GLUCOSE ASSIMILATION BY RUMEN MICROBES AND EFFECT OF PENICILLIN

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Donald H. Steyert

1965

ABSTRACT

GLUCOSE ASSIMILATION BY RUMEN MICROBES

AND EFFECT OF PENICILLIN

By Donald H. Steyert

Strained rumen fluid, collected from fistulated cows fed a hay and grain ration, was fermented with 500 mg glucose per 100 ml fermentation fluid. Twenty μc uniformly labeled glucose-C^{14} was added. Penicillin treatment was 2.5 units penicillin G added per ml fermentation fluid. After three hours bacterial and "protozoal" fractions were prepared by centrifugation and saline washings. The two fractions were subjected to cold-TCA, ethanol, ether and hot-TCA treatment to determine the radioactivity in the transient intermediate compounds, the lipids, nucleic acids and protein residue. Volatile fatty acids accounted for approximately 33% of the total added activity. The most complete set of data showed the bacterial fractions to contain about 1.1% of the added activity, while the "protozoal" fraction contained about 30%. The fractional

distribution of activity in the bacteria and protozoa respectively were: 0.24 and 0.07 in the cold-TCA extract; 0.07 and 0.01 in the ethanol-ether extract; 0.27 and 0.86 in the nucleic acid (hot-TCA) fraction; 0.43 and 0.05 in the protein residue. Thus, protozoa incorporated almost 30 times as much glucose carbon as the bacteria with the nucleic acid fraction accounting for most of the difference. Penicillin decreased the amount of activity in the bacteria to 63-80% that of the control without drastically altering the distribution among the fractions. Penicillin increased the activity present in the "protozoal" fraction to 108% of that in the control with no effect upon the distribution among the fractions.

GLUCOSE ASSIMILATION BY RUMEN MICROBES AND EFFECT OF PENICILLIN

By

Donald H. Steyert

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Dairy

1965

ACKNOWLEDGEMENTS

The author appreciates the assistance of Dr. R. S. Emery, Associate Professor of the Department of Dairy, during the course of this study and the preparation of this thesis.

Appreciation is also expressed to Dr. C. A. Lassiter, Head of the Department of Dairy, for making available a Graduate Research Assistantship.

TABLE OF CONTENTS

Table of contents/continued.

LIST OF TABLES

LIST OF FIGURES

GLUCOSE ASSIMILATION BY RUMEN MICROBES AND EFFECT OF PENICILLIN

I. INTRODUCTION

The ruminant animal has been important to man throughout history because of its ability to utilize normally indigestible feedstuffs and produce animal products which can be utilized by mankind. The ability to convert roughages to animal products depends almost soley upon the microbial population present in the forestomach of these animals. The microbes not only produce compounds which can be utilized by the host animal, but themselves become a source of good nutrition to the animal.

Various workers have shown energy requirements for the maintenance and growth of microorganisms. Bacteria obtain and utilize energy by a number of processes. In the ruminant animal a somewhat specialized fermentation is utilized for energy production from organic compounds.

This study was undertaken to determine in what manner a common energy source, glucose, is utilized, and into what classes of compounds the carbon atoms from such

a source are incorporated by the microbial cells of the ruminant animal. A second aspect of the study was to determine what effect penicillin, a drug used to prevent bloat, has upon the distribution of the carbon from the assimilated glucose.

II. REVIEW OF LITERATURE

Microbial Cellular Metabolism

Energy for cellular maintenance and growth.

Exothermic chemical reactions liberate energy, usually in the form of heat. The total energy in the chemical bonds of the reactants must be greater than the energy in the bonds of the products before a reaction will occur. The energy difference is lost as heat. Biological reactions follow the same rules and therefore the bond energy available to an organism must exceed the amount in the products of its metabolism, including synthesized cell materials. All organisms, with exception of those capable of photosynthesis, rely upon the chemical bond energy of their nutrients. Many cellular constituents, having higher energies than the products from which they were formed, must be produced by reactions that also yield products of lower energy value than the starting nutrient. These are excreted as waste products including heat. Normal metabolism of all organisms involves thousands of separate

chemical reactions closely coupled by enzymes which catalyze these reactions. All the reactions involve the energy that keeps the cells alive.

Bacteria, like any other living organisms, require a source of energy to remain viable and perform their normal metabolic functions. One aspect of this energy requirement is that amount needed for endogenous metabolism.

Endogenous metabolism is customarily described as those processes occurring when the organism is deprived of exogenous energy sources (65). As the starvation proceeds, the processes of endogenous metabolism may change qualitatively or quantitatively. The chemical reactions of endogenous metabolism may either be essential to the vitality of the organism, or they may occur merely because certain enzymes and their substrates are present and in contact with each other (65). If the cell is to maintain normal function, the energy reserves consumed by the endogenous metabolic reactions must be replaced by an exogenous source of energy (65). Study of the energy required for endogenous metabolism leads us into a concept termed "energy of maintenance."

Mallette (65) credits Rahn (101) with defining energy of maintenance as that level of exogenous energy

just sufficient to replace the energy used endogenously, but without inducing growth. Mallette (65) mentions that although the energy of maintenance concept has been shown and accepted in higher animals and in plants, experimental demonstration in bacteria has been difficult. Lamanna and Mallette (58) pointed out that energy of maintenance may have a direct origin in endogenous metabolism.

Mallette (65) reviews many of the indirect demonstrations of energy of maintenance which have been made (28, 32, 127).

Direct demonstration of the energy of maintenance has been undertaken in a number of ways. The most direct means to reveal the phenomenon would be to extrapolate plots of growth versus exogenous energy source to zero growth. Most studies (24, 25, 63, 79) reviewed by Mallette (65) which applied this approach have given negative or inconclusive results.

McGrew and Mallette (76), using Escherichia coli, sought to overcome some of the faults of the former researchers and to determine a threshold value for growth. They used a moderately dense population of E. coli still readily capable of growth and subjected it to low substrate concentrations which permitted only slight growth relative

to the population. For increased sensitivity they extrapolated the data to the inoculum level and used turbidimetric procedures for growth measurement.

Under their experimental conditions, McGrew and Mallette found a threshold value for growth of about 0.55 μmole glucose in 10 ml medium containing 5×10^9 E. coli. To study energy of maintenance, McGrew and Mallette then fed their cultures 0.28 μmole glucose every six hours, a quantity small enough to maintain a constant turbidity, thereby revealing a level of energy of maintenance. The higher level yielded increased turbidity, the lower level maintained turbidity, and the control (no glucose) decreased in turbidity. The lower level (0.28 μmole) appears to be an energy of maintenance level of E. coli under the experimental conditions used. Viability studies performed on the cultures showed a definitely increased viability over the control value due to increased frequency of glucose addition. Energy of maintenance implies an increased viability of a culture when that level of energy is supplied to it. The results of McGrew and Mallette show that increased viability does indeed occur and that the addition of 0.28 μmole glucose every six hours will "maintain" (growth of some cells equal to the turbidimetric decrease

in others) a culture in a fairly constant turbidimetric state. Their work definitely indicates an energy of maintenance for E. coli and the presumption can probably be made that other bacteria behave similarly with some quantitative differences.

Energy of maintenance is only that amount required by the cell for survival. For normal growth and reproduction the cell needs additional available energy.

Energy yielding processes.

Every aspect of growth and normal function is related to metabolism of energy-yielding compounds and utilization of the energy produced. Synthesis of the macromolecular polymers so vital to growth and reproduction of the cell is just one of the phases of the cellular function that requires input of energy. The subunits of macromolecules must be activated by the cell so that the polymerization can proceed with the liberation of energy (112). Chemical derivatives of the subunits, each having more bond energy than the bonds of the polymer must be produced. The most important single compound to produce the activated subunits is adenosine triphosphate (ATP).

ATP is involved in almost every aspect of cellular metabolism. It is formed by liberation of energy from nutrients and organic compounds of the cell and is utilized as a source of energy to "drive" chemical reactions requiring the input of energy. Much energy is required by an organism to form an ATP bond and when the bond is split, much energy is available as heat or chemical energy in the products of the reaction. ATP and other compounds able to release large amounts of energy are really the vital link in the energy supply of the cell. The exogenous energy source so vital to bacterial development is used by the organism to form high energy chemical bonds which in turn are utilized in every aspect of cellular synthesis. The net result of abundant available energy in bacterial colonies is growth and reproduction.

All of the research cited by Mallette (65) for energy of maintenance (8, 24, 25, 28, 32, 63, 79, 127) revealed some aspect of the increased energy requirement for growth and reproduction. In most cases, the relationship was found to be linear (8, 24, 25, 63, 79).

Different species of bacteria obtain energy in varied ways. Some species are capable of aerobic respiration because they have a cytochrome system for transporting

electrons from the oxidizable donor (energy source) to molecular oxygen. The oxygen is reduced to water. Organisms utilizing respiration to oxidize organic compounds generally produce carbon dioxide as the sole or principal carbonaceous waste product (112). Incomplete oxidation will yield partially oxidized organic compounds as the principal waste products.

Cell materials are normally a major product of microbial respiration. As much as two-thirds of the total carbon of an organic nutrient may be used for growth or oxidative assimilation, only one-third being evolved as CO_2 (112). This is not "complete" oxidation in the sense of complete combustion, but the assimilated materials are hardly to be considered waste products. Incomplete oxidation, although generally a fraction of the substrate is assimilated for biosynthesis, produces oxidized organic waste products which accumulate in the medium. Several end products of incomplete oxidation are acetic, gluconic, pyruvic, α-ketoglutaric, fumaric, citric, glycollic, and oxalic acids (112).

Many bacteria obtain energy through anaerobic respiration. Anaerobic respiration utilizes an inorganic substance other than oxygen as the external electron acceptor.

Nitrate, sulfate, or carbonate is the final acceptor (112).

Some bacteria are capable of utilizing photosynthetic reactions for energy. Light energy is transformed into chemical bond energy and the bond energy is then available for synthesis of cell materials from CO_2 or organic nutrients.

Fermentation is a fourth means by which bacteria are capable of obtaining energy for vital processes. Many species of bacteria are capable of carrying on fermentative processes. Fermentation constitutes the class of energy-yielding biological oxidation-reduction reactions in which organic compounds serve as the final electron acceptors (112). End products of fermentation always include reduced organic compounds as well as oxidized products.

Both the electron donor and acceptor are organic compounds in fermentation, and both are usually generated from a single organic substrate during the course of intermediary metabolism. A "fermentable" compound must yield both oxidizable and reducible intermediates, therefore must not be too oxidized or too reduced to begin with. Sugars are the most common compounds used by organisms during fermentation. Many bacteria are also capable of utilizing a variety of organic acids, amino acids, purines,

and pyrimidines. Fermentation end products depend upon the substrate and organism and often upon environmental factors such as temperature and pH (112).

Rumen fermentation.

A very special fermentation is found in the rumen. The rumen is essentially an anaerobic highly reducing system buffered at a slightly acid pH, at a temperature of 39°C and under a gas phase composed mainly of carbon dioxide, methane and nitrogen (1). Under these conditions a specialized, but highly complex, population of microorganisms develops. The complex bacterial population is accompanied by a population of rumen ciliate protozoa.

The rumen protozoa are obligate anaerobes that digest soluble sugars, starch, fibrous matter, and cellulose (1, 112). The rumen ciliate protozoa are mainly of three classes: the large species of the genus Diplodinium and other related types such as the Metadinium which ingest fibrous matter and starch, the smaller Entodinium which digest starch, and the species that do not ingest plant materials, e.g., Isotrichia and Dasytricha (1). These protozoa are hard to maintain in in vitro cultures and

little is known of their growth requirements. The bacterial-protozoan relationships in the rumen are very complex and there may be a true symbiosis which makes nutritional study of the entire protozoal population presently impossible in the absence of the rumen bacterial population.

Although the protozoa are quite important as a source of carbohydrate and protein, the bacterial population provides the bulk of the microbial fermentation in the rumen (1).

The ruminant animal is peculiar in the extent to which it can utilize coarse, fibrous and normally indigestible foodstuffs as sources of nutrients. Grass alone, usually an important constituent of ruminant rations, is composed of the following carbohydrates: glucose, fructose, sucrose, the oligosaccharides tachyose, raffinose and melibiose, pentosans, fructosans of various meolecular weights, and celluloses and hemicelluloses containing glucose, xylose, glucuronic acid, galactose, galacturonic acid, and arabinose. Cereal grains, another important nutrient source for ruminants, are rich in starch (1). The rumen microbial population is principally responsible for the ability to digest all of the above compounds. The ability to digest cellulose, a major constituent of ruminant rations, is perhaps the most

important function of the rumen microbes. Ovine ruminal microbes can render 70-95% of all forms of cellulose soluble in three days (41).

The main products of ruminant carbohydrate fermentation are acetic, propionic, and butyric acids, carbon dioxide, and methane (1, 112). Lactic acid, produced in less quantity, is also an important energy source in ruminants (96).

The production of the short-chained fatty acids (VFA) is a vital aspect of ruminant microbial fermentation. Whereas humans and most other monogastric animals utilize glucose as their primary energy source, the ruminant is organized to utilize VFA's as its primary energy source. Very little carbohydrate is absorbed from the intestine (96). Most of it is fermented in the rumen. Bacterial and protozoal polysaccharide and the little starch that may escape fermentation appear to be the only form of carbohydrate available to the animal. The quantities of these substances entering the abomasum and intestine appear to be relatively insignificant (96).

The lower volatile fatty acids are produced in copious amounts in ruminants. Data from various sources, compiled by Blaxter (10), indicate that sheep on maintenance

levels of feeding produce two moles acetic acid per day. Comparable studies show 10 times greater production of acetic acid in cattle than in sheep (10). Balch (6) has estimated the possible production of acetic acid in a cow to be 1.5 kg per day. Davis et al. (26) using isotope dilution studies, showed good agreement with Balch's value. Carroll and Hungate (15) have shown that 6,000-12,000 kcal become available from the VFA's produced by rumen fermentation in cattle. Sheep fed maintenance rations absorbed almost 900 kcal per day from acetic, propionic, and butyric acids alone (10). This represents two-thirds to three-fourths of the total energy absorbed (10). Glucose in comparison only provided about 75 kcal per day. The remaining one-fourth to one-third of the energy absorbed is accounted for in large part by the energy of amino acids and long-chain fatty acids. A host of minor food constituents and minor microbial fermentation products account for the remainder of the total energy absorbed by the ruminant (10).

Many workers have shown direct absorption of VFA's (3, 4, 29, 56, 69, 94, 95) from the rumen. Some of the fatty acids disappearing from the rumen are partially metabolized by the rumen epithelium (2, 29, 43, 91, 92, 93).

The extent of this epithelial metabolism is least for acetate and greatest for n-butyric acid (29). The VFA's are carried by the portal blood to the liver where they can be metabolized more fully.

McCarthy, et al., (71) using perfused goat livers, showed rapid and complete removal of propionate, butyrate, and valerate from the blood by the liver. Perfusion of the liver with labeled acetate gave increased blood acetate. These workers concluded that acetate is not metabolized appreciably by the liver, and is therefore available to extrahepatic tissues. The liver acts upon propionate and butyrate to form mainly carbohydrate which is then available to the body (71).

Microbial action upon ingested protein and other nitrogenous compounds is another important aspect of the rumen fermentative processes. In monogastric animals nitrogen requirements are met by ingestion of proteins with their subsequent breakdown to and absorption as peptides and amino acids in the stomach and small intestine. Ruminants differ in that ingested proteins are subjected to microbial degradation before passage to the abomasum and intestine. The rumen microbes alter and supplement the amino acids of the ingested protein and modify the amount of

nitrogen which finally becomes available to the animal (1).

Pearson and Smith (90) in 1943 first clearly demonstrated that both synthesis and breakdown of protein occurred in the rumen. McDonald (72, 73) showed that ammonia was a major end-product of the degradation of several different proteins and was the main compound of the nonprotein nitrogen fraction of the rumen contents. The amount of ammonia formed was dependent on both the nature of the ingested protein and the relative amount of carbohydrate in the diet. Free ammonia was formed rapidly from the soluble protein, casein, whereas zein, being relatively insoluble, was only slowly attacked in the rumen. Protein is degraded by the action of the microbial proteolytic enzymes yielding peptides and amino acids which are then attacked by deaminases to give ammonia (1).

Protein synthesis is closely related to the degradation of ingested proteins. The degradative reactions provide a large supply of peptides, amino acids and ammonia for the growth of the rumen microorganisms. This growth naturally involves synthesis of microbial protein.

McDonald (74) has estimated that 40% of the zein ingested by sheep fed only zein as a nitrogen source was used for synthesis of microbial protein. McDonald and Hall

(75) have estimated that 90% of ingested casein, which constituted 87% of the nitrogen intake, was degraded in the rumen and utilized for the synthesis of microbial protein.

The microbial synthesis of protein can become extremely significant in that nonprotein nitrogen sources can replace dietary protein to an appreciable extent. A wide variety of nonprotein compounds can be utilized by rumen microbes for protein synthesis (9). Urea is probably the most extensively studied compound. Ammonia is rapidly formed from ingested urea by the urease activity of the rumen microbes (1). This ammonia, as with that formed by protein degradation, can be utilized for microbial protein synthesis.

To adequately evaluate the protein nutrition of the ruminant the nutritive value of the microbial protein must be considered. Protein quality has been studied by amino acid analysis and by measuring digestibility and biological value when fed to the rat.

Amino acid content of the rumen microbial protein does not fluctuate greatly and is generally similar to that of other microorganisms (125). Some synthesis of amino acids which may be deficient in the dietary protein has been shown to occur (1). Amino acid analyses of rumen

microbial protein hydrolysates indicate that methionine and isoleucine contents may be inadequate, but such evidence is very limited (1).

Feeding experiments with the rumen microbial protein indicate true digestibility values of approximately 70, and biological values of approximately 80 (1). The bacterial and protozoal fractions have similar biological values for rats. The digestibility of the protozoal fraction is somewhat greater than the bacterial portion (1). Annison and Lewis (1) conclude that rumen microbial proteins have a good, but not outstanding, feeding value.

Action upon lipids by rumen microbes is somewhat limited. Leaves of pasture plants and other forages contain 4-6% lipids in the form of glycerides, free fatty acids, sterols, waxes, and phospholipids (34). Fatty acids account for approximately one-half of the weight of the lipids and the larger share occur as glycerides (34). Garton (34) mentions that Weenink (124) showed the glycerides to exist as galactoglycerides. Data compiled by Shorland et al. (111) and Garton (33) reveal that 84.4% and 80.4% of the fatty acids in clover-rich pasture and mixed pasture grasses, respectively, are unsaturated.

One of the major aspects of rumen microbial action upon lipids is the hydrogenation of unsaturated fatty acids (34). Work done by Reisser and Reddy (102), Shorland et al. (111), Garton et al. (35), and Garton (33) show up to six-fold decrease in iodine value between dietary lipids of various rations fed to sheep and goats, and rumen lipids. Shorland et al. (111) also showed that the linoleic acid of the pasture grass lipids was very effectively hydrogenated with over 50% of it being converted to stearic acid. Wright (130, 131) has shown that the hydrogenating effect is due to both bacteria and ciliate protozoa. Polan et al. (97) have shown Butyrvibrio fibrisolvens to be capable of biohydrogenation.

Rumen microorganism can also hydrolyze the ester linkage between glycerol and the fatty acids (35). Garton et al. (35) observed that 50-60% of the rumen lipids of a sheep fed various rations were as free fatty acids. They also showed that 92% of the lipids in the rumen of a sheep fed supplemental linseed oil occurred as free, partially-hydrogenated, higher fatty acids seven hours after the last feeding.

After hydrolysis of the glycerides, the rumen microbes are able to ferment the glycerol released. Garton

(34) reports that Johns (53) found propionic acid produced by the fermentation of glycerol by rumen contents *in vitro* and *in vivo*. Garton (34) reports 50% disappearance, in four hours, of 500 mg glycerol incubated with 100 ml rumen contents. The remainder was utilized over about 20 hours. He found that VFA's, calculated as propionate, accounted for no more than half the glycerol fermented. There is also some evidence that small amounts of lactic and succinic acids can be produced along with the propionic acid (34).

The extreme importance of the rumen microbial population is clear, considering the fermentation of carbohydrates to yield volatile fatty acids for energy, the synthesis of microbial protein, and the hydrogenation, hydrolysis and glycerol fermentation of lipids. The ruminant animal is dependent upon that population for its energy and protein. The concentration of microbes and the nature of the population present are therefore of paramount importance in the nutrition of that animal. In this report, microbial cellular composition and microbial metabolic activity upon fermentable sources of energy such as glucose becomes very important for consideration.

Cell Fractionation and Composition

Most of the extensive work on bacterial cellular composition and metabolic activity has been done with pure cultures of a number of different organisms. One extensive compilation of such research was concerned with studies of biosynthesis in *E. coli* by Roberts, Abelson, Cowie, Bolton, and Britten (104).

By modifying standard methods, Roberts *et al.* (104) resolved *E. coli* cellular constituents into broad classes of compounds. They then proceeded to identify some of the components of each fraction. Their primary interest was into what fractions *E. coli* metabolized a number of radioactive substrates.

The fractions are referred to as the cold-trichloroacetic acid (TCA)-soluble fraction, the alcohol-soluble fraction, the alcohol-ether-soluble fraction, the hot-TCA-soluble fraction, and the principal protein fraction.

The cold-TCA-soluble fraction contains most of the transient intermediates of the cell. Paper chromatography shows many regions which react with ninhydrin, but do not correspond with locations of known amino acids. One

prominent region has been identified as glutathione. Glutathione was found to account for 20% of the carbon of the cold-TCA fraction. No intermediates of carbohydrate metabolism have been identified in this fraction. Such compounds may be so highly transient as to be lost during the washing of the cells.

Chromatograms of the hydrolyzates of the cold-TCA fraction show many components, with glutamic acid being the most prominent and containing 20% of the total carbon. Glycine and cystine are found in quantities corresponding to the glutathione, alanine is less plentiful, and aspartic acid is barely detectable. Two of the regions from the hydrolyzates correspond to adenine and uracil.

Other researchers have also done limited analyses of the cold-TCA-soluble fraction to identify compounds which may be present or absent in various species. Campbell *et al.* (13) found no poly-β-hydroxybutyric acid or glycogen in the cold-TCA-fraction of *Pseudomonas aeruginosa*.

Crater and Mikolajcik (22, 23), working with three strains of *Streptococcus lactis*, studied nucleotide content of the cold-TCA. In the cold-TCA-soluble portion of the cell-free extract they found adenosine, guanosine, uridine,

NAD, AMP, ADP, GMP, UMP, and UDP with most of the compounds present as carbohydrate complexes.

Roberts et al. (104) found two major components in the alcohol-soluble fraction. One is lipid and the other protein. Identification of the lipid components was not made other than chromatographic separation of lipid hydrolyzates into five groups. The alcohol-soluble protein differed from the principal protein fraction in solubility characteristics and in the way sulfur-deficient E. coli synthesized and utilized it. This fraction was essentially free of nucleic acids. Hydrolysis increased free amino acid groups by a factor of eleven and chromatograms of the hydrolyzates show the usual amino acids with no significant alteration from the proportion found in the principal protein fraction. Apparently one-sixth of the total protein of the cell was alcohol-soluble. Ribbons and Dawes (103) reported that a nonreducing carbohydrate was in the alcohol-soluble fraction of Sarcina lutea cells. The alcohol-ether-soluble fraction appears to be a small residual quantity of the alcohol-soluble material (104).

The hot-TCA-soluble fraction consists of the solubilized nucleic acids (104).

The residual (principal) protein fraction consisted mainly of protein. Hydrolysis of the residual precipitate and subsequent chromatography show that known amino acids accounted for 90% of the carbon of this fraction (104).

Mandelstam (67) mentions that polysaccharides and cell wall mucopeptides could be expected to appear in the hot-TCA residue. Clifton (17) states that the hot-TCA-insoluble material is composed of complex polysaccharides, protein, and poly-β-hydroxybutyrate.

Roberts et al. (104) found the bulk of the carbon of E. coli in lipids, nucleic acids and protein. The remainder of the carbon, eight percent, was distributed among the compounds of the cold-TCA fraction. Although Roberts et al. (104) identified no polysaccharide in any of the fractions, they recognized that some of the unknown components of the cold-TCA fraction could be polysaccharides. They also stated that polysaccharides may survive the fractionation and appear in the protein residue. In either case, they conclude that the polysaccharide content of their E. coli cells was less than 5% and probably less than 2%.

Roberts et al. (104) found the cold-TCA fraction to be most variable probably indicating a critical balance

with the synthesizing systems of the cell. The composition of the other fractions was much more stable even though changes in cellular environment could alter the cellular composition somewhat.

Much of the work on cellular metabolism of various substrates has been carried out utilizing the above fractionation scheme to resolve cellular components into various classes of compounds.

Hoover (44, 45) has studied the utilization of an energy source, glucose, by rumen bacteria. Following the removal of protozoa and larger feed particles he conducted ten hour _in vitro_ fermentations with labeled glucose. The cells were fractionated by a procedure similar to that of Roberts _et al_. (104). One percent glucose was utilized at a rate of 55.6 mg/100 ml per hour, while at the two percent level it was used at a rate of 115.5 mg/100 ml per hour. The average incorporation of labeled glucose into the hot-TCA precipitable "protein" at both levels of added glucose was 2.9%. An average of 9.7% of the activity disappearing from glucose appeared as nucleic acids. The specific activity of the nucleic acids was in each case at least eleven times less than that of the "protein" (hot-TCA residue). Hoover found 22-24% contamination in the

hot-TCA precipitate and thought it to be polysaccharide. Barium hydroxide hydrolyzation of the bacteria showed the polysaccharides to have over one-half as much activity as the amino acids from the same cells. If the contamination in the hot-TCA residue is actually polysaccharide it could be a real source of error in estimating incorporation of label into the protein. No consideration was given to what effect the protozoa might have had and no attempt was made to account for the manner in which all the label from glucose was utilized.

Penicillin

Use in bovines.

Mature ruminant animals, especially cattle, are often fed antibiotics for the prevention and treatment of the condition known as bloat. The most widely used and tested antibiotic for this purpose is penicillin. Many aspects of bloat and its treatment have been studied. The cause appears to be production of a stable foam within the rumen that prevents the eructation of metabolically (microbially) produced fermentation gases. The causative factor

involved in producing the stable foam is as yet unidentified although much progress is being made in the isolation of factors that under experimental conditions cause stable foam formation (37, 70, 99).

Many investigators in the field of bloat study are convinced that there are different types of bloat. Pasture bloat and feedlot bloat are two broad categories of bloat that appear to be initiated by different causative factors.

Penicillin has been found effective in combating pasture bloat.

Mode of inhibition of bacteria.

Although there is a wealth of information concerned with the reduction of the incidence of bloat by penicillin treatment, there is a noticeable lack of work concerned with the effect of penicillin upon the ruminal microbial population itself. Available information on the mode of action of penicillin upon microbial cells has to date been performed mainly with pure cultures and has been more concerned with the specific means of inhibition in the cell rather than how the penicillin affects the metabolism of treated cells.

Strominger (118), in a review article, stated that Duguid (31) in 1946 was one of the first to recognize the importance of the morphological changes that susceptible cells undergo upon subjection to penicillin. In 1956 Lederberg (61) observed that penicillin-treated E. coli cells in hypertonic sucrose broth underwent a transformation to spherical forms. Hahn and Ciak (40) confirmed the prevention of lysis by hypertonic sucrose concentrations. When the penicillin was washed out of Lederberg's cultures the cells reverted to their normal bacillary forms. He recognized that the spherical forms produced by penicillin were similar to the protoplasts produced by digestion of the bacterial cell wall with lysozyme. Strominger (118) mentioned that Lark (59) found spherical forms of at least one penicillin-treated bacillus grew and divided as spheres in the presence of penicillin. Lederberg (61) has shown cellular viability during penicillin treatment when the cells were in agar medium. Apparently the action of penicillin is upon the bacterial cell wall with subsequent weakening and disruption, or lysis,of the cell when in a nonprotective medium. Lederberg found the penicillin-formed protoplasts produced in hypertonic sucrose broth to be osmotically fragile and easily lysed when diluted

into water or ordinary broth. This induced osmotic fragility could itself explain the bactericidal properties of penicillin.

Hancock and Fitz-James (42) have shown that penicillin has no effect upon the protoplasts of Bacillus megaterium. Shockman and Lampen (110) have shown penicillin to have no effect upon Streptococcus faecalis protoplasts.

Non-growing cells are not killed by penicillin. This would indicate that blockage of new wall formation, rather than existing wall destruction, is the mode of action of penicillin.

Lower concentration of penicillin may have a bacteriostatic effect by virtue of the inhibition of cell division. Lederberg and St. Clare (62) have shown that dividing cells in lower concentrations of penicillin usually swell first from the point of incipient separation suggesting that the division septum of the cell is especially sensitive to penicillin. Long filamentous forms of the cells are then produced due to blockage of the septum formation without impairment of the synthesis of the outer wall.

The second line of reasoning that led to the conclusion that penicillin blocked cell wall synthesis started with the observation of Park and Johnson (88) that an organic labile phosphorus compound accumulated in Staphylococcus aureus cells subjected to penicillin treatment. Park (83, 84, 85, 86) later identified the previously found compounds as uridine nucleotides. Park (85) reported that his nucleotide compound and the nucleotide of UDP-glucose, isolated by Caputto et al. (14) are structurally the same. The essential difference between the two compounds appears to be the sugar attached to the nucleotide. The coenzyme contains glucose whereas the compounds Park isolated had a 2-acetylamino sugar. Strange and Powell (115) later found this sugar to be a major component of bacterial cell walls. Strominger (118) reported that Strange and Dark (113) isolated, and Strange and Kent (114) synthesized, the compound and identified it as a 3-0 lactic acid ether of N-acetylglucosamine, with only the D-configuration of lactic acid represented. A peptide made up of three D-amino and two L-amino acids is attached to the lactic acid moiety. The N-acetylamino sugar of these complexes is muramic acid. Strominger (119) proposed that the significance of the D-amino acids is to make the peptide resistant to

digestion by ordinary proteolytic enzymes. The arrangement of the D- and L- acids could also provide for structurally strong interaction among the methyl groups of the first four amino acids in the peptide, thereby making the peptide unusually stable, both biologically and chemically.

Strominger (116) showed a rapid and marked increase in the accumulation of this nucleotide compound in penicillin-treated S. aureus cells but not in cells treated with other antibiotics. He also failed to show accumulation of N-acetylamino sugar esters in penicillin-resistant strains of S. aureus and S. faecalis. He concluded that the immediacy of this response to penicillin, its specificity (penicillin causes marked accumulation, other antibiotics do not), and its relation to the threshold concentration for growth inhibition suggest that the nucleotide accumulation is a primary rather than a secondary effect of penicillin. Strominger observed that the nucleotides which accumulate are normal metabolites and are themselves not toxic to the cells. Ito et al. (51) observed a variety of uridine diphosphate amino sugar compounds in both normal and penicillin-treated S. aureus cells indicating the nucleotides are normal constituents although there was a manifold increase in the penicillin-treated cells above the normal values.

Strominger (118) pointed out that many investigators have analyzed the hydrolyzates of bacterial cell wall material and deduced that all bacterial cell walls contain a basal structure with the invariable constituents of acetyl glucosamine, the lactic acid ether of acetyl glucosamine, and alanine, glutamic acid and either lysine or α, ϵ-diaminopimelic acid.

Careful quantitative analyses of S. aureus cell walls by Strominger and Threnn (120) led to the hypothesis that the accumulated nucleotide in penicillin-treated cells was a precursor of the bacterial cell wall and its accumulation was due to inhibition of cell wall synthesis by penicillin. Strominger (118) cited a number of workers who have since supported this hypothesis by direct isotopic measurements of cell wall synthesis.

Strominger (117, 118, 119) has shown the uridine nucleotide to be involved in a cycle whereby sugar fragments can be activated, built upon and then incorporated into bacterial cell wall material of S. aureus. Various antibiotics can be shown to block the cycle at various points. Strominger indicates that penicillin does not interfere with the synthesis of the compounds to be incorporated but somehow blocks the actual step of incorporation

of those compounds into the cell wall material, hence the accumulation of the uridine nucleotides and their derivatives.

Strominger (116) has also shown cytidine-5'-phosphate compounds to accumulate concurrently with uridine nucleotide accumulation during penicillin treatment. The cytidine-5'-phosphate accumulation was doubled by penicillin. Saukkonen (109) has found that increase in cytidine diphosphate derivatives in many cases surpassed the increase of any individual uridine nucleotide during penicillin treatment of the S. aureus strain he was working with.

Baddily et al. (5) have shown a number of cytidine compounds to be normally present in cell wall material in Lactobacillus arabinosus. Salton (108) suggests that at least one of these, cytidine diphosphate ribitol, is involved in the synthesis of the teichoic acid polymer found in cell wall material.

Strominger (117) has also found accumulation of cytidine nucleotides in S. aureus inhibited by Gentian violet.

Many investigators hypothesize that the primary site of action of penicillin is upon the cell wall synthesizing mechanism. Cooper (21) showed that at least one,

and perhaps all, of the transport mechanisms of the cell wall had abruptly ceased to function before cessation of glucose oxidation or fermentation and synthesis of protein, peptide and nucleic acid. His conclusion was that some generalized damage to the osmotic barrier could account for many of the changes induced by penicillin.

Strominger (116) also showed that protein synthesis continues during the cessation of cell wall mucopeptide synthesis and the subsequent accumulation of uridine and cytidine nucleotides. Park and Strominger (89) conclude that the selective toxicity of penicillin is due to its interference in a metabolic sequence not found in animal cells--namely, the biosynthesis of the cell wall. Although they subscribed to the idea that penicillin blocks the incorporation of the N-acetyl amino sugar peptide into the wall, they concede that it could block the synthesis of the acceptor site in the cell wall. Strange and Kent (114) asserted that muramic acid in the cell wall could be attached to an amino acid by a peptide linkage and to hexosamine by a glycosidic linkage. Salton (108) considered the uridine pyrophosphate glycosyl compound to provide a transglycosidation mechanism for transferring the N-acetyl muramic acid peptide to the cell wall polymer. Similar

uridine compounds are engaged in the biosynthesis of other structural polysaccharides such as cellulose and chitin. Davis and Feingold (27) stated that penicillin interferes with transfer of the muramic acid peptide from its uridine diphosphate carrier to its polymerized position in the wall. However, the mechanism is unknown. They suggest that the high energy β-lactam ring in penicillin could infer an acylating action, linking penicillin either to transglycosidase or to the receptor involved in the muramic acid-peptide transfer.

Cooper (21) has proposed a somewhat different hypothesis. He pointed out that many workers have shown an irreversible binding of S^{35}-labeled penicillin to the bacterial cell. Quantitative estimates of amount bound range from 80-1600 molecules bound per cell. Hancock and Fitz-James (42) report about 500 molecules penicillin bound per cell in B. megaterium. The compound or compounds, responsible for the sulfur binding were named PBC, or penicillin binding compounds, by Cooper (21). Cooper's review (21) stated that PBC is probably located in the external interface of the osmotic barrier or within a small metabolic zone which may exist outside the osmotic barrier but within the confines of the cell wall. Cooper proposed that PBC is

a normal component of the cell and is involved in cellular uptake of sulfur. Penicillin when present, irreversibly binds to the PBC thus eliminating the PBC ability to reversibly bind sulfur. PBC cannot be synthesized fast enough by the cell to overcome the binding by penicillin and the cell subsequently suffers from a sulfur deficiency.

Growth is required for penicillin to be bactericidal because the penicillin must be able to bind all the PBC of the cell. In the resting cell only part of the PBC would be bound while the remainder would still be available for sulfur transport upon initiation of further growth.

Cooper hypothesized that 30-60 minutes after initiation of penicillin treatment the PBC and sulfur stock piles are so depleted that upon removal of penicillin the cell needs a period longer than the time exposed to penicillin to be able to start growing again. This is presumably to allow the cell to synthesize sufficient PBC to once again build up the sulfur required for growth. The osmotic barrier, during the 30-60 minute period after exposure to penicillin, shows some functional damage while many other cellular metabolic functions continue quite normally. Sixty to seventy-five minutes after exposure to penicillin, nucleic acid, and peptide syntheses begin

to slow. At this point many reactions can be expected to decrease due to shortage of metals, phosphate, glutamate, and perhaps other nutrients essential to cellular growth. Seventy-five minutes after initiation of penicillin treatment the osmotic barrier commenced to lose its osmotic properties and cellular dissolution began.

Collins and Richmond (20) proposed that a similarity of structure between penicillin and N-acetyl muramic acid could be a basis for the antibiotic action of penicillin. N-acetyl muramic acid is part of the uridine nucleotide complex which accumulates during penicillin treatment of cells. Collins and Richmond realized that many of the atomic configurations in the two molecules were similar and the penicillin could quite conceivably bind an active site that would normally accept the N-acetyl muramic acid molecule.

Many observations of the action of penicillin have been made in attempts to prove or disprove the hypothesis that cell wall mucopeptide synthesis is the primary site of action for penicillin. The many observations of spheroplast formation (47, 54, 59, 61, 62) are the first good evidence in support of the above hypothesis. Sensitive cells, when penicillin treated, lose their cell walls to

form spheroplasts or protoplasts which subsequently seem immune to further action by penicillin (54, 59, 61, 62, 110). The accumulation of uridine and cytidine nucleotides is another indication of penicillin's action upon cell walls although in at least one strain of S. aureus there was no accumulation of those nucleotides normally found to accumulate in penicillin-treated S. aureus cells (68).

Permeability changes should also be evidence of cell wall and osmotic barrier damage. Such changes have been shown often (100, 122) and under normal conditions proceed to the point of leakage of macromolecules (122) and subsequent lysis of the cell (118). Some cells show no signs of gross damage although there is leakage of macromolecules occurring (122).

Studies on rate of incorporation of various labeled compounds into the cell wall should provide solid evidence for or against the above hypothesis; however, the results of different workers are confusing and apparently conflicting. Trucco and Pardee (122) utilized C^{14}-glucose to measure synthesis of cell wall material of E. coli. They found no specific penicillin inhibition of cell wall synthesis, within an experimental error of 10% in either hypotonic or hypertonic medium. Paper chromatograms of hydrolyzates of

penicillin-treated and control wall material showed similar activity in all major ninhydrin spots although there were some faint spots in the control chromatograms that did not appear in the penicillin-treated ones. Electron micrographs showed that cell walls from the penicillin-treated cultures looked similar in shape and structure to control walls, but they seemed larger on the average. They found no indication of damage or abnormal structure caused by the penicillin treatment. Protein per cell wall in the treated cell walls was 1.6 times that in the controls because division was inhibited but not cell wall synthesis. In hypertonic medium, with 97% of the cells damaged enough by penicillin to be rendered nonviable upon plating, the specific activities of cell wall and cytoplasm were observed to be almost identical in both the penicillin-treated cells and the controls.

Rogers and Mandelstam (106) worked with E. coli to show that the action of penicillin is the same in gram-negative as in gram-positive bacteria. They showed inhibition of cell wall mucopeptide synthesis by using two types of penicillin and measuring incorporation of C^{14}-glucose label into diaminopimelic acid (DAP) of cell wall mucopeptide. Trucco and Pardee (122) had reported no inhibition

of C^{14}-glucose incorporation into cell wall material, but Rogers and Mandelstam pointed out that Trucco and Pardee (122) had measured the incorporation into whole walls of E. coli. Mandelstam (66) showed E. coli walls to be constituted of about 3% mucopeptide. Since penicillin would not be expected to affect other substances, its effect on glucose incorporation into mucopeptide would be very difficult to detect by measuring whole wall material.

Meadow (77) measured the incorporation of C^{14}-glucose, C^{14}-lysine and C^{14}-α, ϵ-diaminopimelic acid (DAP) into cell wall material of an E. coli mutant requiring DAP. She found that in the first 30 minutes after penicillin treatment of exponentially growing cells viable counts fell and material absorbing at 260 mμ was released; there was no change in the amounts of glucose, lysine or DAP incorporated into cell walls or into intracellular protein. The fact that viable counts decreased prior to any decrease in the amount of DAP incorporated into wall material implies that the effect of penicillin on viability is not primarily a function of inhibition of cell wall synthesis. Hugo and Russell (48) observed that loss of viability exceeded the incidence of sphereoplast formation and as with Meadow's work the conclusion drawn was that the lethal action of

penicillin is not due to increased osmotic fragility. Rogers and Mandelstam (106) pointed out, however, that Hugo and Russell (48), in reporting that viability decrease exceeded spheroplast formation, had used more than 1% (w/v) penicillin in their media. At that extremely high concentration penicillin may well have had other lethal effects upon cells than producing osmotic fragility. At a level of 1000 units/ml penicillin spheroplast formation and viable counts agree quite well.

Prestige and Pardee (100), although they did not work with labeled compounds, also concluded that the primary site of action of penicillin was not inhibition of cell wall synthesis. In E. coli they observed leakage of large molecules and other signs of permeability change 10 minutes after addition of 150 μg/ml penicillin G. In 20 minutes the RNA content decreased, and at 25 minutes protein, RNA, DNA, and enzyme synthesis halted. After 20-30 minutes of penicillin treatment the cells could not form colonies. Similar results were seen in B. megaterium. They showed the viability loss to be correlated in time with the RNA breakdown and also observed that inhibitors of protein synthesis prevented the leakage of molecules from penicillin-treated E. coli. Prestige and Pardee concluded from their

data that a specific protein synthesis occurs in the first few minutes after penicillin addition to E. coli. They proposed that formation of a lytic enzyme, or substance much like an enzyme, is induced by penicillin the first few minutes after exposure to penicillin and that this "enzyme" attacks the bacterial membrane and cell lysis ensues. They indeed did find a lytic action in extracts of E. coli cells. Penicillin-treated cells produced much greater lytic action than the control cells and the difference could be accentuated by centrifugation or heating. The pellet produced by centrifugation of penicillin-treated cells contained much more lytic activity than the control pellet and the penicillin lytic factor was more heat stable than that of the control. These lytic extracts of E. coli caused lysis of B. megaterium protoplasts. They conclude that damage to the cell membrane rather than the wall is responsible for the leakage and permeability changes which have been shown upon penicillin treatment (82). Penicillin has been shown to induce at least one enzyme, penicillinase (98), and could conceivably be responsible for inducing another such as Prestige and Pardee suggest.

More recent work again agrees that the primary site of action of penicillin is the cell wall synthesizing

mechanism. Park (87) reported an 80% decrease of lysine incorporation into cell wall by 5 μg penicillin per milliliter. Nathensen and Strominger (80) found benzyl penicillin (penicillin G) to inhibit incorporation of tritiated-DAP into E. coli cell walls to the extent of 72%. This is in total disagreement with Meadow's work (77). Rogers and Mandelstam (106) later reported that Meadow repeated her work with the addition of sucrose to the medium and did then find penicillin inhibition of DAP-incorporation into cell walls of E. coli.

Rogers and Mandelstam (106) also used a labeled compound to measure penicillin inhibition of incorporation into cell wall material. They measured incorporation of C^{14}-glucose into the DAP of mucopeptide isolated from the cell walls of E. coli. Benzyl penicillin inhibited the incorporation into DAP, but not into amino acid components of protein. This is a similar inhibition of cell wall synthesis to that found in S. aureus by Mandelstam and Rogers (68) even to the extent of inhibition which was approximately 70%. E. coli required 500 times the concentration needed by sensitive S. aureus to produce the same effect.

Rogers and Jeljaszewicz (105) studied inhibition of mucopeptide synthesis and found that of the three major

biosynthetic processes of the cell (synthesis of protein, nucleic acids and cell wall mucopeptides), only formation of the mucopeptides is affected by high concentrations of the antibiotic. They also point out that Hugo and Russell (48) used 10^3-10^4 times the concentration of benzyl-penicillin required to inhibit mucopeptide formation by penicillin-sensitive S. aureus and they ponder whether Hugo and Russell may have been observing more widespread side effects on the cells due to the extremely high concentrations of penicillin.

Rogers and Jeljaszewicz (105) showed that the concentration of benzyl penicillin required to inhibit cell wall mucopeptide formation by S. aureus strain Oxford is of the magnitude as that required to prevent growth. Selected benzyl-penicillin-resistant cells required much higher concentrations of the antibiotic to inhibit cell wall mucopeptide formation although the composition of their cell walls was the same. The inhibitory effects upon mucopeptide synthesis of three other types of penicillin are as would be predicted from their relative potencies when tested as antibiotics. Cells made resistant to benzyl penicillin do not require increased amounts of a penicillinase-resistant penicillin (Methicillin), to

inhibit mucopeptide synthesis. Methicillin, like benzyl penicillin, does not inhibit protein or nucleic acid synthesis. Methicillin does inhibit, almost completely, the mucopeptide synthesis of the penicillinase-producing strain S. aureus 524/SC at a concentration similar to that required to inhibit the penicillin-sensitive strain Oxford. All these facts are strong evidence that in the staphylococci the site of lethal action of penicillin is the system responsible for the formation of cell wall mucopeptides.

Kaufmann (55) has viewed the inhibition of mucopeptide synthesis as a consequence of the inhibition of one or more defined enzymatic reactions. He described an enzymatic reaction that cleaves the side chain of penicillin. Test of 22 penicillins as to influence upon rate of the described reaction and susceptibility to enzymatic cleavage of the side chains showed these two properties to be correlated with the antibiotic activity of the various compounds towards Gram-negative bacteria. A strong affinity for the enzyme appeared to characterize an antibiotically potent penicillin. Kaufmann regards the reaction he described to be a model for a synthetic reaction in one step of cell wall formation whereby penicillins would inhibit the normal function of some essential enzyme.

A number of workers have recently published material concerning cell wall synthesis at the enzyme level.

Ito and Strominger (49, 50) have worked out five ATP and manganese or magnesium-requiring reactions whereby the amino acids are added to uridine diphosphate N-acetylglucosamine lactate. Nathenson and Strominger (81) have isolated a particulate enzyme preparation that catalyzed transfer of acetylglucosamine residues from uridine diphosphoacetylglucosamine to an acceptor prepared from teichoic acid.

Meadow et al. (78) isolated a particulate enzyme which utilized UDP-acetylmuramyl pentapeptide together with UDP-acetylglucosamine to form a polymer which, like cell wall glycopeptide, can be hydrolyzed by egg white lysozyme. Struve and Neuhaus (121) considering their work along with that of Meadow et al. (78) proposed a reaction whereby the N-acetylmuramyl pentapeptide is bound through a phosphate to an acceptor in the cell wall with concomitant release of UMP. The next step would be polymerization of the complex with UDP-glucosamine with the elimination of UDP and inorganic phosphate. Meadow et al. (78) tested the effect of a number of antibiotics upon the reaction. They found that penicillin and bacitracin had no physiological effect

upon the reaction. They propose that penicillin must induce nucleotide accumulation in some other manner, e.g., by interfering with synthesis or competency of the acceptor, with access of the substrate to the enzyme or the acceptor, or even with the replication of a cell wall synthesizing particle.

Although the research on penicillin is extensive and becoming more and more refined, the specific means of inhibition of bacterial cells remains to be completely elucidated.

Effects upon protozoa.

Penicillin has very little effect upon protozoal cells. Evidently protozoa, being animal cells, are not affected by an antibiotic with a selective toxicity for bacterial cell wall synthesis inhibition. Penicillin is used to retard bacterial growth in culture media designed for protozoa. In this capacity it is frequently used to culture rumen protozoa where the bacterial population is overwhelming in proportion to the protozoa.

Coleman has used 1250 units (19) and 1429 units (18) benzylpenicillin per ml of fluid in cultures propagating

Entodinium caudatum. Wright (130) studying the hydrogenation of lipids by rumen protozoa, used penicillin and neomycin to inhibit bacteria associated with the protozoa. Gutierrez and Davis (38) used a combination of procaine benzylpenicillin and dihydrosteptomycin sulfate to grow cultures of Epidinium ecaudatum (Crawley) and reported no deleterious effects upon the protozoa by the antibiotic treatment.

Gutierrez et al. (39) also used these two antibiotics, each at a level of 0.5 mg per ml incubation medium, while studying fatty acid uptake by Isotricha prostoma and Entodinium simplex isolated from rumen material. Williams et al. (126) used 1 mg per ml each of the same two antibiotics for isolation and Warburg respiratory studies of Ophryoscolex caudatus Eberlein.

Jensen and Hammond (52) used 1000 units penicillin and 1 mg streptomycin per ml of medium for the routine cultivation of Trichomonads and other related flagellates from the bovine digestive tract. They also used 5000 units per ml of penicillin, along with streptomycin and polymixin B sulfate, to develop axenic cultures of these organisms. They found that pentatrichomonads occasionally survived as much as 6000 units penicillin per ml of medium and

tetratrichomonads survived up to 3000 units penicillin per ml.

In vivo studies by Bryant et al. (12) found that three daily 75 mg doses of procaine penicillin helped establish a normal protozoal population in a steer with a subnormal number of protozoa.

Conflicting results have been reported by Clark (16). He found potassium benzylpenicillin and penicillin G killed all the ciliates in his cultures in 2-24 hours when added to a final concentration of 50 μg/ml. At lower concentrations the antibiotics were not toxic to the oligotrichs but neither did they inhibit bacterial growth.

The bulk of penicillin research has been concerned with the specific mode of action of inhibition of cell wall synthesis. Very little work has even considered whether or not penicillin affects the metabolic processes of the cell other than those concerned with wall synthesis. Recently, Vaichulis et al. (123) have shown that penicillin, in concentrations of 100,000 units per ml inhibits the endogenous metabolism of Mycobacterium smegmatis. The action of isoniazid on this bacterium was also shown to be potentiated by penicillin.

Ruminal studies.

The greatest amount of literature on the action of penicillin upon the ruminal microbiota concerns its effect with relation to its bloat-inhibiting properties. In such cases the ruminal flora and fauna are treated as one whole population and the effect of penicillin upon metabolism cannot be easily clarified.

Wiseman et al. (129) showed that 50 and 150 mg daily doses of penicillin administered to Ladino clover-fed steers gave no persistent changes in the numbers of ruminal lactobacilli, streptococci, or coliform bacteria during a two week period. In one steer the lactobacilli numbers decreased then increased. This may have indicated development and proliferation of a penicillin resistant strain. In spite of the in vivo results they showed ten isolates of streptococcal bacteria and isolates of lactobacilli to be susceptible to 1 ug penicillin per ml. Paracolon bacteria were able to destroy penicillin and these workers hypothesize that the destruction of the antibiotic may have been the reason for the persistance of the streptococci and lactobacilli. Relating their findings to bloat, they hypothesize that the penicillin-destroying bacteria are reduced in number with the onset of bloat thereby

allowing the antibiotic to act on the bacteria responsible for the bloat.

Bryant et al. (12) obtained results indicating that appropriate levels of penicillin had a drastic effect on numbers and kinds of ruminal bacteria when they are first exposed to it. Their steers went off feed with three daily 75 mg doses of procaine penicillin and they suggest that it was penicillin's adverse effect upon the ruminal microflora that caused the steers to stop eating. In these steers the flora was shown to be abnormal before the animals first refused feed. As soon as the steers went back on full feed the microbial population returned to normal.

Horn et al. (46) found that 100 mg procaine penicillin daily for 21 and 33 days, and 200 mg daily for 12 days, gave a typical microfloral population for adult ruminants and very similar to that of the control. Four hundred and 800 mg daily for the last 4 and 8 days of the trial yielded a distinctly different floral population. They found that 100 and 800 mg levels gave exceptionally dry ruminal contents and somewhat redder ruminal linings although there was no difference between the two levels. All the steers remained in a thrifty, normal condition although a decrease in nitrogen retention was caused by 32,

100 and 400 mg levels of penicillin and at the 400 mg level the urine became a milky-looking suspension with a yellowish-green cast on the third and fourth days of feeding.

Bryant et al. (11) with one 50 mg dose of procaine penicillin given orally, prevented bloat in steers on Ladino clover pasture although there was little apparent effect on the numbers or species of ruminal bacteria they were able to culture. The numbers of facultatively anaerobic streptococci were significantly depressed but the numbers of streptococci were low in relation to the anaerobic bacteria and Bryant et al. thought it extremely unlikely that they would have contributed to bloat under those conditions. The mean of total anaerobic counts in the penicillin-treated steers was lower, though not significantly, than the mean of counts of the same animals not treated with penicillin. These workers concluded that the effectiveness of penicillin in controlling bloat could not be explained on the basis of gross differences in numbers of species of rumen bacteria and they suggest the possibility that the metabolism of the flora may have been altered.

Klopfenstein et al. (57) assumed gas production to be a measure of microbial activity. By manometric technique they measured the effect of exposure time to antibiotic on

gas production *in vitro*. Their work involved a penicillin-streptomycin mixture, so not all effects are attributable to penicillin. Gas production was measured for four hours. It was depressed to a level of about 68% of the control value by a high level of antibiotic (400 mg streptomycin and 40,000 units penicillin per 20 ml fluid). A low level of antibiotic, 0.001 of the high level, depressed gas production to 90% that of the control. The low level of antibiotic produced about 26% more gas than the high level when both were added at the beginning of the fermentation. When added at 100 min the control produced only about 12% more gas. An experiment concerning addition of nutrients at various times showed the antibiotic combination to depress gas formation from approximately 49-79% that of the control depending upon time of nutrient addition to the fermentation mixture.

The following work was undertaken in order to elucidate, in a general manner, what effect penicillin has upon ruminal bacterial and protozoal populations in regard to their metabolism of glucose.

III. EXPERIMENTAL PROCEDURE

A total of four *in vitro* fermentations were conducted. Fermentations I, II, and III each had one control flask and one treatment flask. Fermentation IV had two control and two treatment flasks. Fermentations I, III, and IV were allowed to proceed for 3 hr,while fermentation II continued for 4 hr.

Rumen fluid used in fermentation I was combined from two cows receiving 8 lb hay and 4 lb grain per day. Fermentation II rumen fluid was from one cow receiving 8 lb hay per day. Rumen fluid used for both fermentations III and IV was combined from two cows being fed 10 lb hay and 10 lb grain per day on a twice daily feeding. In every case, rumen fluid was collected from rumen fistulated cows, strained through four layers of cheesecloth, and held at 38-39 C until initiation of the *in vitro* fermentations.

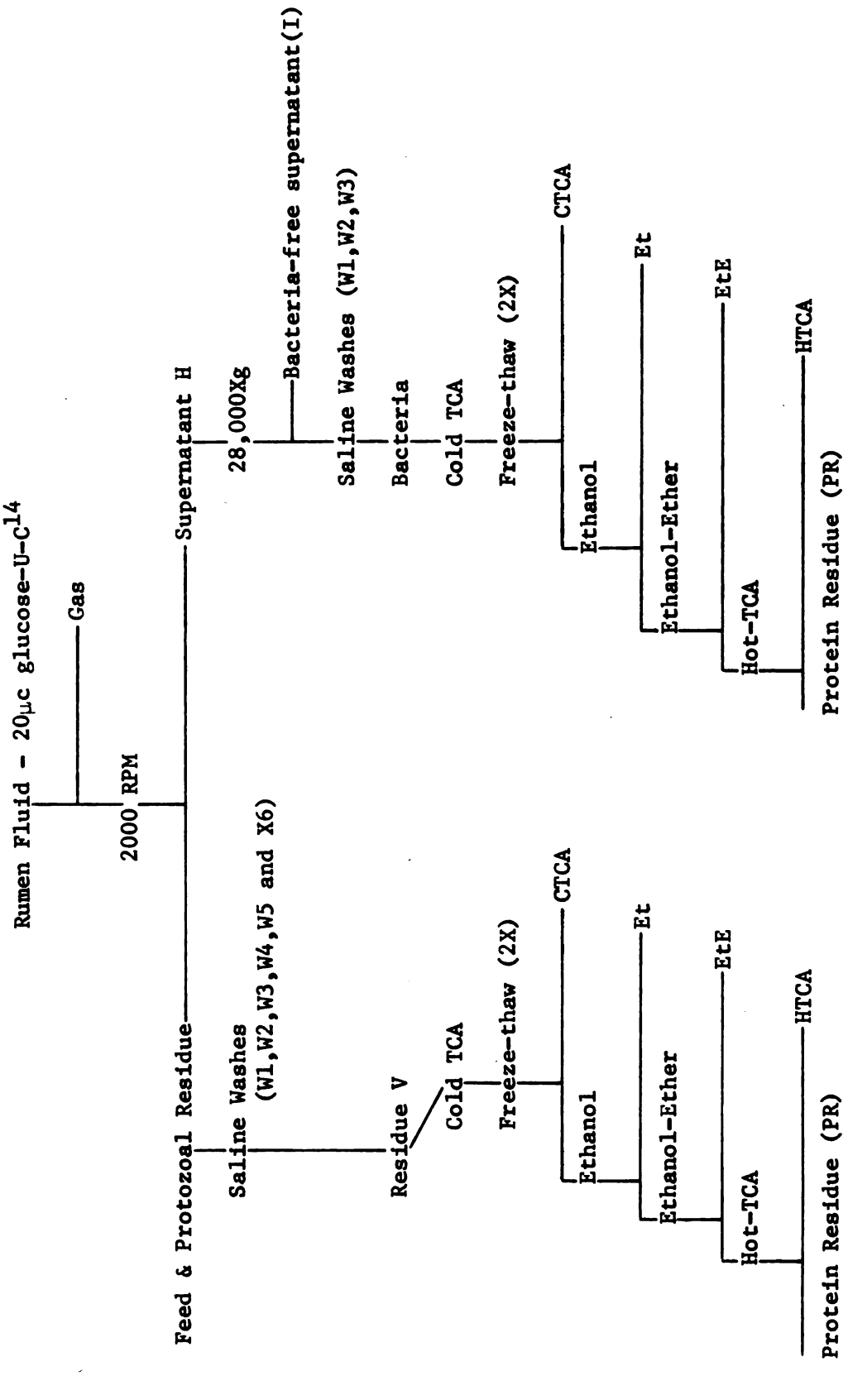
The *in vitro* fermentations were carried out with three parts of this strained rumen fluid and one part of 0.09 M phosphate buffer containing 0.03 M sodium carbonate and 0.033 M urea. The buffer was bubbled with carbon dioxide to pH 6.9-7.0 and two grams of glucose per 100 ml was

added to the buffer. Final glucose concentration of the fermentation mixture was 500 mg/100 ml, excluding that added as labeled glucose.

The fermentations were carried out in Erlenmeyer flasks connected to gas collecting burettes. To each flask was added rumen fluid and buffer in a 3:1 ratio. Total volumes were 100, 80, and 40 ml for fermentations I, II, and III, respectively. Fermentation IV involved 40 ml volumes with two control and two treatment flasks. Twenty microcuries of 2.1 μc/mg uniformly labeled glucose was added to each flask in fermentations I, II, and III. The 20 μc labeled glucose added to each fermentation IV flask had a specific activity of 47.4 μc/mg. Sodium penicillin G was added to treatment flasks at a level of 2.5 units/ml. These flasks were always paired with a control flask. Fermentation proceeded for the designated length of time in a 39 C water bath. Gas production was measured every fifteen minutes.

At the end of 3 hr (4 hr for fermentation II) the flasks were emptied into chilled centrifuge bottles, rinsed with an equal volume of cold 0.85% sodium chloride (NaCl) solution, and that in turn was added to the centrifuge bottle according to the protocol given in figure 1.

Figure 1. Fractionation scheme for *in vitro* fermentation mixtures



The suspension was centrifuged fifteen minutes at 2,000 RPM to remove feed particles and protozoa. The supernatant was either decanted or drawn off under vacuum yielding a bacteria-rich supernatant (H) and a residue.

A large volume of cold 0.85% NaCl was added to the residue, the pellet broken up, suspension mixed, and centrifuged again to yield saline wash W1 and residue. Another large volume of cold saline was added to the residue and the procedure repeated to yield wash W2 and residue. In fermentation IV three more repetitions of the foregoing procedure yielded washes W3, W4, W5, and residue V.

An aliquot of supernatant H was acidified with 50% sulfuric acid and refrigerated for volatile fatty acid (VFA) analyses. The remainder of the initial supernatant (H) was centrifuged for 20 minutes at 28,000 X g to remove the bacteria. This supernatant was decanted and the bacteria washed three times with cold saline.

The fractionation procedure used was a very slight modification of that used by Roberts et al. (104). To the bacterial cells was added cold 5% trichloroacetic acid (TCA). Treatment of the protozoal and feed fraction was done with 7% TCA instead of 5% TCA. The pellet was broken up and the suspension frozen and thawed a number of times to disrupt

the cell membranes. Centrifugation yielded the cold-TCA soluble fraction (CTCA). The residue was then treated with 95% ethanol for 30 minutes at 45 C to give the ethanol soluble fraction (Et). The residue was then treated with a 1:1 ethanol, diethyl ether mixture for 15 minutes at 45 C to give the alcohol-ether soluble fraction (EtE). The residue was next treated with 5% TCA at 100 C in a water bath for 30 minutes to give the hot-TCA soluble fraction (HTCA). The residue of the last treatment is the principal protein fraction (PR).

Chloroform extraction of PR in fermentation II was performed to remove any poly-beta-hydroxybutyric acid from the protein residue. Sulfuric acid treatment and subsequent spectrophotometric analysis according to the method of Law and Slepecky (60) was performed to identify the presence of poly-beta-hydroxybutyric acid.

All fractions were measured for radioactivity by the liquid scintillation method using a 5:5:3 scintillation mixture of xylene, dioxane, and ethanol to which was added 50 mg/l alpha-naphthylphenyloxazole α-NPO), 5 g/l 2, 5-diphenyloxazole (PBO), 80 g/l naphthalene, and 40 g/l Cab-O-Sil (36). In some cases the Cab-O-Sil was omitted. Samples were counted in polyethylene counting vials.

Benzoic acid-C^{14} was utilized for internal standardization. One thousand forty-six disintegrations per minute were added as internal standard. Counting was done in a Packard Tri-Carb liquid scintillation spectrometer.[1]

Volatile fatty acids in fermentations I and II were determined by separation on celite columns by the method of Wiseman and Irvin (128). VFA in fermentations III and IV were isolated by steam distillation. Titrations subsequent to these procedures were performed using approximately 0.02N alcoholic potassium hydroxide standardized against potassium acid phthalate. Gas chromatographic VFA analyses on fermentation IV fluid were done with a Carbowax 20M[2] column at 135 C in an Aerograph Model 600 gas chromatograph[3] equipped with a hydrogen flame detector.

Lactic acid was determined by the method of Barker and Summerson (7). Total carbohydrates were determined by the phenol-sulfuric procedure (30) with glucose used as the standard. Protein was determined by the method of

---

[1]Packard Instrument Co., Inc., LaGrange, Illinois.

[2]Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, California.

[3]Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, California.

Lowry (64). Glucose was determined by the use of Glucostat reagent.[4]

An attempt to characterize the hot-TCA fraction of the "protozoal" portion of the fermentation mixture involved a procedure outlined by Sakami (107). The hot-TCA fraction was subjected to alkaline hydrolysis, acid hydrolysis and extraction with dioxane-water (95:5). Spots of the dioxane-water extract were developed on paper chromatograms using an ethyl acetate, water, acetate acid (3:3:1) solvent. Ribose was detected by m-phenylenediamine hydrochloride and glucose by 1% potassium permanganate in 1% sodium carbonate solution. The residue left after extraction with dioxane-water was dissolved in 0.1N HCl and counted.

---

[4]Worthington Biochemical Corporation, Freehold, New Jersey.

IV. RESULTS

Fermentations I and II were preliminary work performed to make improvements in technique. Fermentation I (100 ml total volume) utilized mixed rumen fluid from two cows receiving 8 lb hay and 4 lb grain per day. The fermentation was allowed to proceed for 3 hr.

Fermentation II was a 4 hr fermentation with 80 ml volumes utilizing rumen fluid collected from one cow receiving 8 lb hay per day.

Fermentation III and IV, which yielded the most complete sets of data, were 3 hr, 40 ml fermentations. Each fermentation involved a penicillin-treated flask and control flask except fermentation IV which had two of each.

The only adequate gas collection measurements were made on fermentations I, II, and IV. In I and II penicillin treatment gave larger volume of gas initially and then gas production decreased below the level of the control. In fermentation IV each penicillin flask had continuously more gas production than the controls.

Volatile fatty acid productions in fermentations I and II were conflicting. The discrepancies may be contributed to by poor technique. In fermentation I the penicillin treated flask produced a larger amount of total VFA than the control. Expressed as percentages of corresponding control values, the penicillin treatment yielded 99, 126, 128, 100, and 219% the amount of respective control acetate, propionate, butyrate, lactate, and valerate. Total VFA production of penicillin treated samples was 112% of control VFA production.

Penicillin treatment in fermentation II yielded only 84.6% the amount of VFA produced by the control. Acetate, propionate, butyrate, lactate, and valerate gave respective percentages of 92, 92, 61, 114, and 32 when penicillin treatment VFA production is expressed as percent of the control VFA production for the respective fatty acids.

Radioactivity measurements on the VFA from fermentation II indicate much higher activities in the acetate and lactate fractions of penicillin treatment VFA than in the control. With the penicillin treatment activity expressed as a percentage of control values, the acetate, propionate, butyrate, valerate, and lactate gave respective percentages of 163, 96, 79, 29.5, and 169. If the data are

accurate, then there is a much higher specific activity in the penicillin treatment acetate and lactate than in the control. Total radioactivity in the penicillin treatment VFA was 133.3% that in the control VFA.

Fractionation and subsequent radioactivity measurements of the cells contained in 15 ml of the control fermentation fluid from fermentation II showed 29% of the bacterial activity to be in fractions CTCA, Et, and EtE, whereas 30% was in the nucleic acid fraction (HTCA) alone. Forty percent remained in the principal protein residue (PR) after extraction of that fraction with hot chloroform. The chloroform extraction accounted for only 0.87% of the total bacterial activity. This could conceivably be residual activity from fraction HTCA that was removed by the chloroform. Spectrophotometric analysis of the sulfuric acid treated extract revealed no poly-beta-hydroxybutyric acid.

Total carbohydrate analyses were performed on some of the bacterial fractions in fermentation I. Carbohydrate was detectable in the first wash, the second and third washes, and in fractions CTCA and HTCA. Fractions Et, EtE, and PR had either negligible amounts of, or no carbohydrate present. The first wash accounted for about 50% of the

total carbohydrate determined to be in the three washes, CTCA, and HTCA. The second and third washes had about 25% and CTCA accounted for about 7.4%. The quantities present in these three fractions were similar for both penicillin and control. Penicillin treatment reduced the amount of carbohydrate in the nucleic acid fraction to an average of 75% that of the control. The radioactivity of penicillin treatment HTCA decreased less in proportion to the control HTCA than did the carbohydrate content of penicillin treatment HTCA compared to the carbohydrate content of control HTCA fraction.

The most complete data on radioactivity distribution were obtained in fermentations III and IV. However, gas was lost during fermentation III due to leaky apparatus.

Low speed centrifugation of the fermentation III mixture revealed that slightly less than one-half of the radioactivity added as glucose was in the supernatant fraction (H). Washing of the residue (V) with large volumes of 0.85% saline washed much of the microfloral population away from the protozoal and feed particle residue. The first saline wash (W1) removed almost 5% of the total added activity from the residue and the second saline wash (W2) removed over 1%. If the radioactivity of the

two saline washes is assumed to be distributed in a manner similar to the initial supernatant H and the washes are lumped in with the low speed supernatant the three fractions (H, W1, W2) account for 51% of the total initial activity.

The residue of the initial low speed centrifugation of fermentation IV was subjected to five 100 ml saline washes. If these are totaled with supernatant H, 40-44% of the total activity is accounted for. For somewhat cleaner separation a portion of supernatant was left on the feed and protozoal residue. When this was removed it accounted for another 1% of the total activity.

High speed centrifugation of H removed the bacteria from suspension. Glucose determination performed on the cell-free supernatant (I) from fermentation III showed over 99.8% utilization of the glucose during the 3 hr fermentation. Volatile fatty acid (VFA) analysis of the cell-free supernatant (I) of fermentation III revealed 97.85 and 98.60 μM VFA per milliliter of fermentation fluid for the control and penicillin treatments, respectively. Specific activities of the VFA were 35.81 DPM/μM for the control and 36.38 DPM/μM for the penicillin treatment.

Gas chromatographic analysis of supernatant H for fermentation IV showed 71.86 μM acetic plus propionic plus butyric acids per ml in the control and 77.55 μM/ml in the penicillin flask. Steam distillation gave values of 57.12 and 56.30 μM VFA/ml for controls and penicillin, respectively. The discrepancies among total VFA are hard to resolve as more would be expected in steam distillation due to at least partial distillation of lactic acid and valeric acid, but data from both fermentations III and IV show that there is little difference between the control and the penicillin VFA production. In each of the five cases the radioactivity present in the penicillin VFA was slightly higher than that in the control. The penicillin VFA activity always accounted for 1-2% more of the total added activity than did the control.

If it is assumed that the distribution of activity in the saline washes is very similar to that in supernatant H then control VFA accounts for 69.86%, and penicillin for 69.22%, of the activity in W1 and W2 of fermentation III. In fermentation IV the control VFA would account for 77.04% of the activity in the first five washes and penicillin VFA would account for 78.58%. The last supernatant removed from the residue V was not considered due to the good

possibility that the distribution of activity was not like that of H. Some of the material from residue V was decanted into the last supernatant.

The activity in the residue V washes assumed to be due to VFA plus the VFA in the initial bacterial suspension (after low-speed centrifugation) accounted for 37-38% of the total added activity in fermentation III and 33-34% in fermentation IV. There was about 1% difference between penicillin and control with penicillin consistently having slightly higher activity. A single lactic acid determination in fermentation III showed three times as much lactic acid in the penicillin flask as in the control. VFA separation on celite columns in fermentation II also showed increased lactic acid production in the penicillin treatment. Lactic acid determinations were not performed on fermentation IV material.

Data showing the removal of activity from the microflora by washing and subsequent fractionation of the cells is presented in table I. The data are expressed as percent of bacterial total (CTCA+Et+EtE+HTCA+PR) and as percent of added 20 μc.

Table I. Activity removed by washing and fractionation of bacterial cells - Fermentations III and IV

Washes	Control		Penicillin	
	% of total	% of 20 μc	% of total	% of 20 μc
Fermentation III				
First wash		1.58		2.42
Second, third washes		0.07		0.08
Fermentation IV				
First wash		0.75-1.15		0.67-0.99
Second, third washes		0.05-0.09		0.03-0.06
Cell fractionation				
Fermentation III				
CTCA	23.4-27.9	0.13-0.14	22.7-30.1	0.11
Et	4.9- 5.2	0.02-0.03	5.2- 5.6	0.02-0.03
EtE	0.15	----	0 0.1	----
HTCA	27.5-40.0	0.16-0.19	26.3-42.3	0.13-0.15
Protein	27.1-43.8	0.13-0.26	22.5-45.3	0.08-0.22
Total		0.47-0.59		0.36-0.48
Fermentation IV				
CTCA	20.8-30.7	0.21-0.32	24.1-33.5	0.16-0.22
Et	4.9- 9.7	0.05-0.10	8.0- 8.8	0.05-0.06
EtE	0.2- 0.4	----	0.2- 0.6	----
HTCA	21.2-29.3	0.22-0.30	20.6-26.7	0.13-0.18
Protein	40.9-43.9	0.42-0.45	36.3-39.9	0.23-0.26
Total		1.02-1.03		0.64-0.65

The initial washing of the bacterial pellet with saline shows removal of considerable activity. In fermentation III the three washes *in toto* accounted for 1.6% and 2.5% of added activity for control and penicillin respectively. In fermentation IV only the control washes accounted for 1% while the penicillin washes accounted for only 0.85%. Differences of this sort can be at least partially explained as the fault of technique. Any residual supernatant fluid not decanted from the bacterial pellet would easily increase the activity of the three washes.

Examination of the tables showing results of cold-TCA (CTCA), ethanol (Et), ethanol-ether (ETE), and hot-TCA (HTCA) treatments reveal some rather wide variation between duplicates. Generally, the lipid (Et and EtE) fractions were in agreement among replicates with about 5% of the bacterial fraction total activity present in these fractions in fermentation III and 7-8% in fermentation IV with the penicillin in each case showing slightly higher percentage of activity in the two fractions.

Cold-TCA extraction of the bacterial pellet in fermentation III removed 23% and 28% of the total control bacterial activity while 23% and 30% were removed in the penicillin treatment. Triplicate determinations in fermentation

IV showed cold-TCA to remove 21-31% of the control bacterial activity and 24-33% of the activity of the penicillin treated cells. The high value in each case seems to be somewhat unrealistic. There appears to be a definite relationship between the amount of activity removed by cold-TCA and the activity removed by hot-TCA. In the case of the high values for cold-TCA there was less activity left to be removed by the hot-TCA. This may be a fault of the extraction technique. If the TCA during the extraction was not kept quite as cold as in the other determinations there is always the possibility of solubilizing some compounds which would otherwise not become soluble until hot-TCA treatment.

The most outstanding discrepancy is the amount of activity present in the hot-TCA and protein residues of duplicates in fermentation III. The control had 27% of the bacterial activity in the hot-TCA and 44% in the protein residue. A duplicate, fractionated on another date, had 40% of the bacterial activity in the hot-TCA and 27% in the protein residue. The penicillin treated cells showed the same pattern.

Protein determinations on the protein residue showed the control cells to have 94.7% of the amount

of protein present in the penicillin treated cells. Duplication between replicates was good, thereby indicating that the discrepancies in the radioactivity data were not due to varying composition of this fraction.

A feasible explanation of the difference in the activities of the nucleic acid and protein fractions could be that the pellets yielding the less activity to hot-TCA treatment were not sufficiently broken up prior to heating with TCA. This could very likely have lessened the amount of nucleic acids solubilized and they would then carry their activity into the protein residue. Although the activity of these "unbroken" protein residues was very much greater, the protein determinations indicated no difference in the amount of protein between the duplicates. The pellets of those duplicates which had greater activity in the hot-TCA were well disrupted prior to hot-TCA treatment and in this case, the data were probably much more reliable.

The extent of disruption of the pellet was probably responsible for the pattern of radioactivity distribution in HTCA and protein residue (PR) of fermentation I and II. Fractionation of the bacteria in fermentation I showed five to ten times as much activity in the hot-TCA fraction as in PR. With one exception, the data from fermentation II

confirmed the presence of more activity in HTCA than in PR. The exception occurred when using an exceptionally large volume of cells in the fractionation procedure. The most probable reason for the higher activity in PR in this instance was that the pellet was too large and was not sufficiently broken up to allow complete solubilization of the nucleic acids present.

Fermentation IV showed 21.2% to 29.3% of the control bacterial activity to be removed by hot-TCA. The 21.2% figure corresponds to the replicate that had more than average activity removed by the cold-TCA treatment as mentioned previously. The other two replicates were very similar giving an average of 28.7% in HTCA. Penicillin treatment yielded 20.6% to 26.75% in HTCA. The low value again corresponded to the high activity cold-TCA replicate. The other two replicates averaged 26.7%. The activity in the protein residue ranged from 40.9% to 43.87% (average 41.9%) in the controls and 36.3% to 39.93% (average 37.87%) in the penicillin treatment. Although the differences were slight the data showed slightly increased activity in CTCA and lipid fractions, and slightly decreased activity in HTCA and PR due to penicillin treatment. Penicillin decreased amount of incorporated glucose to 63%

that of the control in fermentation IV and to 80% in fermentation III.

Table I also shows the percentage of the initial 20 μc added activity accounted for by each of the fractions in fermentations III and IV.

The total activity of the bacterial fractions can be expressed as a percentage of supernatant H. If that percentage of the saline washes of the feed and protozoal residue is considered to be bacteria then the total bacteria can be said to account for 0.5-0.7% of the total added activity in fermentation III. In fermentation IV similar calculations show bacteria to account for 1.14% of the total activity in the control and 0.72% with penicillin treatment.

Analysis of the protozoal fraction could not be accomplished as well as that of the bacterial fraction. The saline washes were an attempt to remove most of the bacteria by means of dilution. Although microscopic examination of the washes and residue revealed greatly decreased numbers of bacteria it would be foolish to assume that all the bacteria had been separated out. Those bacteria intimately associated with feed particles and those within the protozoa would probably be rather unsusceptible to removal

by the washes. The presence of the feed particles also makes analysis of the protozoal fraction very difficult.

The saline washes accounted for about 12.5% of the total added activity in fermentation III and almost 12% in fermentation IV. Fermentation IV had the most cleanly separated fractions and was the only really complete fractionation with respect to radioactivity data on the protozoal fraction.

The activity data collected on the protozoal fraction of fermentation IV is shown in table II. The data show a tremendous amount of activity in HTCA. It accounts for 86% of the total activity in the protozoal fractions in both control and penicillin treatments. The attempt to characterize the hot-TCA fraction of the protozoal residue was very unquantitative. Only a few general remarks can be made concerning the data obtained. Chromatography of known ribose and glucose spots showed the ribose to move only a very short distance while the glucose remained at the origin. These known sugars were not dissolved in dioxane-water as was the unknown mixture. The paper chromatogram, cut according to the known spots chromatographed on the same paper, was then counted. Over ten times as much activity remained at the origin as moved to the region

Table II Activity removed by fractionation of protozoal and feed particle residue - Fermentation IV

Protozoal fractions	Control		Pencillin	
	% of total	% of 20 μc	% of total	% of 20 μc
CTCA	7.3	2.35	7.3	2.53
Et	1.1	0.36	1.1	0.38
EtE	0.06	0.02	0.05	0.02
HTCA	86.2	27.66	86.0	29.83
Protein	5.3	1.71	5.6	1.93
Total		33.1		34.7

where ribose would be expected. A conclusion that could be drawn is that much of the activity was actually in hexose (from hydrolyzed glycogen). There is, however, still the possibility that the dioxane or some chemical carried through the extraction procedure could have remained at the origin of the paper chromatogram and then reacted with the scintillator solution to artificially produce "counts."

The residue which remained after the dioxane-water extraction had almost twenty times the activity of that dioxane-water extract. This material could conceivably have been non-basic amino acids, peptides, or purine and pyrimidine bases. All should have been carried through the procedure to some extent.

Control and penicillin treatment gave practically identical distribution of activity among the fractions. Penicillin increased the total incorporation of activity into the protozoal fractions to 108% that of the control. Protozoa accounted for about 33% of the added activity.

Gas production measurements in fermentations I and II showed initially increased gas production in the penicillin flasks with a decreasing rate of production so that they ended up less than the controls. In fermentation IV the penicillin treatment gave continuously higher gas

production. In none of the fermentations was the gas production drastically different between treatment and control.

Data on gas production during fermentation IV showed 27-30 ml gas produced by the control flasks and 28-32 ml by the penicillin treatment flasks. Passage of the gas through 50% KOH indicated 27-47% CO_2 in the gas mixtures with penicillin treatment consistently showing a CO_2 content 5-20% lower than that of the controls. Attempted gas chromatographic analysis for methane revealed 0-5% methane with inconsistent values among flasks and treatments. Data on radioactivity of the gas produced is quite unreliable. The fermentation mixture foamed somewhat during the fermentation and some of the foaming material, undoubtedly radioactive, was pushed into the gas tubing and was carried over into the gas burettes. Part of this foam material that remained in the tubing was then carried into the KOH during absorption of the CO_2. Solubilization of any of this material could distort the data on activity present in the CO_2. The extreme alkalinity also created problems with the liquid scintillator and often the number of counts per minute was quite irreproducible. Dilution of one of the cleaner CO_2 samples to 1/50 showed 951,600 disintegrations per minute for the control CO_2 and 985,050

for penicillin treatment CO_2. This amounts to 2.1% and 2.2% of the initial activity added as glucose, accounted for by control and penicillin CO_2, respectively.

Partition of activity accounted for in fermentation IV appears in Table III. Only about 70% of the activity could be accounted for. Internal standardization with benzoic acid gave counting efficiencies from about 35-80%. Efficiency values either side this range were not accepted. Although internal standardization should have alleviated many of the counting difficulties, reproducible data on many of the lesser clarified samples were difficult to obtain. Most of the radioactivity unaccounted for was probably "lost" due to irreproducible sample counts and even rather wide discrepancies among channels in the spectrometer.

Table III Radioactivity accounted for in Fermentation IV expressed as percent of initially added 20 μc

	Control	Pencillin
Gas	2.1	2.2
VFA	33.1	34.3
Bacteria	1.14	0.73
Protozoa	33.1	34.7

V. DISCUSSION

The data obtained must be considered only as indicative of what occurs in a three hour *in vitro* fermentation of glucose. Metabolites are not being continually removed from the system as they would be *in vivo*. Coarse feedstuffs are not available as they are in the rumen and saliva, water, and feed are not continually entering the system as they do *in vivo*.

In spite of these drawbacks the three hour fermentation time is both short enough and long enough to give an indication of how the cells will assimilate the glucose and the effect of penicillin upon this assimilation.

At the end of three hours the bacterial population has about one-fourth of its radioactive label in the cold-TCA fraction. These are the low molecular weight metabolic intermediate compounds. The activity in this fraction could reflect the initial utilization of the glucose before it is incorporated into more stable portions of the cellular material. After three hours, however, it could also reflect activity being released from the more stable

fractions of the cell during turnover. Nucleotides and peptides released during turnover of the nucleic acids and protein would be expected to appear in the cold-TCA fraction.

The lipid-alcohol-soluble protein fraction accounts for only a small amount of the activity present in the cells. Some of the activity counted as being in these fractions may well be some residual activity from the preceding TCA extraction. The activity in this fraction ranges from 5-10% of the total bacterial activity and expressed as a percentage of the initial added 20 μc, only 0.1%, at the most, is present in this fraction.

The nucleic acid and protein fractions of the total bacterial population both have a fairly large amount of activity in them. Both these fractions probably reflect the amount of cellular growth and reproduction which is occurring in the bacterial population. Study of incorporation of label into these fractions in relation to time would have been very meaningful, but even without such data the extent of incorporation after three hours reveals how very important the bacterial cells themselves are as a source of nutrients for the ruminant animal. The bacteria are actually taking a relatively simple carbon chain,

glucose, and utilizing it to synthesize a wide variety of molecules and polymers which are eventually available to the host animal. The extent of incorporation into the bacterial protein shows how the bacteria are able to take carbon from one source, add nitrogen from another source, and build amino acids and protein which are so vital to the host animal.

Penicillin does have an effect upon the incorporation of glucose into bacterial cells. The effect appears to be quantitative rather than qualitative. Although there may be a slight trend toward increased incorporation of glucose carbon, expressed as a percent of total incorporation, into the cold-TCA and lipid fractions due to penicillin treatment, the differences are very slight and due to variation among duplicates no emphasis can be placed upon these differences. The same is true of the apparently slight decrease of glucose carbon incorporation into the nucleic acid and protein fractions. This means penicillin decreased glucose carbon incorporation into CTCA and Et-EtE fractions less than it decreased the incorporation into HTCA and PR.

Although penicillin appears to have no significant effect upon the glucose carbon distribution among bacterial

fractions, there is a very marked effect upon the total amount of glucose carbon incorporated into the bacterial population. Penicillin reduced the amount of incorporation to 80% and 63% that of the control in fermentation III and IV, respectively. There is, however, no means of determining whether the decreased incorporation caused by penicillin is due to decimation of the initial population of bacteria, inhibition of reproduction of the original bacteria, or interference with the metabolic pathways by which the bacteria metabolize glucose and/or compounds derived from the glucose.

The mode of action of penicillin appears to be interference in reproduction of susceptible bacterial cells. If this is the only factor involved then the population of susceptible cells should not increase in numbers although they may be able to continue metabolism of the glucose. The nonsusceptible population of bacteria should then be free to increase in numbers due to the decreased competition for substrate. If these cells are able to utilize the glucose in a manner similar to the suceptible cells the amount of incorporation of the glucose should not fall too far behind that of the control. This is not the case however. Possibly the nonsusceptible population

is not able to utilize glucose as well as the penicillin-susceptible cells, or perhaps the nonsusceptible cells are not able to reproduce optimally if the susceptible cells, having been inhibited by penicillin, are not producing metabolites required by the nonsusceptible cells for growth. This could mean a general decline in bacterial number and could happen if the cells, both susceptible and nonsusceptible, are living in some sort of symbiotic relationship.

The increased amount of glucose carbon appearing as VFA after penicillin treatment indicates that the glucose is indeed being metabolized but it is not being incorporated into bacterial cellular material to the same extent as in the nontreated cells. Again only speculation can be made as to the cause. A penicillin-induced alteration of bacterial metabolism may have induced VFA production from carbon which normally would have been incorporated into cellular material. A penicillin-induced change in type of population could yield a situation whereby the uninhibited cells do not have to compete for the glucose to the same extent as they would in an untreated population. If the nonsusceptible cells normally would metabolize glucose to VFA rather than cellular materials, the

reduced competition would give them more substrate to metabolize to VFA. This effect should be revealed as increased glucose carbon incorporation into VFA and decreased incorporation into cellular fractions. It should also mean increased quantity of VFA and this was not clearly shown.

The complexity of the rumen microbial population is such that no clear answer is possible as to the effect of penicillin. The most feasible answer, based on pure culture penicillin research, is that the composition of the bacterial population is changed to some extent due to inhibition of the susceptible species of bacteria by the penicillin. Although the possibility of altered metabolism has definitely not been ruled out, the only concrete evidences shown by this study are that total incorporation of glucose carbon into bacterial cellular materials is decreased, that incorporation of glucose carbon into VFA is increased slightly, and that distribution of glucose carbon among bacterial fractions is not, or is insignificantly, altered by penicillin treatment. These observations could all be due to changed populations.

The distribution of glucose carbon in the protozoa is quite different from that of the bacteria. While there is undoubtedly bacterial material in the protozoal fraction

the magnitude of the differences in carbon-14 distribution show undeniably that during a three hour fermentation the protozoa assimilate the glucose carbon in a drastically different manner than bacteria. The question as to whether or not the nucleic acids actually account for 86% of the incorporated carbon is very valid. The protozoal cells could probably not be reproducing rapidly enough or turning over the nucleic acids fast enough to account for the extensive labeling that appears in this hot-TCA fraction. Coleman (19) states, without referring to published evidence, that protozoal "glycogen" would be expected to appear in the hot-TCA soluble fraction. If this is the case much of the activity appearing in this fraction could be due to storage polysaccharide. After three hours of fermentation with abundant glucose available the protozoa may very well have built up a storage polysaccharide reserve. However, most procedures for the extraction of glycogen from animal tissue utilize a cold-TCA extraction for the solubilization of the glycogen. There should be no reason why the protozoal "glycogen" would not appear in the cold-TCA fraction.

A second possible explanation for the high activity in the hot-TCA is that the thirty minute hot-TCA treatment may have solubilized some of the protozoal protein.

Roberts et al. (104) indicate that none of the bacterial protein they were working with was solubilized by the hot-TCA treatment. The protozoal protein, however, may be more labile and may be partially hydrolyzed by the hot-TCA. Animal tissue nucleic acids are generally solubilized by hot-TCA using lower temperatures and shorter exposure times. The idea that some of the activity in the protozoal hot-TCA fraction may be due to partially hydrolyzed proteins is entirely conceivable.

In the presence of abundant glucose the metabolism of the protozoa may be increased. This would undoubtedly lead to increased enzyme synthesis which would in turn require increased ribonucleic acid synthesis. The activity in the hot-TCA fraction would thereby be increased. Although a portion of the activity could have arisen from such a situation, the evidence does not point to such being the case. If the levels of ribonucleic acid had been greatly increased there should have been a concomitant increase in the level of activity found in the protein fraction. The sheer magnitude of the increased activity points out that more than increased nucleic acid synthesis is involved. Hydrolyses and paper chromatography of this fraction indicate less activity present in ribose, from

the nucleic acids, than in two other parts of the hot-TCA fraction. The other two parts could have represented activity in glycogen and amino acids-peptides.

Even if most of the hot-TCA activity is due to hydrolyzed protein, the protozoal distribution of glucose carbon is different from that of the bacteria. Both the cold-TCA and the lipid-alcohol-soluble protein fractions have considerably less activity than the corresponding bacterial fractions.

Penicillin had no effect upon the distribution of activity in the protozoa. This is to be expected on the basis of work performed with protozoal cultures.

Penicillin did, however, increase the total incorporation of label into the protozoal fraction. This may be due to an increased amount of glucose available to the protozoa due to inhibition of the bacterial metabolism. Another possibility is that the bacterial cells may have been rendered more susceptible to ingestion by the protozoa and in this manner the protozoa may have received more of the glucose carbon.

The differences in total activity incorporated into the bacterial versus the protozoal fractions is the most outstanding aspect of this study. The protozoa, after

three hours, had almost thirty times the amount of label the bacteria had. Studies of incorporation related to time would have been very meaningful. Without such information little speculation can be made as to whether the protozoa are incorporating the label directly from glucose or are obtaining it from bacterial material, either by absorption of bacterial products or ingestion of bacterial cells.

The protozoal fraction accounted for about one-third of the total added 20 μc. This is almost as much activity as is present in the VFA. The VFA are considered an extremely important source of energy for the ruminant animal but the protozoa, at least in the presence of such a readily fermentable sugar, are able to incorporate into cellular materials as much carbon, from that sugar, as goes into VFA. The protozoa are not only an energy source,but also provide a variety of other nutrients valuable to the host animal. The importance of the protozoa in producing nutrient for the host animal is strikingly shown by the data obtained in this study. Much more research into this aspect of ruminant metabolism should be conducted in order to clarify the role of protozoa with respect to various other substrates and with respect to incorporation related to time.

VI. SUMMARY

Three hour *in vitro* rumen fermentations with a C^{14}-labeled glucose substrate showed about 1% incorporation of label into bacteria, 33% into VFA, and 33% into the "protozoal" fraction.

Transient intermediate compounds, nucleic acids, and protein accounted for most of the activity present in the microbial cells. Little activity was found in the lipid-alcohol-soluble protein fractions. The "protozoal" fraction had almost 86% of its total activity present in the "nucleic acid" fraction.

The "protozoal" fraction had incorporated almost thirty times the amount of label found in the bacteria.

Penicillin appeared to have no effect upon distribution of label in either the bacterial or protozoal cells. Penicillin decreased the amount of total label incorporation to 63-80% that of the control in the bacteria. Penicillin increased the incorporation of label in the "protozoal" fraction to 108% that of the control.

LITERATURE CITED

(1) Annison, E. F. and D. Lewis. Metabolism in the Rumen. John Wiley and Sons, Inc., New York. 1959.

(2) Annison, E. F. and R. J. Pennington. The metabolism of short-chain fatty acids in the sheep. 3. Formic, n-valeric and some branched-chain acids. Biochem. J. 57:685. 1954.

(3) Armstrong, D. G. and K. L. Blaxter. The heat increment of steam-volatile fatty acids in fasting sheep. Brit. J. Nutr. 11:247. 1957

(4) Armstrong, D. G., K. L. Blaxter and N. McC. Graham. The heat increments of mixtures of steam-volatile fatty acids in fasting sheep. Brit. J. Nutr. 11:392. 1957.

(5) Baddiley, J., J. G. Buchanan and B. Carss. The presence of ribitol phosphate in bacterial cell walls. Biochim. Biophys. Acta 27:220. 1958.

(6) Balch, D. A. An estimate of the weights of volatile acids produced in the rumen of lactating cows on a diet of hay and concentrate. Brit. J. Nutr. 12:18. 1958.

(7) Barker, S. B. and W. B. Summerson. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138:535. 1941.

(8) Bauchop, T. and S. R. Elsden. The growth of micro-organisms in relation to their energy supply. J. Gen. Microbiol. 23:457. 1960

(9) Belasco, I. J. New nitrogen feed compounds for ruminants--a laboratory evaluation. J. Animal Sci. 13:601. 1954.

(10) Blaxter, K. L. The Energy Metabolism of Ruminants. Charles C. Thomas, Springfield, Illinois. 1962.

(11) Bryant, M. P., B. F. Barrentine, J. F. Sykes, I. M. Robinson, C. V. Shawver and L. W. Williams. Predominant bacteria in the rumen of cattle on a bloat-provoking ladino clover pasture. J. Dairy Sci. 43:1435. 1960.

(12) Bryant, M. P., I. M. Robinson and I. L. Lindahl. Note on the flora and fauna in the rumen of steers fed a feedlot bloat-provoking ration and the effect of penicillin. Appl. Microbiol. 9:511. 1961.

(13) Campbell, J. J. R., A. F. Gronlund and M. G. Duncan. Endogenous metabolism of pseudomonas. in: Lamanna, C. (ed.), Endogenous Metabolism with Special Reference to Bacteria. Annals N. Y. Acad. Sci. 102:669. 1963.

(14) Caputto, R., L. F. Leloir, C. E. Cardini and A. C. Paladini. Isolation of the coenzyme of the galactose phosphate-glucose phosphate transformation. J. Biol. Chem. 184:333. 1950.

(15) Carroll, E. J. and R. E. Hungate. The magnitude of the microbial fermentation in the bovine rumen. Appl. Microbiol. 2:205. 1954.

(16) Clarke, R. T. J. The cultivation of some rumen oligotrich protozoa. J. Gen. Microbiol. 33:401. 1963.

(17) Clifton, C. E. Endogenous metabolism and oxidative assimilation of typical bacterial species. in: Lamanna, C. (ed.), Endogenous Metabolism with Special Reference to Bacteria. Annals N. Y. Acad. Sci. 102:655. 1963.

(18) Coleman, G. S. Effect of penicillin on the maintenance of rumen oligotrich protozoa. Nature 187:518. 1960.

(19) Coleman, G. S. The metabolism of C^{14}-glycine and C^{14}-bicarbonate by washed suspensions of the rumen ciliate Entodinium caudatum. J. Gen. Microbiol. 35:91. 1964.

(20) Collins, J. F. and M. H. Richmond. A structural similarity between N-acetylmuramic acid and penicillin as a basis for antibiotic action. Nature 195:142. 1962.

(21) Cooper, P. D. Site of action of radio penicillin. Bacteriol. Rev. 20:28. 1956.

(22) Crater, P. L. and E. M. Mikolajcik. Intracellular nucleotide contents of lactic streptococci. J. Dairy Sci. 47:M20. (Abstr.) 1964.

(23) Crater, P. L. and E. M. Mikolajcik. Nucleic acid derivatives associated with group N streptococci. I. Cell-free fraction. J. Dairy Sci. 48:1. 1965.

(24) Dagley, S. and C. N. Hinshelwood. Physiochemical aspects of bacteria growth. I. Dependence of growth of Bact. Lactis Aerogenes on concentration of medium. J. Chem. Soc. P. 1930. 1938.

(25) Dagley, S., E. A. Dawes and G. A. Morrison. The effect of aeration on the growth of Aerobacter aerogenes and Escherichia coli, with reference to the Pasteur mechanism. J. Bacteriol. 61:433. 1951.

(26) Davis, C. L., R. E. Brown, J. R. Staubus and W. O. Nelson. Availability and metabolism of various substrates in ruminants. I. Absorption and metabolism of acetate. J. Dairy Sci. 43:231. 1960.

(27) Davis, B. D. and D. S. Feingold. Antimicrobial agents: mechanism of action and use in metabolic studies. in: S. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria. p. 343. Vol. 4. Academic Press, Inc., New York. 1962.

(28) Dawes, E. A. and D. W. Ribbons. Endogenous metabolism of Escherichia coli. Biochem. J. 82:49P. 1962.

(29) Dobson, A. Absorption from the rumen. in: Lewis, D. (ed.), Digestive Physiology and Nutrition of the Ruminant. p. 68. Butterworths, London. 1961.

(30) Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350. 1956.

(31) Duguid, J. P. Edinburgh Med. J. 53:401. 1946.

(32) Freidlein, F. Der quantitative Verwendungsstäff wechsel des Paratyphus-B-Bazillus, des Bacterium coli und des Bacillus pyocyaneus. Biochem. Z. 194:273. 1928.

(33) Garton, G. A. Fatty acid composition of the lipids of pasture grasses. Nature 187:511. 1960.

(34) Garton, G. A. Influence of the rumen on the digestion and metabolism of lipids. in: Lewis, D. (ed.), Digestive Physiology and Nutrition of the Ruminant. p. 140. Butterworths, London. 1961.

(35) Garton, G. A., P. N. Hobson and A. K. Lough. Lipolysis in the rumen. Nature 182:1511. 1958.

(36) Gordon, C. F. and A. L. Wolfe. Liquid scintillation counting of aqueous samples. Anal. Chem. 32:574. 1960.

(37) Gupta, J. and R. E. Nichols. A possible enzymatic cause of viscid ruminal contents--its relationship to legume bloat. Amer. J. Vet. Res. 23:128. 1962.

(38) Gutierrez, J. and R. E. Davis. Culture and metabolism of the rumen ciliate Epidinium ecaudatum (Crawley). Appl. Microbiol. 10:305. 1962.

(39) Gutierrez, J., P. P. Williams, R. E. Davis and E. J. Warwick. Lipid metabolism of rumen ciliates and bacteria. I. Uptake of fatty acids by Isotricha prostoma and Entodinium simplex. Appl. Microbiol. 10:548. 1962.

(40) Hahn, F. E. and J. Ciak. Penicillin-induced lysis of Escherichia coli. Science 125:119. 1957.

(41) Halliwel, G. Cellulolytic preparations from micro-organisms of the rumen and from Myrothecium verrucaria. J. Gen. Microbiol. 17:166. 1957.

(42) Hancock, R. and P. C. Fitz-James. Some differences in the action of penicillin, bacitracin and vancomycin on Bacillus megaterium. J. Bacteriol. 87:1044. 1964.

(43) Hird, F. J. R. and M. J. Weidemann. Transport and metabolism of butyrate by isolated rumen epithelium. Biochem. J. 92:585. 1964.

(44) Hoover, W. H. Carbon sources for in vitro protein synthesis by rumen bacteria. Ph.D. Thesis. Pennsylvania State University, 1962.

(45) Hoover, W. H., E. M. Kesler and R. J. Flipse. Carbon sources for in vitro protein synthesis by rumen bacteria. J. Dairy Sci. 46:733. 1963.

(46) Horn, L. H., R. R. Snapp and L. S. Gall. The effect of anti-biotics upon the digestion of feed nutrients by yearling steers with bacteriological data. J. Animal Sci. 14:243. 1955.

(47) Hugo, W. B. and A. D. Russell. Quantitative aspect of penicillin action on Escherichia coli in hypertonic medium. J. Bacteriol. 80:436. 1960.

(48) Hugo, W. B. and A. D. Russell. Action of penicillin on Aerobacter cloacae. J. Bacteriol. 82:411. 1961.

(49) Ito, E. and J. L. Strominger. Enzymatic synthesis of the peptide in a uridine nucleotide from Staphylococcus aureus. J. Biol. Chem. 237:2689. 1962.

(50) Ito, E. and J. L. Strominger. Enzymatic addition of lysine to a uridine nucleotide. J. Biol. Chem. 237:2696. 1962.

(51) Ito, E., N. Ishimoto and M. Saito. Uridine diphosphate aminosugar compounds from Staphylococcus aureus inhibited by penicillin. Nature 181:906. 1958.

(52) Jensen, E. A. and D. M. Hammond. A morphological study of trichomonads and related flagellates from the bovine digestive tract. J. Protozool. 11:386. 1964.

(53) Johns, A. T. Fermentation of glycerol in the rumen of sheep. N.Z. J. Sci. Tech. 35:262. 1953.

(54) Kandler, O., A. Hund and C. Zchender. Cell wall composition in bacterial and L forms of Proteus vulgaris. Nature 181:572. 1958.

(55) Kaufmann, W. The possible implication of a bacterial enzyme in the biochemical mode of action of penicillins on gram negative bacteria. Biochem. Biophys. Res. Comm. 14:458. 1964.

(56) Kiddle, P., R. A. Marshall and A. T. Phillipson. A comparison of the mixtures of acetic, propionic and butyric acids in the rumen and in the blood leaving the rumen. J. Physiol. 113:207. 1951.

(57) Klopfenstein, T. J., D. B. Purser and W. J. Tyznik. Effect of exposure time to antibiotic on rumen microbial gas production in vitro. J. Animal Sci. 23:126. 1964.

(58) Lamanna, C. and M. F. Mallette. Basic Bacteriology. The Williams and Wilkens Co., Baltimore, Md. 1959.

(59) Lark, K. G. Abnormal growth induced by penicillin in a strain of Alcaligenes fecalis. Canad. J. Microbiol. 4:165. 1958.

(60) Law, J. H. and R. A. Slepecky. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82:33. 1961.

(61) Lederberg, J. Mechanism of action of penicillin. J. Bacteriol. 73:144. 1957.

(62) Lederberg, J. and J. St. Clair. Protoplasts and L-type growth of Escherichia coli. J. Bacteriol. 75:143. 1958.

(63) Lodge, R. M. and C. N. Hinshelwood. Physiochemical aspects of bacterial growth. IV. Conditions determining stationary populations and growth rates of Bact. Lactis Aerogenes in synthetic media. J. Chem. Soc. p. 1683. 1939.

(64) Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265. 1951.

(65) Mallette, M. F. Validity of the concept of energy of maintenance. in: Lamanna, C. (ed.), Endogenous Metabolism with Special Reference to Bacteria. Annals N. Y. Acad. Sci. 102:521. 1963.

(66) Mandelstam, J. Preparation and properties of the mucopeptides of cell walls of gram negative bacteria. Biochem. J. 84:294. 1962.

(67) Mandelstam, J. Protein turnover and its function in the economy of the cell. in: Lamanna, C. (ed.), Endogenous Metabolism with Special Reference to Bacteria. Annals N. Y. Acad. Sci. 102:621. 1963.

(68) Mandelstam, J. and H. J. Rogers. The incorporation of amino acids into the cell-wall mucopeptide of Staphylococci and the effect of antibiotics on the process. Biochem. J. 72:654. 1959.

(69) Masson, M. J. and A. T. Phillipson. The absorption of acetate, propionate and butyrate from the rumen of sheep. J. Physiol. 113:189. 1951.

(70) McArthur, J. M., J. E. Miltimore and M. J. Pratt. Bloat investigations. The foam stabilizing protein of alfalfa. Canad. J. Animal Sci. 44:200. 1964.

(71) McCarthy, R. D., J. C. Shaw and S. Lakshmanan. Metabolism of volatile fatty acids by the perfused goat liver. Proc. Soc. Exptl. Biol. Med. 99:560. 1958.

(72) McDonald, I. W. The absorption of ammonia from the rumen of sheep. Biochem. J. 42:584. 1948.

(73) McDonald, I. W. The role of ammonia in ruminal digestion of protein. Biochem. J. 51:86. 1952.

(74) McDonald, I. W. The extent of conversion of food protein to microbial protein in the rumen of the sheep. Biochem. J. 56:120. 1954.

(75) McDonald, I. W. and R. J. Hall. The conversion of casein into microbial proteins in the rumen. Biochem. J. 67:400. 1957.

(76) McGrew, S. B. and M. F. Mallette. Energy of maintenance in Escherichia coli. J. Bacteriol. 83:844. 1962.

(77) Meadow, P. Effects of penicillin on a mutant of Escherichia coli requiring diamino-pimelic acid. Biochem. J. 76:8P. 1960.

(78) Meadow, P. M., J. S. Anderson and J. L. Strominger. Enzymatic polymerization of UDP acetylmuramyl-L-Ala.-D-Glu.-L-Lys.-D-Ala.-D-Ala. and UDP-acetylglucosamine by a particulate enzyme from Staphylococcus aureus and its inhibition by antibiotics. Biochem. Biophys. Res. Comm. 14:382. 1964.

(79) Monod, J. Recherches sur la Croissance des Cultures Bacteriennes. Hermann et Cie, Eds. Paris, France. 1942.

(80) Nathenson, S. G. and J. L. Strominger. Effects of penicillin on the biosynthesis of the cell walls of Escherichia coli and Staphylococcus aureus. J. Pharmacol. Exptl. Therap. 131:1. 1961.

(81) Nathenson, S. G. and J. L. Strominger. Enzymatic synthesis of N-acetylglucosaminyl ribitol linkages in teichoic acid from Staphylococcus aureus, strain Copenhagen. J. Biol. Chem. 238:3161. 1963.

(82) Pardee, A. B. The synthesis of enzymes. in: I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria. Vol. 3, p. 577. Academic Press, Inc., New York. 1962.

(83) Park, J. T. Isolation of three labile phosphate compounds containing uracil from penicillin treated Staphylococcus aureus cells. Fed. Proc. 9:213. 1950.

(84) Park, J. T. Pyrophosphate derivatives. I. Isolation from Staphylococcus aureus. J. Biol. Chem. 194:877. 1952.

(85) Park, J. T. Pyrophosphate derivatives. II. A structure common to three derivatives. J. Biol. Chem. 194:885. 1952.

(86) Park, J. T. Pyrophosphate derivatives. III. Amino acid-containing derivatives. J. Biol. Chem. 194:897. 1952.

(87) Park, J. T. Inhibition of cell-wall synthesis in Staphylococcus aureus by chemicals which cause accumulation of wall precursors. Biochem. J. 70:2P. 1958.

(88) Park, J. T. and M. J. Johnson. Accumulation of labile phosphate in Staphylococcus aureus grown in the presence of penicillin. J. Biol. Chem. 179:585. 1949.

(89) Park, J. T. and J. L. Strominger. Mode of action of penicillin. Science 125:99. 1957.

(90) Pearson, R. M. and J. A. B. Smith. The utilization of urea in the bovine rumen. 2. The conversion of urea to ammonia. Biochem. J. 37:148. 1943.

(91) Pennington, R. J. The metabolism of short-chain fatty acids in the sheep. 1. Fatty acid utilization and ketone body production by rumen epithelium and other tissues. Biochem. J. 51:251. 1952.

(92) Pennington, R. J. The metabolism of short-chain fatty acids in the sheep. 2. Further studies with rumen epithelium. Biochem. J. 56:410. 1954.

(93) Pennington, R. J. and T. M. Sutherland. The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. Biochem. J. 63:618. 1956.

(94) Pfander, W. H. and A. T. Phillipson. The rates of absorption of acetic, propionic and n-butyric acids. J. Physiol. 122:102. 1953.

(95) Phillipson, A. T. Digestion of cellulose by the ruminant. Symp. Biochem. Soc. 11:63. 1953.

(96) Phillipson, A. T. The general physiology of the ruminant. in: Lewis, D. (ed.), Digestive Physiology and Nutrition of the Ruminant. p. 3. Butterworths, London. 1961.

(97) Polan, C. E., J. J. McNeill and S. B. Tove. Biohydrogenation of unsaturated fatty acids by rumen bacteria. J. Bacteriol. 88:1056. 1964.

(98) Pollock, M. R. Induced formation of enzymes. in: P. D. Boyer, H. Lardy and K. Myrback (ed.), The Enzymes, 2nd Ed. Vol. 1, p. 619. Academic Press, Inc., New York. 1959.

(99) Pressey, R., S. H. Synhorst, J. Bertram, R. S. Allen and N. L. Jacobson. Foaming Properties of alfalfa and their relationships to bloat. J. Animal Sci. 22:970. 1963.

(100) Prestige, L. S. and A. B. Pardee. Induction of bacterial lysis by penicillin. J. Bacteriol. 74:48. 1957.

(101) Rahn, O. Physiology of Bacteria. P. Blakiston's Son and Co., Philadelphia, Pa. 1932.

(102) Reiser, R. and H. G. R. Reddy. J. Amer. Oil Chem. Soc. 33:155. 1956.

(103) Ribbons, D. W. and E. A. Dawes. Environmental and growth conditions affecting the endogenous metabolism of bacteria. in: Lamanna, C. (ed.), Endogenous Metabolism with Special Reference to Bacteria. Annals N.Y. Acad. Sci. 102:564. 1963.

(104) Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten. Studies of Biosynthesis of Escherichia coli. Carnegie Institution of Washington Publication 607. Washington, D.C. 1955.

(105) Rogers, H. J. and J. Jeljaszewicz. Inhibition of the biosynthesis of cell-wall mucopeptides by the penicillins. A study of penicillin-sensitive and penicillin-resistant strains of Staphylococcus aureus. Biochem. J. 81:576. 1961.

(106) Rogers, H. J. and J. Mandelstam. Inhibition of cell-wall mucopeptide formation in Escherichia coli by benzylpenicillin and 6-D(-)-alpha-aminophenylacetiamido penicillanic acid (Ampicillin). Biochem. J. 84:299. 1962.

(107) Sakami, W. (ed.), Handbook of Isotope Tracer Methods. Western Reserve University, School of Medicine. Cleveland, Ohio. 1955.

(108) Salton, M. R. J. Surface layers of the bacterial cell. in: I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria. Vol. 1, p. 97. Academic Press, Inc., New York. 1962.

(109) Saukkonen, J. J. Acid-soluble nucleotides of Staphylococcus aureus: Massive accumulation of a derivative of cytidine diphosphate in the presence of penicillin. Nature 192:816. 1961.

(110) Shockman, G. D. and J. O. Lampen. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bacteriol. 84:508. 1962.

(111) Shorland, F. B., R. O. Weenink and A. T. Johns. Effect of the rumen on dietary fat. Nature 175:1129. 1955.

(112) Stanier, R. Y., M. Doudoroff and E. A. Adelberg. The Microbial World. p. 259. Prentice-Hall, Inc., Englewood Cliffs, N. J. 1963.

(113) Strange, R. E. and F. A. Dark. The composition of the spore coats of Bacillus megaterium, B. subtilis and B. cereus. Biochem. J. 62:459. 1956.

(114) Strange, R. E. and L. H. Kent. The isolation, characterization and chemical synthesis of muramic acid. Biochem. J. 71:333. 1959.

(115) Strange, R. E. and J. F. Powell. Hexosamine-containing peptides in spores of Bacillus subtilis, B. megaterium and B. cereus. Biochem. J. 58:80. 1954.

(116) Strominger, J. L. Microbial uridine-5'-pyrophosphate N-acetylamino sugar compounds. I. Biology of the penicillin-induced accumulation. J. Biol. Chem. 224:509. 1957.

(117) Strominger, J. L. Accumulation of uridine and cytidine nucleotides in Staphylococcus aureus inhibited by gentian violet. J. Biol. Chem. 243:1520. 1959.

(118) Strominger, J. L. Biosynthesis of bacterial cell walls. Fed. Proc. 21:134. 1962.

(119) Strominger, J. L. Biosynthesis of bacterial cell walls. in: I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria. Vol. 3, p. 413. Academic Press, Inc., New York. 1962.

(120) Strominger, J. L. and R. H. Threnn. The optical configuration of the alanine residues in a uridine nucleotide and in the cell wall of Staphylococcus aureus. Biochim. Biophys. Acta 33:280. 1959.

(121) Struve, W. G. and F. C. Neuhaus. Evidence for an initial acceptor of UDP-NAc-muramyl-pentapeptide in the synthesis of bacterial mucopeptide. Biochem. Biophys. Res. Comm. 18:6. 1965.

(122) Trucco, R. E. and A. B. Pardee. Synthesis of Escherichia coli cell walls in the presence of penicillin. J. Biol. Chem. 230:435. 1958.

(123) Vaichulis, E. M. K., E. E. Vicher and M. V. Novak. Inhibition of the endogenous metabolism of Mycobacterium smegmatis by penicillin and isoniazid. Nature 202:726. 1964.

(124) Weenink, R. O. A note on the acetone-soluble lipids of forage grasses and clovers. N.Z. J. Sci. 2:273. 1959.

(125) Weller, R. A. The amino acid composition of hydrolysates of microbial preparations from the rumen of sheep. Aust. J. Biol. Sci. 10:384. 1957.

(126) Williams, P. P., R. E. Davis, R. N. Doetsch and J. Gutierrez. Physiological studies of the rumen protozoan Ophryoscolex candatus Eberlein. Appl. Microbiol. 9:405. 1961.

(127) Windisch, F. and W. Nordheim. Anoxygene Reahtivierung der Zellvermehrung nach anoxybiotisch hervorgerufener Zytostase (Anabiose), Experientia 13:155. 1957.

(128) Wiseman, H. G. and H. M. Irvin. Determination of organic acids in silage. Agr. Food Chem. 5:213. 1957.

(129) Wiseman, R. F., D. R. Jacobson and W. M. Miller. Persistance of lactobacilli and streptococci in the bovine rumen during penicillin administration. Appl. Microbiol. 8:76. 1960.

(130) Wright, D. E. Hydrogenation of lipids by rumen protozoa. Nature 184:875. 1959.

(131) Wright, D. E. Hydrogenation of chloroplast lipids by rumen bacteria. Nature 185:546. 1960.