes nitrate and nitrite as substrates. Interestingly, while the classical NO synthase pathway is oxygen dependent and dysfunctional during ischaemia/hypoxia, the nitrate-nitrite-NO axis is instead greatly enhanced during these conditions. Two components are central in the generation of NO from nitrate and nitrite: The commensal bacteria and the diet.

SOURCES AND ENTEROSALIVARY CIRCULATION OF NITRATE

The main dietary source of nitrate (NO_3^-) is vegetables, which account for 60-80% of the daily nitrate intake in people on a typical western diet (*Lundberg* and *Weitzberg*, 2005). Nitrite (NO_2^-) is also found in some foodstuff. For example, it is used as a food additive in meat to prevent botulism and to enhance its appearance. The main source of endogenous nitrate in mammals is the L-arginine-NO pathway, which is constitutively active in numerous cell types throughout the body. NO is produced from the amino acid L-arginine and molecular oxygen by NO synthases (NOSs). Although in simple aqueous systems NO is oxidized to nitrite, in mammals NO predominantly reacts with oxidized haemoglobin and other compounds to form nitrate.

After ingestion, nitrate is rapidly and effectively absorbed proximally from the gastrointestinal tract into the bloodstream, where it mixes with endogenously synthesized nitrate. Peak plasma concentrations are seen within 60 minutes of nitrate ingestion and the half-life of nitrate in plasma is about 5 hours. For as-yet-unknown reasons, the concentrations of nitrate excreted in saliva are exceptionally high; up to 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva, and the resulting salivary nitrate concentrations are at least 10 times higher than the concentrations in plasma (*Spiegelhalder* et al., 1976).

In the oral cavity salivary nitrate is rapidly reduced to nitrite by facultative anaerobic bacteria residing mainly at the dorsal part of the tongue. The levels of nitrite in fasting saliva are typically 50-150 μM but rise dramatically (to 1-2- mM) following ingestion of nitrate rich food (*Spiegelhalder* et al., 1976).

INTRAGASTRIC NO GENERATION AND ITS PHYSIOLOGICAL ROLE

In 1994 two groups independently discovered that great amounts of NO are being generated constantly in the stomach lumen (*Lundberg* et al., 1994; *Benjamin* et al., 1994). It was immediately realised that this NO was not a result of NO synthase activity but rather a non-enzymatic reduction of nitrite present in swallowed saliva. Nitrite is protonated in the acidic stomach to form nitrous acid (reaction 1), which then decomposes to a variety of nitrogen oxides including NO. Clear evidence of this pathway for NO formation came from human studies in which pre-treatment with the proton pump inhibitor omeprazole (thereby increasing gastric pH) abolished gastric NO (*Lundberg* et al., 1994). Additional *in vitro* experiments with different concentrations of nitrite in acid, or mixing of saliva and gastric juice, confirmed this (*Lundberg* et al., 1994).

$$\mathbf{NO_2^-} + H^+ \leftrightarrow HNO_2 \qquad (1)$$

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \leftrightarrow \mathbf{NO} + NO_2$$

The concentrations of NO in the stomach lumen (20 - 400 ppm.) are several orders of magnitude higher than those that are required for vasodilatation. As NO is known to easily travel across biological membranes and as NO-donating drugs are gastroprotective, it has been proposed that nitrite-derived NO, acting from the luminal side, could be involved in the regulation of

gastric mucosal blood flow (*Lundberg* et al., 1994). Several recent studies from different laboratories support this idea. *Bjorne* and colleagues (2004) studied gastric mucosal blood flow and mucus secretion in a rat *in vivo* model after local application of human saliva to the gastric mucosa. Mucosal blood flow and mucus secretion were increased after luminal application of nitrite-rich saliva, whereas saliva from a fasting individual had no effect. These effects were associated with the generation of NO and *S*-nitrosothiols. In addition, pre-treatment with an inhibitor of guanylyl cyclase markedly inhibited nitrite-mediated effects on blood flow. This indicates that the observed effects were mediated by NO.

Several other recent animal studies indicate that dietary nitrate has gastroprotective activity through the generation of NO in the stomach. We recently pre-treated rats with nitrate in the drinking water for one week and then challenged them with diclofenak; a non-steroidal anti-inflammatory drug (NSAID) known to produce gastric ulcers in animals and humans. Interestingly, the nitrate pre-treated animals were dose-dependently protected against the ulcerogenic effects of this drug (*Jansson* et al., 2007). *Miyoshi* et al. (2003) examined the effects of oral nitrate supplementation on stress-induced gastric injury in rats. Pre-treatment with inorganic nitrate was strongly protective and the effects were paralleled by intragastric generation of NO. Interestingly, NO generation and the protective effects of dietary nitrate were abolished when the oral microflora was removed by topical antibiotic treatment before the experiment.

In addition to the effects of NO on the gastric mucosa, the extremely high luminal levels of NO in the stomach also seems to be involved in the defence against swallowed pathogens *Benjamin* et al., 1994).

Taken together, these studies clearly indicate that dietary nitrate has important gastroprotective effects. The crucial step in the bioactivation of inorganic nitrate is the reduction to nitrite, which is carried out by the oral microflora.

NO GENERATION BY GUT COMMENSAL BACTERIA

The intragastric formation of NO from nitrite in saliva is non-enzymatic and a result of acid-dependent reduction of nitrite. We recently examined if NO could be generated also in lower parts of the GI tract where pH levels are much higher. Interestingly, when human faeces were incubated anaerobically in the presence of nitrate or nitrite considerable amounts of NO were produced (*Sobko* et al., 2004). In parallel *in vivo* experiments we could detect significant NO levels throughout the GI tract in conventional rats but not in germfree animals, thereby confirming the need for bacteria in this process. In addition, caecal and small intestinal NO levels increase in the rat after supplementing the diet with nitrate for one week (*Sobko* et al., 2005). When studying isolated strains of bacteria *in vitro* we found that bifidobacteria and lactobacilli generated large amounts of NO in the presence of nitrite while NO production from *E. coli* and *C. difficile* were negligible. In fact, further experiment showed that *E. coli* and *S. aureus* effectively consumed NO (*Sobko* et al., 2006). Clearly, at this stage we can say that NO is being produced by bacteria in the lower GI tract and its level will depend on the balance

between production and consumption by different bacteria. We also know that levels can be increased by increasing the dietary intake of substrate (nitrate). However, the physiological significance of this locally generated NO remains to be elucidated. In the stomach luminal NO clearly affects the host mucosa as discussed above but one should keep in mind that the levels in the stomach are orders of magnitude higher than those normally found in lower parts of the GI tract.

NITRITE REDUCTION TO NO IN THE SYSTEMIC CIRCULATION AND TISSUES

The generation of NO from nitrite occurs spontaneously in highly acidic or reducing environments. Interestingly, such non-enzymatic generation of NO can also occur systemically. In ischaemic tissues in which the pH value is decreased, NO is formed from nitrite by similar mechanisms (*Weitzberg* and *Lundberg*, 1998; *Lundberg* et al., 2004; *Gladwin* et al., 2005; *Zweier* et al. 1995). In addition, recent research indicates that nitrite can be converted to NO by several other pathways, which involve mammalian enzymes or proteins (*Weitzberg* and *Lundberg*, 1998; *Lundberg* et al., 2004; *Gladwin* et al., 2005; *Zweier* et al. 1995). It has now been shown that physiological concentrations of nitrite can dilate blood vessels through conversion to NO (*Cosby* et al., 2003). With this new knowledge, nitrite might be considered an important vascular storage pool of NO. Interestingly, it was recently found that the levels of nitrite in plasma increase 4-5-fold after ingestion of inorganic nitrate (*Lundberg* and *Govoni*, 2004). This increase was abolished if the test subject avoided swallowing after the nitrate intake, thereby illustrating its salivary origin. By extrapolation, this could in fact indicate that the commensal oral flora contributes not only to the local regulation of gastric function, as discussed above, but also to systemic NO-mediated effects, such as the regulation of vascular tone, platelet function and leukocyte adhesion. In strong support of this idea we recently noted a reduction in systemic blood pressure in healthy normotensive volunteers after a 3 day dietary supplementation with nitrate (sodium nitrate) in an amount corresponding to a daily intake of 100-300g of a nitrate rich vegetable such as spinach or lettuce (*Larsen* et al., 2006). This asks the intriguing question whether the high nitrate content in vegetables contributes to their beneficial effects on the cardiovascular system. Emerging data from numerous laboratories now also suggest that direct administration of nitrite can be therapeutically useful e.g. in treatment of ischaemia reperfusion injury (*Weitzberg* and *Lundberg*, 1998; *Gladwin* et al., 2005; *Zweier* et al. 1995). As an example, *Duranski* and colleagues (2005) pre-treated mice with extremely low doses of nitrite and then subjected them to cardiac ischaemia. Remarkably, nitrite treatment reduced their myocardial infarction by up to 70%. Interestingly, the dose that afforded the greatest protection is equivalent to what is achieved by ingestion of no more than 100 g of spinach or lettuce.

CONCLUSION

Nitrate is generally considered a water pollutant and an undesirable fertilizer residue in the food chain. Research in the 1970s indicated that, by reducing nitrate to nitrite, commensal bacteria might be involved in the pathogenesis of gastric cancers and other malignancies, as nitrite can enhance the generation of carcinogenic *N*-nitrosamines. More recent studies indicate that the bacterial and host metabolism of nitrate to nitrite can lead to formation of nitric oxide (NO) which could have beneficial roles not only locally in the stomach but also systemically. There is now accumulating evidence that nitrate-reducing commensals have a true symbiotic role in mammals and facilitate a previously unrecognized but potentially important aspect of the nitrogen cycle. This could lead to a paradigm shift in the view of dietary nitrate in relation to human health. It may be that the high nitrate content of vegetables explains some of their well known beneficial effects on health including a decreased risk of cardiovascular disorders.

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MICROBIAL PRODUCTION AND HOST DISPOSITION OF INTESTINAL CO_2: INFLUENCE OF DIET AND PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL EFFECTS IN THE LARGE INTESTINE

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SUMMARY

High luminal Pco_2 levels occur in the caecum and anterior large intestine of many species with conventional microflora, while Pco_2 levels equivalent to normal tissue Pco_2 occurs in germfree animals. Serosal Pco_2 is higher than normal tissue Pco_2 in conventional mice, which is caused by the large intestinal microflora metabolism and the inability of the caecal circulation to absorb the exogenous CO_2, produced by the microflora, at sufficient speed.

Lower Pco_2 levels in the caecal and colonic wall of larger species (guinea pigs, rabbits and dogs), despite luminal Pco_2 levels comparable to or higher than in mice, are considered to be a result of a more effective vascular absorption and physical containment of the CO_2 produced by the large intestinal microflora.

When added to normal peripheral partial pressures of other blood gases, the high serosal Pco_2 levels contributes to tissue gas supersaturation in the entire depth of the affected regions of the caecal wall in mice. If blood passage through the affected parts of the caecum leaves sufficient time for equilibration of dissolved gases, gas carrier contrast agents (GCAs) used as ultrasound contrast agents present in the plasma phase of the blood will experience gas supersaturation. Under such conditions, and particularly when a significant proportion of the gases is CO_2, microbubble growth will be rapid. Upon administration of GCAs in mice, vascular clearance of CO_2 will be compromised by vascular obstruction of microvascular beds. Other microbubbles present in the affected vascular bed will experience increased equilibration time with the supersaturated tissues, resulting in a cascading worsening and expansion of the caecal wall region affected by vascular obstruction and ischaemia in mice. The hepatic lesions observed in mice are caused by embolic distribution of large gas bubbles from the caecum via the portal vein.

The effects in rodents of exogenous CO_2 of microbial origin illustrate the unique features of CO_2 in relation to bubble growth and that local Pco_2 levels may deviate substantially and transiently from the normal systemic blood Pco_2 levels. Endogenous and exogenous sources (e.g. from intestinal microflora) of CO_2 may therefore have dramatic local effects on the initiation of bubble growth, even at marginally increased tissue concentrations. The contribution of CO_2 to gas bubble growth is particularly important during hypobaric decompres-

sion, but has also been implicated during hyperbaric decompression. As the initiation of bubble growth is essential for the clinical outcome of decompression in both humans and diving mammals, transiently increased local Pco_2 levels and the contribution of CO_2 by the intestinal microflora should receive more attention in decompression medicine.

INTRODUCTION

The intestinal microflora is vital to the host, both in health and disease. By its close integration in the host physiology, the distinction between host and host-derived characteristics and those of the intestinal microflora is often not only quite uncertain but also ignored. Microbial generation of nutrients, vitamins and gases, and intestinal anatomy, physiology and immune function are just some of the characteristics known in humans and conventional animals, which are dependent upon and/or interact with the intestinal microflora. By complete lack of all microflora, including that of the intestinal tract, germfree (GF) animals are fundamentally different. Germfree animals thereby serve as an important resource and baseline reference that lend them to investigations of the importance and effects of the intestinal microflora. The fact that GF animals do not eliminate H_2, CH_4 or H_2S gas (*Levitt* and *Bond*, 1970) is strong evidence for the intestinal microflora origin of these gases in conventional animals and humans. Microbial numbers and their metabolism of fermentable dietary substrate is supposedly low in the normal small intestine of conventional animals and at it's highest in the large intestine, where CO_2, H_2, CH_4 and H_2S gases are produced. Regional differences in large intestinal bacterial numbers are not indicated when measured as CFU/g intestinal content, but it is important to realise that CFU numbers reflect microbial *viability* but not *activity*. Microbial mRNA concentrations, which reflect actively dividing and hence metabolically active bacteria, are markedly higher along the mucosal surface of the caecum in mice (*Poulsen* et al., 1995). The higher bacterial metabolism in the caecum is logically associated with the higher concentrations of dietary substrates entering the anterior part of the large intestine. As the intestinal gas production is directly related to microbial metabolism, the caecum and anterior parts of the large intestine is the anatomical region where the intestinal tract microflora contributes the most exogenous gas to the host. The CO_2, CH_4, H_2 and H_2S gases are produced in the large intestine and the relative contribution in flatus is very variable (*Levitt* and *Bond*, 1970; *Calloway*, 1968; *Levitt*, 1971; *Danhof* et al., 1963; *Saltzman* and *Sieker*, 1968; *Steggerda*, 1968; *Levitt* and *Ingelfinger*, 1968). Reported gas concentrations vary markedly between individuals, not only due to different microflora compositions and activities and dietary substrate, but also as a result of the gas sampling technique. Most references have analysed the gas composition of the posterior parts of the large intestine via rectal sampling tubes (*Danhof* et al., 1963; *Levitt*, 1971; *Steggerda*, 1968). Due to the sampling techniques and differences in the gas characteristics (i.e. gas solubility and diffusivity); data from the posterior parts of colon and rectum are representative for these large intestinal segments only. Experimental access to, and gas sampling from, the anterior large intestine

(caecum and cranial colon) is difficult, and impossible without removing the intestinal content in a clinical setting. The composition and volumes of intestinal gases produced during normal filling in this part of the intestinal tract is hence effectively unknown in humans and rarely documented in animals.

Reports on microbial production of CO_2 in the large intestine of animals are scarce. Intra-colonic Pco_2 levels in conventional, germfree and conventionalised rats are reported to be significantly higher in conventional versus germfree and conventionalised animals (*Bornside* et al., 1976). The measurements were based on rectal introduction of a gas-sampling cannula into the colon lumen and the exact anatomical point of sampling is hence unknown. As the anterior large intestine has the best supply of fermentable dietary substrates and highest microbial metabolism of the entire intestinal tract, the true contribution of metabolic gases from this part of the intestinal tract to the host organism is therefore largely unknown. However, recent introduction of microelectrodes has enabled measurement of Pco_2 for various purposes in experimental animal studies (*Antonsson* et al., 1990; *Rozenfeld* et al., 1996; *Tønnessen* and *Kvarstein*, 1996) and our results from measurements in the intestinal tract of various species indicate that microbial production of CO_2 is very high in some intestinal compartments.

SOLUBILITY AND DIFFUSION OF GASES

The gases dissolved in tissues originate from atmospheric gases inhaled or ingested, and gases formed during metabolism. Both the host and intestinal microflora metabolism contribute to the metabolic gases, which are eliminated via the lungs or as flatulence. The gases CO_2, H_2, CH_4 and H_2S are produced by the intestinal microflora, particularly in the large intestine (*Saltzman* and *Sieker*, 1968; *Levitt*, 1971). Also H_2S has recently been identified as a gas produced by the host and with a multitude of effects (e.g. cardiovascular, neurological and inflammatory effects) (*Olson* et al., 2006; *Zanardo* et al., 2006; *Sivarajah* et al., 2006; *Lee* et al., 2006). Although nitric oxide is also a metabolic gas produced by the host, it is ignored in this context as the concentrations are significantly lower than other gases of exogenous and endogenous metabolic origin.

If gas exchange is primarily blood flow-limited, the absorption rate will be determined primarily (but not exclusively) by effective blood solubility (and blood flow rate). If exchange is diffusion-limited, the exchange rates will vary primarily (but not exclusively) according to the gas diffusion coefficients in tissue. Absorption of intestinal gases is primarily blood flow-limited, while the absorption of gases from a subcutaneous gas pocket is an example of a primarily diffusion-limited gas exchange (*Van Liew*, 1962, 1968). Parameters describing tissue gas exchange are diffusion coefficients (e.g. Fick's diffusion coefficient [D] and Krogh's diffusion coefficient [K]) and tissue solubility (e.g. Ostwald solubility coefficient [L]). As described by (*Langø* et al., 1996), K is the product of D and L and K arises from the steady state diffusion equation (Equation 1).

Reported diffusion coefficients and tissue solubility values vary quite significantly, dependent upon the type of

Equation 1: Steady state gas diffusion in tissues.

$$\frac{dV}{dt} = -D \times L \times A \times \frac{dP}{dz} = -K \times A \times \frac{dP}{dz} \qquad (\mathbf{1})$$

V: volume of gas measured at ambient temperature, A: area through which the diffusion flux is confined, P: partial pressure of the diffusing gas, and z: distance of diffusion.

tissue and conditions applied (*Langø* et al., 1996). Values of D, L and K from relevant tissues are included in Table 1. Intestinal gases will exchange across tissue-tissue and tissue-gas interfaces according to partial pressure gradients and according to gas specific transfer coefficients. Experimental exchange rates of CO_2, H_2, CH_4 and H_2S between isolated intestinal segments (with intact blood supply) and surrounding tissues have been described by (*Forster*, 1968; *Saltzman* and *Sieker*, 1968) and are presented in Table 1 as equilibration half-time, transfer coefficient (k) of gas exchange, and *measured* relative rates of gas absorption. The equilibration half times, transfer coefficients and relative absorption rates of Table 1 are experimental data, measured in the small intestine. These values compare well, and best to K (and L), and are assumed to be relevant for large intestinal gas exchange. The majority of the CO_2 generated in the large intestinal tract is hence absorbed by the mucosal circulation and eliminated by pulmonary exhalation. Relative diffusion velocity has also been *computed* from the physical characteristics of each gas. The computed relative diffusion velocity primarily reflects the gas-specific diffusion coefficients and is hence more relevant for diffusion-limited gas exchange (e.g. subcutaneous gas pocket). Measured and computed exchange rates of O_2 and CO_2 therefore differ. In addition to different dependence on solubility versus diffusion coefficient, this difference most likely also reflects that chemical interaction (inside RBCs) significantly increase the effective solubility of both gases and that the intestinal blood flow is related to the intraluminal Pco_2, such that increasing Pco_2 cause increased intestinal blood flow (*Pals* and *Steggerda*, 1966).

MICROBIAL CO_2 PRODUCTION AND INFLUENCE OF DIET

Initial measurements of the luminal Pco_2 in *HsdHan:NMRI* mice confirmed that the caecum is the primary site of fermentation, with remarkably higher Pco_2 levels in the caecum (52 kPa) versus stomach, jejunum and colon (18-23 kPa) (*Rasmussen* et al., 1999a). In all subsequent experiments, the Pco_2 micro-electrodes were introduced into the lumen and onto the serosa of the jejunum and caecum/colon after midline laparotomy. The caecum was used in rats, guinea pigs and mice while the cranial colon was used in dogs. The tip of the intraluminal Pco_2 sensor was placed in the centre of the intestinal contents through anti-mesenteric incisions, generally avoiding contact with the mucosa. Two different diets were used in experiments with mice. The standard diet, used if not otherwise noted, was the SDS diet. The experimental diet was the *Diet 4012.01*. The fat, protein and energy content of the

Table 1: Calculated and measured tissue gas diffusion and solubility values

Gas	D 10^{-5} cm^2/s	L ml gas/ml tissue	K 10^{-5} ml gas x cm^2/ml tissue x s at 1 ATA	Equilibration half-time min	Transfer coefficient, k 10^{-3} ml/min x mmHg	Measured relative rates of absorption †	Computed relative diffusion velocity ‡
CO_2	1.4	0.58	0.90	5	3.7	160	35
H_2S	ND	ND	ND	12	1.6	69	130
O_2	1.5	0.024	0.042	50	0.38	13-14	1.8
H_2	ND	0.018	0.054	90	0.22	7-8	5.0
N_2	1.3	0.013	0.019	97-280	0.07-0.2	1-2	1
CH_4	ND	ND	ND	185	0.1	4-5	2.5

D measured in rat skeletal muscle, except O_2 (rat myocardium). K measured in rat skeletal muscle, except N_2 (cat urinary bladder). L measured in human blood plasma. D, L and K values at 37°C.
ND: no data.
†: Equilibration halftime, transfer coefficient and relative rates of absorption are measured in isolated intestinal segments (with intact blood supply) in cats.
‡: Relative diffusion velocities computed from physical characteristics of the gases.
D, L and K values from (*Langø* et al., 1996), other data from (*Forster*, 1968) and (*Saltzman* and *Sieker*, 1968).

two diets are practically identical, but while the standard *SDS* diet contains 14% dietary fibres, 46% starch and 7% "sugars" (including glucose), the custom made *Diet 4012.01* contains no dietary fibre, no starch and 73% glucose as the only carbohydrate source.

In vivo Pco_2 levels in mice, rats, guinea pigs and dogs were remarkably high in the caecal and/or colonic lumen of all species with a normal microflora metabolism (*Rasmussen* et al., 1999a, 2002) (Table 2). The highest luminal Pco_2 levels were recorded in the colon/caecum of dogs and mice, intermediate levels were recorded in rats, guinea pigs, conventionalized germfree *KI:NMRI* mice and *HsdHan:NMRI* mice on *Diet 4012.01*, and Pco_2 levels equivalent to or slightly higher than normal tissue Pco_2 were recorded in mice with no or very limited microflora metabolism (germfree and gnotobiotic *KI:NMRI* mice and *HsdHan:NMRI* mice after removal of the caecal content by saline flushing) (Table 1). Pco_2 levels, equivalent to normal tissue Pco_2, were also recorded in the lumen and on the serosal side of the ileum in all species. The lumen Pco_2 levels in both ileum and caecum/colon were unaffected during the 5 min post mortem observation time by the blood flow stop after death. The results clearly demonstrate that Pco_2 levels in the caecum/colon lumen are determined primarily by microflora and dietary substrate composition. When the microflora is absent (GF) or reduced in numbers and/or metabolic activity (flushed *HsdHan:NMRI* mice), or the dietary substrate entering the caecum is reduced (*HsdHan:NMRI* mice on *Diet 4012.01*), significantly lower luminal Pco_2 values are observed.

Table 2: *In vivo* Pco_2 levels in caecum and colon at t=0, Jco_2 and sensitivity of different species and strains for GCA-induced lesions

Species/strain	N	GCA-lesions	Jco_2 (kPa/min)	Caecum/colon Pco_2 (kPa)	
				Lumen	Serosa
HsdHan:NMRI mice [1]	10	+	4.3 ± 0.5	54.5 ± 4.2	26.5 ± 2.9
HsdHan:NMRI mice [2]	8	-	1.7 ± 0.2	18.2 ± 0.9	9.7 ± 2.9
Hsd:ICR mice [1]	5	+ ①	3.7 ± 0.7	58.1 ± 10.8	12.9 ± 1.9
BK:NMRI mice [1]	6	+ ①	4.5 ± 1.0	75.7 ± 6.8	26.2 ± 3.9
"Irrigated *HsdHan:NMRI*" mice [1]	6	NA	1.2 ± 0.2	10.0 ± 0.8	8.7 ± 0.4
Germfree *KI:NMRI* mice [3]	7	-	0.4 ± 0.1	8.7 ± 0.7	9.2 ± 0.4
Gnotobiotic *KI:NMRI* mice [3] †	4	NA	1.0 ± 0.2	11.0 ± 0.8	11.2 ± 0.7
Gnotobiotic *KI:NMRI* mice [3] ††	3	NA	0.7 ± 0.2	9.3 ± 0.3	9.6 ± 1.3
Gnotobiotic *KI:NMRI* mice [3] †††	3	NA	1.1 ± 0.4	11.9 ± 0.5	12.0 ± 0.6
Conventionalized germfree *KI:NMRI* mice [3]	8	-	2.0 ± 0.6	28.8 ± 2.7	14.4 ± 2.3
Mol:SPRD rats [1]	8	+ ②	5.3 ± 0.6	52.2 ± 2.5	8.5 ± 0.5
Hsd:DH guinea pigs [4]	7	-	3.0 ± 0.2	36.7 ± 2.3	8.9 ± 0.4
Mongrel dogs [5, *]	4	+ ③	0.2 ± 0.1	69.8 ± 8.9	3.9 ± 0.9
Mongrel dogs [5, **]	4	+ ③	0.3 ± 0.2	14.1 ± 2.8	6.3 ± 0.8

All measurements were performed in the caecum, except in dogs where colon was sampled. Pco_2 values are stable *in vivo* values immediately before the animals were killed. Jco_2 is $dPco_2$(serosa)/dt for 5 min after death. All values are mean ± SEM. 1: *SDS* diet, 2: *Diet 4012.01*, 3: *Lactamin R36* diet, 4: *B&K Rabbit and Guinea Pig Maintenance* Diet, 5: *Purina Fit & Trim*® diet, *: intestinal content in colon, **: no intestinal content in colon, n: number of animals. Gnotobiotic mice were mono-associated with †: *E. coli*, ††: *Cl. difficile*, †††: *L. acidophilus*. +: Affected by GCA-induced lesions, -: Not affected by GCA-induced lesions, NA: Not applicable. ①: Markedly lower incidence of GCA-induced lesions than in *HsdHan:NMRI* mice, ②: Caecum/colon lesions only, ③: Caecum/colon lesions only and only after 28 days repeated dosing. Table content extracted from (*Dirven* et al., 2003; *Rasmussen* et al., 1999a; *Rasmussen* et al., 2002), except Jco_2 data from all other mice strains that *HsdHan:NMRI*, which are previously unpublished data (H. Rasmussen).

HOST DISPOSITION OF INTESTINAL CO_2

The serosal Pco_2 levels in dogs and mice were significantly lower and higher, respectively, than all other species tested (Table 2). In the dog, serosal Pco_2 levels were equivalent to normal tissue Pco_2 and hence according to expected normal physiology. The serosal Pco_2 of dogs remained virtually constant for 5 min after the circulation had stopped. In addition to the high lumen/serosa Pco_2 ratio, the effective washout of CO_2 in the colon of dogs was also demonstrated by simultaneous measurement of mucosal and luminal Pco_2 in two dogs. Luminal-mucosal-serosal Pco_2 values of 58-6-3 kPa and 51-7-4 kPa, respectively, demonstrate an extremely efficient removal of luminal CO_2 by the mucosal circulation.

Contrary to dogs, *in vivo* serosal Pco_2 levels markedly above normal tissue Pco_2 were recorded in mice and

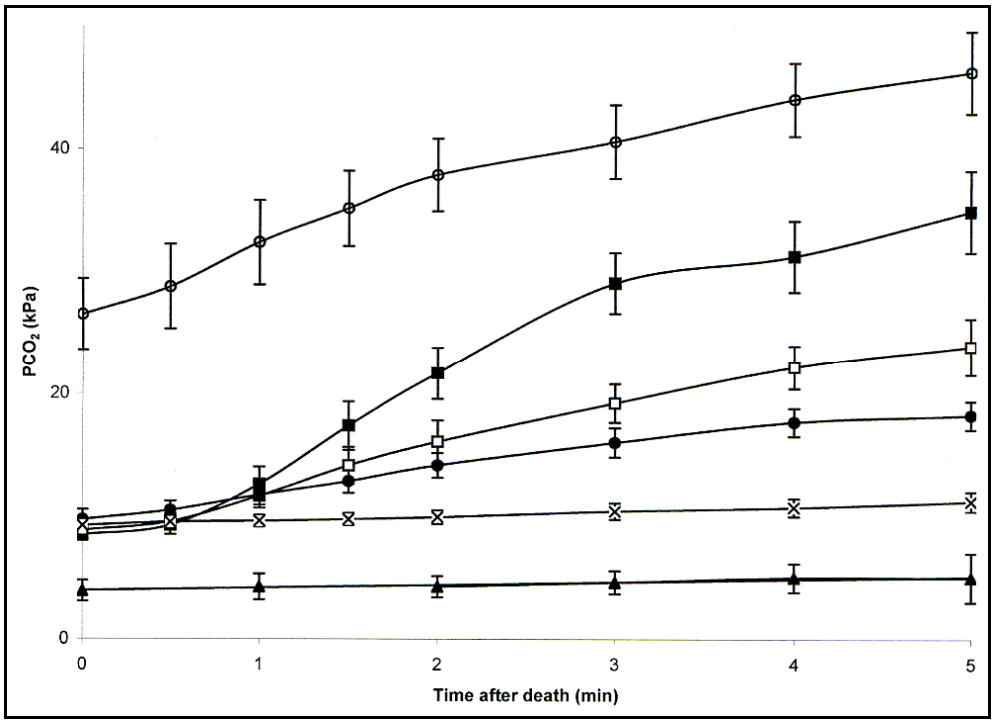

Figure 1: Serosal Pco_2 in caecum and colon.
Pco_2 measured in caecum in all species, except dogs (colon). Values are mean ± SEM.
○: *HsdHan:NMRI* mice on *SDS* diet (n=10), ■: *Mol:SPRD* rats (n=8), □: *Hsd:DH* guinea pig (n=7), ●: *HsdHan:NMRI* mice on *Diet 4012.01* (n=8), x: GF *KI:NMRI* mice (n=7), ▲: Mongrel dog (n=4). Values at *t*=0 are stable *in vivo* values. Values from 0 to 5 min are post mortem values. When error bars are not shown, they are smaller than the symbols of the graph. The inclination of the serosal Pco_2 lines from *t*=0 to *t*=5 constitutes Jco_2 for each group. Figure previously presented in (*Rasmussen* et al., 2002) (without GF data) and at the Bengt E. Gustafsson Symposium, November 1, 2003, Stockholm, Sweden (unpublished).

the serosal Pco_2 levels increased rapidly after death. Serosal Pco_2 levels in rats, guinea pigs and *HsdHan:NMRI* mice on *Diet 4012.01* glucose diet were in-between these two extremes, and significantly higher than normal tissue Pco_2. The lumen and serosal Pco_2 in rats were lower than that of mice, but the post mortem transmural increase in serosal Pco_2 (Jco_2) was markedly and significantly higher. The lumen and serosal Pco_2 and Jco_2 were lower in guinea pigs and *HsdHan:NMRI* mice on *Diet 4012.01* glucose diet. The lumen and serosal Pco_2 levels in GF, conventionalised GF and flushed *HsdHan:NMRI* mice were equivalent to that of normal tissue and the absence of a transmural Pco_2 gradient resulted in a low Jco_2. These observations also confirmed that the anaesthetic (*Svendsen* and *Carter*, 1985) and surgical protocol, which was the same across all rodents, contributed little or not at all to the tissue Pco_2 levels recorded. The *in vivo* serosal Pco_2 levels and the rate of increase during the first 5 min after death (= Jco_2) are included in Table 2 and illustrated in Figure 1.

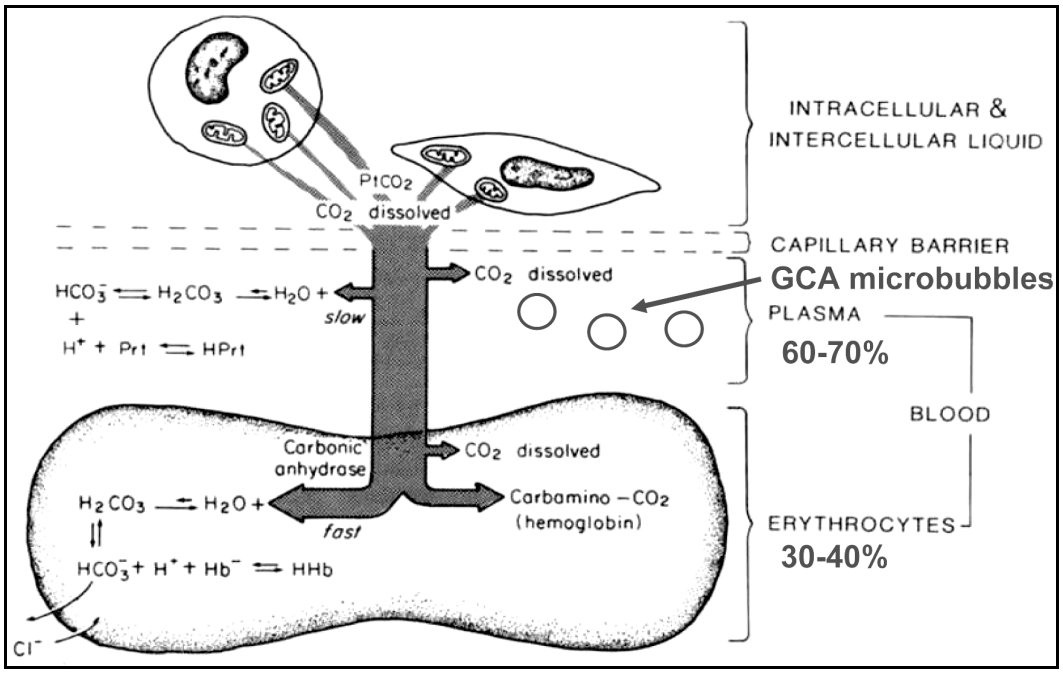

Figure 2: Different forms of CO_2 and its transportation in different blood compartments.
Only dissolved CO_2 can cross the capillary barrier. The halftime for hydration of CO_2 to carbonic acid is slow without carbonic anhydrase (plasma) and rapid with carbonic anhydrase present (RBC). Dissociation of carbonic acid is spontaneous and rapid, without any enzymatic acceleration. GCA microbubbles are illustrated in the plasma space, which constitute 60-70 volume % of the whole blood. Figure modified from (*Staub*, 1991).

PHYSIOLOGICAL EFFECTS OF MICROBIAL CO_2 PRODUCTION IN THE COLON

The physiological effects of increased serosal and intramural Pco_2 in mice (and other rodents) during normal conditions are unknown. The high effective solubility and blood buffering of CO_2 is well known to maintain systemic blood Pco_2 levels within relatively narrow limits and one may therefore question if high CO_2 production in the caecal lumen will be able to affect the total gas tension in the blood as it passes through the caecal wall? The intracellular effective solubility of CO_2 is high due to hydration/dehydration of CO_2 to/from carbonic acid (H_2CO_3), and dissociation of carbonic acid to form bicarbonate (HCO_3^-) and hydrogen (Equation 2). While the dissociation of carbonic acid is spontaneous and immediate (microseconds) without

Equation 2: Carbon dioxide reactions

$$CO_{2,\,dissolved} + H_2O \leftrightarrow^{①} H_2CO_3 \leftrightarrow^{②} H^+ + HCO_3^- \qquad (2)$$

①: Halftime in seconds without and milliseconds with carbonic anhydrase. ②: Spontaneous reaction with half time of microseconds.

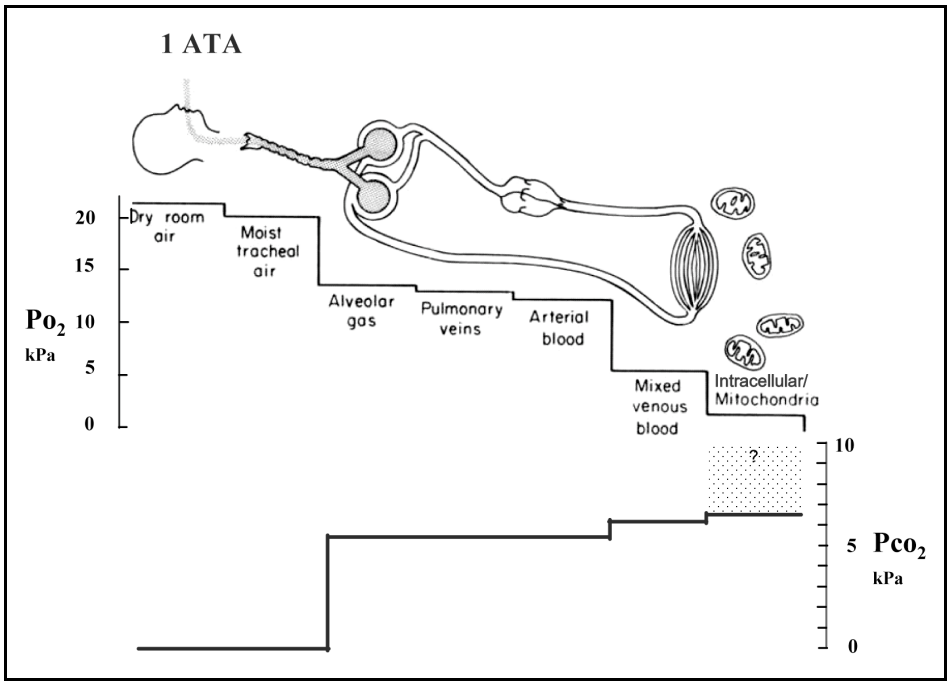

Figure 3: Regional O_2 and CO_2 tensions in different tissue compartments.
The high effective solubility of CO_2 results in only minor Pco_2 increases as oxygen is consumed. Uncertainty regarding the intracellular Pco_2 range (6-12 kPa) is indicated. The mitochondrial Pco_2 levels are unknown. Figure modified from (*Staub*, 1991; *Hempleman*, 1993).

any enzymatic acceleration, the half-time for hydration of dissolved CO_2 is relatively slow without carbonic anhydrase (CA) (*Staub*, 1991). Carbonic anhydrase reduces the hydration half-time to milliseconds and the ability of blood to solubilize and carry large quantities of CO_2 is due to the CA in RBCs. Despite the ability for rapid hydration/dissociation of CO_2 in intra- and intercellular compartments, it is important to point out that CO_2 can only cross cellular and organelle membranes as molecular CO_2 (*Klocke*, 1987; *Henry*, 1996). Carbon dioxide can therefore only enter the blood stream across the capillary endothelium as dissolved molecular CO_2 and above considerations about effective solubility are important, but yet irrelevant in this context when it occurs in the extravascular space.

In the vascular space, CA enzyme occurs in high quantities inside the RBCs but is completely absent in the plasma (*Staub*, 1991; *Maren*, 1967; *Lumb*, 2000). The half-time for hydration of CO_2 is therefore >5 sec in the plasma without CA, while the half-time is in milliseconds (*Staub*, 1991) and some $13x10^3$ - $1x10^6$ times faster inside the RBCs because of the CA enzyme (*Klocke*, 1987; *Maren*, 1967; *Lumb*, 2000). Although different CA iso-enzymes occur in various tissues (*Sly* and *Hu*, 1995; *Maren*, 1967), including the capillary endothelium and the colon, the activity and importance of these iso-enzyme is negligible when compared to the importance of CA in RBCs (*Klocke*, 1987). Tissue Pco_2 levels equal to or higher than those measured on the caecal serosal in the mouse may therefore *transiently* apply to the

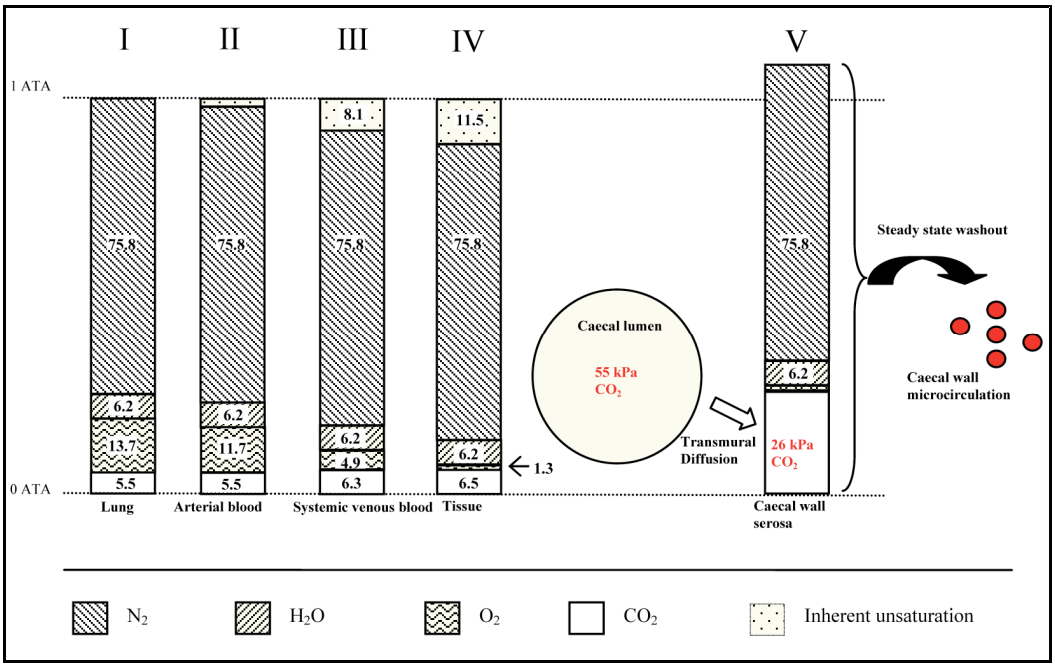

Figure 4: Tissue gas tensions and effects of exogenous influx of CO_2 into the caecal wall.
The Pco_2 levels (kPa) of the caecal lumen and caecal wall serosa at 1 ATA are from paper II (*HsdHan:NMRI* mice). Other tissue gas tensions according to (*Hempleman*, 1993; *Hills*, 1975). Column numbers (I-V) indicated above columns. Figure presented with slight variations at the XIII International Symposium on Gnotobiology, June 19-24, 1999, Stockholm, Sweden (*Rasmussen* et al., 1999b), the American Ultrasound in Medicine 45th Annual Convention, March 11-14, 2001, Orlando, Florida, USA (*Rasmussen* et al., 2001) and the 2002 IAG-SOMED Joint Congress, June 14-18, 2002, Raleigh, North Carolina, USA (Unpublished).

plasma *during* capillary passage through *discrete* volumes of the caecal wall (Figure 2), while the CA enzyme in the RBCs and the haemoglobin buffering of the hydrogen ions formed during carbonic acid dissociation maintains the narrow range of Pco_2 in *systemic* whole blood.

Partial pressures of O_2 and CO_2 are regionally different (Figure 3) and are affected by metabolic consumption/ production, respectively. At isobaric conditions the total tissue gas tension is hence affected by cellular metabolism, and uptake of exogenous gases originating from the intestinal tract microflora or other sources. If capillary plasma Pco_2 becomes transiently identical to or higher than serosal Pco_2 during passage through the affected caecal wall, a transient state of isobaric gas supersaturation may occur in the blood plasma.

The body is exposed to an external atmospheric gas pressure of 1 ATA or 101.3 kPa at sea level altitudes, composed in dry air of 20.2 kPa O_2, 81.0 kPa N_2 (including 0.9 kPa argon) and a mere 36.5 Pa CO_2 (*Hempleman*, 1993; *Nunn*, 1993). After humidification of the inhaled air (Figure 4, column I) and the normally occurring ventilation/perfusion mismatch, O_2, N_2, H_2O and CO_2 tensions are 11.7, 75.8, 6.2 and 5.5 kPa, respectively, and the total gas tension in the arterial blood leaving the

lungs is 2 kPa lower than that of the air inhaled (column II) (*Hempleman*, 1993; *Hills*, 1975). From this point onwards, water vapour and nitrogen remains the same throughout all tissues and further changes in total gas tension are caused by changes in O_2 and CO_2 tensions only. During tissue metabolism and O_2 consumption, a typical respiratory coefficient of 0.8-0.9 results in production of 0.8-0.9 moles of CO_2 for every mole of O_2 consumed. However, because the *effective* solubility of CO_2 (which includes both physical solubility and chemical dissociation) is considerably higher than that of O_2, the resulting decrease in total gas tension is almost identical to the decrease in Po_2. This difference in solubility (see Table 1 and Figure 3) result in an increasing difference between the atmospheric pressure and the total gas tension of the tissues. This difference is also known as the "inherent unsaturation" or "oxygen window" (*Van Liew* and *Raychaudhuri*, 1997; *Van Liew* et al., 1993; *Aksnes* and *Rahn*, 1957; *Hills* and *LeMessurier*, 1969; *Lategola*, 1964; *Hempleman*, 1993). After passage through the capillary circulation, where O_2 is supplied to and CO_2 removed from the tissues, the inherent unsaturation of the venous blood has increased to 8.0 kPa (column III). In the tissue cells (column IV), Po_2 and Pco_2 have been reported to be 1.3 and 6.5 kPa, respectively (*Hempleman*, 1993), resulting in a *computed* sum of partial pressures of 90 kPa and hence an inherent unsaturation of approximately 11 kPa during normal aerobic metabolism (*Hills* and *LeMessurier*, 1969; *Lategola*, 1964; *Hempleman*, 1993; *Hills*, 1975). Others have reported a normal tissue Pco_2 of 8-12 kPa (*Tønnessen*, 1997) and hence a *computed* maximum inherent unsaturation of approximately 6-10 kPa. An inherent tissue unsaturation of 6-11 kPa is therefore considered to be normal during aerobic metabolism at normal atmospheric pressure.

No exogenous influx of gases is included in the above considerations about inherent unsaturation. However, if one or more exogenous gases constantly diffuse into a tissue region at sufficiently high rates, the total gas tension may increase. If the exogenous gas (e.g. CO_2 from the caecal lumen) constantly diffuses at high rates into a tissue region (e.g. the caecal wall) with an otherwise normal perfusion, the tissue Pco_2 levels may increase substantially above the normal 6-12 kPa. The increase in tissue Pco_2 may occur despite constant vascular absorption and disposition of CO_2 in the caecal wall. This exogenous CO_2 may be sufficient to exceed or "fill" the normal 6-11 kPa oxygen window, pushing the total tissue gas tension above 1 ATA, as illustrated in Figure 4, column V. If this CO_2 influx occurs in areas with a low hydrostatic blood pressure, such as the capillary vessels, post-capillary venules, veins and surrounding interstitium, gas supersaturation will occur as the total gas tension exceed the total hydrostatic pressure (atmospheric pressure + blood pressure).

PATHOPHYSIOLOGICAL EFFECTS OF MICROBIAL CO_2 PRODUCTION IN THE COLON

While the normal physiological consequences of caecal gas supersaturation in mice is unknown, characteristic pathophysiological effects will occur if mice or rats are dosed with a single dose of pre-formed gas bubbles, such

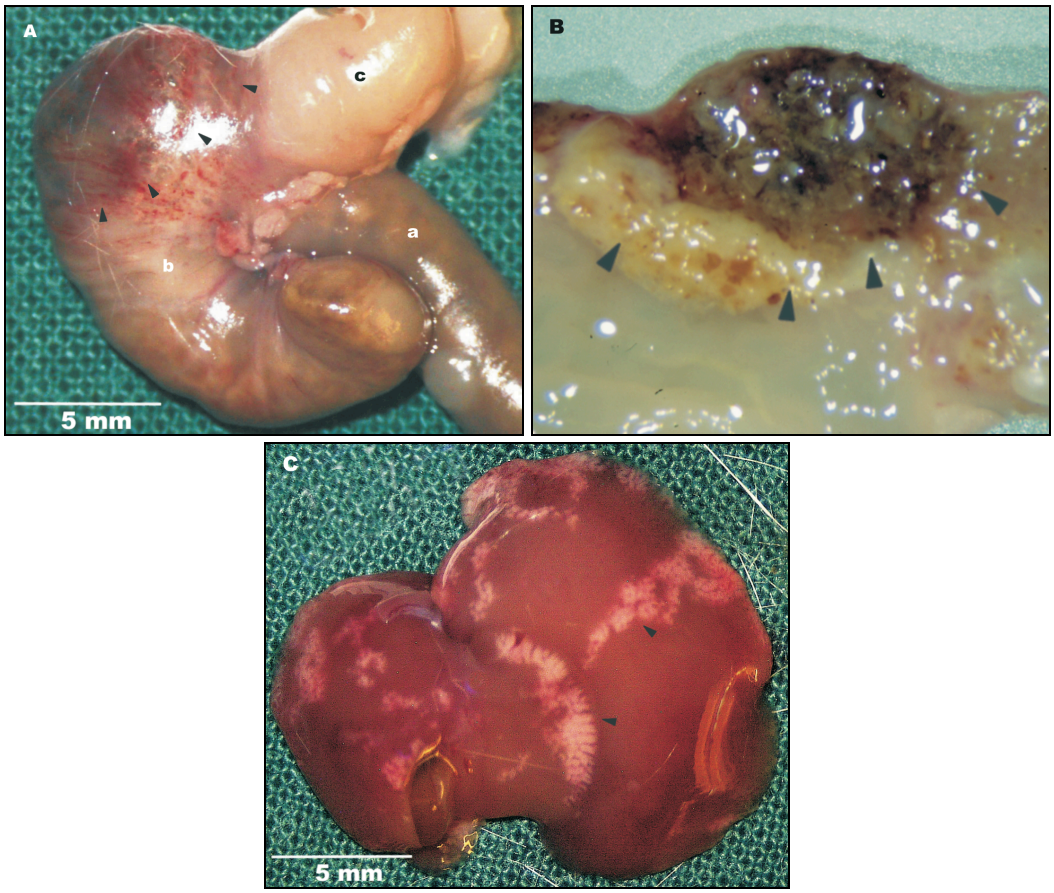

Figure 5: Typical macroscopic appearance of GCA-induced lesions in mice, 24 h after GCA dosing.
Ampulla coli caecum and caecocolonic area (A), caecal mucosa (B) and liver (C) of affected *HsdHan:NMRI* mice after a single i.v. injection of *Optison* (A and C) and *Sonazoid* (B) (7.5 µl microbubbles/kg). In A, ileum (a) and cranial colon (c) is normal, while arrowheads indicate oedema and haemorrhage in the antimesenteric wall of the ampulla coli caecum (b). In B, arrowheads indicate the corresponding mucosal ulceration of caecum. In C, arrowheads indicate pale, irregular necrotic areas, often affecting the edges and surface of the liver lobes. Figure was previously published in (*Dirven* et al., 2003).

as gas-carrier contrast agents (GCAs). Gas-carrier contrast agents, which are gas microbubbles that are small enough to pass the pulmonary circulation and sufficiently pressure stable to allow passage through the left ventricle after intravenous administration, are used for contrast ultrasound imaging of the vascular system.

Characteristic necrotic lesions are observed within 24 h in the caecum, colon and liver of mice after a single iv administration of GCAs such as Optison®, Levovist® and Sonazoid™ (*Dirven* et al., 2003). The caecal lesions, consisting of submucosal oedema, inflammation and necrosis, appeared from 15 min and multifocal liver necrosis from 2 h after administration of GCAs (Figures 5-6). Antimesenteric and segmental predilection of submucosal oedema and lymphatic

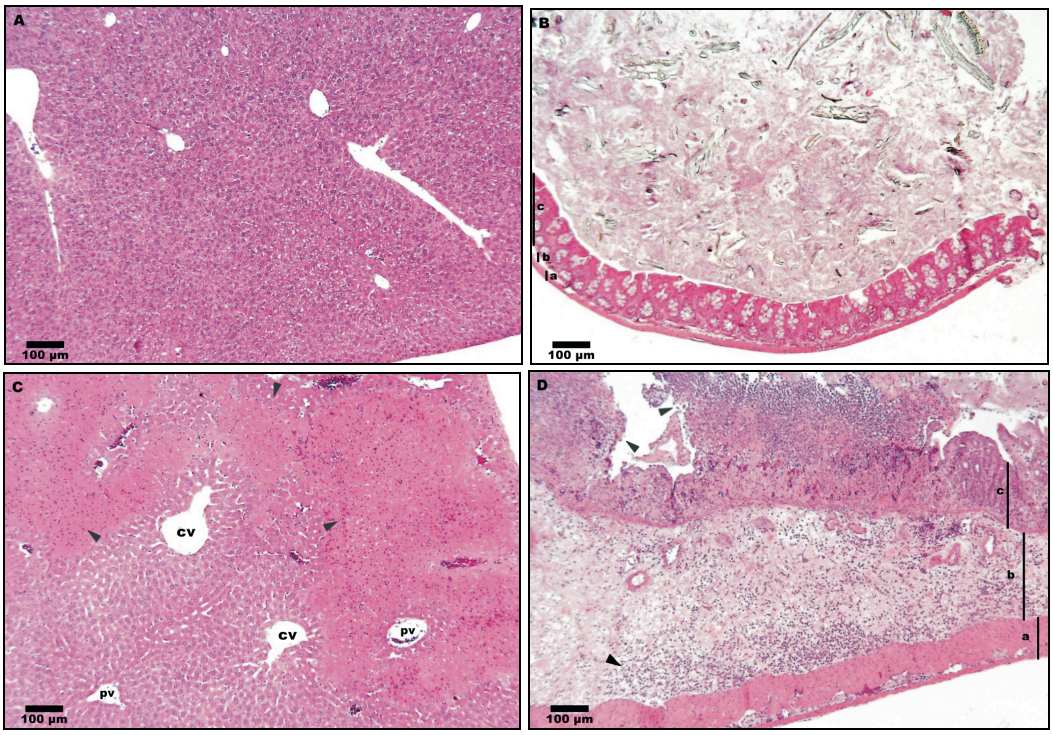

Figure 6: Histological appearance of liver and caecum in HsdHan:NMRI mice, 24 h after intravenous injection of Sonazoid or glucose.
Liver (A) and caecum (B) after injection of control substance (glucose). Liver (C) and caecum (D) after injection of *Sonazoid* (7.5 µl microbubbles/kg). Centrilobular coagulative necrosis indicated with arrowheads between central vein (cv) and portal vein (pv) in liver. Normal muscle layer (a) and arrowheads indicating oedema and inflammation of submucosa (b) and erosion and inflammation of mucosa (c) in caecum. This figure was previously published in *Dirven* et al., 2003.

vessels dilatation was characteristic, particularly at the earlier time points, indicating that venous occlusion was central to the pathology of caecum and colon (*Marcuson* et al., 1972; *Polk*, 1966; *Noonan* et al., 1968; *Khanna*, 1959). When studied by a modified fluorescein flowmetry (FF) method 5-101 min after GCA administration, characteristic intravascular gas bubbles in and hypofluorescence of the affected caecal wall were observed in mice (*Rasmussen* et al., 2003) (Figure 7-8). The appearance and location of the entrapped and embolic intravascular gas bubble observed by FF supported that the venous occlusion primarily occurred in capillary and postcapillary venules (*Rasmussen* et al., 1999a). The observation of intravascular gas bubbles flowing freely in intestinal veins towards the portal vein in mice, and the time course of the intestinal and liver lesion, indicate that the liver lesions are caused by embolic gas bubbles of caecal/colonic origin. This is supported by the observation that the liver lesions in mice never occurred without concurrent caecal/colonic lesions, i.e. never without a source of embolic gas bubbles. The multifocal liver abnormalities in mice, observed macroscopically as either hypofluorescence during FF or pale necrosis during 24 h pathology,

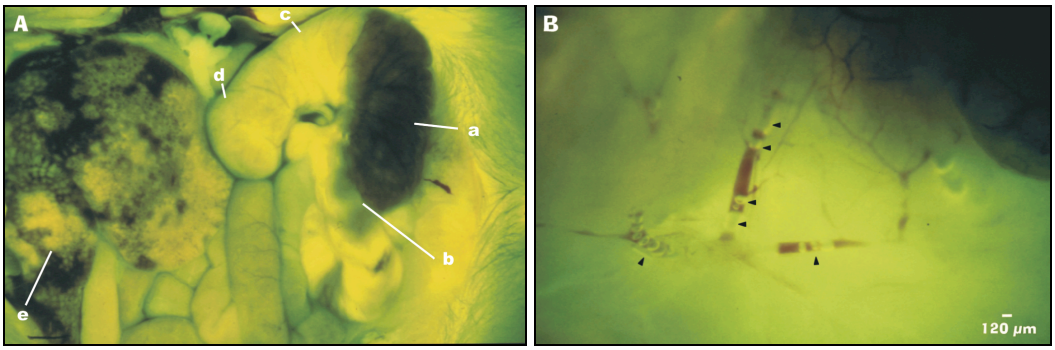

Figure 7: Fluorescein flowmetry in mice after intravenous administration of *Sonazoid*. Representative post mortem observation in blue light, *HsdHan:NMRI* mouse 58 min after *Sonazoid*. The animal was dosed with sodium fluoride 10 sec before it was killed. A, Abdomen, 6.5 x magnification, note non-fluorescence of antimesenteric basis caecum (a) and caecocolonic junction (b) and normal fluorescence of corpus (c) and apex caecum (d). Uniform fluorescence of all other intestines. Irregular sharply demarcated lobular fluorescence in liver (e). B, Border zone between fluorescent and non-fluorescent caecum wall, 40 x magnification. Note numerous intravascular gas bubbles in subserosal veins (arrowheads). Fluorescein flowmetry image of control animals, dosed with glucose, is illustrated in Figure 8. Figure was previously published in (*Rasmussen* et al., 2003). Video sequences, demonstrating intravascular gas bubbles and their relation to the fluorescence distribution, are available as supplementary material via www.sciencedirect.com.

had a characteristic peripheral distribution along the edges and surface of the liver lobes. This characteristic location of the liver lesions is consistent with that observed by ultrasound in patients with hepatic portal venous gas, in which hyperechoic signals from embolised gas bubbles are observed primarily in the periphery of the liver lobes. The peripheral and superficial location of liver gas emboli is explained by the centrifugal flow of the portal vein and is distinctly different from the deeper location and characteristic US images of gas bubbles in the centripetally flowing biliary system (*Peloponissios* et al., 2003; *Oktar* et al., 2006; *Sebastia* et al., 2000; *Liebman* et al., 1978). Intravital microscopy in the rat cremaster muscle after retrograde arterial administration into the femoral indicates that the intravascular behaviour and rheology of Sonazoid is not different from that of the white blood cells (*Braide* et al., 2006). The vast majority of the Sonazoid microbubbles passed the field of view as free flowing microbubbles and the discrete and temporary plugging of the capillary circulation, which comprised 2% of the microbubbles and lasted for 3-18 seconds, was in all cases comparable to the naturally occurring leukocyte plugging (*Bagge* and *Brånemark*, 1977).

Histopathologically, the centrilobular hepatocyte necrosis observed in mice from 2 hours after administration of GCAs is consistent with the dual blood supply via the portal vein and the hepatic artery (*Butler* and *Morris*, 1995), and reperfusion injury. The centrilobular distribution is consistent with the liver necrosis of reperfusion observed in dogs 24-48 hours after transient (3 hours) episodes of severe hypovolaemia and hypotension (*Levin* et al., 1996). The centrilobular region has the lowest oxygen tension in hepatic

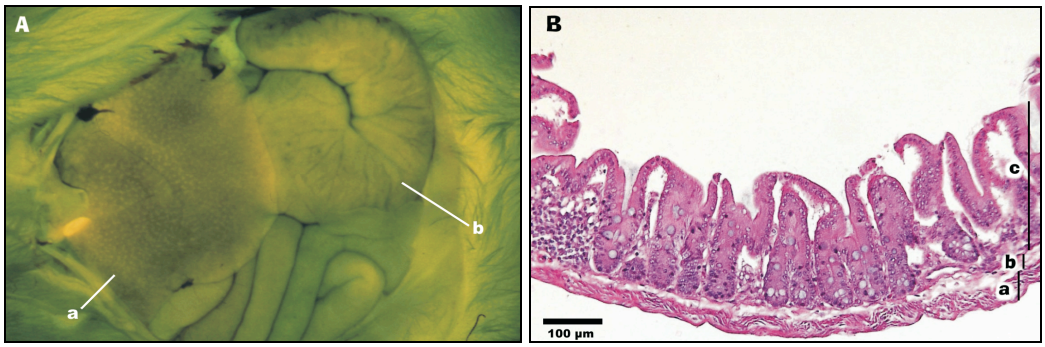

Figure 8: Fluorescein flowmetry in mice after intravenous administration of glucose.
A, Representative post mortem observation of abdomen in blue light, 6.5x magnification, *HsdHan:NMRI* mouse 60 minutes after administration of control substance (glucose). Sodium fluoride was dosed 10 sec before the animal was killed. Note the uniform lobular fluorescence pattern in liver (a) and uniform fluorescence of all intestines, including caecum (b). B, Representative histology of the caecum after administration of control substance (glucose), 100 x magnification, H&E. Note uniformity of the muscle layer (a), submucosa (b) and mucosa (c). Narrow space between submucosa and muscle layer is due to histological processing. Figure was previously published in (*Rasmussen* et al., 2003).

lobules and this is assumed to be the reason why this region is most severely affected by reperfusion injury following gas emboli entering the liver via the portal vein.

That presence of gas supersaturation in the caecal wall of normal mice and the causal relationship of the intestinal lesions with the disposition of the microbial CO_2 are also indicated by the GCA reaction in mice with reduced microflora activity. No intestinal or hepatic lesions were observed if the mice were maintained on *Diet 4012.01* for a minimum of 2 days before administration of *Sonazoid* and no lesions were observed in GF mice after administration of *Sonazoid*.

The absence of caecal lesions 24 h after single or repeated dosing of *Sonazoid* for up to 2 weeks in guinea pigs, rabbits and dogs, and the similarity of the macroscopic and microscopic caecal lesions in mice and rats divided the species into two distinct groups. Mice and rats are obviously more sensitive and although the liver is unaffected and no abnormalities other than post mortem presence of intravascular bubbles were observed by FF in the caecum of rats, the mechanism of action leading to identical intestinal pathology at 24 h obviously shared a common mechanism in these two species. By nature and location of the scattered foci of minimal-mild submucosal inflammatory infiltration in the caecum and colon of dogs, observed after 28 days of repeated dosing of 30-1000 the clinical dose of *Sonazoid*, dogs are in principle affected by with intestinal lesions after GCA administration. However, the lesions in dogs are fundamentally different in severity, does not include liver lesions and occur only after prolonged and repeated administration of relatively high dose levels, obviously beyond clinical relevance.

What can explain the sensitivity of the mouse and rat and is this explanation relevant for the observations in dogs? The FF results clearly indicated that the GCA gas bubbles grow *in situ* in the caecum and colon wall of the

mouse and this was consistent with the observed vascular obstruction and pathology. As the volume of enlarged and entrapped gas bubbles observed in a part of the caecal wall equalled or exceeded the volume of gas contained within the administered dose of *Sonazoid*, local coalescence and/or local entrapment could not in itself explain the pathology. The most likely source of exogenous gas responsible for bubble growth is hence CO_2 of bacterial origin. The nature of the initiating insults must therefore be associated with the diffusion of CO_2 beyond the intestinal mucosa and through the intestinal wall.

WHAT IS EXPECTED TO OCCUR IN HUMANS?

Based on our experience with *Sonazoid*, *Optison* and *Levovist* and a number of internal and external GCA candidates (proprietary information), and the publicly available data on *Sonovue* and *Echogen*, the large intestinal and hepatic lesions are considered to be a generic trait to GCAs in the appropriately sensitive species and strain. This generic trait is observed despite quite different gas-liquid interface membranes, gas content and nature of the microbubble formation (pre-formed or de novo formation upon reconstitution). How relevant is then this generic trait of GCAs to human health?

In our measurements of large intestinal Pco_2, 50-70 kPa were measured in mice, rats and dogs. In the dog (the species least sensitive to caecal/colonic lesions) 28 days repeated dosing was needed to create scattered foci of minimal-mild granulocytic foci, despite luminal Pco_2 levels of 70 kPa. If we assume that the hydrostatic pressure in the expandable intestine remains equal to ambient pressure, these Pco_2 values represent 50-70 % of the dissolved gases in the caecum and cranial colon. When the gas solubilities are also considered, gas supersaturation and intravascular growth of *Sonazoid* microbubbles in the large intestinal wall due to bacterial production of other gases than CO_2, such as H_2S, H_2 and/or CH_4, is an unlikely event in human subjects. In the event that gas supersaturation does occur in the inner-most layers of the cranial colon in some individuals, the clinical experience with *Levovist* in more than 100,000 patients (*Schlief* et al., 1999) indicate that this is without clinically discernible consequences.

The heterogeneous delayed liver enhancement during US imaging, observed in 6 out of approximately 1500 patients dosed with different GCAs were considered a normal variant, independent of liver disease (*Okada* et al., 2002). The anatomical location and pattern was speculated to be compatible with GCA growth or fusion in the blood vessels upstream from the portal vein. It is noted that the two GCAs (*Levovist* and *Echogen*) administered to the 6 affected patients are fundamentally very different formulations, both when compared to other GCAs and each other. Both agents are formed *de novo* immediately before administration and the degree of gas bubble membrane stabilisation is uncertain for both agents. Fusion of *de novo* forming gas microbubbles may hence be more likely than with other GCA formulations, which are all pre-formed and ready to use formulations. *Levovist* gas microbubbles are presumed to be stabilised by palmitic acid and contains relatively rapidly diffusing room air. *Echogen* contains the relatively slowly diffusing dodecafluoropentane gas and

membrane stabilisation, if any, is unknown.

Irrespective to these formulation differences, intravascular GCA growth in humans should also be considered as a possible explanation to these normal variant imaging results. When compared to the theories about pre-existing gas bubbles, intravascular GCAs represent ideal gas nuclei due to their relatively large size and the low surface tension caused by their gas-liquid interface. Lower levels of gas supersaturation will hence be needed to cause GCA growth, particularly if CO_2 constitutes a larger than normal proportion of the gas composition. However, the 0.4 % incidence of heterogeneous delayed liver enhancement (*Okada* et al., 2002) and the otherwise uneventful clinical experience after GCA administration (*Schlief* et al., 1999; *Okada* et al., 2002) make these rare observations fundamentally different from the large intestinal and hepatic lesions observed in mice and rats.

Another way of judging the human relevance is by comparing the intestinal anatomy of humans to that of the species evaluated here. Metabolically active bacteria are actively dividing bacteria and the actively dividing bacteria are often associated with the mucosal mucous layer in the anterior part of the large intestine of mice (*Poulsen* et al., 1995). The mucosal area, A, of the anterior large intestine does therefore affects the total *active* bacterial count in this intestinal segment. The volume, V, of an intestinal segment determines the volume of dietary substrate available to these active bacteria on the mucosa. As the area/volume ratio is inversely related to the size of an intestinal segment, the active bacteria/dietary substrate ratio will be higher in a small caecum (e.g. mice) versus a large caecum (e.g. dogs and humans). It is also important to realize that the mucosal epithelium is one cell layer thick in all species, whilst the submucosal and muscular tunic thickness increase with increasing thickness of the intestinal wall, and the size of the species and its intestinal tract volume. Based on these anatomical differences, the observed susceptibility to large intestinal and hepatic lesions of smaller species and resistance in larger species is consistent. Decreased microflora numbers and/or metabolism as seen by dietary and antimicrobial intervention will hence reduce/eliminate the possibility of these lesions, but it is the nature of the intestinal wall which determines if and how severe the lesions will occur in a given species, even if microflora numbers and/or metabolism are increased.

A WIDER PERSPECTIVE OF LOCAL Pco_2 LEVELS AND ITS IMPORTANCE FOR *IN VIVO* BUBBLE GROWTH

The nature and properties of inert gases in breathing mixtures used for diving are of primary concern in decompression medicine. While the inert gases are indeed the gases of highest concentration and partial pressure in both tissues and the relatively large intravascular gas bubbles associated with clinical symptoms of decompression sickness, the importance of other and more rapidly diffusing gases during *initial* growth of micronuclei and microbubbles is largely ignored in hyperbaric decompression medicine. The solubility of CO_2 is exceptional among all gases present in the body, being some 20-50 times more soluble than O_2 and N_2. While systemic Pco_2 is nor-

mally maintained within narrow limits, even these modest partial pressures reflect relatively high concentrations of CO_2 and the gas *concentration* difference between CO_2 and the inert gases is therefore lower than the corresponding *partial pressure* difference. It has been demonstrated during gas supersaturation that the gases surrounding the micronucleus will leave the liquid phase and enter the gas micronucleus according to the concentration, not the partial pressure, difference across the gas-liquid interface (*Hemmingsen*, 1970). Carbon dioxide will hence be relatively more important in this process than indicated by the Pco_2. The rate of gas transport in the tissues, towards the gas bubble, and across the gas-liquid interface is dependent upon the diffusion coefficient for each of the gases in question. As demonstrated earlier (Table 2), CO_2 is in a class of its own with respect to solubility (L) and diffusion (K). At comparable conditions, carbon dioxide will enter gas bubbles 20-50 times faster than that of other biologically relevant gases, both during states of gas supersaturation and normal levels of inherent unsaturation (*Van Liew* and *Burkard*, 1995). Comparable dynamic or static conditions are important requirements for this comparison, as this will affect the microbubble microenvironment and hence the gas diffusion and bubble growth kinetics.

Gas bubble growth is dependent upon the initial bubble size and will occur earlier with larger bubbles (*Van Liew* and *Raychaudhuri*, 1997). The critical bubble diameter, above which the bubble grows irreversibly, depends on a number of factors, such as surface tension, hydrostatic pressure and gas concentration difference across the bubble membrane. Decreased surface tension and blood pressure, and increased gas concentration gradient will all promote a decrease in critical diameter and hence increased chance of bubble growth at a given level of gas supersaturation. It follows from this that a sufficiently high degree of gas concentration gradient will cause bubble growth, irrespective of the bubble size. As CO_2 diffuse more rapidly than other gases, the entry of CO_2 into the gas bubble will promote early bubble growth by increased bubble diameter and decreasing the concentrations of other gases in the bubble. Enlargement beyond the critical bubble diameter and irreversible bubble growth will therefore occur at lower degrees of gas supersaturation when Pco_2 is increased and constitutes a larger than normal proportion of the gas mixture external to the gas nucleus/bubble.

Although slower, the inert gases will also enter the gas bubble and the bubble concentration of both CO_2 and inert gases will eventually attain those of the surrounding tissue. Therefore, even at normal 5-6 kPa Pco_2 in the systemic blood, the *initial* concentration of CO_2 in an intravascular gas bubble occurring after decompression may be considerably higher than Pco_2 in systemic blood should indicate, and yet be equivalent to or lower than the systemic blood Pco_2 shortly after the bubble has attained its final "stable" size.

Unfortunately, obtaining and analyzing gas bubbles immediately after formation in the tissue of origin and Pco_2 values in the capillary plasma compartment is practically impossible. Intravascular gas bubbles are most often obtained in larger conductive blood vessels and at the earliest minutes after experimental decompression. The gas composition of these "mature" and established gas bubbles therefore reflect the systemic blood gas tensions in the larger blood vessels, rather than the initial gas concentrations in the plasma compartment of the capillary and post-

capillary circulation. The CO_2 concentration in the matured bubble will hence be largely equivalent to that of the systemic blood and extrapolation is needed to estimate the CO_2 concentration immediately after formation in the microcirculation. Extrapolation of bubble gas concentrations to the time of decompression start was included in a study of bubble formation in rabbits after severe and rapid hyperbaric decompression (*Ishiyama*, 1983). Gas bubbles were recovered from the vena cava at 5-15 min after decompression and analyzed so as to avoid contamination/dilution of the sampled bubbles with other gases. Gas bubble CO_2 concentrations and venous blood Pco_2 were 6-8 % and approximately 7-9 kPa, respectively, at t = 5-15 min after severe and rapid decompression, but 23% and approximately 6 kPa, respectively, at t = 0. Whilst these are extrapolated data from a rather limited study that should be judged cautiously, the importance and transient nature of CO_2 during early bubble is illustrated by this study.

The very early effects of mixed gases, including CO_2, on bubble growth after decompression may also be mathematically modelled at microbubble diameters relevant for GCAs and the microcirculation (few μm) (*Kislyakov* and *Kopyltsov*, 1988). Hyperbaric decompression to 1/10 or 1/5 of the compressed values (corresponding roughly to 10 and 5 ATA supersaturation, respectively) and standard partial pressures of blood gases (e.g. 6.7 kPa CO_2) were used. The modelling demonstrated bubble growth from 1 to 4.6 μm radius in approximately 0.12 sec after 1/10 decompression and that CO_2 enters the microbubble within 0.05 sec under these conditions. The rapid growth was modelled despite that the Pco_2 immediately after decompression were 90% of the standard 6.7 kPa or lower, and hence not significantly higher than normal. Although based on mathematical modelling and instantaneous decompression, the applied gas mixture, bubble size and bubble density is more relevant than seen in most studies on bubble growth after decompression. The importance of CO_2 during initial bubble growth and the relatively higher importance of CO_2 at lower degrees of decompression/gas supersaturation are demonstrated.

The facilitating effects of CO_2 on bubble growth may also be associated with increased systemic and local levels of Pco_2, as demonstrated very convincingly by *Mano* and *D'Arrigo* (1978). In a study of the DCS incidence occurring during compressed-air work in a caisson structure down to 56 m depth and 18°C on reclaimed sea bed, the rate of DCS was observed to be related to the concentration of CO_2 inspired during the hyperbaric decompression. The workers were experienced personnel, selected by their previous experience with caisson work and prior history of DCS. Little physical exercise was associated with the caisson work itself and the workers rested during decompression in the decompression lock, but going up a spiral stair from the caisson bottom to the decompression lock at the end of each shift was a physical strain that increased with depth and compression. Maximum compression was 3.9 ATA and signs of DCS stated to occur from 2.7 ATA. The compression lock temperature was 30°C. At compression below 3.2 ATA, the decompression lock was not ventilated and the CO_2 concentrations were therefore 1.8-2.3%. At compression above 3.2 ATA, the compression lock was ventilated and the CO_2 concentrations dropped to 0.3-0.8%. The normal atmospheric CO_2 concentration is 0.03%, which was also the CO_2 concentration in the

caisson structure. Despite the increasing compression, the modest reduction in inspired CO_2 concentration caused the DCS incidence to drop from 3.05% at 3-3.2 ATA to 0.96% at 3.2-3.4 ATA. The DCS incidence increased at increasing depths above 3.2 ATA, but the DCS incidence at 3.2-3.9 ATA was still below that observed at 3-3.2 ATA. The vast majority of symptoms occurred in the knees, which was thought to be associated with the hard physical exercise associated with climbing up to the decompression lock. As the workers rested in the decompression lock, local CO_2 production during climbing, rather than decreased hydrostatic pressure associated with muscle activity (tribonucleation), is considered to be the primary factor associated with bubble growth and the observed cases of DCS. Even at these modestly increased CO_2 concentrations of the breathing gas in the decompression lock, the results indicate that presumed reduced pulmonary elimination (systemic effect) and increased production (local effect in muscles around knee joint) of CO_2 facilitated increased tissue concentrations of CO_2 and caused early bubble growth during hyperbaric decompression. In addition, although the core body temperature was probably not decreased while in the caisson, the physical exercise of climbing the stairs and the markedly increased compression lock temperature may have increased local and/or systemic body temperature, causing the solubility of CO_2 (but not N_2) to decrease. This principle of temperature-dependent gas solubility is well known in deep sea saturation diving, where hypothermia and physical exercise contributes to an increased partial pressure of metabolic gases when normal body temperature is regained in the compression chamber.

Diving mammals are able to dive to extreme depths and lengths (*Kooyman* and *Ponganis*, 1998) and it is quite controversial if they are affected by DCS (*Jepson* et al., 2003; *Piantadosi* and *Thalmann*, 2004). The ability to undertake such extreme physical exercise is primarily associated with a higher volume of blood and blood cells, haemoglobin and myoglobin, such that 80-90 % of the O_2 stores are located in the blood and muscles and not in the lungs (*Kooyman* and *Ponganis*, 1998). Redistribution of blood perfusion from the visceral organs to the central nervous system and the heart has been observed in seals during prolonged restraint dives (*Kooyman* and *Ponganis*, 1998). This “diving reflex” is assumed to enable the extreme diving capabilities of some seals and to occur in other mammals during unrestrained diving, but experimental verification is lacking. Uptake of gases at increased pressure occur from start of diving and until the lung collapse and repeated diving to the depth of lung collapse or lower has been shown to cause gas supersaturation in unrestrained dolphins (*Ridgway* and *Howard*, 1979). Based on simulation in other whale species, the degree of gas supersaturation developing in diving mammals will hence dependent upon frequency and depth of diving, surface time between diving, rates of descent and ascent and depth of lung collapse (*Houser* et al., 2001). As bubble growth is known to be enhanced by rectified diffusion when exposed to acoustic energy of sufficient magnitude (*Crum* and *Hansen*, 1982), exposure of diving mammals (including humans) to high acoustic pressure may represent a risk (*Crum* and *Mao*, 1996; *Houser* et al., 2001). Mass stranding of whales has been associated with the use of military sonar (*Jepson* et al., 2003; *Fernández* et al., 2005) and the observation of high volumes of intravascular gas bubbles in the liver,

intestines, mesenteric lymph nodes and other visceral organs has been suggested by *Jepson* et al. (2003, 2005) and *Fernández* et al (2005) to indicate that a DCS-related aetiology may be involved. Contrary to this view, the location large gas bubble volumes of both chronic and acute nature in the liver and the diving reflex has been argued to contradict an aetiology of DCS by nitrogen gas supersaturation (*Piantadosi* and *Thalmann*, 2004).

The pathogenesis of these distinctive intestinal and hepatic lesions of intravascular gas bubbles remains unknown, but a theory of facilitation by metabolic gases can be discussed. If metabolic gases, and not only nitrogen, were considered in diving mammals, the distinctive intestinal and hepatic location of the intravascular gas bubbles could perhaps be associated with metabolic gases of intestinal microflora origin, in principle as outlined in the present thesis. The intestinal tract of whales is obviously very different from rodents, but fermentation of the dietary substrate and production of CO_2 by the intestinal microflora will be largely constant during diving and it is therefore interesting that large amounts of fresh gastric content were observed in stranded whales with the above lesions in liver and intestinal tract (*Fernández* et al., 2005). This metabolic gas production will accumulate locally in the intestinal tract tissue and in the systemic circulation until the animal again can exhale the gases upon surfacing. If the diving reflex applies to whales, the CO_2 will primarily accumulate in the splanchnic blood and tissues and relatively high Pco_2 levels will hence enter the portal circulation as splanchnic perfusion is normalised. If the diving mammal is exposed to high energy sonar shortly before or after normalization of the splanchnic perfusion, the increased Pco_2 in splanchnicus and/or systemically may lower the threshold for bubble growth(*Van Liew* and *Raychaudhuri*, 1997; *Mano* and *D'Arrigo*, 1978) and intravascular bubble growth may be triggered in the portal tract and liver. If such sonar triggering of portal bubble growth in whales occurs repeatedly, a mixed chronic and acute pattern of hepatic cavitation by gas emboli, as observed by (*Jepson* et al., 2003, 2005; *Decker*, 1990), may eventually occur and may contribute to the death and/or stranding of the animal.

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GASEOUS MEDIATORS IN THE ENTERIC NERVOUS SYSTEM

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SUMMARY

The enteric nervous system is an independent nervous system that controls vital gut functions including motility and secretion. Enteric neurons use a variety of neurotransmitters to modulate neural as well as muscle and epithelial activity. Among them are three gaseous mediators which are nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S). All three have potent inhibitory effects on smooth muscle. While NO and CO are released from terminals of enteric neurons to directly affect muscle or epithelial cells, H_2S exerts its effect via extrinsic afferent neurons. At least in the guinea pig and human gut H_2S appears to activate transient receptor potentials vanilloid receptor 1 (TRPV1) expressing extrinsic afferent neurones. This mode of action is important for the prosecretory effect of H_2S.

INTRODUCTION

The enteric nervous system (ENS) is considered to be an independent nervous system that controls and coordinates motility, blood flow and secretion. The ENS is thereby crucial to maintain vital functions such as transit of luminal contents, secretion and absorption of ions water and nutrients, microcirculation and barrier functions. One of the hallmarks of the ENS is its similarity to the brain hence its alias "little brain of the gut". The ability to function as a little brain implies that the ENS, like the CNS, uses a variety of neurotransmitters to coordinate nervous activity as well as activation and inhibition of the various non-neuronal effector cells in the gut (*Schemann* and *Neunlist*, 2004). Beside the classical neurotransmitters, the ENS uses the three known gaseous mediators nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S) to modulate neural activity as well as activity of muscle and epithelium. The three gasomediators share some common features. For example the concentration of the three gasomediators has to be tightly controlled, as all of them are potentially toxic and lethal. In addition, NO, CO and H_2S are lipophilic and thereby easily cross the cell membrane. They activate or inhibit signalling cascades inside the cell, as their action does not involve "classical" neurotransmitter receptors. Last but not least all three of them are synthesised by enzymes close to their site of release and action. While their final action may be similar for some effector systems, NO, CO and H_2S have specific mode of actions and their relevant contribution varies according to the physiological and pathophysiological state of the organ or body (see Table 1).

Table 1: Summary of synthesis, functions and putative mode of actions of nitric oxide (NO, carbon monoxide (CO) and hydrogen sulphide (H_2S)

Gasomediator	Synthesis	Functions	Mode of action
NO	By neuronal NOS [1]: mostly in myenteric and less in submucous neurones	Decrease nerve activity Muscle realaxation Weak secretagogue	Presynaptic inhibition cGMP↑ and muscle hyperpolarisation involves nerves and prostanoids
CO	By HO-2 [2]: mostly in myenteric and less in submucous neurones	Neuronal effect ? Muscle relaxation Effect on secretion ?	cGMP↑ via NO and muscle hyperpolarisation
H_2S	By CSE [3] and CBS [4]: mostly in submucous and less in myenteric neurones	No direct effect on enteric neurones Relaxation of smooth muscle Strong secretagogue	? TRP [5]channel expressing extrinsic afferents

[1]nitric oxide synthase; [2]haem oxygenase-2; [3]cystathionine gamma-lyase; [4]cystathionine beta-synthase; [5]transient receptor potential.

NO AS A GASEOUS MEDIATOR IN THE GUT

In the ENS, nitric oxide is mainly synthesised by inhibitory muscle motor neurones of the myenteric plexus. Only a very few interneurones, some of which also synthesise acetylcholine, express nitric oxide synthase (NOS), the enzyme which produces NO. In some species including humans NO is also synthesised by enteric neurones of the submucous plexus. NO relaxes the smooth muscle in the stomach, small and large intestine of all species studied so far (*Salzman*, 1995; *Sanders*, 1996). Interstitial Cells of Cajal (ICC) are crucial in this relaxation as the nitric oxide released from enteric neurons uses the ICC network to spread the inhibitory signal to the smooth muscle. The inhibitory action of nitric oxide depends on increase in cGMP, opening of potassium channels and finally hyperpolarisation of smooth muscle. NO also has an inhibitory action on enteric neurones. At least in the guinea pig it consists of a pre-synaptic inhibition of transmitter release (*Tamura* et al., 1993). Interestingly, this affects only synapses involved in slow excitatory synaptic transmission but not those that mediate fast excitatory postsynaptic potentials. The physiological relevance of NO released from submucous neurons is not as clear as that described for the smooth muscle. NO does increase secretion probably involving neural actions and release of prostaglandins, respectively.

CO AS A GASEOUS MEDIATOR IN THE GUT

CO is generated by haem-oxygenase-2 (HO-2), which is constitutively expressed in many inhibitory neurones of the ENS (*Gibbon* and *Farrugia*, 2004). The membrane potential gradients along and across the muscle layers of

the gastrointestinal tract require the generation of CO by HO-2. The presence of CO is also necessary for normal inhibitory neurotransmission in circular smooth muscle and appears to permit nitric oxide-mediated inhibitory neurotransmission. Loss of HO-2 activity slows gut transit. It is striking that neurons expressing haem oxygenase by far outnumber NOS expressing neurons in the ENS, the functional meaning of which is unknown. There is not report on the effect of CO on the activity of enteric neurons, yet the data from neurochemical coding studies would suggest that CO is present in some enteric interneurones.

H_2S AS A GASEOUS MEDIATOR IN THE GUT

While the role of CO and in particular NO in gut functions is well established, the role of the newest member of the gaseous mediators, namely H_2S in the gut is just emerging. So far it seems clear that H_2S exerts widespread functions on neurons, muscle lamina propria and epithelial cells. Synthesis of H_2S mainly involves two enzymes, cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS). Strikingly, more than 90% of guinea pig and human submucous and myenteric neurons express both CSE and CBS. Myenteric ICC were CSE-immunoreactive. While the role of H_2S in ICC is not understood, its role in the ENS has been thoroughly studied. Thus the exogenous H_2S donor NaHS relaxes guinea-pig gut smooth muscle (*Teague* et al., 2002). This effect does not involve K_{ATP} channels which appear to mediate the H_2S induced relaxation of vascular smooth muscle.

Possible role of hydrogen sulphide as a pre-secretory modulator has been studied in human and guinea-pig gut (*Schicho* et al., 2006). NaHS increased chloride secretion in human and guinea-pig colon. This effect requires intact nerves, as it is not observed in the colonic epithelial cell line T84. The secretory response was reduced significantly by the nerve blocker tetrodotoxin, by capsaicin desensitization, and the TRPV1 antagonist capsazepine. The endogenous H_2S donor L-cysteine mimicked the effect of NaHS and this secretion was also diminished significantly by capsaicin desensitization, the CBS inhibitor amino-oxyacetic acid, and the CSE inhibitor propargylglycine. NaHS increased spike discharge in 23% of guinea pig and 36% of human submucous neurons, but had no effect on Ca^{++} mobilization in isolated cultured guinea-pig enteric neurons. This excitatory response was reduced significantly by capsaicin desensitization and capsazepine. The presence of H_2S producing enzymes in human and guinea-pig enteric neurons, the excitatory action in the ENS, and the prosecretory effects of NaHS strongly suggest H_2S as a gut-signalling molecule. Its action mainly involves TRPV1 expressing extrinsic afferent terminals, which in turn activate enteric secretomotorneurons.

H_2S production is also important under pathological conditions (*Attene-Ramos* et al., 2006). Thus persistent sulphate-reducing bacterial colonization and high luminal and faecal H_2S levels have been reported in ulcerative colitis and colorectal cancer. Whether this is primary or secondary to the disease needs to be investigated. The potent anti-inflammatory actions of H_2S in the upper gut open new clinical applications. Initials results suggest that addition of H_2S releasing moiety to

NSAID significantly improves side effects and thereby tolerability without affecting efficacy (*Wallace*, 2007).

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INTESTINAL GASES AND THE MUCOSAL BARRIER FUNCTION

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INTRODUCTION: THE GASTRO-INTESTINAL BARRIER

The gastro-intestinal (GI) barrier is a complex functional unit that separates the environment, i.e. the gastro-intestinal lumen, from the host. The barrier is by far the largest of the body comprising about 200 m^2, compared to the respiratory mucosa (100 m^2) and the skin (2 m^2). This barrier is also the most challenged one, because the respiratory mucosa is either equipped with squamous epithelium that protects much better compared to GI mucosa, or is sterile under normal conditions, e.g. in the lung. The skin mucosa consists of keratinized squamous epithelium that forms a hardly penetrable barrier. In contrast, the intestinal epithelium is the most permeable barrier because one of the major tasks of the GI tract is uptake of nutrients and fluids. Thus, the dilemma of the gut is that it has to fulfil two opposite tasks:

1. Uptake of nutrients and fluids that requires a high permeability, and
2. Protection against microbes, toxins and other harmful agents that requires a tight barrier.

Both task are indispensable for life and can be achieved simultaneously only if the GI barrier function is carefully balanced. Otherwise, either malabsorption (in case of loss of permeability and uptake functions) or inflammatory diseases (in case of loss of the barrier integrity) would be the consequences. Recent studies clearly showed that any impairment of the GI barrier is a crucial step for the development of acute diseases such as systemic inflammatory response syndrome (SIRS) and sepsis in the critically ill, and chronic diseases such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), allergic diseases, joint diseases etc. The mechanisms of selection of the particular type of disease that develops following GI barrier impairment are unclear, but likely dependent on the rapidiness and the extent of GI barrier disturbance.

After birth, there is a phase in which the GI barrier is not developed yet; therefore, newborns are characterized by a kind of physiological immaturity of the GI barrier. This might explain why the first months of life are obviously a particular sensitive time window for protection against diseases or the development of allergy and other immune diseases. It fits also with the known fact that pre-term babies are at particular risk for the development of *Candida* infections and Gram-negative sepsis. It s clear now that the GI tract, which is primary sterile, needs to be adequately colonized in order to develop a normal GI immune system and, finally, a normal GI barrier function. Any disturbance of this colonization will at last delay the development of the mucosal defence system, or even impair it for longer times. This observation made clear that the bacterial flora of the GI tract has a central role

for the GI barrier. Only the controlled interaction between bacterial flora and GI immune cells allows the development and maintenance of an intact GI barrier.

In recent years, great attempts have therefore been made on studying the GI barrier and the GI bacterial flora, and on modulating this functional unit by diet, life-style changes, by pre- and probiotics and by other means. However, the role of gases has not been addressed so far in this respect. This is surprising, considering the fact that many individuals suffering from symptoms related to an impaired GI function such as IBD, IBS etc. report bloating and flatulence. Therefore, the possible interactions between intestinal gases and the GI barrier will be discussed in the present review.

DO INTESTINAL GASES IMPAIR THE GI BARRIER?

Gases could affect the GI barrier by mechanical distension once high amounts of gases are generated that cause the feeling of distension, discomfort or even pain. Gases could also affect the GI barrier by their possible pharmacological effects, since cellular receptors have been identified for particular gases that mediate pharmacological effects.

Mechanical distension could lead to an impairment of the mechanical barrier (epithelial injury) resulting in bacterial translocation, enhanced secretion, leaky gut and malabsorption. In more advanced stages, impairment of the mucosal immune system and the enteric nervous system (ENS) may occur. The latter can lead to abdominal pain, enhanced secretion and motility, diarrhoea, and finally to depression and other psychological effects.

A number of potential targets of action of intestinal gases on the GI barrier are imaginable. They can be classified into three areas:

1. The secretory components of the GI barrier (impaired mucin, IgA, or chloride secretion, enhanced bacterial attachment to the epithelium, reduced production of antimicrobial agents such as bacteriocins or defensins),
2. The mechanical components of the GI barrier (effects on cell-cell contact, tight junctions, APC regions, effects on epithelial growth and apoptosis), and
3. The immunologic components of the GI barrier (modulation of the T cell response, activation of lymphocytes, mast cells or macrophages, support and priming of dendritic cells).

However, data confirming such effects of intestinal gases are lacking until now; therefore, any consequences remain speculative. The question is what do we know at all about intestinal gases? It is surprising to note that many patients that consult a gastroenterologist consider "gases" as their main problem.

KNOWLEDGE ABOUT INTESTINAL GASES IN THE PAST AND AT PRESENT

A particular interesting source of information on gases and bloating is a monograph dated 1831 and published in Germany by an unknown practitioner (*Anonymous*, 1831). He reports the intestinal gases known at this time which, apart from NO, have not changed until now:

- Wasserstoffgas: Hydrogen (H_2)
- Sauerstoffgas: Oxygen (O_2)
- Stickgas: Nitrogen (N_2)
- Kohlenwasserstoffgas: Methane (CH_4)
- Schwefelwasserstoffgas: Sulphur Hydrogen (H_2S)
- Kohlensaures Gas: Carbomonoxyde (CO) and Carbodioxide (CO_2)

Moreover, this practitioner reports on the thought on causes of bloating 175 years ago:

- Gases are mostly a result of bacterial digestion
- Gas production is dependent of what you eat (in particular, raw food is of risk)
- Gas production increases in individuals that lack exercise (increase of age and wealth)
- Gas production increases if the power of the digestive organ decreases (maldigestion, malabsorption)
- Normal versus abnormal is a question of gas composition rather than amount (e.g. N_2 is increased in patients)

Finally, he stated some additional important facts such as "Bloating remains substantially ignored, without proper clinical classification, known pathophysiology, and effective treatment. It is not even clear to what extent the complaints of individual patients correlate with objective evidence of abdominal distension. This uncertainly regarding the subjective or objective origin of the complaints further adds to confusion." How true this is until today!

What can be added since then? Until today, bloating as a medical problem remains substantially ignored, without proper clinical classification, known pathophysiology, and effective treatment. It is still not even clear to what extent the complaints of individual patients correlate with objective evidence of abdominal distension. This uncertainly regarding the subjective or objective origin of the complaints still exists and therefore, confusion could not be reduced.

EXPERIMENTAL DISTENSION OF THE GI TRACT BY GASES

The simple question whether increasing amounts of intestinal gases cause increasing intensities of GI symptoms cannot be answered easily. It is generally believed that patients with functional gut disorders manifest poor tolerance to intestinal gas loads but the mechanism of this dysfunction is unknown. *Harder* et al. (2003) therefore explored the relationship between amount of intestinal gas load versus its distribution on symptom production and gut motility. To do this, the group examined 14 healthy subjects with no GI symptoms, and infused them a gas mixture either into the jejunum or rectum for one hour during blocked rectal gas outflow. They visualized gas infusion by scintigraphic images of 133xenon labelled gas and measured abdominal perception, distension, and gut tone by duodenal and rectal barostats. By doing this, they found that a similar magnitude of gas retention (720 ml) produced significantly more abdominal symptoms with jejunal compared with rectal infusion whereas abdominal distension was similar. Jejunal gas loads were associated with proximal contraction and colonic loads with distal relaxation. According to these data, the volume of gas within the gut determines abdominal distension whereas symptom perception depends on intraluminal gas distribution and possibly also on the gut motor response to gas loads. This might, at least in part, explain the enormous variability

in symptoms the patients with bloating are suffering from.

In large animal studies, morphologic effects of experimental distension of small intestine have been studied (*Allen* et al., 1988). Intraluminal hydrostatic pressures of 0, 9, and 18 cm H_2O were induced in jejunal segments by installing Tyrode's solution for different time intervals (0, 1, 4 hours). Analysis of morphologic changes in the bowel wall was performed by light and electron microscopy (LM, EM). On decompression of the intestinal segments, progressive peristaltic contractions resumed in all segments. Experimental distension of equine small intestine resulted in oedema of the villi and submucosa, separation of the epithelial cells adjacent to the basement membrane in all distended segments, and thus impairment of the intestinal barrier, which may lead to bacterial translocation and inflammation.

Secondly, the intestinal microcirculation and the intramural vascular patterns of the small intestine were evaluated after intraluminal distension (25 cm of H_2O, 120 min) and decompression (60 min) in anesthetized horses (*Dabareiner* et al., 1993, 2001). The readouts were micro-angiography (by injection of a blue-coloured radiopaque medium), micro-corrosion (by injection of methyl-methacrylate for scanning EM), and vascular filling (LM). After intraluminal distension and decompression, the distended segments had short villi, which were separated by expanded crypts, and had mesothelial cell loss, neutrophil infiltration, and oedema in the sero-muscular layer. The number of perfused vessels was significantly decreased in the sero-muscular layer and, to a lesser extent, in the mucosal layer of the distended segments, compared with controls. After decompression, the morphologic lesions progressed in mucosal and serosal layers and the number of observed vessels increased in all intramural layers; however, vascular density did not return to the predistension state. Evaluation of the intestinal blood flow of the equine small intestine after intraluminal distension and decompression revealed a significant decrease in mesenteric blood flow to the distended intestine (from 21.4 to 13.4 ml/min per kg). Blood flow increased significantly during the decompression period (340% of baseline blood flow). An increase in microvascular permeability was documented by the determination of the osmotic reflection coefficient. Oxygen delivery and oxygen content decreased significantly during the distension period and increased during decompression. This process was accompanied by a significant increase in oedema and neutrophil infiltration after distension and decompression.

Third, the role of nitric oxide (NO) was studied in ischaemia-reperfusion experiments in feline small intestine to address the question of whether NO synthesis inhibition affects intestinal barrier function after ischaemia-reperfusion. *Kubes* (1993) showed that ischaemia-reperfusion-induced mucosal and microvascular permeability increases were dramatically augmented by NG-nitro-L-arginine methyl ester (L-NAME) infusion, and this effect was reversed by infusion of L-arginine (125 $nmol.ml^{-1}.min^{-1}$) suggesting that indeed NO plays a significant protective role for microvascular barrier function. Another approach to study effects of intraluminal distension of the small intestine is to prepare extracorporeal circuits from the jejunum of healthy horses. This allows to subject one segment to distension (intraluminal pressure, 25 cm H_2O) followed by decompression, and another segment without distension control segment) *ex vivo*. Using these means, *Nieto* et al.

(2002) showed that intestinal vascular resistance increases during intraluminal distension and returns to baseline values after decompression. Albumin clearance rate increased after distension, compared with baseline and control values, whereas the contractile response induced by cisapride, erythromycin, and metoclopramide decreased following distension.

Several conclusions can be drawn from these equine experiments. Intestinal pressure leads to oedema of the villi and submucosa, expansion of the crypts, disruption of the epithelial barrier and neutrophil infiltration into the seromuscular layer. Intestinal pressure changes vessel functions by a decrease of perfused vessels that persists after decompression, by oedema of the villi and submucosa, and by expansion of the crypts resulting in a decrease in mesenteric blood flow (followed by a compensatory increase during decompression), in oxygen delivery and tissue oxygen content. Intestinal pressure causes an increase in vascular resistance, in microvascular permeability, in albumin clearance rate, while the contractility response to prokinetics is decreased. The clinical impact of such findings cannot be judged definitively, because analogous human experiments are lacking and the experimental approaches in horse studies may be somewhat artificial. However, the likeliness of similar mechanisms in humans must be considered.

MECHANISM OF DISTENSION – HUMAN STUDIES

The number of human studies is limited. Therefore, only preliminary conclusion can be drawn. An intra-abdominal volume load, produced by colonic gas infusion, induces in healthy subjects an increment in tonic activity of the abdominal muscles that can be measured by electromyography (*Tremolaterra* et al., 2006) and this response is probably mediated via viscerosomatic reflexes (*Martinez* et al., 1999). This adaptation is impaired in patients complaining of bloating who fail to contract their abdominal muscles. Hence patients with bloating do have objective abdominal distension but it may not necessarily be due to a true increment in intra-abdominal volume, but to abdominal wall dystony with abdominal redistribution and protrusion of the anterior wall. Under normal conditions, the abdominal wall likely adapts to its content.

Does "pathological gas production" exist? Everybody produces intestinal gases. Gas production is a "physiological" consequence of digestion and bacterial fermentation, and by far not a pathological condition per se. However, if huge amounts of gases are produced, they might cause problems. The amount of intestinal gases depends on diet, composition of the colonic flora, small bowel bacterial overgrowth, small bowel malabsorption (e.g. lactose intolerance), and functional outlet obstruction (impaired anal evacuation of gases, faecal retention prolonging the fermentation process) (*Azpiroz*, 2005). Clinical evidence suggests that healthy subjects propulse and evacuate also large intraluminal gas loads without symptoms whereas IBS patients who do not necessarily produce more gas than healthy subjects might have an impaired evacuation or an impaired perception of distension. Hence, intestinal distension is not a cause of bowel dysfunction but rather a consequence of impaired adaptation.

GASES AND IRRITABLE BOWEL SYNDROME – IS THERE A UNIFYING FRAMEWORK?

There is clinical and experimental evidence that small intestinal bacterial overgrowth (SIBO) may explain bloating occurring in 92% of patients suffering from IBS. For example, it could be shown that IBS patients have an enhanced total hydrogen excretion after lactulose ingestion. The prevalence of abnormal lactulose breath test is 84% in IBS patients. There is a correlation between the pattern of bowel movement and the type of excreted gas. Most importantly, eradication of SIBO results in a 75% improvement of IBS symptoms (*Lin* et al., 2004).

Results from scintigraphic studies using gas labelled with radioactive xenon confirmed that indeed the small bowel is responsible for impaired gas transit (*Salvioli* et al. 2005; *Azpiroz*, 2005). The ileo-caecal region is an area with sphincteric function likely implicated in this dysfunction. Gas retention is due to impaired propulsion in more proximal parts of the small bowel. Interestingly, impaired gas clearance in IBS patients is related to abnormal gut reflexes: the prokinetic effect of gut distension is impaired and the inhibitory effect of intestinal lipids is upregulated, further arguing for an impaired adaptation in IBS patients suffering from bloating and pain, possibly triggered by bacterial overgrowth of the small intestine. The cause of such an overgrowth remains obscure. Possibly, immunological impairments might be responsible.

CONCLUSIONS

According to our current knowledge, four major origins/mechanisms of intestinal gases, bloating, and discomfort must be anticipated, which can cause impairment of the GI barrier:

1. Impaired adaptation (intestinal muscle/nerve failure leading to an functional outlet obstruction),
2. Abnormal composition of the colonic flora,
3. Small intestinal bacterial overgrowth
4. Diet (beans etc.) and/or small bowel malabsorption (e.g. lactose intolerance).

A number of possible effects of intestinal gas must be considered. These include bloating and distension that may lead to discomfort and pain (although the correlations are weak), impairment of the mechanical barrier including epithelial injury, bacterial translocation, enhanced secretion, leaky gut syndrome and malabsorption, impairment of the mucosal immune system, and impairment of the ENS leading to pain, diarrhoea, and psychological effects such as depression.

The ultimate question whether intestinal gases truly matter must remain open. Likely, gas is not necessarily the offending element, but rather other intraluminal components that could trigger the abnormal responses and thus be responsible for the abdominal symptoms that patients misinterpret and attribute to intestinal gas.

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