Succinic Acid Turnover and Propionate Production in the Bovine Rumen

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Received for publication 15 October 1962

ABSTRACT

BLACKBURN, T. H. (University of California, Davis) AND R. E. HUNGATE. Succinic acid turnover and propionate production in the bovine rumen. Appl. Microbiol. **11**:132–135. 1963.—High velocity constants for conversion of added succinate to propionate, together with estimations of pool size, showed that extracellular succinate is the major precursor of the propionate formed in the rumen. Some bacteria give off succinate as a final fermentation product which is decarboxylated by others to propionate.

Many pure cultures of rumen bacteria form succinic acid as a final fermentation product (Hungate, 1960), but it does not accumulate in the rumen (Sijpesteijn and Elsden, 1952). Added succinate is rapidly converted to propionate in the rumen, by washed rumen bacteria (Sijpesteijn and Elsden, 1952), by *Veillonella gazogenes* (Johns, 1951*a*, *b*), and slowly by species of the genus *Propionibacterium* (Delwiche, 1948; Johns, 1951*c*).

The object of this investigation was to determine the importance of succinate as a precursor of rumen propionate. This has been attempted by comparing the rate of succinate turnover with the rate of propionate production in rumen contents removed from the animal and incubated in vitro for a brief period.

MATERIALS AND METHODS

Succinate estimation. The assay procedure was essentially that of Rodgers (1961), using a washed pigeon breast muscle preparation as a source of succinic dehydrogenase and 2,6-dichlorophenol-indophenol as the indicator. The reagents were those described by Rodgers (1961), with the exception that the 2,6-dichlorophenol-indophenol was purified (Punnet, 1959) and the stock solution (0.15%; w/v) diluted 15× with 0.06 M phosphate buffer (pH 7.0) immediately before use.

Pigeon breast muscle was prepared, washed (Rodgers, 1961), and weighed out in 5-g amounts onto squares of aluminum foil. These were closed up and stored in a screw-cap jar at −15 C for up to 6 months. Before use, the washed muscle (5 g) was homogenized for 1 min in a Waring Blendor in 60 ml of 0.06 M phosphate buffer (pH 7.0) and treated sonically for 15 min in a 10-kc Raytheon ultrasonic disintegrator. The preparation was then centrifuged at 800 × *g* at 4 C. The supernatant contained adequate succinic dehydrogenase activity.

Extraction of succinic acid from rumen fluid. Since it was essential to measure the extracellular succinate, a separation of the liquid from the rest of the rumen contents was necessary. A large representative sample (500 g) of rumen digesta was removed via the fistula to a Dewar flask, flushed with CO_2, and immediately transported to the laboratory. A 100-g portion was filtered through muslin into a round-bottomed flask and immediately chilled to 2 C in a solid CO_2-alcohol bath. The drop in temperature from 38 to 2 C was achieved in 30 to 60 sec. The chilled rumen fluid was immediately centrifuged at 20,000 × *g* for 15 min at 2 C to clarity.

The supernatant was removed and acidified with 12 N HCl (2 to 100 ml of fluid). Samples (50 ml) were brought to a 1.0-ml volume under reduced pressure at 70 C. To ensure a low pH, 0.1 ml of 10 N H_2SO_4 was added, and the sample was taken up quantitatively in 6 g of the Celite cap material of Wiseman and Irwin (1957). This cap material was transferred to prepared columns (Wiseman and Irwin, 1957), and the columns were extracted with the appropriate mixed hexane-acetone solution. The fraction containing succinate was collected; the succinate was extracted with water, reduced in volume to 1 ml, acidified as before, and run on a second column. This was necessary to remove impurities, especially those reducing the indicator. The succinate was again extracted from the organic solvent with water, neutralized to cresol red end point with 0.01 N NaOH and brought to dryness. The cresol red at pH 7.0 had a low optical density (OD) at 600 mμ.

The dried extract was made up to 2 ml with 0.06 M phosphate buffer (pH 7.0) for analysis. The sample (not more than 1.0 ml) was mixed in small Pyrex-stoppered tubes with 0.5 ml of the Rodgers (1961) cyanide reaction mixture, 0.5 ml of 0.01% 2,6-dichlorophenol-indophenol, and enough 0.06 M phosphate buffer (pH 7.0) to bring the volume to 2 ml. The enzyme preparation (1.0 ml) was added; after incubation at room temperature for 20 min, the OD was measured at 600 mμ using a Beckmann DU spectrophotometer. With each set of estimations, a standard curve was prepared using 0.01, 0.02, 0.03, 0.04, and

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0.05 μmoles of succinic acid. The enzyme preparation had a considerable reducing effect on the indicator but the OD blank − OD test increased linearly with succinate, 0.05 μmoles of succinate producing an OD difference of ca. 0.15 at 600 mμ.

Succinate turnover and propionate production. Portions (about 15 g) of rumen digesta from the large sample removed for the succinate determination were transferred under oxygen-free CO_2 to weighed 30-ml screw-capped bottles in a 38-C water bath, care being taken to include adequate solids as substrate. These subsamples were left for 10 min to equilibrate the temperature, and then 1 μmole of 2,3-C^{14}-succinate with 1 μc of activity was added with vigorous mixing. At 5, 10, 20, or 120 sec, a subsample was killed by adding 0.3 ml of concentrated H_2SO_4, again with rapid mixing. The zero time sample was acidified prior to addition of the succinate-C^{14}.

The subsample bottles were weighed; 1.0-g portions were removed and mixed with 6 g of cap material and immediately fractionated on Celite columns (Wiseman and Irwin, 1957) into butyric, propionic, acetic, and succinic acids. Each acid fraction was mixed with a magnetic stirrer during titration with 0.01 N NaOH under a stream of CO_2-free air.

Each fractionated acid salt in aqueous solution was brought to dryness and then redissolved in 0.5 ml of water. Portions (0.1 ml) were plated on stainless-steel planchettes and counted on either a Tricarb Geiger counter or a Tricarb gas-flow counter. The amounts of acid plated never exceeded 0.4 mg, except for acetate, and no correction for self-absorption was necessary. Since the quantities of rumen digesta varied, results are expressed as background corrected average counts per minute for the total amount of acid in the sample.

The rate of propionate production was calculated from the amount of propionate produced by control subsamples incubated for 30 and 60 min (Hungate, Mah, and Simesen, 1961).

A single fistulated 7-year-old Jersey heifer was used in these experiments. It was fed alfalfa hay at 8 AM and 4 PM, with no concentrates. Samples were obtained between 9:30 and 11:30 AM, or, in one case, at 12:30 PM.

Results

The technique of Wiseman and Irwin (1957) did not separate lactic from succinic acid, but good separation of butyric, propionic, acetic, formic, and a combined succinic-lactic fraction was obtained. Standards containing 2.5 to 25 micro-equivalents of each acid were recovered with ± 7% accuracy. In the studies with C^{14}-labeled acids, no evidence of cross contamination of bands was observed (Table 1). The rate of propionate production varied from 2.0 to 7.0 μmoles per hr. Since the fermentation was only moderately active, the reported rates for propionic acid production are not highly accurate but all are of the same order of magnitude and provide a reliable average.

The percentages of total counts per minute of each of the acid fractions after adding 2,3-C^{14}-labeled succinate are seen in Table 1. It is clear that there was a very rapid conversion of the succinate-C^{14} and a slight lag in the appearance of label in the propionate. All the added succinate did not reappear as propionate, even after 10 min. The succinate recovered as propionate in the two longest experiments (2 and 8) averaged 75%. Long incubation was not practiced in each experiment. After 2 min, about 70% of the succinate was converted to propionate (Table 1).

The velocity constant k for succinate conversion was calculated from the equation

$$1n\frac{x_0}{x} = kt$$

in which x_0 is the initial succinate count and x is the count at time t. For each experiment, x was plotted against t on semilog graph paper and an average value of k estimated from the slope of the line. This velocity constant for each experiment is given in Table 1.

In keeping with the observations of Rodgers (1961), it was found that the slope of the standard succinate curve, though constant between 0 and 0.08 μmole in any one experiment, varied considerably between experiments. It was necessary to perform a standard curve for each determination.

The recovery of 1.0 μmole of succinate added to 50 ml of acidified centrifuged rumen fluid was examined. When the columns were throughly extracted with 70 ml of 30% acetone and 70 ml of 50% acetone in mixed hexanes (Wiseman and Irwin, 1957), 90 to 96% of the added succinate was recovered, after subtracting the succinate in a control without any addition. In these experiments, there was an over-all tenfold concentration of the sample in the extraction process.

The effect of the extract on the estimation of 0.05 μmole of succinate was also examined. There was a net inhibition of the dehydrogenation, and it was necessary to apply a correction factor, which varied between 1.04 and 2.20 according to the particular experiment. The per cent recovery with the corrected figure was 95 to 136%.

The corrected values for total succinate (i.e., intra- plus extracellular) in acidified rumen fluid varied from 0.013 to 0.029 μmole per ml in 11 separate samples taken between 9:30 and 11:30 AM. Values of 0.054, 0.058, and 0.058 μmole per ml were obtained for triplicates of one sample taken at 2:30 PM. There was complete liberation of succinate from the microbial cells on acidification.

For calculating succinate turnover, the concentration of extracellular succinate is the pertinent value. Considerable trouble was experienced in the first measurements, because of the very low concentration of extracellular succinate. The direct reducing activity on the 2,6-dichlorophenol-indophenol in the absence of enzyme diminished the sensitivity of the analysis, and the extract was colored

even after two fractionations on Celite columns. Both the color and reducing activity were removed in part by mixing the concentrated rumen fluid at pH 10.0 with 6 g of Celite and extracting with 100 ml of 50% acetone in hexane prior to the acid fractionation. Ether extraction could not be employed, since it gave a preparation with high OD containing lipids.

Malonate (20 μmoles) almost completely inhibited the reduction by the test samples, indicating that only succinate reduction was involved. In those cases where the test sample still retained some direct reducing activity in the absence of enzyme, the reduction due to succinate was taken as the difference between the reduction of 2,6-dichlorophenol-indophenol in the presence and absence of malonate. The extracellular succinate concentration was so low that this OD difference was very small, approaching the limits of the method.

Three careful attempts to measure extracellular succinate concentration were completed in experiments 6, 7, and 8. The pool sizes, derived from the corrected malonate inhibition figures, are shown in Table 1.

The estimated rates of propionate production from the extracellular succinate are given in Table 1. They were obtained by multiplying the rate constant for succinate

TABLE 1. *Succinic acid and propionate activity in bovine rumen contents*

Expt no.	Incubation time*	Per cent activity in fractions†					k	Extracellular succinate	Succinate turnover to propionate	Total propionate producition	Measured succinate turnover as percentage of propionate production
		But.	Prop.	Acet.	Succ.	Total					
	sec							*μmole/ml*	*μmoles per g per hr*	*μmoles per g per hr*	
1	0	0.1	1.5	0.1	98.4	100.0	13.7/min	0.0036	2.2	3.0	73
	5	0.6	28.5	1.1	3.6	33.7		(estimated)			
	10	0.1	52.0	2.8	1.0	55.9					
	15	0.2	51.2	2.8	2.0	56.2					
2	0	0.0	1.1	0.6	98.4	100.0	12.0	0.0036	2.0	7.1	28
	5	0.0	53.4	1.9	27.5	82.8		(estimated)			
	10	0.4	63.1	5.0	16.1	84.5					
	20	0.0	59.9	3.9	10.5	74.2					
	40	0.0	75.2	5.0	5.1	85.2					
	120	3.5	72.2	2.7	7.8	86.1					
	480	0.5	85.6	8.6	2.5	97.1					
3	0	0.0	0.8	0.2	99.0	100.0	6.8	0.0036	1.1	3.5	31
	5	0.0	20.0	0.8	59.0	79.8		(estimated)			
	10	0.0	45.0	0.8	29.2	75.1					
	120	0.0	68.8	2.5	0.6	71.9					
4	0	0.1	0.1	0.1	99.7	100.0	12.1	0.0036	2.0		
	10	0.1	49.7	0.7	14.5	64.1		(estimated)			
	10‡	0.1	49.9	0.6	12.8	63.5					
5	0	0.0	0.0	0.0	100.0	100.0	8.8	0.0036	1.4	2.1	67
	10	0.0	57.6	4.6	22.0	84.3		(estimated)			
	20	0.0	74.5	1.3	6.9	82.8					
6	0	0.0	0.0	0.0	100.0	100.0	7.2	0.0058	1.9	3.9	40
	10	0.0	54.4	2.5	30.4	87.4		(measured)			
	20	0.0	67.9	3.6	8.8	80.3					
	120	0.0	72.1	2.5	3.8	78.5					
7	0	1.7	0.8	0.8	96.7	100.0	8.9	0.0033	1.3	4.2	31
	10	0.6	49.9	1.7	21.3	73.3		(measured)			
	20	0.3	56.6	2.5	5.3	64.7					
	120	0.6	51.3	1.7	14.9	68.4					
8	0	0.3	0.0	0.3	99.4	100.0	8.9	0.0017	0.7	3.6	19
	10	0.6	38.4	8.1	6.7	53.9		(measured)			
	20	0.0	53.9	4.8	5.1	63.8					
	600	0.0	66.0	3.9	5.1	75.0					

* Time interval between the addition of 2,3-C^{14}-labeled succinate to ca. 15 g of rumen digesta, and the stopping of the reaction with acid.

† Abbreviations: But. = butyric; Prop. = propionic; Acet. = acetic; Succ. = succinic.

‡ Sample was homogenized in a Waring Blendor prior to incubation.

times the extracellular succinate pool size (in μmoles per ml) times the fraction of succinate which went to propionate (0.75) times 60.

Discussion

The concentrations of acetic, propionic, and butyric acids in the Jersey rumen contents averaged 80, 17, and 16 μmoles per g, and the average increments after 1 hr of incubation were approximately 5, 4, and 3 μmoles, respectively. The rate of propionate production in these experiments (4 μmoles per g per hr) was low compared with the 6 to 10 μmoles found by Hungate et al. (1961). The difference can be explained by the ration; the animal in these experiments was fed alfalfa hay and no concentrates, whereas the earlier rates were for milk-producing Holstein cows on a ration including concentrates.

The demonstrated rates of succinate conversion to propionate constituted, on the average, 41% of the total rate of propionate production.

Because of the very small concentration of extracellular succinate in the rumen, the added labeled material increased the succinate concentration about ten times. There was little evidence that k was affected by this increased concentration. The slope of the curve $1n\ x$ against t was reasonably constant. This would be expected if the succinate-utilizing systems of the rumen contents were not saturated by the added tracer succinate.

The main error in these studies was in measuring the succinic pool. With the high velocity constants for succinate turnover, the extracellular succinate concentration could easily have diminished during preparation of the liquid sample. Straining the rumen digesta removed the solids, the chief substrate for succinate production, and, although the liquid was cooled to 2 C within 30 to 60 sec, some extracellular succinate could have been used during the early stages of cooling, without a compensating production. Values obtained must therefore be regarded as minimal. The fact that, even under these circumstances, over 40% of the propionate was accounted for indicates that a major portion of propionate in the rumen arises from extracellular succinate.

Jayasuriya and Hungate (1959), investigating lactate conversions in the bovine rumen, concluded that lactate was not a major precursor of propionate, acetate being the main end product. Baldwin, Wood, and Emery (1962) confirmed this observation and showed that the small quantity of lactate converted to propionate goes through the acrylate route. These results confirm the earlier postulate that succinate rather than lactate is the source of rumen propionate. Succinate is a major fermentation product of *Bacteroides*, *Ruminococcus*, *Succinimonas*, *Succinivibrio*, and spirochetes in the rumen.

The extracellular succinate pool figures of 0.0017 to 0.0058 μmole per ml were only a small fraction of the total succinate pool (0.013 to 0.058 μmole per ml). The figures for total pool size are considerably lower than the figures reported by Sijpesteijn and Elsden (1952) of 0.22 to 3.6 μmoles per ml.

Although its fate was not followed experimentally, the labeled succinate not converted to propionate (about 25%) was presumably assimilated by the rumen microbes. Such assimilation would be quantitatively important.

In addition to calculating the rate constant from labeled succinate, it was calculated also from the rate of accumulation of the label in propionate. The propionate count was subtracted from the final propionate count, and the logarithms of the resulting values were plotted against time. The rate constants thus obtained were almost identical with those calculated from succinate, provided these latter values were multiplied by the fraction of the label which finally appeared in propionate (about 0.75). There was no indication from the graph of propionate activity that the values of k for the first 5-sec interval differed significantly from the values for later periods.

These kinetics could indicate that any propionate-forming intracellular succinate pool fed from extracellular succinate is extremely small, perhaps only a few rumen bacteria absorb and decarboxylate extracellular succinate, or, more probably, that the decarboxylating enzymes act directly on the external succinate pool, being extracellular or part of the cell envelope.

Acknowledgments

This investigation was aided in part by research grant E 1266 from the National Institutes of Health. One of us (T.H.B.) wishes to thank The Wellcome Trust for receipt of a traveling fellowship.

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