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Propionate Formation from Cellulose and Soluble Sugars by Combined Cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*

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Succinate is formed as an intermediate but not as a normal end product of the bovine rumen fermentation. However, numerous rumen bacteria are present, e.g., *Bacteroides succinogenes*, which produce succinate as a major product of carbohydrate fermentation. *Selenomonas ruminantium*, another rumen species, produces propionate via the succinate or randomizing pathway. These two organisms were co-cultured to determine if *S. ruminantium* could decarboxylate succinate produced by *B. succinogenes*. When energy sources used competitively by both species, i.e. glucose or cellobiose, were employed, no succinate was found in combined cultures, although a significant amount was expected from the numbers of *Bacteroides* present. The propionate production per *S. ruminantium* was significantly greater in combined than in single *S. ruminantium* cultures, which indicated that *S. ruminantium* was decarboxylating the succinate produced by *B. succinogenes*. *S. ruminantium*, which does not use cellulose, grew on cellulose when co-cultured with *B. succinogenes*. Succinate, but not propionate, was produced from cellulose by *B. succinogenes* alone. Propionate, but no succinate, accumulated when the combined cultures were grown on cellulose. These interspecies interactions are models for the rumen ecosystem interactions involved in the production of succinate by one species and its decarboxylation to propionate by a second species.

Propionic acid is a major end product of the fermentation of plant polysaccharides by the rumen microbial population. Pure cultures of certain predominant rumen bacteria produce propionate directly from carbohydrates other than cellulose. For example, *Selenomonas ruminantium* and *Megasphaera elsdenii* both produce propionate from carbohydrates and lactate, the former by the succinate or randomization pathway (13) and the latter by the acrylate pathway (2). Several important species of rumen bacteria produce succinate as a major, pure-culture product of carbohydrate fermentation. *Ruminococcus flavefaciens* and *Bacteroides succinogenes*, two of the three major cellulolytic species in the rumen, produce succinate. Succinate, however, does not accumulate in the rumen ecosystem, but it is known to be produced and rapidly decarboxylated to propionate in the rumen. Quantitative studies have shown that succinate is a major precursor of propionate in the rumen (3). A likely explanation for the conversion of succinate to propionate is that a species like *S. ruminantium* decarboxylates succinate produced by other rumen organisms. Resting cell decarboxylation of succinate to propionate and CO_2 by bacteria that use the succinate pathway for production of propionate from carbohydrates or lactate is well established (10, 11). *S. ruminantium* presumably would obtain energy for growth in the rumen by the conversion of carbohydrates to propionate, and the grown cells would then carry out what could be considered a resting cell decarboxylation of succinate, produced by other organisms, to propionate and CO_2.

The purpose of this study was to obtain experimental evidence for the decarboxylation of succinate produced by rumen cellulolytic bacteria when they are grown together with *S.*

ruminantium. Initial studies were carried out by co-culturing *B. succinogenes* and *S. ruminantium* in media containing glucose or cellobiose, sugars that both species use as an energy source. It was subsequently found that *S. ruminantium*, a non-cellulolytic species, can grow together with the cellulolytic *B. succinogenes* on a medium containing cellulose as an energy source. *B. succinogenes* provides *S. ruminantium* with an energy source from cellulose, and the latter organism decarboxylates succinate produced by the former organism. The net result is a two-species cofermentation of cellulose to propionate, acetate, and CO_2. The results of these fermentation interaction studies provide evidence for the explanation of the mode of conversion of succinate to propionate in the rumen discussed above.

MATERIALS AND METHODS

Organisms and cell growth. *B. succinogenes* S-85 and *S. ruminantium* HD4 were used. Independent and combined cultures were usually grown at 37 C in 10 ml of medium in 18 by 150 mm rubber-stoppered test tubes. The atmosphere was CO_2 freed of trace amounts of O_2 by passing over heated copper filings. A complex medium (4, 6) was slightly modified and used for routine transfers of both organisms. It was made by first adding the following ingredients and distilled H_2O to a final volume of 93 ml: Trypticase, 0.5 g; yeast extract, 0.1 g; dithiothreitol, 0.054 g; glucose, 0.2 g; cellobiose, 0.2 g; starch, 0.2 g; clarified rumen fluid, 20 ml; 4 ml each of minerals no. 1 (0.6% K_2HPO_4) and no. 2 (0.6% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 1.2% NaCl, 0.24% $MgSO_4 \cdot 7H_2O$, 0.16% $CaCl_2 \cdot 2H_2O$); and 0.1 ml of 0.1% resazurin. After adjusting the pH to 6.5 and autoclaving at 15 lb/in^2 for 15 min under CO_2 in a sealed flask, 5 ml of sterile 8% Na_2CO_3 and 2 ml of sterile 2.5% cysteine·hydrochloride were added. The medium was then tubed under O_2-free CO_2 for use. The procedures were essentially those previously described (4–6). In most experiments, the same medium was used except for the use of glucose, cellobiose, or cellulose as energy sources as indicated. A defined medium was used for some experiments, which was the same as the complex medium, except for the omission of rumen fluid, Trypticase, and yeast extract and the addition of vitamins, isobutyric, isovaleric, 2-methyl-butyric, and *n*-valeric acids as previously described (16). All cultures were incubated on a reciprocal shaker at 120 strokes per min.

Direct counts. A Petroff-Hauser chamber was used. It was possible to enumerate both species in combined cultures because of their distinctly different morphologies.

Manometric experiments. Cells for manometric analysis were grown for 24 h at 37 C in 100 ml of the complex medium, with 0.2% each of cellobiose and glucose, under an atmosphere of CO_2. The cells were harvested by centrifugation (12,000 × *g*) for 5 min under CO_2 by using screw cap tubes and were suspended in approximately 5 ml of an anaerobic mineral salt dilution buffer. The dilution buffer previously described (16) was used, but was modified to delete the glucose and sodium sulfide and to contain 0.01 M dithiothreitol.

Double-sidearm Warburg vessels (15 ml total volume) were used. The reaction mixtures contained 50 mM potassium phosphate buffer, at pH 6.5, approximately 10^{10} cells per flask, 2 μg of biotin per ml, and 10 mM sodium succinate in a final volume of 2.9 ml. The succinate, in 0.3 ml, was tipped in from one sidearm to start the reaction. After 40 min, the reaction was stopped by the addition of 0.1 ml of 6 N H_2SO_4 from the second sidearm, and the flasks were shaken for an additional 10 min to release and measure dissolved CO_2. After centrifugation, the supernatant solutions were analyzed for acids (see below). Incubations were at 37 C in an atmosphere of argon.

Fermentation analyses. Cellobiose was determined by the ferricyanide reduction method of Park and Johnson (12). Glucose was determined with glucose oxidase as described in Bulletin 510 of the Sigma Chemical Co. Culture supernatant solutions were clarified by the Somogyi procedure (15) prior to analysis for glucose or cellobiose. The cellulose used was ball milled Whatman no. 1 filter paper in a 2% (wt/vol) aqueous slurry as described by Hungate (8). The concentration of the slurry was determined gravimetrically, and complete cellulose disappearance from cultures was estimated by microscopy observation of the disappearance of the cellulose particles.

For fermentation acid analysis, 2 ml of culture supernant solution was acidified with 0.1 ml of 6 N H_2SO_4 and centrifuged for 15 min at 15,000 × *g* to remove any precipitate. The silicic acid column and methods of Ramsey (14) were modified for batch collection (9). The solvents and elution order were (in milliliters) benzene, 56; $CHCl_3$, 100; 1% tert-butanol (t-B) in $CHCl_3$ (C), 100; 2% t-BC, 200; 5% t-BC, 250; and 8% t-BC, 180. All solvents were equilibrated with H_2SO_4 and used at room temperature. Samples were collected in graduated cylinders with 10 ml between batches to check for any trailing. The collection schedule was designed to obtain butyrate in the first 95 ml, propionate in the next 55 ml, acetate in the next 70 ml, formate in the following 240 ml, lactate in the next 165 ml, and succinate in the final 180 ml. The acids were titrated to a phenolphthalein end point by using 0.01 N ethanolic KOH.

RESULTS

Decarboxylation of succinate by S. ruminatium. Before carrying out studies with combined cultures, experiments were performed to determine if *S. ruminantium* decarboxylates succinate to propionate and CO_2. The results in Table 1 show that resting cells decarboxylate succinate to propionate and CO_2. In another experiment, *B. succinogenes* was grown for 48 h in the complex medium with cellobiose. A 24-h

culture of *S. ruminantium* was then centrifuged aseptically in a CO_2 atmosphere, the cells were resuspended in the 48-h *B. succinogenes* culture, and the tubes were incubated for an additional 24 h. Succinate, but no propionate, was present in the *B. succinogenes* culture, and propionate, but no succinate, was present after incubation of the culture with the added *S. ruminantium* cells (Table 2). This experiment also showed that *S. ruminantium* decarboxylated succinate to propionate and that changes in the medium caused by growth of *B. succinogenes* did not prevent the decarboxylation. Identical results were obtained when glucose was the energy source for *B. succinogenes*.

Concurrent fermentation of cellobiose or glucose by B. succinogenes and S. ruminantium. The question of whether both organisms could grow together and carry out a combined fermentation of carbohydrate to propionic acid was examined. When cellobiose or glucose are used, the two species are competing for energy source. If competition is significantly skewed in the direction of *B. succinogenes*, no significant growth of *S. ruminantium* will take place in the combined cultures, and the fermentation would essentially be the same as the independent *B. succinogenes* fermentation. If competition for substrate is strongly in favor of *S. ruminantium*, the fermentation would be the same as the independent *S. ruminantium* fermentation and the presumptive competitive cofermentation by the two species. Because of the inability to distinguish between an independent *S. ruminantium* fermentation and a truly competive cofermentation simply on the basis of product formation, the contribution of the individual species to the cofermentation process was estimated. This was done by determining cell numbers of each species in the combined culture and calculating the expected amounts of products produced by each species from their respective per cell activities in independent, single-species fermentation. Table 3 shows the results of independent and combined fermentations of cellobiose, and Table 4 shows the results obtained when glucose was the energy source. It can be seen that succinate, but no propionate, was produced by *B. succinogenes* alone and that propionate, but no succinate, was produced by *S. ruminantium* alone. In the combined cultures, propionate but no succinate was found, although significant amounts of succinate would have been expected on the basis of the independent activity of the concentration of *B. succinogenes* found in the combined cultures. The results strongly suggest that the species use cellobiose or glucose at similar rates when they are co-cultured under the conditions of these experiments. This results in a combined fermentation of cellobiose or glucose to propionate, acetate, and CO_2 without succinate accumulation.

TABLE 1. *Propionate and CO_2 production by resting cells of S. ruminantium*[a]

Additions	Propionate	CO_2
None	0.0	0.0
Succinate	13.0	13.7

[a] The protocol was as described in Materials and Methods, and values are expressed as μmoles per 10^{10} cells per hour.

TABLE 2. *Decarboxylation of succinate produced in a B. succinogenes culture*

Supernatant solution	Products (mM)[a]	
	Propionate	Succinate
B. succinogenes culture[b]	0	4.5
B. succinogenes culture plus *S. ruminantium*[c]	5.5	0

[a] The amounts in the uninoculated medium were subtracted.

[b] A 48-h culture of *B. succinogenes* grown with 0.1% cellobiose.

[c] Washed cells from a 24-h *S. ruminantium* culture were resuspended in a 48-h *B. succinogenes* culture and incubated an additional 24 h at 37 C.

The amount of propionate formed in the combined cultures was significantly greater than the amount expected on the basis of the amount of *S. ruminantium* present and was also greater than the amount expected on the basis of the estimated amount of succinate produced by *B. succinogenes* in the combined cultures. A possible reason for the larger than calculated amount of propionate obtained in the combined cultures has not been definitely established, but the discrepancy may be due to differences in product formation by *B. succinogenes* in single and combined cultures. Relatively good carbon recoveries were obtained in fermentation balance studies with the single *S. ruminantium* and the combined *B. succinogenes-S. ruminantium* fermentations, but not with *B. succinogenes* alone. In the single *S. ruminantium* fermentation, the only products were propionate, acetate, CO_2 (calculated as equal to acetate), and small amounts of lactate. The combined fermentation yielded only propionate, acetate, small amounts of formate, and

TABLE 3. *Production of succinate and propionate from cellobiose*[a]

Organism	10^8 cells/ml	Products (mM)		Micromoles per 10^8 cells	
		Propionate	Succinate	Propionate	Succinate
S. ruminantium HD4	6.4	10.2	0.0	1.6	0.0
B. succinogenes S-85	7.3	0.0	6.5	0.0	0.9
Mixed HD4 and S-85					
Expt 1					
HD4	2.1	8.6 (3.4)[b]	0.0 (2.3)[c]	4.1	
S-85	2.5				0.0
Expt 2					
HD4	2.8	9.7 (4.5)[b]	0.0 (2.4)[c]	3.5	
S-85	2.7				0.0

[a] Initial cellobiose concentration was 4.4 mM.
[b] Calculated value based on micromoles of propionate per 10^8 cells of *S. ruminantium* in single culture.
[c] Calculated value based on micromoles of succinate per 10^8 cells of *B. succinogenes* in single culture.

TABLE 4. *Production of succinate and propionate from glucose*[a]

Organism	10^8 cells per ml	Products (mM)		Micromoles per 10^8 cells	
		Propionate	Succinate	Propionate	Succinate
S. ruminantium HD4	5.6	10.6	0.0	1.9	0.0
B. succinogenes S-85	6.6	0.0	6.4	0.0	1.0
Mixed HD4 and S-85					
Expt 1					
HD4	2.5	9.5 (4.7)[b]	0.0 (2.9)[c]	3.8	
S-85	2.9				0.0
Expt 2					
HD4	2.8	9.0 (5.3)[b]	0.0 (2.7)[c]	3.2	
S-85	2.7				0.0

[a] Initial glucose concentration was 8.3 mM.
[b] Calculated value based on micromoles of propionate per 10^8 cells of *S. ruminantium* in single culture.
[c] Calculated value based on micromoles of succinate per 10^8 cells of *B. succinogenes* in single culture.

CO_2 (calculated as acetate minus formate). *B. succinogenes* alone produced succinate, acetate, and small amounts of formate, but significant amounts of carbon disappeared that could not be accounted for by the products or calculated CO_2. Table 5 shows a comparison of fermentation balances for glucose. Similar results were obtained when cellobiose was used. These results suggest that either an unidentified product is produced by *B. succinogenes* alone which can be converted to propionate by *S. ruminantium* or that co-culturing of *S. ruminantium* and *B. succinogenes* prevents the formation of the unidentified compound by *B. succinogenes*.

Fermentation of cellulose. *B. succinogenes* used cellulose as an energy source and fermented cellulose in the complex medium to succinate, acetate, formate, and CO_2 (Table 6). *S. ruminantium* grew only slightly in the same medium without degrading cellulose, but good growth of *S. ruminantium* was obtained when it was co-cultured with *B. succinogenes* on the cellulose medium. No succinate was produced in the combined fermentation, and cellulose was fermented to propionate, acetate, and CO_2 (Table 6). As shown in Table 6, similar results were obtained when a defined medium was used, except that the base growth of *S. ruminantium* alone was eliminated. The carbon recovered in the synthetic medium (assuming CO_2 equal to acetate minus formate) represented 94 and 110% of the original cellulose carbon for *B. succinogenes* alone and the mixture of *B. succinogenes* and *S. ruminantium*, respectively. There is probably some inaccuracy in the original cellulose concentration because

the cellulose was pipetted from a suspended slurry and the actual concentrations in the fermentation media were not measured. It appears, however, that most of the carbon of the cellulose was recovered in the indicated products. When grown alone, the amount of *B. succinogenes* cells per milliliter of synthetic medium was 6.5×10^8, and the respective concentrations of cells in the mixed culture were 4.4×10^8 for *B. succinogenes* and 1.0×10^8 for *S. ruminantium*. The combined cultures were serially transferred in the synthetic medium at 72-h intervals by using 0.5% inocula, and the combined culture fermentation of cellulose to propionate, acetate, and CO_2 was maintained through at least seven serial transfers.

DISCUSSION

These experiments show that it is highly likely that the conversion of succinate to propionate in the rumen is carried out by bacteria that form propionate via the succinate pathway. *S. ruminantium* is probably a major factor in the conversion although, under certain circumstances, other species such as *Veillonella alcalescens* in the sheep rumen (10) may play a similar role. Dehority reported that high concentrations of rumen fluid caused the succinate-producing *B. ruminicola* to produce small amounts of propionate (7), but it was subsequently shown that the propionate is formed by the acrylate pathway (17). It is, therefore, highly unlikely that *B. ruminicola* is capable of decarboxylating succinate.

The rate of succinate decarboxylation by resting cells of *S. ruminantium* was about 13.0 μmol per h per 10^{10} cells. The rate of conversion of succinate to propionate by bovine rumen contents was measured by Blackburn and Hungate (3) and was found to be approximately 1.6 μmol per h per g of rumen contents. By using the resting cell rate determined in these experiments it would have taken approximately 1.2×10^9 selenomonads per ml to account for the turnover number reported by Blackburn and Hungate. It is not possible to directly extrapolate from the cell suspension decarboxylating activity to the activity of the selenomonads in the ecosystem because of the differences in the conditions for succinate decarboxylation. The rate of succinate decarboxylation by cell suspensions leaves the question of whether bovine rumen selenomonads can account for all of the ecosystem conversion of succinate to propionate an open one. We estimate the cell suspension decarboxylating activity (on a dry-weight basis) of *S. ruminantium* HD4 to be about 87 times greater than that reported for propionibacteria (11), but only one-third of that reported for *Veillonella* (10).

We suggest that the model presented in Fig. 1 is a fairly accurate representation of the microbial interactions that result in propionate

TABLE 5. *Fermentation balances for glucose fermentations*[a]

Determination	Organism[b]		
	S. ruminantium	*B. succinogenes*	*Mixed*
Products			
Propionate	10.6	0.0	9.5
Acetate	5.7	2.5	4.5
Formate	0.0	1.7	2.0
CO_2[c]	5.7	0.8	2.5
Succinate	0.0	6.4	0.0
Carbon recovery	104%	56%	90%
O-R index	1.07	0.52	0.79

[a] Initial glucose concentration was 8.3 mM. The data were corrected for a small amount of fermentation by *S. ruminantium* in a glucose-free medium.
[b] Product values are expressed in millimolarity.
[c] Calculated as equal to acetate minus formate.

TABLE 6. *Fermentation of cellulose by B. succinogenes and S. ruminantium*

Organism	Medium	Products (mM)			
		Succinate	Propionate	Acetate	Formate
S. ruminantium HD4	Complex[a]	0.0	2.8	4.5	1.2
B. succinogenes S-85	Complex[a]	13.5	0.0	6.2	2.8
HD4 plus S-85	Complex[a]	0.0	15.9	10.1	0.2
S. ruminantium HD4	Synthetic[b]	0.0	0.0	0.0	0.0
B. succinogenes S-85	Synthetic[b]	6.5	0.0	5.3	1.9
HD4 plus S-85	Synthetic[b]	0.0	8.5	5.4	1.7

[a] Initial cellulose concentration was 0.2%.
[b] Initial cellulose concentration was 0.1%.

formation when cellulose is the major source of carbohydrate fed to a ruminant. It is known that when cellulose dominates as a dietary carbohydrate source, the succinate or randomization pathway is the dominant pathway for making propionate in the rumen (1). When starch is the dominant carbohydrate, there is a shift to formation of propionate via the acrylate pathway, but there is still a significant amount of propionate formed by the randomization pathway during starch fermentation (1). Fig. 2 describes the types of interactions that may occur to produce propionate via the randomization pathway when starch or soluble carbohydrates are fermented in the ecosystem in addition to the interactions depicted in Fig. 1. *S. ruminantium*, depending on the strain, can ferment starch, lactate, and a variety of soluble carbohydrates to propionic acid directly. Non-starch fermenting strains could feed off starch breakdown products, either sugars or lactate produced by starch-fermenting organisms. Microbial interactions that lead to propionate formation from starch and soluble sugars are probably more complex than those interactions involved in propionate formation from cellulose.

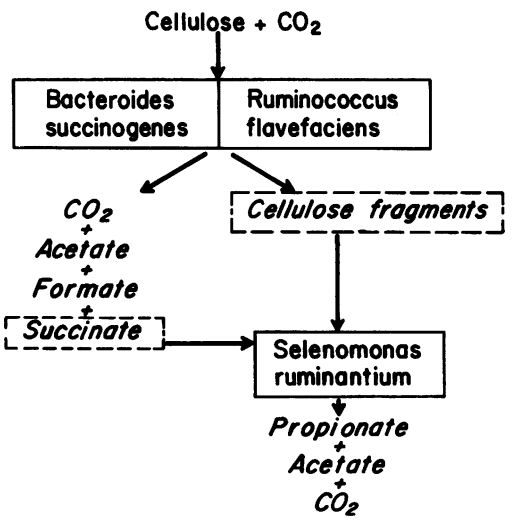

FIG. 1. *Combined species production of propionate from cellulose via succinate.*

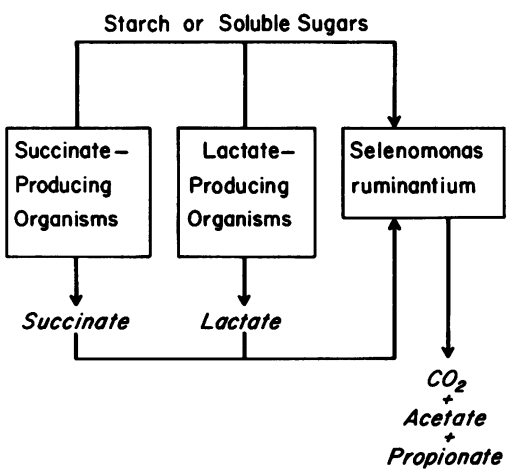

FIG. 2. *Combined species production of propionate from starch or soluble sugars via succinate.*

The spin-off of carbohydrate from cellulose by major cellulolytic rumen bacteria to non-cellulolytic major rumen species has been logically assumed to be a significant means of providing energy to the latter species. To our knowledge, however, the present experiments represent the first direct demonstration of this type of interaction. The interaction between *B. succinogenes* and the HD4 strain on cellulose was duplicated with other selenomonas strains, both lactate and nonlactate-fermenting strains, and the results were essentially the same as with the lactate-fermenting HD4 strain. *R. flavefaciens* has also been substituted for *B. succinogenes* in the cellulose system with the HD4 strain with essentially similar results.

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LITERATURE CITED

1. Baldwin, R. L., W. A. Wood, and R. S. Emery. 1963. Conversion of glucose-^{14}C to propionate by the rumen microbiota. J. Bacteriol. 85:1346–1349.
2. Baldwin, R. L., W. A. Wood, and R. S. Emery. 1965. Lactate metabolism by *Peptostreptococcus elsdenii*: evidence for lactyl coenzyme A dehydrase. Biochim. Biophys. Acta 97:202–213.
3. Blackburn, T. H., and R. E. Hungate. 1963. Succinic acid turnover and propionate production in the bovine rumen. Appl. Microbiol. 11:132–135.
4. Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205–217.
5. Bryant, M. P., and I. M. Robinson. 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. 44:1446–1456.
6. Bryant, M. P., and I. M. Robinson. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. 84:605–614.
7. Dehority, B. A. 1966. Characterization of several bovine rumen bacteria isolated with a xylan medium. J.

Bacteriol. **91**:1724–1729.
8. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
9. Iannotti, E. L., D. Kafkewitz, M. J. Wolin, and M. P. Bryant. 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes:* changes caused by interspecies transfer of H_2. J. Bacteriol. **114**:1231–1240.
10. Johns, A. T. 1951. The mechanism of propionic acid formation by *Veillonella gazogenes*. J. Gen. Microbiol. **5**:326–336.
11. Johns, A. T. 1951. The mechanism of propionic acid formation by propionibacteria. J. Gen. Microbiol. **5**:337–345.
12. Park, J. T., and M. J. Johnson. 1949. A submicrodetermination of glucose. J. Biol. Chem. **181**:149–151.
13. Paynter, M. J. B., and S. R. Elsden. 1970. Mechanism of propionate formation by *Selenomonas ruminantium*, a rumen microorganism. J. Gen. Microbiol. **61**:1–7.
14. Ramsey, H. A. 1963. Separation of organic acids in blood by partition chromatography. J. Dairy Sci. **46**:480–483.
15. Somogyi, M. 1930. A method for the preparation of blood filtrates for the determination of sugar. J. Biol. Chem. **86**:655–663.
16. Tiwari, A. D., M. P. Bryant, and R. S. Wolfe. 1969. Simple method for isolation of *Selemononas ruminantium* and some nutritional characteristics of the species. J. Dairy Sci. **52**:2054–2056.
17. Wallnöfer, P., and R. L. Baldwin. 1967. Pathway of propionate formation in *Bacteroides ruminicola*. J. Bacteriol. **93**:504–505.