CXCVII. THE PRODUCTION AND UTILIZATION OF LACTIC ACID BY CERTAIN PROPIONIC ACID BACTERIA¹

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LACTIC ACID has long been recognized as a substrate for the propionic acid bacteria. Fitz [1880], the first to present an equation for the propionic acid fermentation, suggested that it might be expressed as

Only recently has this compound been regarded as a possible end product. Foote *et al.* [1930] reported that under certain conditions some cultures of the propionic acid bacteria accumulate this acid. Other investigators [Wood & Werkman, 1937; Fromageot & Tatum, 1933] have reported other conditions which yield similar results. The amount of lactic acid produced varies with pH, nutrients and probably with other factors. The optical properties of the lactic acid produced by these organisms have not been determined. While lactic acid has often been used as a substrate the availability of the optical isomerides of this acid has not been studied.

The present studies were undertaken to determine conditions under which lactic acid would accumulate and to isolate and characterize the lactic acid formed by some of the propionic acid bacteria. The availability of the optical isomerides was also studied.

Cultures and methods

The cultures used in this study were *Propionibacterium pentosaceum*, P₁₁; *P. technicum*, P₁₂; and *P. shermanii*, P₁₉.

The racemizing action of resting cells upon the Ca salt of *d*(-)-lactic acid was determined at pH 6.8 and 5.0 by the method of Christensen *et al.* [1939].

For most of the experiments a medium containing 2% substrate, 0.5% peptone, 0.5% Na acetate, 100 ml. yeast water² and distilled water to make 1 l. was used. 150 ml. of the medium were fermented in 250 ml. Erlenmeyer flasks. In some fermentations sterile CaCO₃ was added immediately before inoculation and in others sterile indicator and alkali were added periodically as required. 1 ml. of a 24–36 hr. culture was used as inoculum. All flasks were incubated aerobically at 30°. Approximately 25 ml. samples for sugar and lactic acid determinations were aseptically removed at the intervals indicated.

Lactic acid was determined by the method of Friedemann & Graesser [1933] upon the ethyl ether extract of an acidified (pH 2) aliquot of the fermented medium. Sugars were determined by the method of Schaffer & Hartmann as modified by Stiles *et al.* [1926].

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² The clear extract from 200 g. pressed yeast autoclaved in 1 l. water.

The basal medium for the study of the availability of the various forms of lactic acid contained 200 ml. yeast water, 0.5 % peptone, 0.004*M* phosphate and distilled water to make 1 l. The substrates *l*(+), *d*(−) and *dl*-lactates and glucose were incorporated in 0.45 % concentrations. The unsupplemented basal medium was also tested. Triplicate tubes of these five media were inoculated with 2 drops of a 5 ml. suspension of washed cells from 10 ml. of a 24 hr. culture which had been grown upon the *dl*-lactate medium. The incidence and rate of growth were determined by following the increase in turbidity as determined with an Evelyn photoelectric photometer.

The specificity of the lactic dehydrogenase was determined by the Thunberg technique. Cells from 24 hr. cultures on the *dl*-lactate medium were washed twice by centrifuging in 0.01*M* pH 7 phosphate buffer and suspended in distilled water in such a concentration that 1 ml. of the cell suspension at pH 6 (the optimum for this dehydrogenase) and 40° would, in the presence of *l*(+)-lactate, reduce 1 ml. of 1 : 5000 methylene blue in approximately 5 min. 1 ml. each of the methylene blue solution, 0.04*M* substrate and 0.2*M* pH 6 phosphate-phthalate buffer were placed in the tubes and 1 ml. of the cell suspension was placed in the cap. The contents of the tube and cap were mixed after 3–4 min. equilibration.

Racemization

Racemizing activity could not be demonstrated at either pH 6.8 or 5.0. The absence of this activity suggested that an optically active lactic acid rather than a racemic mixture should be formed by these organisms.

Lactic acid accumulation

Lactic acid accumulation, as indicated by serial analyses of representative fermentations by culture P₁₁ on several media, is shown in Table I. The addition

Table I. *Lactic acid production by P. pentosaceum*

Time and analyses	Glucose					Arabinose	
	CaCO ₃		Neutralized with NaOH		Not neutralized No. 13	CaCO ₃	Neutralized with NaOH
	No. 1*	No. 11	No. 2*	No. 12		No. 3*	No. 14
	Period I						
Days of incubation	4	4	5	4	4	5	4
Sugar fermented, % of total	15.9	42.1	56.1	31.8	15.2	39.5	24.8
Lactic acid, %:							
of utilized sugar	15.9	16.4	16.4	31.2	47.6	2.6	24.8
mg. per 100 ml.	47	33	184	199	145	20	155
	Period II						
Days of incubation	9	7	7	7	8	7	7
Sugar fermented, % of total	77.3	90.2	74.7	50.6	19.6	68.2	46.8
Lactic acid, %:							
of utilized sugar	8.2	1.0	14.9	23.2	40.0	1.5	16.9
mg. per 100 ml.	13	18	222	235	157	20	157
	Period III						
Days of incubation	11	11	10	11	—	10	11
Sugar fermented, % of total	82.2	99.0	80.1	71.2	—	89.3	54.2
Lactic acid, %:							
of utilized sugar	4.7	0.7	11.8	6.1	—	1.0	5.4
mg. per 100 ml.	8	13	189	87	—	18	59

* Medium 2 % sugar, 500 ml. yeast water, distilled water to 1 l.

of CaCO_3 (nos. 1, 11 and 13) to the basal medium resulted in a rapid, almost complete fermentation with a very low lactic acid production. With daily neutralization (nos. 2, 12 and 14) the fermentation was almost as rapid as in the presence of carbonate, while the lactic acid accumulation was markedly increased. The reaction of these fermentations varied from pH 5 to 7. In the unneutralized basal medium high percentages of the utilized sugar appeared as lactic acid but, because of the slow rate of fermentation, the percentage of total sugar converted into lactic acid was less than in the preceding fermentations. Replacement of Na acetate with other buffers, addition of concentrates of propionic growth factors and variation in method of neutralization failed to yield significant increases in yield of lactic acid.

From the data for fermentations 2 and 12, Table I, it will be noted that the concentration of lactic acid is maximal near 7 days. After that time the lactic acid concentration tends to decrease while the percentage of lactic acid, based upon the sugar fermented, steadily decreases throughout the period of incubation. This latter fact, as well as the decrease in total weight of lactic acid, tends to substantiate the theory that lactic acid is an intermediate which is formed during the early stages of the fermentation and later utilized.

Isolation of the lactic acid

Four litres of the basal medium were inoculated with 200 ml. of a 36 hr. culture and neutralized daily with NaOH. After 7 days' incubation at 30° the medium was concentrated to 400 ml., acidified to pH 2 and extracted with ethyl ether for 48 hr. Propionic and acetic acids were removed from this extract by steam distillation and the neutralized (pH 6.5) residue was treated with Ag_2O to remove succinic acid. The remaining solution was acidified and the lactic acid extracted with ethyl ether. The Zn salt of the lactic acid was prepared and purified by recrystallization from 50 % alcohol.

The data, Table II, show that both species form *l*(+)-lactic acid and that the optical configuration is independent of the sugar fermented.

Table II. *Properties of Zn salts of lactic acid*

Organism	Sugar	Water of crystallization %	Zn* content %	Specific rotation†
P ₁₁	Glucose	12.85	33.55	-8.26
P ₁₁	Arabinose	12.92	33.40	-8.26
P ₁₂	Glucose	12.87	33.45	-8.23
Zn salt of <i>l</i> (+)-lactate		12.89	33.40	-8.25
Zn salt of <i>dl</i> -lactate		18.18	33.40	0.00

* Value for anhydrous salt.

† Determined in 4 % concentration.

Utilization of the optical isomerides of lactic acid

In view of the production of only one form, the *l*(+)-acid, it seemed of interest to determine the availability of each form of lactic acid to these organisms. The growth of *P. pentosaceum* upon glucose, the isomeric lactates, and the basal yeast-water, peptone, phosphate medium is indicated in Fig. 1. Photometer readings have been converted into millions of cells per ml. to facilitate comparison.

Development was more rapid on the *l*(+)-lactate than on the *d*(-)-lactate while the racemic lactate supported faster growth than did either of the optically active forms. The differences in cell count were greatest at 45 hr. A greater rate of growth and approximately double total growth proved glucose to be a better medium than any form of lactate in the same concentration.

The growth of *P. shermanii* and *P. technicum* upon these media was identical with that of *P. pentosaceum*. The differences between the rates of growth upon the *l*(+) and *d*(-)lactates were approximately the same for each culture. Although these differences were small, they are considered significant because in each of the three cultures they were much greater than the differences between

Fig. 1. The growth of *P. pentosaceum* upon glucose and the optical isomerides of lactic acid.
● Glucose, Δ *dl*-Lactate, ○ *l*-(+)Lactate, □ *d*-(-)Lactate, ■ Basal medium.

triplicate tubes. The ultimate level of growth seems to be independent of the optical form of the acid and exhibits a remarkable constancy of magnitude for not only the three acid forms, but also for the three cultures. The average of the final cell counts upon the three lactate media were *P. pentosaceum*, 698; *P. technicum*, 717; and *P. shermanii*, 709 million cells per ml.

Because of the differences in the growth rates upon the optical isomerides of lactic acid the activity of the lactic dehydrogenases of these species of the propionic acid bacteria was investigated. These data are shown in Table III. The

Table III. Activity of the lactic dehydrogenases of propionic acid bacteria

Substrate	Culture	Cell activity*		
		P ₁₁	P ₁₂	P ₁₁
Endogenous		0.000	0.000	0.000
<i>l</i> (+)Lactate		0.505	0.154	0.203
<i>dl</i> -Lactate		0.295	0.100	0.193
<i>d</i> (-)Lactate		0.068	0.072	0.091

* Reciprocal of decoloration time in min.

results are expressed in suspension activities, i.e. the reciprocal of the observed decoloration time expressed in minutes. No endogenous respiration was detectable in these cell preparations within 18–24 hr. These data indicate the presence, in each of the organisms studied, of specific *l*(+) and *d*(-)lactic dehydrogenases. As suggested by the growth response, the activity of the *l*(+)lactic dehydrogenase of each organism is significantly greater than that of the *d*(-)enzyme. The activity upon the racemic substrate, however, is intermediate.

SUMMARY

1. The influence of certain cultural conditions upon lactic acid accumulation was studied.

2. *P. pentosaceum* produced only *l*(+)lactic acid from glucose and arabinose and *P. technicum* only *l*(+)lactic acid from glucose.

3. *P. technicum*, *P. pentosaceum* and *P. shermanii* failed to racemize optically active lactic acid.

4. The growth rates upon the optical isomerides of lactic acid varied in the order $d(-) < l(+) < dl$. The ultimate level of growth was the same in each case.

5. The presence of both $l(+)$ and $d(-)$ lactic dehydrogenases in these organisms was demonstrated. The $l(+)$ enzyme possessed the greater activity.

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