FERMENTATION OF ALPHA KETO ACIDS BY *MICROCOCCUS AEROGENES* AND *MICROCOCCUS LACTILYTICUS*[1]

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The anaerobic micrococci can be divided into groups on the basis of metabolic activity (Whiteley, 1957). In this laboratory, particular attention has been given to studies of *Micrococcus aerogenes* and *Micrococcus lactilyticus* (also called *Veillonella gazogenes*). These are representatives of 2 separate metabolic groups of anaerobic micrococci. *M. lactilyticus* is characterized by its ability to ferment purines and organic acids but not amino acids, while *M. aerogenes* degrades purines, a limited number of organic acids, and certain amino acids. Although both organisms ferment purines and α-keto acids, distinct differences have been found between the 2 organisms in the metabolism of purines. In the present paper, *M. lactilyticus* and *M. aerogenes* are compared with respect to the fermentation of certain α-keto acids.

METHODS

Strain 228 of *M. aerogenes* was grown either in "peptone medium" or "glutamate medium" and strain 221 of *M. lactilyticus* was ordinarily grown in lactate medium. A few experiments were performed with cells of *M. lactilyticus* grown in a medium low in thiamin and lactate. These media, the conditions of growth, and preparation of cell suspensions, dried cell preparations, and cell free extracts have been described (Whiteley, 1957; Whiteley, 1953*c*). To obtain extracts of maximum activity, the cell debris and intact cells remaining after grinding with alumina were ground a second time with additional alumina and the cell paste-alumina mixture was eluted with the extract obtained from the first grinding. Some experiments were performed with extracts of *M. lactilyticus* obtained by shaking a thick cell suspension with small glass beads in a Mickle apparatus manufactured by H. Mickle, Hampton, Middlesex, England. The shaking was performed in the cold for 30 to 45 min, the preparation was centrifuged at 10,000 rpm for 20 min in a Servall centrifuge and the supernatant liquid ("Mickle extract") was tested without further treatment. All preparations contained a reducing agent in a final concentration of 0.005 to 0.01 per cent. Some experiments were performed with extracts aged by storage at -4 C for 3 to 21 days.

Manometric experiments were carried out with 10 to 30 mg cell protein, 50 μmoles phosphate buffer (pH 6.5 for pyruvate and α-ketoglutarate fermentations and pH 6.8 for α-ketobutyrate), 10 to 50 μmoles of substrate, and other additions as noted, in an atmosphere of oxygen-free nitrogen or hydrogen. Lactic dehydrogenase activity was estimated from the time required to decolorize a standard amount of methylene blue at pH 6.8 in the agar modification of the Thunberg technique (Umbreit *et al.*, 1949) and by following the reduction of diphosphopyridine nucleotide (DPN) by extracts under anaerobic conditions. Succinic and malic dehydrogenases were estimated from the rate of reduction of 2,6-dichlorophenolindophenol at 600 mμ at room temperature in reaction mixtures containing 5 to 10 mg protein, 10 μmoles phosphate buffer pH 6.5, and 20 μmoles substrate. The activation of acids was determined by measuring the amount of hydroxamic acid formed in 30 min in a reaction mixture containing 20 mg of extract protein, 50 μmoles sodium fluoride, 10 μmoles adenosine triphosphate (ATP), 20 units of coenzyme A (CoA), 50 μmoles freshly neutralized hydroxylamine, and 50 μmoles of tris (hydroxy-amine) methane buffer ("tris") at pH 7.5 in a volume of 1.0 ml. Carbon dioxide production from succinate was measured under the conditions described earlier (Whiteley, 1953*a*, 1953*b*); CoA transphorase activity according to Lieberman (1954).

Methods used for the estimation and identification of volatile acids, lactate, hydroxamic acids, α-keto acids, formate, and protein have

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TABLE 1

End products of α-ketobutyrate fermentation by extracts of Micrococcus aerogenes and Micrococcus lactilyticus

End Products*	*M. aerogenes*	*M. lactilyticus*
Carbon dioxide	9.8	9.6
Hydrogen	9.6	9.7
Propionate	9.8	10.1
% Carbon recovery	98	101
O/R Index	0.98	1.00
μL Gas/10 mg protein/10 min	11	15

* End products reported as μmoles/10 μmoles substrate. Reaction mixture contained: 10 μmoles substrate, 25 mg cell protein, 2 μmoles cysteine and 50 μmoles phosphate buffer at pH 6.8 in 2.2 ml.

TABLE 2

End products of pyruvate fermentation by Micrococcus aerogenes

End Products*	Suspension of Cells Grown in "Peptone Medium"	Extract of Cells Grown in "Peptone Medium"†	Suspension of Cells Grown in "Glutamate Medium"
Carbon dioxide	7.4	9.9	9.8
Hydrogen	4.7	9.8	9.7
Acetate	6.9	9.8	9.6
Butyrate	0.2	0	0
Lactate	2.5	0	0
% Carbon recovery	98	98	97
O/R Index	0.97	1.01	1.03
μL Gas/10 mg protein/10 min	17	29	26
Lactic dehydrogenase‡	20	12	16

* Quantities of end products reported as μmoles/10 μmoles of substrate. Reaction mixture as for table 1 except that phosphate buffer pH 6.5 was used.

† Extract or dried cell preparation.

‡ Minutes required to decolorize 0.04 mg methylene blue by 10 mg protein in the presence of 40 μmoles lactate, 50 μmoles phosphate buffer pH 6.5, and 0.5 ml 2% agar in a total volume of 2.0 ml.

been cited previously (Whiteley, 1957). Acrylate was measured by addition of iodine and back titration of the excess iodine with thiosulfate. Citrate was determined according to Weil-Malherbe and Bone (1949).

ATP and CoA were obtained from Pabst and Co. and DPN from Sigma Chemical Co., Inc.

RESULTS

The fermentation of α-ketobutyrate by M. lactilyticus and M. aerogenes. Both anaerobic micrococci are capable of carrying out an oxidative decarboxylation of α-ketobutyrate to form equimolar amounts of carbon dioxide, hydrogen, and propionate (table 1). Maximal activity is found with extracts from freshly grown cells. However, the rate of fermentation of α-ketobutyrate is always less than that of pyruvate. In a hydrogen atmosphere the rate of gas production from both keto acids is reduced, that of α-ketobutyrate more so than that of pyruvate. Various types of preparations, although not equally active, produce the same end products. Preparations characterized by a low rate of fermentation do not normally ferment α-ketobutyrate to completion. Preparations from both organisms show a rapid loss in activity on aging or on dialysis but this effect is more noticeable with *M. aerogenes* than with *M. lactilyticus*. Four hours of dialysis against 0.001 per cent reducing agent or 1 day of storage at −4 C leads to a loss of activity of about 50 per cent. The activity cannot be restored by the addition of cofactors or by prolonged incubation.

The fermentation of pyruvate by M. aerogenes. Pyruvate is fermented to equimolar quantities of carbon dioxide, hydrogen and acetate by freshly prepared extracts, dried cell preparations and suspensions of cells grown in glutamate medium (table 2). However, suspensions of cells grown in peptone medium degrade pyruvate more slowly and form lactate and small amounts of butyrate in addition to carbon dioxide, hydrogen, and acetate (table 2).

Cells grown in peptone medium show a greater lactic dehydrogenase activity than extracts prepared from these cells or cells grown in glutamate medium. In contrast, extracts of cells grown in peptone medium, and whole cells grown in glutamate medium show a relatively high rate of oxidative decarboxylation of pyruvate. Hence, it is possible that formation of lactate is dependent upon the relative activities of lactic dehydrogenase and the enzymes concerned in the cleavage of pyruvate.

Lactate is not produced from serine by cells grown in peptone medium even though pyruvate is an intermediate product in serine degradation (Whiteley, 1957). This can be accounted for if the rate of deamination of serine is less than that

of pyruvate decomposition so that free pyruvate does not accumulate to serve as hydrogen acceptor and all "available hydrogen" is released as hydrogen gas.

The fermentation of pyruvate by M. lactilyticus. Dried cell preparations, and extracts of *M. lactilyticus* prepared by sonic disintegration or alumina grinding, ferment pyruvate completely to carbon dioxide, hydrogen and acetate (table 3). As noted above, these are also the products of fermentation of pyruvate by similar preparations of *M. aerogenes*. However, suspensions of intact cells, and Mickle extracts of *M. lactilyticus* are able to form propionate in addition to carbon dioxide, hydrogen, and acetate (table 3). Preparations of this type will be referred to as type I, whereas preparations of *M. lactilyticus* which are unable to form propionate from pyruvate will be referred to as type II.

In order to account for the production of propionate from pyruvate by type I preparations, a general consideration of the pathway of propionate formation is pertinent. It has been postulated (Delwiche, 1948; Johns, 1951*a*, 1951*b*) that propionate is formed by a sequence of reactions involving the condensation of pyruvate and carbon dioxide to oxalacetate, followed by conversion through malate and fumarate to succinate, which is then decarboxylated to propionate and carbon dioxide. Further work (Whiteley, 1953*a*, 1953*b*; Delwiche *et al.*, 1953) has shown that succinyl-CoA rather than free succinate is decarboxylated and that a C_1 fragment other than carbon dioxide may be formed (Delwiche *et al.*, 1953, 1956; Phares *et al.*, 1956). Since preparations of *M. lactilyticus* contain the condensing enzyme, a possible alternative pathway might involve citrate, *cis*-aconitate and α-ketoglutarate. Although the latter two compounds are readily fermented, citrate is not degraded by preparations from lactate grown cells, and therefore this pathway appears unlikely. As indicated below, the inability of type II preparations of *M. lactilyticus* to form propionate from pyruvate could be explained on the basis of an impairment of one of the enzymatic steps in the sequence proposed by Delwiche and Johns.

Type I preparations form propionate from pyruvate and also from oxalacetate, malate, fumarate, succinate and α-ketoglutarate. Type II preparations, although unable to produce propionate from pyruvate, ferment α-ketoglutarate, succinate, fumarate, malate and oxalacetate with the production of propionate. The fermentations of the last 3 substrates, in contrast to those of α-ketoglutarate and succinate, do not proceed to completion. It is noteworthy that activated succinate can be detected during the fermentation of all substrates listed above when propionate is an end product. Both type I and II preparations activate succinate and produce carbon dioxide from succinate at comparable rates. Succinic and malic dehydrogenase activities are also comparable. On the other hand, type II preparations degrade oxalacetate at about 60 per cent of the rate shown by type I preparations.

TABLE 3

End products of pyruvate fermentation by Micrococcus lactilyticus

End Products*	Cell Suspension	Cell Free Extract†	Cell Suspension in Presence of 5 μmoles Acrylate
Carbon dioxide	7.2	9.7	10.2
Hydrogen	3.6	9.5	9.8
Acetate	8.7	9.8	9.7
Propionate	1.5	0.0	0
% Carbon recovery	97	98	99
O/R Index	0.93	0.99	1.04
μL Gas/10 mg protein/ 10 min	22	25	

* Quantities of end products reported as μmoles/10 μmoles of substrate. Reaction mixture as for table 2.

† Extract prepared by grinding with alumina.

The amount of propionate produced by type II preparations from oxalacetate, malate, and fumarate may be increased when an atmosphere of hydrogen is substituted for nitrogen. This effect is not seen with type I preparations. A further increase in the amount of propionate formed by type II preparations in an atmosphere of hydrogen may be achieved by the addition of semicarbazide. Under these conditions, fumarate is converted almost quantitatively to carbon dioxide and propionate.

It has been found that acrylate has an interesting effect on the fermentation of pyruvate by type I preparations of *M. lactilyticus* (table 3). Although acrylate is not degraded or reduced under any conditions of testing, and can be quantitatively recovered from reaction mixtures, its presence prevents the formation of propionate from pyruvate, so that all of the pyruvate under-

TABLE 4

End products of α-ketoglutarate fermentation by Micrococcus lactilyticus

End Products*	Fresh Extracts†	Aged Extracts‡
Carbon dioxide	19.5	20.6
Hydrogen	9.7	13.4
Acetate	0	1.8
Propionate	9.3	8.4
% Carbon recovery	95	98
O/R Index	1.00	0.97
μL CO_2/10 mg protein/10 min produced from 40 μmoles succinate	35	19

* End products reported as μmoles/10 μmoles of substrate. Reaction mixture as for table 2.

† Fresh extract of cells grown in the optimal medium.

‡ Aged extract or extract of cells grown in suboptimal medium.

goes oxidative decarboxylation. Acrylate has a like effect on the fermentation of lactate but under these conditions this substrate is not fermented to completion.

Acrylate may be activated by the CoA transphorase which is found in extracts of both *M. aerogenes* and *M. lactilyticus*. Therefore, it was considered possible that an indirect effect on the availability of CoA might account for the inhibition in propionate production from pyruvate and lactate. This explanation does not seem likely, however, in view of the observation that propionate, butyrate, and formate are activated more readily than is acrylate yet their addition does not affect propionate formation from pyruvate or lactate. Furthermore, acrylate has no effect on the fermentation of the dicarboxylic acids by *M. lactilyticus* or on the degradation of pyruvate by *M. aerogenes*. The finding that all of the acrylate can be recovered from reaction mixtures precludes the possibility of direct reduction of acrylyl-CoA to propionyl-CoA. Acrylyl-CoA has been implicated as an intermediate in the oxidation of propionate by *Clostridium propionicum* (Stadtman, 1955).

The fermentation of α-ketoglutarate by M. lactilyticus. α-Ketoglutarate is fermented by *M. lactilyticus* but not by *M. aerogenes*. The end products of the fermentation of α-ketoglutarate by 2 extracts of *M. lactilyticus* are given in table 4. Freshly prepared extracts degrade this substrate to carbon dioxide, hydrogen and propionate. As mentioned above, activated succinate can be detected during this fermentation. The rate of fermentation is slightly reduced if an atmosphere of hydrogen is substituted for nitrogen.

If extracts are aged by prolonged storage in the cold, less propionate is formed, and a small amount of acetate is produced as an additional end product (table 4). Comparable results are obtained with freshly prepared extracts of cells grown in a medium low in thiamin and lactate. Little or no acetate is formed by these preparations when malonate is added and the quantities of end products are almost the same as those obtained from fresh extracts of cells grown in the optimal medium. As indicated in table 4, carbon dioxide is produced from succinate at a greater rate by preparations which do not produce acetate as an end product.

DISCUSSION

When α-ketobutyrate is used as substrate, the products of fermentation by both *M. aerogenes* and *M. lactilyticus* are propionate, carbon dioxide and hydrogen, regardless of growth medium or type of preparation employed. Maximum activity was found only with fresh preparations. On aging, a rapid loss of fermentative capacity occurred, particularly with *M. aerogenes*. With less active preparations, the fermentations did not go to completion. However, residual α-ketobutyrate could always be recovered and no additional end products were found.

Only *M. lactilyticus* is able to ferment α-ketoglutarate and the degradation of this compound depends on the medium in which the cells are grown and the type of preparation employed. The products formed by freshly prepared extracts of cells grown in an optimal medium are propionate, carbon dioxide, and hydrogen. These can be assumed to arise as a result of the oxidative decarboxylation of α-ketoglutarate to carbon dioxide, hydrogen and succinyl-CoA in a manner analogous to that shown to occur in mammalian tissue (Sanadi and Littlefield, 1951). This is followed by the decarboxylation of succinyl-CoA to propionyl-CoA and carbon dioxide (Whiteley, 1953*a*, 1953*b*). In line with this hypothesis, succinyl-CoA has been found as an intermediate product in the fermentation of α-ketoglutarate by *M. lactilyticus*. The decarboxylation of succinyl-CoA occurs at a greater rate under hydrogen than under nitrogen, while the rate of decomposition of α-ketoglutarate is reduced in a hydrogen

atmosphere. Hence, this reduction in rate may be attributed to an effect on enzymes concerned in oxidative decarboxylation.

Aged extracts and preparations from cells grown in a suboptimal medium form small amounts of acetate and less propionate from α-ketoglutarate and decarboxylate succinate at a lower rate. The production of acetate is most logically explained by assuming that part of the succinate formed as an intermediate from α-ketoglutarate is oxidized to fumarate and then via malate and oxalacetate to pyruvate, with the eventual production of acetate. Experiments with malonate support this explanation in that essentially no acetate is formed from α-ketoglutarate or succinate in the presence of this inhibitor.

The end products of the fermentation of pyruvate by *M. aerogenes* and *M. lactilyticus* depend on the medium used for growing cells as well as on the type of preparation. Differences in the behavior of intact cells and cell preparations may in part be due to differences in permeability barriers. Most preparations oxidatively decarboxylate pyruvate to carbon dioxide, hydrogen, and acetate. Some preparations of *M. aerogenes* produce lactate and traces of butyrate as additional end products whereas certain preparations of *M. lactilyticus* produce propionate in addition to carbon dioxide, hydrogen, and acetate.

Intact cells of *M. aerogenes* which produce lactate show a more active lactic dehydrogenase and a lower rate of fermentation of pyruvate than do cells carrying out a simple oxidative decarboxylation. Freshly prepared extracts degrade pyruvate at a high rate but dehydrogenate lactate at a low rate. These extracts do not produce lactate. These results suggest that lactate formation depends upon the relative activities of lactic dehydrogenase and of the enzymes concerned in oxidative decarboxylation of pyruvate. Aged extracts or extracts tested at an alkaline pH produce some lactate. Under these conditions, hydrogenase activity is not optimal and lactate formation could be ascribed to a change in the fate of transferable hydrogen.

The ability of intact cells of *M. lactilyticus* to form propionate from pyruvate does not appear to be affected by the composition of the culture medium. However, the property of propionate formation is lost when cells are dried or disrupted by sonic disintegration or by alumina grinding, but not by disintegration in a Mickle apparatus. It is likely that propionate formation in *M. lactilyticus* occurs by the pathway proposed by Delwiche (1948) and Johns (1951*a*, 1951*b*). Various types of extracts were examined with respect to the activities of enzymes operating in this pathway. Comparable activities were found in all extracts with respect to activation and decarboxylation of succinate, and to succinic and malic dehydrogenase activity. Extracts differed markedly in that oxalacetate degradation was sharply reduced in those preparations which were unable to form propionate from pyruvate. All extracts produced propionate from fumarate, malate, and oxalacetate, although extracts unable to form propionate from pyruvate were not able to ferment these dicarboxylic acids to completion in an atmosphere of nitrogen. Slightly greater yields of propionate were produced from these substrates in an atmosphere of hydrogen. When fumarate was fermented in hydrogen in the presence of semicarbazide, hydrogen was taken up and the yield of propionate was markedly increased.

These findings indicate that the formation of propionate from fumarate, malate and oxalacetate is strongly influenced by the availability of substrate hydrogen, and that molecular hydrogen may substitute for substrate hydrogen. The enzymes required for propionate formation from dicarboxylic acids are present in both types of extracts. Hence the failure to produce propionate from pyruvate may be due to impairment in the mechanism for the synthesis of C_4 dicarboxylic acids from pyruvate and carbon dioxide. The production of propionate from pyruvate and lactate by suspensions and Mickle extracts of *M. lactilyticus* is inhibited by acrylate, although this compound is not degraded. Acrylate does not inhibit the production of propionate from dicarboxylic acids. Therefore the effect of acrylate, too, is most logically explained on the basis of interference in the synthesis of C_4 dicarboxylic acids.

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SUMMARY

Cell suspensions, dried cell preparations and extracts of either *Micrococcus aerogenes* or *Micrococcus lactilyticus* ferment α-ketobutyrate to equimolar amounts of carbon dioxide, hydro-

gen, and propionate, but not all preparations ferment this substrate to completion.

Pyruvate is degraded entirely via oxidative decarboxylation by cell free extracts, dried cell preparations and suspensions of cells of *M. aerogenes* grown in "glutamate medium." Suspensions of cells grown in "peptone medium" produce some lactate and traces of butyrate in addition to acetate, carbon dioxide, and hydrogen. It is suggested that the production of lactate is determined by the relative activities of lactic dehydrogenase and the enzymes involved in oxidative decarboxylation.

Some cell free extracts and all dried cell preparations of *M. lactilyticus* degrade pyruvate entirely via oxidative decarboxylation, whereas cell suspensions and certain extracts form propionate as an additional end product. Data are presented which support the view that differences with regard to propionate formation are related to the synthesis of C_4 dicarboxylic acids. It was found that the addition of acrylate prevents the formation of propionate from pyruvate and lactate although acrylate is not metabolized.

M. lactilyticus grown in the optimal medium degrades α-ketoglutarate, via succinyl-CoA as an intermediate product, to carbon dioxide, hydrogen and propionate. Aged extracts and preparations from cells grown in suboptimal medium produce small amounts of acetate in addition. In these preparations, the production of acetate is correlated with a reduction in the rate of decarboxylation of succinyl-CoA.

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