

Citric Acid Metabolism in the Bovine Rumen

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Rumen microorganisms rapidly metabolize citric acid to carbon dioxide and acetic acid. The rate of metabolism varied between 0.00008 and 0.76 μ moles per g per min, the rate becoming higher as the citric acid concentration increased. The addition of potassium chloride to rumen contents decreased the rate of utilization. The results indicate that dietary citric acid is unlikely to accumulate in the rumen to a sufficiently high level to be an important factor in hypomagnesemia, except where other factors such as very high potassium levels in the food influence its metabolism.

With the notable exception of the fatty acids and succinic and lactic acids, little is known about the importance or metabolism of organic acids in the rumen. The dicarboxylic and tricarboxylic acids are common constituents in plants and microorganisms where they are metabolic intermediates or sometimes major end products of metabolism (6). Hungate and co-workers have studied the utilization of lactic (9) and succinic acids (1), and Lacoste-Bastié (Ph.D. Thesis, Univ. of Toulouse, France, 1963), using washed bacteria from the sheep rumen, has determined gas and acid production from several acids.

Interest in the metabolism of citric acid arose from recent work showing that the presence of large amounts of citric acid and potassium chloride in the diet of cattle (Wright and Young, unpublished data) caused hypomagnesemia. An earlier study has shown a slight reduction in levels from 2.11 to 1.86 mg of magnesium per 100 ml of serum in calves fed on a ration containing 1% sodium citrate (4).

The effectiveness of mixtures of citric acid and KCl in lowering blood magnesium and causing tetany has not been explained. Since tetany can be observed within 2 hr of oral dosing with the mixture (2), it seems unlikely that the effect can be explained solely on the basis of reducing the absorption of dietary magnesium by the formation of magnesium citrate in the intestinal tract. A more likely but as yet untested explanation is a mobilization of tissue magnesium by citric acid absorbed through the rumen or lower down in the intestinal tract. An essential feature of either mechanism is the accumulation of citric acid in the rumen.

Studies on the rate of metabolism of citric acid and the metabolites formed are reported in this

paper. Unfiltered rumen contents were incubated for brief periods to maintain the experimental conditions close to those found in the rumen.

MATERIALS AND METHODS

Preparation of samples. Rumen contents were collected from rumen-fistulated identical twin Jersey cows grazed on a mixed clover and ryegrass pasture. In one experiment, one of these cows was stall-fed on hay for 2 weeks before sampling. Rumen samples (50 g) were incubated under O₂-free N₂ or CO₂ in a shaking water bath at 39 C. Experiments were started within 10 min of collecting rumen contents. The radioactive citric acid was injected through rubber enclosures, and metabolism was stopped by adding 0.05 volumes of 10 N H₂SO₄. In experiments measuring radioactivity in CO₂, N₂ was bubbled through the acidified samples and CO₂ was trapped in 1 M hyamine in methanol. Rumen fluid was centrifuged at 20,000 \times g for 30 min at 2 C before steam distillation and ion-exchange chromatography.

Chromatography. Volatile fatty acids were steam-distilled and separated on columns of Celite buffered at pH 6.5 with butanol-chloroform mixtures (3). Citric acid in rumen fluid was isolated by using a column (14 ml) containing Amberlite CG-400. The column was prepared by running through 100 ml of 1 N sodium formate and then washing with water. The sample (1 to 3 ml) was added and the column was developed with a linear gradient of formic acid, the mixing chamber originally containing 100 ml of water and the reservoir containing 94 ml of 6 N HCOOH. Citric acid was eluted between 140 and 165 ml, with recoveries between 93 and 96%. Before chromatography, the acidified rumen samples were neutralized to pH 7.0 with 1 N NaOH. In this chromatography system, volatile acids were eluted near the solvent front with recoveries between 97 and 99%. Citric acid was identified by high-voltage paper electrophoresis (7) and located by scanning for radioactivity and by spraying with bromophenol blue indicator (7).

Radioactivity in the samples was estimated by using standard liquid scintillation techniques with

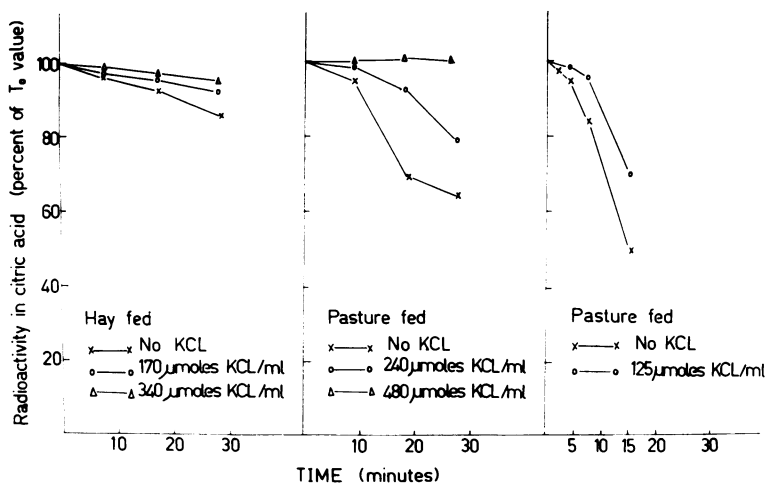


FIG. 1. Effect of KCL on citric acid metabolism by bovine rumen contents.

TABLE 1. Metabolism of citric acid-1,5-¹⁴C by rumen contents

Expt	Citric acid added (counts/min)	Incubation time (min)	Citric acid remaining (counts/min)	Carbon dioxide (counts/min)	Volatile fatty acids (counts/min)	Recovery of radioactivity (%)
1	19,400,000	15	3,000,000	5,800,000	10,400,000	98.9
2	6,540,000	10	1,000,000	1,735,000	3,310,000	92.4
3	1,000,000	10	NE ^a	164,000	355,000	

^a Not estimated.

0.8% 2,5-diphenyloxazole in toluene-methoxyethanol (2:1). Citric acid-1,5-¹⁴C was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

The products of citric acid metabolism were measured in three experiments. The results (Table 1) show that carbon dioxide and volatile fatty acids were the major products. The recoveries of radioactivity accounted for 92.4 to 98.9% of the added carbon-14. Electrophoresis of rumen fluid in these experiments, followed by spraying and scanning for radioactivity, showed that citric acid was the only radioactive non-volatile acid present in the samples. Analysis of the volatile fatty acids showed that acetic acid contained most (96%) of the radioactivity in these fractions, with propionic and butyric acids containing the remaining 4%. In experiment 2, 52,000 counts/min were found in the bacteria, a figure accounting for less than 1% of the label added. Other possible metabolites such as methane, which was not collected, or acids derived from citric acid metabolism did not contain significant radioactivity.

The rate of citric acid metabolism was meas-

TABLE 2. Rate of metabolism of citric acid-1,5-¹⁴C

Expt	Citric acid concn (μmoles/g)	Incubation time (min)	Citric acid metabolized	
			Per cent	Amt (μmoles per g per min)
5	0.0185	15	83	0.001
6	0.0083	10	85	0.0007
8	0.0079	10	14	0.00011
8	0.0079	40	39	0.00008
9	16.2	5	8	0.126
9	16.2	10	12	0.194
9	16.2	40	40	0.162
10	25.8	8	14	0.44
10	25.8	16	47	0.76
11	28	20	21	0.29
12	35.3	30	27	0.32
13	27.2	10	6	0.161
14	27.0	30	16	0.140

ured in several experiments. Attempts to measure pool sizes were not successful, since citric acid could not be detected after electrophoresis following chromatography of 10 ml of clarified rumen contents on Amberlite CG-400. Since the

indicator spray could detect as little as 5 μg of citric acid (0.026 μmole), the total citric acid content in these samples of rumen fluid must have been less than 0.003 $\mu\text{mole/ml}$ and was disregarded in the calculations of rates.

The rates of metabolism varied widely in the experiments, the rate being fastest in those samples containing the highest concentration of citric acid (Table 2). The values ranged from 0.0008 to 0.001 μmole of citric acid per g per min at initial concentrations of 0.0079 to 0.0185 μmole per g, 0.162 to 0.194 μmole per g per min at an initial concentration of 16.2 μmole per g, and 0.140 to 0.76 μmole per g per min at concentrations between 25.8 and 35.3 μmole per g. The rate of utilization in rumen fluid taken from the hay-fed animal (0.140 to 0.160) was lower than the values for its pasture-fed twin (0.20 to 0.44).

The effect of adding potassium chloride on citric acid metabolism is seen in Fig. 1. In all three experiments, the presence of potassium chloride reduced metabolism. When 480 μmoles of potassium chloride was added per g of rumen, no citric acid was used.

DISCUSSION

With suspensions of washed bacteria from the sheep rumen, Lacoste-Bastié (Ph.D. Thesis, Univ. of Toulouse, France, 1963) found that citric acid was fermented to carbon dioxide, methane, and volatile fatty acids. Similar results were achieved in this study by using unprocessed rumen contents containing a normal solids to liquid ratio and both bacteria and protozoa. Carbon dioxide and acetic acids were the major products, accounting for nearly all of the radioactivity added in citric acid- $I,5\text{-}^{14}\text{C}$. Acetic acid contained approximately twice the radioactivity found in carbon dioxide. The significance of this ratio is uncertain, since the well-recognized pathways for citric acid metabolism in microorganism through citric lyase, by the reactions of the citric acid cycle, or by the action of iso-citric lyase (5) would be expected to convert equivalent quantities of carbon-14 from citric acid- $I,5\text{-}^{14}\text{C}$ into carbon dioxide and acetic acids. A study of the enzymes responsible for citric acid metabolism in rumen microorganisms should resolve this question.

The fact that citric acid did not accumulate in rumen fluid is explained by the capacity of the rumen microbes to rapidly degrade this acid. With a potential capacity to metabolize as much as 0.76 μmole per g per min, the highest rate in these experiments, the substrate can quickly be utilized. The citric acid content in perennial ryegrass varies between 0.4 and 0.7% of the dry matter (8), and, in a mixed pasture or Ruanui ryegrass in New Zealand, it varies between 0.88 and 1.34% (13). Assuming a daily intake of 12 kg of dry matter, about 170 g of citric acid could be ingested daily. The potential metabolic capacity in a rumen containing 50 kg of contents could be as high as 38 g of citric acid per min. Obviously, under these conditions, the amount of citric acid ingested will be much lower than the potential rate of citric acid metabolism.

The reduced in vitro microbial metabolism of citric acid in the presence of KCl suggests that, in vivo, the turnover of citric acid may be reduced to the extent that this acid is spared from metabolism in the rumen and subsequently absorbed into the circulation. It is possible that plant potassium which can vary widely between 2 and 5% of dry matter could reach high enough levels to lower (11, 12) the metabolism of citric acid in vivo.

The levels of KCl added in these experiments were high, and the inhibition of metabolism could well be nonspecifically caused by an osmotic effect. Khan et al. (10) have found that KCl added at 200, 500, and 1,000 mM/liter reduced glucose metabolism in rumen fluid. Since 1,000 mM/liter represents a concentration of 7.5% KCl, the salt concentration is very high.

Walker (14) has discussed some effects of salts on rumen fermentation. With heat production measurements, sodium chloride at levels below 1% inhibited microbial activity. He further reported that 1.4% NaCl did not affect the metabolism of pyruvate or lactate but inhibited cellobiose fermentation by 45%.

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