On the Oxidation of Propionic and Pyruvic Acids by *Propionibacterium arabinosum*

Hideo KATAGIRI and Yoshio ICHIKAWA*

It is found that propionate is oxidized by the cell suspension of *P. arabinosum* under aerobic condition, although the physiological significance of the reaction remains uncertain. This oxidation is concluded to occur through pyruvate, and some possible pathways are suggested between propionate and pyruvate. Further oxidation of pyruvate is revealed at least in two ways, the one to acetate and the other to T.C.A. cycle according to the cultural conditions of the bacterial cells. The T.C.A. enzyme system of this bacterium seems to be adaptive, since the activity of enzyme producing acetate from pyruvate is observed to be constant.

INTRODUCTION

Besides acetate, succinate and CO_2, propionate is one of the main end products in the anaerobic decomposition of glucose by propionic acid bacteria. But under the aerobic condition *P. arabinosum* can utilize propionic acid, if the bacterium is previously adapted to this acid[1].

The oxidation of propionic acid is observed with liver[2], muscle or *Torula*[3], and it is considered that there are some differences in metabolic pathway between yeast and higher organisms.

Using the cell suspension of *P. arabinosum*, we recognized that propionic acid was remarkably oxidized and that the end products were different according to the cultural conditions of the bacteria.

METHOD

P. arabinosum, isolated from cow milk and classified to the species by us[4], was used. A cultural medium for bacteria containing 0.5 % of glucose, yeast extract and peptone, was autoclaved for 15 minutes at 15 pounds per sq. inch. The pH was adjusted to approximately 7. Ten ml of test tube culture of the same medium, incubated statically for 48 hours at 30°, was added to 200 ml of the medium and incubated under following cultural conditions:

1) Shaking culture: 500 ml of shaking flask, containing 200 ml of the medium, was kept shaking for 48 hrs at 30°. The rate and amplitude of the shaker were 110 r.p.m. and 5 cm, respectively.

* 片桐　英郎，市川　吉夫

2) Static culture: 300 ml of Erlenmeyer flask, containing 200 ml of the medium, was kept in the air for 48 hrs at 30°.

At the end of the cultivation, the bacterial cells were centrifuged and washed twice with 0.9 % KCl solution and weighed, and then resuspended in distilled water.

The oxygen-uptake was measured by Warburg's manomenter at 30°, and Thumberg's tube was used for the test of pigment decolourization.

RESULT AND DISCUSSION

(1) Oxygen-uptake by the Wet Cell Suspension of *P. arabinosum*

The rates of oxygen-uptake by two sorts of cells cultivated under different conditions mentioned above were compared.   Each cup of Warburg's manometer contained 1 ml of cell suspension (100 mg of wet cell) and 1 ml of M/15 phosphate buffer (pH 6.0) in main chamber, 0.2 ml of KOH solution in center well and 0.1 ml of Na-salt of the substrate (pH 6.0) in side arm.   The curves of oxygen-uptake are given in Fig. 1.   From these results, it is reco-
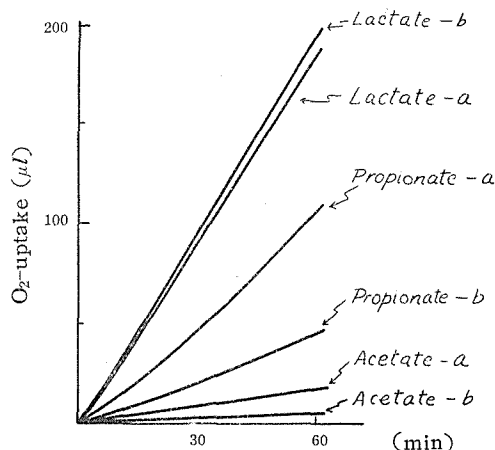

Fig. 1.   Difference of the rate of oxygen uptake according to the cultural condition.

a : O_2-uptake by shaking cultured cell.

b : O_2-uptake by static-cultured cell.

gnized that the remarkalbe oxidation of lactate is found to be similar in both types of the cell, but that the decomposition of acetate seems to be weak especially in static-cultured cell.   The oxidation of propionate is observed in both cells, and the shaking cultured cell is also found to be superior to static-cultured one.   The fact that the oxidation of acetate in static-cultured cell was never accelerated by adding yeast extract or glucose (Table 1) shows that the loss of activity is due to the absence of enzyme

system of oxidation of acetate.

Table 1. The effect of glucose or yeast extract in the oxidation of acetate in static-cultured cell of *P. arabinosum*.

Substrate	nil	acetate	glucose	glucose + acetate	yeast ext.	yeast extract + acetate
O$_2$-uptake in 30 min.	19.4	22.9	53.4	50.5	45.0	49.0
Net O$_2$-uptake by acetate		3.4		− 2 9		4.9

(2) Differences in Respiratory Quotient and the Inhibition of Propionate Oxidation with Malonate between Two Types of Cell

If propionate is oxidized completely in the following scheme; $C_2H_5 \cdot COOH + 7O = 3CO_2 + 3H_2O$ by T.C.A. system[1], respiratory quotient (R.Q.) should be 0.857 and the reaction would be inhibited by the addition of malonate because succinate dehydrogenation process exists in the pathways of propionate metabolism. With the two types of bacterial cell, respiratory quotients and the degree of inhibition with malonate were measured. Results are given in Fig. 2 and Table 2 and 3.

In the experiment shown in Fig. 2, 300μM of malonate in the side arm was poured into the main chamber after a requisite time of shaking, while in the experiment shown in Table 2, malonate was added to the main chamber at
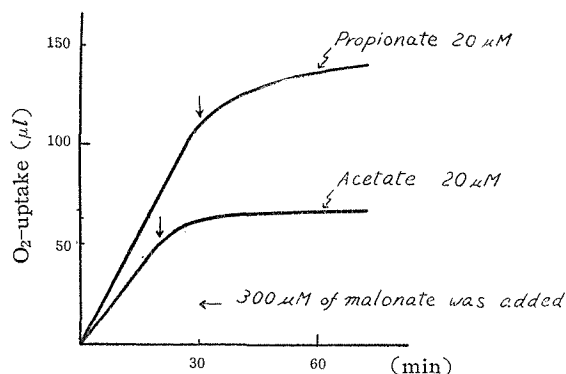

Fig. 2. Malonate inhibition on the oxygen uptake.

Table 2. The inhibition of propionate oxidation with malonate in static-cultured cell.

Substrate	Propionate 20μM	propionate 20μM + malonate 30μM	propionate 20μM + malonate 300μM
O$_2$-uptake in 30 min.	151.1	131.2	111.8

Table 3. Respiratory quotients in each type of cell.

Substrate	R.Q.	
	in shaking-cultured cell	in static-cultured cell
Propionate	0.78	0.65
Succinate	1.45	—
Lactate	0.88	—

the beginning of the experiment. The degree of inhibition with malonate was found to be less in static-cultured cell, and this shows that the pathway of succinate to fumarate does not reveal a main route. The complete inhibition of acetate oxidation with malonate in shaking-cultured cell was observed, but the propionate oxidation was still detectable after the addition of malonate, though the rate of oxygen-uptake was much decreased.

These facts suggest the existence of several routes in the propionate oxidation, in one of which malonate does not reveal a noticeable inhibition.

From these results, it is concluded that in shaking cultured cell the T.C. A. enzyme system will operate and propionate will be oxidized chiefly by this system. On the contrary, there is no remarkable activity of the T.C.A. system in static-cultured cell and propionate will be partially oxidized with lese R.Q. value.

(3) Intermediates in Propionate Oxidation

Succinate or pyruvate seems to be the gateway to the T.C.A. system in the oxidation of propionate.

Barban-Ajil[5] reported that labelled succinate was formed from succinate and labelled CO_2 in the presence of the cell suspension of *P. pentosaceum*, and that the interconversion of propionate and succinate might occur.

Mahler-Huennekens[2] reported that propionate was completely oxidized through lactate or pyruvate by liver preparation. Of course there may be another pathway to which unknown C_3 compound is relating[6]. When pyruvate is formed, acetate may be produced by its decarboxylation which is a normal reaction revealed by propionic acid bacteria, and this reaction would not be affected by malonate.

The mixture of 5 ml of M/10 Na-propionate (pH 6.0), 5 ml of cell suspension and 10 ml of M/15 phosphate buffer (pH 6.0) was placed in Erlenmeyer's flask and shaked for 5 hrs at 30°. After removing the cell by centrifugation, organic acids in the solution were extracted with ether, and they were subjected to paper partition chromatography with ether-acetic acid-water

mixture (13 : 3 : 1). Three spots (Rf = 0.10, 0.64 and 0.73) were obtained. The authentic pyruvate and lactate gave the spots Rf = 0.64 and Rf = 0.74 respectively, but Rf = 0.10 did not correspond to any spot appeared by authentic citrate, α-ketoglutarate, succinate, fumarate or malate.

The same experiment as was mentioed above was carried out in the presence of 5 ml of M/5 Na-bisulphite. When 2N-HCl solution saturated with 2, 4-dinitrophenylhydrazine was added to the acid solution, yellow precipitate was formed. This precipitate, after being washed with 2N-HCl solution and then with water, was dissolved in a small amount of N-Na$_2$CO$_3$ solution and developed on paper with butanol saturated with 3 % aqueous ammonia. 2, 4-Dinitrophenylhydrazone of pyruvate was detected (Rf = 0.35), and the same hydrazone was obtained when lactate was used as substrate instead of propionate.

From these results, it may be concluded that propionate will be oxidized through lactate to pyruvate, but Baker-Lipmann[7] denied the conversion of pyruvate to propionate through lactate by *P. pentosaceum*. Moreover we were unsuccessful to find any condition on the accumulation of lactate in the course of propionate oxidation by *P. arabinosum*. When propionate is converted into CH$_3$·CH$_2$·CO-SCoA in static-celtured cell, it is reasonable to consider that in the first step dehydrogenation in α-β position[8] would take place.

In order to verify this supposition, the oxygen-uptake was tested with acrylic acid, and the rate of oxygenuptake was found much slower than

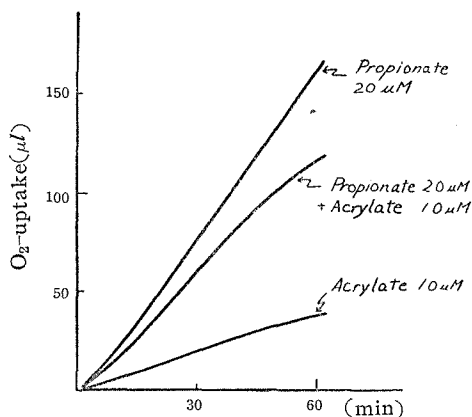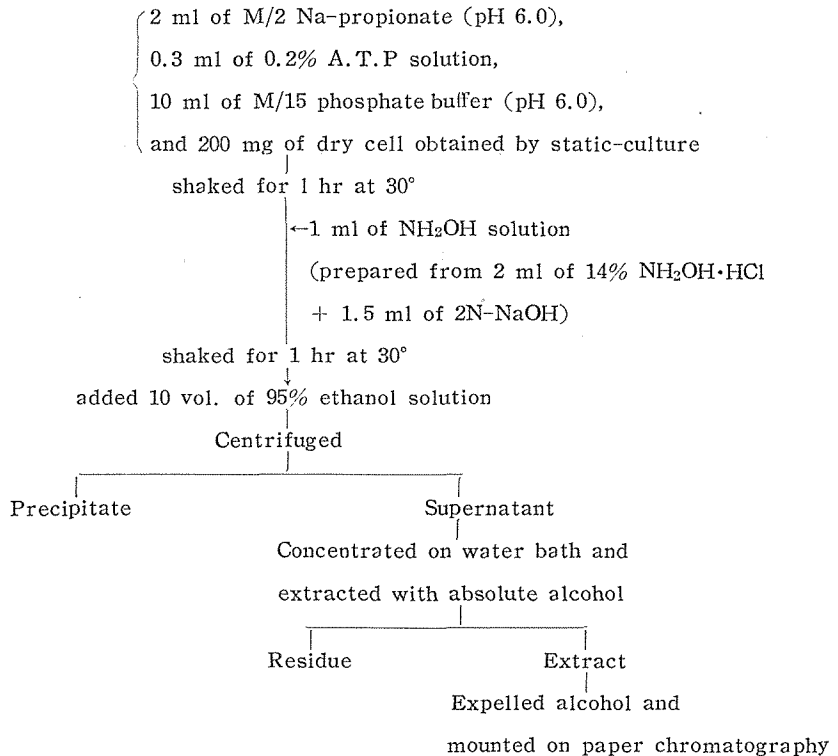Fig 3. Oxygen uptake by propionate and acrylate.

that in propionate (Fig. 3). So the existence of free acrylate in the process of oxidation of propionate is doubtful, though Mahler-Huennekens[9] insisted that the value of oxygen uptake could not be the obligate proof to exclude acrylate from intermediates of the pathway of propionate oxidation.

There remains the possibility that pyruvate formation from propionate is

carried out not through lactate but through other unknown acid.

Succinate is oxidized in shaking-cultured cell with the same rate as pro-
pionate, so if the reaction of propionate to succinate does really exist, it may
be a possible pathway of propionate oxidation. When hydroxylamine is
present in the reaction of succinate decarboxylation by this organism under
anaerobic condition, the formation of succinyl hydroxamate is recognized[10].
With propionate, similar hydroxamate was again observed, as was shown
in Fig. 4.

Fig. 4. The formation of hydroxamates in the propionate oxidation.

    ⎧ 2 ml of M/2 Na-propionate (pH 6.0),
    ⎪ 0.3 ml of 0.2% A.T.P solution,
    ⎨ 10 ml of M/15 phosphate buffer (pH 6.0),
    ⎩ and 200 mg of dry cell obtained by static-culture

        shaked for 1 hr at 30°

            ←1 ml of NH₂OH solution
            (prepared from 2 ml of 14% NH₂OH·HCl
            + 1.5 ml of 2N-NaOH)

        shaked for 1 hr at 30°

    added 10 vol. of 95% ethanol solution

            Centrifuged

    Precipitate   Supernatant

                    Concentrated on water bath and
                    extracted with absolute alcohol

                Residue   Extract

                        Expelled alcohol and
                        mounted on paper chromatography

Rf of chemically synthesized
hydroxamic acids[11]   Rf developed with water-saturated butanol
                                            16
    Acetic   52   51
    Propionic   65   63
    Butyric   78

The spot of Rf=0.16 is suggested to be one of the succinyl hydroxamates,
because the same spot appeared when hydroxylamine was added on the start of
incubation to the mixture of succinate, A.T.P. and cell suspension and kept
under anaerobic condition, although the evolution of CO_2 was not detectable.

From the results of the decolorizing test of pyocyanine under vacuum with or without malonate (see Table 4), it is suggested that some dehydrogenating processes would take place prior to succinate dehydrogenation, so that the conversion to succinate is also not to be the sole pathway of the propionic acid oxidation.

Five ml of cell suspension (containing 500 mg of wet cell) and the mixture of 5 ml of M/10 substrate (Na-salt), 5 ml of M/15 phosphate buffer (pH 6.0) and 0.5 ml of M/100 pyocyanine are added in Thumberg's tube separately. After evacuated, they were mixed each other, incubated at 30° and the time of decolorization was measured, as was shown in Table 4.

Table 4. Reduction of pyocyanine by shaking-cultured cell of *P. arabinosum*.

Substrate	without malonate	with 1.5 mM of mlonate
Propionte	within 3 min.	within 3 min.
Latate	within 2 min.	within 2 min.
Succinate	within 2 min.	more than 20 min.

From the fact that propionyl and acetyl hydroxamates were detected in the presence of hydroxylamine (Fig. 4), it is suggested that the conversion of propionate to acetate is carried out by static-cultured cell. This reaction is not inhibited with malonate and gives the respiration quotient of 0.667. This reaction was verified by determining acetate and propionate after the incubation of static-cultured cell with propionate.

The mixture of 10 ml of cell suspension, M/10 Na-propionate (pH 6.0) and M/15 phosphate buffer (pH 6.0) was incubated for 4 hrs at 30° (surface ratio ≒ 1). After the incubation, the supernatant of the reaction mixture, made slightly alkaline, was concentrated to 10 ml, and then steam-distilled with a few drops of sulphuric acid. Carbon dioxide in the solution was expelled previously by boiling with reflux condenser, and 100 ml of distillate was collected. The amounts of propionic and acetic acids in the distillate were determined by Osburn-Wood-Werkman's partition method[12]. For steam-distillation, the apparatus suggested by Pozzi-Escot[15] which was slightly modified was used.

Exp. No.	I	II	III
Steam-distillate taken (ml)	30	30	25
Ether added (ml)	50	100	0
Acids found in 25 ml of the water layer (μM)	72	44	228
Partition constant (K)	31.5	19.3	—

Values of K for pure propionic and acetic acids are found to be 58.2 and

27.7 in the same experimental condition of Exp. No. I, and 42.2 and 16.2 in the condition of No. II, respectively.

From $K_I = 31.5$

$$\left.\begin{array}{l} P + A = 100 \\ 58.2P + 27.7A = 31.5 \end{array}\right\} \quad P = 86.2 \text{ mol } \% \quad A = 13.8 \text{ mol } \%$$

From $K_{II} = 19.3$

$$\left.\begin{array}{l} P + A = 100 \\ 42.2P + 16.2A = 19.3 \end{array}\right\} \quad P = 88.0 \text{ mol } \% \quad A = 12.0 \text{ mol } \%$$

$$P_{av} = 87.1 \text{ mol } \% \quad P_{av} = 12.9 \text{ mol } \%$$

The partition constant in the condition of No. II is calculated (K_{IIo}) from the observed value of each acid in the condition of No. I.

$$K_{IIo} = 42.2 \times 0.138 + 16.2 \times 0.862 = 19.8$$
$$K_{II} - K_{IIo} = 19.3 - 19.8 = -0.5$$

The value of $K_{II} \sim K_{IIo}$ shows that the above value of propionate and acetate is reliable.

From there values the acids can be determined.

Propionate added	95.4 mM
Propionate remained	78.5 mM
″ consumed	16.9 mM
Acetate formed	12.7 mM
Non volatile acid formed	2.0 mM
(calculated as succinate)	

A part of propionate would be converted to cell metarial or completely oxidzed, but most of the oxidized propionate would change to acetate.

Considering that R.Q. of the cell is 0.65, the reaction will follow to the next formula

$$C_2H_5 \cdot COOH + 3O = CH_3 \cdot COOH + CO_2 + H_2O$$

(4) Possible Pathways of Propionate Oxidation

From these experiments, it is concluded that when *P. arabinosum* is incubated with sufficient supply of oxygen, the enzyme ststem of T.C.A. cycle becomes active and most of propionate is oxidized completely to CO_2 and water. But when oxygen supply is limited during the incubation, the activity of T.C.A. system in the cropped cells is not sufficient to react with pyruvate which is formed from propionate, so that pyruvate is oxidatively decarboxylated to acetate and CO_2, and this enzyme system is thought to be constitutive. The behavior of pyruvate under aerobic condition would be decided by the compartive activity of the two systems mentioned above.

The pathway from propionate to pyruvate is still unable to be completely explained. The formation of succinate would be the main pathway when malonate is absent, because acetate is thought to be formed from succinate at a reasonable rate[13]. The fact that the inhibition in propionate oxidation was recognized in the presence of 30 μM of malonate (Table 2) suggests that the pathway of succinate formation will really operate, although this pathway would not be applicable to anaerobic conditions shown in Table 4. On the other hand, succinate may be derived from pyruvate through Wood-Werkman's reaction coupled with other oxidizing reactions as is already pointed out in the propionate forming process[14].

The pathway on the formation of pyruvate by the oxidation of lactate through acrylate was proposed with liver cyclopholase system by Mahler-Huennekens[2]. In the case of propionic acid bacteria, however, Kalckar[16] observed that the reduction of acrylate never revealed by the bacteria and Barker-Lipmann[7] verified that this organism can produce propionate from pyruvate under the condition in which lactate is not metabolised at all, namely in the presence of 1/80 M NaF. By our experiment, however, it was found that acrylate could be oxidized though quite slowly, and that the inhibiting effect of NaF on propionate oxidation was greater than that on lactate. So that, from our experiments the pathway through acylate cannot be decidedly ex cluded.

Table 5. Influence of sodium fluoride on the oxidation of propionate and lactate.

| | O_2-uptake in 30 minutes | |
	Propionate 20 μM	lactate 20 μM
Nil	94	125
NaF 20 μM	87	111
NaF 100 μM	16	82

In Fig. 5, the possible pathways of propionate oxidation which were hitherto discussed are illustrated. As is already cited, the pathway of propionate oxidation through succinate will be possible, but it cannot be the sole pathway of propionate oxidation since propionate oxidation is still carried out when the dehydrogenation of succinate is inhibited with malonate. Lactate and pyruvate are formed on the oxidation of propionate, and as the conversion of propionate to lactate or pyruvate will not be one step reaction, some substances must be produced as intermediates. From our experimental results, not free acrylate but some drivatives of acrylate may be formed during the pathway of propionate oxidation. From the present knowledge,

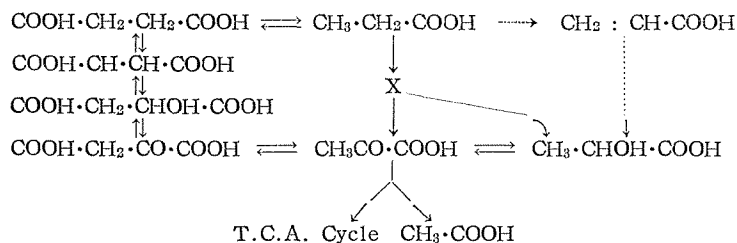we must set up unknown intermediates between propionate and pyruvate in the process of propionate oxidation.

$$
\begin{array}{l}
\text{COOH·CH}_2\text{·CH}_2\text{·COOH} \rightleftharpoons \text{CH}_3\text{·CH}_2\text{·COOH} \longrightarrow \text{CH}_2 : \text{CH·COOH} \\
\quad\quad\uparrow\downarrow \\
\text{COOH·CH·CH·COOH} \\
\quad\quad\uparrow\downarrow \\
\text{COOH·CH}_2\text{·CHOH·COOH} \quad\quad\quad \text{X} \\
\quad\quad\uparrow\downarrow \\
\text{COOH·CH}_2\text{·CO·COOH} \rightleftharpoons \text{CH}_3\text{CO·COOH} \rightleftharpoons \text{CH}_3\text{·CHOH·COOH} \\
\\
\quad\quad\quad\quad\quad\quad \text{T.C.A. Cycle CH}_3\text{·COOH}
\end{array}
$$

Fig. 5. The possible pathway of propionate oxidation by propionic acid bacteria.

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