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In Vitro Ruminal Fermentation of Organic Acids Common in Forage

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Mixed rumen bacteria from cows fed either timothy hay or a 60% concentrate were incubated with 7.5 mM citrate, *trans*-aconitate, malate, malonate, quinate, and shikimate. Citrate, *trans*-aconitate, and malate were fermented at faster rates than malonate, quinate, and shikimate. Acetate was the primary fermentation product for all six acids. Quinate and shikimate fermentations gave rise to butyrate, whereas malate and malonate produced significant amounts of propionic acid. High-pressure liquid chromatography of fermentation products from *trans*-aconitate incubations revealed a compound that was subsequently identified as tricarballylate. As much as 40% of the *trans*-aconitate acid was converted to tricarballylate, and tricarballylate was fermented slowly. The slow rate of tricarballylate metabolism by mixed rumen bacteria and its potential as a magnesium chelator suggest that tricarballylate formation could be an important factor in the hypomagnesemia that leads to grass tetany.

Organic acids, primarily citric, malic, *trans*-aconitic, malonic, shikimic, and quinic, can account for as much as 10% of the dry matter in forages (5), and high levels of these acids have been associated with toxic responses in ruminant animals (2, 3, 8, 9, 16). Stout et al. suggested that organic acids could form complexes with magnesium and decrease the availability of dietary magnesium (16). Subsequent experiments by Bohman et al. indicated that oral administration of *trans*-aconitic or citric acid could induce symptoms of hypomagnesemia, commonly termed grass tetany (2). A 1% concentration of *trans*-aconitic acid in the diet is generally considered toxic if magnesium status is low (8).

In spite of the potential impact of organic acids on animal performance, organic acid fermentation by rumen microorganisms has received little attention. Packett and Fordham (14) and Wright (18) found that the ruminal fermentation of citric acid in vivo and in vitro was rapid. Kennedy studied the fermentation of aconitic acid in vivo and likewise noted a rapid disappearance (11).

Organic acids have been analyzed by gas, paper, thin-layer, and partition-column chromatography, as well as by a variety of spectrophotometric, fluorimetric, polarographic, and chemical methods, but these procedures are often tedious, nonspecific, or nonquantitative (13). Recent advances in high-pressure liquid chromatography have yielded separations that require little or no sample preparation while allowing quantitation that is within the normal biological range (6). The following experiments describe the in vitro fermentation of citric, *trans*-aconitic, malic, malonic, shikimic, and quinic acids by mixed rumen bacteria. Rates of organic acid disappearance and product formation were monitored by resin-based, ion-exchange, high-pressure liquid chromatography.

MATERIALS AND METHODS

Inoculum. Rumen contents were obtained from rumen-fistulated dairy cows that were fed timothy hay or a 60% concentrate and 40% mixed grass hay diet (Table 1). At 1.5 h after feeding, contents were squeezed through eight layers of cheese cloth and purged with O_2-free carbon dioxide. Large feed particles and protozoa were removed by slow-speed centrifugation (150 × *g* for 5 min at 15°C).

Cell growth. The mixed rumen inoculum (above) was added to anaerobic medium that contained 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, and 7.5 mmol of organic acid per liter. Rumen fluid inoculum represented one-third of the total volume (500 ml). Incubation vessels were placed in a 39°C water bath and were continuously purged with O_2-free carbon dioxide. Final pH was 6.7.

Sampling. After 0, 1, 3, 6, 10, 15, 21, and 28 h of incubation, a 25-ml sample was withdrawn through a butyl rubber septum. Samples were centrifuged (10,000 × *g* for 15 min at 0°C) to remove cells, and the supernatant was frozen to −15°C. The cell pellet was washed with 0.9% NaCl, centrifuged, and frozen until time of analysis.

Analyses. Supernatant samples were centrifuged (13,000 × *g* for 5 min at 25°C), and 20 μl was injected into a high-pressure liquid chromatograph (model 334; Beckman Instruments, Inc., Fullerton, Calif.) that was equipped with a resin-based, ion-exchange column (HPX-87H; Bio-Rad Laboratories, Richmond, Calif.). Samples were eluted from the column with 0.013 N H_2SO_4 (25°C at a rate of 0.7 ml/min), and resulting peaks were detected by refractive index (Beckman model 156).

The unknown acid was precipitated from a 15-h incubation of *trans*-aconitic acid. Lead acetate (27 g) was added to 2 liters of cell-free medium, and the precipitate was washed once in distilled water. Lead was subsequently precipitated with an excess of hydrogen sulfide, and the supernatant was lyophilized to dryness. The lyophilized sample was dissolved in 95% ethanol, and precipitating salts were discarded. The ethanol fraction was evaporated to dryness on a steam bath, suspended in an aqueous solution containing an excess of EDTA, and adjusted to pH 7.0 with KOH. Soluble magnesium and calcium were removed by EDTA, but it also precipitated a significant portion of the unknown. Because recovery was poor in this step, EDTA was precipitated by decreasing the pH to 1.7 with HCl. The pellet was discarded, and the supernatant was adjusted to pH 9.0 with NH_4OH. The sample was then reprecipitated with lead acetate, solubilized with hydrogen sulfide, and lyophilized to dryness.

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TABLE 1. Composition of diets fed to fistulated cows[a]

Component	% of component[b] in:	
	Timothy hay	60% Concentrate
Neutral detergent fiber	59.9	41.2
Acid detergent fiber	32.3	24.8
Cellulose	28.0	20.4
Hemicellulose	27.6	16.4
Crude protein	6.7	22.8
Lignin	4.3	4.4
Ash	6.4	5.9

[a] By the methods of Goering and Van Soest (7).
[b] Calculated on a dry matter basis.

The concentration of the unknown was monitored after each step by high-pressure liquid chromatography, and the total purification scheme resulted in a 30% recovery of the unknown compound.

A methyl derivative of the unknown was prepared by dissolving 30 mg of the lyophilized residue (as described above) in 1 ml of distilled water–2 ml of methanol–14 ml of 50% H_2SO_4 and placing it in a 55°C water bath for 30 min. After cooling, 1 ml of distilled water and 0.5 ml of chloroform were added. Inversion of the tube yielded an emulsion that was broken up by centrifugation (5,000 × *g* for 10 min at 0°C).

Methylated acid from the chloroform layer (as described above) was then analyzed by gas chromatography. One microliter was injected into a Varian model 3700 gas chromatograph that was equipped with an S/P 1000, 1% H_3PO_4 on a Chromosorb WAW column (Supelco Chromatography Supplies, Bellefonte, Pa.). Conditions for gas chromatography were as follows: column temperature, 155°C; detector temperature, 180°C; injector temperature, 180°C; carrier gas (N_2) and H_2 flow, 30 ml/min; air flow, 300 ml/min; range, 10^{-11}; attenuation, 32×.

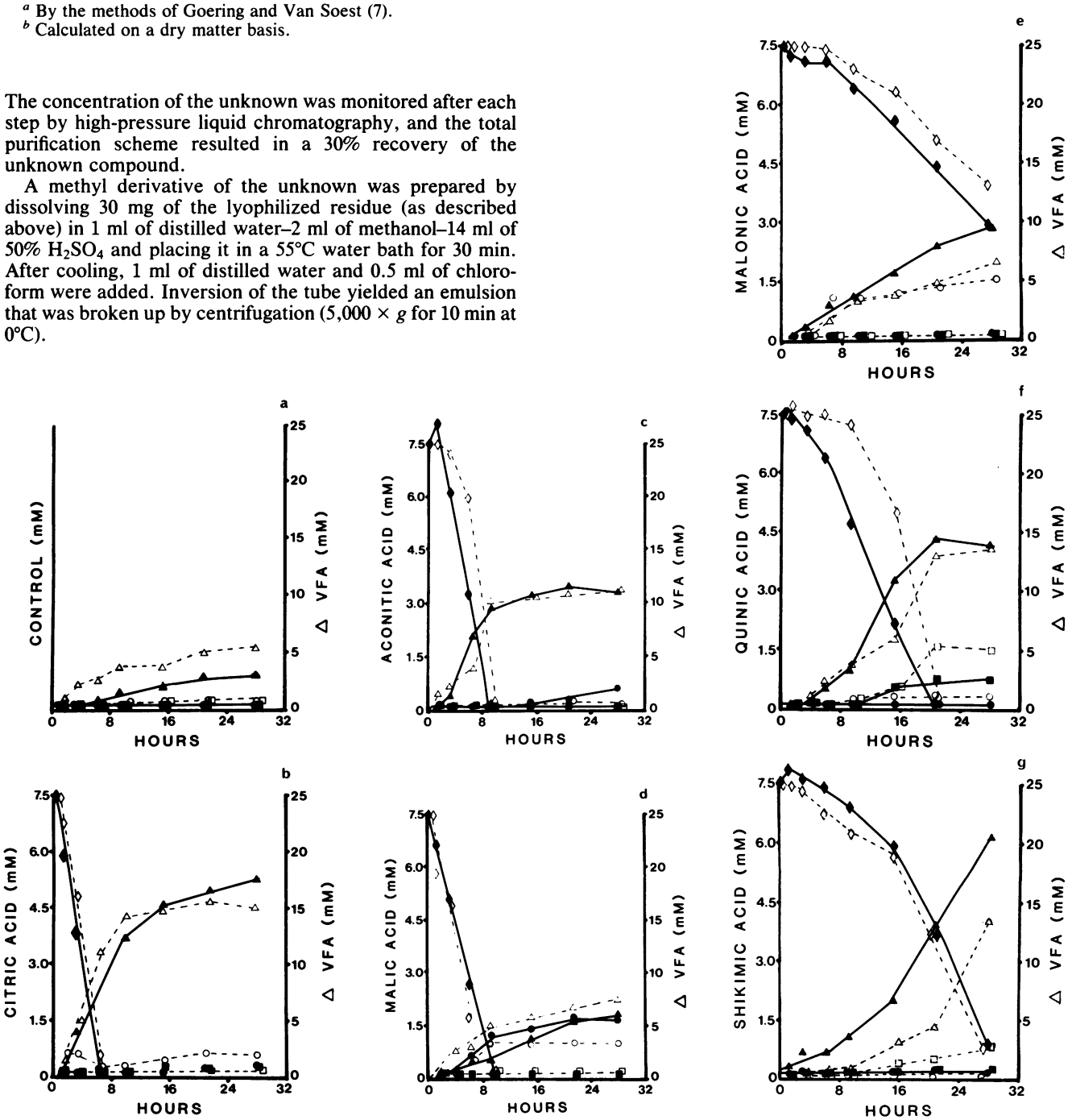

FIG. 1. Fermentation of organic acids (◆, ◇) by mixed cultures or rumen bacteria and the production of acetate (▲, △), propionate (●, ○), and butyrate (■, □). Closed symbols refer to the timothy hay inoculum and open symbols refer to the 60% concentrate inoculum.

The dominant peak appearing on the gas chromatograph (as described above) was later analyzed on a Finnegan 3300 gas chromatograph mass spectrograph equipped with an OV17 fused silica column. Mass spectrometry was conducted from 80 to 250°C at 10°C/min. Eluting compounds were subjected to electron impact or chemical ionization (CH_5^+).

Bacterial protein was measured by recently described methods (15). All incubations were performed at pH 6.7, and the final pH never deviated by more than 0.2 units. Organic acids were obtained from Sigma Chemical Co., St. Louis, Mo. The *trans* isomer of aconitic acid was used and hereafter is referred to simply as aconitic acid.

RESULTS

When a 30% rumen fluid inoculum from a cow fed timothy hay was incubated without any added organic acid, there was a small increase in the concentration of acetic acid and no appreciable increase in either propionic or butyric acid (Fig. 1a). The 60% concentrate inoculum produced higher levels of acetic acid, and this difference probably resulted from a greater contamination of small feed particles in the bacterial fraction (Fig. 1a).

Citric acid was rapidly fermented by both rumen inocula, and fermentation resulted in a significant increase in acetic acid (Fig. 1b). With the timothy hay inoculum there was little increase in propionate or butyrate, but small increases in propionic acid could be detected when the inoculum was obtained from a cow fed 60% concentrate (Fig. 1b). The kinetics of aconitic acid disappearance were also rapid, but complete fermentation of aconitate produced less acetic acid (Fig. 1c) than did similar incubations with citric acid (Fig. 1d). Examination of the high-pressure liquid chromatographic traces revealed a peak that did not have the same retention time as either aconitic acid or volatile fatty acids (Fig. 2). Concentration of this unidentified compound increased during periods of rapid aconitic acid fermentation and decreased slightly thereafter (Table 2 and Fig. 1c).

Malic acid fermentation was rapid and gave rise to both acetate and propionate (Fig. 1d). The molar ratio of propionate to acetate was ca. 1.0 when the timothy hay inoculum was used. The concentrate inoculum had a propionate to acetate ratio of ca. 0.5. Malonic acid, another dicarboxylic acid, was fermented at a much slower rate than malic acid, and fermentation products once again differed with the inoculum used (Fig. 1e). With the timothy hay inoculum, acetate was the only product, but the 60% concentrate inoculum yielded nearly equal concentrations of acetate and propionate.

The phenolic acids, quinate and shikimate, were fermented at faster rates than malonate (Fig. 1f and g). Acetate was the primary product of quinic acid fermentation, but significant production of butyrate was also observed. Shikimic acid disappearance also yielded acetate, and a small increase in butyrate was seen with the concentrate inoculum.

TABLE 2. High-pressure liquid chromatographic peak heights of unknown compound formed in aconitic acid incubations

Time (h)	Peak heights (mm) with:	
	Timothy hay inoculum	60% Concentrate inoculum
0	0	0
1	2	2
3	6	4
6	14	9
10	19	21
15	18	21
21	16	19
28	10	15

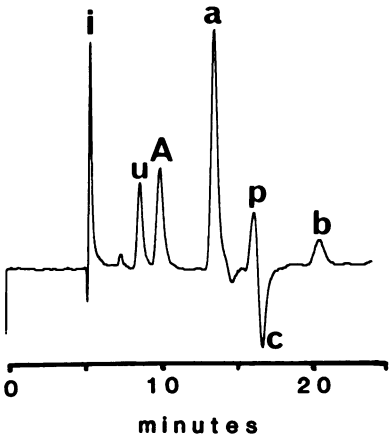

FIG. 2. High-pressure liquid chromatogram from an aconitic acid fermentation after 6 h showing the injection peak (i), acetate (a), propionate (p), butyrate (b), carbonate (c), aconitate (A), and the unidentified compound (u). Inoculum was obtained from a cow fed timothy hay.

Subsequent incubations of aconitic acid likewise produced an unidentified peak on high-pressure liquid chromatographs, and we proceeded to identify the unidentified compound (Fig. 2). Other organic acid standards were analyzed to determine whether retention times were similar to that of the unidentified compound. Citric, malonic, shikimic, quinic, pyruvic, oxaloacetic, succinic, fumaric, lactic, itaconic, citraconic, and mesaconic acids all had high-pressure liquid chromatographic retention times that were significantly different from the unidentified peak. The retention of malic acid was similar, but when samples were mixed with the malic acid standard, the unidentified peak eluted ca. 0.08 min earlier.

After 15 h of incubation, cell-free medium was treated with excess lead acetate. Further purification steps (see above) with hydrogen sulfide, ethanol, EDTA, and additional lead acetate yielded a lyophilized sample that was reasonably free of salts and other medium contaminants. A gas chromatogram of the methylated derivative is shown in Fig.

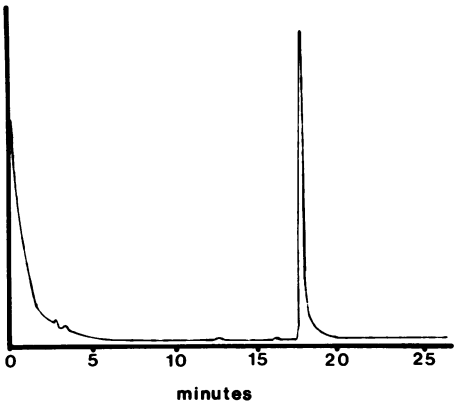

FIG. 3. Gas chromatogram after partial purification of the methylated, unidentified acid that was produced during aconitic acid fermentation.

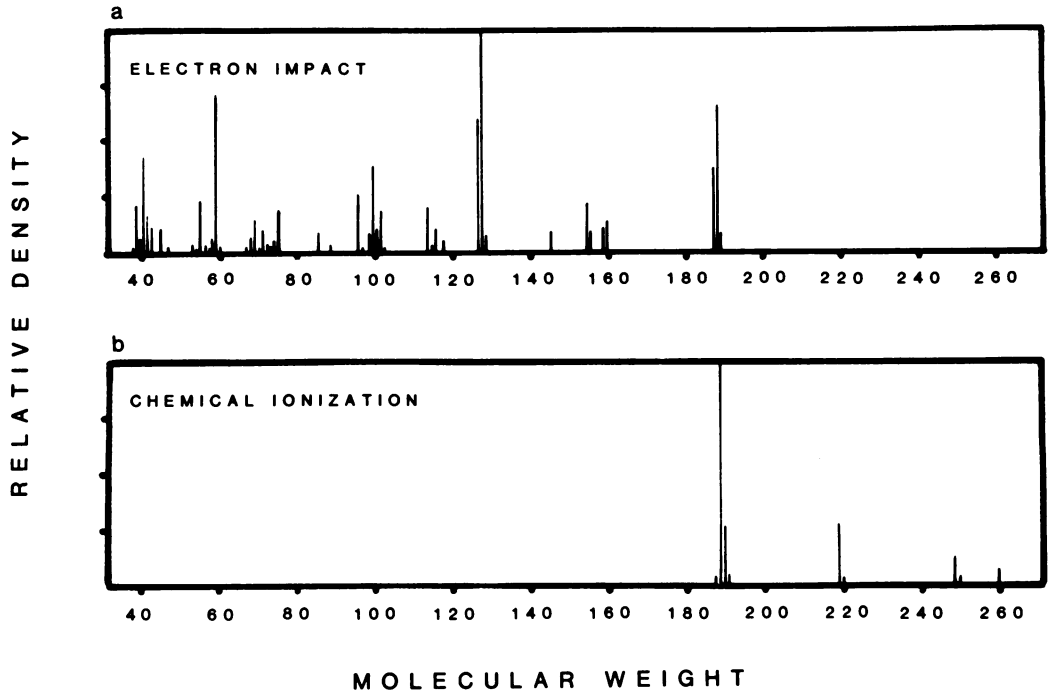

FIG. 4. Electron-impact (a) or chemical-ionization (b) mass spectrometry of the methylated, unidentified acid that was produced during aconitic acid fermentation.

3. When the methylated derivative was subjected to electron-impact mass spectrometry, decomposition occurred (Fig. 4a). More gentle chemical-ionization mass spectrometry with CH_5^+ yielded a dominant peak at a molecular weight of 219 (Fig. 4b). Other peaks can be explained by background levels of ^{13}C isotope, loss of $HOCH_3$ and OCH_3^-, or combination with $C_2H_5^+$ and $C_3H_5^+$. Given a molecular weight of 219 (Fig. 4b) for the protonated, methylated derivative, the most probable molecular weight for the original compound was 176 [219 − 1 − 3(14)]. This molecular weight corresponded to tricarballylic acid, and further high-pressure liquid chromatography, gas chromatography, electron-impact mass spectrometry, and chemical-ionization mass spectrometry analyses indicated that the unknown acid and tricarballylic acid were identical compounds.

An incubation of tricarballylic acid or aconitic acid with the concentrate inoculum is depicted in Fig. 5. Aconitic acid fermentation gave rise to tricarballylic acid, and nearly half of the aconitate was converted to tricarballylate by 15 h. From 15 to 18 h, very little tricarballylic acid was fermented, and this disappearance rate was similar to that seen with a commercial preparation (Fig. 5).

Bacteria from each incubation were analyzed for protein and compared with controls not receiving added organic acid (data not shown). In all cases, changes in cell protein were similar to those in the controls. Such results indicated that many of the bacteria were unable to grow on these organic acids or that the ATP yields from the fermentation were low.

DISCUSSION

The kinetics of organic acid disappearance by the two inocula were remarkably similar, and this would indicate that both animals contained comparable numbers of organic acid-fermenting bacteria. The six organic acids tested were not detected in rumen fluid from either cow. At this time it is impossible to say whether the rumen population could adapt to higher levels of organic acid in the diet.

On a molar basis, acetate was the primary fermentation product for each of the six organic acids (Fig. 1). Quinate and shikimate gave rise to butyrate, and significant levels of

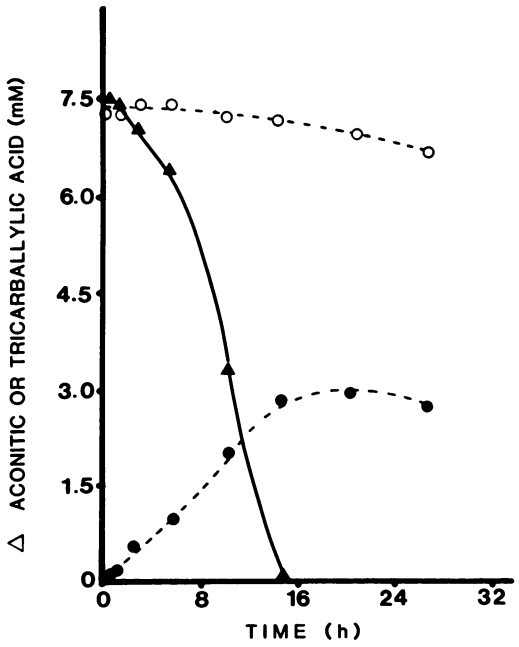

FIG. 5. Fermentation of a commercial preparation of tricarballylic acid (○) or aconitic acid (▲) and the resulting tricarballylic acid (●). Inoculum was obtained from a cow fed 60% concentrate.

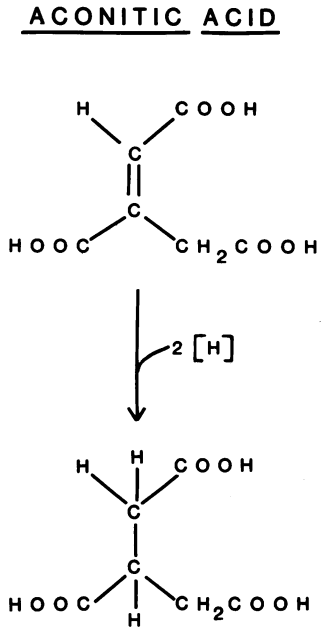

FIG. 6. Structure and likely pathway of aconitic acid conversion to tricarballylic acid.

propionate production were observed with malate and malonate. The two inocula did not always produce the same pattern of fermentation products. Changes in the fermentation product could result from differences in the types of organic acid-fermenting bacteria or changes in their metabolic pathways.

With malate, small amounts of lactate (less than 1 mM) were detected between 0 and 6 h (data not shown). Lactate was not detected in control incubations, and this suggests that lactate was an intermediate during mixed culture fermentation. Lactate turnover in vivo gives rise to acetate and propionate (10).

Aconitic acid fermentation yielded a nonvolatile acid that was subsequently identified as tricarballylic acid. Formation of tricarballylic acid from aconitic acid could take place by simple reduction (Fig. 6), and similar reactions are known to occur in the rumen (1, 4, 12, 17).

Wright (18) and Kennedy (11) observed that citrate and aconitate, respectively, were rapidly metabolized by rumen bacteria, and they questioned the importance of these organic acids in hypomagnesemia. In our experiments, citrate and aconitate were, likewise, fermented at a rapid rate, and it seemed unlikely that they would be present in the rumen for a long enough time to decrease the availability of dietary magnesium. Aconitate fermentation, however, led to an accumulation of tricarballylic acid. Because tricarballylic acid was fermented slowly by mixed rumen bacteria, it could remain in the rumen for a much longer period of time. Magnesium binding studies have not been performed, but the chemical structure, with three exposed carboxyl groups, suggests that it would be an effective chelator of magnesium (Fig. 6). Given its slow rate of metabolism by mixed rumen bacteria and its potential as a magnesium chelator, tricarballylic acid formation could be an important factor in the hypomagnesemia that leads to the clinical symptoms of grass tetany. Studies are currently under way to assess the toxicity and metabolism of tricarballylic acid in sheep.

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