

Determination of rumen microbial growth in vitro from ^{32}P -labelled phosphate incorporation

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1. The extracellular phosphate pool in incubations of rumen fluid or washed cell suspensions of mixed rumen bacteria (WCS) was labelled with ^{32}P . From the constant extracellular phosphate pool specific activity and the amount of radioactivity incorporated during incubation, the amount of P incorporated in the microbial fraction was calculated. From the value for nitrogen : P determined in microbial matter, the amount of N incorporated was calculated as a measure of microbial growth.

2. Incorporation of soluble non-protein-N in incubations devoid of substrate protein was 50 and 80 % of the values obtained using the isotope method for rumen fluid and WCS respectively. It is suggested that results obtained using the former method reflect 'net growth' of micro-organisms which is the result of simultaneous growth and degradation. The isotope method measures 'total growth', as isotope incorporation is not affected by degradation of non-growing cells.

3. Incorporation of ^{32}P in P-containing microbial components (mainly nucleic acids) was compared with net synthesis of these components in incubations of WCS. The results showed different specific rates of synthesis and degradation for all components studied. It is concluded that the composition of microbial matter changed during growth.

4. When N incorporation, calculated from results obtained using the isotope method in incubations with rumen fluid, was compared with the amount of carbohydrate substrate fermented and the type of fermentation, values between 18.3 and 44.6 g N incorporated/kg of organic matter fermented were obtained. Low values were associated with large proportions of the substrate being fermented to lactate and the use of glucose instead of disaccharides as substrate. Part of the variation could also be attributed to differences in incubation period, reflected in different proportions of polysaccharide formed.

5. The use of isotopes for determination of rumen microbial growth in vitro is critically discussed.

The relationship between the amount of microbial matter synthesized and the amount of food organic matter (OM) fermented in the rumen is not completely understood (Smith, 1975). The study of this relationship in vivo involves the use of markers to measure flow of digesta in animals fitted with cannulas in the omasum or duodenum, and calculation of the contribution of microbial matter in digesta flow from the content in the digesta of a microbial marker such as 2,6-diaminopimelic acid (Hutton, Bailey & Annison, 1971), RNA (Smith, 1969) or ^{35}S -labelled microbial S. (Beever, Harrison, Thomson, Cammell & Osbourn, 1974). Experimental values (g nitrogen incorporated in microbial matter/kg OM apparently digested in the rumen ($\text{g N}_i/\text{kg DOM}_R$) where DOM_R is the net loss of OM between the point of entry and the point of exit of digesta flow through the rumen (Egan, 1974) obtained using such methods vary between 10.8 (recalculated from Leibholz, 1972) and 70.4 (recalculated from McMeniman, Ben-Ghedalia & Armstrong, 1974). Part of this variation has been related to differences in the molar proportions of the volatile fatty acids (VFA) present in the rumen (Ishaque, Thomas & Rook, 1971; Jackson, Rook & Towers, 1971; Harrison, Beever, Thomson & Osbourn, 1975), to the presence of protozoa (Lindsay & Hogan, 1972), to differences in the dilution or clearance rate of the rumen fluid (Roberts & Miller, 1969; Harrison *et al.* 1975) and to the rate of digesta flow through the rumen (Walker, Egan, Nader, Ulyatt & Storer, 1975). However, the methods used are elaborate (MacRae, 1975) and calculation of rate of flow of digesta (Faichney, 1975) and of microbial contribution to digesta (Harrison, Beever, Thomson & Osbourn, 1973; Ling & Buttery, 1975) is susceptible to considerable experimental error.

Determination of rumen microbial growth in vitro involves less elaborate methods and

allows straightforward determination of the amount of substrate fermented. Frequently, determination of microbial matter formed is based on the incorporation of isotopes from the labelled extracellular pool of an inorganic precursor X of microbial constituents. During incubation of rumen contents the change of isotope concentration in microbial matter or in the extracellular pool, or both, is followed by radioactivity measurement or mass spectrometry and the amount of precursor X incorporated is calculated from these concentration changes. From the amount of precursor X incorporated, the amount of N incorporated is calculated using the ratio, $X:N$ in microbial matter. Such determinations have been carried out using ^{35}S or ^{15}N to label the pool of sulphide, sulphate and ammonia, precursors of microbial amino acids (Walker & Nader, 1968; Al-Rabbat, Baldwin & Weir, 1971*a*). However, these methods suffer from the fact that considerable amounts of microbial N and S may be derived from direct incorporation of extracellular peptides or amino acids or both (Al-Rabbat, Baldwin & Weir, 1971*b*; Bryant, 1974; Gawthorne & Nader, 1976; Nolan, Norton & Leng, 1976). Determination of ^{35}S -labelled sulphide is subject to technical difficulties (Walker & Nader, 1968; Nikolic, Jovanovic & Filipovic, 1975), and the value for S:N in microbial matter is low and may vary between 0.046 (Bird, 1973) and 0.081 (Walker & Nader, 1975). As it is known that phosphorylated compounds such as nucleotides cannot penetrate into microbial cells (Kepes & Cohen, 1962; Yagil & Beacham, 1975), but nucleic acid-phosphorus forms the major part of microbial P, it may be accepted that all microbial P is derived from the extracellular phosphate pool. Also, Smith (1969) reported that the value for the ratio, nucleic acid-N:total N in rumen microbial matter is 'reasonably constant' (c. 0.19). Bucholtz & Bergen (1973) studied ^{33}P -labelled phosphate incorporation into rumen microbial phospholipids (PL) and converted their results into N incorporation using a ratio PL-P:N in microbial matter, the values varying between 0.016 and 0.029. It is evident that PL-P contributes much less to total microbial P than nucleic acid-P. For these reasons, we decided to use the incorporation of ^{32}P -labelled extracellular phosphate in total microbial P as the measure of microbial growth. This paper gives experimental details and a critical evaluation of the technique adopted. Some of the results have been reported previously (Van Nevel & Demeyer, 1973; Demeyer & Van Nevel, 1974; Van Nevel, Demeyer & Maeng, 1976).

EXPERIMENTAL

Animals

Rumen contents were obtained from four individually penned wethers provided with a rumen fistula and given hay (400 g) and commercial concentrates (200 g) twice daily. One of the sheep was given a molasses-based diet (Marty & Demeyer, 1973). Samples were withdrawn from the rumen for incubation or analysis of microbial matter using the apparatus described by Hungate (1950) after food had been withheld for at least 24 h. It was accepted that essentially all food protein had then disappeared from the rumen.

Incubations

Rumen samples were filtered through stainless-steel wire gauze (16 mesh) and 40 ml portions of rumen fluid were transferred anaerobically (carbon dioxide flushing) to incubation flasks containing 10 ml distilled water with 1 mmol glucose (reagent grade; E. Merck, A.G. Darmstadt, W. Germany), 5 mg N as ammonium bicarbonate (reagent grade; UCB, Brussels, Belgium) and 1–2 μCi (maximum volume 0.5 ml) $\text{H}_3^{32}\text{PO}_4$ (2 mCi carrier-free $\text{H}_3^{32}\text{PO}_4$ was diluted to 25 ml with 0.01 M- H_3PO_4). The isotope was obtained from IRE, Fleurus, Belgium. Incubation was done at 39° under CO_2 in a shaking water-bath for periods between 1 and 4 h as indicated for each experiment. Fermentation was stopped at the end of incubation by injection, of 1 ml 5 M-sulphuric acid. As lactic acid accumu-

lated with glucose as substrate, a mixture of cellobiose (250 μmol) and maltose (250 μmol) was used in later experiments. Incubation flasks were fitted with a silicon-rubber septum which facilitated the sampling of gases. Blank values were obtained by adding 1 ml 5 M- H_2SO_4 to the flasks before incubation to completely inhibit microbial activity. Washed cell suspensions (WCS) were prepared as described by Demeyer & Henderickx (1967), but Hungate's (1969) solutions A and B were used instead of 0.066 M-phosphate buffer, giving an extracellular phosphate pool size comparable with the value obtained for rumen fluid incubations.

Analysis

After acidification of the incubations, gas samples were obtained using a gas-tight syringe (Precision Sampling Corp., Baton Rouge, Louisiana 70815, USA) and methane and hydrogen contents were determined as described previously (Demeyer & Henderickx, 1967). Incubation contents were then centrifuged (10 min at 22000 g and 2°) (MSE High Speed 18 centrifuge; Measuring and Scientific Equipment Ltd, Crawley, Surrey) and the supernatant fraction was used for determination of VFA and lactic acid (Van Nevel, Henderickx, Demeyer & Martin, 1969), ammonia-N (AN) (Conway, 1957), total N referred to as soluble total N (STN) (micro-Kjeldahl method) and inorganic P as phosphate determined by the phosphomolybdate method (Herbert, Phipps & Strange, 1971). It was found that $98.2 \pm 0.9\%$ (mean value \pm SE; n 11) of total P in the supernatant fraction was present as phosphate. For calculation of the extracellular pool specific activity (SA), phosphate was determined in the supernatant fraction of acidified, non-incubated rumen fluid. The precipitate was washed five times with 15 ml sodium chloride solution (8.5 g/l) to remove extracellular radioactivity. The final pellet was re-homogenized in water using a 20 ml Teflon pestle tissue grinder (Thomas Co., Pennsylvania 19105, USA) and the suspension diluted to a final volume of 50 ml. Portions (5 ml) of this suspension were slowly digested with 5 ml perchloric acid (700 g/l) in micro-Kjeldahl flasks until colourless, then were diluted with water to 25 ml. Portions (10 ml) of this mixture were then counted to determine the amount of radioactivity incorporated in the microbes. Duplicate samples of re-homogenized microbial matter were transferred to preweighed aluminium-foil dishes and dried at 105° for at least 24 h and were also used to determine total N (micro-Kjeldahl method) and total P contents after digestion with HClO_4 (phosphomolybdate method). After digestion with HClO_4 P was also determined in PL, separated as described by Sutherland & Wilkinson (1971) and in nucleic acids separated as described by Maeng, Van Nevel, Baldwin & Morris (1976), whereas other portions of the digested samples were used for determinations of radioactivity content. Polysaccharides were determined on other samples of the re-homogenized microbial matter using the phenol- H_2SO_4 method (Herbert *et al.* 1971). Radioactivity was determined using a liquid-scintillation apparatus (Corumatic 200 Tracerlab, Belgium) by Cerenkov counting, requiring no addition of scintillators (Parker & Elrick, 1970). The external-standard, channels-ratio method (Kamp & Blanchard, 1971) was used to correct for colour quenching and a quench correction curve was constructed using yellow-coloured, incompletely digested samples.

RESULTS AND DISCUSSION

Incorporation of radioactivity

After incubation of 40 ml rumen fluid for 2 h with approximately 2 μCi ^{32}P -labelled phosphate, about 1% of the radioactivity added was incorporated into the microbial precipitate. In the fifth and last washing of this precipitate the amount of radioactivity recovered was 0.024 ± 0.007 and $3.6 \pm 0.8\%$ of total radioactivity added and incorporated respectively (mean \pm SE, n 6). Blank incorporation (microbes inactivated by the addition of H_2SO_4 to the

Table 1. *Distribution of ^{32}P incorporated into P-containing cell components during incubation of rumen fluid and washed cell suspensions of mixed rumen bacteria (WCS) from sheep**

(Mean values with their standard errors; no. of replicates in parentheses)

sample	Amount of radioactivity recovered (%) in:				Amount of total radioactivity incorporated (% recovered)
	Inorganic P	Phospholipid	RNA	DNA	
WCS (10)	1.1 ± 0.1	18.4 ± 0.8	71.9 ± 0.7	8.6 ± 0.7	111.6 ± 2.0
Rumen fluid (2)	3.9 ± 0.1	21.4 ± 0.8	72.0 ± 0.7	2.7 ± 0.1	88.8 ± 4.3

* WCS or rumen fluid (40 ml) was incubated with 10 ml phosphate buffer (Hungate, 1969) or water respectively which contained, respectively 500 or 250 μmol cellobiose, 500 or 250 μmol maltose, 10 or 5 mg nitrogen as NH_4HCO_3 , 2–3 $\mu\text{Ci } ^{32}\text{PO}_4^{3-}$. Samples were incubated for 4 h (WCS) or 2 h (rumen fluid) under carbon dioxide.

Table 2. *Size and specific activity (SA) of the extracellular phosphate pool and incorporation of ^{32}P in microbial matter of rumen fluid from sheep**

(Mean values with their standard errors for one observation; no. of replicates in parentheses)

Expt no.	Samples	Pool size				Pool SA (dis-integrations/min ($\times 10^3$) per mg P)		Amount of ^{32}P incorporated			
		mg P		dis-integrations/min ($\times 10^3$)		Mean	SE	dis-integrations/min		$\mu\text{g P}$	
		Mean	SE	Mean	SE			Mean	SE	Mean	SE
1	Blank (3)	31.7	0.3	2535	38	79.9	1.4	991	191	—	—
	Incubated samples (5)	31.2	0.4	2565	14	82.3	1.2	20645	293	254†	6
2	Blank (3)	35.9	0.1	3663	59	102.0	1.7	1513	91	—	—
	Incubated samples (5)	35.4	0.2	3621	16	102.3	0.5	25220	1176	248†	12

* Rumen fluid (40 ml) was incubated with 10 ml H_2O containing 1 mmol glucose, 5 mg nitrogen as NH_4HCO_3 , 1–2 $\mu\text{Ci } ^{32}\text{PO}_4^{3-}$. Samples were incubated for 2 h under carbon dioxide. Fermentation was stopped by injection of 1 ml 5 M-sulphuric acid. Blank samples were treated exactly as incubated samples, but 1 ml 5 M- H_2SO_4 was added before incubation.

† Corrected for incorporation obtained with blank samples (micro-organisms inactivated by H_2SO_4).

incubation medium) represented about 5% of the normal values obtained for incorporation of radioactivity. Table 1 shows the distribution of radioactivity incorporated into the different cellular fractions: it is clear that nucleic acids incorporated most of the radioactivity, whereas cell components other than those studied (e.g. polyphosphates) were obviously of minor importance.

Incorporation of P

In Table 2 it can be seen that any change in the SA of P in the extracellular phosphate pool was too small to be detected; this was the result of the very low fractional turnover-rate of this pool (approximately 0.004/h) and, therefore, the amount of P incorporated in microbial matter could be calculated using the following equation:

$$P_i = \frac{\text{dpm}_i}{\text{SA}_p}, \text{ where } P_i \text{ is the amount of P incorporated (mg),}$$

Table 3. Effect of varying the specific activity (SA) of the extracellular phosphate pool on incorporation of ^{32}P in microbial cells of rumen fluid from sheep*

(Mean values with their standard errors for two replicates)

Expt no.	SA of extracellular pool (counts/min per mg P)		Amount of radioactivity incorporated (counts/min per flask)		Amount of P incorporated ($\mu\text{g P/flask}$)	
	Mean	SE	Mean	SE	Mean	SE
1	30943	210	23630	276	7647	
	57974	429	44041	8617	760149	
2	58369	655	31110	467	5408	
	115345	587	62242	757	5337	

* Rumen fluid (40 ml) was incubated with 10 ml water containing 1 mmol glucose, 5 mg nitrogen as NH_4HCO_3 , 250 μmol cellobiose, 250 μmol maltose, 1–2 $\mu\text{Ci } ^{32}\text{PO}_4^{3-}$. Samples were incubated for 3 h under carbon dioxide. Fermentation was stopped by injection of 1 ml 5 M-sulphuric acid.

dpm_i is the amount of radioactivity incorporated (corrected for the blank value), SA_p is the SA of the extracellular phosphate pool (disintegrations/min per mg P).

The values given in Table 2 can also be used to evaluate the reproducibility of the method. Changing the amount of extracellular phosphate pool SA resulted in a perfect response in the amount of radioactivity incorporated (Table 3), suggesting an equilibrium between extracellular and intracellular phosphate pool. Bucholtz & Bergen (1973) obtained a different value for SA for the intra- and extra-cellular inorganic phosphate pool. However, accurate determination of the intracellular pool SA is difficult because of the presence of very labile organic P components in the cell (Herbert *et al.* 1971).

Incorporation of N

From the amount of P incorporated, the amount of N incorporated can be calculated using as the conversion factor the value for N:P in microbial matter. It is known, however, that the chemical composition of microbial matter is not constant and may vary with the animal, depending on the diet and environment (Smith & McAllan, 1974). The value for N:P as well as the amounts of P and N in microbial dry matter (DM) (g/kg) was determined for a period of nearly 2 years using rumen microbial matter, separated by acidification, centrifugation and washing of rumen fluid taken before morning feeding from four sheep given hay and concentrates (sheep A, 6 and 7) or a molasses-based diet (sheep B). The results presented in Table 4 showed that the amounts of N and P (g/kg DM) varied more than the value for N:P, for which an over-all mean value (\pm SE for one observation, n 40) was 8.37 ± 0.75 . For WCS, mean values (\pm SE for one observation, n 11, three sheep) for the amounts of N and P (g/kg DM) and for N:P were 84.8 ± 8.6 , 14.2 ± 1.7 and 6.05 ± 0.80 respectively. The differences between values for WCS and those for rumen fluid were obviously related to the presence of protozoa and possibly undigested food particles in the rumen fluid material. In the calculation of the amount of N incorporated using values for P-incorporation, a value for N:P of 8.37 was used for rumen fluid and a value of 6.05 for WCS.

Values for N incorporation calculated from values for P incorporation, determined using incubations of strained rumen fluid or WCS in the absence of substrate protein, were compared with values for the incorporation of AN or STN obtained using the same incubations. The latter values were calculated from the decrease, during incubation, in AN and STN concentrations determined using the supernatant fraction obtained after acidification and centrifugation. From the values presented in Table 5, it is clear that STN incorporation

Table 4. *Phosphorus and nitrogen contents of rumen microbial dry matter (DM) from sheep*
 (Mean values with their standard errors, no. of determinations, each carried out in triplicate, in parentheses)

Sheep	Period of study	DM		P				N				N:P in DM		
		(g/l rumen fluid)		mg/l		mg/g DM		mg/l		mg/g DM		Mean		SE
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
A (7)	28 Aug.-8 Oct. 1972	11.43	2.44	83	6	8.3	2.5	689	52	66	25	8.29	0.09	
A (7)	9 May-7 Nov. 1973	13.73	1.46	96	17	7.0	1.4	756	242	66	11	7.86	0.28	
A (6)	11 Oct. 1973-16 Jan. 1974	15.24	2.35	94	12	6.2	1.3	846	127	62	11	9.15	0.46	
7 (8)	1 Oct. 1973-16 Jan. 1974	8.41	1.82	52	9	6.1	1.7	806	114	53	14	8.55	0.17	
6 (6)	15 Oct. 1973-21 Jan. 1974	24.66	1.52	211	37	8.6	1.6	433	82	52	15	8.50	0.39	
B (6)	20 Sept. 1972-6 Feb. 1974							1768	345	72	15	8.17	0.30	
Over-all mean (40)												8.37	0.75*	

* Mean value with its standard error for one observation.

Table 5. Comparison of values for nitrogen incorporation in microbial matter of rumen fluid and washed cell suspensions of mixed rumen bacteria (WCS) from sheep, obtained by calculation from ammonia-N (AN), soluble total N (STN) or ^{32}P incorporation

(Mean values with their standard errors for six determinations for rumen fluid and four determinations for WCS)

Sample	Incubation period (h)	Amount of N incorporated (mg N/incubation flask)						Difference (C) - (B)	
		AN (A)		STN (B)		N from ^{32}P (C)		mg N/incubation flask	mg N/h per incubation flask
		Mean	SE	Mean	SE	Mean	SE		
Rumen fluid*	1	0.57	0.12	0.94	0.28	2.00	0.23	1.06	1.06
	2	1.31	0.06	1.64	0.23	3.86	0.24	2.22	1.11
	3	1.75	0.08	2.41	0.21	5.30	0.25	2.89	0.96
WCS†	4	1.54	0.25	2.08	0.05	2.60	0.18	0.52	0.13

* Rumen fluid (40 ml) was incubated with 10 ml water containing 250 μmol cellobiose, 250 μmol maltose, 5 mg N as NH_4HCO_3 , 2 μCi $^{32}\text{PO}_4^{3-}$ under carbon dioxide.

† WCS (40 ml) was incubated with 10 ml phosphate buffer (Hungate, 1969) containing 500 μmol cellobiose, 500 μmol maltose, 10 mg N as NH_4HCO_3 , 2-3 μCi $^{32}\text{PO}_4^{3-}$ under CO_2 .

was higher than AN incorporation, indicating the presence of N sources in the incubation other than the NH_4HCO_3 added.

However, both values were considerably lower than those for N incorporation calculated from P incorporation. Ammonia production by degradation of food protein or amino acids, or both, still present in the rumen at the time of sampling cannot completely be excluded. However, such production can only account for a small fraction of the discrepancy, as rumen samples were obtained after a fasting period of at least 24 h. Furthermore, differences in values were obtained with WCS as well as with rumen fluid. Also, for the same reason, the fact that protozoa do not use AN (Smith, 1975) in contrast to bacteria, whereas both protozoa and bacteria probably incorporate phosphate, cannot offer the only explanation for the discrepancy. When corrected for the period of incubation, however, differences in values obtained with WCS were considerably smaller (Table 5), suggesting an important role of protozoa in this phenomenon. It is known that protozoa are the most important agents responsible for the degradation of bacteria in the rumen, resulting in N recycling (Coleman, 1964; Jarvis, 1968). Also, it should be realized that isotope incorporation was not affected by degradation of non-labelled cells, whereas incorporation of AN or STN as determined in the present study was the result of microbial growth and degradation and could be defined as 'net incorporation or growth'. N incorporation as determined by isotope incorporation could then be defined as 'total incorporation or growth'.

In addition to degradation of non-labelled cells (extracellular turnover), another reason for the difference between total and net growth obtained in the present study could have been the intracellular turnover of phosphate in the cell components studied. It was found previously that during growth, little or no turnover of nucleic acid phosphorus occurs (Mitchell & Moyle, 1953), and total cellular PL are also quite stable (Kanemasa, Akamatsu & Nojima, 1967). Both fractions represent about 90 % of the total microbial P. It is known that messenger-RNA is rapidly turning over in cells, but this would not prevent the use of labelled phosphate incorporation as a measure of net intracellular rate of RNA synthesis, because messenger-RNA represents only a minor cell constituent (Koch, 1971). In our

Table 6. *Specific rates of synthesis and degradation of various microbial organic phosphorus components of washed cell suspensions of mixed rumen bacteria (WCS) from sheep**

(Mean values with their standard errors for ten determinations, except phospholipids where six determinations were done; values are expressed on a per 100 ml WCS basis)

Component	Present initially		Net incorporation†		Total incorporation‡		Degradation‡		Total incorporation		Degradation		Net incorporation	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	Specific rates (h(× 100)) of:													
DM (mg)	265	15	133	8	—	—	—	—	—	—	—	—	—	—
Organic P (μg)														
Total-P	3154	187	803	38	1082	58	279	32	8·6	0·5	2·2	0·3	6·4	0·3
Phospholipid-P	373	14	165	5	198	4	33	4	13·3	0·7	2·2	0·2	10·0	1·6
RNA-P	1771	103	566	40	853	32	287	45	12·1	0·5	3·6	0·8	8·0	0·6
DNA-P	774	64	93	30	123	9	45	23	4·0	0·2	1·3	0·7	3·0	1·0
Total P in DM (mg/g)	11·9	0·2	6·4	0·5	—	—	—	—	—	—	—	—	—	—

DM, dry matter.

* WCS (40 ml) was incubated with 10 ml phosphate buffer (Hungate, 1969) containing 500 μmol cellobiose, 500 μmol maltose, 10 mg nitrogen as NH₄HCO₃, approximately 3 μCi ³²P₄³⁻. Samples were incubated for 4 h under carbon dioxide.

† Calculated from chemical determination of cell components.

‡ Calculated from ³²P incorporation in cell components.

§ Degradation = total increase - net increase.

Table 7. Phosphorus distribution (mg/g total P in cell components) in synthesized and degraded rumen bacterial matter*

(Mean values with their standard errors)

Cell component	Initially present		Net increase		Total increase		Degradation	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Phospholipids	128	3	202	16	163	3	88	16
RNA	593	23	662	18	726	4	836	34
DNA	279	18	136	29	111	7	76	50

* Calculated from values given in Table 6.

Table 8. Nucleic acid and polysaccharide formation (mg) during incubation of rumen fluid from sheep*

(Mean values with their standard errors for seven determinations for nucleic acids and four determinations for polysaccharides)

Incubation period (h)	Nucleic acids†		Polysaccharides	
	Mean	SE	Mean	SE
1	1.3	0.3	18.2	3.2
2	2.9	0.1	34.9	8.1
3	4.3	0.4	36.0	5.7

* Rumen fluid (40 ml) was incubated with 10 ml water containing 250 μmol maltose, 250 μmol cellobiose, 5 mg nitrogen as NH_4HCO_3 , 1–2 μCi $^{32}\text{PO}_4^{3-}$. Samples were incubated under carbon dioxide.

† Calculated from phosphorus incorporation, assuming nucleic acids account for 700 mg/g P incorporated and contain 98.5 mg P/g.

experiments, we could not distinguish between intra- and extra-cellular turnover of P-containing cell components, but the effect on growth measurements was the same, irrespective of the site of turnover. It seems likely that the discrepancy between N incorporations calculated from ^{32}P and STN incorporation was mainly due to the occurrence of cell degradation during incubation. A fourth reason for this discrepancy, however, may have been a lower value for N:P in the newly-synthesized microbes as compared to the organisms present initially, as it is known, for example, that the value protein:RNA decreases with increasing growth rate (Nierlich, 1974). In order to account completely for the differences observed with strained rumen fluid incubations, a decrease in N:P from an initial value of 8.37 to approximately 4 should be involved (Table 5), which we consider to be unlikely.

In order to study further the existence of cell degradation and possible changes in cell composition during growth, incubations with WCS were carried out. Total incorporation of P in RNA, DNA, PL and in total microbial P was calculated from ^{32}P incorporation, whereas net incorporation was determined by chemical methods. The results, summarized in Table 6, clearly indicated that simultaneous synthesis and degradation of all components studied occurred at different specific rates; PL and RNA were synthesized and, to a lesser extent, degraded much faster than DNA.

From these values the distribution of P in the different fractions synthesized and degraded was calculated and the results indicated a changing chemical composition of microbial matter during incubation (Table 7).

Values shown in Table 6 indicated that the P content of the newly formed microbial DM (net increase) was lower than the value for material present initially, indicating higher

Table 9. *Phosphorus incorporation and lactate formation by rumen fluid from sheep**

Expt no.	Hexose fermented† ($\mu\text{mol C}_6$ /incubation flask)	(No. of replicates in parentheses)						P incorporation‡ ($\mu\text{g}/\text{mmol C}_6$)	Growth efficiency§ (g N _i /kg OM _r)
		Fermentation end-products ($\mu\text{mol}/\text{mmol C}_6$)‡							
		Acetate	Propionate	Butyrate	Lactate	Methane			
1 (2)	543	760	670	110	360	260	606	31.3	
2 (2)	566	640	780	90	400	180	630	32.5	
3 (5)	491	620	610	110	550	220	509	26.3	
4 (5)	689	460	550	100	790	110	438	22.6	
5 (2)	629	440	430	150	840	140	423	21.9	
6 (2)	648	500	500	110	770	90	354	18.3	

* Rumen fluid (40 ml) was incubated with 10 ml water, containing 1 mmol glucose, 5 mg N as NH_4HCO_3 , 1–2 $\mu\text{Ci } ^{32}\text{P}$. Samples were incubated for 2 h under carbon dioxide. Although the incubation period was the same for all experiments, a different fermentation pattern was obtained; the reason for this finding is not known.

† Calculated as: hexose fermented = acetate/2 + propionate/2 + butyrate + lactate/2 = C_6 (Demeyer & Van Nevel, 1975).

‡ $\mu\text{mol}/\text{mmol}$ hexose fermented.

§ Calculated from amount of ^{32}P incorporated, N/P in microbial matter and OM_r as described on p. 111.

Table 10. Effect of incubation period on the efficiency of 'total growth' of microbial matter in rumen fluid from sheep*

(Mean values with their standard errors for five determinations for Expt 1 and for six determinations for Expt 2)

Incubation period (h)	Hexose fermented† (μmol)				Growth efficiency ($\text{g N}_i/\text{kg OM}_F$)‡			
	Expt 1		Expt 2		Expt 1		Expt. 2	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	303	31	324	19	30.5	6.7	31.3	4.6
2	445	34	498	20	44.6	7.1	43.5	6.1
3	488	26	570	46	57.7	7.1	50.8	5.2

OM_F, amount of hexose fermented (μmol) $\times 0.162$; N_i, amount of nitrogen incorporated.

* Rumen fluid (40 ml) was incubated with 10 ml water containing 250 μmol cellobiose, 250 μmol maltose, 5 mg N as NH_4HCO_3 , 2 μCi ^{32}P $_4^{-3}$. Samples were incubated under carbon dioxide.

† Calculated as: hexose fermented = acetate/2 + propionate/2 + butyrate + lactate/2 = C₆ (Demeyer & Van Navel, 1975) (see Table 9).

‡ Calculated from amount of ^{32}P incorporated (see Table 9).

specific rates of synthesis of non-P-containing microbial fractions such as polysaccharides. Values from incubations using rumen fluid indicated that the amounts of polysaccharide formed were ten times greater than the amounts of nucleic acids formed, as calculated from P incorporation (Table 8), at least in the first 1 h of incubation.

Relation to fermentation

Results given in Table 9 indicated that total incorporation of P and N, determined and calculated as described, was closely related to the energy yield of the fermentation. Total P incorporation, expressed per unit amount of substrate (glucose) fermented, decreased when the amount of lactate formed per unit amount of substrate fermented increased.

It is indeed well known that energy yield in lactate production is less efficient than in VFA production (Walker, 1968). From the amounts of P incorporated and fermentation end-products formed total microbial growth efficiency can be calculated as $\text{g N}_i/\text{kg DOM}_R$, where DOM_R only differs from the fraction fermented (OM_F) when the outflow of the rumen contains endproducts of fermentation such as VFA or endogenous additions, or both, or when food OM is directly absorbed through the rumen wall (Egan, 1974), and OM_F was calculated from the amount of hexose fermented (cf. footnote to Table 9), using a molecular weight of 162. Values were obtained which were within the range of many other reported values obtained in vivo for net growth: $32.2 \pm 13.9 \text{ g N}_i/\text{kg DOM}_R$ (mean value \pm SE calculated from 126 individual results obtained by other workers (Hogan & Weston, 1969; Hume, 1970*a, b*; Hume & Bird, 1970; Hume, Moir & Somers, 1970; Hogan & Weston, 1971; Ishaque *et al.* 1971; Jackson *et al.* 1971; Ørskov, Fraser & McDonald, 1971, 1972; Mathison & Milligan, 1971; Coelho da Silva, Seeley, Thomson, Beever & Armstrong, 1972; Leibholz, 1972; Lindsay & Hogan, 1972; Hagemester & Pfeffer, 1973; Hagemester & Kaufmann, 1974; Beever, Thomson & Harrison, 1974; Hume & Purser, 1975; Sutton, Smith, McAllan, Storry & Corse, 1975; McMeniman *et al.* 1976) and in vitro for total growth: $43.7 \pm 22.6 \text{ g N}_i/\text{kg DOM}_R$ (mean value \pm SE, eighteen individual values obtained by other workers (Walker & Nader, 1968; Al-Rabbat *et al.* 1971*b*; Bucholtz & Bergen, 1973; Nikolic *et al.* 1975)). When growth efficiency values were calculated for incubations using cellobiose and maltose as substrates, higher values were obtained, which increased with increasing period of incubation (Table 10). The higher values obtained after a 2 h incubation period compared with those presented in

Table 9 were not surprising because lactate was absent in the former incubations, and additional energy may be released in the fermentation of a disaccharide as compared to a monosaccharide, by phosphorylytic cleavage (Walker, 1968). The increase in growth efficiency with increasing period of incubation (Table 8) was obviously related to preferential synthesis of polysaccharides during the first hours of incubation (Walker & Nader, 1970). However, other changes in cell composition, e.g. in N:P, may have been involved.

Summarizing, the results indicated that extent of synthesis of rumen microbial P can easily be determined quantitatively from incorporation of ^{32}P -labelled phosphate. The extracellular pool size is large, resulting in a low fractional turnover-rate and a constant SA, enabling simple calculation. This is in contrast to the small pool size of e.g. sulphide, where the pool SA changes during incubation (Davis & Hall, 1970). Chemical determination of P is simple and accurate, and radioactivity determination by Cerenkov counting does not require expensive scintillation mixtures. N incorporation can be calculated from P incorporation, using values for N:P determined for rumen microbial matter. Using this method, however, as with all isotope methods for the determination of rumen microbial growth *in vitro*, some basic assumptions are made. (1) All synthesis of the microbial fraction studied is derived from the labelled inorganic precursor pool; (2) the SA of the intracellular precursor pool should equal the SA of the extracellular pool; (3) there is no degradation of non-labelled cells; (4) cell composition remains constant during growth. Our results show that assumptions 3 and 4 are not valid in incubations with rumen micro-organisms.

The results presented illustrate well-known changes in polysaccharide content as well as in PL and nucleic acid contents during growth, as indicated by the different specific rates of synthesis and degradation. Similar differences may exist for the rates of synthesis and degradation for different amino acids and proteins, rendering interpretation of results obtained using $^{15}\text{NH}_3$ or $^{35}\text{S}^{2-}$ incorporation difficult. Also, the existence of simultaneous synthesis and degradation of microbial matter should be realized. Short-term, *in vitro* isotope incorporation measures 'total growth' rather than 'net growth', which is the balance of synthesis and degradation. The difference between total and net incorporation (calculated from STN) can be used as a measure of degradation. Table 5 shows that degradation with strained rumen fluid was approximately 50% of total incorporation, compared to values of approximately 30% calculated from results obtained *in vivo* by Nolan *et al.* (1976). Engulfment by protozoa is not the only factor determining degradation, as this was also observed in incubations with WCS although to a lesser extent. Other factors, e.g. the presence of bacteriophages (Adams, Gazaway, Brailsford, Hartman & Jacobson, 1966; Paynter, Ewert & Chalupa, 1969), mycoplasma (Robinson & Hungate, 1973), damage to bacteria due to manipulation of rumen fluid before incubation (temperature and osmotic shock, oxygen-damage) and during incubation (substrates not available for all bacteria, absence of essential nutrients in the incubation) are obviously involved.

Total growth is mainly determined by the yield of energy in the fermentation of food OM, and it may be affected by the type of fermentation (Jackson *et al.* 1971; Harrison *et al.* 1975) and by the efficiency of utilization of energy for growth, influenced by the specific growth rate of the micro-organisms (Harrison *et al.* 1975; Isaacson, Hinds, Bryant & Owens, 1975). Net growth, although the more relevant index in economic considerations of the rumen, is determined by an additional complex of factors, quite independent from energetic considerations and mainly related to microbial antagonisms and toxic effects on the microbes in the rumen environment. In extreme situations, degradation of microbial matter may become more important than synthesis, resulting in negative net growth values (Naga & Harmeyer, 1975).

In a series of incubations with strained rumen contents devoid of food protein, Durand,

Van Nevel, Dumay, Tassencourt, Beaumatin & Kumaresan (1976) calculated a regression equation for the relationship between results for P incorporation obtained using the method described in the present paper and results for AN incorporation obtained by chemical determination. The regression was then used to calculate microbial N incorporation in incubations containing food protein. It should be understood that such a procedure is of limited value only, as it assumes a constant value for N:P in microbial DM, as well as a constant value for net growth: total growth.

Because of the complexity of the interactions determining net yields of rumen microbial matter, critical interpretation of rumen microbial growth yields determined from isotope incorporation in vitro is indicated and methods determining both net and total growth should be used wherever possible.

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