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Energy Utilization for Polysaccharide Synthesis by Mixed Rumen Organisms Fermenting Soluble Carbohydrates

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Synthesis of reserve polysaccharide by mixed rumen organisms fermenting glucose, maltose, cellobiose, and xylose has been studied in relation to the adenosine triphosphate energy calculated to be available from substrate fermentation. About 80% of the energy available from glucose and xylose was used for polysaccharide synthesis, whereas, assuming hydrolytic cleavage of the disaccharides, more than 100% was used when cellobiose and maltose were the substrates. If, however, phosphorolytic cleavage of the disaccharides, for which there is evidence, was involved, the energy from both maltose and cellobiose fermentation was used with about the same efficiency as that from glucose and xylose fermentation. The rumen fluid used was collected 24 hr after feeding, and growth of microorganisms in such samples was sufficient to account for utilization of less than 10% of the total energy becoming available during the 40-min incubation period.

For the microbial population of the rumen to maintain its numbers in the face of dilution by incoming feed and water and against continual loss of cells from passage of ingesta from the rumen to distal parts of the digestive tract, energy must be made available for microbial growth. This energy is obtained largely by microbial degradation of dietary carbohydrate to volatile fatty acids (VFA). The efficiency with which the microbes use the energy becoming available has considerable bearing upon the cost to the animal of having most of the utilizable carbohydrate in its diet degraded to VFA.

Since the useful energy becoming available to the microbes can be assessed in terms of adenosine triphosphate (ATP) and since the pathways of formation of the end products of ruminal fermentation are fairly well defined (13), it is possible to study some aspects of the efficiency of energy utilization by the rumen microflora.

Immediately after feeding, there is a phase of rapid ruminal fermentation associated with the degradation of readily available carbohydrate (13). Despite the fact that during this time a great deal of energy must become available, data obtained by Warner (18) suggest that microbial growth rates are not correspondingly high. It then becomes important to know whether the energy is uncoupled and wasted or whether a mechanism exists for its conservation.

In view of the known capacity of many species of rumen organisms to accumulate reserve polysaccharide, this investigation was designed to study the energetic relationship between soluble carbohydrate fermented and that laid down as microbial polysaccharide.

MATERIALS AND METHODS

Bacteriological. Rumen fluid was collected via fistula 24 hr after feeding from a sheep maintained on a ration of equal parts of wheaten hay chaff and lucerne hay chaff. Large particles were removed after the rumen fluid had been allowed to stand for 10 min in a separating funnel. A 30-ml amount of the remaining microbial suspension was then incubated at 39 C for 10 min to achieve temperature equilibration before adding 3.0 ml of substrate solution also warmed to 39 C. Control incubations containing water instead of substrate solution were included in each experiment. At all times after collection of the rumen fluid, a gas phase of nitrogen-40% CO_2 was maintained.

Sampling. After thorough mixing, 5-ml samples were withdrawn from the incubation mixture at zero-time and at 10, 20, 30, and 40 min after the addition of substrate and were mixed with 1 ml of 10 N H_2SO_4. The acidified samples were allowed to stand at 4 C for 30 min and then were centrifuged at 22,000 $\times$ *g* for 5 min. The supernatant fluid was retained for assay of residual substrate and lactic acid, whereas the cell debris was washed twice with 1 N H_2SO_4 and finally resuspended evenly in 4 ml of water.

Substrates. Glucose and xylose were added at a concentration of 0.1 M, and cellobiose and maltose were added at a concentration of 0.05 M.

Residual substrate. Duplicate 0.3-ml samples of acidified supernatant fluid were carefully neutralized with 0.5 N NaOH, and reducing sugar was estimated by the titrimetric method of Somogyi (11).

Lactate. Lactate was determined by the method of Elsden and Gibson (5).

Polysaccharide. The washed cell debris suspension was diluted 1:10 with water, and duplicate 0.5-ml samples were used in the cysteine-H_2SO_4 method for hexose polysaccharide described by Dische (4). Glucose was used to prepare a standard curve for each assay.

Washed cell suspensions. After separating off large particles as described above, rumen fluid was centrifuged at 22,000 × *g* for 10 min and the deposited cells were washed twice with oxygen-free 0.01 M KH_2PO_4-K_2HPO_4 buffer (*p*H 6.2) containing 0.08 M $NaHCO_3$ and saturated with CO_2. The cells were finally resuspended in the washing solution to give a cell density about twice that in the original rumen fluid.

VFA. VFA were recovered from acidified reaction mixtures by steam distillation. Neutralized distillates were concentrated to small volume and the proportions of the individual VFA were determined after separation by the gas-chromatographic technique of James and Martin (9).

Microbial growth. Estimates of microbial growth during incubation with added carbohydrate were obtained by measuring microbial protein synthesis with the incubation technique of Walker and Nader (16); this technique relies on the incorporation of radioactive sulfur from the rumen liquor sulfide pool.

RESULTS AND DISCUSSION

Utilization of substrate, synthesis of polysaccharide, and production of lactate for fermentations of cellobiose, maltose, glucose, and xylose are shown in Fig. 1. All of the changes measured were represented by linear relationships with time over the 40-min incubation period used. Repeat incubations with rumen liquor collected on different days gave substantially the same results with all four substrates. The data from these experiments have been used to assemble the figures for ATP availability given in Table 1. To increase precision, the slopes of the lines (Fig. 1) were used to obtain rates of ATP production and utilization. This was desirable since fairly small differences in large quantities were being measured in the case of substrate utilization and polysaccharide production. The initial amount of microbial polysaccharide in the reaction mixtures was about 90 μmoles, expressed as glucose, and the increases over 40 min amounted to between about 60 and 100 μmoles.

Although the copper reduction method used for the estimation of residual substrate is not always specific, glucose was metabolized at a greater rate than were the other substrates used. It is therefore safe to assume that, for the disaccharides and xylose, the residual reducing sugar was the original substrate and not accumulating glucose.

It was necessary for rates of VFA production to be calculated as shown in Table 1, since the relatively small increments in VFA over the short sampling period used represented a very small increase in total VFA concentration above that originally present in the rumen fluid. Meaningful measurements of the rate of VFA production were therefore difficult to obtain.

Lactate accumulated during the fermentations of both glucose and cellobiose. Although this is not a normal event within the rumen, it can occur when an animal is presented with a large quantity of readily fermented carbohydrate (14). For the purposes of this communication, since the ATP yield as a consequence of lactate formation is well defined, lactate accumulation does not affect the validity of the calculations.

In calculating the ATP available, the assumption was made that 2 moles of ATP are gained for each mole of VFA produced. The rationale for this has been previously elaborated (13). Insofar as lactate is concerned, since the Embden-Myerhof (EM) pathway of hexose degradation operates exclusively in the rumen (3), 1 mole of ATP is gained for each mole of lactate produced. In none of the experiments did the control incubation mixtures show a detectable fall in polysaccharide content; thus, any energy derivable from this source could be ignored. In other experiments, not reported here, a small reduction in microbial polysaccharide was observed after 2 hr of incubation.

Calculations for the fermentation of xylose were treated separately, since the complexities of

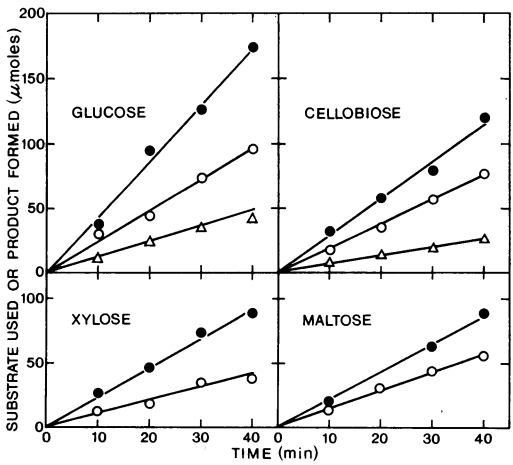

FIG. 1. *Polysaccharide formation from added carbohydrates. Symbols:* ●, *substrate utilization;* ○, *polysaccharide synthesis;* △, *lactate accumulation.*

TABLE 1. *Rates of energy production and utilization during the fermentation of soluble carbohydrates*[a]

Determination	Glucose	Cellobiose	Maltose	Xylose
Rate of substrate utilization[b,c] [*A*]	3.72 (3.05–4.30)	2.86 (2.80–2.92)	1.95 (1.80–2.10)	2.15 (2.00–2.30)
Rate of polysaccharide synthesis[c] [*B*]	2.11 (1.74–2.20)	1.89 (1.86–1.90)	1.32 (1.18–1.45)	1.10 (1.00–1.20)
Rate of lactate accumulation [*C*]	1.15 (0.8–1.40)	0.53 (0.44–0.70)	0	0
Rate of conversion of substrate to VFA [$A - (B + \frac{1}{2}C)$]	1.13 (0.90–1.58)	0.71 (0.67–0.78)	0.62 (0.60–0.65)	
Rate of production of ATP from VFA formation	4.49 (3.60–6.32)	2.84 (2.68–3.12)	2.50 (2.40–2.60)	4.20 (4.05–4.35)
Rate of production of ATP from lactate formation	1.15 (0.80–1.40)	0.53 (0.44–0.70)	0	0
Overall rate of ATP production	5.67 (4.44–7.56)	3.37 (3.14–3.56)	2.50 (2.40–2.60)	4.20 (4.05–4.35)
Rate of ATP utilization for polysaccharide synthesis	4.22 (3.48–4.80)	3.78 (3.60–3.80)	2.62 (2.36–2.90)	3.59 (3.34–3.84)
Per cent of ATP available used for polysaccharide synthesis	75 (64–88)	112 (110–115)	106 (95–111)	85 (82–88)

[a] Results are average of three experiments with each substrate, except for results with xylose which are for two experiments. Range given in parentheses.
[b] Rates expressed as micromoles per minute.
[c] Expressed as micromoles of hexose; with xylose, however, results were calculated as shown in text.

the transaldolase-transketolase pathway must be invoked (13, 17) as follows:

$$3.0 \text{ xylose} + 3.0 \text{ ATP} \xrightarrow{\text{kinase}} 3.0 \text{ xylose-5-P} + 3.0 \text{ ADP}$$

$$3.0 \text{ xylose-5-P} \xrightarrow[\text{epimerase}]{\text{isomerase}} 2.0 \text{ xylulose-5-P} + 1.0 \text{ ribose-5-P}$$

$$1.0 \text{ xylulose-5-P} + 1.0 \text{ ribose-5-P} \xrightarrow{\text{transketolase}} 1.0 \text{ sedoheptulose-7-P} + 1.0 \text{ triose-P}$$

$$1.0 \text{ sedoheptulose-7-P} + 1.0 \text{ triose-P} \xrightarrow{\text{transaldolase}} 1.0 \text{ fructose-6-P} + 1.0 \text{ erythrose-4-P}$$

$$1.0 \text{ erythrose-4-P} + 1.0 \text{ xylulose-5-P} \xrightarrow{\text{transketolase}} 1.0 \text{ fructose-6-P} + 1.0 \text{ triose-P}$$

$$\text{Sum: } 3.0 \text{ xylose} + 3.0 \text{ ATP} \rightarrow 2.0 \text{ fructose-6-P} + 1.0 \text{ triose-P} + 3.0 \text{ ADP}$$

Microbial polysaccharide synthesized from xylose, which has been shown to consist of hexose units (8), can be considered as arising from fructose-6-phosphate (P) after conversion to glucose-6-P, then to glucose-1-P, and finally attaching to to the growing polysaccharide chain with the expenditure of one ATP. Polysaccharide synthesis from the other substrates required 2 moles of ATP per mole of hexose polymerized:

$$\text{hexose} + \text{ATP} \rightarrow \text{hexose-1-P} + \text{ADP}$$

$$\text{hexose-1-P} + \text{UTP} \rightarrow \text{UDP-hexose} + \text{P-P}$$

$$\text{UDP-hexose} + (\text{hexose})_n \rightarrow (\text{hexose})_{n+1} + \text{UDP}$$

$$\text{UDP} + \text{ATP} \rightarrow \text{UTP} + \text{ADP}$$

Less than the maximal amount of ATP expected from glucose and xylose was used in polysaccharide synthesis (Table 1). On the other hand, polysaccharide was synthesized from both cellobiose and maltose in amounts greater than would have been possible with the calculated ATP yield. This latter effect was probably a result of ATP conservation by phosphorylase activity, for which there is evidence in rumen organisms degrading both of these substrates (1, 7). If ATP yields are

TABLE 2. *Efficiency of utilization of ATP for polysaccharide synthesis assuming phosphorolysis of cellobiose and maltose*

Determination	Cellobiose	Maltose
	%	%
ATP yield[a]	3.37	2.50
ATP yield corrected for phosphorolysis	4.80	3.48
Rate of ATP utilization	3.78	2.62
Per cent of corrected ATP used in polysaccharide synthesis	79	75

[a] As calculated in Table 1.

recalculated on the basis of conserving one ATP for the cleavage of each disaccharide molecule, both cellobiose and maltose yield less polysaccharide than could have been formed with the ATP available (Table 2). In fact, for all four substrates, the calculated ATP available appeared to be used with about 80% efficiency.

Fermentation of maltose and cellobiose was further studied in the calorimeter described by Walker and Forrest (15). In these experiments, 2 mmoles of disaccharide were incubated with 250 g of rumen fluid and heat production was recorded continuously until the rate of fermentation had returned to that before addition of substrate. Heat production from 2 mmoles of substrate was 56 cal for cellobiose and 66 cal for maltose. The amount of heat expected if all of the substrates are fermented depends upon the proportions of the end products formed. To obtain an accurate assessment of VFA proportions, washed cell suspensions were used to lower the initial VFA content of the fermentation mixture. Cellobiose gave rise to 61% acetate, 35% propionate, and 4% butyrate, whereas maltose gave rise to 67% acetate, 31% propionate, and 2% butyrate. The proportion of propionate produced from both substrates was higher than that normally produced in the rumen, but recent work in this laboratory with untreated rumen fluid has indicated that maltose gives rise to about 37 to 42% propionate and cellobiose gives rise to between 25 and 30% propionate (M. F. Hopgood, *unpublished data*). Similarly, Sutton (12) observed substantial increases in the proportion of propionate when bovine rumen liquor was incubated with a number of soluble carbohydrates.

Thus, with the proportions of individual VFA given above, the expected heats of fermentation were 180 cal for cellobiose and 178 cal for maltose (W. W. Forrest, *personal communication*) when all of the substrate was degraded. In fact, heat production was only 31% of the maximum for cellobiose and 37% of the maximum for maltose, which compares well with the estimates chemically derived (Table 1) of 34% of the cellobiose and 32% of the maltose being fermented. Thus, the calorimetric data support the general estimates derived from the chemical analyses of the proportions of substrate fermented and polymerized.

The calorimetric estimate of about one-third of the substrate being degraded is consistent with the cleavage of the disaccharides by a hydrolytic mechanism and with the maximal utilization of the ATP produced for polysaccharide synthesis (Fig. 2). However, the direct chemical assays indicated that hydrolytic cleavage of maltose and cellobiose could not generate sufficient energy to account for the polysaccharide synthesized. Similarly, the ATP available from glucose and xylose appeared to be used at about 80% efficiency. Thus, if the proportion of the substrate degraded as determined calorimetrically is taken as representing an 80% efficient system in terms of polysaccharide synthesis, the metabolism of both maltose and cellobiose is consistent with phosphorolytic cleavage of the disaccharide; i.e., with complete utilization of the calculated ATP, one-quarter of the substrate would require fermentation, whereas in an 80% efficient system, one-third of the substrate would have to be degraded.

Two mechanisms alternative to phosphorolysis could result in energy conservation during disaccharide metabolism. First, some disaccharide may be incorporated directly into polysaccharide without prior degradation to monosaccharides. This mechanism does not appear to operate in the case of cellobiose, since the polysaccharide isolated from rumen organisms after cellobiose fermentation had no detectable content of the 1,4-β-glycosidic linkages present in cellobiose itself (Walker, *unpublished data*).

By hydrolysis:

3.0 disaccharide + 3.0 H_2O → 6.0 glucose
2.0 glucose ⟶ 4.0 VFA + 8.0 ATP
4.0 glucose + 8.0 ATP + $(hexose)_n$ ⇢ $(hexose)_{n+4}$

Sum: 3.0 disaccharide + 3 H_2O + $(hexose)_n$ → 4.0 VFA + $(hexose)_{n+4}$

By phosphorolysis:

2.0 disaccharide + 2.0 HOP → 2.0 glucose + 2.0 glucose-1-P
1.0 glucose ⟶ 2.0 VFA + 4.0 ATP
1.0 glucose + 1.0 ATP → 1.0 glucose-1-P
3.0 glucose-1-P + 3.0 ATP + $(hexose)_n$ → $(hexose)_{n+3}$

Sum: 2.0 disaccharide + 2.0 HOP + $(hexose)_n$ → 2.0 VFA + $(hexose)_{n+3}$

FIG. 2. *Polysaccharide yields based upon models involving hydrolysis and phosphorolysis of glucose disaccharides. HOP, orthophosphate.*

3.0 disaccharide + $(hexose)_n \rightarrow$ 3.0 glucose + $(hexose)_{n+3}$
1.0 glucose $\dashrightarrow$ 2.0 VFA + 4.0 ATP
2.0 glucose + 4.0 ATP + $(hexose)_{n+3} \dashrightarrow$ $(hexose)_{n+5}$

Sum: 3.0 disaccharide + $(hexose)_n \rightarrow$ 2.0 VFA + $(hexose)_{n+5}$

FIG. 3. *Synthesis of polysaccharide involving transglycosylation.*

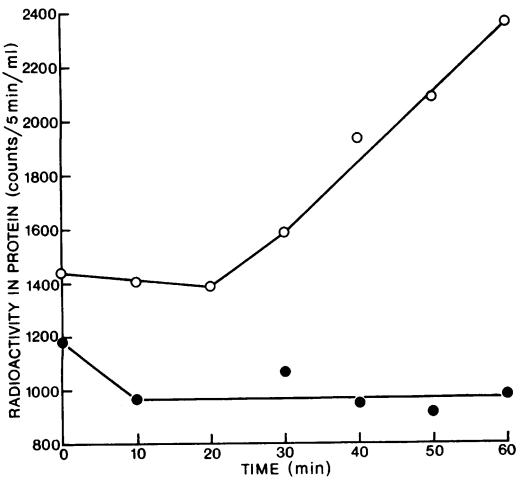

FIG. 4. *Incorporation of ^{35}S sulfide into protein during incubation of rumen fluid with maltose. Symbols: ●, control; ○, plus maltose.*

Second, transglycosylation would enable transfer of glucose units from the disaccharides to a growing polysaccharide chain without utilization of ATP. Such a mechanism would be very efficient in conserving energy, since only 16.5% of the disaccharide would have to be fermented to provide energy for polymerization of the remainder (Fig. 3) in a completely efficient system and 21% of the disaccharide would have to be fermented in an 80% efficient system. However, neither the calorimetric data nor the direct chemical assays reported here support a major transglycosylation mechanism since over 30% of the substrate was fermented.

The reasons for an apparently low efficiency of energy utilization may be twofold. Either the estimates of ATP available may be too high, or energy is being utilized for other purposes such as growth. There is good evidence that the yields of ATP calculated for the conversion of hexose and pentose to acetate are accurate, but there is yet some uncertainty regarding the ATP yield associated with propionate production (2, 10, 13). Therefore, it may be erroneous to assume that for each mole of propionate produced, 2 moles of ATP become available.

To assess the amount of growth which might have occurred, protein synthesis during maltose fermentation was measured by the method of Walker and Nader (16). In two separate experiments, it was found that no synthesis of protein occurred for the first 20 min of incubation. Thereafter, protein synthesis proceeded in a linear fashion (Fig. 4). In these experiments, a total of 46.7 and 23.4 μg of protein were synthesized in 40 min, equivalent to 78 and 39 μg of dry microbial cell material. The synthesis of these quantities of microbial cell materials would require about 8 and 4 μmoles of ATP or about 9 and 5% of the ATP calculated to be available. Thus, up to about 10% of the total ATP available may be used for growth and about 80% for polysaccharide synthesis.

These studies provide evidence that the rumen microorganisms utilize the energy made available from carbohydrate fermentation with a high degree of efficiency in that they are able to store carbohydrate in excess of their immediate needs in the form of reserve polysaccharide. Thus, the rapid fermentation of soluble dietary carbohydrate immediately after feeding is not a nutritional loss to the animal, since the reserve polysaccharide synthesized could presumably be used as a source of energy for cell synthesis when energy supply from the less readily fermentable dietary materials becomes a growth-limiting factor. In addition, a proportion of the reserve polysaccharide will supply glucose directly to the animal as a consequence of overspill from the rumen.

A further indication arising from this work concerns the mode of fermentation of the substrates used. Both cellobiose and maltose, intermediates in the breakdown of two of the major dietary polysaccharides, appear to be split to monosaccharides largely by an ATP-conserving phosphorolytic mechanism.

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