CCLXVI. ENZYMIC RACEMIZATION OF OPTICALLY ACTIVE LACTIC ACID.¹

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(Received 19 August 1936.)

It has been shown in a previous publication [1932] that certain lactic acid bacteria which by themselves form optically active lactic acid produce inactive (*dl*) lactic acid when grown in association with the acetone butyl alcohol organism *Cl. acetobutylicum*. This was thought to be due to an associative modification of the metabolism of the lactic acid bacteria, causing them to produce both forms of lactic acid rather than the one form produced normally. It was stated that the formation of inactive lactic acid took place in association with *Cl. acetobutylicum* but not with the closely related organism *Cl. butylicum*.

Further investigation has shown that *Cl. butylicum* when grown together with lactic acid bacteria does lead to the production of inactive lactic acid. It has further been found that the racemization is caused by the butyric organisms themselves, rather than by their effect on the lactic acid bacteria. Finally, it has been shown that the racemization of optically active lactic acid by *Cl. acetobutylicum* and *Cl. butylicum* is enzymic in character.

EXPERIMENTAL.

Cultures and media. Pure cultures of the following organisms were used: *Strep. lactis*, R.; *Lactobacillus delbrückii*, 3; *Cl. acetobutylicum*, S.; and *Cl. butylicum*, 21 (*Cl. sp.* 21 in previous report). A 3 % glucose, 3 % malt sprouts medium containing an excess of calcium carbonate was used for associated growth of the lactic cultures and the butyric organisms and for some of the enzyme work, but a medium containing 0.5 % glucose and 0.5 % "tryptone" was also used in the latter experiments.

Analytical methods. Volatile and non-volatile acids were determined by steam-distillation and extraction with ether respectively, as described in a previous publication [Pederson *et al.* 1926]. The form of lactic acid recovered was determined as in the previous report and was based on the percentage of water of crystallization and the specific rotation of the zinc salts.

In every case the zinc salts were crystallized from 50 % alcohol to ensure as nearly complete recovery as possible. Over 90 % of the lactic acid in each sample was recovered as the salt. In this way truly representative samples for determination of water of crystallization and specific rotation were assured.

Effect of association of lactic acid bacteria and Cl. butylicum on the form of acid produced.

It was reported in the previous paper that *Cl. butylicum* had no effect on the form of acid produced in association with lactic cultures. This work has been

¹ This work was supported in part by a grant from the Special Research Fund of the Graduate School.

repeated. The results in Table I show that the associated growth of *Cl. butylicum* with either *Strep. lactis*, R. or *L. delbrückii*, 3, caused the production of inactive lactic acid. The acid formed by the lactic acid bacteria alone was dextro-rotatory. The previous negative results were probably due to the failure of

Table I. *Effect of association with Clostridium butylicum on the form of lactic acid produced by bacteria.*

Organism	Water of crystallization of zinc salt of lactic acid %	Form of lactic acid produced
<i>Strep. lactis</i>	11.3	Dextro
<i>Strep. lactis</i> and <i>Cl. butylicum</i>	17.2	Inactive
<i>Strep. lactis</i> and <i>Cl. butylicum</i>	18.2	Inactive
<i>L. delbrückii</i>	12.9	Dextro
<i>L. delbrückii</i> and <i>Cl. butylicum</i>	18.1	Inactive
<i>L. delbrückii</i> and <i>Cl. butylicum</i>	18.2	Inactive
<i>L. delbrückii</i> and <i>Cl. butylicum</i>	18.19	Inactive

Cl. butylicum to grow appreciably, as indicated by the low volatile acid production. Because of the sensitivity of this organism to acid it is difficult to establish a balance between the two cultures in a fermentation. These results indicate that *Cl. butylicum* has the same racemizing effect in associated growth on the form of lactic acid as does *Cl. acetobutylicum*. This would be expected from their close relationship.

Racemization of d-lactic acid by fermentation.

In the previous paper [1932] it was suggested that the associated organism caused the lactic culture to produce both forms of lactic acid rather than only one as it did normally. An alternative explanation which was not considered at that time is that the lactic acid as formed is active but that the associated organism, *Cl. acetobutylicum*, brings about the racemization.

The validity of the latter hypothesis was tested by growing the butyric acid bacteria in a medium containing glucose (3%), malt sprouts (3%), calcium carbonate and the calcium salt of *d*-lactic acid. After several days' incubation the fermentations were stopped and the unfermented lactic acid was recovered and the form determined. Table II gives the results. In 2 days

Table II. *Racemization of d-lactic acid* by fermentation.*

300 ml. medium: 3% glucose, 3% malt-sprouts, CaCO₃.

Organism	Period of fermentation days	Volatile acid as acetic g.	Lactic acid recovered		Water of crystallization of zinc salt of recovered lactic acid %	Form of lactic acid recovered
			g.	%		
None	Sterile	—	5.16	100	12.9	Dextro†
<i>Cl. acetobutylicum</i>	2	1.97	4.54	88	14.7	Mainly dextro
<i>Cl. acetobutylicum</i>	3	2.98	2.78	54	18.6	Inactive
<i>Cl. butylicum</i>	2	1.17	2.68	52	17.0	Mainly inactive
<i>Cl. butylicum</i>	3	1.53	2.25	44	18.1	Inactive

* Lactic acid added as calcium salt.

† Specific rotation = -7.9°.

Cl. acetobutylicum destroyed only 12% of the lactic acid and had only a slight racemizing effect. After 3 days, however, more volatile acid was produced, and 54% of the lactic acid was recovered. It was entirely inactive. *Cl. butylicum* had

a similar effect. In 2 days 52 % of the lactic acid was recovered. It was mainly inactive. Only 44 % was recovered after 3 days and it was completely racemized. These results indicated definitely that the formation of inactive lactic acid in association was due to the direct action of the associated cultures on the active lactic acid produced by the lactic acid bacteria.

Enzymic racemization of lactic acid.

It seemed possible that the racemization of lactic acid by the butyric acid bacteria might be due to enzymic activity. Therefore, instead of fermenting calcium lactate directly, the cultures were grown for 24 hours and active lactic acid was then added as a solution of the calcium salts. Toluene and chloroform were added at the same time. The mixtures were incubated for 3 days at 37°.

Table III. *Enzymic racemization of active lactic acid.*

Medium* ml.	Treatment	Lactic acid				Recovered zinc lactate	
		Added		Recovered		Water of crystalli- zation %	Form of acid
		Form	g.	g.	%		
Culture, <i>Cl. acetobutylicum</i> :							
300 (M)	Antiseptics	Dextro	5.40	5.72	105.0	18.2	Inactive
Culture, <i>Cl. butylicum</i> :							
300 (M)	Antiseptics	Dextro	5.16	4.54	88.0	18.2	Inactive
300 (M)	Antiseptics	Dextro	5.16	4.86	94.2	17.3	Inactive
75 (T)	Antiseptics	Laevo	0.82	0.74	90.5	18.1	Inactive
75 (T)	Antiseptics	Laevo	0.82	0.68	82.4	17.8	Inactive
300 (M)	Heated, 68°	Dextro	5.40	5.18	93.0	13.1	Dextro
300 (M)	Heated, 87° antiseptics	Dextro	5.40	4.80	89.0	12.9	Dextro†

* (M) = malt-sprouts, 3 %; glucose, 3 %. (T) = "tryptone", 0.5 %; glucose, 0.5 %.

† Specific rotation of zinc salt, -6.8°.

The results in Table III show that from 82 to 100 % of the added lactic acid was recovered and that in every case it had been completely racemized. Both cultures were effective and both *d*- and *l*-lactic acids were changed to the inactive form. Cultures of *Cl. butylicum* failed to bring about the change after they had been heated to 68 or 87°.

Racemization by components of racemizing enzyme system.

In order to determine something about the nature of the enzymes responsible for racemization of active lactic acid, *Cl. butylicum* was grown in 0.5 % glucose, 0.5 % "tryptone" medium. After 24 hours' incubation the cells were removed by centrifuging. This treatment threw out most of the cells. The cell-free medium and the cells were separately made up to the original volume of the culture with distilled water. Active lactic acid (calcium salt), toluene and chloroform were added to samples of the cell suspension, the cell-free medium and a mixture of the two. Sufficient distilled water was added to the samples containing only the cell suspension or the cell-free medium to make the final concentration of each component one-half that in the original culture before centrifuging. After 3 days' incubation at 37° the lactic acid was recovered and the form determined. The results are given in Table IV. Neither the cell suspension nor the cell-free medium alone had any effect on the form of lactic acid. A combination of the two, however, was effective, since the recovered lactic acid was inactive. Both *d*- and

Table IV. *Enzymic racemization of lactic acid by cells and medium of Cl. butylicum.*

Agent	Lactic acid				Recovered zinc salt		
	Form	Added		Recovered	Water of crystallization %	Specific rotation $[\alpha]_D^{25}$ °C.	Form of acid
		g.	g.				
Cells	Dextro	2.7	2.4	89	13.3	—	Dextro
Cells	Dextro	2.7	2.3	85	13.2	-6.8	Dextro
Medium	Dextro	2.7	2.7	100	13.2	-7.0	Dextro
Medium	Dextro	2.7	2.5	92	13.0	-6.5	Dextro
Cells and medium	Dextro	2.7	2.6	97	17.0	—	Inactive
Cells and medium	Dextro	2.7	2.7	100	18.1	—	Inactive
Cells	Laevo	0.82	0.70	85	13.1	+7.8	Laevo
Medium	Laevo	0.82	0.69	84	13.4	+7.0	Laevo
Cells and medium	Laevo	0.82	0.71	86	17.8	—	Inactive

l-lactic acids were racemized by the combined cells and medium. This indicates that at least two factors are concerned in the racemization. One seems to be extracellular and the other intracellular.

Heat-stability of components of racemizing enzyme system.

The separation into two components of the enzyme system responsible for racemization of active lactic acid suggested that the system might be similar to an enzyme-coenzyme system. Since many coenzymes are heat-stable, the heat-stability of the component cell and cell-free medium fractions was investigated. The fractions were prepared as detailed before, and portions of each preparation were heated for 10 min. at 87°. Combinations of heated medium with unheated cells, and of heated cells with unheated medium were tested for their racemizing actions on *l*-lactic acid (Table V). The activity of the cell-free medium fraction

 Table V. *Heat-stability of components of racemizing enzyme system of Cl. butylicum.*

Components and heat treatments	Lactic acid			Recovered zinc lactate	
	Laevo-acid added g.	Recovered		Water of crystallization %	Form of acid
		g.	%		
Heated medium and unheated cells	0.82	0.80	98	13.1	Laevo*
Heated medium and unheated cells	0.82	0.72	88	13.4	Laevo
Unheated medium and heated cells	0.82	0.80	98	16.6†	Mixture, laevo and inactive
Unheated medium and heated cells	0.82	0.74	90	18.0	Inactive

* Specific rotation of zinc salt, +7.3°.

† Sample fractionally recrystallized: first crop: H₂O, 18.2%; last crop: H₂O, 13.4%; specific rotation, +7.8°.

was destroyed by the heat treatment. However, the active component in the cell suspension fraction seemed to be heat-stable. The zinc salt from one sample of recovered lactic acid had a water of crystallization content of 16.6% indicating a mixture of active and inactive acids. The sample was fractionally recrystallized. The first crop of salt was the pure inactive form, and the last

crop was pure active salt. Another sample of recovered lactic acid had been completely racemized. By analogy with other enzyme systems, this racemizing enzyme system seems to be composed of an extracellular heat-labile enzyme and an intracellular heat-stable co-enzyme.

DISCUSSION.

The racemization of active lactic acid by enzyme systems of *Cl. acetobutylicum* and *Cl. butylicum* seems to explain fully the results obtained in the previous paper. During that work an attempt was made to separate the two associated organisms by a viscose membrane. No change in the form of acid was observed. A racemizing enzyme produced by *Cl. acetobutylicum* should have been incapable of penetrating such a membrane, and any lactic acid which diffused through the membrane into contact with the enzyme would probably have been completely fermented. The production of active lactic acid in a sterilized *Cl. acetobutylicum* culture on inoculation with a lactic culture was due to the destruction of the racemizing enzyme by heat.

The work of Hammer [1920] with cheese starters suggests that other organisms may cause the production of inactive acid in association with *Strep. lactis*. It seems possible that the general production of inactive lactic acid in starters and in commercial lactic acid fermentations may be due primarily to the racemizing activities of contaminating organisms, which may or may not themselves produce lactic acid. If such were the case, the racemization might be a function of the enzyme concentration and activity rather than of the numbers of contaminating organisms. The growth of the contaminants and the elaboration of the enzymes would not necessarily lower the yield of lactic acid materially.

Pasteur in his classical work on optical activity found that micro-organisms such as moulds were capable of producing active lactic acid from the inactive form by selective fermentation of one enantiomorph. This does not involve any actual change in the configuration of the asymmetric molecules. In contrast, the results in this paper show that some bacteria can change optically active lactic acid quantitatively into the inactive form. This racemization necessitates a reversal of the configuration of the asymmetric molecules. This enzymic Walden inversion proceeds until the two enantiomorphs are in equilibrium. The resulting lactic acid consists of a mixture of both forms present in equal quantities. The enzymic attainment of such a state of equilibrium between the two optical isomerides would explain the complete racemization of either the dextro- or the laevo-rotatory form of lactic acid.

SUMMARY.

Inactive lactic acid was formed in associated growth of *Strep. lactis* or *L. delbrückii* with the butyric acid organisms *Cl. acetobutylicum* or *Cl. butylicum*.

The production of inactive acid in associated growth is probably due to the racemizing effect of the butyric organisms on the lactic acid after it has been formed by the lactic acid bacteria. In the absence of the lactic acid bacteria both *Cl. acetobutylicum* and *Cl. butylicum* partially fermented active lactic acid and changed the remainder to inactive acid.

The racemization seems to be brought about by an enzyme system elaborated by *Cl. acetobutylicum* and *Cl. butylicum* since cultures of these organisms changed either *d*- or *l*-lactic acid quantitatively into *dl*-acid in the presence of antiseptics.

There seem to be at least two components of this racemizing enzyme system, one extracellular and the other intracellular. Neither cell suspensions nor cell-free media of *Cl. butylicum* were able to racemize active lactic acid, but the two components were effective in combination.

The extracellular component was found to be heat-labile (87°, 10 min.) and the intracellular fraction was stable to this heat treatment.

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