On the Metabolism of Organic Acids by *Clostridium acetobutylicum*

Part II. Lactic Acid Metabolism and Relating Role of Racemiase

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With racemiase free bacterial cells of *Clostridium acetobutylicum*, distinguishable mode of the metabolism of both D- and L-lactic acids was found. Racemiase rich cells, however, metabolized both types of lactic acids to a same extent. It was indicated that this organism originally possessed D-lactic dehydrogenase, and anaerobically dehydrogenated D-lactic acid, and that the organism oxidized aerobically L-lactic acid by a lactic acid oxidizing enzyme which was proposed in this paper.

INTRODUCTION

Lactic acid, a minor product in normal culture of *Clostridium acetobutylicum*, is accumulated in adequate conditions as much as eighty to ninety per cent of glucose consumed. Moreover, lactic acid supplied to the growing culture of this organism is thoroughly fermented together with glucose. Not only under anaerobic circumstances, but also in the presence of air oxygen, *Cl. acetobutylicum* is capable of metabolizing lactic acid to some extent.

In the previous paper¹) the formation of lactic acid by *Cl. acetobutylicum* was discussed in several aspects. In all cases, in which lactic acid was formed, racemiase activity was detected in some measure, and lactic acid obtained was always optically inactive. In this investigation, the resolution of characteristics of lactic dehydrogenase in this organism was carried out, and finally indicated the existence of D-type lactic dehydrogenase in this organism.

As previously described, lactic acid added culture showed potent acceleration of racemiase formation, besides, almost whole amount of lactic acid supplemented to the culture medium disappeared during the fermentation. In this connection, it seems interesting to compare the metabolic sequence of D- and L-type lactic acids in relation to racemiase activity. It was revealed that *Cl. acetobutylicum* possessed D-lactic dehydrogenase and L-lactic oxidizing system, and the former was capable of transporting hydrogen from D-lactic acid to atmospheric oxygen via methylene blue as a potent carrier, meanwhile, the latter operated aerobically upon L-lactic acid in forming acetic acid and carbon dioxide without any intermediary carriers supplemented. In the presence of sufficient amount of racemiase, however, such a distinction of action of the organism on both types of lactic acid was vanished. Finally it was concluded that racemiase, in the metabolism of lactate by *Cl. acetobutylicum*, would play a role in connecting both D-lactic dehydrogenase and L-lactic oxidizing enzyme system.

MATERIALS AND METHODS

Microorganism and culture.

A strain of *Clostridium acetobutylicum*¹) was used. According to the modification in Part I, the following composition of basal medium was employed; glucose 2%, peptone 1%, monobasic potassium phosphate 0.05%, dibasic potassium phosphate 0.05%, magnesium sulphate 0.02%, sodium chloride 0.001%, manganous sulphate 0.001% and ferric sulphate 0.001%. Others remained the same as stated in the previous paper unless indicated.

TABLE I. **INTRODUCTION OF LACTIC ACID INTO GLUCOSE FERMENTATION OF *Clostridium acetobutylicum***

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Glucose (mg%)</td>
<td>1249.0</td>
<td>1040.6</td>
<td>625.0</td>
<td>144.0</td>
<td>0</td>
</tr>
<tr>
<td>D,L-Lactic acid (mg%)</td>
<td>0</td>
<td>327.5</td>
<td>982.5</td>
<td>1637.5</td>
<td>1965.0</td>
</tr>
<tr>
<td>Glucose consumed (mg%)</td>
<td>1249.0</td>
<td>1040.6</td>
<td>625.0</td>
<td>144.0</td>
<td>no growth</td>
</tr>
</tbody>
</table>

**Products (mg%)**
- Butyl alcohol: 252.0
- Ethyl alcohol: 61.5
- Acetone: 78.2
- Butyric acid: 44.5
- Acetic acid: 149.1
- Lactic acid: 3.7

**RESULTS AND DISCUSSION**

**Introduction of lactic acid into glucose fermentation.**

Glucose fermentation supplemented with lactic acid proceeded in some variations of the products. In this case, initially subjected lactic acid was rapidly decreased accompanying with the metabolism of glucose, and complete consumption of glucose was acquired. On the other hand, no growth could be observed in a medium employing D,L-lactic acid as a sole carbon source. Table I represents the introduction of lactic acid into glucose fermentation of *Clostridium acetobutylicum*. From the results in Table I, it is evident that a large quantity of lactic acid added to

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**Analysis of fermentation products and assay of racemase activity.**

Each products in cultured solution and in reaction mixture of cell-suspensions were analysed by the methods similar to those described in Part 1. To assay racemase activity, employed the manometric methods by Kitahara and Fukui.

**Assay of lactic dehydrogenase.**

Dehydrogenation of lactic acid was estimated in evacuated Thunberg-Borsook’s tube using methylene blue as a hydrogen acceptor. Aerobically, amount of oxygen uptake was measured in Warburg’s manometer with the reaction mixture similar to Thunberg-Borsook’s method; that is, the dehydrogenation of lactic acid was translated into oxygen uptake, the latter was corresponding to reoxidation of reduced methylene blue, which accepted hydrogen from lactic dehydrogenase system.
On the Metabolism of Organic Acids by Clostridium acetobutylicum. Part II.

Glucose fermentation was consumed incompletely despite of complete disappearance of glucose, and both butyric acid and ethyl alcohol were increased in their rate to glucose consumed, while the rate of butyl alcohol was depressed. On the whole, the rate of two-carbon compounds were remarkably increased with addition of lactic acid to glucose fermentation of this organism. These results may suggest the decomposition of added lactic acid to two-carbon intermediate, common precursor of acetic acid and ethyl alcohol in any way.

Effect of methylene blue on the formation of lactic acid in glucose fermentation.

As a probable mechanism of the formation of lactic acid from glucose by Cl. acetobutylicum, the reduction of pyruvic acid accumulated by blocking further degradation was presented in the previous paper. It was further revealed that methylene blue added to glucose fermentation of this organism was capable of promoting lactic acid formation, moreover, preventing normal fermentation of both glucose and added lactic acid. The results were shown in Table II. In these experiments, supplemented methylene blue was converted to leuco-form (reduced form) in early stage of fermentation and maintained colorless state at least for forty hours thereafter. After forty hours the fermented broth recovered blue color of methylene blue (oxidized form) just by heating. These observations indicate that the reduction systems of this organism can easily reduce methylene blue. In other words, hydrogen generating sites, such as triose phosphate dehydrogenase and pyruvic acid splitting system, are capable of transferring hydrogen to methylene blue as a substitute for hydrogenase and many other reducing enzymes, in which butyl and ethyl alcohol-forming enzymes and lactic dehydrogenase are included. In this point of view, it will be expected that the accumulation of hydrogen by methylene blue protects the liberation of molecular hydrogen but accelerates the reducing enzyme in some extent. The data in Table II indicate predominant effect of methylene blue merely on the accumulation of lactic acid. Hydrogen atoms transferred to hydrogenase are destined to be liberated as hydrogen gas from the medium. Two reasons are adopted for the accumulation of lactic acid by methylene blue. One of them is the block of pyruvate degrading enzyme, and the other is the preferential transfer of hydrogen to lactic dehydrogenase for reducing pyruvic acid.

In acetone-butanol fermentation the redox potentials at pH 7 of various reducing systems are as follows: diphosphopyridine nucleotide = -320 mV, four-carbon compounds reducing system = -280 to -290 mV, ethyl alcohol forming system = -190 to -200 mV and lactic acid forming system = -175 mV. Since the redox potential of methylene blue is about +10 mV, it is expected to draw the potential of medium near zero by supplementing with methylene blue. Such an ascent of redox potential will cause disadvantage for the reducing enzymes of lower redox potential, for instance four-carbon compounds forming system. If the redox potential of the medium were kept about -175 to -190 mV, reducing system of higher potential than that of the e.g. lactic acid forming system of -175 mV, would function preferentially. Accumulation of lactic acid would consequently occur. Utilization of leuco-methylene blue as a hydrogen donor in lactic acid formation, however, would not be probable, because the redox potential of methylene blue is much higher than that of lactic acid forming system. In other words, it is difficult to explain such an effect of methylene blue on the formation of lactic acid with the aid of the operation of lactic dehydrogenase of redox potential of -175 mV or so. In this connection, it is presumable that a new lactic acid forming system of redox potential higher than that of methylene blue might be occupied in

Lactic dehydrogenase of *Cl. acetobutylicum*.

Intact cells of *Cl. acetobutylicum* obtained from basal medium as well as 0.15 per cent lactic acid added medium were used as a source of lactic dehydrogenase. Results of assays in Thunberg-Borsook's tube are indicated in Table III and Table IV. With glucose-grown cells the dehydrogenation of D(−)-lactic acid proceeded far rapidly than that of L(+)-lactic acid. Lactic dehydrogenase activity of glucose-DL-lactate grown cells, meanwhile, was higher than that of glucose-grown cells and no appreciable difference in dehydrogenation velocities between D- and L-lactic acids was observed. The same facts were ascertained in manometric investigations as shown in Figs. 1 and 2. On the occasion in Fig. 1, when glucose-grown racemase-deficient cells were used, the dehydrogenation velocity of L-lactic acid was below the half of D-lactic acid. Glucose-lactate-grown racemase-rich cells, however, dehydrogenated not only D-lactic acid in much higher velocity but also L-lactic acid to nearly equal extent. More distinct results were obtained from manometric assay of lactic dehydrogenase of racemase-deficient cells grown in cyanide containing medium, as indicated in Fig. 3. The effect of cyanide on dehydrogenation of any optical isomer of lactic acids would be out of consideration, since very much the same.
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Fig. 3. Dehydrogenation of Lactic Acid by Cl. acetobutylicum grown in Cyanide-added Speakman's Culture Medium.

Each Warburg vessel contained cell-suspension 1.0 ml (74 mg dried cells, racemase activity 6.490), 0.2 M phosphate buffer (pH 7.0) 0.5 ml, 0.1% methylene blue 0.5 ml and Na-lactate 90 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

Extent of inhibition of cyanide on dehydrogenation velocities was observed with each optical isomers of lactic acid as is shown in Table V.

Table V. Inhibitory Action of Potassium Cyanide on the Dehydrogenation of Lactate by Cl. acetobutylicum

Each Warburg vessel contained glucose-grown cell-suspension of Cl. acetobutylicum 0.5 ml (dry matter 35 mg), 0.2 M phosphate buffer (pH 7.0) 0.5 ml, 0.1% methylene blue 0.5 ml and Na-lactate 0.5 ml (200 μM) to final volume 2.5 ml. Potassium cyanide was added to final concentration 0.01 M. The gas phase was air. Oxygen uptake was read for 130 minutes at 37°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>D(-)-Lactate</th>
<th>L(+)-Lactate</th>
<th>DL-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ uptake for 130 min. (μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without KCN</td>
<td>108</td>
<td>46</td>
<td>89</td>
</tr>
<tr>
<td>with KCN</td>
<td>32</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>Rate of inhibition (%)</td>
<td>70</td>
<td>76</td>
<td>71</td>
</tr>
</tbody>
</table>

Accordingly, it could be postulated that, Cl. acetobutylicum possesses D-lactic dehydrogenase in proper, and in racemase-sufficient cells L-lactic acid would be dehydrogenated followed by conversion to D-form through the action of racemase, which is affecting simultaneously in the reaction mixture. About this problem further discussion is presented below.

Oxidation of lactic acid by cell-suspension of Cl. acetobutylicum.

As described above, a new lactic acid forming system of redox potential higher than that of methylene blue was suggested. In this connection, the oxidation of both D- and L-lactic acids by cell-suspensions of Cl. acetobutylicum was investigated manometrically in the presence or absence of methylene blue. Figs. 4a and 4b as well as Figs. 5a and 5b indicate the results.

Fig. 4. Oxidation of Lactic Acid by Cl. acetobutylicum grown in Speakman's Culture Medium (pH 7.0).

Each Warburg vessel contained cell-suspension 1.0 ml (45 mg dried cells), 0.2 M phosphate buffer (pH 7.0) 0.5 ml and Na-lactate 30 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

a. Without methylene blue.
b. Supplemented with 0.5 ml of 0.1% methylene blue.
FIG. 5. Oxidation of Lactic Acid by Cl. acetobutylicum Grown in Speakman's Culture Medium (pH 5.2).

Each Warburg vessel contained cell-suspension 1.0 ml (43 mg dried cells), 0.2 M phosphate buffer (pH 5.2) 0.5 ml and Na-lactate 30 μM. The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C.

a. Without methylene blue.
b. Supplemented with 0.5 ml of 0.1% methylene blue.

Oxygen uptake with L-lactic acid was independent of the existence of methylene blue at each pH, and its velocity at pH 5.2 was somewhat higher than that at pH 7.0. On the contrary, methylene blue revealed intensive effect upon the oxygen uptake of D-lactic acid. In the absence of the dye, oxygen uptake of D-lactic acid was very few. At pH 7.0, oxygen uptake of D-lactic acid in the presence of the dye increased linearly in process of time far rapidly than at pH 5.2. In various experiments, it was pointed out that the maximum oxygen uptake of D-lactic acid in the presence of methylene blue was observed at pH 7.0 to 7.2, and the optimal pH range for the oxidation of L-lactic acid was below pH 6.0. It is presumable that the responsibility of the dye for the oxidation of D-lactic acid is generally due to reoxidation of leucodye formed in the conjugation with D-lactic dehydrogenase as follows:

\[
\begin{align*}
\text{D-Lactate} & \xrightarrow{\text{DPN}} \text{F} \xrightarrow{\text{Mb}} \text{H}_2\text{O}_2 \\
\text{Pyruvate} & \xrightarrow{\text{DPNH}} \text{H}_2\text{O}_2 \\
\text{D-Lactic Diaphorase} & \text{(Air oxygen)} \text{ (I) dehydrogenase}
\end{align*}
\]

\[
\begin{align*}
\text{Pyruvic acid} + \text{H}_2\text{O}_2 & \rightarrow \text{Acetic acid} + \text{CO}_2 \\
\text{Pyruvic acid} + \text{O}_2 & \rightarrow \text{Acetic acid} + \text{CO}_2 \\
\text{H}_2\text{O}_2 & \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \\
\text{Pyruvic oxidase} & \text{ (II) (Non-enzymic)} \\
\text{Catalase} & \text{ (IV)}
\end{align*}
\]

and this organism is negative in catalase activity.

FIG. 6. Pyruvic Oxidase of Cl. acetobutylicum. Each Warburg vessel contained cell-suspensions obtained from Speakman's culture medium, 60 mg as dry matter, 100 μM Na-pyruvated and 0.5 ml of 0.2 M phosphate buffer (pH as indicated). The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C. Curves of CO₂ output were omitted from the figure.

Curve I. O₂ uptake at pH 5.2. R.Q. was 2.3.
Curve II. O₂ uptake at pH 6.4. R.Q. was 1.8.
Curve III. O₂ uptake at pH 7.2. R.Q. was 1.7.
and pyruvic oxidase of this organism shows merely few activity at pH 7 as indicated in Fig. 6, so that the reactions (I) and (II) would be actual in the experimental conditions described here.

On the whole, *Cl. acetobutylicum* seems to contain the two lactic acid metabolizing systems, one of them is independent to methylene blue and the other is corresponding to the dye. Baker\(^6\) reported the existence of L-α-hydroxy acid oxidase in animal tissues, which was assumed to require flavin-monomonucleotide as the cofactor and was distinguished from classical lactic dehydrogenase. *Mycobacterium phlei* was pointed out to possess L-lactic oxidase, which contained prosthetic flavin-adenine-dinucleotide and catalyzed direct oxidation of L-lactic acid to acetic acid\(^7-9\). Such lactic oxidases in other bacteria were further reported by Yamamura et al.\(^{10}\) and Yagi et al.\(^{11,12}\). The foregoing fact that *Cl. acetobutylicum* oxidizes L-lactic acid without aid of any redox dyestuff would suggest the participation of such a lactic oxidase differing from classical lactic dehydrogenase. In this respect the responsibility of riboflavin, flavin-monomonucleotide and flavin-adenine-dinucleotide for L-lactic acid oxidation by this organism was investigated, but no appreciable effect was observed. The isolation of L-lactic acid oxidizing system has been unsuccessful.

**Distinctive action of Cl. acetobutylicum on D- and L-lactic acid.**

Table VI indicates the summary of stoichiometric analysis in the metabolism of D- and L-lactic acid by glucose-lactate-grown cells of *Cl. acetobutylicum*. Following is the conclusion from the data in Table VI; i) ferrous ion slightly accelerated the disappearance of L-lactic acid, ii) L-lactic acid is consumed to a greater extent than D-isomer, iii) remaining lactic acid contained both types of optical isomers, and the proportion of optical antipode to total remainder (e.g. the proportion of L-lactic acid to the total in the experiment employing D-lactic acid as the substrate) is raised by pre-existing ferrous ion, iv) significant amount of pyruvic acid can be delivered from D-lactic acid, but the accumulation of this acid from L-lactic acid is negligible despite of larger consumption of L-lactic acid more than D-isomer, and finally, v) both isomers of lactic acid are converted to equimolar acetic acid.

**Responsibility of racemiase for lactic acid metabolism by Cl. acetobutylicum.**

In the preceding articles it was pointed out that glucose-lactate-grown cells of *Cl. acetobutylicum* showed dehydrogenation efficiency on D- and L-lactic acid in the same extent, despite of the predominant dehydrogenation of D-lactic acid by glucose-grown cells. Apparent velocity of dehydrogenation of D-lactic acid was accelerated in parallel with the content of racemiase activity. On the contrary, the possession of particular L-lactic acid oxidizing enzyme in

<table>
<thead>
<tr>
<th>Substrate</th>
<th>D(-)-Lactate</th>
<th>L(+)-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial lactic acid</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Final lactic acid</td>
<td>5.32</td>
<td>5.30</td>
</tr>
<tr>
<td>D-Lactic acid</td>
<td>4.82</td>
<td>4.32</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>0.50</td>
<td>1.18</td>
</tr>
<tr>
<td>Lactic acid consumed</td>
<td>0.68</td>
<td>0.50</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.60</td>
<td>0.45</td>
</tr>
<tr>
<td>Acetic formed/Lactic consumed</td>
<td>0.87</td>
<td>0.90</td>
</tr>
</tbody>
</table>

\(^{9}\) F. B. Cousins, *Biochem. J.* 64, 297 (1956).
\(^{10}\) M. Kasunosu, E. Kusunosu and Y. Yamamura, *Kekkaku (Japan)* 27, 73, 243 (1952).
TABLE VII. INFLUENCE OF HYDROXYLAMINE ON THE DEHYDROGENATION OF LACTIC ACID

Cells of Cl. acetobutylicum harvested from DL-lactate-added culture and Leuc. mesenteroides from malt extract culture were used. Each Warburg vessel contained cell-suspension 1.0 ml (corresponding to 47 mg of Cl. acetobutylicum and 20 mg of Leuc. mesenteroides as dry matter), 0.2 M phosphate buffer (pH 7.2) 0.3 ml, 0.02% methylene blue 0.5 ml and Na-lactate 0.3 ml (30 μM). The total volume was 2.3 ml. Gas phase was air. The vessels were incubated at 37°C for 150 minutes (Cl. acetobutylicum) and 90 minutes (Leuc. mesenteroides) respectively.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cl. acetobutylicum</th>
<th>Leuc. mesenteroides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>D(−)-Lactate</td>
<td>L(+)−Lactate</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>added</td>
</tr>
<tr>
<td>O₂ uptake (μM)</td>
<td>10.8</td>
<td>12.6</td>
</tr>
<tr>
<td>Lactate consumed (μM)</td>
<td>9.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Pyruvate found (μM)</td>
<td>1.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Per cent inhibition of racemiase by hydroxylamine otherwise examined was 73 and 95 with final concentration of 0.001 and 0.01 M respectively.

this organism was assumed. To resolve this characteristic behaviour of this organism in relation with racemiase, a potent inhibitor of racemiase was employed in the dehydrogenation of lactic acid by glucose-lactate-grown cells. Cyanide and hydroxylamine inhibit racemiase most intensively among various inhibitors13). Here, hydroxylamine was used because it revealed no action on D-lactic dehydrogenase of Cl. acetobutylicum despite of the considerable extent of inhibitory action of cyanide on the dehydrogenase as indicated in Table V. Results were shown in Table VII. Dehydrogenation of L-lactic acid was markedly suppressed by the addition of hydroxylamine to final concentration 0.01 M, though the dehydrogenation of D-lactic acid was not affected. Results with a D-lactic acid producing organism, Leuconostoc mesenteroides, were presented in the same table so as to compare with others.

From these results two cases would be suggested as follows. The first is that Cl. acetobutylicum possesses two types of lactic dehydrogenases, one of them is hydroxylamine sensitive L-lactic dehydrogenase and the other is non-susceptible D-lactic dehydrogenase. The former would somewhat resemble to D-lactic dehydrogenase of Leuc. mesenteroides in its behaviour towards hydroxylamine. The second case is that L-lactic acid is racemized followed by dehydrogenation by D-lactic dehydrogenase, and the suppression of dehydrogenation of L-lactic acid in the presence of hydroxylamine is due to inhibition of racemiase action preceding to D-lactic dehydrogenase action. In order to settle the problem, partial resolution of lactic dehydrogenase as well as racemiase was carried out. Cell-free racemiase was prepared from cultured filtrate of Cl. acetobutylicum by salting-out with ammonium sulphate (confer subsequent report).

As shown in Table IX, crude preparation of lactic dehydrogenase acted predominantly to D-lactic acid, and dehydrogenated L-lactic acid by...

TABLE IX. EFFECT OF RACEMIASE ON LACTIC DEHYDROGENASE OF *Clostridium acetobutylicum*

Evacuated Thunberg-Borsook's tube contained crude preparation of lactic dehydrogenase (see Table VIII) 0.8 ml, boiled extract of *Clostridium acetobutylicum* 0.3 ml (corresponding to 100 mg dried cells), 0.2 M phosphate buffer (pH 7.2) 0.3 ml, 0.02% methylene blue 0.1 ml and Na lactate 0.3 ml (30 μM). The total volume was 20 ml. Racemiase was prepared by salting-out from 100 ml filtrate of lactate-added culture and lyophilized. The tubes were incubated at 37°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Racemiase</th>
<th>Decoloration time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>&gt;180</td>
</tr>
<tr>
<td>D(-)-Lactate</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td>L(+)-Lactate</td>
<td>—</td>
<td>120</td>
</tr>
<tr>
<td>L(+)-Lactate</td>
<td>added</td>
<td>70</td>
</tr>
<tr>
<td>D(-)-Lactate</td>
<td>added*</td>
<td>&gt;180</td>
</tr>
<tr>
<td>L(+)-Lactate</td>
<td>added*</td>
<td>&gt;180</td>
</tr>
</tbody>
</table>

* Lactic dehydrogenase was omitted from the reaction mixture.

supplementing with racemiase though its velocity was still lower than that of d-lactic acid dehydrogencation. It was therefore assumed that this organism dehydrogenated L-lactic acid through the serial action of racemiase and d-lactic dehydrogenase.

It was also discussed in preceding articles that this organism was capable of oxidizing L-lactic acid by any specific enzyme system which was different from d-lactic dehydrogenase.

Summarizing the facts described above, the following metabolic sequence of both D- and L-lactic acid, considering the interaction of D-lactic dehydrogenase and L-lactic oxidizing system through racemiase, could be postulated.

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Glucose  \downarrow Glycolytic enzymes (Embden-Meyerhof-Parnas pathway)
D-Lactic  \rightleftharpoons Pyruvic acid  \rightarrow Acetic acid + CO2
           \downarrow Racemiase
L-Lactic acid  \rightarrow L-Lactic acid oxidation system
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In this scheme, it is still doubtful whether pyruvic acid would be an intermediate in the oxidation sequence of L-lactic acid to acetyl compound or acetic acid, though pyruvic acid could scarcely be detected in L-lactic acid metabolizing system as indicated in Table VI. Distinction of optimal pH supports the individuality of two types of lactic acid oxidizing (or dehydrogenating) systems. Moreover, it would emphasize the reliability of this scheme that the preference of oxidizing action on any one of the optical isomers of lactic acid was reversed by the addition of methylene blue, and that there could be observed no appreciable difference in dehydrogenation velocities between both optical isomers in the presence of sufficient amount of racemiase. Investigations on L-lactic acid oxidation system are now in succession.

**SUMMARY**

Behaviours of acetone-butanol fermentation bacterium, *Clostridium acetobutylicum*, towards optically active lactic acids were investigated. As a rule, cells harvested from lactic acid supplemented culture medium surpassed in metabolizing lactic acid as compared with glucose-grown cells. It was pointed out that *Clostridium acetobutylicum* possessed D-lactic dehydrogenase by nature, and that L-lactic acid could be dehydrogenated after its conversion to D-isomer through the action of racemiase. In addition to D-lactic dehydrogenase, it was further indicated that this organism had another type of lactic dehydrogenase or oxidase, which was capable of oxidizing L-isomer with air oxygen in liberating acetic acid and carbon dioxide. Physiological action of racemiase as a mediator between two lactic enzymes, D-lactic dehydrogenase and d-lactic acid oxidizing enzyme, was ascertained.

In addition, it is mentioned about pyruvic oxidase and partial inhibition by cyanide to lactic dehydrogenase.