Microbial Phospholipid Synthesis as a Marker for Microbial Protein Synthesis in the Rumen[1]

H. F. BUCHOLTZ[2] AND W. G. BERGEN

Ruminant Nutrition Laboratory, Department of Animal Husbandry, Michigan State University, East Lansing, Michigan 48823

Phosphate uptake into intracellular inorganic phosphorus and cellular phospholipids and the relationship between cell growth and phospholipid synthesis were studied with suspensions of washed ruminal bacteria in vitro with ^{33}P-phosphorus. It was shown that ruminal bacteria accumulated inorganic phosphate at a low rate when incubated without substrate. Upon the addition of substrate, the rate of inorganic phosphorus uptake into the cells increased markedly, and phospholipid synthesis and cell growth commenced. There was a highly significant relationship (r = 0.98; $P < 0.01$) between phospholipid synthesis and cell growth. The specific activity of the intracellular inorganic phosphorus did not equilibrate with phosphorus medium. When ruminal contents from sheep fed a high or low protein diet were incubated in vitro, the rate of ^{33}P incorporation into microbial phospholipids was higher for the high protein diet. Since there was a high relationship between phospholipid synthesis and growth, rumen contents were collected before and various times after feeding and incubated with ^{33}P-phosphorus in vitro. The short-term, zero time approach was used to measure the rate of microbial phospholipid synthesis in whole rumen contents. In these studies the average specific activity of the intracellular inorganic phosphorus was used to represent the precursor pool specific activity. Microbial phospholipid synthesis was then related to protein (N × 6.25) synthesis with appropriate nitrogen-to-phospholipid phosphorus ratios. Daily true protein synthesis in a 4-liter rumen was 185 g. This represents a rate of 22 g of protein synthesized per 100 g of organic matter digested. These data were also corrected for ruminal turnover. On this basis the rate of true protein synthesis in a 4-liter rumen was 16.1 g of protein per 100 g of organic matter digested. This value represents a 30-g digestible protein-to-Mcal digestible energy ratio which is adequate for growing calves and lambs.

Hungate (19) calculated adenosine triphosphate (ATP) yields from known pathways of ruminal fermentation of carbohydrates. By using a Y ATP = 10 (3, 34), he concluded that about 10 g of microbial protein (about 15 g of total cell mass) can be synthesized for each 100 g of carbohydrate fermented. This value was thought to represent an upper limit of the synthetic capacity of anaerobic ruminal fermentation (19). This level of protein synthesis in the rumen is equivalent to 18.3 g of digestible protein per Mcal of digestible energy (36) and is clearly below the requirement of growing ruminants (36). From a husbandry viewpoint,

this result implied that, for optimal growth or production of ruminants, procedures to allow dietary protein to escape ruminal degradation must be developed.

Extensive direct experimental work on quantitative aspects of ruminal microbial protein synthesis indicates, however, that the limits set by Hungate (19) were too low. Thus, growth yield studies with strains of rumen bacteria (11, 12, 18) and ingesta passage studies with sheep by using purified diets or microbial cell markers (14, 15, 17, 27) indicated that ruminal microbial protein synthesis ranged from 15 to 22 g of microbial protein per 100 g of organic matter fermented. Other recent reports (13, 21) have also suggested that the ATP yield per mole of hexose fermented in the rumen is higher than previously anticipated.

504

The experiments described below were initiated to measure absolute bacterial and protozoal protein synthesis rates in whole rumen contents at various times after feeding with short-term, zero time in vitro (6) incubations. Since membrane (phospholipids) synthesis and growth are directly related (31, 33) and since growth and protein synthesis are directly related (28) in microorganisms, protein synthesis rates of ruminal microorganisms were assessed by measuring the rate of microbial phospholipid synthesis.

MATERIALS AND METHODS

Radioactive phosphorus-labeling pattern of cellular phosphorus containing constituents during in vitro incubations of mixed ruminal bacteria: experiment 1. The labeling patterns of cellular phosphorus by ^{32}P-inorganic phosphorus were studied in suspensions of mixed ruminal bacteria in a high and low phosphorus medium in vitro. Rumen contents were collected as outlined by Purser and Moir (37) from sheep fitted with Jarret cannulae (fed ration 1, Table 1) before the morning feeding, placed in a vacuum bottle warmed to 39 C, gassed with oxygen-free CO_2, and transported quickly to the laboratory. Rumen contents from sheep are quite homogenous, and samples of 100 g or more are representative of whole rumen contents (37). The rumen contents were subsequently squeezed through two layers of cheesecloth. Rumen liquor (500 ml for high PO_4 and 800 ml for low PO_4 media) was then centrifuged at 500 × g for 15 min at 2 C. The pellet was discarded, and the supernatant was centrifuged at 10,000 × g for 15 min at 2 C. The resulting pellet (bacterial fractions; 300 to 400 mg dry weight for the high PO_4 and 150 to 200 mg dry weight for the low PO_4 media) was resuspended to the original rumen liquor volume in reduced anaerobic buffer (AB; Table 2) containing either 180 or 35 μg of phosphorus, pH 6.7, per ml. During the centrifugation steps, all tubes and beakers were gassed with O_2-free CO_2 to insure anaerobiosis. The ruminal bacteria fractions were then transferred into prewarmed fermentation flasks (1 liter) and incubated at 39 C under O_2-free CO_2. Radioactive phosphorus (^{32}P-H_3PO_4; ca. 60 × 10^6 DPM) dissolved in AB was then added to the fermentation flask, and initial subsamples (80 ml) were secured. For the experiments with the high PO_4 media, substrate (0.66% glucose, 0.5% soluble starch, and 0.15% urea; expressed as percent weight of the final volume of the fermentation system) was added and further subsamples were obtained at 30, 60, 120, 180, and 240 min. For the experiments with the low PO_4 media, inorganic phosphorus uptake by resting bacteria and actively growing bacteria was studied in the manner outlined by Mitchell and Moyle (31). Thus, substrate (as above) was added after 90 min of incubation. Subsamples (as above) were obtained at 30, 60, 90, 120, 150, 210, 270, and 330 min for the low PO_4 media fermentations. To stop bacterial activity, subsamples were placed in prechilled beakers which were then placed in a solution of solid CO_2 in ethanol.

TABLE 1. *Experimental rations fed to sheep*[a]

Ingredients	Ration (%)		
	1	2	3
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
Corn starch		14.0	17.5
Urea (feed grade)	0.5	3.5	
Molasses	9.0	10.0	10.0
Mineral-vitamin mix[b] ..	2.5	2.5	2.5
Crude protein (N × 6.25)[c]	14.5	15.9	6.1
Estimated digestible energy (kcal/kg)[c]	3,162	3,580	3,727

[a] Sheep were fed at the rate of 158 kcal of digestible energy per bodyweight 0.75 kg per day (27) with equal portions offered at 8 a.m. and 5 p.m.

[b] Contents (in percent): 42.29 dicalcium phosphate; 42.29 high Zn trace mineral salt; 15.0 Na_2SO_4; 0.32 vitamin A premix [10,000 IU/g], and 0.10 vitamin D premix [9,000 IU/g]).

[c] Based on crude protein and digestible energy values from NRC publication 1684, U.S.-Canadian Tables of Feed Composition, 1969.

TABLE 2. *Anaerobic buffer*[a]

Ingredients	Milliliters per 100 ml	
	High phosphorus[b] media	Low phosphorus[c] media
Mineral solution I[d]	7.5	
Mineral solution II[e]	7.5	
Mineral solution III[f]		7.5
Water	82.34	90.84
12% Na_2CO_3	1.11	1.11
3% Cysteine-hydrochloride (wt/vol)	0.56	0.56

[a] The mineral-water solution was heated to 95 C and gassed with O_2-free CO_2, followed by the addition of Na_2CO_3 and cysteine; equilibrated under CO_2 at 39 C, and then finally adjusted to pH 6.7 with 1 N NaOH or 1 N HCl.

[b] Phosphorus, 180 μg/ml.

[c] Phosphorus, 35 μg/ml.

[d] K_2HPO_4, 0.3% (wt/vol).

[e] Contents: 0.3% KH_2PO_4; 1.2% Na_2SO_4; 0.6% NaCl; 0.6% $MgSO_4 \cdot 7H_2O$; 0.06% $CaCl_2 \cdot 2H_2O$ (wt/vol).

[f] Contents: 0.58% tris(hydroxymethyl)aminomethane (mol wt 121.0); 1.28% Na_2SO_4; 1.2% NaCl, 0.12% $MgSO_4 \cdot 7H_2O$; 0.12% $CaCl_2 \cdot 2H_2O$; 7.47% K_2HPO_4 (wt/vol).

When the temperature of the subsample reached 2 to 5 C, the subsamples were centrifuged at 18,000 × g for 15 min at 3 C. The pellet was then resuspended and washed with a small quantity of deionized water and recovered by centrifugation. The pellets were then frozen, lyophilized, and stored for phospholipid, intracellular and total phosphorus, and ^{33}P activity analysis. Increases in cellular dry matter and protein (N × 6.25) content were determined on separate subsamples. A sample of cell-free media was secured and frozen for inorganic phosphorus and ^{33}P analysis.

Radioactive phosphorus incorporation into microbial phospholipids of rumen contents in vitro from sheep: experiment 2. Sheep (fitted with Jarret cannulae) were placed on ration 2 (high protein) or 3 (low protein) (Table 1, one sheep per ration) for a 3-week period. Rumen contents were collected, as described above, from the sheep before (0 h) and 2 and 4 h after the morning feeding. Upon arrival at the laboratory, 150 g of rumen contents was weighed into a wide-mouth fermentation flask (800 ml) (under an O_2-free CO_2 atmosphere), and 6 ml of a solution containing ^{33}P-H_3PO_4 (13.2 × 10^7 DPM) was added. The flask was sealed and then shaken for 30 s, and an initial subsample was removed. Preliminary work showed that 6 ml of 1% crystal violet could be well mixed with 150 g of rumen contents within 30 s under these conditions. Further subsamples were obtained at 30, 60, 120, 180, and 240 min of the in vitro incubation. After each sampling the flask was flushed with O_2-free CO_2 and resealed. The microorganisms were killed by the addition of 0.05 volume of saturated $HgCl_2$. After washing to remove unincorporated ^{33}P, the samples were frozen and lyophilized.

Microbial phospholipid synthesis and microbial protein synthesis in whole rumen contents of sheep: experiment 3. Rumen contents were collected from sheep fed ration 1 (Table 1) before (0 h) and 2, 4, and 9 h after the morning feeding. One sheep served as the donor of rumen contents for two separate experiments. Short-term, zero time method (6, 20) in vitro incubations were conducted with rumen contents as described above, except 750 g of rumen contents was placed in the fermentation flasks. After the additions of ^{33}P-H_3PO_4 (about 44 × 10^7 DPM), an initial subsample (350 g) was obtained. The final sample was obtained after 60 min. The microorganisms were killed with saturated $HgCl_2$. Each subsample was divided in the following manner. From the well-mixed subsample, a 20-g sample was used to determine ^{33}P incorporation into total microbial phospholipids. The particulate matter was spun down and then resuspended and washed in saline and centrifuged at 18,000 × g three times to remove unincorporated, free, and nonspecifically bound ^{33}P. The residue was then frozen and lyophilized. The remaining rumen contents from the subsamples (305 g) were squeezed through two layers of cheesecloth. The particulate matter was then resuspended in 0.85% NaCl, stirred, and squeezed again through two layers of cheesecloth. Microscopy examination revealed that this procedure removed all except a few small protozoa from the residual plant

matter and rumen contents. It was therefore assumed in subsequent calculations that total protozoal protoplasmic mass had been recovered quantitatively from rumen contents by this procedure. A small sample was removed from the rumen liquor for phosphorus and volatile fatty acid (VFA) analysis. The rumen liquor and extract from the second squeeze were then combined. The protozoa and bacteria were separated from the rumen fluid by differential centrifugation as described by Bergen et al. (4). Microscopy examination of the high-speed (bacterial) and 150 × g (protozoal) fractions were done in a separate (i.e., no isotope) study. It was found that the 150 × g (protozoal) and the high-speed (bacterial) fractions were devoid of gross plant material contamination. Previous work had shown that this differential centrifugation system (4) resulted in 150 × g (protozoal) and high-speed (bacterial) fractions which contained only 1.5 and 0.7% (based on dry matter) crude fiber (W. G. Bergen, unpublished data). The 150 × g (protozoal) and high-speed (bacterial) fractions were then frozen and lyophilized.

Analytical procedures. Preliminary experiments were conducted to assess the efficacy of various extraction procedures for phospholipids from ruminal microorganisms and the salt wash procedure of Folch et al. (10). During these studies, microbial preparations were extracted and reextracted in organic solvents, and the extracts were then monitored for phospholipids with thin-layer chromatography. Similarly, the salt wash procedure was checked to assure that only inorganic, but not phospholipid, phosphorus was lost during the wash. In a number of trials, upon addition of ^{33}P-H_3PO_4 to chloroform-methanol (2:1), after the salt wash only 0.3% of the initial radioactivity remained in the solvent. For the experiments described above, microbial phospholipids were extracted from lyophilized rumen contents, high-speed (bacterial), or 150 × g (protozoal) preparations by a modification of the method described by Katz and Keeney (24). Approximately 30 mg of sample was extracted with 5 ml of chloroform-methanol (2:1; vol/vol) in 15 ml of Teflon-lined screw-capped culture tubes. The tubes were rotated for 16 to 20 h at room temperature. The extracts were filtered through a fritted glass Buchner funnel and the nonlipid impurities were removed by the salt wash procedure (10). Sonic treatment of microbial preparations did not improve the yield of total lipid extraction. Phospholipid phosphorus was determined in the washed extract by the ascorbic acid procedure of Chen et al. (7) as modified by Rhee and Dugan (39). Total cellular phosphorus and phosphorus concentration in incubation media were determined by the procedure of Chen et al. (7). Intracellular inorganic phosphorus (IC-P_i) was extracted from 30 to 100 mg of lyophilized microbial preparation with 4.0 ml of 5% perchloric acid (PCA) for 30 min at 5 C in an all-glass Potter-Elvehjem homogenizer (25, 41). The homogenates were centrifuged at 18,000 × g for 15 min. Inorganic phosphorus in the PCA extract was then determined by the isobutanol-benzene-ammonium molybdate procedure of Lindberg and Ernster

(26). Preliminary studies showed that this procedure recovered between 97 and 98% of the phosphorus from standard phosphorus solutions in the presence of 5% PCA.

Nitrogen was determined with a micro-Kjeldahl procedure. VFA were determined from media treated with metaphosphoric acid (9) on a Teflon column (198 by 0.05 cm) packed with Chromosorb 101 (60–80 mesh) at 188 C with nitrogen as carrier gas and a hydrogen flame detector. Dry matter of subsamples was determined by drying at 110 C to a constant weight (this took 20–24 h). Suitable samples of all phosphorus-containing extracts (i.e., phospholipids and intracellular phosphorus) were placed in vials containing 10 ml of scintillation fluid (5 g of 2,5-diphenyloxazole; 0.05 g 1,4 bis-[2-(4 methyl-5-phenyloxazole)]-benzene, 500 ml of toluene, and 500 ml of Triton X-100) and counted in a Nuclear Chicago 6848 liquid scintillation spectrometer. ^{33}P-phosphorus activity (T$^{1/2}$ = 25.3 days) in the counted samples was corrected for counting efficiency and decay, with decay factors derived by Robinson (40). The ^{33}P-phosphorus was obtained from New England Nuclear Corp., Boston, Mass., as H$_3$-^{33}PO$_4$ in 0.02 N HCl.

All glassware used in the experiments was washed with detergent, rinsed in deionized water-12 N HCl (2:1), followed by a final rinse in deionized water.

Calculations. To calculate the rate of microbial phospholipid phosphorus synthesis in whole rumen contents (experiment 3), the IC-P$_i$ specific activity (SA) was used as an indicator of precursor pool SA. Since the SA of IC-P$_i$ may differ between protozoa and bacteria, these were assessed separately. In this incubation system (experiment 3), the SA of the IC-P$_i$ was measured at the start (SA $\cong$ 0) and the end of the incubation time. The rate of ^{33}P uptake into cellular phospholipids is dependent on the rate of phospholipid synthesis as well as the rate of change of the SA of the precursor pool (IC-P$_i$) during the incubation. To properly describe the SA of the IC-P$_i$, more frequent sampling would have been necessary. Thus, an approximation was made to determine the effective SA of the IC-P$_i$ pool during the incubation period. The following formulae describe the calculations:

(i) Intracellular phosphorus SA = (final IC-P$_i$-SA + initial IC-P$_i$-SA)/2.

(ii) Total ^{33}P uptake into high-speed pellet (bacterial) phospholipids = total ^{33}P uptake into rumen contents phospholipids – total ^{33}P uptake into 150 × g pellet (protozoal) phospholipids.

(iii) Total phosphorus (micrograms) incorporation into microbial phospholipids = phosphorus incorporation into high-speed pellet (bacterial) phospholipids (net counts/min of ^{33}P in the PL-P$_i$/SA of the IC-P$_i$) + phosphorus incorporation into 150 × g pellet (protozoal) phospholipids (net counts/min of ^{33}P in the PL-P$_i$/SA of the IC-P$_i$).

(iv) Total microbial protein (N × 6.25) synthesis in the system = [μg of P$_i$ incorporation into high-speed pellet (bacterial) PL-P$_i$ × high-speed pellet (bacterial) N/PL-P$_i$] + [μg of P$_i$ incorporation into

150 × g pellet (protozoal) PL-P$_i$ × 150 × g pellet (protozoal) N/PL-P$_i$].

RESULTS

Physical separation of microbial fractions. Sampling of rumen contents and the subsequent fractionation of the rumen contents into microbial fractions is one of the most difficult aspects of microbial investigations in the rumen. Throughout this work rumen samples were withdrawn from the ventral and dorsal sac of the rumen. More importantly, at least 1 liter (from a 4-liter rumen volume of sheep) of contents was removed to insure that a representative sample had been secured. For experiment 1, mixed ruminal bacteria were prepared similar to the procedure outlined by Baldwin and Palmquist (2). It must be recognized that many large bacteria and clumps of bacteria may be lost during the initial low-speed centrifugation step. As Hungate (19) pointed out, suspensions of washed mixed ruminal bacteria contain all kinds of rumen bacteria, but they are not necessarily in the same proportions as found in the total contents. In experiment 3, the 150 × g pellet was designated the protozoal fraction. Although, the protozoa could be nearly quantitatively removed from the rumen fluid, this fraction may also contain sizeable amounts of bacteria (49). These bacteria are large bacteria as well as those harbored within the protozoa. The metabolic activity of these organisms will contribute to the total activity of the 150 × g (protozoal) fraction. In the remainder of the paper, the 150 × g pellet will be called the protozoal fraction, whereas the high-speed pellet will be called a bacterial fraction.

Experiment 1. The metabolism of ^{33}P-H$_3$PO$_4$ in mixed ruminal bacteria incubated in high or low phosphate medium was studied. Figure 1 depicts the results for the high phosphate medium studies. The SA of inorganic phosphorus medium stayed constant throughout the incubation period. In the presence of substrate there was a linear rise in ^{33}P incorporation into phospholipids and in ^{33}P uptake into IC-P$_i$ and total cellular phosphorus. The increase in dry cell mass and cellular protein (N × 6.25) were significantly correlated (r = 0.99; P < 0.01), and the rate of ^{33}P incorporation into cellular phospholipids and the increase in dry cell mass or cellular protein were significantly correlated (r = 0.91, P < 0.01; r = 0.94, P < 0.01). This high correlation indicates that cellular phospholipid synthesis can be
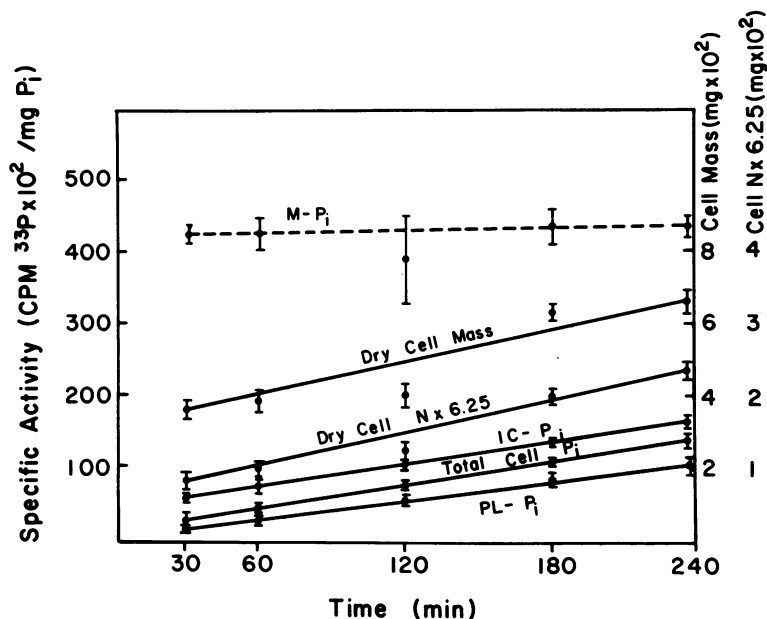
FIG. 1. *Uptake of ³³P-phosphorus into cellular phosphorus constituents and changes in cell mass of mixed ruminal bacteria during an in vitro incubation in high phosphorus buffer. (Mean of three separate experiments; bars represent ± standard error.)*

used as a marker of total cellular growth. During the 240-min incubation period, the SA of the IC-P_i did not equilibrate with the SA of the inorganic phosphorus medium, indicating that, if phospholipid synthesis is to be measured, the SA of the inorganic phosphorus medium will overestimate the SA of the phosphorus precursor pool.

Incubation of mixed ruminal bacteria in the low phosphate medium (Fig. 2) in the absence of substrate (0–90 min) resulted in some uptake of ³³P into the IC-P_i pool. ³³P-phosphorus was not incorporated into the phospholipids during this period.

The addition of substrate caused a more rapid uptake of ³³P into the IC-P_i pool, the incorporation of ³³P into PL-P_i, and an increase (growth) of the bacterial cell mass. The SA of inorganic phosphorus medium showed little change. Again, the SA of the IC-P_i pool did not reach the SA of the phosphorus medium after 240 min of incubation after substrate addition. The rate of ³³P incorporation into cellular phospholipids and the increase in cell mass were significantly correlated (r = 0.98; $P < 0.01$), indicating that cellular phospholipid synthesis can be used as a marker of total cellular growth. Mitchell and Moyle (31) showed similar patterns of ³²P uptake into phosphorus containing constituents of *S. aureus*. In this organism, some ³²P uptake
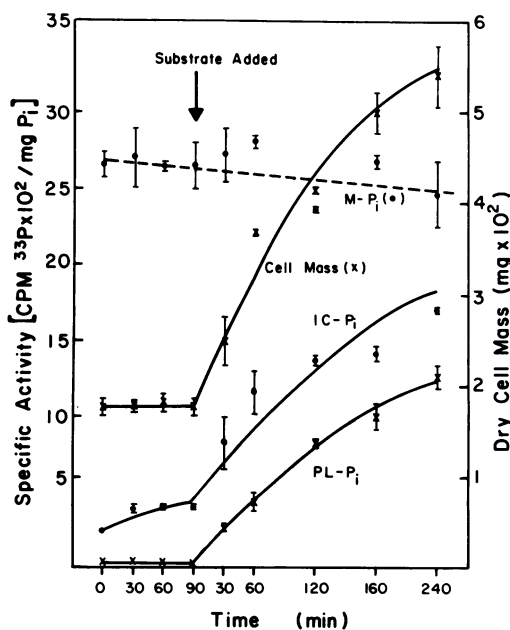

FIG. 2. *Uptake of ³³P-phosphorus into cellular phosphorus constituents and changes in cell mass of mixed ruminal bacteria during an in vitro incubation in low phosphorus buffer. (Mean of three separate experiments; bars represent ± standard error.)*

occurred into the IC-P_i pool, but ³²P incorporation into phospholipid did not occur in the

absence of a carbon source. After the addition of substrate there was ^{32}P uptake into both PL-P$_i$ and IC-P$_i$. As was the case for mixed ruminal bacteria, in *S. aureus* the SA of IC-P$_i$ did not equilibrate with the SA of the inorganic phosphorus in the incubation medium (31).

Experiment 2. Since experiment 1 showed that phospholipid synthesis was highly correlated with increases in cellular mass or protein content in mixed ruminal bacteria, experiment 2 was conducted to investigate whether rates of phospholipid synthesis in whole rumen contents (determined in vitro) could be related to dietary protein intakes of sheep. Under these experimental circumstances a significant amount of ^{33}P incorporation into protozoal as well as bacterial phospholipid would be expected. Although protozoal phosphorus metabolism was not studied in detail as for mixed ruminal bacteria, it was assumed that for these organisms phospholipid synthesis would also reflect cellular growth. When a diet containing low levels of protein (N-limiting) is fed to sheep, an increase and then a decline in microbial growth (activity in the rumen) would be expected, whereas for a diet with adequate (or excess) protein a more sustained rate of microbial growth would be expected. Previous work had shown that, when urea is added to a low protein ration, microbial protein synthesis (16) and animal performance (23) are increased. As the results show (Fig. 3), before feeding and at 2 h after feeding the rate of ^{33}P incorporation into microbial phospholipids (determined in vitro) did not differ between the two rations; however, for the high protein ration at 4 h after feeding the rate of ^{33}P incorporation into phospholipids was still high, whereas for the low protein diet the rate of ^{33}P incorporation into phospholipids declined to the prefeeding level.

Experiment 3. Experiment 1 showed that phospholipid synthesis and increases in cellular protein were highly correlated and experiment 2 showed that, as expected, the addition of a NH$_3$ source to a low protein diet resulted in more sustained (increased) microbial activity (growth). Thus, these studies indicated that phospholipid synthesis can be used as a marker of microbial growth in whole rumen contents. In this experiment microbial protein synthesis, at various times after feeding, in the rumen of a sheep fed a standard ration was studied. The results were expressed as grams of protein (N × 6.25) synthesized per hour per hypothetical 4-liter rumen. Sheep used in this work showed a rumen volume of 3.5 to 4.0 liters from polyethylene glycol disappearance curves (21).
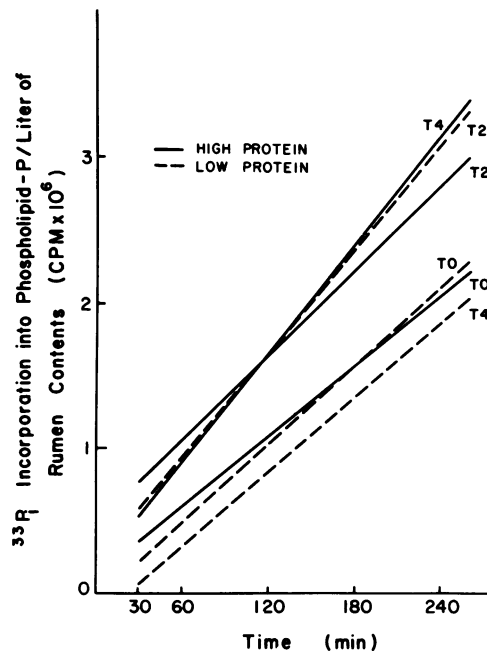

FIG. 3. *Phosphorus ^{33}P incorporation into total rumen microbial phospholipids during a 240-min in vitro incubation of rumen contents from sheep fed a high or low protein ration. (Mean of two separate experiments for each rumen sampling time. Results presented from the following regression equations: low protein T_0: Y = 0.91 × −6.50, r = 0.97; low protein T_2: Y = 1.21 × +20.87, r = 0.99; low protein T_4: Y = 0.85 × −19.18, r = 0.97; high protein T_0: Y = 0.81 × +28.89, r = 0.99; high protein T_2: Y = 1.25 × +28.89, r = 0.97; high protein T_4: Y = 1.40 × +9.94, r = 0.98.)*

To calculate microbial protein synthesis from phospholipid synthesis, the two parameters had to be related. Walker and Nader (45) used a nitrogen-sulfur ratio to relate ^{35}S incorporation to protein synthesis. Similarly, a nitrogen-to-phospholipid phosphorus ratio (N/PL-P$_i$) was used to relate inorganic phosphorus incorporation into phospholipids to protein synthesis. Extensive preliminary studies showed that the N/PL-P$_i$ was not constant between various strains of pure culture rumen bacteria or microbial preparations isolated from sheep (Table 3). Thus, the N/PL-P$_i$ ratio had to be determined separately for each in vitro incubation to insure meaningful calculations. ^{33}P-phosphorus and total phosphorus incorporation rates into microbial phospholipids and protein (N × 6.25) synthesis by whole rumen contents during 60-min in vitro incubation periods are given in Table 3. The apparent protein synthesis rate of the 150 × *g* (protozoal) fraction was equal to the rate noted for bacteria. Although

TABLE 3. *Radiophosphorus (^{32}P) incorporation and phosphorus incorporation into microbial phospholipids and microbial protein synthesis during a 60-min in vitro incubation of whole rumen contents*[a]

Time after feeding (h)	^{32}P incorporation into phospholipids[b] (counts/min)[c]		Phosphorus incorporation (mg)		Protein (N × 6.25) synthesis (mg × 10^2)		N/PL-P$_1$[d]	
	Bacteria	Protozoa	Bacteria	Protozoa	Bacteria	Protozoa	Bacteria	Protozoa
0[e]	13.2 ± 2.3	4.6 ± .6	1.07 ± .07	1.80 ± .43	3.50 ± .01	3.58 ± .64	52.5	34.4
2[e]	21.5 ± 1.5	4.3 ± 1.2	1.09 ± .09	1.80 ± .62	4.19 ± .01	4.21 ± .79	61.5	40.3
4[e]	13.1 ± 4.6	4.2 ± .3	0.92 ± .16	2.12 ± .17	2.43 ± .08	4.74 ± .27	43.8	36.1
9[f]	12.1	3.3	0.59	0.99	2.26	2.17	61.1	35.1

[a] On the basis of 750 g of rumen contents.

[b] Bacterial ^{32}P-phospholipid equals whole rumen contents ^{32}P-phospholipids minus protozoal ^{32}P-phospholipid.

[c] Counts per minute (10^4).

[d] Nitrogen to phospholipid-phosphorus (N/PL-P$_1$) for 12 pure culture strains of ruminal bacteria was 63.2 ± 10.9.

[e] Mean ± standard error from two separate experiments.

[f] Single experiment.

protozoa per se were isolated by the physical separation methods used in this study, the extent of bacterial contamination of the 150 × g fraction was not ascertained. However, it is plausible that one-fifth to one-fourth or more of the cellular mass in the 150 × g fraction was of bacterial origin. Thus, it can be concluded that bacteria contribute 60% or more to overall protein synthesis in the rumen. Recent results by Hungate et al. (21) suggested that the rate of protozoal protein synthesis was nearly equal to bacterial protein synthesis. Overall, the highest rate of protein synthesis was observed at 2 h after feeding, whereas at 9 h after feeding the rate was substantially lower than the rate noted before feeding. There are no apparent reasons for a higher microbial protein synthesis rate at 15 h (0 h in Table 3, Fig. 4) than at 9 h after feeding. Since the 9-h rate was from a single experiment, the rate of protein synthesis may have been underestimated. These results expressed in terms of a 4-liter rumen are depicted in Fig. 4. The relative VFA production rate is plotted on the same graph and is parallel to the changes found for microbial protein synthesis. It is evident that immediately after feeding there was an increase in microbial activity (VFA production) and cellular growth (phospholipid and protein synthesis). Despite differences in rations, these results are in disagreement with the contention of Walker and Nader (46) that microbial protein synthesis in the rumen declines initially after feeding and then increases again to prefeeding rates.

DISCUSSION

Rumen microbial protein represents a major source of amino acids to the ruminant animal.

Quantitative aspects of ruminal microbial protein synthesis have thus been the subject of extensive research efforts. In the past, procedures based on fermentation balances (19), ingesta passage studies with or without microbial cell markers (14), zero time in vitro incubations of rumen contents with ^{35}S or ^{15}N (1, 45), single dose or continuous infusion of ^{15}N-urea into the rumen (32, 35), and rumen fermentation-turnover models with C, N, and H balance studies (21) have been used to assess microbial protein synthesis.

Hungate (19), from known pathways of ruminal VFA production and supposed ATP yields and using Y ATP = 10 (3), calculated that about 10 g of microbial protein can be synthesized for each 100 g of carbohydrate fermented. This value represented an upper limit of the synthetic capacity in the anaerobic ruminal fermentation. This level of protein synthesis in the rumen is equivalent to 18.3 g of digestible protein per Mcal of digestible energy (36). As Purser (36) pointed out, this protein-calorie ratio is below the required protein-calorie ratio to meet the protein requirements of growing ruminants and less than such ratios established from empirical balance studies with growing ruminants.

Growth yield studies with pure cultures of rumen bacteria (11, 12, 18) and ingesta passage studies with sheep, by using nonprotein nitrogen diets or markers for microbial cells (15, 17, 27), indicated that the microbial protein yield in the rumen was higher than predicted by Hungate (19). Overall, the above studies indicate that 15 to 22 g of microbial protein is formed per 100 g of organic matter fermented. There are a number of alternatives to explain these results. First, the Y ATP value may not

be universal among bacteria (however, this has been rejected by Payne [40]), or substrate phosphorylation (43) may result in an extra energy yield to the microorganisms. Although the latter process has been implicated in ruminal organisms (44), a more tenable alternative appears to be the recent suggestion that the Y ATP value of 10 is universal but that previously assumed ATP yields per mole of VFA are too low (13, 21).

To further evaluate the potential of microbial protein synthesis in the rumen, we decided to use the short-term, zero time method. Walker and Nader (45) had used this approach with ^{35}S, but their actual results on protein synthesis rates were quite low. They were able to correct their data by determining a VFA-to-protein synthesis ratio from the in vitro incubation and then multiplying this ratio by an assumed VFA yield. The major problem in the approach of the above authors is that they assumed that the SA of the intracellular precursor pool (in this case ^{35}S-cysteine or ^{35}S-methionine) for protein synthesis equalled the SA of medium ^{35}S-sulfide. Al-Rabbat et al. (1) made similar assumptions in their ^{15}N incorporation studies with ruminal microorganisms. In the present work, it was decided to use phospholipid synthesis as a marker for cellular growth since membrane synthesis is closely related to total cell growth (33, 38, 50), and since IC-P$_i$ equilibrates readily with phospholipid precursors (5, 48) and can be used to determine the SA of the intracellular precursor pool for phospholipid synthesis.

The results of experiment 1 showed clearly that, with ruminal bacteria, the SA of inorganic phosphorus medium did not equilibrate with the IC-P$_i$ pool. A very high correlation between cell growth and phospholipid synthesis was noted for mixed ruminal bacteria similar to the observations of Ohki (33) for $E.$ $coli.$

Preliminary work had shown that the N/PL-P$_i$ ratios of the ruminal 150 × g fractions were not similar to the N/PL-P$_i$ of ruminal bacteria. Since the 150 × g fraction is largely composed of protozoa, it was decided that the SA of the IC-P$_i$ must be established for the 150 × g fraction and bacterial fraction separately. For all the calculations below, the rate of protein synthesis by both fractions have been combined to negate the implication (Table 3) that (apparent) protozoal protein synthesis was equal in magnitude to the bacterial protein synthesis.

The results from Table 3 and Fig. 4 were recalculated to estimate daily protein synthesis. The data (Table 4) are expressed as grams of protein produced in a 4-liter rumen per day.

To estimate daily protein synthesis, the various measured rates were arbitrarily designated to represent an average rate of various time intervals (summation interval). The estimated daily rate of microbial crude protein (N × 6.25) and true protein (N × 6.25 × 0.85) (42) synthesis in the rumen of a sheep with a 4-liter rumen consuming 158 kcal of digestible energy per 0.75 kg body weight per day was 218 and 185 g, respectively. These values represent a rate of microbial crude and true protein synthesis of 26 and 22 g per 100 g of organic matter digested (fermented; DOM) in the rumen, respectively. These values are higher than the theoretical upper limit proposed by Hungate (19) but similar to the upper values of ruminal microbial protein synthesis per 100 g of DOM in the range of values reported from direct measurements (15–17, 27).

The system used in the present work to assess microbial protein synthesis measured absolute but not net rates of microbial protein synthesis which are obtained from ingesta passage studies. The values given above can be recalculated by taking microbial protein turnover into account. Although the extent of turnover of microbial protein and bacterial-protozoal protein interconversions has not been accurately defined, it may be estimated from stud-
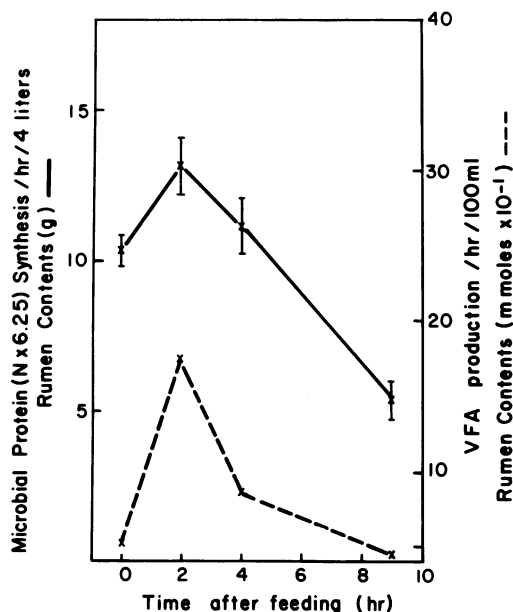

FIG. 4. Microbial protein (N × 6.25) synthesis and relative rate of volatile fatty acid production in rumen contents at various times after feeding. (Protein synthesis results, mean of two separate experiments; VFA production represents a single experiment; bars represent ± standard error.)

TABLE 4. *Estimated rate of ruminal microbial protein synthesis*[a]

Inoculation time after feeding (h)	Summation interval (h)	Summation time (h)	Protein synthesis rate (g per h per 4-liter rumen)		Total microbial protein synthesis during summation interval (g)	
			$N \times 6.25$	True protein	$N \times 6.25$	True protein
0	(T_0-T_1)	1	10.66 ± 0.70	9.06	10.66	9.06
2	(T_1-T_3)	2	13.17 ± 1.64	11.19	26.34	22.38
4	(T_3-T_7)	4	11.14 ± 1.70	9.47	44.56	37.88
9	(T_7-T_{12})	5	5.45	4.63	27.50	23.15
Total	(T_0-T_{12})	12			109.00	92.47

[a] True protein = $N \times 6.25 \times 0.85$ (42). Estimated rates of microbial crude protein and true protein synthesis per day equals 218.0 and 184.9 g, respectively. Estimated microbial crude protein and true protein synthesis per 100 g of organic matter digested in the rumen [75% digestion (1)] equal 26.0 and 22.0 g, respectively.

ies of bacterial lysis in the rumen (22), from protozoa-bacteria interrelationships in the rumen (8, 47), from the recent report on N turnover in ruminants (32), and from microbial turnover data (29) that 25 or more of the total protein synthesized in the rumen may be involved in turnover. On a 25 turnover basis the estimated ruminal protein synthesis rate was 16.1 g of true protein synthesis/100 g of DOM. This value represents a 30-g digestible protein-to-Mcal of digestible energy ratio which is adequate for growing calves and lambs (36).

LITERATURE CITED

1. Al-Rabbat, M. F., R. L. Baldwin, and W. C. Weir. 1971. In vitro ¹⁵nitrogen-tracer technique for some kinetic measures of ruminal ammonia. J. Dairy Sci. 54:1150-1161.
2. Baldwin, R. L., and D. L. Palmquist. 1965. Effect of diet on the activity of several enzymes in extracts of rumen microorganisms. Appl. Microbiol. 13:194-200.
3. Bauchop, T., and S. R. Eldsen. 1960. The growth of micro-organisms in relation to their energy supply. J. Gen. Microbiol. 23:457-469.
4. Bergen, W. G., D. B. Purser, and J. H. Cline. 1968. Effect of ration on the nutritive quality of rumen microbial protein. J. Anim. Sci. 28:1497-1501.
5. Bolton, E. T., and R. R. Roberts. 1964. Phosphorus incorporation. In R. R. Roberts (ed.), Studies of macromolecular biosynthesis. Carnegie Institute Washington Publication 624., Washington, D.C.
6. Carroll, E. J., and R. E. Hungate. 1954. The magnitude of the microbial fermentation in the bovine rumen. Appl. Microbiol. 2:205-214.
7. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. 28:1756-1758.
8. Eadie, J. M., and P. N. Hobson. 1962. Effect of the presence or absence of rumen ciliate protozoa on the total rumen bacterial count in lamb. Nature (London) 193:504-505.
9. Erwin, E. S., G. J. Marco, and E. M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J. Dairy Sci. 44:1768-1771.
10. Folch, J., M. Lee, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226:497-509.
11. Hobson, P. N. 1965. Continuous culture of some anaerobic and facultatively anaerobic rumen bacteria. J. Gen. Microbiol. 38:167-180.
12. Hobson, P. N., and R. Summers. 1967. The continuous culture of anaerobic bacteria. J. Gen. Microbiol. 47:53-65.
13. Hobson, P. N., and R. Summers. 1972. ATP pool and growth in Selenomonas ruminantium. J. Gen. Microbiol. 70:351-360.
14. Hogan, J. P., and R. H. Weston. 1970. Quantitative aspects of microbial protein synthesis in the rumen, p. 474-485. In A. T. Phillipson (ed.), Physiology of digestion and metabolism in the ruminant. Oriel Press Ltd., London.
15. Hogan, J. P., and R. H. Weston. 1971. The utilization of alkali-treated straw by sheep. Aust. J. Agr. Res. 22:951-962.
16. Hume, I. D., R. J. Moir, and M. Somers. 1970. Synthesis of microbial protein in the rumen. I. Influence of the level of nitrogen intake. Aust. J. Agr. Res. 21:283-296.
17. Hume, I. D. 1970. Synthesis of microbial protein in the rumen. III. The effect of dietary protein. Aust. J. Agri. Res. 21:305-314.
18. Hungate, R. E. 1963. Polysaccharide storage and growth efficiency in Ruminococcus albus. J. Bacteriol. 86:848-854.
19. Hungate, R. E. 1966. The rumen and its microbes. Academic Press Inc., New York.
20. Hungate, R. E., R. A. Mah, and M. Simesen. 1961. Rates of production of individual volatile fatty acids in the rumen of lactating cows. Appl. Microbiol. 9:554-561.
21. Hungate, R. E., J. Reichl, and R. Prins. 1971. Parameters of rumen fermentation in a continuously fed sheep: evidence of a microbial rumination pool. Appl. Microbiol. 22:1104-1113.
22. Jarvis, B. D. W. 1968. Lysis of viable rumen bacteria in bovine rumen fluid. Appl. Microbiol. 16:714-723.
23. Karr, M. R., U. S. Garrigus, E. E. Hatfield, and H. W. Norton. 1965. Factors affecting the utilization of nitrogen from different sources by lambs. J. Anim. Sci. 24:459-468.
24. Katz, I., and M. Keeney. 1966. Characterization of the octadecenoic acid in rumen digesta and rumen bacteria. J. Dairy Sci. 49:962-966.
25. Krishran, P. S., S. P. Damle, and V. Bajaj. 1957. Studies on the role of "metaphosphate" in molds. II. The formation of "soluble" and "insoluble" metaphosphates in Aspergillus niger. Arch. Biochem. 67:35-52.

26. Lindberg, O., and L. Ernster. 1956. Determination of organic phosphorus compounds by phosphate analysis, p. 1–33. *In* D. Glick (ed.), Methods of biochemical analysis, vol. III. Interscience Publishers, Inc., New York.

27. Lindsay, J. R., and J. P. Hogan. 1972. Digestion of two legumes and rumen bacteria growth in defaunated sheep. Aust. J. Agr. Res. 23:321–330.

28. Maaløe, O., and N. O. Kjeldgaard. 1966. Control of macromolecular synthesis. W. A. Benjamin, New York.

29. Mandelstam, J. 1960. The intracellular turnover of protein and nucleic acids and its role in biochemical differentation. Bacteriol. Rev. 24:289–308.

30. Meyer, J. H., W. C. Weir, and D. T. Torell. 1962. Response of immature sheep to partial starvation. J. Anim. Sci. 21:916–923.

31. Mitchell, P., and J. M. Moyle. 1953. Paths of phosphate transfer in *Micrococcus pyogenes*: phosphate turnover in nucleic acids and other fractions. J. Gen. Microbiol. 9:257–272.

32. Nolan, J. W., and R. A. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. Brit. J. Nutr. 27:177–194.

33. Ohki, M. 1972. Correlation between metabolism of phosphatidylglycerol and membrane synthesis in *Escherichia coli*. J. Mol. Biol. 68:249–264.

34. Payne, W. J. 1970. Energy yields and growth of heterotrophs. Ann. Rev. Microbiol. 24:17–52.

35. Pilgrim, A. F., F. V. Gray, R. A. Weller, and C. B. Belling. 1970. Synthesis of microbial protein from ammonia in the sheep's rumen and the proportion of dietary nitrogen converted into microbial nitrogen. Brit. J. Nutr. 24:589–598.

36. Purser, D. B. 1970. Nitrogen metabolism in the rumen: microorganisms as a source of protein for the ruminant animal. J. Anim. Sci. 30:988–1001.

37. Purser, D. B., and R. J. Moir. 1959. Ruminal flora studies in sheep. IX. The effect of pH on the ciliate population of the rumen in vivo. Aust. J. Agr. Res. 10:555–564.

38. Randle, C. L., P. W. Albro, and J. C. Dittmer. 1969. The phosphoglyceride composition of gram-negative bacteria and the changes in composition during growth. Biochim. Biophys. Acta 187:214–220.

39. Rhee, K. R., and L. R. Dugan. 1967. The implication of acid concentration in lipid phosphorus determinations. Anal. Biochem. 19:157–165.

40. Robinson, J. R. 1969. ³²P: A superior radiotracer for phosphorus. Int. J. Appl. Radiat. Isotop. 20:531–535.

41. Scherbaum, O. H. 1963. Acid-soluble phosphates in nucleated and enucleated *Acetabularia*. Biochim. Biophys. Acta 72:509–515.

42. Smith, R. H. 1969. Reviews of the progress of dairy science. Section G. General. Nitrogen metabolism and the rumen. J. Dairy Res. 36:316–331.

43. Stadtman, E. R. 1966. Some consideration of the energy metabolism of anaerobic bacteria. *In* N. O. Kaplan and E. P. Kennedy (ed.), Current aspects of biochemical energetics. Academic Press Inc., New York.

44. Walker, D. J. 1968. Energy utilization for polysaccharide synthesis by mixed rumen organisms fermenting soluble carbohydrates. Appl. Microbiol. 16:1672–1677.

45. Walker, D. J., and C. J. Nader. 1968. Method for measuring microbial growth in rumen content. Appl. Microbiol. 16:1124–1131.

46. Walker, D. J., and C. J. Nader. 1970. Rumen microbial protein synthesis in relation to energy supply: diurnal variation with once-daily feeding. Aust. J. Agr. Res. 21:747–754.

47. Wallis, O. C., and G. S. Coleman. 1967. Incorporation of C¹⁴ labeled components of *Escherichia coli* and of amino acids into *Isotricha intestinalis* and *Isotricha prostoma* from the sheep rumen. J. Gen. Microbiol. 49:315–324.

48. Weissbach, H., E. Thomas, and H. R. Kabock. 1971. Studies on the metabolism of ATP by isolated bacterial membranes: formation and metabolism of membrane-bound phosphatidic acid. Arch. Biochem. Biophys. 147:249–254.

49. Weller, R. A., F. V. Gray, and A. F. Pilgrim. 1958. The conversion of plant nitrogen to microbial nitrogen in the rumen of the sheep. Brit. J. Nutr. 12:421–429.

50. White, D. C., and A. N. Tucker. 1969. Phospholipid metabolism during bacterial growth. J. Lipid Res. 10:220–233.