MICROBIOLOGY OF THE RUMEN AND SMALL AND LARGE INTESTINES

INTRODUCTION

The GI tract of higher animals is colonized by microbes (i.e. microscopically small living organisms) soon after birth. Many different varieties of microbes enter the gut; they occupy different sites on the basis of their ability to compete under the conditions that exist at each site. Microbial habitats change as the animal matures and may be differentiated both vertically (i.e. from the mouth to the anus) and horizontally (i.e. from the centre of the lumen to the depths of the crypts). Each habitat is dynamic; conditions are continuously modified by the diet, by the host, and by the metabolic activity of the microbial inhabitants. Homeostatic control operates, however, to ensure that gastrointestinal environments are relatively stable compared with soil or aquatic habitats. Interactions between the host animal and its gut microbes are complex and poorly understood; they are nonetheless very important to the host, and must be considered in any comprehensive study of digestive physiology.

No enzymes capable of degrading cellulose and related plant polymers are produced by mammals; utilization of fibrous vegetation therefore depends upon the metabolic activities of resident gastrointestinal microbes. Microbial cellulose digestion is a slow process, and fibrous foods must therefore be retained in the gut for a long time. This is facilitated in vertebrate herbivores by the development of mechanisms for the selective retention of large particles, and by enlarged fermentation compartments or blind sac pouches.

PREGASTRIC FERMENTATIONS

Microbial fermentation occurring in the simple stomach is generally limited to an ethanolic or lactic acid type of fermentation that has little impact on the digesta or the nutrition of the animal. Foregut fermentations that significantly modify the digesta generally depend on the development of a sac or chamber (such as a rumen) that provides for physical separation of the digesta from the acid-secreting region of the stomach.

The cellulose-degrading bacteria of the rumen can be cultivated only in anaerobic conditions. The bacterial species found in other parts of the gastrointestinal system are very similar to those found in the rumen but we know most about the microorganisms found in the rumens of sheep and cattle.

NUMBERS AND KINDS OF RUMINAL MICROBES

It is hard to estimate concentrations of ruminal bacteria and protozoa. Various microscopical techniques may be used for counting, but they are difficult to apply because microorganisms tend to become attached to plant fibres, and bacteria form tiny dense clusters within plant particles. Bacterial concentrations are usually estimated from culture counts. Estimates of about 10,000 million to 50,000 million bacteria per gram of sample are quoted from culture counts for samples containing typical proportions of solid and liquid rumen contents. Microscopic counts, which will include dead bacteria and some organisms which fail to grow on a culture medium, may be 2 to 3 times greater. Table 1 lists some of the most important ruminal bacterial species in cattle and sheep and their fermentative properties. The relative proportions of these species are profoundly influenced by the composition of the diet, but we know little about the biochemical processes underlying the selection of particular species.

Anaerobic mycoplasmas, quite distinct from mycoplasmas found in any other habitat, are present at 105 to 107 organisms per gram of ruminal contents of cattle and sheep. Bacterial viruses (phage) are also present. The significance of these microorganisms is not known.

Although protozoa are much less numerous than bacteria, they are so much larger than bacteria that they may occupy an equal volume. The most important rumen protozoa are anaerobic ciliates; these are classified according to their morphology. Most belong to one of two families: the Isotrichidae, in which the entire body surface is covered by cilia (holotrich protozoa), and the Orphyroscolecidae, in which most of the body surface is naked apart from regions where cilia are concentrated in tufts or syncillia (entodiniomorphid protozoa). Diet exerts a marked effect on the numbers and kinds of protozoa present in the rumen; protozoal populations alter more with time and between animals than do bacterial populations. Generally speaking, ciliate species are less diverse in browsing ruminants; the latter feed less selectively on more fibrous foods. Organisms originally thought to be flagellate protozoa are now thought to be a stage in the life cycle of Phycomycete fungi. Cellulolytic activity has been demonstrated with some of these fungi; they attach to and invade plant particles, and may be more important than is suggested by their low concentrations in the fluid phase of ruminal contents.

ECOLOGY OF RUMINAL MICROBES

Many of the important features of the rumen as a microbial habitat have been described (see chapter on Fermentation and Digestion in the Ruminant Stomach). The rumen is, to all intents and purposes, an open ecosystem; virtually all microbial species have had an opportunity to grow there. This is probably true for the small and large intestine as well; in this case the acid stomach presents an appreciable barrier, but it is frequently hurdled.

Close or direct contact between ruminants is necessary for transmission of protozoa, but normal rumen bacteria may be isolated from aerosols (i.e. tiny air-borne fluid droplets) in stables and are threfore more readily transmitted between animals. With the passage of time, microbes best adapted to survive and compete in the rumen have been selected and transmitted to succeeding generations. Humans will therefore probably find it difficult to select and establish in the rumen of animals fed traditional rations an exotic microbe with what might be deemed to be desirable physiological capabilities. Perhaps, however, genetic engineering experiments, designed to introduce desirable capabilities into existing ruminant species, may be successful.

Since the rumen is an open system accessible to many different microorganisms, criteria have been proposed to distinguish truly functional rumen organisms from transient species. We talk of autochthonous microbiota when referring to the indigenous flora of an animal species; organisms that may frequently be found in the same habitat, but which are essentially transients, are referred to as allochthonous. Autochthonous gastrointestinal microbes (1) grow anaerobically and produce end-products that are found in the habitat or that are intermediates in the formation of those end products, (2) are always found in normal adults, (3) colonize particular areas of the gastrointestinal tract, (4) colonize their habitats during succession in infant animals (succession being the orderly sequence by which habitats are invaded by fauna and flora) (5) maintain stable population levels in climax communities in????? normal adults, and (6) may associate intimately with the mucosal epithelium.

The rumen may be considered as a fermentation vat in which a complex and dense population of bacteria and protozoa converts plant materials to VFAs, methane, carbon dioxide, ammonia and microbial cells. Concepts developed by chemical engineers for the analysis of chemically stable, continuous-culture fermenters can be applied to the reticulo-rumen system, even though the latter deviates in many respects from a true continuous-culture system.

Organisms that exist in a steady state in a completely mixed continuous fermenter must have a specific growth rate (u) that is a function of the doubling time (td)

log 2u = -------td

and is equivalent to the dilution rate D, where

D is the volume entering the fermenter/culture volume. The rumen, however, is not a homogeneous, completely mixed system, and D for the fluid fraction is approximately 2 to 4 times greater than for the particulate fraction. Among rumen bacterial species, there are large differences in maximum specific growth rates (umax) as determined in vitro in the presence of single energy sources. For example, values for umax with glucose as substrate were 2.0 h-1 (td=20 min), 0.56 h-1 (td=74 min), and 0.39 h-1 (td=106 min) for Streptococcus bovis, Bacteroides ruminicola, and Butyrivibrio fibrisolvens, respectively. Values for u obtained under different conditions for some of the more slowly growing microbes include a range from 0.055 h-1 (td=12.6 h) to 0.036 h-1 (td=19.2 h) for five species species of ciliate protozoa, and a value of 0.014 h-1 (td=50 h) for the large organisms now called Eadie's oval. If these slowly growing organisms were restricted to the fluid fraction, with its very rapid turnover (its D value may range from about 0.2 to 0.04 h-1 depending on diet and other factors) they would be rapidly washed out of the rumen. They must attach to particulate matter, which is more slowly moving and therefore lingers longer in the rumen, and to epithelial tissue if they are to survive.

In the rumen, bacteria rarely grow at umax. Growth is usually limited by the concentrations of energy-yielding substrates, but pH, end-product inhibition, and other factors may also be involved. Substrate affinity determines ability to grow when substrate concentrations are low; it is important determining the outcome of the competition between species for nutrients. Measurement of substrate affinity have not been made for many rumen bacteria; S. bovis has however been shown to have a fairly low affinity for glucose, a finding that is consistent with the observation that S. bovis becomes a predominant member of the population only after glucose concentrations become elevated after overfeeding with concentrates.

A diverse microbial population is to be expected, given the diversity of substrates, particulate surfaces, and therefore of ecological niches within the rumen. Some rumen bacteria utilize one or only a few substrates as energy sources, while other species are more versatile. Bu. fibrisolvens and Bac. ruminicola are examples of species that ferment a variety of sugars, polysaccharides, and glycosides, as well as saponin and levan from grass. In a study of substrate preference within five important species of rumen bacteria, the metabolic activity of all five was repressed by one or more of the substrates tested (glucose, xylose, maltose, sucrose, cellobiose, and lactate). For example, with Bu. fibrisolvens, utilization of maltose and sucrose was not repressed by the presence of other substrates, but utilization of cellobiose, glucose, and xylose was inhibited when either maltose or sucrose were added to the culture. Growth rates were also higher with maltose and sucrose. Substrate preferences and sequential substrate utilization patterns were different for each species. These differences are, no doubt, important in the competitive ruminal environment and help to explain why a given microbe with great versatility in the substrates it can attack does not succeed in completely displacing its competitors.

Both symbiotic and predator-prey relationships exist between certain protozoal species and between many protozoa and bacteria. Some species produce mono- and oligosaccharides from polysaccharides, amino acids, ammonia, branched-chain fatty acids, heme and B vitamins. These compounds are utilized by other species, some of which have absolute nutritional requirements for the compounds. Symbiosis may well exist between certain protozoa and the bacteria that have been observed attached to and apparently growing on the surface of the protozoa. The concept of interspecies hydrogen transfer helps to explain a number of the interactions between species

and is of particular significance in consideration of the role of methanogens in the mixed culture. Thus rumen bacteria interact with each other in a multitude of ways. Some of the interactions have been described, but more information is needed if we are fully to understand this this complex ecosystem.

FUNCTIONS OF RUMEN BACTERIA

The major types of rumen bacteria are listed in Table 1. There is overlap between functions of different species, the first five listed all being cellulose digesters. Some species are highly versatile in function, while others are more specialized. End products of the action of some species are further metabolized by other species, and are therefore intermediates, not end products, of the mixed population.

Hexoses are broken down in the rumen mainly by the glycolytic (Emden-Meyerhof) pathway, which converts glucose to pyruvate. Most of the pentose is thought to be metabolized by the pentose phosphate cycle plus glycolysis, with some metabolized by the phosphoketolase pathway. Pyruvate, a key inermediate, is metabolized by a variety of mechanisms to yield inter alia acetate, butyrate, hydrogen, carbon dioxide and propionate (Fig. 3). Propionate is formed by by two distinct pathways; most is produced by the pathway involving succinate (produced by many species of rumen microbe). Succinate does not accumulate significantly in the mixed population but is decarboxylated to yield propionate and carbon dioxide by organisms such as Selenomonas ruminantium. The direct reduction pathway via acrylyl coenzyme A is found in Megasphaera elsdenii, and the relative amount of propionate formed by this pathway increases when high concentrate diets are fed.

Methanogens utilize hydrogen, normally keeping down its partial pressure to a very low level. This allows the hydrogen gas-producing, carbohydrate-fermenting bacteria to release more of the electrons generated in glycolysis as hydrogen gas. They therefore produce more acetate and carbon dioxide from pyruvate, and less propionate and succinate which otherwise must be produced in order to reoxidise (remove electron from) the cellular reduced pyridine nucleotides. This concept of interspecies hydrogen transfer helps to explain the increase in propionate when methanogenesis is inhibited. It also explains why many rumen bacteria that produce ethanol or lactate in pure culture produce little or none of these, and rather produce an increased amount of acetate in the natural mixed-culture system when methanogenesis is active.

As was discussed in Chapter **[20], dietary protein is metabolized by rumen microbes. Ruminant growth and milk production are possible with urea (which is converted to ammonia) as the sole source of dietary nitrogen; this supports the concept that ammonia plays a pivotal role in ruminal nitrogen metabolism. (Fig. 4).

Many rumen bacteria have the enzymes necessary to enable them to synthesize a complete mixture of amino acids, and these bacteria can therefore grow with ammonia as the main nitrogen source. Some rumen bacteria, however, require amino acids, and others require branched-chain or other fatty acids that are the products of ruminal amino acid metabolism. The protozoa are even less metabolically independent; bacterial cells constitute a major part of their nitrogen needs.

Ammonia fixation by bacteria is achieved mainly by the enzymes glutamate dehydrogenase and glutamine synthetase. The latter has the higher affinity for ammonia, and is probably more important when ammonia levels are low. Efficient use of ammonia depends upon a balanced supply of the fermentable carbohydrate that is needed to furnish both (1) the carbon skeletons for amino acid biosynthesis and (2) the energy for this process.

The protein content of microbial cells generally accounts for 40 to 60 percent of the cell dry weight, and the amino acid profiles of hydrolysates from different populations of mixed rumen microbes are similar. The quantity of microbial protein available for digestion is critical when protein demand is high (e.g. during rapid growth or in late pregnancy and in early lactation). It seems that the efficiency of bacterial growth (i.e. bacterial protein synthesis) as a proportion of the available energy, is greater when growth rates are high; under such circumstances, the energy needed for cell maintenance represents a lower proportion of the total energy that is available.

FUNCTIONS OF RUMINAL PROTOZOA

Ruminal ciliate protozoa are metabolically versatile; they can use all the major constituents of a plant diet. They ferment carbohydrates, including starch, hemicellulose, and cellulose. They are proteolytic, and some species hydrogenate unsaturated fatty acids while others are able to desaturate fatty acids. Although bacterial predation is important to rumen protozoa, the quantity of bacteria ingested varies with differences in ration.

Protozoa are not essential to the ruminant digestive system, but several workers have reported that defaunated animals (i.e. animals treated to rid them of protozoa) gained weight less rapidly and less efficiently than faunated animals. Others have reported the opposite effect. Defaunation may be accomplished by treatment with chemicals (e.g. copper sulphate, or surface-active agents). Protozoa-free ruminants may also be maintained by keeping the animals isolated from other animals from birth. Just because they are not essential, however, that does not mean that protozoa do not have a role in the rumen ecosystem. Characterization of the role of protozoa is complicated by the fact that bacteria appear to occupy biological niches vacated by the ciliates, since bacteria are present in significantly greater concentrations in defaunated animals than in animals with ciliate protozoa.

Ruminal ammonia levels tend to be higher in normal than in defaunated sheep. This could be due to the excretion of ammonia by the protozoa, but excretion of peptides and amino acids and their subsequent deamination by bacteria may be as important.

MANIPULATIONS OF RUMINAL MICROBES

Increasing understanding of the functions of rumen microorganisms may enable us to modify these functions for more efficient animal production. Protein available to the ruminant includes both the dietary protein that escapes ruminal degradation and the protein in microbial cells. Ruminal microbes may provide adequate protein for maintenance and during periods of slow growth and early pregnancy. At other times, when protein demand is high, protein availability can be enhanced by increasing the amount of dietary protein that escapes degradation in the rumen. We are now learning more about the extent to which microbes attack the proteins in various foods, and this information is being used in the formulation of rations. Foodstuffs may be treated to decrease rumen proteolysis; treatments include drying, heat treatment, and treatment with chemicals such as formaldehyde. Increased production has been claimed with such treatments, but results are not consistent. Formaldehyde-treated protein has also been used to coat or protect fats from microbial attack in the rumen, leading to increased yields of milk fat or animal fat with higher amounts of unsaturated fatty acids.

A number of chemical agents have been tested for their capacity to modify the proportions and activities of ruminal microbes. They include substances that inhibit methanogenesis and/or increase the ratio of propionate to acetate. Of these, the antibiotic monensin has been most thoroughly tested. The biochemical basis for increased feed efficiency with monensin is not completely understood, but it is known that monensin inhibits food intake, microbial methane production, proteolysis, and amino acid degradation, and that it increases the ruminal propionate:acetate ratio. Selective pressures against ruminal hydrogen-producers (and therefore against methane production) as well as selection in favour of succinate-producers (and therefore towards increased propionate production) seem to be at least part of the basis for the changes observed.

PRODUCTION AND MODIFICATION OF TOXIC SUBSTANCES IN THE RUMEN

Some substances present in plants are more toxic to nonruminants than they are to ruminants because they undergo degradation and detoxification prior to gastric digestion; such substances include oxalic acid, certain goitrogenic thioglucosides, gossypol, and the oestrogens biochanin A and genistein. Other substances (e.g. lactic acid, ammonia, and nitrite) that are usually considered as intermediates of fermentation, are present at low or moderate levels in animals on normal diets, but they may accumulate at levels that are toxic when other diets are fed. Toxic substances which may be end-products, rather than intermediates, of ruminal fermentation include the thiaminase associated with polioencephalomalacia, the goitrogen 3-hydroxy-4(1H)-pyridone (produced from mimosine), HCN (from cyanogenic glycosides), and 3-methylindole (produced from tryptophane). Dietary change can lead to the selection of microbial populations that degrade specific toxic substances more rapidly; such adaptations may be of great importance in the natural selection of ruminants.

In the following consideration of four disorders that arise when excessive quantities of toxic substances accumulate in the rumen, it will be seen that the microbial population of the rumen can adapt to gradual changes in diet without compromising the health of the animal; problems arise if dietary change is too sudden.

Lactic acidosis

Overfeeding on animals with readily fermented carbohydrate (e.g. grain) can result in accumulation of large amounts of lactic acid in the rumen. The microbial population alters drastically away from a preponderance of gram-negative bacteria towards a population dominated by gram-positive lactic acid producers (S. bovis and Lactobacillus sp.) in the rumen, caecum and colon. The rapid rise in numbers of S. bovis is accounted for by the markedly increased concentrations of glucose, and the high specific growth rate of S. bovis when appropriate substrate levels are high. Ruminal pH normally falls from 6 to 5 or less. In this acid environment, normal rumen microbes are unable to compete, and a population dominated by lactobacilli then evolves.

Concentrations of lactic acid in the rumen may exceed 100 mM, and the proportion of lactate present as the D(-) isomer may increase from about 20 percent at pH 6 to 50 percent at a pH less than 5. The D(-)isomer is less rapidly metabolized and cleared by the animal than the L(+) lactate, and this fact is undoubtedly important in the disease. Low pH in the rumen may contribute to the formation of histamine or endotoxin (produced by lysis of gram-negative bacteria at low pH); these may contribute to the disease. Ethanol accumulates in the rumen of overfed animals, but probably does little harm.

If the animal is adapted gradually to high concentrate rations, the drastic shift in microbial population described above can be avoided. There may be selection of higher proportions of VFA-producing organisms that can rapidly utilize the carbohydrate, and increased numbers of lactic acid utilizers may be selected. Certain bacteria and protozoa can assimilate readily available carbohydrate and store it as intracellular polysaccharide; such organisms would reduce the availability of sugar for S. bovis. Transfer of rumen contents from animals adapted to high grain diets to unadapted animals protects against lactic acidosis, though even adapted populations can be overwhelmed. Certain antibiotics, particularly those selective against S. bovis, may have some value as a prophylactic treatment against

Nitrate-nitrite toxicity

Ruminal microbes can reduce nitrate to nitrite and nitrite to ammonia. Moderate levels of nitrate cause no accumulation of toxic nitrite, but high dietary intake of nitrate can cause nitrite accumulation in the rumen. Nitrite, when absorbed, unites with haemoglobin to form methaemoglobin, resulting in impaired oxygen transport. A gradual increase in dietary nitrate causes the selection of ruminal microbes that can reduce both nitrate and nitrite at a greatly increased rate; ruminants thus adapted can tolerate levels of nitrate intake that would kill unadapted animals. The exact basis for the adaptation is not understood, though it is known that reduction of both nitrate and nitrite depends upon the availability of adequate amounts of hydrogen-donating substances (especially from carbohydrate).

Organic nitro compounds, 3-nitropropionic acid and 3-nitropropanol (toxic constituents of various forage plants such as crown vetch and timber milk vetch), are degraded by ruminal microbes. Bacteria which reduce nitrate and nitrite appear to be responsible for detoxification of these nitro compounds with the acid form more readily degraded than the alcohol.

Oxalic acid toxicosis

Salts of oxalic acid are present in low concentrations in most plants, but the oxalate content of some plants may exceed 10 percent of the plant dry weight. Acute toxicity and death may follow an abrupt introduction to an area where these plants are prevalent. Gradual introduction will be associated with toleration of high oxalate intake, because oxalate-degrading bacteria (as yet unnamed) are selected in the rumen.

Acute bovine pulmonary emphysema

When cattle are moved from dry conditions to lush green pasture, acute pulmonary oedema and emphysema may develop. The condition can induced by intraruminal dosage with tryptophane or with either indoleacetic acid or 3-methylindole, which are products of tryptophane metabolism by rumen microbes. The toxic product formed in the rumen appears to be 3-methylindole, since intravenous administration of 3-methylindole also produces the disease, whereas intravenous tryptophane or indoleacetic acid fail to do so. A lactobacillus capable of converting indoleacetic acid to 3-methylindole has been isolated from the rumen. Antibiotics that inhibit these conversions in animals loaded with tryptophane also prevent development of the disease under field conditions.

SMALL INTESTINAL MICROBES

The concentrations of viable bacteria in the small intestinal contents are much lower (104-106 per gram) than are found in the rumen or large intestine. Most of the bacteria found in the small intestine are believed to be transients, and the impact of microbes on digestion in the small intestine is minimal. In a variety of pathological conditions, the small intestine becomes colonized by specific enteric pathogens or by populations similar to those in the colon.

LARGE INTESTINAL MICROBES

Microbial fermentation forms a major part of the digestive process in the large intestine, and the main products include carbon dioxide, acetate, propionate, and butyrate. Methane or hydrogen, or both, may also be formed.

It is harder to generalise about microbial digestion in the large bowel than about ruminal digestion; these difficulties arise from (1) the greater diversity of microenvironments, which may differ according to both the horizontal and vertical dimensions of the intestine, (2) differences amongst animal species, (3) uncertainties concerning effects of diet, and (4) the relative lack of information on large intestinal microbial populations. It seems clear, however, that the large intestines of many different animals are populated by obligate anaerobic bacteria, and that the density of these organisms is about the same as that found in the rumen i.e. 1010 - 1011 per gram. In the large intestine, as in the rumen, bacteria are found in intimate association with epithelial tissue. The significance of this association is not yet understood, but it appears that populations associated with epithelial tissue differ somewhat from luminal populations.

Microbial interactions and other factors that regulate selection of ruminal bacteria are also important in the large intestine, though mixing is less complete in the less fluid environment of the colon, and the large intestine is even less like a continuous culture model than is the rumen.

It is unlikely that faecal bacterial populations are representative of populations of the entire large intestine. In the pig, for example, gram-negative anaerobes constitute a greater proportion of the culturable population in the caecum than is true for the faeces where gram-positive bacteria predominate.

Many of the species of bacteria present in the large intestine of pigs and ruminants are the same as those which predominate in the

rumen. Some species seldom encountered in the rumen, such as Spirochaets of the genus Treponema, are often observed in the large intestine; coliforms, particularly Escherichia coli, may be present at higher concentrations than are found in the rumen.

Anaerobic protozoa, similar but not identical to rumen ciliates, inhabit the large intestines of horses, rhinoceroses, tapirs, and elephants. Anaerobic protozoa have also been found in the gorilla, chimpanzee, and in rodents. It has been claimed that the concentration of ciliates in the large intestine of the horse is about the same as in the rumen (105-106 per ml), but lower values (about 5 x 103) have also been reported.

TABLE 1

Species

Bacteroides succinogenes C,A F,A,S
Ruminococcus albus C,X F,A,E,H,C
Ruminococcus flavefaciens C,X F,A,S,H
Butyrivibrio fibrisolvens C,X,PR F,A,L,B,E,H,C
Clostridium lochheadiiC,PR F,A,B,E,H,C
Streptococcus bovis A,S,SS,PR L,A,F
Bacteroides amylophilus A,P,PR F,A,S
Bacteroides ruminicolaA,X,P,PR F,A,P,S
Succinimonas amylolytica A,D A,S
Selenomonas ruminantium A,SS,GU,LU,PR A,L,P,H,C
Lachnospira multiparusP,PR,A F,A,E,L,H,C
Succinivibrio dextrinosolvens P,D F,A,L,S
Methanobrevibacter ruminantium M,HU M
Methanosarcina barkeriM,HU MC
Spirochaete sp. P,SS F,A,L,S,E
Megasphaera elsdenii SS,LUA,P,B,V,CP,H,C
Lactobacillus sp.SS L
Anaerovibrio lipolytica L,GU A,P,S
Eubacterium ruminantium SS F,A,B,C

*C = cellulose breakdown, X = xylan breakdown, A = amylase breakdown, D = dextran breakdown, P = pectin breakdown, PR = protein breakdown, L = lipid breakdown, M = methane-producing, GU = glycerol-utilizing, SS = major soluble sugar fermenter, HU = hydrogen utilizer.

+F = formate, A = acetate, E = ethanol, P = propionate, L = lactate, B = butyrate, S = succinate, V = valerate, CP = caproate, H = hydrogen, C = carbon dioxide, M = methane.

Fig. 2

Adapt from Church Fig 12-4 page 223