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THE EFFECT OF MONENSIN AND LENGTH OF EXPOSURE ON ADAPTATION, CELLULAR PHYSIOLOGY AND MORPHOLOGICAL CHANGES OF PURE CULTURE RUMINAL BACTERIA

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THE EFFECT OF MONENSIN AND LENGTH OF EXPOSURE ON ADAPTATION, CELLULAR PHYSIOLOGY AND MORPHOLOGICAL CHANGES OF PURE CULTURE RUMINAL BACTERIA

By

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ABSTRACT

THE EFFECT OF MONENSIN AND LENGTH OF EXPOSURE ON ADAPTATION, CELLULAR PHYSIOLOGY AND MORPHOLOGICAL CHANGES OF PURE CULTURE RUMINAL BACTERIA

By

Phoebe Wei-Tsu Gur-Chiang

The metabolic response to the ionophore monensin was studied in anaerobic microorganisms.

Microbial growth dynamics were assessed in batch and continuous cultures to assess the response to an ionophore insult. Pure cultures of S. bovis 24, L. vitulinus B62, B. ruminicola GA33 and S. ruminantium HD4 were utilized in batch cultures; S. bovis 24 and B. ruminicola GA33 were used in continuous cultures. As an experimental approach, pure cultures in batch culture were grown through 6 successive transfers within a treatment medium (pH 6.8). Successive treatments were as follows: Control (C), 0.5 ppm monensin (A), 20 ppm monensin (B) and back to control (C-1). For S. bovis 24, growth was depressed and time to reach maximum growth (T) was increased ($P<0.01$) by A and B; transfer back to C-1 resulted in a recovery of growth but T was longer than C ($P<0.01$). Cell yield (CY) ($P<0.01$) and $Y_{glucose}$ (Y_G) were depressed by A and B. L. vitulinus B62, did not grow in B and barely grew in A. When B. ruminicola GA33 was grown in B there was an initial depression in growth

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and T increased for the first 3 passages; growth recovered and T declined during passages 4-6. Y_G and CY paralleled the growth response of B. ruminicola GA33. S. ruminantium HD4 grew well in B, but T was increased ($P<0.01$) and CY and Y_G declined ($P<0.01$).

Continuous cultures of S. bovis 24 and B. ruminicola GA33 were performed for up to 30 days; dilution rates were set at 5%/h and 10%/h. Y_G nearly doubled for both organisms as dilution rate was increased from 5 to 10%/h. Monensin depressed growth, RNA/protein, CY and Y_G in S. bovis 24 cultures at both dilution rates. Monensin affected B. ruminicola GA33 cultures much like S. bovis 24; the general depression of cell growth dynamics was less severe with the Bacteroides.

Monensin-C^{14} apparent cell membrane binding was studied in S. bovis 24 and B. ruminicola GA33. Apparent binding was about 50% for both organisms.

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INTRODUCTION

Carboxylic polyether ionophores are used by the cattle feeding industry for increasing the performance of feedlot cattle. Monensin is such an ionophore (Goodrich et al., 1976; Goodrich et al., 1984). Monensin has been defined as a Na^+/H^+ antiporter (Harold, 1972) and the cross flux of Na^+ and H^+ is obligatory primarily because monensin's affinity for Na^+ and H^+ is much higher than for other cations (Pressman, 1976).

In general, previous studies show several important physiological effects upon feeding monensin in cattle; for instance, an increase in the molar proportion of propionate, a decline in rumen methane production (Bergen and Bates, 1984; Richardson et al., 1976; Thornton et al., 1976), as well as a lowered ruminal degradation of dietary protein resulting in more escape protein flowing to the lower gut (Poos et al., 1979; Isichei, 1980) and a retardation of common feedlot disorders such as lactic acidosis and bloat (Nagaraja et al., 1981; Nagaraja et al., 1982; Sakauchi and Hoshino, 1981; Bartley et al., 1983).

Chalupa (1980) also indicated that monensin inhibits gram positive organisms that are formate, H_2 producers, this causes a decrease in methane production (Wolin, 1975). This is due primarily to

lowered availability of H_2 and formate as well as depressed interspecies H_2 transfer.

Cattle, upon ingesting monensin, show lower concentration of rumen ammonia (Chalupa, 1980; Van Nevel and Demeyer, 1977) and depressed protease and deaminase activity (Barao, 1983). Thus, monensin might increase feed/dietary protein reaching the lower gastrointestinal tract for digestion and absorption (Poos et al., 1979; Chalupa, 1980; Isichei, 1980). As far as the third aspect of beneficial characteristics is concerned, monensin inhibits growth of the major lactic acid producers (gram positive organisms) within the rumen (Dennis et al., 1981a; Dennis et al., 1981b) such as Streptococcus bovis which proliferates under acidosis conditions and during feedlot bloat (Sakauchi and Hoshino, 1981).

Although extensive explorations on the effects of ionophores on various aspects of the rumen fermentation have been done in our laboratory (Isichei, 1980; Barao, 1983; Bates and Bergen, 1984a; Bergen and Bates, 1984) and elsewhere (Raun et al., 1976; Lemenager et al., 1978; Prange et al., 1978; Short, 1978; Johnson et al., 1979; Romatowski, 1979; Schelling, 1984; Brondani, 1986; Johnson, 1987; Folz et al., 1988; Galyean and Owens, 1988), there is little knowledge about morphological changes in microbial cell membrane as explored by scanning electron microscopy during exposure

to ionophores.

This dissertation describes studies on the effect of monensin on cellular physiology of characterized common rumen bacteria when grown in batch and continuous culture.

The purpose of the studies described below was to determine in both batch culture and continuous culture the effect of a long term adaptation to monensin by pure culture rumen organisms (S. bovis 24, L. vitulinus B62, B. ruminicola GA33 and S. ruminantium HD4) to assess culture growth dynamics, to determine bacterial RNA to protein ratio, to determine substrate utilization rate, $Y_{glucose}$, membrane integrity. Finally, a ^{14}C-monensin binding autoradiography study was conducted to investigate monensin binding to cell membranes in S. bovis 24 and B. ruminicola GA33.

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LITERATURE REVIEW

The Mechanism of Action of Ionophores in Rumen Microbes

A rather general definition of the term "ionophores" is as follows: ionophores are substances with the ability to promote the transfer of ions from an aqueous medium into a hydrophobic phase (Dobler, 1981).

Generally speaking, some ionophores are naturally occurring compounds such as antibiotics and others are purely synthetic compounds. Four classes of ionophores are recognized: (1) natural neutral ionophores, (2) natural carboxylic ionophores, (3) synthetic ionophores and (4) quasi-ionophores (Dobler, 1981). Two groups of antibiotics, depsipeptides and macrotetrolides, constitute natural neutral ionophores, that is, the first class of ionophores. The second class of ionophores, natural carboxylic ionophores, are produced by various streptomyces cultures. Their biological action is very distinct from that of the neutral ionophores. The third class of ionophores, synthetic ionophores, contains five categories: macrocyclic polyethers, linear polyethers, macropolycyclic polyethers containing tertiary amino groups at the bridge heads ("cryptands"), noncyclic ligands for alkaline earth cations, and chirality-recognizing polyethers. The last class of ionophores, quasi-ionophores, is characteristically a three

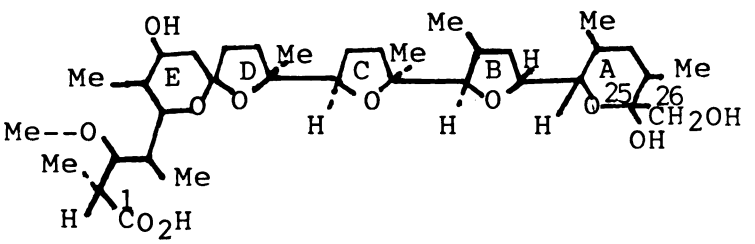
dimensional structural channel-forming ionophore which forms channels across membranes.

Ionophores such as monensin, lasalocid, salinomycin, and narasin belong to the second class. They are used in the cattle feeding industry due to the fact that they can increase the performance of feedlot cattle (Goodrich et al., 1984). However, monensin, lasalocid, or salinomycin are also used by the poultry industry as a coccidiostat (Coban). Monensin (Lilly) is a commonly used feed additive with anti-protozoal, anti-bacterial, coccidiostatic and anti-fungal properties (Blumenthal and Vance, 1988). Monensin is effective in controlling coccidiosis in chickens. Horses are extremely sensitive to monensin and the known toxic effects are related to an ionotropic effect on the heart. Monensin is cytotoxic to HeLa and murine clone NCTC-1742 cells in tissue culture (Blumenthal and Vance, 1988). Whether monensin is a potential neurotoxin has not been established (Oka and Weigel, 1987).

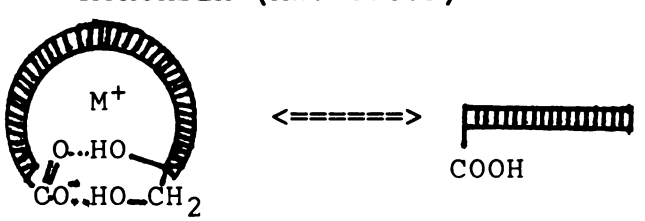
The antimicrobial activity of carboxylic polyether ionophores is related to their ability to modify the movement of cations across biological membranes (Dobler, 1981). In this review, emphasis is placed upon the mechanism of action of monensin in rumen microbes.

Monensin, one of the biologically active, polycyclic, monocarboxylic acids produced by Streptomyces cinnamonensis (Haney and Hoehn, 1967),

has a ring structure under basic conditions and makes a complex with a cation, while it has a chain structure in acidic conditions (Sada et al., 1987). Monensin's chemical chain structure contains a short carbon skeleton of 25 carbon atoms and lacks ring F. The chemical formula of monensin is presented in Fig. 1 (Agtarap et al., 1967; Dobler, 1981; Sada et al., 1987).



Monensin (MW. 670.9)



Basic condition Acidic condition

Fig. 1. The Chemical Formula of Monensin (Dobler, 1981; Sada et al., 1987).

Many investigators (Richardson et al., 1976; Thornton et al., 1976; Van Nevel and Demeyer, 1977; Allen and Harrison, 1979; Poos et al., 1979; Chalupa, 1980; Isichei, 1980; Bergen and Bates, 1984; Martin and Macy, 1985; Newbold et al., 1988) observed various changes in ruminant fermentation of monensin treated animals; a summary of metabolic effects of ionophores on the

Table 1. A Summary of Metabolic Effects of Ionophores on the Rumen Fermentation*

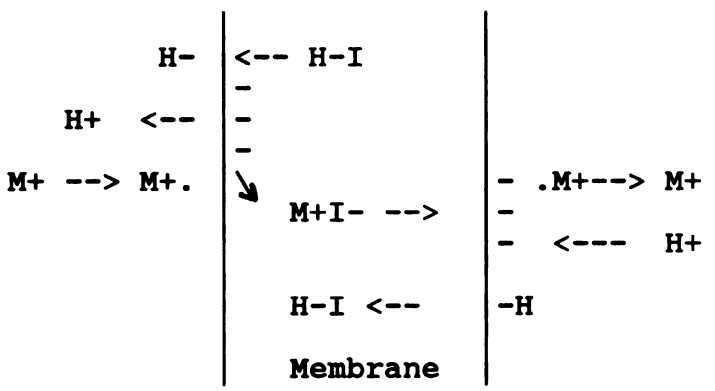
1.	Shift in acetate:propionate ratio toward more propionate.
2.	Some increase of lactate to propionate production via the acrylate pathway.
3.	Decreased ruminal protein breakdown and deamination; lower ruminal ammonia-N.
4.	Primary H_2 or formate producers, gram positive organisms, are inhibited.
5.	Decrease in methane production primarily due to lowered availability of H_2 and formate and depressed interspecies H_2 transfer.
6.	Depression of lactic acid production under acidosis inducing conditions.
7.	Gram negative organisms, of which many produce succinate (source of propionate) or possess capacity for the reductive tricarboxylic acid cycle to use bacterial reducing power, survive.
8.	Some evidence for depressed rumen content turnover.
9.	A mild inhibition of protozoa.
10.	Decrease in rumen fluid viscosity in bloated animals.
11.	Depressed growth yield efficiency of the ruminal microbes.

*Adapted from Bergen and Bates (1984).

rumen fermentation is shown in Table 1 (from Bergen and Bates, 1984).

There are two mechanisms by which ionophores could affect the transfer of ions from a polar region into or across a nonpolar medium (e.g. cellular membrane): the ion carrier and the channel-forming modes (Dobler, 1981). A carrier ionophore forms a complex of well-defined stoichiometry with the ion, carrying the hydrophilic ion into or across the hydrophobic region, whereas channel-forming ionophores form hydrophilic pathways for the ions, spanning a lipid membrane barrier.

Painter et al. (1982) indicated that cation-proton exchange may be mediated by a carboxylic acid ionophore. In this proposed scenario, cation/proton exchange as a basic metabolic effect of monensin in cell membranes is shown in Fig. 2.



M+ = metal cation; I = ionophore; H+ = proton, H-I = protonated ionophore; M+I- = zwitterion of metal cation and anionic form of ionophore

Fig. 2. Carboxylic Ionophore Mediated Cation Transfer Across a Biomolecular Lipid Membrane (Painter et al., 1982).

The transport/exchange cycle begins with the anionic form of the ionophore. As an anion, the ionophore is capable of ion pairing with a metal cation. The binding of a cation mediates the formation of a lipophilic, cyclic cation-ionophore complex that can diffuse through the interior of the biomolecular membrane structure.

Painter et al., (1982) pointed out that the ionophore must be in the anionic form before it is

capable of binding a metal cation (M^+) and diffusion across the membrane occurs only when the ionophore exists in the protonated form (ionophore H^+) or as a zwitterion (M^+ and anionic form of ionophore).

As far as channel-forming characteristics are concerned, ionophores do not display the same affinity for all cations. Monensin has been defined as a Na^+/H^+ antiporter (Harold, 1972) and the cross flux of Na^+ and H^+ is obligatory primarily because monensin's affinity for Na^+ and H^+ is much higher than for other cations (Pressman, 1976).

In general, the primary transport system refers to the processes of translocation of proton and generation of an electro-chemical gradient across membrane. The basic driving force (energy) of primary transport systems such as transport system by the ion carrier and channel-forming modes can be explained by two hypotheses (Dobler, 1981). One such hypothesis is the chemical hypothesis (Pressman, 1965) which postulates a high-energy intermediate , and functions as a common link. The other is chemiosmotic hypothesis (Mitchell, 1961) which postulates that enzymes of the respiratory chain are arranged in such a way that electron transfer down the chain is coupled to a directed transport of protons across a membrane (Fig. 3).

According to Mitchell (1961), such a transport

generates a concentration gradient across the membrane that is able to do osmotic work and thus acts as a store of energy. In addition, because charged particles are transported, a potential difference is also generated and a proton electrochemical gradient is elevated. This

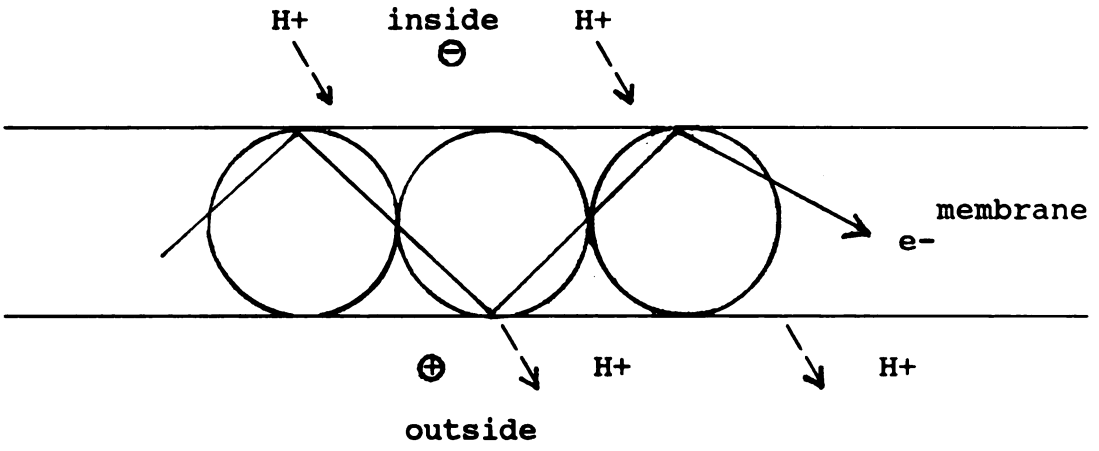


Fig. 3. Coupling of Electron Transfer to Proton Transport (Mitchell, 1961; Dobler, 1981).

potential serves as an energy source for the synthesis of ATP by ATPase. Therefore, it is obvious that any rupture in the membrane would destroy the potential and thus stop the production of ATP (Dobler, 1981). Ionophores can dissipate the ion and electron potential gradients across membranes and destroy primary transport by causing nonphysiological ion leaks. This results in a depression of the proton motive force and eventual depletion of intracellular ATP (Bergen and Bates, 1984). It follows then if the effect of ionophores on rumen bacteria is considered, understanding dissipation of

interaction gradients and mechanisms of ionophore with cellular membranes are paramount importance (Bergen and Bates, 1984).

Bacterial Cell Tolerance to Monensin

Generally speaking, in this review bacterial species that are either gram-positive cocci, gram-positive rods or gram-negative rods will be referred to on discussions of bacterial cell tolerance to the gradient dissipating effect of monensin.

Gram-positive cocci such as Streptococcus bovis, a common facultative anaerobic rumen coccus, is normally present at concentrations of about 10^3 per gram of rumen contents. S. bovis has a capacity to multiply quickly in the presence of readily fermentable carbohydrate (Hungate et al., 1952)

Gram-positive rods, such as Lactobacillus appear to be associated with milk-feeding in infancy in the undeveloped rumen, and the prevalence of these organisms declines on weaning in the developing rumen. Lactobacillus may be present in the adult rumen if the animal's diet is rich in starch (Hungate et al., 1952)

Gram-negative rods, such as anaerobic Bacteroides species, can occur in concentration of more than 10^9 per gram of rumen contents. Almost all species of Bacteroides are strongly saccharolytic and produce various mixtures of volatile fatty acids and succinic acid during fermentation. B. ruminicola is one of the

well-defined rumen Bacteroides species (Hungate, 1966).

Selenomonas ruminantium, an anaerobic rumen rod, has been well characterized (Hobson, 1965b). A few researchers have suggested that Selenomonas are not bacteria and should be classed as protozoa. However, Hobson (1965b) pointed out that the cell wall composition of S. ruminantium is similar to that of other gram-negative bacterial cell walls.

Chen and Wolin (1979) have proposed a model (Fig. 4) which accomodates most of the available information concerning the effect of monensin on rumen fermentation. Their proposal indicates that monensin alters the rumen fermentation pattern by altering the microbial population. In Fig. 4, underlined compounds are end products of the rumen fermentation. The reason for changes in proportions of volatile fatty acids (VFA, i.e., acetate, propionate and butyrate) in rumen contents by monensin, increased propionate, decreased acetate and butyrate and also reduced methanogenesis, is due to *in vivo* selection for an ionophore tolerant microbial community (Van Nevel and Demeyer, 1977; Chen and Wolin, 1979; Bergen and Bates, 1984). The postulated effect of monensin predicts that gram positive bacteria are suppressed while gram negative organisms survive. A shift from gram positive/gram negative mix to a more predominant gram negative population leads to enhanced propionate

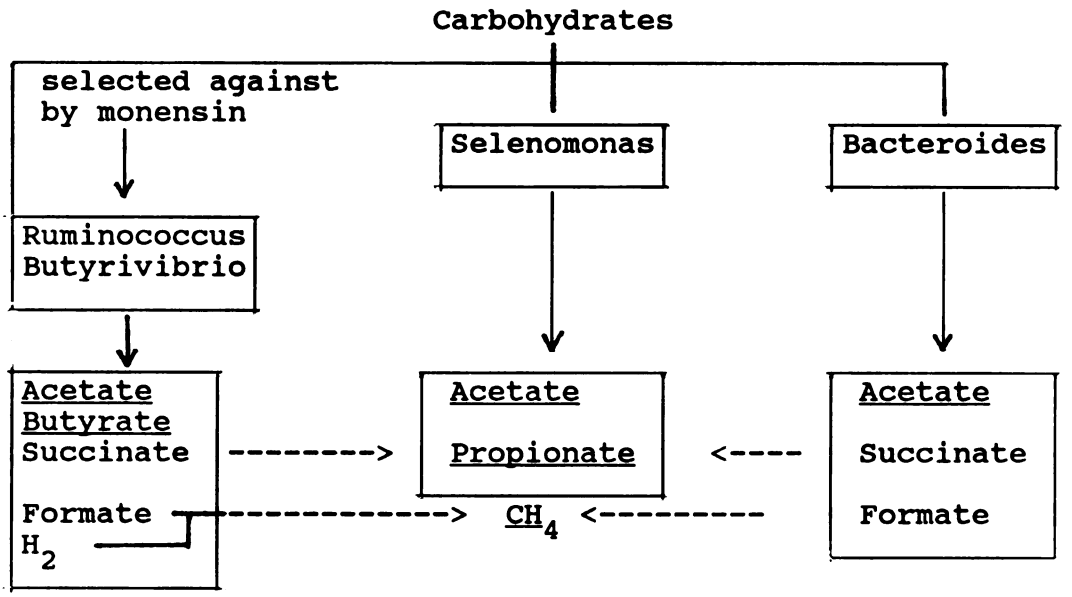


Fig. 4. A Model of The Mode of Action of Monensin (Chen and Wolin, 1979).

and lowered acetate and methane production.

There are 2 major possibilities which may explain bacterial cell tolerance to monensin. One explanation for this phenomenon is the physical structure of gram-negative bacteria. The outer membrane of gram-negative species serves as a penetration barrier which protects these cells from monensin (Chalupa, 1980).

A second possible explanation for this phenomenon involves ATP production, growth energetics and membrane bound proton (electron) transport of gram-negative strains (Bergen and Bates, 1984). Hence, those bacteria which can couple the process of proton extrusion to electron transport, would protect their valuable ATP

stores from dissipation to just maintain a favorable pH, and have a selective advantage over strains (G^+) which depend heavily on direct utilization of intracellular ATP obtained by substrate level phosphorylation reactions (Bergen and Bates, 1984).

A goal of the present work, to be discussed below was to study rumen bacterial tolerance and adaptation to monensin. Growth dynamics of four typical ruminal bacteria were assessed in batch cultures. Adaptation to the ionophore was assessed after six transfer passages of bacteria in media of various composition and ionophore concentrations.

The Nutrient Requirements of Rumen Bacteria

This section is a review of the nutrients required by rumen bacteria and of other factors that influence microbial cell yields. In general, the type of diet and processing affects efficiency of ruminal biomass production (Bergen et al., 1982; Owens and Bergen, 1983). Factors involved in growth and protein synthesis of rumen microorganisms have been delineated, i. e., energy sources, nitrogen sources, carbon sources, mineral elements, organic growth factors such as B-vitamins and physical characteristics of the diet (Owens and Isaacson, 1977). The need of specific nutrients, particularly sulfur and other unidentified factors, such as carbon skeletons also affect ruminal microbial cell yields (Bergen and Yokoyama, 1977).

The nutrients and culture factors most frequently cited (Bryant, 1973; Smith, 1975; Owens and Isaacson, 1977) as influencing the ultimate population mix within a complex microbial ecosystem include 1) maximum growth rate of individual bacterial species, 2) affinity for available substrate, 3) bacterial cell maintenance expenditures and efficiency of cell yield (Y_s or Y_{ATP}), 4) versatility, 5) tolerance to pH and other inhibitory factors (Russell and Hespell, 1981). Bergen et al., (1982) pointed out that when ruminal digesta dilution rate is increased, the extent of digestion in the rumen of dietary organic matter is decreased, and the potential for ATP generation is depressed. This would lower the amount of ATP available for microbial biomass production. The mass of bacteria produced per mole of ATP (Y_{ATP}) varied from 4.7 to 28.5 (Stouthamer and Bettenhaussen, 1973). The values of Y_{ATP} varied markedly with turnover or growth rate of bacteria, accumulation of ash or starch and intraspecies transfer of reducing equivalents. Increased turnover rate of rumen contents appears to enhance bacteria protein production, increase ruminal acetate and methane production, and increase bypass of fiber and concentrate components of the diet (Owens and Isaacson, 1977).

Bacterial yield or output from a continuous flow system, like the rumen, is determined by bacterial population or concentration and by growth rate or

dilution (turnover) of the rumen fluid. Therefore, microbial growth rate must at least equal the ruminal dilution rate, otherwise the population density would change which may results in a new steady state of bacterial numbers or experience wash out (Bergen et al., 1982).

The factors influencing bacterial yield from substrate are ATP yield and efficiency of ATP use. $Y_{glucose}$ is a term for describing the mass of bacteria that will be produced per mole of substrate (glucose) fermented. The values of $Y_{glucose}$ vary markedly from 4.7 to 69.5 (Stouthamer and Bettenhaussen, 1973) when different species of anaerobic bacteria were grown in batch culture using various composition media with glucose as substrate. Thus, in the present work, various physiological parameters of bacterial cell production were used as an index for evaluation of ionophore effects on ruminal bacteria cultures.

Bacterial Energy Distribution and Turnover Rate

Bacteria must spend energy for maintenance first then for growth (Isaacson et al., 1975; Stouthamer and Bettenhausen, 1973). Energy expended for maintenance is used for motility, replacement of lysed cells, sustaining ionic gradients, active transport and for resynthesis during turnover of intracellular components. If any one of these above factors changes, then the maintenance energy needs will also be changed (McGrew and Mallette,

1962).

Generally speaking, in any given culture/ecosystem the microbial population increased when more energy was provided. Tempest and Neyssel (1978) reported that when glucose was pulse fed to a continuous culture growing at a low dilution rate, the rate of metabolism was increased above the value associated with classical operation of a chemostat. Growth efficiency of the pulsed cells was clearly diminished. Batch cultures of R. flavefaciens grown with cellobiose as the primary substrate attained maximum cell density with 40% of the substrate remaining (Pettipher and Loutham, 1979); further increases in fermentation end products were noted until only trace amounts of sugar were detectable. Therefore, at a constant growth or dilution rate, the efficiency of bacterial growth is not always closely coupled to substrate availability (Owens and Isaacson, 1977), but total cell yield overall must be related to available energy.

When turnover rate or dilution rate was increased, the efficiency of bacterial growth increased markedly in the rumen (Owens and Isaacson, 1977). This is because microbial population and bacterial residence time both declined. The relationship between Y_{ATP} and growth rate or dilution rate fits a Michaelis-Menton type curve; thus, as growth rate increased and maintenance expense decreased, the yield approached a theoretical maximum

(Pirt, 1965; Pirt, 1975; Bergen et al, 1982).

Isaacson et al. (1975) combined a number of theoretical and reported efficiencies values of rumen bacterial growth at different dilution rates and found that there is a strong tendency for the efficiency to increase with dilution rate. However, due to the fact that rumen turnover is generally measured by dilution rate of rumen fluids, using soluble or insoluble marker, and not on the absolute actual turnover rate of bacteria, actual growth efficiencies may differ *in vivo* and *in vitro*. Bacterial association with particles would cause bacteria to lag behind the rumen fluid turnover (dilution) rate by 50 to 300% (Mathison and Milligan, 1971).

Dry matter degradation and volatile fatty acid production continue for both maintenance and growth of bacteria (Isaacson et al., 1975; Satter and Slyter, 1974). The production of bacterial protein and use of nonprotein nitrogen are directly proportional to cell yield. As dilution rate increases from 2% to 12% in a chemostat, the bacterial yield (mg/day) and efficiency of mixed rumen bacteria (cells/g glucose) almost doubled (Isaacson et al., 1975).

Many factors influencing ruminal turnover rates of fluids and particles have been delineated (Owens and Isaacson, 1977). One of the factors that influence ruminal turnover rates of fluids is fluid influx

including fluid intake (salts, food), rumen wall influx (osmotic pressure) and salivary flow (Isaacson et al., 1975). Another factor is rumen volume, i.e., total capacity and nonfluid fill (Isaacson et al., 1975). Feed factors that influence ruminal turnover rate of particles are feed intake particle size, particle density and rate of density change (Isaacson et al., 1975).

Fluid enters the rumen as water and in feed, diffusion through the rumen walls is continuous, and influx can be large or small depending on osmolarity of rumen content (Ternouth, 1967; Owens and Isaacson, 1977). Fluid absorbed through the rumen wall does not alter bacterial or particulate turnover while fluid flow through the omasum may alter bacterial growth rate or particulate turnover.

Increased salivary salts in the rumen will increase omasal output. Therefore, roughage added to high concentrate ration might elevate the turnover rate of the ruminal fluid (Cole et al., 1976) due to enhanced saliva flow or reduced fluid space in the rumen. As dilution rate is altered, bacterial populations may change in type or metabolism (Owens and Isaacson, 1977). Pure cultures often produce different end products when grown at faster rates (Hobson, 1965b; Wolin, 1975).

Based on fermentation balance equations (Wolin, 1960; Pirt, 1965, 1975; Baldwin, 1970; Isaacson et al., 1975), with equal substrate supply, an increase in

either acetate or butyrate and a decrease in propionate is accompanied by an increase in methane and heat loss and decrease in ATP production. Increased dilution rate will increase the acetate to propionate ratio, which in turn may alter ruminal and animal energetics (Owens and Isaacson, 1977). Decreased dilution rate will reduce bacterial protein synthesis but may enhance feed efficiency and energy availability through decrease methane and enhancing propionate production (Owens and Isaacson, 1977).

Chemostate - Continuous Culture of Rumen Bacteria

The continuous culture of microorganisms has been studied for many years and the most successful designs of a growth system are the chemostat (Monod, 1950; Novick and Szilard, 1950) and turbidostat (Fox and Szilard, 1955).

The chemostat and turbidostat are essentially the same apparatus. The chemostat has some advantages over the turbidostat mechanically and for growth at low growth rates. Most of the chemostat described have been for the growth of aerobic bacteria and a problem in these designs is to supply sufficient air to the microorganisms, and this has led to the design of apparatus in which a film of culture flows around the walls of a vessel (Monod, 1942; Monod, 1950; Hobson, 1965a).

This type of apparatus is not suitable for anaerobic work as the 'stirred fermenter' type. Most rumen

bacteria are strict anaerobes which require special techniques for handling and are unable to initiate growth unless the media used are highly reduced. Various workers have attempted to compare the rumen fermentation to formalized microbial culture systems. The rumen fermentation can be likened to a continuous microbial culture system with a more or less continuous substrate and buffer supply and a fermentation end product removal system (Bergen and Yokoyama, 1977). The rumen sometimes has been described as a continuous culture (Hungate, 1966), semicontinuous (Wolin, 1979) or discontinuous fermentation (Harrison and McAllan, 1980). It seemed that a better understanding of the behavior of rumen bacteria might be obtained if they were grown in continuous culture rather than batch culture (Hobson, 1965b). A continuous, chemostat system demands a constant volume, a constant microbial population, a growth limiting nutrient, constant dilution rate and steady state (Pirt, 1975; Bergen et al,, 1982).

In the following study, the role of ionophore on bacterial culture physiological parameters was conducted by comparisons of ruminal bacteria growth phenomena at 5%/h and 10%/h dilution rate and by the assessment of the values of fermentation end products, substrate utilization rate, cell yields, and $Y_{glucose}$.

RNA/Protein Ratios

Ribonucleic acid (RNA) may be used as an internal marker of microbial protein. Most RNA reaching the lower gut of ruminants is of microbial origin (Smith, 1969; Smith and McAllan, 1970). As a matter of fact, the nucleic acid/protein values presented here and elsewhere (Bates, 1985; Bates and Bergen, 1984b; Bates et al., 1985) are valuable in an assessment of the use of RNA as a marker for bacterial protein passage to the ruminant gut. Thus, RNA can mark microbial flow and in turn bacterial protein synthesis.

The microbial nitrogen or protein passing to the abomasum can be calculated as follows:

$$\frac{\text{Total N/bacterial dry matter}}{\text{RNA-N/bacterial dry matter}} \times \frac{\text{RNA/N /abomasal dry matter}}{\text{NAN / abomasal dry matter}}$$

Where, NAN is nonammonia nitrogen. Microbial flow is determined by multiplying this value by total NAN passage to the lower gut. Parenthetically, the ratio RNA-N : total-N does not always accurately predict RNA/protein per se (Bates et al., 1985). For instance, as growth rate increases, the proportion of RNA in a bacterial cell also increases. Ribonucleic acid is approximately 14.8% nitrogen (Ling and Buttery, 1978) and this nitrogen will be included in any estimation of total nitrogen. True microbial protein passing to the small intestine will be overestimated by RNA-N/total-N when the RNA/Protein ratio of ruminal bacteria is high (Bates et

al., 1985).

Marcomolecular composition of rumen bacteria as a marker of microbial function and production in the rumen has much potential (Bates et al., 1985). Many investigators have reported digesta passage studies and RNA-N/total-N has been used as a marker for microbial protein synthesis (Theurer, 1979). However, a considerable variation exists in the estimates of microbial yield using this technique. This problem might be due to species variation (Smith, 1969), diet and time after feeding under infrequent feeding conditions, a physiological response of the rumen microorganisms or to difficulties with sample analysis (Bates, 1985).

Most <u>in vivo</u> RNA-N/total-N values in the literature vary from 0.16 to 0.2 (Poos et al., 1979; Isichei, 1980). Gillett et al. (1982) and Barao (1983) reported a value 0.25 when RNA-N/total-N was converted to RNA/protein. RNA/protein values of 0.1 to 0.2 are usually obtained for enteric organisms growing near stationary phase (Koch, 1971). Rumen bacteria are present at 10^{10} to 10^{11} per g rumen contents and grow at an average rate of 0.06 to 0.07 doublings/h (Hungate, 1966; Oxford, 1964), thus the RNA/protein ratios would be expected to be low and constant (Nierlich, 1978; Bergen et al., 1982).

The Impact of Electron Microscope on Rumen Microbial Research

A microscope is an instrument designed to render objects visible which are too small to be seen by the unaided eye. For particles greater than a tenth of a micrometer in diameter, the light microscope is adequate, but for very small objects the light microscope fails because the wavelength of visible light is large compared with the objects to be examined (Agar et al., 1974).

This limitation, called the diffraction limit, is due to the size of the wavelength of light and no further improvement can be expected without using a different illumination of shorter wavelength. The limit of resolution of light microscopy is 0.2 um. Ultraviolet microscopy does not in practice improve this limiting condition (Agar et al., 1974). An electron beam has a wavelength shorter by a factor of 10^5 than visible light. Practical instruments employ magnetic lenses as they can be made with less defects than electrostatic lenses.

For a period of almost forty years from 1949, the multipurpose 100 kV electron microscope has been the standard instrument. The use of electron microscope in studying microorganism has increased enormously in recent years and much information relating structure to function at a sub-cellular level has accrued. The observations obtained by the application of varied techniques to a wide range of specimens include

bacteria, protozoa and virus (Fuller and Lovelock, 1976).

Scanning Electron Microscope Autoradiography

The rational basis of autoradiography is the demonstration of radioactive isotopes in tissue by means of their ability to reduce silver salts in a photographic plate or emulsion (Paul et al., 1970; Budd, 1971; Darley and MacFarlane, 1977).

From the distribution of silver grains, the location of radioactive atoms that have been introduced into the specimen can be determined, allowing localization of many labeled substances (Budd, 1971; Pearse, 1980).

A variety of radioactive labels have been used (Pearse, 1980). Substances occurring naturally in tissues either have been administered in ionic form or combined with organic molecules. Among those isotopes used are ^{3}H, ^{14}C, ^{32}P, ^{24}Na, ^{35}S, ^{131}I and ^{125}I (Pearse, 1980).

Scanning electron microscopy autoradiography (SEM-AR) presents several advantages over transmission electron microscopy autoradiography (TEM-AR) and light microscopy autoradiography (LM-AR) (Petersen, 1984). Sample preparation in SEM-AR is more rapid and easier than bacterial ultrathin section in TEM-AR (Petersen, 1984). Larger sample size in SEM autoradiography allow much shorter exposure times. It is also possible to use smaller crystal emulsions without losing sensitivity, due

to higher levels of radioactivity present in the sample.

Analysis of SEM autoradiograms can be done by several methods. Secondary electrons may be collected to give topographical information, which in some cases is adequate to locate silver grains. Primary electrons are backscattered from the specimen, and the lower the atomic number of a specimen, the greater the absorbance of primary electrons. Silver grains, with a higher atomic number than most biological tissues, have a backscattering coefficient approximately four times as great. Consequently, silver grains appear as bright deposits against a dark background.

^{14}C-Monensin used in these studies was labeled in at least seven positions including the carboxyl side chain and four of the five rings. Monensin is nonvolatile and has no significant UV absorption. Thus, it is not readily adaptable to measurement by conventional gas-liquid chromatography and HPLC. Monensin can be assayed by HPLC with a refractive index detector or it can be detected on thin layer chromatography plates or in methanol solution by reaction with vanillin. Donoho and Kline (1968) conducted a bioautographic method, which was a microbiological assay by using thin layer bioautography for assay of monensin in animal tissues.

The objectives of the present work using scanning electron microscopy were to describe the morphological changes of bacterial membrane surfaces upon ionophore

addition to culture medium and to observe monensin binding to cell membrane surfaces by using scanning electron microscopy autoradiographic technique (Klomparens et al., 1986).

MATERIALS AND METHODS

Experiment 1

In vitro Batch Culture Adaptation Study

This work was designed to answer the question: does repeated exposure to monensin of preexposed ruminal bacteria enhance the eventual growth rate? This question was tested by utilizing a series of six transfers of cultures, the actual experimental approach is presented in a flow chart below (Tables 3 to 6). The hypothesis was that growth phenomena of bacterial cultures grown through six passages in 0.5 or 20 ppm of monensin would not differ from the initial culture to the final culture (via six passages).

Organisms

Bacteroides ruminicola GA33, Lactobacillus vitulinus B62, Selenomonas ruminantium HD4, and Streptococcus bovis 24, were a kind gift from Dr. M. T. Yokoyama, Department of Animal Science, Michigan State University.

B. ruminicola GA33 and S. ruminantium HD4 are gram negative rods, while S. bovis 24 and L. vitulinus B62 are a gram positive cocci and rod respectively. Routine transfers of stock cultures were performed at monthly intervals. Gram stains and wet mounts of the representative microorganisms were examined monthly as a check for culture purity. The fermentation end products

of individual species and the appropriate fermentation pathways are shown in Appendix 1.

Cultivation

The Hungate anaerobic technique (Hungate, 1950) was used in the preparation of media and cultivation of microorganisms. Inoculation and sampling were performed while continuously gassing with O_2-free CO_2 which had been passed through a heated quartz column containing reduced copper. Cultures were grown at a constant 39^0C in an incubator.

A defined anaerobic medium (modified medium 10; Caldwell and Bryant, 1966) was used in this experiment (Table 2). Bacteria were maintained on slants of modified medium 10 and stored at 4^0C

The ingredients of medium were well mixed and adjusted to pH 6, 6.8 and 7.6 with 6 N HCl or NaOH according to the requirement of the specific study. After reaching desirable pH the medium was placed in a round bottom flask; rubber stoppers were used to seal the flask. The rubber stoppers were fastened in place with wire that was tightly twisted over the stoppers and around the neck of the flask to avoid stoppers from popping out during the autoclaving process. Medium was sterilized at 121^0C for 25 minutes in a steam autoclave and then allowed to cool. The wire and rubber stoppers were removed and medium was immediately placed under O_2-free CO_2 gas. Fifteen ml of reduced medium were

transferred anaerobically (under O_2-free CO_2) to test tubes (13 x 150 mm). Upon commencement of the trial, 0.5 ml of culture was added to each broth. All transfers were conducted according to the Hungate technique (Hungate, 1950) for culturing rumen anaerobic bacteria.

The following experiments were carried out to determine the effect of Na-monensin and length of exposure on microbial growth. Na-monensin was a kind gift from Lilly Research Laboratories, Eli Lilly & Co.; for convenience Na-monensin will be referred to monensin in this dissertation. Pure cultures of ruminal anaerobes were grown in batch culture in a defined medium (Table 2) which was a glucose limited and nitrogen rich medium (Bates, 1985). The defined medium contained either 0, 0.5 or 20 ppm of monensin (dissolved in absolute ethanol) plus none or additional NaCl in order to reach 200 mmol/l [Na]. A calculation for reaching 200 mmol/l [Na] is shown in Appendix 1. Final [Na] value was determined by using Atomic Absorption/Emission Spectrophotometer (589 nm; Instrumentation Laboratory Company, model IL 951).

Adaptation Study Protocol

Flow charts of various treatments of <u>in vitro</u> batch culture adaptation study to ionophore are shown in Tables 3 to 6. For each culture condition, bacteria were transferred six consecutive times, e.g. six

Table 2. Experimental Medium[a] for *in vitro* Batch Culture of Ruminal Bacteria

Medium components	g/100 ml medium
Glucose	0.08
Yeast extract	0.05
Trypticase	0.2
Na_2CO_3	0.8
Cysteine-HCl	0.05
Mineral solution I[b]	3.75 ml
Mineral solution II[c]	3.75 ml
Hemin solution[d]	0.1 ml
Resazurin[e]	0.1 ml
Deionized water	to 100 ml

a prepared under O_2-free CO_2; adjusted to various pH based on the requirement of experiments to 6.0, 6.8 and 7.6 with 6 N HCl or NaOH.

b contained 0.6 K_2HPO_4 g/100 ml medium.

c contained in g/100 ml medium: KH_2PO_4, 0.6; $(NH_4)_2SO_4$, 0.6; NaCl, 1.2; $MgSO_4 \cdot 7\ H_2O$, 0.25; $CaCl_2 \cdot 2\ H_2O$, 0.16.

d contained 0.28 g KOH in 25 ml 95% ethanol and 100 mg hemin made up to 100 ml with deionized water.

e contained 25 mg resazurin in 100 ml deionized water.

For more complete media preparation details consult Hungate (1966), Bryant (1973) and Holdeman et al., (1977).

passages, cells from pass No. 6 were used to inoculate the next experimental culture conditions. In Tables 3 to 5, the symbol "X" means the culture failed to grow at the indicated stage.

A preliminary growth curve trial was conducted initially in order to ensure that the various bacteria can grow under defined culture conditions. If a culture failed to grow in the 0.5 ppm monensin medium, further

studies were terminated. A second objective of the preliminary work was to observe time of shift in the growth curve from the exponential phase to stationary phase.

Cultures followed the sequences as described in Tables 3 to 6. For each new passage, 15 ml medium was inoculated with 0.5 ml of previous organism broth and incubated at 39^{0}C. Optical density readings as well as pH values were taken at timed intervals for the various runs. Optical densities were read with a Bausch & Lomb Spectronic 70 at 600 nm and pH values were determined by a PHM 64 Research pH meter.

After each passage, one drop of broth was put on a slide and checked for purity using the gram stain and an Olympus Tokyo light microscope at 1000 X magnification.

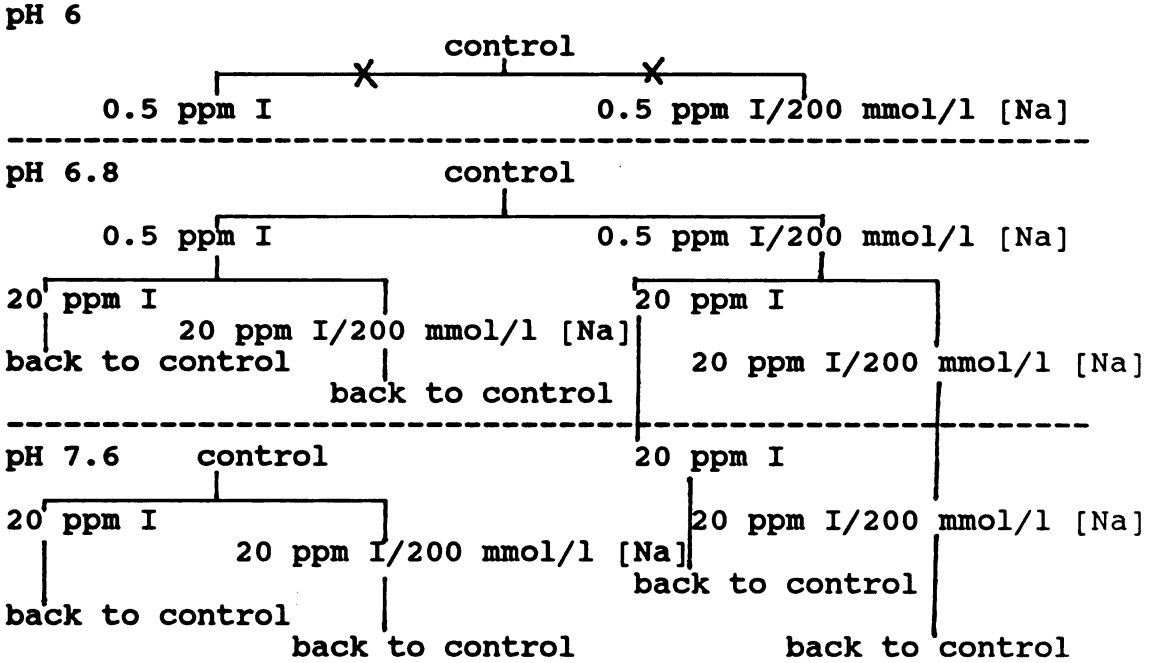
Fermentation Products Determination

Fifteen ml of broth of each run were utilized for VFA, lactate and succinate assays. The fermentation products were analyzed on a GLC, using a method modified from those outlined by Sampugna et al. (1966), Finch (1970), Lambert and Moss (1972) and Salanitro and Muirhead (1975). The detailed analysis procedures are described in Appendix 3. The characteristics of the packing material of GLC column are described in Appendix 4.

Cell Yield Study

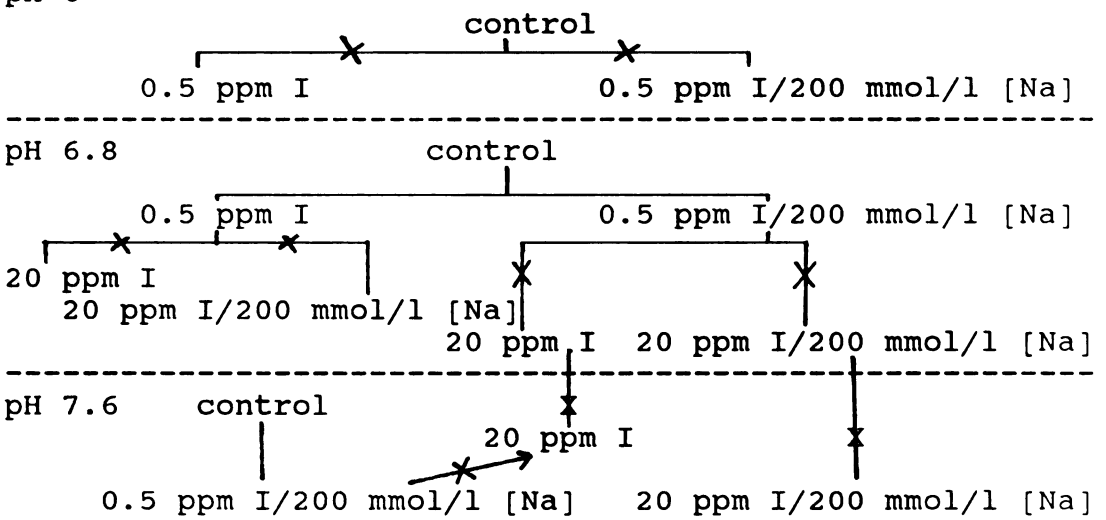
Bacterial cell dry weights were obtained by

Table 3. Flow Chart of Treatments of S. bovis 24 in vitro Batch Culture Study*



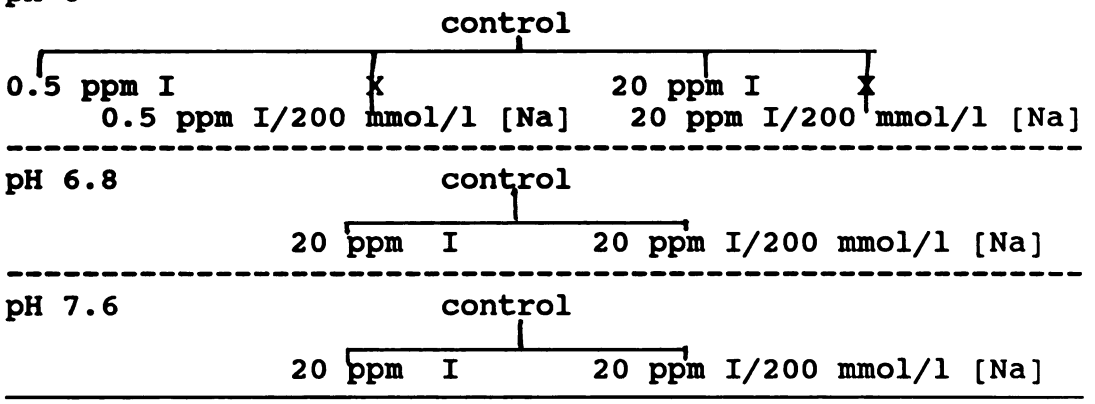
* Follow the sequences of the flow chart and the last passage of the six passages was transferred to the next culture condition. The symbol "X" means the culture failed to grow at the indicated stage and "I" represents ionophore monensin.

Table 4. Flow Chart of Treatments of L. vitulinus B62 in vitro Batch Culture Study*



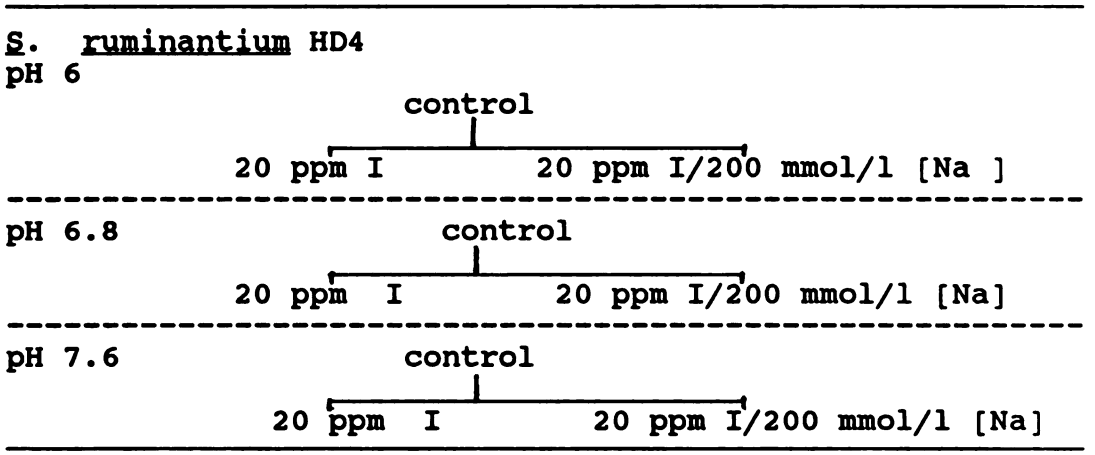
* Follow the sequences of the flow chart and the last passage of the six passages was transferred to the next culture condition. The symbol "x" means the culture failed to grow at the indicated stage and "I" represents ionophore monensin.

Table 5. Flow Chart of Treatments of *B. ruminicola* GA33 *in vitro* Batch Culture Study*



* Follow the sequences of the flow chart and the last passage of the six passages was transferred to the next culture condition. The symbol "x" means the culture failed to grow at the indicated stage and "I" represents ionophore monensin.

Table 6. Flow Chart of Treatments of *S. ruminantium* HD4 *in vitro* Batch Culture Study*



* Follow the sequences of the flow chart and the last passage of the six passages was transferred to the next culture condition and "I" represented ionophore monensin.

modifying the procedure of Isaacson et al. (1975). Culture samples of 15 ml were centrifuged at 12,000 x g for 10 min, the bacterial pellet was washed with deionized H_2O, transferred to a preweighed aluminum dish and dried in 85^0C oven overnight.

Determination of RNA and Protein in Pure Culture

The procedures were a modification of the procedures outlined by Ceruiotti (1955), and Tseng and Johnson (1986). The detailed complete procedures are shown in Appendix 5.

Glucose Assay

Broth culture glucose concentrations were measured by using the glucose (Trinder) kit (Sigma Diagnostics #315). The enzymatic reactions involved are as follows: Glucose is first oxidized to gluconic acid and hydrogen peroxide, this reaction is catalyzed by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye, which has an absorbance maximum at 505 nm.

A glucose standard curve was prepared using a commercial glucose standard solution (Sigma #16-300) of 100 mg/dl. The concentration of glucose in samples were read from the standard curve. Sigma diagnostic glucose [Trinder] reagent is linear to 750 mg/dl.

Cell Surface Morphology Assessment with SEM

One ml of broth of each run was used for cell surface morphological studies using SEM. The experimental procedures were according to Klomparens et al. (1986) and the experiment was done in the Electron Optics Center of the MSU Pesticide Research Center. The detailed procedures are outlined in Appendix 6.

Statistical Methods

The general linear model (GLM) of balanced and unbalanced one way analysis of variance was used (Joyner, 1985). The statistical differences were evaluated among treatments for main effects, interactions and overall means (Gill, 1978a, 1978b). F statistic test was used to compare the differences of the values of total volatile fatty acids, the concentration of lactate and succinate, RNA/protein ratio, glucose utilization rate and $Y_{glucose}$ for the adaptation studies. Only when $P<.05$ are the levels of statistical significance indicated.

Experiment 2

In vitro Continuous Culture Study

The purpose of this work was to test the hypothesis that gram positive bacteria when grown at continuous culture upon sequential addition of .5 ppm monensin, 20 ppm monensin and 20 ppm monensin/[Na] do not overcome ionophore induced growth depression.

Organisms

Five ml of two strains of ruminal bacteria, S. bovis 24 and B. ruminicola GA33, were inoculated from late exponential phase of pure batch cultures into the continuous culture/mini fermenter. The continuous cultures were incubated at 39^0C and allowed to grow overnight as a batch culture.

Cultivation

Continuous cultures of ruminal bacteria, were assigned by monensin dosage, [Na] amount and grown at 5% or 10%/h dilution rate. The continuous culture apparatus was assembled with the medium. The culture was inoculated and allowed to grow as a batch culture overnight, Hungate anaerobic culture methodologies were adapted for the continuous cultures. The fermenter vessel and media reservoir were under a O_2 free CO_2 atmosphere. Measurements of optical densities were made at intervals and a further sample taken for analysis when the culture had been stable for about 2 d at high dilution rates, or about 3 d at low dilution rates. The

sequence of the treatments for the continuous culture studies and the cell harvesting protocol at certain days as indicated are shown in Table 7.

Table 7. Sequence of S. bovis 24 & B. ruminicola GA33 Treatments and the Cell Harvest Days in a Continuous Culture

Treatments	5%/h dilution rate harvest days	10%/h dilution rate harvest days
Control	3, 6	2, 4
.5 ppm monensin	9, 12	6, 8
20 ppm monensin	15, 18	10, 12
20 ppm monensin/200 mM [Na]	21, 24	14, 16
Back to control	27, 30	18, 20

The medium composition was described in Table 2. A pH meter was used to measure pH value as soon as possible after removal of a sample from the fermenter.

Chemostat Apparatus Set Up and Operation

Chemostat continuous culture was as outlined by Slyter et al. (1964) and Hobson (1965a). The fundamental chemostat theory is described in Appendix 7.

The continuous fermentation experiments were set up to establish the effect of monensin concentration, medium sodium concentration and pH (as measured by PHM 64 Research pH meter) on acid production and growth of two ruminal anaerobic bacteria.

In this study, a 150 ml continuous culture minifermenter (Bellco culture flask, #1970-S0027, Bellco

Biotechnology, Bellco glass, Inc.) was utilized. Energy substrate (4.4 mM glucose) level was kept the same for all dilution rates studied. A high precision, constant deliving HPLC pump (Minipump, Milton Roy, Laboratory Data Control Corporation) was used to deliver the medium to the fermenter. This pump was calibrated to deliver 5 and 10% of the culture volume each hour. The complete culture system was composed of a source of O_2 free CO_2 medium reservoir, pump, fermenter, magnetic stirrer (Spin-Master, Model 4802, Cole-Parner instrument company IL 60648), effluent collection vessel, heating tape, temperature probe and temperature controller (Versa-Therm, Proportional Temperature Controller, Cole-Parmer instrument company IL 60648). A heating pad was placed under the medium reservoir, and the medium was continuously bubbled with CO_2.

Temperature control was achieved by regulating the current supplied to a Glas Col^R heating tape wrapped around the fermenter. An ace bandage was wrapped around the heating tape to insulate the system. A teflon coated magnetic stirring bar provided constant mixing within the fermenter. Gas flow was monitored by observing the bubbles coming out a line from the gas outlet to a tube of paraffin oil. Glucose limited basal medium was made in a two liter round bottom flask. The sterilized components were assembled and gas flow was started within one hour of autoclaving.

The tubing to the pump and pump were successively flushed with very large volumes of methanol, autoclaved sterile water and sterile medium solution to keep the tubing and pump free of contamination. For calibration, dilution rates were calculated from the total volume accumulated per unit time (i.e., dilution rates at 0.05 and 0.1 per hr). After the initial 12 hr batch growth at least one turnover of the fermenter (e.g. 150 ml) under continuous culture conditions was allowed before any samples were removed for analysis.

The chemostat experiments involved adding the following treatment: 0.5 ppm and 20 ppm monensin, as well as a preexisting or control concentration of [Na] (158.6 mmol/l, from media ingredients) and a maximum concentration of 200 mmol/l [Na] (Appendix 1) by adding NaCl to the defined media. These treatments were imposed in succession on the continuous cultures.

Cell growth profile during each treatment was assessed twice, i.e., the initial assessment was made once the bacterial culture in the chemostat reached steady state of growth. A 1.5 ml sample was removed and an O.D. reading as well as pH measurement was taken, the culture volume was replenished with medium equal to the amount removed.

Volume of culture needed to obtain O.D. and pH measurements was about 1.5 ml. This volume was about 1% of the total culture volume and would not be expected to

perturb the chemostat. When about 60 ml samples were removed for analysis, the culture was stayed overnight and returned to the initial growth phase.

The continuous cultures continued for long periods needing only occasional adjustments of the CO_2 flow as well as checking pumping rate; the apparatus was routinely left to run overnight without attention.

Determination of Fermentation Products

Fifteen ml of broth of each segment/culture condition were used for VFA, lactate and succinate assays. The fermentation products were analyzed on a GLC using procedures as described above in ***in vitro*** batch culture study (Sampugna et al., 1966; Finch, 1970; Lambert and Moss, 1972; Salanitro and Muirhead, 1975). The detailed analysis procedures are presented in Appendix 3.

Cell Yield Study

Procedures to determined cell yield or cellular dry weight were modified from Isaacson et al. (1975) and were conducted as outlined above.

Determination of RNA and Protein in Continuous Culture

The procedures were a modification from the methods of Ceruiotti (1955), and Tseng and Johnson (1986). The complete procedure is shown in Appendix 5.

Glucose Assays

The principle, methods and material for glucose assays were identical with the one which was used in the

in vitro batch culture studies. Glucose (Trinder) kit was used for glucose assay.

Morphological Study of Cell Surfaces by SEM

One ml of broth of each run was used for morphological studies of cell surfaces using SEM. The experimental procedures were those of Klomparens et al. (1986). The work was completed at Electron Optics Center of the MSU Pesticide Research Center. The detailed procedures are given in Appendix 6.

Experiment 3

^{14}C-Monensin Binding to Membrane Surfaces of Bacterial Cells

The purpose of this work was to determine whether ^{14}C labeled carboxylic polyether ionophore binds to cell membranes of gram positive bacteria and gram negative bacteria. The hypothesis was that ^{14}C-monensin did not bind to cell membranes of both gram positive and gram negative bacteria.

Organisms

Two representative strains of pure culture ruminal bacteria, S. bovis 24 and B. ruminicola GA33, were incubated with ^{14}C-monensin (^{14}C-Monensin was a kind gift from Lilly Research Laboratories, Eli Lilly & Co.). The specific activity of ^{14}C-monensin was .56 uCi/mg. ^{14}C-Monensin was purified by preparative HPLC and had a radiochemical purity of 95% or higher. ^{14}C-Monensin purity was evaluated by TLC and visualized by autoradiography at the Greenfield Laboratory of Lilly Research Laboratories.

Cultivation

Culturing of S. bovis 24 and B. ruminicola GA33 on basal medium (Table 2, 0.2% w/v and 0.08% w/v glucose) has been previously described (experiments 1 and 2).

Supplemental ^{14}C-monensin to a final concentrations 0.5 ppm or 20 ppm (dissolved in absolute ethanol) was

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added to experimental 15 ml cultures and no carrier monensin was used. These additions were made early in the growing phase.

Fixation of Anaerobic Cells for Autoradiography

One ml of ^{14}C-monensin labeled S. bovis 24 or B. ruminicola GA33 broths were fixed individually with 2% glutaraldehyde in phosphate buffer. One drop of fixed culture was placed on a 1% poly-L-lysine fixed carbon planchette. The complete procedures such as dehydration and critical point drying etc. are described in Appendix 6.

Autoradiographic Technique

The autoradiographic technique was based on Klomparens et al. (1986). The work was done at the Electron Optics Center of the MSU Pesticide Research Center. The detailed procedures and the preliminary observation are shown in Appendix 8.

RESULTS AND DISCUSSION

Experiment 1

In Vitro Batch Culture Adaptation Study

The fermentation broth was assayed for organic acids and glucose. Various parameters were measured in microbial pellets. The raw data for fermentation broths are listed in Appendix Tables 45 to 84. Due to problems in sample preparation and other unknown reasons (we are very suspicious of the reliability of the GC), data of fermentation products (organic acids) are difficult to interpret and are contradictory. In the following discussion, all physiological and metabolic parameters measured (except fermentation organic acids) such as cellular RNA/protein ratios, cell yield (CY), rate of glucose utilization (substrate utilization), total glucose percentage utilization and $Y_{glucose}$ (Y_G) will be reported and discussed in detail.

The two key areas of focus in this experiment were: first, to evaluate any potential adaptation of different ruminal microorganisms to ionophore monensin and second, to assess physiological and metabolic parameters of the microorganism during the six passages. These were achieved by first measuring maximum absorbance (OD_{max}), time (T) to reach maximum growth and final pH and then by determining cellular RNA/protein ratios, cell yield

(CY), rate of glucose utilization, total glucose percentage utilization and $Y_{glucose}$ (Y_G).

I. **S. bovis 24**

A. Time Course and Adaptation Study of Six Passages

The data of OD_{max}, time (T) to reach maximum absorbance and final pH from six passages or transfers of S. bovis 24 are presented in Appendix Tables 1 to 19.

Growth of S. bovis 24 after six transfers/passages at same treatment was not different. When transferring from the last (sixth) passage within a treatment to the first inoculation of the next treatment, there were media carry-over effects on bacterial growth which were noted. This is likely due to the transfer of 0.5 ml of inocula but it may be due to true adaptation/re-adaption phenomena. These carry-over residual effects were not noted after the second passage within a treatment and this general pattern was observed for all four organisms studied in Experiment 1 .

At pH 6, S. bovis 24 failed to grow in media containing monensin (0.5 ppm and 0.5 ppm monensin/200 mmol/l [Na]). Thus, no further experiments were done with pH 6.0 culture media (Appendix Tables 1 & 45).

Maximum absorbance (OD_{max}), time (T) to reach maximum absorbance and the final pH for each six passages of S. bovis 24 were determined when cells were grown in pH 6.8 control medium (Appendix Table 2), followed in order by: 0.5 ppm monensin medium (Appendix Table 3), 20

ppm monensin medium (Appendix Table 4), back to control medium (Appendix Table 5), 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 6), then back to control medium (Appendix Tables 7) as well as in 0.5 ppm monensin/200 mmol/l [Na] medium (Appendix Table 8).

Maximum absorbance (OD_{max}), time (T) to reach maximum absorbance and the final pH of six passages of S. bovis 24 were also determined with cells grown in pH 7.6 control medium (Appendix Table 15), followed in order by: 20 ppm monensin medium (Appendix Table 16), 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 18) and back to control medium (Appendix Tables 17 and 19).

Since no significant differences were noted within the six-passages treatment, the data within each treatment were combined and only means are presented to describe the effects of treatments on physiological and metabolic conditions of S. bovis 24 cells. These means for S. bovis 24 grown at pH 6.8 and various media/treatments are shown in Table 8. Each value in Table 8 represents a mean of six passages within a culture under indicated experimental conditions.

The basic statistical comparisons (GLM Method) in Table 8 are: treatment control (C) vs. 0.5 ppm monensin (A), 0.5 ppm monensin/[Na] (A-1), back to control (C-1), 20 ppm monensin (B), 20 ppm monensin/[Na] (B-1) and back to control (C-2); treatment 0.5 ppm monensin (A) vs. 0.5 ppm monensin/[Na] (A-1); treatment 0.5 ppm monensin (A)

vs. 20 ppm monensin (B); treatment 0.5 ppm monensin/[Na] (A-1) vs. 20 ppm monensin/[Na] (B-1); treatment 20 ppm monensin (B) vs. 20 ppm monensin/[Na] (B-1). Table 8 shows that OD_{max} values declined significantly ($p<0.01$) in A, A-1, B and B-1 vs. C; OD_{max} also declined significantly ($p<0.05$) in A vs. A-1 and B vs. A. Time (T) to reach maximum OD values were significantly increased ($p<0.01$) in A-1, B, B-1, C-1 and C-2 vs. C; in B vs. A and in B-1 vs. A-1; but less significantly increased ($p<0.05$) in A vs. C. Final pH stayed constant in all seven treatments.

Table 8 shows that at pH 6.8, OD_{max} values (referring to the growth measured by cell accumulation or the turbidity of culture at 600 nm) declined and the length of time (h) to reach the maximum absorbance increased when S. bovis 24 was transferred from C to A and from A to B. After cells cultured in medium containing 20 ppm monensin were transferred back to control medium, the time required to reach OD_{max} was shortened and the O.D. values were again increased. An overview of the data of S. bovis 24 grown in various media at pH 6.8 is shown in Figures 5 and 6. Each bar represents a mean of six passages when there was no difference among six passages data. The main conclusion is that S. bovis 24 is sensitive to monensin and this sensitivity is not reversed when cells are grown in a higher level (200 mmol/l [Na]) of sodium.

Table 8. Physiological and Metabolic Parameters for S. bovis 24 Grown from Control to 0.5 ppm Monensin, to 20 ppm Monensin and Back to Control; Grown from 0.5 ppm Monensin to 20 ppm Monensin/[Na] and Back to Control; and Grown in 0.5 ppm Monensin/[Na] at pH 6.8 Media*

Metabolic Parameters	Treatments** C	A	B	C-1	B-1	C-2	A-1	SEM
RNA/Protein[a]	.35[h]	.19[i]	.12[i]	.47[h]	.10[i]	.40[h]	.17[i]	.03
CY[b]	472.15[h]	93.81[i]	39.38[i]	87.31[i]	35.48[i]	80.05[i]	73.80[i]	29.49
Rate of glu. util.[c]	20.09[h]	8.42[i]	2.99[i]	5.07[i]	2.44[i]	5.22[i]	4.39[i]	1.44
Total glu. util.%	99.25	99.17	99.22	99.25	99.27	99.27	99.23	.04
Y_{glu}[d]	23.7[h]	11.4[i]	13.4[i]	15.2[i]	14.2[i]	15.3[i]	16.8[i]	1.1
OD_{max}[e]	.67[h,j]	.34[k,o]	.12[i]	.63[h]	.13[i]	.61[h]	.19[i,p]	.02
T[f]	3.83[h,l]	9.00[q]	23.17[m]	13.08[i]	28.00[mk]	12.67[i]	15.33[i,s]	1.40
Final pH	6.07	6.12	6.20	6.12	6.13	6.10	6.07	.02

* Each value represents a mean of 6 passages of cultures.

** Treatment C is control medium.
Treatment A is 0.5 ppm monensin medium.
Treatment B is 20 ppm monensin medium.
Treatment C-1 is back to control medium.
Treatment B-1 is 20 ppm monensin/[Na] medium.
Treatment C-2 is from treatment B-1 to control medium.
Treatment A-1 is 0.5 ppm monensin/[Na] medium.

a means within row with different superscripts differ (h,i, $p<0.01$)

b cell yield, ug/h/15 ml culture; means within row with different superscripts differ (h,i, $p<0.01$)

c umoles/h/15ml culture; means within row with different superscripts differ (h,i, $p<0.01$)

d g of cell/mole of glucose utilized (h,i, $p<0.01$)

e maximum absorbance; means within row with different superscripts differ (h,i, $p<0.01$; j,k, $p<0.01$; o,p, $p<0.05$)

f time to reach maximum absorbance; hr; means within row with different superscripts differ (h,i, $p<0.01$; l,m, $p<0.01$; q,s, $p<0.01$; j,k, $p<0.05$)

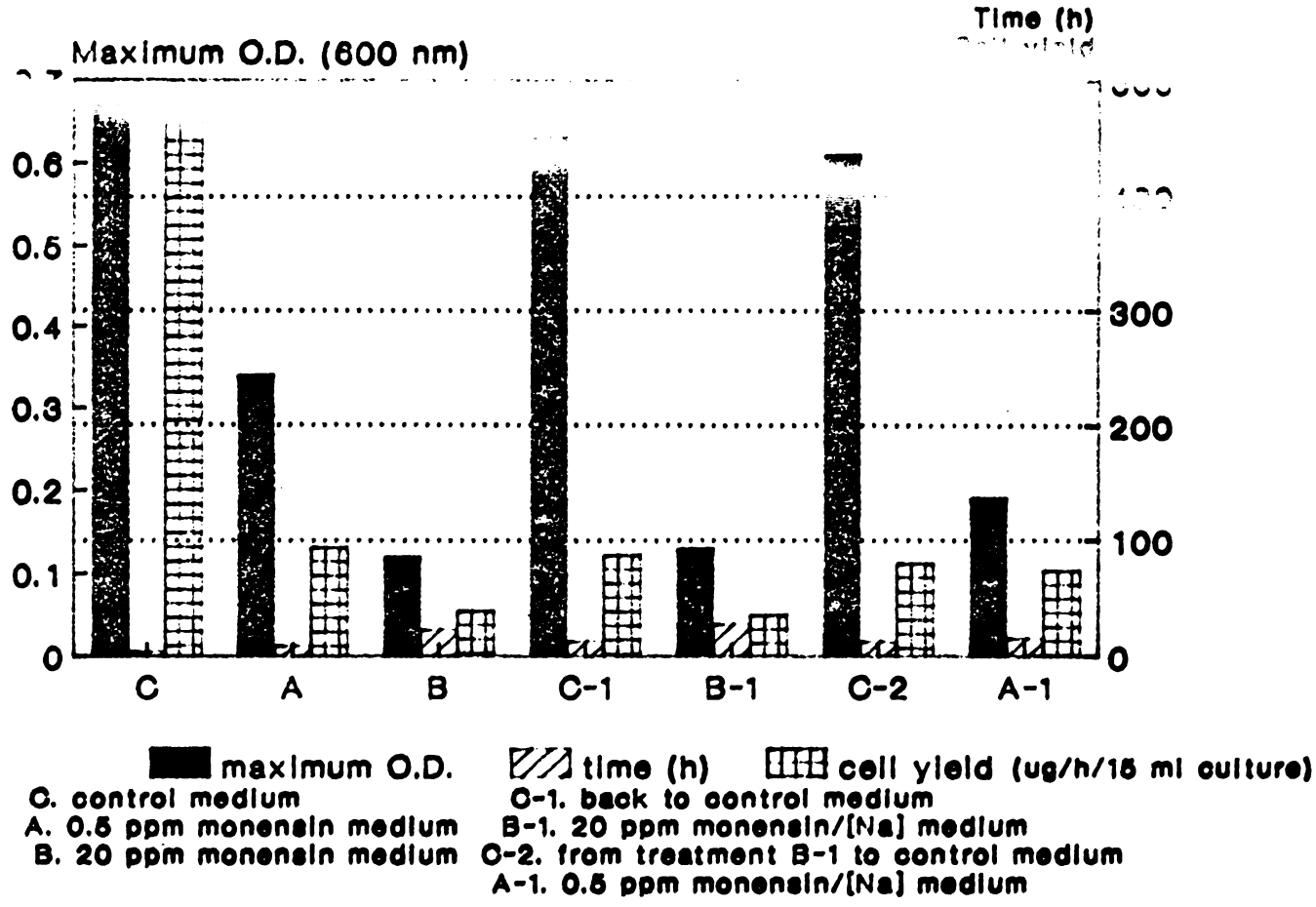


Figure 5. An Overview of S. bovis 24 Grown in Various pH 6.8 Media (Part A)

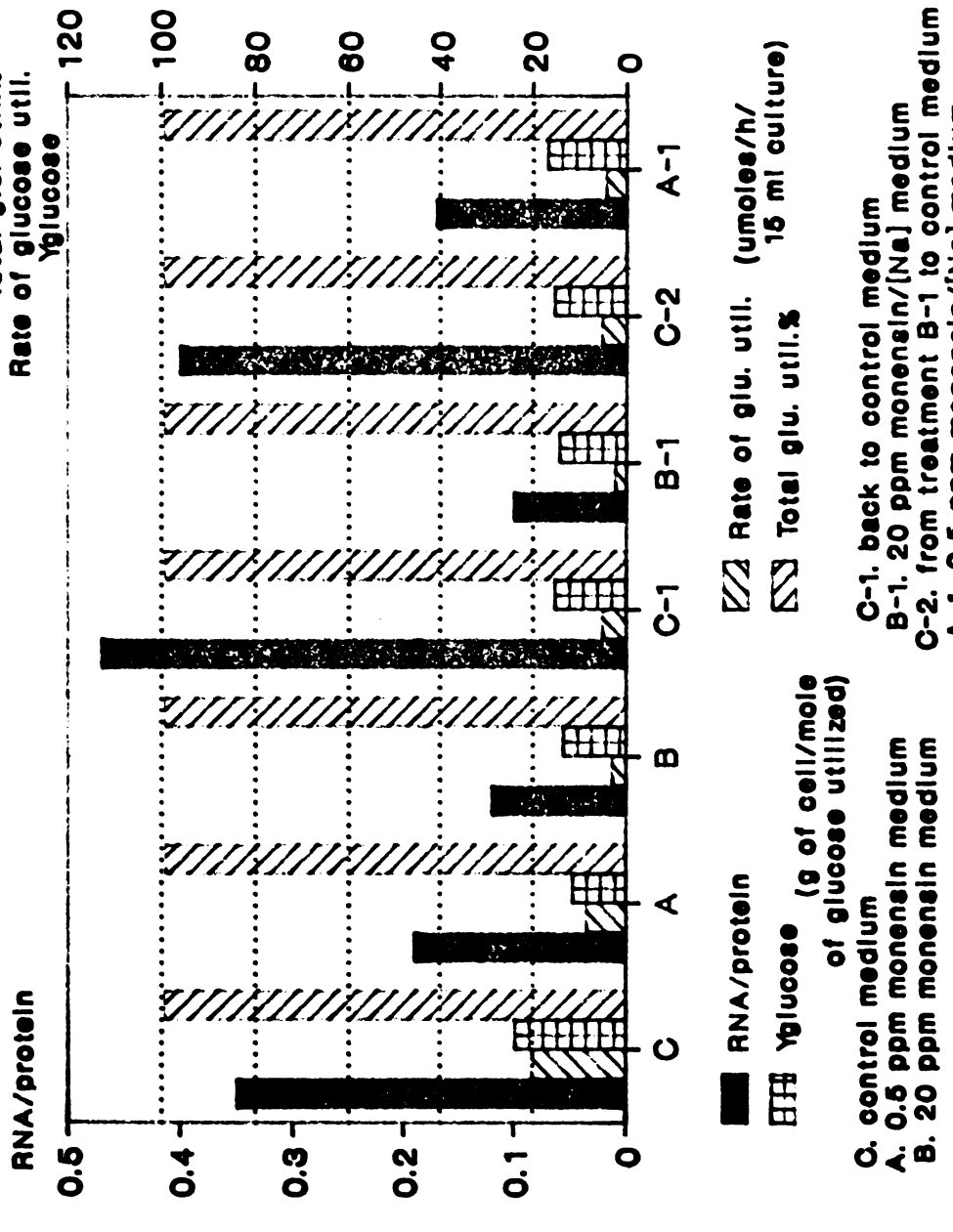


Figure 6. An Overview of S. bovis 24 Grown in Various pH 6.8 Media (Part B)

Basically, the purpose of the experimental design used in these studies for the following listed treatments was to assess whether a switch in pH will enhance cell growth or enable cells to improve their defense system against an ionophore insult. In general, any such finding of differences after repeated six-serial passages for the above experimental variables were not found.

The data for OD_{max}, length of time (T) to reach maximum absorbance and final pH as well as the physiological and metabolic parameters of six passages of S. bovis 24 grown at pH 6.8 from 0.5 ppm monensin/ 200 mmol/l [Na] medium to 20 ppm monensin medium are shown in Appendix Tables 9 & 53; from 0.5 ppm monensin/ 200 mmol/l [Na] medium to 20 ppm monensin/200 mmol/l [Na] medium are shown in Appendix Tables 10 & 54; from 20 ppm monensin pH 6.8 medium to 20 ppm monensin pH 7.6 medium are shown in Appendix Tables 11 & 55; from 20 ppm monensin pH 7.6 medium to control medium are shown in Appendix Tables 12 & 56; from 20 ppm monensin pH 6.8 medium to 20 ppm monensin/200 mmol/l [Na] pH 7.6 medium are shown in Appendix Tables 13 & 57; and from 20 ppm monensin/200 mmol/l [Na] pH 7.6 medium to control medium are shown in Appendix Tables 14 & 58.

The basic statistical comparisons (GLM method) in Table 9 were control medium (treatment C) vs. the other four treatments and 20 ppm monensin medium (treatment B) vs. 20 ppm monensin/[Na] medium (treatment B-1). At pH

7.6 (Table 9), values of OD_{max} significantly declined ($p<0.01$) in 20 ppm monensin and 20 ppm monensin/[Na] medium more than in control medium. Time (T) to reach

Table 9. Physiological and Metabolic Parameters for S. bovis 24 Grown from Control Medium to 20 ppm Monensin Medium and Back to Control Medium; Grown from Control Medium to 20 ppm Monensin/[Na] Medium and Back to Control Medium at pH 7.6*

Metabolic Parameters	Treatments** C	B	C-1	B-1	C-2	SEM
RNA/Protein	.27^{h,j}	.16^{i}	.34^{k}	.18^{i}	.40^{j}	.04
CY[a]	606.15^{h}	76.95^{i}	427.67^{k}	73.32^{i}	225.54^{j}	50.56
Rate of glu. util.[b]	28.91^{h}	4.20^{i}	21.14^{h}	3.38^{i}	10.11^{j}	1.84
Total glu. util.%	99.23	99.19	99.28	99.08	99.23	.26
Y_{glu}[c]	21.5	18.1	19.9	21.4	22.5	2.3
OD_{max}[d]	.46^{h}	.13^{i}	.55^{h}	.13^{i}	.54^{h}	.03
T[e]	2.50^{h}	16.00^{i}	3.30^{h}	19.67^{i}	6.75^{h}	.59
Final pH	6.52	6.55	6.62	6.54	6.60	.02

* Each value represents a mean of 6 passages of cultures.

** Treatment C is control medium.
Treatment B is 20 ppm monensin medium.
Treatment C-1 is back to control medium.
Treatment B-1 is 20 ppm monensin/[Na] medium.
Treatment C-2 is from treatment B-1 to control medium.

a cell yield; ug/h/15 ml culture; means within row with different superscripts differ (h,i, $p<0.01$)

b umoles/h/15ml culture; means within row with different superscripts differ (h,i, $p<0.01$)

c g of cell/mole of glucose utilized

d maximum OD; means within row with different superscripts differ (h,i, $p<0.01$)

e hr; means within row with different superscripts differ (h,i, $p<0.01$)

maximum absorbance values were significantly longer ($p < 0.01$) in 20 ppm monensin medium (16 h) and 20 ppm

monensin/[Na] medium (19.76 h) than in control medium (2.50 h) and back to control medium (3.30 h and 6.75 h). Final pH values were not different among the five treatments.

B. Physiological and Metabolic Parameters of Six Passages

Physiological and metabolic parameters within six serial passages of S. bovis 24 grown at pH 6.8 medium are presented in Appendix Tables 46 to 52.

RNA/protein ratios (Table 8) declined significantly ($p<0.01$) in 0.5 ppm monensin medium (A, 0.19), 0.5 ppm monensin/[Na] medium (A-1, 0.17), 20 ppm monensin (B, 0.12) and 20 ppm monensin/[Na] (B-1, 0.10) vs. control (C, 0.35). Cell yields (CY) were significantly higher ($p<0.01$) in control medium than in A, A-1, B, B-1 or C-2. No difference was observed in the remaining treatment comparisons.

Rate of glucose utilization values were significantly higher ($p<0.01$) in control medium than in A, A-1, B, B-1, C-1 or C-2. No difference was present in other treatment comparisons. Total glucose percentage utilization values were not different among all five treatments. Values for Y_{glu} were significantly increased ($p<0.01$) in C vs. A, A-1, B, B-1, C-1 or C-2.

Total substrate disappearance during fermentation in both control and monensin treated conditions was virtually complete (99.0%) for S. bovis 24. The rate of

glucose disappearance and rate of cellular biomass accumulation was severely inhibited by monensin. Microbial cell yield declined upon monensin insult and then tended to increase again once cells were transferred from 20 ppm monensin or 20 ppm monensin/200 mmol/l [Na] medium back to the control medium. The higher cell yields produced by S. bovis 24 under control conditions or when cell culture was inoculated back to control media from the monensin treated condition indicated that most of the available energy was used by S. bovis 24 for cellular growth purposes, while during the ionophore treatments cell dissipated much energy for cell maintenance or defense and grew very slowly. These findings tend to support the hypothesis of Bergen and Bates (1984) who wrote that any microorganism that can generate sufficient ATP despite the ionophore insult will survive better than those that cannot. Further, organisms that survive poorly in monensin media will expend tremendous amounts of ATP to maintain the H+ and cell maintenance (Bergen & Bates, 1984).

The value of RNA/protein is an indicator of physiological state/growth state of microbes (Bates et al., 1985). Table 8 also shows that values of RNA/protein were significantly higher ($p<0.01$) in control medium (0.35) than in A (0.19), A-1 (0.17), B (0.12) or B-1 (0.10). Values for Y_{glu} (g of cell/mole of glucose utilized) were significantly higher ($p<0.01$) when cells

were grown in pH 6.8 control medium (23.7) than A (11.4), A-1 (16.8), B (13.4) or B-1 (14.2) treated media.

The RNA/protein ratio significantly increased ($p<0.01$) once cell cultures grown from 20 ppm monensin medium were transferred back to control medium (0.47) or from 20 ppm monensin/200 mM [Na] back to control medium (0.40). The values of $Y_{glucose}$ were not different (from 13.4 to 15.2 and from 14.2 to 15.3).

The RNA/protein ratio in bacterial cells is an indirect marker for ruminal microbial growth. RNA/protein ratios are quite variable depending on u (Maaloe and Kjeldgaard, 1966). Cells growing at high u have high RNA to protein ratios while cells growing at low u have low RNA to protein ratios. Bates et al. (1985) studied RNA/protein ratios in ruminal bacteria and found that at stationary phase, the RNA/protein ratio was 0.27. A linear regression calculated from the combined data of reported papers predicted that at u = 0, the RNA/protein ratio was 0.3. In the present dissertation work, RNA/protein ratios were occasionally numerically lower than previous reports. The reason for such observations may be due to lower RNA recoveries in microbial pellets and possibly higher pellet protein values. RNA/protein values as low as 0.2 have also been observed by Bates (1985), and Isichei (1980). This calculated RNA/protein value at u = 0 should not be viewed as an invariant biological constant; what is

important here is that low RNA/protein ratios in bacteria indicate low u.

On the basis of RNA/protein ratios, ruminal bacteria tend to grow *in vivo* at a growth rate which is not near u_{max}, which can be obtained in laboratory cultures (Bergen et al., 1982; Bates, 1985). The slow growth of bacteria in the rumen is not due to some metabolic limitation in these organisms, but rather to a response to environmental (like rumen turnover, attachment to particles), dietary and other factors (Hespell, 1979). A slow rate of growth does not necessarily limit the degradative capacity and the extent of digestion of substrate by the microorganism (Hungate, 1966).

Physiological and metabolic parameters of six passages per treatment of *S. bovis* 24 grown at pH 7.6 media are shown in Appendix Tables 59 to 63. Physiological and metabolic parameters means for *S. bovis* 24 grown in control medium (C), transferred to 20 ppm monensin (B) and transferred back to the control medium (C-1); from control medium transferred to 20 ppm monensin/200 mmol/l [Na] (B-1) and back to control medium (C-2) at pH 7.6 are shown in Table 9. Table 9 shows that rate of glucose utilization values were significantly higher ($p<0.01$) in C than in B or B-1. No differences ($p>0.05$) were observed between B and B-1. Cell yield (CY) values were significantly higher ($p<0.01$) in C than

in the remaining four treatments. There were no differences ($p>0.05$) in total glucose percentage utilization values and Y_{glu} of all five treatments. These results show that S. bovis 24 growth dynamics, when grown at pH 7.6 under these experimental conditions were not different from S. bovis 24 grown in various media at pH 6.8 (Table 8). It had been speculated that when grown at pH > 7.0, S. bovis 24 would survive better. This is based on the observation that adding buffer to feedlot diets often reverses any ionophore effect (Weber, 1979; Bergen & Bates, 1984). I obtained no evidence for such a contention in the present work.

RNA/protein ratios were numerically higher ($p> 0.05$) when bacteria were grown in pH 7.6 control medium (C, 0.27) than in 20 ppm monensin (B, 0.16) and in 20 ppm monensin/200 mmol/l [Na] medium (B-1, 0.18). The RNA/protein ratio increased when cells cultured in B were transferred back to C-1 (0.34), and from B-1 back to C-2 (0.40). The values of $Y_{glucose}$ were numerically higher ($p>0.05$) when S. bovis 24 were grown in C (21.5) than when grown in B (18.1) but not for B-1 (21.4). $Y_{glucose}$ increased again when cell cultures grown in B were transferred back to C-1 (19.9) and from B-1 to C-2 (22.5). None of the differences were statistically significant.

An overview of the data of S. bovis 24 grown in various media at pH 7.6 is shown in Fig. 7. Each bar

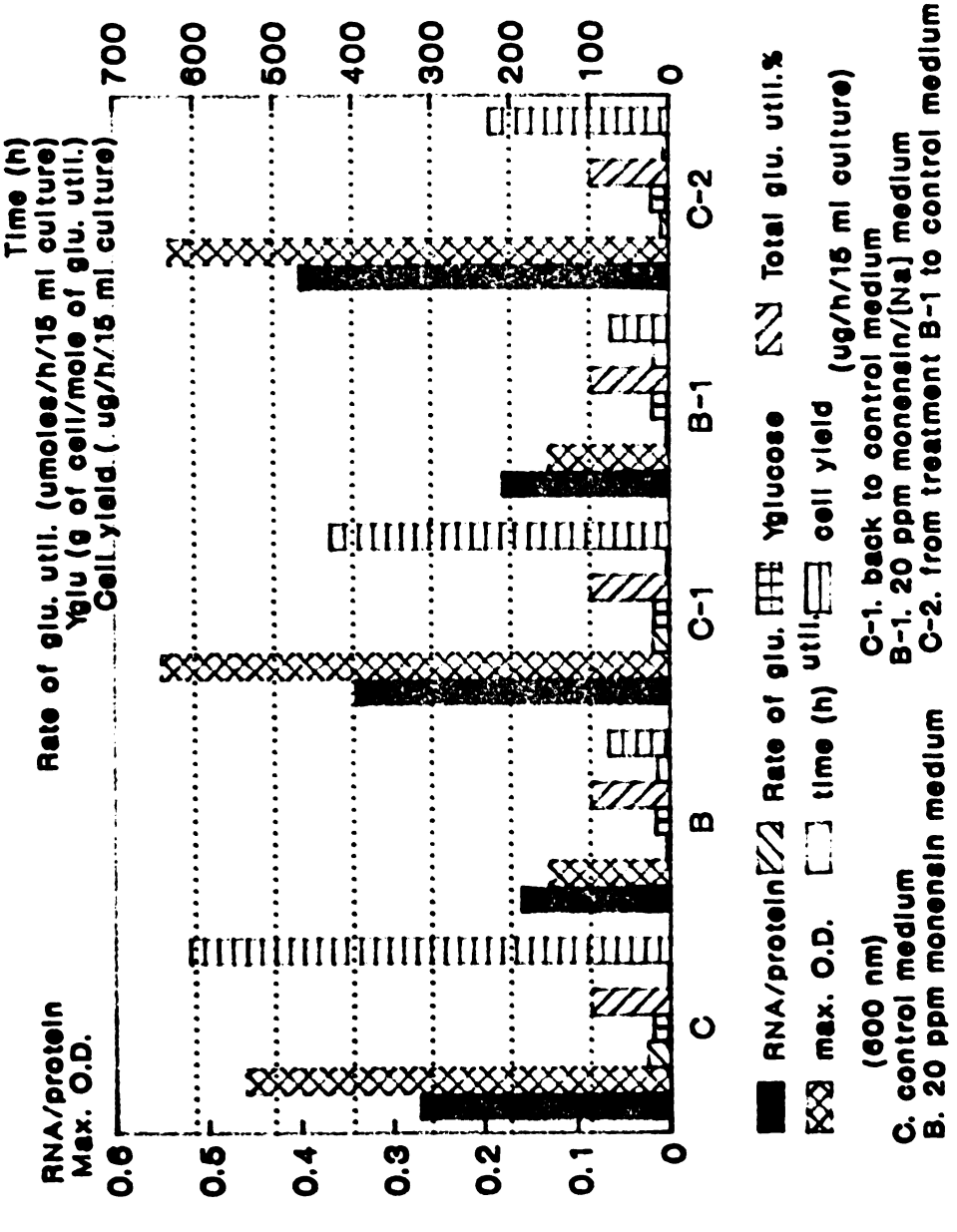


Figure 7. An Overview of S. bovis 24 Grown in Various pH 7.6 Media

represents a mean of six passages when there was no difference among six passages data.

II. L. vitulinus B62

Results for this organisms, as well as the two following organisms, will be reported similar to the format used for S. bovis 24.

A. Time course and Adaptation Study of Six Passages

The data on time (T) to reach maximum absorbance, maximum absorbance (OD_{max}) and final pH of six serial passages of L. vitulinus B62 are presented in Appendix Tables 20 to 24. At pH 6, cell cultures did not grow in any media containing monensin. Thus, no further experiments were done with pH 6.0 culture media (Appendix Table 20).

At pH 6.8 and 7.6, cell cultures of L. vitulinus B62 showed the same trend as those observed for S. bovis 24. In other words, OD_{max} was decreased and T was increased once cells were transferred from control (Appendix Tables 21 and 23) to 0.5 ppm monensin medium (Appendix Table 22 and 24). L. vitulinus B62 cultures grown in 0.5 ppm monensin failed to grow when transferred to 20 ppm monensin medium.

Statistical comparisons, as shown in Table 10, were made for pH 6.8 control medium (treatment C) vs. the remaining four pH 6.8 treatments and for 0.5 ppm monensin pH 6.8 medium (treatment A) vs. 0.5 ppm monensin/[Na] pH 7.6 (treatment A-1) medium. The values of OD_{max} were

significantly higher ($p<0.01$) in A than A-1. The OD_{max} value of C was numerically lower ($p > 0.05$) than pH 6 control medium (treatment 1-C) and higher than for all other treatments.

Time (T) to reach maximum absorbance was shorter ($p<0.01$) in pH 6.8 control medium (8.67 hr) than any of the other treatments.

B. Physiological and Metabolic Parameters of Six Passages

Physiological and metabolic parameters from six serial passages of L. vitulinus B62 grown at pH 6, pH 6.8 or pH 7.6 media are presented in Appendix Tables 64 to 68. The method for analyzing means from these five treatments was identical with the approach used above for S bovis 24.

Table 10 shows that L. vitulinus B62 grown at pH 6 (1-C), pH 6.8 (C) or pH 7.6 control (C-1) media had higher ($p>0.05$) RNA/protein ratios (0.15, 0.11 and 0.08 respectively) than when grown at 0.5 ppm monensin pH 6.8 medium (A, 0.07) or 0.5 ppm monensin/200 mmol/l [Na] pH 7.6 medium (A-1, 0.05). L. vitulinus B62 cell yields were significantly higher ($p<0.01$) in 1-C, C or C-1 than in A or A-1. Rates of glucose utilization were significantly higher ($p<0.01$) in 1-C, C or C-1 than in A or A-1. Total glucose percentage utilization values were significantly higher ($p<0.01$) in organisms grown in A than when grown in A-1. L. vitulinus B62 $Y_{glucose}$

Table 10. Physiological and Metabolic Parameters for L. vitulinus B62 Grown in pH 6 Control Medium; Grown from pH 6.8 Control to 0.5 ppm Monensin; Grown from pH 7.6 Control Medium to 0.5 ppm Monensin/[Na] Medium.*

Metabolic Parameters	Treatments** 1-C	C	A	C-1	A-1	SEM
RNA/Protein	.15	.11	.07	.08	.05	.02
CY[a]	122.23[h]	137.50[h]	46.58[i]	126.85[h,l]	24.60[i,m]	10.29
Rate of glu. util.[b]	6.76[h]	8.07[h]	2.82[i]	7.86[h,l]	2.56[i,m]	.63
Total glu. util.%[c]	99.17[h]	99.26[h]	99.18[h]	99.24[l]	89.07[i,m]	0.98
Y_{glu}[d]	17.9	17.9	17.0	16.7	11.1	1.6
OD_{max}[e]	.47[i]	.23[h,j]	.19[l]	.12[k]	.05[m]	.03
T[f]	10.00[h]	8.67[h]	24.00[i]	9.17[h,l]	27.25[m]	2.42
Final pH[g]	4.87[i]	6.06[h]	6.15[h]	6.53[h]	6.21[h]	.03

* Each value represents a mean of 6 passages of cultures.

** Treatment 1-C is pH 6 control medium.
Treatment C is pH 6.8 control medium.
Treatment A is pH 6.8 .5 ppm monensin medium.
Treatment C-1 is pH 7.6 control medium.
Treatment A-1 is pH 7.6 0.5 ppm monensin/[Na] medium.

a cell yield; ug/h/15 ml culture; means within row with different superscripts differ (h,i, p<0.01; l,m, p<0.01)

b umoles/h/15ml culture; means within row with different superscripts differ (h,i, p<0.01; l,m, p<0.01)

c means within row with different superscripts differ (h,i, p<0.01; l,m, p<0.01)

d g of cell/mole of glucose utilized

e means within row with different superscripts differ (h,i, p<0.01; l,m, p<0.01; j,k, p<0.05)

f time to reach OD_{max}; hr; means within row with different superscripts differ (h,i, p<0.01; l,m, p<0.01)

g means within row with different superscripts differ (h,i, p<0.01)

values in 1-C, C or C-1 (17.9, 17.9 or 16.7) tended to be numerically higher ($p>0.05$) than Y_{glu} for cells grown in A and A-1 (17.0 and 11.1).

An overview of the data of L. vitulinus B62 when grown in 1-C, C, A, C-1 or A-1 media is shown in Fig. 8. Each bar represents a mean of six passages when there was no difference among six passages data.

Addition of [Na] up to 200 mmol/l did not improve L. vitulinus B62 cell growth in any medium. If the hypothesis is correct that high extracellular sodium cation concentration can reestablish cellular proton extrusion via the cation/H^+ antiporter action of monensin in gram positive bacteria (Bergen and Bates, 1984), then raising [Na] should partially (or totally) reverse growth depression. This was not observed here most likely due to the similarity of the levels of initial [Na] and final [Na].

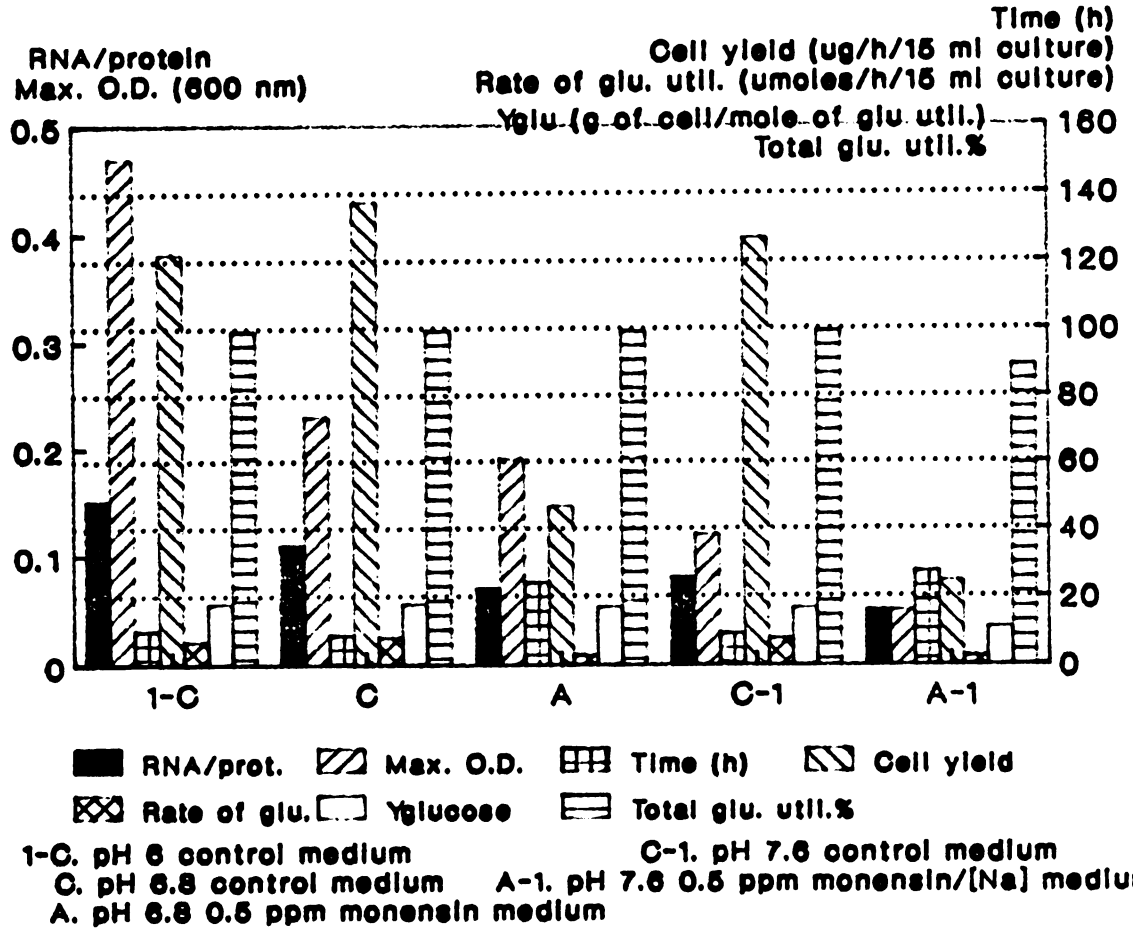


Figure 8. An Overview of L. vitulinus B62 Grown in Various pH 6, pH 6.8 and pH 7.6 Media

III. S. ruminantium HD4

A. Time Course and Adaptation Study of Six Passages

Statistical comparisons for S. ruminantium HD4 grown in pH 6, 6.8 or 7.6 media, respectively, were done and the results are separately given in Tables 11, 12 and 13.

The data on maximum absorbance (OD_{max}), time (T) to reach maximum absorbance, and final pH of six passages of S. ruminantium HD4 grown at pH 6 medium are presented in Appendix Tables 25 to 27.

Few significant adaptation phenomena were observed under repeated six-transfer passages at pH 6 control medium (Appendix Table 25), 20 ppm monensin medium (Appendix Table 26) and 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 27). Mean values for each treatment (pH 6.0) are shown in Table 11. Statistical comparisons in Table 11 were made for pH 6 control medium (treatment C) vs. 20 ppm monensin or 20 ppm monensin/[Na] medium (treatments B and B-1). Values of OD_{max} were significantly higher ($p<0.01$) for cells grown in C than for cells grown in B and B-1 ; T was numerically less ($p>0.05$) for cells grown in C than when grown in B or B-1. Final pH values stayed constant in C, B or B-1.

The data for OD_{max}, length of time (T) to reach maximum absorbance, and final pH of six passages of S. ruminantium HD4 grown in pH 6.8 medium are shown in Appendix Tables 28 to 30.

Table 11. Physiological and Metabolic Parameters for S. ruminantium HD4 Grown from Control to 20 ppm Monensin and 20 ppm Monensin/[Na] media at pH 6*

Metabolic Parameters	Treatments** C	B	B-1	SEM
RNA/Prot.[a]	.35[j]	.25[k]	.32[j]	.04
CY[b]	157.08	137.75	128.61	20.82
Rate of glu. util.[c]	6.78	6.91	6.38	.75
Total glu. util.%	99.28	99.05	99.03	.20
Y_{glu}[d]	23.2	19.9	20.0	1.5
OD_{max}[e]	1.06[h]	.79[i]	.74[i]	.07
T[f]	9.83	10.83	10.83	.09
Final pH	5.12	5.50	5.37	.06

* Each value represents a mean of 6 passages of cultures.
** Treatment C is control medium.
Treatment B is 20 ppm monensin medium.
Treatment B-1 is 20 ppm monensin/[Na] medium.
a means within row with different superscripts differ (j,k, $p<0.05$)
b cell yield; ug/h/15 ml culture
c umoles/h/15ml culture
d g of cell/mole of glucose utilized
e maximum absorbance; means within row with different superscripts differ (h,i, $p<0.01$)
f time to reach OD_{max}; hr

There were few significant differences within the six serial passages for any treatments in pH 6.8 medium (Appendix Tables 28, 29 and 30). As far as OD_{max} and final pH are concerned, the ionophore monensin did not inhibit S. ruminantium HD4 cell growth; however, when C, B and B-1 are compared, the mean value of length of time (T) to reach OD_{max} was significantly longer ($p<0.01$) for the two monensin treatments (Table 12).

The statistical comparisons in Table 12 are for

cells grown in pH 6.8 control medium vs. cells grown in 20 ppm monensin and 20 ppm monensin/[Na] medium. Values of OD_{max} were numerically higher ($p>0.05$) in control medium (treatment C) than in B or B-1. Time (T) to reach OD_{max} values were significantly longer ($p<0.01$) in B and B-1 than in C. The final pH values stayed constant in

Table 12. Physiological and Metabolic Parameters for S. ruminantium HD4 Grown from Control to 20 ppm Monensin and 20 ppm Monensin/[Na] media at pH 6.8*

Metabolic Parameters	Treatments** C	B	B-1	SEM
RNA/Prot.	.35	.31	.30	.04
CY[a]	256.38[h]	112.37[i]	107.30[i]	20.82
Rate of glu. util.[b]	13.17[h]	7.81[i]	7.56[i]	.75
Total glu. util.%[c]	99.20[j]	99.89[j]	98.52[k]	.20
Y_{glu}[d]	20.6[h]	14.3[i]	14.3[i]	1.5
OD_{max}[e]	.91	.87	.82	.07
T[f]	5.08[h]	8.50[i]	10.42[i]	.09
Final pH	6.05	6.19	6.16	.06

* Each value represents a mean of 6 passages of cultures.
** Treatment C is control medium.
Treatment B is 20 ppm monensin medium.
Treatment B-1 is 20 ppm monensin/[Na] medium.
a cell yield; ug/h/15 ml culture; means within row with different superscripts differ (h,i, $p<0.01$)
b umoles/h/15ml culture; means within row with different superscripts differ (h,i, $p<0.01$)
c means within row with different superscripts differ (j,k, $p<0.05$)
d g of cell/mole of glucose utilized; means within row with different superscripts differ (h,i, $p<0.01$)
e maximum absorbance
f time to reach OD_{max}; hr; means within row with different superscripts differ (h,i, $p<0.01$)

treatments C, B or B-1.

There were also few differences among the six serial passages in pH 7.6 control medium (Appendix Table 31), 20 ppm monensin (Appendix Table 32) or 20 ppm monensin/200 mmol/l [Na] media (Appendix Table 33) of S. ruminantium HD4. The means of these six passages are presented in Table 13. The basic statistical comparison

Table 13. Physiological and Metabolic Parameters for S. ruminantium HD4 Grown from Control to 20 ppm Monensin and 20 ppm Monensin/[Na] media at pH 7.6*

Metabolic Parameters	Treatments** C	B	B-1	SEM
RNA/Prot.	.28^{h}	.25^{i}	.23^{i}	.04
CY[a]	268.99^{h}	131.93^{i}	132.69^{i}	20.82
Rate of glu. util.[b]	13.90^{h}	6.53^{i}	6.64^{i}	.75
Total glu. util.%	99.25	99.14	99.22	.20
Y_{glu}[c]	19.1	20.1	21.4	1.5
OD_{max}[d]	.61^{j}	.81^{k}	.82^{k}	.07
T[e]	5.00^{h}	10.67^{i}	10.58^{i}	.89
Final pH	6.51	6.53	6.51	.06

* Each value represents a mean of 6 passages of cultures.
** Treatment C is control medium.
Treatment B is 20 ppm monensin medium.
Treatment B-1 is 20 ppm monensin/[Na] medium.
a cell yield; ug/h/15 ml culture; means within row with different superscripts differ (h,i, $p<0.01$)
b umoles/h/15ml culture; means within row with different superscripts differ (h,i, $p<0.01$)
c g of cell/mole of glucose utilized
d maximum absorbance; means within row with different superscripts differ (j,k, $p<0.05$)
e time to reach OD_{max}; hr; means within row with different superscripts differ (h,i, $p<0.01$)

in Table 13 was made for pH 7.6 control medium (C) vs. 20 ppm monensin (B) and 20 ppm monensin/[Na] (B-1) media. Values of OD_{max} did not differ among treatments; the OD_{max} value in control medium was less ($p<0.05$) than for the other two treatments. The time (T) to reach OD_{max} value in C was about one-half that noted for the other two treatments. Final pH stayed constant among treatments. It seems that at higher pH, monensin induced only a minor inhibition to cell growth when compared to inhibitions noted at pH 6.8 or 6. While the length of time (T) to reach OD_{max} was increased from 5 h to 10 h (C vs. B or B-1), the actual OD_{max} was increased from 0.61 to 0.81 (C vs. B or B-1). Final pH did not differ among treatments ($P>0.05$).

B. Physiological and Metabolic Parameters of Six Passages

The physiological and metabolic parameters within serial passages of S. ruminantium HD4 grown at pH 6, 6.8 or 7.6 are presented in Appendix Tables 69 to 77.

At pH 6, there were few statistical differences in RNA/protein ratios, cell yield (CY) values, rate of glucose utilization, total glucose percentage utilization and Y_{glu}. Cell yield (CY) values, total glucose percentage utilization values and Y_{glu} were numerical higher ($p>0.05$) in control medium (Appendix Table 69) than 20 ppm monensin (Appendix Table 70) or

20 ppm monensin/[Na] (Appendix Table 71) media.

Table 11 shows that RNA/protein values were significantly higher ($p<0.05$) when cells were grown in pH 6 control medium (0.35) than when grown in 20 ppm monensin (0.25) or 20 ppm monensin/200 mmol/l [Na] (0.32). Y_{glu} was numerically higher ($p>0.05$) in cells grown in control medium (23.2) than 20 ppm monensin (19.9) and 20 ppm monensin/200 mmol/l [Na] medium (20.0).

An overview of the data of S. ruminantium HD4 when grown in various pH 6 media is shown in Fig. 9. Each bar is a mean of six passages when there was no difference among six passages data.

There were no significant differences ($p>0.05$) among the metabolic parameters within six serial passages of S. ruminantium HD4 at pH 6.8 control (Appendix Table 72), 20 ppm monensin (Appendix Table 73) or 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 74).

Table 12 shows that the RNA/protein value is numerically higher ($p>0.05$) for cells grown in pH 6.8 control medium (C, 0.35) than for cells grown in 20 ppm monensin (B, 0.31) and 20 ppm monensin/[Na] (B-1, 0.30).

Table 12 also shows that cells utilized from 98.52% to 99.89% of glucose. $Y_{glucose}$ values were also significantly higher ($p < 0.01$) when cells were grown in control medium (20.6 g of cell/mole of glucose utilized) when compared to 20 ppm monensin medium (14.3 g of cell/mole of glucose utilized). Cell yield (CY) values were

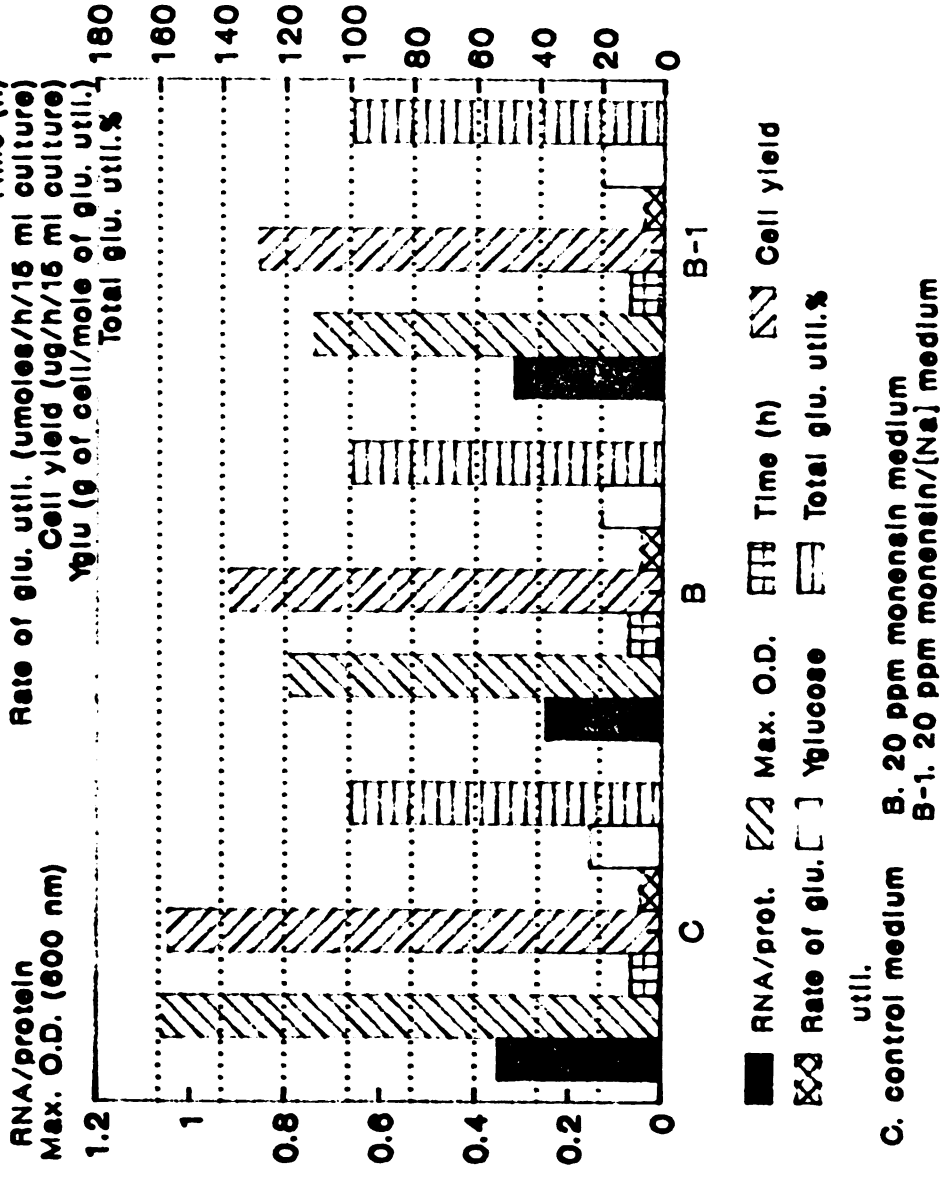


Figure 9. An Overview of S. ruminantium HD4 Grown in Various pH 6 Media

significantly higher ($p<0.01$) for control medium rather than the other two treatments. Rate of glucose utilization values were also significantly higher ($p< 0.01$) in control medium than in the other two treatments.

An overview of the data of S. ruminantium HD4 when grown in various pH 6.8 media is shown in Fig. 10. Each bar is a mean of six passages when there was no difference among six passages data.

The means for physiological and metabolic parameters for S. ruminantium HD4 in pH 7.6 medium (Appendix Tables 75, 76 and 77) are shown in Table 13. RNA/protein values did not differ when S. ruminantium HD4 were grown in C (0.28) compared to B (0.25) or B-1 (0.23). Cell yields (CY) were significantly higher ($p< 0.01$) in C than B or B-1. Rate of glucose utilization values were significantly higher ($p<0.01$) in C than B or B-1. $Y_{glucose}$ did not differ among treatments; the actual values of $Y_{glucose}$ in C, B and B-1 were 19.1, 20.1 and 21.4. These data probably only reflect experimental error.

An overview of the data of S. ruminantium HD4 when grown in various pH 7.6 media is shown in Fig. 11. Each bar is a mean of six passages when there was no difference among six passages data.

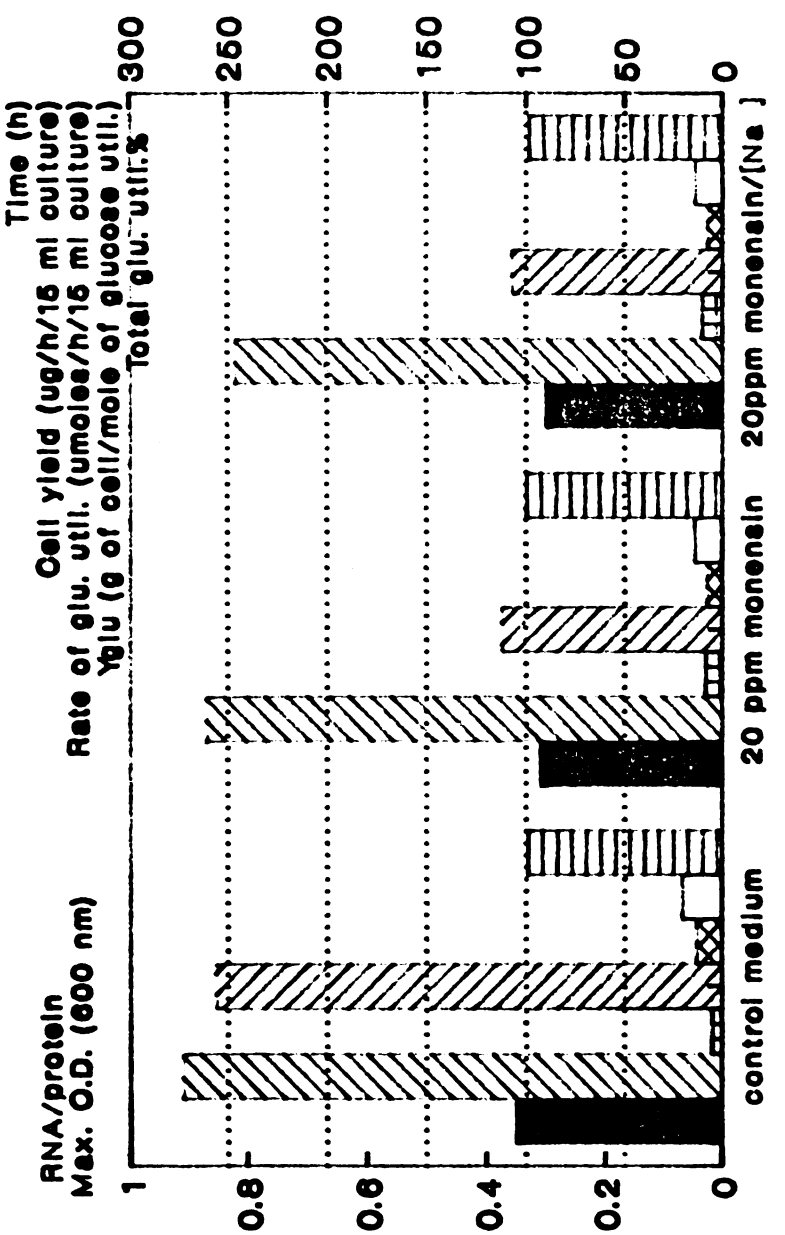


Figure 10. An Overview of S. ruminantium HD4 Grown in Various pH 6.8 Media

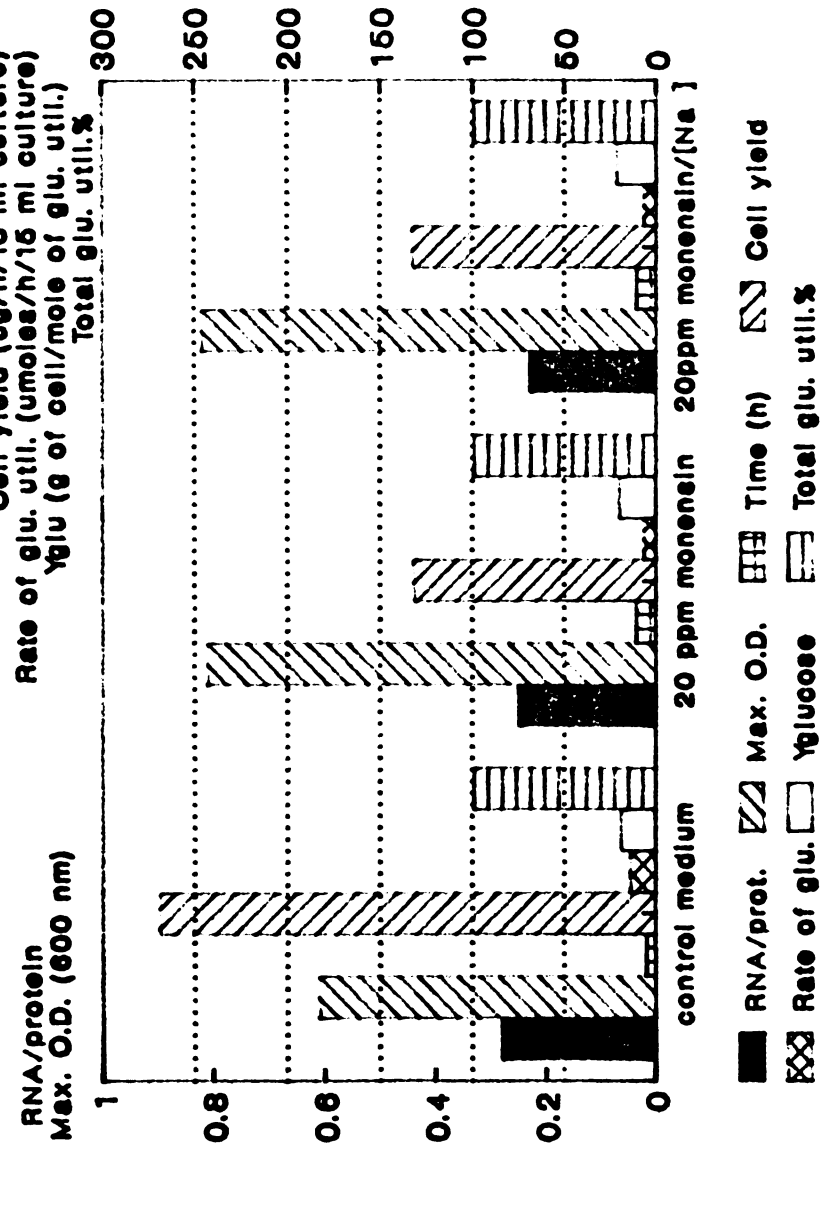


Figure 11. An Overview of S. ruminantium HD4 Grown in Various pH 7.6 Media

IV. B. ruminicola GA33

A. Time Course and Adaptation Study of Six Passages

When B. ruminicola GA33 was transferred from pH 6.8 to 6.0 media, cell growth declined for the first passage but recovered after the third passage (Appendix Table 78). Thus, these results cannot be presented as a single mean of the six passages/treatment and the statistical methods used for the other organisms could not be used for this data analysis.

The data of time (T) to reach maximum absorbance, maximum absorbance (OD_{max}), and final pH of six passages of B. ruminicola GA33 for the various treatments are given in Appendix Tables 34 to 40.

Values of OD_{max}, length of time (T) to reach maximum absorbance, and final pH of B. ruminicola GA33 grown at pH 6 control medium are shown in Appendix Table 34. The cell culture failed to grow under the monensin treated conditions. These results again indicate that B. ruminicola GA33 is pH dependent and monensin sensitive species. Values of OD_{max} of the third and fourth passages in the repeated six passages of B. ruminicola GA33 at pH 6 control medium were 0.19 and 0.95, respectively. These data show that even in the non-monensin-treated condition, cell growth of B. ruminicola GA33 was initially sensitive to the pH of the medium, but then adapted.

Maximum absorbance (growth) for B. ruminicola GA33,

when initially grown in pH 6.8 control medium (Appendix Table 35) and then transferred into (first inoculation) 20 ppm monensin medium (Appendix Table 36) or into (first inoculation) 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 37), was decreased from 0.6 to 0.1 and the length of time to reach the maximum absorbance was increased from 16.5 to 36 h.

Cell growth in 20 ppm monensin and 20 ppm monensin/200 mmol/l [Na] recovered to normal levels in further passages; the initial and the last maximum absorbance were 0.1, 0.105, 0.7 and 0.85, respectively. However, the length of time to reach maximum growth along the six passages did not vary. After a series of six inocula passages were transferred into pH 6.8 medium containing monensin, B. ruminicola GA33 total growth returned to control levels. These results indicate that B. ruminicola GA33 is a monensin-sensitive species, but also has a capacity for adaptation to the ionophore monensin.

The data of maximum absorbance (OD_{max}), length of time (T) to reach maximum absorbance, and final pH of six passages of B. ruminicola GA33 grown at pH 7.6 medium are shown in Appendix Tables 38 to 40.

In contrast to the pH 6.0 and 6.8 media, there were no significant differences, e.g. no growth depression followed by recovery ($p>0.05$), among six serial passages in pH 7.6 control medium (Appendix Table 38), 20 ppm

monensin (Appendix Table 39) or 20 ppm monensin/ 200 mmol/l [Na] media (Appendix Table 40).

Statistical comparisons of growth data for B. ruminicola GA33 grown in pH 7.6 media given in Table 14 are: control medium (C) vs. 20 ppm monensin (B) or 20 ppm monensin/[Na] (B-1) media; 20 ppm monensin medium (B) vs. 20 ppm monensin/[Na] medium (B-1).

Table 14 shows that OD_{max} values were significantly higher ($p<0.01$) in B than in B-1. Time (T) to reach maximum absorbance was significantly less ($p<0.01$) in C than in B or B-1.

B. Physiological and Metabolic Parameters of Six Passages

Data for physiological and metabolic parameters of six serial passages of B. ruminicola GA33 grown at pH 6, 6.8 or 7.6 are shown in Appendix Tables 78 to 84.

When B. ruminicola GA33 was grown in pH 6.0 control media (Appendix Table 78), RNA/protein values were numerically higher ($p>0.05$) in the last three passages (from 0.12 to 0.24) than in the first three passages (0.05); the same pattern was observed for $Y_{glucose}$ values (from 20.2 to 23.0 g of cell/mole of glucose utilized vs. from 9.7 to 13.4 g of cell/mole of glucose utilized). Total glucose-percentage utilization (from 90.69% to 99.23%) was also lower during the first three passages (from 89.36% to 91.05%).

B. ruminicola GA33 likely had a transient metabolic

Table 14. Physiological and Metabolic Parameters for B. ruminicola GA33 Grown from Control Medium to 20 ppm Monensin Medium and 20 ppm Monensin/[Na] medium at pH 7.6*

Metabolic Parameters	Treatments** C	B	B-1	SEM
RNA/Protein[a]	.30[j]	.35[j]	.16[k]	.06
CY[b]	51.21	56.37	45.89	6.95
Rate of glu. util.[c]	3.85[h]	3.22[j]	2.45[i,k]	.24
Total glu. util.%[d]	99.04[j] [h]	99.21[j,o] [i]	95.62[k,p] [i]	1.05
Y_{glu}[e]	13.1	17.5	17.7	1.2
OD_{max}[f]	.32[j]	.44[k,h]	.25[i]	.04
T[g]	17.42[h]	20.75[j]	28.50[i,k]	2.50
Final pH	6.57	6.53	6.67	.02

* Each value represents a mean of 6 passages of cultures.
** Treatment C is pH 7.6 control medium.
Treatment B is pH 7.6 20 ppm monensin medium.
Treatment B-1 is pH 7.6 20 ppm monensin/[Na] medium.
a means within row with different superscripts differ (j,k, $p<0.05$)
b cell yield; ug/h/15 ml culture
c umoles/h/15ml culture; means within row with different superscripts differ (h,i, $p<0.01$; j,k, $p<0.05$)
d means within row with different superscripts differ (j, k, $p<0.05$; o,p, $p<0.05$)
e g of cell/mole of glucose utilized; means within row with different superscripts differ (h,i, $p<0.01$)
f maximum absorbance; means within row with different superscripts differ (h,i, $p<0.01$; j,k, $p<0.05$)
g time to reach maximum absorbance; hr; means within row with different superscripts differ (h,i, $p<0.01$; j,k, $p<0.05$)

adaptation between the third and fourth passages; however, none of the other results pinpoint the reason for these observations.

Results from serial passages of B. ruminicola GA33 grown in pH 6.8 control medium (Appendix Table 79) showed that none of the cell growth parameters differed. However, cells grown at pH 6.8, 20 ppm monensin medium showed that cell growth changed transiently after the fourth passage (Appendix Table 80) and after the third passage of 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 81). Thus, the combined means of these experiments will not be presented.

RNA/protein increased from 0.14 to 0.49 after six passages in 20 ppm monensin medium (Appendix Table 80) and from 0.07 to 0.58 in 20 ppm monensin/[Na] medium after six passages (Appendix Table 81). The range of total glucose percentage utilization was from 86.00% to 99.18% in 20 ppm monensin medium (Appendix Table 80) and from 86.00 to 99.31% in 20 ppm monensin/[Na] medium (Appendix Table 81). $Y_{glucose}$ (g of cell/mole of glucose utilized) increased from 17.9 to 30.9 g of cell/mole of glucose utilized in 20 ppm monensin medium and from 7.0 to 28.1 in 20 ppm monensin/[Na] medium over six passages (Appendix Tables 80 and 81).

The above results show that B. ruminicola GA33 is a monensin-sensitive but adaptable species, at least at pH

6.8. There was no significant response to addition of [Na] in monensin-treated medium. Most likely a greater increase of [Na] may be needed in B. ruminicola GA33 to counteract any early effects of monensin by reversing the proton flow via the proton/Na+ (monensin) antiporter (Bergen and Bates, 1984).

The average cell yield (Appendix Tables 79, 80 and 81) was higher in control medium (71.39 ug/h/15 ml culture) than in 20 ppm monensin (39.21 ug/h/15 ml culture) or 20 ppm monensin/200 mmol/l [Na] medium (53.82 ug/h/15 ml culture). The average rate of glucose utilization was higher in control medium (3.83 umoles/h/15 ml culture) than in 20 ppm monensin (1.80 umoles/h/15 ml culture) or 20 ppm monensin/200 mmol/l [Na] medium (3.35 umoles/h/15 ml culture).

The physiological and metabolic parameters within six serial passages of B. ruminicola GA33 at pH 7.6 medium are presented in Appendix Tables 82 to 84.

There were no differences among these six serial passages of B. ruminicola GA33 at pH 7.6 control medium (Appendix Table 82), 20 ppm monensin medium (Appendix Table 83) or 20 ppm monensin/200 mmol/l [Na] medium (Appendix Table 84); the data are thus combined and presented in Table 14.

Table 14 shows that the RNA/protein ratios and cell yields were not different ($p>0.05$), however, in 20 ppm monensin medium the RNA/protein and cell yields were

numerically higher ($p>0.05$) than in the other two treatments. Cells utilized more glucose in control media (3.85 ug/h/15 ml culture, $p<0.01$) than in 20 ppm monensin medium (3.22 ug/h/15ml) or 20 ppm monensin/200 mmol/l [Na] (2.45 ug/h/15 ml culture). Total glucose utilization was, however, the same for all treatments. $Y_{glucose}$ was significantly different ($p< 0.01$) in control (13.1 g of cell/mole of glucose utilized) than 20 ppm monensin medium (17.5 g of cell/ mole of glucose utilized) and 20 ppm monensin/200 mmol/l [Na] (17.7 g of cell/mole of glucose utilized).

An overview of data of B. ruminicola GA33 grown in pH 7.6 media is shown in Fig. 12. Each bar is a mean of six passages, when there was no difference among six passages data.

Bacterial growth inhibition by monensin is a well-accepted phenomena. Some conjectures were believed only to be true for typical concentrate or roughage diets under physiological pH rumen conditions. How monensin affects cell growth under alkaline pH conditions is rarely considered by current ruminant nutrition researchers. From this study, the monensin effect on cell growth was studied in **in vitro** batch culture experiments. The doses of monensin and the pH conditions were found to be important factors for cell growth.

Monensin acts as a sodium-proton ionophore (Dobler, 1981; Bergen and Bates, 1984). The understanding of

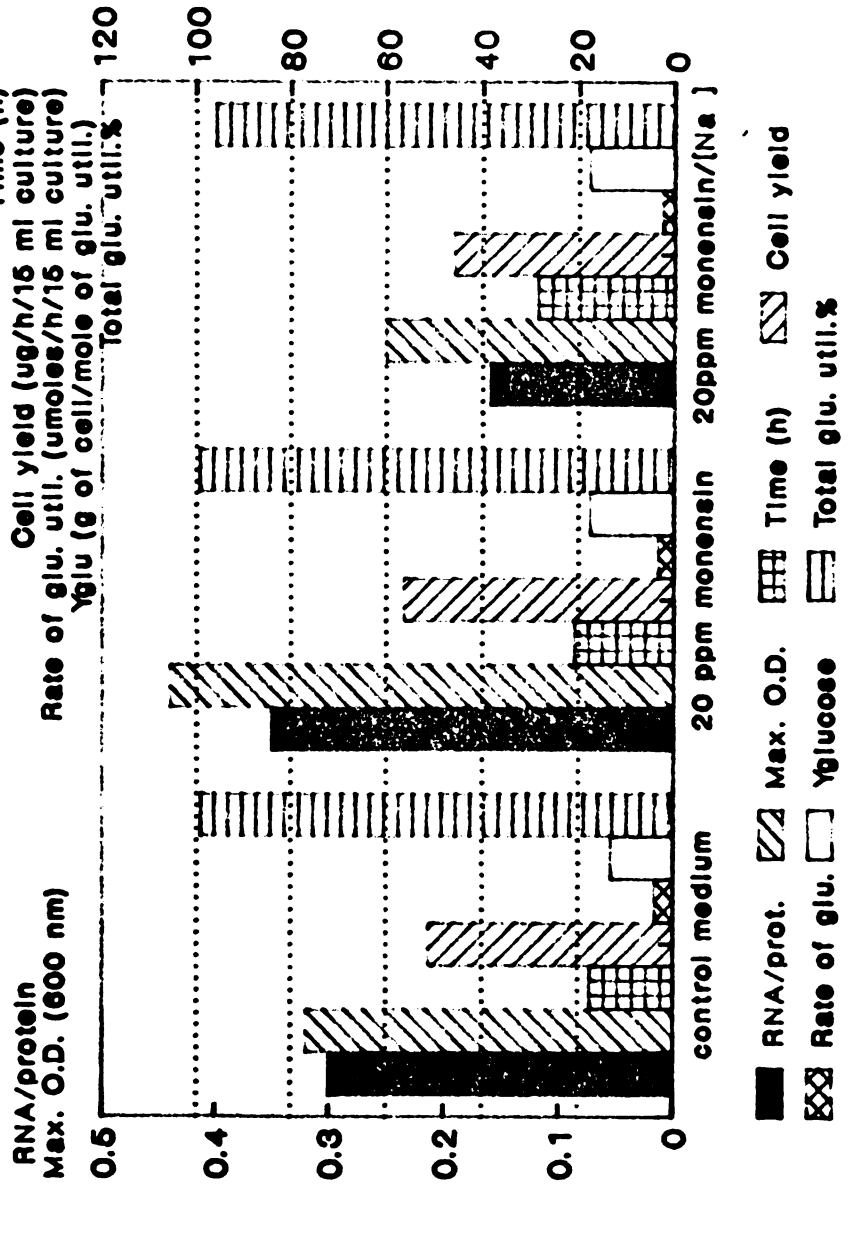


Figure 12. An Overview of B. ruminicola GA33 Grown in Various pH 7.6 Media

sodium concentration and pH values of ruminal bacteria is essential to speculate on the interrelation between monensin and cells.

The concentration of sodium in rumen fluid normally varies between 60 and 120 mM (Caldwell and Hudson, 1974). Romatowski (1979) reported a value closer to 170 mM. Internal sodium can vary with physiological status (Bergen and Bates, 1984). Martinez (1972) pointed out that rumen bacteria contain 21 to 36 mg sodium/g bacterial dry matter. Riebeling et al. (1975) showed that bacteria have 2 to 4 ml intracellular water (volume/g dry matter). Thus, intracellular sodium of ruminal bacteria would be around 5 to 10 g/liter or 250 to 500 mM (Bergen and Bates, 1984).

The pH in the rumen typically ranges from 5.7 to 7.3, with prevailing values around 6.5. The intracellular pH of most bacteria appears to be highly regulated and constant at around 7.6 to 7.8 (Padan et al., 1981; Kobayashi et al., 1982). Assuming basal conditions in the rumen before bacteria are exposed to monensin, rumen fluid (extracellular) Na+ is 100 mM and intracellular sodium is estimated to be 250 mM; the log divided value ($\log [\text{sodium}]_i/[\text{sodium}]_e$) is 0.4. When extracellular pH goes up, the transmembrane proton gradient declines, and vice versa. The distribution of intracellular sodium ($[\text{sodium}]_i$) to extracellular sodium ($[\text{sodium}]_e$) caused by an electrically neutral sodium/H+

antiporter can be thermodynamically related to pH by the equation:

$$\log \frac{[\text{sodium}]_i}{[\text{sodium}]_e} = \Delta \text{ pH}.$$

When the intracellular pH is 7.8 and the typical physiological pH found in the rumen is 6.5 the pH of rumen bacteria is 1.3. This higher proton gradient would then be dissipated (e.g. proton movement into the cells) by monensin until

$$\log \frac{[\text{sodium}]_i}{[\text{sodium}]_e} = \Delta \text{ pH}.$$

The relative concentration for sodium and H+ may play an important role in determining the final physiological effects of monensin. Much of the observed influence of monensin on the physiology of the rumen can be traced back to the dissipation of the primary proton gradient.

General Results of The Adaptation Study

The question of metabolic adaptation of pure cultures of S. bovis 24, L. vitulinus B62, B. ruminicola GA33 and S. ruminantium HD4 to the ionophore monensin was explored. A general result of the adaptation study is shown in Fig. 13.

Figure 13 shows that for S. bovis 24, OD_{max} was depressed by 20 ppm monensin when a pure culture in batch culture was grown through six successive transfers

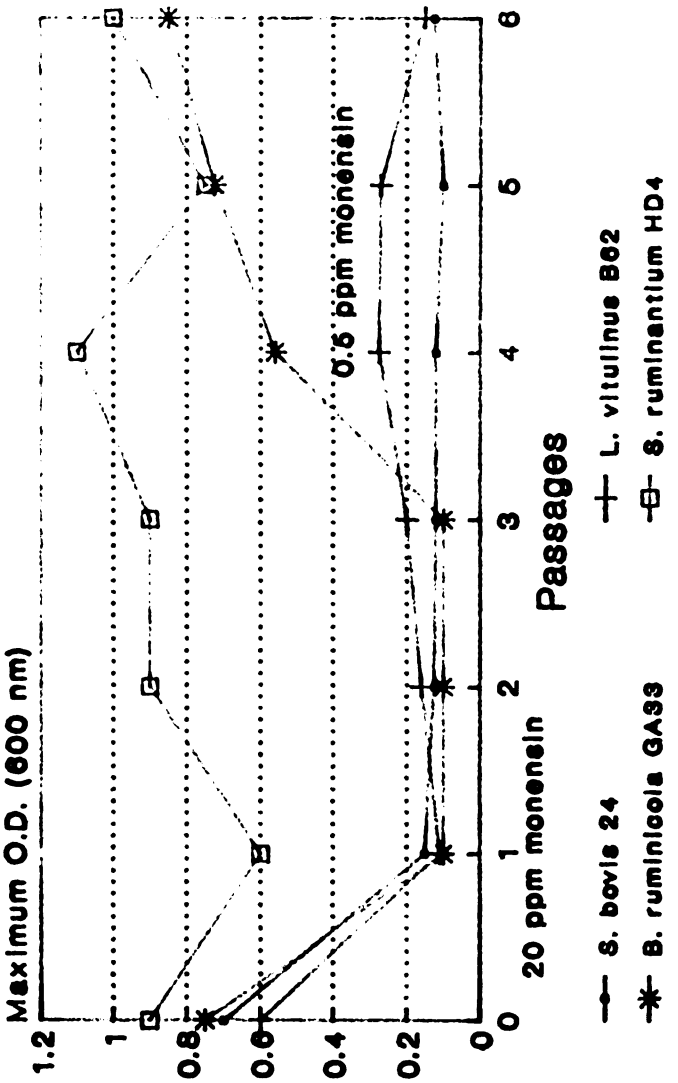


Figure 13. An Overview of S. bovis 24, L. vitulinus B62, B. ruminicola GA33 and S. ruminantium HD4 Grown in pH 6.8 Monensin Media

within a treatment medium (pH 6.8). L. vitulinus B62 did not grown in 20 ppm monensin and barely grew in 0.5 ppm monensin medium. When B. ruminicola GA33 was grown in 20 ppm monensin medium, there was an initial depression in OD_{max} for the first three passages; OD_{max} recovered during the fourth to sixth passages. S. ruminantium grew well in 20 ppm monensin medium. These results provide no evidence of metabolic adaptation of S. bovis 24 to an ionophore monensin insult; they do provide evidence for an initial inhibition followed by an adaptation by B. ruminicola GA33 to monensin and show that S. ruminantium HD4 can survive the ionophore insult by utilizing more available energy for maintenance and less for cell growth.

V. Cell Surface Morphology Assessment With SEM

Results from the experiments on membrane surface assessment with SEM were not sufficient for making any certain conclusions. However, current findings showed that monensin might affect the morphology of B. ruminicola GA33, S. bovis 24 and L. vitulinus B62. The morphology of S. ruminantium HD4 were only slightly affected by monensin. The mechanism of how monensin affects the morphological structure is unknown.

Micrographs of rumen bacteria grown in control or monensin medium are shown in Figures 14 to 21.

Figure 14 is a scanning electron micrograph of S. bovis 24 grown in control medium. Various findings by many other investigators showed that S. bovis are spherical, oval or elongated into rods, 0.9 - 1.0 um in diameter and occurring in pairs, chains and occasionally in long chains in broth (Baker and Nasr, 1947; Baker et al., 1950; Moir and Masson, 1952; MacPherson, 1953; Hobson and Mann, 1955; Bailey and Oxford, 1958; Kane and Karakawa, 1969; Kane et al., 1972; Cheng et al., 1976 and Horacek et al., 1977). The present result regarding large capsules surrounding the cells in the rumen was in agreement with those investigations.

Cell walls (surfaces) of many bacteria are surrounded by a viscous substance known as the capsule or slime layer. A possible function of the capsule is to protect against phagocytosis by macrophages or protozoa

and to serve as a reservoir of stored energy (Ogimoto and Imai, 1981). S. bovis produces extracellular polysaccharides and dextran (Bailey and Oxford, 1958; Barnes et al., 1961), containing glucose, galactose, rhamnose, ribose and glucosamine (Bailey and Oxford, 1958).

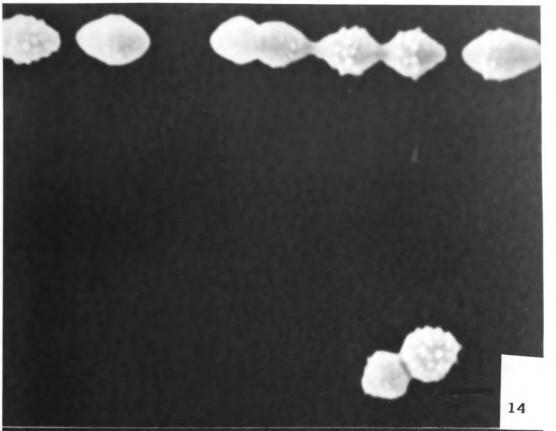
Figure 15 is a micrograph of S. bovis 24 grown in 20 ppm monensin medium. It is apparent that very little or no capsular material surrounded the membrane surface (cell wall). This finding suggests that the ionophore monensin somehow interferes with the cells ability to produce a protective capsule layer. Without the protective capsule layer the gram positive cocci S. bovis 24 may be more easily inhibited by the ionophore, but the mechanism of this protection is unknown.

Figure 16 is a scanning electron micrograph of L. vitulinus B62 grown in control medium. Unknown grain particles in the background were visible. These microscopic observations are consistent with other reports which indicated that L. vitulinus are rods, average size 0.5 - 0.6 by 0.8 - 3.0 um, occurring singly or in pairs (Sharpe et al., 1973).

When L. vitulinus B62 was grown in 0.5 ppm monensin medium, some grain particles still surround the cell surface (Figure 17). This observation of the cell

Figure 14. Scanning electron micrograph of S. bovis 24 grown in pH 6.8 control medium. Note unknown grain materials were present on the cell surface (bar equals 1 um).

Figure 15. Scanning electron micrograph of S. bovis 24 grown in pH 6.8 20 ppm monensin medium. No grain materials were present on the cell surface (bar equals 1 um).



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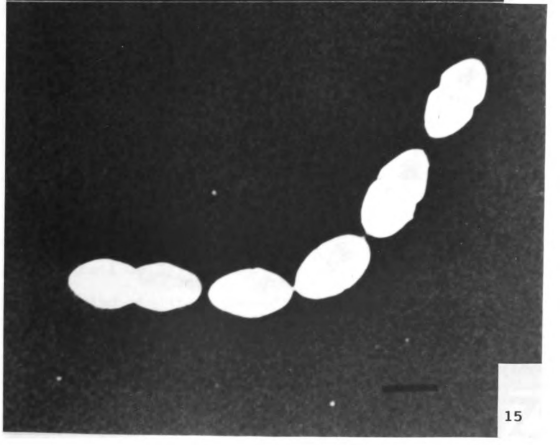

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surface using SEM indicated that gram positive rod, L. vitulinus, capsular material was apparently less affected by lower concentration of ionophore monensin than S. bovis 24. Since L. vitulinus B62 did not grow in 20 ppm monensin, the significance of unknown grain materials in sustaining cellular function could not be assessed in this study.

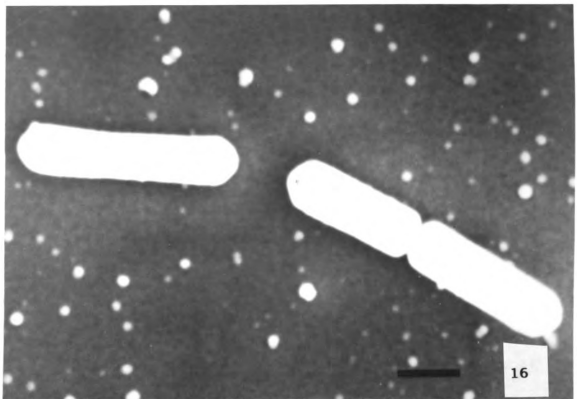
Figure 18 is a scanning electron micrograph of B. ruminicola GA33 grown in control medium. A capsule layer can be seen surrounding the cell surface. This finding was consistent with other reports which indicated that B. ruminicola are occasionally encapsulated (Bryant et al., 1958).

Figure 19 is a micrograph of B. ruminicola GA33 grown in 20 ppm monensin medium. There is noticeably less capsular material surrounding the cell surface. Dawson and Boling (1984) observed that B. ruminicola GA33 is a monensin sensitive species and suggested that part of this sensitivity might be related to the smeller (or less thick) capsule layer around the cell surface (cell wall).

Scanning electron micrographs do not demonstrate any morphological difference in S. ruminantium HD4 when grown in either 20 ppm monensin (Figure 20) or control medium (Figure 21). Wenyon (1926) as well as Ogimoto and Imai

Figure 16. Scanning electron micrograph of L. vitulinus B62 grown in pH 6.8 control medium. Note unknown grain particle attached to the cell surface (bar equals 1 um).

Figure 17. Scanning electron micrograph of L. vitulinus B62 grown in pH 6.8 0.5 ppm monensin medium. Note grain particles attached to the cell surface (bar equals 1 um).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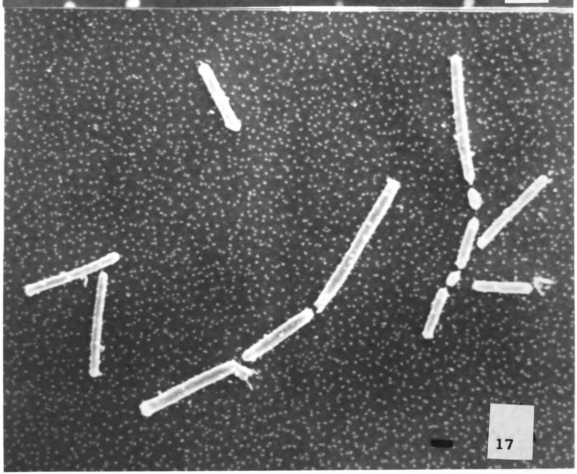
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Figure 18. Scanning electron micrograph of B. ruminicola GA33 grown in pH 6.8 control medium. Note a capsule layer surrounded the cell surface (bar equals 1 um).

Figure 19. Scanning electron micrograph of B. ruminicola GA33 grown in pH 6.8 20 ppm monensin medium. Note no capsule layer attached on the cell surface (bar equals 1 um).

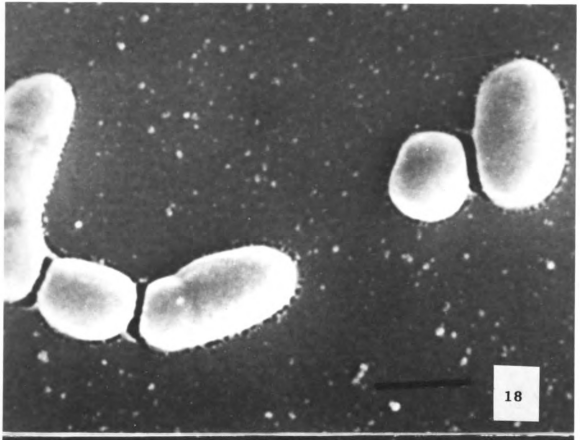
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Figure 20. Scanning electron micrograph of S. ruminantium HD4 grown in pH 6.8 20 ppm monensin medium. Note flagella was present and no capsule layer attached on the cell surface (bar equals 1 um).

Figure 21. Scanning electron micrograph of S. ruminantium HD4 grown in pH 6.8 control medium. Note flagella was present and no capsule layer attached on the cell surface (bar equals 1 um).

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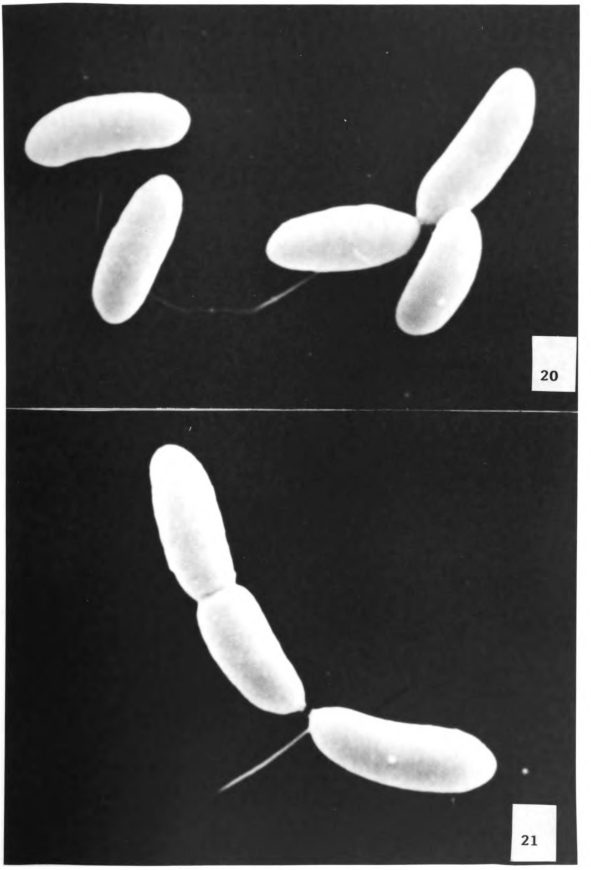


(1981) pointed out S. ruminantium are curved or helical rods, with round ends, crescent-shape cells with tufts of flagella on the concave side, with an average size of 0.8 - 1.0 by 2.0 - 7.0 um arranged singly or in pairs; occasionally in short chains. These previous observation (Wenyon, 1926; Ogimoto and Imai, 1981) on S. ruminantium HD4 morphology were confirmed in present study .

Experiment 2

In Vitro Continuous Culture Study

A continuous chemostat culture represents a system of studying growth regulation of bacteria in steady state at given growth rate (u) by varying the dilution rate (D) and maintaining constant cell concentrations. In a chemostat, u depends on the concentration of a limiting growth substrate [S] in the culture medium. When [S] is kept low in the continuous culture, the rate of medium flow (D) (and hence S availability) regulates u and at steady state (e.g. volume stays constant) u = D (Herbert et al., 1956). High cell densities and accumulation of inhibitor substances, rather than a growth limiting substrate, may well control bacterial growth in the rumen. Thus, whenever the rumen contents are diluted by any means, inhibitory products and cell density decline resulting in faster and more efficient growth of bacteria (Bergen et al., 1982). These fundamental concepts were applied in the following section of results and discussion. Actual data of this study are listed in Appendix Tables 41 to 45 and 85 to 88. Average values of two sets of observations are given in Tables 15 to 22.

This study was limited by the unique continuous culture apparatus in the ruminant nutrition laboratory. It took at least one year to operate and complete the entire experiment. Therefore, in this part of the

experiment, only two sets of observational data were collected. These data are not sufficient for statistical analysis. Hence, a simple numerical comparison method was used in this section of results and discussion.

The concentrations of fermentation products (acetate, propionate, butyrate, lactate and succinate) were determined for all current work as in Experiment 1. Since the samples were either not prepared properly and/or affected by other unknown factors, results of fermentation product values are difficult to interpret and will not be given. Hence, the following section of results and discussion will concentrate on, and discuss in detail, physiological and metabolic parameters such as RNA/protein ratios, cell yield, rate of glucose utilization, total glucose percentage utilization and $Y_{glucose}$.

The central focus of experiment 2 was on two objectives. For each organism, the first objective was to assess u and adaptation to the ionophore monensin in continuous culture. The second objective was to compare the effect of monensin, sodium concentration and length of exposure on adaptation of S. bovis 24 and B. ruminicola GA33 in 5%/h and 10%/h dilution rate continuous culture on physiological and metabolic parameters as listed above.

I. B ruminicola GA33

A. Time Course and Adaptation Study of Five Successive Treatments

The observed data of O.D. of the medium, time of sampling and final pH of B. ruminicola GA33 grown in pH 6.8 medium in a 5%/h continuous culture are given in Table 15. Each value represents an average of two sets of observations. The five sequential medium treatments were examined in the study as follows: bacteria grown in control, 0.5 ppm monensin, 20 ppm monensin, 20 ppm monensin/200 mmol/l [Na], and back to control media.

Table 15 shows that absorbance is lowered when cells grown at steady state in control medium (0.18, 0.13) were exposed to 0.5 ppm monensin (0.07, 0.08) followed by

Table 15. Absorbance, Sampling Time and Final pH of B. ruminicola GA33 Grown in pH 6.8 medium in a 5%/h Dilution Rate Continuous Culture*

Sampling Sequence	Medium	O.D.	Sampling Time (Day)	Final pH
1	Start	.50	0	6.6
2	Control	.18	3	6.5
3	Control	.13	6	6.5
4	.5 ppm monensin	.07	9	6.5
5	.5 ppm monensin	.08	12	6.5
6	20 ppm monensin	.06	15	6.5
7	20 ppm monensin	.04	18	6.5
8	20 ppm monensin/[Na]	.11	21	6.5
9	20 ppm monensin/[Na]	.15	24	6.6
10	Back to control	.29	27	6.6
11	Back to control	.18	30	6.5

* Each value represents an average of two sets of observations.

exposure to 20 ppm monensin (0.06, 0.04). Absorbance increased after cells were grown in a 20 ppm monensin/200 mmol/l [Na] medium phase (0.11, 0.15) and then cultured again in control medium (0.29, 0.18).

The observed data of absorbance, time of sampling and final pH of B. ruminicola GA33 grown in pH 6.8 medium in a 10%/h dilution rate continuous culture are given in Table 16. Each value represents an average of two sets of observations. The examined five sequential medium treatments were examined in the study as follows: bacteria grown in control, 0.5 ppm monensin, 20 ppm monensin, 20 ppm monensin/200 mmol/l [Na], and back to control media.

Table 16. Absorbance, Sampling Time and Final pH of B. ruminicola GA33 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture*

Sampling Sequence	Medium	Absorbance	Sampling Time (Day)	Final pH
1	Start	.6	0	6.8
2	Control	.32	2	6.6
3	Control	.22	4	6.6
4	.5 ppm monensin	.1	6	6.5
5	.5 ppm monensin	.12	8	6.5
6	20 ppm monensin	.07	10	6.5
7	20 ppm monensin	.1	12	6.5
8	20 ppm monensin/[Na]	.08	14	6.5
9	20 ppm monensin/[Na]	.09	16	6.5
10	Back to control	.24	18	6.5
11	Back to control	.38	20	6.5

* Each value represents an average of two sets of observations

Table 16 shows that absorbance declined when cells grown from control medium (0.32, 0.22) were exposed to a 0.5 ppm monensin phase (0.1, 0.12), 20 ppm monensin phase (0.07, 0.1) or 20 ppm monensin/[Na] medium (0.08, 0.09). The absorbance recovered after the switch back to the control medium (0.24, 0.38). The 200 mmol/l sodium level was not able to improve cell growth after depression by monensin, as noted at the 5% dilution rate.

B. **Physiological and Metabolic Parameters of Five Treatments**

Physiological and metabolic parameters of B. ruminicola GA33 grown in pH 6.8 medium in a 5%/h dilution rate continuous culture are given in Table 17. Cells utilized 96.98% to 99.24% glucose (14.08 to 14.41 umoles glucose/h) for growth and maintenance. RNA/protein ratios were not numerically different among five treatments; but during the 20 ppm monensin/[Na] medium phase values were 0.37, 0.27 and the back to control medium phase values were 0.31, 0.24, which were numerically higher than the RNA/protein 0.5 ppm monensin medium phase values of 0.26, 0.26, and the 20 ppm monensin medium phase values of 0.24, 0.26.

Table 17 also shows that cell yield (ug/h) was decreased at 0.5 ppm monensin medium phase (472.32 & 336.04), 20 ppm monensin medium phase (405.13 & 334.92) or 20 ppm monensin/[Na] medium phase (353.13 &

309.67). However, cell yield recovered during the back to control medium phase (485.18 & 429.41). Y_{glu} was decreased at 0.5 ppm monensin phase (15.4 & 10.9), 20 ppm monensin medium phase (13.24 & 11.09) and 20 ppm monensin/200 mmol/l [Na] medium phase (11.70 & 10.04). Y_{glu} is enhanced in the back to control medium phase (15.7 & 13.9).

Table 17. Physiological and Metabolic Parameters of B. ruminicola GA33 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture*

	Metabolic Parameters				
No.[a]	RNA/ protein	Cell yield[b]	Rate of glu. util.[c]	Total of glu. util.%	Y_{glu}[d]
1 Start					
2	.25	513.36	30.87	99.22	16.6
3	.24	513.28	30.87	99.24	16.6
4	.26	472.32	30.78	98.95	15.4
5	.26	336.04	30.87	99.24	10.9
6	.24	405.12	30.60	98.35	13.2
7	.26	334.92	30.20	97.08	11.1
8	.37	353.13	30.17	96.98	11.7
9	.27	309.67	30.83	99.10	10.0
10	.31	485.18	30.83	99.09	15.7
11	.24	429.41	30.86	99.19	13.9

* Each value represents an average of two sets of observations.

a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.

b ug/h

c umoles/h

d g of cell/mole of glucose utilized

An overview of the data of B ruminicola GA33 grown in various 5%/h dilution rate media is shown in Figures 22 and 23. Each bar is an average of two sets of experimental data.

The physiological and metabolic parameters of B. ruminicola GA33 grown in pH 6.8 medium in a 10%/h dilution rate continuous culture are presented in Table 18. Each value represents an average of two sets of observations.

Cell utilized 92.46% to 99.20% glucose (57.53 to 61.72 umoles glucose/h) for growth and maintenance. RNA/protein ratios exhibited the same trends as for 5%/h continuous cultures.

Table 18 shows cell yield (ug/h) was less in .5 ppm monensin medium phase (1371.73 and 1259.64), 20 ppm monensin medium (868.2 & 914.65) or 20 ppm monensin/[Na] media (1193.79 & 1372.05) than in control medium (1735.98 & 2095.92). Cell yield was enhanced in back to control medium phase (1452.99 & 1983.81). Control phase cell yields also doubled when the dilution rate was doubled (5% - 10%/h).

Table 18 also shows that Y_{glu} was less in 0.5 ppm monensin medium phase (22.3 & 20.4), 20 ppm monensin (15.1 & 15.2) or 20 ppm monensin/[Na] medium phase (20.1 & 22.5) than in control medium. However, Y_{glu} recovered during the back to control medium phase (23.6 & 32.2).

The above data showed that the ionophore monensin

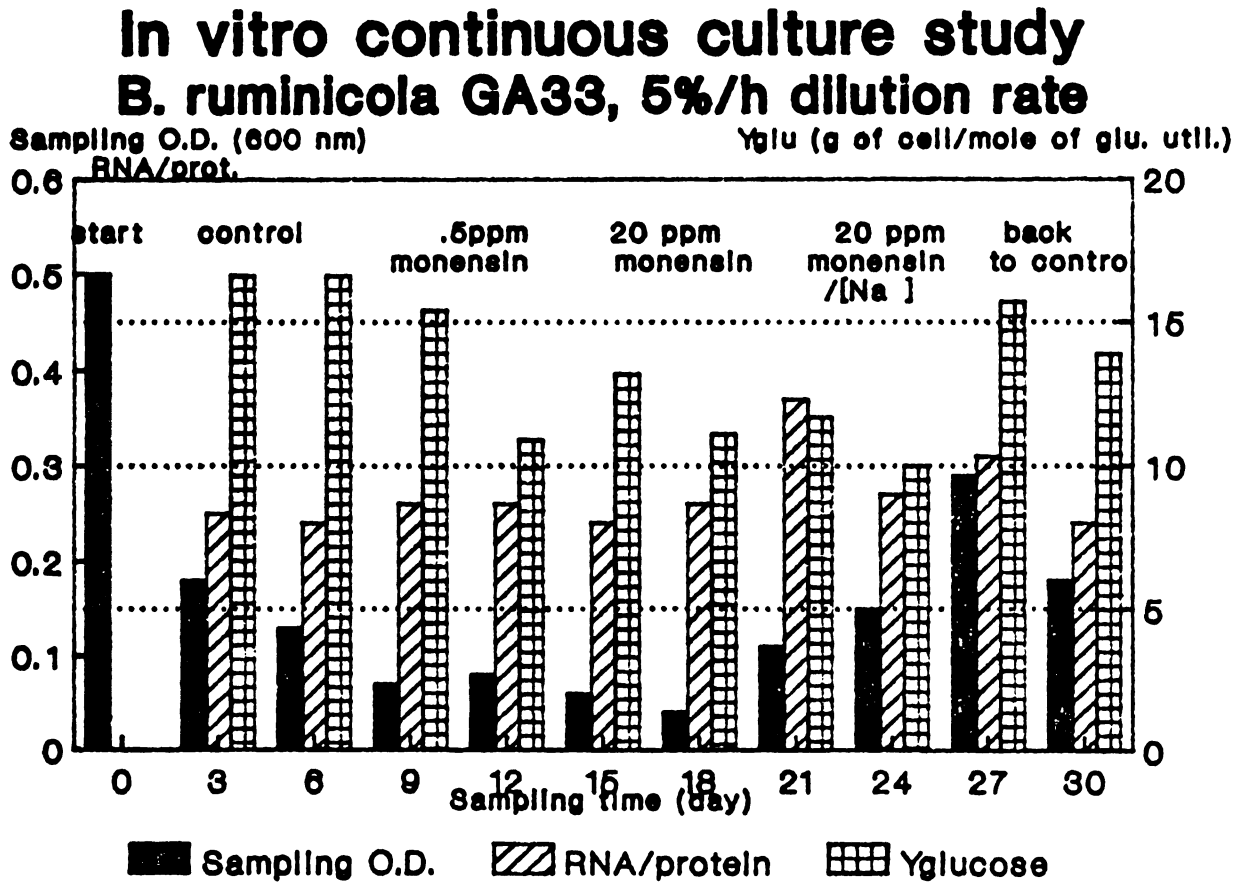


Figure 22. An Overview of B. ruminicola GA33 Grown in 5%/h Dilution Rate Continuous Culture Various Media (Part 1)

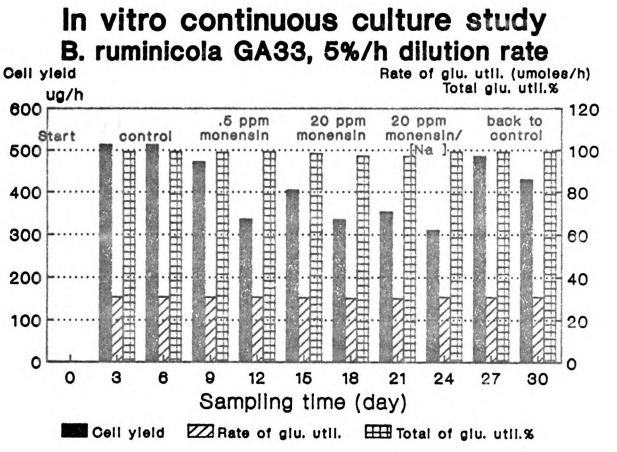


Figure 23. An Overview of B. ruminicola GA33 Grown in 5%/h Dilution Rate Continuous Culture Various Media (Part 2)

depressed the cell yield of B. ruminicola GA33. This was probably due to the nature of the interrelation of the ionophore monensin and B. ruminicola GA33 under steady state conditions (which requires an actively growing population and a constant removal of a portion of the population).

Table 18. Physiological and Metabolic Parameters of B. ruminicola GA33 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture*

	Metabolic Parameters				
No.[a]	RNA/ protein	Cell yield[b]	Rate of glu. util.[c]	Total of glu. util.%	Y_{glu}[d]
1	Start				
2	.28	1735.98	61.72	99.20	28.1
3	.26	2095.92	61.72	99.19	34.0
4	.23	1371.73	61.59	98.99	22.3
5	.22	1259.64	61.67	99.12	20.4
6	.23	868.20	57.53	92.46	15.1
7	.32	914.65	60.23	96.80	15.2
8	.23	1193.79	59.28	95.27	20.1
9	.28	1372.05	60.97	97.99	22.5
10	.26	1455.99	61.70	99.16	23.6
11	.33	1983.81	61.70	99.17	32.2

* Each value represents an average of two sets of observations.
a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.
b ug/h
c umoles/h
d g of cell/mole of glucose utilized

An overview of the data of B. ruminicola GA33 grown in 10%/h dilution rate various media is shown in Figures

24 and 25. Each bar is an average of two sets of observations.

The use of continuous culture makes process control much easier; in a steady state it enables measurements to be made with great accuracy over a long period of time.

II. S. bovis 24

A. Time Course and Adaptation Study of Five Treatments

The observed data of O.D. of the medium, time of sampling, and final pH of S. bovis 24 grown in pH 6.8 medium in a 5%/h continuous culture are given in Table 19. Each value represents an average of two sets of observations.

Table 19. Absorbance, Sampling Time and Final pH of S. bovis 24 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture*

Sampling Sequence	Medium	Absorbance	Sampling Time (Day)	Final pH
1	Start	.55	0	6.8
2	Control	.23	3	6.6
3	Control	.23	6	6.5
4	.5 ppm monensin	.14	9	6.6
5	.5 ppm monensin	.16	12	6.6
6	20 ppm monensin	.09	15	6.5
7	20 ppm monensin	.1	18	6.6
8	20 ppm monensin/[Na]	.11	21	6.5
9	20 ppm monensin/[Na]	.1	24	6.6
10	Back to control	.25	27	6.5
11	Back to control	.19	30	6.6

* Each value represents an average of two sets of observations.

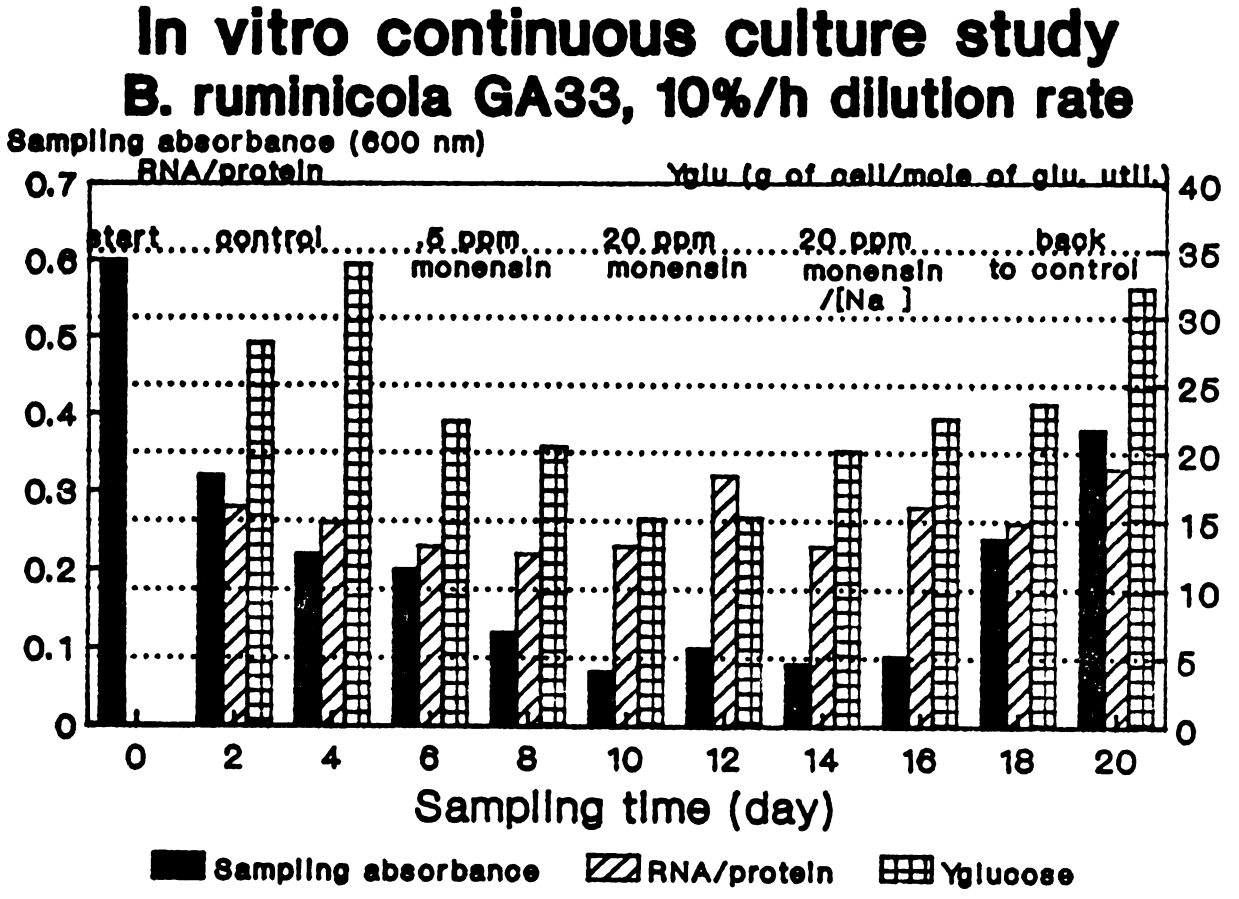


Figure 24. An Overview of B. ruminicola GA33 Grown in 10%/h Dilution Rate Continuous Culture Various Media (Part 1)

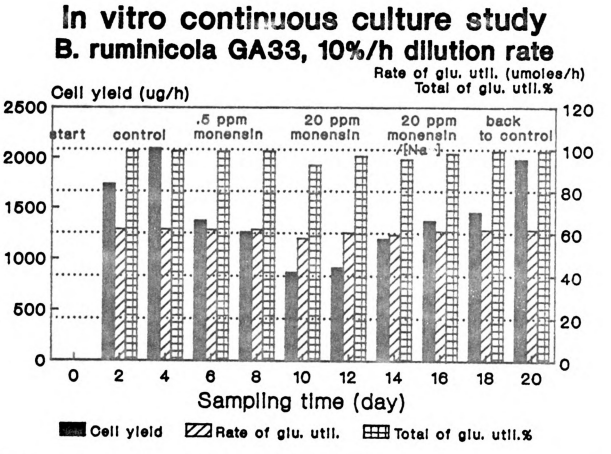


Figure 25. An Overview of B. ruminicola GA33 Grown in 10%/h Dilution Rate Continuous Culture Various Media (Part 2)

The five sequential media treatments examined in the study were as follows: bacteria grown in control, 0.5 ppm monensin, 20 ppm monensin, 20 ppm monensin/200 mmol/l [Na], and back to control media.

Table 19 shows that absorbance declined when cells grown in control medium (0.23, 0.23) were exposed to the 0.5 ppm monensin phase (0.14, 0.16), followed by the 20 ppm monensin phase (0.09, 0.1), and 20 ppm monensin/[Na] medium phase (0.11, 0.1). The absorbance recovered after the switch back to the control medium (.25, .19). There were no significant differences for final pH among these treatments.

The observed data for absorbance, time of sampling, and final pH of S. bovis 24 grown in pH 6.8 medium in a 10%/h dilution rate continuous culture are given in Table 20. The five sequential medium treatments examined in the study were as follows: bacteria grown in control, .5 ppm monensin, 20 ppm monensin, 20 ppm monensin/200 mmol/l [Na], and back to control media.

Table 20 shows that absorbance declined when cells grown in control medium (0.29, 0.21) were switched to the 0.5 ppm monensin phase (0.16, 0.15), followed by the 20 ppm monensin phase (0.06, 0.07) and 20 ppm monensin/[Na] medium phase (0.11, 0.16). Absorbance recovered after the switch back to the control medium (0.21, 0.34). Final pH stayed constant with all of these treatments.

Table 20. Absorbance, Sampling Time and Final pH of S. bovis 24 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture *

Sampling Sequence	Medium	O.D.	Sampling Time (Day)	Final pH
1	Start	.55	0	6.8
2	Control	.29	2	6.2
3	Control	.21	4	6.2
4	.5 ppm monensin	.16	6	6.2
5	.5 ppm monensin	.15	8	6.2
6	20 ppm monensin	.06	10	6.3
7	20 ppm monensin	.07	12	6.2
8	20 ppm monensin/[Na]	.11	14	6.2
9	20 ppm monensin/[Na]	.16	16	6.3
10	Back to control	.21	18	6.3
11	Back to control	.34	20	6.3

* Each value represents of an average of two sets of observations.

B. Physiological and Metabolic Parameters of Five Treatments

Physiological and metabolic parameters of S. bovis 24 grown in pH 6.8 medium in a 5%/h continuous culture are given in Table 21. S. bovis 24 utilized 99% of the glucose (30.84 to 30.89 umoles glucose/h) for growth and maintenance. Cell yield and Y_{glu} were lower in 0.5 ppm monensin medium phase, 20 ppm monensin medium phase and 20 ppm monensin/[Na] medium phase. These values recovered when S. bovis 24 was re-exposed to the control medium. The RNA/protein ratios only showed a slight decline upon monensin treatment.

An overview of the data of S. bovis 24 grown in pH

Table 21. Physiological and Metabolic Parameters of S. bovis 24 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture*

	Metabolic Parameters				
No.[a]	RNA/ protein	Cell yield[b]	Rate of glu. util.[c]	Total of glu. util.%	Y_{glu}[d]
1	Start				
2	.30	765.32	30.85	99.15	24.8
3	.31	641.32	30.84	99.12	20.8
4	.26	429.32	30.87	99.20	13.9
5	.31	560.02	30.87	99.17	18.1
6	.24	504.08	30.85	99.15	16.3
7	.22	410.52	30.85	99.15	13.3
8	.25	728.04	30.85	99.15	23.6
9	.27	757.42	30.85	99.15	24.6
10	.27	653.16	30.89	99.24	21.6
11	.34	970.68	30.89	99.24	31.4

* Each value represents an average of two sets of observations.
a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.
b ug/h
c umoles/h
d g of cell/mole of glucose utilized

6.8 medium in a 5%/h dilution rate continuous culture is shown in Figures 26 and 27. Each bar is an average of two sets of observations.

The physiological and metabolic parameters of S. bovis 24 grown in pH 6.8 medium in a 10%/h continuous culture are given in Table 22. S. bovis 24 utilized 96.11% to 99.30% (59.80 to 61.79 umoles glucose/h) of available glucose for growth and maintenance. Again,

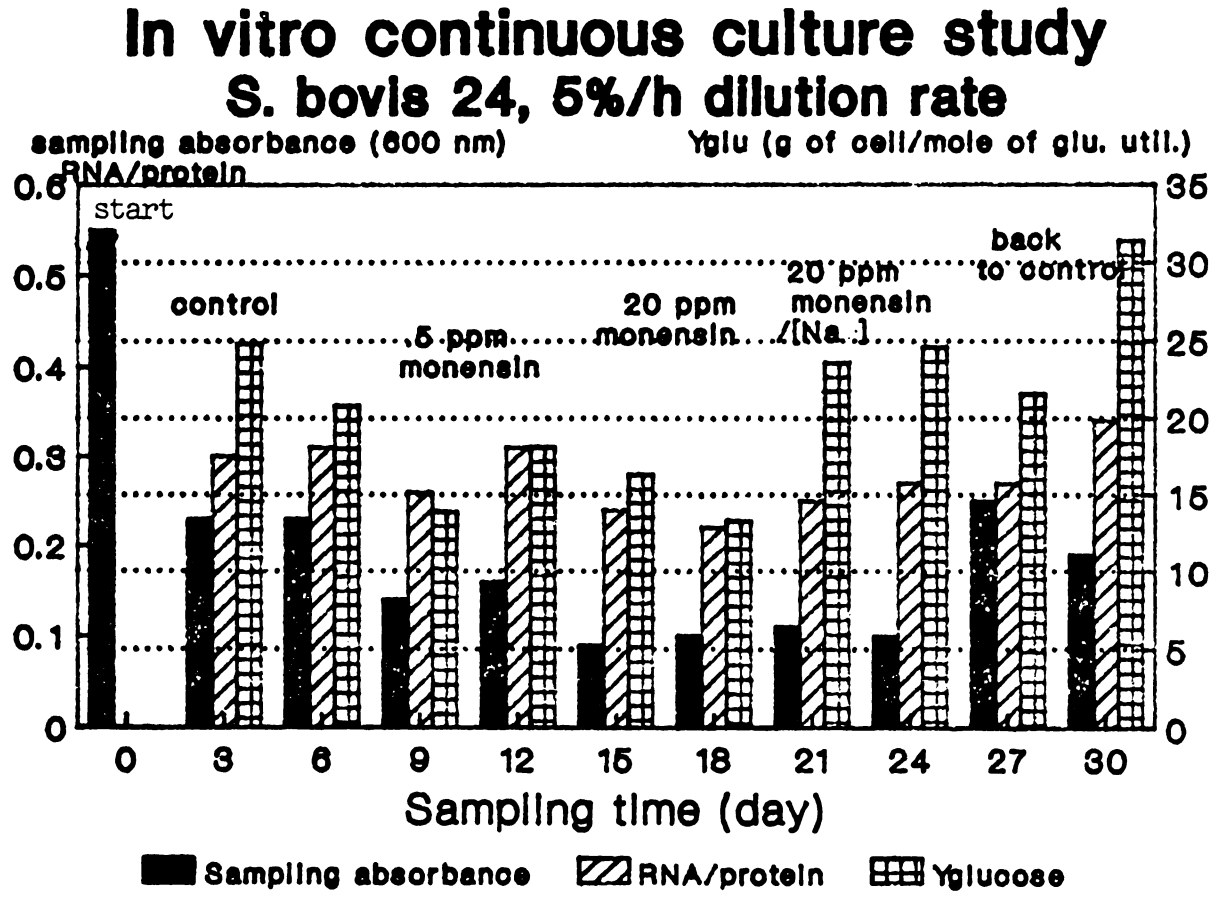


Figure 26. An Overview of S. bovis 24 Grown in 5%/h Dilution Rate Continuous Culture Various Media (Part 1)

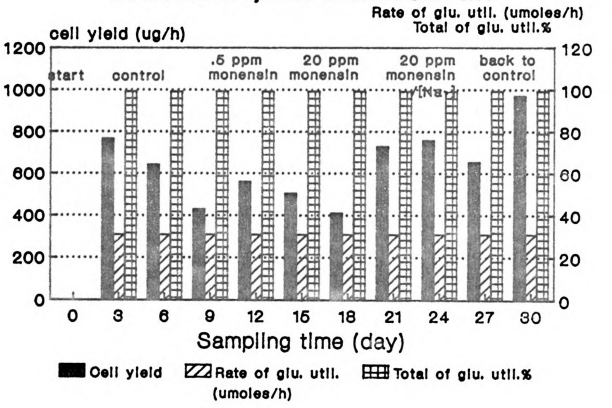


Figure 27. An Overview of S. bovis 24 Grown in 5%/h Dilution Rate Continuous Culture Various Media (Part 2)

increasing dilution rate improved cell yield and Y_{glu} for all treatments. Results in Table 22 also show that RNA/protein ratios, cell yield and Y_{glu} were low in 0.5 ppm monensin medium phase and 20 ppm monensin medium phase. These values recovered when S. bovis 24 was re-exposed to 20 ppm monensin/[Na] medium phase and back to control medium. Thus, at the higher dilution rate, monensin appeared to have a more severe effect on S. bovis 24 growth dynamics as seen by changes in RNA/protein ratios.

An overview of the data of S. bovis 24 grown in 10%/h dilution rate various media is shown in Figures 28 and 29. Each bar is an average of two sets of observations.

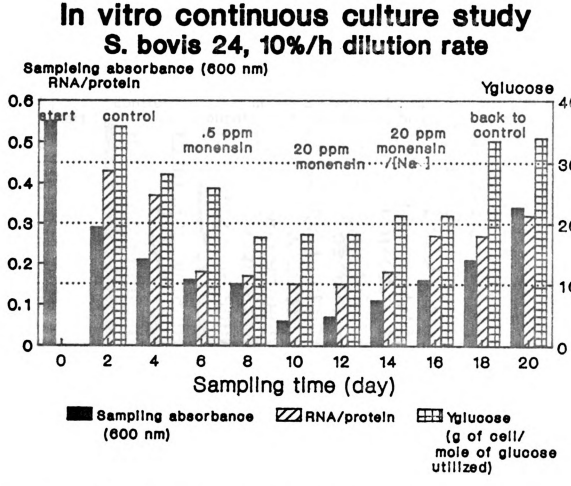


Figure 28. An Overview of S. bovis 24 Grown in 10%/h Dilution Rate Continuous Culture Various Media (Part 1)

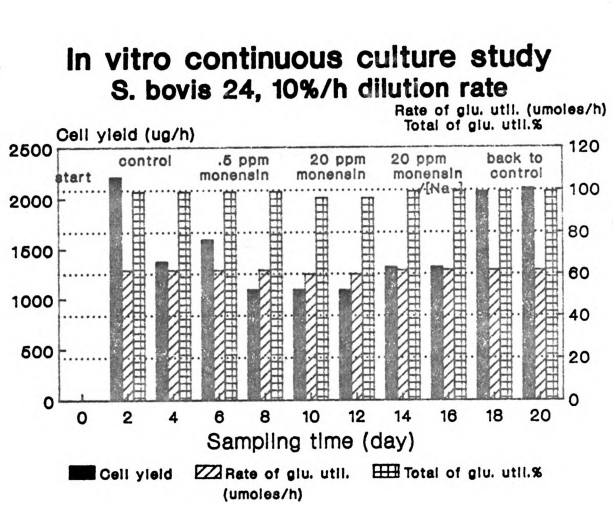


Figure 29. An Overview of S. bovis 24 Grown in 10%/h Dilution Rate Continuous Culture Various Media (Part 2)

Table 22. Physiological and Metabolic Parameters of S. bovis 24 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture*

No.[a]	Metabolic Parameters				
	RNA/ protein	Cell yield[b]	Rate of glu. util.[c]	Total of glu. util.%	Y_{glu}[d]
1	Start				
2	.43	2211.93	61.75	99.24	35.8
3	.37	1373.25	61.74	99.22	28.1
4	.18	1593.91	61.76	99.25	25.8
5	.17	1091.98	61.76	99.25	17.7
6	.15	1091.98	59.88	96.24	18.2
7	.15	1089.14	59.80	96.11	18.2
8	.18	1316.01	61.78	99.29	21.3
9	.27	1316.04	61.79	99.30	21.3
10	.27	2068.11	61.72	99.20	33.5
11	.32	2096.22	61.74	99.22	34.0

* Each value represents an average of two sets of observations.

a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.

b ug/h

c umoles/h

d g of cell/mole of glucose utilized

III. **Morphological Study of Cell Surfaces by SEM**

Since results from the study of cell surface morphology by scanning electron microscope only used one drop of culture from each bacterial sample, I do not have sufficient evidence to make any definite conclusion. However, current findings (Figures 30 to 35) show that monensin might affect the morphological structure of rumen bacteria as well as size (diameters).

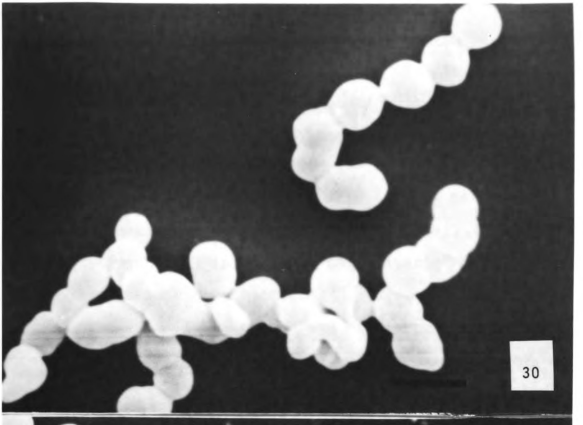
Scanning electron micrographs of S. bovis 24 grown in 5%/h dilution rate continuous culture are presented in Figures 30 and 31.

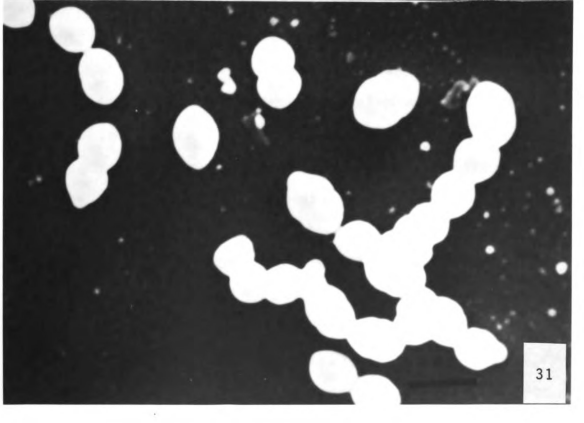
Figure 30 is a scanning electron micrograph of S. bovis 24 grown in 20 ppm monensin (harvested at day 18). A capsule layer was not observed to surround the cell surface. Since in batch culture (control, no monensin), S. bovis 24 possessed some capsular material these results indicate that monensin somehow depresses capsule formation. This result is consistent with previous finding (Fig. 15) in batch culture.

Figure 31 is a scanning electron micrograph of S. bovis 24 grown to steady state in control medium (harvested at day 30) after exposure to 0.5, 20 ppm monensin and 20 ppm monensin/[Na]. A clear capsule layer surrounding the cell surface was not observed. This finding may be due to the limited ability of S. bovis 24 to restore the capsule material, despite the rapidly increased growth rate of S. bovis 24.

Figure 30. Scanning electron micrograph of S. bovis 24 grown in 20 ppm monensin medium in a 5%/h dilution rate continuous culture (bar equals 1 um).

Figure 31. Scanning electron micrograph of S. bovis 24 grown in control medium after exposure to 20 ppm monensin/[Na] medium in a 5%/h dilution rate continuous culture (bar equals 1 um).


30


31

Scanning electron micrographs of S. bovis 24 grown at 10%/h continuous culture were in all respects identical to those from the 5%/h continuous culture study (Figures 30 and 31); therefore, these micrographs are not presented here.

From the micrographs of B. ruminicola GA33 grown in a 5%/h continuous culture (Figs. 32 and 33), I could not distinguish the individual cell clearly and their images do not look like bacteria.

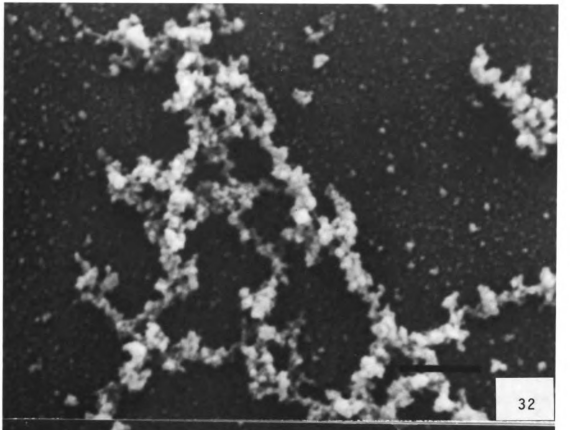
Figure 32 is a micrograph of B. ruminicola GA33 grown in control medium (harvested at day 6). The growth characteristics of B. ruminicola GA33 might be different in 5%/h continuous culture than batch culture. This present finding is consistent with a previous report (Bryant et al., 1958). These investigators found that B. ruminicola were not always encapsulated or covered with a slime layer.

Figure 33 is a micrograph of B. ruminicola GA33 grown in 20 ppm monensin medium (harvested at day 18). The cell size is smaller than the cells grown in 20 ppm monensin medium at batch culture (Fig. 19).

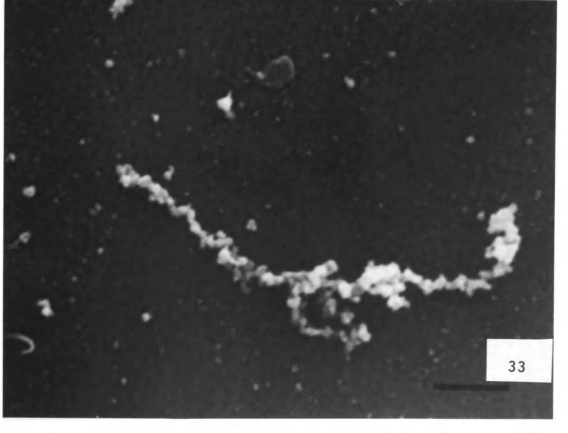
Figure 34 is a micrograph of B. ruminicola GA33 grown in 10%/h dilution rate in control medium continuous culture for 2 days. The cell size (diameter) is smaller than in cells grown in batch culture (Fig. 18); however, cell size (diameter) is larger than cells grown in 5%/h continuous culture control medium for 6 days (Fig. 32).

Figure 32. Scanning electron micrograph of B. ruminicola GA33 grown in control medium in a 5%/h dilution rate continuous culture (bar equals 1um).

Figure 33. Scanning electron micrograph of B. ruminicola GA33 grown in 20 ppm monensin medium in a 5%/h dilution rate continuous culture (bar equals 1 um).



32



33

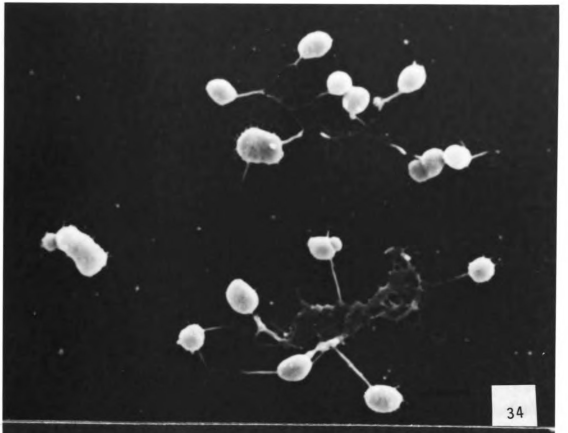
A capsule layer clearly surrounds the cell surface.

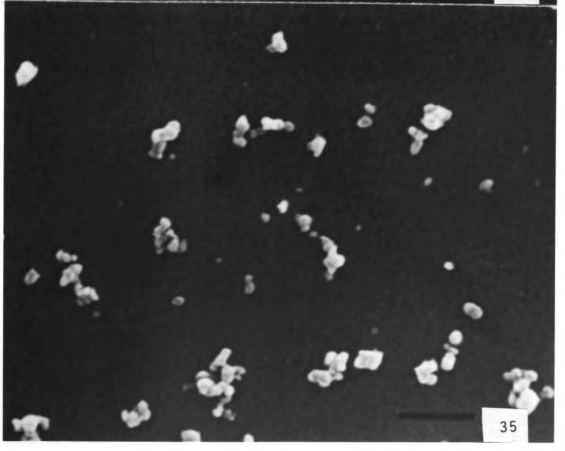
The reasons for the difference between the continuous culture at 5%/h and 10%/h dilution rate are not clear, but this finding might be related to greater nutrient availability to B. ruminicola GA33 within a unit of time at the 10%/h than during 5%/h dilution rate continuous culture.

Figure 35 is a micrograph of B. ruminicola GA33 grown in 20 ppm monensin medium at a 10%/h dilution rate (harvest at day 12). The cell size (diameter) is smaller than cells grown in control medium at 10%/h dilution rate (Figure 34). Again the capsular layer was absent in cells grown in the presence of monensin.

Figure 34. Scanning electron micrograph of B. ruminicola GA33 grown in control medium in a 10%/h dilution rate continuous culture (bar equals 1 um).

Figure 35. Scanning electron micrograph of B. ruminicola GA33 grown in 20 ppm monensin medium in a 10%/h dilution rate continuous culture (bar equals 1 um).


34


35

Experiment 3

^{14}C-Monensin Binding to Membrane Surface of Bacterial Cells

During the autoradiographic process, radioactive decay of the escaping gamma particles passing through the photographic emulsion encounter silver salts. These silver halides, after reduction to elemental silver by the developing process, are detectable in a SEM equipped with a backscatter detector (Petersen, 1984).

Cell surface binding/absorption of ^{14}C labelled monensin to S. bovis 24 and B. ruminicola GA33 cells was directly detected by both secondary electron (SE) and backscattered electron (BSE) analysis. The silver grains deposited during the autoradiographic process appeared as bright deposits on the surface of the cell membrane. In autoradiography, silver is deposited on areas of the cell membrane from which radioactive particles are emitted. The silver signal was produced after the developing process and was detected by the backscattered electron detector. This procedure is capable of distinguishing whether or not ^{14}C-monensin binds on cell membranes.

To prevent possible BSE interference from osmium (atomic number 76), osmium fixation was omitted from sample preparation. SEM samples are usually coated with a thin layer of gold to make the sample conductive, so

that a charge does not build up. However, in SEM-AR, specimens were not coated with gold (atomic number 79) before viewing, since gold would interfere with the detection of silver which atomic number is 47 (Petersen, 1984). A layer of gold coating on the specimen surface could physically block detection of silver, as well as mask the presence of silver signal by its own backscattered electrons signal. Instead of gold coating, specimens were coated with a layer of carbon to overcome charging.

I. Autoradiography Study

Results from cultures of S. bovis 24 and B. ruminicola GA33 incubated with ^{14}C labelled monensin showed that ^{14}C-monensin does bind to both gram positive and gram negative bacterial cell membranes. Micrographs of S. bovis 24 and B. ruminicola GA33 grown with and without ^{14}C-monensin addition are presented in Figures 36 to 39.

A. B. ruminicola GA33

B. ruminicola GA33 was grown in the control medium and upon reaching late log phase, .5 ppm ^{14}C-monensin was added to the culture for 60 min. The culture was sampled and an autoradiographic study was carried out to determine monensin binding to cell surfaces of the bacteria. After developing 21 days, the bacteria were viewed with SEM.

Figure 36 is a scanning electron micrograph of B.

ruminicola GA33 grown in control medium and examined by secondary electron signal. In this micrograph it is clear that the secondary electron signal enabled visualization of the cells. It was not possible to observe cells using the backscattered signal from the BSE micrograph (Fig. 37). The contrast of Fig. 37 is too high and the background color should be black.

Figure 38 is a scanning electron micrograph of B. ruminicola GA33 exposed to 0.5 ppm ^{14}C-monensin. There are a couple of cells image that appear under secondary electron signal detection. The images of B. ruminicola GA33 from BSE detector (Fig. 39) are difficult for comparison of cells and silver spots.

The net apparent binding activity of ^{14}C-monensin to B. ruminicola GA33 cell membrane was 54.5% (of total counts per minute) from preliminary study (Appendix 9). The apparent binding of ^{14}C-monensin to S. bovis 24 was 57% (of total counts per minute).

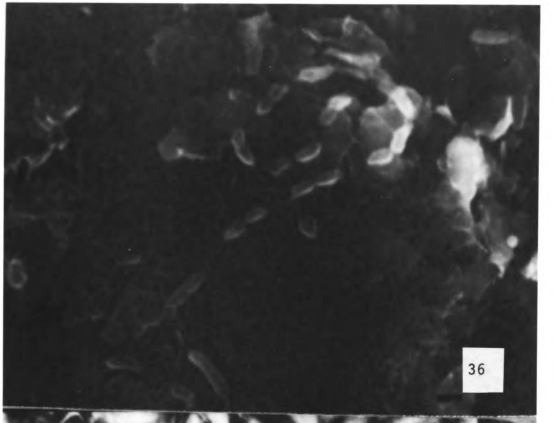
B. **S. bovis 24**

S. bovis 24 was grown to the late log phase in control medium; 0.5 ppm ^{14}C- monensin was then added to the culture as for B. ruminicola GA33 but was then incubated for only 30 min. The S. bovis 24 cells were then harvested followed by the emulsion developing procedure for 28 days (Appendix 9).

A specific cellular location site of ^{14}C-monensin was not found by scanning electron microscopy/

Figure 36. Secondary electron image of B. ruminicola GA33 grown in pH 6.8 control medium (bar equals 1 um).

Figure 37. Backscattered electron image of B. ruminicola GA33 grown in pH 6.8 control medium (an arrow points out cells, bar equals 1 um)


36

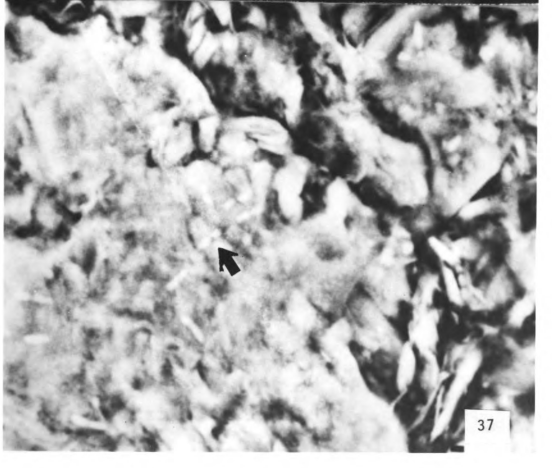
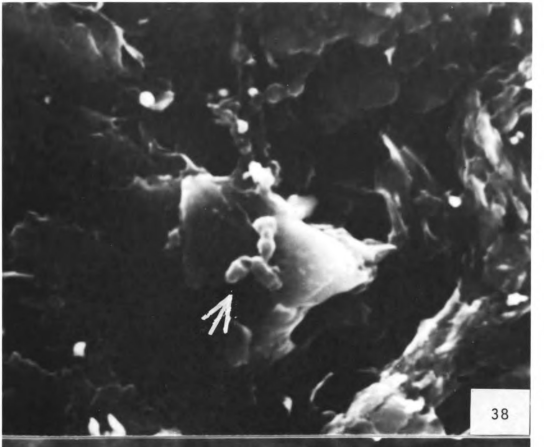
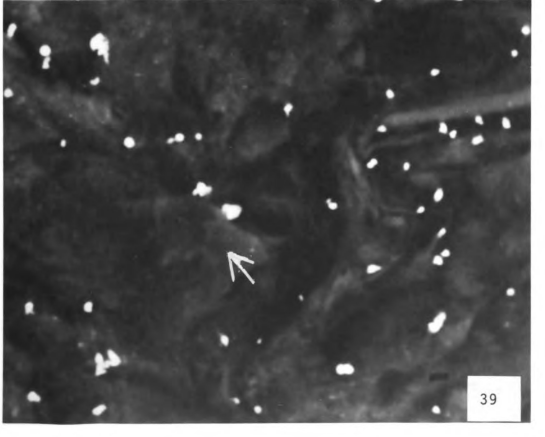

37

Figure 38. Secondary electron image of B. ruminicola GA33 grown in pH 6.8 0.5 ppm ^{14}C-monensin medium (an arrow points out cells, bar equals 1 um).

Figure 39. Backscattered electron image of B. ruminicola GA33 grown in pH 6.8 0.5 ppm ^{14}C-monensin medium (an arrow points out cells, bar equals 1 um).



38



39

autoradiography. Both images of S. bovis 24 from SE signal and BSE studies are not shown in this dissertation.

SUMMARY AND CONCLUSION

This dissertation work was initiated to determine whether a series of transfers of cells grown under monensin treated conditions would affect cell growth. Results from an *in vitro* batch culture adaptation study showed that the cell growth of all four species rumen bacteria examined did not improve after a series of six transfers. Of the four species of typical rumen bacteria chosen for this research, *S. ruminantium* HD4 cell growth was the most stable no matter how variable the media composition was in sodium concentration, monensin levels or the length of exposure time.

The metabolic parameters such as RNA/protein, rate of glucose utilization, cell yield and Y_{glu} were generally lower in organisms during monensin treated conditions than control. Virtually complete substrate utilization by these organisms indicates that they were able to catabolize glucose and grow at some rate. When exposed to monensin, cellular RNA/protein generally declined indicating a depressed growth rate. Since substrate catabolism by bacteria was not depressed by any treatment, energy must either have been used for cellular maintenance or somehow wasted during monensin treatment periods. The high concentration of monensin decreased the maximum cell yields or increased the lag times in cultures of organisms but did not completely

inhibit the growth of these species. The long lag time means that the organism was initially inhibited by monensin and eventually grew, but total growth was also much less.

In the present study, the antimicrobial activity of monensin was sometimes apparently reversed in the presence of a higher [sodium] concentration. The continuous culture experiments, for the most part, produced similar conclusions when compared to the batch culture studies. S. bovis 24 was able to grow sequentially under 0.5 ppm monensin medium first, then at 20 ppm monensin with or without higher [sodium] medium, and eventually back to control medium.

This dissertation also details some practical methods for checking organisms morphological changes by using SEM. Monensin might be able to influence monensin sensitive bacteria by removing the protective layer from the outer cell surface or by reducing the thickness of the layer. At this juncture there is no mechanistic explanation of these results.

Finally, the study of ^{14}C-monensin binding to membrane surfaces of S. bovis 24 and B. ruminicola GA33 shows that apparent binding of ^{14}C-monensin was about 50% for both organisms. A specific cellular location site by scanning electron microscopy/autoradiography was not found. Much work remains to be done to unravel the complexities and interesting interrelationships between

ionophore (monensin) and factors that control bacterial growth in the rumen.

APPENDICES

Appendix 1

A Calculation to Obtain 200 mmol/l [Na]

Reagent	Source	Amount	Real amount [Na]/100 ml medium
NaCl	Medium	1.2 g/ 100 ml solution II	0.0177 g
Na_2CO_3	Medium	0.8 g/ 100 ml medium	0.347 g
Sum			0.3648 g (158.6 mmol/l)
Additional NaCl to reach 200 mmol/l [Na]			0.2418 g

Determination of [Na] by AA Spectrophotometer

Medium	Dilution	By Analysis (ppm)	Total (ppm)	[Sodium] mmol/l
Control	6,000x	.636	3816	165
control/[Na] 20 ppm	5,500x	.821	4515	196.3
Monensin 20 ppm	6,000x	.647	3882	168.8
Monensin/[Na]	5,500x	.854	4697	204.2

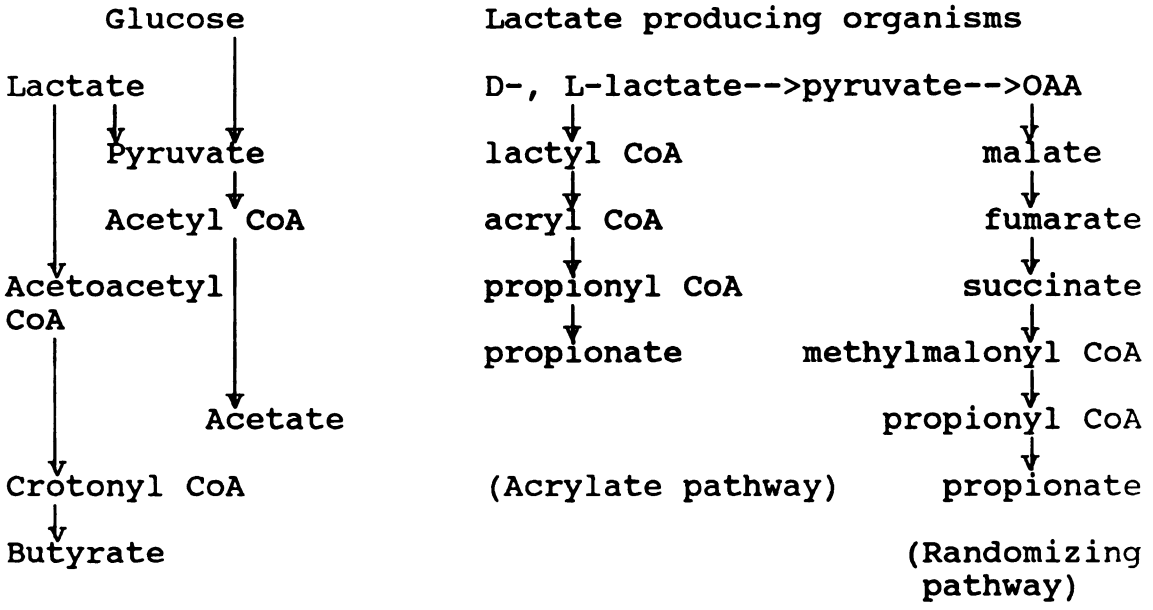
Appendix 2

The Fermentation End Products and the Pathways

Fermentation End Products of Four Representative Ruminal Bacteria

Species	Gram type	End products
S. bovis	Gram positive coccus	Lactic (acetic, formic)
L. vitulinus	Gram positive rod	D-lactic (acetic)
B. ruminicola	Gram negative rod	Succinic, acetic (formic, propionic, butyric, isobutyric isovaleric, lactic)
S. ruminantium	Gram negative rod	Lactic, propionic, acetic, (succinic)

The Fermentation Pathways



Appendix 3

Fermentation Products Determination

Identification and quantification of VFA and non-VFA fermentation acids was accomplished with a gas-liquid chromatograph (model 5840A, Hewlett-Packard Avondale, PA 19311) equipped with auto injector and hydrogen flame ionization detector. Fatty acid butyl esters were separated on a coiled column (183 cm x 0.32 cm) packed with Chromosorb W (80/100 mesh, HP, DMCS, AW) coated with 10% Dexsil 300 GC (Supelco Inc., Supelco Park, Belefonte, PA 16823, Appendix 4). The column was conditioned prior to use by heating to 250^0C overnight under helium as carrier gas.

The following additional chromatographic conditions were employed throughout this study. Gas flow rates of 84, 260 and 50 ml/min for H_2, air and He respectively; injection port temperature, 230^0C; detector temperature, 270^0C and the following temperature program: initial column bath was set at 40^0C for 3 min followed by a 10^0C/min temperature increase to 240^0C and a constant 240^0C for 5 min. The total time for chromatographic separation and integration of each sample was 28 min. A fifteen min column bath cooling period followed after the last ester was detected and integrated. The preparation of fatty acid butyl esters for both standards and sample are described below.

A standard fatty acid mixture (Supelco #46975 and #46985) containing 10 mM of acetic, propionic, butyric and lactic acids, 5 mM of succinic acid and individual acid standards at the same concentration as in standard mixture were prepared in distilled water and the pH was adjusted to 9 to 10 with 10 N NaOH. All standards were made alkaline to convert the free acids to the ionized species and thus prevent their loss during subsequent lyophilization.

Rumen anaerobic bacteria cultures were centrifuged (in a RC-5 Superspeed refrigerated centrifuge, Sorvall, DuPont Instruments) at 12,000 X g for 10 min to remove cells and the supernatant (1 ml) was treated in the same way as the standard acid mixture.

One ml of each ionized fatty acid mixture (standards or samples) was placed in a culture tube, frozen in a freezer and dried overnight on a continuous freeze dryer. Chloroform (1.6 ml) and 0.4 ml of 1-butanol saturated with anhydrous HCl were added to the dry acid salts.

After mixing on a Vortex spinner, tubes were tightly capped and sealed with a layer of Teflon tape. The mixture was then heated at 80^{0}C in a water bath for 2 hr. Tubes were then cooled to room temperature and 0.4 ml of trifluoroacetic anhydride (TFA) was added per tube. The solution was mixed and allowed to react for 1 hr. TFA was used to react with hydroxy acids forming the trifluoroacetyl esters; excess butanol in the reaction

mixture was also reacted with TFA.

Samples were then washed twice with 1 ml deionized water to remove excess TFA reagent and the water layer was discarded. The chloroform layer which contained the butyl esters was placed into 1.5 ml GLC vial and the vial cap was sealed. Two microliters of sample was injected and analyzed by GLC.

Appendix 4

Column for GLC Analysis of Fermentation Acids

Packing Material

Packing material was Chromosorb W (80 to 100 mesh, HP, DMCS, AW) coated with 10% Dexsil 300 GC (Analabs, North Haven, Conn.).

Dexsil 300 GC (Olin Corp.) is polycarboranesiloxane polymer. Upon reaching stationary phase of moderate polarity, Dexsil 300 GC is stabilized against thermal degradation. The maximum temperature limit of Dexsil 300 GC is 450 to 500 ^{o}C.

Appendix 5

Determination of DNA, RNA and Protein in Bacterial Cells

Reagents' Recipes

1. Acetaldehyde solution : 0.4 ml per 250 ml deionized H_2O, store at 4^0C.
2. Bio-Rad protein kit: one bottle containing 450 ml solution of dye, phosphoric acid and methanol.
3. Diphenylamine reagent : 4 g diphenylamine per 100 ml glacial acetic acid.
4. Perchloric acid (PCA): 1%, 2.5%, 5%, and 10%, store at 4^0C.
5. DNA standard: 5 mg DNA (Type I, from Sigma) per 50 ml deionized H_2O, make a 10% solution with concentrated Perchloric acid (70%).
6. Ferric chloride solution: 1 g $FeCl_3 \cdot 6H_2O$ per 1 liter concentrated (37%) HCl, store at room temperature.
7. NaOH: 1.0 N, 40 g NaOH per 1 liter deionized H_2O, store at room temperature.
8. Orcinol reagent: 1 g orcinol (5-methyl resorcinol) monohydrate per 100 ml 0.1% $FeCl_3$-HCl solution.
9. KOH: 0.3 N, 16.8 g KOH per 1 liter deionized H_2O, store at room temperature.
10. RNA standard: 5 mg RNA (Type IV, from Sigma Co.,) per 50 ml deionized H_2O, make a 5% solution with concentrated PCA (70%).

Principle of Bio-Rad Protein Assay

The Bio-Rad protein assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm, when binding to protein occurs. The extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Over a broader range of protein concentrations, the dye-binding method gives an accurate, but not entirely linear response.

Procedures for RNA, DNA and protein analysis

1. Five ml cold 2.5% PCA was added to 30 ml of culture sample, to a centrifuge tube, and the sample was placed on ice for at least 30 min. The sample was vortexed and centrifuged (in a Sorvall RC-5 superspeed refrigerated centrifuge) at 12,000 x g for 10 min.
2. The supernatant was discarded and the precipitate was an intact pellet. This pellet was washed with 5 ml cold 1% PCA and the centrifugation process repeated.
3. The final pellet was hydrolyzed in 4 ml of 0.3 N KOH, at 95^{0}C for 30 min with marbles covering the tubes.
4. After cooling, 2.4 ml cold 5% PCA was added to the hydrolysate, the mixed solution was vortexed and placed on ice for 15 min.
5. The centrifugation process was repeated. The

supernatant was then carefully removed with a Pasteur pipette into 15 ml graduated tube for the RNA fraction.

6. The pellet was washed with 2.5 ml cold 5% PCA and the centrifugation process was repeated. The supernatant was added to RNA fraction which was then made up to 10 ml with 5% PCA for RNA analysis.
7. The remaining (RNA free) pellet was hydrolyzed with 2.5 ml of 10% PCA at 70^{0}C for 25 min. After incubation the hydrolysate was vortexed and placed on ice.
8. The centrifugation process was repeated. The supernatant was carefully removed with a Pasteur pipette into a 15 ml graduate tubes for DNA fraction.
9. The pellet was washed with 2.5 ml cold 10% PCA. The centrifugation process was repeated and the supernatant was combined with the previous DNA fraction and made up to 6 ml with 10% PCA and used for DNA analysis.
10. The remaining (DNA free) pellet was solubilized with 3 ml 1.0 N NaOH at 55 ^{0}C for 1 hr and analyzed for protein by the Bio-Rad protein assay.
11. For RNA analysis, 2 ml of supernatant from step 6 were pipetted into test tubes in duplicates. Standard concentrations were 6.25, 12.5, 37.5 and 50 ug/ml and were made up in duplicate as follows:

Concentration (ug/ml)	Vol. of RNA standard (ml)	Vol. of 5% PCA (ml)
0	0	2.00
6.25	0.25	1.75
12.5	0.50	1.50
25	1.00	1.00
37.50	1.50	0.50
50.0	2.00	0

2 ml of orcinol reagent solution were added to each tube, marbles were placed on tubes and tubes were incubated in a boiling water bath for 30 min. After cooling, the samples were read with a Bausch & Lomb Spectronic 70 at 680 nm.

12. For DNA analysis, 2 ml of supernatant from step 9 were pipetted into test tubes in duplicates. Standards were the same concentration as RNA standards and were made in the same manner except that 10 % PCA was used rather than 5 % PCA. Two ml of the 4 % diphenylamine reagent were added to each tube. One tenth ml acetaldehyde solution was added to each tube and mixed. A marble was placed on each tube and incubated at 30^{0}C for 16 hr. After cooling, the samples were read with a Bausch & Lomb Spectronic 70 at 595 nm.
13. For protein analysis, the Bio-Rad protein kit was used. Bovine serum albumin served as protein standard. One ml of supernatant from step 10 was

pipetted into test tubes in duplicates. Two tenth ml of Bio-Rad kit reagent and 2 ml 1.0 N NaOH were added in both standards and samples. After 10 min. at room temperature, samples were read in a Bausch & Lomb Spectronic 70 at 595 nm.

Appendix 6

Procedures of Sample Preparation for SEM Study

1. One ml of culture broth was combined with 1 ml 5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) in a test tube and the sample was then placed on ice for at least 1.5 hrs.
2. One drop of 1% poly-L-lysine was put on a plastic petri dish, a glass coverslip was placed on the drop for 5 min.
3. The coverslip was removed and washed with several drops of H_2O and drained.
4. One drop of culture was fixed on the coverslip (the side which had previously faced down) for 5 min.
5. The coverslip was washed with H_2O and was put in multiple coverslip holder with the culture fixed side up.
6. The holder was dehydrated through a graded ethanol series, i.e., 25%, 50%, 75%, 95% and 100% for 5 min in each step and the 100% ethanol step was changed three times.
7. After dehydration, the holder was transferred to the critical point dryer for drying.
8. After drying, the coverslip was taken out of the holder and mounted on a stub, and was sputter coated with gold.

Appendix 7

Chemostat theory

A continuous chemostat culture represents a system of studying growth regulation of bacteria in steady state at given growth rate (u) by varying the dilution rate (D) and maintaining constant cell concentrations (Bergen et al., 1982). In a chemostat u depends on the concentration of a limiting growth substrate (S) in the culture medium. When (S) is kept low in the continuous culture, the rate of medium flow (D) regulates u and at steady state, u = D (Herbert et al., 1956). The equations below are applicable as a mathematical formulation of chemostat cultures (Pirt, 1965, 1975).

$$1/Y_{glu} = 1/Y_{glu}^{Max} + M_s/u \qquad (1)$$

$$1/Y_{ATP} = 1/Y_{ATP}^{Max} + M_e/u \qquad (2)$$

Pirt (1965, 1975) derived these equations which relate molar growth yield for glucose (Y_{glu}) and specific growth rate during steady state (u = D), assuming that the consumption of the energy source (or ATP) is partly growth dependent and partly growth independent.

In equation 2, M_e indicates growth dependent and growth independent energy needs. The molar growth yield Y_{ATP} is dependent on u or D. At low u, a greater proportion of the available ATP is utilized for

maintenance.

The type of apparatus considered is the "chemostat"; bacteria are grown in a culture vessel or "fermenter" into which sterile growth medium is at a steady flow rate (f) and from which bacterial culture emerges at the same rate, a constant level device keeping the volume (v) of culture in the fermenter constant. The culture in the fermenter is stirred and temperature is automatically controlled.

For any dilution rate, the culture automatically adjusts itself to a steady state in which the concentrations of microorganisms and nutrients in the culture remain constant, so long as the composition and flow-rate of the incoming medium remain unaltered. In such a steady state the growing rate of the organism (u) must be equal to the dilution rate (D). The stability of the system is due to the fact that it is essentially substrate controlled.

A chemostat is a device for controlling the growth rate through control of the steady state substrate concentration; at each dilution rate substrate concentration is fixed at a value which makes u equal to D.

Successful chemostat operation include obligatory equipment, e. g. temperature control, redox control, provision for replication and ease of measurement as well as the optional parts, efficiency of stirring, removal of

products, provision for measuring gaseous exchanges and provision for sterile condition. The continuous flow type is often referred to an artificial rumen (Hobson, 1965a; Czerkawski, 1976).

The duration for the artificial rumen can be short as 2 - 8 hr, intermediate as to 24 hr, or long to days.

In this study, all joints of the apparatus are constituted of ground glass and rubber tubing was heavy wall butyl rubber and all rubber to glass joints were taped and wired on.

Appendix 8

Autoradiographic Technology

Preliminary Observations

^{14}C-Monensin was a kind gift from Lilly Research Laboratories (Eli Lilly & Co.). Samples were harvested at 10 min, 30 min and 60 min intervals for assessment of specific binding activity with scintillation counter (Isocap/300 6872 liquid scintillation system, Searle Analytic Inc.) which was equipped with Texas instruments and Silent 700 ASR electronic data terminal.

Fifteen ml of culture sample was put in a centrifuge tube and the sample was placed on ice for at least 30 min. The sample was vortexed and centrifuged (in a Sorvall RC-5 Superspeed Refrigerated Centrifuge) at 12,000 X g for 10 min. Ten ml of scintillation solution was added in scintillation vial and one ml of supernatant or intact pellet was separately put in each vial.

The counts per minute of both liquid (free phase) and pellet (bound phase) against time interval are shown below, where "I" represents ionophore monensin. From the radioactive counting results, the best incubation intervals for B. ruminicola GA33 in the basal medium (with .08% glucose W/V) were 30 min and 60 min, and 30 min for S. bovis 24.

Species	Free	Bound	Free	Bound	Free	Bound
	10 min		30 min		60 min	
	--- counts per minute ---					
B. ruminicola GA33						
.2% glucose basal medium						
.5 ppm I	305	853	640	1287	212	1919
Total	1158		1927		2131	
Label%	73.7		66.8		91	
.08% glucose basal medium						
.5 ppm I	327	377	445	530	590	1126
Total	704		975		1716	
Label%	53.5		54.3		65.6	
.2% glucose basal medium						
20 ppm I	12267	7870	15217	10907	16158	13308
Total	20137		26124		29466	
Label%	33.8		33		33.5	
S. bovis 24						
.08% glucose basal medium						
.5 ppm I	-	-	225	304	320	245
Total	-		529		565	
Label%	-		57		43	

Autoradiographic Study

Autoradiographic studies were carried out according to the following procedures: Batch cultures of S. bovis 24 and B. ruminicola GA33 were incubated with ^{14}C-labelled monensin. The organisms were then fixed with 4% glutaraldehyde and one drop of broth was put on the top of 1% poly-L-lysine fixed carbon planchette for 10 min. The dehydration and critical point dry processes were as outlined in Appendix 5. After mounting in a stub, the above samples were coated with a thin layer of carbon in a vacuum evaporator. After carbon coating, the sample stub was coated with a layer of liquid nuclear photographic emulsion (Kodak NTB3) in dark room and

stored in dark at 4^0C for about one month in order to develop.

The incubation time of B. ruminicola GA33 and S. bovis 24 with ^{14}C-Monensin and the emulsion developing time are listed as below.

Species	Sample No.	Emulsion Developing Periods (days)	Incubation Time (min.)	Amounts (ml)
B. ruminicola	GA33 1	7	30	1
	2	17	30	2
	5	21	30	3
	6	28	30	1
	10	28	60	1.5
	12	28	30	2
S. bovis	24 4	7	30	3
	3	17	30	2
	7	21	60	1
	8	28	30	1
	9	28	60	2
	11	28	30	1.5

The incubation intervals of the six culture sample of B. ruminicola GA33 and S. bovis 24 were 30 min or 60 min. The emulsion developing periods were 7, 17, 21 and 28 d. The culture sample's volume was either 1, 1.5, 2 or 3 ml.

The developing of the autoradiogram was completed in dark room by immersing the entire stub in a beaker of half - strength Kodak D-19 for 3 min without agitation.

The stub was then immersed in a beaker with

distilled water for 10 sec, into stop solution for 3 min, into distilled water for 10 sec, into fixing solution for 3 min, and into distilled water for 10 sec twice.

The stubs were drained on their sides, allowed to air dry about 1 hr, and coated with a thin layer of carbon in a rotary vacuum evaporator. After coating, the stub was viewed by JEOL JSM-35C SEM.

Principle of Backscattered Electrons

High energy backscattered electrons are usually utilized since this process tends to give better resolution than secondary electrons. The specific reasons for this are as follows:

The backscattered electron signal is capable improving the spatial resolution. Since the farther an electron travels in the specimen from the primary impact point, the greater will be its loss of energy. The backscattered electrons which have lost only about 1% of their incident energy, e. g. low-loss electron, can only have traveled a few nanometer before being scattered out of the specimen.

The electron detector is placed in the forward scattering direction to maximize collection of the desirable portion of the signal. Images from this detector system, coupled with a high brightness electron gun, show some of the finest structural detail from solid objects may obtained by SEM.

The solid state detector for backscattered electron

also contributes to the high-resolution imaging. Since this detector produces a signal proportional to the electron energy and produce no response below a cutoff energy, it provides some emphasis on the high energy fraction of the signal which is desirable for high-resolution imaging.

Appendix 9

Appendix Tables

Table 1. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.725	.75	.725	.7	.7	.725
Time to reach max. O.D.(h)	4.0	3.0	4.0	4.0	4.0	4.0
Final pH	5.15	4.8	4.7	4.7	4.9	4.7

Table 2. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.7	.7	.7	.56	.7	.7
Time to reach max. O.D.(h)	6.0	3.0	4.5	2.0	5.0	2.5
Final pH	6.0	6.0	6.0	6.25	6.05	6.15

Table 3. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 .5 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.38	.32	.32	.33	.33	.36
Time to reach max. O.D.(h)	9.5	10.0	9.0	15.0	5.0	5.5
Final pH	6.05	6.15	6.15	6.15	6.1	6.1

Table 4. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin to 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.15	.125	.12	.12	.1	.125
Time to reach max. O.D.(h)	26.0	33.0	18.0	22.0	23.0	17.0
Final pH	6.25	6.2	6.2	6.15	6.15	6.25

Table 5. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from 20 ppm Monensin Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.45	.6	.72	.63	.7	.7
Time to reach max. O.D.(h)	14.5	13.0	13.0	12.5	12.0	13.5
Final pH	6.15	6.15	6.1	6.1	6.15	6.1

Table 6. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin Medium to 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.16	.14	.1	.11	.12	.18
Time to reach max. O.D.(h)	27.0	31.0	23.0	21.0	33.0	33.0
Final pH	6.25	6.1	6.15	6.1	6.1	6.1

Table 7. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from 20 ppm Monensin/[Na] Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.56	.6	.6	.6	.6	.7
Time to reach max. O.D.(h)	13.0	12.5	12.0	12.5	13.0	13.0
Final pH	6.05	6.1	6.05	6.15	6.1	6.15

Table 8. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 .5 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.22	.14	.17	.22	.165	.21
Time to reach max. O.D.(h)	12.0	15.0	13.5	17.5	17.5	16.5
Final pH	6.05	6.05	6.15	6.15	6.05	6.0

Table 9. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin/[Na] Medium to 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.14	.1	.08	.08	.11	.12
Time to reach max. O.D.(h)	22.0	27.0	27.0	27.0	18.0	21.0
Final pH	6.2	6.15	6.15	6.0	6.05	6.05

Table 10. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin/[Na] Medium to 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.1	.1	.06	.085	.16	.12
Time to reach max. O.D.(h)	23.0	18.0	19.0	23.0	27.0	28.0
Final pH	6.2	6.2	6.1	6.0	6.2	6.15

Table 11. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown from pH 6.8 20 ppm Monensin Medium to pH 7.6 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.2	.21	.22	.22	.21	.21
Time to reach max. O.D.(h)	27.5	14.0	7.5	9.5	7.5	10.5
Final pH	6.55	6.55	6.6	6.55	6.75	6.65

Table 12. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 7.6 from 20 ppm Monensin Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.29	.42	.38	.315	.39	.4
Time to reach max. O.D.(h)	5.0	2.5	3.0	4.0	4.0	3.0
Final pH	6.75	6.65	6.7	6.65	6.7	6.75

Table 13. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown from pH 6.8 20 ppm Monensin Medium to pH 7.6 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.16	.15	.155	.16	.2	.18
Time to reach max. O.D.(h)	22.0	17.0	21.0	22.0	17.0	16.0
Final pH	6.65	6.65	6.65	6.55	6.65	6.65

Table 14. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown from pH 7.6 20 ppm Monensin/[Na] Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.38	.4	.37	.4	.46	.42
Time to reach max. O.D.(h)	3.0	2.5	2.5	3.0	2.5	3.0
Final pH	6.7	6.65	6.75	6.75	6.7	6.75

Table 15. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 7.6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.47	.45	.38	.38	.40	.36
Time to reach max. O.D.(h)	4.5	2.0	2.5	2.0	2.0	2.0
Final pH	6.5	6.5	6.5	6.5	6.5	6.65

Table 16. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 7.6 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.15	.12	.12	.145	.13	.12
Time to reach max. O.D.(h)	12.0	17.5	17.0	17.0	16.0	16.5
Final pH	6.5	6.55	6.55	6.65	6.55	6.5

Table 17. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown from pH 7.6 20 ppm Monensin Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.4	.54	.58	.59	.62	.57
Time to reach max. O.D.(h)	4.0	4.0	2.0	4.0	3.0	3.0
Final pH	6.65	6.65	6.6	6.6	6.65	6.55

Table 18. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 7.6 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.175	.11	.125	.12	.12	.125
Time to reach max. O.D.(h)	17.5	22.0	21.5	19.0	19.0	19.0
Final pH	6.5	6.5	6.55	6.45	6.65	6.6

Table 19. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. bovis 24 Grown in pH 7.6 from 20 ppm Monensin/[Na] Medium to Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.27	.5	.6	.64	.59	.66
Time to reach max. O.D.(h)	8.0	8.0	7.0	7.0	5.5	5.0
Final pH	6.6	6.5	6.65	6.6	6.6	6.65

Table 20. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of L. vitulinus B62 Grown in pH 6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.3	.5	.6	.4	.6	.42
Time to reach max. O.D.(h)	8.5	11.5	8.5	11.5	8.5	11.5
Final pH	4.9	4.7	4.8	4.9	5.0	4.9

Table 21. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of L. vitulinus B62 Grown in pH 6.8 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.24	.19	.25	.085	.4	.22
Time to reach max. O.D.(h)	8.0	11.5	8.0	11.5	6.5	6.5
Final pH	5.95	6.05	6.05	6.1	6.05	6.15

Table 22. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of L. vitulinus B62 Grown in pH 6.8 .5 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.11	.16	.2	.275	.27	.15
Time to reach max. O.D.(h)	30.0	27.0	25.0	23.0	20.0	19.0
Final pH	6.1	6.2	6.1	6.2	6.1	6.2

Table 23. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of L. vitulinus B62 Grown in pH 7.6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.13	.08	.16	.05	.08	.21
Time to reach max. O.D.(h)	8.0	12.0	8.0	14.0	6.5	6.5
Final pH	6.5	6.6	6.5	6.5	6.55	6.55

Table 24. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of L. vitulinus B62 Grown in pH 7.6 .5 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.035	.04	.04	.06	.05	.06
Time to reach max. O.D.(h)	32.0	40.0	40.0	21.0	15.0	15.0
Final pH	6.15	6.25	6.15	6.15	6.2	6.35

Table 25. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	1.0	.975	1.1	1.1	1.1	1.1
Time to reach max. O.D.(h)	8.5	10.5	9.0	10.5	10.0	10.5
Final pH	5.6	5.0	5.0	5.1	5.0	5.0

Table 26. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.975	.68	.9	.875	.75	.55
Time to reach max. O.D.(h)	8.5	11.5	8.5	10.5	10.0	16.0
Final pH	5.8	5.3	5.3	5.5	5.8	5.3

Table 27. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.9	.64	.85	.75	.775	.55
Time to reach max. O.D.(h)	8.5	11.5	8.5	10.5	10.0	16.0
Final pH	5.7	5.2	5.2	5.3	5.5	5.3

Table 28. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6.8 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	1.25	.75	1.25	.64	.9	.66
Time to reach max. O.D.(h)	4.0	5.0	5.5	5.5	5.0	5.5
Final pH	5.95	6.05	6.0	6.15	6.05	6.1

Table 29. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6.8 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.6	.9	.9	1.1	.75	1.0
Time to reach max. O.D.(h)	7.5	9.0	9.0	9.0	9.0	7.5
Final pH	6.15	6.2	6.25	6.25	6.15	6.15

Table 30. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 6.8 20 ppm Monensin/[Na] medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.66	.85	.8	.975	.67	.95
Time to reach max. O.D.(h)	15.5	8.5	10.0	9.0	12.0	7.5
Final pH	6.05	6.15	6.2	6.15	6.2	6.2

Table 31. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 7.6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.85	.4	.75	.37	.64	.64
Time to reach max. O.D.(h)	3.0	5.5	5.5	5.5	5.0	5.5
Final pH	6.5	6.5	6.5	6.5	6.5	6.55

Table 32. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 7.6 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	1.05	.7	.6	1.0	.85	.68
Time to reach max. O.D.(h)	7.0	12.0	15.0	9.5	9.0	11.5
Final pH	6.55	6.55	6.45	6.5	6.6	6.55

Table 33. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of S. ruminantium HD4 Grown in pH 7.6 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	1.05	.7	.62	.9	.95	.7
Time to reach max. O.D.(h)	7.0	12.0	15.0	9.5	8.5	11.5
Final pH	6.5	6.55	6.5	6.45	6.55	6.5

Table 34. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.35	.145	.195	.95	1.1	.8
Time to reach max. O.D.(h)	41.0	19.0	24.0	53.0	50.0	32.0
Final pH	5.5	5.6	5.9	5.1	5.1	5.3

Table 35. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 6.8 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.7	.63	.6	.75	.6	.64
Time to reach max. O.D.(h)	19.5	18.5	15.0	17.0	16.5	16.5
Final pH	6.05	6.1	6.15	6.05	6.1	6.05

Table 36. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 6.8 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.105	.75	.1	.56	.725	.85
Time to reach max. O.D.(h)	36.0	49.0	26.0	60.0	21.0	48.0
Final pH	6.25	6.2	6.25	6.15	6.2	6.2

Table 37. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 6.8 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.08	.1	.6	.62	.825	.7
Time to reach max. O.D.(h)	12.0	14.5	25.0	34.0	21.5	19.0
Final pH	6.25	6.25	6.15	6.15	6.15	6.15

Table 38. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 7.6 Control Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.42	.225	.31	.3	.34	.36
Time to reach max. O.D.(h)	17.5	18.5	18.5	18.5	13.0	18.5
Final pH	6.5	6.6	6.65	6.5	6.55	6.6

Table 39. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 7.6 20 ppm Monensin Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.46	.45	.435	.5	.39	.41
Time to reach max. O.D.(h)	19.5	25.5	19.5	20.0	18.5	21.5
Final pH	6.55	6.55	6.45	6.5	6.6	6.55

Table 40. Maximum O.D., Time to Reach Max. O.D. and Final pH of Six Passages of B. ruminicola GA33 Grown in pH 7.6 20 ppm Monensin/[Na] Medium

	Passages					
	1	2	3	4	5	6
Max. O.D.	.28	.11	.06	.29	.39	.4
Time to reach max. O.D.(h)	20.0	20.0	20.0	41.0	40.0	30.0
Final pH	6.6	6.65	6.7	6.65	6.75	6.7

Table 41. Absorbance, Sampling Time and Final pH of B. ruminicola GA33 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture

Sampling Sequence	Medium	Absorbance	Sampling Time (day)	Final pH
1	start	.50	0	6.6
		.50		6.6
2	control	.20	3	6.6
		.16		6.4
3	control	.15	6	6.5
		.10		6.5
4	.5 ppm monensin	.08	9	6.4
		.06		6.5
5	.5 ppm monensin	.11	12	6.5
		.05		6.5
6	20 ppm monensin	.07	15	6.5
		.05		6.5
7	20 ppm monensin	.04	18	6.4
		.04		6.6
8	20 ppm monensin/[Na]	.10	21	6.5
		.12		6.5
9	20 ppm monensin/[Na]	.18	24	6.6
		.12		6.6
10	back to control	.29	27	6.6
		.29		6.6
11	back to control	.15	30	6.5
		.21		6.5

Table 42. Absorbance, Sampling Time and Final pH of B. ruminicola GA33 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture

Sampling Sequence	Medium	Absorbance	Sampling Time (day)	Final pH
1	start	.65	0	6.8
		.55		6.8
2	control	.42	2	6.5
		.22		6.7
3	control	.16	4	6.6
		.28		6.6
4	.5 ppm monensin	.10	6	6.5
		.10		6.5
5	.5 ppm monensin	.13	8	6.5
		.10		6.5
6	20 ppm monensin	.04	10	6.5
		.10		6.5
7	20 ppm monensin	.12	12	6.5
		.08		6.5
8	20 ppm monensin/[Na]	.06	14	6.5
		.10		6.5
9	20 ppm monensin/[Na]	.09	16	6.5
		.09		6.5
10	back to control	.16	18	6.5
		.31		6.5
11	back to control	.42	20	6.5
		.24		6.5

Table 43. Absorbance, Sampling Time and Final pH of S. bovis 24 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture

Sampling Sequence	Medium	Absorbance	Sampling Time (day)	Final pH
1	start	.5	0	6.8
		.6		6.8
2	control	.20	3	6.7
		.26		6.5
3	control	.21	6	6.5
		.25		6.5
4	.5 ppm monensin	.17	9	6.65
		.11		6.65
5	.5 ppm monensin	.19	12	6.6
		.13		6.6
6	20 ppm monensin	.08	15	6.5
		.1		6.5
7	20 ppm monensin	.1	18	6.7
		.1		6.5
8	20 ppm monensin/[Na]	.12	21	6.5
		.09		6.5
9	20 ppm monensin/[Na]	.08	24	6.7
		.12		6.5
10	back to control	.28	27	6.5
		.22		6.5
11	back to control	.19	30	6.6
		.19		6.6

Table 44. Absorbance, Sampling Time and Final pH of S. bovis 24 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture

Sampling Sequence	Medium	Absorbance	Sampling Time (day)	Final pH
1	start	.50	0	6.8
		.60		6.8
2	control	.31	2	6.2
		.26		6.2
3	control	.24	4	6.2
		.17		6.2
4	.5 ppm monensin	.17	6	6.2
		.14		6.2
5	.5 ppm monensin	.19	8	6.3
		.11		6.2
6	20 ppm monensin	.08	10	6.3
		.04		6.3
7	20 ppm monensin	.09	12	6.2
		.05		6.2
8	20 ppm monensin/[Na]	.10	14	6.25
		.12		6.25
9	20 ppm monensin/[Na]	.12	16	6.25
		.19		6.45
10	back to control	.22	18	6.4
		.29		6.2
11	back to control	.29	20	6.35
		.39		6.35

Table 45. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
L[a]	1.45	3.52	ND	.74	.68	1.14
RNA/Protein	.21	.11	.11	.11	.14	.17
Cell yield[b]	430.00	493.33	305.00	360.00	355.00	305.00
Rate of glu. util.[c]	16.54	22.06	16.51	16.55	16.54	16.54
Total of glu util.%	99.28	99.28	99.06	99.31	99.28	99.28
Y_{glu}[d]	25.99	22.36	18.47	21.75	21.46	18.43

a mole lactate/mole of glucose utilized
b ug/h/15ml culture
c umoles/h/15ml culture
d g of cell/mole of glucose utilized
ND = Not Detected

Table 46. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
L[a]	ND	ND	1.37	1.13	1.30	ND
RNA/Protein	.43	.25	.36	.43	.37	.29
Cell yield[b]	246.67	573.33	368.89	700.00	320.00	624.00
Rate of glu. util.[c]	11.04	22.06	14.72	33.10	13.19	26.46
Total of glu util.%	99.35	99.28	99.35	99.31	98.94	99.25
Y_{glu}[d]	22.35	25.99	25.07	21.15	24.26	23.58

a mole lactate/mole of glucose utilized
b ug/h/15ml culture
c umoles/h/15ml culture
d g of cell yield/mole of glucose utilized
ND = Not Detected

Table 47. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 in .5 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.69	ND	ND	.55	ND	ND
L[b]	.85	.67	.35	.63	ND	1.32
RNA/Protein	.29	.23	.15	.15	.13	.20
Cell yield[c]	98.95	80.00	62.22	57.33	148.00	116.36
Rate of glu. util.[d]	6.96	6.60	7.35	4.41	13.22	12.00
Total of glu. util.%	99.18	99.08	99.25	99.28	99.18	99.05
Y_{glu}[e]	14.22	12.11	8.46	12.99	11.19	9.69

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 48. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin Medium to 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.51	.53	.23	ND	.56	ND
L[b]	1.11	ND	ND	ND	2.39	ND
RNA/Protein	.18	.13	.13	.10	.07	.11
Cell yield[c]	41.54	26.67	42.22	33.64	48.70	43.53
Rate of glu. util.[d]	2.55	2.00	3.67	3.01	2.87	3.89
Total of glu. util.%	99.35	99.25	99.07	99.31	99.15	99.18
Y_{glu}[e]	16.31	13.30	11.51	11.18	16.95	11.19

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 49. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from 20 ppm Monensin Medium to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	.11	.47	ND	ND	ND
L[b]	1.64	1.28	ND	.62	2.11	1.38
RNA/Protein	.43	.47	.51	.50	.60	.32
Cell yield[c]	55.17	69.23	63.08	99.20	118.33	63.70
Rate of glu. util.[d]	4.57	5.09	5.09	5.29	5.51	4.90
Total of glu. util.%	99.35	99.28	99.28	99.25	99.21	99.25
Y_{glu}[e]	12.08	13.60	12.39	18.74	21.47	12.99

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 50. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin Medium to 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	ND	ND	.79	ND	1.17
L[b]	2.42	2.26	ND	ND	ND	1.99
RNA/Protein	.13	.07	.10	.07	.11	.12
Cell yield[c]	40.00	27.74	33.91	40.95	28.40	33.64
Rate of glu. util.[d]	2.45	2.13	2.88	3.15	2.00	2.01
Total of glu. util.%	99.31	99.25	99.25	99.25	99.18	99.38
Y_{glu}[e]	16.31	12.99	11.79	12.99	14.18	16.75

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 51. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from 20 ppm Monensin/[Na] Medium to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
L[b]	1.78	1.85	1.56	1.23	ND	2.33
RNA/Protein	.36	.46	.34	.36	.36	.51
Cell yield[c]	63.08	78.40	96.67	96.00	55.38	90.77
Rate of glu. util.[d]	5.09	5.29	5.51	5.29	5.09	5.09
Total of glu. util.%	99.35	99.28	99.25	99.25	99.28	99.21
Y_{glu}[e]	12.38	14.81	17.53	18.14	10.88	17.84

b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 52. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 .5 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	.18	.30	.41	.36	ND
L[b]	ND	ND	1.30	1.03	1.32	1.25
RNA/Protein	.31	.19	.14	.13	.16	.12
Cell yield[c]	94.17	55.33	88.89	82.85	63.43	58.18
Rate of glu. util.[d]	5.51	4.41	4.89	3.78	3.78	4.01
Total of glu. util.%	99.25	99.15	99.05	99.31	99.31	99.31
Y_{glu}[e]	17.08	12.56	18.17	21.90	16.77	14.50

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 53. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin/[Na] Medium to 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	1.08	.86	.30	.22	ND	.26
L[b]	ND	ND	ND	ND	ND	1.14
RNA/Protein	.16	.13	.09	.10	.13	.08
Cell yield[c]	33.64	29.63	21.48	25.18	50.00	26.67
Rate of glu. util.[d]	3.01	2.45	2.45	2.45	3.49	3.15
Total of glu. util.%	99.25	99.25	99.31	99.35	94.41	99.25
Y_{glu}[e]	11.18	12.09	8.76	10.27	14.30	8.46

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 54. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 from .5 ppm Monensin/[Na] Medium to 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.37	.28	ND	.25	.37	ND
L[b]	1.10	.94	.93	.96	1.31	ND
RNA/Protein	.12	.04	.08	.13	.15	.08
Cell yield[c]	30.43	81.11	69.47	68.70	38.52	33.57
Rate of glu. util.[d]	2.88	3.68	3.48	2.88	2.45	2.34
Total of glu. util.%	99.31	99.31	99.25	99.25	99.31	98.10
Y_{glu}[e]	10.57	22.05	19.95	23.88	15.71	14.37

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 55. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown from pH 6.8, 20 ppm Monensin to pH 7.6 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.27	.23	.37	ND	.26	.20
L[b]	ND	1.00	ND	ND	ND	.99
RNA/Protein	.10	.08	.11	.07	.08	.17
Cell yield[c]	40.73	57.14	109.33	92.63	69.33	72.38
Rate of glu. util.[d]	2.41	4.73	8.83	6.96	8.82	6.30
Total of glu. util.%	99.31	99.38	99.35	99.25	99.28	99.31
Y_{glu}[e]	16.92	12.08	12.38	13.30	7.86	11.48

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 56. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 7.6 from 20 ppm Monensin to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.22	.48	.75	ND	.71	ND
L[b]	1.07	ND	ND	ND	.96	2.30
RNA/Protein	.09	.14	.09	.13	.11	.13
Cell yield[c]	264.00	456.00	326.67	375.00	255.00	379.99
Rate of glu. util.[d]	13.25	26.48	22.08	16.55	16.56	22.07
Total of glu. util.%	99.38	99.31	99.38	99.31	99.38	99.35
Y_{glu}[e]	19.92	17.22	14.79	22.66	15.39	17.21

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 57. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 6.8 20 ppm Monensin to pH 7.6 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.19	.23	ND	ND	.89	ND
L[b]	1.01	ND	ND	1.12	ND	ND
RNA/Protein	.07	.04	.05	.07	.05	.03
Cell yield[c]	45.45	65.88	45.71	65.45	64.71	62.50
Rate of glu. util.[d]	3.01	3.89	3.15	3.01	3.78	4.13
Total of glu. util.%	99.31	99.31	99.25	99.25	96.42	99.25
Y_{glu}[e]	15.10	16.92	14.51	21.76	17.11	15.11

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 58. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown from pH 7.6 20 ppm Monensin/[Na] to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.26	ND	.87	.22	.25	ND
L[b]	.97	ND	1.01	.99	ND	1.09
RNA/Protein	.22	.10	.11	.07	.07	.09
Cell yield[c]	420.00	336.00	464.00	360.00	400.00	286.67
Rate of glu. util.[d]	22.07	26.50	26.50	22.08	26.50	22.08
Total of glu. util.%	99.31	99.38	99.38	99.38	99.38	99.38
Y_{glu}[e]	19.03	12.68	17.51	16.30	15.09	12.98

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 59. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 7.6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
L[b]	ND	ND	1.59	1.25	1.50	ND
RNA/Protein	.22	.27	.41	.21	.25	.26
Cell yield[c]	368.89	970.00	648.00	520.00	640.00	490.00
Rate of glu. util.[d]	14.71	33.10	26.48	33.10	33.05	33.01
Total of glu. util.%	99.28	99.31	99.31	99.31	99.15	99.05
Y_{glu}[e]	25.08	29.30	24.47	15.7	19.37	14.84

b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 60. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 7.6 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	ND	ND	.84	ND	.88
L[b]	.83	1.27	1.04	1.12	ND	.85
RNA/Protein	.16	.13	.27	.14	.15	.12
Cell yield[c]	133.33	89.14	103.53	47.06	46.25	42.42
Rate of glu. util.[d]	5.51	3.78	3.89	3.89	4.13	4.01
Total of glu. util.%	99.11	99.18	99.11	99.28	99.25	99.25
Y_{glu}[e]	24.22	23.59	26.64	12.09	11.18	10.58

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 61. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown from pH 7.6 20 ppm Monensin to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.27	ND	ND	ND	.32	ND
L[b]	1.52	1.33	.88	2.35	ND	1.05
RNA/Protein	.19	.45	.41	.50	.15	.36
Cell yield[c]	275.00	375.00	720.00	270.00	466.67	460.00
Rate of glu. util.[d]	16.54	16.54	33.09	16.54	22.07	22.05
Total of glu. util.%	99.28	99.28	99.28	99.25	99.35	99.25
Y_{glu}[e]	16.62	22.66	21.76	16.32	21.14	20.86

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 62. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 7.6 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	.22	.21	.30	ND	.22
L[b]	1.52	1.10	ND	ND	1.38	.94
RNA/Protein	.32	.18	.20	.19	.11	.13
Cell yield[c]	93.71	47.28	55.81	82.10	64.21	96.84
Rate of glu. util.[d]	3.78	3.00	3.07	3.48	3.48	3.48
Total of glu. util.%	99.18	99.11	99.18	99.28	99.25	99.25
Y_{glu}[e]	24.81	15.68	18.15	23.57	18.44	27.81

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 63. Physiological and Metabolic Parameters of Six Passages of S. bovis 24 Grown in pH 7.6 from 20 ppm Monensin/[Na] to Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	.11	ND	ND	.36	ND
L[b]	1.03	.95	1.12	.71	1.47	.94
RNA/Protein	.20	.41	.40	.68	.32	.43
Cell yield[c]	130.00	262.50	200.00	240.00	192.73	328.00
Rate of glu. util.[d]	8.27	8.27	9.45	9.45	12.02	13.23
Total of glu. util.%	99.28	99.28	99.21	99.21	99.18	99.25
Y_{glu}[e]	15.71	31.73	21.17	25.40	16.03	24.79

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 64. Physiological and Metabolic Parameters of Six Passages of L. vitulinus B62 Grown in pH 6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.54	.32	1.30	.55	.57	.88
L[b]	6.21	6.29	3.34	7.00	2.16	6.72
RNA/Protein	.27	.14	.08	.15	.10	.14
Cell yield[c]	150.59	111.30	136.47	88.70	157.65	88.70
Rate of glu. util.[d]	7.79	5.75	7.74	5.75	7.78	5.75
Total of glu. util.%	99.28	99.28	98.75	99.23	99.25	99.26
Y_{glu}[e]	19.34	19.34	17.62	15.42	20.25	15.41

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized

Table 65. Physiological and Metabolic Parameters of Six Passages of L. vitulinus B62 Grown in pH 6.8 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.22	.03	.22	ND	.07	ND
L[b]	2.41	1.79	1.31	1.00	1.30	2.74
RNA/Protein	.09	.19	.06	.05	.19	.11
Cell yield[c]	185.00	164.35	100.00	95.65	156.92	123.08
Rate of glu. util.[d]	8.26	5.76	8.27	5.75	10.18	10.18
Total of glu. util.%	99.18	99.35	99.25	99.18	99.31	99.28
Y_{glu}[e]	22.39	28.54	12.09	16.64	15.41	12.09

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 66. Physiological and Metabolic Parameters of Six Passages of L. vitulinus B62 Grown in pH 6.8 .5 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
L[b]	ND	1.24	ND	1.08	1.51	1.14
RNA/Protein	.06	.05	.09	.09	.06	.07
Cell yield[c]	46.40	52.59	49.60	46.52	37.00	47.37
Rate of glu. util.[d]	2.20	2.45	2.64	2.87	3.31	3.48
Total of glu. util.%	99.18	99.18	99.18	99.18	99.18	99.18
Y_{glu}[e]	21.05	21.47	18.75	16.19	11.19	13.61

b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 67. Physiological and Metabolic Parameters of Six Passages of L. vitulinus B62 Grown in pH 7.6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	ND	.252	ND	ND	.12	ND
L[b]	ND	2.31	1.26	1.60	1.19	ND
RNA/Protein	.07	.10	.11	.05	.08	.09
Cell yield[c]	165.00	106.68	112.50	100.00	160.00	116.92
Rate of glu. util.[d]	8.27	5.52	8.27	4.72	10.17	10.18
Total of glu. util.%	99.31	99.31	99.21	99.11	99.21	99.28
Y_{glu}[e]	19.94	19.24	13.61	21.19	15.72	11.48

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 68. Physiological and Metabolic Parameters of Six Passages of L. vitulinus B62 Grown in pH 7.6 .5 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
Acetate[a]	.27	ND	.32	.16	.22	ND
L[b]	1.16	ND	ND	1.57	ND	ND
RNA/Protein	.03	.06	.04	.06	.10	.03
Cell yield[c]	24.36	13.50	15.75	23.33	36.67	34.00
Rate of glu. util.[d]	2.06	1.43	1.52	2.73	3.82	3.82
Total of glu. util.%	99.05	86.00	91.38	86.00	86.00	86.00
Y_{glu}[e]	11.79	11.26	11.35	10.22	11.47	10.63

a mole acetate/mole of glucose utilized
b mole lactate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 69. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.81	.45	.64	.38	ND	.74
L + S[b]	1.12	1.51	1.22	1.34	ND	1.50
RNA/Protein	.30	.31	.30	.32	.43	.45
Cell yield[c]	190.59	180.95	146.67	142.86	150.00	131.43
Rate of glu. util.[d]	7.79	6.31	7.35	6.30	6.61	6.30
Total of glu. util.%	99.28	99.33	99.25	99.31	99.23	99.31
Y_{glu}[e]	24.48	28.69	19.95	22.66	22.68	20.84

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 70. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.76	.79	.66	.20	ND	.18
L + S[b]	.21	ND	.14	ND	.32	ND
RNA/Protein	.31	.12	.17	.24	.36	.28
Cell yield[c]	157.65	113.04	155.29	118.09	138.00	144.44
Rate of glu. util.[d]	7.78	5.75	7.79	6.30	6.52	7.35
Total of glu. util.%	99.26	99.26	99.31	99.28	97.87	99.30
Y_{glu}[e]	20.25	19.65	19.94	18.74	21.15	19.64

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 71. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.77	.91	.57	.66	.53	.75
L + S[b]	.21	.13	.20	.20	1.82	1.22
RNA/Protein	.21	.48	.22	.36	.30	.37
Cell yield[c]	167.06	118.26	171.76	93.33	140.00	81.25
Rate of glu. util.[d]	7.79	5.76	7.78	6.30	6.52	4.14
Total of glu. util.%	99.28	99.31	99.25	99.25	97.82	99.26
Y_{glu}[e]	21.46	20.54	22.07	14.81	21.47	19.65

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized

Table 72. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6.8 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	1.64	.06	.03	1.08	.22	.16
L + S[b]	1.13	1.32	2.04	1.86	1.33	1.27
RNA/Protein	.32	.42	.42	.24	.30	.39
Cell yield[c]	375.00	304.00	221.82	229.09	272.00	236.36
Rate of glu. util.[d]	16.53	13.24	12.04	12.04	13.17	12.03
Total of glu. util.%	99.18	99.31	99.35	99.31	98.78	99.28
Y_{glu}[e]	22.69	22.96	18.42	19.03	20.65	19.64

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized

Table 73. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6.8 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.29	.46	.79	.49	.66	.10
L + S[b]	1.25	ND	1.07	1.22	ND	1.66
RNA/Protein	.20	.43	.31	.42	.28	.20
Cell yield[c]	144.00	102.22	106.67	108.89	71.11	141.33
Rate of glu. util.[d]	8.82	7.35	7.33	7.33	7.28	8.76
Total of glu. util.%	99.25	99.25	98.98	98.94	98.34	98.61
Y_{glu}[e]	16.32	13.91	14.55	14.86	9.76	16.13

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 74. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 6.8 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.07	.44	.49	.51	ND	.13
L + S[b]	.86	1.73	1.12	.91	ND	1.31
RNA/Protein	.17	.43	.30	.31	.39	.22
Cell yield[c]	154.19	84.71	72.00	116.20	105.00	114.66
Rate of glu. util.[d]	8.54	7.78	6.62	7.35	6.47	8.62
Total of glu. util.%	99.25	99.25	99.25	99.25	97.10	97.03
Y_{glu}[e]	18.05	10.83	10.83	15.74	16.54	13.57

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 75. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 7.6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	ND	1.02	ND	.03	.40	.01
L + S[b]	1.08	1.65	ND	1.59	1.30	ND
RNA/Protein	.41	.29	.44	.22	.14	.21
Cell yield[c]	466.67	269.09	200.00	232.73	260.00	185.45
Rate of glu. util.[d]	22.07	12.04	12.04	12.04	13.20	12.02
Total of glu. util.%	99.31	99.33	99.35	99.35	99.01	99.15
Y_{glu}[e]	21.15	22.35	16.61	19.33	19.70	15.43

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 76. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 7.6 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.61	.62	.56	.71	.66	.80
L + S[b]	.33	.31	.41	.45	ND	ND
RNA/Protein	.34	.24	.26	.16	.30	.23
Cell yield[c]	240.00	123.25	98.67	151.58	91.11	86.96
Rate of glu. util.[d]	9.43	5.30	4.39	6.96	7.35	5.76
Total of glu. util.%	99.08	99.25	98.84	99.18	99.18	99.31
Y_{glu}[e]	25.44	23.25	22.46	21.78	12.40	15.10

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 77. Physiological and Metabolic Parameters of Six Passages of S. ruminantium HD4 Grown in pH 7.6 20 ppm/[Na] Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.72	.76	.81	.76	.76	.79
L + S[b]	.23	.25	.26	.25	.27	.25
RNA/Protein	.29	.17	.17	.30	.26	.19
Cell yield[c]	137.14	161.67	118.67	101.05	117.64	160.00
Rate of glu. util.[d]	9.44	5.51	4.41	6.96	7.78	5.76
Total of glu. util.%	99.11	99.25	99.18	99.25	99.21	99.31
Y_{glu}[e]	14.53	29.32	26.92	14.51	15.05	27.79

a mole total volatile fatty acids/mole of glucose utilized
b mole lactate and succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized

Table 78. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.59	.47	.81	.63	.85	1.17
S[b]	2.57	1.20	2.30	4.30	1.08	1.73
RNA/Protein	.05	.05	.05	.24	.16	.12
Cell yield[c]	19.02	42.10	24.17	27.73	30.40	43.75
Rate of glu. util.[d]	1.48	3.15	2.48	1.37	1.32	2.05
Total of glu. util.%	91.05	89.70	89.36	90.69	99.23	98.21
Y_{glu}[e]	12.85	13.38	9.74	20.18	22.98	21.38

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umole/h/15ml culture
e g of cell/mole of glucose utilized

Table 79. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 6.8 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.07	.53	.16	.36	.42	.73
S[b]	2.93	1.23	1.10	2.25	2.71	3.23
RNA/Protein	.13	.21	.28	.31	.15	.19
Cell yield[c]	54.36	72.43	46.67	69.41	89.70	95.76
Rate of glu. util.[d]	3.39	3.57	4.12	3.89	4.01	4.01
Total of glu. util.%	99.25	99.01	92.73	99.18	99.18	99.15
Y_{glu}[e]	16.02	20.30	11.32	17.85	22.39	23.91

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized

Table 80. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 6.8 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.21	.11	.20	ND	ND	.42
S[b]	ND	1.32	1.38	1.30	1.13	ND
RNA/Protein	.15	.14	.14	.22	.41	.49
Cell yield[c]	29.44	41.63	50.77	26.00	62.86	24.58
Rate of glu. util.[d]	1.59	1.35	2.23	1.10	3.15	1.38
Total of glu. util.%	86.00	99.11	87.01	99.01	99.11	99.18
Y_{glu}[e]	18.49	30.88	22.76	23.64	19.98	17.85

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 81. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 6.8 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.35	.46	ND	.21	.20	.51
S[b]	.26	.60	2.76	1.52	1.04	1.36
RNA/Protein	.08	.07	.28	.32	.52	.58
Cell yield[c]	33.33	31.72	56.80	35.29	67.91	97.89
Rate of glu. util.[d]	4.78	4.17	2.64	1.95	3.08	3.48
Total of glu. util.%	86.00	90.71	99.11	99.31	99.31	99.31
Y_{glu}[e]	6.97	7.61	21.49	18.13	22.05	28.09

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umoles/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 82. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 7.6 Control Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	ND	.13	.51	ND	.24	.22
S[b]	1.21	.63	.24	1.04	1.42	.51
RNA/Protein	.16	.08	.25	.22	.57	.54
Cell yield[c]	56.00	43.24	40.00	38.92	81.54	47.57
Rate of glu. util.[d]	3.77	3.57	3.55	3.57	5.09	3.57
Total of glu. util.%	99.11	99.15	98.51	99.11	99.18	99.18
Y_{glu}[e]	14.83	12.10	11.27	10.90	16.03	13.31

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umole/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 83. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 7.6 20 ppm Monensin Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	ND	.43	.71	.50	.28	.27
S[b]	.85	1.03	1.07	.94	1.09	1.02
RNA/Protein	.46	.39	.44	.32	.17	.35
Cell yield[c]	64.87	44.71	57.44	60.00	63.78	47.44
Rate of glu. util.[d]	3.40	2.59	3.39	3.31	3.58	3.08
Total of glu. util.%	99.25	99.05	99.18	99.25	99.28	99.25
Y_{glu}[e]	19.12	17.27	16.94	18.14	17.83	15.42

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umole/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 84. Physiological and Metabolic Parameters of Six Passages of B. ruminicola GA33 Grown in pH 7.6 20 ppm Monensin/[Na] Medium

Metabolic Parameters	Passages 1	2	3	4	5	6
TVFA[a]	.31	.72	.88	.14	.19	.33
S[b]	1.64	.23	ND	.83	.65	.56
RNA/Protein	.13	.02	.07	.08	.46	.20
Cell yield[c]	78.00	57.00	61.00	19.51	22.50	37.33
Rate of glu. util.[d]	3.24	3.11	2.92	1.57	1.65	2.21
Total of glu. util.%	97.30	93.40	87.68	96.76	99.28	99.28
Y_{glu}[e]	24.05	18.31	20.87	12.40	13.60	16.92

a mole total volatile fatty acids/mole of glucose utilized
b mole succinate/mole of glucose utilized
c ug/h/15ml culture
d umole/h/15ml culture
e g of cell/mole of glucose utilized
ND = Not Detected

Table 85. Physiological and Metabolic Parameters of Two Runs of B. ruminicola GA33 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture

	Metabolic Parameters						
No.[a]	TVFA[b]	S[c]	RNA/ Protein	Cell yield[d]	Rate of glu. util.[e]	Total of glu. util.	Y_{glu}[f] %
1	Start						
2	.51	.26	.25	523.33	30.87	99.22	16.95
	.45	.22	.25	503.39	30.87	99.22	16.31
3	.88	1.68	.24	581.46	30.87	99.24	18.83
	.78	1.48	.24	445.1	30.87	99.24	14.42
4	.73	1.67	.26	500.29	31.09	99.95	16.09
	.65	1.49	.26	444.35	30.47	97.95	14.58
5	.96	.35	.265	364.01	30.87	99.24	11.79
	.84	.31	.255	308.07	30.87	99.24	9.98
6	1.76	.31	.24	470.45	30.60	98.35	15.37
	1.62	.29	.24	339.79	30.60	98.35	11.11
7	1.18	1.99	.256	338.94	30.20	97.08	11.22
	1.14	1.93	.264	330.0	30.20	97.08	10.96
8	.63	2.08	.37	400.27	30.17	96.99	13.27
	.61	2.04	.37	305.99	30.17	96.98	10.14
9	.44	7.35	.27	314.76	30.83	99.10	10.21
	.38	6.51	.27	304.58	30.83	99.09	9.88
10	ND	ND	.31	489.87	30.83	99.09	15.89
	ND	ND	.31	480.49	30.83	99.09	15.59
11	.44	1.40	.24	438.76	30.86	99.19	14.22
	.40	1.24	.24	420.06	30.86	99.19	13.61

a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.

b mole total volatile fatty acids/mole of glucose utilized

c mole succinate/mole of glucose utilized

d ug/h

e umoles/h

f g of cell/mole of glucose utilized

ND = Not Detected

Table 86. Physiological and Metabolic Parameters of Two Runs of B. ruminicola GA33 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture

	Metabolic Parameters						
No.[a]	TVFA[b]	S[c]	RNA/ Protein	Cell yield[d]	Rate of glu. util.[e]	Total of glu. util.%	Y_{glu}[f]
1	Start						
2	.89	.18	.28	1745.29	61.72	99.20	28.27
	.79	.16	.27	1726.67	61.72	99.19	27.98
3	.62	5.31	.27	2104.1	61.72	99.19	34.09
	.54	4.69	.25	2087.74	61.72	99.19	33.83
4	.91	.13	.23	1379.7	61.59	98.99	22.40
	.81	.11	.24	1363.76	61.59	98.99	22.14
5	.69	.11	.22	1324.97	61.67	99.12	21.48
	.61	.09	.22	1194.31	61.67	99.12	19.36
6	1.33	.36	.22	914.9	57.53	92.46	15.9
	1.59	.44	.24	821.5	57.53	92.46	14.28
7	1.52	2.70	.32	918.67	60.23	96.80	15.25
	1.50	2.64	.32	910.63	60.23	96.80	15.12
8	.74	.23	.22	1197.81	59.28	95.27	20.21
	.78	.23	.24	1189.77	59.28	95.27	20.07
9	.49	1.42	.29	1377.14	60.97	97.99	22.59
	.45	1.32	.28	1366.96	60.97	97.99	22.42
10	.96	.14	.23	1460.68	61.70	99.16	23.96
	.84	.12	.29	1452.30	61.70	99.16	23.8
11	.28	1.86	.33	1993.16	61.70	99.17	32.3
	.24	.64	.33	1974.46	61.70	99.17	32.0

a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium
No. 10 and 11 are back to control medium.

b mole total volatile fatty acids/mole of glucose utilized

c mole succinate/mole of glucose utilized

d ug/h

e umoles/h

f g of cell/mole of glucose utilized

Table 87. Physiological and Metabolic Parameters of Two Runs of S. bovis 24 Grown in pH 6.8 Medium in a 5%/h Dilution Rate Continuous Culture

	Metabolic Parameters						
No.[a]	Acet.[b]	L[c]	RNA/ Protein	Cell yield[d]	Rate of glu. util.[e]	Total of glu. util.	Y_{glu}[f] %
1	Start						
2	.35	3.47	.31	783.94	30.78	98.94	25.46
	.29	3.07	.29	746.7	30.91	99.36	24.16
3	.51	1.61	.31	504	30.87	99.22	16.32
	.45	1.43	.31	778.64	30.82	99.08	25.26
4	.55	2.08	.24	373.38	30.89	99.29	12.08
	.49	1.84	.28	485.26	30.85	99.15	15.72
5	.49	1.60	.30	429.26	30.87	99.22	13.91
	.43	1.42	.32	690.78	30.87	99.22	22.38
6	.38	.95	.25	410.68	30.85	99.15	13.31
	.34	.83	.26	597.48	30.85	99.15	19.37
7	.69	.86	.22	317.04	30.85	99.15	10.28
	.61	.76	.22	504	30.85	99.15	16.34
8	.50	.74	.25	720	30.89	99.29	23.31
	.44	.64	.25	736.08	30.80	99.01	23.90
9	ND	.38	.27	746.64	30.85	99.15	24.20
	ND	.26	.27	768.2	30.85	99.15	24.90
10	.26	.77	.28	643.88	30.87	99.22	20.86
	.24	.69	.26	662.44	30.91	99.36	21.43
11	.47	2.78	.35	951.98	30.89	99.29	30.82
	.39	2.44	.33	989.38	30.89	99.29	32.03

a No. 2 and 3 are control medium.
NO. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.
b mole acetate/mole of glucose utilized
c mole lactate/mole of glucose utilized
d ug/h
e umoles/h
f g of cell/mole of glucose utilized
ND = Not Detected

Table 88. Physiological and Metabolic Parameters of Two Runs of S. bovis 24 Grown in pH 6.8 Medium in a 10%/h Dilution Rate Continuous Culture

	Metabolic Parameters						
No.[a]	Acet.[b]	L[c]	RNA/ Protein	Cell yield[d]	Rate of glu. util.[e]	Total of glu. util.	Y_{glu}[f] %
1	Start						
2	.305	.82	.43	2221.24	61.75	99.24	35.97
	.335	.72	.43	2202.62	61.75	99.24	35.67
3	.32	.86	.37	1804.43	61.74	99.22	29.23
	.28	.76	.37	1668.07	61.74	99.22	27.02
4	.27	.52	.18	1621.88	61.76	99.25	26.26
	.25	2.18	.18	1565.94	61.76	99.25	25.36
5	.53	2.28	.15	1097.31	61.76	99.25	17.77
	.45	2.00	.19	1086.65	61.76	99.25	17.60
6	ND	.38	.15	1138.68	59.88	96.24	19.02
	ND	.60	.15	1045.28	59.88	96.24	17.46
7	.49	.88	.15	1135.88	59.80	96.11	18.99
	.27	.88	.15	1042.40	59.80	96.11	17.43
8	.39	.90	.18	1320.03	61.78	99.29	21.37
	.33	.80	.18	1311.99	61.78	99.29	21.24
9	.42	.89	.27	1321.13	61.79	99.30	21.38
	.38	.79	.27	1310.95	61.79	99.30	21.22
10	ND	.96	.27	2072.8	61.72	99.20	33.58
	ND	.84	.27	2063.42	61.72	99.20	33.43
11	ND	2.14	.32	2105.57	61.74	99.22	34.11
	ND	1.88	.32	2086.87	61.74	99.22	33.80

a No. 2 and 3 are control medium.
No. 4 and 5 are .5 ppm monensin medium.
No. 6 and 7 are 20 ppm monensin medium.
No. 8 and 9 are 20 ppm monensin/[Na] medium.
No. 10 and 11 are back to control medium.
b mole acetate/mole of glucose utilized
c mole lactate/mole of glucose utilized
d ug/h
e umoles/h
f g of cell/mole of glucose utilized
ND = Not Detected

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