

Nutritional Interdependence Among Rumen Bacteria, *Bacteroides amylophilus*, *Megasphaera elsdenii*, and *Ruminococcus albus*

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Nutritional interdependence among three representatives of rumen bacteria, *Bacteroides amylophilus*, *Megasphaera elsdenii*, and *Ruminococcus albus*, was studied with a basal medium consisting of minerals, vitamins, cysteine hydrochloride, and NH_4^+ . *B. amylophilus* grew well in the basal medium supplemented with starch and produced branched-chain amino acids after growth ceased. When cocultured with *B. amylophilus* in the basal medium supplemented with starch and glucose, amino acid-dependent *M. elsdenii* produced an appreciable amount of branched-chain fatty acids, which are essential growth factors for cellulolytic *R. albus*. A small addition of starch (0.1 to 0.3%) to the basal medium containing glucose and cellobiose brought about successive growth of the three species in the order of *B. amylophilus*, *M. elsdenii*, and *R. albus*, and successive growth was substantiated by the formation of branched-chain amino acids and fatty acids in the culture. Supplementation with 0.5% starch, however, failed to support the growth of *R. albus*. On the basis of these results, the effects of supplementary starch or branched-chain fatty acids on cellulose digestion in the rumen was discussed.

Although most cellulolytic rumen bacteria have been proved to have absolute requirements for branched-chain fatty acids (4, 10, 11, 19) produced by the deamination of branched-chain amino acids by other organisms (2), a large number of cellulolytic bacteria which require these fatty acids (28, 29) as well as a considerable amount of the branched-chain fatty acids (16) have been found in the rumen of animals fed diets devoid of the fatty acids and of their biosynthetic precursor amino acids. Cellulose digestion in vitro by rumen bacteria has also been reported to be stimulated by a small addition of starch to a medium containing urea as the sole source of nitrogen (5, 6, 17). One possible explanation for these phenomena would be that cell proteins of some of the bacteria, such as *Bacteroides amylophilus*, capable of synthesizing protein from starch and NH_4^+ (8, 13), are the sources of branched-chain amino acids, which are decomposed to produce the essential branched-chain fatty acids by deaminase-positive bacteria, such as *Megasphaera elsdenii* and *Bacteroides ruminicola* (3).

The present investigation was undertaken to see if such a nutritional interdependence could take place among *B. amylophilus*, *M. elsdenii*, and cellulolytic *R. albus*.

MATERIALS AND METHODS

Organisms and culture methods. *B. amylophilus* 70, *M. elsdenii* T81, and *R. albus* 7 were obtained from the culture collection of the Department of Dairy Science, University of Illinois. The organisms were grown in medium 10 (15) except that agar was omitted and the concentrations of starch, glucose, and cellobiose were increased to 0.3% (wt/vol) each. The cells were harvested in the logarithmic growth phase by centrifugation at $20,000 \times g$ for 15 min, and the pellet was washed three times with an anaerobic salts solution, which had the same composition as that of the basal medium (see below) except that the vitamin mixture was omitted. The cells were suspended and diluted with the same salts solution to an optical density (600 nm, Hitachi model 100-30 spectrophotometer) of approximately 0.3 in a 1.0-cm cuvette. A 0.1-ml amount of this suspension per 10 ml of culture was used as an inoculum. The basal medium contained the following substrates (in milligrams per 100 ml): thiamine · HCl, calcium-D-pantothenate, riboflavin, nicotinic acid, and pyridoxine, 0.2 each; *p*-aminobenzoic acid, 0.01; biotin, folic acid, and thioctic acid, 0.005 each; cobalamin, 0.002; Na_2CO_3 , 400; K_2HPO_4 and KH_2PO_4 , 45 each; NaCl and $(\text{NH}_4)_2\text{SO}_4$, 90 each; CaCl_2 , 9; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 19; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1 each; resazurin, 0.0001; and cysteine · HCl · H_2O , 50. The composition of the amino acid mixture was essentially the same as that of *B. amylophilus* 70 (27) with slight modifications, and the percentage of amino acids was as follows: L-threonine, 5.7; L-valine, 11.4; L-

leucine, 7.7; L-isoleucine, 6.3; L-serine, 3.5; glycine, 5.9; L-alanine, 5.3; L-methionine, 2.2; L-cystine, 1.2; L-glutamic acid, 10.3; L-aspartic acid, 9.9; L-lysine, 8.3; L-histidine, 2.2; L-phenylalanine, 5.2; L-proline, 3.9; L-tyrosine, 4.2; L-arginine, 5.7; and L-tryptophan, 1.1. The method for preparation of the medium was the same as that described by Bryant and Robinson (13). The bacteria were cultured in 50-ml flasks each containing 25 ml of medium with the exception of a single culture for nutritional requirements in which test tubes (1 by 10 cm) containing 2.5 ml of medium were used. The flasks and test tubes had butyl-rubber stoppers. Medium 10 described by Caldwell and Bryant (15) was supplemented with 0.05% (wt/vol) ferric ammonium citrate and 0.008% (wt/vol) sodium thiosulphate in colony counting by the roll tube method, and the preparation of roll tube media was essentially the same as that described by Bryant and Burkey (12). Colonies were counted 3 days after incubation, and *M. elsdenii* was assigned by black colony formation due to H₂S production. About 30 well-isolated white colonies were then picked up from single roll tubes, and *R. albus* was distinguished from *B. amylophilus* by the requirement for branched-chain fatty acids. All incubations were at 39°C and pH 6.6 to 6.8 under an atmosphere of O₂-free CO₂.

Analytical procedures. Microbial protein (microbial N × 6.25) was estimated by the micro-Kjeldahl method after harvesting the cells by centrifugation at 20,000 × g for 15 min at 0°C. Fractions of volatile fatty acids were obtained by the method of Fenner and Elliot (20) and analyzed with a gas chromatograph (Yanaco G 180) equipped with a flame ionization detector and a glass column (2.0 m) packed with Chromosorb 101 and operated at 180°C with He as a carrier gas. Isovaleric acid could not be separated from 2-methylbutyric acid. Free branched-chain amino acids were determined as follows: a 10-ml culture was centrifuged at 20,000 × g for 15 min, and the supernatant was evaporated to dryness. The residue dissolved in water was membrane filtered (0.22 μm), and the filtrate was chromatographed on a Sephadex G-10 column (100 by 1 cm) with a 0.05 M NaCl elution to remove peptides by the method of Pittman and Bryant (26), followed by chromatography on a column (20 by 0.8 cm) of Amberlite [resin] IR-120 (H⁺) with a 10% (vol/vol) pyridine elution to remove metal salts. Material in the eluate was dissolved in 5% (vol/vol) acetic acid, adsorbed on a column (0.4 by 1 cm) of active carbon (Norit A), and eluted with 5% (vol/vol) acetic acid to remove aromatic amino acids. The concentrated eluate was mixed with a drop of 30% (vol/vol) H₂O₂ and left at room temperature for 1 h to oxidize methionine. After the solution was evaporated to dryness, the residue was dissolved in a small amount of water (50 to 500 μl), and aliquots (5 μl) were applied to cellulose thin-layer plates (10 by 10 cm, Merck), which were developed with butanol-acetic acid-water (4:1:2). The color developed with a ninhydrin-dipping reagent (31) was measured with a Shimadzu Dual-Wavelength TLC scanner CS-900 at 520 and 700 nm. The recoveries of valine and leucine added to the membrane filtrates were 76 and 83%, respectively, against which the concentrations of amino acids were corrected.

RESULTS

Single cultures. *B. amylophilus* had no requirements for amino acids and fatty acids, and growth increased with an increase in starch concentration up to 0.5%. At a growth peak which was reached after 8 to 12 h of incubation, the amounts of microbial protein of *B. amylophilus* in the basal medium supplemented with 0.05, 0.1, 0.3, and 0.5% starch were 0.09, 0.16, 0.38 and 0.44 mg/ml, respectively. When growth ceased, autolysis took place, and a decrease in microbial protein of 30 to 40% was observed after 48 h of incubation. The decrease in microbial protein was accompanied by an increase in branched-chain amino acids, which are precursors of branched-chain fatty acids, but no branched-chain fatty acids were detected up to 48 h of incubation in the medium (Fig. 1).

M. elsdenii required amino acids, and maximal growth was obtained in the medium supplemented with 0.02% or more of amino acids, but the production of branched-chain fatty acids increased with the concentration of amino acids. Whereas the production of *n*-caproic acid was higher and that of *n*-valeric acid was lower in the glucose medium than in the lactate medium, there was little influence by the substrate on the production of branched-chain fatty acids (Table 1).

Cellulolytic *R. albus* required for growth isobutyric acid, which could be replaced by equimolar 2-methylbutyric acid but not by the amino acid mixture containing branched-chain amino acids; the concentration required for maximal growth was more than 0.04 mM.

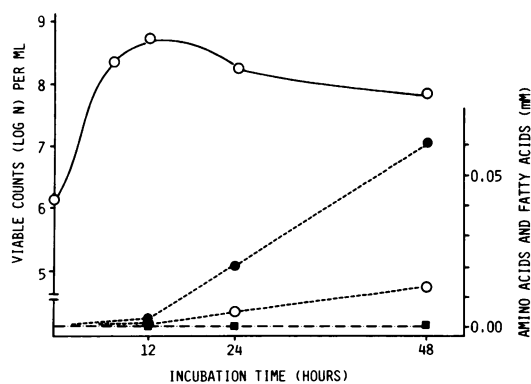


FIG. 1. Production of branched-chain amino acids and fatty acids in the *B. amylophilus* culture in the basal medium supplemented with 0.3% starch. The initial viable count of *B. amylophilus* was 1.1×10^8 /ml. Symbols: ○—○, *B. amylophilus*; ●—●, valine; ○----○, branched-chain C₆ amino acids; ■—■, branched-chain fatty acids.

TABLE 1. Production of branched-chain fatty acids, *n*-valeric acid, and *n*-caproic acid by *M. elsdenii* in the basal medium supplemented with amino acids after 48 h of incubation

Carbohydrate ^a	Amino acid concn (%) ^b	OD ₆₀₀ ^c	Production ^d of:			
			Isobutyric acid	Branched-chain C ₅ fatty acids	<i>n</i> -Valeric acid	<i>n</i> -Caproic acid
Glucose	0.005	0.22	0.01	0.01	0.03	2.60
	0.01	0.77	0.03	0.03	0.03	2.70
	0.02	1.12	0.09	0.08	0.03	2.50
	0.05	1.16	0.23	0.37	0.05	1.00
	0.1	1.08	0.90	0.65	0.15	0.85
Lactate	0.02	0.51	0.11	0.11	6.00	0.19
	0.05	0.58	0.25	0.38	5.30	0.19
	0.1	0.59	0.70	0.70	6.00	0.35

^a Carbohydrates were added to the medium at a concentration of 0.3% (wt/vol).

^b See text for details.

^c OD₆₀₀, Optical density at 600 nm.

^d Results are given as millimolar concentrations.

Coculture of *B. amylophilus* and *M. elsdenii*. In the coculture of *B. amylophilus* and *M. elsdenii* the basal medium did not support growth of the two strains, whereas the basal medium supplemented with starch supported growth of not only *B. amylophilus* but also *M. elsdenii* (Fig. 2). Viable colony counts of *M. elsdenii* increased with an increase in starch concentration in the range from 0.05 to 0.3% and remained constant in the range from 0.3 to 0.5%; colony counts (log *n*) per ml at the maximal growth stage in 0.05, 0.1, 0.2, 0.3, and 0.5% starch media were 6.81 ± 0.06 ($n = 3$), 7.22 ± 0.16 ($n = 3$), 7.72 ± 0.08 ($n = 3$), 7.92 ± 0.04 ($n = 4$), and 7.92 ± 0.10 ($n = 3$), respectively.

The concentrations of branched-chain fatty acids formed in the coculture are shown in Fig. 3. The production of isobutyric acid was usually observed after 36 h of incubation and increased with the time of incubation. Only traces of branched-chain C₅ fatty acids were formed and were not quantitatively analyzed. The concentration of isobutyric acid tended to increase with an increase in starch concentration; approximately 0.04 mM isobutyric acid was formed after 72, 48, and 36 h of incubation with 0.1, 0.2, and 0.3% or more starch, respectively.

Coculture of *M. elsdenii* and *R. albus*. In the coculture of *M. elsdenii* and *R. albus* in the basal medium supplemented with amino acids, growth of *M. elsdenii* was followed by the distinct growth of *R. albus* (Fig. 4). The lower the amino acid concentration in the medium was, the poorer the growth of *M. elsdenii* and the better the growth of *R. albus*. The time required for maximal growth of *R. albus* increased with a decrease in amino acid concentration. The viable colony counts of *R. albus* at the maximal growth stage in the basal medium supplemented

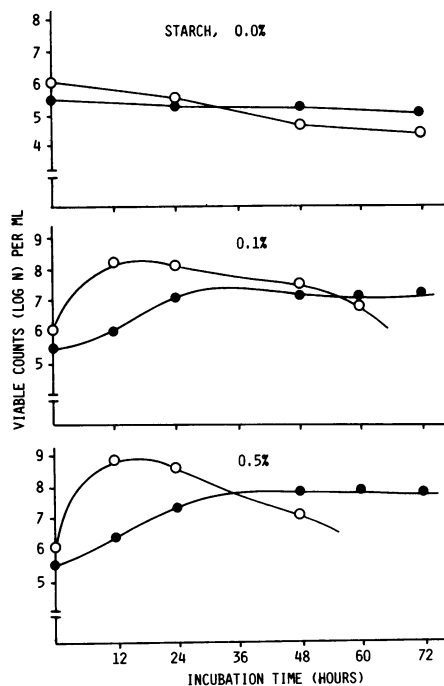


FIG. 2. Growth of *M. elsdenii* cocultured with *B. amylophilus* in the basal medium supplemented with glucose (0.3%) and starch (0.0 to 0.5%). Initial viable counts of *B. amylophilus* and *M. elsdenii* were 1.1×10^6 and 5.5×10^5 /ml, respectively. Symbols: ○, *B. amylophilus*; ●, *M. elsdenii*.

with 0.005, 0.01, 0.02, and 0.05% amino acids were 10^9 , 4.3×10^8 , 3.2×10^8 , and 2.1×10^8 /ml, respectively.

Coculture of *B. amylophilus* and *R. albus*. In the coculture of *B. amylophilus* and *R. albus* in the basal medium containing 0.3% (wt/vol)

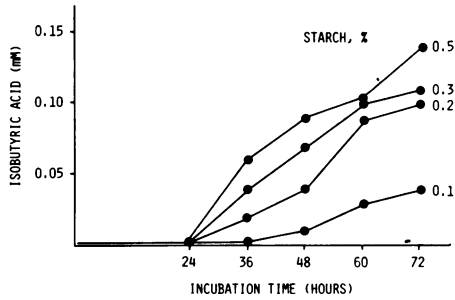


FIG. 3. Production of isobutyric acid in the coculture of *B. amylophilus* and *M. elsdenii* in the basal medium supplemented with glucose (0.3%) and starch (0.1 to 0.5%).

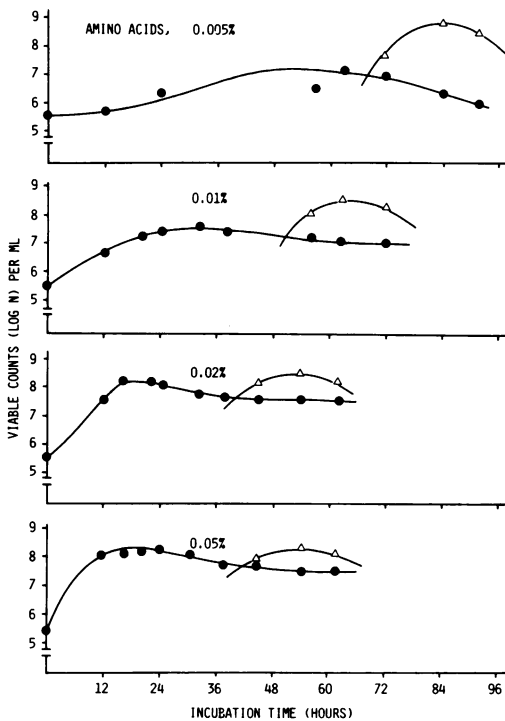


FIG. 4. Growth of *M. elsdenii* and *R. albus* cocultured in the basal medium supplemented with amino acids. The basal medium contained 0.3% each of glucose and cellobiose. Initial viable counts of *M. elsdenii* and *R. albus* were 4.0×10^5 and 1.2×10^6 /ml, respectively. Symbols: ●, *M. elsdenii*; △, *R. albus*.

each of starch and cellobiose, growth of *B. amylophilus* took place, but growth of *R. albus* could not be observed up to 96 h.

Coculture of three strains. When *B. amylophilus*, *M. elsdenii*, and *R. albus* were cocultured in the basal medium containing 0.3% (wt/vol) each of starch, glucose, and cellobiose, growth of the three strains occurred in succession (Fig. 5). The pattern of their viable counts

was virtually a composite of the pattern obtained with the coculture of *B. amylophilus* and *M. elsdenii* (Fig. 2) and that obtained with the coculture of *M. elsdenii* and *R. albus* (Fig. 4). The production of branched-chain amino acids was observed after 12 h of incubation, reaching a maximum of 60 h. However, branched-chain fatty acids were not observed until 36 h of incubation. Effects of the starch concentration on the growth pattern of the three strains were studied next (Fig. 6). In the 0.1% starch medium the second growth peak of *B. amylophilus* appeared. When the starch concentration was increased from 0.1 to 0.3%, the second peak disappeared, and delayed growth of *R. albus* was observed. Upon increasing the starch concentration to 0.5% the growth of *R. albus* failed, and its viable counts never exceeded 4.0×10^6 /ml for all incubation times.

DISCUSSION

The present results that *B. amylophilus* was able to grow well in the basal medium supplemented with starch and that the production of branched-chain amino acids was observed in the medium after growth ceased (Fig. 1) are consistent with earlier findings that *B. amylophilus* is proteolytic (1, 9) and does not require amino acids or fatty acids for growth (8, 13). The amount of branched-chain amino acids produced in the basal medium supplemented with 0.3%

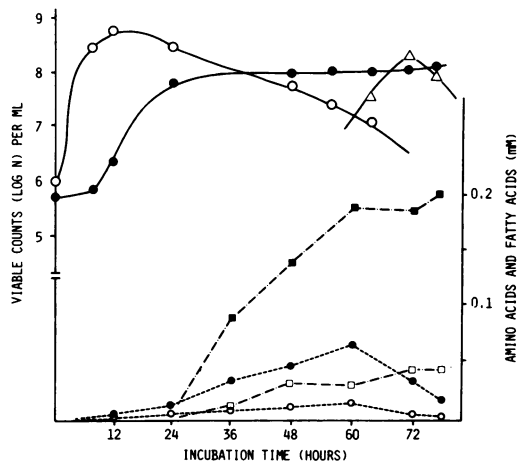


FIG. 5. Successive growth of *B. amylophilus*, *M. elsdenii*, and *R. albus* and production of branched-chain amino acids and fatty acids in the basal medium containing 0.3% each of starch, glucose, and cellobiose. Initial viable counts of *B. amylophilus*, *M. elsdenii*, and *R. albus* were 10^6 , 5.2×10^5 , and 1.4×10^6 /ml, respectively. Symbols: ○—○, *B. amylophilus*; ●—●, *M. elsdenii*; △, *R. albus*; ■, isobutyric acid; □, branched-chain C₅ fatty acids; ●—●, valine; ○—○, branched-chain C₅ amino acids.

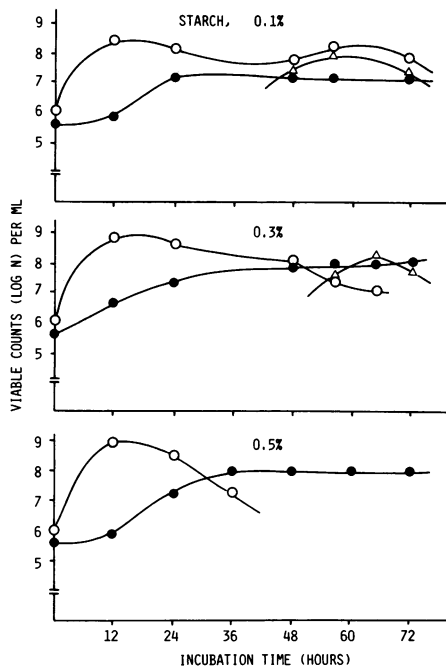


FIG. 6. Influence of starch concentration (0.1 to 0.5%) on the successive growth of *B. amylophilus*, *M. elsdenii*, and *Ruminococcus albus* in the basal medium containing 0.3% each of glucose and cellobiose. Initial viable counts of *B. amylophilus*, *M. elsdenii*, and *R. albus* were 1.1×10^6 , 5.0×10^5 , and 1.4×10^6 /ml, respectively. Symbols: ○, *B. amylophilus*; ●, *M. elsdenii*; △, *R. albus*.

starch was smaller (Fig. 1) than the amount expected (0.1 mM) based on the assumptions that 0.12 mg of microbial protein per ml was completely hydrolyzed to amino acids after 48 h of incubation and that the amounts of valine and total branched-chain C_6 amino acids in the protein of *B. amylophilus* 70 were 11.4 and 14.0% (wt/wt), respectively (27). During the course of the separation of amino acids in the culture, a considerable amount of peptides was found but was not quantitatively analyzed. As stimulatory effects of peptides on the production of branched-chain fatty acids have been reported with *M. elsdenii* (3), the strict amount of peptides required for growth of *M. elsdenii* in our systems remains to be elucidated. The amount of branched-chain C_6 amino acids produced was significantly smaller than that of valine (Fig. 1) in spite of the fact that the molar ratio of leucine plus isoleucine to valine in *B. amylophilus* cells is about 1 (27). This may have been due to the difference in the liberation rate of amino acids from microbial protein (7).

M. elsdenii did not grow alone in the medium devoid of amino acids up to 72 h. This result is

in agreement with the observations of Bryant and Robinson (13) and Forsberg (21), although the latter found that serial transfer in the medium similar to ours permitted growth of this species. The present result that the production of *n*-valeric acid in the glucose medium was very low as compared with that in the lactate medium (Table 1) is in agreement with observations by other workers.

In the coculture of *B. amylophilus* and *M. elsdenii* the requirement of *M. elsdenii* for amino acids seemed to be fulfilled by the hydrolysis products of the microbial protein of *B. amylophilus* because the growth of *B. amylophilus* was essential for that of *M. elsdenii* (Fig. 2) and isobutyric acid was produced (Fig. 3). The concentration of isobutyric acid produced in the basal medium supplemented with starch exceeded the limiting level (0.04 mM) for the maximal growth of *R. albus*, whereas the concentration of branched-chain C_5 fatty acids was very low, probably reflecting the low concentration of branched-chain C_6 amino acids produced by *B. amylophilus* (Fig. 1).

Vigorous digestion of cellulose in the rumen suggests the importance of a similar type of interdependence between the branched-chain fatty acid-producing bacteria and the cellulolytic rumen bacteria because many cellulolytic rumen bacteria require branched-chain fatty acids (4, 10, 11, 19), and the ability to deaminate branched-chain amino acids to branched-chain fatty acids is limited to only a few species, such as *M. elsdenii* and *Bacteroides ruminicola* (3). Bryant and Wolin (14) described an example of such an interdependence in the coculture of *B. ruminicola* and *R. albus*, providing a useful model to illustrate nutritional interdependence with respect to branched-chain fatty acids in the rumen ecosystem. Similarly, the results of the present experiments on the coculture of *M. elsdenii* and *R. albus* showed that the growth of *R. albus* was supported by branched-chain fatty acids which were produced by *M. elsdenii* from the supplementary amino acids (Fig. 4).

A logical deduction from the above results is that when *R. albus* is cocultured with *B. amylophilus* and *M. elsdenii* in the basal medium containing cellobiose or cellulose, the branched-chain fatty acids requiring *R. albus* will be able to grow by supplementation with starch via the mechanism shown in Fig. 7. This deduction is justified by the observations of the successive growth of three species in the basal medium supplemented with 0.3% starch (Fig. 5); the growth pattern of these three species roughly corresponded to the pattern of production of branched-chain amino acids and fatty acids. The

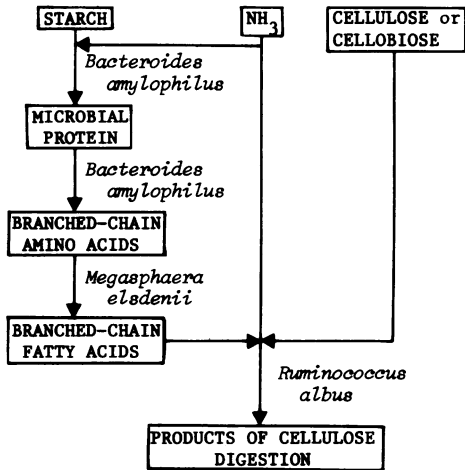


FIG. 7. A possible mechanism of the stimulatory effect of starch on cellulose digestion in the rumen ecosystems.

larger amount of branched-chain fatty acids produced as compared with the amount of branched-chain amino acids produced after 36 h of incubation may have been due to a rapid deamination of branched-chain amino acids.

The interdependence among three rumen species observed in the 0.3% starch medium was reconfirmed in the basal medium supplemented with 0.1% starch (Fig. 6). In this case, however, the second peak of *B. amylophilus* was observed. One possible explanation for this phenomenon could be that *R. albus* had synthesized polysaccharide (24) which was used by amyolytic *B. amylophilus* after the exhaustion of exogenous starch. In the 0.5% starch medium, however, *R. albus* failed to grow, whereas the growth of *M. elsdenii* (Fig. 6) and the concentration of isobutyric acid (Fig. 2) were comparable to those, respectively, in the 0.3% starch medium.

Although changes in the pH of the culture broth were not followed in the present study, it is probable that the delayed growth of *R. albus* with 0.3% starch in the medium and the failure to observe growth with 0.5% starch could be related to a fall in pH due to the greater growth of *B. amylophilus* or *M. elsdenii*. It has been reported that cellulolytic rumen bacteria are sensitive to acid and hardly grow at pH 6.0 (30), whereas *M. elsdenii* can continue to grow even at a pH below 5.0 (23). The phenomenon that the maximal growth of *R. albus* decreased with an increase in the growth of *M. elsdenii* (Fig. 4) may also be explained by a pH change. In any case, this inhibitory effect of a high concentration of starch on the growth of cellulolytic *R. albus* may explain, in part, the depression of

cellulose digestion by a large addition of starch to a ration for ruminants.

As to the stimulatory effect of branched-chain fatty acids on cellulose digestion in vivo, both positive and negative results have been reported in animals fed diets containing urea as the sole source or main source of nitrogen (18, 22, 25). This discrepancy in the results could be partly explained by the present result that only a small addition of starch produced enough branched-chain fatty acids for the requirement of cellulolytic rumen bacteria; supplementation of the branched-chain fatty acids would be effective only when the main components of diets are cellulose and urea, and the effect of the branched-chain fatty acids may be replaced by a small addition of starch to the diet (22).

Of course, nutritional interdependence among microbial populations in the rumen is much more complex than that observed in this study with three representatives of rumen bacteria. However, further work along these lines will offer good insight into the nutritional complexity in the rumen ecosystems.

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