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Use of Adenosine 5'-Triphosphate as an Indicator of the Microbiota Biomass in Rumen Contents

C. W. FORSBERG* AND K. LAM

Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

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A number of techniques were tested for their efficiency in extracting adenosine 5'-triphosphate (ATP) from strained rumen fluid (SRF). Extraction with 0.6 N H_2SO_4, using a modification of the procedure described by Lee et al. (1971), was the most efficient and was better suited for extracting particulate samples. Neutralized extracts could not be stored frozen before assaying for ATP because large losses were incurred. The inclusion of internal standards was necessary to correct for incomplete recovery of ATP. The ATP concentration in rumen contents from a cow receiving a ration of dried roughage (mainly alfalfa hay) ranged from 31 to 56 μg of ATP per g of contents. Approximately 75% of the ATP was associated with the particulate material. The ATP was primarily of microbial origin, since only traces of ATP were present in the feed and none was found in "cell-free" rumen fluid. Fractionation of the bacterial and protozoal populations in SRF resulted in the isolation of an enriched protozoal fraction with a 10-fold higher ATP concentration than that of the separated rumen bacteria. The ATP pool sizes of nine functionally important rumen bacteria during the exponential phase of growth ranged from 1.1 to 17.6 μg of ATP per mg of dry weight. This information indicates that using ATP as a measure of microbial biomass in rumen contents must be done with caution because of possible variations in the efficiency of extraction of ATP from rumen contents and differences in the concentration of ATP in rumen microbes.

Currently used indicators of microbial biomass in rumen contents include the amino acid 2,6-diaminopimelic acid for bacteria and 2-aminoethylphosphonic acid for protozoa (8). 2,6-Diaminopimelic occurs in the bacterial cell wall peptidoglycan and is absent from plant and animal tissues. 2-Aminoethylphosphonic acid is found only in protozoa and animal tissue (1, 12). In protozoa, 2-aminoethylphosphonic acid occurs in both lipids and proteins, whereas in higher animals it is confined mainly to lipids and is probably a result of digestion and absorption of protozoal matter (15). These specific indicators are intended for monitoring the biomass of bacteria and protozoa separately. However, for studies where this separation is unnecessary or undesirable, a more representative and rapid assay would be advantageous. Furthermore, an indicator whose concentration is intimately related to the metabolic activity and viability of cells would be very useful for the study of factors affecting microbial numbers and activity in rumen contents.

Adenosine 5'-triphosphate (ATP) is a well-accepted indicator of microbial biomass in aquatic and terrestrial environments (2, 6, 10). The primary reasons for measuring this cellular constituent include: (i) ATP is present in all living cells and absent from dead cells (11, 20); (ii) ATP is considered to be present in a uniform concentration in all microbes (2, 10); and (iii) the analytical techniques for the extraction and assay of ATP are specific, relatively quick, easy to perform, and inexpensive. Because of these unique features of ATP measurement, the feasibility of using this technique as a reliable indicator of microbial biomass in rumen contents was examined.

MATERIALS AND METHODS

Bacterial cultures, cultural conditions, and media. The anaerobic rumen bacteria listed in Table 9 were generously provided by M. P. Bryant, University of Illinois, Urbana, and K. J. Cheng, Research Station, Agriculture Canada, Lethbridge, Alberta.

The anaerobic technique used for culturing the bacteria was essentially that of Hungate (13) as modified by Bryant (5). The rumen bacteria were cultured anaerobically in the artificial medium described by Scott and Dehority (21). The cultures were stored at −65°C on agar slants. Before use in a

growth experiment, each culture was subcultured two or three times at 24-h intervals at 37°C. When the culture reached the late exponential phase of growth during the last subculture, it was used to inoculate 300 ml of liquid medium to give a final culture density of 20 μg of cells (dry weight)/ml. The cultures were incubated stationary in a water bath at 37°C. At given times of incubation, the culture was mixed and fluid was removed anaerobically for optical density (OD) measurements, calibration of OD readings to dry weight, and extraction of ATP.

The OD measurements were made using matched cuvettes (13 by 100 mm) in a Spectronic 20 at 675 nm. The readings were corrected for deviations from Beer's law using the tables of Toennies and Gallant (22). The OD values of each culture were correlated with the dry weight of cells by means of a separate calibration curve for each culture relating the OD of cells (harvested at an OD of 0.5 to 0.7 and washed twice in water) to the dry weight after drying of an aliquot to constant weight at 105°C. From the curves it was determined that an OD reading of 1.0 was equilvalent to 0.48 mg of *Streptococcus bovis*, 0.37 mg of *Bacteroides ruminicola*, 0.36 mg of *Selenomonas ruminantium*, 0.46 mg of *Butyrivibrio fibrisolvens*, 0.45 mg of *Megasphaera elsdenii*, 0.43 mg of *Ruminococcus albus*, 0.36 mg of *Bacteroides succinogenes*, 0.58 mg of *Bacteroides amylophilus*, and 0.53 mg of *Eubacterium ruminantium* per ml.

Rumen microbiota. Rumen contents were obtained from two fistulated cows. A Holstein cow was initially fed a ration of roughage plus a corn-based supplement (2:1), and later a ration of roughage alone. A Hereford cow used as a source of rumen contents for one experiment was fed a ration of roughage plus rolled oats. The roughage was a mixture of alfalfa and brome grass containing primarily alfalfa. The samples of rumen contents were collected 3 to 4 h after feeding via the fistula and taken to the laboratory in a prewarmed (37°C) Thermos flask. Strained rumen fluid (SRF) was prepared by filtration of rumen contents through four layers of surgical gauze. The plant material retained by the gauze was called the particulate material.

Methods for extracting ATP. (i) "Boiling" Tris buffer. A 0.5-ml sample of SRF was transferred to a screw-cap vial, and 4.5 ml of 0.04 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8, just off the boil, was added. The vial was quickly sealed, and the contents were mixed and then placed in a boiling water bath for 5 min with occasional mixing. After cooling, the contents of the vial were centrifuged at 12,800 × *g* for 10 min (4°C) and the supernatant was saved. A portion of the supernatant was diluted to one-fifth of the original concentration with Tris buffer.

(ii) Chloroform (a modification of the method of Dhople and Hanks [9]). A 0.5-ml sample of SRF was added to a test tube containing 0.15 ml of chloroform. The contents of the tube were mixed for 1 min with a Vortex mixer, and the tube was placed in a boiling water bath for 5 min. During the last minute of heating, the tube was shaken to facilitate vaporization of the chloroform. The solution was then made up to 5 ml with 0.1 M $NaHCO_3$ (pH 8.5) and centrifuged at 12,800 × *g* for 10 min (4°C). A 1-ml sample of the supernatant was vacuum-evaporated to remove residual $CHCl_3$ and then made up to 10 ml with 0.4 M Tris buffer, pH 7.8.

(iii) "Boiling" $NaHCO_3$ buffer (6). Liquid samples were handled as described in (i) except 8 ml of 0.1 M $NaHCO_3$ (pH 8.5), just off the boil, was added in the place of Tris buffer. After the centrifugation step, the supernatant was decanted and a portion was diluted to one-tenth of the original concentration with 0.4 M Tris buffer, pH 8.5. In the case of particulate material (total rumen contents or strained particulate material), a 4- to 7-g sample was washed into a preheated stainless-steel Waring blender jar with 125 ml of 0.1 M $NaHCO_3$ (pH 8.5), just off the boil. The contents were mixed at 10,000 rpm for 4 min. After cooling, a portion was treated as described for liquid samples.

(iv) Cold 0.6 N H_2SO_4 (a modification of the method described by Lee et al. [17]). This is the technique recommended for use. A 1-ml sample of SRF was added to 4 ml of ice-cold 0.6 N H_2SO_4 in a centrifuge tube and mixed intermittently. The tube was maintained for exactly 5 min in an ice bath and then centrifuged at 39,000 × *g* for 10 min (4°C). A 2-ml volume of the supernatant and 1.5 ml of a 10% (wt/vol) suspension of Na^+-saturated cation-exchange resin (Amberlite IR-120) suspended in water were immediately added to a test tube and mixed for 30 s. Two milliliters of 0.5 M Tris buffer (pH 7.8) was added, and the contents were mixed while 1.0 to 1.2 ml of 1 N NaOH was added (i.e., sufficient to adjust the mixture to pH 7.8 in the absence of Tris buffer), followed by 10 to 15 ml of water. Samples were processed to this stage without interruption. The extract was separated from the resin by aspiration, and the resin was washed twice with 2-ml aliquots of distilled water. The combined extract and washes were pooled and made to a volume of 25 ml with water. In the case of particulate material (total rumen contents or separated particulate material), a 4- to 7-g sample of thoroughly mixed material was added to a centrifuge tube, 25 ml of 0.6 N H_2SO_4 was added, and the contents were mixed vigorously. The subsequent manipulations were identical to those for processing samples of SRF.

When a nonradioactive internal standard of ATP was added to a liquid sample, 3 ml of 0.6 N H_2SO_4 was first mixed with the 1-ml sample; then 1 ml of 0.6 N H_2SO_4 containing the standard (100 nM of nonradioactive ATP) was immediately added, and the contents were mixed again. For particulate samples, the sample was mixed with 20 ml of 0.6 N H_2SO_4, and then 5 ml of 0.6 N H_2SO_4 containing the standard (750 nM nonradioactive ATP) was added. The H_2SO_4 solutions of ATP to be used as internal standards were prepared immediately before use.

Radioisotope usage. [U-^{14}C]ATP (ammonium salt, 554 mCi/mmol) was obtained from Amersham/Searle Corp., Oakville, Ontario. It was added without dilution to 0.6 N H_2SO_4 to give specific activities of 0.04 μCi/ml for addition to liquid samples and 0.1 μCi/ml for particulate samples. Neutralized sam-

ples were suspended in a dioxane-base scintillation fluid (4) for counting in a Beckman LS-255 scintillation counter.

Separation of bacteria and protozoa. Two liters of rumen fluid collected from the cow on a hay ration was strained through six layers of surgical gauze into a prewarmed (37°C) insulated container and quickly brought to the laboratory. All apparatus and glassware used were prewarmed to 37°C. A bacterial fraction, free from protozoa, was obtained by first centrifuging SRF at 175 × *g* for 5 min at 37°C to sediment the protozoa and feed particles. The supernatant containing bacteria was aspirated off and sampled for the measurement of ATP. Thirty milliliters was added to each of four tubes, which were then centrifuged at 39,000 × *g* for 5 min at 37°C to sediment the bacteria. The supernatant fluid was poured off, and two cell pellets were separately extracted with 20 ml of 0.6 N H_2SO_4; after mixing, 5 ml of each was added to 100 nmol of ATP (0.1 ml). The extraction procedure was continued as described above for both the two 5-ml samples with internal standard and the two 5-ml samples without. The other two cell pellets were washed twice at 4°C with 25-ml volumes of water, centrifuging each time at 27,000 × *g* for 10 min; then each pellet was made up to 10 ml with water, and 5 ml was dried to constant weight at 105°C.

To obtain an enriched fraction of protozoa, two 750-ml volumes of SRF were added to 1-liter separatory funnels, pregassed with CO_2. The head spaces of the funnels were gassed with CO_2, stoppered, and placed in a 37°C incubator for 60 min. The white sediments in the funnels were collected, pooled, and suspended in 45 ml of freshly prepared "cell-free" rumen fluid (prepared by two successive centrifugations at 27,000 × *g* for 30 min, warmed to 37°C, and gassed with CO_2). The protozoal suspension was divided equally among four tubes and centrifuged at 175 × *g* for 8 min at 35°C to sediment the protozoa, and the culture supernatant was carefully aspirated off each cell pellet. Two cell pellets were resuspended in 10 ml of 0.6 N H_2SO_4 and treated as described for the bacteria. The two remaining cell pellets were washed twice as described for the bacteria, and the dry weights were determined. Lysis of the bacterial and protozoal fractions during the water washes preceding the dry-weight determinations did not appear to occur because similar dry weights were obtained when the cells were pretreated with either glutaraldehyde or formaldehyde. Phase-contrast microscopy revealed that the protozoa were contaminated with a few bacteria. Further purification was not attempted to avoid disturbing the metabolism of the protozoa, which would lead to a changed ATP content.

Estimation of ATP. ATP was determined by the firefly bioluminescence technique. The reaction mixture contained 0.02 to 0.5 nmol of ATP in 0.3 ml of 0.04 M Tris buffer, pH 7.8, and 0.2 ml of a freshly prepared solution of 0.125 M sodium arsenate-0.05 M magnesium chloride solution, pH 7.8. The reaction was started by addition of 0.1 ml of reconstituted firefly lantern extract (one vial of FLE-50 homogenized with 5 ml of water, centrifuged at 12,000 × *g* for 15 min at 4°C to remove particulate material and stored on ice; Sigma Chemical Co., St. Louis, Mo.). The intensity of the bioluminescence was recorded after 30 s, using a Chem-Glow Photometer (American Instruments Co., Silver Spring, Md.). The concentration of ATP in experimental samples was determined by reference to a standard curve relating light intensity to ATP concentration prepared from disodium ATP. All experimental samples were assayed at three or four separate concentrations and in some cases with added internal standards of ATP to check for inhibition of light emission by components present in extracts. Sodium carbonate inhibited luciferase activity, but at the highest concentration present in experimental samples it caused less than 5% inhibition and was disregarded.

Extraction and measurement of the ATP pool sizes of bacteria. A 1-ml volume of culture was mixed with 0.2 ml of 3.6 N H_2SO_4 and set on ice for 20 min. One milliliter of Na^+-saturated cation-exchange resin (Amberlite IR-120) suspended in water was added. The contents were shaken vigorously and then centrifuged at 12,000 × *g* for 15 min at 4°C to remove cell debris and resin. A 1.5-ml volume of the supernatant was removed, and the pH was adjusted to 7.8. The solution was made 0.04 M with respect to Tris buffer (pH 7.8) in a volume of 10 ml. When internal standards of ATP (100 nM in 0.1 ml) were added, they were mixed into the samples immediately after addition of the H_2SO_4. These extracts were assayed for ATP by using minivials in a Beckman model LS-255 scintillation counter because of its increased sensitivity over the Chem-Glow Photometer. The assay reagents used were the same as those used with the Chem-Glow Photometer except that (i) the firefly lantern extract was aged for 2 h at 4°C, (ii) the volume of sample plus 0.04 M Tris buffer (pH 7.8) was increased to 1.0 ml and the volume of the arsenate-magnesium solution was increased to 0.6 ml, and (iii) 0.2 ml of firefly extract was used to start the enzymatic assay. Each sample was mixed and counted for three 0.1-min cycles, using gain and window settings used for counting tritium. The counts per minute recorded for the third 0.1-min cycle was recorded. A standard curve, plotted on log-log graph paper, relating counts per minute to ATP concentration was prepared by assaying a standard solution of ATP. The recovery of added internal standard usually ranged from 70 to 105% and was used to correct the ATP concentrations to 100% recovery.

RESULTS

Extraction techniques. The ATP content of rumen fluid was determined by a variety of techniques with the intention of selecting the best method for the extraction of ATP. The results illustrated in Table 1, experiment 1, are typical of those obtained in a number of experiments and show that hot 0.1 M $NaHCO_3$ (pH 8.5) with heating for 5 min gave the greatest recovery of ATP. The efficiency of extraction by this technique was compared with that of the

TABLE 1. *Effect of extraction technique on the amount of ATP extracted from strained rumen fluid from a cow on a ration of hay plus cereal*

Expt no.	Extraction technique	μg of ATP/ml of SRF
1	(i) Boiling 0.04 M Tris buffer, pH 7.8	69.5
	(ii) Chloroform	31.1
	(iii) $NaHCO_3$-chloroform-blending[a]	40.5
	(iv) Boiling$NaHCO_3$	92.5
2	(iv) Boiling $NaHCO_3$	120.7 ± 6.9[b]
	(v) Cold 0.6 N H_2SO_4	111.0 ± 2.7

[a] Five milliliters of SRF plus 45 ml of 0.1 M $NaHCO_3$ (20°C) were added to a blender. Fifteen milliliters of $CHCl_3$ was added, and the materials were mixed for 5 min.

[b] Mean and the standard deviation. (iv, four samples; v, five samples).

method described by Lee et al. (17) using cold 0.6 N H_2SO_4. In the latter technique, after addition of 0.6 N H_2SO_4 to the sample, the mixture was incubated on ice for 60 min and the sample was centrifuged to remove particulate material. A 2-ml volume of the clarified extract was treated batchwise with two 0.5-ml amounts of cation-exchange resin, and the pH was adjusted to 7.8 by titration with 1 N NaOH. The extract was then made to a volume of 25 ml and 0.04 M with respect to Tris (pH 7.8). There was no significant difference (at the 95% confidence level) in the relative extraction efficiency between the two methods (Table 1, experiment 2). The recoveries of ATP (100 nM) added as internal standards to the $NaHCO_3$ and H_2SO_4 extractions were 94 and 105%, respectively. In separate experiments, the efficiency of the $HClO_4$ extraction technique published by Bagnara and Finch (3) was compared with the H_2SO_4 extraction technique for measuring ATP in the particulate fraction of rumen contents. Extraction with $HClO_4$ gave only 16% of the ATP obtained using H_2SO_4, and a comparable recovery of internal standard was observed. Because it is more convenient, the $NaHCO_3$ extraction technique was used initially, and results using this method are reported in Tables 1, 2, and 3; however, because the H_2SO_4 extraction technique was found to give higher recoveries of ATP from particular samples, it was used in the remainder of the study.

To determine how the time of incubation affected the ATP content of SRF, a sample was extracted at intervals after removal of rumen contents from a cow. There was no detectable change in the ATP content of the SRF over a 160-min period (Table 2).

The use of ATP as a reliable indicator of microbial biomass in rumen contents depends upon there being a low amount of ATP in the feed consumed by the ruminant animal. The ATP content of a number of feed constituents was determined by extraction with hot 0.1 M $NaHCO_3$, using the procedure involving blending. Less than 0.24 μg of ATP per g was extracted from alfalfa hay, and no ATP was detected in either ground corn or the pelleted corn-based supplement containing soybean meal, a complete vitamin mixture, and minerals. Similar results were obtained using the H_2SO_4 extraction technique.

Observed distribution of ATP in rumen contents. The distribution of ATP between the SRF and particulate fraction of rumen contents was determined for the cow on a hay-corn-based

TABLE 2. *Effect of incubation at 37°C on the amount of ATP extracted from strained rumen fluid from a cow on a ration of hay plus cereal*[a]

Time interval (min)	μg of ATP/ml
1	104.3 ± 0.3
5	104.8 ± 3.8
10	103.9 ± 6.8
20	96.1 ± 4.5
40	115.0 ± 1.4
80	95.7 ± 5.7
160	110.6 ± 5.3

[a] A 300-ml volume of strained rumen fluid contained in a 500-ml round bottom flask was incubated under an atmosphere of carbon dioxide. The $NaHCO_3$ extraction technique was used to extract ATP.

TABLE 3. *Distribution of ATP in rumen contents from cows on a ration of hay plus cereal*[a]

Expt no.	Sample	μg of ATP/g of contents observed	Distribution (%) observed
1	Ration of hay + corn-based supplement		
	Mixed rumen contents	84.9[b]	100
	SRF	103.1	60
	Particulate material	48.3	17
2	Ration of hay + rolled oats		
	Mixed rumen contents	61.5 ± 1.7[c]	100
	SRF	37.0 ± 4.0	31
	Particulate material	78.0 ± 1.8	67
	Gauze[d]		2

[a] The $NaHCO_3$ extraction technique was used for experiment 1, and the H_2SO_4 extraction technique of Table 1 was used for experiment 2. In both cases the extracts were assayed for ATP immediately after preparation.

[b] Mean value of duplicates.

[c] Mean and standard deviation of samples extracted and assayed in triplicate.

[d] The moist gauze with associated traces of rumen contents was immersed in 200 ml of 0.6 N H_2SO_4, and the fluid was processed as described for SRF samples.

supplement ration and the cow on a hay-rolled oats ration (Table 3). In both cases the ATP detected in the two fractions accounted for greater than 90% of the ATP in the mixed rumen contents. Very little ATP remained associated with the gauze used for the filtration process. When the separation was repeated with rumen contents taken after the diet of the first cow had been changed to hay, the observed overall recovery of ATP was 42% when using the $NaHCO_3$ extraction procedure and 55% for the H_2SO_4 extraction procedure used in Table 1, thus indicating that both methods gave incomplete recoveries of ATP. To allow for incomplete recovery of ATP, the experiment was repeated with internal standards, which were added during the extraction procedure (Table 4, experiment 1). In this particular experiment the neutralized extracts were stored frozen at −20°C overnight. There was very little binding of [^{14}C]ATP to particulate materials since little radioactivity was lost as a result of the centrifugation step and resin treatment. However, there were large decreases in the amount of nonradioactive ATP internal standard detected in all samples except the SRF sample. When internal standards were added to the luciferase assay system, all the added ATP was detected, indicating that no inhibitors of luciferase were present in the extracts. In the second experiment (Table 4, experiment 2), the loss of internal standard was found to be less when the assays for ATP were conducted immediately. Nevertheless, the overall recovery of ATP was low. In both experiments the greatest loss in detectable ATP was in the particulate fraction.

Factors affecting the amount of ATP detected in rumen contents using the H_2SO_4 extraction technique. Since the extraction of ATP from the particulate fraction was the least efficient, this fraction was examined in the greatest detail. The effect of freezing and thawing of neutralized extracts on the concentration of ATP detected was first examined (Table 5). There was a decrease in the concentration of ATP detected in treated samples of both SRF and particulate material as a consequence of freezing and thawing, and the decreases were greater in extracts from particulate samples. These decreases could not be due to the activity of an acid-stable enzyme that was active while the neutralized samples were thawed, because

TABLE 5. *Effect of freezing and thawing on the amount of ATP detected in neutralizing H_2SO_4 extracts prepared from fractionated rumen contents*[a]

Sample	μg of ATP/g of contents		
	Unfrozen	Frozen once	Frozen twice
SRF	17.3	15.6	11.6
SRF + internal standard	62.0	59.2	35.5
Particulate material	27.1	6.5	2.0
Particulate material + internal standard	79.7	32.1	16.3

[a] Samples of rumen contents, obtained from a cow on a ration of hay, were fractionated, and the SRF and particulate fractions were extracted with 0.6 N H_2SO_4 for 60 min as described for Table 1. The extracts were assayed for ATP, frozen by storage at −20°C for 20 h and then thawed at 22°C, and assayed again for ATP; and the cycle was repeated a second time.

TABLE 4. *Distribution of ATP in rumen contents from a cow on a ration of hay*[a]

Sample[a]	Recovery of internal standard (%)		μg of ATP/g of contents		Distribution (%)	
	[^{14}C]ATP	Nonradioactive ATP	Observed	Calculated[b]	Observed	Calculated
Expt 1						
Mixed rumen contents	84	51	25.7 ± 1.4[c]	50.4	100	100
SRF	92	67	15.2 ± 0.5	22.7	27	21.0
Particulate material	91	23	21.0 ± 0.4	91.3	40	87.0
Gauze	91	53	3.3 ± 0.1	6.2	0.8	0.7
Expt 2						
Mixed rumen contents	—[d]	72	40.3 ± 3.7	56.0	100	100
SRF	—	91	27.3 ± 0.7	30.0	36	29.0
Particulate material	93	64	25.2 ± 1.1	39.4	22	25.0
Gauze		51	9.2 ± 0.2	18.2	1.9	2.6

[a] Samples were extracted with 0.6 N H_2SO_4 as described for Table 1. In experiment 1 the neutralized samples were stored frozen before analysis, and in experiment 2 the samples were assayed immediately.

[b] The calculated values have been corrected for the average loss of nonradioactive internal standard (samples containing internal standards were also extracted in triplicate).

[c] Mean and standard deviation of samples extracted and assay in triplicate.

[d] —, Not determined.

incubation of extracts at 30°C for an extended period of time (15.5 h) (Table 6) resulted in only a minor decrease in ATP content. This might be expected since contaminating microbes could have grown and hydrolyzed some of the ATP in that time interval.

Freezing and thawing of neutralized extracts of particulate samples after heating at 100°C for 5 min sometimes resulted in an increase in the amount of ATP detected. For example, after a portion of sample 2 from Table 6 had been frozen and thawed, the concentration of ATP detected was 54.3 μg/g. An identical sample containing an internal standard treated in parallel increased from 91.3 to 132.0 μg of ATP per g after the freezing and thawing procedure. This effect was not reproducible from one experiment to the next; however, it did increase the amount of ATP detected after one freezing and thawing cycle as compared with unheated samples (Fig. 1).

Since there was an incomplete recovery of ATP in the experiment illustrated in Table 4, experiment 2, even though the samples had been assayed immediately after neutralization, the effect of extraction time with H_2SO_4 was examined. Extraction times longer than 5 min caused a decrease in the amount of ATP detected (Fig. 1). Therefore these experiments showed that a rapid extraction procedure must be used and the neutralized samples should not be frozen before being assayed.

Distribution of ATP in rumen contents from a cow on a ratio of hay. The distribution of ATP in rumen contents from the cow on a ration of hay was determined by using the modified H_2SO_4 extraction procedure. The recovery of internal standard was uniformly high in all fractions, and the overall recovery was 80% based on the observed distribution of ATP (Table 7). Comparison of these results with those in Table 3 shows that the observed ATP concentration in rumen contents was apparently greater for an animal receiving cereal in the diet.

TABLE 6. *Effect of incubation at 30°C on the amount of ATP detected in neutralized H_2SO_4 extracts of the particulate fraction of rumen contents from a cow on a ration of hay*[a]

Sample	μg of ATP/g		
	0 h	4 h	15.5 h
1. Extracted with 0.6 N H_2SO_4[b]	40.7	41.9	34.3
2. Neutralized extract from 1 was heated at 100°C for 5 min	38.5	40.7	39.7
3. H_2SO_4 extract from 1 filtered through a UM-10 Amicon ultrafilter and then neutralized	27.7	29.2	–[c]

[a] Aliquots of samples 1, 2, and 3 were incubated at 30°C immediately after preparation. The assays for ATP were conducted immediately after sampling at each time interval.

[b] Extractions were conducted as described for Table 1.

[c] –, Not determined.

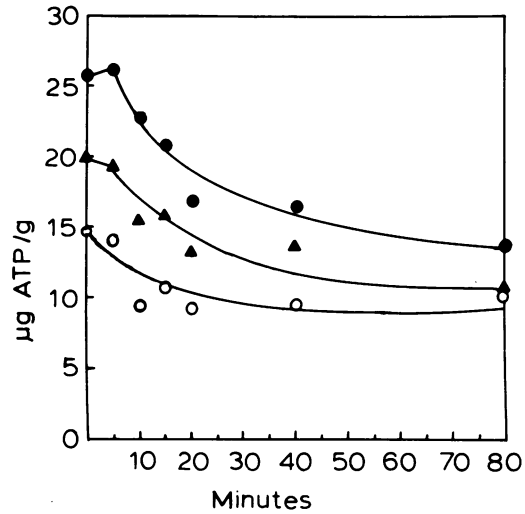

FIG. 1. *Effect of extraction time in 0.6 N H_2SO_4 on the amount of ATP detected in untreated (●), untreated and frozen (○), and heated and frozen (▲) extracts from the particulate fraction of rumen contents obtained from a cow on a ration of hay. After extraction, each sample was rapidly processed as described for Table 1. Aliquots of each neutralized sample were treated as described in Table 5 without or with an initial heating at 100°C for 5 min.*

The proportion of ATP associated with the particulate material was greater when the animal was on the hay ration. There was no ATP present in "cell-free" rumen fluid, probably because of the activity of an ATP hydrolase(s) that was found to be present (Fig. 2).

ATP content of separated bacterial and protozoal fractions. To determine whether the ATP in rumen contents was distributed uniformly between bacteria and protozoa (on a dry-weight basis), enriched fractions of each were prepared. The bacterial fraction was prepared by differential centrifugation of SRF. The recovery of added internal standard in one experiment (Table 8, experiment 1) exceeded 100%. This occurred infrequently for SRF samples but made the inclusion of internal standards essential. A low-speed centrifugation (175 × *g*) to sediment the protozoa and feed particles removed 74% of the ATP (Table 8, experiment 1). By measuring the amount of ATP in the super-

TABLE 7. *Recovery and distribution of ATP in rumen contents from a cow on a ration of hay using the modified H_2SO_4 extraction procedure*[a]

Sample	Recovery of internal standard (%)	μg of ATP/g of contents		Distribution (%)	
		Observed	Calculated[b]	Observed	Calculated
Mixed rumen contents	83.4	25.8 ± 0.6[c]	31.0	100	100
SRF	83.0	11.7 ± 0.3	14.1	23.0	23.3
Particulate material	69.0	30.8 ± 2.9	44.6	53.0	65.0
Gauze	82.4	17.7 ± 0.2	21.5	3.7	3.8
45,000 × *g* SRF supernate		0		0	0
Recovery (%)				79.7	92.1

[a] Extraction procedure is outlined in Materials and Methods.
[b] Calculated values have been corrected as described in Table 4.
[c] Mean and standard deviation of samples extracted and assayed in triplicate.

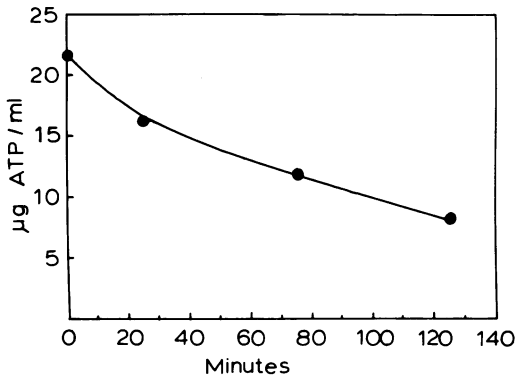

FIG. 2. *ATP hydrolase activity in "cell-free" rumen fluid from a cow on a ration of hay. Six milliliters of rumen fluid supernatant taken after two successive centrifugations at 45,000 × g for 30 min was mixed with 200 nM ATP (0.2 ml), gassed with CO_2, and incubated at 37°C. One-milliliter samples were extracted with H_2SO_4.*

natant and using this to compute the concentration of ATP in bacteria, the value obtained (2.10 μg of ATP/mg) was 28% higher than when the bacterial sediment after a high-speed centrifugation was extracted (1.64 μg of ATP/mg). The apparent decrease in the concentration of ATP in cells after centrifugation could be attributed in part to the loss of slime-encapsulated bacteria that formed a loose top layer over the sedimented cells and as a result was lost when the supernatant was poured off.

The protozoal fraction prepared by a combination gravity sedimentation and a subsequent low-speed centrifugation step, designed to remove residual bacteria, contained 16.9 μg of ATP per mg of dry weight. This concentration of ATP was 10-fold higher than that observed in the bacteria.

ATP pool sizes of pure cultures of rumen bacteria. To obtain information about the variation in ATP pool sizes among rumen bacteria, the ATP concentration was determined for a number of the functionally important species. Figure 3 illustrates the variation of the ATP content of *Selenomonas ruminantium* during growth. The level of ATP was high during the early exponential phase of growth and decreased by half in the late exponential phase; it then dropped off precipitously during the stationary phase. This type of profile for ATP was typical for all the species of bacteria studied (Table 9) except that the ATP pools of some species, e.g., *Bacteroides amylophilus* and *Ruminococcus albus*, remained nearly constant during the exponential phase of growh.

TABLE 8. *ATP pool sizes of bacterial and protozoal fractions from strained rumen fluid from a cow on a ration of hay*

Fraction	Recovery of internal standard (%)	μg of ATP/ml calculated[a]	μg of ATP/mg (dry wt) calculated[a]
Expt 1			
SRF	142	9.3	
SRF after centrifugation at 175 × *g* for 5 min	151	2.4	
Bacteria			
Before centrifugation	151		2.1
Cell pellet	105		1.6
Protozoa	82		16.9
Expt 2			
Bacteria			
Before centrifugation[b]	103		2.2
Cell pellet	84		1.6

[a] After correction of the values to 100% recovery.
[b] ATP content of SRF after a centrifugation of 470 × *g* for 4 min at 37°C to remove protozoa; expressed on the basis of dry weight of washed bacteria prepared from an aliquot of the fluid. All other manipulations in experiments 1 and 2 were carried out as described in Materials and Methods.

DISCUSSION

A number of methods were tested to determine their efficiency in extracting ATP from either SRF or its particulate fraction. The two

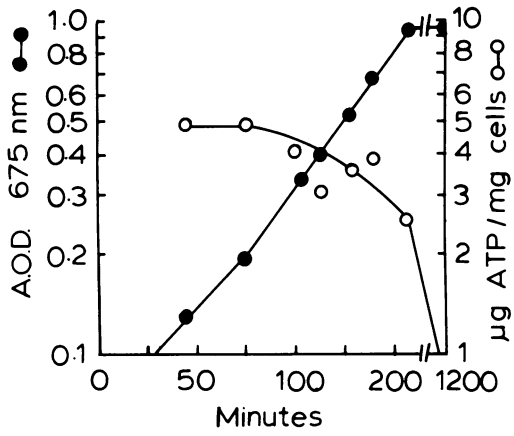

FIG. 3. *ATP pool size of Selenomonas ruminantium during growth.*

most efficient methods for extracting ATP were those using boiling 0.1 M $NaHCO_3$ (modification of the method by Christian et al. [6]) and cold 0.6 N H_2SO_4, described by Lee et al. (17). Both techniques gave incomplete recoveries of ATP when used to measure the ATP in particulate materials from rumen contents of a cow on a ration of hay. The recovery of ATP using the H_2SO_4 extraction procedure was the greater, and, since it was better suited for particulate samples, the factors influencing the recovery of ATP by this technique were examined.

The inclusion of [^{14}C]ATP in the extraction of ATP from whole rumen contents, SRF, and particulate materials revealed that the low recovery of endogenous ATP was not caused by binding of ATP to sedimentable materials. The lowest recovery of added nonradioactive ATP occurred when the strained particulate fraction of rumen contents was extracted, and the losses were increased if samples were frozen before assay. By decreasing the time of extraction with H_2SO_4 and assaying samples immediately after extraction, the low recovery of measurable ATP was largely corrected.

The apparent loss of ATP from neutralized extracts of the particulate materials did not occur when they were incubated at 30°C. Heating the neutralized extracts decreased the amount of ATP lost during freezing and thawing; in fact, in some cases the concentration of ATP was actually increased. These observations are consistent with the loss in ATP being caused by the extraction of a soluble component(s) from plant material, which forms complexes with ATP, either in acid solution or during freezing and thawing, and thereby prevents the reaction with luciferase. The higher recovery of ATP in neutralized extracts prepared from the SRF fraction and the decreased losses resulting from freezing and thawing suggest that the inhibitor was absent from the SRF.

The greater proportion of the ATP in rumen contents of the cow on a ration of hay plus cereal was present in the SRF fraction, whereas the inverse was observed when the cow was on a hay ration. Seventy-four percent of the ATP in the SRF from the latter animal was sedimented by an extremely low centrifugal force (175 × *g* for 5 min), indicating that the bulk of the ATP was present in microbes associated with plant material and protozoa. From an applied point of view, these observations suggest that the proportion of microbial biomass present in SRF may depend upon the animal ration. Since the types of bacteria in the fluid and on the particulate material are different (18), the use of SRF in in vitro experiments could be open to criticism because the population of microbes in the inoculum is changed from whole rumen contents.

To serve as a reliable indicator of microbial biomass, ATP must be present at a uniform concentration in viable microbes and absent from dead microbes and other materials. ATP was absent from the animal ration (dried alfalfa hay and corn-based supplement) fed in these experiments. The ATP content of fresh grass and silage has not been determined. Therefore, no recommendations can be made about the suitability of ATP as an indicator of rumen microbial biomass for animals on rations of fresh grass and silage. There presumably is a continual sloughing of epithelial tissue from the rumen wall; however, because of the increased lysozomal activity in this tissue (16), it probably is partially autolyzed and therefore depleted in ATP.

We found that ATP added to "cell-free" rumen

TABLE 9. *ATP pool sizes of nine species of rumen bacteria during the exponential phase of growth in artificial medium*

Bacterium	Generation time (min)	µg of ATP/mg (dry wt)[a]
Streptococcus bovis S-b-3	24	17.6
Bacteroides ruminicola subsp. *ruminicola* 23	57	6.5
Selenomonas ruminantium strain D	57	3.9
Butyrivibrio fibrisolvens D1	80	2.8
Megasphaera elsdenii B-159	94	4.5
Ruminococcus albus B-199	95	1.1
Bacteroides succinogenes S-85	107	1.8
Bacteroides amylophilus 70	112	2.6
Eubacterium ruminantium GA 195	127	3.5

[a] Each value is the average of three extractions at culture OD readings of approximately 0.20, 0.40, and 0.70 during exponential growth.

fluid gradually disappeared ($t_{1/2}$ = 85 min; Fig. 2). This loss in ATP was probably due to the presence of ATP-hydrolyzing enzymes. Since no attempt was made to identify reaction products, this proposal cannot be confirmed. The observed absence of ATP in the supernatant from centrifuged rumen fluid supports this contention. Thus, under our experimental conditions, the ATP in the rumen is limited to bacteria and protozoa.

The ATP concentration in the isolated protozoal fraction was 10-fold higher than in the bacterial fraction. Although precautions were taken to maintain anaerobiosis and a constant temperature, the possibility cannot be excluded that the values obtained, particularly for the protozoa, were changed as a result of metabolic disturbances that may have occurred during the separation procedure. For example, it has been observed that exposure of *Clostridium acetobutylicum* to aerobic conditions leads to a fall in the ATP pool size (19).

The ATP pool sizes of functionally important rumen bacteria growing exponentially in artificial media varied by a factor of 16, from 1.1 μg of ATP per mg of dry weight for *R. albus* to 17.6 μg of ATP per mg for *S. bovis*. Most of the values were higher than that found for the isolated population of rumen bacteria. Barring some effect related to the extraction procedures, this is expected for a number of reasons. The rumen contains many genera of bacteria that undoubtedly have different ATP pool sizes. The different types of bacteria have different degrees of affinity for particulate material (18), whereas the isolated bacterial fraction contained only those free in solution. In the rumen, microbial growth may be limited by the availability of carbohydrate (14), and for this reason bacteria, and particularly those free in solution, may be expected to have a decreased ATP pool size. This effect possibly is accentuated in animals on a roughage diet. In *Escherichia coli*, glucose-limited growth leads to lower pool levels of ATP (7).

Whether ATP reflects the overall metabolic activity of the rumen microbiota remains to be determined. The correlation of the concentration of ATP in the microbial fraction throughout the day with feed intake and ration composition should shed light on this subject.

These data show that considerable caution must be used in interpreting microbial biomass data in rumen contents based on ATP concentrations. ATP presumably can be used as an indicator of biomass where animals are receiving a uniform ration free from ATP. However, measurement of ATP in fractionated rumen contents can lead to discrepancies.

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