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Role of *Megasphaera elsdenii* in the Fermentation of DL-[2-^{13}C]lactate in the Rumen of Dairy Cattle

G. H. M. COUNOTTE,[1] R. A. PRINS,[1]† R. H. A. M. JANSSEN,[2] AND M. J. A. DEBIE[2]

Laboratory of Animal Nutrition, Zootechnical Department,[1] and Laboratory of Organic Chemistry,[2] State University of Utrecht, Utrecht, The Netherlands

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Since *Megasphaera elsdenii* ferments a variable part of DL-lactate to butyrate, measurement of the percentage of DL-lactate fermented to propionate via the acrylate pathway in rumen contents will underestimate the participation of *M. elsdenii* in the DL-lactate fermentation. The percentage of DL-[2-^{13}C]lactate fermented via the acrylate pathway and the percentage of DL-lactate fermented to butyrate can be measured with ^{13}C-FT (Fourier transform)-nuclear magnetic resonance. On the average, the contribution of *M. elsdenii* to DL-lactate fermentation in the rumen of dairy cattle was found to be 74% (standard deviation, 13%), but differed with animal or diet. After feeding a cow readily fermentable carbohydrates, the contribution of *M. elsdenii* to the fermentation of DL-lactate increased as a consequence of catabolite repression in other DL-lactate-fermenting bacteria.

High-concentrate diets induce a rapid microbial fermentation in the rumen and the accumulation of intracellular (H) within the microbes leads to an increased formation of reduced end products and of lactic acid (4). This in itself should not lead to DL-lactate accumulation in the rumen, provided that the capacity to remove DL-lactate was high enough. DL-Lactate formed in the rumen may be removed in three ways: (i) by passage to the lower gastrointestinal tract, (ii) by absorption directly from the rumen, and (iii) by microbial fermentation.

Megasphaera elsdenii, a predominant DL-lactate-fermenting organism, shows no catabolite repression by carbohydrates such as glucose and maltose, whereas *Selenomonas ruminantium* subsp. *lactilytica*, another predominant DL-lactate-fermenting organism, ferments glucose, sucrose, and xylose first before fermenting DL-lactate (7, 11).

M. elsdenii is commonly considered to be associated with the fermentation of readily fermentable carbohydrates (9, 14). Therefore, it is important to know the participation of *M. elsdenii* in the fermentation of DL-lactate under several feeding conditions, in different animals, and especially after feeding readily fermentable carbohydrates.

M. elsdenii is the only rumen microorganism known to ferment DL-lactate to propionic acid via the nonrandomizing (acrylate) pathway (Fig. 1). The possession of the acrylate pathway makes it possible to measure the participation of *M. elsdenii* in the fermentation of DL-lactate by mixed rumen microorganisms. In this study we describe the use of DL-[2-^{13}C]lactate instead of DL-[2-^{14}C]lactate in the in vitro incubations to determine the percentage of DL-lactate fermented via the acrylate pathway and the percentage of DL-lactate fermented to butyric acid.

† Present address: Department of Bacteriology, University of California, Davis, CA 95616.

MATERIALS AND METHODS

Animals. The animals used in this study were fistulated lactating or nonlactating Friesian-Holstein dairy cows. The animal used in the in vivo experiment was Neel 248 after receiving concentrate mixture III listed in Table 1.

Microorganisms. The bacteria used in this study were isolated from rumen fluid of Friesian-Holstein dairy cows fitted with rumen fistulae. The basal medium was that of Herbeck and Bryant (6), except that fructose was replaced by DL-lactate. Casitone was replaced by Trypticase (BBL Microbiology Systems; 0.20%, wt/vol), and yeast extract (0.20%, wt/vol) was added. Just before inoculation, 0.10 ml of 3.0% (wt/vol) cysteine hydrochloride was added as a reducing agent. Bacteria were isolated by the anaerobic Hungate roll-tube technique. Identifications were made with reference to the *Anaerobe Laboratory Manual* (8) and *Bergey's Manual of Determinative Bacteriology* (3).

Preparation of DL-[2-^{13}C]lactate. The reaction mixture contained 0.75 g (15.3 mmol) of NaCN and 0.50 g (11.1 mmol) of [1-^{13}C]acetaldehyde dissolved in 2.5 ml of water at 0°C. Six milliliters of 1.25 M H_2SO_4 was added dropwise with constant stirring for 1 h at 0°C. The reaction mixture was then stirred for a further hour at 20°C. Twenty milliliters of 12 M HCl

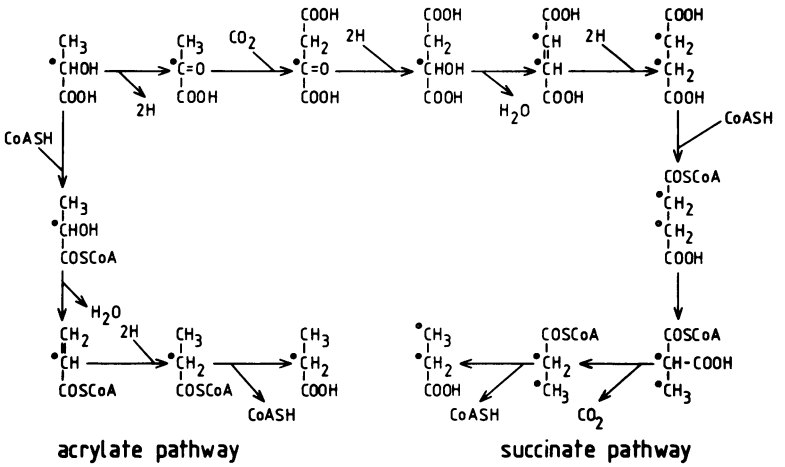

FIG. 1. *Two different pathways of the fermentation of* DL*-lactate to propionate in the rumen.*

was added dropwise with constant stirring over a 30-min period. After refluxing for 10 min, the DL-lactic acid was subjected to continuous ether extraction overnight with 100 ml of $(C_2H_5)_2O$. The ether was removed (by evaporation), and a 0.20 M DL-lactate solution (pH 7.0) was prepared with NaOH. The DL-lactate solution was depolymerized by heating at 120°C for 20 min and could then be used for incubations.

Incubation with DL-[2-^{13}C]lactate. (i) Mixed rumen microorganisms. A total of 0.50 ml of 0.20 M DL-[2-^{13}C]lactate was added to a 10-ml suspension of mixed rumen microorganisms and incubated at 39°C for 3 h. After incubation, 10 ml of 5% (wt/vol) $ZnSO_4 \cdot 7H_2O$ was added, and after 10 min 10 ml of 0.17 M $Ba(OH)_2$ was added together with 0.10 ml of 8 M NaOH. After centrifugation (1,000 × *g*, 15 min) the supernatant was freeze-dried.

The part of DL-lactate fermented by *M. elsdenii* in the mixed culture with *S. ruminantium* (Table 2) was calculated by using a flow scheme (G. H. M. Counotte, Ph.D. Thesis, University of Utrecht, 1981) in which mixed-order kinetics were assumed and in which allowance was made for the decrease in DL-lactate concentration. Calculations were based on 0.05-h intervals during a 3-h incubation period.

(ii) Pure cultures. A total of 0.50 ml of 0.20 M DL-[2-^{13}C]lactate was added to 10 ml of basal medium. When the organisms reached the stationary growth phase, the medium was deproteinized as described above and freeze-dried.

^{13}C-FT-NMR measurements. All ^{13}C-FT (Fourier transform)-NMR measurements were performed with a Varian CFT-20 spectrometer. The experimental conditions were: spectrum width, 4,000 Hz; flip angle, 45°; repetition rate, 8 s; number of scans, 3,200; temperature, 20°C; solvent, D_2O.

Calculations. The ^{13}C peak height (h_i) in a ^{13}C-FT-NMR spectrum is linearly related to the number of carbon atoms (N_i) responsible for this peak which are in the sensitive volume: $h_i = c_i N_i$. The constant c_i depends on the experimental conditions (flip angle, repetition rate temperature, and the relaxation times T_{1i} and T_{2i} for carbon *i*). For a quantitative evaluation of the concentrations of the individual volatile fatty acids (VFA) (acetic acid [HAc], propionic acid [HPr], butyric acid [HBut]), the c_i constants have to be determined. This was done in preliminary experiments. The constants were found to be: c_{HAc}, 0.719;

TABLE 1. *Percent composition (wt/wt) of the concentrate mixtures used in this study*

IRN[a]	Feedstuff	Mixture		
		I	II	III
4-00-409	Animal fat	2	0	0
4-00-669	Beet pulp	18	0	20
4-01-237	Citrus pulp	11	0	13
5-01-573	Copra meal	12	0	11
5-02-903	Corn gluten meal	21	0	34
6-01-069	Limestone	0	1.5	0
5-02-048	Linseed meal	7	0	0
4-07-911	Maize (corn)	0	70	0
4-00-668	Molasses	8	8	8
4-03-928	Rice meal	11	0	0
5-04-604	Soybean meal	3	19	6
	Vitamin mineral mixture	4	1.5	3
4-05-337	Wheat	3	0	5

[a] IRN, International reference number in the *Atlas of Nutritional Data on United States and Canadian Feeds* (National Academy of Sciences, Washington, D.C., 1971).

TABLE 2. *Fermentation of* DL*-[2-^{13}C]lactate in mixtures of M. elsdenii strain 57 and S. ruminantium subsp. lactilytica strain 73*

No. of cells per g (×10^8)		%Acryl	% of DL-lactate fermented by *M. elsdenii*	
M. elsdenii	*S. ruminantium*		Theoretical	From ^{13}C experiment[a]
8.34	2.35	62	82	75
5.90	5.43	36	58	56
2.20	7.70	18	27	29

[a] Calculated as [%Acryl × (1 − %HBut/100)] + %HBut (see text).

c_{HPr}, 1.313; and c_{HBut}, 1.930. The fraction of HPr (fN_{HPr}) can be calculated from

$$fN_{HPr} = (h_{HPr}/c_{HPr})/(h_{HAc}/c_{HAc} + h_{HPr}/c_{HPr} + h_{HBut}/c_{HBut})$$

However, when fN_{HPr} is known, h_{HPr} can be calculated

$$h_{HPr}/c_{HPr} = fN_{HPr}(h_{HAc}/c_{HAc} + h_{HPr}/c_{HPr} + h_{HBut}/c_{HBut})$$

Rearranging reveals

$$h_{HPr} = \{fN_{HPr}[(h_{HAc})(c_{HPr}/c_{HAc}) + (h_{HBut})(c_{HPr}/c_{HBut})]\}/(1 - fN_{HPr})$$

Thus

$$h_{HPr} = fN_{HPr}[(h_{HAc} \times 1.83) + (h_{HBut} \times 0.68)]/(1 - fN_{HPr})$$

The procedure to calculate the percentage of DL-lactate fermented via the acrylate pathway to propionic acid and the percentage of DL-lactate fermented to butyric acid is as follows.

After measurement of the ^{13}C-FT-NMR spectrum, the molar composition of VFA was calculated after measuring the VFA concentrations. An example is given to explain the procedure (see Fig. 5). The measured ^{13}C-NMR peak heights are: $h_{C\text{-}2HAc}$, 24; $h_{C\text{-}2HPr}$, 250; $h_{C\text{-}3HPr}$, 30; $h_{C\text{-}2HBut}$, 8; $h_{C\text{-}3HBut}$, 25; $h_{C\text{-}4HBut}$, 8. The molar composition of the VFA is: fN_{HAc}, 0.6695; fN_{HPr}, 0.2412; fN_{HBut}, 0.0893. The peak height of C-2HPr and C-3HPr without fermentation of DL-[2-^{13}C]lactate therefore would be (background correction)

$$h_{HPr} = 0.2412[(24 \times 1.83) + (8 \times 0.68)]/(1 - 0.2412) = 15.7$$

The peak height of C-2HPr as result of fermentation of DL-[2-^{13}C]lactate is therefore 250 − 15.7 = 234.3, whereas the peak height of C-3HPr is 30 − 15.7 = 14.3.

The percentage of propionic acid formed from DL-lactate via the acrylate pathway (%Acryl) (Fig. 1) can be calculated from the formula

$$\%Acryl = (h_{C2HPr} - h_{C3HPr})/(h_{C2HPr} + h_{C3HPr}) \times 100$$

The %Acryl therefore is (234.3 − 14.3)/(243.3 + 14.3) × 100 = 88%.

The percentage of DL-[2-^{13}C]lactate fermented to butyric acid (%HBut) is

$$\%HBut = (1.5 \times h_{C3HBut})/[(1.5 \times h_{C3HBut}) + (h_{C2HPr} + h_{C3HPr})] \times 100$$

which results in this example in [1.5 × (25 − 8)]/(25.5 + 234.3 + 14.3) × 100 = 9%.

The label of DL-[2-^{13}C]lactate will be measured only in the C-3 carbon of butyric acid. The peak hight of C-3HBut has to be multiplied by about 1.5 to correct for possible asymmetry in butyrate synthesis (Fig. 2). ^{13}C recovered from DL-[2-^{13}C]lactate in propionic acid and butyric acid was put at 100%. The fraction of total DL-lactate converted to propionic acid via the acrylate pathway can be obtained from the %Acryl by multiplying with (100 − %HBut) in which %HBut is the fraction of ^{13}C recovered in butyric acid of the total ^{13}C recovered in propionic and butyric acid. The participation of *M. elsdenii* to DL-lactate fermentation can be derived from [%Acryl × (1 − %HBut/100)] + %HBut.

Analysis of DL-lactate, VFA, and carbohydrates. D-Lactate and L-lactate were determined enzymatically (2). VFA (HAc, HPr, HBut, HVal, and the isomers) were determined on a Becker gas chromatograph (model 430, Packard-Becker) fitted with a flame ionization detector as described by Salanitro and Muirhead (13).

The method used to determine pentoses, hexoses, and di- and trisaccharides was according to the Handbook and General Catalog of Pierce (Pierce Chemical Co.; method 21, p. 182–184). Hexa-methyldisilanazane was used as a silylating reagent after oximation of the carbohydrates with $NH_2OH \cdot HCl$ in pyridine. The column was packed with 2% OV-17 on Chromosorb W (HP) 80/100 mesh. The detector and injector temperature were 260 and 230°C, respectively. The oven temperature was programmed from 150 to 260°C at 3°C/min; one run lasted 35 min. A Packard-Becker gas chromatograph was used to determine the concen-

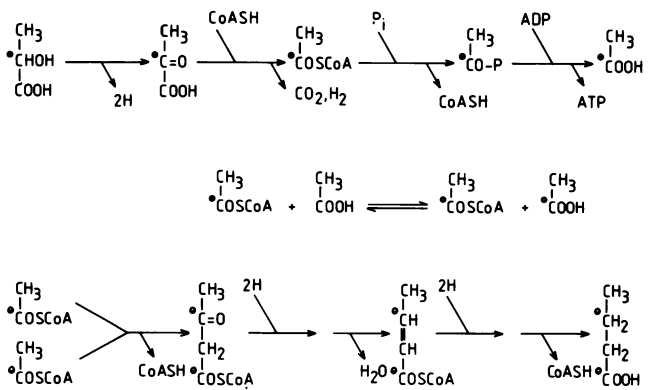

FIG. 2. *Fermentation of DL-lactate to acetate and butyrate in the rumen.*

trations of carbohydrates; β-phenyl-D-glucopyranoside was used as internal standard.

Chemicals. [1-^{13}C]acetaldehyde (90%) was obtained from Merck Sharp & Dohme Canada Limited Isotope Division.

RESULTS

Pure culture studies. Most DL-lactate-fermenting bacteria isolated from the rumen of dairy cattle in this study (*S. ruminantium* subsp. *lactilytica* strains 63 and 73, *Veillonella parvula* strain 803, *Propionibacterium acnes* strain 81, and an unidentified strain 467) fermented DL-lactate via the succinate pathway with randomization of the ^{13}C over C-2 and C-3 in propionic acid without producing butyric acid. Only *M. elsdenii* strain 57 was found to ferment DL-lactate to butyric acid and via the acrylate pathway to propionic acid.

Before starting measurements in mixed cultures of unknown composition, experiments were performed with known constituted mixed cultures of *M. elsdenii* and *S. ruminantium* to determine the outcome of incubation with 10 mM DL-[2-^{13}C]lactate. Mixed cultures of *M. elsdenii* strain 57 and *S. ruminantium* strain 73 were prepared in three different ratios. The mixtures were incubated with 10 mM DL-[2-^{13}C]lactate in a nongrowth situation (without protein) for 3 h, and the percentage of DL-lactate fermented to propionic acid via the acrylate pathway and the percentage of DL-lactate fermented to butyric acid were calculated after deproteinization and ^{13}C-FT-NMR measurements. The number of cells present at the start of the incubation was counted in a Helber counting chamber. *M. elsdenii* fermented more DL-lactate per cell (V_{max}, 5.49×10^{-14} mol cell^{-1} h^{-1}) than did *S. ruminantium* (V_{max}, 2.42×10^{-14} mol cell^{-1} h^{-1}); the K_m for DL-lactate was 17.5 mM for *M. elsdenii* and 10.5 mM for *S. ruminantium*. This was taken into account in calculating the percentage of DL-lactate fermented by *M. elsdenii*. The results of this experiment (Table 2) show that it is possible to predict the participation of *M. elsdenii* in the fermentation of DL-lactate by a mixture of DL-lactate-fermenting bacteria with DL-[2-^{13}C]lactate.

Studies with mixed rumen microorganisms. The results of some in vitro incubations of mixed rumen microorganisms with 10 mM DL-[2-^{13}C]lactate are given in Table 3. These results show that 60 to 95% of the DL-lactate was fermented by *M. elsdenii*. Continuous infusion of 1 mol of DL-lactate per h for 4 weeks into the rumen of a cow did not change the percentage of DL-lactate fermented by *M. elsdenii* (Table 3).

TABLE 3. *Influence of diet and inoculum donor on the percentage of DL-lactate fermented by M. elsdenii in the rumen during incubations of ruminal samples taken 4 h after feeding*

Daily intake[a] (kg)	Animal	%Acryl	%HBut	% of DL-lactate fermented by *M. elsdenii*[b]
Concentrate mix I (12)	Saar 218	43	55	73
Concentrate mix II (12)	Saar 218	96	33	97
Soybean meal (6)	Saar 218	69	16	74
Hominy feed (6)	Saar 218	59	15	65
Concentrate mix II (12)	Neel 248	27	46	61
Beet pulp (6)	Neel 248	54	18	62
Hay (8)	Ommen	82	22	86
Hay + lactate[c]	Ommen	88	9	89
Soybean meal (6)	Ida 332	72	29	80
Hominy feed (6)	Neel 240	61	9	65

[a] Hay was given ad libitum only with diets containing the concentrate mixtures.

[b] See footnote *a* of Table 2.

[c] Cow Ommen after a continuous intraruminal infusion of 1 mol of DL-lactate per h for 4 weeks.

Concentrate feeding experiment. High concentrations of soluble mono- and disaccharides were measured in vitro when a concentrate mixture rich in sugars was incubated with mixed rumen microorganisms (Fig. 3). Results from in vivo experiments can differ from those obtained in vitro due to dilution rate of ruminal contents and absorption of VFA and lactate by the rumen epithelium. Therefore, the concentration of mono- and disaccharides, VFA, and DL-lactate in the rumen of a cow was measured after the cow had received 6 kg of concentrate mixture III. The animal consumed the concentrates within 6 min. The total VFA concentration, D- and L-lactate concentrations, pH, and carbohydrate concentrations are listed in Table 4. The percentage of DL-lactate fermented by *M. elsdenii*, the percentage of DL-lactate converted to propionic acid via the acrylate pathway (%Acryl), and the percentage of DL-lactate fermented to butyric acid (%HBut) are also included in Table 4.

The concentrations of soluble sugars were maximal 30 min after feeding. After 90 min no sugars could be measured in the rumen fluid. The detection limit of the carbohydrate analysis was 0.01 mM for monosaccharides.

M. elsdenii fermented 64% of the DL-[2-^{13}C]lactate in the nonfed animal. When carbo-

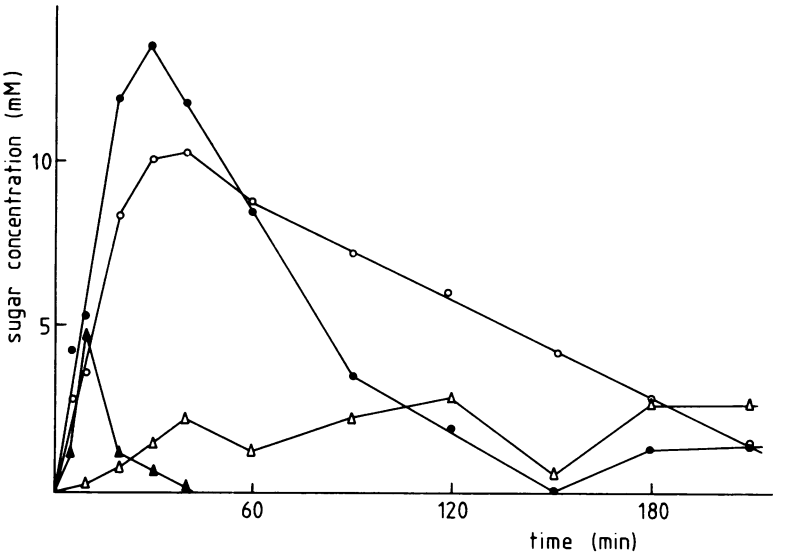

FIG. 3. *Concentrations of soluble sugars at different times after 12 g of concentrate mixture III was dissolved in 200 ml of rumen fluid obtained from cow Neel 24. Symbols: ●, fructose; ○, glucose; ▲, sucrose; △, cellobiose.*

TABLE 4. *Effects of feeding 6 kg of readily fermentable carbohydrates*[a]

Time after feeding (min)	pH	Concn (mM) of:						%Acryl	%HBut	% of DL-lactate fermented by *M. elsdenii*[b]
		Total VFA	Fructose	Glucose	Other carbohydrates	D-Lactate	L-Lactate			
0	6.90	65.56	0	0	0	0.21	0.17	54.3	22.2	64
15	6.41	72.65	1.31	0.61	0.07	10.27	5.24	ND[c]	ND	ND
30	5.98	108.23	4.23	3.33	1.22	18.69	10.61	72.9	35.3	82
45	5.86	129.43	0.24	0.16	0.10	14.75	8.47	ND	ND	ND
60	5.81	100.21	0.29	0.89	0.27	9.85	6.75	76.8	36.5	85
90	6.12	107.48	0	0	0	0.41	0.50	ND	ND	ND
120	6.04	117.48	0	0	0	0.13	0.08	49.1	31.2	65
150	6.30	117.70	0	0	0	0.15	0.10	ND	ND	ND
180	6.22	99.42	0	0	0	0.14	0.08	55.0	29.2	68

[a] Concentrate mixture III of Table 1.
[b] See footnote *a* of Table 2.
[c] ND, Not determined.

hydrate concentrations were measurable, *M. elsdenii* fermented an increasing part of the DL-lactate. When the pH decreased, more DL-lactate was fermented to butyric acid than at pH 6.90 (Table 4).

DISCUSSION

At present only *M. elsdenii* and *Clostridium propionicum* are known to ferment DL-lactate via the acrylate pathway. The use of DL-[2-^{14}C]lactate to determine the percentage of lactate fermented via the acrylate pathway is time consuming and potentially dangerous to the investigator, because the propionic acid produced from DL-[2-^{14}C]lactate has to be subjected to a stepwise degradation procedure (Schmidt degradation, as described by Sakami [12]). Furthermore, *M. elsdenii*, a predominant DL-lactate-fermenting organism in the rumen of dairy cattle, ferments a variable portion of the DL-lactate to butyric acid, depending on pH (Fig. 4). Therefore, the percentage of DL-lactate fermented by the acrylate pathway is an underestimation of the total contribution of *M. elsdenii* to DL-lactate fermentation, as can be seen in Table 2. The percentage of acetic acid produced by DL-lactate-fermenting bacteria varies between 35 and 45%, provided that no electron acceptors such as

nitrate are present (results not shown).

The results of Table 2 show that the percentage of DL-lactate fermented to butyric acid plus the percentage of DL-lactate fermented to propionic acid via the acrylate pathway is a good approximation of the participation of *M. elsdenii* in the fermentation of DL-lactate in mixed cultures of DL-lactate-fermenting organisms.

The results of Table 3 show that 27 to 96% (average, 63%) of DL-[2-^{13}C]lactate is fermented via the acrylate pathway which is in agreement with results obtained by other investigators: 57

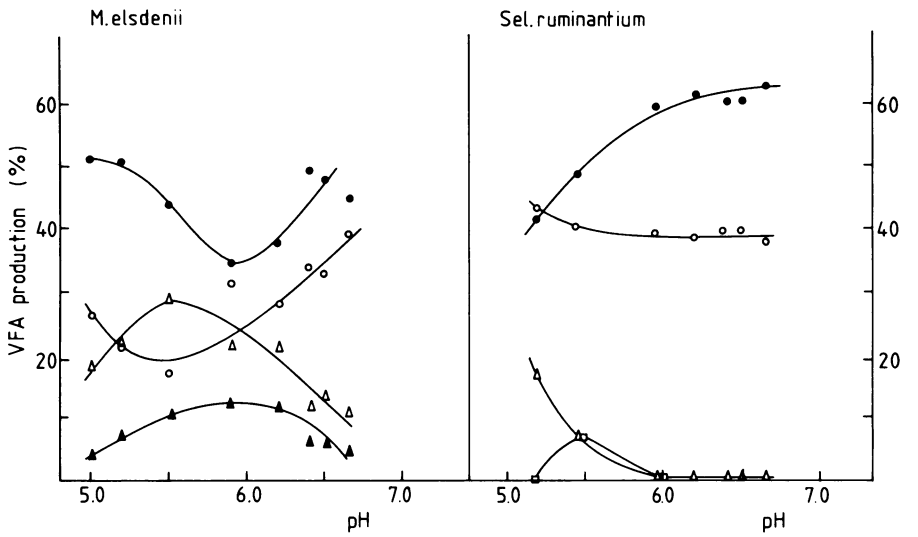

FIG. 4. *Effect of pH on product formation of M. elsdenii strain 57 and S. ruminantium subsp. lactilytica strain 73 with* DL*-lactate as substrate. Tubes with 5-ml quantities of medium were sterilized after the pH of the medium was adjusted to different values in triplicate. After inoculation, one tube of each triplicate was opened, and the pH was measured. During the stationary phase of growth and after measurement of pH, 1 ml of medium was withdrawn from each of the two remaining tubes to determine the concentrations of fermentation acids; pH values before and after growth differed by <0.1 unit. Symbols: ●, propionic acid; ○, acetic acid; ▲, valeric acid; △, butyric acid; □, succinic acid.*

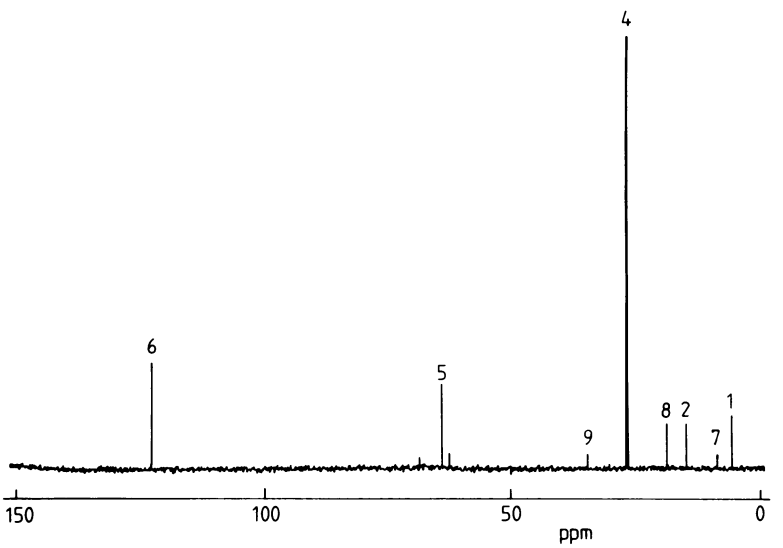

FIG. 5. *^{13}C-FT-NMR spectrum obtained after incubation of mixed rumen microorganisms with* DL*-[2-^{13}C]lactate for 3 h. Major peaks: 1, *CH_3CH_2COOH; 2, CH_3*CH_2CH_2COOH; 4, CH_3*CH_2COOH; 5, CH_3*CHOH-COOH; 6, internal standard (benzene); 7, *$CH_3CH_2CH_2COOH$; 8, *CH_3COOH; 9, CH_3CH_2*CH_2COOH; asterisk indicates the position of the ^{13}C-atom.*

to 88% (1); 30 to 65% (15); and 42 to 85% (10). However, the total participation of *M. elsdenii* in the DL-lactate fermentation is somewhat higher: 74 ± 13% (± standard deviation). Whanger and Matrone (16) showed that the mixed rumen microorganisms from sheep fed sulfur-deficient purified diets hardly converted DL-lactate to propionic acid via the acrylate pathway. This agrees with the known requirement for sulfur in the nutrition of *M. elsdenii* (5).

Therefore, we conclude that in ruminants fed normal diets *M. elsdenii* ferments 60 to 80% of the DL-lactate fermented in the rumen.

Most DL-lactate-utilizing bacteria do not ferment DL-lactate when carbohydrates are present at the same time (7, 11). When ruminants are fed diets containing readily fermentable carbohydrates, mono- and disaccharides can be measured shortly after feeding. The presence of soluble sugars would therefore result in a relative increase in the participation of *M. elsdenii* in the fermentation of DL-lactate since *M. elsdenii* shows no catabolite repression by glucose or maltose (11). This is demonstrated in Table 4: when the sugar concentration increased after feeding, the percentage of DL-lactate fermented by *M. elsdenii* also increased.

Thus, in vitro incubations with DL-[2-^{13}C]lactate are a simple but accurate way to determine the participation of *M. elsdenii* in the fermentation of DL-lactate in the rumen of dairy cattle.

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