The Mechanism of Acetate Oxidation by *Corynebacterium creatinovorans* [1]

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Introduction

The mechanism of oxidation of acetic acid has been the object of intensive search in several laboratories. In animal tissues acetic acid oxidation starts with the formation of citric acid through condensation with oxalacetic acid (1,2,3). In some plant tissues (potato tuber, tomato stems) the same pathway seems to be followed, *i.e.*, oxidation via the tricarboxylic acid cycle (4). The oxidation of acetic acid by *Aspergillus niger* occurs also, according to Lynen and Lynen (5), via citric acid formation. The same path seems to be followed by yeast (6,7). According to Nord and Vitucci (8,9), some wood-destroying molds oxidize acetic acid through two pathways: via succinic acid, and via glycolic acid. Less is known about the mechanism of oxidation of acetic acid in bacteria. Karlsson and Barker (10) presented some evidence against the occurrence of a tricarboxylic acid cycle in *Azotobacter agilis;* whereas, Novelli and Lipmann (11) demonstrated the synthesis of citric acid by the condensation of acetyl phosphate and oxalacetate in cell-free extracts of *Escherichia coli*. Indications of acetate oxidation via succinic acid were given by Slade and Werkman (12) working with *Aerobacter indologenes*. These divergent observations show that the oxidation of acetic acid in bacteria is not as uniform as it seems to be in animal tissues, but proceeds by a variety of routes. We present here studies made in this laboratory over a number of years on the oxidation of acetate by the soil bacterium, *Corynebacterium creatinovorans*. The oxidation of acetic acid seems to proceed via the dicarboxylic acid cycle, *i.e.*, oxidation through the oxidative condensation to succinic acid.

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Experimental

The soil microorganism *Corynebacterium creatinovorans* was obtained from the American Type Culture Collection. It was grown at first in "tryptone broth" (2 g. tryptone + 0.5 g. NaCl + 2.0 ml. 1 *M* phosphate pH 7, + 98 ml. water). When growth became heavy, the bacteria were washed three times under sterile conditions with distilled water, and were grown in flat 1-l. bottles containing 50 ml. of an acetate medium made as follows: *Artificial tap water:* 1.5 ml. of 3% $MgSO_4$, 5 ml. of 1% $CaCl_2$, 4 ml. of 1% Na_2CO_3, 2 ml. of 1% $NaHCO_3$, 0.3 ml. of 1% $FeCl_3$, 1 ml. of 0.1% $MnCl_2$, 1 ml. of 0.02% HNO_3, 1 ml. of 0.01% $(NH_4)_2SO_4$, and distilled water to 1 l. To 1 l. of this solution were added 12 g. $NaC_2H_3O_2 \cdot 3\ H_2O$; 1 g. $(NH_4)_2SO_4$; 5 g. NaCl; 0.2 g. Difco bacto-yeast extract; 25 ml. 1 *M* phosphate buffer, pH 7.0. Essentially, $(NH_4)_2SO_4$ was the source of nitrogen, and acetate the source of foodstuff. This medium with creatinine instead of acetate was used by Miller *et al.* (13). Yeast extract could be replaced by a mixture of vitamins containing pantothenic acid, riboflavin, and thiamine. Of these three vitamins, the first two were essential. In the absence of thiamine, growth was possible, although extremely slow. The organisms were grown generally at about 35°, sometimes at 38°. At the end of 1 or 2 days' growth, the microorganisms were centrifuged in a Servall centrifuge at 3° for 20 min. at 5000 r.p.m., and washed twice with distilled water. Then, after being suspended in distilled water, the bacteria were ready for use. Oxygen uptake measurements were made with the usual Warburg-Barcroft vessels and manometers in a water bath at 38°, unless otherwise indicated. The following methods were used: respiratory quotient measurements, Warburg and Yabusoe (14); citric acid, Speck *et al.* (15); succinic acid, enzymatic oxidation with pigeon muscle succinoxidase; acetaldehyde, Stotz (16).

The Extent of Acetate Oxidation

Krebs and Eggleston (17) in their study of oxidations produced by *Corynebacterium creatinovorans* (named by them *Corynebacterium ureafaciens*) found that acetate was slowly oxidized by this microorganism. When the bacteria were grown in a medium with acetate as the sole substrate, the rate of acetate oxidation increased steadily until it became ten times the O_2 uptake of the washed bacteria (Table I). However, bacteria grown even for a long time in acetate medium showed no uniform rate of oxidation of acetate. In general, the rate of oxidation was highest in 24-hr. cultures; the rate diminished steadily so that 5-day-old cultures oxidized acetate at a very slow rate. The enzyme for the oxidation of acetate (initial step of oxidation) seems to belong to the group of *partly adaptive enzymes* of Karström (18), since acetate may be oxidized even when the bacteria are grown in a medium with no acetate as substrate. The bacteria grown in the acetate medium lost completely the power to oxidize creatinine. When acetate-grown bacteria were

TABLE I

Relation of the Rate of Acetate Oxidation with the Length of Time of Growth in Acetate Medium

Culture started to be grown in acetate on Oct. 1, 1947. Figures give cu. mm./mg. dry wt./hr.

Date	O_2 uptake
Oct. 15	15
Nov. 27	34
Dec. 15	44.2
Jan. 15	57.0
Feb. 15	79.0
March 30	90.6

transferred into the Dubos and Miller (19) creatinine medium, the rate of oxidation of acetate reverted to that found before the initial transfer to the acetate medium. The same reversibility was found with the creatinine oxidation; the enzyme, lost in the acetate medium, appeared again when the bacteria were grown in the creatinine medium (Table II).

When the extent of acetate oxidation was measured, it was found that only from 68 to 75% of the added acetate was oxidized (Fig. 1). The end of oxidation was not due to the production of some inhibitory oxidation product, for the O_2 uptake started at the same rate as formerly on addition of more acetate.

TABLE II

Dependence of Acetate and of Creatinine Oxidation on the Availability of these Substances in the Culture Media

Buffer, 0.01 *M* phosphate, pH 7.0. Acetate and creatinine, 0.01 *M*. Temp. 38°. Figures give cu. mm./mg. dry wt./hr.

Exptl. conditions	O_2 uptake		
	No substrate	Acetate	Creatinine
	cu. mm.	*cu. mm.*	*cu. mm.*
I. Bacteria grown in acetate medium for 5 years	10	76	10
II. Same bacteria grown in creatinine medium for 2 months	10	23	52
III. Bacteria grown in "tryptone" broth	7	15	6

The oxidation of the fraction of acetate which was oxidized was complete, to CO_2 and H_2O, according to the equation

$$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O.$$

This is shown in the experiments of Table III, where the respiratory quotient for acetate oxidation was one. The figures given in this table

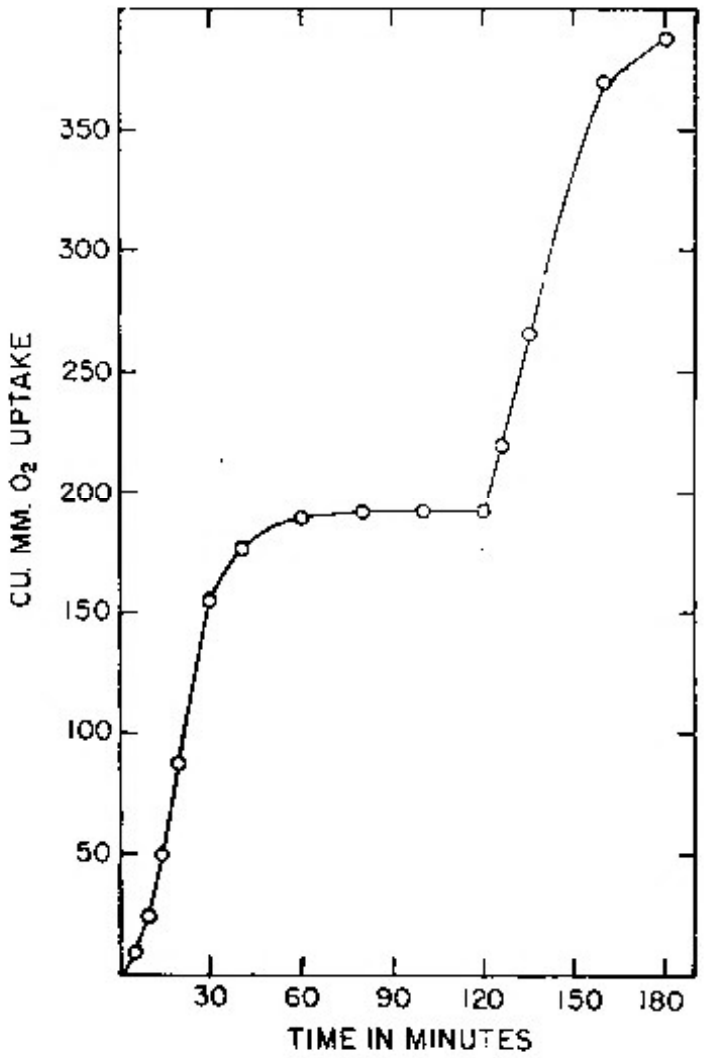


FIG. 1. Oxidation of acetate by *Corynebacterium creatinovorans*. Buffer, 0.03 *M* phosphate, pH 7.0. Acetate 6 μmoles. At the end of 120 min., 7 μmoles of acetate added. Temp. 38°. Abscissa, time in minutes; ordinate, O_2 uptake in cu. mm. Figures given are blank subtracted. Calculated O_2 uptake for complete oxidation of acetate, 269 cu. mm.; found 195 cu. mm. (72.5% oxidation).

are all blank-subtracted, *i.e.*, the endogenous respiration was subtracted from the O_2 uptake or CO_2 production in the presence of acetate.

Effect of pH

The optimum pH value for the oxidation of acetate was 7.0–7.8. From this plateau the activity dropped sharply toward the acid side

TABLE III

Respiratory Quotient for the Oxidation of Acetate by M creatinovorans

Buffer, 0.03 *M* phosphate buffer, pH 7.0. Acetate, 0.01 *M*. Temp. 38°. Duration of experiment, 1 hr.

Expt. no.	O_2 uptake	CO_2 output	R.Q.
	cu. mm.	cu. mm.	
I	144	155	1.07
II	150	164	1.09
III	119	121	1.01
IV	119	118	0.99
V	181	187	1.03

(at pH 4.9 there was no oxidation at all) and somewhat less sharply toward the alkaline side (there was no acetate oxidation at pH 10.8) (Fig. 2).

When the pH value of the suspension medium was kept constant, the rate of oxidation increased in the presence of phosphate (Fig. 3). It was, however, impossible to stop the oxidation of acetate by mere withdrawal of phosphate, even after washing the bacteria five to six times with large amounts of distilled water. Of course, it may be claimed that

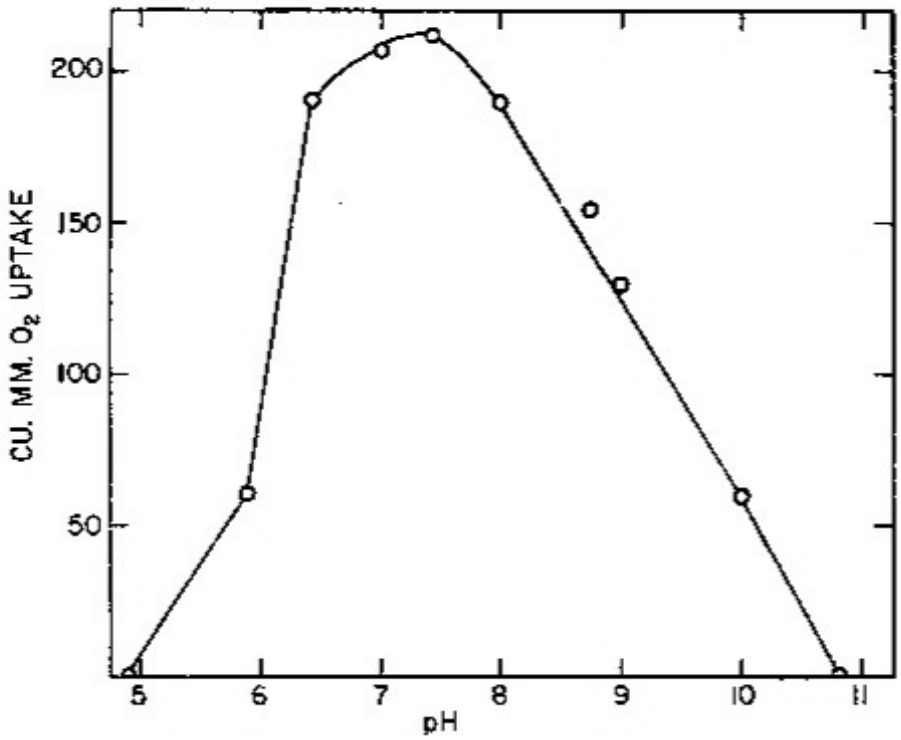


FIG. 2. Effect of pH on the oxidation of acetate by *Corynebacterium creatinovorans*. Buffer, Teorell universal buffer. Abscissa, pH values. Ordinate O_2 uptake in cu. mm./2 mg. dry wt. of bacteria/hr., blank subtracted. Acetate, 30 μmoles. Temp. 38°.

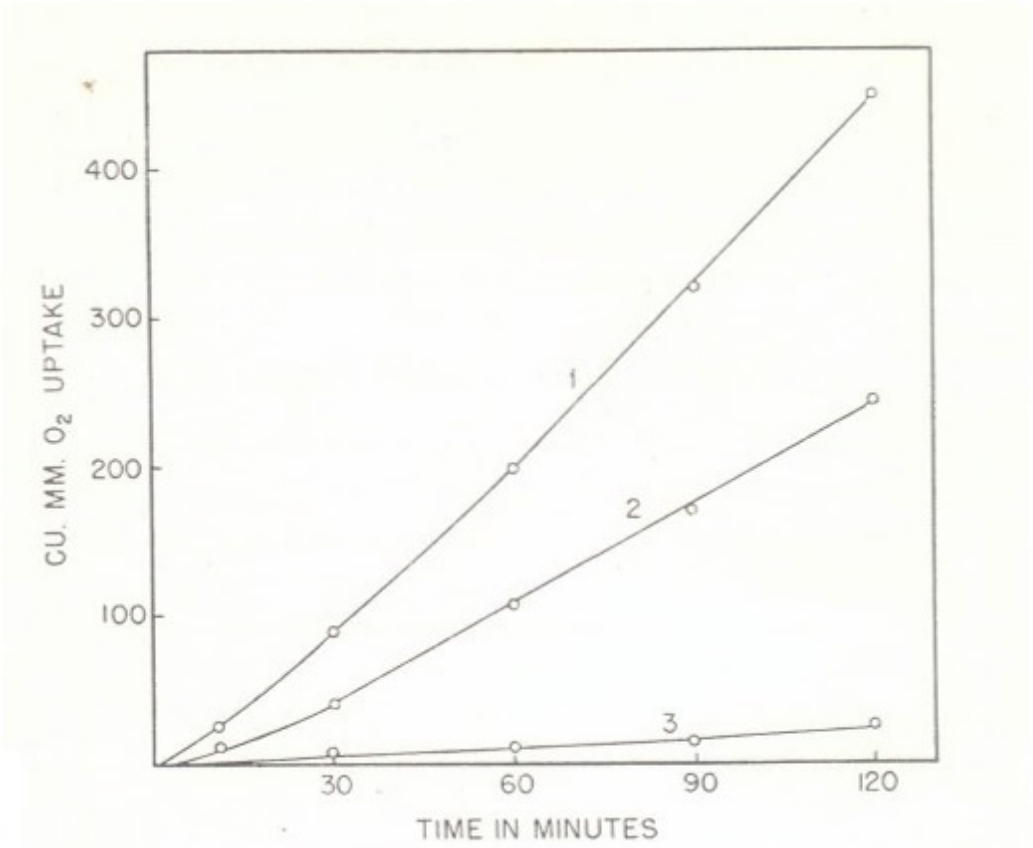


FIG. 3. Effect of phosphate on acetate oxidation by *Corynebacterium creatinovorans*. Three-times-washed bacteria suspended in buffer. Acetate, 30 μmoles. 1. 0.03 *M* phosphate buffer + acetate; 2. 0.01 *M* veronal buffer + acetate; 3. 0.03 *M* phosphate buffer, no acetate. pH, 7.43. Temp. 28°.

the amount of intracellular phosphate was enough for the continued oxidation of acetate.

As is well known, the oxidation of acetate by animal tissues and by yeast is increased in the presence of Mg^{++}; furthermore, Ba^{++} ions increase citrate formation from acetate. None of these cations had any effect on the rate of oxidation of acetate by *Corynebacterium creatinovorans*. These experiments were performed with glycerophosphate as buffer.

TABLE IV

Rate of Oxidation of Saturated Fatty Acids by M. creatinovorans

Buffer, 0.03 *M* phosphate, pH 7.0. Substrate concentration 0.01 *M*. Figures give cu. mm./mg. dry wt./hr.

Fatty acid	O_2 uptake
Formic	3
Acetic	75
Propionic	24
Butyric	19
Caproic	13

Rate of Oxidation of Saturated Fatty Acids

The rate of oxidation of acetate by acetate-grown *Corynebacterium creatinovorans* was higher than that of any other saturated fatty acid, being three times as great as that of propionic acid, the next highest. Then came in decreasing order, butyrate, caproate, and formate (Table IV).

Effect of Starvation and of Acetaldehyde

Lynen (6) found that the oxidation of acetate by baker's yeast that had been kept bubbling with oxygen for 24 hr. ("starved yeast") showed a long induction period, which was broken on addition of alcohol, acet-

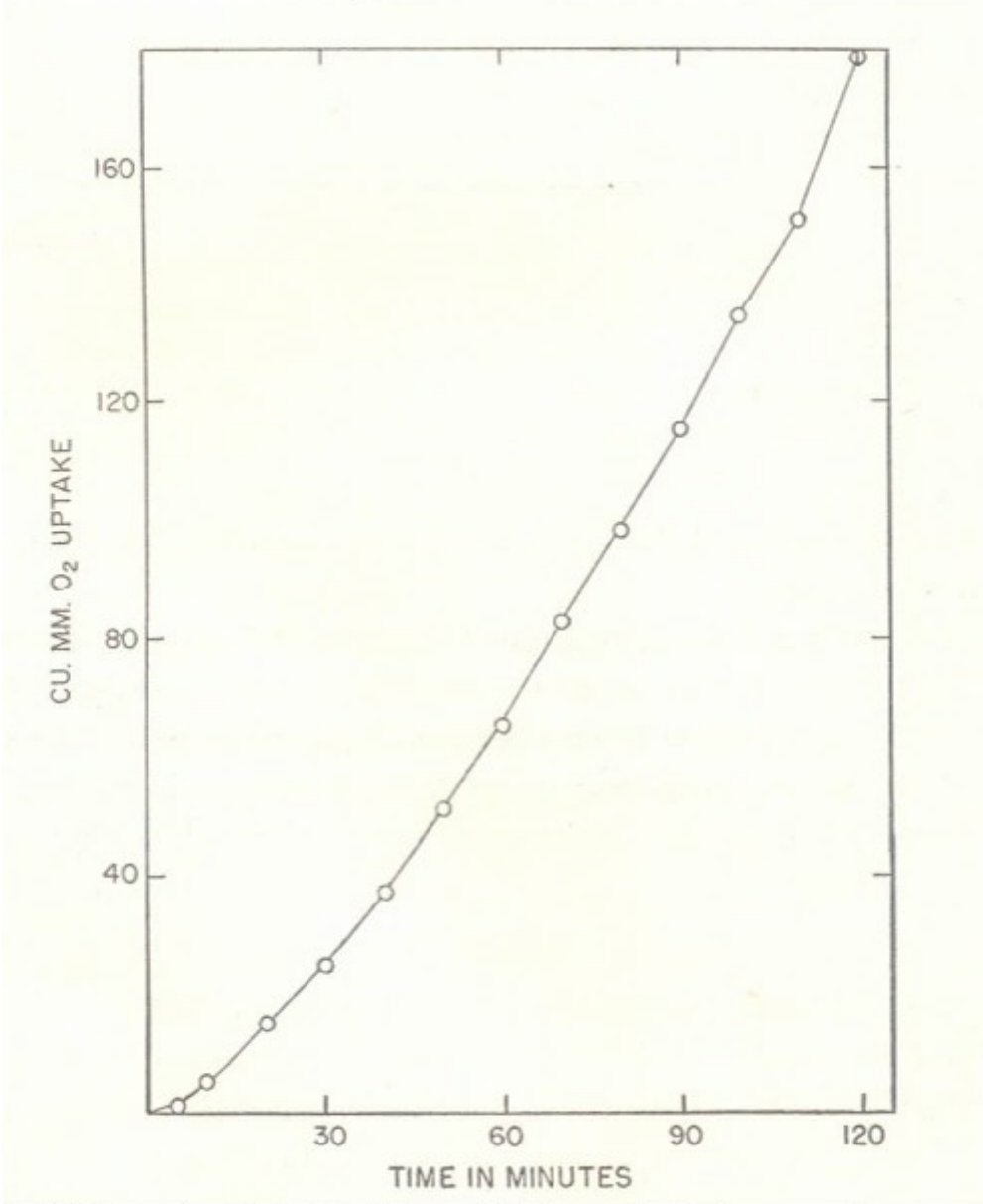


FIG. 4. Oxidation of acetate by "starved" *Corynebacterium creatinovorans*. Washed bacteria suspended in water, bubbled with O_2 for 24 hr. at 25°. After centrifugation suspended in 0.03 *M* phosphate, pH 7.0. Acetate, 30 μmoles. Figures give cu. mm. O_2 uptake/mg. dry wt., blank subtracted. Abscissa, time in minutes; ordinate, O_2 uptake in cu. mm. Temp. 38°.

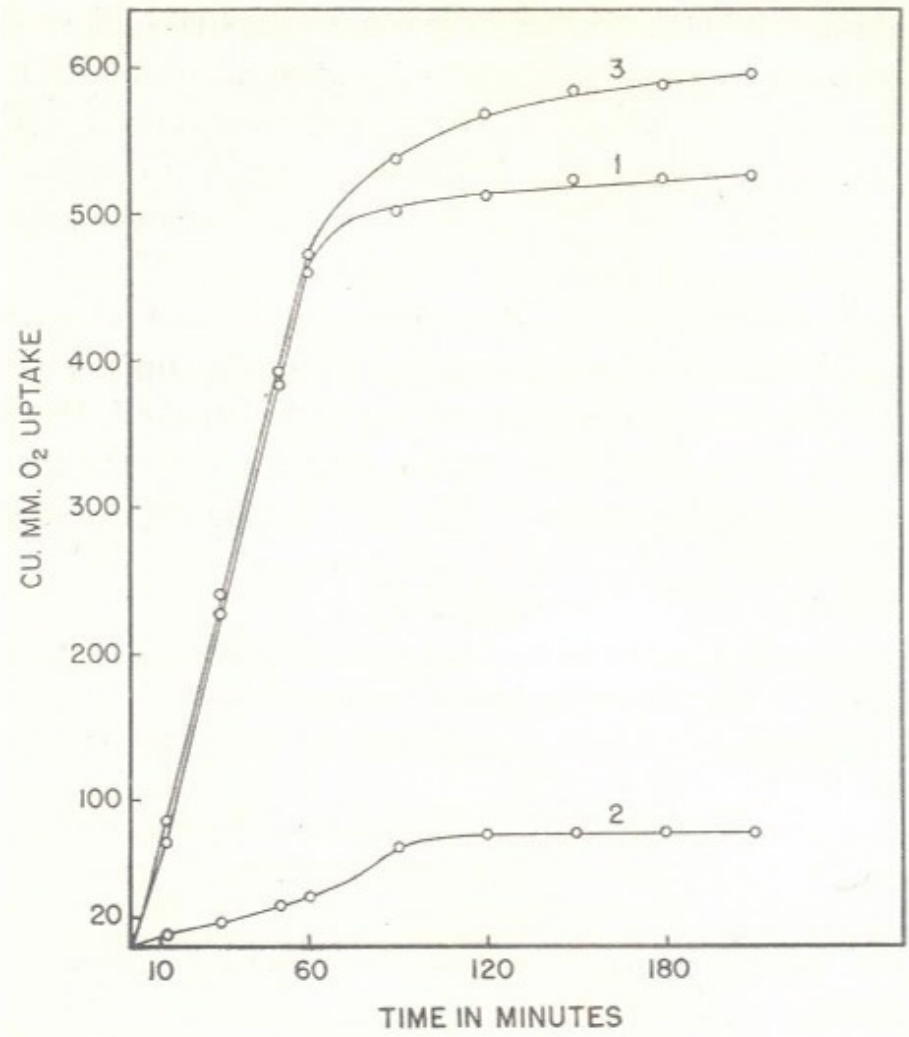


FIG. 5. The effect of acetaldehyde on the oxidation of acetate by *Corynebacterium creatinovorans*. Buffer, 0.03 *M* phosphate buffer, pH 7.04. Acetaldehyde, 3 μmoles; acetate, 30 μmoles. Temp. 28°. Abscissa time in minutes; ordinate, O_2 uptake in cu. mm. 1. Acetate; 2. acetaldehyde; 3. acetate + acetaldehyde. All figures are blank subtracted.

aldehyde, succinate, *etc.* The same procedure was applied to *Corynebacterium creatinovorans* to diminish as much as possible the endogenous respiration. The twice-washed bacterial suspension was bubbled with oxygen for 24 hr., and then centrifuged, and washed again. Although the endogenous respiration was decreased by half, and the rate of acetate oxidation increased by one-third, the induction period was too short to be measurable, and could be detected only by the steadily increasing rate of O_2 uptake (Fig. 4).

Addition of acetaldehyde in amounts one-tenth that of acetate produced no increase in the rate of oxidation of acetate, and the total O_2 uptake was only the sum of the O_2 uptake with either substrate alone (Fig. 5). Identical results were obtained on addition of ethyl alcohol.

In contradistinction to these negative effects was the effect of bisulfite, an aldehyde-fixative agent, which at a concentration of 0.005 *M* inhibited completely acetate oxidation. However two other aldehyde fixative-reagents were less effective: 0.01 *M* hydroxylamine inhibited 17%, and 0.04 *M* semicarbazide, 29% (Table V). It might be argued that the last two reagents did not penetrate the cell membrane. Whatever the effect of bisulfite may be, it seems to be exerted in the initial process of oxidation; once this barrier is overcome the effect disappears. This can be observed when the bisulfite concentration is diminished. With 5×10^{-4} *M* the inhibition lasted for 1 hr., at the end of which the rate of oxidation proceeded as in the control (Fig. 6). At the end of these experi-

TABLE V

Effect of Some Ketone-Fixation Agents on the Oxidation of Acetate by Corynebacterium creatinovorans

Buffer, 0.03 *M* phosphate, pH 6.8. Acetate, 0.01 *M*. Temp. 38°. Duration of experiments, 1 hr.

Reagent	conc.	No substrate	Inhibitor	Substrate	Inhibitor
	M	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
$NaHSO_3$	0.005	18	14	238	17
Hydroxylamine	0.01	118	124	740	642
Semicarbazide	0.04	18	20	315	105

ments, the bacterial suspensions were centrifuged, and acetaldehyde estimations were made after steam distillation of the solutions made alkaline with $NaHCO_3$. No acetaldehyde was found.

Fluoroacetate Inhibition

It had been shown previously that fluoroacetate inhibits acetate oxidation, and that this inhibition is accompanied, in baker's yeast, by an inhibition of citrate synthesis (20). The inhibition of acetate oxidation by fluoroacetate resembles closely the inhibition by bisulfite in that it is reversible when the inhibitor concentration is diminished. With 1×10^{-6} *M* fluoroacetate, the inhibition of acetate oxidation lasted for 2 hr., at the end of which acetate oxidation started at a constantly increasing rate. The competitive nature of this inhibition was shown when fluoroacetate and acetate were added simultaneously; there was no inhibition at all (Fig. 7).

Effect of Dinitrophenol, Pyocyanine, Azide and HCN

The oxidation of acetate by *Corynebacterium creatinovorans* was completely inhibited by 0.001 *M* HCN, an indication that electron transfer to molecular oxygen occurs through the cytochrome–cytochrome oxidase system. Azide, however, failed to inhibit, perhaps due to lack of penetration. Dinitrophenol was found to be a powerful inhibitor of acetate

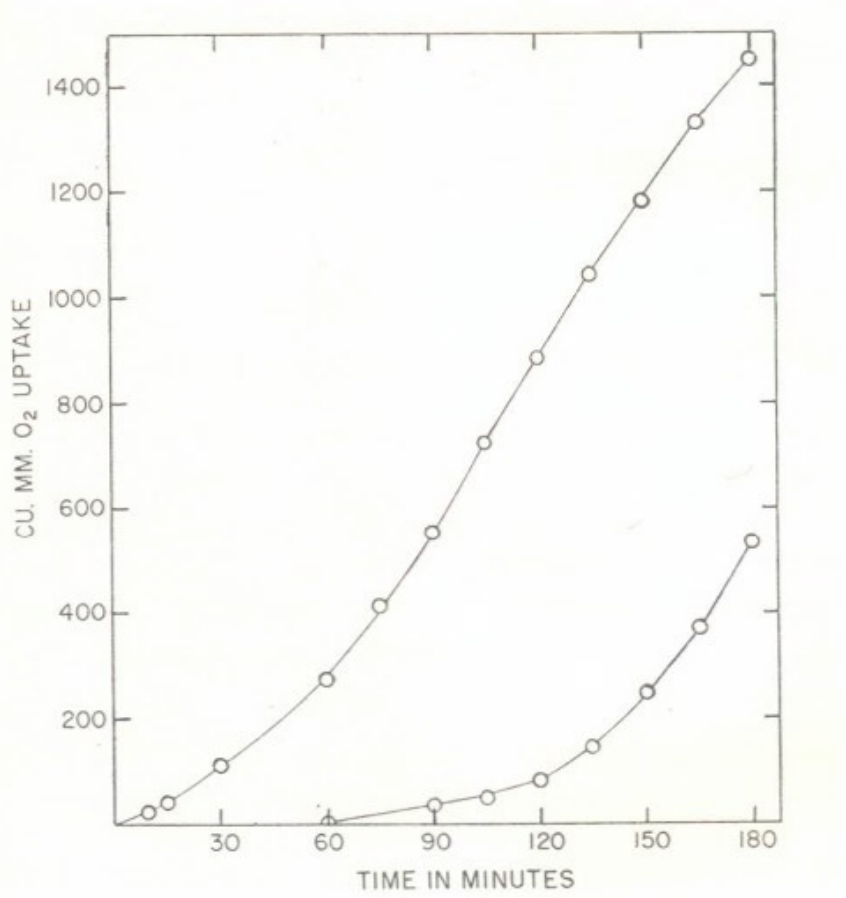


FIG. 6. Inhibition of acetate oxidation by $NaHSO_3$. Buffer, 0.03 *M* phosphate, pH 6.18. Acetate, 90 μmoles. $NaHSO_3$ 1.5 μmoles (5 ± 10^{-4} *M*). Temp. 28°. Abscissa, time in minutes. Ordinate, O_2 uptake in cu. mm. 1. Acetate; 2. acetate + $NaHSO_3$. (All figures blank subtracted.)

oxidation when the pH value of the buffer was lowered to 5.5. Under these conditions, 5×10^{-5} *M* dinitrophenol inhibited the oxidation completely, and 1×10^{-6} *M*, 20%. At pH 7.0, however, dinitrophenol had almost no effect, an indication that only the undissociated molecule penetrates the cell membrane (Table VI). This complete inhibition of acetate oxidation is the more striking when compared with the increased O_2 uptake produced on the endogenous respiration. Similar results, *i.e.*,

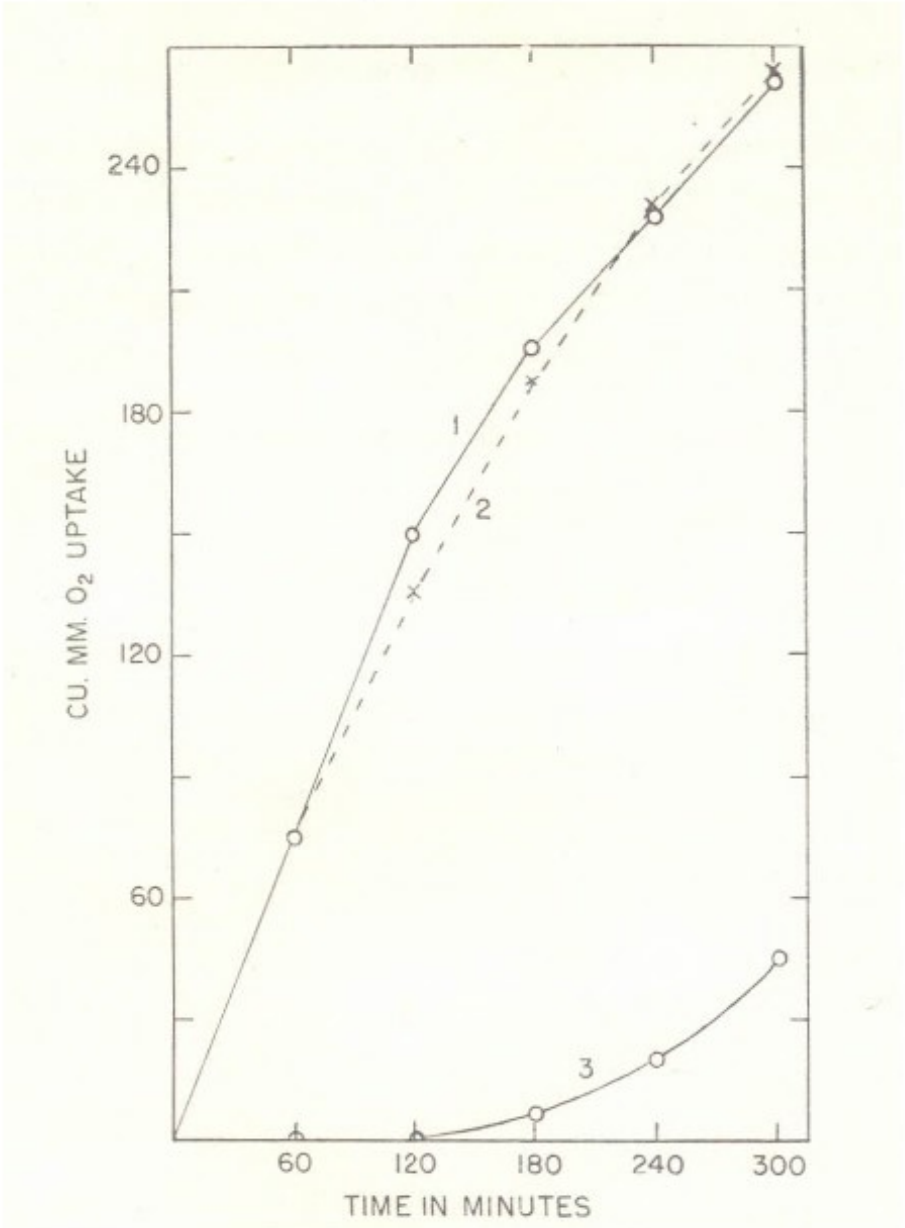


FIG. 7. Fluoroacetate inhibition of acetate oxidation by *Corynebacterium creatinovorans*. Buffer, 0.03 *M* phosphate, pH 7.0. Acetate, 0.01 *M*; fluoroacetate, 1×10^{-6} *M*. Temp. 38°. Abscissa, time in minutes; ordinate, O_2 uptake in cu. mm. 1. Acetate; 2. acetate + fluoroacetate added simultaneously; 3. fluoroacetate + acetate, fluoroacetate added 15 min. before acetate addition. All figures blank subtracted.

inhibition of acetate oxidation in baker's yeast, were found by Stoppani (21) when the cells were suspended in a buffer of pH 4.8, although at pH 7, dinitrophenol had no effect at all. Pyocyanine at a concentration of 5×10^{-4} *M* inhibited acetate oxidation by 60%, while it increased the endogenous respiration by 23% (Table VI).

Rate of Oxidation of Substrates of the Tricarboxylic Acid Cycle

The fact that inhibition of acetate oxidation by dinitrophenol occurs only when the pH of the buffer is on the acid side of neutrality shows

TABLE VI

Effect of Dinitrophenol, Azide, Pyocyanine and HCN on the Oxidation of Acetate

Buffer, 0.03 *M* phosphate. Acetate, 0.01 *M*. Temp. 38°. Duration of experiments, 2 hr.

Inhibitor	pH	O_2 uptake			
		No substrate	Inhibitor	Substrate	Inhibitor
M		*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
Dinitrophenol, 5×10^{-5}	5.5	120	190	226	100
5×10^{-5}	7.0	133	153	1146	914
1×10^{-5}	5.5	130	160	226	132
1×10^{-6}	5.5	130	130	226	207
Pyocyanine, 5×10^{-4}	7.0	60	74	565	278
Azide, 0.001	6.8	40	65	740	724
HCN, 1×10^{-3}	7.0	50	2	190	0

the great influence that the cell membrane has on the metabolic reactions of *Corynebacterium creatinovorans*. This influence of the cell membrane was most strikingly demonstrated on measuring the rate of oxidation of substrates belonging to the Krebs tricarboxylic acid cycle. At pH 7, if the rate of acetate oxidation is taken as 100, that of pyruvate was 57.4; of oxalacetate, 43.3; of malate, 24.4; of fumarate, 2.33; and of α-ketoglutarate, 11.1. Citrate and succinate were not oxidized. When the pH value of the buffer was lowered to 5.5, and again the rate

TABLE VII

Rate of Oxidation of Substrates of the Tricarboxylic Acid Cycle

Buffer, phosphate, 0.03 *M*, pH 5.5. (When the pH value of the suspensions was measured at the end of the experiments, the pH of the samples containing oxalacetate was 6.5.) Duration of experiments, 1 hr. Substrate concentration, 0.01 *M*.

Substrate	O_2 uptake	
	pH 5.5 *cu. mm.*	pH 7.0 *cu. mm.*
Acetate	38	90
Succinate	45.6	0
Fumarate	54.4	21
Malate	32	22
Oxalacetate	63.5	39
Pyruvate	42	52
Citrate	0	0
α-Ketoglutarate	15	10

of acetate oxidation taken as 100, that of oxalacetate was 167; of fumarate, 143; of succinate, 120; of malate, 84; of α-ketoglutarate, 39.4. The relatively high rate of oxalacetate oxidation must be due to the increased pH value that was found at the end of the experiments in the vessels containing oxalacetate and to the rapid change in oxidation rate with increase of pH from pH 5 to pH 7. On addition of citrate there was no increased O_2 uptake (Table VII).

The influence of the cell membrane was also observed on studying the effect of malonate on the oxidation of acetate. At pH 7, the optimum pH value for acetate oxidation, malonate had no effect at all. In fact, this negative effect, as well as the lack of succinate oxidation (observed at this laboratory by J. Meyer and T. P. Singer), was a

TABLE VIII

Malonate Inhibition of Acetate and Succinate Oxidations by Corynebacterium creatinovorans. *The Effect of pH*

Buffer, 0.03 *M* phosphate. Duration of experiments, 1 hr.

Exptl. conditions	Conc.	O_2 uptake in 1 hr.	
		pH 7.0	pH 5.3
	M	*cu. mm.*	*cu. mm.*
No substrate		21.6	14.9
Acetate	0.01	112	89.8
Acetate + malonate	0.04	116	17.0
Succinate	0.01	22	80.0
Succinate + malonate	0.04	30	15.0

stumbling block for a tentative hypothesis on the mechanism of acetate oxidation. When the pH value was lowered to 5.3, 0.04 *M* malonate inhibited completely the oxidation of acetate as well as that of succinate (Table VIII).

Since a possible mechanism of acetate oxidation, though unlikely, is via glycolic and glyoxylic acids, the rate of oxidation of these acids was also compared with that of acetate. At pH 7, the rate of oxidation of glyoxylic acid was 11, and that of glycolic acid, 6. At pH 5.5, the rate for glyoxylic acid was 30, and for glycolic acid, 26. The O_2 uptake in 1 hr. on addition of 0.01 *M* acetate was again taken as 100. It is, therefore, unlikely that oxidation of acetate occurs through this pathway.

Effect of Fumarate and of Oxalacetate on the Malonate Inhibition of Acetate Oxidation

Lynen (22) found that malonate inhibition of acetate oxidation by baker's yeast was reversed on addition of fumarate. Addition of fumarate to suspensions of *Corynebacterium creatinovorans* containing malonate and acetate reversed the inhibition by 40% (Table IX). When

TABLE IX

Effect of Fumarate on the Malonate Inhibition of Acetate Oxidation

Buffer, phosphate, pH 5.37, 5 ml. Acetate, 0.1 *M*, 0.6 ml. Fumarate, 0.1 *M*, 0.3 ml. Malonate, 0.3 *M*, 0.4 ml. Temp. 38°. Duration of experiment, 3 hr. Figures given are amount of blank substrated.

Exptl. conditions	O_2 uptake *cu. mm.*	Inhibition *per cent*
No substrate	133	
Substrate + malonate	160	None
Fumarate + malonate	512	
Acetate	1795	
Acetate + malonate	206	98
Acetate + fumarate	2097	
Acetate + fumarate + malonate	960	59

malonate was added to a bacterial suspension containing oxalacetate, the inhibition produced by malonate was small: 29% instead of 98%; the latter was the inhibition produced by malonate in the presence of acetate alone (Table X).

Numerous attempts were made to detect the formation of citric acid in large-scale experiments similar to those described in Tables IX and X. The bacterial suspensions containing acetate, acetate + fumarate, acetate + fumarate + malonate, acetate + oxalacetate, and acetate + oxalacetate + malonate were boiled for 1 hr. in the presence of 0.1

TABLE X

Acetate and Oxalacetate Oxidation in the Presence of Malonate

Buffer, 0.1 *M* phosphate, 1.4 ml. Malonate, 0.1 *M*, 1.0 ml.; or water, 1 ml. Acetate, 0.1 *M*, 0.5 ml. Oxalacetate, 0.1 *M*, 0.5 ml. Bacteria, 43 mg. dry wt. All solutions brought to pH 5.36. Temp. 38°. Duration of experiment, 120 min.

Substrate	O_2 uptake *cu. mm.*
None	2830
Acetate	2150
Acetate + oxalacetate	2830
Malonate + acetate + oxalacetate	2005

M H_2SO_4, and citric acid was then determined according to Speck *et al.* (15). In no case was there any citric acid formation. These negative experiments, added to the lack of oxidation of citric acid, indicate that oxidation of acetic acid by *Corynebacterium creatinovorans* does not start, as in animal tissues and some plant tissues, with the condensatoin reaction with oxalacetate to give citric acid.

The Formation of Succinate

The rejection of the tricarboxylic acid cycle, and of oxidation via glycolic acid, as well as the complete inhibition of acetate oxidation by

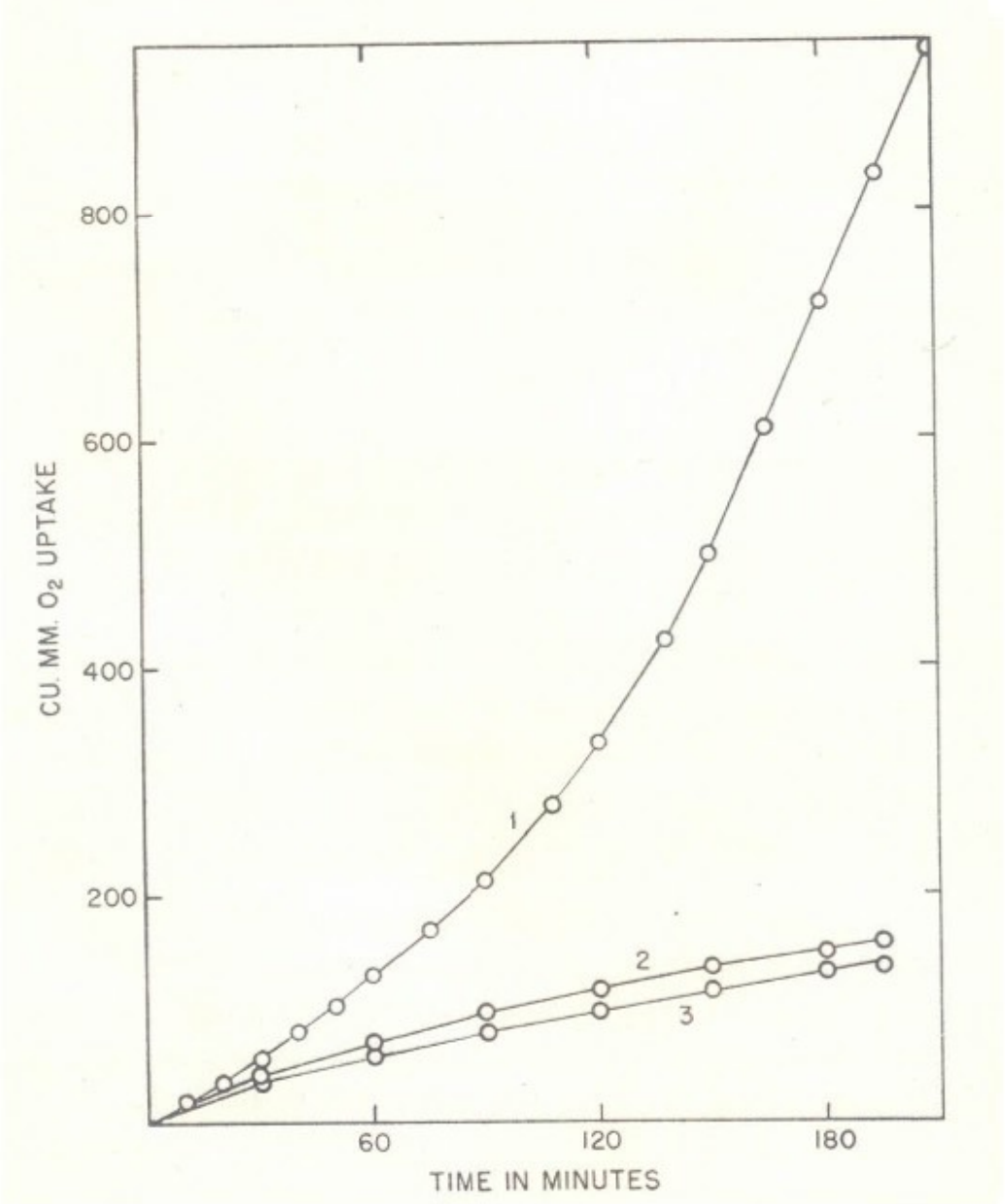


FIG. 8. Complete inhibition of acetate oxidation by malonate. Buffer, 0.03 *M* phosphate, pH 5.5. Acetic acid, 0.01 *M*, and malonic acid, 0.04 *M* brought to pH 5.5 by addition of NaOH. Temp. 38°. Abscissa, time in minutes; ordinate, O_2 uptake in cu. mm.

malonate when the experiments are conducted so as to allow rapid penetration of malonic acid (Fig. 8) brought forth the old hypothesis of Thunberg (23) and Wieland (24) that oxidation takes place by means of the oxidative condensation of acetate to succinate. Acceptance of this hypothesis alone could explain the complete inhibition by malonate. It was therefore necessary to demonstrate the formation of succinic acid during the oxidation of acetate. The bacteria were suspended in phosphate buffer, pH 5.37, and a malonate concentration was used so as to give partial inhibition of acetate oxidation (63%). There were three series of vessels: (*a*) bacteria with no substrate; (*b*) bacteria with acetate; and (*c*) bacteria with acetate and malonate. At the end of 2 hr., 0.36 ml. of 50% H_2SO_4 was added to each vessel, and all were left standing overnight. On the following day, 2 ml. of 10% sodium tungstate and 0.34 ml. of water were added, making a total volume of 10 ml.

TABLE XI

Succinate Formation on Oxidation of Acetate by Corynebacterium creatinovorans

Buffer, phosphate, 0.03 *M*, pH 5.37. Bacteria suspended in buffer, 5 ml. Malonate, 0.3 *M*, 0.4 ml. Acetate, 0.1 *M*, 0.6 ml. H_2O, 1.3 ml.

Exptl. conditions	O_2 uptake *cu. mm.*	Succinate formation *cu. mm.*
No substrate	594	None
Acetate	1318	None
Acetate + malonate	864	64

The suspensions were centrifuged, malonate was oxidized with $KMnO_4$ at 50°, and the supernatant fluids from all vessels were extracted with ether for 4 hr. in the Kutscher-Steudel extractors. Succinate was determined enzymatically with succinoxidase. The fluid from vessels 1 and 2 contained no succinate; vessel 3 contained 64 cu. mm. of succinate (28.5 μmoles) (Table XI).

Metabolism of Oxalacetate and of Pyruvate

It had been shown previously that all the substrates belonging to the dicarboxylic acid cycle of acetate oxidation were oxidized at a rapid rate once the ionic environment was made favorable for their penetration into the cells (Table VII). If the dicarboxylic acid cycle does occur, the oxalacetic acid produced on oxidation of malic acid must be decarboxylated by the bacteria. In these experiments, *Corynebacterium creatinovorans* was suspended in 0.03 *M* phosphate buffer, pH 5.27, and was

bubbled with purified nitrogen; oxalacetic acid was adjusted to the same pH value. The vessels were saturated with nitrogen, and the rate of oxalacetic acid decarboxylation was measured at 28° to diminish the spontaneous decarboxylation. At the end of 1 hr., there were 80 cu. mm. of CO_2 produced by oxalacetate in the absence of bacteria, while in the presence of bacteria there were 180 cu. mm. (Fig. 9). In the absence of oxalacetic acid there was slight and erratic production of CO_2 which never exceeded 15 cu. mm.

It is known that the metabolism of pyruvic acid by bacteria occurs either by direct oxidation to acetic acid, by dismutation to lactic and

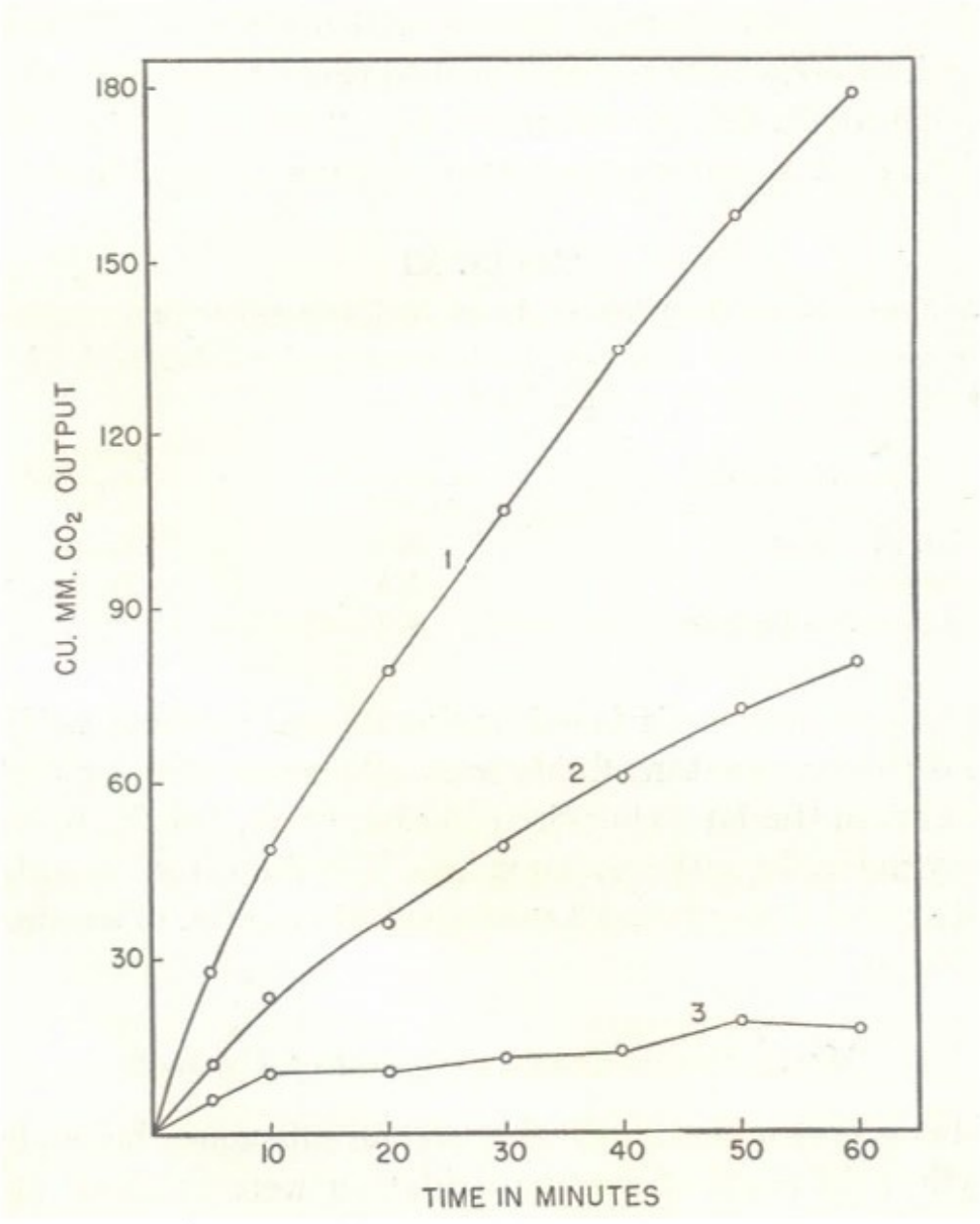


FIG. 9. Decarboxylation of oxalacetic acid by *Corynebacterium creatinovorans*. Buffer, 0.03 *M* phosphate, pH 5.27; 0.01 *M* oxalacetic acid adjusted to pH 5.3. Gas phase, N_2. Temp. 28°. 1. Bacteria + oxalacetic acid; 2. oxalacetic acid (no bacteria); 3. bacteria.

acetic acids, by decarboxylation to acetaldehyde, or by hydrolysis to acetic acid and formic acid (25,26). The first process can be measured by the O_2 uptake in the presence of pyruvate; the last three, by the CO_2 formation in the absence of oxygen. *Corynebacterium creatinovorans* oxidized pyruvate in air as the gas phase, but not in the absence of oxygen, as demonstrated by the lack of CO_2 formation (Fig. 10). Further evidence of the lack of utilization of pyruvate in the absence of oxygen was found on analysis of pyruvic acid, acetaldehyde, and lactic acid. Added pyruvic acid was recovered, and no acetaldehyde or lactic acid was found.

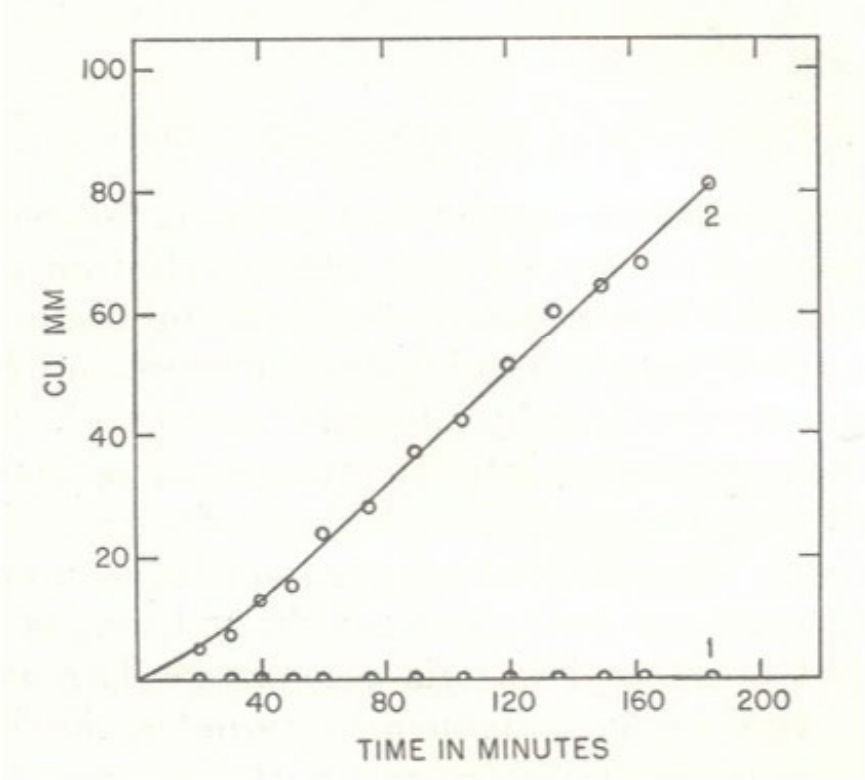


FIG 10. Pyruvate metabolism by *Corynebacterium creationovorans*. Buffer, 0.03 *M* phosphate, pH 5.54. Pyruvic acid, 0.01 adjusted to pH 5.5. 1. Bacteria saturated with nitrogen; gas phase of vessels, N_2. 2. Bacteria saturated with oxygen; gas phase of vessels, air. Temp. 38° (figures are blank subtracted). In *1*, CO_2 formation was measured; in *2*, O_2 uptake.

Some attempts were made to explore the possibility that the metabolism of malic acid may proceed by oxidative decarboxylation to pyruvic acid: $HOOCCHOHCH_2COOH + \frac{1}{2}\ O_2 = CH_3COCOOH + CO_2 + H_2O$, without previous oxidation to oxalacetic acid. Lwoff and Cailleau (27) gave evidence for this reaction in the mutant *S* of *Moraxelle Lwoffi*. They found that oxalacetic acid oxidation was completely inhibited by malonic acid at a ratio of malonic acid: oxalacetic acid of 10. They also found that dinitrophenol (DNP) inhibited greatly pyruvic acid oxida-

tion. The bacteria in the presence of malic acid, malonic acid, and DNP produced pyruvic acid. The oxidation of oxalacetic acid by *Corynebacterium creatinovorans* at pH 5.8 was not affected at all at a ratio of malonic acid : oxalacetic acid of 30. No inhibitors were found specific for oxalacetic acid oxidation, and thus it was impossible to differentiate between an oxidative decarboxylation of malic acid to pyruvic acid and a dehydrogenation to oxalacetic acid followed by decarboxylation to pyruvic acid.

The Initial Step in the Oxidation of Acetate

If the oxidation of acetic acid starts with an oxidative condensation to succinic acid:

$$2CH_3COOH - 2e \rightleftharpoons HOOCCH_2CH_2COOH + 2H^+,$$

there must be an oxidation enzyme that performs this reaction in the absence of oxygen with the aid of a suitable electron acceptor. To obtain this enzyme it was necessary to break the bacterial cell wall. Freezing in liquid nitrogen and thawing at 50° repeatedly had no influence at all. Bacterial cytolysis by ultrasonic waves broke the cells, but neither the whole suspension, nor the cell-free extract oxidized acetic acid. Vacuum-drying produced dried bacteria with no oxidative power toward acetic acid. The cell membrane was so altered, however, by this procedure that succinate penetrated readily and was oxidized. Some experiments with these dried bacteria were previously reported (20).

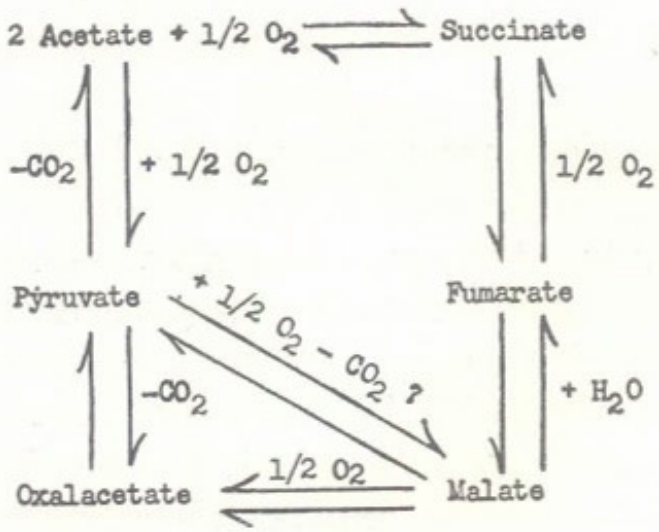
A cell-free extract was finally prepared by grinding the thrice-washed bacteria for 3 hr. in a mechanical mortar in the presence of Pyrex glass powder. The ground Pyrex glass was sifted through a sieve, No. 60 mesh, was washed twice in hot cleaning solution, was rinsed several times in distilled water, and dried at 105°. To 1 vol. of bacterial paste were added 1 vol. of glass and one vol. of 0.1 *M* phosphate buffer, pH 7.4. This mixture was ground at 3° (cold room) in a mechanical mortar for 4 hr. During the grinding procedure drops of water were added to prevent the paste from becoming dry. When the grinding process was finished the paste was transferred to a cellophane centrifuge tube, the mortar was washed with 2 ml. water, and this fluid was added into the centrifuge tube. The suspension was centrifuged for 2 hr. at 16,000 r.p.m. at 3°. The supernatant fluid was perfectly clear and had a golden-yellow color. Anaerobic oxidation of acetic acid was tested in Thunberg tubes evacuated three times, and submerged in a water bath

at 38°. The tubes containing acetate were decolorized in 15 min., while those without acetate did not reduce methylene blue. Addition of adenosine triphosphate (ATP) had no influence on the speed of methylene blue reduction (Table XII).

Discussion

The complete lack of citrate oxidation, the failure to obtain citric acid formation on addition of acetate and oxalacetate, and the complete inhibition of acetate oxidation by malonate left no other obvious pathway for the oxidation of acetate by acetate-grown *Corynebacterium creatinovorans* than the initial oxidative condensation to succinate postulated 30 years ago by Thunberg (23). The formation of succinate by acetate in the presence of malonate, and the reduction of methylene blue by cell-free extracts of the bacteria, anaerobically and in the presence of acetate, speak in favor of this hypothesis. The further oxidation of acetate seems to proceed, via the *dicarboxylic acid cycle,* as represented in the following scheme:

The Dicarboxylic Acid Cycle of Acetate Oxidation.



The first process must be performed by a reversible oxidation–reduction enzyme probably present in the cell-free bacterial extract. The free energy of this reaction can be adequately calculated from the free energies of formation of aqueous acetate, and succinate, which are given by Parks and Huffman (28). The $-\Delta F$ value of the acetate ion at 1

TABLE XII

Anaerobic Oxidation of Acetate by Cell-Free Extracts of Corynebacterium creatinovorans

I. One ml. cell-free extract + 0.5 ml. 0.1 *M* phosphate buffer, pH 7.4 + 0.9 ml. H_2O + 0.3 ml., 0.1 *M* Mg acetate.
II. One ml. cell-free extract + 0.5 ml. 0.1 *M* buffer + 1.0 ml. H_2O + 0.2 ml. 0.01 *M* Na adenosine triphosphate + 0.3 ml. 0.1 *M* acetate.
III. One ml. cell-free extract + 0.5 ml. buffer + 1.2 ml. H_2O.

The side arm of all vessels contained 0.3 ml. 1.34×10^{-3} *M* methylene blue. Temp. 38°.

Vessel no.	Methylene blue reduction *min.*
I	15
II	15
III	>?

mole and at 25° is 89,720 cal.; the $-\Delta F$ of the succinate ion is 165,090 cal. Therefore, the $-\Delta F$ value for the reaction

$$2CH_3COO^- - 2e \rightleftharpoons {}^-OOCCH_2CH_2COO^- + 2H^+$$

will be: $2 \times 89{,}720 - 165{,}090 = 14{,}350$ cal.

The E_0 value of this oxidation–reduction system at pH 0 can be calculated by means of the equation, $-\Delta F = n\mathbf{F}E$, where n is the number of equivalents, **F** the Faraday (96,500 coulombs or 23,068 cal./v.-equiv.), and E, the electromotive force in volts:

$$E_0 = \frac{14{,}350}{2 \times 23{,}068} = 0.311 \text{ volt.}$$

At pH 7, the E'_0 value of the system would be −0.109 volt. This rather high value makes the pyridine nucleotides unlikely oxidizing agents for the enzymatic oxidation of acetic acid to succinic acid, since the E'_0 of diphosphopyridine nucleotide (DPN) at pH 7.0 is −0.282 v. (29). A flavoprotein is the most likely acceptor. Labilization of the hydrogens of the α-C atoms of acetic acid requires a high energy of activation and it is most probable that this process is facilitated by introduction of some electronegative groups into the molecule. The increase of acetate oxidation by phosphate, and the complete inhibition of this oxidation by dinitrophenol speak in favor of a previous phosphorylation process of acetate. It would be this phosphorylated acetate which undergoes oxidative condensation. It is unlikely that the active compound is

acetyl phosphate, for replacement of acetate by acetyl phosphate had no influence on the rate of oxidation or on the induction period. Kharasch and Gladstone (30) found that acetyl peroxide when heated in the presence of glacial acetic acid gave succinic acid. The formation of succinic acid was explained as dimerization of the free radicals $\cdot CH_2COOH$. Labilization of the hydrogen of the α-C atoms was attributed to the action of the free methyl radicals $\cdot CH_3$. It is unlikely that reactions of this kind occur in biological fluids.

The lack of succinate formation in the presence of acetate alone resembles the behavior of yeast cells, where Kleinzeller (31) reported no succinate formation on addition of pyruvate or acetate. Succinate was found only when its oxidation was inhibited by malonate (32). The succinate formation on oxidation of acetate in the presence of malonate can be calculated from the experiments in Table XI. The increase in O_2 uptake in the presence of acetate + malonate was 270 cu. mm. which if converted entirely to succinate would give 135 cu. mm. There were 65 cu. mm. actually found, *i.e.*, 48% yield.

The further oxidation of acetate through the dicarboxylic acid cycle is indicated by the respiratory quotient of one as provided in the cycle, and by the rates of oxidation of the intermediary substrates which in general are equal to or higher than the rate of oxidation of acetic acid. The anaerobic decarboxylation of oxalacetic acid and the *obligatory* oxidative pathway of pyruvate metabolism are further indications in support of the dicarboxylic acid cycle. In contrast to most bacteria, which utilize some pyruvate in the absence of oxygen *Corynebacterium creatinovorans* used none at all. It may be recalled, as Krebs and Kleinzeller found, that these bacteria do not ferment glucose, *i.e.*, they do not utilize glucose in the absence of oxygen.

Corynebacterium creatinovorans possesses a cell membrane quite impermeable to some dicarboxylic acids, a property which produced an apparent lack of effect of malonate on the rate of oxidation of acetate when the experiments were performed at pH 7, the optimum pH of acetate oxidation. This impermeability was also shown by the lack of oxidation of succinate. When the pH of the buffer was diminished so as to allow the presence of the undissociated acids, both malonate inhibition of acetate oxidation and oxidation of succinate were demonstrated. Inhibition of acetate oxidation by dinitrophenol occurred at pH 5.5, while at pH 7 it had almost no effect; this is another example of the influence of the cell membrane.

Some indication of the oxidative condensation reaction of acetate to succinate has been given in the experiments with cell-free extracts, where acetate was oxidized anaerobically with methylene blue as the hydrogen acceptor. Isolation of the enzyme, and the potentiometric measurement of the oxidation–reduction potential remain as problems for further study. Also for further study remains the demonstration of the existence of the dicarboxylic acid cycle of acetate oxidation in other bacteria, and in cells of other origin.

Acknowledgments

In the initial phases of these studies, Drs. J. Meyer and T. P. Singer collaborated.

Summary

The soil bacterium *Corynebacterium creatinovorans*, which oxidizes acetate at a very low rate, increased considerably the rate of oxidation when grown in a medium with acetate as the sole substrate. From 65 to 75% of the added acetate was oxidized completely to CO_2 and H_2O. The pH optimum of this oxidation was from 6.9 to 7.5. There was no acetate oxidation at pH 4.9 and at pH 10.8. These bacteria did not oxidize creatinine. When grown in a medium containing creatinine, acetate oxidation was diminished considerably, while creatinine oxidation appeared.

At the optimum pH of acetate oxidation, there was no inhibition by malonate and no oxidation of succinate. At pH 5.3 the oxidation of acetate was completely inhibited by a proper concentration of malonate; succinate was oxidized. When the concentration of malonate was reduced to give partial inhibition of acetate oxidation, there was succinate formation. No succinate was formed in the absence of malonate. At pH 5.5 the rate of oxidation of succinate, fumarate, malate, oxalacetate, and pyruvate was equal to or higher than the oxidation of acetate. There was no oxidation of citrate, and the rate of α-ketoglutarate oxidation was slow. Cell-free extracts oxidized acetate anaerobically with methylene blue as the hydrogen acceptor.

The oxidation of acetate was inhibited by HCN, $NaHSO_3$, and fluoroacetate, the last two inhibitions being reversed with time when small concentrations of the inhibitor were employed. At pH 5.5, acetate oxidation was inhibited by dinitrophenol, but scarcely affected at pH 7.0. It was also inhibited by pyocyanine.

It is postulated that the oxidation of acetate by *Corynebacterium creatinovorans* occurs *via the dicarboxylic acid cycle*, the process starting with the oxidative condensation to succinate.

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