CITRIC ACID FERMENTATION BY STREPTOCOCCI AND LACTOBACILLI

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A study of lactic acid bacteria has revealed an active citric acid fermentation system in a number of homofermentative streptococci and lactobacilli. The present report includes data on the suitability of citric acid as an energy source and fermentation balances for representative strains.

In a paper dealing specifically with the heterofermentative cocci—genus *Leuconostoc*—Slade and Werkman (1941) have reviewed the literature on citric acid fermentation by various organisms. The scheme which was suggested by Brewer and Werkman (1939) to account for fermentation balances of the *Aerobacter* genus was included and its applicability to the data for the heterofermentative cocci pointed out. Data on the disappearance of citric acid from citrate, or glucose-citrate, cultures of homofermentative cocci are reported by Hucker and Pederson (1930). Rudert (1940) noted an increased carbon dioxide production on the addition of sodium citrate to tomato-glucose cultures of *Lactobacillus casei*. With certain strains of *Streptococcus liquefaciens*, Long and Hammer (1936) have found an increase in volatile acid and acetylmethylcarbinol production on the addition of citrate to milk cultures.

EXPERIMENTAL

Bacteriological methods. The streptococcus strains, which were from the departmental culture collection, conformed to the usual characteristics for the species listed (Sherman, 1937). The enterococci have previously been studied and resting cell suspensions found to convert 90 to 98 per cent of the glucose fermented to lactic acid (Smith and Sherman, 1941). Growing cultures also conform to the homolactic fermentation though some, especially strain 815, may be partially converted away from the characteristically high lactic acid yields by alkaline growth conditions (Gunsalus and Niven, 1942). The lactobacilli, with the exception of strain 19, were received from the Wisconsin collection through the kindness of Dr. McCoy. Strain 19 is one of Rudert's cultures. The BC1 strain (ATC No. 7469) is the organism now commonly employed in microbiological assay methods.

The fermentation balances and growth response measurements were run in a medium containing 0.5 per cent tryptone, and 0.5 or 1.0 per cent yeast extract. Citric acid was added to the medium aseptically as sodium citrate. Since 1 per cent citric acid inhibited the growth of the lactobacilli, it was added to these cultures in two portions. In growth experiments, turbidity was measued with an instrument previously calibrated by microkjeldahl. The scale is nearly linear for the quantity of growth—each scale unit approximating 6 micrograms of bacterial nitrogen per 10 ml.

No further buffer was added to the medium since the pH remained favorable for growth throughout the fermentation. After inoculation the oxygen was displaced from the flasks with nitrogen and the cultures incubated at 35 C until the substrate was exhausted, ordinarily a period of about 3 days. The fermentation was stopped by the addition of sufficient 10 normal H_2SO_4 to bring the pH below 3.0 and the carbon dioxide was swept from the flasks to potash bulbs with a stream of CO_2-free air.

Chemical methods. Residual citrate was determined by the colimetric method of Pucher, Sherman, and Vickery (1936). Lactic acid was determined on Somogyi filtrates according to Barker and Summerson (1941). The volatile acids were removed from the medium by steam distillation and an aliquot titrated for total volatile acids. Formic acid was determined on another aliquot by the A.O.A.C. (1940) method, and acetic acid calculated by difference after suitable correction for recoveries in the steam distillation. The absence of volatile acids above acetic was confirmed by the partition coefficient method of Osburn, Wood, and Werkman (1936). The carbon dioxide was determined by weighing the potash bulbs mentioned above. Acetylmethylcarbinol was determined iodometrically on the third quarter of the neutral volatile distillate by the method of Langlykke and Peterson (1937).

Quantity of growth. The marked increase in growth of a number of lactic acid bacteria resulting from the addition of citric acid to a tryptone-yeast-extract medium indicates its availability as an energy source for these organisms (table 1). The enterococci showed the greatest stimulation in growth, that is, up to 100 scale units or a 5- to 6-fold increase. With 0.5 per cent glucose the growth amounted to 1.5 to 2.0 times that with 0.5 per cent citric acid. The lactobacilli, which grew much more poorly in the base medium than did the enterococci, showed a much smaller increase in growth with citric acid. Pro portionally, however, about a five-fold stimulation occurred on the addition of citric acid.

An increase in the initial citrate concentration to 1.0 per cent resulted in an inhibition of the growth of some of the lactobacilli. This was especially marked in the case of *Lactobacillus delbrueckii*, strain No. 3. The strains of *Streptococcus mastitidis* tested—one of which is shown in table 1—gave less growth in the presence of citrate than in the basal medium. Inhibition was also apparent on the addition of citrate to glucose broth which otherwise supported good growth.

Streptococcus lactis and one of the *Streptococcus fecalis* cultures tested failed to show citrate utilization as indicated by growth.

The utilization of citric acid as an energy source for growth in the absence of a fermentable carbohydrate is in contrast to the results for the heterofermentative cocci which require an available carbohydrate to attack citrate in growing cultures. This ability of homofermentative organisms to utilize citric acid as an energy source allows a study of its decomposition without the complications caused by the presence of a second substrate.

Fermentation balances. The predominant products from the fermentation of citric acid are acetic acid and carbon dioxide (table 2). For all 5 of the organisms

TABLE 1

Citric acid as an energy source

Base medium: 1% tryptone; 0.5% yeast extract; 0.2% K_2HPO_4; pH 7.4

ORGANISM	STRAIN	GROWTH (NEPHELOMETER READING*)	
		Base medium	+0.5% citric acid
S. mastitidis	70b	17	2
S. lactis	21	7	7
S. fecalis	24	14	10
	10C1	32	175
S. liquefaciens	815	27	140
S. zymogenes	26C1	34	150
	H69D5	15	40
L. casei	19	8	24
L. casei (E)	BC1	9	58
L. delbrueckii	Ld3	9	50
	Ld5	8	60
L. lactis	BL1	12	60

* Each scale unit ≙ 6 micrograms bacterial Nitrogen/10 ml.

TABLE 2

Fermentation products from citric acid

Growth medium: 0.5% tryptone; 1% yeast extract; 1% citric acid; pH 7.2

Products in millimoles per 100 millimoles citrate fermented

PRODUCTS	S. LIQUEFACIENS (815)	S. ZYMOGENES (H69D5)	S. FECALIS (10C1)	L. CASEI (BC1)	L. DELBRUECKII (LD5)
Carbon dioxide	137	130	106	115	119
Acetic acid	155	157	176	175	172
Formic acid	38	34	80	73	64
Lactic acid	35	42	4	12	16
Acetylmethyl carbinol	.1	1	2.6	.6	.4
Ethyl alcohol				3	2
Carbon recovery %	98	101	95	97	97
O/R balance	1.02	.97	.94	.96	.99
Final pH	6.4	7.0	6.7	6.2	7.2

studied the yields of these compounds were in excess of one mole per mole of substrate fermented. The majority of the remaining carbon can be accounted for as formic and lactic acids. Only traces of other compounds were formed under

the conditions of the experiments. The small amounts (table 2) of acetylmethylcarbinol and ethyl alcohol found are in agreement with the general opinion that acid reaction and reducing conditions, respectively, are required for the formation of these compounds. Succinic acid was not present in an appreciable amount, nor was hydrogen among the fermentation products.

The yields of lactic acid—4 to 42 moles per 100 moles of citrate fermented—are low for organisms generally considered to be homofermentative lactic acid types. The alteration of fermentation is not, however, too surprising when one considers the oxidized nature of the substrate.

Although a comparison of the fermentation products of the 5 cultures can not yield information as to the fermentation mechanism of any given culture, certain suggestions of relationships are apparent. Among these is a correlation in the quantity of lactic acid formed with the yield of CO_2, in excess of one mole of CO_2 per citrate fermented. A similar relationship, although not so direct, exists between the total formic acid produced and the quantity of acetic acid beyond the first mole per mole of citrate. These correlations in the yield of products indicate the possibility of their formation from a common precursor. There is also an apparent reciprocal relationship between the yields of formic and lactic acids (table 2). This would suggest that they may be formed by competition for a common precursor.

When the products from glucose fermentation at alkaline reaction are compared to those obtained from citric acid, the reciprocal relationship between the yields of lactic acid and volatile acids appears in both. The correlation of the lactic acid with the carbon dioxide yield is, however, peculiar to the oxidized substrates. The results in table 2 suggest that metabolic studies on an oxidized substrate, such as citric acid, may yield more information of the potential enzymic activities of these homofermentative lactic acid organisms than will studies with glucose as substrate.

DISCUSSION

Perhaps the most striking feature of the fermentation balances is the almost complete absence of lactic acid among the fermentation products—only 2 to 20 per cent of the carbon from the citric acid decomposed. This may be partially due to the inherent requirement of oxidized products to balance the reactions with so oxidized a substrate. On the other hand, the presence of additional enzyme systems which actively compete for the intermediate substrates must be a factor.

The very considerable yields of carbon dioxide indicate the presence of an active decarboxylation system among the enzymes of these organisms. Thus they can not be differentiated from the heterofermentative lactic acid bacteria merely by the lack of a decarboxylation system. The contrast between the products of glucose fermentation in the two groups suggests that the difference may lie in the type of enzyme system responsible for the carbon dioxide formation. Perhaps the mixed lactic organisms possess a yeast-type carboxylase whereas the homofermentative cultures possess only the so-called animal type.

The latter is known to occur in some strains of *Lactobacillus delbrueckii* (Lipmann, 1939), and in some *Streptococcus fecalis* cultures (Krebs, 1937; Miller, 1942). Although this system may be operating in the fermentation of citric acid, by far the larger portion of the carbon dioxide formed must be from another reaction —possibly from the decarboxylation of oxalacetic acid (see Brewer and Werkman, 1939).

The parallel yields of formic and acetic acids may be due to the hydroclastic reaction, since these compounds are known to be formed from pyruvic acid, and from glucose, by certain streptococci (Barron and Lyman, 1938; Gunsalus and Niven, 1942). More direct information on the mechanism of citric acid decomposition by the homofermentative lactic acid bacteria must await controlled variation in the metabolism of a given culture.

A comparison of the fermentation balances, as shown in table 2, with the balances for resting cells of heterofermentative cocci (Slade and Werkman, 1941) and to those for *Aerobacter aerogenes* (Brewer and Werkman, 1939) reveals striking similarities. This is especially true when one compares the yields of formic and acetic acids and carbon dioxide. The main differences lie in the absence of succinic acid and hydrogen among the fermentation products of the homofermentative lactics, whereas they have been reported among the products of heterofermentative cocci and *Aerobacter aerogenes*. The carbon which is found in succinic acid with the heterofermentative organisms is accounted for largely as lactic acid with the organisms studied here. The products of citric acid fermentation by these three groups of organisms have much more in common than might be expected from a knowledge of their fermentation products from glucose.

The appreciable quantities of formic acid produced by the 5 cultures studied contrast with the results of van Beynum and Pette (1939) for *Streptococcus citrophilus*. This culture, which is apparently homo-lactic on glucose, is reported to ferment citric acid at neutral reaction with the production of carbon dioxide, acetic acid, and ethyl alcohol. Since these workers did not analyze for formic acid, it is possible that some of the acid which they recorded as acetic is actually formic.

SUMMARY

Several species of homofermentative lactic acid bacteria, cocci and rods, have been studied and shown to utilize citric acid as an energy source for growth in the absence of fermentable carbohydrate.

Fermentation balances with growing cultures of representative strains of enterococci, *Lactobacillus delbrueckii*, and *Lactobacillus casei*, have shown the main products in neutral cultures to be acetic acid and carbon dioxide, with formic and lactic acids accounting for most of the remaining carbon. Traces of acetylmethylcarbinol and ethyl alcohol were also formed.

With citric acid as substrate, the fermentation products of the homofermentative lactic acid bacteria show several similarities to those formed by members of the *Leuconostoc* and *Aerobacter* genera.

It is suggested that oxidized substrates, such as citric acid, will give more information as to the potentialities of the homofermentative organisms than will the more conventional hexoses.

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