MICROBIAL PROTEIN SYNTHESIS IN THE RUMEN: ASSESSMENT OF RADIOACTIVE PHOSPHROUS AS A MARKER FOR CELLULAR GROWTH

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ABSTRACT

MICROBIAL PROTEIN SYNTHESIS IN THE RUMEN:
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By

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The protein (amino acid) requirements of ruminants is met by microbial cells and undegraded dietary protein that passes from the rumen to the small intestine. The microbial cells are produced during the fermentation of dietary carbohydrates in the rumen. Ammonia is the primary nitrogen for microbial protein synthesis in the rumen.

To further improve the productivity of ruminants, the level of ruminal microbial protein production must be determined. Since dietary and microbial protein is not easily distinguishable there have been few studies on the rate of protein synthesis by ruminal microorganisms. To date, procedures developed to measure ruminal protein synthesis have been inadequate.

Seven experiments were conducted to determine the feasibility of using radioactive phosphrous (^{33}P) incorporation into microbial phospholipids (PL) as a marker

of microbial growth (protein synthesis) in whole rumen contents. Since microbial protein synthesis had to be related to PL synthesis a suitable nitrogen to phospholipid-phosphrous (N/PL-Pi) ratio had to be determined. Results from experiments 1, 2 and 3 showed that N/PL-Pi ratios were not similar for 12 strains of pure rumen bacteria, mixed rumen bacteria or rumen protozoa. The N/PL-Pi ratio also differed in rumen bacteria collected from the same sheep at different times after feeding and incubated in vitro with different levels of substrate. These experiments showed that a N/PL-Pi ratio would need to be determined separately for bacteria and protozoa in each experiment designed to determine the rate of microbial cell (protein) synthesis.

The metabolism of ^{33}P by rumen bacteria was studied in vitro in experiments 4 and 5. ^{33}P uptake and incorporation into the intracellular phosphrous (IC-Pi) and PL-Pi fractions were linear with time and paralleled changes in cell growth. Before substrate addition to the incubation medium, ^{33}P uptake was noted into the IC-Pi fraction but ^{33}P was not incorporated into the PL-Pi fraction. To relate ^{33}P incorporation into PL, to the µg of phosphrous uptake into PL, the specific activity (SA) of the IC-Pi pool was used. The SA of the IC-Pi pool was assumed to represent the phosphrous precursor pool for microbial PL synthesis.

In experiment 6 the rate of ^{33}P incorporation into microbial PL was studied during *in vitro* incubations of whole rumen contents obtained from sheep fed either a high (15.7%) or a low (6.1%) protein ration of similar digestable energy. The incubations were conducted at 0 (before) 2 and 4 hours after the sheep were fed. Rate of ^{33}P incorporation into microbial PL in the rumen contents collected from the sheep fed the high protein ration was highest for the 2 and 4 hour (before) after feeding incubations. These rates of ^{33}P incorporation into microbial PL are indictive of microbial cell growth rates that occur *in vivo* when low or high nitrogen diets are fed to ruminants. Results of experiments 1 through 6 showed that ^{33}P incorporation into microbial PL can be used to determine microbial cell growth in systems using whole rumen contents.

In experiment 7 the rate of rumen microbial cell (protein) synthesis was measured using 60-minute *in vitro* incubations of whole rumen contents collected from a sheep at 0, 2, 4, 9 and 11 hours after the am feeding. After the 9 hour sample was obtained the sheep was refed thus the 9 and 11 hour incubations were actually at 0 and 2 hours after the pm feeding. The amount of rumen microbial protein (N x 6.25) synthesized (adjusted to a sheep with a four liter rumen volume) was: 10.66, 13.16, 11.14, 5.45 and 8.61 g protein per hour for the 0, 2, 4

9 and 11 hours after feeding incubations respectively. These results represent a total microbial protein synthesis of 109.6 g per 12 hours or a daily rate of 219.3 g. Expressed in another manner the daily rate of 219.3 g microbial protein synthesis gave an estimated rate of protein synthesis of 26.0 g/100 g organic matter digested in the rumen.

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INTRODUCTION

Ruminant animals are unique in that they can utilize both protein and nonprotein nitrogen through their rumen microorganisms. Proteins enter the rumen mainly from the diet and are first digested by microbial proteolytic enzymes to peptides and free amino acids. The free amino acids are then degraded by microbial fermentation to form carbon dioxide, volatile fatty acids and ammonia (Hungate, 1966). Nonprotein nitrogen sources enter the rumen from the diet and from endogenous secretions of urea via the saliva and across the rumen wall (Allison, 1970; Houpt, 1970). The nonprotein nitrogen sources in the rumen are degraded by microbial enzymes to ammonia (Lewis, 1960).

Pearson and Smith (1943) and Loosli *et al.* (1949) showed that ammonia was utilized as a nitrogen source for the synthesis of rumen microbial amino acids and protein. A number of investigators have shown that rumen microbes require ammonia as their nitrogen source for growth (Burroughs, 1951, Belasco, 1954; Bryand *et al.*, 1959; Bryant and Robinson, 1961; Dehority, 1963). Rumen bacteria have also been shown to utilize amino acid, and peptides in addition to ammonia for growth (Bryant and Robinson, 1962; Wright and Hungate, 1967).

Oltjen (1967) and Virtanen (1969) showed that dairy cows, when fed rations in which nonprotein nitrogen was the sole dietary nitrogen source, produced approximately 2600 to 3500 kg milk per year. For the cows fed the above diets, the dietary protein requirements for milk production were met by the synthesis of ruminal microbial protein and their utilization of the nonprotein nitrogen. Chalupa (1972) calculated that in the lactating cow ruminal microbial protein synthesis could support the protein requirements for approximately 10 kg milk production per day.

The above discussion has indicated that protein and nonprotein nitrogen that enters the rumen is degraded by microbial action. The rumen microbes utilize the nitrogen from the degraded protein and nonprotein nitrogen as a nitrogen source for synthesis of their cellular proteins.

Thus, the logical question to ask is: How much microbial protein is synthesized in the rumen per day? The quantatative estimation of the rate of ruminal microbial protein synthesis has long been regarded as a very difficult problem. Essentially the difficulty has involved the differentiation of plant material from microbial cells. A number of techniques have been developed to measure the rate of ruminal microbial protein synthesis with all of the approaches having had varying degrees of

success in the differentiation between microbial and plant material in estimating microbial protein synthesis in the rumen (Chalmers and Synge, 1954; Walker and Nader, 1968).

The objective of the research presented in this thesis was to develop a method to measure the quantatative rate of rumen microbial protein synthesis. The method developed utilized ^{33}P incorporation into microbial phospholipids as a marker to differentiate between microbial cell growth and dietary protein. The metabolism of ^{33}P in rumen bacteria was studied to determine if the incorporation of ^{33}P into microbial phospholipids could actually be used as a marker of microbial protein synthesis.

LITERATURE REVIEW

Methods Developed to Measure Ruminal Microbial Protein Synthesis

Review of Early Work

The first researchers to experimentally estimate the amount of microbial protein synthesized in the rumen were Pearson and Smith (1943) while studying ruminant urea utilization *in vitro*. The amount of microbial protein synthesized during the first two hours of incubation of strained rumen fluid in the presence of starch and urea substrates was equivalent to 8 mg nitrogen per 100 g rumen fluid. If this rate was maintained for 24 hours they calculated that *in vitro* the protein synthesized would be equivalent to 72 g nitrogen or 450 g protein for a cow having a rumen capacity of 72 kg. Smith and Barker (1944) estimated the amount of rumen microbial protein synthesized in rumen fluid by the increase in dry matter after a two-hour *in vitro* incubation. They estimated that 102 mg of microbial protein was synthesized/100 g rumen fuild in the two-hour incubation when a carbohydrate substrate was added (88 g protein/24 hr./72 kg rumen capacity.

Assuming an average weight for bacteria, but not protozoa, and a bacterial turnover rate of once per day, Thaysen (1945) calculated the synthesis of 404 g dry bacterial substance per 100 liters of strained rumen fluid. The crude protein content of the bacterial cells was found to be 45%, and thus he calculated that 180 g bacterial protein could be synthesized in the rumen per day. He thought this to be a very conservative figure since pure cultures of bacteria in the logarithmic growth phase have a generation time of 1.5 to 2 hours. Smith (1945), while studying the utilization of nonprotein nitrogen in ruminants, calculated that 300 g of ruminal microbial protein could be synthesized in the bovine rumen per day. McNaught and Smith (1947), using the assumption derived by Schwarz (1925), that 12% of the rumen contents is bacterial protein, calculated that approximately 75 g of bacterial protein passed from the bovine rumen per day.

Feeding rumen fistulated calves purified rations containing urea as the sole protein source, Agrawala et al. (1953) measured the amount of microbial protein synthesized by completely removing the rumen contents. The removed rumen contents were weighed before feeding and at six hours after feeding to determine the increase in microbial protein (N x 6.25) during that time period. They calculated that 33 to 109 g of microbial protein was

synthesized in the six-hour period after the rations were fed. However the authors stressed that this was only an approximate and an underestimate since microbial cell loss via rumen turnover and passage of digesta from the rumen was not accounted for.

Rate of Passage Studies

To determine the quantity of microbial protein which passes from the rumen to the omasum or abomasum two factors must be known: 1) the rate or amount of rumen digesta passing to the omasum or abomasum, and 2) the microbial protein in the passing digesta must be distinguished from dietary protein (McDonald, 1954). Gray et al. (1953) estimated the conversion of dietary nitrogen into microbial nitrogen in the rumen of hay fed sheep to be 50% using the indigestable lignin of the forage as a marker to differentiate between microbial and dietary protein. Their results indicated that the nitrogen in the forage per gram lignin was about equal to the nitrogen in the rumen contents per gram lignin. They thus assumed that 50% of the rumen contents were of microbial origin and that the conversion of dietary to microbial nitrogen was 50%. Chalmers and Synge (1954) challenged these data stating that several of the assumptions on which the calculations were based were not clearly defined by Gray et al. (1953). McDonald (1954) conducted a similar study in sheep fed a

semi-purified diet in which zein constituted 94% of the total dietary nitrogen. Zein was used in the diet because zein is soluble in ethanol which makes it extractable from microbial protein and since zein is practically devoid in lysine, microbial growth could be accessed from increases in microbial lysine synthesis. Utilizing these characteristics of zein the authors found that 40% of the zein protein was converted into microbial nitrogen and that 60% of zein protein was undegraded in the rumen. These results showed that zein protein was very insoluble in the aqueous rumen fluid and for this reason McDonald and Hall (1957) considered that zein would be less effectively utilized by the rumen microbes than a more soluble protein. Chalmers and Synge (1954) and McDonald (1952) showed that casein was very soluble in the rumen, thus McDonald and Hall (1957) devised a procedure to study the conversion of casein nitrogen into microbial nitrogen. When casein provided 87% of the nitrogen in a semi-purified diet fed to sheep, 90% of the casein was degraded in the rumen and utilized for microbial protein synthesis. The analytical procedure for casein analysis in the abomasal fluid was by the determination for the phosphate group in casein. However, in both the zein and casein experiments conducted by McDonald determination of protein synthesized was not possible since the flow of digesta from the rumen to the abomasum was not determined.

Hogan and Weston (1967) estimated rumen microbial protein synthesis in sheep fed every three hours either a high (19.8%) or low (7.8%) protein diet consisting of hay, corn and a protein supplement. Dietary lignin and infused ^{51}Cr-EDTA were used as reference substances to calculate the flow of digesta from the rumen to the abomasum. The protein synthesis estimates were made from material collected at the abomasum by assuming that: 1) 1 g/day of endogenous nitrogen was secreted into the abomasum, 2) the non-ammonia nitrogen in the abomasum is mainly microbial nitrogen, and 3) microbial cells contain 10.5% nitrogen. Daily microbial crude protein synthesis amounted to 49 g for the high protein diet, this is equivalent to 15 g crude protein synthesized/100 g organic matter digested in the rumen. For the low protein diet the daily microbial protein synthesized was 44 g or 15.6 g/100 organic matter digested in the rumen. The authors believed that these values were likely to be an overestimate since some of the nitrogen reaching the abomasum was probably of dietary origin.

The most complete direct measurements of daily production of ruminal microbial protein synthesis *in vivo* were conducted by Hume (1970a,b) and Hume *et al.* (1970a,b). Sheep with permanent cannulas located in the rumen, omasum and abomasum were fed at two-hour intervals with one of a series of virtually protein-free diets in which urea

provided 2, 4, 9 and 16 g nitrogen per day. To maintain the sheep in a constant nitrogen status while varying the dietary nitrogen intakes casein was continuously infused into the abomasum. Polyethylene glycol was used as a reference to estimate the rate of digesta flow from the rumen and samples for estimation of protein synthesis were obtained from the omasum.

Nitrogen flow from the rumen showed a net increase over the dietary intake of 2 and 4 g of nitrogen, but no change at 9 g and a loss at 16 g of nitrogen intake. At the low nitrogen intakes, nitrogen was recycled and utilized by the rumen microorganisms but there was an efflux of nitrogen at the higher nitrogen intakes. Yield of microbial protein increased from 9.1 g to 13.3 g/100 g organic matter digested in the rumen for the sheep fed the diets containing 2 g and 16 g dietary nitrogen respectively (Hume et al., 1970a). In subsequent investigations using the same techniques as discussed above, Hume (1970a,b) studied the effect of adding to the diet mixtures of branched-chain volatile fatty acids (VFA) and the additions of purified proteins on the amount of microbial protein synthesized in the rumen. The addition of a 10 g mixture of branched-chain VFA to the purified diet of sheep increased the mean daily microbial protein synthesized from 71 g to 81 g. The yield of microbial protein synthesized per 100 g organic matter digested

was 12.5 g for the sheep not receiving branched-chain VFA's, and 13.4 g for the sheep receiving branched-chain VFA's. However, the addition of branched-chain VFA's did not result in any appreciable increase in the efficiency of protein production. Branched-chain VFA's have been shown to be essential for growth of cellulolytic bacteria (Allison, 1965). Hume (1970a) hypothesized that factors other than branched-chain VFA such as natural proteins are essential for maximum protein production in the rumen.

Thus in a following experiment Hume (1970b) studied the effect on microbial protein synthesis of adding a purified protein to the urea, branched-chain VFA supplemented purified diets which were fed to sheep. The author theorized that the added purified protein would supply additional growth factors that would promote enhanced microbial protein synthesis. The purified proteins added seperately to the sheep diets were casein, gelatin and zein. Casein and gelatin are soluble and zein is relatively insoluble in rumen fluid. Total microbial protein flow from the rumen to the omasum was 89.5, 90.5, 101.4 and 104.3 g/day for the iso-nitrogenous urea, gelatin, casein and zein supplemented diets. The gelatin supplemented and urea diets both allowed for similar amounts of microbial protein synthesis. The author theorized that microbial protein synthesis in the gelatin fed sheep may have been limited by the slow rate of synthesis

of one or more amino acids by the rumen bacteria, since gelatin is deficient in several amino acids including methionine. The microbial protein production was greater for both the casein and zein diets as compared to the gelatin and urea diets because of a more efficient utilization by the microbial population of the ammonia released in the rumen. Protein synthesis per 100 g organic matter digested in the rumen was 17.1, 19.8, 23.3 and 22.5 g for the urea, gelatin, casein and zein supplemented diets respectively.

Specific Cellular Constituents as Markers of Microbial Cell Synthesis

α, ε-diaminopimelic acid (DAP) an amino acid, has been used as an indicator to differentiate between dietary and bacterial protein in the rumen. This amino acid has been found to be absent in feed material but a normal cell wall constituent of many bacteria (Synge, 1953; Work and Dewey, 1953; Weller *et al.*, 1958; Purser and Buechler, 1966).

Weller *et al.* (1958) utilized the presence of DAP in rumen bacteria to determine the amount of bacterial nitrogen present in rumen contents of sheep fed a "wheaten" hay (dry wheat plant) ration. Rumen contents were obtained from sheep slaughtered at intervals ranging from 2 to 24 hours after feeding. The rumen contents were first squeezed through cheesecloth, and bacteria and

protozoa were isolated from the resulting rumen fluid by differential centrifugation. However, the bacteria could not be quantatatively recovered from the rumen content sample so the correction for the bacterial protein remaining on the plant material was made on the basis of the DAP content of the plant fiber fraction. The authors found that at 2 to 24 hours after feeding, 63-82% of the total nitrogen was present as microbial nitrogen, 11-27% as plant nitrogen and 5-10% as nonmicrobial soluble nitrogen. Using the same procedure as stated above, Weller et al. (1962) studied nitrogen digestion in sheep fed two types of roughage rations and found that in three hours, 80% of the plant nitrogen was converted to microbial nitrogen. For sheep fed a wheaten hay-lucerne hay ration, containing 1.4% nitrogen, ruminal microbial nitrogen increased 6 g, six hours after feeding whereas plant nitrogen decreased 7 g. For an all lucerne-hay ration containing 2.9% nitrogen, microbial nitrogen increased 7 g as plant nitrogen decreased 17g, six hours after feeding. These workers concluded that rumen microbial attack on plant nitrogen was very rapid and the nitrogen in the low nitrogen ration was more effectively utilized by the microbial population. However the amount of microbial protein synthesized per day was not calculated by the authors, but if the 6 g and 7 g increase in microbial nitrogen reported for the six hours was recalculated

150 g and 175 g of protein (N x 6.25) would be synthesized per day for the sheep fed the wheaten hay-lucerne hay and lucerne hay rations, respectively.

El-Shazley and Hungate (1966) measured changes in the DAP content in rumen digesta samples incubated *in vitro* for which the growth rate had previously been estimated *in vitro* by measuring changes in the fermentation capacity. The average net microbial growth rate was 7.5% per hour as determined by changes in the fermentation capacity and 6% as measured by changes in the DAP content. By knowing the DAP content of the *in vitro* rumen digesta samples and also knowing the ratio of nitrogen to DAP, which was established previously in samples of bacteria, the authors were then able to indirectly calculate the bacterial growth rate in the rumen digesta sample. According to the authors, the lower estimated growth rate as measured by the DAP method resulted from sampling and analytical errors and protozoal activity.

Hutton *et al.* (1971) utilized DAP as a marker of bacterial nitrogen that enters the duodenum from the rumen. In that experiment a cow was fed chromic oxide in its ration as a marker of rumen digesta entering the duodenum per day and they found that about 270 g of total nitrogen flowed into the duodenum of which approximately 133 g or about 50% was of bacterial origin.

Ruminal microbial protein synthesis was measured by Hogan and Weston (1970) by estimating the amount of DAP in the rumen digesta and multiplying the nitrogen content of the digesta by a nitrogen to DAP ratio value. They estimated in sheep fed every three hours that 3.7 g bacterial nitrogen was synthesized (N x 6.25 = 13.0 g crude protein) per 100 g organic matter digested in the rumen. When allowances were made for protozoal protein, these workers felt that 150 g of microbial protein could be synthesized daily for sheep fed under normal ad libitum feeding conditions.

Lindsay and Hogan (1972) estimated the synthesis of rumen bacterial protein in defaunated (protozoa free) sheep by measuring the DAP content of the rumen digesta leaving the rumen and multiplying it by the established nitrogen to DAP ratio. These workers found that in sheep which were fed either lucerne hay or red clover hay at three-hour intervals, that 23 g of bacterial protein were synthesized in the rumen per 100 g organic matter digested in the rumen.

The major criticism of using DAP as a marker to distinguish bacterial from plant protein is the fact that the amino acid is not present in all bacterial cell walls (Work and Dewey, 1953; Synge, 1953; Hoar and Work, 1957). Purser and Buechler (1966) showed that in addition to DAP being absent in some rumen bacteria, its concentration

varied from 0.6 to 3.4 g/100 g total amino acids for 22 strains of bacteria analyzed. Coupled with variations in bacterial concentrations, DAP is absent from rumen protozoa. This limits the usefulness of DAP in assessing the total rate of microbial protein synthesis in the rumen.

Smith (1969) conducted an extensive review of rumen nitrogen metabolism and stated that nucleic acid content of rumen microorganisms could serve as a possible marker of microbial contribution to the total protein present in the rumen. According to Belozersky and Spirin (1960) the amount of DNA in pure bacterial cultures reflects the number of organisms present. DNA content of bacterial cells is relatively constant except for a period at the end of the lag phase of growth. However, there does exist a correlation between rate of protein synthesis and RNA content of the cell. Cellular RNA content varies in accordance with growth of the cell with maximum RNA concentrations occurring during or just prior to the lag phase of growth (Maaloe and Kjeldgaard, 1966). This information can be interpreted as follows; the amount of DNA reflects the number of organisms present but RNA is more closely associated with protein synthesis.

Ellis and Pfander (1965) fed sheep diets essentially devoid of nucleic acids and noted an increased nucleic acid content in samples of rumen fluid incubated <u>in vitro</u>,

the nucleic acids accounted for about 15% of the microbial nitrogen formed.

A procedure for nucleic acid analysis of rumen microorganisms in rumen digesta was developed by McAllan and Smith (1969) who adapted methods devised for animal tissue and pure bacterial cultures. Using the above method and by comparing nucleic acid nitrogen to total nitrogen ratios in rumen fluid and bacteria, Smith and McAllan (1970, 1971) found that non-ammonia nitrogen in rumen fluid from calves and cows was 55 to 80% and 40 to 50% from microbial origin respectively. Smith and McAllan (1970) observed variations in DNA values and stated that RNA may be a better index of microbial nitrogen in rumen digesta than DNA or total nucleic acids.

Coleman (1968) found that washed cell suspensions of the rumen protozoa, Entodinium caudatum, grown in vitro incorporated ^{14}C nucleic acids. E. caudatum suspensions were also shown to incorporate purine and pyrimidine bases, ribose and phosphate from bacterial nucleic acids into protozoal nucleic acids. There was no evidence that the protozoa could synthesize ribose from other carbohydrates. These observations indicate that the quantatative assessment of bacterial protein synthesis by DNA or RNA analysis would have been theoretical limitations since some nucleic acids synthesized by bacteria would never be measured because of protozoal engulfment of bacteria.

Radioactive Tracers as Markers of Microbial Protein Synthesis

Hendrickx et al. (1962) reviewed advantages of using radioactive tracers in metabolism studies of rumen microorganisms. Radioactive ^{14}C-carbon has been extensively used in the measurement of VFA production rates. However, use of ^{14}C for estimating microbial protein synthesis has been limited because the metabolic pathways for carbon in rumen microorganisms is not specific and the carbon atom is incorporated into many cellular constituents other than protein. This total flux of carbon atoms in the rumen make the use of ^{14}C unrealistic as a marker for microbial protein synthesis.

Block et al. (1951) fed ^{35}S in the form of sodium sulfate to a sheep along with the regular diet. Results of that experiment showed that rumen microorganisms can synthesize sulfur containing amino acids from inorganic sulfate in the diet. Hendrickx et al. (1962) incubated rumen fluid in vitro with known levels of radioactive Sodium ^{35}S-sulfate and withdrew samples from the incubation flask at hourly intervals to measure the increase in microbial protein and the associated increment of ^{35}S incorporated into the sulfur containing amino acids of the microbial protein. The uptake of ^{35}S into microbial amino acids was positively correlated to the increases in total protein during a seven-hour incubation.

Roberts and Miller (1969) infused a solution of ^{35}S-sulfate, PEG and water into the rumen of a sheep fed a high energy ration containing 13.3% crude protein. These workers measured the rate of passage of rumen digesta and the ^{35}S incorporation into bacterial protein which was seperated from protozoa and feed particles by differential centrifugation. The authors assumed that ruminal bacteria pass from the rumen in the liquid phase and that when water input into the rumen was 76.8 ml/hr., the conversion of dietary protein to bacterial protein was 53% whereas at a water input rate of 115 ml/hr. the conversion was 73%. They did not determine or estimate the rate of ruminal microbial protein synthesis in this study. However, the feed intake was 720 g per day and the protein content of the diet was 13.3% or 95.7 g protein per day, the rate of microbial protein synthesized was then calculated to be 50.7 g and 69.9 g per day when the water flow rate was 76.8 ml and 115 ml per hour respectively.

Emery et al. (1957a) however, noted that ^{35}S-sulfate was not incorporated into a majority of rumen organisms during a three-hour in vitro incubation of rumen fluid. Emery et al. (1957b) also surveyed 10 pure cultures of rumen bacteria and found only five incorporated significant amounts of ^{35}S-sulfate into microbial protein. When cysteine was present in the culture medium, incorporation of ^{35}S-sulfate was partly inhibited and cysteine was the

preferable sulfur source. Sulfate must be reduced to sulfide prior to its incorporation into sulfur containing amino acids and this reduction process has been found to occur in the rumen (Anderson, 1956; Hungate, 1966). Walker and Nader (1968) noted a relatively slow conversion of sulfate to sulfide in the rumen and abandoned the idea of using sulfate as a possible marker for microbial protein synthesis.

Conrad et al. (1967a,b) estimated the daily ruminal methionine synthesis in cows given oral doses of sodium ^{35}S-sulfide or barium ^{35}S-sulfide. A series of regression equations were used to determine the amount of feed methionine and the amount of synthesized methionine in a given rumen sample. For cows fed an alfalfa hay ration at a constant level of intake, daily ruminal methionine synthesis ranged from 32.8 to 46.2 mg/kg body weight, however from the data presented the determination of the rate of microbial protein synthesis was not possible.

Walker and Nader (1968) reported the development of an in vitro method using sodium ^{35}S-sulfide incorporation into microbial sulfur containing amino acids to estimate the rate of microbial protein synthesis in rumen digesta from sheep fed under practical feeding conditions. The in vitro incubation procedure was conducted as follows: whole rumen contents were incubated for two hours after 1) a 30-minute preincubation period to reestablish

the H_2S concentration similar to that occurring *in vivo* and 2) a 15-minute period to allow for a nonenzymatic binding phenomenon to occur after the introduction of sodium ^{35}S-sulfide to the incubation. The rate of microbial protein (N x 6.25) synthesis was determined by calculating the sulfur incorporation into microbial sulfur containing amino acids and multiplying the sulfur incorporation rate by a microbial nitrogen to sulfur ratio. The nitrogen to sulfur ratio established was a constant 11:1 in both rumen bacteria and protozoa. These workers calculated that 80.3 to 94 mg microbial protein was synthesized per g of dry rumen digesta per hour. Protein synthesis per hour can be recalculated if one assumes a four-liter rumen volume, 10% dry matter for the rumen contents and a rate of microbial protein synthesis of 90 mg/hr./g dry rumen digesta. The recalculated rate of protein synthesis was 0.36 g microbial protein synthesized per hour and 8.46 g per 24 hours. In addition to using the ^{35}S incorporation rate to estimate microbial protein synthesis, Walker and Nader (1968) also measured the VFA production rate. By using an average ratio for protein to VFA production rate, these authors calculated the rate of microbial protein synthesis to be 92 g per day. In another paper Walker and Nader (1970) reported 5.9 g microbial protein synthesized per mole of ATP produced during the microbial fermentation process. These

calculations assumed that one mole of VFA produced yielded two moles of ATP and that 10 to 11 g dry cell caterial containing 60% protein was synthesized per mole of ATP. Further, in the same paper using the ^{35}S procedure these workers found 26 to 113 mg protein synthesized per gram dry rumen digesta per hour. If these data are recalculated using the same assumptions as stated earlier except that 75 mg of protein was synthesized per hour per g dry rumen digesta, the microbial protein synthesized per day would be 7.2 g. The VFA production rates in rumen contents incubated in vitro reported by Walker and Nader (1968, 1970) were low compared to those reported by Gray et al. (1967) who measured the rates in vivo for sheep fed similar rations with similar intakes. Walker and Nader (1970) compared estimates of microbial protein synthesis calculated from the VFA production rates to values obtained in vivo by Hogan and Weston (1967). They agreed with Hogan and Weston (1967) that about 15 to 16 g microbial protein could be synthesized per 100 g organic matter digested in the rumen. However, the actual data presented by Walker and Nader (1968) on the rate of microbial protein synthesis were much lower than those obtained by indirect procedures (Hume, 1970a,b), and thus their work appears to contribute little to the quantatative knowledge on ruminal microbial protein synthesis.

Most of the earlier workers employed ^{15}N-ammonia to obtain evidence that the rumen microorganism could utilize ammonia as a nitrogen source and to quantitate the amount of ammonia incorporated into microbial protein and various amino acids (Warner, 1956; Williams, 1958; Phillipson, et al., 1962).

Pilgrim et al. (1970) continuously infused $(^{15}NH_4)SO_4$ into the rumen of a sheep for periods of 78 to 98 hours and was able to calculate the fraction of bacterial nitrogen and protozoa nitrogen derived from ammonia nitrogen. When a low nitrogen (12.5 g N/day) wheaten hay diet was fed, 76 to 78% and 43 to 64% of the ammonia nitrogen was converted to bacterial nitrogen and protozoal nitrogen, respectively, and 8.5 g microbial nitrogen (5.3 g protein/day) was synthesized per day. However, when a lucerne hay diet was fed (22.9 g N/day) 62 to 64% and 35 to 44% of the ammonia nitrogen was converted to bacterial nitrogen and protozoal nitrogen, respectively and 12.3 g microbial nitrogen (76.9 g protein/day) synthesized per day.

Nolan and Leng (1972) infused ^{15}N-urea into the rumen of sheep receiving a lucerne hay diet (23.4 g N/day) and observed that 80% of the nitrogen incorporated into microbial cells came from ammonia nitrogen and that 20% came from amino acid nitrogen. The amount of bacterial protein synthesized from both ammonia and amino acids was estimated to be 17 g nitrogen per day (106 g protein/

day) which is greater than the estimate by Pilgrim et al. (1970). In these trials 4.3 g ammonia nitrogen per day was recycled in the rumen and this could occur, according to the authors from 1) lysis of viable bacteria, 2) engulfment of bacteria by protozoa, and 3) death of bacteria. They suggested that 30% of the ammonia nitrogen incorporated into microbial protein may have been recycled through amino acid and ammonia pools.

Mathison and Milligan (1971) studied the quantatative importance of ammonia as a nitrogen source in the synthesis of microbial cells by infusing $^{15}NH_4Cl$ continuously into the rumen of sheep for periods of 120 to 216 hours. Sheep were fed four diets with nitrogen contents of 1.4 to 1.6 g/100 g dry matter for a grass-hay diet and 1.8 to 2.5 g/100 g dry matter for a barley-hay diet. These workers found that 55 to 65% and 31 to 55% of the nitrogen incorporated into bacterial and protozoa protein nitrogen respectively originated from ammonia nitrogen. However, these workers stated that only the ammonia which equilibriated with the extracellular rumen ammonia pool was measured as contributing to microbial nitrogen and that if all recycling of ammonia was accounted for the reported percent incorporation of ammonia nitrogen to microbial nitrogen would represent only a minimum value. The amount of microbial nitrogen passing through the abomasum per day was 7.8 to 9.4 g and 9.2 to 12.9 g in the sheep given

the hay and barley-hay diets, respectively. The authors calculated microbial nitrogen yield of 1.7 to 2.6 g/100 g dry matter digested (10.6 to 16.3 g protein).

Al-Rabbat *et al.* (1971a,b) reported the development of an *in vitro* technique using $(^{15}NH_4)_2SO_4$ to determine the dependence of rumen microbial growth on ammonia nitrogen and also to estimate the amount of microbial cell growth in the rumen. *In vitro* incubations of whole rumen contents, collected from a sheep and a cow at various times after feeding, were conducted for 60 minutes and the rate of ammonia nitrogen incorporation into rumen microbes and rates of VFA production were determined. They reported for a cow fed an alfalfa pellet or an alfalfa-barley pellet ration that 1,125 g and 771 g microbial cells and 675 g and 426 g of microbial protein was synthesized per day for the respective diets using the ammonia incorporation procedure. The microbial cell yield estimated from VFA production data was 841 g and 517 g per day for the alfalfa and alfalfa-barley rations, these values are 75 and 67% of those obtained using the ammonia incorporation data. The authors noted however that the microbial cell yields obtained using the VFA production rates were approximately half of the microbial cell yields reported by Hume *et al.* (1970b). They also stated that the VFA production rates obtained were lower than generally reported in the literature. This often

occurs with *in vitro* incubations, the process of removing rumen contents for *in vitro* incubation is sufficient to cause reduction in the activity of rumen microorganisms (Whitelaw, *et al.*, 1970; Warner, 1964).

Thermodynamics of Ruminal Microbial Protein Synthesis

Until now this review has been concerned with the methods to measure microbial protein synthesis, however, a discussion of the biochemical thermodynamics of ruminal microbial cell synthesis is desirable in order to understand the capacity for cell synthesis. According to Hungate (1966) the amount of microbial cell synthesis depends on two things, 1) the usable high-energy compounds that can be derived from the substrate (expressed as ATP) and 2) the amount and nature of intermediates which can be synthesized into microbial cells. In the rumen the energy-yielding material and material transposed into microbial cells are the same. Carbohydrate is the substrate, in that it serves as a source of energy and a source of chemical compounds that can be built into cell bodies.

Aerobic microorganisms can synthesize into cell bodies 60 to 70% of the carbon from substrate whereas most anaerobic bacteria can synthesize only about 10% and rarely 20% (Hungate, 1966). The anaerobiosis that occurs

in the rumen according to Hungate (1966), limits the extent to which substrate can be synthesized into cell material.

In ruminant diets the major carbon substrate components are hexose polymers (cellulose, starch, fructosans), pentose polymers (mostly xylan) and protein, of these compounds the carbohydrate fraction is the largest (Walker, 1965).

The pathways of substrate metabolism by rumen microorganisms and the ATP yields have been established (Walker, 1965; Baldwin, 1965). The major fermentation pathway of hexose and pentoses converted to hexose, is the Embden-Meyerhof glycolytic pathway which involves the transformation of hexose monophosphate to pyruvate (Baldwin, 1965). The conversion of pyruvate to the end products of ruminal microbial fermentation (acetate, propionate, butyrate, carbon dioxide and methane) proceed by a number of pathways according to the microbial population found in the rumen. The ATP yield from substrate by anaerobic organisms is less than by aerobic organisms, chiefly because during anaerobic glycolysis the low energy yield per substrate is caused by an incomplete breakdown of the substrate, due to limited electron acceptors, whereas in aerobic organisms, excess oxygen acts as an electron acceptor. Aerobic respiration is generally considered to occur at maximal rates at lower substrate concentrations

than does anaerobic fermentation (Gunsalus and Shuster, 1961).

Bauchop and Elsden (1960) showed that 10.5 g of dry cells could be synthesized per mole of ATP (Y_{ATP}) generated by microorganisms. However, the value obtained by Bauchop and Elsden (1960) was obtained with nonruminant anaerobic bacteria and values reported for ruminant bacteria have been found to be different. Hobson and Summers (1967) found that Bacteroides amylophilus had a Y_{ATP} value of 20 and Bacterium 5S, a rumen lipolytic bacteria had a Y_{ATP} value of 15. Similarly, Hungate (1963) obtained a high Y_{ATP} value for Ruminococcus albus. Walker and Nader (1968) calculated a Y_{ATP} value of 14 for rumen bacteria by basing yield on an estimated rate of cell synthesis.

Payne (1970) recently reviewed the Y_{ATP} subject and noted that a sufficient number of workers have confirmed the Y_{ATP} value of approximately 10.5. He also stated that the question of the number of moles of ATP produced by cells growing at low substrate concentrations could still be disputed. A higher ATP yield may result in mixed cultures of rumen bacteria than as compared to pure cultures due to a greater number of ATP-yielding reactions (Hungate, et al., 1971).

Phospholipid Metabolism in Microorganisms

Since the method to measure the rate of ruminal microbial protein synthesis in this thesis involves the use of microbial cellular phospholipids as a marker, the metabolism of phospholipids in microorganisms will be reviewed.

Most of the lipid in microorganisms is located in the cell membrane with very little being found in the cell walls or cytoplasma (Lennarz, 1966; Rothfield and Finkelstein, 1968). All cellular membranes, bacterial, protozoal or animal, are known to contain phospholipids, up to 70 to 90% of the total lipids and these phospholipids are in a hydrophobically bound complex with membrane proteins (Lennarz, 1966; Sulton, 1967; Rothfield and Finkelstein, 1968).

The structure of microbial cell membranes has been extensively studied by electron microscopy. Such data show that Gram-positive bacteria contain a cell wall and a plasma membrane whereas the Gram-negative bacteria contain an outer membrane (envelope), dense intermediate layer and a plasma membrane. The plasma membrane of the Gram-positive and the cell envelope of the Gram-negative bacteria are the components of the bacterial cells that contain a majority of the cellular phospholipids (Lennarz, 1966; Sulton, 1967; Glavert and Thornley, 1969).

Getz (1970) stated that since polar phospholipids are found almost exclusively in cellular membranes they would be useful markers of membrane synthesis and degration. The interrelationship between protein and lipid synthesis appears to be under the same genetic control as RNA and protein synthesis in Escherichia coli. Analytical studies of the membrane lipids, in E. coli have shown them to be very constant in quantity and characteristic in composition and with only limited variations occurring due to dietary changes (Haest, et al., 1969).

The phosphrous moiety of phospholipids in E. coli has been shown to turnover only slightly, since no radioactive phosphrous ^{32}P that was incorporated into phospholipids could be detected in the incubation medium after several generations of rapid growth (Kanfer and Kennedy, 1963; Ames, 1968). Kanemasa et al. (1967) using E. coli observed only about an 8% loss of ^{32}P from membrane phospholipids in 13 hours after a ^{32}P pulse label. However, they did observe a change in the composition of the phospholipids after the pulse of ^{32}P. From the above discussion it appears that ^{32}P once incorporated into bacterial phospholipids would not be recycled.

Phospholipids are synthesized from phosphatidic acid with the phosphrous moiety ultimately being derived from a cytidine nucleotide (Kennedy, 1963; Lennarz, 1970). A number of workers have used radioactive phosphrous ^{32}P

to study the metabolism of phospholipids in microorganisms (Mitchell and Moyle, 1953; Harold, 1960; Kanfer and Kennedy, 1963; Kanemasa, et al., 1967; Okuyama, 1969; White and Tucker, 1969). Mitchell and Moyle (1953) found that inorganic phosphrous moves into the cell (Micrococcus pyogenes) during the lag phase of growth but phosphrous incorporation into cellular phospholipids occurs only during the growth phase. However, to date radioactive phosphrous has not been used to study phospholipid metabolism in rumen microorganisms.

The discussion on phosphrous and phospholipid metabolism in bacteria indicates that phospholipids are an integral part of the cell structure and that ^{32}P or inorganic phosphrous incorporated into bacterial phospholipids is not recycled from the lipid moiety. Getz (1970) stated that cellular lipid synthesis is under the same genetic control as RNA and protein synthesis. He also stated that cellular phospholipids would be useful as markers of cell membrane synthesis.

The above information lead to the question: Could radioactive phosphrous incorporation into rumen microbial phospholipids be used as a marker of microbial cell synthesis. This hypothesis would involve measuring the radioactive phosphrous incorporation into microbial phospholipids to obtain the total rate of phosphrous incorporation into microbial phospholipids. By using a

nitrogen to phospholipid phosphrous ratio it would then be possible to calculate the microbial nitrogen synthesized.

A number of experiments were designed to study the theoretical aspects of this hypothesis and an experiment was conducted to measure the rate of rumen microbial protein synthesis.

MATERIALS AND METHODS

Analytical Methods

Quantatative Extraction of Phospholipid from Rumen Bacteria and Protozoa

Rumen bacterial and protozoal lipids were extracted by a modification of the method described by Katz and Keeney (1966). Approximately 30 mg of lyophilized bacteria or protozoa were extracted with 5 ml chloroform-methanol 2:1 (V/V) in 15 ml screw-capped culture tubes, which were rotated for 16 to 20 hours at room temperature. The extracts were filtered through a fritted glass Buchner funnel and the nonlipid impurities removed by the salt wash procedure of Folch *et al*. (1957). Total lipid yields were determined by evaporating the extracting solvent to a constant weight in a forced air oven at 110 C. Experiments were conducted to determine the validity of the procedures described above.

Hoogenraad and Hird (1970) reported the use of sonification in a method used to extract cell wall constituents from rumen bacteria. The length of extraction time and the effect of cell disruption by ultrasonification was studied. In a first trial, rumen protozoa were

extracted for 4, 12 and 24 hours with half the samples being sonified for three minutes in chloroform-methanol 2:1 (V/V) with a Sonified Cell Disruptor, model W 185 D, Heat Systems-Ultrasonics, Inc., Plainview, New York, operated at optimum wattage. The mean results are shown below.

Treatment	Extraction Time, hr. in 2:1 $CHCl_3$: MEOH	Lipid Extracted, % of Dry Cell Wt. (Protozoa)	SE
Sonified	4	2.95	±0.03
Not Sonified	4	3.52	±0.08
Sonified	12	4.78	±0.10
Not Sonified	12	8.66	±0.09
Sonified	24	11.73	±0.03
Not Sonified	24	12.56	±0.19

The results showed that sonification of the protozoa cells yield less lipid than the cells not sonified. However, this study did not adequately determine if the extraction times were sufficient to obtain maximum lipid extraction. Thus to determine if complete lipid extraction occurred, two different bacteria and protozoa samples were extracted for 12 hours in 2:1 chloroform-methanol, the extracts were filtered and the residues reextracted for 24 hours with either chloroform-methanol, hexane (Skelly Solve B), or diethyl ether. Results of these extraction procedures are presented below.

Organism	Number of Replicates	Extraction Time, hr.	Lipid Extracted, % of Dry Cell Wt.	SE
Bacteria 1	5	12	11.13	±0.26
Bacteria 2	5	12	9.26	±0.63
Protozoa 1	5	12	9.02	±0.21
Protozoa 2	5	12	9.57	±0.53

Reextraction of the residues with the above mentioned solvents yielded by weight, no additional lipid, however, to determine if small amounts of lipid were re-extracted, the whole sample was analyzed by thin layer chromatography. Thin layer plates were prepared with silica Gel G, 0.5 mm thick and the spotted plates developed with either hexane (Skelly Solve B), diethyl ether, acetic acid, 90:10:1 (V/V) or chloroform, methanol, water, 80:25:3 (V/V). Lipids were detected with a 50% sulfuric acid spray and phospholipids detected using a molybdenum blue reagent spray as described by Dittmer and Lester (1964). The results of the thin layer chromatographs of the re-extracted samples showed no visible detection of lipids or phospholipids on the plates when the different development solvents and detection sprays were used.

The results of the aforementioned experiment indicated that maximum lipid extraction would occur by 12 hours and that the modified procedure of Katz and Keeney

(1966) would quantatatively extract all lipid from the microbial samples used.

The nonlipid impurities were removed from the microbial lipid extracts by the salt wash procedure described by Folch et al. (1957). Since quantatative determination of the phospholipid-phosphrous content of lipid samples was necessary, this required that all of the impurities (especially inorganic phosphrous) and none of the phospholipids were removed during the salt wash. To examine the Folch salt wash under our conditions pure egg phospholipid of known phosphrous content was prepared by the method of Ansell and Hawthorne (1966) and was used in the experiments described below.

To determine if any phospholipid was removed into the salt wash from a lipid mixture in 2:1 chloroform-methanol, the phospholipid-phosphrous content was measured in an egg phospholipid mixture in 2:1 chloroform-methanol before and after the salt washing procedure, also the wash extract was analyzed for phosphrous.

Phosphrous content of phospholipids extracted from either egg (or microorganisms) was determined by the ascorbic acid procedure described by Chen et al. (1956), and confirmed for phospholipid-phosphrous determination by Rhee and Dugan (1967).

Results for the above mentioned experiment are shown below.

Fraction	Total µg Pi
Before salt wash	20.65
After salt wash	20.10
Salt wash extract	Trace

The above experiment was conducted again except this time the different fractions were spotted on thin layer chromatography plates and handled by the procedures described above. Development of the chromatograms revealed that phospholipids were present in the spots corresponding to the before and after salt wash fractions, but none were present in the salt wash extract spots. Results of these experiments showed that the phospholipids in lipid extracts were not removed by the salt wash.

To further determine if the salt wash would remove all the inorganic phosphrous impurities from the lipid extract, inorganic phosphrous ^{33}P was added to lipid-free 2:1 chloroform-methanol and then subjected to the salt wash. The wash extract and the 2:1 chloroform-methanol fraction were separated and the different fractions analyzed for radioactivity by methods described on page 40.

Fraction	Mean Total CPM of Sample
Chloroform-methanol ^{33}P mixture before wash	1,304,751
Extract after salt wash	1,290,675
Chloroform-methanol after wash	3,157

These results showed that the salt wash was capable of removing the inorganic phosphrous ^{33}P and that only 3,157 CPM or 0.24% of the original radioactivity was found in the 2:1 chloroform-methanol fraction after the salt wash procedure.

The above experiments showed that the salt wash procedure of Folch et al. (1957) removed nonlipid impurities from the lipid extract and allowed for quantatative determination of phospholipid-phosphrous from the lipid fraction.

Extraction of Intracellular Phosphrous from Rumen Bacteria and Protozoa

Intracellular phosphrous was extracted from 30 to 100 mg of lyophilized rumen bacteria or protozoa with 4.0 ml of 5% perchloric acid for 30 minutes in a Potter-Elvejhem homogenizer with a glass pestle at 5 C (Krishran et al., 1957; Harold, 1960; Scherbaum, 1963). The homogenates were placed in "Corex" glass tubes and then centrifuged at 18,000 xg for 15 minutes.

The inorganic phosphrous was extracted from the intracellular phosphrous extract by a modification of the method described by Lindberg and Ernster(1955). The centrifuged extracted supernant was filtered through Whatman No. 1 filter paper and 3.0 ml of the filtrate, 3.5 ml of 1.5% ammonium molybdate in 0.5 NH_2SO_4 were

mixed vigorously for exactly 30 seconds after 4.0 ml isobutanol-benzene 1:1 (V/V) was added. The two phases were allowed to separate; the inorganic phosphrous was found in the upper-solvent phase. One half ml of the upper layer was placed in 1) scintillation vials for radioactivity analysis by the procedure which will be described later in this section and 2) into test tubes for phosphrous analysis using the method of Chen et al. (1956).

To determine if the method described by Lindberg and Ernster (1955) using ammonium molybdate-isobutanol-benzene did extract all of the inorganic phosphrous from a sample, an experiment was conducted using anerobic dilution solution (ADS) medium which contained 180 µg phosphrous per ml (Table 2, p. 47). The phosphrous content of the ADS was determined first by calculation and by the method described by Chen et al. (1956). The phosphrous content of the ADS was extracted using the method of Lindberg and Ernster (1955) followed by phosphrous analysis using the method described by Chen et al. (1956). The results are shown below.

The phosphrous determination of ADS by the method described by Chen et al. (1956) showed a somewhat higher phosphrous content than by calculations. The extraction of inorganic phosphrous using the method of Lindberg and Ernster (1955) and subsequent analysis for phosphrous by

the method of Chen et al. (1956) showed that the phosphrous content of ADS + Resazurin and ADS was 97.4 and 98.4% respectively of the same ADS samples when analyzed by the method of Chen et al. (1956).

Medium	Method of Analysis	µg phosphrous/ml
ADS	Calculated	180.0
ADS + Resazurin	Chen et al. (1956)	190.0
ADS	Chen et al. (1956)	182.5
ADS + Resazurin	Extraction by Lindberg and Ernster (1955),	185.0
ADS	analysis by Chen, et al. (1955)	177.2

Other Analytical Procedures

Total phosphrous was determined by digesting about 10 mg of lyophilized bacteria or protozoa in perchloric acid according to the method of Chen et al. (1956).

Phosphrous concentration of the medium was determined by drying about 0.2 ml of medium in a test tube and analyzing for phosphrous by the method of Chen et al. (1956).

Nitrogen content of the bacterial and protozoa cells was determined by the micro Kjeldahl procedure.

Volatile fatty acid content of the in vitro incubation medium was determined by taking 5 ml of medium and adding 1 ml meta-phosphoric acid to acidify the samples, centrifuged at 18,000 xg for 15 minutes and the supernatant

seperated and frozen until analyzed. VFA were determined in a Packard gas chromotograph model 840 equipped with a Packard hydrogen flame ionization detector model 803. Samples were injected into a 1.98 m x 0.05 cm teflon column packed with Chromsorb 101. Nitrogen carrier gas flow rate was 40 ml per minute and column oven temperature was 188 C. Peak areas were converted to mm moles/ml by comparing with peak areas of standard VFA solutions determined at the same time.

Phosphrous ^{33}P was obtained from New England Nuclear, Boston, Mass., as $H_3{}^{33}PO_4$ diluted in 0.02 N HCl. Radioactivity, ^{33}P was analyzed by placing aliquots of samples in scintillation fluid (5 g, 2, 5-diphenyl oxazole, 0.05 g 1, 4-bis-(2-(4 methyl-5-phenyl oxazole))-benzene, 500 ml toluene, 500 ml triton X-100) and counting in a Nuclear-Chicago model 6848 liquid scintillation counter. Phosphrous ^{33}P content of the counted samples was determined by comparison with standard solutions of ^{33}P. The half life of ^{33}P is 25.3 days and appropriate decay factors derived by Robinson (1969) were used in calculating the data. Machine counting efficiency was determined to be 83.5% using internal standards.

All glassware used in the experiments was washed with soap and water, rinsed in deionized-distilled H_2O-Conc. HCl (2:1) followed by a final rinse in deionized-distilled water.

Experimental Animals

Three suffolk wethers (wt. 60 kg each) fitted with rumen cannulae (Jarrett, 1948) were maintained on one of the rations described in Table 1. The sheep were fed at the rate of 158 kcal calculated feed digestible energy/ kg $BWT^{\cdot 75}$ per day (Meyer et al., 1962) with the ration being offered in equal proportions twice daily at 8 am and 5 pm.

TABLE 1

Composition of Rations for Sheep

Ingredients	Ration 1[b]	Ration 2[c]	Ration 3[d]
	%	%	%
Corn cob pellets	10.0	--	--
Alfalfa meal (dehy)	15.0	--	--
Rolled oats	26.0	10.0	10.0
Ground corn	22.0	40.0	40.0
Wheat bran	10.0	--	--
Soybean meal	5.0	--	--
Ground wheat straw	--	10.0	10.0
Glucose monohydrate	--	10.0	10.0
Starch	--	14.0	17.5
Urea	0.5	3.5	--
Molasses	9.0	10.0	10.0
Mineral-vitamin mix[a]	2.5	2.5	2.5

[a]Mineral-vitamin mix contained in %: 42.29 Dicalcium phosphate; 42.29 high zine tract mineral salt: 15.0 $Na_2 SO_4$; 0.32 (10,000 IU/g) vitamin A; 0.10 (9,000 IU/g) vitamin D.

[b]14.5% crude protein, 3162 kcal digestible energy/kg.

[c]15.9% crude protein, 3580 kcal digestible energy/kg.

[d]6.1% crude protein, 3727 kcal digestible energy/kg.

Crude protein and digestible energy values were calculated from NRC Publication 1684, 1969. United States-Canadian Tables of Feed Composition.

EXPERIMENTS

Experiment 1

Walker and Nader (1968) utilized the nitrogen to sulfur ratio which was found to be constant in rumen bacteria and protozoa to determine the amount of microbial nitrogen synthesis. By determining and multiplying the amount of sulfur incorporated into microbial cells by the nitrogen to sulfur ratio, they were able to indirectly determine the amount of microbial nitrogen (protein) synthesized in the rumen of sheep.

Since phosphrous incorporation into rumen microbial phospholipid-phosphrous was to be used as a marker of cell growth, and to determine the amount of microbial nitrogen synthesized, the amount of phosphrous incorporated would need to be multiplied by a nitrogen to phospholipid-phosphrous (N/PL-Pi) ratio of the cells. The following experiment was conducted to determine the N/PL-Pi ratio in 12 pure strains of rumen bacteria and to ascertain if the N to PL-Pi ratio would be similar for the 12 strains studied.

The pure culture rumen bacteria were grown by Dr. B. A. Dehority, Department of Animal Science, Ohio

Agricultural Research and Development Center, Wooster, Ohio 44691, according to techniques described by Hungate (1950) and Dehority (1969). The bacteria were isolated from the 1% cellulose rumen fluid medium by centrifuging at 150,000 xg for 30 minutes. The supernant was discarded and the pellet was resuspended in 0.82% NaCl and centrifuged at 150,000 xg for 30 minutes. The remaining pellet in the centrifuge tube was then washed with a small amount of distilled-deionized H_2O to remove excess NaCl. The pellet was then frozen and lyophilized. The lyophilized bacteria were then analyzed for nitrogen and phospholipid-phosphrous content by the procedures described previously.

Experiment 2

Measurement of rumen microbial protein synthesis would ultimately be conducted with whole rumen contents which contain both bacteria and protozoa. Thus it seemed desirable to determine the nitrogen to phospholipid-phosphrous (N/PL-Pi) ratio in mixtures of bacteria and protozoa obtained from rumen contents.

The N/PL-Pi ratio was determined for rumen bacteria and protozoa fractions isolated from rumen contents collected from a sheep fed ration 1 (Table 1), and a cow fed hay and grain.

The bacteria and protozoa were isolated from rumen contents as follows: 1) the rumen contents were squeezed through two layers of cheesecloth to separate the large particles from the rumen fluid, 2) the fluid was centrifuged at 500 xg for 15 minutes to remove bacteria and feed residue (supernant) from the protozoa (pellet), 3) the supernant was recentrifuged at 2500 xg for 15 minutes to separate bacteria (supernant) from feed residue (pellet) and 4) the supernant containing the bacteria was centrifuged at 18,000 xg for 15 minutes to isolate the bacteria (pellet). The bacteria and protozoa fractions were both resuspended individually in approximately 100 ml 0.82% NaCl, to remove any medium or foreign material that the microbial cells might attach to, and centrifuged at 18,000 xg for 15 minutes, the supernant was discarded, this procedure was repeated three times. The resulting pellet was washed with a small amount of distilled-deionized H_2O to remove excess NaCl in the pellet, then the pellet was frozed and lyophilized. The lyophilized bacteria and protozoa were analyzed for nitrogen and phospholipid-phosphrous by the methods described earlier and N/PL-Pi ratios were calculated for the bacteria and protozoa separately.

Experiment 3

To further study variations in the microbial N/PL-Pi ratios and to determine if the ratio would be different

for bacteria obtained at different times after feeding the following study was conducted. In vitro fermentations were conducted using washed cell suspensions of mixed rumen bacteria collected at various times after feeding and incubated with or without substrate additions. Sheep rumen contents were collected for the 180-minute in vitro incubations at 4, 24 and 48 hours after feeding of ration 1 (Table 4, p. 59).

Rumen digesta was collected from the sheep in a vacuum bottle warmed to 39 C, gassed with oxygen-free CO_2 (Hungate, 1966) and transported quickly back to the laboratory. The bacteria were isolated from the rumen contents as follows: 1) rumen contents were squeezed through two layers of cheesecloth and the rumen fluid collected, 2) 595 ml of rumen fluid was then centrifuged at 500 xg for 15 minutes and 3) the supernant centrifuged at 10,000 xg for 15 minutes with the resulting pellet containing the isolated bacteria. All centrifuge tubes and beakers were gassed with oxygen-free CO_2 to assure anaerobiosis. The isolated bacterial pellet was resuspended in 595 ml of reduced anaerobic dilution solution medium (ADS) (Table 2) and the pH adjusted to 6.7. The prewarmed fermentation flasks containing the resuspended bacteria were placed in a 39 C water bath and the incubation medium was bubbled with oxygen-free CO_2.

TABLE 2

Anaerobic Dilution Solution[a]

Composition	ml/liter
Mineral Solution I[b]	7.5
Mineral Solution II[c]	7.5
H_2O	85.0

The mineral-water solution was heated almost to a boil, gassed with oxygen-free CO_2 before the addition of Na_2CO_3 and cysteine.

Composition	ml/liter
12% Na_2CO_3	1.11
3% Cysteine HCl	0.56

[a]Phosphrous content 180 µg/ml.

[b]Mineral Solution I, 0.3% K_2HPO_4 (W/V per liter).

[c]Mineral Solution II, 0.3% K_2HPO_4, 1.2% Na_2SO_4, 0.6% NaCl, 0.6% $MgSO_4.7H_2O$, 0.06% $CaCl_2.2H_2O$ (W/V per liter).

Since the bacteria were isolated in a refrigerated centrifuge the starting time of the incubations was delayed until the incubation medium reached 39 C which usually took 15 to 30 minutes. This assured that all incubations were conducted under similar conditions. At that time the substrate, 0.66% glucose, 0.5% soluble starch and 0.15% urea (% weight of the final volume) was added to half the incubation flask before the initial subsample was obtained. Subsamples of 85 ml were obtained from the incubation medium at 0, 60, 120 and 180 minutes after the substrate was added. To stop bacterial activity the subsamples were immediately placed in cold beakers which were then placed in a solution of 95% ethanol and solid CO_2 until the temperature of the incubation medium was 5 C, which usually required 1-2 minutes. To collect the bacteria, the subsamples were centrifuged at 18,000 xg for 15 minutes. The pellet containing the bacteria was resuspended in about 25 ml of 0.82% NaCl to remove any medium that might adhere to the cells and centrifuged at 18,000 xg for 15 minutes. The supernant was then discarded and the washing procedure was repeated three times. The final pellet was washed with a small quantity of distilled-deionized water to remove excess NaCl and the pellet was then frozen and lyophilized. The lyophilized bacteria were then analyzed for nitrogen and phospholipid phosphrous.

The data were statistically analyzed for differences between means using the "Duncan's New Multiple Range Test" as described by Steel and Torrie (1960).

Experiment 4

Before conducting experiments designed to measure the rate of rumen microbial protein synthesis, utilizing radioactive phosphrous ^{33}P as a marker, the metabolism of ^{33}P was studied in washed cell suspensions of mixed rumen bacteria. Metabolism of ^{33}P in the following cellular fractions was studied; intracellular phosphrous (IC-Pi), phospholipid-phosphrous (PL-Pi) and total cell phosphrous. Also studied was the ^{33}P content of the medium, and changes in dry cell mass and nitrogen content with time.

The preparation of washed cell suspensions of mixed rumen bacteria and the procedures for the in vitro incubation were as described in experiment 3 with two exceptions. The following exceptions to the previous method were: 1) the incubations were 240 minutes in length, subsamples were obtained at 0, 30, 60, 120, 180 and 240 minutes, and 2) 45 ml of ^{33}P tracer containing approximately 60 x 10^6 DPM (depending on age of isotope) was added in place of 45 ml of medium at 0 time, before substrate addition. Subsamples were collected and processed as described in experiment 3 except that 2 ml of medium

was saved for phosphrous and ^{33}P analysis after the first centrifugation. The lyophilized bacterial samples were analyzed for nitrogen content, phosphrous, and ^{33}P content of IC-Pi, PL-Pi, total cell phosphrous and medium phosphrous. Changes in dry cell mass content of the incubation was determined by drying an aliquot of the subsample for 24 hours at 110 C. Changes in dry cell mass were expressed in mg of the total dry matter of the subsample.

Experiment 5

Mitchell and Moyle (1953) studied phosphrous metabolism in Micrococcus pyogenes and conducted an experiment in which the substrate was added to the medium 90 minutes after the start of the incubation. These workers noted that ^{32}P uptake into the IC-Pi fraction occurred at all times but incorporation of ^{32}P into the PL-Pi fraction did not occur until the substrate was added.

The metabolism of ^{33}P in rumen bacteria was studied following the incubation time schedule outlined by Mitchell and Moyle (1953). The phosphrous content of the medium was also lowered to 35 µg/ml from 180 µg/ml and the medium composition is shown in Table 3. The purpose of reducing the phosphrous content of the medium was to see if the specific activity of the IC-Pi would equilibriate with the specific activity of the medium. If this occurred then the medium specific activity could be used as the

TABLE 3

Low Phosphrous Anaerobic Dilution Solution[a]

Composition	ml/liter
Mineral Solution[b]	7.5
H_2O	92.5

The mineral-water solution was heated almost to a boil, gassed with oxygen-free CO_2 before the addition of Na_2CO_3 and cysteine.

Composition	ml/liter
12% Na_2CO_3	1.11
3% Cysteine NCl	0.56

[a]Phosphrous content 35 µg/ml.

[b]Mineral Solution, 0.58% Trishydroxymethyl-amino methane (Tris) 3 x Cystalline (MWT 121.0), 1.28% Na_2SO_4, 1.2% NaCl, 0.12% $MgSO_4.7H_2O$, 0.12% $CaCl_2.2H_2O$, 7.47% K_2HPO_4 (W/V per liter).

^{33}P precursor pool thus making the analysis of the precursor pool easier and less time consuming.

The incubation time was 330 minutes with subsamples taken at 0, 30, 60 and 90 minutes before substrate addition, and 30, 60, 120, 180 and 240 minutes after substrate addition. Since the incubation time was longer the incubation volume was 850 ml, and 850 ml of rumen fluid was used for collection of the washed cell suspension of rumen bacteria. The procedure for the preparation of washed cell suspensions of mixed rumen bacteria and the method of conducting the *in vitro* incubation were the same as in experiment 3.

Analytical parameters were the same as those enumerated in experiment 4.

Experiment 6

The maximum production of VFA and growth of rumen microorganisms has been shown to occur shortly after the animal has been fed. The synthesis of VFA and cells by ruminal microorganisms depends on an adequate supply of substrate (energy, nitrogen and mineral cofactors). Volatile fatty acid production or microbial cell synthesis is dependent upon the substrate level in the rumen (Hungate, 1966).

The effect of dietary protein level ration on ^{33}P incorporation into microbial phospholipids was studied

during a 240-minute *in vitro* incubation of whole rumen contents collected from sheep fed either a high (15.7%) or a low (6.1%) crude protein ration containing similar levels of digestible energy (Table 1, rations 2, 3).

The *in vitro* incubations were conducted with rumen contents collected at 0 (before), 2 and 4 hours after feeding. Twenty g subsamples from the *in vitro* incubation were collected at 0, 30, 60, 120, 180 and 240 minutes after the start of the incubation. The procedure for collection of rumen contents from the sheep and the pre-incubation treatment of the fermentation flasks were as described in experiment 3. Immediately upon arriving in the laboratory with the collected rumen contents 150 g of rumen contents were weighed into the fermentation flask and 6 ml of ^{33}P tracer containing approximately 13.2×10^7 DPM was added. The flask was then shaken for 30 seconds and the zero time subsample obtained. To determine if the 6 ml of radioactive tracer was quickly mixed with the rumen contents, 6 ml of 1% crystal violet dye was added to 150 g of rumen digesta in a separate experiment. After the 6 ml of dye was added to the flask about 20 seconds of shaking was required to obtain complete mixing of the dye on to the digesta particles.

The microbial population of the subsamples was killed immediately by the addition of 1 ml saturated $HgCl_2$ and the samples were then frozen and lyophilized.

Phospholipids were extracted from the lyophilized whole rumen digesta and ^{33}P incorporation into the microbial phospholipids was determined by the procedures described in the analytical methods section.

Experiment 7

The previous preliminary experiments conducted showed that ^{33}P incorporation into rumen microbial phospholipids could be used as a marker of microbial cell synthesis and protein synthesis. The rate of rumen microbial protein synthesis was measured *in vitro* at different times after feeding using incubations of whole rumen contents. Rumen contents were collected from the sheep fed ration 1 (Table 1) and the rate of protein synthesis was assessed at 0 (before) 2, 4, 9 and 11 hours after the am feeding. In this trial at 9 hours the sheep was refed so the 11-hour incubation represented again a 2 hour after feeding sample. This type of collection schedule was designed to provide information as to when the maximum and minimum rates of microbial protein synthesis occur after feeding.

The incubations were conducted according to the *in vitro* "zero-time rate method," developed by Carroll and Hungate (1954) and Hungate *et al.* (1961). In this experiment the rumen contents were incubated for 60 minutes with subsamples being collected at the start (T_0), and the end (T_{60}), of the incubation. Collection and handling of the rumen contents and preparation of the fermentation

flasks was the same as described in experiment 6. Seven hundred fifty g of rumen contents were weighed into a 800 ml mason jar (fermentation flask) and 20 ml of ^{33}P, approximately 44 x 10^7 DPM were added to the contents. The jar was stoppered and shaken for 30 seconds and immediately a 325 g subsample was weighed into a cold beaker containing 20 ml saturated $HgCl_2$. The final subsample was removed in the same manner at the end of the incubation (60 minutes). The 325 g subsample was then divided into different fractions, 20 g of contents were obtained to determine the amount of ^{33}P incorporation into the total microbial phospholipids. For this analysis 20 g of contents were placed into a 200 ml centrifuge tube and about 100 ml of 0.82% NaCl was added to wash nonincorporated ^{33}P from the digesta. The tube was centrifuged at 18,000 xg for 15 minutes and procedure repeated three times followed by a final wash using a small volume of deionized-distilled H_2O to remove traces of NaCl. The sample was then frozen and lyophilized. The bacteria and protozoa were isolated from the remaining rumen contents by first squeezing the contents through two layers of cheesecloth and then resuspending and mixing the squeezed contents in about 500 ml of 0.82% NaCl to remove microorganisms adhering to the plant material. The resuspended contents were squeezed again and the extract fluid pooled with the rumen fluid from the first squeezing. However,

before the resuspended digesta was resqueezed, 2 ml and 5 ml of the first rumen fluid obtained was collected for analysis of phosphrous (1 ml) and VFA (5 ml). The bacteria and protozoa were isolated by differential centrifugation from the pooled volumes of rumen fluid by the procedure described in experiment 3. The resulting bacterial and protozoal fractions were then frozen and lyophilized. Microscopic examination of the bacterial and protozoal fractions obtained through the differential centrifugation method were done in a separate study. The protozoal fraction was found not to be contaminated with plant material or free bacteria and the bacterial fraction was not contaminated with plant material or protozoa. Microscopic examination of the squeezed and resuspended contents showed absence of protozoa but presence of a number of bacteria. These observations indicated that the protozoa could be quantatatively recovered from rumen contents but the bacteria could not.

Phospholipids were extracted from the lyophilized whole rumen digesta and ^{33}P incorporation into the microbial phospholipids was determined by the procedures described in the analytical methods section. Intracellular phosphrous and phospholipids were extracted from the lyophilized bacteria and protozoa and ^{33}P uptake and incorporation into those fractions was determined by the procedures described in the analytical methods section.

The nitrogen and phospholipid-phosphrous (N/PL-Pi) ratios for bacteria and protozoa were calculated for the T_0 and T_{60} subsamples. Volatile fatty acids (VFA) were determined from the rumen fluid collected at the T_0 and T_{60} samples. Production rates of VFA were calculated by the difference between the T_{60} and T_0 samples.

The following procedure was used to calculate the rate of rumen microbial protein synthesis using ^{33}P incorporation into microbial phospholipids.

1. Incorporation of ^{33}P into Bacterial Phospholipids. Quantatative incorporation of ^{33}P into bacterial phospholipids could not be measured, since quantatative isolation of bacteria from the rumen contents using the differential centrifugation procedure described above was not possible. Thus incorporation of ^{33}P into bacterial phospholipids was determined by subtracting the amount of ^{33}P incorporated into protozoal phospholipids from the amount of ^{33}P incorporated into the total microbial phospholipids which were extracted from the whole rumen contents. Incorporation of ^{33}P into the phospholipids of whole rumen contents would only be bacteria and protozoa phospholipids and not feed phospholipids.

2. The Amount of Phosphrous Incorporated into Bacteria and Protozoa. The amount of phosphrous incorporated into the bacteria and protozoa during the 60-minute incubation period was calculated by dividing the

counts per minute of ^{33}P incorporated into bacterial or protozoal phospholipids by the respective mean specific activity of the intracellular-phosphrous fractions. The specific activity of the intracellular-phosphrous fraction was taken to be representative of phosphrous percursor pool for microbial phospholipid synthesis.

3. Amount of Nitrogen Synthesized. The amount of bacterial or protozoal nitrogen containing compounds synthesized were calculated by multiplying the amount of phosphrous incorporated (step 2) by the appropriate N/PL-Pi ratio which was established for each incubation.

4. Amount of Microbial Protein Synthesized. Protein synthesized was calculated by multiplying the amount of nitrogen containing compounds by 6.25.

Table 4 presents the various equations used to calculate ruminal microbial protein synthesis.

TABLE 4

Calculation of Microbial Protein Synthesis

(1) Incorporation of ^{33}P into bacterial phospholipids = (CPM PL-Pi/mg WRC x mg WRC) - (CPM PL-Pi/mg protozoa x mg protozoa)

(2) Amount (µg) of phosphrous incorporated into bacteria and protozoa =

µg Pi into bacteria = (CPM protozoa PL-Pi/SA bacterial IC-Pi)

µg Pi into protozoa = (CPM protozoa PL-Pi/SA protozoal IC-Pi)

(3) Amount nitrogen synthesized

µgN = mg Pi incorporated into bacteria x N/PL-Pi ratio

µgN = mg Pi incorporated into protozoa x N/PL-Pi ratio

(4) Protein synthesized

µg Protein = Total µg N synthesized x 6.25

Abbreviations:

CPM, counts per minute; PL-Pi, phospholipid phosphrous; WRC, whole rumen contents; SA, specific activity; IC-Pi, intracellular phosphrous; N, nitrogen.

RESULTS AND DISCUSSION

Experiment 1

The nitrogen (N) and phospholipid-phosphrous (PL-Pi) content was determined and the N/PL-Pi ratio calculated for eight species (12 strains) of pure culture rumen bacteria. The results are shown in Table 5. These data show that both nitrogen and PL-Pi content differed between the different species of bacteria. These differences resulted in variations in the N/PL-Pi ratios. Similar differences in nitrogen and phosphrous content were also noted for different strains of the same species. The mean nitrogen content of bacteria in this study was 73.8 µg/mg dry sample or 49.9% crude protein (N x 6.25) with a range from 59.0 to 92.8 µg nitrogen/mg dry sample or 36.9 to 58.0% crude protein (N x 6.25). The crude protein of rumen bacteria is often assumed to be 60% (Luria, 1960) however, the data presented above suggest that the protein level is lower than 60% in rumen bacteria.

The N/PL-Pi ratios ranged from 31.7 to 137.5 with a mean value of 63.2 for all bacteria analyzed. This wide variation in the ratio between bacteria would indicate that a standard N/PL-Pi ratio calculated only once, such

TABLE 5

Nitrogen (N) and Phospholipid-Phosphrous (PL-Pi) Content of Pure Cultures of Rumen Bacteria

Species	Strain	µg N/mg	µg PL-Pi/mg	N/PL-Pi
R. albus	7	92.8	0.96	96.1
E. ruminatium	B_1C_{23}	76.4	1.11	68.8
S. dextrinosolvens	24	77.5	1.03	75.2
P. elsdenii	B 159	60.5	.71	85.1
L. multiparus	D 15d	70.2	.50	137.5
B. ruminicola	H 15a	59.0	3.21	18.4
	D 31d	69.8	1.79	38.9
	H 8a	79.6	2.01	39.6
B. succinogenes	A 3c	82.0	2.54	32.3
	S 85	66.2	2.65	24.9
R. flaveflaciens	B 1a	81.9	.75	109.3
	B 34b	70.1	2.21	31.7
Mean		73.8	1.62	63.2
SE ±		2.8	0.26	10.9

as done by Walker and Nader (1968) for the nitrogen to sulfur ratio in rumen bacteria could not be employed when phosphrous incorporation into PL-Pi is used as a marker for cellular growth. The results showed that a N/PL-Pi ratio would have to be determined separately in every experiment to accurately assess microbial incorporation of nitrogen (into protein) when PL-Pi is used as a marker of cellular growth.

Experiment 2

The objective of this experiment was to determine if variations in the N/PL-Pi ratio occurred in preparations of mixed cultures of bacteria and protozoa obtained from the same sample of rumen contents. The N/PL-Pi ratios determined for bacterial and protozoal preparations from sheep and cow rumen contents are shown in Table 7. The variations in nitrogen content were much less marked than the variations in the PL-Pi content of the ruminal bacteria and protozoa. The ratios shown in Table 6 indicated that variations occurred between bacteria and protozoa from the same rumen contents as well as between rumen contents from different animals. Differences in rations might explain the variations in N/PL-Pi ratios of microorganisms obtained from the two species of animals. The variations in the N/PL-Pi ratios noted in this experiment and in experiment 1 support the conclusion

TABLE 6

Nitrogen (N) and Phospholipid-Phosphrous (PL-Pi) Content of Sheep and Cow Rumen Bacteria and Protozoa

Animal	Organism	µgN/mg	µg PL-Pi/mg	N/PL-Pi
Sheep	Bacteria	40.3	.67	60.3
	Protozoa	54.0	.74	72.6
Cow	Bacteria	57.8	1.21	47.8
	Protozoa	49.0	.35	141.2

that a standard single N/PL-Pi ratio does not exist for either ruminal bacteria or protozoa.

Experiment 3

Rumen bacteria were obtained from sheep rumen contents collected at 4, 24 and 48 hours after feeding to investigate possible time after feeding effects on the N/PL-Pi ratio of bacteria. Rumen bacteria collected at these times were incubated for 180 minutes *in vitro*. Energy and nitrogen substrate were added to one half of the incubations to also study the effect of substrate addition on bacterial N/PL-Pi ratio. Results of this experiment are presented in Table 7 as mean values of subsamples taken from the *in vitro* incubation at 0, 60, 120 and 180 minutes. The complete data are presented in

TABLE 7

Mean Nitrogen (N) and Phospholipid-Phosphrous (PL-Pi) Content of Mixed Rumen Bacteria Collected from a Sheep at 4, 24 and 48 Hours After Feeding and Incubated *In Vitro*[1]

Fermentation Treatment	Sampling Time After Feeding	N	PL-Pi	N/PL-Pi
	(hr.)	(µg/mg dry sample)		SE
Substrate Added	4	29.7± 1.16	.69± .03	42.8[b] ± .91
	24	23.1± .84	.58± .02	38.7[a] ± .45
	48	23.2± 1.00	.64± .02	36.6[a] ±2.03
No Substrate Added	4	71.6± 1.33	1.39± .02	51.1[c] ±1.47
	24	69.9± .89	1.59± .03	43.9[b] ± .67
	48	67.5± .62	1.15± .02	58.7[c] ± .87

[1]Incubations were conducted for 180 minutes with subsamples obtained at 0, 60, 120 and 180 minutes. Values reported are mean values for the 4 subsamples, and presented as mean SE±.

[2]Values in that column carrying different superscripts are significantly different at $P < 0.01$.

Appendix 1. Comparison of the N/PL-Pi ratios showed that the 24 and 48 hour "substrate added" values were significantly different ($P < .01$) from the 4, 24 and 48 hour "nonsubstrate added" values. The 4 hour "substrate added" and the 24 hour "nonsubstrate added" values were not significantly different at the $P < .01$ or the $P < .05$ level. Some of the values within a substrate treatment were also significantly different ($P < .01$) from each other. This finding again indicated that the N/PL-Pi ratios of rumen bacteria change under different conditions and that a standard or single value for N/PL-Pi ratio does not exist in ruminal bacteria.

The nitrogen content of bacteria incubated in the "nonsubstrate added" medium was approximately three times higher and the PL-Pi content was twice the respective values found in the bacteria incubated in the "substrate added" medium. The lower values observed for the bacteria incubated in the "substrate added" medium could be due to increased storage of carbohydrate polymers by these bacteria since they were grown in a substrate rich medium (Luria, 1960).

The results of experiments 1, 2 and 3 showed that N/PL-Pi ratios for ruminal bacteria and protozoa are not constant and that the ratio differed for bacteria and protozoa. Thus when the incorporation of labelled phosphrous into microbial phospholipids is to be used as an

indirect marker of microbial protein synthesis (N incorporation) by multiplying phosphrous incorporated by a N/PL-Pi ratio, an appropriate N/PL-Pi ratio must be determined for each such experiment. These results also indicated that the often quoted crude protein value of 60% for rumen bacteria (Luria, 1960) may be open to question. The crude protein value of 60% was derived from nonruminal bacteria and Luria (1960) did not indicate that rumen bacteria contain 60% crude protein. Luria (1960) states however that the micro-Kjeldhal method as commonly employed recovers only about 80-90% of the cellular nitrogen since nitro and azo groups or nitrogen in rings of purines and pyrimidines are often refractory to the H_2SO_4 digestion.

Correction of all nitrogen content values of rumen microorganisms analyzed in experiments 1, 2 and 3 to account for incomplete nitrogen recovery (Luria, 1960) would still not raise the nitrogen x 6.25 content to an average of 60%.

Experiment 4

Before experiments designed to measure the rate of rumen microbial protein synthesis utilizing ^{33}P as a marker of cellular growth could be conducted, the metabolism of ^{33}P in rumen bacteria required investigation in this study. Parameters of ^{33}P metabolism studied

were ^{33}P incorporation into intracellular-phosphrous (IC-Pi), phospholipid-phosphrous and total cell phosphrous of bacteria. The changes of medium ^{33}P content and changes in dry cell mass and cellular nitrogen content were also determined during the incubation. The results of three in vitro incubations are given in Table 8 and depicted in Figure 1. A complete listing of the raw data is presented in Appendix 3. The data in Table 8 and Figure 1 show that the uptake of ^{33}P into the IC-Pi and the total cell phosphrous fractions of the cell were linear with time as was the ^{33}P incorporation into cellular phospholipids during the 240 minute incubation.

The specific activity (SA) of the medium did not change during the time of the incubation and it can be noted that the SA of the IC-Pi did not approach or equilibriate with the medium SA. The SA of the IC-Pi was anticipated to equilibriate rapidly with the SA of the medium. If the above had occurred, the SA of the medium phosphrous could have been used to determine phosphrous incorporation into cellular phospholipids and eliminate the necessity for the more difficult IC-Pi determination.
The results (Table 8, Figure 1) of these trials showed the IC-Pi must be determined to calculate cellular phosphrous incorporation into PL-Pi.

The dry cell mass and cellular nitrogen content increased with time, however the changes were only slight

TABLE 8

Mean Changes in Cellular Constituent Specific Activity,[2] Dry Cell Mass, and Nitrogen of Mixed Rumen Bacteria During a 240-Minute *In Vitro* Incubation

Subsample Times	Cellular Constituents					
	Phosphrous Fractions					
	PL-Pi[3]	IC-Pi[4]	Medium	Total Cell Phosphrous	Dry Cell Mass	Dry Cell Nitrogen
(min)	(SA)	(SA)	(SA)	(SA)	(mg)	(mg)
0	10.1	56.2	411.3	26.3	359	112
30	20.9	64.7	423.7	27.7	365	113
60	34.4	88.9	428.7	39.5	382	120
120	65.7	106.6	453.3	74.7	394	131
180	89.9	142.5	424.7	90.3	629	199
240	106.7	164.2	434.7	122.2	657	216

[1]Mean values from three separate *in vitro* incubations.

[2]Specific Activity (SA) = $\frac{\text{CPM-}^{33}\text{P}}{\text{unit phosphrous}}$

[3]PL-Pi, Phospholipid-Phosphrous.

[4]IC-Pi, Intracellular-Phosphrous.

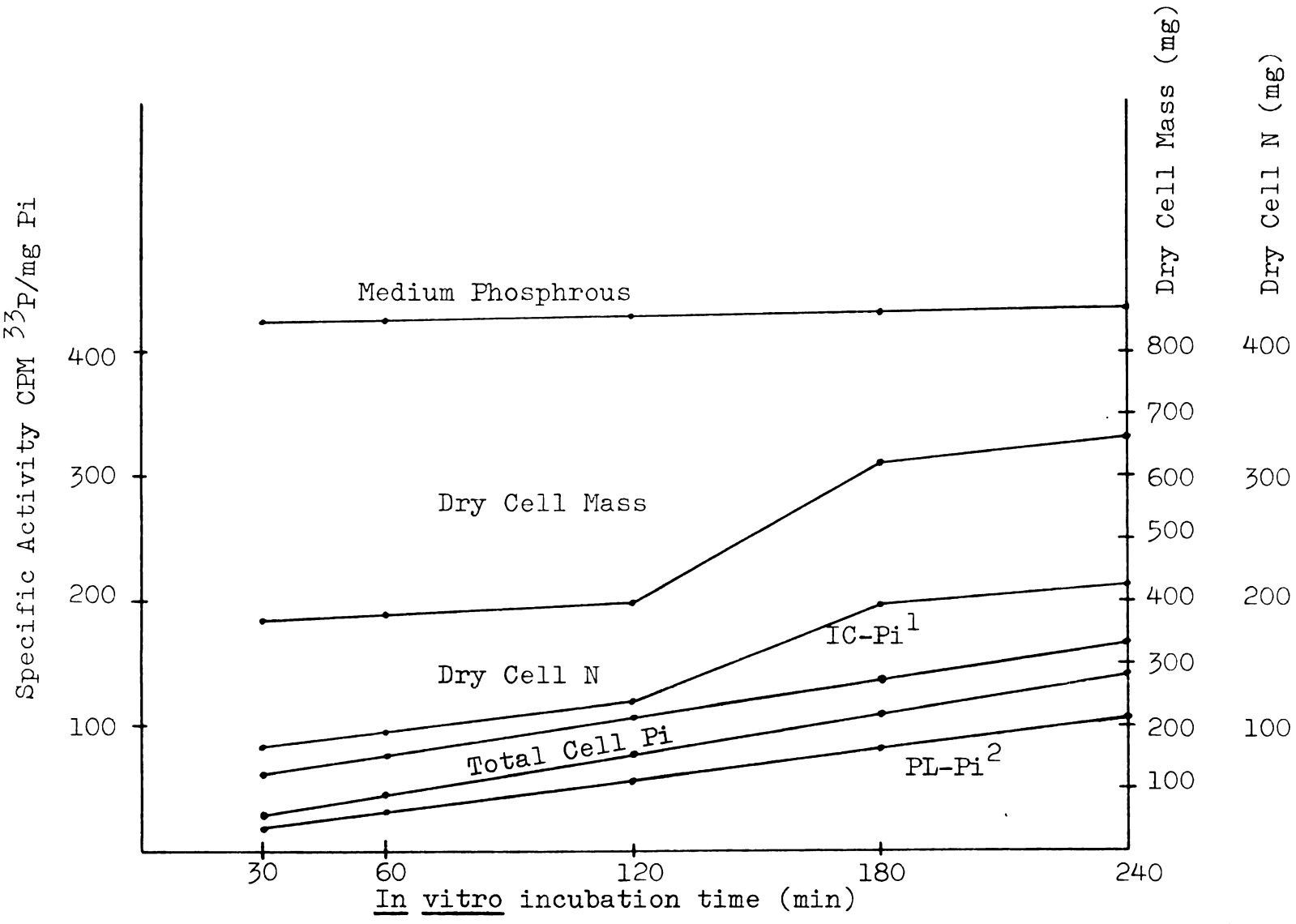

Figure 1. Mean changes in specific activity, dry cell mass and nitrogen (N) of mixed rumen bacteria during a 240-minute in vitro incubation

[1]IC-Pi, Intracellular-Phosphrous. [2]PL-Pi, Phospholipid-Phosphrous.

until 120 minutes after the start of the incubation. This may be due to a lag effect on the bacteria caused by the preparative steps in obtaining washed cell suspensions of rumen bacteria. This procedure included centrifugation steps at low temperatures (5 C). The slight changes in total dry cell mass early in the incubation may be also due to an increase in bacterial numbers in the absence of synthesis of carbohydrate polymers. After 120 minutes the rapid increases in cell mass noted was probably due to the bacteria entering the log phase of growth and the depositing of larger amounts of carbohydrate polymers. This explanation for the rapid increase in dry cell mass that occurred between 120 and 180 minutes of incubation seems feasible since the change in the SA of the PL-Pi fraction was linear with time during the entire incubation. A linear increase in the SA of the PL-Pi and the IC-Pi fractions would be probable even if bacterial synthesis of storage carbohydrate occurred. The synthesis of storage carbohydrates would not affect the ^{33}P uptake or incorporation into cellular constituents but would only affect the weight of the cells.

Experiment 5

The metabolism of ^{33}P in rumen bacteria was studied following the incubation time schedule designed by Mitchell and Moyle (1953) who reported that in Micrococcus pyogenes,

^{32}P uptake occurred into the IC-Pi fraction before substrate addition but that incorporation of ^{32}P into the PL-Pi fraction did not occur until after substrate addition which was when cellular growth started. In this study the phosphrous content of the medium was reduced from 180 µg/ml to 35 µg/ml. This change in the medium was made to investigate if a lower phosphrous content of the medium would enhance the equilibriation of the SA between the medium and IC-Pi pool.

The mean results of two in vitro incubations are shown in Table 9 and a complete listing of the raw data is presented in Appendix 4. The data is also shown graphically (Figure 2). The curves depicted in Figure 2 are from quadratic and linear regression equations calculated from the raw data. These equations are presented in Appendix 5. The IC-Pi SA depicted in Figure 2 showed that ^{33}P uptake into cells occurred before substrate was added. There was no incorporation of ^{33}P into the PL-Pi fraction in advance of rapid cell growth; however, after substrate addition there was a rapid increase in ^{33}P incorporation into the PL-Pi fraction (Table 9). Changes in dry cell mass were only slight before the addition of substrate but after substrate was added dry cell mass of the incubations increased. The increase of ^{33}P incorporation into PL-Pi and increase in cell mass were linear after substrate addition. Mitchell and Moyle (1953)

TABLE 9

Mean Changes in Cellular Constituent Specific Activity and Dry Cell Mass of Mixed Rumen Bacteria During a 300-Minute *In Vitro* Incubation

Subsample Times	Cellular Constituents[2]			Dry Cell Mass
	PL-Pi	IC-Pi	Medium	
(min)	SA[2]	SA	SA	mg
0	13.2	220.4	2660.0	175.9
30	14.5	387.4	2676.0	178.3
60	12.0	405.1	2648.0	182.9
90[3]	16.5	393.2	2636.0	186.6
30	272.0	788.5	2738.0	246.8
60	431.7	1137.3	2811.0	368.8
120	813.6	1353.7	2373.0	413.6
180	988.0	1401.2	2678.0	498.3
240	1234.4	1678.1	2484.0	540.8

[1]Mean values from two separate *in vitro* incubations.

[2]Specific Activity (SA) = $\frac{\text{CPM } ^{33}\text{P}}{\text{unit phosphrous}}$

[3]Substrate added to incubation medium.

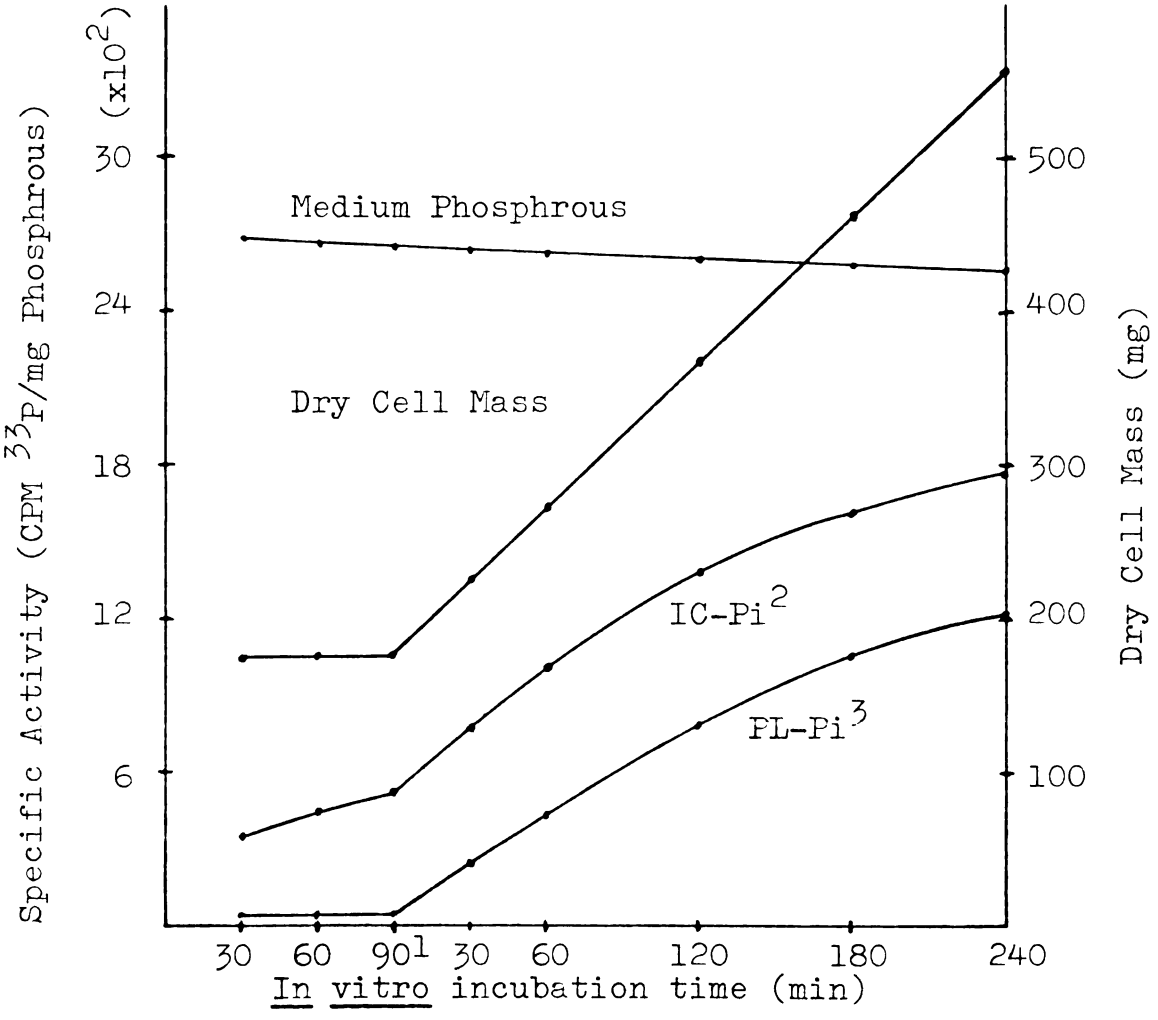

Figure 2. Mean changes in cellular constituent specific activity and dry cell mass of mixed rumen bacteria during a 300-minute in vitro incubation

[1]Substrate added to incubation medium. [2]IC-Pi, Intracellular-Phosphrous.
[3]PL-Pi, Phospholipid-Phosphrous.

reported that during the lag phase of growth of M. pyogenes ^{32}P moved freely into the IC-Pi fraction but ^{32}P incorporation into bacterial phospholipids occurred only during the growth phase. The SA for IC-Pi and PL-Pi increased after the substrate was added and that the increase in PL-Pi ^{33}P incorporation corresponded closely with the increases in dry cell mass (Table 9, Figure 2). This indicates that the synthesis of rumen bacterial phospholipids occurred only during growth and that the synthesis of rumen bacterial phospholipids can be measured by the incorporation of ^{33}P.

The SA of the IC-Pi fraction did not equilibriate with the medium SA even though it did approach it. This eliminates the possibility of using the SA of the medium phosphrous as an estimate of the IC-Pi pool SA. Since IC-Pi, SA equilibriates rapidly with intracellular free phosphrous containing compounds (Bolton and Roberts, 1964; Weissbach et al., 1971), the SA of the IC-Pi pool was used to determine the SA of the phosphrous precursor for phospholipid synthesis.

Experiment 6

The extent of microbial cell synthesis or VFA production depends on the level of substrate in the rumen (Hungate, 1966). The effect of dietary protein level on ^{33}P incorporation into microbial phospholipids was studied

using a 240-minute *in vitro* incubation of whole rumen contents collected from sheep fed either high (15.7%) or low (6.1%) crude protein rations containing similar levels of digestable energy. ^{33}Phosphrous incorporation rates into microbial phospholipids during the 240-minute *in vitro* incubations are presented graphically in Figures 3 and 4. The complete raw data and the linear regression equations are given in Appendixes 6 and 7.

Maximum rate of ^{33}P incorporation into the microbial phospholipids for the sheep fed the low protein ration occurred in rumen contents removed two hours after feeding. Rates of ^{33}P incorporation into rumen contents microbial phospholipids, before and 4 hours after feeding were similar. Sheep fed low protein rations do not exhibit extensive growth of rumen microorganisms since substrate (nitrogen in this case) availability limits growth (Hungate, 1966) and since the limiting substrate (nitrogen) was available to rumen microorganisms for only a short time after feeding. Low rates of ^{33}P incorporation were anticipated for the 0 and 4 hour incubations with a higher rate of ^{33}P incorporation expected for the 2 hour incubation of rumen contents.

The results for incubations of the rumen contents from the sheep fed the high (15.7%) crude protein ration are shown in Figure 3. The maximum rates of ^{33}P incorporation into microbial phospholipids occurred in rumen

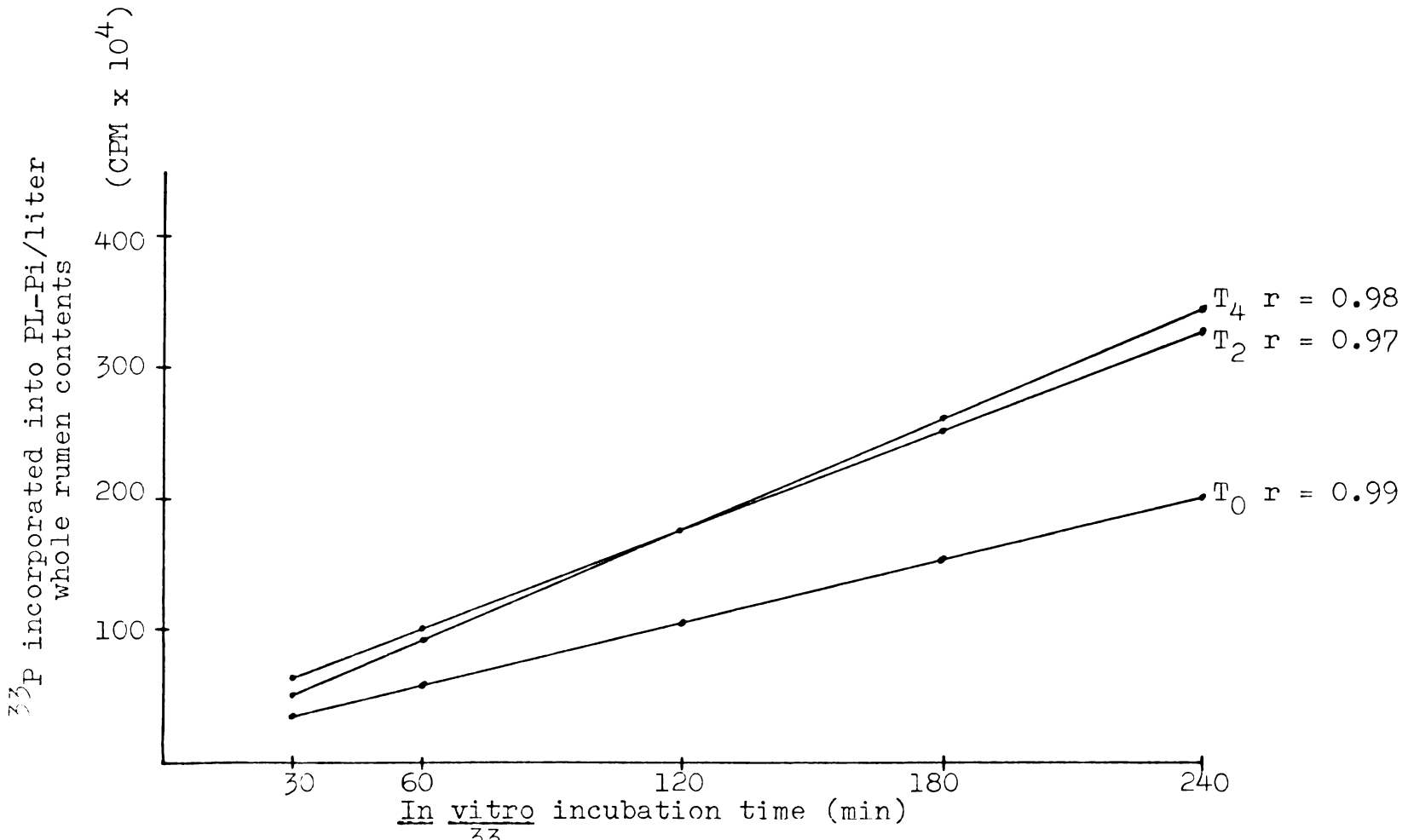

Figure 3. Incorporation of ^{33}P into microbial phospholipids during an in vitro incubation of rumen contents from a sheep fed a high protein (15.7%) containing ration

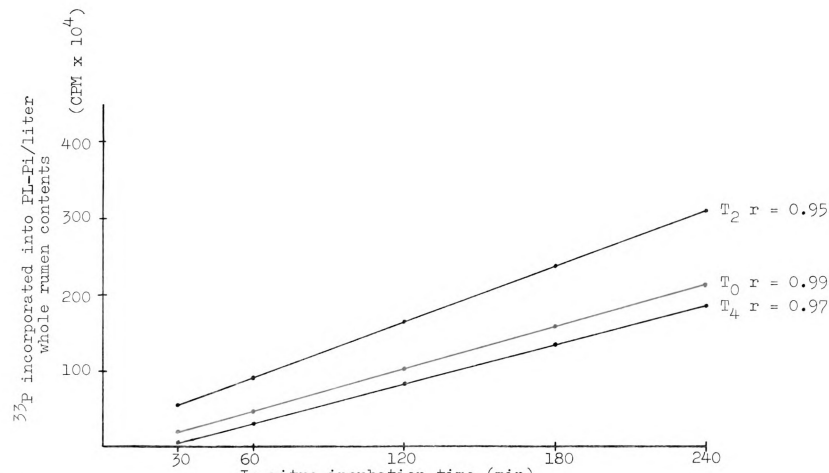

Figure 4. Incorporation of ^{33}P into microbial phospholipids during an in vitro incubation of rumen contents from a sheep fed a low protein (6.1%) containing ration

contents obtained at 2 and 4 hours after feeding. The rate of ^{33}P incorporation for the rumen contents removed before feeding was similar to the rate for the 0 and 4 hour after feeding incubations for the sheep fed the low protein ration (Figure 4). When high protein containing rations are fed to sheep, substrate (especially nitrogen) would not become limiting and thus a sustained level of microbial growth (as monitered by ^{33}P incorporation into phospholipids) would occur for a longer period.

The above data therefore indicate that when different dietary protein levels are fed to sheep, ^{33}P incorporation rates into microbial phospholipids of whole rumen contents reflect expected changes in cell growth.

Experiment 7

Experiments 4, 5 and 6 showed that ^{33}P incorporation into rumen microbial phospholipids could be used as a marker of cell growth. The rate of rumen microbial protein synthesis was measured using 60-minute *in vitro* incubations of whole rumen digesta collected from a sheep at 0 (before) 2, 4, 9 and 11 hours after the am feeding. The quantity of microbial protein synthesized using the method of calculation described in the materials and methods section and the estimated VFA production rates at various times after feeding from the *in vitro* incubations are also shown in Table 10. A complete presentation

TABLE 10

Calculated Microbial Protein Synthesis and Volatile Fatty Acid Production During *In Vitro* Incubations of Sheep Rumen Digesta Collected at Various Times After Feeding

Incubation Time After Feeding	Microbial Protein Synthesized Nitrogen x 6.25/hr/ 4 liter Rumen Volume	Mean	VFA Production m moles/hr/4 liter Rumen Volume
(hr)	(g)	SE	(m moles)
0	9.97	10.66 ± .70	20.4
	11.36		
2	14.49	13.16 ± 1.64	69.6
	11.52		
4	12.84	11.14 ± 1.70	34.8
	9.44		
9	5.45		13.8
11[a]	8.61		16.0

[a]Sheep was refed after the 9-hour incubation sample was obtained, 11 hour incubation is actually 2 hours after feeding.

of the raw data used to calculate the values presented in Table 10 is made in appendixes 8, 9, 10 and 11. Synthesis of microbial protein was expressed in terms of hourly synthesis in a sheep with a rumen volume of 4 liters. The rates of microbial protein synthesis were determined twice for rumen contents collected at 0, 2 and 4 hours after feeding but only once for rumen contents collected at 9 and 11 hours after the am feeding.

The maximum rate of microbial protein synthesis (13.16 g/hr.) occurred at 2 hours after feeding. This was followed by a rate of 11.14 g/hr. at 4 hours after feeding in (Table 10).The lowest rate of microbial protein synthesis (5.45 g/hr.) occurred at 9 hours after feeding just before the pm feeding of the sheep. The rate of microbial protein synthesis of 10.66 g/hr. before the am feeding was much higher than the 5.45 g/hr. that occurred at 9 hours after feeding. Both the 0 and the 9 hour incubations were conducted with rumen contents obtained just before the sheep was fed and it would seem that the rate of microbial protein synthesis would be similar in both instances. The rates of VFA production at 0 and 9 hours after feeding (as estimated in vitro) were also different. The VFA production rates were 20.4 m moles/hr. and 13.8 m moles/hr. for the 0 and 9 hour incubations respectively. The respective VFA production rates do parallel the different microbial protein synthesis

rates noted for rumen contents obtained at 0 and 9 hours after feeding, but the VFA data do not explain why difference in microbial activity or protein synthesis occurred. Replicate incubations were conducted for rumen contents obtained at 9 and 11 hours after feeding but improper laboratory handling of the IC-Pi analysis for these incubations made it impossible to use the data. However, incorporation of ^{33}P into bacterial and protozoal fractions per unit dry cell weight were similar for the first and second set of *in vitro* incubations with rumen contents obtained at 9 and 11 hours after feeding. Thus it can be tentatively concluded that the differences in microbial protein synthesis rates for the 0 and 9 hour incubations must be due to the rumen contents and the microbial populations and not due to large errors in experimental technique.

The rate of microbial protein synthesis at 11 hours after feeding was 8.61 g/hr. which was lower than the rate of 13.16 g/hr. that occurred 2 hours after feeding. Differences in rates of protein synthesis at 2 and 11 hours (2 hours after pm feeding) after the am feeding are reflected by parallel differences in VFA production rates (Table 10). This relationship is similar to that occurring for the 0 and 9 hour incubations. There may be differences in the productive capacity and metabolic activity of rumen microbial populations in rumen contents

either before or after the am feeding (0 and 2 hours) as compared to rumen contents collected either before or after the pm feeding (9 and 11 hours). This probable phenomenon needs to be investigated further.

To determine a daily rate of microbial protein synthesis from the results of *in vitro* incubations of rumen contents at various times after feeding as reported in Table 10, a series of summation intervals were established for the periods between feeding (Table 11). The summation intervals were established as follows: at 0 hours before feeding the summation interval established was from T_0 to T_1 or the time before feeding to 1 hour after feeding for a total summation time of 1 hour. At the 2 hour after feeding incubation the summation interval was from T_1 to T_3 for a total summation time of 2 hours. Summation intervals were established for the 4, 9 and 11 hour incubations and the total summation time for all the incubations was 12 hours. The established summation times were then multiplied by the rates of protein synthesis (Table 10) for the appropriate *in vitro* incubations to calculate total g of protein synthesized. The totals for the summation intervals were then added. Based on this protocol an estimated rate of microbial protein synthesis for a 12 hour period was 109.6 g. If this estimated 109.6 g of microbial protein synthesized in 12 hours is doubled then 219.3 g of microbial protein was estimated to be

TABLE 11

Estimated Rate of Microbial Protein Synthesized During Established Summation Times and for 12 and 24 Hours

Incubation Time After Feeding	Summation Interval	Summation x Time	Protein Synthesized Nitrogen x 6.25/hr./ 1 liter Rumen Volume	=	Total Microbial Protein
(hr.)		(hr.)	(g)		(g)
0	(T_0-T_1)	1	10.66 $\pm$ 0.70		10.66
2	(T_1-T_3)	2	13.17 $\pm$ 1.64		26.34
4	(T_3-T_6)	3	11.14 $\pm$ 1.70		33.42
9	(T_6-T_{10})	4	5.45		22.0
11	$(T_{10}-T_{12})$	2	8.61		17.22

Estimated Microbial Protein Synthesized per 12 hours = 109.6 g.

Estimated Microbial Protein Synthesized per 24 hours = 219.3 g.

synthesized in the 4 liter rumen of a sheep during 24 hours. It must be kept in mind that these rates are estimates, since in vitro incubations were not conducted every hour for 12 or 24 hours and that arbitrary summation intervals were used to calculate the daily rates of ruminal microbial protein synthesis.

The estimated rate of 219.3 g microbial protein synthesized per day was compared in Table 12 to the rates found by other workers. The data were all expressed as g of microbial protein synthesized per 100 g organic matter digested in the rumen. The estimated rate of microbial protein synthesis was calculated to be 26.0 g microbial protein synthesized per 100 g organic matter digested in the rumen. This rate is higher than net rates based on passage studies as reported by Hume (1970b), Hogan and Weston (1970, Lindsay and Hogan (1972), and Walker and Nader (1970). The 26.0 g rate however is a measure of the absolute rate of microbial protein synthesis which includes the turnover and protozoal degradation of bacterial cells. Thus, the absolute rate of microbial protein synthesis should be higher than the rates obtained by the authors mentioned above, since they measured the amount of microbial protein that would be made available to the host animal and not the absolute rate of bacterial and protozoal protein synthesis that occurred in the rumen.

TABLE 12

Grams of Microbial Protein Synthesized per 100 g Organic Matter Digested in the Rumen

Microbial Protein Synthesized/100 g OMD (g)	References
13.3	Hume (1970a)
14.4	Walker and Nader (1970)
15-16	Hogan and Weston (1970)
23.0	Lindsay and Hogan (1972)
23.3	Hume (1970c)
26.0	Present Study[1]

[1]Assumed a value of 75% organic matter digested in the rumen (Al-Rabbat et al., 1970b).

GENERAL DISCUSSION

The objective of this research was to develop a method to measure the quantatative rate of rumen microbial protein synthesis. The estimated 26.0 g of microbial protein synthesized per 100 g organic matter digested in the rumen is the end product of the method development. However, the methods used to derive the final value of 26.0 g should be evaluated and discussed.

The major difficulty encountered in measuring the rate of microbial protein synthesis in rumen contents is in the differentiation between dietary protein and microbial cells (protein) (McDonald, 1954). To differentiate between microbial protein and dietary protein, Hume et al. (1970a,b) fed sheep protein-free diets in which nonprotein nitrogen (urea) was the only dietary nitrogen source. Thus all protein that was found in rumen contents of sheep was of microbial origin. This method employed by Hume (1970a,b) and Hume et al. (1970a,b) did allow for differentiation between dietary and microbial protein but it also required that a purified diet be fed. Such diets are not fed under practical situations and do not accurately reflect on-farm feeding situations.

Hogan and Weston (1970) used α, ε-diaminopimelic acid (DAP), an amino acid that is found only in bacterial cell walls but not in plants, to differentiate between microbial protein and dietary protein. The use of DAP as a marker of bacterial protein synthesis requires that the DAP be extracted from a mixture of microbial cells and dietary protein and that a ratio of bacterial nitrogen to DAP be established on an isolated purified preparation in rumen bacteria. To calculate the amount of bacterial protein in a sample of rumen contents, the DAP is extracted and the quantity of DAP is multiplied by the nitrogen to DAP ratio. A major limitation to the use of DAP is that DAP is found in only some rumen bacteria and not at all in rumen protozoa (Purser and Buechler, 1966), although Mason (1969) showed that the nitrogen to DAP ratio is constant in rumen contents from sheep fed a given ration.

Walker and Nader (1968) used ^{35}S incorporation into rumen microbial cells in a system similar to the one used in experiment 7 to estimate rates of ruminal protein synthesis. Their method relied on a nitrogen to sulfur ratio to calculate nitrogen incorporation (protein synthesis) by ruminal microorganisms. They found that the nitrogen to sulfur ratio was the same and constant in both bacteria and protozoa under different conditions. However, the rates of microbial protein synthesis obtained using the ^{35}S method were very low.

The method developed in this thesis utilized ^{33}P incorporation into microbial phospholipids as a marker to differentiate between microbial cell growth and dietary protein. The method required that a nitrogen to PL-Pi ratio be established in order to calculate the microbial protein synthesis from phosphrous incorporation into microbial phospholipids. In experiments 1, 2 and 3 the N/PL-Pi ratio was not the same in all bacteria and protozoa and the ratio varied with time after feeding and level of substrate in the in vitro incubation medium. This variation in part appears to be related to the physiological state of rumen microbial cells and the extent of carbohydrate polymer storage (Luria, 1960).

The metabolism of ^{33}P in rumen bacteria cellular fractions was studied in order to determine if the incorporation of ^{33}P into microbial phospholipids could actually be used as a marker of microbial protein synthesis. In experiment 4 the ^{33}P uptake and incorporation into IC-Pi and PL-Pi fractions of the cell were linear with time and followed the changes in cell growth. Specific activity of the IC-Pi fraction was assumed to represent the SA of the phosphrous precursor pool for phospholipid synthesis since IC-Pi rapidly equilibriates with compounds such as purine and pyrimidine nucleotide conenzymes (Bolton and Roberts, 1964; Weissbach et al., 1971). In experiment 5 uptake and incorporation of ^{33}P into the IC-Pi and PL-Pi

fractions of washed cells of ruminal bacteria was studied before and after substrate additions to an in vitro system. Uptake of ^{33}P into the IC-Pi fraction occurred before substrate addition, however ^{33}P incorporation into the PL-Pi fraction occurred only after substrate addition. The incorporation of ^{33}P into the PL-Pi fraction paralleled changes in cell growth. Mitchell and Moyle (1953) reported similar results from their studies of phosphrous metabolism in Micrococcus pyogenes.

The SA of the IC-Pi fraction was never near zero for the initial (0 time) subsample (Figure 2, Appendix 5). The method of inhibiting bacterial activity (rapid cooling to 5 C) was first thought to allow for ^{33}P uptake into the IC-Pi fraction. However in experiment 7 the bacteria and protozoa were killed with saturated HgCl and the SA of the IC-Pi fraction still was not 0 in the initial (0 time) subsample. In experiments 4, 5 and 7 the SA of the IC-Pi pool was noted to increase with time but never reached the SA of the medium-Pi. Roberts et al. (1955) and Bolton and Roberts (1964) noted that the SA of most precursor pools in yeast and bacteria reached equilibrium shortly after a radioactive isotope was added to the incubation medium. Since the IC-Pi, SA, did not equilibriate or standardize, in experiment 7 the mean SA of the IC-Pi fraction between the initial (0 time) and 60-minute subsamples were used as the SA of the phosphrous precursor

pool for microbial phospholipid synthesis. The phosphrous moiety of phospholipids has been shown to be derived from CTP (Kennedy, 1963; Lennarz, 1970). Perhaps the use of cytidine triphosphate (^{33}P) as the precursor pool in any future work might be desirable.

CONCLUSIONS

1. The crude protein (N x 6.25) content of rumen bacteria is not always equal to 60%.
2. The N/PL-Pi ratio in rumen bacteria and protozoa are not the same and the ratio changes between rations fed, incubation conditions and experiments.
3. ^{33}P phosphrous incorporation into bacterial phospholipids was parallel with cell growth.
4. The SA of the IC-Pi pool was assumed to represent the phosphrous precursor pool for microbial phospholipid synthesis. The use of IC-Pi SA as the phosphrous precursor pool for microbial phospholipid synthesis can be questioned since the SA of the IC-Pi fraction did not equilibriate with the SA of the medium phosphrous and is not easily determined.
5. The quantatative rate of rumen microbial protein synthesis can be estimated by using ^{33}P as a marker of microbial phospholipid synthesis.
6. The rate of ruminal microbial protein synthesis was estimated to be 219.3 g/day or 26.0 g/100 g organic matter digested in the rumen of a sheep.

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APPENDIXES

APPENDIX 1

Nitrogen (N) and Phospholipid-Phorphrous (PL-Pi) Content of Mixed Rumen Bacteria Incubated *In Vitro*

Fermentation Treatment	Sampling Time After Feeding	Subsample Time	N	PL-Pi	N/PL-Pi
	(hr.)	(min.)	µg/mg	µg/mg	
Substrate Added	4	0	28.6	.70	40.8
		60	26.9	.63	42.5
		120	31.1	.68	45.2
		180	32.6	.76	42.5
	24	0	20.9	.54	38.4
		60	24.5	.65	37.7
		120	21.6	.56	38.7
		180	22.9	.57	39.8
	48	0	21.3	.66	31.9
		60	25.9	.66	39.2
		120	22.1	.64	34.5
		180	23.3	.57	40.7
No Substrate Added	4	0	67.9	1.36	47.6
		60	73.7	1.38	53.3
		120	73.6	1.37	53.7
		180	71.2	1.43	49.7
	24	0	71.6	1.59	45.1
		60	67.5	1.53	44.1
		120	69.9	1.66	41.9
		180	70.3	1.59	44.3
	28	0	69.2	1.18	58.4
		60	67.3	1.12	59.8
		120	67.1	1.19	56.4
		180	66.4	1.10	60.2

APPENDIX 2

Statistical Treatment of N/PL-Pi Ratios Presented in Appendix 1

	d.f.	Mean Square	Level of Significance
Treatment	5	285.79	.01
Error	18	5.95	

Standard Error $5.95/_5 = 1.19$

Duncan's New Multiple Range Test.

Significant Level	P = 2	P = 3	P = 4	P = 5	P = 6
.05	2.97	3.12	3.21	3.27	3.32
.01	4.84	5.08	5.21	5.31	5.39

APPENDIX 3

Mean[1] Changes in Cellular Constituent Composition and Phosphrous Fractions CPM[2] of Mixed Rumen Bacteria During A 240-Minute *In Vitro* Incubation

Subsample Times	Cellular Constituents				Medium Phosphrous
	N	PL-Pi	IC-Pi	Total Cell Phosphrous	
(min)		µg/mg	µg/mg	µg/mg	µg/ml
0	31.3	.98	.96	5.84	228.3
30	31.0	.88	.90	5.96	225.1
60	31.4	.84	.86	7.93	227.1
120	33.3	.86	.82	6.00	212.9
180	31.6	.82	.68	6.67	227.7
240	32.9	.96	.84	7.74	228.8
$\bar{x}$	31.7	.89	.84	6.69	225.1
SE±	.5	.03	.04	.38	2.5

[1]Mean values from three separate *in vitro* incubations.
[2]Counts per minute.

APPENDIX 3 (Continued)

Subsample Times	Cellular Constituents			Medium Phosphrous
	PL-Pi	IC-Pi	Total Cell Phosphrous	
(min)	CPM/mg	CPM/mg	CPM/mg	CPM/ml
0	9.4	53.9	153.7	93,894
30	18.3	58.3	165.0	95,382
60	28.8	75.9	313.2	97,364
120	56.5	87.8	447.9	96,504
180	73.7	96.9	602.0	96,669
240	102.4	131.9	945.5	99,465

APPENDIX 4

Mean[1] Changes in Cellular Constituent Composition and Phosphrous Fractions CPM[2] of Mixed Rumen Bacteria During A 300-Minute *In Vitro* Incubation

Subsample Times	Cellular Constituents				Medium Phosphrous	
	PL-Pi[4]		IC-Pi[5]			
(min)	µg/mg	CPM/mg	µg/mg	CPM/mg	µg/ml	CPM/ml
0	1.18	15.6	1.07	220.4	29.8	2,660
30	2.02	29.3	1.10	387.4	29.8	2,676
60	1.74	20.8	1.08	405.1	30.5	2,648
90[3]	1.67	27.6	1.13	393.2	30.8	2,636
30	1.17	318.1	.84	788.5	25.0	2,738
60	.86	371.3	.56	1137.3	24.8	2,811
120	.75	610.2	.61	1153.8	22.5	2,373
180	.71	701.5	.58	1401.2	20.0	2,678
240	.61	753.0	.57	1678.1	19.5	2,484

[1]Mean values from two separate *in vitro* incubations.

[2]Counts per minute.

[3]Substrate added to incubation medium.

[4]PL-Pi Phospholipid-Phosphrous.

[5]IC-Pi Intracellular Phosphrous.

APPENDIX 5

Quadratic and Linear Regression Equations of the Mean Changes in Cellular Constituents Presented in Table 10

Phospholipid Phosphrous--		
90 to 240 minutes	$y = 33.18 + 7.49x + -.01x^2$	
Intracellular Phosphrous--		
0 to 90 minutes	$y = 226.38 + 6.25x + -.04x^2$	
90 to 240 minutes	$y = 483.54 + 9.87x + -.02x^2$	
Medium--		
0 to 240 minutes	$y = -.51x + 2705.67$	$R = -.43$
Dry Cell Mass--		
0 to 240 minutes	$y = 1.28x + 130.58$	$R = .97$

APPENDIX 6

Incorporation of ^{33}P into Microbial Phospholipids During an In Vitro Incubation of Rumen Contents from a Sheep Fed a Low (6.1%) or a High (15.7%) Protein Containing Ration

Incubation Time After Feeding	Subsample Time	Sheep Fed a Low Protein (6.1%) Ration			
		DM[1]	g DM in 150 g Whole Rumen Contents	Microbial Phospholipid	
(hr)	(min)	%		CPM[2]/mg sample	CPM/kg whole rumen contents
0	0	10.84	16.26	0	0
	30			1.65	178,860
	60			4.23	458,532
	120			9.85	1,067,740
	180			15.47	1,676,948
	240			18.82	2,040,088
2	0	14.73	22.10	0	0
	30			2.79	411,060
	60			7.59	1,118,826
	120			11.91	1,754,740
	180			14.61	2,152,540
	240			21.58	3,179,453
4	0	11.25	16.88	0	0
	30			1.29	145,168
	60			3.31	372,485
	120			6.50	731,467
	180			9.88	1,111,829
	240			18.32	2,061,611

[1]Dry Matter.

[2]Counts per minute.

APPENDIX 6 (Continued)

Incubation Time After Feeding	Subsample Time	Sheep Fed a High Protein (15.7%) Ration			
		DM	g DM in 150 g Whole Rumen Contents	Microbial Phospholipid	
(hr)	(min)	%		CPM/mg sample	CPM/kg whole rumen contents
0	0	12.10	18.15	0	0
	30			2.37	286,770
	60			5.72	692,120
	120			8.76	1,059,960
	180			12.55	1,518,550
	240			17.08	2,066,680
2	0	12.25	18.38	0	0
	30			3.24	397,008
	60			11.65	1,427,513
	120			13.11	1,606,412
	180			21.80	2,671,227
	240			26.25	3,216,500
4	0	11.00	16.50	0	0
	30			3.45	379,500
	60			8.84	972,400
	120			16.29	1,791,900
	180			26.88	2,956,800
	240			29.01	3,191,000

APPENDIX 7

Linear Regression Equations of the ^{33}P Incorporation into Microbial Phospholipids Presented in Figures 3 and 4 and Appendix 6

Incubation Time After Feeding (hr)		
	Low Protein Ration (6.1%)	
0	y = .9122x + (-6.4954)	R = 966
2	y = 1.2021x + (20.8669)	R = 985
4	y = .8542x + (-19.1753)	R = 974
	High Protein Ration (15.7%)	
0	y = .8063x + (10.8819)	R = 996
2	y = 1.2499x + (28.8902)	R = 971
4	y = 1.3960x + (9.9416)	R = 983

APPENDIX 8

Dry Matter Content and ^{33}P Incorporation into Bacteria, Protozoa and Whole Rumen Contents During 60-Minute Incubations of Whole Rumen Contents

Incubation Time After Feeding	Size of Subsample	Dry Matter	WRC Dry	WRC[1] PL-Pi[4]	Protozoa	Protozoa[2] PL-Pi	Bacteria[3]
(hr)	(g)	(%)	(g)	CPM/mg	(g)	CPM/mg	(Total CPM x 10^5)
0	250	11.8	29.5	5.45	5.10	10.25	1.08
—	375	11.9	44.6	4.40	7.18	5.70	1.54
2	250	17.8	44.5	5.86	5.17	6.03	2.29
	375	14.8	55.5	4.60	7.49	7.30	2.01
4	250	14.8	36.9	4.49	5.88	6.69	1.26
	375	14.2	53.3	3.40	6.84	6.64	1.36
9	375	13.3	49.7	3.10	6.63	5.10	1.21
11	250	14.2	35.5	4.70	4.76	6.78	1.34

[1]Total whole rumen contents (WRC) PL-^{33}Pi = WRC (mg) x CPM PL-^{33}Pi/mg WRC.

[2]Total Protozoa PL-^{33}Pi = Protozoa (mg) x CPM PL-^{33}Pi/mg Protozoa.

[3]Total Bacteria PL-^{33}Pi = a-b.

[4]PL-Pi Phospholipid Phosphorous.

APPENDIX 9

Intracellular Phosphrous ^{33}P Uptake, Nitrogen, N/PL-Pi Ratio of Bacteria and Protozoa During 60-Minute Incubations of Whole Rumen Contents

Incubation Time After Feeding	Bacteria IC-Pi[1] SA[2] Subsample 0 (min)	Subsample 60 (min)	0 + 60 / 2	Protozoa IC-Pi SA Subsample 0 (min)	Subsample 60 (min)	0 + 60 / 2	Nitrogen Bacteria	Nitrogen Protozoa	N/PL-Pi Bacteria	N/PL-Pi Protozoa
(hr)							µg/mg			
0	117.3	115.7	136.5	16.4	20.4	18.4	65.2	85.2	64.4	34.5
	71.1	155.6	113.4	22.2	54.1	38.1	82.3	74.8	41.0	34.3
2	154.1	244.7	199.4	19.9	25.6	22.7	59.5	63.3	66.8	33.3
	153.5	236.5	195.0	23.5	30.2	26.9	74.0	65.0	56.9	47.2
4	78.5	154.4	116.5	14.3	26.0	20.2	64.8	61.1	34.9	36.6
	154.8	201.3	178.0	15.7	23.8	19.8	77.5	67.2	52.6	35.6
9	166.8	242.1	204.5	22.2	45.1	33.7	87.7	79.7	61.1	35.1
11	52.5	223.6	138.0	19.1	36.9	28.0	63.1	72.3	38.6	31.4

[1]IC-Pi, Intracellular Phosphrous.

[2]SA, Specific Activity.

APPENDIX 10

Contents of PL-Pi,[1] IC-Pi[2] and CPM[3] of ^{33}P for These Fractions Obtained from Bacteria and Protozoa During 60-Minute Incubations of Whole Rumen Contents

Incubation Time After Feeding	Bacteria						Protozoa						
	PL-Pi			IC-Pi			PL-Pi			IC-Pi			
	0 (min)	60 (min)	0 (min)	60 (min)	0 (min)	60 (min)	0 (min)	60 (min)	60 (min)	0 (min)	60 (min)	0 (min)	60 (min)
(hr)	µg/mg		µg/mg		CPM/mg		µg/mg		CPM/mg	µg/mg		CPM/mg	
0	.93	1.97	.32	.13	36.9	41.1	2.38	2.56	5.10	1.51	1.50	24.5	30.3
	1.69	2.52	.36	.26	25.3	41.4	2.27	2.13	7.18	.81	.45	17.9	24.4
2	.84	.95	.25	.11	37.5	25.4	1.94	1.87	5.17	.78	.78	15.0	20.0
	1.30	1.31	.25	.12	39.0	27.2	1.42	1.34	7.49	.73	.80	18.7	24.0
4	1.80	1.92	.32	.24	24.9	36.9	1.67	1.66	5.88	.73	.71	10.4	18.3
	1.29	1.66	.41	.24	63.1	48.4	1.92	1.85	6.84	.87	.72	13.8	17.4
9	1.39	1.47	.57	.45	96.0	109.1	2.17	2.35	6.63	.91	.65	20.4	29.0
11	1.48	1.77	.29	.30	15.1	66.2	2.31	2.27		.86	.64	16.2	24.4

[1]PL-Pi Phospholipid Phorphrous.

[2]IC-Pi Intracellular Phosphrous.

[3]CPM, Counts Per Minute.

APPENDIX 11

^{33}Phosphrous Incorporation into Phospholipids, Total Phosphrous and Nitrogen Content of Bacteria and Protozoa at 60 Minutes after a 60-Minute Incubation of Whole Rumen Contents

Incubation Time After Feeding	Total ^{33}P in Phospholipids		Total Phosphrous Incorporation		Nitrogen Synthesized	
	Bacteria	Protozoa	Bacteria	Protozoa	Bacteria	Protozoa
(hr)	CPM[1]		µg	µg	mg	
0	108,340	52,000	995.4	1373.9	40.8	47.1
	155,444	40,928	1139.0	2224.0	73.2	67.7
2	229,814	31,155	1178.5	1158.2	66.9	54.7
	200,555	54,740	1005.3	2407.2	67.2	80.0
4	126,476	39,379	1085.6	1949.5	37.5	71.5
	135,607	45,442	761.8	2296.3	40.1	81.7
9	120,656	33,382	591.5	990.6	36.1	34.7
11	134,548	32,302	975.0	1153.6	37.6	36.2

[1]CPM, Counts Per Minute.