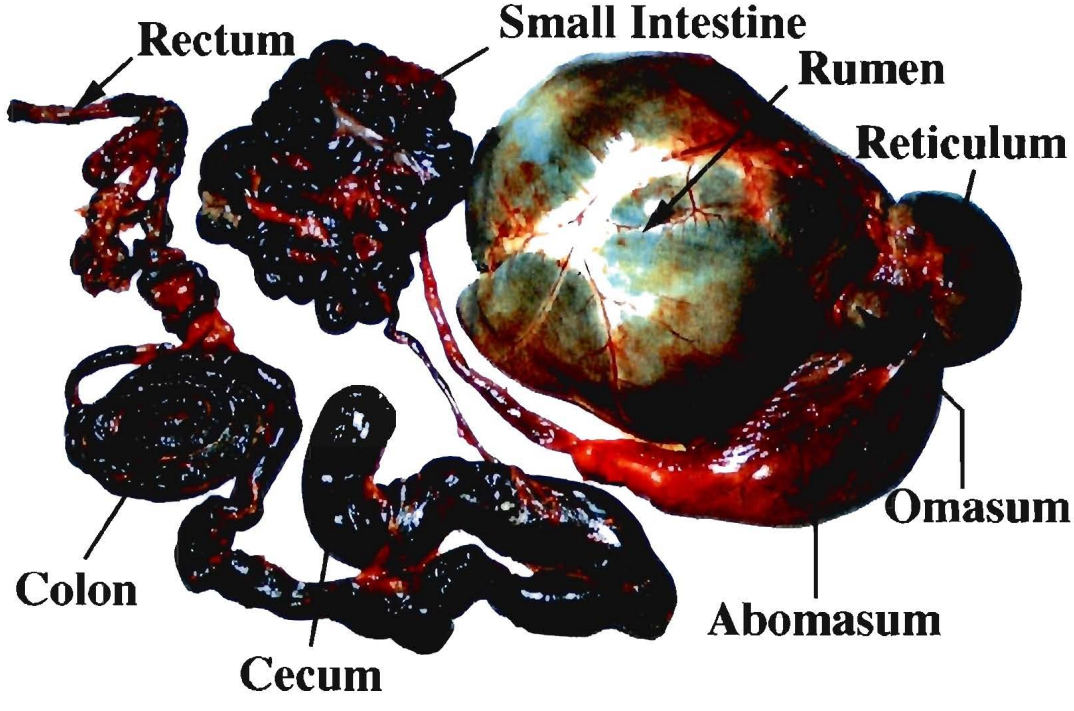
Rumen Microbiology
and Its Role In Ruminant Nutrition



A Textbook by James B. Russell

Rumen Microbiology

and Its Role in Ruminant Nutrition

Comments from recognized experts on ruminal fermentation:

"I enjoyed the opportunity to read the chapters you sent for your proposed book. In general, I thought it was concise, well written and should be of value to a wide range of scientists and veterinarians. " Burk Dehority, Professor of Animal Science, Ohio State University, Wooster

"I am sure that the advantages of a single authored book will be appreciated by many readers." Milton Allison, Professor of Microbiology, Iowa State University, Ames

"Overall, I thought it was a clear and concise presentation . . . . " Ron Teather, Research Microbiologist, Agriculture Canada, Lethbridge, Alberta

"This is going to be a very useful book for my ruminant nutrition class as I'm sure it will be for others." Jeff Firkins, Professor, Professor of Animal Science, Ohio State University, Columbus

"I think that your text is going to get wide spread use . . . . " Tim McAllister, Research Animal Scientist, Agriculture Canada, Lethbridge, Alberta

The picture on the cover and Fig 2-1 were reprinted with permission from Russell, J.B., and J.L. Rychlik 2001, Factors that alter rumen microbial ecology. Science vol. 292 pages 1119-1122, American Association for the Advancment of Science; Russell, J.B. 2000, Rumen Fermentation. Encyclopedia of Microbiology, vol 4, pages 185-194, Academic Press; Rural Research in CSIRO, June 1971, CSIRO of Australia.

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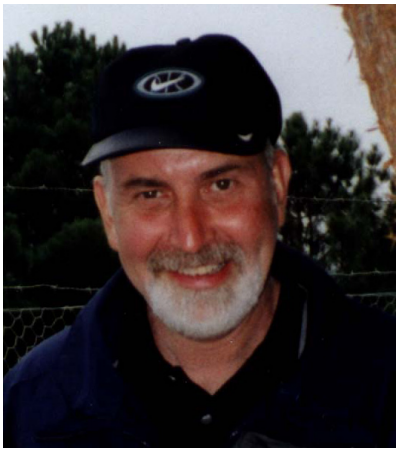
Forward

More than 35 years ago, Robert Hungate published *The Rumen and Its Microbes*. This book forecast the ability anaerobic bacteria to transfer reducing equivalents (a dominant theme in many microbial habitats), and it was read by a much wider audience than the title dictated. Hungate had little formal training in nutrition, animal science or veterinary medicine, but he made sure that the rumen and animal were well described. *The Rumen and Its Microbes* remained in press for approximately 30 years. In the 1990's, Dr. Hungate stated that he had never contemplated writing a second edition. The book was a "classic," and it did not need a follow up. His only reservation about book was that it needed "a better index."

The Rumen and Its Microbes and the following book have some similarities, but the goals are distinctly different. Hungate was able to summarize most of research on rumen microbiology, but since this time the field has expanded significantly. In recent years, a variety of edited books have been published, and many of them are referenced herein. Edited books are useful tools for people with a background, but they are generally too advanced for beginning students or people that simply want an overview. *Rumen Microbiology and Its Role in Ruminant Nutrition* is aimed at this latter audience.

This book had an arbitrary guideline of 100 pages, but the task of writing a short book is not an easy one. Former students will remember the John Steinbeck phrase (from *Travels With Charlie)*, 'I am sorry I wrote you such a long letter, I didn't have time to write short one.'* However, one can use method of Kurt Vonnegut. Vonnegut said, "Our power is patience. We have discovered that writing allows even a stupid person to seem halfway intelligent, if only that person will write the same thought over and over again, improving it just a little bit each time. It is a lot like inflating a blimp with a bicycle pump. Anybody can do it. All it takes is time."

*The earliest instance of this quotation (as given by Oxford Dictionary of Quotations, 1999) was Blaise Pascal (1623-62), in Letters Provinciales (1657): " I have made this [letter] longer than usual, only because I have not had the time to make it shorter)."



About the Author

The author was born in Livermore, California in 1951, but his father, Lincoln Russell, returned to upstate New York in 1954 and became a dairy farmer. Dr. Russell's first goal was to be a veterinarian, but this career choice did not persist. As a 'pre-vet' at Cornell, he took introductory microbiology and changed his major. In the summer of 1972, he read *The Rumen and Its Microbes* and eventually moved to the University of California at Davis where Professor Robert Hungate was residing. Dr. Hungate retired in 1973, but Dr. Russell was able to work for approximately one year in his lab. Dr. R.L. Baldwin served as Dr. Russell's Ph.D advisor, and it was during this time he was first exposed to computer models.

Jobs as a rumen microbiologist were not abundant, but the FDA's approval of *Rumensin* in 1976 created an increased demand for people with this training. Dr. Russell's first job was as an Assistant Professor in the Department of Animal Science at the University of Illinois, and his first teaching assignment was nutrition for first year veterinary students. Other course assignments included *Intermediary Metabolism of Animals* and *Microbiology of the GI Tract* with Drs. Hespell and Bryant.

When the USDA established a new position at Cornell University in 1981, Dr Russell returned to his *alma mater*. He taught *'Microbiology of the Rumen'* in the Department of Animal Science at Cornell from 1982 until 1990. In 1991, he moved to the Department of Microbiology where is a professor. He has performed research on many aspects of rumen microbiology, trained more than 20 graduate students and published more than 185 journal articles. He is a member of the American Academy of Microbiology, was a recipient of the American Feed Industry Award, has received numerous USDA awards and is regularly sought as a speaker. From 1991 to 1997 he was General Chairman of the Rumen Function Conference. He has served on the editorial boards of *Microbiology, Applied and Environmental Microbiology* and *Journal of Dairy Science*.

Dedication

This book is dedicated to my son, Aaron T. Russell, father, Lincoln A. Russell, dedicated laboratory technician, Mrs. Kathy Dusinberre, former teacher, Carole Rehkugler, and Mrs. Shirley Cramer, the friend that solved my computer problems.

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U.S. Dairy Forage Research Center

Chapter 1

Ruminants and Mankind

Mankind and domestic ruminants have shared a common history for more than 10,000 years, and this relationship has allowed man to maintain stable food supplies, harvest the photosynthetic potential of the grasslands, expand his geographical range, and develop stable cultures. Ancient man often used ruminants in his religious ceremonies, and ruminants were a common symbol of wealth, power and prestige. The Bible recognized the value of ruminants as livestock, and the Old Testament stated, "Whatsoever parteth the hoof, and is clovenfooted, and cheweth the cud among the beasts, that shall ye eat."

Aristotle described the four compartments of the ruminant stomach, but the role of microorganisms was not recognized until van Tappeiner (1884) added antiseptics to ruminal fluid and showed that fiber digestion was inhibited. Ruminant animals are commonly defined as four footed, even toed, cud chewing mammals that have a four compartment stomach, but this simple definition ignores the basic feature that differentiates ruminants from other animals, namely the ability of ruminants to exploit fermentation as a method of feed digestion. The evolutionary path of ruminant animals did not entail an inherent capacity for fiber (cellulose and hemicellulose) digestion, but ruminants were able to develop a symbiotic relationship with ruminal microorganisms. The animal provides a habitat for microbial growth, and the microbes, in turn, provide the animal with nutrients that would otherwise be unavailable.

Zoologists have described more than 180 species of ruminants, but man's efforts in ruminant domestication have largely been devoted to cattle (bovines), sheep (ovines), and goats (caprines). Camels have a slightly different digestive anatomy than "true ruminants", but their pre-gastric fermentation is remarkably similar. Some non-ruminant species (e.g., horses, zebras, rabbits, and rodents) also have a large fermentative capacity, but this fermentation occurs post-gastrically and the microbial protein can only be harvested by coprophagy.

Modern societies tend to emphasize their technologies and industries, but these developments are in large part a result of increased agricultural efficiency. Ruminant animals provide the bulk of our meat and milk. In 2001, yearly milk sales from American dairy cattle were $21 billion, and meat from beef cattle production was estimated at $36 billion.

Barnes, J. 1984. *The Complete Works of Aristotle*. Princeton Univ. Press, Princeton, NJ.

Clutton-Brock, J. 1999. *A Natural History of Domesticated Mammals*. Cambridge Univ. Press., Cambridge, UK.

Hungate, R.E. 1966. *The Rumen and its Microbes*. Academic Press, New York, NY.

Leviticus 11:3-4, *The New English Bible* (Oxford Univ. Press, United States, 1970), pp. 119.

Chapter 2

Ruminant Anatomy

The gastrointestinal tract of ruminant animals is an ideal habitat for gut micro-organisms. The ruminant stomach is composed of four compartments, the rumen, reticulum, omasum and abomasum.

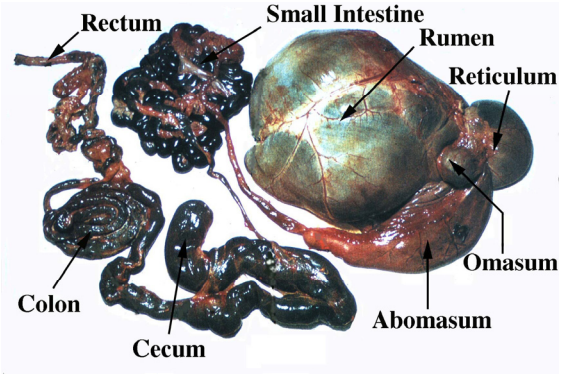

Fig. 2-1. The digestive tract of an adult sheep showing its various compartments.

These compartments function in a coordinated fashion to provide a site for fermentation, mix ingested feed with microorganisms, reduce the particle size of feed materials, eliminate fermentation gases, and regulate the flow of digesta to the lower gut. The ruminant stomach comprises more than 50% of the digestive tract volume and at least 1/10 of the mass of the animal. Food that travels down the esophagus is deposited in the rumen, the largest compartment and primary site of fermentation (Fig. 2-1). The rumen is lined with a filiform and foliate epithelium, and this extended surface area facilitates the absorption of fermentation acids (see Chapter 3, *Digesta Flow)*. The rumen does not secrete mucous or enzymes, but it is strongly buffered by salivary secretions.

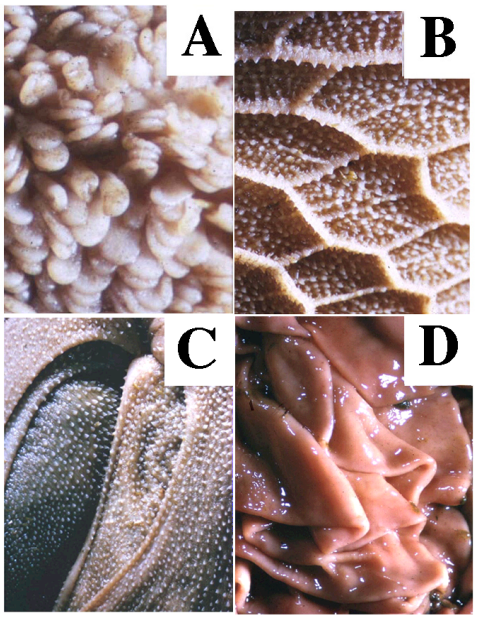

Fig. 2-2. Epithelial linings of the rumen (A), reticulum (B), omasum (C), and abomasum (D).

The rumen has pillars or baffles, and contractions force the digesta back and forth across these structures in mixing movements. Mixing movements inoculate the feed with microorganisms and transfer fermentation acids to the epithelial surface so they can be absorbed. Pressure in the rumen triggers the extension of the cranial pillar, and this structure holds digesta away from the esophagus so fermentation gases can be eliminated by a process known as eructation. Rumen movements are coordinated by the activity of the vagus nerve (the 10th cranial nerve). The vagus nerve is stimulated by the tactile stimulation of feed in the pharynx and rumen. Blood is supplied to the rumen wall by the celiac artery that runs from the aorta. Blood flow is stimulated by feeding and the absorption of volatile fatty acids.

Table 2-1. The digestive compartments of a mature sheep and cow (values are expressed as liters).

	Sheep	**Cow**
Rumen	23	84
Reticulum	2	16
Omasum	1	8
Abomasum	3	27
Small Intestine	9	66
Cecum	1	10
Large Intestine	5	28
Total	44	239

The reticulum is a small but distinct out-pouch of the rumen, and its epithelial surface has a honeycomb or reticular appearance. Small particles are transferred from the rumen and reticulum to the omasum, but large and potentially digestible feed particles are forced back up the esophagus to the mouth for further particle size reduction (rumination). In this regard, the reticulum plays a key role in both rumination and digesta flow to the lower gut.

The omasal epithelium is arranged as a series of folds (laminae) that act as a filtering device. The muscular action of the omasum creates a vacuum that sucks digesta through the reticulo-omasal orifice. Small particles and bacteria pass through the orifice, but large feed particles are trapped in the laminae of the omasum. Closure of the omasal-abomasal orifice and contraction of the omasum flushes the large feed particles back into the rumen so they can be fermented and ruminated. The omasum does not have an absolute particle size cut-off, but the average particle size of materials passing through the omasum is

less than 1 mm. The omasum absorbs water, volatile fatty acids (VFA) and buffer salts, but it does not secrete mucous or enzymes.

Fig. 2-3. Fistulated cow consuming hay (Courtesy H.C. Mantovani).

The abomasum is a gastric stomach, but its pH is not as low as the gastric stomach of non-ruminant species. The epithelium of the abomasum is covered by a mucous that protects it from pepsin and hydrochloric acid. Undigested feed and microbes entering the abomasum undergo the first steps of protein digestion and are then transferred to the small intestine for more extensive breakdown via intestinal proteinases, amylases, lipases, etc.

Fig. 2-4. The esophageal (reticular) groove of a new-born calf.

The ruminant stomachs are arranged so that the rumino-esophageal, rumino-omasal and omasal-abomasal orifices are in close proximity, and in neonate ruminants, milk can be shuttled directly from the esophagus to the abomasum via a structure known as the esophageal (reticular) groove. The esophageal groove is a muscular fold that extends to form a tube when the animal is suckling. If the calf consumes solid food, the groove is not formed, and the feed is deposited in the rumen. Once the ruminant is weaned, the reflex is lost, and food and liquids are always deposited in the rumen. However, the response can be retained by adult animals that are allowed to suckle.

Habel, R. E. 1965. Anatomical and histalogical nomenclature of the ruminant stomach, p. 15-23. *In* R. W. Dougherty (ed.), Physiology of Digestion in the Ruminant. Butterworths, Inc., Washington, DC.

Langer, P. 1984. Anatomical and nutritional adaptations in wild herbivores., p. 140-195. *In* F.M.C. Gilchrist, and R.I. Mackie (eds), Herbivore Nutrition in the Subtropics and Tropics. Science Press, Johannesburg, South Africa.

Chapter 3

Digesta Flow

In nature, ruminants consume feed rapidly and re-chew (ruminate) the cud later. Long feed particles that cannot pass from the rumen through the rumino-omasal orifice are forced back up the esophagus to the mouth and ruminated. Grazing ruminants ruminate for as long as 10 hours each day. Because rumination increases the surface area of feed particles, fermentation rate and the extent of digestion increase.

Some feed materials are so tightly bound to lignin and other indigestible materials that they can never be degraded by ruminal microorganisms, but most carbohydrates and proteins can be digested if they remain in the rumen for a sufficient period of time. The disappearance of potentially digestible feed materials is a dynamic process that is controlled by two competing rates (fermentation, k_f, versus passage, k_p). Both rates can be described by first order constants that have the units of h^{-1} (per h).

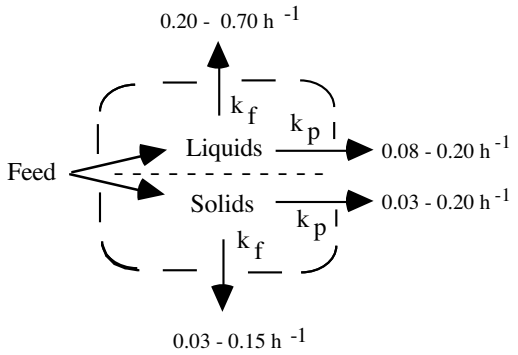

Fig. 3-1. The passage rates (k_p) of feed (solid versus liquid) from the rumen, and the fermentation rates (k_f) of material of each fraction.

If the digestion of potentially digestible feed is calculated from the simple relationship, mass times $K_f \div (K_f + K_p)$, one can see that the impact of K_p is most important when K_f is low and vice versa. Fermentation rate is an inherent property of each dietary component, but it can be influenced by rumination and feed processing. Passage rate is determined by the type of feed that the animal consumes (forage versus concentrate, buoyancy, digestion kinetics, etc.), processing (chopping, grinding, etc.) and intake.

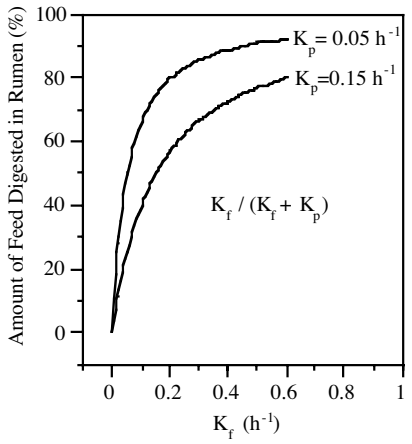

Fig. 3-2. The impact of fermentation rate (K_f) on the disappearance of digestible feed from the rumen at two different passage rates (K_p).

Passage rate calculations are complicated by the fact that the rumen has a least two major dilutions, the liquid/small particle pool and the large particle pool. Liquid passage rates can be estimated from the dilution of markers (e.g, Co-EDTA) that cannot be fermented or absorbed directly from the rumen. A dose of marker is added to the rumen, the contents are mixed thoroughly, and samples are withdrawn over time.

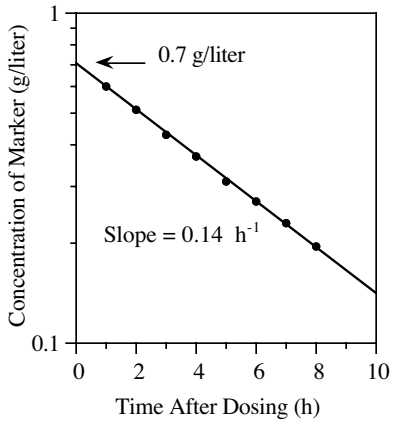

Fig. 3-3. The dilution of a soluble marker from the rumen and the use of a log transformation to calculate the ruminal fluid dilution rate.

By plotting the decline in marker concentration as a logarithmic function, it is possible to calculate the passage rate of the marker:

$$(ln\ M_0 - ln\ M)/time = K_p$$

where M_0 is the concentration at time zero and M is the concentration at some later time.

Solid dilution rates are inherently more difficult to estimate, but nutritionists have used lignin and rare earth elements (e.g., ytterbium) as markers. It should be noted that the binding of earth elements to fiber is not an irreversible process. Rare earth elements have a higher affinity for phosphates than fiber carbohydrate, and they can be displaced by the low pH of the abomasum.

Faichney, G. J. 1986. Kinetics of particulate matter in the rumen., p. 173-195. *In* L. P. Milligan, W. L. Grovum, and A. Dobson (eds.), Control of Digestion and Metabolism in Ruminants. Prentice Hall, Eglewood Cliffs, NJ.

Van Soest, P. J. 1982. Nutritional Ecology of the Ruminant. O & B Books, Corvallis OR.

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Chapter 4

Buffering and Acid Absorption

Dairy cattle can produce as many as 160 moles of fermentation acids per day, and these acids must be buffered by salivary secretions. Bicarbonate is the dominant ruminal buffer, and it has a pK_a of approximately 6.7:

$$CO_2 + H_20 <-> H_2CO_3 <-> HCO_3- + H^+$$

If the ruminal pH is less than 5.7, the ability of bicarbonate to act as a buffer declines, but VFA can prevent additional reductions in pH.

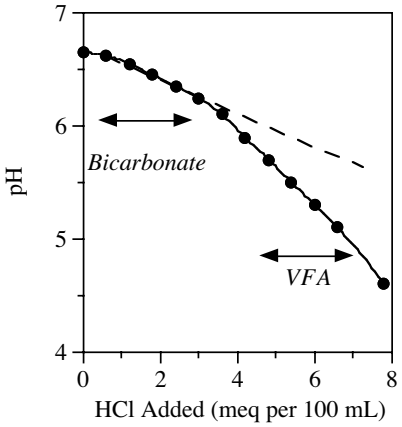

Fig. 4-1. The effect of HCl addition on the pH of ruminal fluid from a cow fed hay .

Because cattle fed forage secrete more saliva and have a higher ruminal pH than cattle fed grain, some people hypothesized that their ruminal fluid might have a stronger buffering capacity. However, there is little evidence to support this hypothesis. Bicarbonate enters the rumen as either a sodium or potassium salt. Because homeostatic mechanisms insure that the osmotic pressure of rumen is always in close proximity to the osmotic pressure of the plasma and interstitial fluids, the bicarbonate concentration of the rumen cannot change significantly.

The impact of fiber on ruminal pH is most easily explained by its effect on fluid dilution rate. When cattle are fed forage, rumination and saliva flow are stimulated, the fluid dilution rate is as high as 20% per h, and the large amounts of acid are washed out of the rumen to the abomasum where the pH is lower, a greater fraction of the acid is undissociated and the absorption rate is faster.

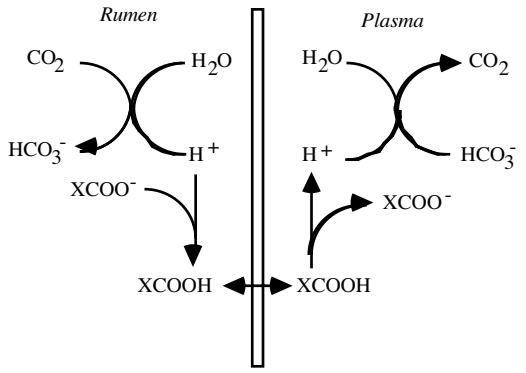

Fig. 4-2. Absorption of undissociated VFA (XCOOH) from the rumen or abomasum and the action of bicarbonate to buffer pH.

The situation is much different in cattle fed grain. The rate of acid production is greater, but there is little fiber to trigger rumination and saliva flow, the fluid dilution rate is low (only 5% per h), and a even greater fraction of the acid must be absorbed from the rumen. Because VFA absorption is a passive process, and only undissociated acids are readily absorbed, absorption rate does not increase until VFAs accumulate and the pH declines.

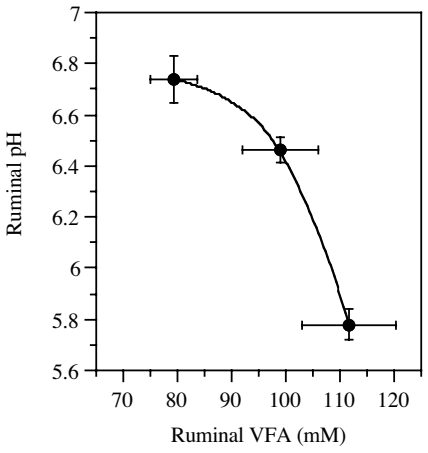

Fig. 4-3. The relationship between ruminal VFA concentration and the pH.

The effect of ruminal fluid dilution rate on VFA production and passage is illustrated by simple calculations (see **Table 4-1**). This example assumes that cattle fed hay will consume 10 kg of dry matter per day, and cattle fed a grain-based ration will consume 15 kg. If the ruminal digestion coefficients for the two diets are 50 and 66%, respectively, organic matter digested in the rumen will be 5,000 and 10,000 g per day, respectively. If ones assumes that: 1) most of the carbohydrate is hexose (180 g/mol), 2) the molar ratio of VFA to hexose is 1.8, 3) the rumen volume is 70 liters, 4) 66% of the organic matter is fermented (33% incorporated in microbes), and 5) there are 24 h in a day, the VFA production rates for the cattle fed hay and grain will be 20 and 40 mmol per liter per h, respectively. The amount of VFA that passes from the rumen can then be estimated by multiplying the VFA concentration in the rumen by the fluid dilution rate. These calculations indicate that 72% of the VFA in the cow fed hay are washed from the rumen, but only 18% of the VFA leave the rumen in the fluid dilution of the cow fed grain.

Table 4-1. Hypothetical calculations showing ruminal VFA production and passage from the rumen.

	Hay	Grain
Organic Matter Digestion (g/day)	5,000	10,000
VFA Production Rate (mmol/liter/h)	20	40
Fluid Dilution Rate (per h)	0.18	0.06
Ruminal VFA Concentration (mM)	80	120
VFA Passage (mol/liter/h)	14.4	7.2
Passage ÷ Production (%)	72	18

VFAs are the normal end-products of ruminal fermentation, but lactate can accumulate if the rate of carbohydrate (e.g., starch) fermentation is rapid and the animals have not been acclimated to the diet. Because lactate is a much stronger acid than VFA (pK_a 3.9 versus ~4.7, respectively), lactate accumulation in the rumen will cause an even more pronounced decrease in pH. If animals are shifted slowly from forage to starch containing rations, lactate is converted to acetate and propionate, and lactate does not accumulate.

Allen, M.S. 1997. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. J. Dairy Sic. 80:1447-1462.

Ash, R.W., and A. Dobson. 1963. The effect of absorption on the acidity of rumen contents. J. Physiol. 169: 39-61.

Russell, J.B., and J.M. Chow. 1993. Another theory for the action of ruminal buffer salts: Decreased starch fermentation and propionate production. J. Dairy Sci. 76:826-830.

Chapter 5

Feed Analysis and the Balance of Digestion Rates

Feed composition. Ruminants evolved as grazing animals, and forages are still an important feedstuff for domestic livestock. Forages have a cell wall that thickens as the plant matures. This wall is composed of pectin, cellulose and hemicellulose, and the hemicellulose may be covalently linked to lignin, a complex polymer that is not digested in the rumen. The cell wall provides structural support for the plant and insect resistance.

The cell wall surrounds a cytoplasm of soluble enzymes, and the photosynthetic enzyme ribulose 1,5 *bis* phosphate carboxylase, is usually the most abundant protein. Because the cytoplasm contains most of the protein and cell wall has little protein, the protein content of a forage decreases as the plant matures and the cell wall expands.

The outside sheath of the plant is covered by a waxy cuticle that helps prevent dehydration. Because this waxy cuticle is resistant to degradation, ruminal microorganisms digest plant material from the "inside out." Vascular bundles allow the plant to transport water, minerals and sugars from the leaves to the roots, and these bundles have cellulose and lignohemicelluose fibers running through them. The 'parenchyma' is made up of cells that were once photosynthetic but via the process of cell wall thickening have become primarily structural.

Rapidly growing, immature plants have an abundance of sugar (primarily sucrose) in their cytoplasm, and the sugar can account for as much as 18% of the dry matter. When forages are cut and wilted, much of this sugar is depleted by plant respiratory enzymes. If forage is prepared as a silage, the remaining sugar is used by fermentative bacteria that produce silage acids.

Because lignin accumulates when the plant cell wall thickens, there can be a pronounced decrease in fiber digestibility. Some of this decline can be explained by spatial hindrance. When the fiber contains lignin, microorganisms and their cell associated cellulases cannot reach and degrade all of the carbohydrate. Another impact of lignin is mediated by rumination and passage. If the forage is highly lignified (woody), it cannot be easily ruminated into small particles that have a large surface area. Immature plants that have thin cell walls and little lignin are easily ruminated, have a large fiber surface area and are more digestible.

Lignin is synthesized from aromatic acids, but these acids (e.g., *p*-coumaric, ferulic and sinapic acids) can accumulate in some forages. Aromatic acids are toxic to ruminal bacteria and cause a depression in fiber digestion. Plants can also convert aromatic acids into tannins. Tannins may be divided into two groups. Hydrolyzable tannins are esters of sugars with one or more trihydroxybenzene carboxylic acids, whereas condensed tannins are flavanol derivatives. Tannins have a high affinity for proteins and protect them from ruminal degradation. However, if the tannin content is high, overall digestibility declines.

It has long been recognized that feeds can be heated (e.g., roasted, pelleted, extruded, etc.) to alter their rate of ruminal degradation. When soluble proteins (e.g., enzymes) are heated, the tertiary structure is destroyed, and hydrophobic amino acid residues that were buried deep inside the molecule come to the surface. The hydrophobic residues then bind to each other and precipitate as insoluble complexes that are not as accessible to microbial proteinases. Some plants store starch and other polymers in their seeds, but these granules are often encapsulated by proteins. Some of these proteins (e.g., zein from corn) are not easily digested by ruminal microorganisms. When cereal grains are heated, the protective protein coating is ruptured, and the starch becomes more accessible to microbial amylases.

Feed analysis. In the early 1900's, feeds were analyzed by a system of 'proximate analysis' that was based on harsh chemical extractions. Feeds were treated with strong acid and base to determine the crude fiber and incinerated to determine the ash. The feeds were treated with sulfuric acid to estimate crude protein (Kjeldahl nitrogen times 6.25), and fats were extracted with ether. By subtracting the ether extract, crude protein, ash and crude fiber from the original dry matter, it was then possible to estimate the nitrogen free-extract (NFE).

Proximate analysis gave farmers a general assessment of nutritive quality, but it eventually became apparent that better methods were needed. Crude fiber is primarily cellulose and lignin, but it also has some hemicellulose and alkali labile lignin. NFE is primarily sugars, starch and pectin, but it also has some hemicellulose and even small amounts cellulose. Crude protein assumes that proteins are always 16% nitrogen (w/w), and Kjeldahl nitrogen does not differentiate soluble from insoluble proteins or true protein from non-protein nitrogen sources like urea.

In the 1960's and 70's, Van Soest and his colleagues used detergents (laurel sulfate or sodium dodecylsulfate (SDS)) to fractionate feeds. Neutral detergent and heat only liberated the cell solubles and pectin, but acid detergent and heat was needed to solublize the hemicellulose. Based on these observations, the acid-detergent insoluble residue (ADF) was defined as cellulose plus lignin, and the neutral detergent insoluble residue (NDF) was defined as the hemicellulose, cellulose and lignin. The difference between ADF and NDF was the hemicellulose. This scheme provided a more accurate description of fiber, but there were still discrepancies. Lignin estimates varied with the method of analysis, and the ADF often had residual nitrogen and pectin. Acid-detergent insoluble N (ADIN) has been used as an index of unavailable N, but even this assumption was not always clear-cut.

The neutral detergent soluble fraction has little hemicellulose and virtually no cellulose or lignin, but this pool is heterogeneous and not easily defined. Sugars, starch, pectin, organic acids and many proteins are soluble in neutral

detergent, but these components supply different amounts of energy to ruminal microorganisms. Detergent analyses can be used to determine the pool size (amount) of a particular component, but these methods do not provide an estimate of fermentation rate. The NDF fraction of by-product feeds (e.g. distillers grains) has residual starch and protein, but these components can be solubilized by heat, stable amylases and sulfite.

Because the rumen operates as a flow through system, ruminal availability is dictated by the balance of fermentation and passage rates, not just pool size (see Chapter 3, *Digesta Flow)*. This point is illustrated by a simple example. Ruminal bacteria grow most efficiently when approximately 14% of their organic matter is peptides and amino acids and the remainder is soluble carbohydrate. This ratio of amino N to carbohydrate is typical of medium quality forages, but the rates carbohydrate and protein fermentation are not well balanced. Because protein is fermented at a faster rate than the carbohydrate, the bacteria have more nitrogen than they can utilize, and large amounts of ammonia accumulate.

Nutritionists can counteract ruminal ammonia accumulation by adding a rapidly fermented carbohydrate to the diet (e.g., finely ground high moisture corn). When the bacteria are supplied with additional carbohydrate, microbial protein synthesis is enhanced, and ammonia production declines. Conversely, if the bacteria have an abundance of ruminally degraded carbohydrate, and peptides and amino acids are deficient, a rapidly degraded protein source can be added to the ration to decrease energy spilling (See Chapter 18, *Growth, Maintenance and Energy Spilling)*. Until recently fermentation rates could only be derived from in vitro experiments or in situ studies (dry matter disappearance from nylon bags), and even these estimates had to be interpreted carefully. Near infrared (NIR) spectrometry has been used to determine feed composition, and it appears that these complex spectra may also be analyzed to estimate the fermentation rate.

Effective fiber. Ruminants evolved as grazing herbivores, but livestock are often fed an abundance of cereal grain. Grain feeding increases productivity, but fiber-deficient diets can cause a variety of problems including ruminal acidosis, laminitis (founder) and liver abscesses (see Chapter 22, *Rumen Disorders and Toxicities)*. The question then arises, do ruminants have a fiber requirement, and if so, how can it be met? Fiber promotes rumen mixing motions, rumination, saliva flow and fluid dilution rate. If the diet has little fiber, the rumen becomes a stagnant pool, VFA removal via the fluid dilution rate declines, and the animal is more prone to acidosis and related problems.

In the 1980's, Mertens and others coined the term 'effective NDF' to describe the ability of dietary fiber to promote normal rumen function, and they used chewing time as an index. Cattle fed long hay will chew their cud for as long as 700 minutes each day, but the chewing time decreases to as little as 400 minutes if the ration is fiber-deficient. Physically effective NDF is computed by dividing the chewing time by the total intake of NDF:

chewing time ÷ kg NDF

Feed processing can have a profound effect on effective NDF. For example, NDF from long hay stimulates chewing to a much greater extent than NDF from finely chopped hay or cereal grains. These differences are accounted by assigning each NDF fraction an effectiveness factor. Long hay is given an effectiveness factor of 1.0, but this value can decrease to as little as 0.3 if the hay is finely ground or pelleted. High moisture corn that has been rolled is assigned a value of 0.80, but high moisture corn that is ground has a value of only 0.4.

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Chapter 6

The Rumen as a Microbial Habitat

Weimer estimated that the ruminal volume of domesticated cattle, sheep and goats (approximately 2 billion animals) was nearly 100 billion liters and concluded that on a volume basis - the rumen is the "world's largest fermentation process." The rumen is an ideal habitat for the growth of anaerobic microorganisms. Ingested feed supplies nutrients, the temperature is carefully regulated, water and saliva create a moist environment that is well buffered, volatile fatty acids are absorbed across the rumen wall so end-products do not become inhibitory, and autoregulatory processes keep the food moving at a pace that allows relatively rapid rates of microbial growth. Oxygen taken in with the food is expelled with the fermentation gases or is consumed by a small but highly active population of facultative anaerobes.

Ruminal fluid is in equilibrium with the plasma and interstitial fluid of the animal, and this sodium rich environment has been called an 'inland sea.' Sea water has more salt than the rumen (approximately 460 versus 120 mM sodium), and many ruminal bacteria have an absolute requirement for sodium. The sodium and potassium content of ruminal fluid varies with diet. Cattle fed forage have a higher potassium content than cattle fed grain, but the potassium plus sodium content remains more or less constant (approximately 150 mM). The overall ion concentration is less than 400 mOsmol per liter.

Ruminal fluid is highly reduced, and the oxidation-reduction potential (Eh) is always less than -0.35 and often as low as -0.42 volts, where the half reaction for $H^+ + e^- \rightarrow 1/2\ H_2$ is assigned a value of 0.00 volts. The low Eh is due to highly reduced end-products (e.g., H_2S) and the propensity of ruminal microorganisms to use electron carriers with a very low potential. Soon after feeding, ruminal fluid has sugars and other soluble carbohydrates, but these substrates are difficult to detect 2 or 3 hours later. Soluble feed proteins are rapidly converted to peptides and amino acids, and peptide concentrations can be as high as 1200 μg/ml immediately after feeding. However, most peptides are also utilized rapidly, and the peptide concentration declines to approximately 200 μg/ml. The rumen has an abundance of insoluble material (approximately 10% of the dry matter). This feed is composed of large and sometimes complex polymers that must be degraded by extracellular enzymes before it can be utilized. The rumen has some large feed particles, but average particle size is relatively small (< 2 mm). In cattle fed forage, the rumen has a dense mat of feed at the top. The ruminal mat is buoyed by gas production from the fermentation.

Bacterial density in the rumen is very great, and direct counts can be as high as 10^{10} cells per g ruminal contents. Bacterial mass has not been precisely determined, but values are typically in the range of 14 to 18 mg bacterial dry weight per ml (7 to 9 mg bacterial protein per ml). The bacteria are approximately 10% N (62.5% crude protein), but only 80% of the crude protein is true protein. The remainder is nucleic acid nitrogen. First order rates of gas production indicate that approximately 3/4 of the rumen bacteria are bound to feed particles, and only 1/4 are free-floating, planktonic bacteria. Ruminal bacteria range in size from approximately 1 to 5 μm. Based on an internal volume of approximately 4 μl per mg protein, the internal volume of ruminal bacteria would be 32 μl per ml or 0.3% of the total volume.

Rumen protozoa are large microorganisms that vary in size from 20 to 200 μm. Protozoa numbers in the rumen do not often exceed 10^7 cells per ml, but they can account for as much as half of the mass. When protozoa numbers increase, bacterial mass declines and vice versa. Protozoa associate with the feed particles, and this association prolongs their residence time in the rumen. Rumen fungi are even more difficult to quantify. They have a complex lifecycle that varies from zoospores to a mycelium that covers the feed particles. The highest estimates for fungi appear to be in the range of 6% of the total biomass.

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Chapter 7

Oxygen Toxicity and the 'Roll Tube Technique'

By the early 1900's, microbiologists knew that some bacteria were sensitive to oxygen, but these species could be cultivated on agar plates, so long as the plates were placed in an incubator that had been flushed with nitrogen or carbon dioxide. These techniques were, however, not sufficient to grow the most important (predominant) ruminal bacteria. Most ruminal microorganisms are strict anaerobes and cannot sustain viability or grow if the oxygen concentration is greater than 1 part per million and the oxidation-reduction potential is greater than -0.3 volts. The oxygen sensitivity of ruminal microorganisms is due to reactive oxygen molecules and the lack of detoxification enzymes.

Oxygen reacts with protons to form peroxide:

$$O_2 + 2H^+ + 2e^- <-> H_2O_2$$

Most aerobes have catalase, an enzyme that detoxifies peroxide, but many ruminal bacteria lack this enzyme or have low activity:

$$H_2O_2 \xrightarrow{\text{catalase}} H_20 + 1/2\ O_2$$

Oxygen reacts with free electrons to form an even more reactive species, superoxide:

$$O_2 + e^- \dashrightarrow O_2^{-}\cdot$$

Superoxide reacts with a variety of cellular components (reduced flavins, quinones, flavoproteins, thiols and iron sulfur proteins) and oxidizes them. Because ruminal bacteria need SH containing molecules for their metabolism, they are more sensitive to oxygen than aerobes. Aerobes have an enzyme that protects them from superoxide:

$$O_2^{-}\cdot + O_2^{-}\cdot + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

The combined action of superoxide dismutase and catalase detoxifies oxygen, superoxide and peroxide.

In the 1940's, two microbiologists, R.E. Hungate and A.K. Sijpsteijn, worked independently to devise methods for cultivating strictly anaerobic ruminal bacteria. Because Hungate used agar that was rolled on the inside surface of a tube, the technique was called the 'roll tube technique.' Hungate's technique was based on a series of practical observations and simple solutions:

1) Commercial gases are typically 99.998% pure, but these preparations have enough residual oxygen to prevent the growth of most ruminal bacteria, but these gases can be further purified by passing them over hot copper filings in a furnace:

$$Cu + 1/2\ O_2 \rightarrow CuO$$

The oxidized copper is then reduced with hydrogen so the copper can be re-used:

$$CuO + H_2 \rightarrow Cu + H_2O$$

2) Because the solubility of all gases is zero at the boiling point of water, liquids can be brought to a boil and cooled under an atmosphere of O_2-free gas to prepare a strictly anaerobic preparation.

3) Liquid media and molten agar can be transferred from one vessel to another using a pipette connected to a mouth tube. The pipette and the tube are filled with O_2-free gas, and this gas provides an anaerobic barrier between the person's mouth and the medium.

4) Tubes sealed with natural rubber will eventually become oxidized, but butyl rubber is much more resistant to oxygen penetration. Tubes sealed with butyl rubber stoppers will remain anaerobic for long periods of time.

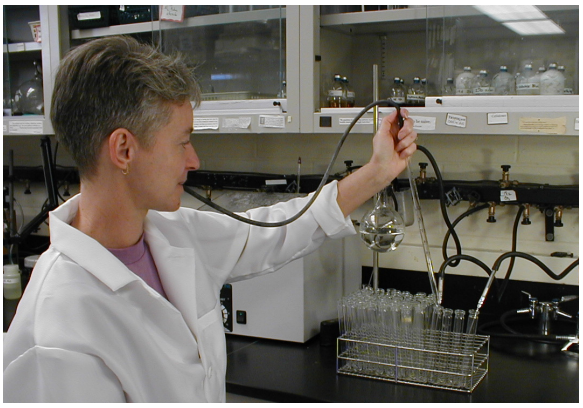

Fig. 7-1. The use of mouth tube and pipette to anaerobically dispense ruminal medium (courtesy of K. Dusinberre).

5) Medium brought to a boil lacks O_2, but it does not have a low Eh. However, reducing agents (e.g., cysteine, hydrogen sulfide) can be added to create a highly reduced environment.

6) The redox dye, resazurin, indicates if the tubes remained anaerobic. Resazurin is purple when it is exposed to oxygen and colorless if anaerobic.

7) Some ruminal bacteria require nutrients that are not found in standard microbiological media (e.g., branched chain VFA), but Hungate added 1/3 ruminal fluid to create a 'habitat simulating medium.'

8) If ruminal fluid is serially diluted into molten agar, the tubes can be rolled in an ice bath to create a film of agar. Once isolated colonies have grown in the agar, the tubes are opened and flushed with O_2-free gas. Isolated colonies are then transferred with a needle to broth.

9) In the 1960's, anaerobic glove boxes capable of maintaining a strictly anaerobic environment were engineered, and these systems allow rumen microbiologists to use Petri plates and traditional schemes of isolating bacteria. However, these boxes must be monitored carefully to make sure that the catalyst is working properly and partial pressure of O_2 is low.

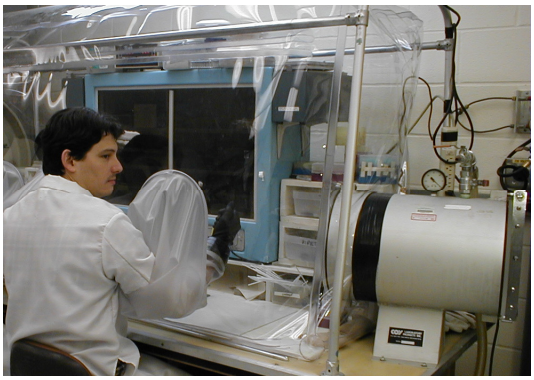

Fig. 7-2. A Coy Laboratory Products (Ann Arbor, MI) anaerobic glove box that is equipped with a catalyst system that uses hydrogen to remove oxygen (courtesy of H.C. Mantovani).

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Chapter 8

Bacterial Diversity

Hungate noted that the rumen had a complex population of strictly anaerobic bacteria, and he explained this diversity in three ways: 1) the complexity of feedstuff materials that are ingested by ruminants, 2) the assumption that specialization would allow 'selection for maximum biochemical work', and 3) the hypothesis that once the 'best fitted' bacterium had displaced all others, the 'most fitted' type would itself alter the niche and create new opportunities for yet other bacteria.

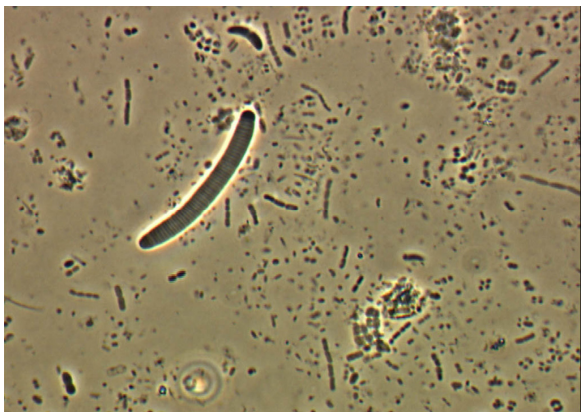

Fig. 8-1. A photograph showing mixed ruminal bacteria from a cow fed hay. (Courtesy of S.A. Zinder).

Because bacteria are unicellular organisms that reproduce asexually, the definition of a bacterial species is not clear-cut. Traditionally, microbiologists used morphological traits, substrate utilizations, end-products, and simple biochemical tests to create a system of numerical taxonomy, but these schemes gave equal emphasis to all the differences and did not have a true genetic basis. Numerical taxonomy is most often used to classify strains within a genus. The strains are scored according to phenotypic characteristics (e.g., 25 different traits) and the scoring is a simple + or -. Each strain is then compared to every other strain, and a similarity (S_A) coefficient is computed:

$$S_A = \frac{[+,+] + [-,-]}{[+,+] + [-,-] + [+,-] + [-,+]}$$

The strains are then organized into a dendrogram (Fig. 8-2). Highly related strains have high S_A values while those that are not so closely related have lower S_A.

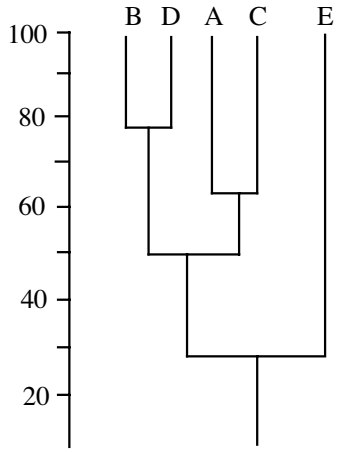

Fig. 8-2. A dendrogram of relatedness for bacterial strains (A-E). Strains B and D are 78% related while strains B and E are only 28% related.

With the discovery that DNA was the molecule responsible for genetic variation, microbiologists started to use DNA as a tool for bacterial classification. DNA is a double stranded linear molecule that is composed of four bases (adenosine (A), thymine (T), cytosine (C), and guanine (G)). The strands are held together by hydrogen bonds so that A is complementary with T and G is complementary with C. Because A-T has 2 hydrogen bonds and G-C has 3 hydrogen bonds, there is an inherent difference in thermal stability. If the DNA has mostly A-T bonds, the strands can be dissociated at a lower temperature than if it is G-C rich.

When purified DNA is subjected to an increasing gradient of temperature in a thermal cuvette, DNA dissociation (denaturation) can be monitored by measuring optical density changes. When the strands separate, there is an increase in optical density, and T_m is defined as the point where half of the DNA is dissociated. The relationship between T_m and mol %G-C is linear over the range of 30-75%, and mol % G-C ranges from 37 to 72% depending on the bacterium. Mol %G-C is a useful tool for assigning bacteria to a genus, but it does not differentiate species within a genus. Because DNA from high G-C bacteria is denser than is A-T rich DNA, mol %G-C can be determined by

centrifugation (cesium chloride density gradients). The bases have also been separated and determined by HPLC methods.

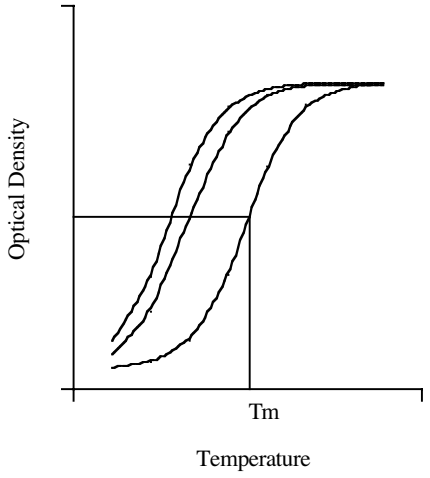

Fig. 8-3. The dissociation of DNA (optical density) that has been subjected to an increasing gradient of temperature. T_m is defined as the temperature at which half of the DNA is dissociated.

Interspecies relationships (phylogenies) can also be based on the ability of DNA from one species to bind to the DNA another via a process known as 'DNA hybridization.' When DNA is heated, it dissociates into single stranded molecules, and single stranded DNA binds tightly to variety of membrane filters (e.g., nitrocellulose). When this reference DNA is flooded with labeled DNA fragments from another bacterium, the label hybridizes in a complementary fashion. If the bacteria are closely related, there is a high degree of DNA hybridization. DNA hybridization can also be estimated from the thermal stability of hybridized (annealed) DNA. In this latter case, the reference DNA is washed at increasing temperatures, and the liberation of 'melted' DNA is monitored spectrophotometrically. Most ruminal bacteria were classified in the 1950's, and at this time, DNA homology was not a routine technique. In the 1980's, Mannarelli and his colleagues examined the DNA homology of ruminal strains. Because strains within the same species had values as low as 20%, it appeared that new names were needed.

In the 1970's, Woese, speculated that ribosomal RNA could be used as a tool for classifying bacteria and determining their phylogeny. All bacteria have ribosomes, and ribosomes are such large and complicated structures that mutations are only rarely advantageous. Thus, evolutionary changes in rRNA are relatively slow. Bacterial ribosomes account for approximately 20% of the bacterial dry weight, and each ribosome is composed of protein as well as rRNA. Bacterial ribosomes have a sedimentation coefficient of 70S, but each ribosomal particle can be further separated into smaller particles. Most of our information on bacterial phylogeny is based on 16S rRNA and corresponding rDNA gene sequences. The first studies of bacterial phylogeny were based directly on rRNA and the ability of reverse transcriptase to polymerize bases from an RNA template. More recently, microbiologists sequenced the rDNA genes directly. Because the rDNA genes are highly conserved, it is possible to construct specific primers (17 to 21 bp). Once the primers have been synthesized, the genes can be amplified by the polymerase chain reaction (PCR), and sequenced.

Woese demonstrated that methanogenic 'bacteria' were more closely related to eucaryotes (plants, animals, etc.) than other bacteria, and he proposed that living organisms should be re-classified into three domains: 1) eubacteria (true bacteria), 2) eucaroytes, and 3) archaea (ancient bacteria). By comparing 16S rRNA sequences, Woese separated the eubacteria into 11 groups, and ruminal eubacteria are found in at least 4 of these groups. The rumen also has archaea and two distinct populations of eucarya (protozoa and fungi).

In the 1960's, microbiologists noted that bacteria had specialized enzymes that hydrolyzed DNA at specific sites. The specificity of these restriction enzymes provided a basis of 'DNA fingerprinting.' When genomic DNA or DNA from the polymerase chain reaction is treated with restriction enzymes, DNA fragments can be applied to gels and separated according to size. By comparing the sizes of the fragments, one can determine restriction fragment length polymorphisms (RFLP) of bacterial species or strains. The number and size of the fragments can be compared to create dendrograms of relatedness that are based on DNA rather than simple

phenotypic characteristics.

The ability of molecular biologists to use DNA as a taxonomic tool was enhanced by the discovery of the polymerase chain reaction (PCR). When isolated DNA is denatured briefly and flooded with deoxynucleoside triphosphates, heat stable DNA polymerase (*Taq* I) can replicate (amplify) the DNA in a sequential fashion (typically 25 to 30 cycles), but only if suitable primers (at the '5 and 3' ends) are present. These primers are short segments of complementary DNA that bind to the DNA template at distinct sites (e.g., rDNA genes). Some bacteria (e.g. streptococci) have repetitive segments of DNA that are scattered throughout the genome, and these 'BOX elements' have been amplified to determine the phylogeny of *S. bovis* strains.

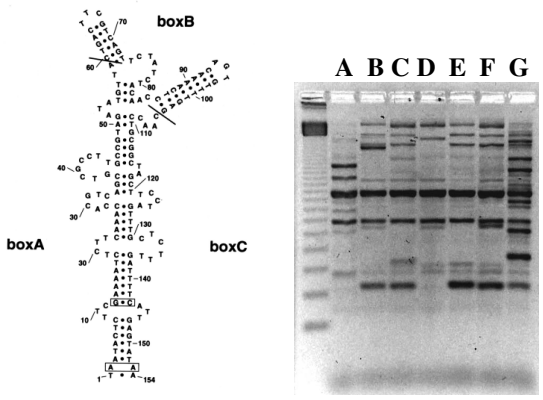

Fig. 8-4 Repetitive DNA (BOX) sequences and a gel showing PCR products of these elements from *S. bovis* (left panel is taken with permission from Belkum and Hermans (2000) right panel is courtesy H.C. Mantovani).

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Chapter 9

Predominant Ruminal Bacteria and Archaea

Early work indicated the culturable (viable) cell count of ruminal bacteria was typically 10-fold lower than the total microscopic count, and 'shot gun' cloning experiments support the idea that many ruminal bacteria have not yet been cultivated. As many as 90% of 16S rDNA genes that were cloned into *E. coli* did not have sequences that matched previously isolated bacteria, and fluorescent probes (to these sequences) indicate that some of these uncultivated bacteria are abundant. However, bacteria and archaea capable of performing most of the major transformations known to occur in the rumen have been isolated, and these species have provided a model of rumen bacterial ecology.

Ruminococci. Cellulolytic cocci can be isolated from the rumen on agar containing ball-milled cellulose, and these bacteria were first isolated by Hungate. He called the non-pigmented (white) types *Ruminococcus albus*, and the pigmented (yellow) isolates were named *Ruminococcus flavefaciens*. *R. albus* produces acetate, formate and hydrogen, but *R. flavefaciens* produces succinate and

less ethanol than *R. albus*. 16S rDNA sequencing indicates that ruminococci are closely related to other species of Gram-positive bacteria, but they do not have a typical Gram stain. *R. albus* cells often stain Gram-negative, and *R. flavefaciens* is Gram-variable. Some strains of *R. albus* produce a bacteriocin that inhibits *R. flavefaciens,* and this finding is consistent with the observation that *R. albus* is often more prevalent than *R. flavefaciens*. Most ruminococci cannot grow on pentoses; however, many of them can utilize hemicellulose as a source of energy. Growth on glucose, when possible, is often quite slow. They prefer to use cellobiose (a disaccharide of glucose) that is produced by cellulase(s).

Table. 9-1. Characteristics of predominant ruminal bacteria and archaea.

Species	Products	Primary Niches
Fibrobacter succinogenes	S, F, A	CU
Ruminococcus albus	A, F, E	CU
Ruminococcus flavefaciens	S, F, A	CU
Butyrivibrio fibrisolvens	B, F, L, A	CU, HCU, ST, PC, SU
Ruminobacter amylophilus	S, F, A	ST
Selenomonas ruminantium	L, A, P, B, H_2	SU, ST, L
Prevotella sp.	S, A, F, P	ST, HCU, PC, ß GL, PT
Succinomonas amylolytica	S, A, P	ST
Succinivibrio dextrinosolvens	S, A, F, L	MD
Streptococcus bovis	L, A, F, E	ST, SU
Eubacterium ruminantium	A, F, B, L	MD, SU
Megasphaera elsdenii	P, A, B, Br	L, MD, AA
Lachnospira multiparus	L, A, F	PC, SU
Anaerovibrio lipolytica	A, S, P	GY, L
Peptostreptococcus anaerobius	Br, A	PEP, AA
Clostridium aminophilum	A, B	AA, PEP
Clostridium sticklandii	A, Br, B, P	PEP, AA
Wolinella succinogenes	S	MAL, FUM
Methanobrevibacter ruminantium	CH_4	H_2, CO_2, F

(A, acetate; B, butyrate; P, propionate; F, formate; L, lactate; E, ethanol; S, succinate; Br, branched chain VFA; CU, cellulose; HCU, hemicellulose; ST, starch; SU, sugars; MD, maltodextrins; AA, amino acids; GY, glycerol; PT, protein; PEP, peptides; PC, pectin; MAL, malate; FUM, fumarate; βGL, β-glucans).

Fibrobacter succinogenes. Hungate also isolated a Gram-negative, cellulolytic rod. Based on the observation that the cells were pleomorphic and produced large amounts of succinate, it was originally classified as *Bacteroides succinogenes*. However, *B. succinogenes* was not closely related to colonic bacteroides, and 16S rRNA indicated that it needed to be placed in a new genus, *Fibrobacter*. Fibrobacters isolated from the rumen are typically called *succinogenes,* but there are two recognized species. *F. intestinalis* has been isolated from the large intestine, but this habitat distinction is not a clear-cut indicator of the species. *F. succinogenes* attaches very tightly to cellulose, and this feature has confounded its isolation from the rumen. 16S rDNA probes indicated that *F. succinogenes* was not as numerous as *R. albus*. *F. succinogenes* does not utilize pentoses, and it must be 'trained' to grow rapidly on glucose.

Butyrivibrios. Bryant isolated motile butyrate producing rods from the rumen, and classified these bacteria as *Butyrivibrio fibrisolvens*. Butyrivibrios are extremely versatile organisms, and they can utilize pentoses, hexoses, pectin, starch, xylans, and hemicellulose. Some strains are even weakly cellulolytic. Hungate noted that some strains of *B. fibrisolvens* produced lactate while others did not. He indicated that the non-lactate producers should be designated as *B. alactacidigens*, but this designation was not retained.

16S rDNA sequencing indicates that strains classified as *B. fibrisolvens* are not always closely related, and they have different methods of butyrate production. Lactate production is related to the method of butyrate formation. Strains that use the butyryl CoA-acetyl CoA transferase need acetate in the growth medium. If acetate is not available, butyrate cannot be produced, and the carbon is diverted to lactate. Those butyrivibrios that have butyrate kinase, do not need acetate and never produce lactate. Recent work indicates that the genus *Butyrivibrio* should be divided into two genera. In this most recent scheme of classification, *Butyrivibrio* species produce butyrate with a kinase, and the *Pseudo-butyrivibrio* species utilize a transferase.

***Prevotella* species.** Bryant isolated pleomorphic rods that produced large amounts of succinate, did not digest cellulose and could utilize a variety of substrates. Based on these characteristics the bacteria were classified as *Bacteroides ruminicola*, but DNA hybridizations indicated that these strains shared as little as 20% homology. In 1990, Shah and Collins noted that ruminal *Bacteroides* species were sensitive to bile salts and lacked the hexose monophosphate pathway. Based on these characteristics, *B. ruminicola* was reclassified as a new genus, *Prevotella (e.g., P. ruminicola)*. However, only a few years later, 16S rDNA indicated that many of the strains should be re-grouped into different species. Strains that did not use xylose, were CMCase negative, produced DNAase and had 45 to 52 G-C mol% were called *P. brevis*. Those that were CMCase positive, used xylose, produced DNAase and had 39 to 43 G-C mol% were *P. bryantii*. CMCase positive strains that did not produce DNAase and had 45-51 G-C mol % were still called *P. ruminicola* while those having little CMCase or DNAase activity and having 39-43 G-C mol% were classified as *P. albensis*.

The 16S rDNA cloning experiments of Whitford et al. indicated that approximately 30% of the ruminal bacteria from cattle fed a forage/grain diet were related to bacteria in the *Prevotella-Bacteroides* group, and clones containing signature sequences for *P. bryantii* and *ruminicola* were relatively abundant. *Prevotella* species seem to play an important role in ruminal protein catabolism. Most strains can hydrolyze proteins, utilize peptides and deaminate amino acids. The deamination rates of *Prevotella* species are not as high as obligate amino acid fermenting ruminal bacteria, but they are found at high numbers in the rumen.

Selenomonas ruminantium. These crescent shaped organisms have a tuft of flagella on the inside surface, and they can turn on both axes like a 'whirling dervish.' When sugars are abundant, *S. ruminantium* grows rapidly, and its fermentation is nearly homolactic. However, if sugars are not abundant and its growth rate is slow, acetate and propionate are produced, and it can even utilize the lactate previously produced. Early work indicated that *S. ruminantium* produced formate, but later work showed that it can also produce hydrogen and carbon dioxide, particularly if a methanogen is present to utilize the hydrogen. Most strains do not degrade starch, but they can utilize dextrins that are produced by other bacteria. *S. ruminantium's* primary niche is sugar utilization (e.g., sucrose), but it can also utilize lactate. *S. ruminantium* is a Gram-negative bacterium, but 16S rDNA sequencing indicates that its closest relatives are Gram-positive species.

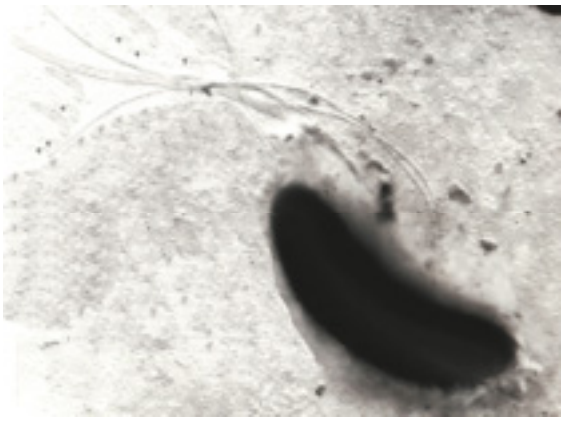

Fig. 9-2. A scanning electron micrograph of *S. ruminantium* showing its flagella (Courtesy H.J. Strobel).

Streptococcus bovis. This facultative anaerobe was isolated from the rumen before strictly anaerobic techniques were developed. *S. bovis* grows aerobically if the culture medium is rich, but it grows faster if the medium is anaerobic and reduced. *S. bovis* is one of the fastest growing bacteria in nature, and its doubling time can be as short as 24 minutes. *E. coli* grows faster than *S. bovis* if oxygen is available, but *S. bovis* grows faster than *E. coli* under anaerobic conditions.

Many strains of *S. bovis* have an orange pigmentation, but this characteristic varies with the growth condition. When cells are harvested by centrifugation, the orange pigment stays in the culture supernatant (it does not adhere to the cell pellet). Like most streptococci, *S. bovis* is not a true coccus, and the cells are distinctly ovoid in shape. The cells can form chains of 2 to 4 cells.

S. bovis was originally described as a 'homolactic' bacterium, but its fermentation shifts to acetate, formate and ethanol if the glucose consumption rate is slow (e.g., continuous culture). Many

lactic acid bacteria have complex growth requirements and need at least 8 preformed amino acids. *S. bovis* can use ammonia as its sole source of nitrogen, and it still grows rapidly (50 minute doubling time). *S. bovis* is an opportunistic bacterium that only becomes a dominant species if large amounts of soluble carbohydrate are added to the diet. When soluble carbohydrates are abundant, *S. bovis* outgrows other ruminal bacteria, and its lactate production decreases ruminal pH. Because *S. bovis* is more acid tolerant than other ruminal bacteria, ruminal ecology can be drastically altered.

S. bovis is also found in the colon of humans and it has been linked to the early stages of colon cancer. When the colon deteriorates, sugars are not as readily absorbed, and *S. bovis* has additional substrates to exploit. If *S. bovis* migrates from the colon into blood, it can cause endocarditis, meningitis, and septicemia like other streps. In the 1980's, many streptococci were re-classified into other genera (e.g., lactococci and enterococcci), but the re-classification of *S. bovis* was problematic. *S. bovis* and *Streptococcus equinus* are so closely related that they cannot be easily differentiated. Human strains of *S. bovis* appear to differ from bovine strains, but until recently this distinction was not clear-cut. *S. bovis* (like other streptococci) has repetitive DNA sequences (BOX elements) scattered throughout the genome, and the number of these elements and distance between them varies from strain to strain.

Megasphaera elsdenii. This species was isolated from the rumen by S.R. Elsden in the 1950's, and it is a relatively aerotolerant bacterium. Based on its shape (very large coccus) and fermentation characteristics, it was originally classified as *Peptostreptococcus elsdenii*. However, Rogosa noted that it had uncharacteristic features. Peptostreptococci are Gram-positive, but *M. elsdenii* has an outer membrane. Because its method of propionate production is distinctly different from that of *Veillonella,* a new genus, *Megasphaera,* was created. *M. elsdenii* stains Gram-negative, but its closest relatives (like *S. ruminantium*) are Gram-positive species. *M. elsdenii* ferments a variety of simple sugars (not usually starch), but its primary niche is lactate utilization. *M. elsdenii* has a relatively high affinity for lactate. The ^{14}C-labeling studies of Counotte et al. indicated that it accounted for approximately 80% of the lactate turnover in cattle fed large amounts of cereal grain. *M. elsdenii* produces ammonia from protein hydrolysate, but it only uses a few amino acids (e.g., serine and threonine) and grows slowly if carbohydrates are not provided. Some strains can deaminate branched chain amino acids and produce branched chain VFA, but this catabolism does not provide enough energy for growth.

Ruminobacter amylophilus. Hamlin and Hungate isolated this starch digesting, succinate producing bacterium, and originally called it as *Bacteroides amylophilus*. However, 16S rDNA sequencing indicated that it should be transferred to a new genus, *Ruminobacter*. *R. amylophilus* is completely dependent on starch for its energy. It uses maltose, dextrins or starch, but not glucose. It is proteolytic but only seems to use ammonia nitrogen. Some strains are very unstable in the laboratory and must be transferred frequently. If the transfer interval is longer than a day or two, the cultures lyse and die out.

Anaerovibrio lipolytica. Stanley Mann isolated this bacterium from sheep fed linseed meal. It hydrolyzes triglycerides and ferments the glycerol that is released. *A. lipolytica* also ferments lactate, but its ability to utilize other substrates is very limited. The only sugar that it can ferment is fructose. It is a Gram-negative, curved rod with a polar flagellum, and it produces succinate and propionate.

Succinomonas amylolytica. Bryant isolated *S. amylolytica* from cattle fed hay and grain, and its niche in the rumen appears to be starch utilization. It is a Gram-negative rod that produces succinate, acetate and small amounts of propionate.

Succinivibrio dextrinosolvens. *S. dextrinosolvens* was also isolated by Bryant from cattle fed starch, but this species cannot digest intact starch. It uses extracellular maltodextrins that are released by the amylases of other bacteria. It has Gram-negative, helically shaped cells and produces succinate, acetate and small amounts of formate and lactate.

Spirochetes. Gram-negative, spirochetes are often observed in ruminal fluid, but these motile bacteria can penetrate plant materials. Ruminal spirochetes have been classified as *Treponema (bryantii* and *saccharophilum)*. Because they are

versatile, their niche has not been precisely defined, but pectin utilization seems to be an important characteristic. When continuous cultures having an abundance of Trypticase are inoculated with mixed ruminal bacteria, spirochetes predominate and ammonia accumulates. Acetate, formate and succinate are major end-products.

Obligate amino acid fermenting bacteria. In the 1960's, Balden et al. examined the ability of carbohydrate fermenting ruminal bacteria to produce ammonia from protein hydrolysate. Most strains did not produce any ammonia, and those that did were less active than mixed ruminal bacteria. Hungate (in his book) indicated that "rumen bacteria able to digest casein and requiring no carbohydrate have recently been encountered by the author," but he did not isolate these bacteria.

In the 1980's, ruminal enrichments with high concentrations of Trypticase yielded three previously unrecognized bacteria. 16S rRNA indicate that the large coccus was *Peptostreptococcus anaerobius* and the short rod was *Clostridium sticklandii*. The football-shaped bacterium was not closely related to other bacteria, and it was named *Clostridium aminophilum*. The obligate amino acid fermenting bacteria cannot use carbohydrates as an energy source for growth, but they deaminate amino acids at least 20-fold faster than other ruminal bacteria.

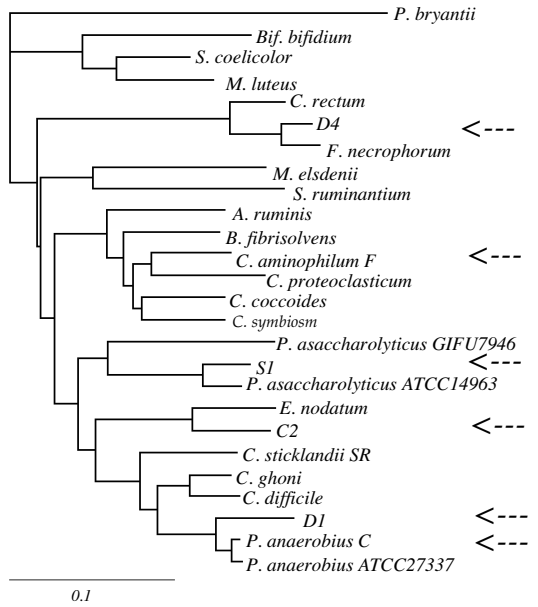

Fig. 9-3. 16S rDNA phylogeny of obligate amino acid fermenting ruminal bacteria (arrows). The bar is a 10% difference (Courtesy J. L. Rychlik).

The original isolates were all closely related to clostridia, but later work indicated that the diversity was greater. Attwood et al. isolated obligate amino acid fermenting bacteria from sheep, deer and cattle. Isolate D1 was closely related to *P. anaerobius*. *C. aminophilum* and *C. sticklandii* SR were not isolated or detected using 16S rRNA probes, but several of their isolates were also found in the 'low G+C Gram-positive' branch of bacteria. One isolate (D4) stained Gram-negative, but it was 98.8% similar to *Fusobacterium necrophorum*. *F. necrophorum* causes liver abscesses in cattle and is found between high and low G+C Gram-positive species.

Wolinella succinogenes. Wolin isolated and classified this bacterium as *Vibrio succinogenes*, but it was later re-named in his honor. *W. succinogenes* has a very narrow niche. It uses H_2 and either malate or fumarate to make succinate. It must compete with methanogens for H_2, and it is not normally a predominant ruminal bacterium. Because it is less sensitive to oxygen than methanogens, it was a convenient model for studies of inter-species hydrogen transfer (see Chapter 15, *Redox, Fermentation Balances and Interspecies Hydrogen Transfer).*

Archaea. Methane is a major end-product of ruminal fermentation, and cattle can produce as much as 17 liters of methane each hour. Because this production represents a loss of feed energy (as much as 12% of the gross energy), and ruminants are a significant source of 'greenhouse' gas, nutritionists have sought methods of inhibiting methane production (see Chapter 22, *Manipulation of Rumen Fermentation).* Methane is produced by a specialized group of archaea. In 1957, Opperman isolated a methanogenic bacterium from the rumen that converted either formate or hydrogen and carbon dioxide to methane, but its abundance was not defined. One year later, Smith and Hungate isolated several strains of methane-producing bacteria, and these isolates were present in the 10^{-7} dilution of ruminal fluid. Based on cell shape and colony morphology, they were named *Methanobacterium ruminantium.* In the 1980's, Grant et al. renamed *M. ruminantium* as *Methanobrevibacter ruminantium*. This re-classification was based on differences in polar lipids.

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Chapter 10

Ruminal Protozoa and Fungi

Protozoa. The rumen has a diverse population of ciliate protozoa, but the role of these eucaryotic organisms in ruminal fermentation is still not clear. Defaunation studies indicate the protozoa are not essential, but protozoa can account for as much as half of microbial mass in the rumen. Ruminal protozoa appear to be both advantageous and detrimental to the host. If the diet has little nitrogen, bacterial predation and protozoal lysis leads to a recycling of microbial protein in the rumen, and there is a direct relationship between the presence of protozoa and ruminal ammonia. However, if the ration is based on grain, protozoal engulfment of starch grains can modulate pH and protect the animal from acidosis. Rumen protozoa can be divided into two classes. The 'holotrichs' have cilia over their entire bodies, but the 'entodinomorphs' only have cilia in discreet regions. All entodinomorphs have oral cilia, and some have dorsal cilia. The location of the cilia has been used as a taxonomic tool.

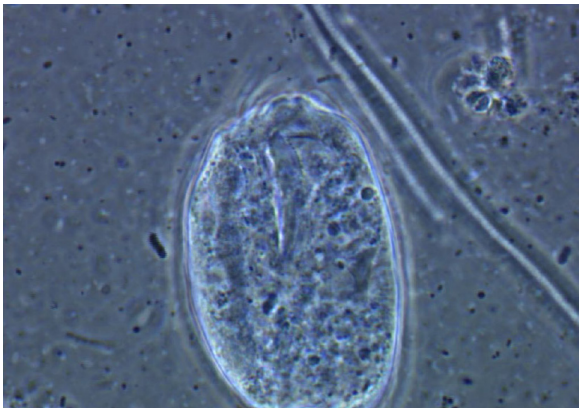

Fig. 10-1. A protozoa collecting ruminal bacteria and feed materials with its cilia. (Courtesy of S.H. Zinder).

Holotrichs utilize sugars and are prevalent in animals consuming fresh forage (e.g., red clover). Because holotrichs cannot control their rate of sugar uptake, they can become so filled with glycogen that they burst. If animals are fed starch, holotrichs are typically out-numbered by entodinomorphs. The entodinomorphs are found in close proximity to feed particles, and use starch as well as a variety of plant materials.

Holotrichs and entodinomorphs both use their cilia for locomotion and to bring food, bacteria and smaller protozoa to the oral cavity. In vitro experiments indicated that a single protozoan consumed 12,000 bacterial cells per hour. Predation rates in vivo are probably not this high, but it is clear that protozoa have considerable capacity to 'graze' bacteria. Whether the protozoa selectively engulf certain types of bacteria have not been definitely proven, but Kurihara et al. noted that re-faunated sheep had fewer small, amylolytic bacteria.

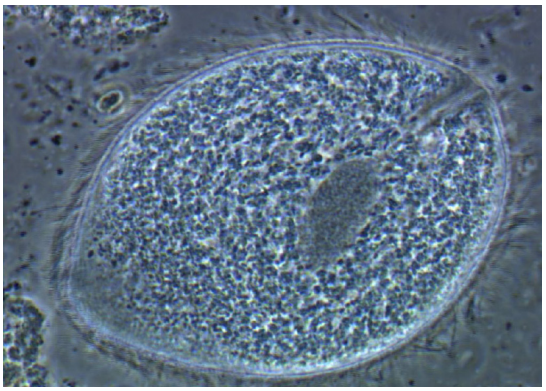

Fig. 10-2. A holotrich grazing on feed particles and bacteria in the background. (Courtesy of S.H. Zinder).

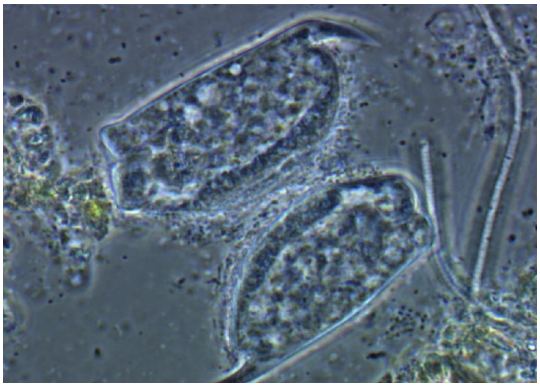

Fig. 10-3. Entodinomorphs grazing on feed particles and bacteria in the background. (Courtesy of S.H. Zinder).

Ruminal protozoa, and in particular entodinomorphs, can also 'cannibalize' other protozoa. Margaret Eadie noted that sheep tended to have stable populations that could be designated as "A" and "B." When ruminal fluid from a sheep having an A population was inoculated into a sheep having a B population, the B population disappeared (only A types were observed). The A population was dominated by relatively large protozoa (*Polyplastron, Diploplastron* and *Orphyoscolex*), while the B types were predominantly smaller ones (*Eudiplodium, Epidinium, Eremoplastron* and *Ostracodinium*). In vitro studies indicated that protozoa can adapt. When *Polyplastron* was introduced into sheep that contained *Eudiplodia,* the *Eudiplodia* increased in size, presumably in an attempt to evade predation. Another antagonism occurs between *Ent. bursa* and *Ent. caudatum*. *Ent. bursa* is the larger of the two, and it can engulf *Ent. caudatum*. However, if *Ent. bursa* is present, *Ent. caudatum* grows spines and resists uptake.

Ruminal protozoa grow so slowly (doubling times of 15 to 24 hours) that they could be washed out of the rumen. However, protozoa by associating with the slow moving particle fraction prolong their residence time in the rumen. Because many protozoa lyse before they leave rumen, they contribute directly to the microbial protein turnover (see Chapter 19, *Microbial Death and Turnover).* Rumen ciliate protozoa cannot be isolated on agar plates, and cultures are obtained by picking individual cells with a long dissecting needle. The study of rumen protozoa has been confounded by their fastidious nutritional requirements. They do not grow if bacteria are not present in the culture medium. Some researchers have used antibiotics, but some viable bacteria were always present. When *Ent. caudatum* was provided with dead bacteria, the culture did not persist for more than 22 days. Because protozoal cultures have always had a large number of contaminating bacteria, it has been difficult to say with certainty if a particular function was performed by protozoa or bacteria.

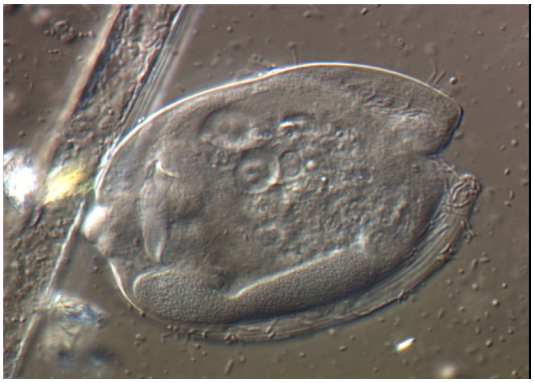

Fig. 10-4. A micrograph showing the internal structure of a ruminal entodinomorph. (Courtesy of S.H. Zinder)

Protozoa are eucaryotes that have an organized nucleus, contractile vacuoles and, in some cases, skeletal plates. *Orphyoscolex* is the most complex ciliate protozoan, it has as many as 3 skeletal plates. Ruminal protozoa reproduce asexually via binary fission, but they can mate and exchange genetic material. Sexual reproduction occurs sporadically, and factors affecting this activity have not been defined. Many protozoa have bacteria on their outside surfaces (exosymbionts). Because methanogens are epifluorescent and most eubacteria are not, it is possible to observe attached methanogens. Attached methanogens derive H_2 directly from specialized organs (hydrogenosomes).

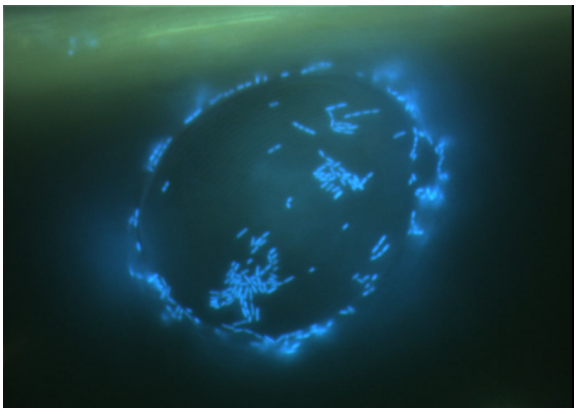

Fig. 10-5. Epifluorescence micrograph of methanogenic bacteria attached to the surface of a ruminal protozoan. (Courtesy of S.H. Zinder)

If new-born ruminants are removed from their mother and reared in isolation, maternal grooming is not possible, and the animals lack a protozoal fauna. Because protozoa are sensitive to harsh chemicals (1,2-dimethyl-5-nitroimizole), it is also possible to defaunate mature animals, but the procedure can kill the animal. In recent years, organic defaunation procedures have been reported, but these methods are still proprietary. Positive effects of defaunation on the animal appear to be mediated through nitrogen metabolism. Protozoal protein has a higher biological value than bacterial protein, but little protozoal protein leaves the rumen. Because protozoal lysis and bacterial predation increases microbial protein turnover and ammonia accumulation, animals on diets lacking protozoa can benefit.

Table 10-1. Characteristics of entodinomorph protozoa.

Genus	Dorsal Cilia	Skeletal Plates	Macro-nucleus	Length (um)	Width (um)	Operculum	Vacuoles	Caudal Spines
Entodinium	0	0	V	22-95	11-68	N	1	0-6
Parentodinium	0	0	C	26-39	14-21	N	1	0
Diplodinium	1	0	B	55-210	41-136	W	2	0-6
Eudiplodinium	1	1 narrow	H	105-198	56-120		2	0
Ostracodinium	1	1 wide	V	58-133	36-54	V	2-6	0-2
Metadinium	1	2 narrow	R	110-288	61-165	S	2	0
Enoploplastron	1	3 narrow with window	E	60-140	32-90	S	2	0
Elytroplastron	1	4 narrow	E	110-160	67-97	W	4	0
Polyplastron	1	5 narrow	R	123-205	98-123	L	7-9	0
Epiplastron	1	5 variable width	E	90-140	41-60	W	2	0
Epidinium	1	3 variable width	E	105-150	44-72	S	0-5	0-5
Opisthotrichum	1	1 cylinder	E	60-80	21-28		2	1
Caloscolex	1	1 complex	E	130-160	73-90		7	0-5

V, variable; R, rod; B, bent; H, hook; C, circular; E, elongated; N, none; S, small; W, wide; L, large.

Protozoal taxonomy is a classical exercise that depends on morphological traits, and there is more than one system for classifying them. Holotrichs can be divided into two major groups. *Isotricha* are large oval shaped organisms (approximately 135 x 70 μm), and the *Dasytricha* are smaller (60 x 30 μm). Entodinomorphs are typically identified by: 1) overall shape, 2) location of cilia on the body, 3) presence and shape of the region between the cilia (operculum), 4) number of skeletal plates, 5) shape of the skeletal plates, 6) shape of the macro-nucleus, 7) location of the micro-nucleus, 8) number of contractile vacuoles, and 9) number of caudal spines. Because these traits are not always constant, the taxonomy is at best arbitrary. 18S rDNA and rRNA is another approach, but few people have used these methods with ruminal protozoa.

Fungi. Small flagellated organisms were observed in the rumen, but these creatures were misclassified as flagellated protozoa. In the 1970's Colin Orpin, noted that the flagellates had a cell wall that contained chitin and a reproductive life cycle typical of fungi. The flagellates were fungal zoospores that eventually colonized plant surfaces to produce a mycelium. The mycelium gives rise to sporangia that release more zoospores, and the cycle continues.

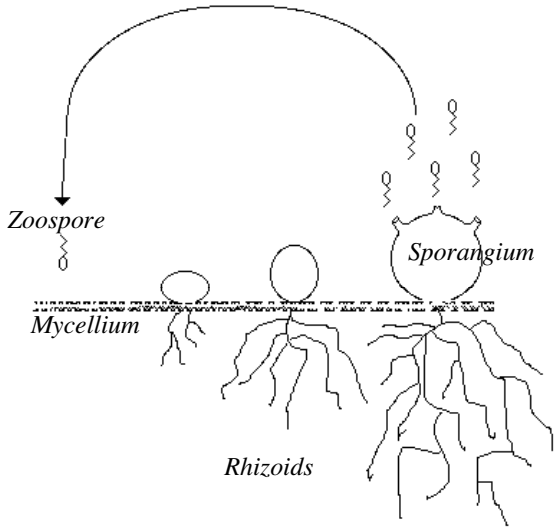

Fig. 10-6. Life cycle of ruminal fungi.

The life cycle of ruminal protozoa is approximately 24 h, but the fungi (like the protozoa) have increased their residence time by attaching to feed particles. Zoospores are small (6 to 10 μm), but the sporangia can be as large as 100 μm. The mycelia can be as long as 450 μm. Because fungi attach to feed particles, it has been difficult to estimate their biomass. Some workers have counted zoospores (as high as 10^6 per ml) or sporangia on leaf edges, but these estimates are not highly reliable. Chitin is more quantitative, and these measurements indicated that fungi can account for as much as 8% of the microbial biomass.

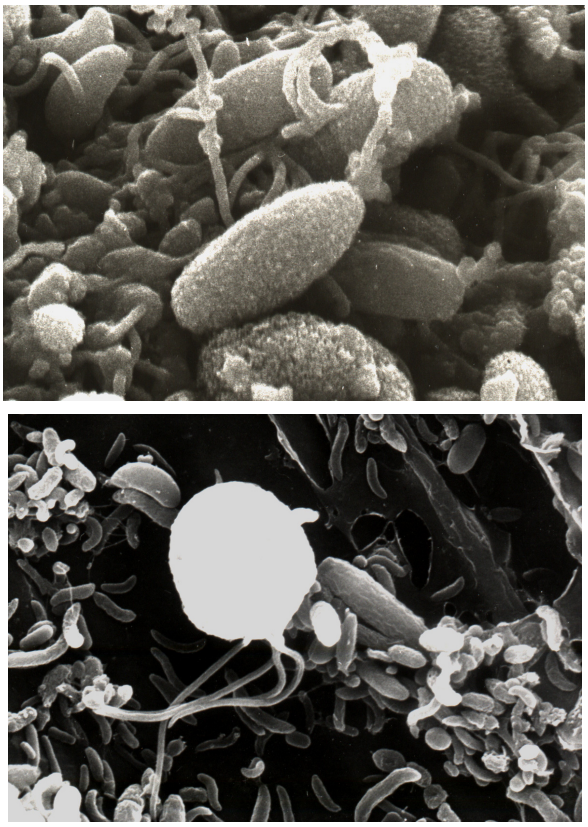

Fig. 10-7. Ruminal sporangia (top) and a zoospore (bottom).

Ruminal fungi digest cellulose and other fibrous materials, and fungi appear to have better enzymes than the bacteria. Because the mycelium penetrates deep into feed particles and breaks the fibers apart, there is an increase in surface area for fungi as well as bacteria. Fungal prevalence is diet-dependent, and fungi are more important when the diet is fibrous. There is an inverse relationship between ruminal fungi and bacteria. Dehority and Tirabasso showed that ruminal bacteria produced a bacteriocin-like-substance that inhibited fungi.

Because fungi were previously thought to be obligate aerobes, the discovery of anaerobic ruminal fungi was an important step in fungal phylogeny. 5S and 18S rRNA analyses supported the idea that these organism were, indeed, fungi, but their classification has been largely based on morphological traits. *Neocallimastix frontalis* and *N. patriciarum* both have polyflagellated zoospores, but *N. patriciarum* has more flagella and different fine structure than *N. frontalis*. *N. hurleyensis* have ring shaped hydrogenosomes. Vegetative *Piromyces communis* is smaller than *Neocallimastix,* and it has rhizoids that are highly branched. *Caecomyces communis* and *equi* lack a branching mycelia rhizoid system. *Oprinomyces joyonii* was once called *N. joyonii*, and it has a polycentric thalli, raised sporangia as well as polyflagellated zoospores. *Anaeromyces* is a mono-flagellated genus that was once called *Ruminomyces,* but the former name "takes precedent by prior publication."

Ruminal fungi have simple nutritional requirements and can be easily grown in the laboratory as axenic cultures. The zoospores are attracted to simple sugars and they have catabolite regulatory systems that can differentiate sugars. When cellobiose is provided, most species produce lactate, acetate, hydrogen, carbon dioxide and trace amounts of formate and ethanol. Because ruminal fungi are abundant in animals fed fibrous rations, there has been considerable interest in their cellulases. Xylanases, endo- and exo-β-glucanases, a β-glucosidase, a β-xylosidase, and arabinosidase activities have been identified and cloned, but the nature of the cellulose degrading system has not been clearly defined. *Clostridium thermocellum,* a non-ruminal bacteria, has cellulases that are organized into discrete structures, cellulosomes. Wood and his colleagues indicated that ruminal fungi may have similar structures.

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Chapter 11

Enumerations and Activities

Direct counts. Protozoa are large enough to be counted directly, but their lysis and association with feed particles is a complicating factor. Protozoa can, however, be fixed with formaldehyde and counted later. Protozoa have a unique amino acid, aminoethylphosphonic acid, but it is relatively difficult to measure, can vary with the type of protozoa present and was found in the rumen of a defaunated sheep. Fungi are also large enough to be counted directly, but they have more than one life form. Zoospores and sporangia on leaf edges have been used to assess fungal mass, but the standard deviations can be larger than mean value. Chitin is unique to

fungi, and it has also been used to estimate fungal numbers.

Serial dilution. Because bacterial numbers in natural habitats are often very large, microbiologists have used serial dilution as a method for determining number. Liquid samples are serially diluted (e.g., 10-fold increments) until growth is no longer observed. Numbers based on serial dilution can give the researcher an estimate that is accurate in the 'log' range, but it is not usually accurate with in the nutritional range (e.g., a 10% difference). Many ruminal bacteria attach so tightly to feed particles that they cannot be removed, and bacterial enumeration is usually based on the free-floating or loosely attached population rather than the total.

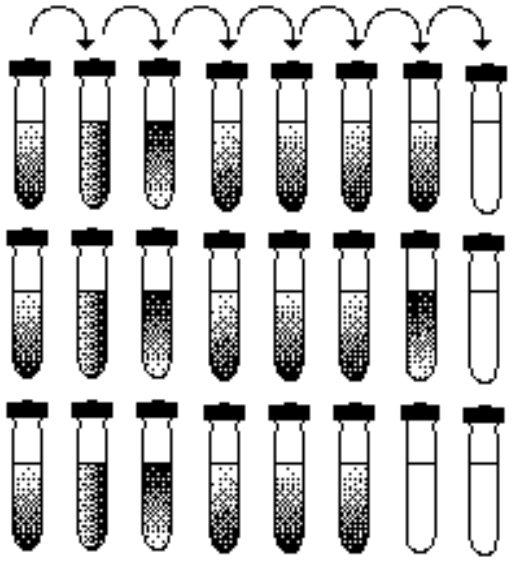

Fig. 11-1. Serial dilution of a bacteial culture. Growth occurs until cells do not reach the next dilution tube.

Plate counts and MPN. Serially diluted samples can be spread on the surface of agar plates to determine colony forming units (CFU), but some bacteria do not grow well on agar surfaces. When *P. bryantii* $B_1 4$ was serially diluted in an anaerobic glove box, the plate count was 10- to 100-fold lower than the one obtained in liquid broth (unpublished results). Plate counts are also confounded by the fact that some bacteria spread across the surface of the agar and cannot be counted as individual cells. When a plate count cannot be performed, it is possible to determine the most probable number (MPN) in liquid broth.

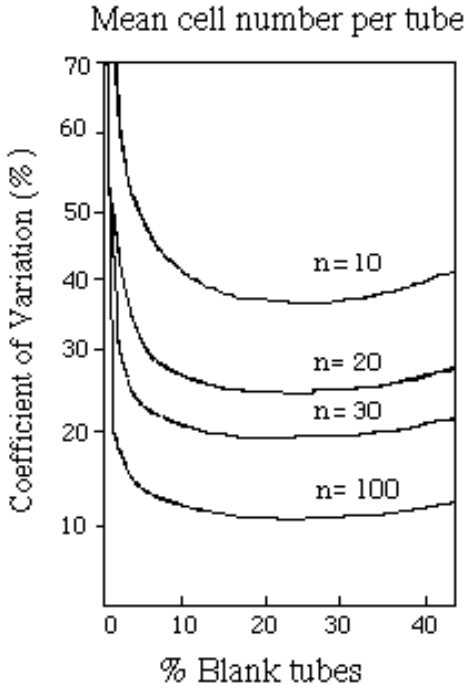

Fig. 11-2. The use of most probable number (MPN) method to determine bacterial count.

The MPN method is based on a chi distribution, and the values are derived from a table of codes. For example:

3 tubes grew at 10^{-6}

2 tubes grew at 10^{-7}

0 tubes grew at 10^{-8}

Code = 3 2 0 = 0.053

MPN = 0.053×10^{-7} *or* 5.3×10^{-5}

Plate counts are only as good as the selection pressure of the growth medium, and the total count is not necessarily the count of a specific species. However, if selection and enrichment is not possible, end-products in a MPN tube can be measured even if contaminating bacteria are present.

Table 11-1. Codes for most probable number (MPN) determinations. Taken with permission from Koch (1981).

CODE			X	CODE			X
5	5	0	0.128	3	3	0	0.064
5	4	1	0.127	3	2	1	0.064
5	4	0	0.114	3	2	0	0.053
5	3	1	0.113	3	1	2	0.064
5	3	0	0.100	3	1	1	0.053
5	2	2	0.113	3	1	0	0.043
5	2	1	0.099	3	0	2	0.052
5	2	0	0.086	3	0	1	0.042
5	1	2	0.099	3	0	0	0.032
5	1	1	0.086	2	4	0	0.062
5	1	0	0.073	2	3	0	0.051
5	0	2	0.085	2	2	0	0.041
5	0	1	0.072	2	1	1	0.040
5	0	0	0.060	2	1	0	0.030
4	5	0	0.106	2	0	2	0.040
4	4	0	0.093	2	0	1	0.030
4	3	1	0.092	2	0	0	0.020
4	3	0	0.080	1	3	0	0.038
4	2	1	0.080	1	2	1	0.038
4	2	0	0.068	1	2	0	0.029
4	1	2	0.080	1	1	1	0.028
4	1	1	0.068	1	1	0	0.019
4	1	0	0.056	1	0	1	0.019
4	0	2	0.067	1	0	0	0.110
4	0	1	0.056	0	2	0	0.018
4	0	0	0.045	0	1	1	0.018
3	4	0	0.076	0	1	0	0.009
3	3	2	0.086	0	0	1	0.009
3	3	1	0.075				

First order kinetics. Bacterial numbers can also be estimated from the rate of product formation. When the reaction is first order and substrate is in excess, the amount of product produced is proportional to the enzyme (bacterial) concentration and time (*see Appendix,* page 110). If the time or amount of enzyme (bacteria) is doubled, twice as much product is produced. In the 1950's, Hungate used first order kinetics to estimate the number of bacteria that were free floating bacteria and those that were attached to feed particles. He obtained a sample of ruminal digesta and centrifuged it briefly to separate the feed particles (pellet) from the free-floating bacteria (supernatant). The pellet was re-suspended in anaerobic buffer, and both fractions were given excess glucose. Because the fraction containing feed particles (pellet) produced gas 3-fold faster the supernatant, he concluded that 3/4 of the bacteria were attached to feed particles.

Molecular probes. With the advent of molecular techniques, researchers used specific probes to monitor species or strains within a mixed population. The first studies used rRNA but more recently rDNA has been used as a target molecule. The technique is, in principle, quite simple. The RNA or DNA is isolated from the mixed population and annealed to a filter (e.g., nitrocellulose or nylon). The filter is washed with a labeled probe (specific to the target population). If the sample is diluted and the target probe is compared to a universal probe (reacts with all the bacteria), it is possible to estimate the percentage of bacteria in the population.

In the early days, cell breakage and nuclease activities were problematic, but lysis buffers and a device known as the 'bead beater' greatly improved the extraction steps. Pure culture can be used to make sure that the probes do not react with other species. However, it is almost impossible to guarantee that the probes can react with all of the strains in a species. If the probes are strain-specific, the population size can greatly underestimated (See Chapter 22, *Models of Rumen Fermentation).* Because the detection method is based on a dilution of macro-molecule (e.g., slot blot), quantitation can still be a problem.

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Chapter 12

Bacterial Attachment and Extracellular Degradation

The rumen has an abundance of feed (approximately 10% w/v), but this material is primarily composed of large, relatively insoluble, and sometimes complex, polymers. These polymers must be degraded to low molecular weight substances (sugars, oligosaccharides, amino acids, peptides, etc.) by extracellular enzymes before they can be utilized. When bacteria secrete enzymes into the cell-free fluid, bacteria that did not produce the enzyme can also use the products. If the bacteria attach tightly to feed particles and use cell associated enzymes, they can (at least in theory) capture a larger fraction of the product for themselves.

Attachment. The easiest compartment to sample is the liquid fraction. Free-floating bacteria, protozoa and fungal zoospores utilize soluble nutrients (sugars, peptides, amino acids, etc.), but these nutrients are scarce except in the period immediately after feeding. Most free-floating (planktonic) types appear to be in a phase of transit, namely daughter cells of attached cells that are seeking a new site of attachment.

Some ruminal bacteria attach to the rumen wall. These typically proteolytic bacteria digest epithelial cells, play a role in urea hydrolysis and scavenge oxygen that might diffuse into the rumen from the portal blood, but they only account for a small fraction of the total population (approximately 1 to 2%). A much larger population of the bacteria (approximately 70% of the total) are found in biofilms on the surface of feed particles.

Bacteria attached to feed can be sub-divided into those that are loosely attached and those that are firmly bound. The outside surface (glycocalyx) of the bacteria has an excess of negatively charged groups (e.g., COO^-), and plant cell walls also have considerable 'cation exchange capacity.' These properties could lead to a repulsion, but the opposite seems to be the case. The negatively charged surfaces are bridged by divalent cations and plant lectins. Loosely attached bacteria can be liberated from plant surfaces by blenders, salts and cold shocks.

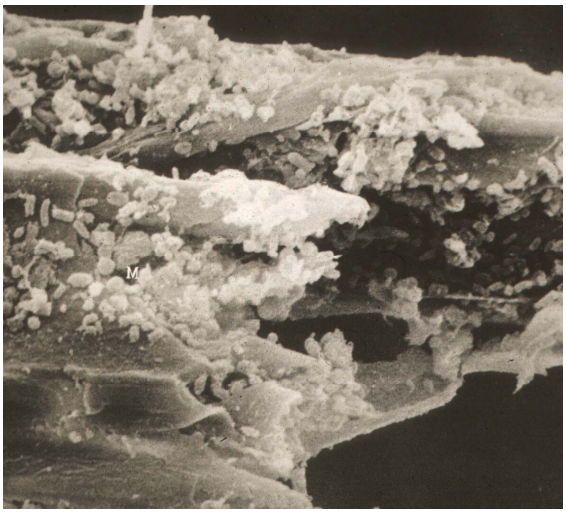

Fig. 12-1. Rumen bacteria attached to the inside surfaces of a forage particle. Taken with permission from Ogimoto and Imai (1981)

Less is known about the firmly attached bacteria, but adhesion seems to be mediated by specific receptors or cell-associated degradative enzymes. The idea that bacteria can selectively attach to certain surfaces is supported by microscopic observations. Ruminal bacteria do not bind to the waxy surfaces (cuticle) of forage particles. Instead, they prefer to attach to the inside material which is more easily degraded. Ruminococci cells that were treated with proteinases, dextrinases, periodate, formaldehyde, carboxymethylcellulose or methylcellulose lost their ability to bind cellulose, but those given large amounts of cellobiose or treated with heat and arsenate (a metabolic inhibitor) could still bind. However, these effects could be species specific. *F. succinogenes* cells that were treated with cellobiose, did not bind cellulose.

In the 1980's, Stack and Hungate re-examined the observation that *R. albus* strain 8 could not digest cellulose unless ruminal fluid was added to the medium, and they showed that the missing factor was phenylpropionate (PPA). Cultures not supplemented with PPA had less glycocalyx and could not adhere to cellulose, but those given PPA had more glycocalyx and bound tightly. In the rumen, PPA is derived from the

fermentation of phenylalanine or perhaps phenolics. The precise role of PPA in attachment is not known, but Hungate hypothesized that PPA was a "glue" that held a "raft" of enzymes together in a large complex. Pegden et al. showed that PPA stabilized the cellulase complex and prevented dissociation.

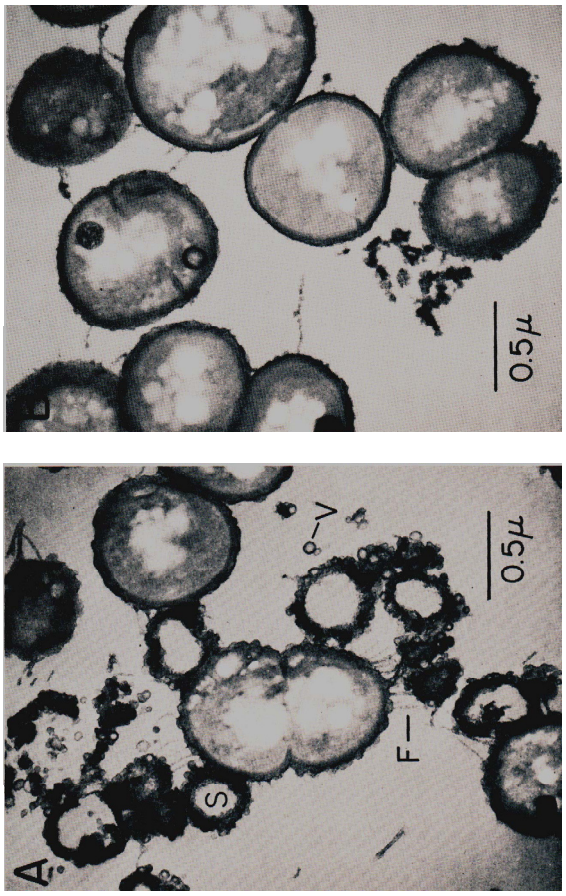

Fig. 12-2. *R. albus* strain 8 cells that had been grown without (top) and with (bottom) phenylpropionate. Taken with permission from Stack and Hungate (1984).

Ruminal bacteria bind to insoluble starch and a variety of other feed materials. Anderson found that *R. amylophilus* starch binding sites could be removed with proteinase K or saturated with maltose and maltodextins. Based on these results, it appears that *R. amylophilus* has a specific binding site for starch. Starch molecules are then transported through outer-membrane porins to the periplasm where they are hydrolyzed.

Enzymatic hydrolysis. The study of ruminal enzyme activity is complicated by the fact that the most active populations are found in biofilms that encompass many species. This consortium has enzymes that operate in a synergistic fashion. These enzymes have been classified according to the type of substrate they hydrolyze (e.g., cellulases, hemicellulases, pectinases, amylases, proteinases, etc.). However, it should be realized that individual species often produce more than one enzyme, and several enzymes may be needed to hydrolyze the entire polymer complex.

Cellulases. In the 1960's Halliwell and Bryant attempted to extract cellulases from the three most active species of cellulolytic bacteria (*R. albus, R. flavefaciens* and *F. succinogenes).* Cell-free extracts of the ruminococci solubilized some of the native cellulose, but the extracts from *F. succinogenes* had virtually no activity. Unpublished experiments performed by David Wilson and the author, indicated that only growing cultures of *F. succinogenes* could digest cellulose. If the cultures were limited by nitrogen or branched chain VFAs, cellulose digestion ceased.

Studies of cellulose digestion by *F. succinogenes* are complicated by the fact that the cultures die rapidly once they reach stationary phase. However, cultures actively growing on cellobiose have cellulase activity, and it is possible to use the cellulose-dependent succinate production as an index of first order rate. Such experiments indicated thiocellobiose (a non-metabolizable analog of cellobiose) was a strong feedback inhibitor. Based on these results. it appears that the cellulases of *F. succinogenes* cannot function unless the cells take up and metabolize the end-products of cellulose digestion.

Because cellulose is often digested slowly and incompletely in the rumen, it has generally been assumed that cellulose was refractory to hydrolysis. However, experiments with different types of cellulose indicated that surface area is a more important characteristic than crystallinity per se. When *F. succinogenes* was provided with finely ground ball milled filter paper, the initial rate of cellulose digestion was as fast as the rate of cellobiose fermentation. The rate of cellulose digestion only declined when the

surface area was saturated and the daughter cells could no longer bind.

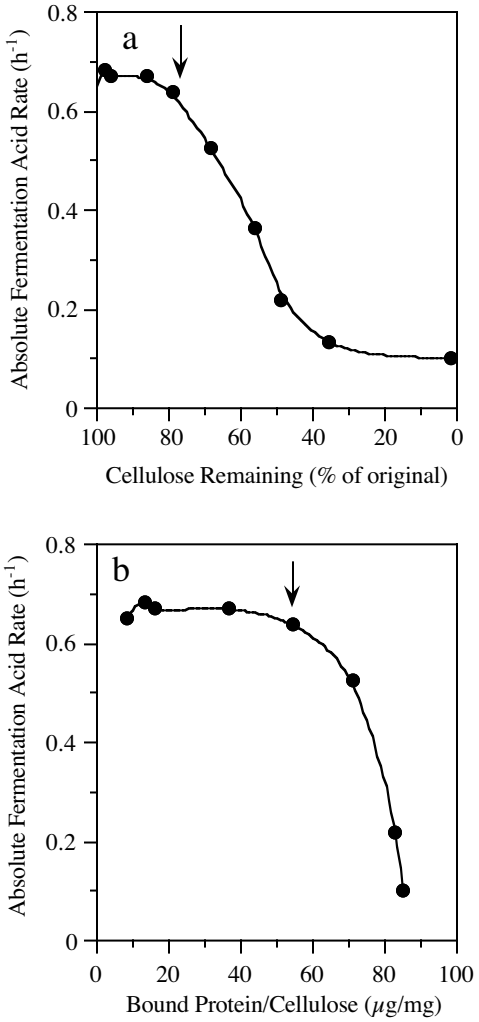

Fig. 12-3. The digestion of finely ground ball milled Whatman filter paper by *F. succinogenes* S85. The arrows show the point at which the rate starts to decline. Taken with permission from Fields et al. (2000).

The cellulosomes of *Clostridium thermocellum* are large enough to be observed with a scanning electron microscope, and genetic studies indicate that they have two functional domains, a catalytic domain that hydrolyzes the β-1,4 bonds of cellulose, and a 'scaffoldin' skeleton that anchors the cells to cellulose. The scaffoldin protein in turn has a cellulose binding domain (CBD) which facilitates the activity of the active site. Because *C. thermocellum* and ruminococci are closely related, Gram-positive bacteria, there was considerable interest in CBDs and the possibility of a similar paradigm. Ruminococci and *F. succinogenes* have proteins with CBDs, but evidence for a cellulosome is much less convincing. The cellulases of ruminococci are embedded in very large complexes, and further work is needed to define this architecture.

'Cellulases' have in many cases often defined by carboxymethylcellulose (CMC) hydrolysis, but CMCases do not necessarily give a bacterium the ability to utilize native, insoluble cellulose. Cellulolytic and non-cellulolytic ruminal bacteria have enzymes that can degrade CMC, but these CMCases (β-1,4 endoglucanases) lack CBDs. Work with non-ruminal bacteria indicates that CMC-ases can operate in a synergistic fashion with exocellulases. However, few (if any) ruminal exocellulases have been identified. The definition of cellulases is further complicated by the observation that many CMCases are actually xylanases. Xylose and glucose have similar structures, and many xylanases lack the specificity to differentiate xylan, CMC or β-glucan.

β-glucanases, xylanases and mannanases, etc. CMCases hydrolyze cellodextrins (released by cellulases), but their real role is probably in β-glucan utilization. A variety of cereal grains (e.g., barley) have water soluble β-glucans, and this material can account for a significant fraction of the total dry weight (> 5%). A mutant strain of *P. bryantii* lacking CMCase activity lost its ability to grow on β-glucans, but it was still was able to grow slowly on cellodextrins. However, it should be realized that non-cellulolytic ruminal bacteria appear to have two distinctly different methods of β-glucan utilization. All CMCases hydrolyze β-glucans, but not all β-glucanases can hydrolyze CMC. *S. bovis* has this latter mechanism of β-glucan utilization.

Hemicellulose is a complex polymer that contains xylans and mannans as a core, but the core may be substituted with arabinose, glucose, other sugars, uronic acids or acetyl groups. The xylan chain may also be attached to lignin, and this ether linkage is highly resistant to ruminal degradation. Cellulolytic ruminal bacteria play a key role in hemicellulose digestion, but at least some of them are unable to

utilize the end-products (xylans, mannans, arabans, etc.). These products are hydrolyzed by a different group of enzymes, and some of these proteins have been purified and cloned (see Teather et al.).

Amylases. Modern rations often contain an abundance of cereal grain. Because starch grains are covered with a protein coat, protein degradation is typically the rate limiting step in starch utilization. If the protein coating is broken by mechanical or heat treatments, the rate of starch fermentation is generally increased (Chapter 22 *Manipulation of Ruminal Fermentation*). Because corn starch is encapsulated by zein, a hydrophobic protein, the rate of corn fermentation is often slower than other cereal grains.

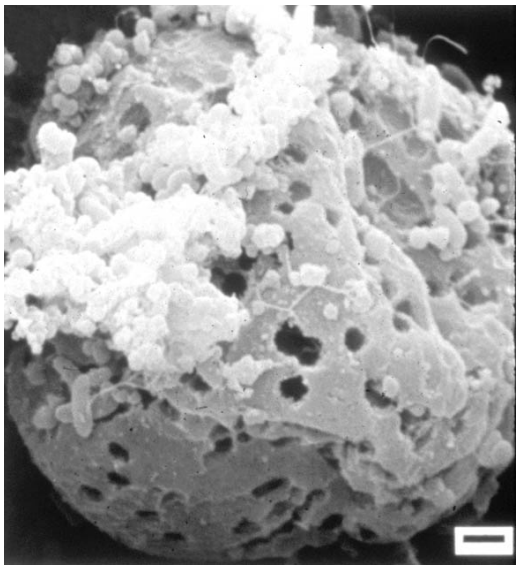

Fig. 12-4. Scanning electron micrograph of a starch granule from sectioned maize after 24 h of exposure in the rumen. The starch granule is colonized by bacteria of various morphotypes. Bar = 1 μm. (Courtesy of T. A. McAllister)

In the 1980's, Mike Cotta studied the amylolytic activity of ruminal bacteria (*R. amylophilus, P. ruminicola and bryantii, B. fibrisolvens,* and *S. bovis)*. These amylases were either cell associated or secreted into the cell-free supernatant. All of the amylases were endo-splitting enzymes that produced maltose and maltodextrins, and their production was induced by maltose and repressed by glucose. *S. bovis* and *R. amylophilus* had the highest activity, and *S. bovis* degraded even the most recalcitrant forms of starch. Starch is frequently substituted at the 6 position (1,6 linkage), and these linkages must be degraded by separate enzymes (e.g., amyloglucosidase).

Pectinases. Pectin is a polymer of galacturonic acid that can be methylated. Pectin acts as a 'cement' in plant cell walls, but demethylated pectin molecules are soluble in water and can be rapidly fermented. Ruminal pectin enrichments often yield *L. multiparus,* but it should be realized that a variety of other ruminal bacteria degrade pectin (e.g., *B. fibrisolvens* and *Prevotella* species). *S. bovis* has pectinolytic activity, but it is not able to utilize the galacturonic acid that is released.

Proteinases and peptidases. Because ruminally degraded protein is often wasted as excess ruminal ammonia, nutritionists have sought methods of decreasing proteolysis and deamination. Heat treatments that denature proteins and make them less soluble are an important tool for decreasing ruminal proteolysis, but some polypeptide chains are inherently more resistant to proteolysis than others. Mahadevan showed that circular proteins (e.g., ovalbumin) and those with disulfide bridges were hydrolyzed more slowly than other types. In the 1960's, Blackburn and his colleagues tried to purify proteinases from ruminal bacteria, but they had little success. The proteinases were bound together in large complexes and could not easily be separated.

Ruminant nutritionists have sought to use proteinase inhibitors as feed additives, but this approach is confounded by the number of species involved, the diversity of enzymes and the observation that proteinases are generally in excess. Brock et al. examined the effect of 21 proteinase inhibitors on the activity of mixed ruminal bacteria. All of the inhibitors had some effect, and no single inhibitor completely prevented hydrolysis. Serine proteinase inhibitors were most effective, and later work showed that 30 to 50% of the bacteria had extracellular proteolytic activity. *B. fibrisolvens*, *Prevotella* species, selenomonads, eubacteria, lachnospiras and even succinivibrios can be important. *S. bovis* has a proteinase that works synergistically with those of other bacteria.

Proteinases attack long chain polypeptides, but they do not hydrolyze the peptides that are released. Peptide con-

centrations in the rumen vary from approximately 1200 µg/ml shortly after feeding to 200 µg/ml pre-feeding. Some peptides are derived from the turnover of microbial protein, but feed proteins are a more important source. As much as 5% of the dietary protein can escape the rumen as peptide nitrogen. 'Hydrophobic' peptides rich in proline are more resistant to peptidase activity than hydrophilic peptides. Because proline introduces an angle into the peptide bond, special peptidases are needed to hydrolyze proline containing peptides. When lysine and methionine were linked to proline, their degradation rates decreased substantially.

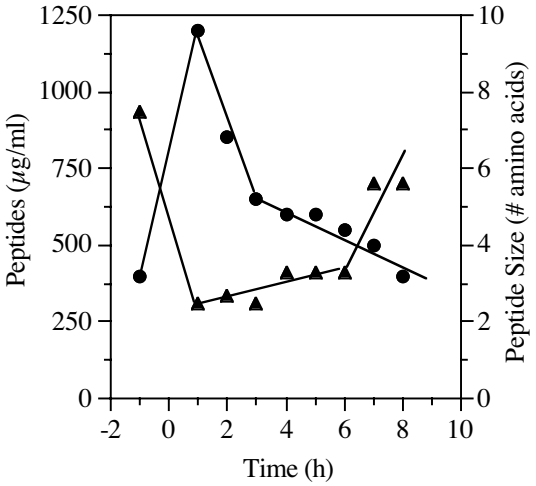

Fig. 12-5. The ruminal peptide concentrations (●) of a cow fed hay and the average size of these peptides (▲).

Pitmann and Bryant suggested that *Prevotella* species could take up peptides that had 10 amino acids, but it is unlikely that bacteria ever take up peptides longer than 5 amino acids. A variety of ruminal bacteria degrade peptides, and *Prevotella* species are important. Wallace identified a peptidase that produces dipeptides, and ruminal bacteria transport short peptides at a rapid rate.

Urea. Because most ruminal micro-organisms can utilize ammonia, non-protein nitrogen (e.g., urea) can be an important source of crude protein. Urea is hydrolyzed rapidly in the rumen, and ammonia accumulation can be so rapid that the animal is killed. Urea toxicity can be combated by making sure that there is an adequate supply of ruminally degraded carbohydrate to drive ammonia uptake and assimilation. Ruminal ureases are stimulated by nickel and are similar to the one produced by Jack beans. Ruminal urease is produced by bacteria that reside in the lumen of the rumen as well as on the rumen wall. The urease of *Selenomonas ruminantium* has been isolated, purified and studied in the most detail, but it should be noted that many other ruminal bacteria produce urease.

Nucleic acids. Nucleic acids can account for 5 to 10% of the crude protein in grasses, and the carbohydrate backbone of RNA and DNA (ribose and deoxyribose) is a lucrative substrate for ruminal bacteria. In vitro experiments with purified DNA indicate that nucleic acids are hydrolyzed rapidly by mixed ruminal bacteria, but feed processing methods can decrease this hydrolysis. When McAllan and Smith incubated mixed ruminal bacteria with nucleic acids, the purines were converted to hypoxanthine and xanthine, and the pyrimidines were converted to uracil and thymidine, but these intermediates did not accumulate in vivo.

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Chapter 13

Transport and Phosphorylation

Because bacterial densities in the rumen are very high, and the rate of polymer degradation is often slow, success of individual species is largely dependent on their ability to take up and compete for nutrients. Many non-ruminal bacteria can grow when nutrients are present at μM concentrations, and ruminal bacteria also have highly efficient transport systems. The rumen has been called a "inland sea," and ruminal bacteria often have transport mechanisms that are sodium-dependent.

Bacterial envelopes. The bacterial cell is defined by the cell membrane, a bilayer of phospholipids. The cell membranes of bacteria are morphologically similar, but they can alter their fatty acids so the membrane remains fluid over a wide range of environmental conditions. *E. coli* varies the fluidity of its membrane by regulating the activity of a special desaturase that alters that ratio of saturated and unsaturated fatty acids. Ruminal bacteria do not have any unsaturated fatty acids, but they can alter membrane fluidity by methylation. When the membrane fatty acids are methylated (branched-chain fatty acids), the phospholipids do not pack as tightly, and fluidity increases.

Cell membranes have proteins as well as phospholipids, and proteins can comprise 75% of the weight. Peripheral proteins can be removed from the cell membrane by chelating agents, but some membrane proteins are embedded in the lipid bilayer via hydrophobic domains. Proteins which traverse the cell membrane (e.g., transport proteins) often have several hydrophobic regions. Since the membrane is fluid, membrane proteins can move in a lateral fashion.

Bacteria have high concentrations of intracellular potassium and other solutes, and the intracellular pressure can be as high as 1000 psi. Because membrane lipids have little tensile strength, bacteria need other components to maintain their integrity. Most eubacteria (not archaea) have a rigid layer of N-substituted glucosamine and muramic acid linked together by 1,4 β bonds. These glucan chains are joined together by short peptides that often contain diaminopimelic acid. The entire structure is called the peptidoglucan.

Gram-positive bacteria have a thick peptidoglycan layer (approximately 30 nm), and it accounts for 40 to 90% of the cell wall weight. The peptidoglycan has lipoteichoic acid chains (LTAs) extending outwardly from the cell surface to make a glycocalyx. Glycocalyx protects the cell, but it is not as good a barrier as the outer membrane of Gram-negative organisms.

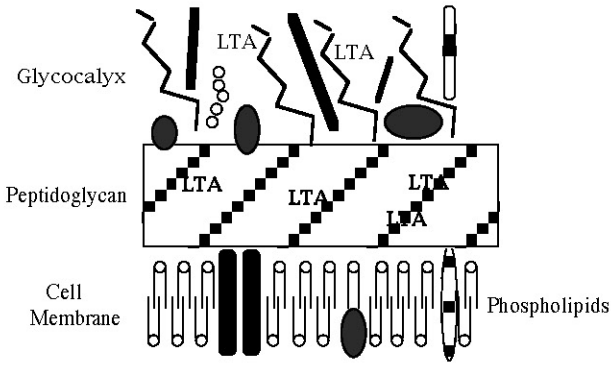

Fig. 13-1. The cell wall structure of a Gram-positive bacterium (LTA are lipoteichoic acids).

Gram-negative bacteria have less intracellular potassium content than Gram-positives, and they have a thinner peptidoglycan (only 5 to 10% of the cell wall weight). Gram-negative bacteria, however, have an outer membrane that excludes high molecular weight substances. The inner surface of this membrane consists of phospholipids, and the outer surface is coated with irregular lipopolysaccharide chains. The outer membrane is stabilized and joined to the underlying peptidoglycan by lipoproteins. Porins traverse the outer membrane and act as channels for low molecular weight compounds. In *E. coli*, porins are peptide trimers with a molecular weight cut off of approximately 600 daltons. The outer membrane also has transport proteins for specific high molecular weight substances (maltodextrins, cobalamin, etc.). The region between the cell membrane and the outer membrane is the periplasm. In *E. coli*, the periplasm contains more than 50 proteins and has a density which resembles a gel rather than an aqueous solution. Periplasmic binding proteins have a high affinity for substrates and transfer these

substrates to transport proteins located in the cell membrane.

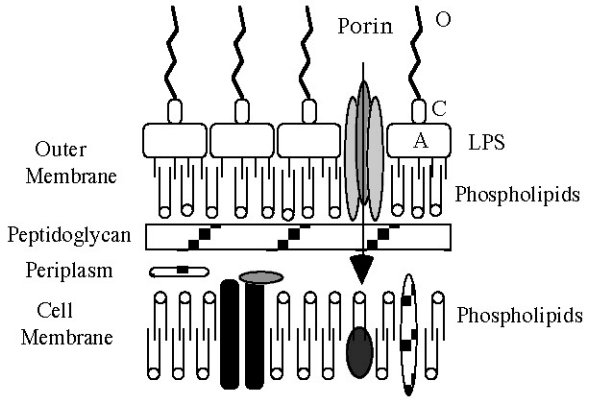

Fig. 13-2. Cell wall structure of Gram-negative bacteria (LPS are lipopolysaccharides).

Transport mechanisms. The lipid bilayer of cell membranes is impermeable to polar molecules, but small lipophilic compounds can cross by simple (passive) diffusion. Since passive diffusion rates are proportional to the concentration gradient, continued flux is dependent on substrate removal by internal metabolism, and in this case, cellular affinity is determined by the first enzyme in the pathway of utilization. Uncharged substrates like un dissociated acetate (CH_3COOH) and dissociated ammonia (NH_3) can enter ruminal bacteria by passive diffusion.

Many solutes are too lipophobic or large to pass freely across the lipid bilayer, but they may be taken up by a diffusion process involving specific protein carriers. Facilitated diffusion only allows net flux if the extracellular concentration is greater than the intracellular concentration. However, because a protein carrier is involved, facilitated diffusion can exhibit saturation kinetics, directionality and asymmetric velocities (i.e differential affinities). Facilitated diffusion systems have very high capacities (V_{max}) and can be advantageous when the extracellular concentration is high (e.g., soon after feeding). *S. bovis* has a facilitated diffusion system for glucose, and obligate amino acid fermenting bacteria have facilitated diffusion systems for some amino acids. When the extracellular concentration of solute is low, bacteria use active transport mechanisms to create a gradient across the cell membrane. The work of active transport may be driven by chemical bond hydrolysis (e.g., ATP) or ion gradients. Transport mechanisms driven directly by chemical bond energy are called primary active transport, and those coupled to ion gradients are known as secondary active transport.

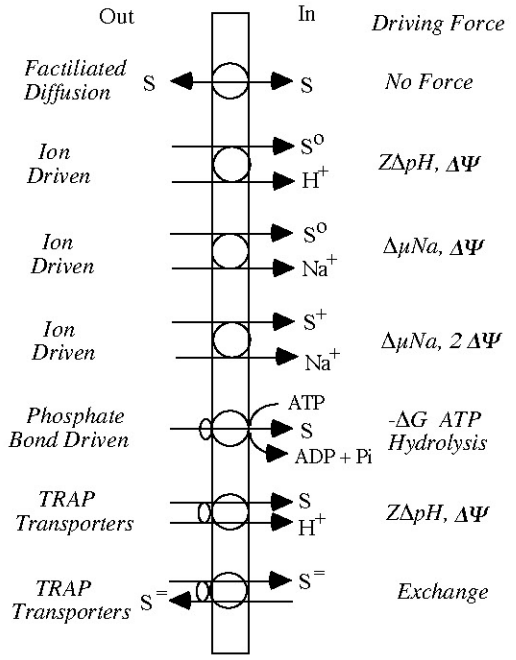

Fig. 13-3. Bacterial transport mechanisms and their driving forces.

Ion driven transporters are simple structures, and their small size allows for increased numbers in membranes that are already crowded with other proteins. Because primary active transporters are inherently large, they are often inducible systems. Primary transporters have very high affinities, and this characteristic can be crucial if the substrate concentration is low. Secondary transporters can catalyze substrate efflux as well as influx, but primary systems are typically irreversible. Substrate efflux would not be advantageous during starvation.

Primary active transport (driven directly by ATP hydrolysis) can create a very large concentration gradient across the cell membrane (as much as 1,000,000-fold). In the 1960's, Leon Heppel noted that Gram-negative bacteria lost

periplasmic proteins after they were subjected to an osmotic shock, and these cells did not have primary active transport activity. Periplasmic proteins bind substrates and donate them to carriers in the cell membrane. Gram-positive bacteria also have primary active transporters, but in this case the 'periplasmic' component is fused to the membrane carrier.

Recent work indicates that eubacteria and archaea have another method of secondary active transport (tripartite-ATP-independent periplasmic or TRAP transporters). TRAP transporters have binding proteins similar to those found in primary active transport. These binding proteins increase the substrate affinity of carrier, but ion gradients (not ATP) are the driving force for transport. In some cases, TRAP transporters act as antiport mechanisms that couple the uptake of one tricarboxylic acid to the efflux of another. For example, the nonruminal bacterium, *Shewanella,* takes up fumarate, respires it, and excretes succinate via a TRAP transporter that acts as an antiporter. TRAP transporters have not yet been detected in ruminal bacteria.

The driving force of ion driven (secondary) transport is dependent on the coupling ion (e.g., H^+ or Na^+), the net charge of the overall transport process, and the magnitude of individual driving forces. These driving forces are the electrical potential ($\Delta\Psi$), the chemical gradient of protons ($Z\Delta pH$) and chemical gradient of sodium ($\Delta\mu Na$). When the overall transport process is electroneutral (e.g., H^+ coupled with S^-), $\Delta\Psi$ is not a driving force, but $\Delta\Psi$ can be a driving force if the process is electrogenic (e.g., H^+ coupled with S^0). If the transporter is a proton symport system, $Z\Delta pH$ is a driving force. $\Delta\mu Na$ is a driving force if the carrier is coupled to sodium. The study of secondary active transport was enhanced by the work of Kaback. He noted that membranes from lysed bacteria re-assembled to form small spheres or vesicles. Because the vesicles lacked enzymes and other internal 'machinery,' transport mechanism could be differentiated from catabolism.

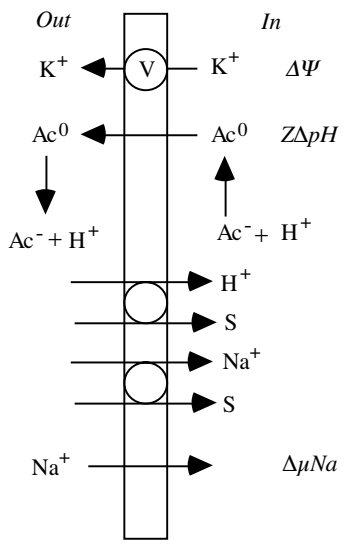

Fig. 13-4. The use of valinomycin (V) and potassium diffusion to create an artificial $\Delta\Psi$. An artificial $Z\Delta pH$ can be created by acetate diffusion, and a $\Delta\mu Na$ can be created by adding sodium to the extracellular buffer. These driving forces can then be used to drive substrate transport.

Transport driving forces can be artificially generated. When membranes are treated with the ionophore, valinomycin, they become permeable to potassium but not other ions. If membrane vesicles or de-energized cells are loaded with potassium and diluted into a buffer lacking potassium, potassium efflux creates an artificial $\Delta\Psi$. If vesicles are loaded with acetate and diluted into buffer lacking acetate, the efflux of un-dissociated acetate and its dissociation in the extracellular space creates an artificial $Z\Delta pH$. An artificial $\Delta\mu Na$ is generated by adding sodium to the extracellular buffer.

In the 1970's, it became apparent that many bacteria had a mechanism of transport that was linked to the hydrolysis of phosphoenolpyruvate (PEP) rather than ATP or ion gradients. The PEP phospho-transferase system (PTS) is technically a group translocation mechanism not active transport, but it, too, can lead to an accumulation of substrate inside the cell. *S. bovis, S. ruminantium* and *M. elsdenii* have PTSs for sugars.

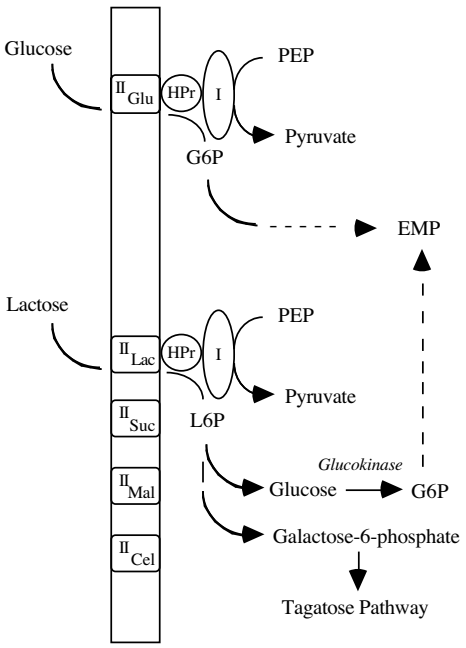

Fig. 13-5. The PTSs of *S. bovis*. *S. bovis* has at least 5 different enzyme IIs (glucose, lactose, sucrose, maltose and cellobiose).

When PEP is dephosphorylated by the PTS, the phosphate is donated to a soluble enzyme (enzyme I). The phosphate is then transferred to a histidine containing protein (HPr) and in some cases to still another protein (enzyme III). The cascade of phosphorylation and dephosphorylation leads to enzyme II, a substrate specific protein in the cell membrane. The sugar or sugar alcohol is then phosphorylated by enzyme II as it passes across the cell membrane. Because enzyme IIs differ in their affinity for HPr, the PTS can be a mechanism of catabolite regulation. For instance, when *S. bovis* is provided with glucose or sucrose, it does not take up other sugars (see Chapter 20, *Regulation of Metabolism)*.

Phosphorylases and hydrolases. Monosaccharides taken up by the PTS are also phosphorylated as they pass through the cell membrane, but those entering the cell by active transport or facilitated diffusion must be phosphorylated by kinases (e.g., gluco-kinase). Disaccharides taken up by active transport must be degraded to mono-saccharides and these reactions can be catalyzed by hydrolases or phosphorylases. Cellobiose phosphorylase was first recognized in *Clostridium thermocellum*, but it was soon detected in *R. flavefaciens*. *F. succinogenes* also has cellodextrin and cellobiose phosphorylases. Because phos-phorylases are reversible, they can also catalyze sugar efflux from the cell (see Chapter 16, *Crossfeeding)*. *S. bovis* has a phosphorylase that cleaves sucrose and maltose even though it has PTS systems for both of these sugars.

If a disaccharide is hydrolyzed, the free energy of the bond is dissipated as heat, and both sugars must then be phosphorylated by a kinase. If disaccharides are cleaved by a phosphorylase, free energy from the bond is conserved, and one of the sugars is phosphorylated. The phosphorylation is at the carbon-1 position, but the phosphate can be transferred from the 1 to the 6 position by a mutase. Since one of the sugars is phosphorylated by the cleavage step, only half as much ATP is devoted to a kinase reaction as would occur with a hydrolase and active transport.

Table 13-1. Different strategies of transporting and cleaving sugars and subsequent costs of creating a sugar phosphate (e.g., glucose-6-phosphate) that can enter the EMP pathway.

Substrate, Transport, Cleavage	Active Transport	Kinase	PTS	~P per Hexose
Hexose, Active Transport	1/3 ATP	1 ATP	-------	1.33
Hexose,PTS	-------	-------	1 PEP	1.0
Disaccharide, Active Transport, Hydrolase	1/3 ATP	2 ATP	-------	1.17
Disaccharide, PTS, Sugar Phosphate Hydrolase	-------	1 ATP	1 PEP	1.0
Disaccharide, Active Transport, Phosphorylase	1/3 ATP	1 ATP	-------	0.67

*Values are expressed as ATP equivalents (~P). The cost of secondary active transport is assumed to be 1/3 ATP equivalent per molecule transported. If primary active transport were employed, the cost would be 1 ATP per molecule.

The PTS conserves energy when monosaccharides are the substrate, but the

advantage is not nearly so great for disaccharides. When disaccharides are taken up by the PTS, one of the sugar residues is phosphorylated, and the other one must still be phosphorylated by a kinase. If the disaccharide is taken up by primary active transport, and cleaved by a phosphorylase, the cells expend approximately the same amount of phosphate bond energy (ATP or PEP). However, if the disaccharide is taken up by ion driven transport and cleaved by phosphorylase, the cells expend less energy than the PTS and a hydrolase.

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Chapter 14

ATP Production

Ruminal fermentation is an exergonic process the converts carbohydrates and other substrates to partially oxidized fermentation end-products, and some of the change in free-energy is trapped as ATP. This ATP is then used to drive anabolic reactions (bacterial biomass formation) and maintain vital cell functions. Introductory textbooks of biology often describe ATP as the ‘energy currency’ of the cell. The idea that ATP is an 'energy rich' compound is a valuable teaching tool, but it is not entirely correct. In 1982, Nichols corrected this misconception. "It is frequently, and misleadingly, supposed that the phosphate anhydride bonds of ATP are 'high energy' bonds which are capable of storing energy and driving reactions in otherwise unfavorable directions. However, it should be clear that it is the extent to which the observed mass action ratio is displaced from equilibrium which defines the capacity of the reactants to do work, rather than the attribute of a single component." The re-definition of bioenergetics as chemical equilibria provides a theme for understanding the once disjointed concepts of electron transport, ATP formation, and transport.

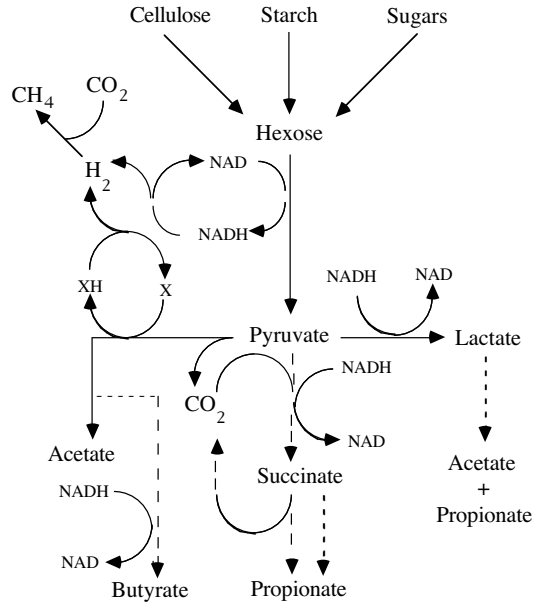

Fig. 14-1. The metabolism of hexoses by the various ruminal fermentation schemes. "X" denotes undetermined electron carrier.

EMP Pathway. The Embden-Meyerhof-Parnas (EMP) pathway is the most common pathway of hexose metabolism in microorganisms. The initial steps of the EMP require an input of ATP rather than its release, but phosphorylated derivatives are less permeable to the cell membrane and thus more easily "trapped" by the cell. After the 6th position of glucose is phosphorylated by an ATP-dependent kinase or the phospho-transferase system of transport, the phosphorylated derivative is isomerized and phosphorylated again at the carbon-1 position by another ATP-dependent kinase. Aldolase cleaves a carbon-carbon bond of fructose 1,6 diphos-phate, and the resulting triose-phosphates are oxidized by two dehydrogenases and dephosphorylated by kinases which produce ATP.

The ATP yield of the EMP pathway is typically two, but *Propionibacterium shermanii* has a phosphofructokinase that uses pyrophosphate as the phosphoryl donor rather than ATP. This substitution is not a complete case of 'getting something for nothing.' The pyrophosphate bond has a free energy of 4.6 kcal. Pyrophosphate is otherwise hydrolyzed to help 'assure the completeness of certain biosynthetic reactions like fatty acid and nucleoside syntheses.

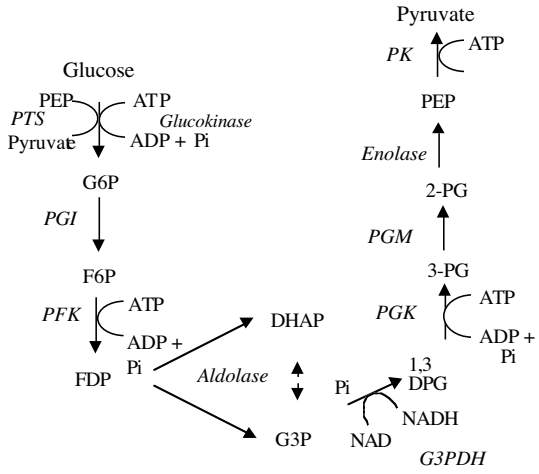

Fig. 14-2. Embden-Meyerhof-Parnas (EMP) pathway. Structures and abbreviations are shown in the *Appendix* on page 104.

Many non-ruminal bacteria have alternative catabolic routes (Entner-Doudoroff, pentose and phosphoketolase pathways). When glucose is metabolized by these pathways, glucose-6-phosphate is dehydrogenated and decarboxylated, and the labeling patterns are distinctly different. When Baldwin incubated mixed ruminal bacteria with ^{14}C-labeled glucose (1, 2, or 6 positions), the labeling pattern in acetate was consistent with the EMP pathway. More $^{14}CO_2$ arose from [1-^{14}C] glucose than from [2-^{14}C] glucose or [6-^{14}C] glucose, but more than 90% of the glucose was degraded by the EMP pathway. The EMP is advantageous to anaerobic bacteria because it maximizes the yield of ATP.

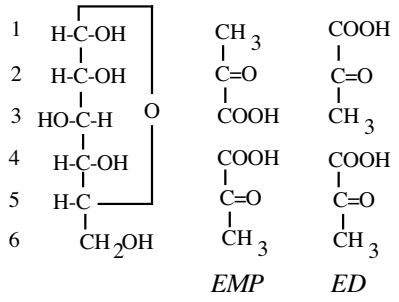

Fig. 14-3. Labeling pattern of glucose via the Embden-Meyerhof-Parnas (EMP) or Entner-Doudoroff (ED) pathways.

Pentose metabolism. Feedstuffs are primarily made up of hexose, but hemicellulose contains varying amounts of pentose. Pentoses can be metabolized by the transketolase and transaldolase reactions of the pentose cycle (see *Appendix* page 107) or by a phosphorolytic cleavage via phosphoketolase. The ATP yield of the pentose cycle is greater than the pathway involving phosphoketolase, and labeling studies indicated that 75% of the xylan was fermented by the pentose pathway while 25% would have been fermented via phosphoketolase.

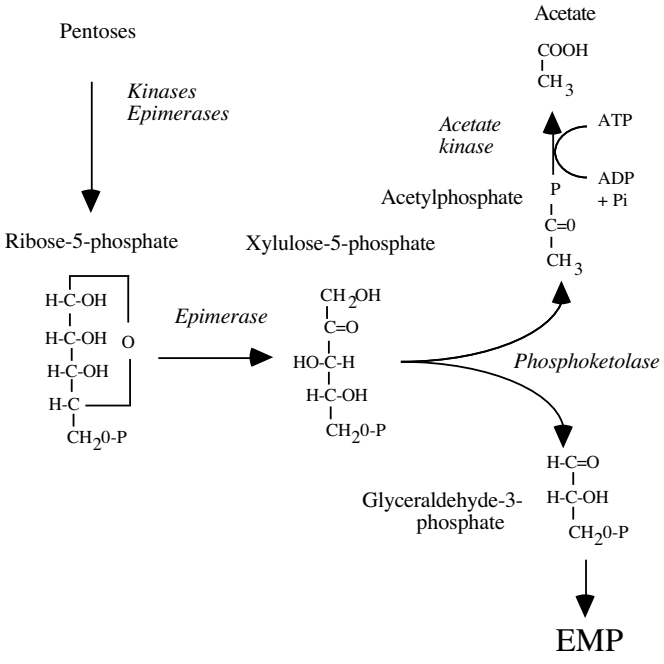

Fig. 14-4. Pentose catabolism via phosphoketolase.

Pyruvate metabolism. Pyruvate is a central intermediate in ruminal bacteria, and it can be converted to a variety of fermentation end-products. Reducing equivalent disposal is the primary factor determining its fate. During glycolysis, NAD is converted to NADH, and this NADH must be re-oxidized so fermentation can continue. The simplest mechanisms of re-oxidizing NADH are lactate or ethanol formation. Pyruvate can also be converted to acetyl CoA. Aerobes produce acetyl CoA by an NAD linked pyruvate dehydrogenase, but anaerobes do not have this enzyme.

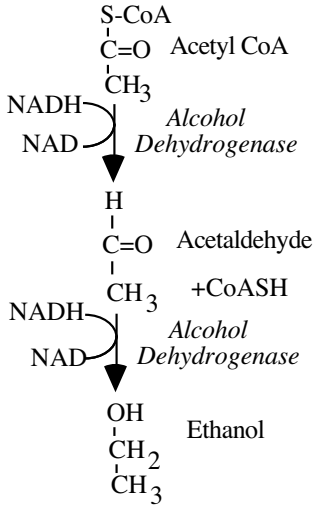

Fig. 14-5. Conversion of acetyl CoA to ethanol.

E. coli, *S. bovis* and butyrivibrios have pyruvate-formate lyases that produce acetyl CoA and formate. Alternatively, some bacteria (e.g., clostridia and selenomonads) have a pyruvate-ferredoxin oxidoreductase that produces reduced ferredoxin and CO_2 rather than formate.

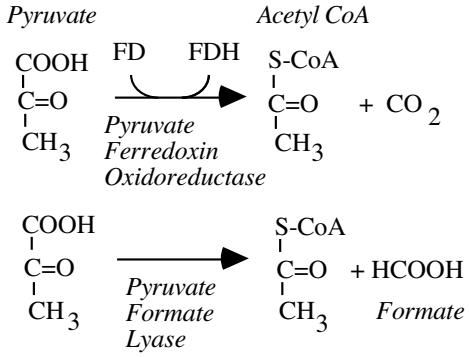

Fig. 14-6. Alternative schemes of pyruvate metabolism in ruminal bacteria.

Acetate and butyrate. Acetyl CoA arising from pyruvate metabolism can be converted to either acetate or butyrate. If the bacteria have phosphotransacetylase, energy of the CoA bond is conserved as a phosphate ester (acetyl phosphate). Acetyl phosphate can then be converted to acetate by acetate kinase, and ATP is produced.

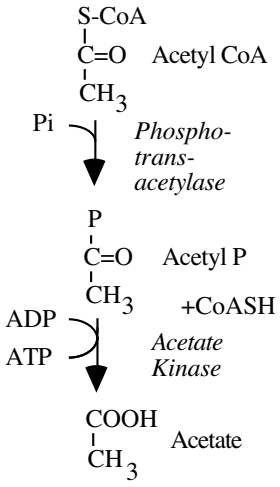

Fig. 14-7. Conversion of acetyl CoA to acetate via phosphotransacetylase and acetate kinase.

Alternatively, two acetyl CoA molecules can be condensed to produce acetyl-acetyl CoA. The acetyl-acetyl CoA can then be converted to butyryl CoA by a reversal of β-oxidation, and this scheme allows the bacteria to dispose of excess reducing equivalents (e.g., NADH or NADPH). Butyrivibrios are the most important butyrate-producing bacteria in the rumen, but they have two different schemes of butyrate production. Some species convert butyryl CoA to butyryl phosphate and use a butyrate kinase to obtain more ATP directly. Other species lack butyrate kinase and use a butyryl CoA acetyl CoA transferase. Because acetate is ultimately converted to additional acetyl CoA, phosphotrans-acetylase and acetate kinase drive additional ATP formation. *S. ruminantium* produces butyrate via a scheme employing butyrate kinase.

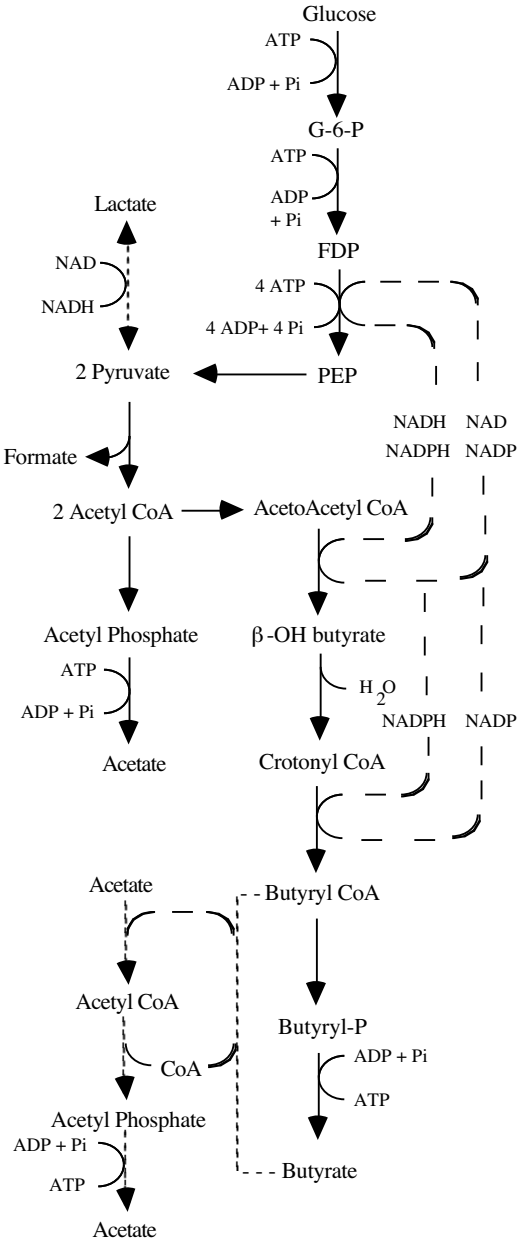

Fig. 14-8. Pathways of butyrate production in butyrivibrios.

Propionate and succinate. Propionate can also be synthesized by two different pathways, and these pathways can be differentiated by ^{14}C labeling patterns. When the label from [2-^{14}C] glucose is metabolized by the EMP pathway, the label is found in the number two position of pyruvate. If pyruvate (or phosphoenolpyruvate) is metabolized by the 'randomizing pathway,' the intermediates (fumarate and succinate) are symmetrical molecules, and the label is seen in the second and third positions of propionate.

Oxaloacetate is formed from either pyruvate or PEP via carboxylation reactions, and the OAA is then reduced to malate by a dehydrogenase. In some lactobacilli, pyruvate is reduced and carboxylated directly to malate by the malic enzyme. The conversion of malate to fumarate is a simple dehydration, but the fumarate reductase step is a complicated reaction that involves cytochromes, electron transport and ATP formation. Some bacteria produce succinate as an end-product, but succinate can be converted in stepwise fashion to propionate by other species.

The energetics of the randomizing pathway were at first precarious: 1) there were no obvious sites of substrate level phosphorylation, and 2) the carboxylation and formation of succinyl CoA could have caused a loss of ATP. This dilemma was solved when researchers showed that OAA synthesis was catalyzed by a biotin-dependent, transcarboxylation reaction that conserved the energy of succinyl CoA decarboxylation. Propionyl CoA hydrolysis does not lead to a phosphate intermediate or ATP formation by a kinase, but this free energy is conserved by a CoA transferase that drives the synthesis of succinyl CoA. It had been generally assumed that strict anaerobes did not possess electron transport systems capable of catalyzing ATP formation, but the discovery of a cytochrome-linked fumarate reductase reaction in *P. ruminicola* challenged this assumption.

In the 1960's, it became apparent that ruminal bacteria had another pathway of propionate production that did not involve succinate as an intermediate. When mixed ruminal bacteria were incubated with [2-^{14}C] glucose, the label only showed up in the second carbon of propionate, and subsequent work by Baldwin and his colleagues showed that propionate was being produced by a direct reductive pathway that involved acrylyl CoA.

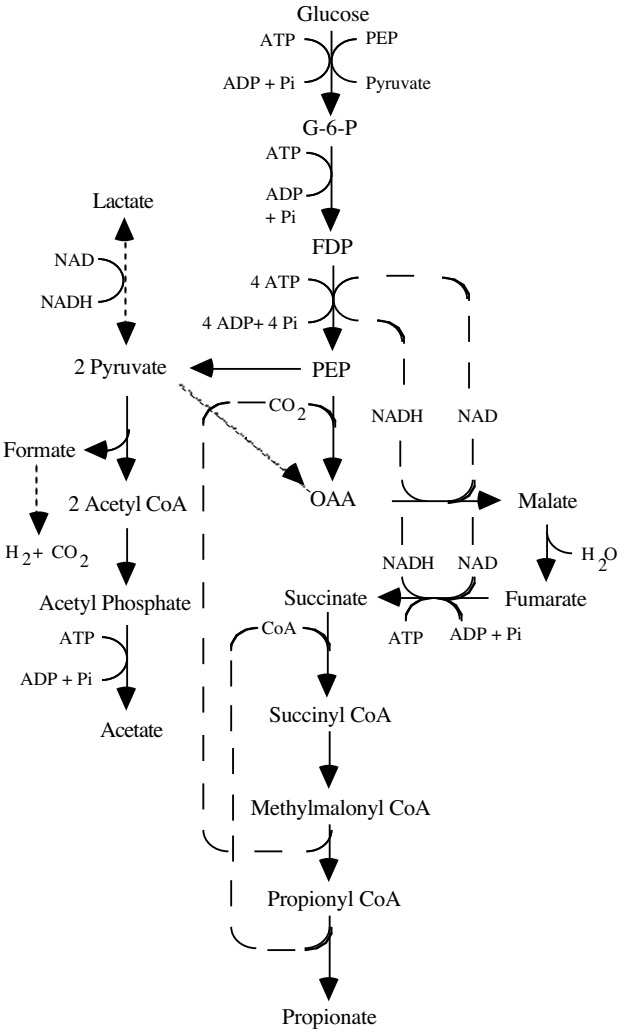

Fig. 14-9. Propionate production by selenomonads via the randomizing pathway.

In the direct reductive pathway, lactate (or pyruvate from glucose) is converted to a lactyl CoA ester which is subsequently dehydrated and reduced by a flavoprotein. The direct reductive pathway does not have a carboxylation or decarboxylation step. Acrylyl-CoA reduction involves an electron transport chain employing ferredoxin and an "electron transferring" flavoprotein, but ATP synthesis has not yet been demonstrated. *M. elsdenii* is the only ruminal bacterium known to have the direct reductive pathway.

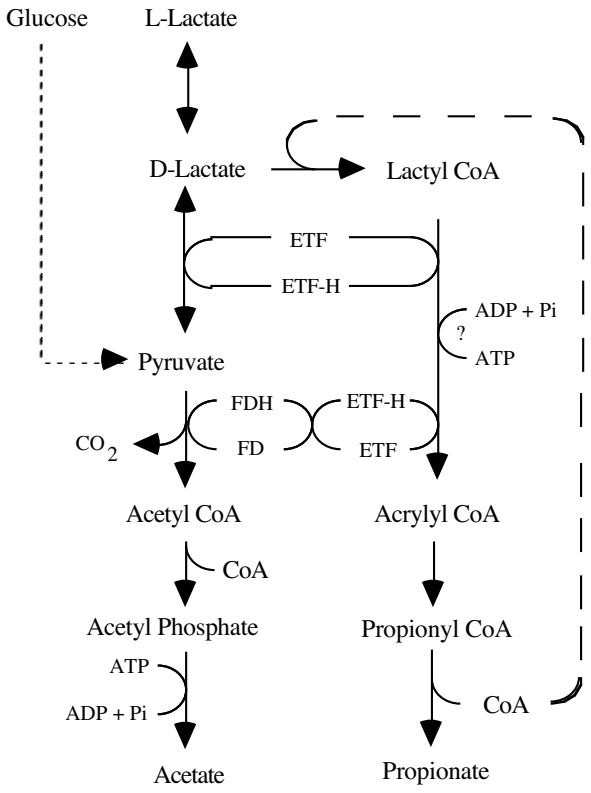

Fig. 14-10. The direct reductive pathway of propionate production in *M. elsdenii*. ETF denotes electron transferring flavoprotein that has not been carefully defined.

Higher volatile fatty acids (valeric, caproic, etc.) are formed by the condensation of acetyl-CoA and/or propionyl-CoA. The reversal of β-oxidation which leads to their formation represents another means of reducing equivalent disposal. As in butyrate synthesis, the ATP yield is decreased because the free energy of the CoA ester is used for carbon-carbon bond formation rather than phosphate ester and ATP formation.

Methanogenesis. Ruminal methane production involves the uptake of H_2 and the stepwise reduction of CO_2. Formate can also serve as a substrate for ruminal methanogenesis, but Hungate showed that most of the ruminal formate is first converted to H_2 and CO_2 by formate-hydrogen lyase. This conclusion was based on the observation that the K_m for formate was much higher than the ruminal concentration, and the rate of methane production was more closely correlated with dissolved H_2 than formate. In sewage and many other anaerobic habitats, volatile fatty acids act as substrates for methanogens, but these organisms grow so slowly that they are washed out of the rumen. As a result, little ruminal acetate is converted to methane. Short chain alcohols (from pectin breakdown), methylamine, and triethylamine can also be converted to methane.

Until the 1990's, the energetics of methane production were not well understood. The initial steps of CH_4 formation have low or even positive free energy changes, and only the last step, methanol reduction has a free energy change (-26.9 kcal) sufficient to drive ATP formation. However, the free energy change of the overall process is very negative (-32.4 kcal), and ATP can be produced by a chemiosmotic mechanism.

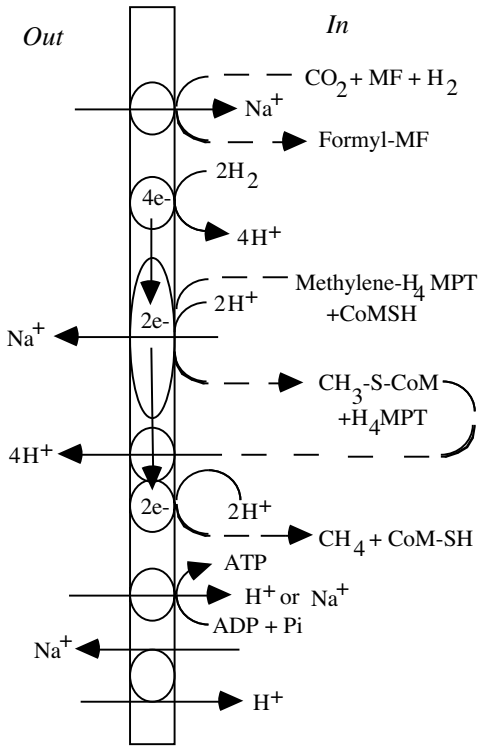

Fig. 14-11. The conversion of carbon dioxide and hydrogen to methane. Adapted with permission from Blaut et al. (1990).

CO_2 reduction is initiated by methanofuran (MF), a C1 carrier catalyzes the formation of formyl methanofuran. Tetrahydromethanopterin (H_4MPT) accepts the formyl group from formylmethanofuran (formyl MF). The carbon is then reduced to a methyl group via methenyl

and methylene derivatives. The reduction of methyl to methylene is mediated by F_{420}, an electron transport carrier unique to methanogens. The last step of CO_2 reduction to CH_4 is mediated by coenzyme M (2-mercaptoethane sulfonic acid), and it serves as the terminal acceptor for the methyl group. The reduction of methyl-coenzyme M is the most exergonic step of carbon dioxide conversion to methane, and this step creates a protonmotive force that then drives ATP synthesis.

Amino Acids. Amino acids can also serve as a substrate for ATP formation, but the yield is low. Only specialized bacteria can utilize amino acids as a sole energy source. In the 1930's, Stickland noted that some clostridia only fermented amino acids rapidly if they were provided as pairs of highly reduced (alanine, leucine, isoleucine, and valine) and oxidized (glycine, proline, arginine) amino acids. However, the Stickland reaction is not an important feature of ruminal deamination. Methanogenesis is the dominant mechanism of reducing equivalent disposal in the rumen. When mixed ruminal bacteria were treated with the hydrogenase inhibitor, carbon monoxide, ammonia, branched-chain volatile fatty acids and methane decreased.

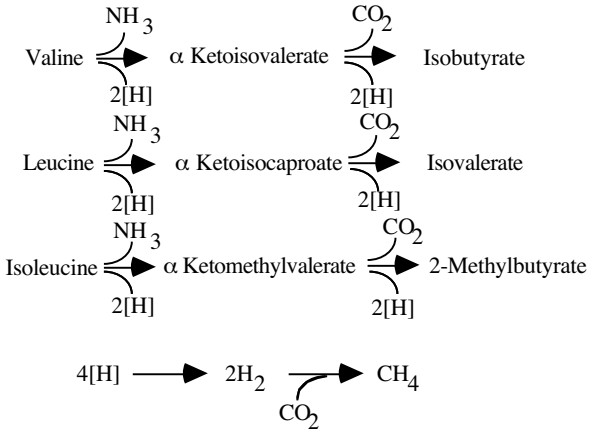

Fig. 14-12. Deamination and decarboxylation of branched chain amino acids and the utilization of the reducing equivalents by methanogens.

Obligate amino acid fermenting bacteria ferment amino acids rapidly, but the ATP yield is low. They ferment 20 to 25 amino acids to gain enough energy to polymerize a single amino acid into protein. Other ruminal bacteria (e.g., *M. elsdenii* and *Prevotella* species) can ferment amino acids, but the rate of ammonia and ATP production is slow. *M. elsdenii* grows on serine and threonine, but most other amino acids are not utilized rapidly. Branched chain amino acids provide maintenance energy to *M. elsdenii,* but the rate of ATP production is too slow for growth.

The obligate amino acid fermenting bacteria have energy conserving mechanisms. *P. anaerobius* and *C. sticklandii* use facilitated diffusion mechanisms to decrease the cost of transport. *C. aminophilum* has a membrane glutaconyl CoA decarboxylase that expels sodium. *C. sticklandii* uses ornithine efflux to create a sodium gradient that drives arginine or lysine uptake.

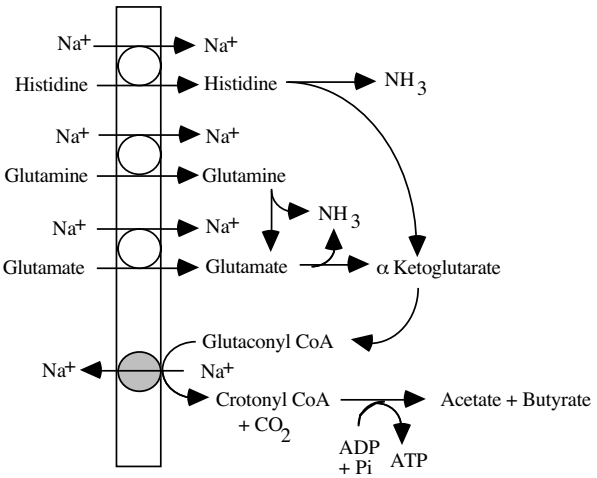

Fig. 14-13. Amino acid uptake and catabolism by *C. aminophilum*.

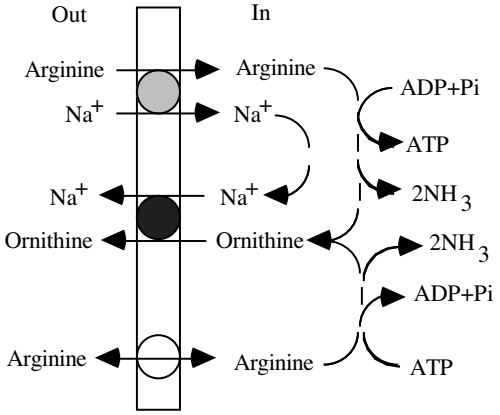

Fig. 14-14. The uptake of arginine by *C. sticklandii* and its conversion to ornithine. Ornithine efflux generates a sodium gradient that drives arginine uptake.

Organic acids. Organic acids have a positive redox state, and the ATP yield is typically lower than carbohydrates. Mixed ruminal bacteria degrade a variety of organic acids common in fresh forage, but the bacteria responsible for these degradations have not been identified. Silages can have as much as 10% lactate. Lactate serves as an energy source for *M. elsdenii* and *S. ruminantium,* but the yield is low.

Table 14-1. Reactions Producing ATP (~P) or Reducing Equivalents (2H).

Enzyme	Lactate	Acetate	Propionate	Butyrate	Ethanol	Valerate
Glucokinase	-1	-1	-1	-1	-1	-1
Phosphofructokinase	-1	-1	-1	-1	-1	-1
Glycertate kinase	2	2	2	2	2	2
Pyruvate kinase	2	2	2	2	2	2
Acetate kinase	-	2	-	-	-	-
Fumarate reductase	-	-	2	-	-	-
Butyrate kinase	-	-	-	1	-	-
Total (~ P)	2	4	4	3	2	-
G-3-P dehydrogenase	2	2	2	2	2	2
Lactate dehydrogenase	-2	-	-	-	-	-
Pyruvate oxidoreductase	-	2	-	2	2	1
Alcohol dehydrogenase	-	-	-	-	-4	-
Malate dehydrogenase	-	-	-2	-	-	-1
Fumarate reductase	-	-	-2	-	-	-1
β-OH butyrate dehydrogenase	-	-	-	-1	-	-
Butyryl CoA dehydrogenase	-	-	-	-1	-	-
β-OH valerate dehydrogenase	-	-	-	-	-	-1
Valeryl CoA dehydrogenase	-	-	-	-	-	-1
Total (2H)	0	4	-2	2	0	-1

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Chapter 15

Redox, Fermentation Balances, and Interspecies Hydrogen Transfer

Oxidation is the primary means of ATP generation in living organisms, but strict anaerobes cannot utilize oxygen as a terminal electron acceptor. If oxygen is not available, oxidations must be simultaneously coupled with reductions. Oxidation-reduction state (redox) is determined arbitrarily by assigning each [H] in the compound a value of -1/2 and each [O] a value of +1. As a consequence, highly reduced compounds have negative values while highly oxidized compounds have positive values.

Table 15-1. The empirical formulas and redox states of various compounds.

Compound	Formula	Redox
Valerate	$C_5H_{10}O_2$	-3
Methane	CH_4	-2
Ethanol	C_2H_6O	-2
Butyrate	$C_4H_8O_2$	-2
Propionate	$C_3H_6O_2$	-1
Acetate	$C_2H_4O_2$	0
Water	H_20	0
Glucose	$C_6H_{12}O_6$	0
Lactate	$C_3H_6O_3$	0
Formate	CH_2O_2	+1
Carbon dioxide	CO_2	+2
Succinate	$C_4H_6O_4$	+1
Tricarballylate	$C_6H_8O_6$	+2
Aconitate	$C_6H_{10}O_6$	+3
Citrate	$C_6H_8O_7$	+3
Cells	$C_{4.44}\ H_{8.88}\ O_{2.35}$	-1.88

The importance of redox is illustrated by the conversion of glucose to lactate. Glucose ($C_6H_{12}O_5$) and lactate ($C_3H_6O_3$) have the same overall oxidation-reduction state, but the molecules have a different distribution of this redox. All of the carbon atoms in glucose have a relatively neutral oxidation-reduction state. In contrast, the methyl carbon of lactate is highly reduced while the carboxyl carbon is highly oxidized.

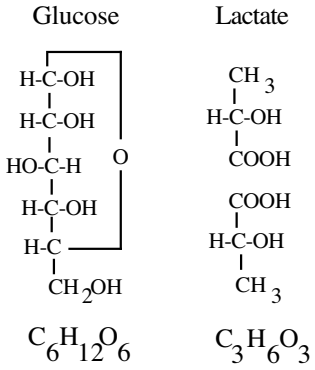

Fig. 15-1. The chemical and empirical formulas of glucose and lactate.

Fermentation balances. In 1960, Wolin devised a system for calculating fermentation balances. His scheme allows researchers to estimate the amount of carbon dioxide and methane that a ruminal fermentation would produce by measuring volatile fatty acids.

Step 1. Determine the mM concentration of the major VFA.

Example: 65 mM acetate, 19 mM propionate, 16 mM butyrate

Step 2. Multiply concentration by the redox state of VFA, CO_2 and CH_4. The sum of these values must be equal to zero, the redox state of glucose.

Example:

$$65(0)+19(-1)+16(-2)+CO_2(2)+CH_4(-2)= 0$$

$$or \quad -19-32+2\ CO_2\ -2CH_4 = 0 \quad or$$

$$2\ CO_2\ -2CH_4\ = 51 \quad or$$

$$CO_2\ -\ CH_4 = 25.5$$

Step 3. When glucose is converted to acetate, CO_2 is produced, and the ratio of CO_2 to acetate is 1 to 1. If butyrate is produced, the ratio is 2 to 1.

Thus, the sum of C1 compounds (CO_2 plus CH_4) is equal to sum of acetate plus butyrate:

$$CO_2 + CH_4 = 65 + 2(16) \text{ or}$$

$$CO_2 + CH_4 = 97$$

Step 4. Use simultaneous equations to solve for CO_2 and CH_4. There are two equations and two unknowns:

$$CO_2 - CH_4 = 25.5$$

$$\underline{CO_2 + CH_4 = 97}$$

$$2CO_2 = 122$$
$$CO_2 = 61$$
$$61 - CH_4 = 25$$
$$CH_4 = 36$$

Step 5. Write a balanced equation based on redox and concentration:

$$2(65)^{Ac} + 3(19)^{Pr} + 4(16)^{Bu} + 1(61)^{CO2} + 1(36)^{CH4} = 6(C_6H_{12}O_6)$$

$$348 = 6(C_6H_{12}O_6) \text{ or } 58 = (C_6H_{12}O_6)$$

$$58C_6H_{12}O_6 = 65Ac + 19Pr + 16Bu + 61\ CO_2 + 36CH_4$$

The Wolin fermentation balance has several inherent limitations:

1) Only hexoses are accommodated (ignores pentoses, amino acids, organic acids etc).

2) Cells are not considered as an end-product. Because cells have membranes that are composed of fatty acids and are relatively reduced (O/R = -1.88), methane is over estimated.

3) Hydrogen and other end-products are ignored.

Hydrogenase activity. Ruminal methanogens can use formate, but H_2 and CO_2 are the primary sources of ruminal methane. Non-methanogenic bacteria can use membrane bound hydrogenases to produce H_2 from reduced electron carriers, but this process can be thermodynamically unfavorable. Hydrogenases linked to reduced ferredoxin produce some hydrogen, but hydrogenases linked to NADH can be inhibited by even small amounts of hydrogen. Methanogens scavenge H_2 and keep the partial pressure of H_2 low enough so the NADH-linked hydrogenases can operate.

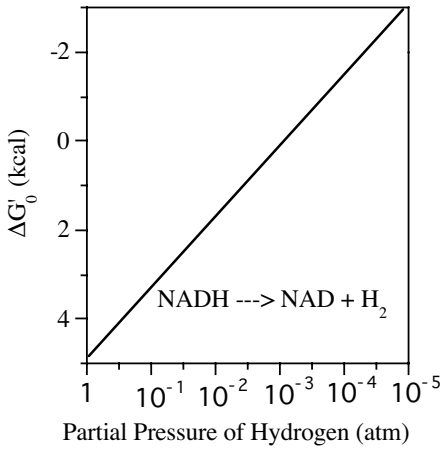

Fig. 15-2. The effect of H_2 partial pressure on the ΔG of NADH conversion to hydrogen by membrane bound hydrogenases.

Interspecies hydrogen transfer. In the rumen, methane is the dominant means of reducing equivalent disposal, and the interspecies transfer of hydrogen from carbohydrate fermenting bacteria to methanogens enhances acetate production and increases ATP. For example, when *R. albus* is grown in pure culture, some of the hexose is converted acetate, but the hydrogenase is unable to oxidize NADH arising from glycolysis. Alcohol production provides an alternative method of reducing equivalent disposal, but the ATP yield is lower.

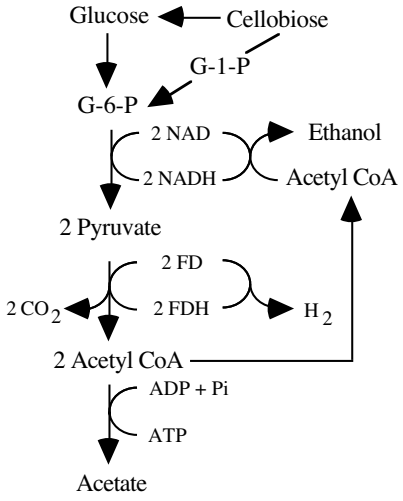

Fig. 15-3. The fermentation of cellobiose by *R. albus* grown as a pure culture. FD is ferredoxin.

In contrast, if *R. albus* is co-cultured with a methanogen, the partial pressure H_2 of is low, the membrane bound hydrogenase oxidizes NADH, more hexose can be converted to acetate, and the ATP yield increases.

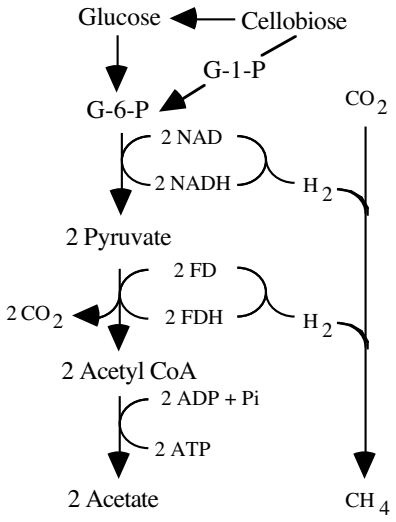

Fig. 15-4. The fermentation of cellobiose by *R. albus* in co-culture with a methanogen.

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Chapter 16

Crossfeeding

Early work with pure cultures of ruminal bacteria indicated that many species produced products not detected in ruminal fluid. The absence of some products (e.g., ethanol) could be explained by the inability of the pure cultures to transfer H_2 to methanogens. In other cases, the end-product of one species is simply substrate for another.

Many ruminal bacteria produce succinate, but succinate is subsequently decarboxylated to propionate by other species. Labeling studies indicate that as much as 50% of the ruminal propionate arises from succinate cross-feeding. Some strains of *S. ruminantium* can take up succinate and decarboxylate it, but it is unclear if the specific activity of *S. ruminantium* is high enough to explain ruminal flux.

The energetics of succinate decarboxylation are not well defined. *S. ruminantium* has a succinate transport system that is driven by protonmotive force, but this system is repressed by glucose and lactate. Non-ruminal bacteria can link methyl-malonyl CoA decarboxylation to sodium expulsion, but evidence for a similar reaction in *S. ruminantium* or other ruminal bacteria is lacking.

A variety of ruminal bacteria can produce lactate, but the concentration of lactate in vivo is usually very low. Three factors contribute to this observation. First, because lactate is an alternative mechanism of reducing equivalent disposal, interspecies H_2 may decrease its production. Secondly, bacteria such as *S. bovis* and *S. ruminantium* only produce lactate if their fermentation rates are very high. Thirdly, lactate can be used by a secondary population of bacteria (*M. elsdenii, Veillonella alcalescens,* and *S. ruminantium).*

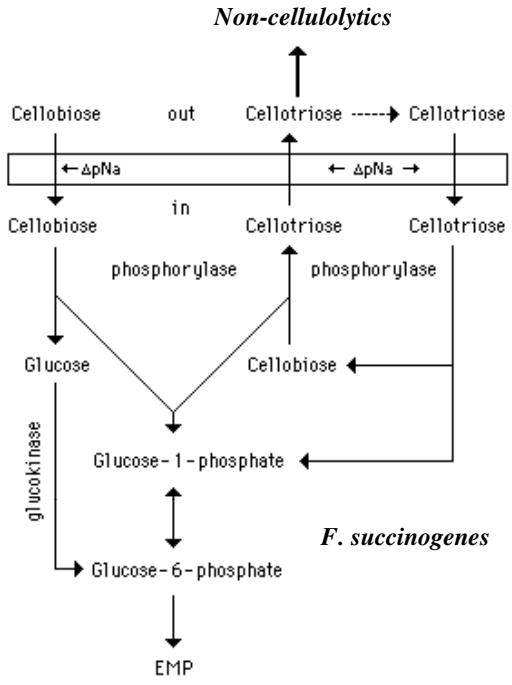

Fig. 16-1. Uptake of sugars by *F. succinogenes*, phosphorylation and efflux of sugars via reversible phosphorylases.

Cellulolytic bacteria are out-numbered by non-cellulolytics even if the diet is mostly fiber, and co-culture experiments showed that non-cellulolytic species like *S. ruminantium* could be co-cultured with cellulolytic species (e.g., *R. albus*) even if cellulose was the only energy source. Wolin hypothesized that the non-cellulolytic species were living on the extracellular products of cellulose digestion, but more recent work indicates that at least some cellulolytics have a reversible cellodextrin phosphorylase. Cellodextrins excreted by cellulolytic bacteria can be "stolen" by non-cellulolytics.

The interaction between cellulolytic and non-cellulolytics is even more complicated. All three of the prominent cellulolytic species *(R. albus, R. flavefaciens* and *F. succinogenes)* are unable to synthesize branched chain amino acids unless they have branched chain VFA as a carbon skeleton. Branched chain VFA are supplied by amino acid-fermenting bacteria that deaminate branched chain amino acids.

Another example of crossfeeding is seen in protein degradation. Obligate amino acid fermenting bacteria cannot hydrolyze proteins, but other proteolytic species can degrade proteins and produce peptide and amino acids. Conversely, *R. amylophilus* is a proteolytic bacterium, but it does not use amino acids. The utility of this seemingly futile activity can be explained by the fact that starch grains are often encapsulated in protein (e.g., zein). In order for *R. amylophilus* to get to the starch, it must first digest the protein layer.

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Chapter 17

Ruminal Fat Metabolism

Effects of fat on fermentation. Grazing ruminants consume a diet that is low in fat (< 5% of the dry matter), and it has long been recognized that large amounts of fat can be detrimental. However, fats can be pre-treated with calcium to produce an insoluble complex that is not toxic to ruminal bacteria, and this complex dissociates in the abomasum to release fatty acids (see Chapter 22, *Manipulation of Rumen Fermentation)*. In recent years, the value of supplemental fat has been recognized, and it can be an important nutrient for lactating dairy cattle and rapidly growing beef cattle. When supplemental fat is added to the ration, it is possible to decrease starch and prevent acidosis.

Most naturally occurring fats are present in plants as triglycerides, phospholipids or glycolipids, and these esters can be hydrolyzed by ruminal bacteria. Glycerol arising from this de-esterification is used by a variety of ruminal bacteria (*Anaerovibrio lipolytica,*

M. elsdenii and some strains *of S. ruminantium* and *B. fibrisolvens)*. Some non-ruminal bacteria degrade fatty acids, but these syntrophic bacteria grow very slowly and are found at low numbers in the rumen.

Non-esterified fatty acids can alter membrane fluidity, disrupt transport proteins and react with bacterial magnesium and calcium to form soaps. Gram-negative bacteria are more resistant to fatty acids than Gram-positive species that do not have an outer membrane to protect the cell membrane (e.g., ruminococci). The effect of fatty acids on pure cultures of ruminal bacteria is consistent with the overall effect of fat on ruminal fermentation, namely a decrease in fiber digestion, acetate to propionate ratio and methane (see Chapter 22, *Manipulation of Rumen Fermentation)*. The effect of fatty acids on methane production is dual fold. Fatty acids inhibit bacteria that produce hydrogen, a precursor of methane, and biohydrogenation is an alternative method of reducing equivalent disposal. This latter effect is, however, not as important as the former.

Fatty acid synthesis. In mammals, acyl groups (e.g. acetyl CoA) that give rise to fatty acids are found in combination with a multi-enzyme complex that serves as a carrier, but bacteria have a single acyl carrier protein (ACP). In bacteria, four steps are required to elongate the fatty acid chain: 1) acetyl-ACP reacts with malonyl-ACP to form acetoacetyl-ACP, 2) the acetoacetyl-ACP is reduced to produce β-hydroxybutyryl-ACP, 3) the β-hydroxybutyryl-ACP is dehydrated to produce crotonyl-ACP, and 4) the crotonyl-ACP is then reduced to produce butyryl-ACP. Butyryl-ACP and even longer chain derivatives can react with malonyl-ACP, and the cycle continues until C16 and C18 acids are produced. Because ruminal bacteria can substitute propionyl CoA and valeryl CoA for acetyl CoA as a starting point for fatty acid synthesis, ruminal bacteria often have a significant amount of odd chain fatty acid.

Aerobic bacteria (e.g. *E. coli*) have a special desaturase that can introduce double bonds into the fatty acid chain, and this desaturation increases membrane fluidity when the temperature is low. Ruminal bacteria lack this enzyme and have a much lower concentration of unsaturated fatty acids. Ruminal bacteria increase membrane fluidity by methylating their fatty acids. When the fatty acids are methylated, the chains are spread further apart, and fluidity increases.

Biohydrogenation. Fatty acids with double bonds are more toxic to bacteria than saturated fatty acids, and many ruminal bacteria use biohydrogenation as a detoxification reaction. (e.g., *B. fibrisolvens, Prevotella* species, and ruminococci). Tove studied the biohydrogenation mechanisms and showed that *B. fibrisolvens* had an unusual electron carrier (tocopherol-quinol, THQ). THQ shuttles electrons from flavins and NADH to unsaturated fatty acids via reductases found in the cell membrane. *B. fibrisolvens* produces *trans*-11 octadecenoic acid (C18:1) from linoleic acid, but it produces very little stearic acid (C18:O).

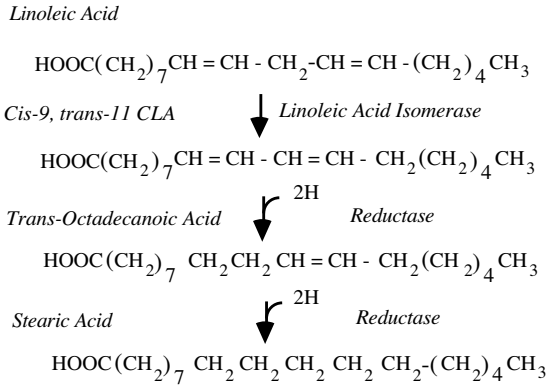

Fig. 17-1. Biohydrogenation of linoleic acid by *B. fibrisolvens*.

It had been assumed that *trans*-9, octadecanoic acid conversion to stearic acid occurred without an isomerase, but work with mixed ruminal bacteria indicates that *trans*-monene intermediates can arise (9, 10, and 11). Forages often have fatty acids with 3 double bonds (α or γ linolenic), and these acids are also hydrogenated via conjugated intermediates.

cis-6, cis-9, cis-12 γ-linolenic acid ->

cis-6, cis-9, trans-11 octadecatrienoic acid ->

trans-11 octadecenoic acid

or

cis-9, cis-12, cis-15 α-linolenic acids ->

cis-9, trans-11, cis-15 octadecatrienoic acid ->

trans-11 octadecenoic acid

The rumen appears to have two distinct populations of biohydrogenating bacteria. The 'A' group is made up of butyrivibrios, spirochetes and some intestinal bacteria, and they convert C18:2 to C:18:1, but not significantly to C18:0. The 'B' group bacteria convert C18:1 to C18:0, but they have not been definitively classified. Two of the B type bacteria belong to the genus *Fusocillus* and one was an unnamed Gram-negative rod. In vitro experiments indicated that the A group was more active than the B group, and Keeny noted that C18:1 accounted for approximately 7% of the fatty acids in rumen contents of cattle fed hay. The effect of ruminal pH on biohydrogenation has not been studied in great detail, but Latham et al. noted that diets deficient in fiber increased the proportion of unsaturated acids in rumen contents and milk.
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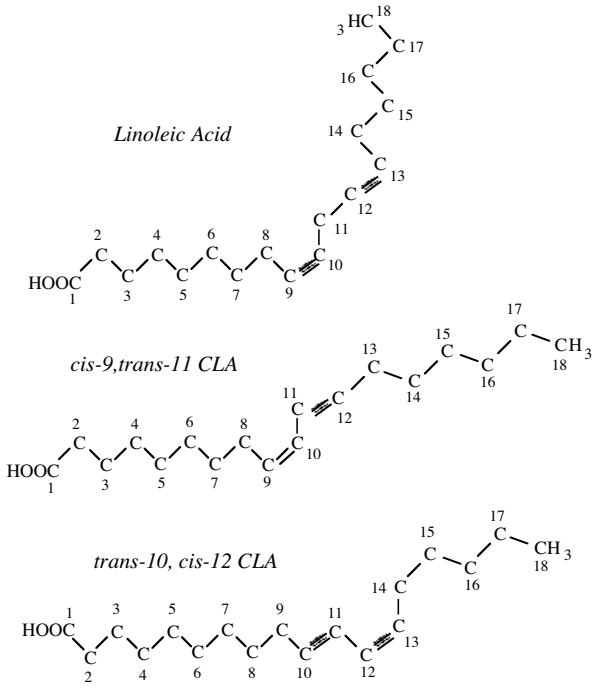

Fig. 17-2. The structure of linoleic acid and its conjugated analogs.

CLA. The first step in biohydrogenation is typically an isomerization that moves the double bond one position to produce a 'conjugated' intermediate prior to the reduction steps. In the 1930's, Booth noted cows fed grass in the summer had a different milk composition and subsequent work by Boer showed that rats fed summer milk grew better than those fed winter milk even though the fat content was similar. In 1963, Riel noted that summer milk fat had more conjugated (dienoic) acids than winter milk.

In recent years, there has been a keen interest in conjugated linoleic acid (CLA). CLAs inhibit chemically-induced tumors, prevent atherosclerosis and improve the protein to fat ratio of animals. *B. fibrisolvens* has often been used as a model for ruminal CLA production, and it produces the *cis*-9, *trans*-11 CLA, the one most likely to be produced by grass fed cows. When *B. fibrisolvens* cultures were treated with low concentrations of linoleic acid, the linoleic acid was converted to *trans*-octadecenoic acid (*trans*-C18:1) and to a lesser extent stearic acid (18:0). CLA only accumulated if the linoleic acid concentration was high enough to inhibit growth, metabolism and reductase activities. Because CLA production was enhanced by oxygen, it appeared that CLA was a by-product of dying cells that could no longer metabolize glucose and produce reducing equivalents. Washed cell suspensions of *B. fibrisolvens* produced CLA very quickly, but the isomerase did not recycle like a normal enzyme to catalyze the production of more CLA. CLA could be increased by adding more cells, but time was without effect.

The *cis*-9,*trans*-11 CLA content of milk can be increased by feeding grain supplements and polyunsaturated fats to cattle, but the study of *cis*-9,*trans*-11 CLA production in ruminants is complicated by the fact that ruminal fermentation is not the only source of CLA. If biohydrogenation is incomplete and *trans*-C18:1 passes from the rumen, *trans*-11 C18:1 can be converted to CLA by the Δ-9 desaturase of the mammary and adipose tissues. Mammalian Δ-9 desaturase can be inhibited by sterculate, and post-ruminal infusions of sterculate reduced the CLA content of milk 40 to 65%. Based on these results, it appeared that endogenous synthesis of CLA from *trans*-11 C18:1 was 60 to 75% of the total.

Milk fat depression. Dairy cattle are frequently fed large amounts of grain to increase ruminal fermentation rate and energy availability, but grain feeding can have a negative impact on ruminal pH, milk fat percentage and yield. Because milk fat depression was in many cases correlated with a decreased ratio of ruminal acetate to propionate, ruminant nutritionists suspected that this shift was either directly or indirectly altering the cow's ability to synthesize milk fat.

Several questions then arose. Was by-passed starch causing an increase in insulin and diverting fatty acids from the mammary gland to adipose tissue? Was propionate causing an increase in glucose and insulin? Were vitamin B_{12} deficiencies or microbially derived vitamin B_{12} analogs preventing normal propionate metabolism (via methylmalonyl CoA)? Despite more than two decades of work, definitive answers were not forthcoming, and the condition known as milk fat depression was still a problem.

Davis and Brown noted that dietary oil supplements accentuated the milk fat depression of cattle fed low fiber rations, but abomasal infusions of the same oil did not. These results suggested that milk fat depression was associated with changes in ruminal metabolism rather than a direct effect of the oils on the mammary gland. These same authors also noted that oil supplements increased the amount of *trans*-C18:1 and other isomers of unsaturated fatty acids leaving the rumen. They concluded that these "unacceptable substrates" might be rejected by the mammary gland.

The ability of nutritionists to increase the CLA content of milk has been thwarted by the propensity of fat supplements to decrease total milk fat percentage. For example, fish oil supplements can increase CLA, but they often decrease total milk fat. When diets are supplemented with fish oil, there is an increase in the ratio of *trans*-10 to *trans*-11 C18:1 fatty acids in milk fat. This shift is probably caused by ruminal microorganisms, but the nature of this effect was not defined.

More recently, Baumgard et al. demonstrated that abomasal infusion of *trans*-10, *cis*-12 CLA caused a significant (up to 50%) decrease in milk fat yield. *Trans*-10, *cis*-12 CLA is not derived from animal metabolism, but until recently bacteria capable of producing this CLA had not been isolated. It has long been recognized that grain can increase lactate turnover in the rumen, but only a few ruminal bacteria utilize lactate (e.g *M. elsdenii* and *S. ruminantium*). When mixed ruminal bacteria were enriched with lactate, *trans*-10, *cis*-12 CLA increased, and large cocci that resembled *M. elsdenii* were isolated. 16S rDNA indicated that these *trans*-10, *cis*-12 CLA producing cocci were indeed *M. elsdenii,* but some laboratory strains of *M. elsdenii* did not make this CLA. Further work will be needed to define more clearly the contribution of *M. elsdenii*, but the observation that grain feeding promotes the growth of *M. elsdenii* in vivo is consistent with the idea that this species may be significant. Not all cows fed high grain rations have low milk fat, and not all strains of *M. elsdenii* produce *trans*-10, *cis*-12 CLA.

Ruminant fat composition. Simple stomached animals have fatty acids that are not completely saturated, the double bonds are arranged with a *cis* configuration, and carbon number is always even. By contrast, ruminant fats are more completely saturated, and unsaturated fatty acids that are produced often have a *trans* configuration. Because ruminal bacteria can use propionyl CoA as a precursor for fatty acid synthesis, ruminant fats can also have a significant amount of odd chain fatty acid.

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Chapter 18

Growth, Maintenance and Energy Spilling

Growth rates. Because bacteria grow by binary fission, bacterial growth is an exponential function. If "N" equals the number of cells, the specific growth rate (μ) can be calculated from the rate of increase in N:

$$\frac{dN}{dt} = \mu N.$$

Where μ is an absolute rate constant with the units of h^{-1}. One can solve for μ by re-arranging the expression:

$$dN/dt/N = \mu$$

By integrating, we see that the number of cells formed depends on the initial cell number, N_o, the time that growth has occurred (0 to t), and the specific growth rate constant, μ:

$$\int_{N_o}^{N} dN/N = \mu \int_{o}^{t} dt.$$

By converting the integral into a function based on natural logarithms:

$$ln\ N - ln\ N_o = \mu t$$

Then, by rearranging, it is possible to achieve the equation of a straight line that has an ordinate of ln N, an abscissa of t, a slope of μ, and an intercept of the ordinate, ln N_o :

$$ln\ N = \mu t + ln\ N_o$$

The growth rate of a bacterium is inversely related to its doubling time (g). In the simplest case, if we start with one cell and measure the time (t) necessary for it to become two cells, it is possible to solve for g (doubling time):

$$ln\ 2 = \mu t + ln\ N_o$$
$$ln\ 2 = \mu t + 0$$
$$ln\ 2/\mu = t$$
$$0.693/\mu = t = g.$$

Bacterial doubling times range from approximately 20 min (2.1 h^{-1}) for *E. coli* growing aerobically in rich medium to as long as several days or weeks for soil bacteria.

Bacterial number and mass. In the laboratory, bacteria are often grown on agar plates, but this method of growth is not conducive to growth rate determinations. When bacteria are grown in broth, bacterial number or mass can be estimated from the optical density of the culture so long as the optical density is not too high. If the optical density is high, light is scattered secondarily, and the relationship between optical density and bacterial mass is no longer linear. Because optical density is an easy and highly reliable measurement, bacterial growth rate is typically estimated from "growth curves" that are based on increases in optical density.

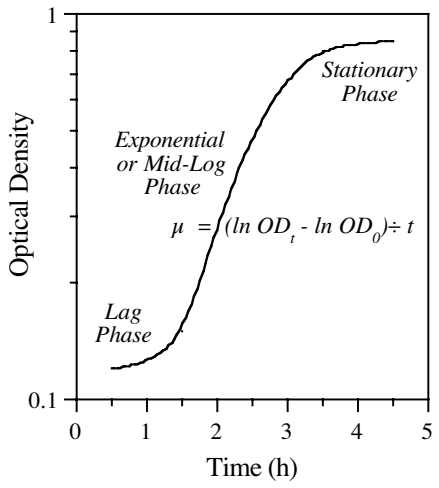


Fig. 18-1. Optical density of a bacterium growing in liquid broth.

If the culture is not fresh, there may be a lag phase before exponential growth is observed. This lag can be caused by non-viable cells. Once the non-viable cells are outnumbered by viable ones, the relationship between log optical density and time becomes linear, and it is possible to estimate μ. After the culture has depleted one of the nutrients (typically the energy source), growth ceases and the culture enters stationary phase. If the inoculum size is significant (5 to 10%), the bacteria only double 3 to 5 times before they enter stationary phase.

Many bacteria have the potential to grow very rapidly, but in nature they can only sustain these growth rates for short periods of time. Some nutrient (often energy) is invariably depleted. The power of exponential growth is illustrated by the following example. If a bacterium has an internal volume of 1 μm^3 and the doubling time is 20 min, the biomass volume would increase rapidly and to a very high value:

3 doublings (1 h) $2^3 = 8\ \mu m^3$

36 doublings (12 h) $2^{36} = 6.87 \times 10^{10}\ \mu m^3$

144 doublings (48 h) $2^{144} = 2.23 \times 10^{43}\ \mu m^3$

How big is 2.23 x 10^{43} μm^3?

It takes 1000 x 1000 x 1000 μm^3 to make 1 mm^3, so 2.23 x 10^{43} μm^3 = 2.23 x 10^{34} mm^3.

It takes 1000 x 1000 x 1000 mm^3 to make 1 m^3, so 2.23 x 10^{34} mm^3 = 2.23 x 10^{25} m^3.

It takes 1000 x 1000 x 1000 m^3 to make 1 km^3, so 2.23 x 10^{25} m^3 = 2.23 x 10^{16} km^3.

How big is 2.23 x 10^{16} km^3?

A cube with 281,471.84 km on each side!

The diameter of the earth is less than 13,000 km.

Bacterial composition. Protein is the most abundant polymer in bacteria, and it accounts for approximately 50% of the biomass dry weight *(see page 115)*. Ribosomal RNA is the next most abundant substance, but rRNA content can vary with growth rate. Rapidly growing bacteria often have more rRNA than slow growing cells, but this feature is species-dependent. *E. coli* has as much as 4% DNA, but the DNA content of ruminal bacteria is usually only 1 to 2%. Lipid accounts for approximately 10% of the biomass. Bacteria can accumulate polysaccharide on their outside surfaces as a glycocalyx or as an intracellular energy reserve. In some cases, polysaccharide can account for 50% of the dry weight. When bacteria are cultured in the laboratory for long periods of time they often loose their glycocalyx.

Ammonia assimilation. Many bacteria can utilize ammonia as a nitrogen source for growth, but ammonia concentrations in the rumen fluctuate considerably. If the diet has an abundance of soluble protein or large amounts of urea, the ammonia concentration can be greater than 50 mM. Because excess ruminal ammonia is converted to urinary urea, the animal must expend energy to excrete this nitrogen. Conversely, if the diet has little ruminal degraded protein or is supplemented with large amounts soluble carbohydrate, ruminal ammonia concentrations can be very low.

Ammonia assimilation is typically mediated by glutamate dehydrogenase (GDH). Mixed ruminal bacteria have high GDH activities and NAD-linked activity is more prevalent than NADP-linked activity. Work with non-ruminal bacteria indicates that GDH has a K_m for ammonia of approximately 5 mM, but ruminal ammonia concentrations are usually sufficient for GDH to operate rapidly:

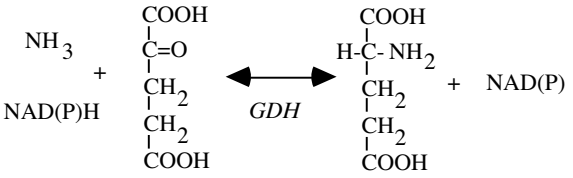


Fig. 18-2. Ammonia assimilation via glutamate dehydrogenase (GDH).

In the 1970's Satter and Slyter used artificial rumen devices to determine the effect of ammonia on the amount of microbial protein that was being produced. Because there was little increase in microbial protein when ammonia was greater than 3 mM (5 mg per 100 ml), they concluded that urea should not be added if the diet already had 14% true protein. A short time later Orskov and his colleagues supplemented sheep with urea and examined the effect of ammonia on starch disappearance from nylon bags suspended in the rumen. In this case, maximal disappearance was not observed until the ammonia concentration was greater than 14 mM.

When Schaefer and his colleagues grew pure cultures of ruminal bacteria in ammonia-limited continuous culture devices, the amount of ammonia needed to achieve half maximal growth rate was only 6 to 125 μM, and it appeared that the bacteria had a much better affinity for ammonia than mixed cultures. Because these values were 40 to 1000-fold lower than the K_m of GDH for ammonia, it appeared that ruminal bacteria had an alternative method of assimilation when the ammonia concentration was low. Ruminal ammonia concentrations are not normally low, but ammonia can be 'undetectable' if the diet is rich in starch and has little ruminally degradable protein or urea.

Experiments with marine bacteria indicated that there was an alternative mechanism of ammonia assimilation that employed glutamine synthetase.

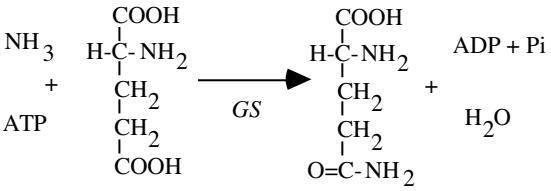


Fig. 18-3. Ammonia assimilation via glutamine synthetase (GS)**.**

Glutamine synthetase works in a cyclic fashion with a novel enzyme, glutamate synthase (GOGAT) to produce glutamate:

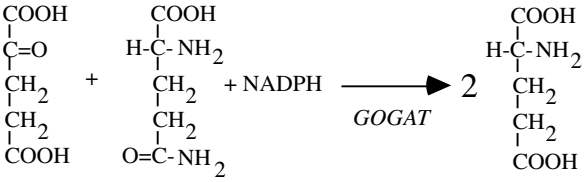


Fig. 18-4. Glutamate formation via glutamate synthase (GOGAT)**.**

The cyclic action of GS and GOGAT lowers the K_m for ammonia (glutamine synthetase has a K_m of 0.2 mM), but there is an input of ATP.

ATP+Glutamate+NH_3 -> Glutamine+ ADP+Pi

Glutamine + α-KG + NADPH ->
2 Glutamate + NADP

α-KG + NH_3 + ATP + NADPH ->
glutamate + NADP + ADP + Pi

Differences in ammonia and ATP illustrate the advantages of each system:

1) GDH is advantageous when ammonia concentrations are high and carbohydrates (ATP) are limiting.

2) GS/GOGAT cycle is advantageous when ammonia concentrations are low and carbohydrates (ATP) are not the factor limiting growth.

GS is a very large enzyme that is composed of 12 identical subunits, and each subunit is capable of being adenylated by ATP. Because the adenylated form is less active than the non-adenylated form, GS can sense the energy status of the cell.

GS+(1-12) ATP -> GS-(1-12)AMP+(1-12)PPi

GS can be de-adenylated by snake venom phosphodiesterase in vitro, and this treatment increased the GS activity of *S. dextrinosolvens*. *S. ruminantium* cells from an ammonia-limited chemostat had GS activity that was enhanced by phospho-diesterase and inhibited by AMP.

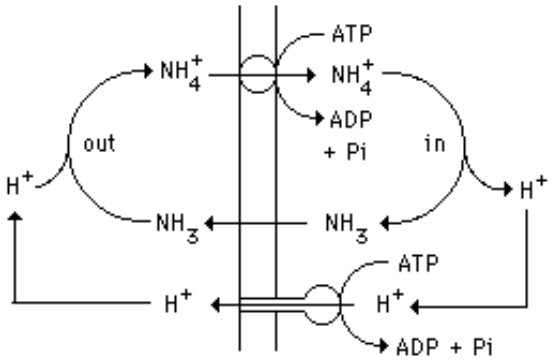


Fig. 18-5. Active uptake of ammonium ion by bacteria, its dissociation to ammonia and its ability to promote energy spilling.

Dissociated ammonia is a lipophilic substance that can pass across cell membranes by facilitated diffusion, and for many years it appeared that this was the only method of ammonia uptake in bacteria. However, *E. coli* and marine bacteria have a high affinity potassium carrier that can also concentrate ammonium ion. Because ammonium and potassium ions have similar size and valence, the carrier cannot differentiate them. Active transport of ammonia ion has not yet been demonstrated in ruminal bacteria, and it is a highly inefficient process. When the ammonium ion reaches the inside of the cell membrane, it dissociates, and ammonia can then leak back out of the cell. Work with marine bacteria indicates that as many as six molecules leak back out of the cell before a single ion is fixed. Ammonia recycling can be a mechanism of energy spilling.

Amino acid synthesis. Ammonia assimilated by either GDH or the GS/GOGAT cycle can be transaminated to produce other amino acids so long as the appropriate carbon skeletons are available. These skeletons are derived from inter-

mediates in central metabolism (pyruvate, oxaloacetate, α-ketoglutarate, acetyl CoA and succinyl CoA) (*see Appendix, page 114)*. The biosynthetic pathways of ruminal bacteria have not been studied and are presumed to be similar to those found in *E. coli*. An exception seems to be branched chain amino acids. Cellulolytic ruminal bacteria cannot synthesize branched chain amino acids de novo and must have branched chain volatile fatty acids as a precursor (see Chapter 16, *Crossfeeding)*.

Table 18-1. Amount of ATP needed to synthesize each component. Calculations were performed with (+AA) and without (-AA) preformed amino acids. Adapted from the data of Stouthamer (1973).

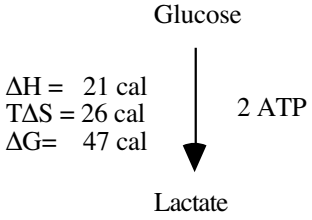
		ATP Requirement	
Macromolecule	% Dry Weight	mmol per g -AA	mmol per g + AA
Polysaccharide	16.6	2.1	2.1
Protein	52.2		
amino acid formation		1.4	0.0
polymerization		19.0	19.0
Lipid	9.4	0.1	0.1
RNA	15.7		
nucleoside formation		1.5	1.5
polymerization		0.9	0.9
DNA	3.2		
nucleoside formation		0.4	0.4
polymerization		0.2	0.2
mRNA turnover		1.4	1.4
Transport functions			
ammonium ions		0.0	0.0
amino acids		0.0	4.7
potassium		0.2	0.2
phosphate		0.8	0.8
Total (mmol/g)	97.1	28.0	31.2
Y_{ATP} (1/total x 1000) (g/mol)		35.7	32.0

ATP yield. Bauchop and Elsden studied the growth of anaerobic bacteria and correlated biomass production with ATP availability (Y_{ATP}). They obtained an average value of 10.5 g cells/mole ATP, but the range was actually 8.3 to 12.6. Despite this more than 50% variation, the 10.5 value was treated as a biological constant, and it still appears in textbooks of microbiology. By the 1970's, however, the notion of a constant Y_{ATP} was being questioned. Y_{ATP} values ranged from 4.7 to 28.5 g cells/mole ATP, and the average value was considerably greater than 10.5. Stouthamer calculated the amount of ATP that would be needed to synthesize bacterial biomass, and these calculations indicated three things: 1) protein was the most costly polymer to synthesize, and it accounts for approximately 1/2 of the total ATP requirement, 2) polysaccharide is inexpensive to synthesize, and 3) the Y_{ATP} of bacteria should be much greater than 10.5 g cells/mole ATP.

Table 18-2. ATP needed to synthesize protein, nucleic acid or polysaccharide.

Macromolecule	*ATP Requirement (mmol/g)*
Protein	36.4
RNA	15.3
DNA	18.8
Polysaccharide	12.6

The question then arises, why is protein synthesis so inefficient? The inefficiency of protein synthesis is illustrated by a simple example involving homolactic fermentation. Because the conversion of glucose to lactate has an enthalpy of 21 cal per mmol, 10.5 cal of heat would be generated for each mmol of ATP synthesized:



21 cal ÷ 2 ATP = 10.5 cal / ATP

If 5 ATP equivalents are needed to synthesize a peptide bond:

10.5 cal /ATP x 5 ATP/peptide bond = 52.5 cal/peptidebond

Since a peptide bond only has an enthalpy of 3 cal/bond, the efficiency of energy capture as peptide bond energy is very low

(3 cal / 52.5 cal = 6%)

The inefficiency of protein synthesis may be related to the information protein

contains and the 'willingness' of living organisms to invest a large amount of energy to make sure that the information (sequence) is correct. This principle is supported by a 'thought experiment" called 'Maxwell's Demon." In 1868, James Maxwell hypothesized a Demon that was able to operate a frictionless door that connected two rooms separated by a partition. Whenever air molecules looked as if they would pass through the opening, the Demon opened the door. Afterwards he closed the door to trap the molecule. The demon did this over and over until there was a concentration gradient of air molecules between the two rooms. A concentration gradient is energy. Where did the energy come from? The demon had information when the door should be opened and closed. Hence, energy and information are interconvertible!

Maintenance energy. The calculations of Stouthamer assumed that all of the ATP available to a bacterium could be used to synthesize biomass, but bacteria must expend a portion of their energy to 'maintain' their cells. Bacterial maintenance energy has not been precisely defined, but at least three functions are known to contribute: 1) motility, 2) turnover of macromolecules (e.g., protein), 3) and re-establishment of ion gradients across the cell membrane. Because membranes have an inherent 'leakiness,' the third component is clearly the most important one. Bacterial maintenance is estimated indirectly from growth rate-dependent changes in bacterial yield, and it is possible to compare maintenance to the overhead of a small business. When the rate of cash flow (glucose consumption) is slow, overhead (maintenance) makes a larger proportion of the total budget (total ATP availability). Thus, the contribution of maintenance is more pronounced when fermentation and growth rates are slow. Because ruminal bacteria in vivo grow slowly, they can expend as much as 30% of their ATP on maintenance.

Continuous culture. Maintenance energy is easily demonstrated in continuous culture. In batch cultures, the bacteria grow as fast as they can, maintenance only makes up a small part of the total ATP consumption, and the composition of the growth changes. In continuous culture, steady states can be maintained, and better estimates of cell composition (including the specific activity of enzymes) can be achieved.

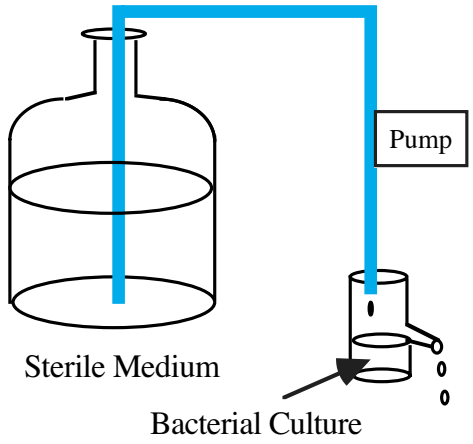


Fig. 18-6. A diagram showing a simple chemostat.

The principles of continuous culture were developed by Novick and Monod. Both groups published their findings in 1950. Monod was a Frenchman, and he called his device a chemostat. In a chemostat, medium is added to the growth or culture vessel at a constant rate from a medium reservoir. Because the culture vessel has a constant volume, every time a drop of medium is added to the culture vessel, one drop of cell containing material leaves the vessel. The dilution rate of the vessel *(D)* is computed by dividing the flow rate of medium leaving the reservoir *(F)* by the volume of the culture vessel *(V)* and has the units of h^{-1}.

$$\frac{F(ml/h)}{V\,(ml)} = D(h^{-1})$$

At steady state, some nutrient must be limiting, and the rate of cell production (μx) must be equal to the rate of cell loss through overflow (Dx):

$$\mu x = Dx.$$

If $\mu x = Dx$, the growth rate, μ, must be equal to the dilution rate D:

$$\mu = D$$

In the late 1940's, Monod noted that the growth rate of bacteria was related to the concentration of limiting substrate, and that the two parameters followed the Michaelis-Menten saturation kinetics:

$$\mu = D \frac{\mu_{max} S}{K_S + S}$$

Solving for *S,* we can see that the amount of substrate left in the chemostat is proportional to the dilution rate, *D,* the maximum growth rate, μ_{max}, and the substrate affinity constant, K_S:

$$S = K_S \frac{D}{\mu_{max} - D}$$

Thus, as *D* increases so does *S*. When $D = \mu_{max}$, the maximum growth rate, the bacterium washes out and $S = S_R$ the amount of *S* that was originally in the medium reservoir:

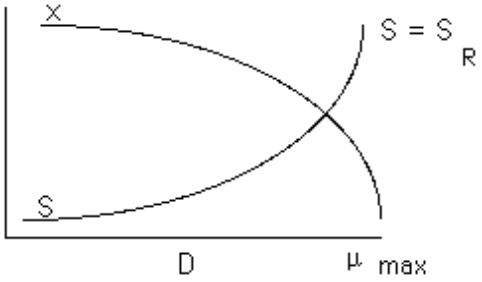


Fig. 18-7. The relationship between limiting substrate concentration *(S),* bacterial mass *(X)* and the dilution rate of a chemostat. When *S* increases, *X* declines. The bacterium washes out when *S* equals S_R the concentration of substrate in the reservoir.

Growth efficiency can be estimated by measuring cell material and dividing this value by the amount of energy source consumed. This term is commonly called "yield" or *Y:*

Y = g bacteria (x) ÷ g substrate used (S)

Because cattle often consume large meals, the rumen does not operate as a closely regulated continuous culture device, but it can reach a steady if the animal is fed continuously with a rotary feeder (see Chapter 23, *Models of Rumen Fermentation).* When large meals are consumed, the rumen operates as a batch culture, but the soluble materials are rapidly depleted. Thereafter, bacterial growth rate is dependent on the degradation rates of insoluble materials, and these rates can be very slow.

Maintenance derivations. Maintenance is estimated from growth rate-dependent declines in yield (growth efficiency):

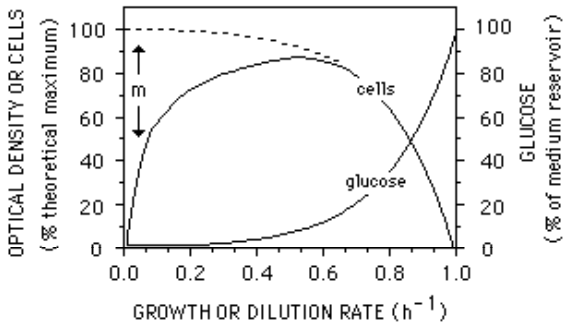


Fig. 18-8. The impact of maintenance (m) on bacterial growth. The dotted line shows the cell mass that would be present if there was no maintenance energy.

In the derivation of Marr, maintenance is expressed as an absolute rate (h^{-1}), and he assumed that bacteria would grow even faster if they did not have this non-growth function.

$dS/dt \cdot Y = \mu x + ax$, where:

dS/dt = rate of hexose consumption

Y = yield (g cells/g hexose)

μ = rate growth (h^{-1})

a = maintenance rate (h^{-1})

x = cell mass (g)

Pirt express maintenance as a specific rate of energy consumption (mmol ATP or glucose per mg cells per h):

$$dS/dt = dS/dt_m + dS/dt_g$$

$$\mu x/Y = mx + \mu x$$

dividing by μx:

μ = rate of growth (h^{-1}), where:

$$1/Y = m/\mu + 1/Y_g$$

Y= yield (g cells/g hexose)

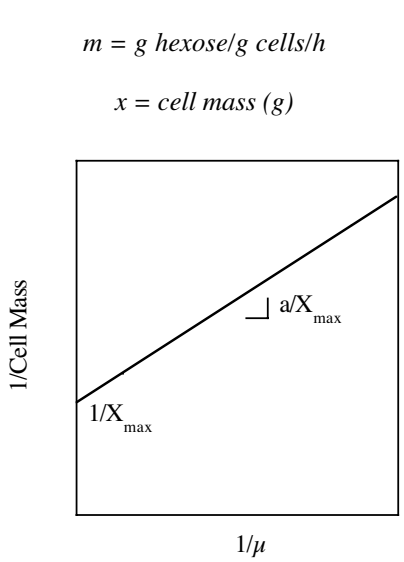


Fig. 18-9. Double reciprocal plot of Marr.

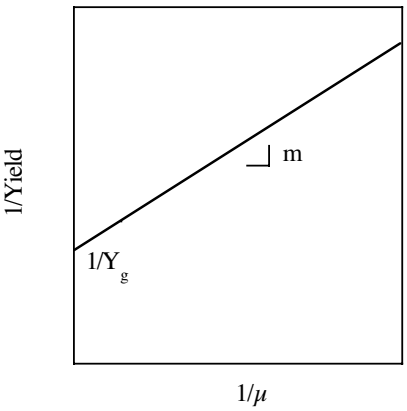


Fig. 18-10. Double reciprocal plot of Pirt.

The Cornell Net Carbohydrate Protein System (CNCPS) uses Pirt plots to estimate maintenance expenditures and calculate the amount of bacterial protein that flows from the rumen.

Energy spilling. Maintenance energy explains why bacteria have lower yields at low growth rates, but maintenance cannot always explain why the Y_{ATP} is lower than the value proposed by Stouthamer. Even Y_{ATP} values corrected for maintenance can be significantly lower than 32 g cells/mol ATP, and it appears that many bacteria (including ruminal bacteria) have mechanisms of spilling energy. When the ATP supply from catabolism exceeds needs for maintenance and growth, bacteria can spill energy and generate heat.

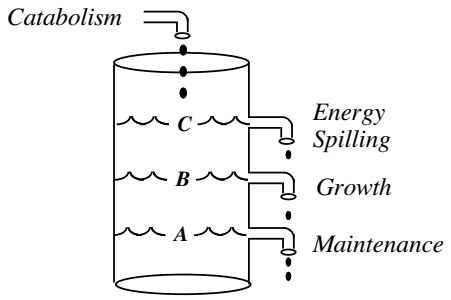


Fig. 18-11. Bucket model of energy expenditures by bacteria (A, maintenance energy; B, growth; C, energy spilling).

S. bovis has often been used as a model of energy spilling. Non-growing *S. bovis* cells can spill ATP as fast as those that are growing rapidly. Energy spilling is triggered by antibiotics like chloramphenicol and monensin or a deficiency of amino acids. In *S. bovis*, energy spilling is mediated by a futile cycle of protons through the cell membrane and the activity of the membrane bound ATPase that pumps protons out of the cell.

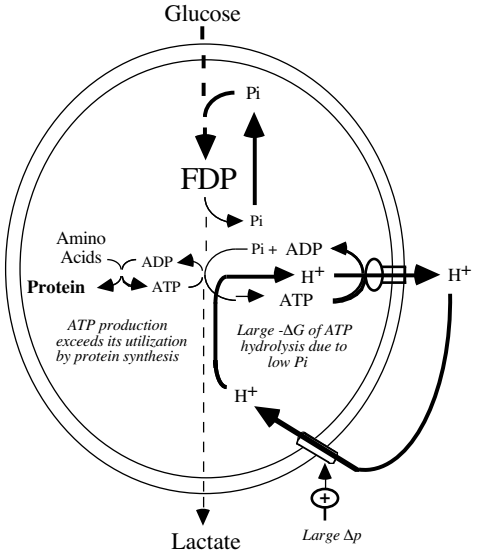


Fig. 18-12. The futile proton cycle of *S. bovis* and its regulation.

When the glycolytic rate of *S. bovis* is high, fructose 1,6 diphosphate (FDP) accumulates, and this accumulation causes a decline in intracellular phosphate. The decline in intracellular phosphate, in turn, leads to an increase in the free energy of

ATP hydrolysis (more negative ΔG):

$$K_{eq}\ ATP = [ATP] \div [ADP]\ [Pi]$$

$$-\Delta G'p = -\Delta G^{\circ}p/F + Z\log\ [ATP]\ /[ADP]\ x\ [Pi]\ mV$$

Because the free energy of ATP hydrolysis is greater, the cells can increase the protonmotive force (Δp). When the Δp increases, membrane resistance to protons declines, and the activity of the futile cycle increases.

Not all bacteria use a futile cycle of protons to spill energy. For example, in *E. coli* energy spilling is mediated by a futile cycle of potassium or ammonium ions. *E. coli* has two transport systems for potassium, a high affinity proton symport mechanism and a transporter that is driven directly by ATP hydrolysis. This latter transporter recognizes either potassium or ammonium ion. When potassium is limiting, the high affinity carrier takes up the potassium, but potassium can leave the cell via the reversible proton symport system to create a cycle. In the case of ammonia limitation, ammonium ion is taken up by the ATP-driven transporter, but it dissociates to ammonia in the more alkaline interior. Ammonia then diffuses out of the cell. Whether ruminal bacteria have similar mechanisms of energy spilling has yet to be determined.

It has long been recognized that most bacteria grow more efficiently when they are supplied with amino acids in addition to ammonia, but the nature of this stimulation was not readily apparent. The calculations of Stouthamer *(see Table 18-1)* indicated that the cost of amino acid biosynthesis is small. Indeed, the cost of taking up an amino acid can be nearly as great as synthesizing one. However, if bacteria are forced to grow with ammonia as their sole energy source, their growth rate is typically 50% slower. When the anabolic rate is low, there is an increased chance that the catabolic rate will be greater than the anabolic rate. If amino acid N (e.g., ruminally degraded protein) is supplied, the catabolic rate better matches the anabolic rate, less energy is spilled, and the efficiency of bacterial growth is improved. Until recently the benefit of energy spilling was not entirely clear, but it should be noted that ruminal bacteria that do not spill energy can be killed by 'excess carbohydrate.' Conversely, bacteria that spill energy may be better suited for rapid growth when their limitation is relieved.

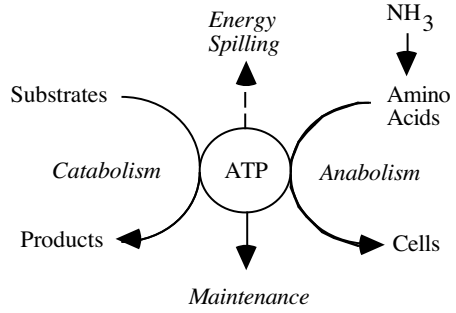


Fig. 18-13. Avenues of ATP generation and utilization in bacteria and the balance of anabolic and catabolic rates.

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Chapter 19

Microbial Death and Turnover

In many ecosystems (e.g., soil), substrate availability is so restricted that the bacterial doubling time is as long as one year. Because cattle consume feed regularly, ruminal bacteria are among the fastest growing microbes in nature (mean doubling time is approximately 7 h). Nonetheless, individual organisms must withstand periods when exogenous substrates are not available.

^{15}N turnover. Radioactive isotopes of nitrogen (^{13}N) have very short half-lives, but ^{15}N, is a stable isotope, and it can be differentiated from ^{14}N by mass spectrometry. Nolan and Leng added ^{15}N labeled ammonia to the rumen, and concluded that 35% of the microbial mass in the rumen was turning over. However, their studies were confounded by fact that: 1) the NH_3 pool in the rumen is very large, 2) urea from the animal is recycled back to the rumen to produce NH_3, and 3) the calculations are prone to compounding errors.

Protozoal predation and lysis. Because ruminal protozoa graze and digest ruminal bacteria, protozoal predation has been cited as a key factor causing bacterial turnover. This assumption is supported by the observation that the ruminal ammonia concentrations are greater when protozoa are present, but the impact of protozoa on protein turnover is not entirely clear. Jouany and others concluded that they are an important factor in turnover, but Krebs et al. reported that defaunation did not dramatically decrease protein recycling. Weller and Pilgrim noted that little protozoal protein ever seems to leave the rumen, and this result is consistent with the idea that protozoa are themselves very prone to lysis ands turnover.

Bacterial lysis. Bacteria must expand their cells while they are growing, and this process is much like driving across a bridge while you are building it. Cell wall-degrading enzymes (autolysins) cut the cell wall so it can be expanded, but bacterial cells have a very high turgor pressure. If autolytic activity is low, growth rate is sacrificed, but excessive autolytic activity can cause lysis. In the surface stress model of Koch, Gram-positive rods first deposit peptidoglycan at the inner surface. The older, outer layers are then cut by autolytic enzymes, and the stress is gradually transferred to more recently synthesized portions of the peptidoglycan. With cocci, the cell wall expansion occurs at the poles of the cell.

Ruminal bacteria have different types of autolytic regulation. *F. succinogenes* uses a proteinase to degrade its autolysins once it reaches stationary phase, and this degradation is triggered by energy source depletion. If nitrogen or some other factor limits growth, the autolysins are not degraded, and it lyses quickly. Strains of *B. fibrisolvens* and *R. amylophilus* lyse even faster than *F. succinogenes,* but little is known about their autolytic regulation. In *S. bovis*, the autolysins are inactivated after the cells reach stationary phase. The autolytic inactivation of *S. bovis* autolysins is mediated by an unusual sugar residue (kojibiose) in its lipoteichoic acids. Kojibiose is a glucose disaccharide with a 1,2 linkage. If *S. bovis* is cultivated with 2-deoxyglucose, kojibiose is not produced, the autolysins are not inactivated, and the cultures lyse.

Endogenous metabolism. When exogenous energy sources are depleted,

some bacteria have endogenous sources to sustain viability. A variety of intracellular molecules can be used as an energy source for endogenous metabolism, but glycogen is utilized most efficiently. Starving bacteria use their glycogen reserves, but the size of the glycogen pool is not directly proportional to the survival time. When *F. succinogenes* cultures are starved, the initial rate of glycogen utilization is 10-fold faster than the endogenous rate needed to maintain cell viability. Because glycogen is pre-maturely degraded, *F. succinogenes* has a short half-life.

The survival of ruminal bacteria can, in some cases, be explained by the method of transport. *F. succinogenes* does not have a phosphotransferase system (PTS) to take up sugar, and it uses sodium symport mechanisms. When the endogenous metabolic rate is too slow to generate a membrane potential or sodium gradient, transport is no longer possible, and viability decreases rapidly. *S. bovis* does not store glycogen, but *S. bovis* can use PEP to drive sugar transport and re-initiate growth. *S. bovis* survives for long periods of time even if intracellular ATP and membrane potential are too low to be measured. *S. ruminantium* also has a PTS for glucose and stores glycogen, but it lyses when the sugar is depleted.

Because some ruminal bacteria are very sensitive to starvation, the question arises, can feeding schedules affect the metabolic activity of ruminal bacteria? When mixed ruminal bacteria were starved in vitro, intracellular glycogen reserves decreased exponentially, but their ability to re-initiate fermentation did not decrease for more than 12 h. Because bacteria from cattle fed only once a day initiated fermentation as fast as those from cattle fed twice a day, bacterial starvation does not seem to have a large impact on ruminal fermentation.

Methylglyoxal toxicity. When bacteria are nitrogen-limited and carbohydrate is in excess, protein synthesis is arrested, ATP accumulates, and ADP is no longer available to the kinase reactions of the glycolytic scheme. Work with *E. coli* showed that energy excess, nitrogen-limited cells diverted carbon to the methylglyoxal shunt, a pathway that does not have kinases and does not require ATP. In *E. coli* and *Klebsiella aerogenes*, carbon that passes through the methylglyoxal shunt is eventually converted to D-lactate, but methylglyoxal can accumulate. Methylglyoxal denatures DNA and protein, and it kills bacteria and animal cells.

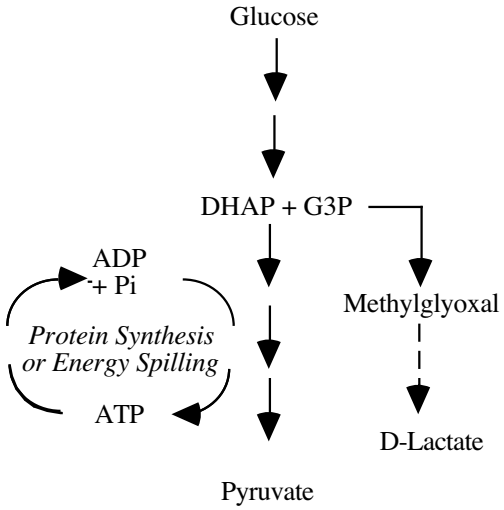

Fig. 19-1. The methylglyoxal shunt and its role in metabolizing excess glucose.

S. ruminantium also has the ability to convert methylglyoxal to D-lactate, but continuous culture studies indicated that *P. bryantii* could not. When glucose was in excess, methylglyoxal accumulated, and viability decreased dramatically. Not all *Prevotella* species produced methylglyoxal, but nearly half of them produced more than 1 mM (a concentration that kills cells). *F. succinogenes* is also killed by excess carbohydrate, but this toxicity could not be explained by methylglyoxal production. N-limited, cellobiose-excess *F. succinogenes* cells had a very large pool of glycogen, but the intracellular ATP pools and membrane potential were very low. *S. bovis* does not produce methylglyoxal when glucose is in excess, and this observation supports the idea that energy spilling is a protective mechanism

Bacteriocins. In the 1920's and 30's, cheese makers noted that some lactococci had antibacterial activity. These antibacterial substances were at first called "antibiotics," but the term "bacteriocin" was introduced to differentiate these small peptides from classical antibiotics. A variety of Gram-positive bacteria produce bacteriocins, and these peptides may play a role in regulating the growth and ecology of ruminal microorganisms.

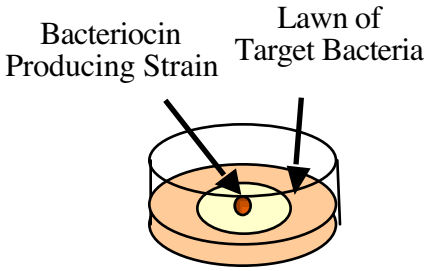

Fig. 19-2. The use of agar overlays to monitor bacteriocin production.

The best understood bacteriocin is nisin, the one produced by the cheese starter, *Lactococcus lactis*. Nisin is a relatively small peptide (34 amino acids) with five unusual sulfur-containing (lanthionine) rings. Nisin molecules assemble in the cell membrane to form a barrel-like structure that facilitates the loss of intracellular solutes (e.g., potassium). Nisin can also inhibit peptidoglycan synthesis, but the independence of this activity from protonmotive force dissipation has not been clearly established. Because nisin had effects on ruminal fermentation in vitro that were similar to monensin (see Chapter 22, *Manipulation of Rumen Fermentation)*, it appeared that nisin might be an alternative ruminal additive.

Some bacteriocins (e.g., nisin) have a broad spectrum, but many can only inhibit a narrow group of closely related strains. This latter characteristic indicates that bacteriocins may have specific receptors on the cell surface. When *Lactococcus cremoris* cells were treated with trypsin, they became 10-fold less sensitive to the bacteriocin, lactostrepcin 5. Nisin does not need a specific receptor, but it binds to the lipid II of cell membranes. The nisin activity was enhanced when the amount of lipid II in membrane vesicles was increased.

Bacteriocin activity can be detected on agar plates. The bacteriocin-producing strain is cultivated until a colony is observed. The plate is then overlayed with molten agar inoculated with target bacterium (e.g., 10^6 cells per ml). The plate is re-incubated so the target bacterium can grow as a 'lawn' in the top layer of agar. When the bacteriocin diffuses from the producing strain, growth is inhibited and there is a distinct zone of clearing.

Many bacteriocins are so tightly bound to the cell surface that they will not diffuse through agar, but Tween can be added to promote bacteriocin release. Bacteriocins bound to the cell surface may be a form of 'arm to arm combat.' By inhibiting or killing competing bacteria (often their closest relatives), they promote their own success. The first ruminal bacteriocins were obtained from *S. bovis* by Iverson and Mills in the 1970's, and half of the *S. bovis* strains from the rumen produce bacteriocins. Bacteriocins have also been isolated from strains of *B. fibrisolvens, R. albus, Lactobacillus fermentum*, and *Enterococcus faecium.*

Not all ruminal bacteria are sensitive to bacteriocins, and some initially sensitive strains can become resistant. For example, *S. bovis* JB1 becomes resistant to nisin after only a few hours of exposure. The nisin resistance of *S. bovis* JB1 is mediated by an alteration of lipoteichoic acids on the cell surface. Nisin is a positively charged molecule. Because nisin-resistant cells are more positively changed than nisin-sensitive cells, they repel the nisin molecule. The idea that nisin-resistant *S. bovis* could exclude nisin was supported by several observations, the nisin-resistant cells are less hydrophobic, more lysozyme-resistant and have more lipoteichoic acid than nisin-sensitive cells. When de-esterified lipoteichoic acids were separated on polyacrylamide gels, extracts from nisin-resistant cells migrated more slowly than those from nisin-sensitive cells. Nisin-resistant *S. bovis* JB1 cells are also resistant to the bovicin of *Streptococcus gallolyticus,* but they are susceptible to the bacteriocin of *S. bovis* HC5.

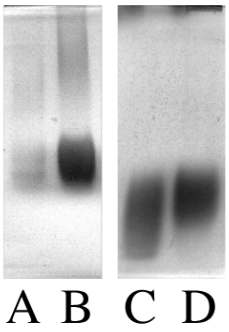

Fig. 19-3. Lipoteichoic acids from nisin-sensitive (A) and nisin-resistant *S. bovis* cells (B). Lanes C and D show extracts treated with sodium hydroxide to remove lipid. Taken with permission from Mantovani and Russell (2000).

Many bacteriocins have specialized amino acids and linkages, but they can be degraded by peptidases and proteinases. Nisin is rapidly degraded by enzymes in cell-free ruminal fluid, but this degradation does not prevent its ability to bind to mixed ruminal bacteria and catalyze potassium efflux. Because intact nisin can be recovered from mixed ruminal bacteria by acidic sodium chloride treatment, it appears that bacteriocins in the membrane are protected from proteolytic degradation.

Batch cultures of ruminal bacteria do not typically produce bacteriocin until the rate of growth has slowed and the cells are entering stationary phase, and work with *S. bovis* HC5 indicated that bacteriocin production is catabolite repressed by glucose. When the dilution rates of glucose-limited chemostats were decreased to values typical of the rumen, the cells produced nearly as much bacteriocin as stationary phase cultures. Acidic pH and amino acids stimulated bacteriocin production, but these effects were less than 3-fold. Acidic pH, however, can have a large impact on the activity of the bacteriocin that has already been produced, and in this case the effect was more than 10-fold.

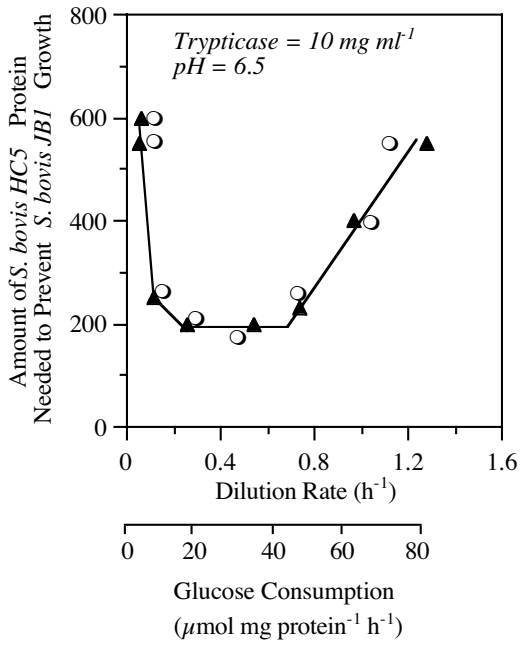

Fig. 19-4. The effect of continuous culture dilution rate on the amount of *S. bovis* HC5 cell protein that was needed to prevent the growth of *S. bovis* JB1 (▲). The glucose consumption rate is also shown (○).

Bacteriocin production does not seem to be a phylogenetically conserved trait. When the repetitive DNA (BOX) sequences of bacteriocin-producing *S. bovis* strains were compared, bacteriocin-producing strains had similarity indexes as low as 40%. 16S rDNA sequencing likewise indicated that butyrivibriocin production was not confined to a particular group, and even highly related species differed in their ability to produce bacteriocins. Recent work by Teather and colleagues showed that non-bacteriocin producing butyrivibrios carried homologue genes.

The role of bacteriocins in ruminal ecology has not been clearly defined. Because producing and sensitive strains can be readily isolated from the same cattle, bacteriocin production does not confer an absolute growth advantage. However, cell-associated bacteriocins could be a critical factor in colonization. The importance of bacteriocins is supported by several observations: 1) in vivo enumerations indicated that *R. albus* (a species that produces a bacteriocin) out-numbered *R. flavefaciens* (a bacteriocin-sensitive species) even though *R. flavefaciens* grew faster on cellulose than *R. albus;* 2) when cattle were adapted in stepwise fashion to increasing amounts of grain, there was an inverse relationship between *S. bovis* and bacteriocin producing lactobacilli that could not be explained by pH; 3) obligate amino acid fermenting bacteria are very sensitive to bacteriocins; and 4) ruminal bacteria produce an antifungal substance that seems to explain the inverse relationship between ruminal bacteria and fungi.

Anaeroplasma and bacteriophage. Some bacteria are intracellular parasites (e.g mycoplasmas), and their lifecycle causes bacterial lysis. In the 1970's, Hungate and Robinson isolated bacteriolytic bacteria that promoted the lysis of heat-killed, bacteria. However, anaeroplasma capable of infecting live bacteria were present at less than 10,000 cells per ml ruminal fluid, and it was not clear if they were truly parasitic. Viruses can also attack bacteria and cause lysis, but temperate phage incorporate into the chromosome and pass from one generation to another without causing lysogeny. Bacteriophage have been found at 10^7 to 10^{10} per ml of ruminal fluid, but

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Chapter 20

Regulation of Metabolism

Bacteria have a variety of mechanisms for adapting to a changing environment. The metabolic regulation of ruminal bacteria has not been extensively defined, but some examples can be described. These regulatory mechanisms can be classified as either short- or long-term. Short-term regulation (e.g., allosteric effects, feedback inhibition, PTS-regulation or inducer exclusion) modulates the *activity* of proteins that have already been synthesized. Long-term effects (e.g., induction and repression) control the *amount* of protein that is synthesized. In this regard, one can use the analogy of controlling water supply in the house. The faucets can be opened or closed (short-term regulation) or the plumbing replaced with larger or smaller pipes (long-term regulation).

Induction and repression. Bacteria produce many different proteins, but only some of these proteins are synthesized constitutively. Proteins produced in response to a particular stimulus are called 'inducible' proteins, and the molecules that stimulate their production are called 'inducers.' Inducers are small compounds that facilitate the binding of RNA polymerase to DNA and promote transcription. If an inducer is the end-product of an extracellular enzyme, the bacterium has a mechanism for sensing potentially lucrative substrates. For example, *S. bovis* only produces amylase if starch is present. Starch is too large a molecule to be transported into the cell, but *S. bovis* has a low level of amylase production that produces maltose. Because maltose is only derived from starch, maltose uptake signals the cells to make amylase.

Growth-rate dependent shifts. Some ruminal bacteria change their fermentation products in a growth rate-dependent fashion. The fermentation shift of *S. bovis* is mediated by an increase in fructose 1,6 diphosphate (FDP). When the fermentation rate is slow, FDP is less than 1 mM. However, if the glycolytic rate increases, FDP accumulates (as much as 30 mM). The increase in FDP has a profound impact on lactate production. *S. bovis* has a lactate dehydrogenase (LDH) that is made up of 4 subunits, but these subunits cannot bind to form active tetramers until they first bind FDP. When the FDP is high, lactate is the end-product, but pyruvate is converted to acetate, formate and ethanol if the FDP declines.

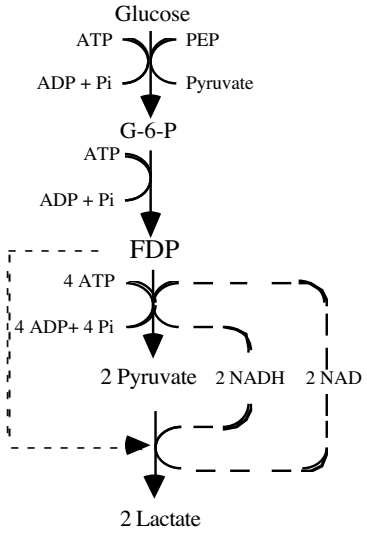

Fig. 20-1. The carbohydrate fermentation scheme of *S. bovis* when carbohydrates are plentiful and the fermentation rate is rapid.

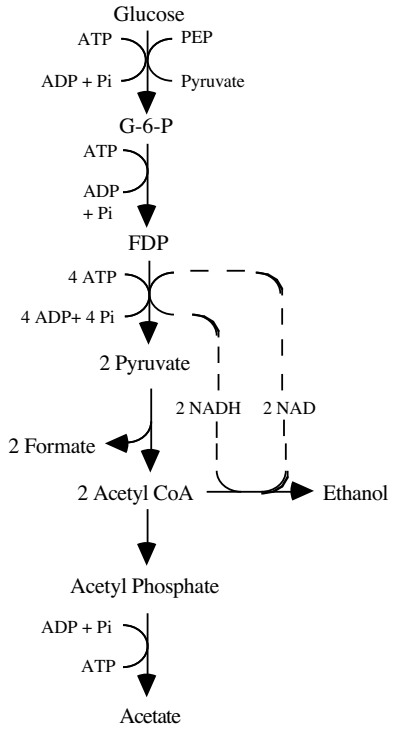

Fig. 20-2. The carbohydrate fermentation scheme of *S. bovis* when carbohydrates are limiting and the fermentation rate is slow.

At least some strains of *B. fibrisolvens* have LDHs that are activated by FDP, but it is unlikely that FDP plays a significant role in vivo. Only butyrivibrios that have acetyl CoA/butyryl CoA transferase produce lactate, and they only produce lactate if acetate is not available. If acetate is present (as it would be in vivo), pyruvate is converted to acetate and butyrate.

The FDP-dependent activation is a common feature in low G+C Gram-positive bacteria (e.g., streptococci, lactococci, enterococci and clostridia), but it cannot explain the fermentation shift of *S. ruminantium*. *S. ruminantium* switches from acetate and propionate to lactate when the fermentation rate increases, but the LDH of *S. ruminantium* is not activated by FDP. Many LDH's have more than one binding site for pyruvate (Hill plots greater than 1.0), but there has been little indication that intracellular pyruvate increases.

ATP per hour versus ATP per hexose. In the 1950's, Hungate contemplated the observation that *S. bovis,* a bacterium that produces only 2 ATP per glucose via homolactic fermentation, could out grow bacteria that produced twice as much ATP per glucose via acetate formation. Because this overgrowth only occurred if sugar was plentiful, he concluded that "ATP per unit time" was a more critical feature of biological work than "ATP per hexose."

At the time that Hungate formulated his ideas on ATP per hour, continuous culture devices had not yet been used to study ruminal bacteria, and he did not realize that *S. bovis* could alter its fermentation in a growth rate-dependent fashion. The question then arises, why doesn't *S. bovis* produce acetate, formate and ethanol all the time and produce even more ATP per hour by increasing ATP per hexose? The answer seems to be a simple matter of rate limitation. The pathway of acetate, formate and ethanol production can only catabolize glucose at a rate of 10 μmol hexose/mg protein/h, but the rate of lactate fermentation can be as great as 80 μmol hexose/mg protein/h. If *S. bovis* relied solely on the acetate, formate and ethanol pathway, ATP per hexose would be higher, but the rate of ATP production would be much slower.

Catabolite repression. In the 1940's, Monod noted that *E. coli* utilized glucose preferentially, and lactose did not decrease until all of the glucose had been depleted. The phenomenon was originally called the 'glucose effect,' but the term 'catabolite repression' was introduced when it was found that substrates other than glucose could repress substrate utilization. The catabolite repression of *E. coli* was mediated by an increase in cyclic AMP. When cyclic AMP binds to a catabolite activating protein (CAP or sometimes referred to as CRP, catabolite repression protein), cAMP-CAP binds to DNA and promotes transcription of the lactose operon. Because there is a shift in protein synthesis, growth pauses before lactose is utilized. Monod coined the term 'diauxie' to describe the two phases of feeding. *B. fibrisolvens* and *S. bovis* have diauxic growth curves and utilize some substrates to the exclusion of others, but most ruminal bacteria have very low concentrations of cAMP.

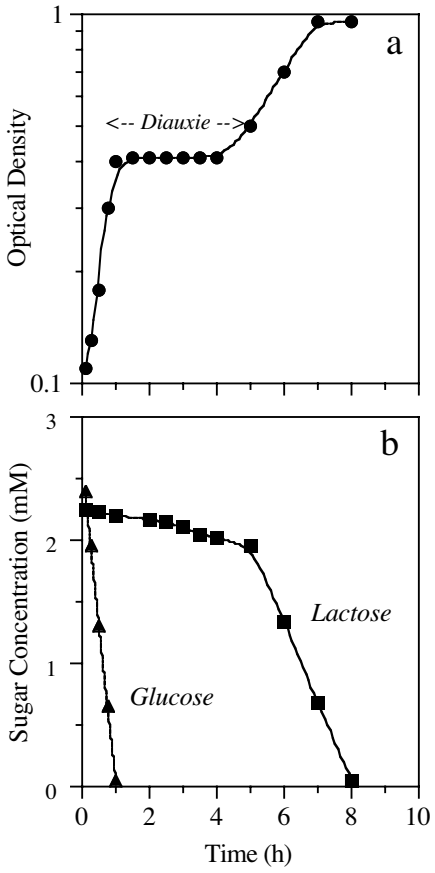

Fig. 20-3. The diauxic growth scheme of *S. bovis*. The culture was provided with glucose and lactose, but glucose was used preferentially.

PTS repression. Because classical catabolite repression is mediated by protein synthesis, the switch from 'non-preferred' to 'preferred' substrates is a gradual process that depends on the dilution of catabolite repressed proteins in subsequent daughter cells. Some bacteria switch from 'non-preferred' to 'preferred' substrates almost immediately, and this process was initially called 'catabolite inhibition.' The term catabolite inhibition was dropped when people showed that it was mediated by the PTS.

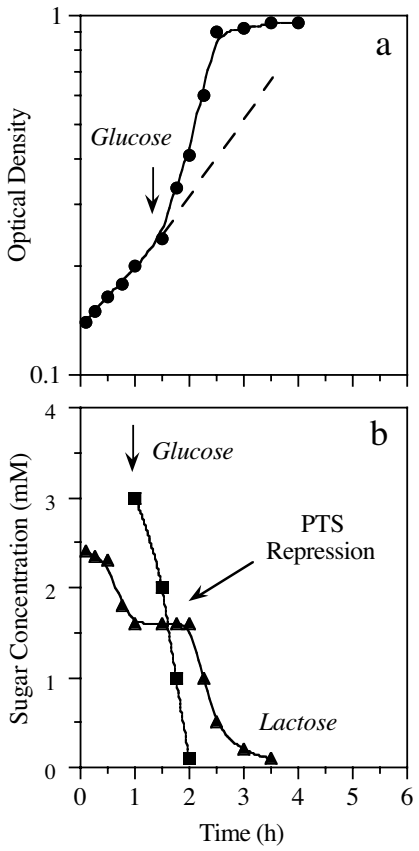

Fig. 20-4. The PTS repression of *S. bovis*. The culture was provided with lactose at time zero. After some lactose was used, glucose was injected into the culture. Glucose caused an increase in growth rate and an inhibition of lactose utilization. (Taken with permission from Kearns and Russell, 1996)

The PTS has soluble non-sugar-specific proteins as well as sugar-specific proteins that are in the cell membrane (enzyme IIs). Enzyme II proteins differ greatly in their affinity for phosphorylated HPr (see Fig. 13-5, page 40). For instance, glucose enzyme II outcompetes lactose enzyme II of *S. bovis,* and this feature allows *S. bovis* to utilize glucose preferentially. When glucose is depleted, glucose enzyme II is no longer able to bind phosphorylated HPr, and HPr-P then binds lactose enzyme II.

Regulatory glucokinases. Genetic studies with non-ruminal bacteria indicated some species had more than one glucokinase. If only one of the glucokinases was deleted (mutation), the strains continued to use glucose, but they lost their catabolite repression. Based on these results, it appeared that some glucokinases acted in a regulatory fashion to control gene expression, but the nature of this regulation was not biochemically defined.

In *P. bryantii* $B_1 4$, the β-glucanase and mannanase genes are encoded by the same operon. Both genes are catabolite repressed by glucose but not mannose or cellobiose. Because *P. bryantii* does not have PTS activity, has very low levels of cAMP, and uses the same carrier for glucose and mannose, the most common mechanisms of catabolite regulation could not explain this repression.

P. bryantii $B_1 4$ has a glucokinase that phosphorylates either glucose or mannose, but the rate of glucose phosphorylation is 4-fold faster. The glucomannokinase is competitively inhibited by the glucose analog, 2-deoxyglucose (2DG), and simple growth experiments indicated that 2DG alleviated glucose-dependent repression of mannanase and β-glucanase. Because β-glucanase mRNA decreased before the glucose consumption rate increased, it appears that β-glucanase production is being regulated at the level of transcription. The involvement of the glucomannokinase was supported by the observation that 2-DG decreased the glucose consumption rate and increased β-glucanase expression.

Some Gram-positive bacteria have more than one glucokinase, and mutant studies indicated that "regulatory glucokinases" play a role in catabolite repression. Because the *P. bryantii* $B_1 4$ glucomannokinase gene DNA sequence had similarity with the regulatory glucokinases of Gram-positive bacteria, it appears that this enzyme may play a role in catabolite regulation.

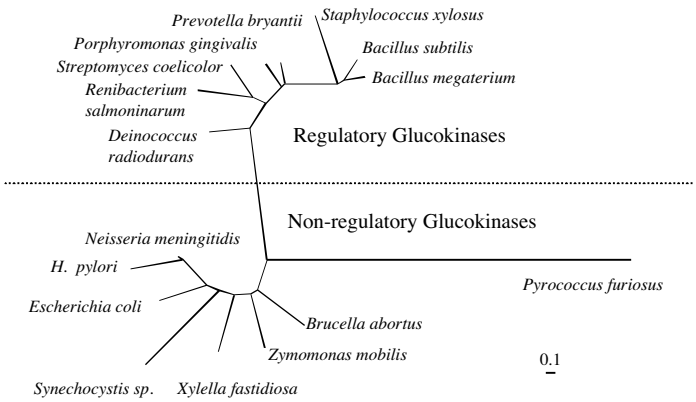

Fig. 20-5. An amino acid sequence tree showing the phylogenetic relationships of presumptive regulatory and non-regulatory bacterial glucokinases. The bar shows 0.1 difference in sequence.

Intracellular pH. Many bacteria can grow over a wide range of extracellular pH, but it had been assumed that intracellular pH was always near neutral. This assumption was based on experiments with *E. coli* K-12, a strain maintained in the laboratory for more than 70 years. Work with acid-resistant ruminal bacteria indicated that intracellular pH can decrease significantly, and this decline seems to be a survival mechanism.

Bacteria are too small for pH electrodes, but intracellular pH can be estimated from uptake of radiolabeled acids and bases. The principle is quite simple. Extracellular pH is measured, and the ratio of dissociated [A^-] to undissociated label [HA] in the extracellular space is estimated from the Henderson-Hasselbalch equation:

$$pH_e = pK_a + log\ [A^-]/[HA]$$

$$pH_e - pK_a = log\ [A^-]/[HA]$$

$$10^x\ (pH_e - pK_a) = [A^-]/[HA]$$

The total uptake of label is then measured after the cells have been centrifuged through silicon oil. Because HA can diffuse freely though the cell membrane, the concentration of HA is the same inside and out:

$$HA_{ex} = HA_{in}$$

By subtracting intracellular HA from the total, it is then possible to estimate intracellular A^-:

$$Total - HA_{in} = A^-_{in}$$

Once you know the intracellular concentration of HA and A^-, the Henderson Hasselbalch equation is used to estimate intracellular pH.

Most bacteria have an intracellular volume to protein ratio of 3 to 4 μl/mg, and this value is estimated from the uptake of tritiated water (^3H_2O). Most of the ^3H_2O that passes through the silicon oil is in the intercellular volume, but the bacteria have an extracellular glycocalyx that traps some ^3H_2O. In Gram-negative bacteria, the periplasm can account for as much as half of the total volume, and this volume is not technically 'intracellular' space. Extracellular volume in the silicon pellet is estimated with labeled compounds that are not transported or metabolized. In the case of *E. coli,* sucrose is often used as an extracellular marker, but many bacteria utilize sucrose. Taurine is often a good alternative.

Anion accumulation. Fermentation acids are toxic to bacteria if the pH is low, and fermentation acids have long been used as food preservatives (pickles, yogurt, etc.). Because undissociated fermentation acids diffuse through cell membranes and dissociate in the more alkaline interior, it had often been supposed that fermentation acids act as uncouplers. Uncouplers are highly hydrophobic substances (e.g., CCCP and TCS) that remain in the membrane and shuttle protons until the cell is completely de-energized. However, the analogy between uncouplers and fermentation acids is flawed. Undissociated fermentation acids pass through the cell membrane, but the dissociated species are highly charged and membrane impermeable. If the undissociated species cannot pass back though the cell membrane to pick up another proton, the futile cycle of de-energetization cannot continue.

The uptake and accumulation of fermentation acid anions can, however, itself be toxic to the bacterium. The cell can counteract the accumulation of protons by pumping protons back out of the cell with ATPases or electron transport chains, but this activity will increase ΔpH. ΔpH causes a logarithmic accumulation of intracellular anions. If the concentration of fermentation acid is 100 mM outside, and the ΔpH is 1.0 unit, the intracellular concentration of fermentation acid anion

is 1000 mM! If the ΔpH is 2.0 units, the accumulation would in theory be 10,000 mM!!!!

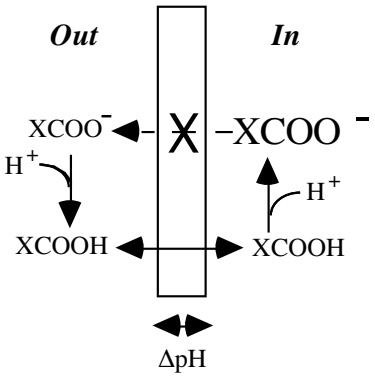

Fig. 20-6. The uptake of a fermentation acid (XCOOH), its dissociation in the more alkaline intracellular pH, and accumulation of the undissociated fermentation acid anion ($XCOO^-$).

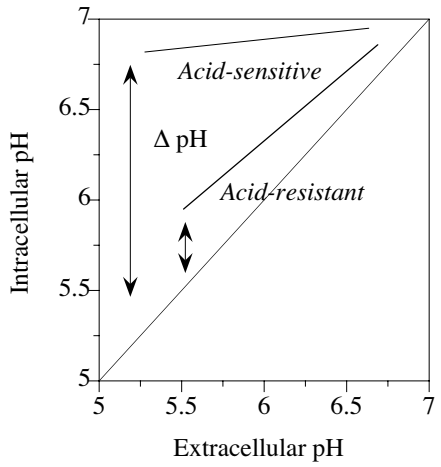

Fig. 20-7. Strategies of intracellular pH regulation in fermentative bacteria. Some bacteria try to maintain a near neutral intracellular pH, but this strategy leads to a large increase in ΔpH. Conversely, if ΔpH is low, intracellular pH declines.

Acid-resistant ruminal bacteria (e.g., *S. bovis, lactobacilli, P. ruminicola, S. ruminantium, and C. aminophilum*) counteract fermentation acid anion accumulation by allowing intracellular pH to decline. Because ΔpH is never greater than 0.7 units, the intracellular concentration of fermentation acid anion is never greater than 500 mM. This strategy of allowing intracellular pH to decrease, however, necessitates a metabolism that can operate at acidic pH. When its fermentation rate is slow and extracellular pH is near neutral, *S. bovis* produces acetate, formate and ethanol. However, pyruvate formate lyase (PFL) is a pH sensitive enzyme. If extracellular and intracellular pH decline, the PFL can no longer function, but pyruvate can be converted to lactate even if the pH is low. Some ruminal bacteria do not let their intracellular pH decline or have a metabolism that is pH-resistant. For example, when *F. succinogenes* was incubated at pH values less 5.8, ΔpH increased, but its membrane potential declined. When the membrane potential declined, cells were no longer able to transport cellobiose.

Genomics. In recent years, molecular biologists have made great advances in the study of bacterial genomes, and entire DNA sequences of many bacteria have been determined (including *F. succinogenes* and *R. albus*). Because individual genes can be isolated and placed on solid surfaces to create gene arrays, transcription can be monitored. By monitoring gene expression, the effect of environmental conditions (or stresses) can be more carefully defined. Comparative genomics provides insight into the evolution and distribution of genetic materials. For example, microbiologists had assumed that bacterial evolution was a 'tree of life' with distinctly different paths. However, we now know that genes, and even gene clusters, can move horizontally from one bacterial species to another. Thus, evolution is better depicted as a 'web of life.' Our understanding of rumen genomics is as yet very limited, but the technology has many potentials.

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Chapter 21

Ruminal Disorders and Toxicities

Acute acidosis. Cattle develop severe ruminal acidosis if they are switched abruptly from forage to grain, and this acute indigestion can be explained by an overgrowth of *S. bovis*. *S. bovis* has a very active amylase, and it can out grow other ruminal bacteria when starch is plentiful. *S. bovis* produces acetate, formate and ethanol when the carbohydrate concentration is low, but its fermentation is homolactic if the rate of carbohydrate fermentation is rapid. Because lactate is a stronger acid (lower pK_a) than VFA, lactate accumulation causes a marked and sudden decrease in ruminal pH. If lactate concentrations are very high, water is pulled from blood into the rumen to equilibrate the ionic concentration. Animals die from hemo-concentration and metabolic acidosis.

Fig. 21-1. Beef cattle consuming grain.

Acidic pH decreases the growth rate of *S. bovis*, but it is more pH-resistant than other ruminal bacteria. When other rumen bacteria are inhibited, *S. bovis* has less competition for substrate. *S. bovis* produces acetate, formate and ethanol when its fermentation rate is slow, but only if the pH is neutral. When the pH is acidic, pyruvate formate lyase is inhibited, and it can only produce lactate. This latter regulation leads to spiraling decrease in ruminal pH that is difficult to reverse.

Lactate utilizing species such as *M. elsdenii* can alleviate the acidosis by converting lactate to acetate and propionate, but these bacteria do not grow well at pH values less than 5.6. When the pH of the rumen becomes very low, lactobacilli displace *S. bovis*. Hungate explained this latter population shift with the observation that lactobacilli are even more pH-resistant than *S. bovis*. However, bacteriocin producing lactobacilli can displace *S. bovis* even if the pH is greater than 6.0.

Chronic acidosis. Cattle gradually adapted to grain do not develop acute acidosis, but they may have a chronically low ruminal pH. Cattle with chronic (subacute) acidosis do not ruminate as much, produce less saliva and have a slow ruminal fluid dilution rate. When ruminal fluid dilution declines, fewer fermentation acids are washed from the rumen to the abomasum (see Chapter 4, *Buffering and Acid Absorption)*. If the production rate of acids is high and out flow is low, the rumen is forced to absorb more acid. Because VFA absorption is a passive process, acids must accumulate before the absorption rate can increase. The net result is a chronically low ruminal pH.

Fig. 21-2. A scarred rumen wall that was caused by ruminal acidosis.

Chronic acidosis causes a variety of maladies. Because the rumen wall is not protected by mucous, the acids and increased osmolality cause ulceration and scarring. When the rumen wall is ulcerated, the lactate using bacterium *Fusobacterium necrophorum* migrates from the ruminal ulcers to the liver via the portal blood. Once in the liver, it embeds and causes absesses. *F. necrophorum* produces a leukotoxin and endotoxic lipoplysaccharide. Beef cattle are routinely fed antibiotics to suppress liver abscesses, but 13% of the livers are still condemned. Dairy rations contain less grain than feedlot rations, but the life span is longer and antibiotics are not normally fed. The incidence of liver abscesses in dairy cattle is approximately 25%.

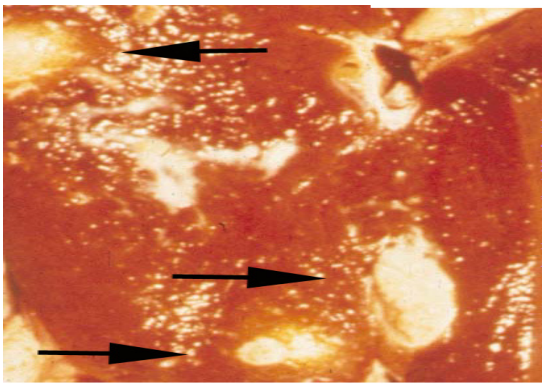


Fig. 21-3. Abscesses in the liver of a steer that was fed a grain-based ration.

Histamine. Animal tissues under stress produce histamine, but histamine can also be derived from the decarboxylation of dietary histidine. Histamine is an inflammatory agent that causes laminitis (sore feet) in cattle and horses, and lamintitis is a chief reason why lactating dairy cattle leave the milking herd. Dougherty noted that the "histamine content of rumen ingesta can be increased markedly by feeding protein rich feeds," but only a small conversion of the histidine to histamine can be harmful. Cattle consume as much as 70 g histidine each day (20 kg dry matter, 17% crude protein, 2% histidine), but as little as 70 mg of histamine per liter of ruminal fluid is toxic.

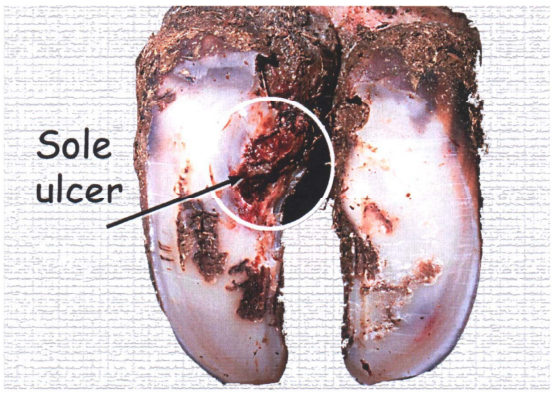


Fig. 21-4. The sole ulcer of a dairy cow with laminitis. (Courtesy of Christer Bergsten)

In the 1950's, Rodwell isolated lactobacilli from the rumen that produced histamine, but recent work indicates that a novel bacterium *(Allisonella histaminiformans)* is probably more important. Cattle fed commercial dairy rations had few if any histamine producing lactobacilli, but they had significant numbers of *A. histaminiformans*. *A. histaminiformans* is a monensin-resistant bacterium, but 16S rDNA indicates that its closest relatives are Gram-positive species. The histidine decarboxylation reaction consumes an intracellular proton, and this consumption creates a protonmotive force. A variety of non-ruminal bacteria decarboxylate histidine, but only *A. histaminiformans* uses histidine as its sole energy source for growth.

A. histaminiformans grows at pH values as low as 4.0, but pH alone does explain its niche in the rumen. Dairy rations often have large amounts silage, and silage extracts stimulate the growth of *A. histaminiformans*. Because non-ensiled alfalfa did not stimulate histamine production nearly as much, it appears that *A. histaminiformans* needs a growth factor that can be derived from silage fermentation. Further work will be needed to assess the involvement of *A. histaminiformans* in laminitis, but the observation that alfalfa silage stimulates histamine production could have practical significance. Logue et al. noted that dairy cattle fed grass silage had a significantly greater incidence of laminitis and foot lesions than cattle that were fed non-fermented dry forage.

Bloat. Ruminal gases (CO_2 and CH_4) escape from the rumen via eructation. Eructation is initiated by the stiffening of the cranial pillar, a reticulo-ruminal fold that holds feed particles away from the cardia. Small bubbles coalesce to form larger ones, and gases pass up the cardia into the esophagus. Mature cattle produce as much as 60 liters of gas per hour. Ruminants do not usually have problems eructating, but those fed lush legumes (e.g., clover or alfalfa) or grain can bloat.

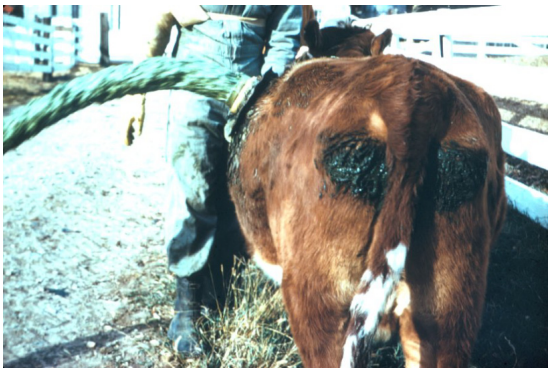


Fig. 21-4. Digesta escaping from the fistula of a bloated steer. (Courtesy T.G. Nagaraja).

Immature legumes and lush grasses (e.g., wheat) have little fiber to trigger rumination and eructation, and denatured chloroplast proteins can also produce a froth which entraps small gas bubbles. Because ruminants do not have a reflex that triggers vomiting, gas accumulates in the rumen, and eructation is inhibited. When bloat is severe, gas pressure in the rumen can be as high as 70 mm Hg. The rumen compresses the lungs, blood flow is cut off, and the animal suffocates. Cattle fed grain-based diets can also bloat, but ruminal fluid is not usually frothy. The mucopolysaccharide (slime) of starch-fermenting bacteria produces a foam, but some cattle feedlot cattle have a 'dry' bloat. In most cases, feedlot bloat only depresses feed intake and performance, but some animals die.

Certain cattle are more susceptible to bloat than others, and studies with identical twins support the idea that bloat has a genetic determinant. Bloat-resistant cattle secrete more saliva and their saliva contains more mucin, a substance that acts as an antifoam. Conversely, cattle more prone to bloat seem to secrete less saliva and have greater populations of mucinolytic bacteria. However, further investigation is needed to assess the roles of microbial populations and the animal in

bloat susceptibility. Abnormalities in physiological development may also play a role bloat. Gas also accumulates in the intestines of cattle fed grain, and this accumulation can also cause death.

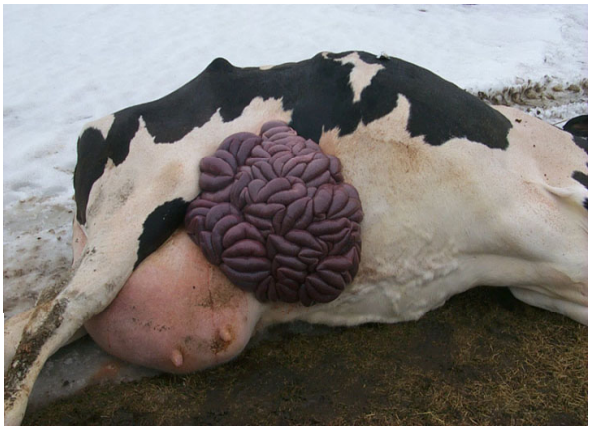


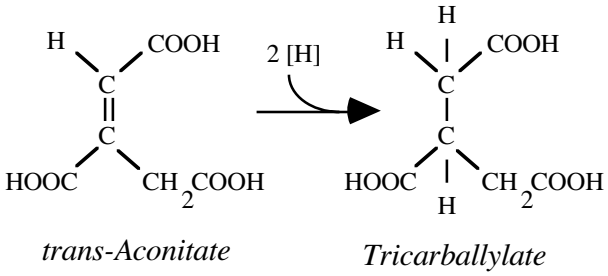
Fig. 21-5. Intestinal bloat in a cow that was fed a grain-based ration. The abdomen was incised and the intestines were then exposed. (Courtesy T.V. Muscato).

Grass Tetany. Cattle and sheep that graze lush grass in the spring can suffer from a hypomagnesemia known as 'grass tetany.' When grasses (e.g., crested wheat grass or winter wheat that is grazed as forage) grow rapidly, the magnesium content of the plant is often low, and the potassium content is high. In animals, potassium is a competitive inhibitor of magnesium absorption, and this feature aggravates the hypomagnesemia. When blood magnesium is very low, the central nervous system is subjected to hyper-excitability, and coma and death are likely.

In the 1960's, Stout and co-workers noted that grasses causing grass tetany also had high concentrations of *trans*-aconitate. Plants use *trans*-aconitate as an anion to counteract increases in potassium. It was initially hypothesized that *trans*-aconitate bound magnesium and decreased its availability, but later work showed that *trans*-aconitate was fermented rapidly by ruminal bacteria. Some of the *trans*-aconitate was fermented to acetate, but much of it was converted to another tricarboxylic acid, tricarballylate.

Fig. 21-6. A cow with grass tetany being treated with an infusion of calcium and magnesium gluconate. (Courtesy of D. Grunes).

Tricarballylate is absorbed but not metabolized by the animal. Tricarballylate is a competitive inhibitor of the citric acid cycle enzyme, aconitase, but its effect on metabolism has not been examined in any detail. Studies with rats showed that tricarballylate could chelate divalent cations in blood and promote urinary excretion. Rats fed tricarballylate had lower blood magnesium, calcium and zinc levels than rats fed citrate, a metabolizable analog.



trans-Aconitate *Tricarballylate*

Fig. 21-7. The conversion of *trans*-aconitate to tricarballylate.

S. ruminantium is the most important producer of tricarballylate, and it uses this reaction for reducing equivalent disposal. Because *S. ruminantium* has a high affinity for sucrose and rapidly growing plants have an abundance of this sugar, *S. ruminantium* numbers in the rumen are already high. Some ruminal bacteria can be inhibited by ionophores, but *S. ruminantium* is monensin-resistant.

Grass tetany is a disease that comes on suddenly, and surviving animals are not chronically affected. In vitro enrichments with *trans*-aconitate yielded a bacterium identified by 16S rDNA as *Acidaminacoccus fermentans*. *A. fermentans* converts *trans*-aconitate to acetate. When cattle were ruminally inoculated with a large dose of *A. fermentans* and the ruminal fluid was incubated in vitro, the conversion of *trans*-aconitate to tricarballylate decreased more than 50%. In vivo enrichment was confounded by the fact that *A. fermentans* only uses a narrow range of substrates. *A. fermentans* ferments citrate, other bacteria are better suited to use this substrate.

Nitrate Toxicity. Nitrates from drinking water (contaminated by nitrate fertilizers) or plant materials can be toxic to livestock including ruminants. Nitrate itself is not highly toxic, but it is rapidly converted to nitrite by ruminal bacteria. Once absorbed, nitrite oxidizes the ferrous iron of hemoglobin. Methemoglobin does not transport oxygen. Nitrite causes metabolic anoxia, rapid breathing, trembling, and in extreme cases, death. Many plants can accumulate nitrate. Immature oats prepared as hay, mangolds or sorghum or sorghum-Sudan hybrids that have been harvested after a killing frost can have large amounts of nitrate. Denitrifying bacteria convert nitrite to nitric oxide, nitrous oxide and ultimately to nitrogen gas, but this process is slower than the conversion of nitrate to nitrite. Nitrite is converted to ammonia by a process known as assimilatory nitrate reduction, but this pathway is repressed by ammonia.

Oxygen is not a terminal electron acceptor for respiration in the rumen, but some ruminal bacteria reduce nitrate. Anaerobic respiration is facilitated by nitrate reductase, a molybdenum containing membrane bound enzyme that is coupled to electron transport carriers (e.g., cytochromes). When sheep were fed large amounts of nitrate, methanogenesis was inhibited. Addition of nitrate to ruminal fluid from un-adapted animals caused a large increase in nitrite and lactate and a marked decrease in propionate, butyrate and even acetate. Some bacteria (e.g., *Clostridium botulinum*) are sensitive to nitrite, but in vitro and in vivo studies indicate that ruminal populations are highly adaptive. When sheep were given gradual increases in nitrate, little nitrite accumulated. Continuous cultures that were treated with nitrate produced less methane, and bacterial biomass tended to increase.

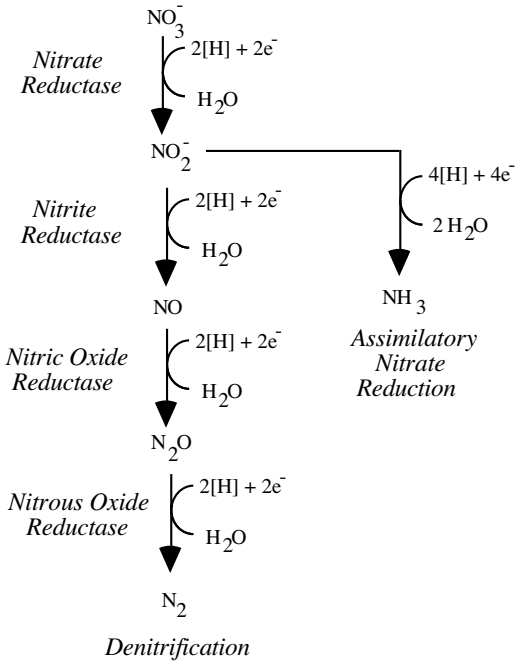


Fig. 21-8. Bacterial pathways of nitrate metabolism.

The studies of Reddy indicated that a variety of ruminal bacteria have nitrate reductase activity, but *S. ruminantium* is the most easily identified type. Nitrate reducing anaerovibrios and Gram-positive rods were also isolated. These later bacteria fell into two groups, those that used formate and produced lactate and lesser amounts of acetate and succinate, and those that produced mostly acetate and lesser amounts of succinate.

Sulfide toxicity. When large amounts of sulfate are added to ruminant rations (or drinking water is contaminated), animals can die from hydrogen sulfide poisoning. Sulfide is not rapidly absorbed from the rumen, but it is found in ruminal gases that are passed to the lungs during

eructation. Absorbed sulfide is a potent inhibitor of mitochrondrial electron transport (cytochrome a to cytochrome a_3), and animals can die from metabolic anoxia. *Desulfovibrio desulfuricans* was isolated from the rumen at sufficient numbers (> 10^8 cells per ml) to account for published rates of sulfide production in vivo. Sulfate reduction proceeds via the formation of adenosylsulfate:

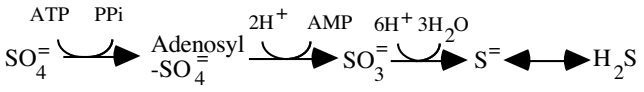


Fig. 21-9. Bacterial sulfate reduction.

Sulfate reduction, like nitrate reduction, is an alternative method of reducing equivalent disposal, and sulfate reducers compete with methanogens. Many sulfate reducers use lactate as an energy source and convert it to acetate:

$$2\ Lactate + 2H_20 \rightarrow 2\ Acetate + 2CO_2 + 8H^+$$

$$8H^+ + SO_4^{-2} \rightarrow S^{-2} + 4H_2O$$

Sulfide also arises from the fermentation of sulfur amino acids, but the production does not usually exceed the biosynthetic rate of sulfur amino acids. If ruminants are subjected to chronically high concentrations of hydrogen sulfide, they develop polioencephalomalacia (PEM) and brain lesions virtually identical to viral PEM or a thiamin deficiency. Because ruminal bacteria and ruminants need dietary sulfate to grow, PEM is not easy to prevent. Nutritionists recommend sulfur to nitrogen ratios of less than 1 to 10, but this arbitrary guideline does not differentiate whether the sulfur is coming from sulfate or other sources. Bacteria producing hydrogen sulfide can be inhibited by anthraquinones, but these compounds have not yet been approved for cattle diets. Because anthroquinones are fat soluble compounds, they may accumulate in meat or milk.

Oxalate toxicity. Some plants such as *Halogeton glomeratus* accumulate as much as 25% oxalate, and this poisonous compound causes gastroenteritis and renal damage. In humans, chronic oxalate ingestion (e.g., spinach and rhubarb) causes kidney stones (calcium oxalate). In the 1970's, Allison and is colleagues isolated an oxalate degrading bacterium from the rumen. *Oxalobacter formigenes* takes up oxalate and converts it to formate and carbon dioxide.

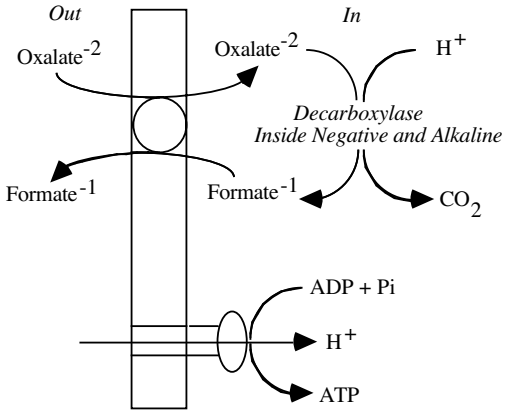


Fig. 21-10. The oxalate metabolism of *O. formigenes.*

The decarboxylation consumes a proton and generates ZΔpH. Because the uptake of $oxalate^{-2}$ is coupled to the efflux of $formate^{-1}$, the overall reaction also generates a membrane potential (inside negative). Oxalate metabolism does not generate ATP directly via substrate level phosphorylation, but membrane bound ATPases use the protonmotive force as a driving force to synthesize ATP.

Un-adapted cattle are more susceptible to oxalate toxicity than those that have been consuming it. If cattle are adapted gradually, *O. formigenes* numbers increase, and the oxalate is degraded. *O. formigenes* is widely distributed in herbivores and humans, but some humans are not colonized. Absence of these bacteria (due to diarrhea or antibiotic treatments) causes an increased absorption of oxalate and an increased risk of kidney stones. Preliminary results indicate that people can be inoculated with *O. formigenes* to alleviate these problems.

Mimosine toxicity. Tropical trees and leguminous shrubs belonging to the genus *Leucaena* originated in Central America, but *Leucaena leucocephal*a is now widely distributed in tropical and sub-tropical areas. Leucaena grows rapidly and its leaves are rich in protein. However, the leaves and the seeds have a toxic amino acid, mimosine. Mimosine is converted to 3-hydroxy-4-(1H)-pyridone (3,4 DHP) by enzymes present in both leucaena and ruminal bacteria.

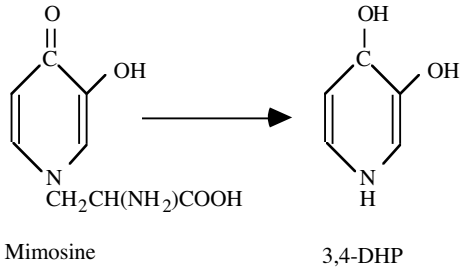


Fig. 21-11. The conversion of mimosine to 3,4-DHP.

DHP causes low weight gain, hair loss, goiter and esophageal ulceration in cattle. However, Ray Jones noted that cattle and sheep in Hawaii could consume large amount of leucaena without showing signs of toxicity. Australian cattle inoculated with ruminal fluid from a leucaena-adapted, Indonesian goat recovered, and this observation supported the theory that DHP degrading ruminal bacteria were geographically isolated. Allison isolated the DHP degrading bacterium *Synergistes jonesii*. *S. jonesii* is a Gram-negative, strictly anaerobic rod that does not use carbohydrates, but it utilizes histidine and arginine. *S. jonesii* is widely distributed in animals where leucaena is a native plant, but it could not be detected in cattle in Florida. Inoculation of cattle in Florida with *S. jonesii* protected them from the toxicity.

Bovine emphysema. When beef cattle in the Western United States are taken off range and allowed to graze lush irrigated pastures, some of the animals developed acute pulmonary emphysema. Elsewhere, similar symptoms have been noted with cattle grazing rapidly growing rape, kale, turnip tops, small grains, alfalfa and a variety of grasses. Work by James Carlson and his colleagues showed that this type of emphysema was caused by the ruminal conversion of tryptophan to the lung toxin, 3-methylindole also called skatole. Tryptophan is first deaminated and decarboxylated by the normally occurring microorganisms that produce indoleacetate. Indoleacetate is then converted to 3-methylindole by lactobacilli that grow on the sugars in lush grass. Because lactobacilli are Gram-positive bacteria that are inhibited by monensin, a common feed additive, the problem has largely been alleviated.

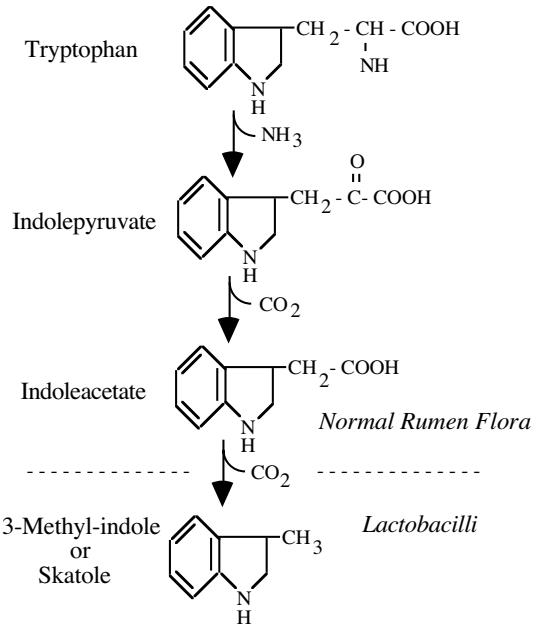


Fig. 21-12. The conversion of tryptophan to 3-methylindole (skatole) by the combined action of the normal rumen flora and lactobacilli.

Miserotoxins. Many legumes including species of *Astragulus* (e.g. crown vetch), *Lotus, Cornilla* and *Indigofera* have nitrotoxins that cause increased heart and respiration rates, frothy salivation and a lack of coordination. Affected animals can also have elevated blood concentrations of nitrate and methemoglobin, but the exact etiology of this toxicity has not been precisely defined. Nitrotoxins are present in the plant as glycosides, but the carbohydrate moieties are rapidly removed and fermented by ruminal bacteria. Glycoside hydrolysis releases 3-nitro-1-propionate, 3-nitropropanol or nitroethane. Cheng and his colleagues showed that cattle given nitroethane, the least toxic analog, adapted so they could metabolize 3-nitropropanol at a faster rate. This latter observation supports the idea that the rumen microflora can be selected or adapted to enhance this detoxification. Bacteria capable of detoxifying nitrotoxins were isolated, and they were placed in a new genus and species, *Denitrobacterium detoxificans*. H_2, formate and lactate serve as reductants for the conversion of the nitro groups to their corresponding amines. Thus, 3-nitro-1-

propionate is converted to β-alanine and 3-nitropropanol is converted to 3-amino-propanol, but the pathways have not been defined.

Fluoroacetate toxicity. Plants such as *Oxylobium parviforum* accumulate fluoro-acetate in their leaves and seeds, and fluoroacetate has also been used as a "pesticide" to combat rabbits and wild dogs (dingo) in Australia. The toxicity of fluoro-acetate is mediated by its conversion to fluorocitrate by mammalian enzymes. Fluoro-citrate is a non-metabolizable analog of citrate, and it inhibits the citric acid cycle enzyme, aconitase. Animals can tolerate some fluoro-citrate, but the toxicity is enhanced by exercise or the ingestion of large amounts of water. Bacteria degrading fluorocitrate could not be easily enriched. However, in the 1980's, Gregg and his colleagues used recombinant engineering to transfer a 'dehalogenating gene' from a non-ruminal organism to *B. fibri-solvens*. Concerns over genetically engineered bacteria have delayed its practical application. (see Chapter 19, *Manipulation of Ruminal Fermentation).*

Cyanide toxicity. Some plants (e.g., sorghums, clover and cassava) have significant amounts of cyanide containing glycosides, and this accumulation is enhanced by adverse growing conditions (e.g., drought or frost). Because these glycosides are hydrolyzed to release cyanide, toxicity and death can result. Cyanides can be detoxified via their con-version to thiocyanates, asparagine, aspartate or formate and ammonia, but bacteria respon-sible for this conversion have not yet been identified.

Selenium toxicity. Selenium is an essential mineral for mammals, but large amounts can be toxic. Some plants (e.g., *Astragulus*) accumulate selenium and drinking water can also have high concentrations. Selenite is water soluble, is more readily absorbed, and is more toxic than selenate. The ruminal conversion of selenite to selenate is a detoxification mechanism. Several species of ruminal bacteria (e.g., *S. ruminantium, W. succino-genes,* and *P. ruminicola)* are able to use selenite, and this reaction is the reverse of nitrate metabolism:

$$SeO_3 + H_2O \rightarrow SeO_4 + 2[H] + 2e^-$$

The feed additive monensin inhibits selenite conversion to selenate in vitro, but it is not clear if this occurs in vivo.

Tannins and gossypol. Some plants produce polyphenolics, and use these compounds to combat insects and other pests. Tannins and other polyphenolics bind to proteins and denature them. Ruminants are not as sensitive to polyphenolics as simple stomached animals, and it is now thought that low concentrations of tannins may protect feed proteins from wasteful degradation.

Ruminal bacteria differ greatly in their sensitivity to tannins. Gram-negative bacteria have an outer membrane that offers some protection. Ruminal enrich-ments indicated that some streptococci (*Streptococcus gallolyticus)* tolerated high concentrations. These bacteria decarbox-ylate gallic acid and produce pyrogallol. Some strains of *S. ruminantium* have a tannin acyl-hydrolase that may be invol-ved in a more complete detoxification scheme.

Cotton and okra have a polyphenolic yellow pigment known as gossypol, and seed concentrations can be as great as 0.2%. Gossypol is very toxic to simple-stomached animals, and it affects a variety of physiological systems. Recommenda-tions for poultry indicate that the ration should have less than 0.02% gossypol. Because gossypol is an aromatic hydrocarbon, the gossypol content of cotton seed meal can be significantly reduced by heat treatments. Ruminants tolerate larger amounts of gossypol than non-ruminants. Ruminal microorganisms can bind gossypol, but specific detoxification reactions per se have not yet been demonstrated.

Toxic alkaloids. More than 6000 toxic alkaloids have been identified in plants, and their accumulation can be a serious problem to livestock. Physiological effects include liver, lung and kidney damage as well as a decrease in food intake and a general malaise. Ruminants are often more resistant to toxic alkaloids than non-ruminants, but detoxification reactions have not been demonstrated. An exception to this generalization is helio-trine. In the 1970's, Lanigan isolated a Gram-positive bacterium (*Peptococcus heliotrinreducans*) that converted pyrroly-zidine alkaloids into non-toxic methylene derivatives.

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Chapter 22

Manipulation of Ruminal Fermentation

Ideal Rumen Fermentation. Ruminants are dependent on fermentation products for most of their nutrients, and nutritionists have sought to modify or manipulate the fermentation. These modifications include changes in diet, feed treatments and additives. To evaluate these effects, we should review the characteristics of an ideal fermentation:

1. Rapid rates of fiber digestion. Fiber is an important part of many ruminant rations, but ruminal fiber digestibility is often less than 50%. When fiber digestion rate is slow, food intake decreases.

2. Rapid and efficient production of microbial protein. Microbial protein is an important amino acid source for the animal, but microbial protein yield is dependent on the amount of energy that is diverted to maintenance and energy spilling.

3. Little ammonia accumulation. Protein fermentation in the rumen deprives the animal of amino acids. Because excess ammonia must be converted to urea, the animal must expend energy to compensate for wasteful amino acid degradation.

4. Little methane production. As much as 11% of the feed energy can be belched away as methane. If reducing equivalents can be diverted from methane production to propionate, energy retention is increased.

5. Optimal ratio of VFAs. Propionate is a precursor of blood glucose, but acetate and butyrate cannot be converted to glucose. If the animal does not have enough propionate, amino acids must be converted to glucose.

6. Little lactate. Lactic acid is a 10-fold stronger acid than the VFAs. Lactate can decrease ruminal pH, restrict food intake, inhibit microbial protein synthesis, cause founder or even kill the animal.

7. Fewer toxins. Some ruminal bacteria produce toxins, but this production can in many cases be arrested. If toxic dietary compounds are degraded by ruminal microorganisms, the animal is protected.

Diet formulations. In the early days of ruminant nutrition, nutritionists often tried to focus on specific components of the diet (e.g., fiber, protein, energy, vitamins or minerals). Such specialization is, however, not well suited to the manipulation of rumen fermentation. This point is illustrated by protein and carbohydrate interactions Microbial protein is an amino acid source for the animal, but the synthesis of microbial protein is dependent on the availability of carbohydrates to drive protein synthesis. Thus, it is possible to utilize carbohydrates as a protein supplement (so long as there is an adequate supply of ammonia).

Ruminants versus Simple-Stomached Animals

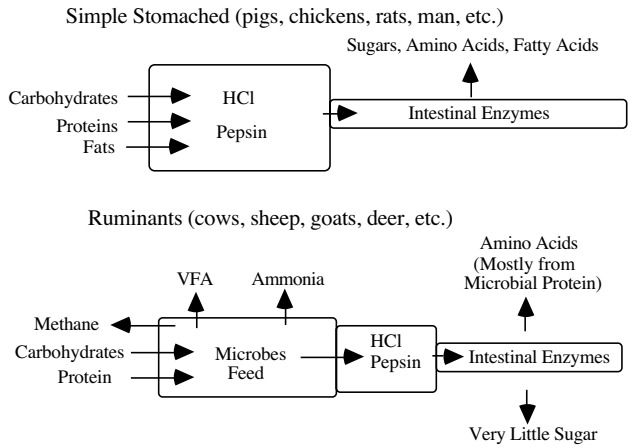

Fig. 22-1. Feed digestion in simple stomached animals versus ruminants.

When ruminants are fed forages rich in protein, ammonia accumulates in the rumen, and this excess nitrogen is eventually lost as urinary urea. Early work by Lewis and others showed that starch supplements decreased ammonia accumulation. This decrease can be explained in two ways. First, if the bacteria have additional carbohydrate they grow and assimilate more ammonia. Secondly, if carbohydrates are available, the bacteria can incorporate amino acids directly into microbial protein, and less ammonia is produced.

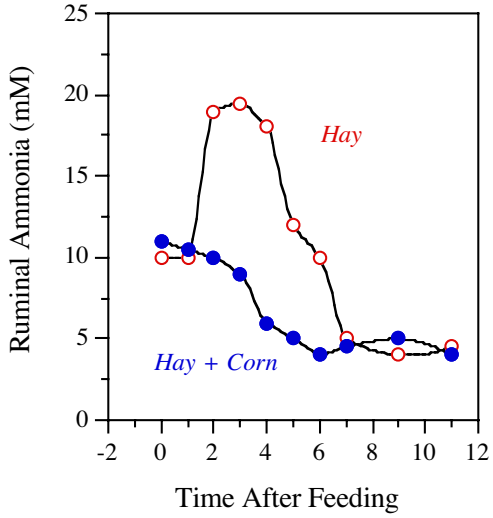

Fig. 22-2. Ammonia accumulation in cattle fed hay or hay and corn. Re-drawn from the data of Lewis (1957).

Feed treatments. Feed processing can have a profound impact on ruminal fermentation end-products. Heat due to pelleting, flaking, extruding, and toasting alters the balance of protein and carbohydrate degradation rates. When moist feeds (e.g., grain supplements) are heated, the proteins become less soluble and degradable, but the starches become more soluble and available. Dry heat (e.g. micronization) decreases the rate of starch digestion, but it does not always decrease protein degradation.

Feeds that are chopped, shredded or ground have increased surface area, and this increase often causes an increase in fermentation rate. However, smaller particles may pass unfermented from the rumen. The overall benefit of particle size reduction is feed-dependent. When a hay is ground, fiber digestibility decreases because the increase in passage rate exceeds the increase in fermentation rate. However, if high moisture corn grain is ground, the increase in fermentation rate is greater than the increase in passage rate, and digestibility increases.

Some feed ingredients are so valuable (e.g., lysine and methionine) or potentially toxic (e.g., fats or urea) that nutritionists have developed strategies of protecting them from ruminal degradation. This protection can be mediated by the pH

difference between the rumen and abomasum. If the coating or product (e.g., calcium and magnesium salts of fatty acids) is soluble at neutral pH, material is protected from ruminal degradation. However, once these compounds reach the low pH of the abomasum, the protective layer or salt solubilizes, and the nutrient is available for intestinal absorption. This strategy of ruminal protection can, however, be confounded by acidic silages. If acid-sensitive materials are mixed with silages, the protection can be lost before it reaches the rumen. Another recent strategy of ruminal protection has been to use polyurethane capsules. These capsules have small pores that release compounds like urea slowly. This protection is, however, a delicate procedure not without problems. Polyurethane capsules can be damaged by harsh feed mixing or even rumination.

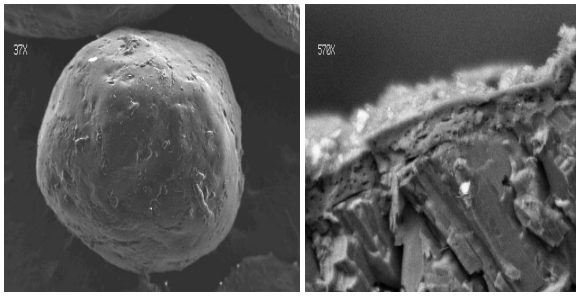

Fig. 22-3. Urea (Optigen) that has been encapsulated in a polymer to slow its rate of ruminal degradation (approximately 10X magnification). The right hand panel shows a particle that had been sectioned (Courtesy of C.R. Holtz).

Buffer salts. In the 1960's, it became apparent that the milk fat percentage of lactating dairy cattle could be increased by adding sodium bicarbonate or carbonate to the ration, and ruminal pH often increased. Because sodium bicarbonate is the dominant buffer in the rumen, these supplements were marketed as 'ruminal buffers.' However, there has been little indication that buffering capacity of ruminal fluid actually increases. The question then arises, why does sodium bicarbonate increase ruminal pH?

When sodium bicarbonate is added to a fermentation flask in vitro, the sodium and dissolved bicarbonate concentrations both increase, and there is an increase in buffering capacity. However, the situation in vivo is not the same. When cattle ingest sodium bicarbonate or sodium carbonate, ruminal sodium increases, but this increase is only transient. The animal either drinks water or water moves into the rumen from the blood to equilibrate the ion concentration. Because homeostatic mechanisms of the animal prevent an increase in sodium, bicarbonate and buffering cannot increase appreciably. The impact of sodium bicarbonate on ruminal pH is most easily explained by fluid dilution rate. If the animal drinks more water, ruminal fluid dilution rate increases, and this dilution carries VFAs and undigested starch from the rumen to the abomasum and lower gut (see Fig. 4-1, page 9). If ruminal VFA concentrations decrease, pH increases.

Chlorinated hydrocarbons. In the 1970's Van Nevel, Demeyer and Prins noted that methanogenic bacteria were sensitive to chlorinated hydrocarbons (e.g., chloroform and carbon tetrachloride), and even trace amounts inhibited methane production in vitro and in vivo. In fact, I once heard that three medicated cough drops had enough chloroform to inhibit methane production in a sheep, but I never validated this claim.

Because chlorinated hydrocarbons attack methanogens directly, some H_2 accumulates, but hydrogenase is then inhibited (see Fig. 15-2, page 50). When hydrogenase is inhibited, acetate production declines, propionate increases and the animal can, in theory, retain more energy. In vitro and short-term in vivo experiments were so encouraging that a large chemical company developed a process for chlorinating starch and constructed a pilot plant. However, long term feeding experiments showed little benefit. As time progressed, it became apparent that ruminal bacteria could adapt and inactivate these compounds. The adaptation process has not been thoroughly defined, but reduction is the most likely mechanism. Pyromellitic diimide was also developed as a ruminal methane inhibitor, but it is susceptible to a similar adaptation and inactivation. The coenzyme M analog, bromoethanesulfonic acid, has a different target site, but some cows have methanogenic flora that are resistant to this compound.

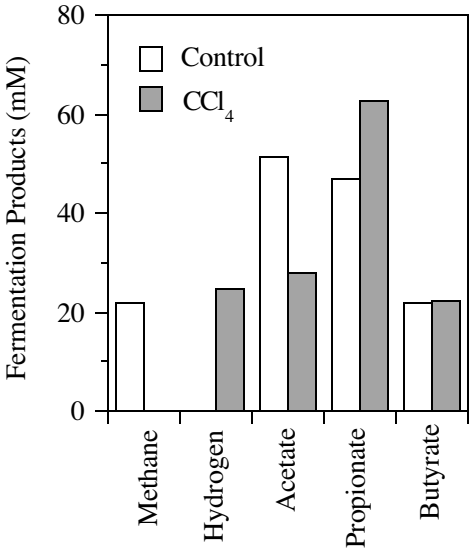

Fig. 22-4. The effect of carbon tetrachloride on the in vitro ruminal fermentation. Re-drawn from the data of Van Nevel et al. (1970).

Ionophores. Monensin is an antibiotic that was originally developed as a coccidostat (anti-protozoan) for chickens. However, research showed that this polyether also had a positive impact on ruminants. Feedlot cattle fed monensin consumed less feed and had a 6% greater feed efficiency, and grazing animals gained weight 15% faster. Within a short time after its FDA approval (1976), most feedlot cattle in the United States were being fed monensin as a feed additive. Monensin is the most commonly used ionophore, but the FDA has also approved lasalocid, laidlomycin and salinomycin.

Monensin affects several key aspects of ruminal fermentation. Methane declines 30 to 50%, and this effect only accounts for one third of the increase in feed efficiency. Early work by Van Nevel and Demeyer indicated that monensin did not cause a decrease in methane production if hydrogen and carbon dioxide were the substrates. This observation indicated that monensin did not have a direct impact on methanogens. Monensin inhibits carbohydrate fermenting bacteria that produce H_2. Because H_2 producing bacteria (rather than the methanogens) are inhibited, H_2 does not usually accumulate. Monensin decreases methane and the ratio of ruminal acetate to propionate, but this effect is dependent on diet. Cattle fed forage have an acetate to propionate ratio as high as 4 to 1, and under these conditions monensin decreases the ratio to 2.5 to 1. When cattle are fed grain-based rations, the acetate to propionate ratio is already low (1 to 1) so the effect is not nearly so great.

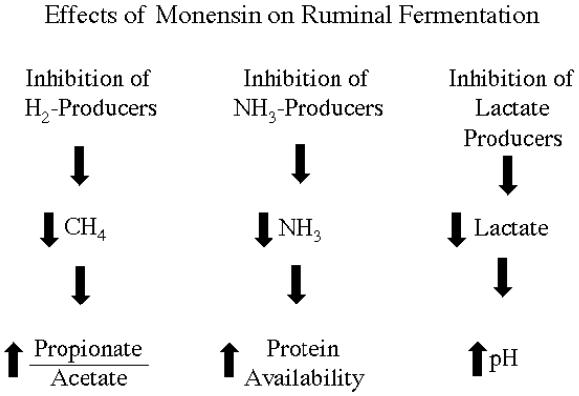

Fig. 22-5. The ability of monensin to alter ruminal fermentation.

Early work indicated that monensin decreased ruminal ammonia concentrations, but the amino acid sparing effect of monensin has sometimes been difficult to demonstrate.

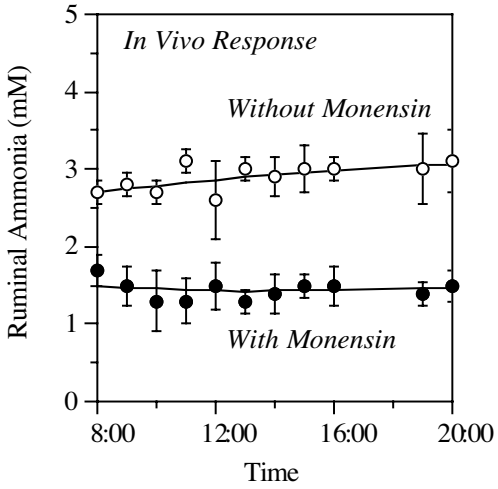

Fig. 22-6. The effect of monensin on steady state ruminal ammonia concentration of cows fed hay.

When cattle were fed timothy hay or hay plus soybean meal, monensin caused a large decrease in ammonia, but a similar effect could not be demonstrated when alfalfa hay was the forage even though the protein content was similar. The idea that

ionophores can spare protein is supported by the observation that steers supplemented with soybean meal had an improved feed efficiency when monensin was fed, but those given only urea did not. In vitro studies showed that some lactate producing ruminal bacteria are sensitive to monensin, but this effect has been difficult to demonstrate in vivo. Increases in ruminal pH are partly due to more consistent intake of feed. Monensin also seems to prevent bloat, but the mechanism of this action has not been determined.

Ionophores are hydrophobic molecules that are produced by *Streptomyces* species (e.g *S. cinnamnensis*). Ionophores dissolve in membranes and destroy ion gradients. Monensin is a metal/proton antiporter that is shaped like a 'donut.' The interior of the molecule shields a monovalent cation (e.g., sodium or potassium), and the carboxyl group binds a proton.

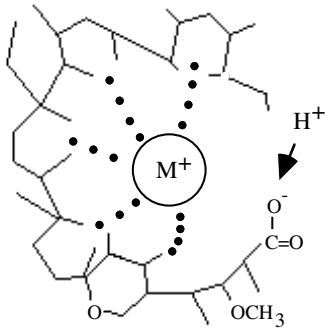

Fig. 22-7. The reactive center of monensin.

Monensin can only move across the cell membrane if it is bound to a metal ion or proton. Monensin shuttles back and forth across the cell membrane to move these ions in opposite directions. The direction of ion movement is dictated by the magnitude of the ion gradient. Monensin only moves monovalent cations across, but lasalocid is able to move divalent cations (e.g. Ca^{++} and Mg^{++}) as well as monovalant ions. Ionophores have different preferences (selectivities) for specific ions, but the direction of metal and proton movement ultimately is dictated by the concentration gradient of ions across the cell membrane in accordance with the Nernst equation:

$$Z \log_{10} [M^+]_{in} / [M^+]_{out}$$

where Z has the value of 62 mV at 39° C, and M^+ denotes a specific metal ion that is translocated across the cell membrane to establish a concentration gradient.

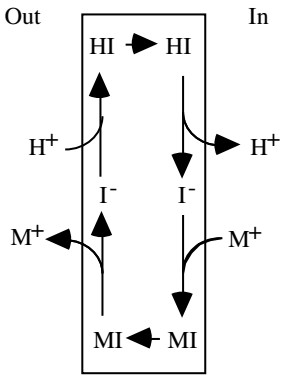

Fig. 22-8. The ability of an ionophore (I) to shuttle metal cations (M^+) and protons (H^+) across a cell membrane.

Most living organisms maintain a higher concentration of potassium inside the cell than outside, and they expel sodium and protons. When glycolyzing *S. bovis* cells are treated with monensin, there is a rapid efflux of potassium and an influx of sodium and protons.

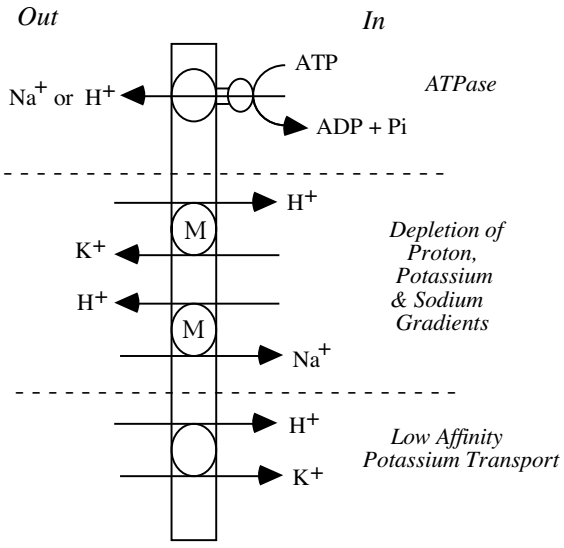

Fig. 22-9. The effect of monensin (M) on the ion gradients and ATPase activity of *S. bovis*.

Because the sodium gradient is smaller than the potassium gradient, there is a net accumulation of protons and intracellular pH declines. *S. bovis* then uses its membrane bound ATPase to re-establish its protonmotive force (Δp), and this activity leads to a decline in intracellular ATP. Within a short period of time the cells are de-energized, and *S. bovis* is unable to grow.

The carboxyl group of monensin has a pK_a of 7.95 (Pressman, 1973), this group is dissociated over normal ranges of ruminal pH. However, continuous culture studies indicated that pH was having a profound impact on activity. When the pH was adjusted from 6.7 to 5.7, the amount of monensin needed to cause a 50% decline in cell yield decreased almost 10-fold. *S. bovis* persisted until the monensin concentration was 10 μM, but cell yield was very low. Because the specific rate of glucose consumption increased more than 5-fold, it appeared that monensin was promoting a futile cycle of energy spilling (see Chapter 18, *Growth, Maintenance and Energy Spilling).*

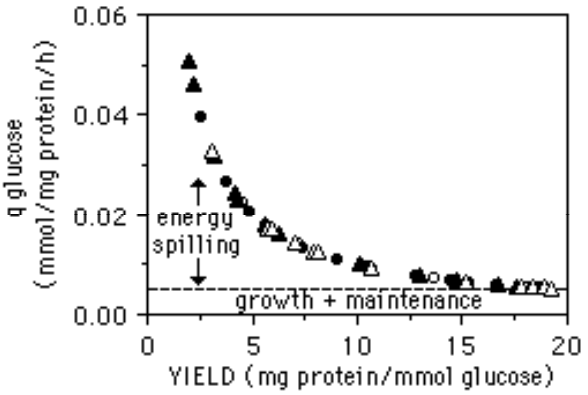

Fig. 22-10. The effect of monensin (circles) and lasalocid (triangles) on the glucose consumption rate of *S. bovis* cells. Open symbols show pH 6.7 and closed symbols are pH 5.7. (Taken with permission from Chow and Russell, 1990)

Not all ruminal bacteria are sensitive to monensin. If they were, ruminal fermentation would be indiscriminately inhibited, and there would be little improvement in feed efficiency. Monensin resistance can be correlated with differences in cell wall structure. Gram-positive bacteria lacking an outer membrane to protect the cell membrane, are generally more sensitive to monensin than Gram-negative species. The outer membrane model of monensin resistance is, however, not always clear-cut. Some Gram-negative strains are as monensin-sensitive as Gram-positive species.

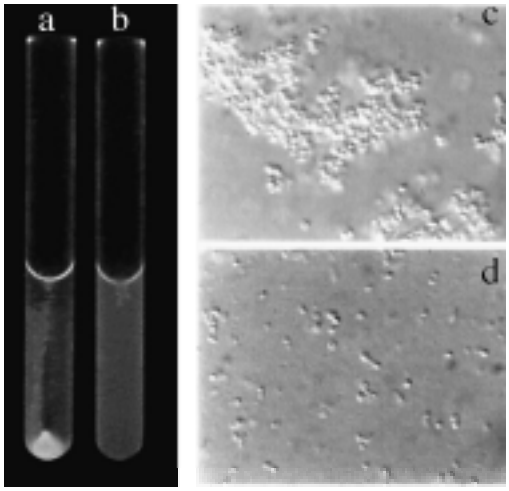

Fig. 22-11. The ability of lysozyme to agglutinate wild-type (a,c) and monensin-selected (b,d) *P. bryantii* B_14 cells. Taken with permission from Callaway and Russell (1999).

Pure culture studies indicate that monensin resistance is mediated by carbohydrates on the outside surface of the cells. Monensin-resistant *P. bryantii* B_14 cells had more polysaccharide and were no longer agglutinated by lysozyme, a positively charged protein that binds to lipid A of the outer membrane. Similar results were reported for *C. aminophilum,* a Gram-positive bacterium that also becomes monensin-resistant.

Monensin alters fermentation end-products in vitro and in vivo, but the correlation of in vitro and in vivo effects was confounded by inherent differences. If a steer with a 70 liter rumen is fed 350 mg monensin per day, the monensin concentration would in theory be 7 μM, but this simple calculation does not account for: 1) ruminal dilution rate, 2) the fact that the density of bacteria in vivo is much greater than in vitro, and 3) the binding of monensin to feed particles. Based on these latter comparisons, the in vitro concentration of monensin has often been larger than the physiological dose.

Potassium depletion studies, however, supported the idea that monensin does in deed alter microbial ecology in vivo.

When cattle were fed a timothy hay diet, the amount of monensin needed to cause 1/2 maximal potassium depletion (K_d) was only 0.2 μM (200 μg bacterial protein per ml), but this value increased to 1.6 μM as soon as the animals are fed 350 mg monensin per day. When monensin was withdrawn, K_d decreases rapidly to its original value. These experiments indicated that: 1) monensin resistance is prevalent before monensin is given, 2) increases in resistance occur very rapidly, 3) the resistance does not persist once the antibiotic is withdrawn.

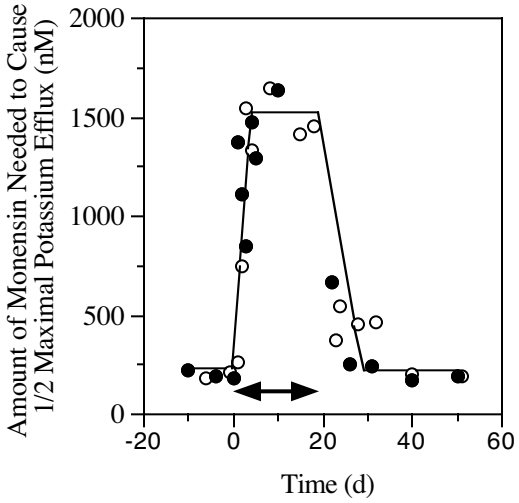

Fig. 22-12. The effect of monensin (during the arrow) on the amount of monensin that is needed to cause 1/2 maximal potassium depletion (K_d) from mixed ruminal bacteria. Taken with permission from Lana and Russell (1996).

Monensin is the most commonly fed ionophore, but other ionophores have also been approved by the FDA (e.g., lasalocid, salinomycin, and laidlomycin). Lasalocid has approximately the same mode of action as monensin, but it binds divalent ions (e.g., calcium and magnesium) as well as mono-valent ones. In vitro studies indicated that it was even more potent than monensin, but in vivo studies indicated the reverse. When cattle fed timothy hay were given 350 mg of lasalocid per day, there was little change in the potassium diffusion constant (K_d) or acetate to propionate ratio. Differences between in vitro and in vivo response may be related to solubility. Lasalocid is less soluble in water than monensin, and it is more likely to bind to feed particles and ionophore-resistant bacteria.

In recent years, there has been an increased perception that animals should not be fed antibiotics routinely. This perception is based on the observation that: 1) animals fed antibiotics often have more antibiotic-resistant bacteria than those not fed antibiotics, and 2) antibiotic-resistant genes can be transferred to food borne pathogens. However, ionophores are not used in human medicine, and there is little indication that ruminal bacteria are becoming more resistant. Ionophore resistance does not seem to be mediated by plasmid-mediated genes that can be easily transferred to other species of bacteria.

Essential oils. Steam and solvent extracts of plant materials contain a group of compounds called essential oils (e.g, eugenol and limonene). These oils are not "essential" in a traditional sense, but they have a pleasant odor or essence. Essential oils are found in herbs, spices and perfumes, and many of them also have antimicrobial activity. Essential oils have been fed to chickens, swine and more recently to ruminants as an alternative to antibiotics. In vitro experiments indicate that essential oils spare amino acids. When mixed ruminal and obligate amino acid (hyper-ammonia-producing) bacteria were treated with essential oils, ammonia declined. Essential oils also decreased the ability of *R. amylophilus* to colonize feedstuffs and degrade protein. However, further work will be needed to see if similar effects effect can be demonstrated in vivo.

Malate supplementation. In the 1970's, Linehan et al. noted that *S. ruminantium* could not utilize lactate until malate, aspartate or fumarate was added to the culture medium. This stimulation can be explained by the pathway of lactate conversion to acetate and propionate. When lactate is converted to acetate and propionate, excess reducing equivalents can be recycled by the conversion of malate to fumarate and ultimately to propionate. Some strains of *S. ruminantium* use lactate, and Martin and his colleagues showed that malate supplementation caused a decrease in ruminal lactate and an increase in the ruminal pH

of cattle fed feedlot rations. Some forages are rich in malate.

Yeast. Yeast has in some cases improved milk production and body weight gain, but the mechanism of this effect has not been clearly defined. In vitro experiments indicated that fiber digestion was enhanced, but these improvements were detected late in the incubation (e.g., 96 hours). When nylon bags containing straw were suspended in the rumen, the rate of fiber digestion was also increased, but this trend was not statistically significant if hay was used. However, more recent in vitro experiments indicate that live yeast can compete with *S. bovis* for glucose and prevent a decrease in pH. The yeast also stimulated the growth of *M. elsdenii*. These results indicate that yeast may prevent lactic acidosis, and in vivo experiments supported this conclusion.

Wallace hypothesized that live yeast products might scavenge oxygen and protect fiber digesting bacteria. This hypothesis was based on the observation that non-respiratory mutants did not stimulate the growth of cellulolytic bacteria in an artificial rumen device as much as wild-types. Immature lambs that were supplemented with yeast had a lower ruminal redox potential than untreated controls, but it is not clear if this effect persists in adult animals. Some yeast products have significant amounts of malate, and this characteristic may enhance lactate utilization. Because yeast products have a very attractive aroma, they may enhance food intake.

Enzymes. Fiber digesting enzymes (e.g., hemicellulases, xylanases, and β-glucanases) can be produced industrially and added to ruminant rations, but their value has been difficult to assess. Commercial preparations often have more than one enzyme, and the composition is not always consistent. Ruminal bacteria produce proteinases that can degrade enzymes, but at least some commercial preparations are highly resistant to ruminal degradation. Enzyme application is complicated by the fact that ruminal bacteria already produce similar enzymes, but fiber digesting ruminal bacteria can be inhibited when the ruminal pH is low. Many of the commercial enzymes have a low pH optimum, and the greatest benefits have been observed with grain-based rations.

When enzymes are applied to feeds as a pre-treatment, the exposure time can be significantly increased. In vitro fermentation studies indicated that feeds pre-treated with enzymes were "more indigestible" than those that were not treated. However, only the most easily digested parts of the feed are typically hydrolyzed, and few studies have documented a statistically significant increase in total tract digestibility. Studies with chickens indicated that xylanases could decrease digesta viscosity, prevent constipation and increase food intake. Similar activity has been demonstrated in ruminants, but the viscosity of ruminant colonic contents is lower and not likely to impede nutrient intake or absorption.

Ruminal inoculation. The ability of researchers to counteract mimosine toxicity by cross inoculation (see Chapter 18, *Ruminal Disorders and Toxicities*) contradicts the assumption that bacteria are ubiquitous, but the success of ruminal inoculation in solving other problems is very limited. In the 1980's, several groups attempted to inoculate the rumen with acetogens, and hoped that acetogens, by converting CO_2 and H_2 to acetate, would increase energy availability to the animal. However, methanogens have a better affinity for H_2 and they out-compete acetogens. Methanogens can be transiently inhibited by chlorinated hydrocarbons, but these compounds are as toxic to acetogens as methanogens. The coenzyme M analog, bromoethanesulfonic acid (BES), is more selective, but some methanogenic populations are not affected. The strategy of inoculating the rumen with acetogens was also complicated by the fact that ruminal acetogens have the ability to use other substrates (e.g., sugars). They only use H_2 and CO_2 if these other substrates are unavailable.

Acetogens are more abundant in the intestinal tract of termites than methanogens, and this observation introduces yet another question. Why do acetogens proliferate in the termite gut while methanogens dominate the rumen? The answer may be related to the protozoa rather than the bacteria. In the rumen, many methanogens are found on the surface of protozoa. Termites have even more protozoa than ruminants, but these protozoa may not be suited to a symbiotic interaction with methanogens.

When cattle are switched abruptly from forage to feedlot rations, lactate increases and ruminal pH declines. However, little lactate is observed in properly adapted animals. The adaptation of cattle to grain

can be explained by an increased number of lactate utilizing bacteria. In vivo experiments indicated that *M. elsdenii* inoculations increased the ruminal pH of cattle fed grain-based rations, but field applications were complicated by the fact that *M. elsdenii* is an oxygen-sensitive bacterium that is difficult to store and apply.

When cattle graze lush grass in the spring, they can die from a hypomagnesemia that is caused by *trans*-aconitate and its conversion to tricarballylate (see Chapter 18, *Ruminal Disorders and Toxicities*). Cattle have low numbers of *A. fermentans,* a bacterium that converts *trans*-acontitate to acetate, but this population is initially too low to prevent grass tetany. Cook and Russell proposed that ruminal inoculations with *A. fermentans* might afford animals some protection against hypomagnesemia, but this idea has not yet been tested.

Genetic engineering. With the advent of recombinant DNA technology and the ability of microbiologists to genetically alter bacteria, the question arose, could ruminal bacteria be modified to improve ruminal fermentation? In the 1980's, rumen microbiologists cloned a variety of genes from ruminal bacteria into *E. coli,* and at least four projects were proposed: 1) improvement of fiber digestion by enhancing cellulase production, 2) the addition of genes to *F. succinogenes* so it could use xylans, 3) transfer of fluoroacetate degradation genes to *B. fibrisolvens,* and 4) the creation of an acid-resistant ruminal bacterium that could digest at low pH.

Improvements in fiber digestion were thwarted by the inability of the researchers to clone critical enzymes (native cellulases with activity against insoluble cellulose), and the observation that surface area (not the amount of enzyme) is the factor that usually limits fiber digestion. The project to increase cellulose digestion at low pH was based on the observation that *P. bryantii* was highly acid-resistant, and it already produced a carboxymethylcellulase (CMCase). The CMCase did not bind or digest insoluble cellulose, but the CMCase was reconstructed to include a cellulose binding domain. *E. coli* clones had native cellulose activity, but the reconstructed gene could be not successfully returned to *P. bryantii*. Similar problems thwarted the *F. succinogenes* project. *F. succinogenes* lacked so many components of xylose utilization that a simple and straightforward scheme was not possible. Greater success was seen with the fluoroacetate degradation. Gregg and his colleagues successfully transferred a dehalogenase gene from *Moraxella* to *B. fibrisolvens*, and the recombinant persisted in the rumen for several months. However, concern over the release of genetically engineered bacteria into the environment, has, to date, prevented this application.

Early work indicated that rumen microbial ecology was complicated, but the true extent of this diversity was not evident until the 1990's when molecular typing methods were perfected. This diversity has itself been a complication. If individual strains only occupy small niches and are only found in some animals, it is unlikely that recombinantly engineered bacteria would have a significant impact. Genetic engineering is likewise complicated by the fact that not all ruminal bacteria have standard methods of transcription or genetic regulation. For instance, CMCase of *P. bryantii* $B_1$4 was cloned into *E. coli,* but the initial clones recognized fortuitous promoters and produced a highly truncated enzyme. The truncated enzyme hydrolyzed CMC, but it lacked the N-terminus that tethered it to the cell surface. By sequencing upstream, the entire gene was eventually cloned, but *E. coli* did not recognize the *P. bryantii* promoter and vice versa.

Most recombinant DNA schemes have sought to add a gene (or genes) to the rumen microflora, but many problems are due to genes already present in ruminal bacteria (e.g., excessive ammonia and lactate production). Mutants lacking specific functions can be created, but the wild-types would need to be excluded. In the laboratory, antibiotics are often used as a counter selection, but antibiotic use in animal rations has been criticized on the basis that it leads to an increase in resistance.

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Chapter 23

Models of Rumen Fermentation

Pure cultures. In the 1950's, there were at least two 'schools of thought' on the future of rumen microbiology. One group was devoted to the isolation and characterization of pure cultures, but others felt that enzyme profiles of the mixed population might be more fruitful. The latter group was thwarted by the observation that diet had little impact on the enzymatic profile of mixed ruminal bacteria, and they were unable to measure critical activities (e.g., cellulases).

Many of the pure cultures that we use today were isolated by Bryant, but Hungate did not share Marvin's enthusiasm for a culture collection. Hungate felt that it was better to re-isolate fresh strains each time they were needed. Bryant argued that his strains varied considerably, and felt that they should be preserved so this diversity could be more fully explored.

Because most ruminal bacteria can only be maintained on agar slants for 3 weeks in a refrigerator, and liquid nitrogen storage is tedious, few people had the resources to maintain a large collection. In the 1980's, Teather exploited techniques commonly used in artificial insemination and showed that ruminal bacteria could be preserved in glycerol for long periods in a normal freezer.

In 1960, Hungate stated that the ecological analysis of a microbial habitat entailed: 1) a description of the habitat and its environmental conditions, 2) an assessment of the kinds and numbers of organisms present, 3) a knowledge of the activities and requirements of these organisms, and 4) some quantification of the extent to which these activities are expressed. "In many cases, the rare bacteria are quantitatively unimportant, performing in small measure the activities accomplished chiefly by the abundant types. But there is a chance that numerically insignificant species may be unique in performing an essential function."

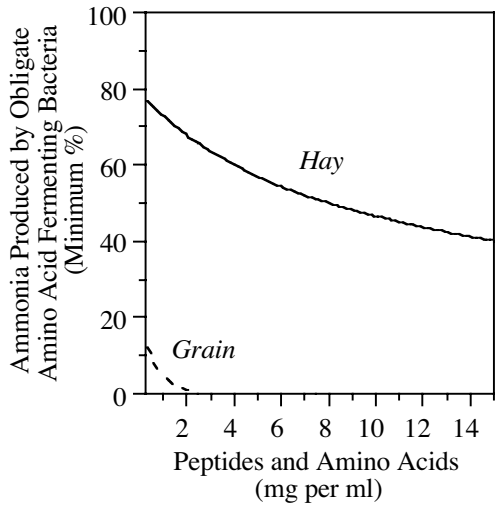

Fig. 23-1. The role of obligate amino acid fermenting bacteria in ruminal ammonia production. Values were simulated from a model based on specific activity (Rychlik and Russell, 2000).

Specific activities can be used to demonstrate the importance of a pure culture. These calculations are based on the assumption that the mass of an individual bacterium, species or strain (mg protein per liter) and its specific activity (nmol product per mg bacterial protein per liter) should approximate the activity of the total population (nmol product per min per liter).

nmol/mg protein/min x mg protein/liter = nmol/liter/min

By using this strategy, it was possible to demonstrate that obligate amino acid fermenting ruminal bacteria accounted for a large fraction of the ammonia in cattle fed hay even though they accounted for only a small portion of the total population. The question then arises, are the cultures that we have isolated and studied in the laboratory good models for the bacteria that are important in vivo? When Weimer and his colleagues used 16S rRNA to enumerate cellulolytic bacteria, the probes hybridized with less than 2% of the total RNA, a value that seems abnormally low. Other workers obtained values that were 2 to 3 fold higher, but the possibility that the probes were only reacting with a portion of the population could not be excluded.

Table 23-1. The abundance of cellulolytic species in the rumen. The values were derived from 16S rRNA specific to each species and a universal probe that reacts with all eubacteria. The data were taken fromWeimer et al. (1999).

Bacterium	24% Fiber	32% Fiber
R. albus	0.91%	1.35%
R. flavefaciens	0.15%	0.18%
F. succinogenes	0.19%	0.26%
Total	1.25%	1.79%

However, pure cultures can be useful even if they only represent a portion of the total population. For example, pure cultures of ruminal cellulolytic bacteria have an absolute requirement for branched chain VFA, use ammonia as a nitrogen source for growth, do not grow if the pH is less than 5.8 and digest ball-milled cellulose at rates greater than or equal to 10% per hour. Because the mixed population has similar characteristics, the pure cultures have provided useful models of cellulose digestion in vivo.

Mixed cultures. Mixed cultures can be used to examine the effect of dietary treatments on fermentation end-products, but it is difficult to maintain the same diversity in vitro as is found in vivo. For example, if mixed cultures are provided with excess hexose, *S. bovis* will outgrow other ruminal bacteria, and lactate is the dominant end-product. The diversity is greater if solid substrates are used, but once again there is often a marked simplification.

Short term, mixed culture incubations circumvent the problem of enrichment and selection. However, even short term, mixed culture studies must be interpreted carefully. In vitro experiments often have bacterial cell mass values that are 3 to 10-fold lower than would be present in vivo, and they often lack protozoa. Microbial mass is particularly important with compounds such as ionophores. Ionophores are lipophilic substances that accumulate in cell membranes, and the ratio of ionophores to cells is more important than the absolute concentration.

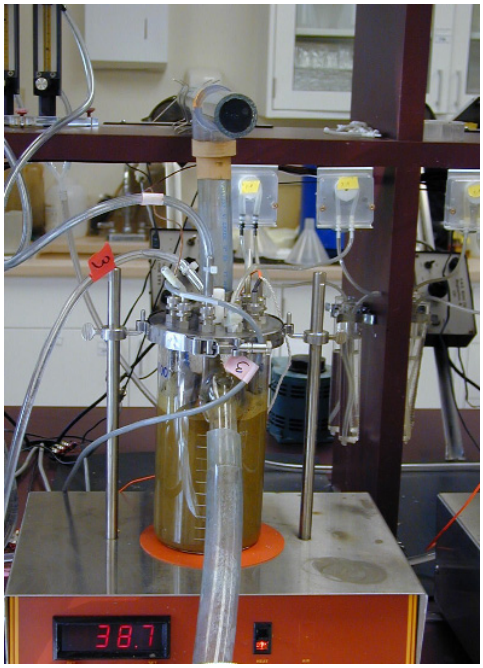

Fig. 23-2. The fermentation vessel of a dual flow 'artificial rumen.' (Courtesy M.D. Stern)

Artificial rumens. In the 1960's and 70's, Slyter, Hoover, Stern, Varga and their colleagues constructed mechanical devices that could be inoculated with mixed ruminal microorganisms, handle solid as well as soluble substrates, and in some cases, maintain dual passage rates for solids and liquids. These devices also maintained protozoal populations for extended periods of time. Because microbial protein can be determined with markers (e.g., purines or daminopimelic acid), it is possible to estimate the efficiency of microbial protein synthesis. NaOH was added to control pH, but it should be noted that 'artificial rumens' have glass walls that cannot absorb VFA. If the dry matter content is as high as those found in vivo (approximately 10% dry matter), VFA concentrations can be abnormally high.

***In situ* experiments.** Another avenue for studying ruminal fermentation is the *in situ* or 'nylon (or dacron) bag' approach. *In situ* methods use bags with pores large enough to let ruminal microorganisms pass but small enough to retain solid feed materials. If the bags are removed from the rumen at various times and the residue is weighed, it is possible to estimate the rate of dry matter disappearance. *In situ* experiments have several advantages. Because the bags are incubated directly in the rumen, the feed materials are exposed to a representative population of microorganisms. However, if the pores are too small to let micro-organisms pass through or too large to retain the feed materials, problems can arise. Accuracy is improved by rinsing the undigested feed residue to remove micro-organisms, but these procedures must be carefully monitored to make sure that feed is not lost and microbes are not still attached to feed.

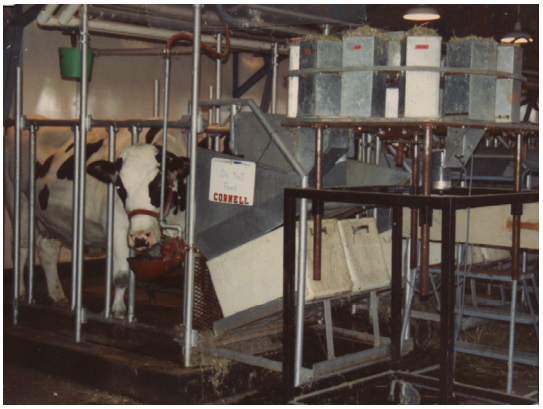

Fig. 23-3. A fistulated cow being fed with a rotary feeder to obtain steady state ruminal fermentation conditions.

Rotary feeders. In vivo studies are complicated by the fact that animals often consume large meals and have different eating behavior. Because the rumen is not at steady state, many samples must be taken to determine fermentation patterns, and standard deviations can be larger than the experimental differences. Most of these problems can be counteracted by using rotary feeders. If animals are fed 12 times per day at 95% of ad libitum intake, feed is consumed almost continuously, and the rumen is similar to continuous culture. Because the kinetics are steady state, the number of samples needed is dramatically decreased (see Fig. 22-6, Chapter 21, *Manipulation of Rumen Fermentation)*. If the animals are equipped with duodenal cannula, it is also possible to measure the flow of microbial protein from the rumen.

Germ-free ruminants. When calves or lambs are delivered by Cesarean section and reared in isolation from other ruminants, they will develop a rumen ecosystem that has bacteria but lacks

protozoa. Researchers at the Rowett Research Institute in Scotland and the INRA Research Institute (Clermont-Ferrand) in France expanded the scope of such experiments so the effects of bacteria and fungi could also be monitored. In their studies, lambs delivered at birth by hysterectomy were passed through an antiseptic bath or given large doses of antibiotics to kill bacteria. The lambs were then placed in a sterile isolator, given sterile food and water and inoculated in stepwise fashion with pure cultures of bacteria or fungi.

Many species of bacteria readily colonized the rumen of germ-free lambs, but an active cellulolytic population was difficult to establish. *F. succinogenes* only persisted if 182 strains of other bacteria had already been added. If fewer strains of other bacteria were given, *F. succinogenes* did not reside long or its numbers were very low. The observation that cellulolytic populations were difficult to establish, however, gave rise to another animal model that was easier to control. If new-born lambs were left outside the isolator for 24 hours, they developed a microflora that did not contain cellulolytic bacteria or fungi. When these animals were inoculated with pure cultures of cellulolytic bacteria and fungi, the fermentation was similar to conventional animals.

Results from selectively inoculated animals supported the idea that *F. succinogenes* could digest cellulose from straw more completely than either *R. albus* or *flavefaciens*. Lambs inoculated with only fungi did not digest cellulose as completely as those inoculated with cellulolytic bacteria, and *R. flavefaciens* increased the cellulose digestion of animals harboring fungi. In vitro studies indicated that fungi were proteolytic, but this effect could not be demonstrated in selectively inoculated lambs. Lambs not placed in the isolator until 24 hours after birth lacked methanogens, and this result allowed the researchers to examine the effect of methane production in vivo. Lambs inoculated with methanogens had greater enzyme activities (especially xylanase), faster rates of dry matter disappearance and greater VFA concentrations than those lacking methanogens.

Need for mathematical models. In simple stomached livestock (pigs and chickens), the prediction of weight gain from dietary ingredients is often very good. However, until recently, feeding standards for ruminants were notoriously inaccurate. In ruminants, feeds are subjected to a fermentation in the rumen prior to gastric and intestinal digestion, and this feature greatly complicates diet formulations. This point is illustrated by microbial protein synthesis. In ruminants, microbial protein is the dominant amino acid source for the animal, but the magnitude of this synthesis is dependent on a variety of factors.

Microbial protein synthesis is dependent on the amount of dry matter that the animal consumes, but not all of this material is digested. Whole tract digestibility provides a better prediction of microbial protein than dry matter intake alone, but microbes can only use feeds that are degraded in the rumen. If feed is digested further down the tract, it does not generate microbial protein that can be utilized. Microbial protein synthesis is also dependent on the rate at which the material is digested. If the fermentation is slow, much of the energy is used for maintenance, and bacterial yield declines. The estimation of microbial protein production is further complicated by the fact that ruminal bacteria spill more energy and grow less efficiently when peptides and amino acids are not available and the pH is acidic.

Types of mathematical models. With the advent of computer technology in the 1960's, the ability of biologists to describe biological relationships mathematically was greatly enhanced, and mathematical models have been constructed to describe various aspects of ruminal fermentation. Mathematical models are classified as either *empirical* or *mechanistic, deterministic* or *stochastic* and *dynamic* or *static*.

Empirical models describe the relationship of two or more variables, and they are usually based on a regression. *Empirical* models are only as strong as the data set used to develop the regression and should not be extrapolated beyond the range of the original data. These models often provide little causal insight. For example, the correlation between dry matter intake and digestible nutrients is relatively high, but this relationship does not explain why some diets are more digestible than others or why some diets generate more microbial protein per unit of digestible energy. Empirical models can be modified or adjusted to account for additional variables, but this approach is not necessarily mechanistic.

Mechanistic (sometimes called theoretical) models seek to explain why relationships vary and are derived from natural behaviors and biological principles. Mechanistic models are sometimes less accurate than empirical ones, but properly constructed mechanistic models are often better over a broader range of conditions. In making a comparison of models, one should remember the debate that occurred over Ptolemaic and Copernican views of our solar system. When Copernicus first proposed a sun-centered system, people argued that the earth-centered, Ptolemaic system, gave a more accurate prediction of planetary movement than the new one he was proposing.

Deterministic models are based on the assumption that solutions can be derived from exact equations or functions, but biological data often has a high degree of intrinsic variability. S*tochastic* (*probabilistic)* models use statistical relationships to account for this inherent variation, and results change according on the amplitude of the variance in each component. *Static* models ignore time effects, but *dynamic* models describe temporal relationships in a time-dependent fashion (e.g., ruminal pH changes during the course of a feeding cycle).

Most empirical, and many mechanistic models are static, and the outcome of one relationship does not necessarily provide the starting point for the next calculation, cycle or step time. *Dynamic* models accommodate such interactions, but they are prone to compounding errors. If one element of the model is wrong, this error can occur over and over in an additive fashion until the final prediction is ridiculous. When this occurs, the dynamic model can literally 'spin out of control.' This out-of-control behavior can be dampened by making sure that the model is robust enough to exclude abnormalities. Such behavior only occurs if the relationship between 'stocks' and 'flows' is inadequate.

Validation. Models are often analyzed by comparing predicted outputs with experimental data, but they should be validated with data sets that did not directly contribute to the original model. These comparisons are typically a linear regression, but it should be realized that not all regressions are well suited to this type of analysis. If the regression does not take into account the fact that both the model and the experimental data have 'errors,' the regression coefficient can be unrealistic (biased). Techniques have been developed to account for the inherent variability of each point of the data set. Such techniques use a weighted least square regression algorithm to compute the standard deviation of the regression parameters (observed versus predicted).

Once the model has been compared to experimental data, the researcher can then perform a sensitivity analysis by changing critical constants or equations by some arbitrary amount (e.g., 10%). This analysis will tell the researcher if a particular component of the model is critical or only marginally important. This exercise is much easier if the model is a simple one (fewer interactions to track). Model improvement is then contingent on the ability of the researcher to pin point areas of the original algorithm that are either inaccurate or do not adequately address some important aspect of the biology.

In the last 15 years, nutritionists have striven to model rumen fermentation in a mechanistic fashion so the impact of dietary changes on fermentation products and microbial protein availability can be more accurately predicted. A variety of computer simulation models have been published and evaluated as research tools (see Baldwin et al., 1987; Dijkstra et al., 1992; Tamminga et al. 1994; Sauvant, 1997), but few of them have been used in a practical way to evaluate and formulate farm rations. The one exception to this generalization is the Cornell Net Carbohydrate Protein System (CNCPS). The CNCPS and its derivative (Cornell, Penn, Minor--CPM Dairy) have been used by farmers, ranchers and consultants to design better rations. The NRC Recommendations for Beef Cattle have a mechanistic model (level 2) that was adopted from the CNCPS. The rumen sub-model of the CNCPS accommodates many basic features of ruminal fermentation, and experience indicates that it is a good teaching tool for students.

CNCPS. The Cornell Net Carbohydrate Protein System (CNCPS) is a deterministic, mechanistic and static model that uses relative rates of feed digestion and passage to estimate the extent of ruminal fermentation and microbial protein flow. The CNCPS has been used successfully by farmers to evaluate and formulate rations, and feed cost savings as great as 17% have been documented.

Fig. 23-4. A computer disc of the 4.0 version of the Cornell Net Carbohydrate Protein System (CNCPS).

The CNCPS calculates the ruminal availability of each feed from the relative rates of fermentation and passage (see Chapter 5, *Feeds and the Balance of Digestion Rates)*. Each component is assigned a first order rate constant of disappearance (Kd). The passage rate (Kp) is computed from dry matter intake, metabolic body weight ($BW^{0.75}$), and forage content of the ration. The Kp of each feed is adjusted according to type of feed (concentrate versus forage) and by the effectiveness of its NDF (eNDF). Ruminal availability is then a simple function of digestion versus passage (i.e., Kd/(Kd +Kp).

Once the ruminally available pool of each component has been calculated, the magnitude of bacterial growth is computed from the carbohydrate fractions. The CNCPS assumes that fats do not drive microbial growth, and ruminally degraded proteins (and resulting peptide and amino acids) only stimulate bacterial mass that is derived from carbohydrates. The rumen also has a pool of obligate amino acid fermenting bacteria that produce ammonia at a rapid rate, but these bacteria are found at low numbers in the rumen and are not a significant source of microbial protein.

The CNCPS assumes that bacteria are the only source of microbial protein leaving the rumen. This assumption is a major simplification, but *in vivo* studies support the idea that ruminal protozoa lyse and account for less than 10% of the microbial protein entering the abomasum (see Chapter 10, *Ruminal Protozoa and Fungi)*. The impact of ruminal protozoa is addressed by decreasing the theoretical maximum growth yield of the bacteria by 20%. This adjustment seeks to account for protozoal predation, protozoal competition with bacteria as well as protozoal lysis.

A	B_1	B_2	C
NH_3& NPN Amino Acids Peptides	Rapidly Degraded Proteins	Slowly Degraded Proteins	Lignin Bound Proteins
Immediately Soluble in Water	Soluble in Buffer	Insoluble in Buffer	Insoluble in ADF

Proteins in Various Feedstuffs

Fermentation versus Passage ------->

NFC and FC in Various Feedstuffs

Soluble in NDF	Insoluble in NDF	Soluble in ADF	Insoluble in ADF
Sugars Organic Acids Starches Pectin	Hemicellulose	Cellulose	Lignin Residual CHO

A	B_1	B_2	C

Fig. 23-5. System of carbohydrate and protein characterization used by the Cornell Net Carbohydrate Protein System (CNCPS).

Because each carbohydrate fraction is described by a first order rate constant, it is possible to estimate bacterial growth rates and correct the theoretical maximum growth yield for maintenance energy (see Chapter 18, *Growth, Maintenance and Energy Spilling)*. The CNCPS divides the bacteria into two pools based on distinct metabolic differences. The fiber carbohydrate (FC) bacteria: 1) only utilize neutral detergent fiber (NDF) as an energy source, 2) have low maintenance energy requirements, and 3) derive all of their nitrogen from ammonia. The non-fiber carbohydrate (NFC) bacteria: 1) use starch, pectin, sugars, organic acids, etc., 2) have a threefold higher maintenance energy requirement, 3) can use either ammonia or amino nitrogen, and 4) can produce ammonia if carbohydrates are lacking. The rumen sub-model was constructed before obligate amino acid fermenting bacteria were

isolated, and these bacteria are currently part of the NFC bacteria.

The CNCPS recognizes the fact that ruminal bacteria have different patterns of nitrogen utilization. NFC (but not FC) bacteria are stimulated by the availability of peptide and amino acids, and amino nitrogen availability is a function of protein degradation rates, the peptide uptake rate and the relative utilization of ammonia and amino nitrogen by NFC bacteria. FC bacteria only use ammonia nitrogen, but NFC bacteria can derive as much as 2/3 of their nitrogen from amino N, if amino nitrogen is available. The impact of amino nitrogen on NFC yield is based on the ratio of peptide and amino acids to organic matter (peptide and amino acid plus carbohydrates). NFC yield can be increased by as much as 18.7% if the peptide and amino acids are available.

The peptide stimulation function does not directly address the impact of amino nitrogen on the microbial growth rate or the impact of growth rate on maintenance. Because peptide stimulation is invoked even if the fermentation rate (Kd) of NFC is slow, the relationship between amino nitrogen and energy spilling is simplistic. Energy spilling is more apt to occur if the rate of energy source degradation is fast, and NFC bacteria are being restricted by amino acids (not energy).

Original versions of the CNCPS noted that pH could have a negative impact on the yield of NFC bacteria if ruminal pH was less than 6.0, but ruminal pH was not predicted. The yield of NFC was decreased 2.5% for every 1% decrease in the NDF when NDF was less than 20% of the ration. Because FC only comprise a small part of the population when the diet is apt to decrease pH, the impact of pH on FC digestion was ignored. Pitt et al. attempted to predict ruminal pH and its effect in a more mechanistic fashion, and three facets of this work were incorporated into later versions of the CNCPS (Fox et al., 2000). First, pH was a function of effective NDF (pH = 5.425 + 0.04229 x eNDF) where eNDF was defined by cell wall content and particle size. Secondly, yield of NFC bacteria was decreased as a function eNDF. Thirdly, as ruminal pH declined, the maintenance energy coefficient of FC bacteria increased and the rate of fiber digestion decreased. These adjustments did not account for the effect of NFC digestion rate on pH or the impact of ruminal fluid dilution rates on VFA concentrations in the rumen. De Veth and Kolver concluded that these adjustments had too high a pH threshold and led to an underprediction of microbial growth and fiber digestion at low pH.

The original CNCPS recognized that a ruminal nitrogen deficiency would have a negative impact on bacterial yield, but it did not quantify this effect. Tedeschi et al. added a series of equations that were designed to accommodate ruminal nitrogen deficiencies. Ruminally available amino and ammonia nitrogen pools are used to determine the 'N allowable microbial growth.' The N allowable microbial growth value is then subtracted from the energy allowable microbial growth to obtain the reduction in microbial mass. This mass reduction is then allocated between FC and NFC according to their original proportions in the 'energy allowable microbial growth.' The reduction in fermented FC is computed from the FC bacterial mass reduction. This reduction is then added to FC escaping the rumen.

The CNCPS uses protein degradation and peptide uptake rates to estimate the amount of amino nitrogen that the NFC incorporate. The relative incorporation of amino N versus ammonia nitrogen into NFC bacteria is computed from the yield equations described above. The remaining amino nitrogen (taken up by the NFC bacteria) is then converted to ammonia. Because the obligate amino acid fermenting bacteria are not partitioned into a separate bacterial pool, it is difficult to assess the effect of additives (e.g., monensin) on amino acid sparing.

The pool of peptide and amino acids that pass out of the rumen undegraded is typically small relative to total nitrogen flux. However, peptides and amino acids can be used as a diagnostic tool to monitor the status of NFC bacteria. If the peptide balance is negative, the addition of ruminally degraded protein will have a positive impact on the flow of NFC bacteria from the rumen. However, if the balance is already positive, supplemental ruminally degraded protein will simply enter the ammonia pool.

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Chapter 24

Concluding Comments

In 1950, Robert Hungate concluded his review of "anaerobic mesophilic cellulolytic bacteria" from the bovine rumen with a statement that is still very appropriate. "In summary, an industrial cellulose fermentation might be profitable if the cost of collection of raw materials could be minimized through the use of numerous small plants, if the small plants could be cheaply constructed, if the operation could be made automatic to decrease necessary personnel, and if the concentration of cellulose fermented could be increased by continuous removal of fermentation products. Although such a situation is at present quite out of the question as an industrial process, it is almost an exact specification of the ruminant animal, a small fermentation unit which gathers the raw materials, transfers it to the fermentation chamber, and regulates its further passage, continuously absorbs the fermentation products, and transforms them into a few valuable substances, meat, milk, etc. To these advantages must be added the crowning adaptation: the unit replicates itself."

In the 50 years since Hungate first isolated strictly anaerobic bacteria from the rumen, animal husbandry, breeding and production have been improved dramatically. Indeed, dairy cattle produce approximately 3 times as much milk as they did in 1950, and beef cattle grow nearly twice as fast. However, cattle must consume more feed, and in particular more cereal grain, to sustain this productivity. For example, many lactating dairy cows produce more than 14,000 kg of milk each year. In order for a cow to produce this much milk, she must consume more than 30 kg of dry matter each day, and half of this feed must be cereal grain. Based on a ruminal digestion of 60%, more than 150 moles (approximately 9 liters) of VFA and 3 kg of microbial protein would be generated in the rumen each day.

The use of grain in cattle rations has in large measure been driven by economic forces. During and shortly after World War II, American farmers were encouraged to increase grain production to feed a world that had been devastated by war and had a rapidly growing population. However, within a few years, the damages of war were repaired, the 'green revolution' had reached the developing world, and grain was in excess. American cattle consume large amounts of grain, but it should be realized that ruminants can and do consume feedstuffs that would otherwise be wasted. Much of our land is not tillable, but it can be grazed by cattle, sheep and goats. Because ruminants can utilize hulls, vines, stalks, straws and other by-products of the human food industry, ruminants will always play a key role in highly efficient food production systems.

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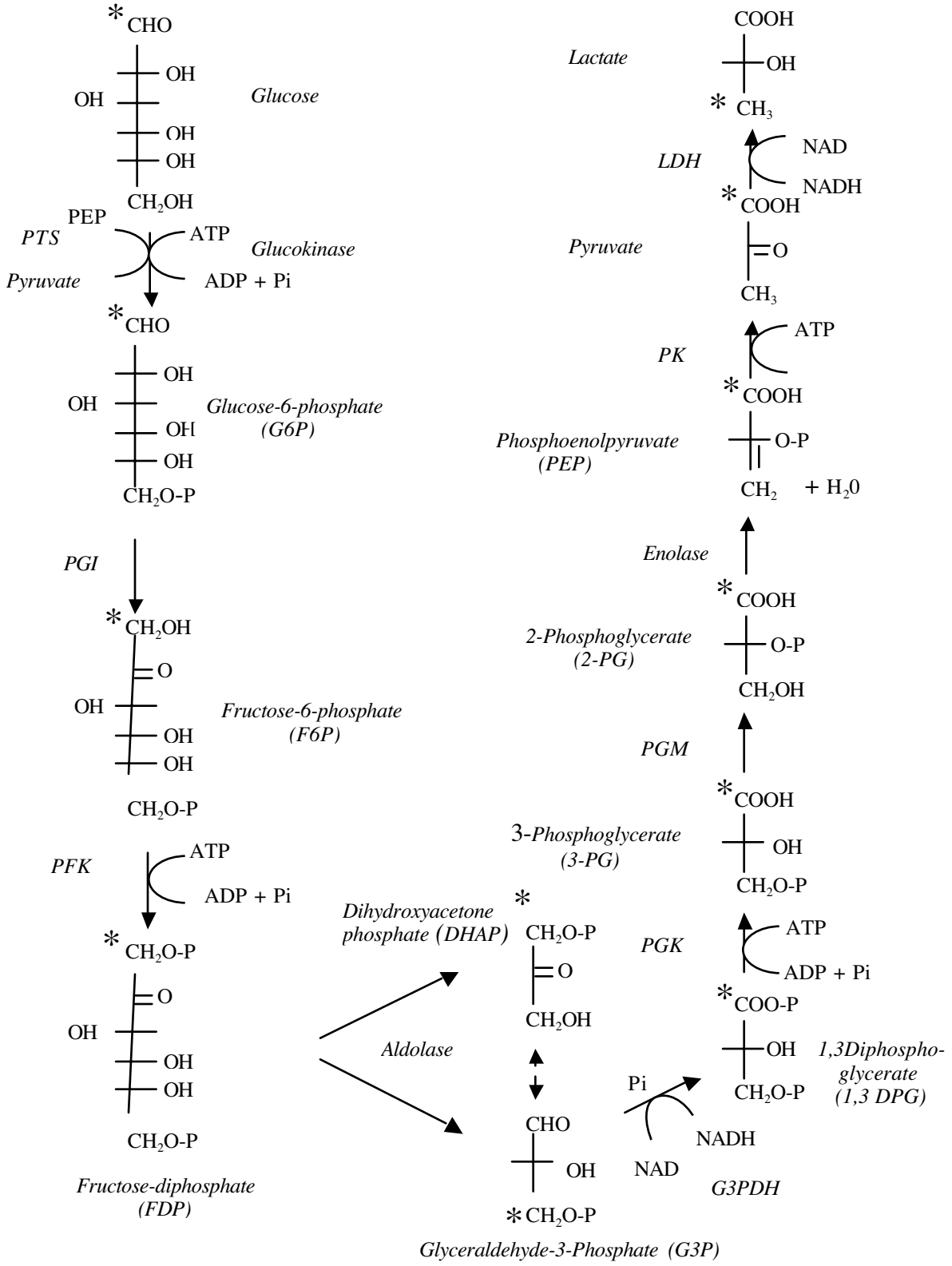
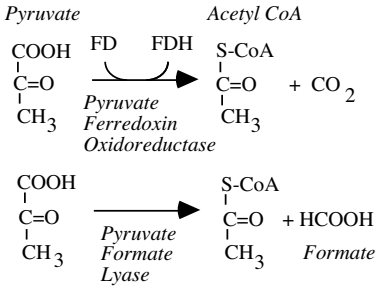
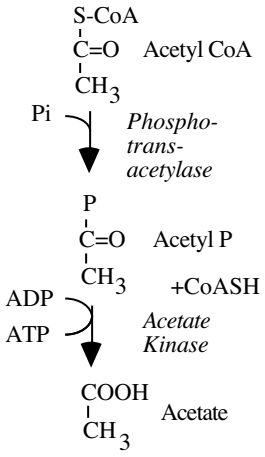
Glucose Catabolism via Embden-Meyerhof-Parnas Scheme



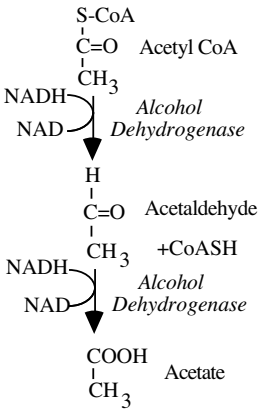
Pyruvate Catabolism



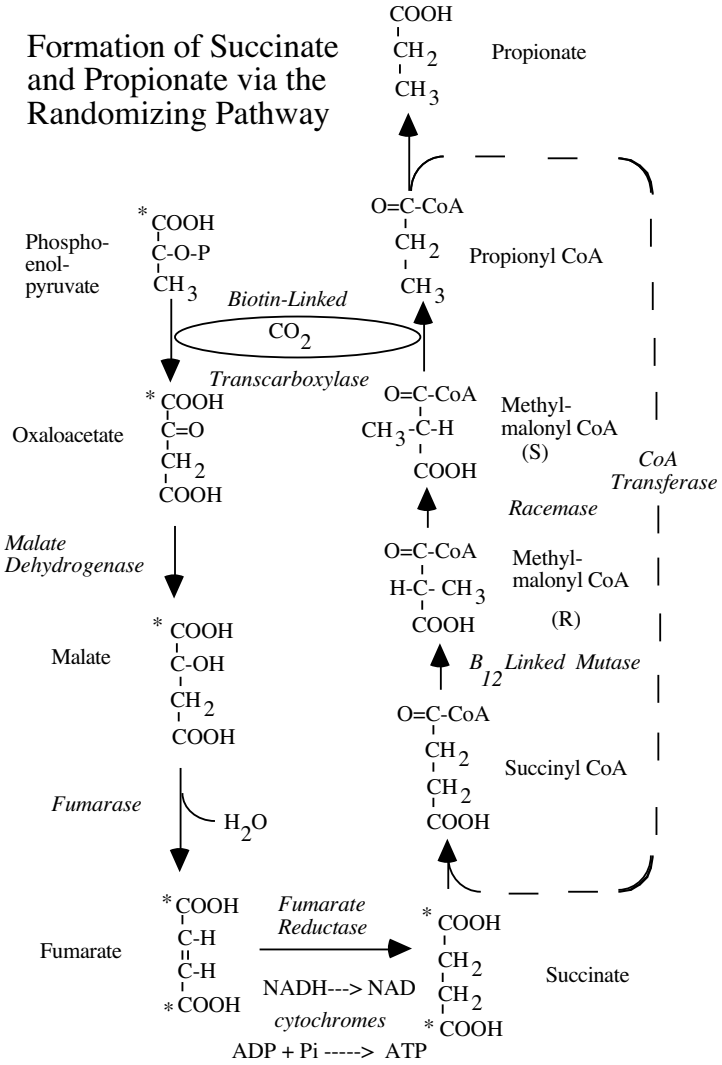
Acetate Formation via Acetate Kinase



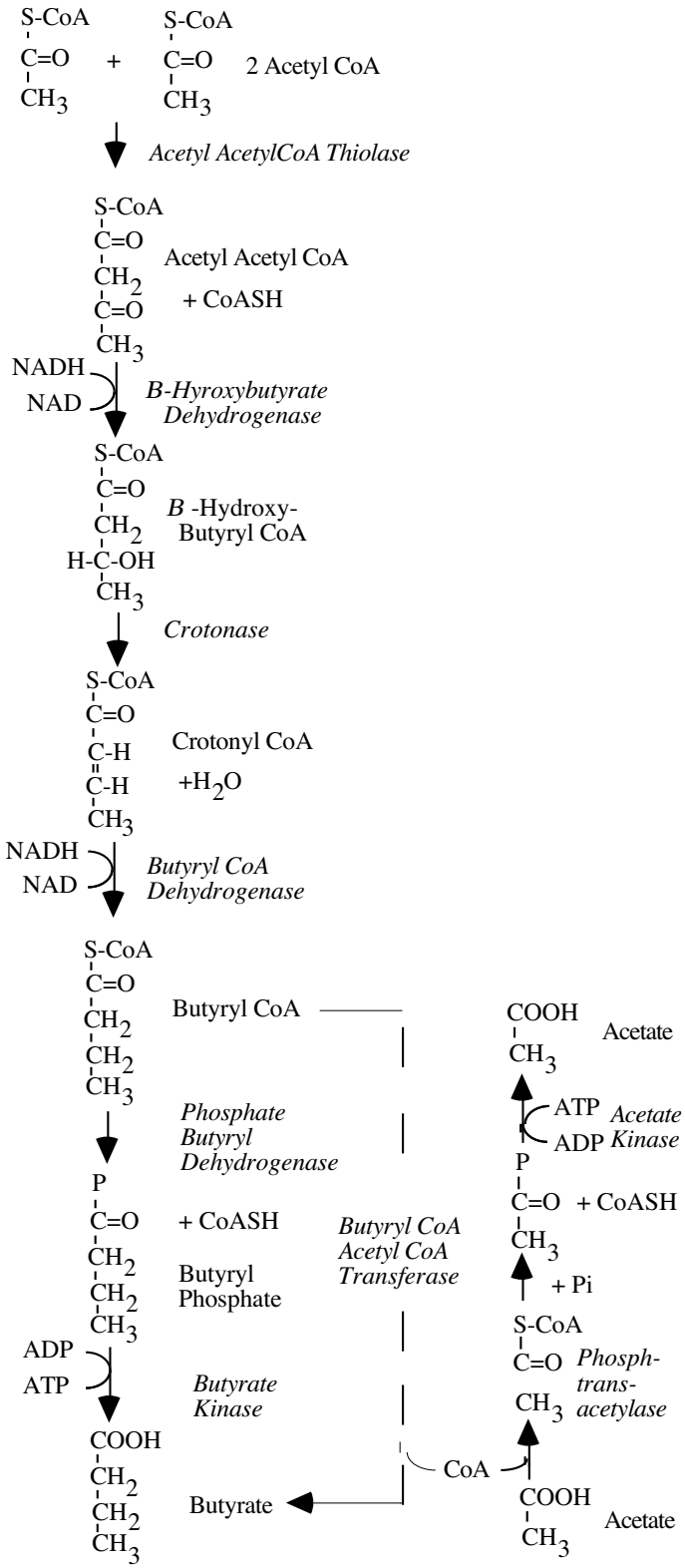
Ethanol Formation



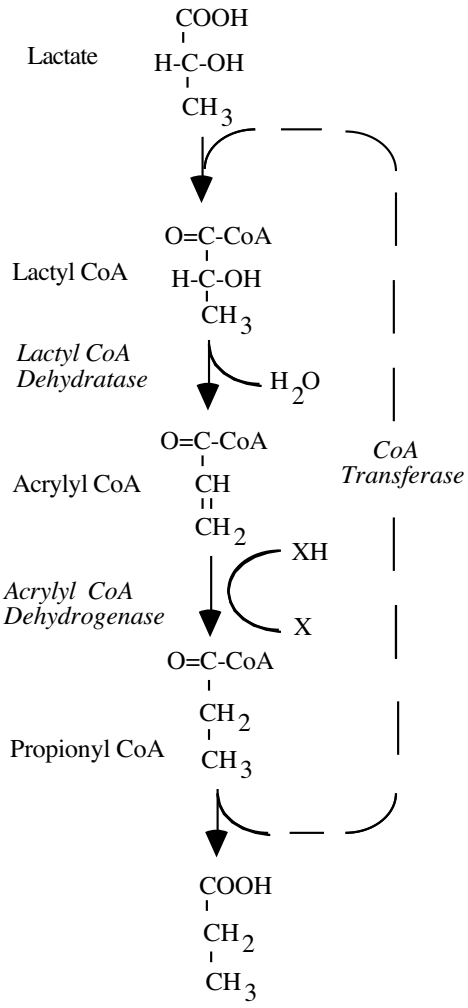
Formation of Succinate and Propionate via the Randomizing Pathway



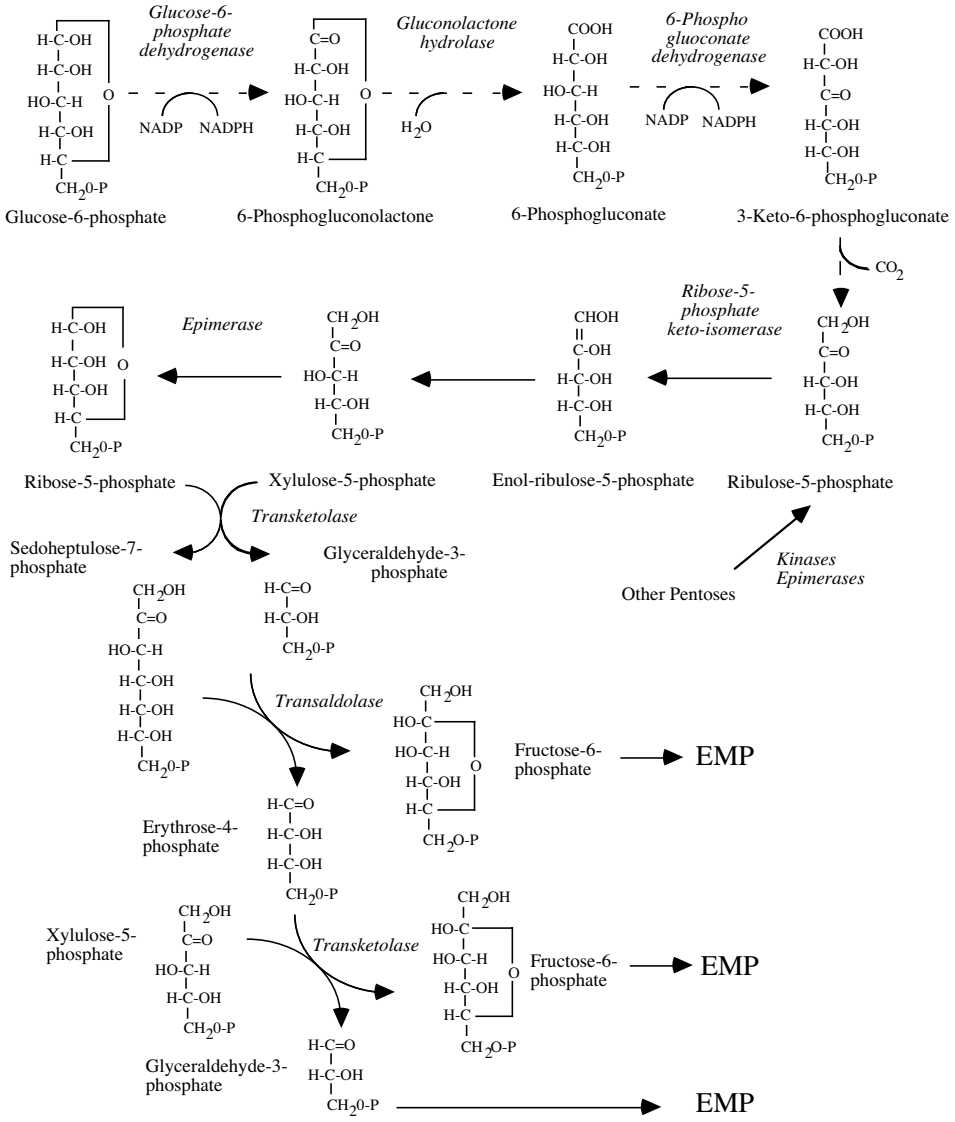
Butyrate Formation via Butyrate Kinase or Transferase



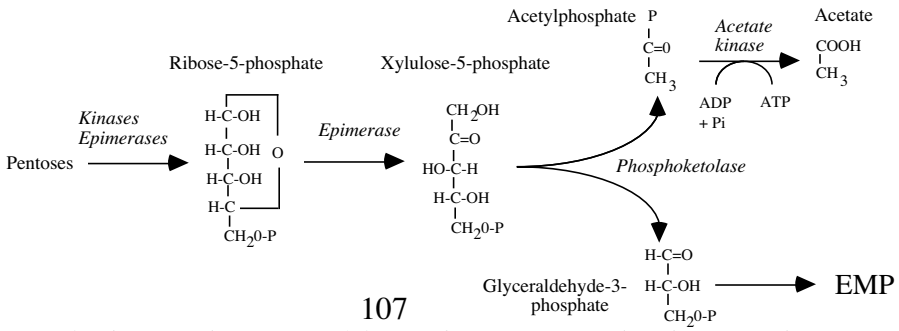
Propionate Formation via Acylate Pathway

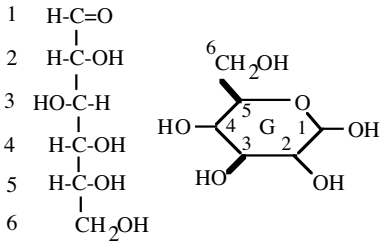


Hexose Monophosphate Shunt and Pentose Pathway

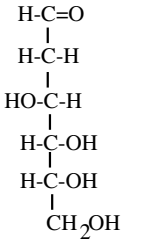


Pentoses via Phosphoketolase

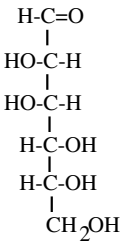




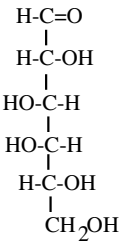
Glucose



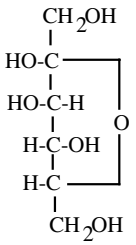
2-Deoxyglucose



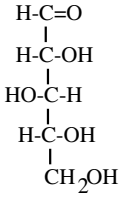
Mannose



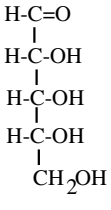
Galactose



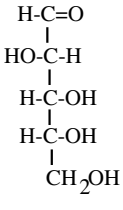
Fructose



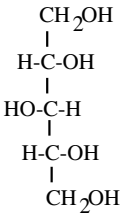
Xylose



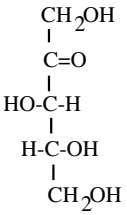
Ribose



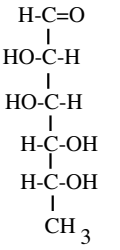
Arabinose



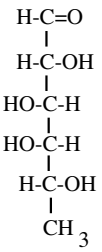
Xylitol



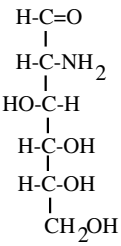
Xylulose



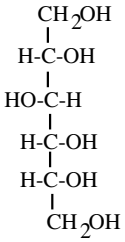
Rhamnose



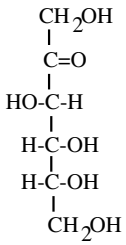
Fucose



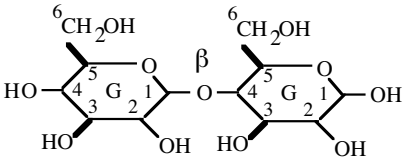
Glucosamine



Sorbitol



Sorbose



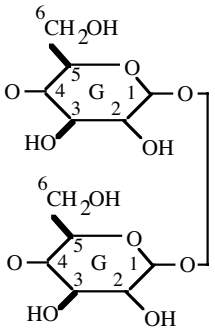
Cellobiose

Galactose - glucose - fructose

Raffinose

Glucose - fructose - glucose

Melezitose



α 1,4
Glucose - glucose

Maltose

β 1,4
Glucose - fructose

Sucrose

α 1,4
Galactose - glucose

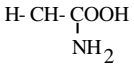
Lactose

α 1,6
Galactose - glucose

Melibiose

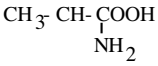
Amino Acid Structures and Properties

Glycine (G)



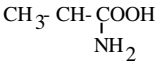
pKs = 2.3, 9.6
pI = 6.0

Alanine (A)



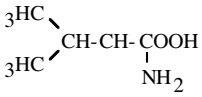
pKs = 2.4, 9.7
pI = 6.0

Valine (V)



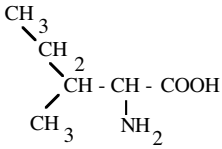
pKs = 2.3, 9.6
pI = 6.0

Leucine (L)



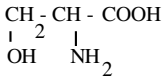
pKs = 2.4, 9.6
pI = 6.0

Isoleucine (I)



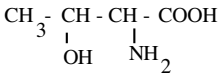
pKs = 2.4, 9.7
pI = 6.0

Serine (S)



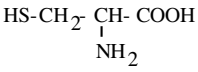
pKs = 2.2, 9.2
pI = 5.7

Threonine (T)



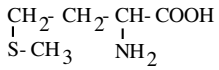
pKs = 2.6, 10.4
pI = 6.5

Cysteine (C)



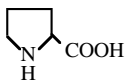
pKs = 1.7, 8.3, 10.8
pI = 5.0

Methionine (M)



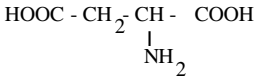
pKs = 2.3, 9.2
pI = 5.8

Proline (P)



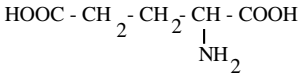
pKs = 2.2, 9.4
pI = 5.9

Aspartate (D)



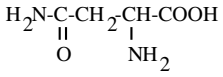
pKs = 2.1, 3.4, 9.8
pI = 3.0

Glutamate (E)



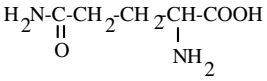
pKs = 2.2, 4.3, 9.7
pI = 3.2

Asparagine (N)



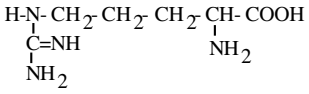
pKs = 2.0, 8.8
pI = 5.4

Glutamine (Q)



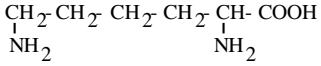
pKs = 2.2, 9.1
pI = 5.7

Arginine (R)



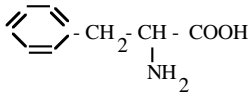
pKs = 2.2, 9.0, 12.5
pI = 10.8

COOH
Lysine (K)



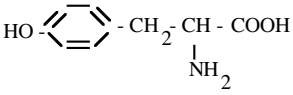
pKs = 2.2, 9.0, 10.5
pI = 9.7

Phenylalanine (F)



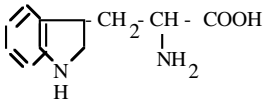
pKs = 1.8, 9.1
pI = 5.5

Tyrosine (Y)



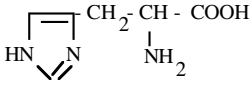
pKs = 2.2, 9.1
pI = 5.7

Tryptophan (W)



pKs = 2.4, 9.4
pI = 5.9

Histidine (H)

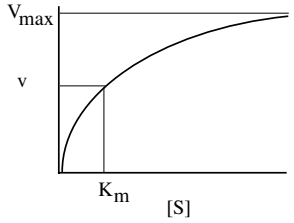


pKs = 1.8, 6.0, 9.2
pI = 7.6

Kinetics

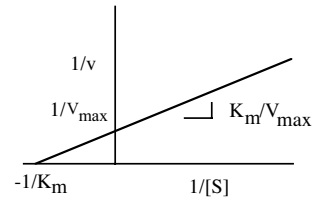
Michaelis Menten

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

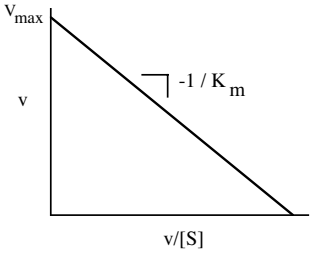


Lineweaver Burk

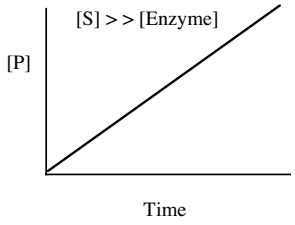
$$1/[S] = K_m/V_{max} \cdot 1/[S] + 1/V_{max}$$



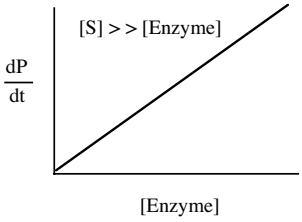
Eadie Hofstee



First Order Kinetics

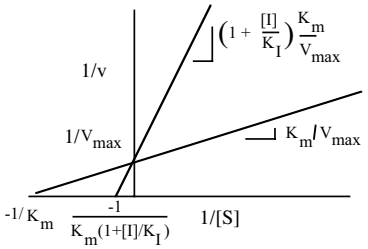


< 10% of S converted to P

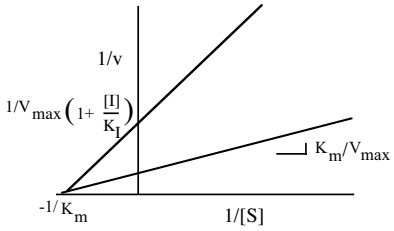


"First order with respect to enzyme concentration"

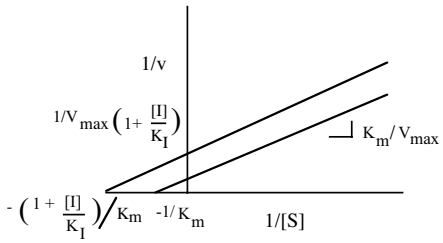
Competitive Inhibition



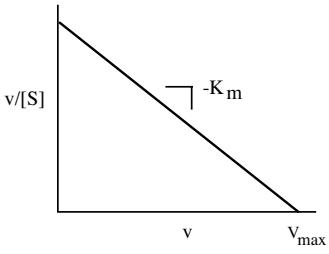
Non-competitive Inhibition



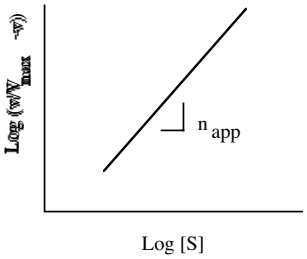
Uncompetitive Inhibition



Eadie Scatchard



Hill Plot



If the slope of the Hill plot (n_{app}) is greater than 1, the enzyme or carrier has more than one binding site.

Acid Data

Acid	Formula Weight	Specifc Gravity	Strength (% w/v)	Molarity (M)	1 mM (µl/L)
HCl	36.46	1.19	37.2	12.1	82.5
HNO_3	63.01	1.42	70.4	15.9	63.0
HF	20.00	1.18	49.0	28.9	34.5
$HClO_4$	100.47	1.67	70.5	11.7	86.5
H_2SO_4	98.08	1.84	96.0	18.0	55.5
H_3PO_4	97.10	1.70	85.5	14.8	67.5
Lactic	90.0	1.07	30.0	10.1	99.0
Formic	46.02	1.22	85.0	22.5	42.5
Acetic	60.05	1.05	99	17.3	57.5
Propionic	74.08	0.99	99	13.2	74.5
Butyric	88.10	0.96	99	10.7	91.8
Isobutyric	88.10	0.96	99	10.7	91.8
Valeric	102.13	0.94	99	9.3	108.6
Isovaleric	102.13	0.93	99	9.3	109.6

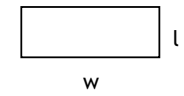
Heats of Combustion

Compound	cal/mmol	j/mmol
Acetate	209	8778
Butyrate	524	2200
Citrate	474	1990
Ethanol	328	1378
Formate	63	265
Glucose	673	2827
Glycerol	397	1667
Isobutyrate	517	2171
Lactate	326	1369
Lactose	1351	5674
Malate	320	1344
Maltose	1350	5670
Methane	211	886
Oxalate	60	252
Palmitate	2398	10046
Phenol	732	3074
Phenyl acetate	930	3906
Propionate	367	1541
Succinate	357	1499
Sucrose	1349	5666
Valerate	682	2864
Xylose	562	2360

Common Elements

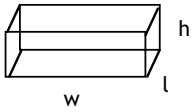
Element	Symbol	Atomic Number	Atomic Weight
Aluminum	Al	13	26.98
Antimony	Sb	51	121.80
Argon	Ar	18	39.95
Arsenic	As	33	74.92
Boron	B	5	10.81
Barium	Ba	56	137.30
Beryllium	Be	4	9.01
Bismuth	Bi	83	209.00
Bromine	Br	35	79.90
Carbon	C	6	12.01
Calcium	Ca	20	40.08
Cadmium	Cd	48	112.40
Chlorine	Cl	17	35.45
Cobalt	Co	27	58.93
Chromium	Cr	24	52.00
Cesium	Cs	55	132.90
Copper	Cu	29	63.55
Fluorine	F	9	19.00
Gallium	Ga	31	69.72
Gold	Au	79	197.00
Hydrogen	H	1	1.01
Helium	He	2	4.00
Iodine	I	53	126.9
Iron	Fe	26	55.85
Lead	Pb	82	207.20
Lithium	Li	3	6.94
Magnesium	Mg	12	24.31
Manganese	Mn	25	54.94
Mercury	Hg	80	200.9
Molybdenm	Mo	42	95.94
Nickel	Ni	28	58.71
Nitrogen	N	7	14.01
Oxygen	O	8	16.00
Phosphorus	P	15	30.97
Platinum	Pt	78	195.10
Potassium	K	19	39.10
Rubidium	Rb	37	85.47
Ruthenium	Ru	44	101.10
Selenium	Se	34	78.96
Silicon	Si	14	28.09
Silver	Ag	47	107.9
Sodium	Na	11	22.99
Sulfur	S	16	32.06
Tin	Sn	50	118.70
Titanium	Ti	22	47.90
Vanadium	V	23	50.94
Wolfram-Tungsten	W	74	183.90

Geometry



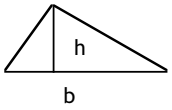
Perimeter = 2(l +w)

Area = l w

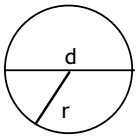


Volume = l w h

Surface = 2 (lh + lw + hw)

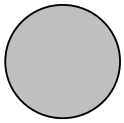


Area = (b h) / 2



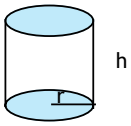
Circumference = 2 πr

Area = πr^2

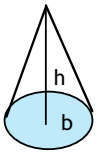


Surface = $4 \pi r^2$

Volume = $4/3 \pi r^3$



Volume = $\pi r^2 h$



Volume = bh/3

pK Values of Common Acids

Compound	pK_1	pK_2	pK_3
Acetate	4.76		
Acrylate	4.26		
Benzoate	4.21		
Butyrate	4.85		
Caproate	4.88		
Citrate	3.13	4.76	6.40
Dithiothreitol	8.9		
Formate	3.75		
Fumarate	3.02	4.38	
Lactate	3.86		
Malate	3.40	5.05	
Oxalate	1.27	4.27	
Phenylacetate	4.31		
Propionate	4.87		
Pyruvate	2.49		
Succinate	4.21	5.64	
Urate	5.40		
Velarate	4.78		
MES	6.15		
ADA	6.60		
PIPES	6.80		
MOPS	7.20		
TES	7.50		
HEPES	7.55		
HEPPS	8.00		
TRICINE	8.15		
BICINE	8.35		
CHES	9.50		
CHAPS	9.50		
CAPS	10.50		
Phosphoric	2.12	7.21	12.32

Empirical Formulas and Redox States of Various Compounds

Compound	Formula	Redox
Valerate	$C_5H_{10}O_2$	-3
Methane	CH_4	-2
Ethanol	C_2H_6O	-2
Butyrate	$C_4H_8O_2$	-2
Propionate	$C_3H_6O_2$	-1
Acetate	$C_2H_4O_2$	0
Water	H_20	0
Glucose	$C_6H_{12}O_6$	0
Lactate	$C_3H_6O_3$	0
Formate	CH_2O_2	+1
Carbon dioxide	CO_2	+2
Succinate	$C_4H_6O_4$	+1
Tricarballylate	$C_6H_8O_6$	+2
Aconitate	$C_6H_{10}O_6$	+3
Citrate	$C_6H_8O_7$	+3

Products and Niches of Some Predominant Ruminal Bacteria

Species	Products	Primary Niches
Fibrobacter succinogenes	S, F, A	CU
Ruminococcus albus	A, F, E	CU
Ruminococcus flavefaciens	S, F, A	CU
Butyrivibrio fibrisolvens	B, F, L, A	CU, HCU, ST, PC, SU
Ruminobacter amylophilus	S, F, A	ST
Selenomonas ruminantium	L, A, P, B, H_2	SU, ST, L
Prevotella sp.	S, A, F, P	ST, HCU, PC, ß GL, PT
Succinomonas amylolytica	S, A, P	ST
Succinivibrio dextrinosolvens	S, A, F, L	MD
Streptococcus bovis	L, A, F, E	ST, SU
Eubacterium ruminantium	A, F, B, L	MD, SU
Megasphaera elsdenii	P, A, B, Br	L, MD, AA
Lachnospira multiparus	L, A, F	PC, SU
Anaerovibrio lipolytica	A, S, P	GY, L
Peptostreptococcus anaerobius	Br, A	PEP, AA
Clostridium aminophilum	A, B	AA, PEP
Clostridium sticklandii	A, Br, B, P	PEP, AA
Wolinella succinogenes	S	MAL, FUM
Methanobrevibacter ruminantium	CH_4	H_2, CO_2, F

(A, acetate; B, butyrate; P, propionate; F, formate; L, lactate; E, ethanol, Br, branched chain VFA; CU, cellulose; HCU, hemicellulose; ST, starch; SU, sugars; MD, maltodextrins; AA, amino acids; GY, glycerol; PT, protein; PEP, peptides; PC, pectin; MAL, malate; FUM, fumarate; βGL, β-glucans).

Different Strategies of Transporting and Cleaving Sugars

Substrate, Transport, Cleavage	Active Transport	Kinase	PTS	~P per Hexose
Hexose, Active Transport	1/3 ATP	1 ATP	-------	1.33
Hexose,PTS	-------	-------	1 PEP	1.0
Disaccharide, Active Transport, Hydrolase	1/3 ATP	2 ATP	-------	1.17
Disaccharide, PTS, Sugar Phosphate Hydrolase	-------	1 ATP	1 PEP	1.0
Disaccharide, Active Transport, Phosphorylase	1/3 ATP	1 ATP	-------	0.67

Values are expressed as ATP equivalents (~P).

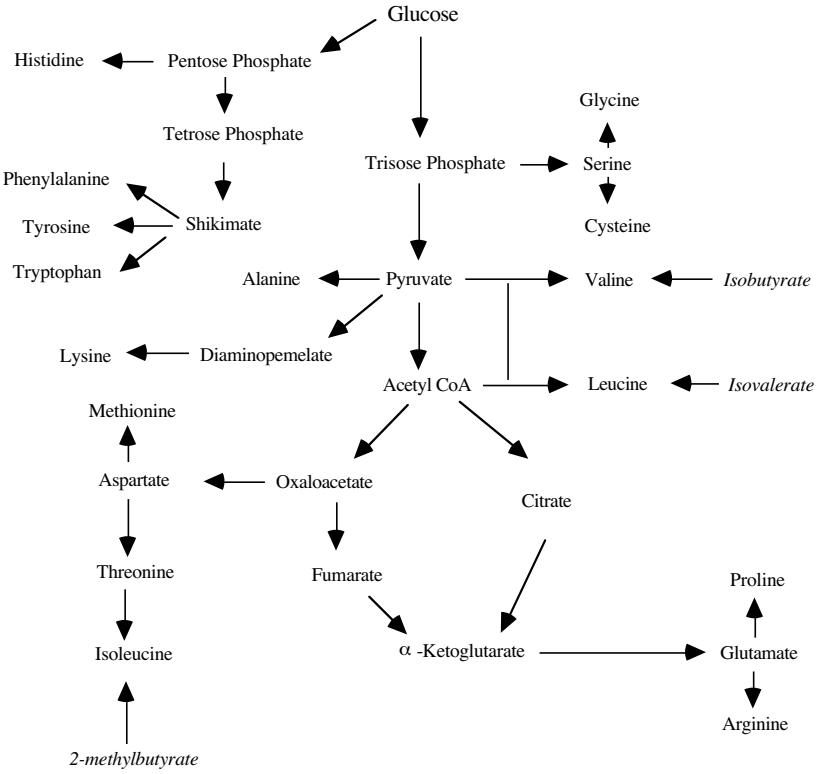
The cost of secondary active transport is assumed to be 1/3 ATP equivalent per molecule transported.

If primary active transport were employed, the cost would be 1 ATP per molecule.

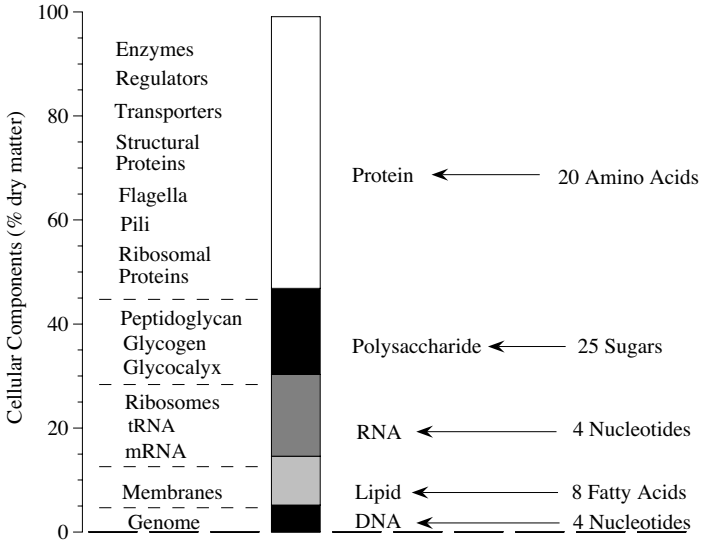
Reactions Producing ATP (~P) or Reducing Equivalents (2H)

Enzyme	Lactate	Acetate	Propionate	Butyrate	Ethanol	Valerate
Glucokinase	-1	-1	-1	-1	-1	-1
Phosphofructokinase	-1	-1	-1	-1	-1	-1
Glycertate kinase	2	2	2	2	2	2
Pyruvate kinase	2	2	2	2	2	2
Acetate kinase	-	2	-	-	-	-
Fumarate reductase	-	-	2	-	-	-
Butyrate kinase	-	-	-	1	-	-
Total (~ P)	2	4	4	3	2	-
G-3-P dehydrogenase	2	2	2	2	2	2
Lactate dehydrogenase	-2	-	-	-	-	-
Pyruvate oxidoreductase	-	2	-	2	2	1
Alcohol dehydrogenase	-	-	-	-	-4	-
Malate dehydrogenase	-	-	-2	-	-	-1
Fumarate reductase	-	-	-2	-	-	-1
β-OH butyrate dehydrogenase	-	-	-	-1	-	-
Butyryl CoA dehydrogenase	-	-	-	-1	-	-
β-OH valerate dehydrogenase	-	-	-	-	-	-1
Valeryl CoA dehydrogenase	-	-	-	-	-	-1
Total (2H)	0	4	-2	2	0	-1

Pathways of Amino Acid Biosynthesis in Bacteria



Macromolecular Components of Bacteria and Precursors From Which They Were Derived



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