PROPERTIES OF THE LACTIC ACID-RACEMIZING ENZYME OF CLOSTRIDIUM BUTYRICUM*  

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In previous publications (1-3) it was shown that the acetone-butyrol alcohol organisms Clostridium acetobutylicum and Clostridium butylicum have the curious property of converting d- or l-lactic acid into the inactive dl form. Katagiri and Kitahara (4, 5) found that many lactic acid bacteria and Staphylococcus urea also possess this racemizing ability, but that other bacteria and many yeasts and molds do not have it.

In a preceding paper (3) racemization was attributed to an enzyme which was thought to consist of two components: one found in the cells and heat-stable; the other liberated into the medium and heat-labile. Neither cells nor medium functioned separately, and hence it was assumed that the racemizing system consisted of an enzyme-coenzyme complex. More extended study of the problem has shown that it is not necessary to assume the existence of an extracellular component and an intracellular component. The earlier results may be explained by the distribution of enzyme between cells and medium and by the pH requirements of the enzyme.

The present paper deals with these and other factors influencing the activities of the enzyme. Such a study, it was hoped, would lay the groundwork for elucidation of the function of the enzyme.

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Culture and Medium—The organism used in this investigation was Clostridium butylicum, No. 21. It was always grown in a 0.5 per cent glucose-0.5 per cent tryptone medium. This is the same organism and the same medium used in the studies reported by Tatum et al. (3).

Preparation of Calcium Lactate—Dextrorotatory lactic acid in the form of its calcium salt was used. It was prepared in large quantities by fermenting glucose with a strain of Lactobacillus delbrückii, according to the method of Tatum and Peterson (6). Calcium lactate obtained from different fermentations, after several recrystallizations from water, always showed a specific rotation of $-6.1^\circ$ to $-6.3^\circ$ in a 4 per cent solution. Calcium determinations gave practically theoretical results for calcium lactate.

Methods

All of the work so far reported on racemization of active lactic acid has been based on water of crystallization of the zinc lactate as a measure of the change. This method, however, requires much time and is too inaccurate for a quantitative study of the enzyme activity. For this reason a shorter and more accurate method was needed. Such a method, based on the optical rotation of calcium $d$-lactate, was developed.

Rotation of Calcium $d$-Lactate—Preliminary experiments on the optical properties of calcium $d$-lactate showed that the specific rotation decreases with increasing concentration. However, during racemization experiments the total concentration of calcium lactate remains the same, although the concentration of the $d$-lactate decreases. It was thought that under such conditions the rotation might be closely proportional to the concentration of the dextro acid and hence permit calculation of the $dl$ acid present. Mixtures of calcium $d$- and $dl$-lactate in various proportions, but always totaling 4 per cent, were made up and read in a 4 dm. tube in the saccharimeter. From the observed readings the composition of the mixtures was determined and compared with the calculated values. The data are given in Table I and show good agreement between observed and calculated values.

It was thus evident that if the method could be applied to cell
suspensions and cultures the tedious process of extracting the acid, preparing the zinc salts, and determining the water of crystallization could be eliminated.

_Treatment of Cultures_—The method employed for preparing the cultures for analysis was as follows: Calcium d-lactate was added in solution to the culture or fraction to be tested. The amount was usually 2 gm. of the anhydrous salt in 50 cc. of solution. The volume was adjusted as desired, toluene was added, and the flask was tightly stoppered, shaken, and incubated at 37°. After incubation the solution was boiled to remove the toluene, and the pH was adjusted to about 7. After addition of norit to decolorize the solution and remove the cells, and of diatomaceous earth to aid filtration, the solution was filtered on a small Buchner funnel, and the residue well washed. The filtrate and washings were boiled down and made up to a volume such that the resulting concentration of calcium lactate was 4 per cent. The readings on a 4 dm. tube were taken in a saccharimeter.

With cell suspensions the above treatment always yielded water-clear solutions. Many control experiments in which the enzymes were inactivated before incubation gave complete recovery of the active calcium lactate. Similar treatments of solutions of active calcium lactate alone gave the same results. Therefore, the procedure does not racemize active lactate nor does it remove lactate from solution.

With the cultures or with the cell-free medium certain difficulties

<table>
<thead>
<tr>
<th>Composition of solution</th>
<th>[α]₀</th>
<th>Racemization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca d-lactate per cent</td>
<td>Ca dl-lactate per cent</td>
<td>degrees</td>
</tr>
<tr>
<td>4.00</td>
<td>0.00</td>
<td>-6.13</td>
</tr>
<tr>
<td>3.52</td>
<td>0.48</td>
<td>-5.30</td>
</tr>
<tr>
<td>3.00</td>
<td>1.00</td>
<td>-4.44</td>
</tr>
<tr>
<td>2.00</td>
<td>2.00</td>
<td>-3.03</td>
</tr>
<tr>
<td>1.08</td>
<td>2.92</td>
<td>-1.73</td>
</tr>
<tr>
<td>0.48</td>
<td>3.52</td>
<td>-0.54</td>
</tr>
<tr>
<td>0.00</td>
<td>4.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Enzyme of *Clostridium butylicum*

were encountered. Following the above treatment duplicate solutions often showed differences in rotation as high as 10 per cent. Control experiments showed the same discrepancies. The differences were finally attributed to optically active substances which were removed from the medium in unequal amounts by the above treatment. For the quantitative study of the enzyme system, however, it was possible to dispense with the medium as a source of the enzyme, since as will be shown later, the cells under prescribed conditions are active alone.

**Distribution of Enzyme between Medium and Cells**

10 liters of culture were sampled at intervals, and the racemizing activity at pH 5 of the cells and of the cell-free medium was determined. The per cent racemization produced by the cells was determined in the usual manner. The medium, however, after incubation with calcium lactate, was acidified and the lactic acid was extracted with ether. The calcium salt of the extracted acid was then made, and its specific rotation determined. The results are shown in Fig. 1. It is seen that the enzyme content of the cells increased up to 20 hours and then decreased as the culture aged. That the initial increase in activity does not parallel in-
crease in cell weight is illustrated by the fact that in from 9 hours to 20 hours the enzyme content increased more than 5-fold, while the cell weight only doubled. The enzyme content of the medium rose rapidly as the culture aged, the enzyme very obviously being liberated into the medium from old cells.

**Racemization Studies with Cell Suspensions**

In order to study some of the properties of the racemizing enzyme a large quantity of cells was grown. The cells were centrifuged from four different cultures of 14 liters each. The wet cells obtained, about 100 gm., were kept in a frozen condition until used. These cells have been kept over a period of 8 months, during which time they have been repeatedly thawed, and the activity has remained quite constant.

**Effect of pH**—In preliminary experiments on effect of pH, the solutions were adjusted with hydrochloric acid and calcium hydroxide. It was found, however, that upon readjusting the solutions to pH 7 the rotations of control solutions containing inactivated cells were considerably decreased. However, the use of sulfuric acid and calcium hydroxide for pH adjustments gave theoretical recovery of the rotations of control solutions upon readjustment to pH 7 with the same reagents. In this case the excess calcium and sulfate ions were precipitated upon adjusting and readjusting the solutions, while in the other the presence of excess calcium or chloride ions brought about the decrease in rotations. In all experiments, therefore, sulfuric acid and calcium hydroxide were used as needed for pH control.

Because of the danger of influencing the rotations, as above, no buffers were used. It was found that at pH 5 and below, the lactate had sufficient buffer capacity to hold the pH at the desired value. Above pH 6, where the solutions had very little buffer capacity, the greatest fluctuation was about 1 pH unit. The pH of each solution was determined before and after incubation.

Fig. 2 shows that there is a sharp rise in activity from pH 3 to pH 5 followed by a more gradual decrease in activity as the pH increases. The optimum is clearly at about pH 5. In view of this pronounced effect of pH on the velocity of the racemization reaction, it is possible that the negative results obtained by Kata-
giri and Kitahara (5) with many microorganisms may have been due to unfavorable pH.

From the pH curve it appears that the substrate for the racemizing enzyme may be the lactate ion rather than the undissociated lactic acid. There is appreciable racemization at the higher pH values, where the substrate is present entirely as lactate ion. At the lower pH values, much of the substrate is present as free lactic acid, and consequently the concentration of available substrate is lower. In the acid range, therefore, the pH activity curve represents the effect of pH on the available substrate concentration as well as on the racemization reaction itself.

![PERCENT RACEMIZATION](image)

**Fig. 2.** Effect of pH on enzyme activity. 75 cc. of reaction mixture, containing 2 gm. of calcium lactate and 0.25 gm. of wet cells, were incubated 40 hours at 37°.

**Effect of Enzyme Concentration**—In Fig. 3 the effect of enzyme concentration on racemization is shown. The solid curve is a theoretical curve for a first order reaction and the experimental points fall upon or close to it.

It may be shown on the following theoretical basis that such a first order reaction curve is to be expected with the racemizing enzyme. Since the product of the reaction is dl-lactic acid, the affinity of the enzyme for the d and l acids must necessarily be identical. Therefore, since the total lactate concentration remains constant, the fraction of the total enzyme present as enzyme-substrate complex remains the same. Of this combined
enzyme, the percentage combined with the \textit{d} acid is always equal to the percentage of \textit{d} acid in the mixture. Thus it follows that a first order reaction is to be expected regardless of the substrate concentration.

\textit{Effect of Substrate Concentration} In Fig. 3, the racemization given by a cell suspension is plotted against molar concentration of lactate. It will be noted that the enzyme did not approach maximal activity until a very high substrate concentration was reached (0.5 M). While the data of the figure do not allow an exact determination of the Michaelis constant, its value apparently lies in the neighborhood of 0.14, a value much higher than that for most respiratory enzymes.

\textit{Effect of Cyanide}—As shown in Table II, 0.05 M cyanide completely inhibits racemization. Iron- or copper-containing enzymes are known to be cyanide-sensitive, while most dehydrogenases are not.

\textit{Effect of Incubation Temperature}—Because of the long incubation periods necessary, thermal inactivation of the enzyme limited measurement of the racemization velocity at high temperatures.
The data of Table III show appreciable inactivation at 45°, and probably some inactivation at 40°. However, the apparent energy of activation of the racemization process is less than that of most enzymatic reactions, which usually ranges from 10,000 to 20,000 calories.

**Clostridium butylicum Dehydrogenases**—While the racemizing activity of washed cells is extremely stable, preliminary experi-
ments showed the cell dehydrogenases to be extremely unstable. The very considerable activity possessed by freshly harvested cells disappeared within a few hours. Table IV gives representative data. It will be noted that even the fresh cells had very little lactic acid dehydrogenase activity, although growing cultures of the organism are able to metabolize lactic acid (3).

**TABLE IV**

*Dehydrogenase Activity of Washed Cells*

4 cc. of reaction mixture contained cells from 5 cc. of a 22 hour culture, 1 cc. of 0.02 per cent methylene blue, and 1 cc. of pH 6.4 phosphate buffer. The incubation was carried out at 40° in evacuated Thunberg tubes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration of substrate</th>
<th>Decolorization time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fresh cells</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>&gt;215</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.025</td>
<td>7</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>0.05</td>
<td>22</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.025</td>
<td>156</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.075</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

It seems probable that the racemizing enzyme has, in the living cell, a function other than that of racemization.

Any enzyme capable of combination with lactic acid could, under the proper conditions, act as a racemase, provided that the enzyme possessed an equal affinity for the two optical forms of lactic acid and that the enzyme-substrate reaction destroyed the asymmetric structure of the α-carbon atom. This might take place by momentary, reversible conversion of the lactic acid molecule into acrylic acid, pyruvic acid, or methylglyoxal. The extreme instability of the cell dehydrogenases, as well as the cyanide sensitivity of the racemizing enzyme, seems to indicate that the latter is not a lactic acid dehydrogenase. Moreover, bacterial dehydrogenases are usually strongly cell-bound, while the racemizing enzyme is readily secreted into the medium. The substrate affinity of the racemizing enzyme is also much lower than that of a dehydrogenase.
Enzyme of *Clostridium butylicum*

Since growing cultures of *Clostridium butylicum* are able to ferment added lactic acid (3), and will actually produce lactic acid from glucose when grown at high pH values (7), it seems probable that the racemization studied in the present paper is due to an enzyme concerned in the normal lactic acid metabolism of the organism.

**SUMMARY**

Additional data on the pH requirements and distribution of the racemizing enzyme of *Clostridium butylicum* show that the enzyme does not necessarily consist of two components as was previously concluded.

The enzyme acts most rapidly at pH 5 and at a lactate concentration of 0.5 M. It is completely inhibited by 0.05 M cyanide. While cells from young cultures are high in enzyme, in old cultures most of the activity is found in the culture medium.

**BIBLIOGRAPHY**

PROPERTIES OF THE LACTIC ACID-RACEMIZING ENZYME OF CLOSTRIDIUM BUTYLICUM
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